CORPUSCLES OF STANNIUS AND STANNIOCALCIN IN THE MORE ANCIENT BONY FISHES: PHYLOGENETIC IMPLICATIONS OF MORPHOLOGICAL AND MOLECULAR DIFFERENCES

By

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A thesis submitted in conformity with the requirements
For the degree of Doctor of Philosophy
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The corpuscles of Stannius [CS], small perirenal endocrine glands implicated in calcium and phosphate regulation in bony fishes, show interspecific variation in number, anatomical distribution, and fine structure. Whereas the more ancient members of Neopterygii possess a large number of CS scattered throughout the kidney proper, the Euteleostei demonstrate a reduction in number and a more posterior and independent distribution of the glandular bodies with respect to the renal tissue. Stanniocalcin [STC], the only active principle characterized to date from the CS, appears to be more widely distributed among the vertebrates than was previously believed. Our examination into STC of the more ancient Actinopterygii suggests that this hormone has undergone considerable evolutionary pressure at the level of the molecule and in tissue distribution. Molecular approaches reveal differences in the protein core, the primary transcript size, and perhaps glycosylation of the STC molecule between species. Evidence at the
molecular level suggests that STC mRNA may be subject to posttranslational processing and that tissue-specific expression of the STC gene is possible. This finding is interesting in light of the recent identification of STC in many mammalian tissues. Further, molecular approaches utilized to examine one member of the Osteoglossomorpha, one of the most ancient of teleosts, provide preliminary evidence that multiple forms of STC molecules may be present in representative species. Multiple sequence alignments of piscine STC revealed highly conserved structural elements found also in mammals. However, our results suggest that the variation in CS number, distribution, and fine structure, and differences in the STC molecule among Actinopterygii may be a consequence of their evolutionary history.
DEDICATION

To the memory of my father

Marino Marra
This doctoral dissertation reports the morphological analysis of the corpuscles of Stannius [CS] and the molecular analysis of stanniocalcin [STC] in several extant bony fishes with ancient lineage. Routine light and electron microscopy, several immunolabelling paradigms, and molecular approaches were used to examine the corpuscles of Stannius and stanniocalcin in the bowfin [Amia calva], the gar [Lepisosteus osseus], the silver arawana [Osteoglossum bicirrhosum], the butterflyfish [Pantodon buchholzi], the featherfin knifefish [Chitala chitala], the elephantnose [Gnathonemus petersii], and the white sucker [Catastomus commersoni]. The thesis consists of three parts: a general introduction, eight chapters in the format of individual manuscripts, and a general discussion. The general introduction provides a review of CS and STC biology, and outlines the major objectives of this study. Eight chapters form the core of the thesis, and their interrelationship is introduced at the beginning of Part II. The chapters are divided into two general sections. The first section deals with the morphology of the CS, in particular the study of corpuscular and extracorpuscular STC-like immunoreactivity in the representative specimens [Chapters 1-5]. The second section deals with the molecular analysis of STC in these specimens [Chapters 6-8].

All research reported here, was conceived by myself or in collaboration with colleagues, and planned and conducted by L.E. Marra, who is responsible for the design, analysis, and interpretation of the data. This research program was conducted under the supervision of Dr. John H. Youson, and the three other members of my committee: Dr. H. Elsholtz, Dr. M. Filosa, and Dr. A. Jorgensen.
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PART I

GENERAL INTRODUCTION
Calcium and phosphate play important roles in a number of vital biological processes, and the maintenance of their ionic levels within specified limits is mandatory for all vertebrates [Aurbach, 1988]. Therefore, it is not surprising that homeostatic systems have evolved in the various vertebrate groups which control the level of calcium and phosphate in the extracellular fluid [Cohn et al., 1972; Aurbach, 1998]. The activity of specialized target tissues, which may include the gills, skin, kidney, intestine, and bone, maintain plasma levels of these ions in each class of vertebrate at a characteristic level [Feinblatt, 1982]. These tissues, in turn, are regulated by the actions of several well-known hormones and, most probably, still unknown factors.

Although much is known about calcium and phosphate metabolism in mammals, it is by no means conclusive [Feinblatt, 1982; Aurbach, 1988]. Further, information on the regulation of these ions in other vertebrate species is relatively limited. Both within and between species the vertebrate subphylum consists of heterogeneous members encompassing different environments and life styles. Therefore, the obvious approach of attempting to examine a particular response based on studies of a particular group of animals and then trying to demonstrate a similar effect on this parameter in another species may not always be successful [Feinblatt, 1982]. In other words, findings in one species may not always be transposable to another species. In the field of endocrinology the difficulty in applying the knowledge gained in one animal model to another animal system needs to be interpreted in view of the fact that endocrine glands may be present in one group and absent from another [Aurbach, 1988; Feinblatt, 1982]. Furthermore, the same gland may be present throughout the different groups, however, the specific hormones may be present but may have acquired different biological functions [Aurbach, 1988; Feinblatt, 1982]. There may have also been the development of new target tissues or sensitivities throughout vertebrate evolution. Without a frame of phylogenetic reference it is difficult to assess an adapted character within and between different vertebrate groups. Therefore, an evolutionary
examination of hormones and the glands that produce them will not only lead to a better understanding of the role of these factors in the ancient vertebrates but will also yield insight as to their function in more modern vertebrates, including man.

Bentley [1998] points out that for comparative endocrinology “the prime academic objective is to reconstruct evolutionary pathways by the study of extant species”. Since the phylogenetic distribution of hormones and the glands that produce them is reflective of the evolutionary process, such comparative knowledge can be used to expand our understanding of both the phylogenetic relationships between vertebrates and to reconstruct the course of evolution of the endocrine system in question [Bentley, 1998]. To this end, the focus of this study is to examine a unique endocrine tissue referred to as the corpuscles of Stannius [CS] and its mineralohomeostatic active principle, stanniocalcin [STC], in representative specimens of the three extant groups of the more ancient bony fishes – Amiidae, Lepisosteidae, and Teleostei [Nelson, 1994].

Since these groups of fishes occupied an early position in the evolution of vertebrates, a comparative analysis of the CS and STC could be of fundamental significance in the development of a conceptual framework for the understanding of both the nature of this hormone and mineral metabolism in other vertebrates. Further, an examination of STC and the CS in extant ancient representatives would be significant in understanding the evolution of these elements in the extinct fishes. In addition, because of incomplete and unknown fossil species, and the frequent difficulty in determining from osteology what are shared specializations versus derived characters, it has been difficult to clarify basal vertebrate evolution. Therefore, such “soft anatomy” characters, along with molecular data, may provide important evidence when comparing living species [De Pinna, 1996].
Basal Neopterygian relationships

The phylogenetic relationships of the Amiidae, Lepisosteidae, and Teleostei, the three extant groups of Neopterygii, have been controversial for many years. Most commonly, Amiidae are placed as either the sister group to Lepisosteidae, forming the Holostei [Huxley, 1861; Nelson, 1969; Normark et al., 1991] [Fig. 1a], or as the sister group to Teleostei, forming Halecostomi [Patterson, 1973; Wiley and Schultze, 1984; Nelson 1994; Gardiner et al., 1996] [Fig. 1b]. Whereas the name 'Holostei' [Huxley, 1861] was accepted for many years as a taxon which included the living Amiidae and Lepisosteidae it was not until well after the passage of 100 years that Patterson's [1973] studies challenged this notion. In a detailed study, Patterson [1973] suggested that amiids were more closely related to teleosts than to lepisosteids thus supporting the amiid-teleost monophyly – the Halecostomi [Fig. 1b]. However, proponents of the phylogenetic validity of Holostei found evidence based on morphological data [Jollie, 1984] and molecular data [Normark et al., 1991] to support their hypothesis. In a revisitation of the halecostome/holostean controversy, Patterson [1994] concluded that the problem is unresolved based on the available morphological and molecular data and suggested the need for more extensive fossil and molecular data [Fig. 1d]. Gardiner et al. [1996] reported that molecular data supported a holostean monophyly whereas their morphological data validated Halecostomi. Olsen [1984] was the first to propose that the Lepisosteidae may be more closely related to the Teleostei than the Amiidae [Fig. 1c], however, later interpretations [Olsen and McCune, 1991] also suggested that more sophisticated fossil evidence and molecular data would be required to resolve neopterygian relationships [Fig. 1d].

It appears obvious, then, that a better understanding of Amiidae, Lepisosteidae, and Teleostei morphology, along with corroborating molecular data, could more clearly discern what are general character states and help resolve the Holostei versus Halecostomi controversy. At the
**Figure 1a-d.** Four different phylogenies indicating the relationships of amiids, lepisostids, and teleosts [modified from Grande and Bemis, 1998]. The two most accepted phylogenies place amiids and lepisostids as a monophyletic grouping forming Holostei [a] or amiids and teleosts as a natural grouping forming the Halecostomi [b]. Olsen [1984] was the first to propose the unnamed relationship depicted in c. However, more recent analyses consider the lepisosteid-amiid-teleost phylogeny to be unresolved [d].
very least, a comparative analysis of the CS, an endocrine tissue unique to certain bony fishes [Wendelaar Bonga and Pang, 1991] and STC, a glycoprotein hormone likely to have a long evolutionary history among the vertebrates [Wagner et al., 1995, 1998] may provide insight into the phylogeny of these elements among the fishes.

**The Corpuscles of Stannius**

The corpuscles of Stannius [CS], first described by Stannius [1839], are unique among the vertebrate endocrine glands in that they only occur in amiid, lepisosteid, and teleostean fishes. After their discovery they were often equated as homologs of the adrenal glands of the terrestrial vertebrates. However, they were recognized as distinct glands when the interrenal tissue in the head kidney of teleost fishes was identified as the true homolog of the adrenal cortex [Chester-Jones et al., 1969; Butler, 1973]. In fact, the ontogeny of the CS was demonstrated to be distinct from that of the adrenocortical homolog [Giacomini, 1933; Garrett, 1942]. Whereas the CS arise as outgrowths of the pronephric [Hout, 1898; Garrett, 1942; Ford, 1959; Kaneko et al., 1992], mesonephric [Garrett, 1942; Ford, 1959; Krishnamurthy, 1967] and opisthonephric ducts [De Smet, 1962], depending on the species, the adrenocortical tissue is derived from coelomic epithelium [Giacomini, 1933]. The embryological origin of the CS from renal tissue makes them unique among the vertebrate endocrine glands. An examination of the CS and STC in the most ancient extant species to possess these glands is of interest since investigations may shed some light on the phylogeny of not only this endocrine tissue but also calcium and phosphate regulation among the vertebrates in general. This type of investigation becomes all the more interesting in light of the fact that the first indications of a mammalian form of STC came from immunohistochemical investigations of human kidney where the hormone was localized to certain cells of the renal tubules [Wagner et al., 1995].
Early studies employing tinctorial stains demonstrated that the CS reacted strongly to periodic acid-Schiff [PAS] suggesting that CS cellular products may be glycosylated in nature rather than steroidogenic, as initially speculated [Krishnamurthy, 1976]. In classical fashion Fontaine [1964] demonstrated that the ablation of the CS in the European eel would lead to hypercalcemia which could be alleviated by injections of CS extracts. The stage was now set for the search of a CS active principle, perhaps a glycoprotein, that was involved in calcium metabolism. Soon thereafter, the chemical nature of one active CS product, STC, was well characterized in the more modern bony fishes [Butkus et al., 1987; Wagner et al., 1986, 1992] and in mammals [Chang et al., 1995, 1996; Olsen et al., 1996].

**Anatomical Distribution of the CS**

In the more ancient bony fishes the number of CS is high and varies in the holosteans from several hundred in the bowfin [Amia calva] to between 4 and 8 in the gar [Lepisosteus platyrhynchos] [Garrett, 1942; De Smet, 1962; Youson et al., 1976; Bhattacharyya, 1982]. In teleosts, the more modern bony fishes, the number of CS tend to be lower, however, the number is species specific and variable [Krishnamurthy, 1976]. Bouchot [1953] was the first to suggest that the number and anatomical location of the CS could be of phylogenetic significance. An increase in the number of glands associated with the middle of the kidneys represents a more ancestral organization, whereas, a reduction in CS number associated with a more posterior distribution may be characteristic of more advanced species. The above hypothesis was corroborated by Youson et al. [1976] who also suggested that a phylogenetic significance can be attributed to the variation in CS number, distribution and organization with respect to renal tissue and the adrenocortical homolog. In teleosts, a reduction in number is accompanied by a more peripheral and posterior positioning of the glands with respect to the kidneys. In addition, the CS
and the adrenocortical homolog are widely separated. This morphologic pattern is contrasted with that in the more “primitive” bony fishes. In the bowfin, the CS have been reported mostly concentrated in the posterior region of the kidney and partially intermingled with the adrenocortical homolog, perhaps reflecting a more ancestral organization [Youson et al., 1976; Youson, 1985]. In addition, many CS remain in the interior of the kidney and are closely associated with the distal renal tubules, from which they are believed to arise [Youson et al., 1976]. However, the distribution and numbers of the CS in the gar [Lepisosteus osseus] is strikingly different from that occurring in the bowfin kidney. The CS of the gar are localized in the anterior portion of the kidney and are often associated with the large vessels and occasionally with the adrenocortical homolog in regions of the kidney which have abundant hemopoietic tissue and are devoid of nephric tissue [Bhattacharya et al., 1982].

Whereas much is known about the CS and STC in the more recently evolved bony fishes, relatively few studies have examined these elements in the most ancient extant species to possess these glands, the bowfin and the gar. Furthermore, other than one reported study on the CS in Notopterus notopterus [Belsare, 1973], there have been no examinations of the CS in the Osteoglossomorpha, the most ancient subdivision of the Teleostei [Nelson, 1994]. Because this ancient group of fish occupy a pivotal evolutionary position between Amiidae, Lepisosteidae and the more recent Teleostei, an examination of these glands in representatives of these most ancient teleosts would be useful for the interpretation of evolutionary trends of the CS.

Histology and Fine Structure of the CS

The CS are ovoid-shaped, white, glandular bodies [Youson et al., 1976] surrounded by a connective tissue capsule [Krishnamurthy and Bern, 1969]. The glandular parenchyma may be arranged in follicles, strands, and lobules invaded by connective tissue septa containing all the
vascular and nervous elements [Krishnamurthy and Bern, 1969; Wendelaar Bonga and Pang, 1986]. The CS are well vascularized [Tomasulo, 1970; Belsare, 1973; Wendelaar Bonga et al., 1977; Bhattacharyya and Butler, 1978; Meats et al., 1978] and have a rich autonomic innervation [Heyl, 1970; Krishnamurthy and Bern, 1971; Belsare, 1973; Schreibman and Pang 1975; Wendelaar Bonga et al., 1977; Unsicker et al., 1977]. It has been reported that the nerve fibers appear to be confined to the septa and do not penetrate the parenchyma [Heyl, 1970; Krishnamurthy and Bern, 1971; Belsare, 1973; Schreibman and Pang 1975; Wendelaar Bonga et al., 1977; Unsicker et al., 1977]. There have been no reports of evidence for direct nervous innervation of the CS gland cells.

Both light and electron microscopic observations demonstrate that the gland cells of the CS are histochemically and structurally diverse. Historically, tinctorial staining procedures, have been applied to the CS of numerous teleosts [Krishnamurthy and Bern, 1969]. Despite demonstrating structural diversity, no staining procedure was known to be specific for the secretory material of the CS. Furthermore, whereas histochemical attempts to demonstrate a steroidogenic nature of the secretory material of the CS failed [Bara, 1963; Chieffi and Botte, 1963; Chester-Jones et al., 1974], PAS positive results led to speculations that the products within the CS gland cells might be glycosylated proteins [Krishnamurthy and Bern, 1969].

Electron microscopic studies revealed that the ultrastructure of the CS parenchyma show all the structural characteristics of cells producing proteins or glycoproteins [Ogawa, 1967; Tomasulo, 1970; Youson and Butler, 1976; Wendelaar Bonga et al., 1977; 1980; Bhattacharyya, 1982]. Krishnamurthy and Bern [1969] were the first to show that what were histochemically different cell types [PAS-positive and PAS-negative] in the CS of the trout represented ultrastructurally distinct cells which are now referred to as type-1 and type-2 cells [Wendelaar Bonga and Greven, 1975; Wendelaar Bonga et al, 1977]. Wendelaar Bonga and Pang [1986]
reviewed the cytophysiology of the CS describing the presence of either one or two structurally-different cell types [type-1 and type-2] both involved in protein synthesis and secretion.

Whereas type-1 cells, with their extensive rough endoplasmic reticulum and large secretory granules, are exclusive to marine fish, type-2 cells are characterized by a less extensive rough endoplasmic reticulum and smaller secretory granules and are also found in freshwater and euryhaline fish. In the bowfin, in teleosts like the cod and plaice, and in the toadfish, only the type-1 cell has been identified [Wendelaar Bonga and Greven, 1975; Youson and Butler, 1976; Bhattacharyya and Butler, 1978]. The CS of the gar contain both cell types [Bhattacharyya et al., 1982]. These reports of structural diversity of the CS gland cells suggest that their uniqueness reflects either one functional cell type in different phases of its life cycle or functionally different cells each synthesizing a different product. It was long assumed, based on their structural distinctness and their specific responses under experimental conditions [Cohen et al., 1975; Wendelaar Bonga et al., 1976, 1980; Meats et al., 1978; Aida et al., 1980] that the two cell types in teleosts were physiologically distinct with respect to their function and their secreted products. However, it has now been suggested that both cell types may synthesize and secrete the same product, STC [Wendelaar Bonga et al., 1989]. The isolation and purification of the active principle from the CS and creation of antibodies to the glycoprotein has permitted immunological procedures to be used to demonstrate that both the type-1 and the type-2 cells in teleosts contain stanniocalcin [Gellersen et al., 1988; Wagner et al., 1986, 1988; Kaneko et al., 1988; Wendelaar Bonga et al., 1989].

Despite several reports on the putative CS in bowfin [Garrett, 1942; De Smet, 1962; Youson et al., 1976; Youson and Butler, 1976] and the gar [Bhattacharyya et al., 1982] STC-like immunoreactivity has never been demonstrated in either of these species. As was noted earlier, whereas much is known about the CS and STC in the more recently evolved teleosts,
comparatively little is known about these elements in these ancient extant species of bony fishes. It is not even known if antibodies generated against teleostean STC are capable of recognizing the hormone in the bowfin or the gar. Furthermore, other than one published report [Belsare, 1973], no information exists on the CS or STC-like immunoreactive profile in any member of the osteoglossiformes. Such information, on a group of fish evolutionarily intermediate to the more modern bony fishes and those with a more ancestral lineage, is necessary in order to establish a phylogenetic perspective on the CS and STC.

Identity and Action of Stanniocalcin

The identity of the active principle of the CS had been the subject of some discussion since Fontaine [1964] observed that the removal of these glands in eels resulted in hypercalcemia which could be corrected by injections of CS extracts. Fenwick and his colleagues clearly demonstrated that the CS active principle was an inhibitor of gill calcium transport [Fenwick and So, 1974; So and Fenwick, 1977, 1979]. Studies have shown that the secretion of STC is positively regulated by extracellular levels of ionic calcium in a time and dose-dependent manner [Glowacki et al., 1990; Wagner et al., 1989, 1995]. These studies are corroborated by histophysiological studies that revealed that CS cells respond to elevations in calcium levels under a variety of experimental paradigms [Cohen et al., 1975; Meats et al., 1978; Lopez et al., 1984; Wendelaar Bonga et al., 1976, 1980]. The primary function of STC in fish is to prevent hypercalcemia, and a rise in serum calcium level is the primary stimulus for secretion from the CS [Wagner et al., 1989]. When released, STC lowers calcium uptake by the gills and intestine thereby reducing serum calcium levels [Wagner et al., 1986; Sundell et al., 1992; Lu et al., 1994; Olsen et al., 1996]. Recent evidence suggests that a second function of STC is the stimulation of phosphate reabsorption by the renal proximal tubules [Lu et al., 1994]. This combined effect of
STC on calcium and phosphate movement results in a synergistic effect in lowering serum calcium levels. In mammals, STC has been proven to be an effective stimulator of phosphate transport by the mammalian kidney and calcium and phosphate transport across the mammalian intestine [Olsen et al., 1996; Wagner et al. 1997 Madsen et al., 1998]. The early physiological studies [Fontaine, 1964; Fenwick and So, 1974; So and Fenwick, 1977, 1979] which targeted the gills as the site of CS biological activity, laid the foundation for the ensuing isolation of STC.

Shortly after Fontaine’s [1964] breakthrough in CS physiology, Chester Jones et al. [1966] ascribed a pressor activity to CS extracts injected into European eels and rats which demonstrated similarities to the pressor activity associated with the renin–angiotensin system in mammals. Subsequent studies demonstrated that angiotensin I was produced when chum salmon and Japanese goosefish CS extracts were incubated with homologous plasma [Takemoto, 1983; Hasegawa et al., 1984]. More recently, a cardiovascular role has been ascribed for the CS of North American freshwater eels [Butler and Oudit, 1994, 1995]. Pang et al. [1973] characterized a heat labile and acid stable product of high molecular weight from the CS capable of hypocalcemic activity and called it hypocalcin. The renin–like material associated with CS extracts was later equated with hypocalcin [Ogawa et al., 1978; Pang et al., 1981]. Ma and Copp [1978] proposed a second factor as the CS hypocalcemic hormone, teleocalcin, a small glycoprotein with a molecular weight between 3000 and 4000 daltons, isolated from salmon. In addition, since the CS have been equated with the parathyroid glands of terrestrial vertebrates there has been some presumption of homology based on immunological and functional arguments. Thus, a third factor, referred to as parathyrine of the corpuscles of Stannius, was identified with an antibody raised against the N–terminal [1–84] of bovine parathyroid hormone [Milet et al., 1980, 1982; Lopez et al., 1984]. This substance was present in intact eels but disappeared after stanniectionomy, and the plasma levels of this factor were related to the calcium
metabolism status of the eel [Milet et al., 1989]. Therefore, it was suggested that parathyroidine may represent another hypocalcemic factor released by the CS in response to hypercalcemic states. Milet et al. [1989] identified a 34 kDa protein and a 45 kDa precursor having a potent hypocalcemic response in CS-deprived eels. To date, no examinations of the primary structure of STC have identified any similarities to parathyroid hormone or renin [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995; Chang et al., 1995, 1996; Olsen et al., 1996]. Furthermore, it is also not unreasonable to assume that there may be more than one form of STC or other bioactive compounds in the CS. This suggestion of multiple forms of the hormone is supported by the recent report of Wagner et al. [1998] of the presence of two forms of STC in the Atlantic salmon, earlier reports of multiple STC-like immunoreactive bands in CS extracts of coho salmon [Wagner et al., 1992], and indications that there are at least two bioactive principles in the CS of certain teleosts [Verbost et al., 1993]. Despite recent reports of a second form of mammalian STC, referred to as STC-2 [Chang and Reddel, 1998; Ishibashi et al., 1998], to date, there is no molecular evidence that alternate forms of STC occur in the fishes. Furthermore, relatively few studies would suggest that the CS are capable of producing other biologically active substances.

The nature of STC itself appears to vary only slightly between species and has some salient features which characterize it as a unique hormone. In its native state, STC occurs as a disulfide-linked homodimer. This quaternary arrangement was deduced on the basis of the shift that occurs in the molecular weight of STC in the presence of reducing agents. In the presence of a reductant, STC migrates electrophoretically with an apparent molecular weight of one half of the native molecule [Wagner et al., 1986, 1992, 1994; Butkus et al., 1987; Lafeber et al., 1988]. The molecular weight of the STC molecule varies between species. In the salmon the monomer migrates with an apparent molecular weight of 26 kDa [Wagner et al., 1988] whereas in trout and
eels the respective values are 28 and 30 kDa [Flik et al., 1989]. PAS staining and Concanavalin A-lectin affinity [which forms the basis for its purification] demonstrate the glycosylated nature of the STC molecule [for reviews, see Wagner 1993].

The dimeric and glycoprotein nature of STC along with a well conserved primary structure have been validated by molecular biological protocols in numerous species including mammals [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996]. Despite the variability in size, all STCs described to date share certain structural features. In all species examined a single glycosylation consensus sequence [Asn-Ser-Thr] has been reported for the active form of this hormone [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996]. Recent evidence suggests an unglycosylated or differentially glycosylated form of STC occurs in the CS of the Atlantic salmon which is less potent than the principal form [Wagner et al., 1998]. At present there is no description of the primary structure of such forms of STC. There also appears to be an odd number of cysteine residues among the various vertebrate forms of STC suggesting that one would be free to participate in interchain bonding [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996]. Similarly, mammalian STC-2 has a conserved N-glycosylation site and is rich in cysteines [Chang and Reddel, 1998; Ishibashi et al., 1998]. Most STCs appear to share at least one conserved dibasic pair [Arg-Arg], and numerous monobasic amino acids that can serve as sites of proteolytic modifications [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996]. Numerous reports by Wagner and his colleagues suggest that, at least in salmon, STC may be posttranslationally truncated [Wagner et al., 1988, 1991, 1992, 1993]. Northern blot and in situ hybridization analyses [Butkus et al., 1987; Wagner et al., 1992;Sterba et al., 1993] demonstrate that, whereas the synthesis of piscine STC is confined to the CS, mammalian STC
gene expression [both STC-1 and STC-2] appears to be more widespread and, therefore, the hormone may function in a paracrine rather than in an endocrine manner [Chang et al., 1995; Chang and Reddel, 1998; Ishibashi et al., 1998]. Recently, Wagner et al. [1995] and De Niu et al. [1998] demonstrated that certain cells of the human kidney are one likely source of immunoreactive STC. To date, no extracorporeal sources of STC or STC-like molecules have been characterized in the fishes. However, the possibility that the gene is expressed ectopically in other tissues during development or during physiological challenges, and functioning in a tissue-specific manner, cannot be ruled out.

Whereas much is known about the primary structure of STC [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996; Chang and Reddel, 1998; Ishibashi et al., 1998], and information is accumulating as to the likely target tissues of this hormone [Fontaine, 1964; Fenwick and So, 1974; So and Fenwick, 1977, 1979; Wagner et al., 1986; Sundell et al., 1992; Lu et al., 1994; Olsen et al., 1996], no information exists regarding the nature of the STC receptor. At least one correlational observation indicates that STC may stimulate net renal Pi reabsorption by a cAMP-dependent pathway [Lu et al., 1994]. Although speculative at best, based on the above observation it may be reasonable to suggest that the STC receptor is a G-protein linked receptor type. However, the possibility that the STC receptor is either a catalytic or channel-linked receptor can not be ruled out.

The molecular characterization of STC in these few piscine and mammalian representatives suggest that this hormone has had a long evolutionary history among the vertebrates. However, to date, no information exists on the more ancient forms of this hormone and, therefore, STC lacks an evolutionary perspective. In order to expand our knowledge on the evolution of this hormone an examination of STCs that predate those of the Salmoniforme and Anguilliforme orders are critical. In this study I have chosen to examine the CS and STC in
representatives of the Amiiformes, Lepisosteiformes, and Osteoglossiformes for several reasons. Firstly, the extant species within the Amiiformes and Lepisosteiformes groups demonstrate a uniqueness with respect to number and distribution of the CS as compared to other piscine members and STC has never been characterized in these ancient bony fishes. Secondly, other than one reported study [Belsare, 1973], there is a paucity of information on the CS and STC in the Osteoglossomorpha, the most ancient subdivision of the Teleostei [Nelson, 1994]. An examination of CS and STC in these ancient extant representatives provide the opportunity to study the evolution of this hormone in the most ancient extant species to possess these glands. Since these specimens represent an almost 250 million year evolutionary perspective, they appear to be the most logical choice for examining the early evolutionary development of this hormone and endocrine tissue. I have examined these structures using morphological, biochemical, and molecular approaches. Phylogenetic relatedness with a single methodology is difficult to claim and can only be tentatively inferred but the use of several procedures expands the opportunity for speculation in this regard.

Central Questions and Aims of this Study

Youson et al. [1976] were the first to suggest that a phylogenetic significance can be attributed to the variation in CS number, distribution, and the anatomical organization of these glands with respect to renal tissue and the adrenocortical homolog. Based on the aforementioned proposal, this study was organized to test whether the CS and STC in the lepisostids are more similar to those in the teleosts, as Olsen (1984) suggested for the phylogeny of these bony fishes, or whether these characters support a Holostei or Halecostomi monophyly. In order to carry out such a comparative analysis I examined the morphology, immunoreactive profile, and molecular biology of the CS and STC in representative species since similarities and differences in these
characters are reflective of the evolutionary process. The first aim included the immunohistochemical, immunocytochemical and Western blot analysis of the CS and CS extracts from the bowfin [Amia calva] and the gar [Lepisosteus osseus]. The former is the only surviving representative of the Amiidae [Grande and Bemis, 1998]. In addition, the CS and CS extracts were examined from the silver arawana [Osteoglossum bicirrhosum], the butterflyfish [Pantodon buchholzi], the featherfin knifefish [Chitala chitala], the elephantnose [Gnathonemus petersii], surviving members of the osteoglossomorph superorder [Nelson, 1994]. The white sucker [Catostomus commersoni], a member of the Ostarophysi superorder [Nelson, 1994], served as a representative of the more modern bony fishes. The white sucker, a cypriniforme, is considered to be among the more specialized euteleosts [Nelson, 1994]. For these immunolabelling protocols we used previously well characterized [Gellersen et al., 1988; Wagner et al., 1988; Kaneko et al., 1988, 1992; Wendelaar Bonga et al., 1989] polyclonal antibodies generated against salmonid forms of STC. Our second aim focused on the elucidation of the primary structure of STC from the bowfin, gar, silver arawana, and the white sucker employing PCR-based molecular techniques. It was our hope that such an examination of the morphology of this endocrine tissue and the structure and nature of their secreted principle(s), in the most ancient extant representatives known to possess these unique endocrine glands, would provide definitive evidence on the evolution of the CS and STC among the bony fishes and perhaps contribute to the overall phyletic study of vertebrates. These data could perhaps be indirect evidence of the relationship of the hormone to established functions of calcium and phosphate homeostasis, or other functions, in vertebrates in general.
PART II

RESEARCH CHAPTERS
ORGANIZATION OF CHAPTERS

The eight research chapters which follow are grouped into two general sections. The first section [chapters 1-5] reports on the morphological variation in the corpuscles of Stannius in representatives of the ancient bony fishes including the bowfin [Amia calva], the gar [Lepisosteus osseus], surviving representatives of the Amiidae and Lepisosteidae families, the silver arowana [Osteoglossum bicirrhosum], the butterflyfish [Pantodon buchholzi], the featherfin knifefish [Chitala chitala], the elephantnose [Gnathonemus petersii], surviving members of the osteoglossomorph superorder, and the white sucker [Catostomus commersoni], a representative of the more modern bony fishes. We used immunolabelling techniques to analyze the corpuscles from these representative specimens and to characterize their STCs. The second section analyzes the molecular variation in STC from the bowfin, gar, arowana, and the white sucker and includes three chapters. The study of both phenotypic and molecular variation is unified by a central phylogenetic theme. Each chapter, except chapters 4 and 7, consists of one manuscript prepared and submitted for publication. Chapters 4 and 7 comprise one manuscript, but have been separated in order to maintain the general organization of this study.

Chapter one deals with the qualitative examination of the CS and STC, using antibodies generated against the salmonid form of the hormone, in the most ancient extant species that possess these glands, the bowfin. Prior to this study it was not known whether antibodies to the teleostean form of the hormone could recognize a STC-like molecule in the bowfin. Differences in the distribution of the CS and STC, compared to the more modern fishes, is suggestive of evolutionary pressure.
Chapter two reports the findings of a preliminary assessment of the CS and STC in the gar [Lepisosteus osseus] using immunolabelling techniques and employing heterologous antibodies. Prior to this study, STC-like immunoreactivity had never been demonstrated in this order. Earlier taxonomic investigations grouped the Lepisosteiformes and Amiiformes as a single taxon, the Holostei. Our results suggest that despite obvious similarities between lepisosteid, amiid, and teleostean STC, differences in the distribution of the CS and biochemical nature of their STC molecules suggests a disparate evolutionary history of these elements in these species.

Chapter three deals with the localization of immunoreactive STC in the CS of one of the most ancient teleosts, the silver arawana [Osteoglossum bicirrhosum]. The CS of arawana are similar to those in more recent teleosts with respect to cell structure and anatomical distribution but their STC is more similar in molecular weight to that of the gar, a non-teleost actinopterygian.

Chapter four re-examines STC distribution in several members of the Osteoglossomorpha in an immunolabelling paradigm and using heterologous antisera to several salmonid forms of STC. This is the first report of an extracorpucular source of STC in certain cells of the distal renal tubules of the bony fishes. This renal form of the hormone demonstrates different properties compared to previously described STC molecules during electrophoresis and Western blotting paradigms. In light of the pattern of STC distribution in mammals, this kidney form of the hormone may represent the evolutionary fore-runner of the more modern forms of STC.

Chapter five describes the ultrastructure of the white sucker [Catostomus commersoni] CS and the distribution and nature of immunoreactive STC. Whereas the distribution of the white sucker CS and the nature of its STC most closely resemble those previously described for other more
modern bony fishes, the glandular parenchyma possesses a novel cell-type not previously described in other species. The presence of this cell-type may account for some of the results observed during physiological assays of the corpuscles and STC.

Chapter six reports the deduced primary structure of white sucker STC. This specimen served as a representative of the bony fishes with a more recent lineage and was used as a positive control for subsequent molecular protocols. Chapter 6 includes a multiple sequence alignment of STC from the human, salmon, and white sucker. Areas of divergence in the STC consensus are examined. Functional constraints may account for highly conserved stretches of sequence in the STC molecule. The primary structure of white sucker STC is intermediate to those of the coho salmon and the Australian eel and parallels the accepted taxonomic relationship of these species.

Chapter seven reports on the molecular basis for the corpuscular and extracorpuscular sources of STC-like immunoreactivity in the arawana. Chapter 7 also includes a multiple sequence alignment of piscine and mammalian STC amino acid sequences. The patterns of sequence conservation are suggestive of relatedness. In fact, the similarity between the arawana kidney form of STC and human STC suggests that the renal-specific expression of STC in humans is likely of ancient rather than modern origins. Molecular protocols, using an arawana CS cDNA library as a target template, demonstrated the presence a STC molecule lacking many of the features characteristic of this hormone in other species. We suggest that the STC gene may occur as multiple copies in the arawana genome and that the expression may be tissue-specific.

Chapter eight reports the findings of molecular protocols, using PCR-based techniques, to isolate and sequence a partial clone from a gar CS cDNA library which was highly homologous
to teleostean forms of STC. Further, this chapter also describes our attempt to purify bowfin STC at the protein level using HPLC. The inability to isolate a “recognizable” STC clone from a bowfin kidney-corpuscle cDNA library suggests that in spite of the fact that one or more epitopes of the protein have been preserved, the nucleotide sequence encoding *A. calva* STC has undergone considerable variation from the teleost and gar mRNA. The results suggest that *Amia calva* represents a nodal point in STC evolution.
CHAPTER 1
Stanniocalcin-like immunoreactivity in the corpuscles of Stannius of the bowfin, 

*Amia calva* L.

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ABSTRACT

We used an antiserum against salmon stanniocalcin in an immunohistochemical, immunocytochemical, and Western blot analysis of bowfin [Amia calva] corpuscles of Stannius. The bowfin is one of two extant holostean species with ancient ancestral links to modern-day bony fishes. The corpuscles of Stannius [white corpuscles] of the bowfin were scattered throughout much of the kidney among the adrenocortical homolog [yellow corpuscles] but could be distinguished from the adrenocortical homolog by their positive staining with both periodic acid-Schiff reagent and salmon stanniocalcin antiserum. Immunoreactivity was confined to cytoplasmic granules and was absent when the antiserum was blocked with salmon stanniocalcin or with a crude extract of bowfin corpuscles of Stannius. When bowfin corpuscle of Stannius extracts were subjected to sodium dodecyl sulphate electrophoresis and Western blot analysis, two closely spaced bands were evident (43-45 kDa). Staining of both bands was abolished by pre-absorption of the antiserum with salmon stanniocalcin. In comparison to salmon stanniocalcin, the reputed bowfin hormone migrated faster in sodium dodecyl sulphate gels, suggesting a smaller apparent size. The purification of bowfin stanniocalcin should yield important new information regarding the evolution of this unique calcium-regulating hormone.
Introduction

The corpuscles of Stannius [CS], small endocrine glands associated with the kidneys of holostean and teleostean fish, were first described by Stannius [1839]. Early embryological and morphological studies illustrated that the CS bore no relationship to vertebrate adrenal glands [Hout, 1898; Giacomini, 1908]. Despite these earlier studies, the CS were often equated with the adrenocortical homolog [AH] of terrestrial vertebrates, until the identification of the interrenal tissue in the head kidney of teleost fishes as the site of corticosteroid production [Jones et al., 1961; Butler, 1973]. In most teleosts the number of CS is low [2-5] and the AH and CS are widely separated. In the bowfin [Amia calva], the two endocrine tissues are partially intermingled but the CS can be identified upon dissection as white corpuscles, whereas the more numerous AH appear as yellow corpuscles. Youson et al. [1976] reported 332 CS of highly variable size from serial sections of an entire bowfin kidney, 70% of which were small and located in the posterior half of this organ. In contrast, there are fewer CS in the garpike [Lepisosteus platyrhynchus], the only other extant holostean species [Bhattacharyya et al., 1982].

The bowfin was chosen for the present study because of its uniqueness with respect to the number and distribution of the CS as compared to other fish. Also, this uniqueness suggests a more ancestral organization of the CS in this species [Garrett, 1942; de Smet, 1962; Youson et al., 1976]. In all fishes the CS originate as outgrowths of the pronephric, mesonephric, or opisthonephric ducts [Garrett, 1942; De Smet, 1962]. In teleosts, the glands then assume a peripheral location on the exterior surfaces of the kidney but still maintain close neural and vascular connections to the latter. In the bowfin, however, the CS remain closely associated with the distal renal tubules [Youson et al., 1976]. The physiological significance of this has yet to be established, but it makes the bowfin a unique model for comparative and developmental studies. However, bowfin CS are not only interesting from a developmental perspective. They have also
provided us with the opportunity to study the evolution of stanniocalcin [STC] structure in the most ancient extant species to possess these glands.

Histochemical and fine structural observations on the CS cells of various teleosts first suggested that this gland was involved in protein synthesis and secretion [Ogawa, 1967; Tomasulo et al., 1970; Wendelaar Bonga et al., 1977, 1980; Bhattacharyya and Butler 1978; Aida et al., 1980]. The major cell type in the gland [type-1 cell] was shown to have an extensively developed rough endoplasmic reticulum, a Golgi apparatus, and numerous large secretory granules. On the other hand, the secondary cell type [type-2 cell] had smaller and fewer secretory granules and a less developed rough endoplasmic reticulum and Golgi apparatus. Furthermore, whereas the type-1 cells responded to increases in the ambient calcium content by releasing their granules [Aida et al., 1980], the type-2 cells showed increased secretory activity in media of low osmotic strength and were relatively unresponsive to calcium. Because of these apparent differences it was long assumed that the type-1 and type-2 cells were physiologically distinct with respect to their function and their secreted products. It has now been suggested that both cell types may synthesize and secrete the same product [Wendelaar Bonga et al., 1989].

STC, however, it is also possible that although the products are related they may have different functions. Using immunological procedures it appears that the product common to both cell types is stanniocalcin, an anti-hypercalcemic agent that was formerly known as both teleocalcin [Wagner et al., 1986] and hypocalcin [Lafeber et al., 1988a]. Although it has long been established [Fenwick and So 1974; So and Fenwick 1977,1979] that the function of STC is to inhibit the inward transport of calcium from the aquatic environment by the gills [through a mechanism not yet understood], the active principle was first isolated from sockeye salmon CS only five years ago. STC was shown to be a glycosylated, disulfide-linked homodimer with a unique primary structure among the vertebrate polypeptide hormones. It was also shown to be a
potent inhibitor of gill calcium transport [Wagner et al. 1986]. Since then, progress on the structure and physiology of this hormone has been rapid. The complete primary structure of the STC monomer has been deduced in the Australian eel [Anguilla australis] using molecular biology, and the hormone has been isolated from two other salmonids, the rainbow trout [Lafeber et al., 1988a] and the coho salmon [Wagner et al., 1988a]. The role of calcium in regulating STC secretion has also been examined both in vitro [Gellersen et al., 1988; Wagner et al., 1989; Flik et al., 1986, 1989] and in vivo [Glowacki et al., 1990; Wagner et al., 1991] using salmonids as model systems and validated immunoassays to precisely quantify release [Kaneko et al., 1988; Gellersen et al., 1988]. The results suggest, so far, that STC secretion is positively regulated by plasma calcium levels in a time- and concentration-dependent manner, thereby regulating gill calcium transport at a rate in proportion to metabolic needs.

Whereas much is now known about the CS and STC in the more highly evolved teleost fishes, comparatively little is known about STC in the two extant holosteans, the bowfin and the garpike. It is not even known, for example, if antibodies to the teleost hormone are capable of recognizing STC in the holostean fish. Therefore, the purpose of the present study was to examine the CS cells and STC in a holostean fish, the bowfin, by light and electron microscope immunocytochemistry in conjunction with Western blot analysis by employing a highly specific antiserum to sockeye salmon stanniocalcin [sSTC].

**Materials and methods**

*Animals and tissue collection*

Adult bowfin, *Amia calva* L., were trap-netted in Lake Ontario, Bay of Quinte [Hay Bay], Napanee [Lennox and Addington County], Canada, in September and October, 1989. The specimens, 45-75 cm in length, were transported to the laboratory at the Scarborough Campus of
the University of Toronto. The bowfin were maintained in a single large fiberglass tank containing flow-through, dechlorinated, aerated tap water with the temperature maintained at 10°C. They were fed live minnows. The bowfin were anaesthetized using MS222 and sacrificed with a sharp blow to the head. A longitudinal incision extending from the cloaca to the pericardial cavity was made exposing the retroperitoneal opisthonephros. The kidneys were removed and examined under a dissecting microscope for the characteristic white corpuscles [Youson et al., 1976]. The CS were quickly and carefully excised and immediately fixed for light and electron microscopy. Approximately 2 g CS were also isolated from 54 animals, frozen in liquid nitrogen, and stored at -70°C. These corpuscles were used for the Western blot analysis.

*Partial purification of bowfin stanniocalcin*

Fresh frozen bowfin CS [0.05 g] were homogenized in 10 ml concanavalin A sepharose [Con A] buffer [0.05 M sodium phosphate, 0.5 M sodium chloride, 0.01% sodium azide; pH 7.2], containing 1 mM each of N\(^*\)-p-tosyl-L-arginine methyl ester, N-tosyl-L-phenylalanine and trypsin inhibitor [Sigma. St. Louis, Missouri, USA]. The homogenate was centrifuged at 50 000 x g for 30 min and the pellet was re-extracted twice more as above. The combined supernatants [30 ml] were filtered through Whatman #1 filter paper on a Buchner funnel and applied to a 1.5 x 20 cm column of Con A equilibrated in the same buffer minus the protease inhibitors. The extract was recycled through the column three times after which the column was washed with the Con A buffer until the absorbance of the eluent at a wavelength of 280 nm approached baseline. Bound glycoproteins were eluted with 0.5 M methyl-\(^*\)-D-mannopyranoside. Both the Con A void [proteins which did not bind] and the Con A bound fractions were dialyzed in 0.005 M ammonium bicarbonate and lyophilized. These fractions, as well as a crude bowfin extract, were subsequently used in the immunohistochemical and immunocytochemical blocking studies.
Primary antisera

A polyclonal antiserum generated against sockeye salmon STC was used for the immunocytochemistry and Western blot analysis. This antiserum has been previously characterized by Western blotting, immunocytochemistry, radioimmunoassay, and in vitro translation / immunoprecipitation studies, whereby it was shown to be highly specific for salmon and trout STC [Gellersen et al., 1988; Wagner et al., 1988]. The lyophilized anti-STC serum was reconstituted with phosphate-buffered saline, separated into 20-μl aliquots, and stored at -20°C until use.

Immunohistochemistry and immunocytochemistry

For light microscopic studies, the CS were fixed for 24-48 h in Bouin’s fluid and then stored in 70% ethanol until dehydration in a graded series of ethanols and embedding in Paraplast paraffin. Serial sections at a thickness of 4 μm were mounted on glass slides and the peroxidase antiperoxidase staining procedure was used to localize STC cells [Sternberger et al., 1970]. The sections were first rehydrated and equilibrated in phosphate-buffered saline containing 3% heat-inactivated goat serum, and then incubated for 48 h at 4°C with the primary antiserum at a 1:1000 dilution. Goat anti-rabbit gamma globulin [1:50 dilution] was then applied for 30 min at room temperature. This was followed by rabbit peroxidase antiperoxidase serum [1:50 dilution] for the same period of time. Non-specific immunolabelling was reduced by washing the slides for 20 min with phosphate-buffered saline containing 1% heat-inactivated goat serum after the application of each antiserum. The sites of peroxidase activity were revealed by incubating the tissue sections in buffered [pH 7.6] 0.003% hydrogen peroxide, containing
0.0125% 3,3-diaminobenzidine, for 15 min. The sections were then post-fixed with 1% osmium tetroxide to enhance the contrast.

For electron microscope studies, the CS were excised and placed in 2% glutaraldehyde buffered to pH 7.3 using 0.1 M Millonig's phosphate [0.12 M NaH₂PO₄, 0.1 M NaOH, 0.54% (w/v) glucose; pH 7.3] [Millonig 1961]. The excised CS were trimmed of excess renal tissue while in a drop of fixative before placement in ice-cold fixative for an additional 2 h. After a 30-min wash in phosphate buffer, the tissue was dehydrated in a graded series of ethanols and treated with propylene oxide. The embedding medium used was Araldite. Ultrathin sections were cut with a glass knife on a Reichert OM U3 ultramicrotome, mounted on copper grids, and stained with anti-salmon STC [1:1000 dilution] and protein A-gold [Wendelaar Bonga et al., 1989]. The sections were examined with a Siemens Elmiskop 102 electron microscope.

Control procedures included substitution of the primary antiserum with phosphate-buffered saline, saturation of the primary antiserum before staining with sSTC [100 μg STC/ml of a 1:1000 antibody dilution], or saturation of the primary antiserum with a crude bowfin CS extract prepared in phosphate-buffered saline [25 mg/ml], as well as the bowfin CS Con A bound [100 μg/ml] and Con A void [250 μg/ml] fractions. Adjacent CS sections were stained by the periodic acid-Schiff [PAS] reaction and counter-stained with acid haemalum and orange G.

**Western blot analysis**

For Western blotting, the frozen bowfin CS were homogenized directly in sodium dodecyl sulphate [SDS] sample buffer at a concentration of 140 mg/ml. Crude extracts of salmon CS were prepared in the same manner. The bowfin and salmon CS extracts were subjected to SDS electrophoresis in 10% polyacrylamide gels [Laemmli 1971]. Mercaptoethanol, which
reduces the stanniocalcin homodimer to its monomeric form, was omitted from the sample buffer so that the comparison between species was done with the hormone in the native state. Following SDS electrophoresis, the gels were equilibrated in transfer buffer [0.025 M TRIS, 0.5 M glycine, and 20% (v/v) methanol] and the resolved proteins were transferred to nitrocellulose using a BioRad TransBlot apparatus [0.25 A, 4 h]. The nitrocellulose filters were then equilibrated in antibody diluent buffer [0.25 M TRIS, 0.5 M NaCl, 0.01% Tween-20, 1% normal goat serum, 1% bovine serum albumin; pH 7.5] and incubated overnight at room temperature in a 1:1000 dilution of stanniocalcin antiserum. The filters were washed [4 x 20 min] in TRIS saline [0.25 M TRIS, and 0.5 M NaCl; pH 7.5] and incubated for 1 h in a 1:3000 dilution of peroxidase-coupled goat anti-rabbit gamma globulin [BioRad, Mississauga, Ontario, Canada]. The filters were washed again in TRIS saline and the sites of peroxidase activity were visualized by exposure to 0.004% 4-chloro-l-napthol containing 0.01% hydrogen peroxide. Carbon-14 standards [Amersham, Oakville, Ontario, Canada] were used as molecular weight markers and the dried blot was exposed to X-ray film afterwards in order to identify these marker proteins.

In order to confirm the specificity of the reaction, nitrocellulose filter strips of bowfin CS extract were treated overnight with a 1:1000 dilution of normal rabbit serum or primary antiserum saturated overnight with STC [100 µg/ml] and processed as above.

Results

In all nine specimens examined, the CS appeared as ovoid structures surrounded by a connective tissue capsule. The cells of the glands were arranged into cords separated by septa from the connective tissue capsule. The septa contained all of the vascular and nervous elements. The glandular parenchyma consisted of elongated epithelial cells grouped into convoluted and anastomosing cellular lobules. After the PAS, acid haemalum and orange G reaction, the
epithelial cells had a pale staining spherical nucleus with a small central nucleolus and a basophilic cytoplasm. A portion of the glandular cell cytoplasm also exhibited staining with PAS [Figs. 1a,b, 2a]. In adjacent sections stained for stanniocalcin, immunoreactive material was observed as a brown precipitate [Fig. 1c] which correlated closely with those areas stained by the PAS technique [Fig. 2a,b]. The most intense immunostaining was observed on the periphery of the CS and decreased gradually towards the centre of the gland [Fig. 1c]. There also appeared to be a number of unstained cells within each corpuscle [Fig. 2b]. The immunostaining was specific for the CS as only background staining was observed in hemopoietic tissue, kidney tissue, and adrenocortical tissue [Fig. 1c]. It was also observed that the CS were in close association with distal renal tubules. Often their respective connective tissue capsules appeared fused [Fig. 2a]. Absorption of the antibody with sSTC or a bowfin CS extract [Fig. 2c] abolished the immunostaining. Absorption with the bowfin CS Con A bound fraction had no effect on the intensity of immunostaining [Fig. 2e]. Absorption with the void fraction, however, abolished most of the staining over the CS cells, although some faint staining persisted [Fig. 2d]. The use of phosphate-buffered saline in place of the primary antiserum also resulted in no staining reaction.

In order to maintain the highest possible antigenicity, osmium tetroxide was not employed as a fixative. As a result, poor membrane integrity was observed in the tissue sections. However, the cell morphology observed was consistent with our previous findings [Youson and Butler 1976]. Only type-1 cells were identified in bowfin CS [Fig. 3]. These endocrine cells had a spherical nucleus with a prominent nucleolar region and large secretory granules, none of which were observed in the process of exocytosis. When treated with anti-sSTC, highly specific staining was observed over the secretory granules [Fig. 3].
A typical Western blot of salmon and bowfin CS extracts is illustrated in Fig. 4. The pro and mature forms of STC are usually not well resolved in CS extracts under non-reducing conditions [Butkus et al., 1987, 1989; Wagner et al., 1988a], hence they appeared as one dense band [Fig. 4a] with an apparent molecular weight of approximately 50 kDa. The immunoreactive molecular weight species in the bowfin CS extract were better resolved, however, and appeared as two closely spaced bands with an estimated size of 43-45 kDa. No other immunoreactive bands were visible in bowfin CS extract concentrations as high as 100μg protein/lane. The specificity of antibody staining in bowfin CS was evident by the absence of staining when the primary antiserum was pretreated with sSTC or when normal rabbit serum was used instead.

Discussion

The light and electron microscopic observations made in the present study were consistent with those of other researchers examining the structure of the CS [Ogawa, 1967; Krishnamurthy and Bern 1969; Tomasulo et al., 1970; Carpenter et al., 1973; Youson et al., 1976; Wendelaar Bonga et al., 1977; Bhattacharyya and Butler 1978; Bhattacharyya et al., 1982]. Whereas both type-1 and type-2 cells are present in freshwater fish, Amia calva appears to be an exception as only type-1 cells are present in this species [Youson and Butler 1976].

Our results demonstrated a good correlation between immunocytochemical and PAS-stained regions of the CS cells. However, the immunoreactivity of our antiserum and the absorption controls demonstrated several interesting features. Firstly, the immunostaining appeared to be enhanced about the periphery of the CS. This does not appear to be a fixation artifact as regions of the CS adjacent to kidney tissue also exhibited more intense staining than the centre of the gland. This may reflect an increased concentration of granules in the peripheral areas as has been observed in sockeye salmon [Wagner et al., 1988]. Secondly, some CS cells
did not stain positively with either the PAS reaction or the immunological techniques. These may represent physiologically inactive or immature cells. Thirdly, the fact that we could completely abolish staining with both STC and a bowfin CS homogenate suggests that the bowfin and salmon hormones have a common epitope which is recognized by the antiserum. This viewpoint of a common epitope was further confirmed by our Western blot analysis of salmon and bowfin CS homogenates.

The studies which have been conducted to date in teleost fish have shown that the pro and mature forms of STC have the same approximate size regardless of species [Gellersen et al., 1988; Wagner et al., 1988; Flik et al., 1990]. However, this is the first time that a CS extract has been examined in a holostean fish and is the lowest molecular weight form of STC ever identified by Western blot analysis. Two factors could account for this difference in size: polypeptide chain length and the extent to which the protein core of bowfin STC is glycosylated. Evidence for the first possibility comes from the Australian eel, the only species for which a complete amino acid sequence is known [Butkus et al., 1987]. An Arg-Arg cleavage site has been identified in the core protein of eel STC that would remove 80 residues from the C-terminal, if the site were functionally employed. Presently, there is no evidence that such post-translational modifications occur in the CS of eels or other teleosts to yield a truncated hormone, but the option obviously exists. Secondly, STC is a glycoprotein and this has enabled its purification from various teleosts since it binds to and can be eluted from Con A sepharose. This does not appear to be the case, however, in the bowfin. The immunohistochemical blocking studies revealed that, unlike the salmonid STCs, the bowfin hormone remained in the Con A void fraction. However, we have since established that bowfin STC is capable of binding to soybean lectin [unpublished observations G.F. Wagner 1990]. Hence it appears that the hormone is glycosylated, but that the carbohydrate moiety has a different structure and is therefore
incapable of binding to Con A. This could also account for its faster electrophoretic mobility in sodium dodecyl sulphate gels relative to sSTC. Within the salmonids, STC shows a high degree of sequence similarity [>95%], but there is less similarity with more advanced species such as the eel [80%]. Now, the apparent difference in the size and biochemical properties of STC from the bowfin suggests that there has been evolutionary pressure on this hormone.

This is the first time that the CS in bowfin have been examined with immunological techniques. Our results suggest that there are similarities between the bowfin and salmon hormone on the basis of their common cross-reactivity to salmon STC antiserum. However, it is also apparent that there are major differences in the structure of the carbohydrate moieties and possibly in the size of the protein core. Therefore, the logical next step in our view is to purify the hormone from A. calva the most ancient extant species to possess these glands. This should yield further insight into the evolution of STC structure and function.
Figure 1a-c. a Light micrograph of a corpuscle of Stannius [CS] from *Amia calva* stained with periodic acid-Schiff reaction. Note the pale staining hemopoietic [H] tissue and renal tubules [R]. b An encapsulated corpuscle of Stannius [CS] stained positively with periodic acid-Schiff reaction. Also note the adrenocortical homolog [AH]. c An adjacent section to Fig. 1b, showing the intense staining of the CS with anti-sSTC. Note that the adrenocortical homolog [AH] and the renal tubules [R] are unstained. The cells at the periphery of the CS demonstrate a particularly strong immunoreactivity. Pigment [arrowhead]. X520; Bar: 50 μm
Figure 2a-e. Light micrographs of adjacent sections of a corpuscle of Stannius [CS] from Amia calva showing its close association to a distal renal tubule [R] and the surrounding hemopoietic tissue [H]. Pigment [arrowhead] is also present in each section. a The cells of the CS show variable staining intensity [arrow] with periodic acid-Schiff. b The cells of the CS show variable staining intensity [arrow] for stanniocalcin using anti-sSTC. c There is no staining in this section which had been treated with an antiserum absorbed with a homogenate of bowfin CS. The same result was observed when the anti-sSTC was absorbed with salmon STC before application. d There is decreased staining in this section which had been treated with an antiserum absorbed with the bowfin ConA void fraction. Note that absorption with this fraction abolished most of the staining, although some faint staining persisted. e Staining was not abolished when this section was treated with an antiserum absorbed with the bowfin ConA bound fraction. X1450; Bar: 100 μm
Figure 3a, b. a High magnification of secretory granules [arrowhead] in type-1 cells demonstrating specific localization of gold particles. The presence of gold particles indicates that these granules are the primary site of STC localization. b Low magnification electron micrograph of *A. calva* corpuscle of Stannius showing numerous secretory granules [arrowhead]. Osmium tetroxide was not used as a fixative so the membranes are not readily visible. The tissue sections were incubated with anti-sSTC followed by protein A-gold. X50 000a; *Bar*: 1 μm, X5000b; *Bar*: 10 μm
Figure 4a and b. a Western blot analysis of salmon [S] and bowfin [A] CS extracts [100 μg/lane] under non-reducing conditions. The salmon STC hormone appears as one dense band [50 kDa]. The reputed bowfin STC hormone appears as two closely spaced bands [43 and 45 kDa]. b Control study for Western blot analysis. Normal rabbit serum [a]; sSTC antiserum [1:1000 dilution] [b]; anti-sSTC preabsorbed with sSTC [100 μg/μl] [c]. Note the absence of staining with normal rabbit serum and preabsorbed antiserum.
CHAPTER 2
CHAPTER 2

Immunoreactivity of the corpuscles of Stannius of the garpike, *Lepisosteus osseus* L., to antisera against salmon and trout stanniocalcin

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ABSTRACT

Stanniocalcin-immunoreactive cells were localized in the corpuscles of Stannius of a holostean fish, the garpike [Lepisosteus osseus], using antisera against salmon and trout stanniocalcins and the peroxidase-antiperoxidase and protein A-gold immunohistochemical methods. The stanniocalcin-immunoreactive cells were periodic acid-Schiff positive, and antibody staining was abolished if the antiserum was preabsorbed with corpuscle homogenate. Immunocytochemistry revealed two reactive cell types in the glandular parenchyma, and immunoreactivity was confined to the secretory granules. Staining of the granules was also abolished when the antisera were blocked with crude corpuscle homogenate. When corpuscle extracts from garpike were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis, a single dense band was evident with a molecular weight of ~68 kDa under non-reducing conditions, whereas three bands were observed (~29, 31, 34 kDa) under reducing conditions. Staining of all bands disappeared following preabsorption of the antiserum with salmon stanniocalcin, trout stanniocalcin, or garpike corpuscle extract. The results are compared with stanniocalcins from another extant holostean, the bowfin [Amia calva], and from more modern bony fishes, the teleosts.
Introduction

The corpuscles of Stannius [CS] are kidney-associated endocrine glands that only occur in holostean and teleostean fishes, members of the infraclass Neopterygii in subclass Actinopterygii. These glands produce stanniocalcin [STC], a homodimeric glycoprotein hormone that is 50 kDa in size in teleosts [Wagner et al., 1986, 1988a, 1992; Butkus et al., 1987; Lafeber et al., 1988a]. This hormone is capable of inhibiting calcium transport across the gills [So and Fenwick, 1979; Lafeber and Perry, 1988; Lafeber et al., 1988b], whole body calcium uptake [Wagner et al., 1986, 1988a; Lafeber et al., 1988b], and intestinal calcium uptake in vitro [Sundell et al., 1992]. In most teleosts, the number of CS is low [2-5], while in the bowfin [Amia calva], an extant holostean with direct links to teleosts through the division Halecostomi [Nelson, 1984], several hundred corpuscles have been reported mostly concentrated in the posterior region of the kidney, perhaps reflecting a more ancestral arrangement [Youson et al., 1976]. On the basis of Western blot analysis, the bowfin has the lowest molecular weight form of STC identified to date, with an estimated size of 43-45 kDa [Marra et al., 1992]. The garpike [Lepisosteus osseus] is another surviving holostean but has been placed within the division Ginglymodi, separate from the bowfin and teleosts [Nelson, 1984]. Perhaps in concert with this taxonomic separation, the CS of the garpike show some marked differences compared to the bowfin with respect to number, location, anatomical distribution, and ultrastructure. The 5-7 CS of the garpike are localized in the anterior third of the kidney [Bhattacharyya et al., 1982].

Wendelaar Bonga and Pang [1986] reviewed the cytophysiology of the CS, describing the presence of either one or two structurally discrete [type-1 and type-2] cells, both of which are involved in protein synthesis and secretion. In the bowfin, only type-1 cells are present [Youson and Butler, 1976; Marra et al., 1992], whereas the garpike has both cell types [Bhattacharyya et
Immunological procedures demonstrate that the product common to both cell types in teleosts is STC [Wendelaar Bonga et al., 1989].

Stanniocalcin-like immunoreactivity has never been demonstrated in the garpike. In light of suggestions that the two orders comprising the holosteans [Lepisosteiformes and Amiiformes] have disparate evolutionary histories and the fact that our findings in the bowfin suggest a unique form of STC, it was our goal to study the immunoreactivity of cells of the CS and STC in the garpike for comparison with bowfin and teleosts. We used immunohistochemistry and immunocytochemistry in conjunction with Western blot analysis and highly specific antisera to sockeye salmon and trout STCs in an attempt to provide more information on the phylogeny of the CS and the characterization of stanniocalcin among the fishes.

Materials and methods

Animals and tissue collection

Adult garpike, Lepisosteus osseus L., were trap-netted in Lake Ontario, East Lake, Picton, Canada, in September and October, 1991. The specimens, varying in length from 40-103 cm, were transported to the laboratory at the Scarborough Campus of the University of Toronto. The garpike were maintained in a large fibreglass tank supplied with flow-through, dechlorinated, aerated tap water [10°C]. The garpike were anaesthetized using MS222 and sacrificed by anaesthetic overdose followed by cervical dislocation. A longitudinal incision extending from the cloaca to the pericardial cavity was made, exposing the retroperitoneal opisthonephroi. The kidneys were quickly excised and examined under a dissecting microscope for the characteristic white corpuscles [Bhattacharyya et al., 1982]. The CS from seven animals were carefully teased from surrounding hemopoietic tissue and immediately fixed for either light or electron microscopy. An additional 0.25 g of corpuscle tissue was harvested from 40 animals,
frozen in liquid nitrogen, and stored at -70° C. These corpuscles were used for Western blot analysis.

**Primary antisera**

The immunological procedures in this study were conducted using two polyclonal antisera, one generated against sockeye salmon STC and the other against trout STC. Both the salmon [Gellersen et al., 1988; Wagner et al., 1988b] and the trout [Kaneko et al., 1988; Wendelaar Bonga et al., 1989] STC antisera have been characterized previously.

**Immunohistochemistry and Immunocytochemistry**

Excised garpike CS from four specimens were fixed in Bouin’s fluid for 24-48 h and then stored in 70% ethanol until dehydration in a graded series of ethanols and embedding in paraffin. Immunoreactive STC sites were localized on 4-μm-thick sections using the peroxidase-antiperoxidase procedure [Sternberger et al., 1970]. The sections were first rehydrated and equilibrated in phosphate-buffered saline containing 3% heat-inactivated goat serum to reduce non-specific staining and then incubated for 48 h at 4° C with either salmon or trout STC antiserum [1:1000]. Goat anti-rabbit gamma globulin [1:50] was applied for 30 min at room temperature, followed by rabbit peroxidase-antiperoxidase serum [1:50] for the same period of time. The slides were washed for 20 min with phosphate-buffered saline containing 1% heat-inactivated goat serum after the application of each antiserum. The sites of peroxidase activity were visualized by incubating the sections in buffered 0.003% hydrogen peroxide [pH 7.6] containing 0.0125% 3,3-diaminobenzidine for 15 min. The sections were postfixed with 1% osmium tetroxide to enhance contrast.
For immunocytochemical studies, excised CS from three specimens were fixed for 2 h in 2% glutaraldehyde adjusted to pH 7.3 with 0.1 M phosphate buffer [Millonig, 1961]. The tissue was washed for 30 min in phosphate buffer and then dehydrated in a graded series of ethanols, passed through propylene oxide, and embedded in Araldite. Ultrathin sections were cut with glass knives on a Reichert OM U3 ultramicrotome, mounted on nickel grids, and stained with salmon or trout STC antiserum [1:1000] and protein A-gold [Wendelaar Bonga et al., 1989]. Sections were examined with a Siemens Elmiskop 102 electron microscope.

Control procedures included substitution of the primary antiserum with phosphate-buffered saline and saturation of the primary antiserum before staining with a crude corpuscle extract prepared in either phosphate-buffered or TRIS-buffered saline [25 mg/ml]. Adjacent sections were stained by the periodic acid-Schiff [PAS] reaction and counterstained with acid haemalum and orange G.

**Western blot analysis**

Western blotting, 4-5 frozen CS from five different garpike were homogenized in sodium dodecyl sulphate [SDS] sample buffer [100 mg/ml] and centrifuged at 12000 X g for 30 min. The supernatant (hereafter referred to as “crude extract”) was decanted and stored at -20° C until used. The crude extract from each specimen was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis [SDS-PAGE] in 12% gels in the presence and absence of mercaptoethanol [Laemmli, 1971]. Following SDS-PAGE, the gels were equilibrated in transfer buffer [0.025 M TRIS, 0.192 M glycine, and 20% methanol], and the resolved proteins were transferred to nitrocellulose using a BioRad TransBlot apparatus [0.25 A, 4 h]. The nitrocellulose filters were equilibrated in antibody diluent buffer [0.02 M TRIS, 0.5 M NaCl, 0.05% Tween-20, 1% bovine serum albumin, 1% normal goat serum; pH 7.4] and incubated overnight at room
temperature in salmon or trout STC antiserum [1:40000]. The filters were washed [3 x 15 min] in Tween/TRIS-buffered saline [0.02 M TRIS, 0.5 M NaCl, and 0.05% Tween-20; pH 7.5] and incubated for 30 min in biotinylated goat anti-rabbit gamma globulin [1:1000; Zymed, San Francisco, Calif., USA]. The filters were washed again in Tween/TRIS-buffered saline [3 x 15 min] and incubated for an additional 30 min in alkaline phosphatase streptavidin [1:1000; Zymed]. Following another series of washes in Tween/TRIS-buffered saline [3 x 15 min], the filters were incubated in alkaline phosphatase buffer [0.1 M TRIS, 0.1 M NaCl, 0.05 M MgCl₂; pH 9.5] for 15 min. The sites of alkaline phosphatase activity were visualized by exposure to 0.004% nitroblue tetrazolium chloride and 0.003% 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt.

To confirm the specificity of the results with the Western blotting procedure, the primary antiserum was substituted with either normal rabbit serum [1:40000] or primary antiserum preabsorbed with crude extract.

Results

The garpike CS appeared as white, ovoid structures ranging in width from 1-2 mm and in length from 1-4 mm. Five-to-seven corpuscles were localized in the anterior third of the kidney and were often associated with the large vessels and occasionally with the adrenocortical homolog [yellow corpuscles]. The glands were localized in kidney regions which had abundant hemopoietic tissue but were devoid of nephric tissue. As in other fish species, the garpike corpuscle had a connective tissue capsule, comprised of collagen fibrils and fibroblasts, which invaded the gland and divided it into a variable number of lobes and lobules.

The parenchymal population of each corpuscle consisted of elongated epithelial cells grouped into convoluted and anastomosing cords or islets, the thickness of which was limited to
two cell layers. Each cord was circumscribed by a thin basal lamina [Fig. 1a]. After PAS staining and counterstaining, the glandular cells appeared slightly basophilic, and PAS-positive cytoplasmic granules were scattered about a spherical, centrally located nucleus [Fig. 1a, b]. In adjacent sections stained for STC, the immunoreactive material appeared as a brown precipitate within the cells [Fig. 1b, c]. Areas of presumptive immunoreactive STC correlated closely with those areas stained by the PAS technique. Non-immunoreactive cells were also noted within each corpuscle. The immunostaining was specific for corpuscle cells and only background staining was observed in hemopoietic tissue and other surrounding [connective, vascular, and nervous] tissues [Fig. 1c, d]. Staining was evident with either salmon [Fig. 1c] or trout [Fig. 1d] STC antiserum. The specificity of the reaction was confirmed as immunostaining was completely abolished when the primary antisera were preabsorbed with crude extract or when phosphate-buffered saline replaced the primary antiserum [Fig. 1e].

In preparation of tissue for immunocytochemistry, postfixation with osmium tetroxide was avoided so that the highest possible antigenicity could be maintained. As a result, membranes were poorly preserved in the sections. However, the fine structure of cells in the garpike CS was consistent with previous findings in this species [Bhattacharyya et al., 1982]. Two cell types [Fig. 2a] were identified in garpike CS, comparable to type-1 and type-2 cells in teleosts [Ogawa, 1967; Tomasulo et al., 1970; Wendelaar Bonga et al., 1977, 1980; Bhattacharyya and Butler, 1978]. The more numerous type-1 cells were characterized by an abundance of electron-dense cytoplasmic granules which were either distributed evenly or concentrated toward the cell apex. In sections the granules varied from round to oval in shape and from 0.15 \( \mu \text{m} \) to 0.40 \( \mu \text{m} \) in diameter. A prominent feature of the type-1 cell was a spherical, central nucleus containing an eccentric nucleolus and patchy heterochromatin. Other
characteristic features of the type-1 cell included polymorphic mitochondria, numerous coated vesicles, and abundant free ribosomes.

The type-2 cell was polymorphic and frequently compressed between the more abundant type-1 cells. Type-2 cells contained oblong-to-irregularly shaped nuclei and a paucity of electron-dense, variably-shaped secretory granules with similar dimensions to those of type-1 cells [Fig. 2a-c]. Mitochondria, coated vesicles, and free ribosomes were also evident. The reader is referred to Bhattacharyya et al. [1982] for a detailed description of the fine structure of cells in the garpike CS.

When either salmon or trout STC antiserum was used, highly specific staining was observed over the secretory granules of type-1 [Fig. 2b] and type-2 cells [Fig. 2c]. Absorption of the primary antiserum with a crude extract abolished the immunostaining. The use of phosphate-buffered saline or TRIS-buffered saline in place of the primary antiserum also resulted in no staining reaction. A semi-quantitative, dot-density analysis revealed an average of 12.2 gold particles/μm² over the secretory granules and a background level of less than 2.2 gold particles/μm². In contrast, 0.45 gold particles/μm² were present on control tissues.

Western blot analysis of corpuscle extracts revealed one diffuse, immunoreactive band with an apparent molecular weight of approximately 68 kDa under non-reducing conditions [Fig. 3]. Under reducing conditions, the immunoreactive band was better resolved and appeared as three, closely-spaced bands with sizes of 29, 31, and 34 kDa [Fig. 4]. Identical results with the Western blotting procedure were obtained with salmon and trout STC antisera. The specificity of antibody staining in corpuscle extract was evident by the absence of staining when the primary antiserum was preabsorbed with corpuscle extract or sockeye salmon STC or replaced by normal rabbit serum [Fig. 3].
Discussion

Morphological observations in the present study were consistent with previous studies on garpike CS [Bhattacharyya et al., 1982]. However, when these results are compared to those from the bowfin, *Amia calva* [Youson and Butler, 1976; Youson et al., 1976; Marra et al., 1992], it is apparent that there are some fundamental differences between the CS of the two genera (*Lepisosteus* and *Amia*) comprising the holostean. In agreement with previous studies [Bhattacharyya et al., 1982], 5-7 CS were localized in the anterior third of the kidney in regions devoid of nephric tissue but rich in hemopoietic tissue. In the bowfin, however, several hundred CS were scattered throughout the opisthnonephros in hemopoietic-rich regions and in areas abundant with nephric tissue, still maintaining a close association with the distal renal tubules from which they are assumed to emanate [Youson et al., 1976; Marra et al., 1992]. The number and distribution of bowfin CS perhaps reflect the ancestral organization of the corpuscles of Stannius [Garrett, 1942; de Smet, 1962; Youson et al., 1976]. Both garpike and bowfin CS are closely associated with the adrenocortical homolog [Youson et al., 1976; Bhattacharyya et al., 1982]. In teleosts, the number of CS is low [2-5], the glands assume a peripheral position on the exterior surface of the kidney, maintaining close neural and vascular connection to it [Wendelaar Bonga and Pang, 1986], and the adrenocortical homolog and the CS are widely separated. With respect to ultrastructure, the garpike CS are comprised of two discrete cell types which are more similar to those described in some teleosts [Ogawa, 1967; Krishnamurthy and Bern, 1969; Tomasulo et al., 1970; Wendelaar Bonga et al., 1977, 1989; Wendelaar Bonga and Pang, 1986], whereas in the bowfin only type-1 cells are present [Youson and Butler, 1976; Marra et al., 1992]. Our study supports the view that the distribution and structure of the garpike CS may represent an intermediate position between those of the bowfin and the teleosts [Bhattacharyya et al., 1982].
Our results demonstrated a good correlation between immunostained and PAS-positive regions of the glandular cells in the corpuscles, whether the immunostaining was conducted with salmon or trout STC antiserum. At the electron-microscopic level, immunocytochemistry revealed two structurally distinct cell types in which immunoreactivity was localized in the secretory granules. This same phenomenon was seen in teleosts [trout and flounder] where it was tentatively concluded that the type-1 and type-2 cells were structurally different forms of a single cell-type [Wendelaar Bonga et al., 1989]. In situ hybridization analysis of tissue from salmon CS also suggests that the STC gene is expressed in all corpuscle cells [Sterba et al., 1993].

This is the first time that an extract of garpike CS has been examined, and Western blot analysis has yielded a putative form of STC with a molecular weight of ~68 kDa, the largest identified thus far. Interestingly, our results on bowfin STC have identified one of the smallest molecular weight forms of STC (~45 kDa). Bowfin STC is, however, similar in size to chum salmon STC [Sundell et al., 1992]. Previous studies have shown that STC in teleosts is a homodimeric glycoprotein, with the molecular weight of the monomer varying from 28 kDa in trout to 30 kDa in salmon and 32 kDa in the eel [Wagner et al., 1986, 1992; Lafeber et al. 1988; Flik et al., 1989]. It is the glycosylated nature of STC that has enabled its purification from various teleosts by concanavalin A-Sepharose. However, this method of purification has not been successful with the bowfin [Marra et al., 1992], and the same may be the case with the garpike. Lectin histochemical and lectin chromatographic studies indicate that neither garpike nor bowfin corpuscle extracts are capable of binding to concanavalin A [L.E. Marra, J.H. Youson, G. Zaccone, S. Fasulo, and G.F. Wagner, unpublished observations]. In addition, there is no binding to several other lectins, such as peanut agglutinin, soybean agglutinin, and *Ricinus communis* agglutinin. On the basis of our results, it appears that garpike STC may be glycosylated but that the carbohydrate moiety is different from that in teleosts. Differences in core glycosylation could
account for the variable electrophoretic mobilities of the two holostean STC molecules as well as their variation from teleost STC. Since differences in polypeptide chain length could also account for the molecular weight deviations amongst the species, the future characterization of these hormones should explain their size differences.

Paleontological and morphological evidence on Neopterygii [Olsen and McCune, 1991] indicate that lepisosteids constitute a monophyletic group. Support for this hypothesis comes from an examination and comparison of the amino acid sequences of bowfin and garpike insulins [Conlon et al. 1991]. There are unusual amino acid substitutions in bowfin insulin that are not found in the insulins of garpike and teleosts, suggesting that bowfin and garpike did not descend from a common Mesozoic ancestor, but evolved in parallel. The results of the present study suggest that there are similarities between holostean and teleostean STCs on the basis of their common cross-reactivity to STC antisera. That is, their STC molecules must share common epitopes. However, major differences exist between the biochemical properties of the two holosteans and the teleostean STC molecules. The apparent differences in size and biochemical properties in the STC molecules of teleosts and holosteans of infraclass Neopterygii, and in fact between the two orders [Lepisosteiformes and Amiiformes], suggest that this hormone has undergone considerable evolutionary change.
Figure 1a-e. Light micrographs of adjacent sections of a corpuscle of Stannius [CS] from *Lepisosteus osseus* stained with periodic acid-Schiff [a, b], salmon stanniocalcin antiserum [c], trout stanniocalcin antiserum [d], and salmon stanniocalcin antiserum preabsorbed with crude gar CS extract [e]. Results similar to those in e were obtained when the antiserum was preabsorbed with salmon stanniocalcin or replaced with phosphate-buffered saline. Note that the glands are encapsulated [arrowheads in a, c-e] and are located in areas of the kidney abundant in hemopoietic [h] tissue. Glandular cells react strongly [arrowheads in b] to periodic acid-Schiff staining and show variable staining intensity [arrows in e] for stanniocalcin. X450 [a, c-e], X 1125 [b]. Bars: 25 μm [a, c-e], 10 μm [b].
Figure 2. a Electron micrograph of a corpuscle of Stannius from *Lepisosteus osseus*. Note the presence of two cell types: type-1 \( t_1 \) and type-2 \( t_2 \) cells with abundant and sparse granules [arrowheads], respectively. b Higher magnification of a type-1 cell. Protein A-gold particles are preferentially located over secretory granules [arrowheads] following immunocytochemistry with salmon or trout stanniocalcin antiserum. Low background staining is found in the nuclear [N] and cytoplasmic [arrow] matrices. c Immunoreactivity for stanniocalcin is evident with gold particles over the sparse granules [arrowheads] in type-2 cells. d Antiserum preabsorbed with crude extract reveals low background levels of gold particles. Non-specific binding [arrowhead] was minimal. X3000 [a], X40000 [b], X50000 [c, d]. Bars: 5 \( \mu \text{m} \) [a], 0.25 \( \mu \text{m} \) [b-d].
Figure 3. Western blot analysis of garpike [a] and bowfin [b] corpuscle extracts (100 µg/lane) under non-reducing conditions. The reputed garpike stanniocalcin appears as a single dense band [68 kDa]. Substitution of salmon stanniocalcin or trout stanniocalcin antiserum with normal rabbit serum [c] or preabsorption of antiserum with corpuscle extract [d] abolishes the garpike immunoreactive band.

Figure 4a, b. Western blot analysis of garpike corpuscle extracts [100 µg/lane] under reducing conditions. a Garpike stanniocalcin appears as three closely spaced bands with sizes of ~29, ~31, and ~34 kDa. b Preabsorption of the primary antiserum with corpuscle extract abolishes the immunoreactive bands.
CHAPTER 3
CHAPTER 3

The corpuscles of Stannius in arowana [*Osteoglossum bicirrhosum*], an ancient teleost

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John Youson assisted in the interpretation of the electron micrographs.
The corpuscles of Stannius of arowana \textit{(Osteoglossum bicirrhosum)}, an ancient teleost, were examined by routine light and electron microscopy and following their immunoreactivity to salmon and trout stanniocalcin antisera. Periodic acid-Schiff positive cells of the corpuscles of Stannius had a follicular arrangement and demonstrated a strong immunohistochemical reaction with both stanniocalcin antisera. Fine structural analysis of the paired, posteriorly located, and perirenal ovoid glands revealed two morphologically distinct cell types the basal laminae of which were ramified by nerve terminals. Immunocytochemistry demonstrated that osmiophilic secretory granules in both cell types were immunoreactive to the stanniocalcin antisera. When arowana corpuscles of Stannius were subjected to sodium dodecyl sulphate electrophoresis and Western blot analysis a diffuse molecular weight band was evident [\textasciitilde68 kDa] in the non-reduced condition. In all cases, immunoreactivity was abolished by preabsorption of the antisera with salmon stanniocalcin or with a crude extract of arowana corpuscles of Stannius. The corpuscles of Stannius of arowana are similar to those in more recent teleosts with respect to cell structure and their anatomical distribution but their stanniocalcin is more similar in molecular weight to that present in at least one other nonteleost actinopterygian [the gar] which has an ancient lineage.
Introduction

The corpuscles of Stannius [CS], first described by Stannius [1839], are kidney-associated endocrine organs which have purportedly been developed for the prevention of hypercalcemia in only three groups of fish, the teleosts [the modern bony fish] and the bowfin [Amia calva] and the gar [Lepisosteus osseus]. Previously, the latter two species comprised a single superorder of ancient bony fish, the Holostei but now are separated into the two orders, Amiiformes and Lepisosteiformes [Nelson, 1984]. In teleosts, the CS elaborate stanniocalcin [STC], a potent anti-hypercalcemic hormone [Wagner et al., 1986, 1988; Butkus et al., 1987; Lafeber et al., 1988].

Considerable variation exists in the CS in terms of number, distribution, ultrastructure, and organization among teleosts and between these fishes [Wendelaar Bonga and Pang, 1986] and the bowfin and gar, which in turn differ from one another [Youson et al., 1976; Bhattacharyya et al., 1982; Marra et al., 1992, 1994]. In teleosts the number of CS is low [2-5], and they usually assume a peripheral and posterior position on the exterior surface of the kidney but still maintain close neural and vascular connections to this organ. Interestingly, in all teleost species examined, the CS have a rich nervous supply [Wendelaar Bonga and Pang, 1986]. However, the nerve fibres are usually vasoganglionic units [Heyl, 1970] and synaptic nerve endings on CS gland cells have never been reported [Wendelaar Bonga and Pang, 1986]. This morphologic pattern is contrasted with that in the more "primitive" bony fishes, the bowfin and gar where a divergence in the number of CS occurs between the two genera, Lepisosteus and Amia, respectively [Youson et al., 1976; Bhattacharyya et al., 1982; Marra et al., 1992, 1994]. In the bowfin several hundreds of these glands have been reported mostly concentrated in the posterior region of the kidney perhaps reflecting a more ancestral organization [Youson et al., 1976]. In addition, many CS remain in the interior of the kidney and are closely associated with
the distal renal tubules [Youson et al., 1976; Marra at al., 1992]. In comparison, the 5-7 CS of the
gar are located in the anterior 1/3 of the kidney often in association with the large vessels in
regions of the kidney devoid of nephric tissue [Bhattacharyya et al., 1982; Marra et al., 1994].

Earlier fine-structural observations on the gland cells of the CS in teleosts suggested that
these endocrine organs were involved in protein synthesis and secretion [Ogawa, 1967;
Wendelaar Bonga et al., 1977, 1980; Bhattacharyya and Butler, 1978; Aida et al., 1980]. The
parenchymal population of the CS consists of either one or two structurally different cell types
[type-1 and type-2]. Whereas type-1 cells, with their extensive rough endoplasmic reticulum
[RER] and large secretory granules, are exclusive to marine fish, type-2 cells are characterized
by a less extensive RER and smaller secretory granules and are also found in freshwater and
euryhaline fish [Wendelaar Bonga and Pang, 1986]. It was first assumed that the two cell types
in teleosts were physiologically distinct with respect to their function and secreted products.
However, based on the positive immunoreactivity to antisera generated against teleost STC in
both the type-1 and type-2 cells, it appears that these gland cells may represent varied
expressions of the same cell type [Wendelaar Bonga et al., 1989]. Although a similar
cytophysiological organization to the above is also represented in the CS glands of the gar
[Bhattacharyya et al., 1982; Marra et al., 1994], the bowfin has a single immunoreactive cell
type, namely type-1 [Youson and Butler, 1976; Marra et al., 1992].

The molecular weight of STC seems to vary slightly between species and the hormone
itself appears to occur in a labile dimeric form [Wagner et al., 1986, 1992, 1994; Butkus et al.,
1987; Lafeber et al., 1988]. The monomeric form of the hormone in salmon has been
demonstrated to be a 26 kDa product with a 32 kDa prohormone [Wagner et al., 1988]; in trout
and goldfish the respective values are 28 and 32 kDa, and in eels 30 and 34 kDa [Flik et al.,
1989]. On the basis of periodic acid-Schiff [PAS] positive staining and concanavalin A [Con A]
lectin affinity the putative hormone was suggested to be a glycoprotein. The dimeric form and glycoprotein nature of the STC molecule has recently been validated by molecular biological approaches in which the primary structure of the main secretory protein of the CS of the Australian eel [Butkus et al., 1987] and the coho salmon [Wagner et al., 1992] have been elucidated from cDNA sequences.

In contrast to STC in teleosts, comparatively little is known about the more ancient forms of this molecule. Our previous researches into bowfin and gar STC provide several interesting insights. The bowfin STC molecule appears to be one of the smallest thus far identified with a molecular weight of approximately 45 kDa in its non-reduced form whereas the gar STC [~68 kDa] molecule appears to be one of the largest [Marra et al., 1992; 1994]. PAS staining of histological sections indicated that both the bowfin and gar forms of STC are likely to be glycosylated, however, neither molecule demonstrated affinity to the Con A lectin [Marra et al., 1992; 1994]. Whereas Western blotting and immunohistochemical techniques demonstrated that antisera generated against teleost STC is capable of recognizing bowfin and gar STC [Marra et al., 1992; 1994], full length salmon STC cDNA was unsuccessful in generating a hybridization signal to bowfin CS RNA by northern blot analysis [Wagner et al., 1992]. Therefore, despite the fact that epitopes of the protein have been preserved, alteration of the STC sequence by evolutionary pressure appears likely [Wagner et al., 1992, Marra et al., 1992]. Whether the same holds true for the gar has yet to be determined.

Our recent research into bowfin and gar CS and STC suggest that they may have undergone change during the evolution of fishes. Although present evidence indicates that the physiological importance of the CS is a reality, most comparative experimental and morphological investigations on these glands have been performed on "modern" bony fishes. We supposed that an examination of the CS and STC in representatives of more ancient teleosts,
such as Osteoglossiformes, could prove to be useful for our interpretation of evolutionary trends in this endocrine system. In an attempt to gain such information on this unique endocrine tissue we used highly specific antisera generated against salmon and trout STC in an immunohistochemical, immunocytochemical and Western blot analysis of arowana [Osteoglossum bicirrhosum] CS and STC.

Materials and Methods

Animals and tissue collection

Juvenile arowana, Osteoglossum bicirrhosum, were purchased from a commercial supplier in Toronto, Ontario, Canada in May, 1992. The specimens, 6-10 cm in total length, were maintained at room temperature in a standard glass tank containing dechlorinated, aerated water at the Scarborough Campus of the University of Toronto. The specimens were sacrificed 2 hours after their arrival to the laboratory by an MS222 anaesthetic overdose. The kidneys were exposed by a cloacal-pericardial incision and the characteristic white corpuscles were excised under a dissecting microscope. CS from 5 different specimens were immediately fixed for light and electron microscopy and an additional 20 CS were prepared for Western blot analysis and stored at -70°C until their use. In addition, one adult specimen [58 cm in length], was similarly maintained and sacrificed and one CS was prepared for electron microscopy and the other for Western blot analysis.

Primary Antisera

Previously characterized polyclonal antisera generated against sockeye salmon [Gellersen et al., 1988; Wagner et al., 1988] and trout [Kaneko et al., 1988; Wendelaar Bonga et al., 1989] STC were used for immunohistochemistry, immunocytochemistry, and Western blot analysis.
The lyophilized anti-STC serum was reconstituted with phosphate-buffered saline (PBS) and stored at -70°C in 10 µl aliquots until use.

**Immunohistochemistry**

Excised arawana CS were fixed in Bouin's fluid for a period of 24 h. and then stored in 70% ethanol until dehydration in a graded series of ethanols and embedding in paraffin. Tissue sections (5 µm) were mounted on albumin-coated slides and immunoreactive STC sites were localized using the alkaline phosphatase-antialkaline phosphatase staining procedure. The sites of immunolabelling activity were revealed by a commercial rabbit alkaline phosphatase kit [BIO/CAN Scientific, Mississauga, Ontario, Canada]. Deparaffinized TRIS-buffered saline rehydrated and equilibrated tissue sections were incubated for 90 min at room temperature with anti-salmon STC sera at a 1:1000 dilution. Non-specific immunolabelling was reduced by a 1 h application of the supplied blocking agent. This procedure was followed by a 30 min application of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. Sites of alkaline phosphatase activity were visualized by incubating the tissue sections in the supplied chromogenic substrate for 30 min followed by a 5 min application of Mayer's haematoxylin to enhance contrast. Adjacent sections were stained by the PAS reaction and counterstained with acid haemalum and orange G.

Control procedures included the substitution of the primary antisemum with normal rabbit serum [NRS] and saturation of the primary antibody with crude arawana CS extract.

**Routine electron microscopy**

Portions of CS excised from anaesthetized animals were fixed in 2% glutaraldehyde buffered to pH 7.3 using 0.1 M Millonig's phosphate [0.12 M NaH₂PO₄, 0.1 M NaOH, 0.54%
glucose; Millonig, 1961). They were then washed and postfixed for 1 h at room temperature in 1% OsO4. After dehydration in a graded series of ethanols and propylene oxide treatment the tissues were embedded in Araldite. Thin sections were then stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 102 electron microscope.

**Immunocytochemistry**

The procedures for immunocytochemistry were similar to those described in Wendelaar Bonga et al. [1989] and Marra et al. [1992, 1994]. In brief, the excised CS were placed in 2% glutaraldehyde buffered to pH 7.3 using 0.1 M Millonig’s phosphate [Millonig, 1961]. The tissue was washed for 30 min in phosphate buffer and then dehydrated in a graded series of ethanols, treated with propylene oxide, and embedded in Araldite. In order to maintain the highest degree of antigenicity, postfixation with OsO4 was omitted. Ultrathin sections were cut with a glass knife on a Reichert OM U3 ultramicrotome, mounted on nickel grids, and stained with anti-salmon or anti-trout STC serum [1:1000 dilution] and protein A-gold.

The control procedure involved the preabsorption of the primary antibody from the serum with crude arawana CS extract (~20 mg/ml) prepared in PBS [pH 7.3]. The sections were examined on a Siemens Elmiskop 102 electron microscope.

**Immunoblotting**

For Western blotting, liquid nitrogen-frozen arawana CS were homogenized in PBS [pH 7.3] containing 10 mM phenylmethylsulfonylflouride, centrifuged at 12000 g for 30 min, and the supernatants pooled. The crude CS extract was diluted in sodium dodecyl sulphate [SDS] sample buffer 1:1, heated for 5 min at 95°C, and separated by one-dimensional polyacrylamide gel electrophoresis [PAGE] in a 12% gel devoid of any reducing agents [Laemmli, 1971]. For
comparative purposes, homogenates of bowfin and gar CS were similarly treated. Following SDS-PAGE, the gels were equilibrated in transfer buffer [0.025 M TRIS, 0.192 M glycine, and 20% methanol] for 90 min at room temperature. The resolved proteins were electrophoretically transferred [250 mA, 2 h] onto transfer buffer-presoaked nitrocellulose sheets using a BioRad Transblot apparatus. Subsequently, the blots were soaked for 1 h at room temperature in blocking solution consisting of Tween-TRIS-buffered saline [0.02 M TRIS, 0.5 M NaCl, 0.05% Tween-20; pH 7.4], 1% bovine serum albumin, and 1% normal goat serum. The nitrocellulose blots were incubated at room temperature in anti-salmon or anti-trout STC serum [1:40000 dilution]. The blots were then washed in Tween-TRIS-buffered saline [3 X 15 min] and incubated for 30 min in a 1:1000 dilution of biotinylated goat anti-rabbit gamma globulin [Zymed, San Francisco, USA]. Following an additional series of washes [3 X 15 min] in Tween-TRIS-buffered saline, a 30 min incubation in a 1:10000 dilution of alkaline phosphatase streptavidin [Zymed, San Francisco, USA] was carried out. Subsequent to a final series of Tween-TRIS-buffered saline washes [3 X 15 min], the nitrocellulose blots were incubated in alkaline phosphatase buffer [0.1 M TRIS, 0.1 M NaCl, 0.05 M MgCl2; pH 9.5] for 15 min. The sites of alkaline phosphatase activity were visualized by exposure of the blots to 0.004% nitroblue tetrazolium chloride and 0.003% 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt.

Control procedures included the substitution of the primary antiserum with NRS and saturation of the primary antibody with crude arawana CS extract with similar blots being processed as above.
Results

Histological features and immunohistochemistry

In the juvenile arawana, a pair of the characteristic white [Youson et al., 1976], round to oval CS, approximately 1 mm in diameter, were localized immediately anterior to the bulbous portion of the retroperitoneal kidney. The adult arawana demonstrated a considerably larger [2-4 mm] pair of glands localized to the same area. In either case, gross inspection of the CS [under a dissecting microscope] revealed little about the structure of the glands or their anatomical relationship to the kidney other than having a similar retroperitoneal positioning in the body of the animal.

Similar to that found in other fish species, a light microscopic examination revealed a gland encapsulated with collagen and fibroblasts and invaded by connective tissue septa and capillaries [Fig. 1]. The epithelial cells were arranged into cords and follicles [Fig. 1a, b] and were of variable staining intensity with the PAS reaction [Fig. 1b]. Cells immunoreactive to polyclonal anti-salmon STC [Fig. 1a] and anti-trout STC [results not shown] were observed throughout the CS. The presumptive immunoreactive STC areas [Fig. 1a] in adjacent sections were closely correlated with those areas stained by the PAS technique [Fig. 1b]. Since only background staining was observed in surrounding tissues, the immunostaining appeared to be specific for the gland cells of the CS [Fig. 1a]. As following the PAS reaction, the cells immunostained for STC also demonstrated a variable staining intensity with a gradation occurring from the periphery towards the centre of the gland [Fig. 1a]. Absorption of the primary antibody with crude arawana CS extract before immunostaining abolished staining over the gland cells of the CS [Fig. 1c].
**Fine structure and immunocytochemistry**

Consistent with the view in light microscopy, the epithelium of the CS was often separated by wide spaces [Fig. 2]. The bordering cells frequently had microvilli and/or larger cytoplasmic projections extending into these spaces. Since many of the cells also possessed what appeared to be junctional complexes on the lateral surfaces near these wide spaces [Fig. 2 and inset], the latter were designated lumina. Thus, the cells had a polarity with an apical surface having microvilli and/or other cytoplasmic protrusions and a basal surface bordering the perivascular space [Fig. 2, 3]. The lateral plasma membrane followed a relatively straight course but was interrupted by regions where microvillus-like projections extended into a widened intercellular space. The intercellular space at these sites was separated from the remaining intercellular space by zonulae occludentes [tight junctions], even near the basal surface [Fig. 4]. We visualized these structures as an intercellular canalculus but were uncertain of their relationship to the lumina described above. The basal plasma membrane followed a rather tortuous course, for there were many projections of the basal cytoplasm and a few coated pits [Fig. 5]. A ciliary axoneme was periodically observed extending from any of the cell surfaces. A narrow perivascular space, with sparse collagen fibrils, separated the thin basal lamina of the epithelium from the fenestrated endothelium of capillaries [Fig. 5]. A particularly noteworthy feature was the frequency in which nerve terminals [Fig. 3] and cells with dense-cored vesicles [Fig. 6] were encountered in the perivascular space. These two components also penetrated the basal lamina and thus, were present among the epithelial cells [Fig. 3, 5, 6]. However, no epithelial-dendritic synapse was found. These dense-cored vesicles within the spindle-shaped cells were scattered throughout the cytoplasm and possessed a rather loose-fitting membrane.

Two types of epithelial cells were identified based on the degree of elaboration of their cell organelles but it is possible they were varied expressions of the same cell type. The most
numerous type 1 cells [Fig. 2] had extensive arrays of rough endoplasmic reticulum, an elaborate Golgi apparatus with several sets of saccules and coated vesicles, and numerous membrane-bounded granules of various electron densities and sizes [Fig. 7]. Although type 2 cells had a conspicuous Golgi apparatus, rough endoplasmic reticulum was less extensive and the granules tended to be smaller and fewer in number [Fig. 8]. Mitochondria of both cell types were equivalent in numbers but the matrices of those in type-1 cells sometimes were more electron dense. The nuclei of both types was roughly spherical with moderate quantities of condensed chromatin but with a prominent nucleolus. The osmiophilic nature of the granule matrices of both cells was reflected by the loss of electron density when osmium tetroxide was omitted in the fixation procedure prior to immunocytochemical staining [Fig. 9, 10]. Lead citrate and uranyl acetate did not restore the electron density.

When the gland cells were immunostained for STC, gold particles were almost exclusively localized over the secretory granules in both the type-1 and type-2 cells [Fig. 9, 10]. Preabsorption of the primary antibody with crude arawana CS extract resulted in no gold particles confirming the specificity of the antibody.

**Immunoblotting**

Western blot analysis using a polyclonal anti-salmon STC or anti-trout STC as the probing antibodies, demonstrated that arawana STC-immunoreactive protein migrated at approximately 68 kDa [Fig. 11a], similar to that of the gar [Fig. 11b] and had a 13-15 kDa slower mobility than the presumptive bowfin STC molecule [Fig. 11c] under non-reducing conditions. Preabsorption of the salmonid STC antibodies with crude arawana CS extract [Fig. 11d] or replacement of the primary antiserum with NRS [Fig. 11e] abolished the immunoreactive bands.
Discussion

The location of a pair of ovoid corpuscles of Stannius in a peripheral and posterior position on the exterior surface of the kidney in arawana is consistent with the distribution and number of these glands in the more modern bony fishes [Wendelaar Bonga and Pang, 1986]. However, this morphological pattern is strikingly different from the CS in the more "primitive" bony fish, the gar and the bowfin. In the latter species, the CS are widely scattered throughout the substance of the kidney [Youson et al., 1976; Marra et al., 1992], whereas, the gar demonstrates a reduction in number [5-7] and a regional concentration of the CS towards the centre of the kidney, perhaps indicative of an intermediate phylogenetic organization [Bhattacharyya et al., 1982]. Therefore, it appears that the arawana may be more closely related to other more recent teleosts with respect to the morphology of the CS glands.

The ultrastructural organization of the CS gland cells in the arawana is strongly suggestive of cells involved in the synthesis and secretion of a protein product(s). These features include an elaborate rough endoplasmic reticulum, a prominent Golgi apparatus, and many secretory granules. Immunocytochemistry reveals that the content of the granules is immunoreactive with antiserum against stanniocalcin [STC], formerly known as teleocalcin [Ma and Copp, 1978; Wagner et al., 1986] or hypocalcin [Pang et al., 1974; Lafaber et al., 1988]. The abundance of these stanniocalcin-positive granules explains the strong immunoreactivity to anti-stanniocalcin which was observed in light microscopic immunohistochemistry.

Ultrastructural profiles of the Golgi apparatus, rough endoplasmic reticulum, and the apparent size and numbers of secretory granules revealed that two cell types [type-1 and type-2] make up the parenchymal population of the CS in the arawana. The type-1 and type-2 cells of the arawana are ultrastructurally similar to the major cell types found in other teleosts such as the stickleback, European eel, killifish, trout, flounder, and goldfish [Krishnamurthy and Bern, 1969;
Wendelaar Bonga and Greven, 1975; Meats et al., 1978; Aida et al., 1980; Wendelaar Bonga et al., 1980, 1989] and in the gar [Bhattacharyya et al., 1982; Marra et al., 1994]. The presence of the two cell types in this most ancient teleost, the arawana, and in the extant representative of ancient bony fish, the gar [previously grouped along with the bowfin in the superorder Holostei] is noteworthy since the ultrastructure of the CS glands also hints at a phylogenetic relationship. This observation is especially significant since previous research into the CS and STC of the bowfin and gar revealed a closer relationship between gars and teleosts than between gars and bowfin [Marra et al., 1992, 1994]. Immunocytochemistry reveals that in the arawana, as well as in other species, the product common to both cell types is likely stanniocalcin suggesting that the different cell types of the CS represent structurally different forms of the same functional cell-type such as secretory cells in different phases of the secretory cycle [Bhattacharayya and Butler, 1978; Wendelaar Bonga et al., 1989]. Alternatively, the secretory products of each cell-type share common epitopes but serve different functions.

Although the morphology and fine structure of the arawana CS appears similar to that observed in other teleosts, several features are strikingly different. Firstly, we report that cells border an apparent lumen or large intercellular canaliculi with cytoplasmic projections invading these wide intercellular spaces and cell junctions near the cell apices. The significance of these morphological features could be functional and/or developmentally related but any interpretation would be speculative at best. It is noteworthy that cell junctions and small intercellular spaces were previously noted in the ancient actinopterygian, the bowfin [Youson and Butler, 1976]. Secondly, the osmiophilic nature of the secretory granules in each of the cell types in arawana was surprising and we believe unique at least in comparison to bowfin [Marra et al., 1992] and gar [Marra et al., 1994]. The absence of the electron-dense matrix of granules without osmium tetroxide fixation implies that lipid may be a component of the granules. However, this feature
cannot be rationalized in a secretory granule with strong glycoprotein content. Thirdly, although we did not identify any epithelial-dendritic synapses, some nerve terminals were observed to ramify the glandular parenchyma at the basal lamina. This feature is especially interesting since Cano et al. [1994] hypothesized that type-2 cells in eels may respond to cholinergic stimulation. Although speculative, this finding in arawana implies that a secretory phenomenon could be influenced by either the modification of blood flow to the glands or via a parenchymal stimulation by transmitter release into the pericellular space, a situation similar to thyroid follicular cells. This hypothesis is further supported by Hanssen et al. [1991] who reported that carbachol, a cholinergic agonist, was able to stimulate STC release in vitro while atropine could inhibit the release. It is important to note, however, that this morphological arrangement may likely represent a fine adjustment mechanism since transplanted, denervated CS remain competent in their ability to reverse experimentally induced hypercalcemia [Schreibman and Pang, 1975] and that the stimulatory effect of increases in extracellular calcium on STC release have been well documented both in vitro [Aida et al., 1980; Wagner et al., 1989; Hanssen et al., 1991] and in vivo [Lafeber and Perry, 1988; Wagner et al., 1988; Hanssen et al., 1992].

This is the first time arawana CS extracts have been examined by Western blot analysis. The putative arawana STC molecule migrated with a molecular weight approaching ~68 kDa, identical to that found in the gar [Marra et al., 1994]. Bowfin STC has been shown to have a molecular weight of approximately 45 kDa [Marra et al., 1992]. Previous studies into STC from several teleosts have shown the hormone to be a homodimeric glycosylated molecule which varies in size between species, the monomer ranging from 28 kDa to 32 kDa depending on the species examined [Wagner et al., 1986, 1992; Lafeber et al., 1988; Flik et al., 1989]. In fact, the glycosylated nature of STC has enabled its purification by Con A-sepharose in these teleost species. However, bowfin and gar STC do not bind to Con A lectin suggesting a modification of
the glycomoiety of STC occurred during the evolution of fishes. Considering the taxonomic position of arawana with respect to modern teleosts and both the gar and bowfin it would be interesting to determine where the arawana STC molecule fits in terms of its carbohydrate composition. In combining molecular evidence with morphological and fine structural features, it seems that arawana STC is more similar to gar than to bowfin STC. This view is supported by the earlier work of Tsuneki [1986] and Tsuneki and Nozaki [1989] who demonstrated that the anatomical characteristics of the neurohypophysis of Osteoglossomorpha [especially arawana] is intermediate between Lepisosteiformes and non-osteoglossomorph teleosts.

The results of the present study indicate that the CS of arawana, an Osteoglossiforme, are teleostean in their distribution and number but their STC is more similar to gar, based on the behaviour of their respective STCs under electrophoretic conditions and during immunolabelling procedures. However, the morphology of arawana CS has revealed some interesting characteristics not previously observed in other fish species.
Figure 1a-c. a Adjacent sections of a corpuscle of Stannius [CS] from *Osteoglossum bicirrhosum* stained by salmon stanniocalcin antiserum, the periodic acid-Schiff reaction [b], and salmon stanniocalcin antiserum preabsorbed with arawana crude CS extract [c]. Note that the gland is encapsulated [large arrowhead in a, b] and that the gland cells of the CS have a follicular arrangement. Pigment [*] is present in each section. The glandular cells react strongly to both the antiserum [short arrows in a] and to periodic acid-Schiff [short arrows in b], however, the cells demonstrate a variable staining intensity [small arrowheads in a, b]. Absorption of the primary antibody with arawana crude CS extract abolished the immunostaining. Results similar to those in [a] and [c] were obtained when the probing anti-salmon antibody was replaced with trout stanniocalcin antiserum. X400.
**Figure 2.** Low magnification electron micrograph of apical protrusions [*], microvilli [*arrow*], and junctional complexes [*arrowheads*] of cells bordering on a lumen [*L*] and extending towards a capillary [*C*] at their basal surface. These type-1 cells, with their numerous granules [*GR*] and prominent Golgi apparatus [*GA*] are periodically separated by wide lateral intercellular spaces [*IS*]. X8300. Inset: a zonula occludens from near the apical surface. X110,000.

**Figure 3.** Type 1 [*T₁*] and 2 [*T₂*] cells border a lumen [*L*] and extend to the perivascular space where there are numerous nerve terminals [*arrows*] around a capillary [*C*]. One nerve terminal [**] is among the epithelial cells. X8300.
Figure 4. Lateral intercellular spaces near the perivascular surface contain microvillus-like projections [arrows] and one is limited by a zonula occludens [arrowhead]. C, capillary. X9600.

Figure 5. The basal surface of cells have coated pits [*] and protrusions [arrow] resting upon a thin basal lamina [BL] which is in close proximity to the fenestrated [arrowheads] endothelium of a capillary. A small nerve terminal [NT] is in close apposition to the epithelium. X17200.

Figure 6. A nerve terminal [NT] and a cell [DC] with dense-cored vesicles are located at the perivascular surface of type-1 cells with their many granules [GR] of variable electron density. X18800.
**Figure 7.** A type-1 cell possesses numerous granules [GR], rough endoplasmic reticulum [ER], and a prominent Golgi apparatus with saccules [*] and coated vesicles [arrowhead]. Mitochondria [M] [with their dense matrices] are sparse and the spherical nucleus possesses a nucleolus [Nu]. X18800.

**Figure 8.** A type-2 cell with a large Golgi apparatus [GA], a few small granules [GR], some rough endoplasmic reticulum [ER], and sparse mitochondria [M]. Elaboration of the granules is suggested at the Golgi apparatus [arrow]. X18800.
**Figure 9.** Type-1 cell immunostained for stanniocalcin shows gold particles over the numerous secretory granules [GR] but not over the nucleus [N], mitochondria [M], rough endoplasmic reticulum [ER], a coated pit [arrowhead] or the cytoplasmic matrix [*]. Osmium tetroxide was omitted in the fixation procedure in order to maintain the highest possible antigenicity. X18800.

**Figure 10.** Type-2 cell immunostained for stanniocalcin shows the gold particles over granules [GR] of various sizes but not over mitochondria [M], rough endoplasmic reticulum [ER], coated vesicles [arrowheads], the nucleus [N], a vacuole [V] or the cytoplasmic matrix [*]. X18800.
Figure 11a-e. Western blot analysis of corpuscle of Stannius [CS] extracts from arawana [a], gar [b], and bowfin [c] under non-reducing conditions. Probing of the blots with salmon stanniocalcin antiserum reveals a ~68 kDa immunoreactive band in the arawana similar to that in the gar, whereas the reputed bowfin stanniocalcin appears as a dense 43-45 kDa band. Identical results were observed with trout stanniocalcin antisera. Pre-absorption of the primary antibody with arawana crude CS extract [d] or the replacement of the primary antibody with normal rabbit serum [e] abolishes the immunoreactive bands.
CHAPTER 4

Certain members of the Osteoglossiformes [bony tongue teleosts] exhibit an extracorpuscular source of stanniocalcin-like immunoreactivity

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ABSTRACT

An antiserum to salmon stanniocalcin [STC] was used for immunolabelling studies of the corpuscles of Stannius [CS] and kidneys of one member of each of the families Osteoglossidae [Osteoglossum bicirrhosum], Pantodonidae [Pantodon buchholzi], Notopteridea [Chitala chitala], and Mormyridae [Gnathonemus petersii] of the Osteoglossiforme order of teleosts. Immunohistochemistry revealed STC-like immunoreactivity in the CS parenchyma of each specimen examined. Immunocytochemistry demonstrated that labelling was confined to the secretory granules of the CS gland cells. In addition, in all specimens examined, except G. petersii, STC-like immunolabelling was also observed in certain cells of the distal renal tubules of the kidneys. The morphology of these STC-immunoreactive kidney cells was similar to that of “renal mitochondria-rich cells”. Western blot analysis suggested that this kidney form of STC had a different biochemical profile than that of previously described forms of the hormone.
Introduction

Stanniocalcin (STC) is a homodimeric glycoprotein hormone involved in calcium and phosphate regulation [Wagner et al., 1995]. This hormone was initially identified in the bony fishes [Wendelaar Bonga et al., 1985; Wagner et al., 1986, 1988; Lafeber et al., 1988] where it is produced by small kidney-associated endocrine glands referred to as the corpuscles of Stannius (CS). Recently, recombinant technologies have identified molecules with striking similarities to STC encoded in mammalian genomes [Chang et al., 1995, 1996; Wagner et al., 1995; Olsen et al., 1996] which also appear to play a role in mineral metabolism [Lu et al., 1994; Olsen et al., 1996; Wagner et al., 1997]. Interestingly, whereas the synthesis of piscine STC is confined to the CS, mammalian STC gene expression appears to be more widespread and, therefore, the hormone may function in a paracrine rather than in an endocrine manner [Chang et al., 1995].

Recently, Wagner et al. [1995] and De Niu et al. [1998] demonstrated that certain cells of the human kidney are one likely source of immunoreactive STC. To date, no extracorpuscular sources of STC have been characterized in the fishes.

It was originally suggested that the CS are embryologically derived from the pronephric and/or mesonephric ducts [Garrett, 1942] and subsequently Kaneko et al. [1992] demonstrated that the CS develop from individual epithelial cells of the nephric ducts during salmonid development and that STC-like immunoreactivity was evident as early as 13 days before hatching. These results are interesting in light of results which demonstrated that adults of the bowfin [Amia calva], the most ancient extant species known to possess these glands, has several hundred CS many of which have an intimate association with distal renal tubules [Youson et al., 1976; Marra et al., 1992]. Furthermore, it has been suggested that this type of arrangement reflects a more ancestral organization compared to a reduction in number and a more posterior distribution of this endocrine tissue in the more modern bony fishes, the teleosts [Bouchot, 1953; Youson et al., 1976]. However,
not all of the more ancient orders of teleosts, such as the Osteoglossiformes, have been examined for the distribution of their CS or nature of their STC.

STC was initially identified in fish as a glycosylated, disulfide-linked dimer through Western blotting and other biochemical protocols [Wagner et al., 1986, 1988; Lafeber et al., 1988]. Recently, this biochemical profile has been validated by molecular biology protocols [Butkus et al., 1987; Wagner et al., 1992; Olsen et al., 1996; Chang et al., 1995, 1996]. Despite the considerable similarity in the primary structure of piscine and mammalian STC, variability, indicative of evolutionary pressure, appears to be the norm in the more ancient forms of the hormone. For example, whereas bowfin STC appears to be one of the smallest forms reported, gar [Lepisosteus osseus] and arawana [Osteoglossum bicirrhosum] are two of the largest STC molecules to be identified to date [Marra et al., 1992, 1994, 1995]. In addition, our previous researches into these ancient forms of STC imply that the glycomoiety of bowfin and gar STC may be considerably different than those of the more modern bony fishes [Marra et al., 1992, 1994]. Further, molecular protocols imply that the primary structure of STC has undergone considerable and rapid evolutionary change during the evolution of the teleosts among the Neopterygii [Wagner et al., 1992; 1995]. Wagner et al. [1992] were the first to suggest the possibility of different forms of circulating and bioactive forms of STC. Recent evidence is supportive that multiple forms of STC may indeed be present [Wagner et al., 1998]. However, at present it is difficult to say whether these STC isoforms represent differential posttranscriptional processing of the STC message or the presence of more than one STC-like gene.

The Osteoglossiforme order occupies a unique taxonomic position intermediate to the early Neopterygii, the Semionotiformes [e.g. gar] and Amiiformes [e.g. bowfin] and the more recent Teleostei [Nelson, 1994]. Being the most ancient order of the Teleostei division, an examination of Osteoglossiforme STC is interesting from both an evolutionary and comparative viewpoint. An
analysis of the ancient forms of the CS and STC is necessary in order to develop a conceptual framework for understanding not only the nature of this endocrine tissue and its product(s) but also for understanding both the phylogeny and ontogeny of these elements in higher vertebrates. Our earlier study of one member of the Osteoglossomorpha, the silver arowana [Osteoglossum bicirrhosum], showed a “higher” teleost-like distribution of the CS but a STC with a molecular weight similar to that of the more ancient non-teleost, the gar [Marra et al., 1995]. The present report provides evidence for the presence of an extracorporeal source of STC in certain members of the Osteoglossiform order.

Materials and Methods

Animals and tissue collection

Adult and juvenile silver arowana [Osteoglossum bicirrhosum], butterfly fish [Pantodon buchholzi], featherfin knifefish [Chitala chitala], and elephantnose fish [Gnathonemus petersii] were purchased from a commercial supplier [Toronto, Canada] and transported to the freshwater facilities at the University of Toronto at Scarborough. After a period of acclimatization in tanks containing 18°C, dechlorinated, aerated water, the specimens were sacrificed by an overdose of tricaine methanesulfonate. The CS, kidneys and various control tissues [gut, brain, and muscle] from different specimens were harvested and were either immediately fixed for electron and light microscopy or frozen in liquid nitrogen and stored at -70°C until their use in Western blot analysis.

Primary antiserum

This series of immunolabelling studies employed well characterized polyclonal antiserum generated against chum salmon, trout or sockeye salmon STC. The antiserum employed were
previously well characterized by Western blot analysis, radioimmunoassay, and immunocytochemistry [Kaneko et al., 1988, 1992; Wagner et al., 1988; Wendelaar Bonga et al., 1989].

**Immunohistochemistry**

The specimen tissues, were fixed for 24 h in Bouin's solution, dehydrated in a graded series of ethanols, and embedded in paraffin. Adjacent sections were cut at 7 μm thickness, mounted on glass slides and then deparaffinized, rehydrated and equilibrated in Tris-buffered saline [TBS] before subjecting them to the following procedures. The peroxidase-labeled-[strept]avidin-biotin [Elias et al., 1989] staining method was used to localize the cellular sites of STC immunoreactivity. A 1:1000 dilution of chum salmon STC antiserum and the HISTOSTAIN-SP AEC immunostaining kit [Zymed Laboratories Inc., San Francisco, U.S.A.] was applied to some sections according to the manufacturer's instructions. Negative controls on adjacent sections consisted of either, a similar dilution of antiserum which had been preabsorbed with salmon STC, antiserum preabsorbed with crude CS extract or substitution of the antiserum with normal rabbit serum [NRS] or phosphate-buffered saline [PBS]. Labeled tissue sections were counterstained with haematoxylin to enhance contrast. Adjacent, untreated tissue sections were also stained with the periodic acid-Schiff [PAS] technique as previously described [Marra et al., 1992].

**Immunocytochemistry**

The procedures for immunocytochemistry were similar to those described by Wendelaar Bonga et al. [1989] and Marra et al. [1992, 1994, 1995]. The CS from the different specimens were treated as for routine electron microscopy [see Marra et al., 1992, 1994, 1995, 1998]. Briefly, the
excised tissue was fixed for 2 h in 2% glutaraldehyde buffered to pH 7.3 with 0.1 M phosphate [Millonig 1961]. The tissue was washed for 30 min in phosphate buffer and then dehydrated in a graded series of ethanol, cleared in propylene oxide, and embedded in Araldite. In order to maintain the highest degree of antigenicity, OsO₄ postfixation was omitted. Ultrathin sections were mounted on nickel grids and immunostained with a 1:1000 dilution of chum salmon STC antiserum and protein-A gold. Similarly prepared sections were treated with the same dilution of primary antibody which had been preabsorbed with either salmon STC or with crude CS extract. Unstained sections were examined as above.

In order to characterize STC immunoreactive renal cells at the electron microscopic level, awana kidney tissue proximal to the CS was excised and fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer [pH 7.4] for 3 hr. Following washes [3 X 5 min] in 0.1 M phosphate buffer the tissue was transferred for 1 hr each to a graded series [10%, 20% and 30%] of sucrose in 0.1 M phosphate buffer. After snap freezing in liquid nitrogen the sample tissue was returned to a 30% sucrose in 0.1 M phosphate buffer solution and washed [3 X 5 min] in 0.1 M phosphate buffer. The tissue sample was cut at 50 μm thickness using a vibratome and further washed [2 X 5 min] in 0.1M phosphate buffer. Prior to immunostaining, endogenous peroxidase activity was blocked by incubating the sample in 3% hydrogen peroxide [H₂O₂] in methanol [1 part 30% H₂O₂ to 9 parts absolute methanol] for 30 min at room temperature. After a series of washes [3 X 5 min] in phosphate-buffered saline [PBS, pH 7.4] non-specific immunolabelling was reduced by incubating the tissue slices in 10% normal rabbit serum in PBS for 30 min at room temperature. The tissue sections were stained with anti-salmon STC [1:1000] and incubated overnight at 4°C in a moist chamber. Prior to each antibody or conjugate presentation the tissue sections were washed in PBS [3 X 10 min]. Following a 30 min incubation at room temperature with biotinylated secondary antibody [1:50] the tissue sections were incubated for a further 30 min at room
temperature with a peroxidase-antiperoxidase enzyme conjugate [1:50]. Following another series of PBS washes the sites of peroxidase activity were revealed by incubating the tissue sections in buffered H$_2$O$_2$ containing the electron dense 3,3-diaminobenzidine [15 µl of 30% H$_2$O$_2$ 0.06% 3, 3-diaminobenzidine in 0.05 M PBS pH 7.6, 50 ml]. After the sites of immunoreactivity were visualized by a dissecting light microscopy the tissue sections were trimmed of excess tissue, washed in PBS [3 X 10 min] and postfixed with 1% OsO$_4$ to enhance contrast. Following washes in 0.1 M phosphate buffer [3 X 10 min] the tissue sections were dehydrated in a graded series of ethanols and embedded in epoxy resin.

Control procedure included substitution of the primary antiserum with phosphate buffered saline and saturation of the probing antibody before staining with purified salmon STC [100 µg/ml] prepared in PBS. Routine electron microscopy was carried out using previously established protocols [Marra et al., 1992, 1994, 1995]. Ultrathin sections were cut with a glass knife on a Reichert OM U3 ultramicrotome, mounted on copper grids and examined with a Siemens Elmiskop 102 electron microscope.

**Western blot analysis**

The soluble fractions of the CS [100 µg/ml] and kidneys [200 µg/ml] from the different specimens were subjected to Western blot analysis using polyclonal anti-chum salmon STC serum as described previously [Marra et al., 1995, 1998]. The kidney samples from 5 specimens, excised of CS, flash frozen in liquid nitrogen and stored at -70°C, were pooled and homogenized in PBS [pH 7.3] containing phenylmethyisulfonylflouride. After centrifugation the soluble protein fraction was resolved by polyacrylamide gel electrophoresis [PAGE] in 12.5% gels either in the presence, or in the absence, of reducing agents [β-mercaptoethanol] and electroblotted onto nitrocellulose [Laemmli, 1971]. The procedure for Western blotting was similar to those described in Marra et al.
[1995, 1998] using a 1:40000 dilution of the chum salmon STC antiserum overnight at room temperature followed by a 1:1000 dilution of biotinylated goat anti-rabbit gamma globulin [Zymed, San Francisco, USA] and a 1:10000 dilution of alkaline phosphatase streptavidin [Zymed, San Francisco, USA]. Subsequent to each antibody presentation, the blots were washed [3 X 15 min] in TTBS [0.02 M Tris. 0.5 M NaCl, 0.05% Tween-20, pH 7.4] containing 1% bovine serum albumin and 1% normal goat serum. The sites of immunoreactivity were visualized by exposure to 0.004% nitroblue tetrazolium chloride and 0.003% 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt. Control blots were processed in a similar manner, however, in the absence of the probing antibody.

Results

Each specimen of silver arawana [Marra et al., 1995], butterflyfish, featherfin knifefish, and elephantnose possessed at least 2 oval, discrete, glandular appearing white corpuscles. Each corpuscle was located retroperitoneally in the mid-portion of each of the opisthonephric kidneys. Depending on the size of the specimen examined, the CS measured anywhere between 1-3 mm at their widest diameter, however, smaller corpuscular bodies were occasionally noted.

Immunohistochemistry

Each gland had a connective tissue capsule which ramified the parenchyma as septae containing the supporting neuro-vascular elements. Epithelial cells, grouped into lobules, made up the glandular parenchyma. The periodic acid-Schiff [PAS] reaction [results not shown] demonstrated the gland cells to be of variable staining intensity but the cytoplasmic granules of the majority of cells stained positively. In adjacent sections, cells immunoreactive to the probing antibody [Fig. 1a] were closely correlated with those cytoplasmic areas stained by the PAS
technique and the results were similar to those previously described [Marra et al., 1992, 1994, 1995]. Immunoreactivity of the cells confirm the corpuscles as the CS in each specimen. The specificity of the immunolabelling was confirmed by the low levels of background staining in the surrounding supporting tissues and by the abolition of the reaction in adjacent tissues under control conditions [Fig. 1b]. These results are indicative of the specificity of the chum salmon STC antiserum for the gland cells of the CS of the specimens we examined.

Interestingly, it was also noted that certain cells of the distal renal tubules, in all Osteoglossiformes examined, except the elephantnose [G. petersii], were also STC immunoreactive [Fig. 1c]. In light of these results we attempted immunohistochemistry, under the same conditions described, on Bouin's-fixed renal tissue from the ancient Neopterygii, Lepisosteus osseus, and the more modern teleost Anguilla rostrata. In both cases no extracorpuscular STC-like immunoreactivity was noted. The low levels of background staining in the surrounding connective, vascular, and nervous tissues is indicative of the specificity of the chum salmon STC antiserum for the STC-immunoreactive renal cells. These cells were also stained with an antiserum against trout STC [Wendelaar Bonga et al., 1989] but not with an antiserum made against sockeye salmon STC [Wagner et al., 1988]. The positive staining reaction was abolished in adjacent sections when the primary antiserum was preabsorbed with salmon STC [Fig. 1d].

**Routine electron microscopy and Immunocytochemistry**

At the electron microscopic level two epithelial cell types were identified in the CS of both the butterflyfish [P. buchholzi] and the elephantnose [G. petersii], a result similar to that previously described in the silver arawana [Marra et al., 1995]. The above suggestion was based on structural polymorphisms and on the variance in the appearance, arrangement and amount of their cellular organelles. The more numerous type corresponded to the type-1 cell previously
described in the CS of other actinopterygians [Ogawa, 1967; Youson and Butler, 1976; Wendelaar Bonga et al., 1977, 1980, 1989; Bhattacharyya et al., 1982; Marra et al., 1992, 1994, 1995] having numerous, apically located, membrane-bound secretory granules, abundant RER, free ribosomes, Golgi apparatus, and polymorphic mitochondria [Fig. 2a]. The type-2 cells had a less extensive protein synthesizing machinery and the granules tended to be smaller and fewer in number, similar to those features described for type-2 cells by Marra et al. [1994, 1995] in the gar and arawana. In contrast, the featherfin knifefish [C. chitala] had a single cell type having all the characters of the type-1 cell. Although there was some variation in fine structure between cells having these fine structural features, there did not appear to be a definitive type-2 cell as described in some other fishes [Bhattacharyya et al., 1982; Wendelaar Bonga et al., 1989; Marra et al., 1994, 1995].

When the gland cells were treated with the chum salmon STC antiserum in order to localize STC, gold particles were exclusively localized over the secretory granules of the CS cells [Fig. 2b]. STC immunoreactivity was abolished in the CS cells by preabsorbing the probing antibody with salmon STC or substituting NRS or PBS in place of the chum salmon STC antiserum, indicating that these cells were STC-specific [Fig. 2c]. In kidney tissues from arawana which had been incubated in the primary antibody and then again in DAB, reaction product was primarily localized in the cytoplasmic matrix of specific cells of the distal renal tubules. The cells were characterized by an abundance of mitochondria and smooth tubules. Cells of similar description have been termed mitochondria-rich cells or renal chloride cells in kidneys of other ray-finned fishes [Youson and Butler, 1988]. Adjacent cells, with few mitochondria and smooth tubules were devoid of reaction product. No reaction product was present in the mitochondrial matrix, in the tubular lumina or in any secretory granules. Instead the electron-dense deposits were seen in clumps in the matrix or associated with mitochondrial, tubular or plasma membrane. Deposition of reaction
product was also noted on collagen fibrils of the extracellular matrix and the endothelium of sinusoids below the reactive renal cells. Negative controls showed no reaction product.

Western blot analysis

Western blot analysis of butterflyfish, featherfin knifefish, and elephantnose CS extracts under reducing conditions [presence of β-mercaptoethanol] yielded distinct immunoreactive bands [Fig. 3]. Under these conditions the putative butterflyfish STC monomer migrates with an apparent molecular weight of 24 kDa [Fig. 3b] whereas elephantnose STC migrates as a 23 kDa band under the same reducing conditions [Fig 3e]. The featherfin knifefish putative CS STC monomer appears as 3 immunoreactive bands of 26, 23, and 18 kDa [Fig 3d]. Arawana CS extracts previously subjected to Western blot analysis yielded monomers in the range of 28-32 kDa [Marra et al., 1995]. Interestingly, when the soluble fraction of the kidney samples were examined by Western blotting under non-reducing conditions we were unable to detect an immunoreactive signal. However, in the presence of β-mercaptoethanol an immunoreactive band which migrated in the size range of the putative CS STC monomer was observed [Fig. 3c] in all specimens except G. petersii, the elephantnose [Fig. 3f]. STC-like immunoreactivity in all the bands could be abolished by preabsorption of the primary antiserum with either salmon STC or if normal rabbit serum or PBS was used in place of the probing antiserum.

Discussion

This study is the first to examine in detail the distribution of STC in several representatives of the most ancient group of Teleostei, the Osteoglossiformes. Such an examination of the more ancient forms of this hormone, and the endocrine tissue responsible for its production, has potential value for understanding both the nature and the phylogenetic and
ontogenetic history of STC. In addition, such examinations of "soft-tissue" characters may lend some insight into phylogenetic relationships among similar species and provide independent evidence when comparing living species from an evolutionary viewpoint. This is especially significant in light of the difficulty in clarifying basal vertebrate evolution [De Pinna, 1996].

The light and electron microscopic observations of the present study were consistent with those of previous examinations of the CS in other piscine species [Ogawa, 1967; Youson et al., 1976; Wendelaar Bonga et al., 1977; Bhattacharyya et al., 1982; Marra et al., 1992, 1994, 1995]. The CS of the species of the Osteoglossiforme order that we examined consist of either one or two principal cell types commonly referred to as type-1 and type-2 cells depending upon the species examined. This cellular characterization is based upon the elaboration of their protein synthesizing machinery [see Wendelaar Bonga et al., 1980, 1989 for a more detailed examination]. Immunocytochemistry revealed in each of the specimens examined that the content of the secretory granules was immunoreactive with antiserum generated against chum salmon STC. The fact that we could completely abolish the immunostaining, by preabsorbing the primary antibody with STC, suggests that the species-specific forms of the Osteoglossiforme hormone share a common epitope which is recognized by the antiserum.

This is the first report of renal STC-like immunoreactivity in species that possess the CS. It may be of phylogenetic interest that the CS-extracorpucular distribution is found in Osteoglossidae and Notopteridae families but absent in the Mormyridae family. At present, there is discord with respect to the monophyly of the members of the Osteoglossiformes [Nelson, 1994]. Evidence suggests that the Osteoglossininae subfamily [including Pantodon] form a monophyletic clade and that the notopterids and mormyroids form a monophyletic clade [Lauder and Liem, 1983]. However, our results infer that with respect to the distribution of STC-like
immunoreactivity alone, *G. petersii* [elephantnose] is an out-lier whereas *O. bicirrhosum*, *P. buchholzi*, and *C. chitala* are more closely related.

These results do not provide any insight into what may be a functional specialization of STC in these species. However, we noted that the CS of these species are relatively smaller than those of other species we have examined [e.g. compared to white sucker of similar size, Marra et al., 1998]. Therefore, extracorporeal sites of STC production may compensate for the small size of the CS. Furthermore, this extracorporeal source of STC may function in a paracrine rather than an endocrine manner. The presence of STC-like immunoreactive cells with an ultrastructure similar to renal mitochondria-rich cells suggests a role of the hormone in renal ion movement implicative of the now established role of STC on renal phosphate movement [Lu et al., 1993; Wagner et al., 1997]. Youson and Butler [1988] suggested that renal mitochondria-rich cells might be expected to be involved in mineral metabolism. The effects of STC on renal tissue have now been well established [Lu et al., 1993; Wagner et al., 1997]. Osteoglossiformes are exclusively freshwater in nature [Guo-Qing and Wilson, 1996], and therefore, phosphate homeostasis would be a definite challenge. A renal source of STC would be an asset to this aspect of mineral metabolism and may be important to the phylogenetic development of the Osteoglossomorpha.

Our previous results on the CS of the more ancient Actinopterygii showed STC immunoreactivity to be localized to the CS in the bowfin, gar, and the arowana [Marra et al., 1992, 1994, 1995]. Whereas in the bowfin several hundred CS are scattered throughout the kidney, many still in close association with renal tubules, in the gar 5-7 CS are localized to areas devoid of nephric tissue and abundant with hemopoietic tissue [Marra et al., 1992, 1994]. In the arowana, one of the most ancient teleosts, several CS are more posteriorly localized and autonomous from the kidneys [Marra et al., 1995]. Interestingly, in the mammals, STC immunoreactivity is localized to certain cells of the kidney [Wagner et al., 1995]. Furthermore, it has been suggested that an
increase in the number of CS glands associated with the middle of the mesonephric kidneys represents a more ancestral organization, whereas, a reduction in CS number associated with a more posterior distribution may be characteristic of more advanced species [Bouchot, 1953; Youson et al., 1976]. In light of the above observations these present results may be significant from an evolutionary viewpoint since the STC immunoreactive profile which we now describe among the Osteoglossomorpha may represent an intermediate phylogenetic arrangement between the more ancient vertebrates and those with a more modern lineage. These results are also supportive of recent research on the ontogeny of the CS [Kaneko et al., 1992] which suggests that the CS develop from individual cells which first appear among the epithelial cells of the embryonic nephric ducts.

Our previous results showed that arawana STC is one of the largest STCs, migrating with a molecular weight of 68 kDa, and almost similar to that of the gar with an estimated monomer size between 28-34 kDa [Marra et al., 1995]. Western blot analysis of butterflyfish, featherfin knife fish, and elephantnose CS extracts revealed that the STC monomer from these specimens was between 2-11 kDa smaller than that of the arawana. At present it remains unknown whether these size differences are due to polypeptide chain length or variability in glycosylation. Interestingly, the featherfin knifefish CS extracts demonstrated several molecular weight forms of STC. Although this result could be accounted for by degradative pathways, this is unlikely since all samples were prepared in the same manner. A more likely explanation, however, is that in the featherfin knifefish these different molecular weight species of STC represent post-translationally modified and heterogeneous forms of the hormone. This suggestion is not unreasonable since Wagner et al. [1991, 1992] identified seven forms of the STC monomer in salmon CS extracts and six molecular weight forms circulating in trout plasma.
This is the first time that STC-like immunoreactivity has been reported for piscine renal tissue extracts. Interestingly, we were unable to detect a non-reduced form of osteoglossomorph "renal STC" even though our light and electron microscopic observations demonstrated the presence of an immunoreactive target in the kidney tissue. Initially, it was assumed that this inability to detect a non-reduced form of STC was related to one of sensitivity since it was difficult to estimate the relative content of the STC-like target in the kidney tissue. However, this assumption was rejected since equal loading of the extracts in the presence of a reductant demonstrated robust STC-like immunoreactive bands in all specimens except the elephantnose, a finding which was consistent with our immunohistochemical results. These Western blot observations may infer that the tertiary structure of the renal forms of STC has been modified such that the epitope is somehow unavailable to the primary antibody except when the putative hormone is in the reduced state.

The present results support the earlier finding that suggested that more than one form of STC may be present in some piscine vertebrates [Wagner et al. 1991, 1992, 1998]. These heterogeneous forms of STC could arise from either the STC message being post-transcriptionally modified or by the expression of various STC-related genes. Wagner et al. [1992] were the first to suggest that there may be more than one copy of the STC gene. Whether the STC primary transcript is differentially processed or whether a gene duplication event has occurred during the evolution of this hormone remains to be determined and awaits an examination at the molecular level.

In summary, we have identified STC-like immunoreactivity in the CS and renal cells of certain members of the Osteoglossomorpha subdivision. This immunoreactive profile may be due to the tissue-specific expression of two different STC-related mRNAs or a differentially processed primary transcript. It remains to be determined if this extracorpuscular STC profile is
unique to this order or is more widespread in the bony fishes. It is an intriguing possibility that the arowana kidney form of STC may represent the evolutionary fore-runner of the more modern forms of this hormone, for example, the form found in the mammalian kidney. An examination of arowana STC at the molecular level should provide insight into such a possibility.
Figure 1a–c. a Butterflyfish corpuscle of Stannius [CS] stained with a 1:1000 dilution of anti-salmon STC serum using the peroxidase anti-peroxidase method and counterstained with haematoxylin. The CS gland cells of the silver arawana [Marra et al., 1995], featherfin knifefish, and the elephantnose react equally as strong to the primary antibody. b Adjacent section to [A] stained with anti-salmon STC serum preabsorbed with salmon STC. Note that the immunoreactivity is abolished over the CS. c Certain cells in the distal renal tubules of the butterflyfish kidney react strongly to the primary anti-salmon STC antibody [arrows]. A similar STC immunoreactive profile was also observed in the distal renal tubule cells of the silver arawana and the featherfin knifefish. Interestingly there was no extracorpuscular STC-like immunoreactivity in the kidney of the elephantnose, *Gnathonemus petersii.* X750. d Adjacent section to [C] stained with anti-salmon STC serum preabsorbed with salmon STC. The renal tubule immunostained in [C] is outlined. X400a, b, X750c, d
Figure 2a-d. a Routine electron micrograph demonstrating the follicular arrangement of the butterflyfish CS gland cells. The type-1 cells \( [t_1] \) are characterized by the more numerous, larger and apically located secretory granules \( [arrows] \). The type-2 \( [t_2] \) cells have smaller granules. A similar fine structure was observed in the arawana [Marra et al., 1995]. The featherfin knifefish and the elephantnose CS possess only the type-1 cell. b Protein A-gold particles are preferentially located over the secretory granules \( [arrows] \) of the featherfin knifefish CS gland cells following immunocytochemistry with anti-salmon STC serum. A similar reaction was observed in the CS gland cells of the arawana [Marra et al., 1995], butterflyfish, and elephantnose. c Featherfin knifefish CS gland cell stained with the probing antibody which was preabsorbed with salmon STC. The secretory granules \( [arrows] \) are unstained. d Immunocytochemistry of an arawana STC immunoreactive renal cell. Electron-dense reaction product is localized within mitochondrial rich cells of the distal tubule but is concentrated within the cytoplasmic matrix near smooth tubules \( [t] \). Note the absence of product in adjacent cells with fewer mitochondria. Deposits are associated with collagen \( [arrows] \) in the extracellular matrix and on endothelium. X7000a, X37000b, X36000c, X30000d
Figure 3. Corpuscles of Stannius from butterflyfish [B], butterflyfish kidney [C], featherfin knifefish kidney [D], and elephantnose CS [E] were homogenized directly in SDS sample buffer containing mercaptoethanol and subjected to SDS electrophoresis in a 12% polyacrylamide gel. The resolved proteins were transferred to nitrocellulose and probed with a 1:40000 dilution of anti-chum salmon STC serum. Putative butterflyfish CS STC and kidney STC migrate with an apparent molecular weight of 24 kDa. Featherfin knifefish STC appears as 3 immunoreactive bands of 26, 23, and 18 kDa, whereas elephantnose STC migrates as a 23 kDa monomer. Elephantnose kidney preparations did not yield any immunoreactive bands [F] when processed in the same manner. Arawana CS extracts previously subjected to Western blotting yielded monomers in the range of 28-32kDa [Marra et al., 1995]. Salmon STC [A] served as a positive control. Preabsorption of the probing antibody with salmon STC abolishes the immunoreactive bands.
CHAPTER 5

Corpuscles of Stannius and stanniocalcin-like immunoreactivity in the white sucker (Catostomus commersoni) and evidence for the presence of a new cell type

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ABSTRACT

The distribution of stanniocalcin immunoreactivity was examined in the corpuscles of Stannius of the white sucker \textit{[Catostomus commersoni]} by using a chum salmon stanniocalcin antiserum, Western blotting, and light and electron microscopy. The white sucker possesses at least two stanniocalcin-immunoreactive corpuscles in the most posterior portion of the kidneys. Immunocytochemistry and ultrastructure revealed two cell types making up the corpuscle parenchyma, only one of which was immunoreactive. The nonimmunoreactive cells contained dense-cored vesicles and long processes that extended between the immunoreactive cells and terminated at the perivascular spaces. When corpuscle extracts were subjected to electrophoresis and Western blotting, three non-reduced bands [approximately 56, 61, and 64 kDa] were observed. However, in the presence of a reductant, a diffuse band migrating in the range of 28 kDa to 32 kDa was noted. The results of this study of the white sucker demonstrate the presence of a dimeric stanniocalcin-like molecule and present evidence for the presence of a previously uncharacterized cell type in the corpuscles of Stannius.
Introduction

The corpuscles of Stannius [CS] are small, kidney-associated endocrine glands, which, to date, have only been identified in bony fishes [actinopterygians], namely the Neopterygii. The CS seemingly play an important role in the ability of these animals to efficiently regulate their plasma calcium concentration, despite the varied environments which they often inhabit. The parenchymal cells of the CS, in response to elevations in calcium levels, impart this ability by synthesizing and secreting a homodimeric glycoprotein hormone called stanniocalcin [STC]. This hormone, with an apparent molecular weight ranging from 39 kDa in salmon [Wagner et al., 1986] to 60 kDa in the American eel [Flik et al., 1989] exerts a hypocalcemic effect by blocking calcium transport across the gills [So and Fenwick, 1979; Lafaber and Perry, 1988; Wagner et al., 1988a; Verbost et al., 1993], the gastrointestinal tract [Sundell et al., 1992], and possibly also by stimulating phosphate reabsorption at the nephrons [Lu et al., 1994].

In addition to the implication of a calcium homeostatic role for stanniocalcin, earlier physiological studies showed persistent pressor activity in corpuscle extracts [Chester-Jones et al., 1966]. Indeed, it is necessary to acidify and neutralize corpuscle extracts in order to abolish endogenous pressor effects prior to gill perfusion studies [So and Fenwick, 1982]. Based on the observation that CS of the European eel contain a pressor-like substance, Chester-Jones et al. [1966] speculated that these glands are related to a renin-angiotensin system. Subsequently, it was demonstrated that angiotensin I is produced when chum salmon and Japanese goosefish corpuscle extracts are incubated with homologous plasma [Takemoto, 1983; Hasegawa et al., 1984]. More recently, Butler and Oudit [1994, 1995] provided evidence regarding of a role for the CS in the cardiovascular physiology of freshwater eels. It is not presently known whether any morphologically distinct cell type is present in the CS that could be a site for the synthesis and secretion of a pressor substance or even whether a distinct cell type is necessary. It is possible that
the same parenchymal cell type could produce more than one physiologically distinct product or that the same product [stanniocalcin] can have different physiological effects.

Morphological observations on the CS suggest that the parenchyma consists of one or two structurally distinct cell-types [type-1 and type-2] both demonstrating an ultrastructure consistent with protein-producing endocrine cells [Krishnamurthy and Bern, 1971; Youson and Butler, 1976; Wendelaar Bonga and Pang, 1986; Hirano, 1989; Marra et al., 1992, 1994, 1995]. Whereas type-1 cells are characterized by extensive rough endoplasmic reticulum, a Golgi apparatus, and numerous large secretory granules, type-2 cells have a less extensive protein-synthesizing machinery. Immunocytochemistry has revealed that reactivity is localized over the secretory granules of both type-1 and type-2 cells when an antibody to teleost stanniocalcin is used. This result suggests that the two cells represent different phases of a single cell or two cells producing structurally similar products [Wendelaar Bonga et al. 1989]. There is evidence for the association of nerve terminals with both type-1 cells and type-2 cells [Marra et al. 1995].

We are currently investigating the diversity of both corpuscle morphology and stanniocalcin-like proteins among the Neopterygii. The present study provides evidence for a stanniocalcin-like protein in the CS of the white sucker, a member of the Order Cypriniformes and Superorder Ostariophysi. We also describe a potentially new cell type in these corpuscles. This cell-type has a distinct morphology, including vesicles with no stanniocalcin-immunoreactivity.

Materials and Methods

Animals and tissue collection

Adult white suckers [Catostomus commersoni] measuring from 30-50 cm in length were trap-netted during their spawning migration in the Humber River [Toronto, Ontario, Canada] and transported to the freshwater facilities at the Scarborough Campus of the University of Toronto.
Juvenile white suckers measuring 10-15 cm in length were trap-netted in the Lake Nipissing watershed [North Bay, Ontario, Canada] and obtained live from a local fish supplier. After a period of acclimatization [approximately 1 week] in tanks containing 18°C, dechlorinated, aerated water, the specimens were sacrificed by an overdose of tricaine methanesulfonate. The retroperitoneal kidneys were exposed following a midventral, [cloacal-pericardial] incision and removal of the viscera. The CS from 15 adult and 11 juveniles were removed and immediately prepared for light and electron microscopy or Western blotting.

*Primary antisera*

Immunolabelling studies employed a polyclonal antisera to chum salmon STC. Antisera to sockeye salmon stanniocalcin and rainbow trout stanniocalcin were also used. All antisera were previously characterized by Western blotting or radioimmunoassay and by immunocytochemistry [Kaneko et al. 1988, 1992; Wagner et al. 1988b].

*Immunohistochemistry*

The Peroxidase-labeled streptavidin-biotin method [Elias et al., 1989] was used to localize sites of stanniocalcin immunoreactivity in CS that had been fixed previously in Bouin’s fluid for 24 h, dehydrated in a graded ethanols, and embedded in paraffin. Sections were cut at a thickness of 7 μm and mounted on glass slides. They were deparaffinized, rehydrated and equilibrated in TRIS-buffered saline. Chum salmon stanniocalcin antiseraum [1:1000] was applied to some sections, and an immunostaining kit [Zymed, San Francisco, Calif., USA; catalog no. 95-6143] was used as instructed by the manufacturer. Negative controls on adjacent sections included antiseraum preabsorbed with salmon stanniocalcin, antiseraum preabsorbed with crude corpuscle extract and normal rabbit serum or phosphate-buffered saline substituted for antiseraum. Labeled tissue sections
were counterstained with hematoxylin to enhance the contrast. Adjacent, untreated sections were also stained with the periodic acid-Schiff reaction [Marra et al. 1992].

**Conventional electron microscopy**

CS were fixed in 2% glutaraldehyde [pH 7.3] in 0.1 M Millonig’s phosphate buffer, rinsed in buffer, postfixed in 1% aqueous OsO₄ in the same buffer, dehydrated in a graded ethanols, treated with propylene oxide and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 102 transmission electron microscope.

**Immunocytochemistry**

The procedures for immunocytochemistry were similar to those described by Wendelaar Bonga et al. [1989] and Marra et al. [1992, 1994, 1995]. The CS were treated as for conventional electron microscopy, but postfixation was omitted to optimize antigenicity. Ultrathin sections were mounted on nickel grids and immunostained with chum salmon stanniocalcin antiserum [1:1000] and protein-A gold. Similarly processed sections were treated with antibody preabsorbed with either salmon stanniocalcin or corpuscle extract. Unstained sections were examined as above.

**Western blotting**

The soluble fraction of the CS was subjected to electrophoresis and Western blotting. The CS from 10 specimens, flash frozen in liquid nitrogen and stored at -70°C, were pooled and homogenized in phosphate-buffered saline [pH 7.3] containing phenylmethylsulfonylfluoride. The corpuscle homogenate was centrifuged at 14000 g for 60 min at 4°C, and the supernatant was extracted. Following a 1:1 dilution in sodium dodecyl sulfate sample buffer, the crude corpuscle
extract was heated for 5 min at 100° C, and the proteins were separated by polyacrylamide gel electrophoresis in 12.5% gels [with or without reducing agents] at a concentration of 0.01 mg/lane. The procedure for Western blotting was similar to that of Marra et al. [1995]. Briefly, the gels were equilibrated for 90 min at room temperature in transfer buffer [0.0025 M Tris, 0.192 M glycine and 20% methanol]. After the electrophoretic transfer [250 mA, 2 h] of resolved proteins to nitrocellulose, the blots were blocked for 1 h at room temperature in Tween-TRIS-buffered saline [0.02 M TRIS, 0.5 M NaCl, 0.05% Tween-20, pH 7.4] containing 1% bovine serum albumin and 1% normal goat serum. Subsequently, the blots were incubated with chum salmon stanniocalcin antiserum [1:40000] overnight at room temperature. The blots were then incubated for 30 min in biotinylated goat anti-rabbit gamma globulin [1:1000; Zymed] followed by a 30 min exposure to a alkaline phosphatase streptavidin [1:10000; Zymed]. Subsequent to each antibody incubation, blots were washed [3 X 15 min] in Tween-TRIS-buffered saline containing 1% bovine serum albumin and 1% normal goat serum. Following the last series of washes, the blots were incubated in alkaline phosphatase buffer [0.1 M TRIS, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5] for 15 min and the sites of immunoreactivity were visualized by exposure to 0.004% nitroblue tetrazolium chloride and 0.003% 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt. Control blots were processed in a similar manner, but without the probing antibody.

Results

Adult and juvenile white suckers possess at least two discrete ovoid white corpuscles. The corpuscles usually occupy retroperitoneal positions in the posterodorsal surface of the opisthonephric kidneys. They measure 2-3 mm at their widest points in the adult, although smaller corpuscles are noted occasionally. In juveniles, substantially smaller [0.5-1.0 mm in diameter] CS are observed. Each gland had a collagenous capsule that sends connective tissue septa, containing
the supporting neural and vascular elements, into the parenchyma. Parenchymal cells are grouped into lobules. The periodic acid-Schiff reaction [results not shown] reveals that parenchymal cells have a variable staining intensity, although the cytoplasmic granules of most cells stained positively. In adjacent sections, cells immunoreactive to the probing antibody are correlated closely with those cytoplasmic areas stained by the periodic acid-Schiff reaction [fig. 1a]. Although some nonreactive cells have been observed in each corpuscle [fig. 1b], the periodic acid-Schiff staining and the immunoreactivity of most cells confirm the cell mass to be a CS. Similar results have been observed with sockeye salmon and rainbow trout STC antisera [results not shown]. The low levels of background staining in the surrounding connective, vascular, and nervous tissues are indicative of the specificity of the chum salmon STC antiserum for the parenchymal cells of white sucker. In addition, the staining reaction is abolished in adjacent sections when antiserum is preabsorbed with either salmon STC or corpuscle extract or if normal rabbit serum or phosphate-buffered saline is substituted for the antibody [fig. 1c].

Two cell types have been identified in the CS of both the adults and juveniles, based on structural features and location, arrangement and content of organelles. The more numerous cell-type corresponds to the type-1 cell described in other actinopterygians [Ogawa, 1967; Youson and Butler, 1976; Wendelaar Bonga et al., 1977, 1980, 1989; Bhattacharyya et al., 1982; Marra et al., 1992, 1994, 1995]. These cells are intimately associated with fenestrated capillaries at their perivascular surfaces and are characterized by numerous, apically located, membrane-bound secretory granules of various sizes [Fig. 2, 3]. Further, the cells contain eccentrically located, spherical to ovoid nuclei, often with prominent nucleoli and a moderate amount of patchy heterochromatin [figs. 2, 3]. Abundant rough endoplasmic reticulum, free ribosomes, a Golgi apparatus, and mitochondria of various sizes and shape are prominent components of this cell [figs. 2, 4, 5]. Although there is some variation in ultrastructure between cells having these features,
there does not appear to be a definitive type-2 cell as described in other fishes [Bhattacharyya et al., 1982; Wendelaar Bonga et al., 1989; Marra et al., 1994]. We shall refer to all cells with the general features described above as “principal cells”.

Another cell-type in the white sucker CS extends long processes between the principal cells [fig. 2-6]. The epithelial nature of this cell type is illustrated by junctions with the plasma membranes of adjacent principal cells [fig. 4]. The cytoplasmic processes of this novel cell type extend to the basal laminae near the perivascular spaces where they interdigitate with processes of the principal cells [fig. 2, 3]. This second cell-type, called the “minor cell” since it is less numerous than the principal cell, is characterized by numerous dense-cored vesicles of variable electron density with single loose-fitting membranes. The dense-cored vesicles are particularly concentrated in the cell processes [fig. 2-6]. A large, centrally located nucleus possesses finely dispersed chromatin. This cell in the juvenile frequently has a polymorphic nucleus [fig. 6b]. Numerous free ribosomes, many polymorphic mitochondria, and an extensive internal membrane network of rough endoplasmic reticulum and Golgi apparatus take up most of the remaining cytoplasm [figs. 2-6]. Multivesicular bodies and vacuoles of various shapes and sizes are also present in moderate numbers throughout the cytoplasm. The cytoplasmic matrix contains microtubules and glycogen particles [fig. 3, 5].

When cells were treated with the chum salmon STC antiserum, gold particles localized exclusively over the secretory granules of the principal cells [fig. 7]. A range of antibody dilutions has failed to generate specific staining of the vesicles in the minor cells [fig. 7] in either population or age class of white sucker. Similar results have been obtained when either the sockeye salmon or the rainbow trout STC antiserum is used [results not shown]. STC immunoreactivity is abolished in the principal cells by preabsorbing the antibody with salmon STC or corpuscle extract, or
substituting normal rabbit serum or phosphate-buffered saline for the chum salmon antiserum [fig. 8].

Western blot analysis of corpuscle extracts under non-reducing conditions yields three distinct bands of approximately 56 kDa, 61 kDa, and 64 kDa, perhaps representing the mature form, pre-form, and prepro-form of white sucker STC, respectively [fig. 9]. However, an analysis of the primary structure of the white sucker STC molecule should clarify this interpretation. Under reducing conditions, in the presence of β-mercaptoethanol, a diffuse immunoreactive band migrating between 28 and 32 kDa is observed [fig. 9]. STC-like immunoreactivity in all bands is abolished by preabsorption of the primary antiserum with either salmon STC or corpuscle extract or by the replacement of the probing antiserum with normal rabbit serum and phosphate-buffered saline.

Discussion

In the white sucker a single large CS is located near the posterodorsal surface of each kidney. This distribution pattern is suggestive of a more recent phylogenetic organization of these glands in the bony fishes [Garrett, 1942; de Smet, 1962; Youson et al., 1976]. Immunological observations in the present study are consistent with previous studies on the CS and STC of other teleosts [Wagner et al., 1988b; Kaneko et al., 1988; Lafeber et al., 1988; Wendelaar Bonga et al., 1989; Marra et al., 1995]. Immunohistochemistry has demonstrated a strong reaction of the principal cells to three teleost STC antisera. However, our results also demonstrate that not all parenchymal cells are reactive to the probing antibodies, and electron microscopy provided an explanation for the diversity in immunoreactivity.

Ultrastructural observations have revealed two morphologically distinct cell-types, and only one of these is reactive to anti-STC serum. The ultrastructure of this reactive cell-type
[principal cell] is consistent with previous electron-microscopic observations on type-1 cells [Ogawa, 1967; Krishnamurthy and Bern, 1969; Youson et al., 1976; Wendelaar Bonga et al., 1977, 1989; Bhattacharyya et al., 1982] and the immunoreactivity is confined exclusively to the secretory granules.

We report here the first epithelial cell-type that is not reactive to STC antibodies, in the CS of a bony fish. This cell-type [minor cell] is found in the CS of white suckers from two isolated populations. Earlier ultrastructural observations of parenchymal cells have demonstrated the presence of one or two morphologically distinct, protein synthesizing cell-types [type-1 and type-2], which subsequent studies have revealed to be immunologically similar [Wendelaar Bonga et al., 1989]. Type-1 and type-2 cells have not been discerned in the CS of white suckers, although the distinction may have been masked by the structural diversity of the principal cells. Minor cells appear to be neither morphologically nor immunologically similar to principal cells, and therefore, it is unlikely that there is a functional similarity. The minor cell, with its numerous STC-negative dense-cored vesicles, protein synthesizing organelles, and long interdigitating processes, shows greater similarity to a neuroendocrine or neurosecretory cell [Zaccone et al., 1995]. Since the probing antibody is capable of detecting a shift in molecular weight and structure of STC after glycopeptidase-F removal of the N-linked glycomoiety during Western blotting, this would preclude the product of the minor cells from being a nonglycosylated form of STC [Butkus et al., 1987; Flik et al., 1989]. Furthermore, few if any endocrine tissues are committed to a single function. Whether by post-transcriptional processing, by post-translational modifications, or by the presence of morphologically and functionally distinct cell-types, most endocrine tissues produce a variety of physiologically active and distinct products. Thus, the CS may follow this vertebrate pattern.
This is the first time that a sample of white sucker CS has been examined by immunological techniques. Western blot analysis has demonstrated a putative mature STC form with a molecular weight approximating 56 kDa, similar to that in other teleost species, such as trout, salmon, and eel [Wagner et al., 1986, 1992; Lafeber et al., 1988; Flik et al., 1989]. The dimeric nature [Butkus et al., 1987; Lafeber et al., 1988; Wagner et al., 1986, 1988, 1992] of the white sucker STC molecule was demonstrated by the approximate doubling in apparent molecular weight in the absence of a reductant.

STC has the capacity to reduce plasma calcium levels in fishes by inhibiting net calcium influxes from the water [Wagner et al., 1986; Hanssen et al., 1989; Perry et al., 1989]. Earlier research into the physiology of the CS suggested a pressor role for these glands [Chester-Jones et al., 1966; Ogawa and Sokabe, 1982]. Recently, Butler and Oudit [1994, 1995] have shown that altered patterns of blood flow to osmoregulatory organs such as the gills, kidney, and skin, in addition to a reduction in cardiac output, follows the removal of the CS. The modulation of branchial calcium flux is important for maintaining plasma calcium levels [Flik et al., 1985; Verbost et al., 1993]; however, a significant portion of the whole-body calcium uptake occurs through the skin [Mashiko and Jozuka, 1964; Perry and Wood, 1985; McCormick et al., 1992]. The unique position of the non-reactive minor cells between the reactive principal cells at the vascular space is particularly interesting and might provide an optimal environment for the detection of ionic or cardiovascular changes. Furthermore, the minor cells might function in a paracrine manner, producing bioactive product(s) that influence(s) the STC-containing principal cells, a situation similar to the effects of somatostatin on the insulin- and glucagon-producing cells of the endocrine pancreas [Plisetskaya and Duguay, 1993]. This viewpoint is interesting since it has been demonstrated that STC release is influenced by several factors [Lafeber and Perry, 1988; Glowacki et al., 1990; Wagner et al., 1991; Hanssen et al., 1991; Cano et al., 1994] suggesting
other levels of control for the release of the product(s) of the CS. In addition, it has been demonstrated that the removal of the CS from the eel and killifish leads to hypercalcemia, which can be alleviated by homotransplantation of these glands [Chan et al., 1969; Fenwick and Foster, 1972; Pang et al., 1973; Schreibman and Pang, 1975]. These transplanted CS are able to function normally despite vascular and neural ablation, suggesting that minor cells function in a local sensory control capacity. However, the presence and distribution of this cell-type in other teleosts has not been reported.

Little evidence exists for active agents other than STC being synthesized and released by the CS. Previously, we have identified dense-cored vesicles within cytoplasmic processes or terminals that are interspersed amongst the parenchymal cells of an ancient teleost, the arawana, *Osteoglossum bicirrhosum* [Marra et al., 1995]. In light of our present results, which demonstrate the presence of a cell-type nonreactive to STC antibody in the white sucker CS, a more detailed search for minor cells in the CS of other bony fishes is warranted.
Figure 1a-c. Adjacent sections of a white sucker corpuscle of Stannius stained by salmon stanniocalcin antiserum [a, b] or salmon stanniocalcin antiserum preabsorbed with salmon stanniocalcin [c]. Most parenchymal cells react strongly to the antibody [arrowheads in a, b]; however, some cells are nonreactive STC [arrow in b]. X250a-c, X650b. Bars: 25 μm a, c, 50 μm b.
Figure 2. Principal cells \([P]\) with abundant secretory granules \([g]\) and minor cells \([M]\) with numerous dense-cored vesicles \([\text{arrowheads}]\) are evident. Long processes \([\text{arrows}]\) of the minor cells interdigitate with the principal cells and abut the perivascular spaces of fenestrated blood vessels \([BV]\). \([G\text{ Golgi apparatus}].\ X9000. \ Bar: 10 \mu m.\)

Figure 3. Process from a minor cell \([M]\) extending between principal cells \([P]\) to a perivascular space. A fenestrated capillary \([\text{short arrows} \text{ fenestrae}]\) contains a red blood cell \([RBC]\). Numerous membrane-bounded, dense-cored vesicles \([\text{arrowhead}]\) of variable electron density appear in the minor cell. The cytoplasmic matrix contains microtubules \([\text{long arrow}]\) and glycogen particles \([gl]\). X36000. \ Bar: 0.5 \mu m.
**Figure 4.** A minor cell has a large nucleus [\(Nu\)] with finely dispersed heterochromatin, a network of rough endoplasmic reticulum [\(er\)], polymorphic mitochondria [\(m\)], and dense-cored vesicles [arrowhead]. A junction [arrow] is apparent between a minor cell and a principal cell [\(P\)]. The principal cell has large cytoplasmic granules [\(g\)]. X16800. *Bar: 10 \(\mu m\).*

**Figure 5.** Processes [asterisks] of minor cells extending between principal cells [\(P\)]. Note the variable electron density of the membrane-bounded vesicles [arrowheads], glycogen particles [\(g\)], and mitochondria [\(m\)] in the cytoplasm of the processes. Large cytoplasmic granules [\(g\)] characterize the principal cells. X37500. *Bar: 0.5 \(\mu m\).*
Figure 6a-b. Comparable fields of minor cells from adult [Humber River, a] and the juvenile [Lake Nipissing, b] white suckers. In both populations, minor cells [M], with small dense-cored vesicles [arrowheads], are located amongst the principal cells [P] and abut on the perivascular spaces [v]. The minor cell of the juvenile has a more polymorphic nucleus [Nu] than that of the minor cell of the adult. X10800a, X14400b. Bars: 1 μm.
Figure 7. Protein A-gold particles localized over the secretory granules [g] of the principal cells, indicating a primary site of stanniocalcin localization. No gold particles are seen over the dense-cored vesicles [arrowheads] of the minor cells. X30000. Bar: 0.3 μm.

Figure 8. Absorption of the salmon stanniocalcin antibody with salmon stanniocalcin abolishes immunostaining over secretory granules [g] of the principal cells. [Nu nucleus]. X45000. Bar: 0.5 μm.
Figure 9. Western blot of corpuscles of Stannius under non-reducing [a] and reducing [c] conditions. Putative stanniocalcin appears as three bands [approximately 64, 61, and 56 kDa]. In the presence of mercaptoethanol [c], a diffuse band migrates between 28 and 32 kDa. Preabsorption of the salmon stanniocalcin antibody with salmon stanniocalcin [b], or with corpuscle extract [d] abolishes the bands.
CHAPTER 6
CHAPTER 6

Molecular cloning of white sucker [Catostomus commersoni] stanniocalcin

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ABSTRACT

A description is provided of the molecular cloning and cDNA sequence analysis of stanniocalcin [STC] from mRNA in the corpuscles of Stannius [CS] of the white sucker [Catostomus commersoni] using R.A.C.E. [rapid amplification of cDNA ends]. Sequence analysis of the cDNA revealed that white sucker STC mRNA has an open reading frame of 756 bp encoding a 252 amino acid primary translation product containing an odd number of Cys residues, an N-linked glycosylation site, and several potential post-translational cleavage and phosphorylation sites. White sucker STC shares a considerable degree of sequence similarity with eel, salmon, and human stanniocalcin. Consistent with the presently accepted taxonomic position of the Cypriniforme order, the deduced amino acid sequence of white sucker STC is intermediate to those of the coho salmon and the Australian eel STCs.
Introduction

Stanniocalcin [STC], is the principal hypocalcemic factor originally associated only with the Osteichthyes [bony fishes]. This glycoprotein hormone, the molecular weight of which is variable, is synthesized and secreted by kidney-associated endocrine glands referred to as the corpuscles of Stannius [CS]. Stanniocalcins have been isolated and partially sequenced [N-terminal 40 residues] from several salmonid species [Wagner et al., 1986, 1988; Lafeber et al., 1988a; Sundell et al., 1992] and the Australian eel [Butkus et al., 1987]. The nucleotide sequence of STC mRNA and the deduced amino acid sequence have been reported from the Australian eel [Butkus et al., 1987] and coho salmon [Wagner et al., 1992]. The complete amino acid sequence of STC isolated from chum salmon [Yamashita et al., 1995] has been elucidated. Most recently, the mammalian equivalent of this calcitropic hormone has been described [Chang et al., 1995, 1996; Olsen et al., 1996] suggesting a widespread existence and a long evolutionary history of STC. Interestingly, whereas the synthesis and secretion of STC appears to be confined to the CS in the bony fishes, the expression of the STC gene in mammals is distributed among a variety of tissues [Chang et al., 1995].

STC is a glycosylated homodimeric protein consisting of two identical, disulfide-linked polypeptide chains with a distinct primary structure among known proteins. In response to an elevation in plasma calcium levels [Wagner et al., 1989, 1991], the CS release STC, whereupon, the hormone acts on the gills [So and Fenwick, 1979; Wagner et al., 1986; Lafeber and Perry, 1988], the gut [Sundell et al., 1992], and the kidney [Lu et al., 1994] to appropriately readjust calcium and phosphate levels. The mechanism in fishes may also involve alterations in blood flow [Butler and Oudit, 1995]. In mammals, human STC has been shown to inhibit renal phosphate excretion [Wagner et al., 1997].
Despite the information gained in the recent past regarding the structure and function of STC in some fishes and mammals little is known about the more ancient forms of this hormone. It has been suggested that STC has been subjected to evolutionary pressure during the phylogenetic development of vertebrates [Marra et al., 1992, 1994; Wagner et al., 1992]. This conclusion was drawn from the identification of STC-like immunoreactivity and putative STC which varied considerably in molecular weight in the CS of the Cypriniforme, Osteoglossiforme, Semionotiforme, and Amiiforme orders of ray-finned fishes [Actinopterygii] [Marra et al., 1992, 1994, 1995, 1998]. Stanniocalcin is interesting from both an evolutionary viewpoint and comparative analysis of the molecular structure of hormones can be a useful adjunct to other parameters in taxonomic classification [Bernardi et al., 1993; Graybeal, 1994]. Given the phylogenetic position of the Cypriniforme order, intermediate to both the Salmoniformes and Anguilliformes [Nelson, 1994], the characterization of STC from one of its members [eg. white sucker, *Catostomus commersoni*] should be useful in this type of taxonomic interpretation. A further incentive for studying this species is that its CS is thus far unique among teleosts in that it has a cell type which is nonimmunoreactive to existing STC antisera [Marra et al., 1998]. The focus of this study is to elucidate the primary structure of the STC-like immunoreactive target in the CS of the white sucker.

**Materials and Methods**

*Animals and tissue collection*

Adult white suckers [*Catostomus commersoni*] measuring from 30-50 cm in total length were trap-netted in the Humber River [Toronto, Canada] and transported to the freshwater facilities at the University of Toronto at Scarborough. After a period of acclimatization [approximately one week] in tanks containing 18°C, dechlorinated, aerated water, the specimens
were sacrificed by an overdose of tricaine methanesulfonate. The CS and various tissues
[muscle, kidney, gut] from 6 different adult specimens were removed and immediately frozen in
liquid nitrogen and stored at -70°C until their use.

RNA extraction and cDNA library construction

Total RNA was isolated from frozen white sucker tissues using the guanidinium
thiocyanate/cesium chloride method. White sucker CS Poly(A)+ RNA was isolated from the
total cellular RNA using the Oligotex mRNA isolation kit [Qiagen] according to protocol. The
cDNA library was constructed using the Marathon PCR cDNA amplification kit [Clontech]
following the suggested protocol. Briefly, Poly(A)+ RNA [1µg] was reverse transcribed using
MMLV reverse transcriptase and converted to double stranded cDNA by standard methods. The
oligo dT-primed CS cDNAs were blunt-ended using T4 DNA polymerase and ligated to partially
double stranded synthetic adaptors. The adaptor-ligated C. commersoni CS cDNA was
appropriately diluted in tricine-EDTA buffer and subjected to standard PCR protocols and both
5’ and 3’ R.A.C.E. [rapid amplification of cDNA ends] using touchdown PCR. All PCR
amplification products were subcloned by blunt-ended cloning using the SrfI restriction
endonuclease into pCR-script Amp SK(+) vectors and propagated in Epicurian Coli XL1-Blue
MRF’ Kan supercompetent cells [Stratagene]. Positive clones were selected by blue-white colour
screening and antibiotic resistance, characterized by endonuclease digestion, and sequenced on a
Pharmacia ALF automated sequencer at the University of Toronto Biotechnology Service
Centre.
Standard PCR protocol

In order to determine a partial nucleotide sequence of white sucker STC for the construction of gene-specific primers [GSP] necessary for R.A.C.E., the CS cDNA library was subjected to PCR using degenerate 30 mer primers corresponding to conserved regions of salmon, eel, and human STC [Fig. 3]. The PCR reaction parameters [100 μl volume]: 25 mM each dNTP, 1 μg of each upstream and downstream degenerate primer, 0.32 mM MgCl₂, 1 pg CS cDNA template, 10X PCR reaction buffer [Pharmacia], 1 unit Taq polymerase [Pharmacia], were subjected to 30 cycles consisting of a 20 second 93° C denaturation step, 1 min 48° C annealing step, and a 2 min 72° C extension step on a Perkin-Elmer 2400 thermal cycler [Perkin-Elmer]. Amplification products were resolved on a 1.3% agarose-ethidium bromide gel and viewed with an ultraviolet transilluminator.

R.A.C.E. reaction protocol

The 5' and 3' R.A.C.E. reactions were carried out according to the Marathon cDNA amplification kit protocol [Clontech]. For the PCR reaction parameters see Table 1 and Table 2. The inner antisense primer 5'CTGTTAGCATTCTTGCACCTCTTGATC3'[GSP1] for 5' R.A.C.E. and the inner sense primer 5'GAACCTGGACGTCAATACAGATGGGATGCATG' [GSP2] for 3' R.A.C.E. were designed to have melting temperatures greater than 68° C. The adaptor primer 5'CCATCCTAATACGA CTCACATAGGGC3' [AP1] was supplied by Clontech [Palo Alto, USA] with a melting temperature of 68° C. Amplification of both the 5' and 3' STC fragments was carried out in a Perkin-Elmer 2400 thermal cycler using touchdown PCR under the following conditions: denaturation at 94° C for 1 min, 5 cycles consisting of 94° C denaturation for 20 seconds and 72° C annealing-extension for 4 min, 5 cycles consisting of 94° C denaturation for 20 seconds and 70° C annealing-extension for 4 min, and 25 cycles consisting...
of 94°C denaturation for 20 seconds and 68°C annealing-extension for 4 min. Both the 5' and 3' RACE reactions in the absence of the GSP primers, AP1 primers, or target template served as controls. PCR, using the same parameters and conditions in the presence of both GSP1 and GSP2 primers, generated a region of overlap [215 bp] which served as an internal positive control.

**Northern Blot Analysis**

Total RNA [approximately 15 μg] from each of white sucker CS, kidney, gut [intestine] and muscle was suspended in 10 μl of deionized formamide for 15 min at room temperature. To each suspension 5 μl of 37% formaldehyde and 5 μl of 1x MOPS buffer, pH 7.0, containing 8 mM sodium acetate and 1 mM EDTA were added. The RNA was incubated at 65°C for 15 min, electrophoresed on a 1.0% agarose gel containing 1x MOPS, and transferred to a Hybond-N membrane [Amersham]. A cDNA for STC (~0.5 kb) was labeled with [32P]dCTP by random priming. Membranes were hybridized for 16 h at 42°C and washed twice at 42°C in 2x SSPE containing 0.1% SDS for 15 min followed by a washing at 50°C in 1x SSPE containing 0.1% SDS for 10 min. A final washing occurred at 55°C in 0.1x SSPE containing 0.1% SDS for 10 min. After the final washing the membrane was exposed to X-ray film. Following a 2 h pre-hybridization period blots were probed with the white sucker STC 5' R.A.C.E. amplification product.

**Results and discussion**

In the initial PCR screening of the white sucker CS cDNA library the degenerate STC primers yielded a 217 bp amplification product. A Genebank and SWISS-PROT comparison of the nucleotide and deduced amino acid sequence of this amplification product revealed it to be
highly similar to salmon, trout, eel, and mammalian STC. The R.A.C.E. reactions yielded 5' and 3' products of 500 and 1300 bp, respectively. When the 5' and 3' R.A.C.E. reaction amplification products were used as the target templates for PCR, the GSP1 [inner antisense primer] and the GSP2 [inner sense primer] control reactions generated the expected 217 bp amplification product. The lack of an amplification product during both the 5' and 3' R.A.C.E. reactions in the absence of GSP primers, the AP1 [adapter primer], or in the absence of the target template demonstrated the specificity of the PCR reactions. A diagrammatic representation of the sequencing strategy of white sucker STC is shown in figure 1.

The deduced amino acid sequence of white sucker STC was aligned with those of coho salmon [Wagner et al., 1992], Australian eel [Butkus et al., 1987], and human [Chang et al., 1995] using the Geneworks program [V. 6.0] in order to generate a consensus sequence of this hormone [Fig. 3]. The cDNA and the deduced amino acid sequence of white sucker STC are shown in the figures 2 and 3, respectively. The mRNA encodes a primary translation product of 252 amino acids. Purified white sucker STC has not been subjected to amino acid sequencing and, therefore, the N-terminal sequence of the mature molecule is unknown. If the assumption is made that phenylalanine forms the N-terminus of the white sucker STC, similar to other known piscine forms of this molecule [Butkus et al., 1987; Wagner et al., 1992], then, an endopeptidase cleavage at Arg32–Phe33 yields a mature white sucker STC molecule of 220 amino acids and the first 32 residues form the putative prepro region. However, this remains to be established.

Sequence analysis suggested that the molecular weight of the polypeptide monomer was 28 kDa. Although lower than our Western blot estimates of 64 kDa for the homodimeric prepro form of the white sucker hormone [Marra et al., 1998], the generated product is in line with the suggestion that STC occurs as a homodimeric glycoprotein hormone [Wagner et al., 1992]. Our results from cDNA cloning suggest that the white sucker STC dimeric precursor is
approximately 56 kDa in molecular weight. The difference in molecular weight from our previous estimate may be accounted for by the carbohydrate moiety of the molecule. The presence of an odd number [11] of cysteine [Cys] residues and a single N-linked glycosylation consensus sequence [Asn61-Ser62-Thr63] supports the hypothesis of a homodimeric molecule. It has been suggested that an odd number of Cys residues leaves one residue unpaired and, therefore, free to participate in the formation of a disulfide-linked homodimeric molecule [Butkus et al., 1987; Wagner et al., 1992]. These observations correlate well with the observed electrophoretic profile of the C. commersoni STC in the presence and absence of reducing agents and glycolytic enzymes [Marra et al., 1998]. The spatial conservation of the Cys residues [Fig. 3] among the known STCs is significant, since tertiary structures of proteins are more conserved in evolution than are amino acid sequences [Johnson et al., 1990]. Johnson et al. [1990] suggest that this parameter can be used to infer relationships among proteins. The spatial conservation of the Cys residues and the relatively invariant "protein core" [Phe37-Met193 of the consensus] among the known STCs, suggest that these molecules in general appear to have an overall similar tertiary structure. This feature could account for the relatively large cross-species STC-like immunoreactivity by polyclonal STC antibodies and bioactivity during physiological assays [Marra et al., 1992; Lu et al., 1994; Wagner et al., 1995].

Comparison of the deduced amino acid sequence of white sucker STC using BLAST showed the following homologies with the deduced sequences of STC from other vertebrates: coho salmon [73% identity and 82% similarity]; Australian eel [66% identity and 75% similarity]; human [61% identity and 78% similarity]; mouse [56% identity and 73% similarity]. However, the highest degree of homology occurred with isolated and sequenced STC from chum salmon [80 percent identity and 88 percent similarity with only a 1 percent gap penalty]. Yamashita et al. [1995] found the chum salmon STC hormone to be 44 residues smaller at the C-
terminal region than the deduced coho salmon STC. This underscores a common feature of the known piscine STC forms not seen in the mammalian forms of the hormone, that is, the extreme divergence in the deduced amino acid sequence that occurs in the C-terminus [Wagner et al., 1992]. The white sucker STC molecule demonstrates this same divergence at the C-terminus. In fact, if a regional analysis of sequence homology is conducted, the protein core [Phe_{33}-Met_{189}] of the white sucker STC shares 83% identity to the coho salmon core [Phe_{34}-Met_{189}], and 79% to the eel [Phe_{33}-Met_{188}] and 65% to the human [Val_{34}-Met_{189}] STC cores. A comparison of white sucker STC [Phe_{33}-Met_{189}] to coho salmon STC [Phe_{34}-Met_{189}] demonstrates that there are 32 amino acid differences in this region of the protein core, 15 of which are conserved changes. The same comparison with the Australian eel [Phe_{33}-Met_{188}] demonstrates that out of 33 amino acid differences in this region 19 are conserved changes. This result supports the view [Wagner et al., 1992], and previous studies of calcium transport [Lafeber et al., 1988b; Milliken et al., 1990], that the C-terminus of piscine STC is less important in terms of STC bioactivity than the N-terminus. To date, this divergence in the deduced amino acid sequence of the C-terminal has not appeared in the mammalian forms of the hormone [Chang et al., 1995, 1996].

A closer examination of the protein core of the mature hormone reveals that certain regions are invariant and either intolerant to, or inaccessible to, evolutionary pressure, suggesting that these areas are critical for both the structure and function of STC. However, it is also obvious that other regions are not only susceptible but also tolerant of evolutionary pressure. For example, Phe_{60}-Met_{73}, Gly_{91}-Phe_{111}, Gly_{126}-Tyr_{132} and Asn_{160}-Tyr_{163} of the consensus are almost totally conserved in the species thus far examined. Any changes in these areas of the molecule have generally been for functionally or characteristically similar amino acids i.e., hydrophobic nonpolar for hydrophobic nonpolar residues [HNP], or polar uncharged for polar uncharged [POU] residues. In addition, at positions 42 and 180 of the consensus, the evolutionary trend has
been a change from HNP residues to those that are POU. Notably, Asp$_{64}$ and Asn$_{69}$ in the eel have been replaced by Glu and Asp, respectively, in the white sucker STC molecule. Also, whereas Leu predominates in human, eel, and salmon forms of STC at position 99 [consensus numbering], the white sucker molecule possesses an Ile residue at this position suggesting that this locus has been subjected to several evolutionary events during its history. The trend at positions 43, 49, 58, and 111 of the consensus appears to be from POU residues to those that are HNP. While the trend in some areas of the STC core [positions 139, 142, 192 of the consensus] has been the replacement of charged amino acids with ones that are POU. Still other areas [positions 157, 172, 179, and 186 of the consensus] appear to be able to tolerate the presence of either positively or negatively charged amino acids, polar or nonpolar amino acids, hydrophobic or hydrophilic residues.

Several evolutionary events appear to have influenced changes at the amino acid level which have modified several residues within the core sequence of white sucker STC. The presence of POU residues [positions 54, 59, 78, 88, and 112, consensus numbering], HNP residues [positions 79, 106, and 137, consensus numbering] and charged residues [positions 75, 90, and 122, consensus numbering] suggest that these loci may have been the focus of "tolerable" evolutionary pressure.

Some other notable features within the white sucker STC molecule include a conserved dibasic pair [Arg$_{111}$-Arg$_{112}$] as well as several monobasic residues which serve as potential sites for endopeptidase activity [Fig. 3]. Interestingly, whereas human, salmon and eel STCs are characterized by Ser$_{133}$ [consensus numbering], the white sucker form possesses a positively charged Lys residue at this position providing an additional dibasic site [Lys$_{129}$-Lys$_{130}$] for further potential post-translational cleavage activity in the core of this molecule. Wagner et al. [1991] demonstrated the presence of truncated forms of STC in the plasma of rainbow trout. At
present there is no evidence that such post-translational cleavages of white sucker STC occurs, but the presence of potential proteolytic cleavage sites is suggestive of the possibility of truncated forms of STC in the species. In addition, the putative white sucker prepro leader sequence is one residue smaller than both salmon and human and is similar in size to that of the Australian eel. Also, the white sucker prepro region shares approximately a 50% identity with those of salmon, human, and eel. The protein core of white sucker STC is 4 residues smaller than coho salmon STC, 9 residues smaller than Australian eel STC, and 5 residues larger than the human STC form. Interestingly, there are at least three potential CK2 phosphorylation sites in the N-terminus of white sucker STC [Fig. 3]. One occurs in the prepro region [Ser16-Glu19] and the other two [Ser37-Asp40 and Ser62-Asp65] are localized to the N-terminus of the protein core of the molecule. While the Ser62-Asp65 site is also present in both the coho salmon and human STC molecules, it is conspicuously absent in eel STC. Although the putative phosphorylation site at Ser37-Asp40 appears to be unique only to the white sucker molecule, the Thr39 residue has been replaced by a Ser residue, which is similarly polar and uncharged, in the salmon and eel and may, therefore, remain a viable phosphorylation site. This is the first time that STC phosphorylation has been suggested but it is not an unreasonable assumption since other peptide hormones of ancient lineage, such as adrenocorticotropic hormone, have been shown to undergo phosphorylation events [Eipper and Mains, 1982]. Phosphorylation may provide a level of control for STC not previously considered in the literature. However, to date no evidence exists that such post-translational processing of STC occurs in any of the species examined.

Northern blot analysis demonstrated that a 1.6 kb mRNA from the CS encodes white sucker STC [Fig. 4]. C. commersoni mRNA approximates the size of salmonid [Wagner et al., 1992] STC mRNA [2 kb] and the 2 kb mammalian STC hybridization signal [Chang et al., 1995, 1996]. However, white sucker mRNA is substantially smaller than the 3.5 kb message
encoding Australian eel STC [Butkus et al., 1987] and the 4 kb mammalian STC message [Chang et al., 1995, 1996]. The expression of the STC gene is unique to the CS of the white sucker since no hybridization signal was observed in the control tissues [Fig. 4].

In summary, we have cloned and sequenced a mRNA encoding white sucker [C. commersoni] STC, and therefore provide the first report of a STC-like gene in the Cypriniforme order. An analysis of relatedness of STCs between coho salmon, Australian eel, human, and white sucker using Geneworks [V. 6.0] demonstrates that the white sucker form is more similar to the salmon molecule than to either the eel or human forms of the molecule. The relatedness also suggests that the phylogeny of white sucker STC is intermediate to the Anguilliforme and Salmoniforme orders. This view is consistent with the present thinking about the taxonomic relatedness of these orders of Actinopterygii [Nelson, 1994]. However, it also appears that the white sucker STC gene has been exposed to evolutionary pressure since the deduced amino acid sequence of the primary transcript demonstrates certain structural modifications including deletions, substitutions, and residue changes. However, in general the deduced primary structure of white sucker STC shares a considerable degree of sequence similarity with other known STC molecules. This similarity is more fully appreciated if only the relatively invariant portion of the molecule, which we refer to as the STC protein core, is considered. The present study complements our previous characterization studies of the white sucker CS and STC using antibodies generated against chum salmon STC [Marra et al., 1998]. We suggest the reason for cross-reactivity of salmon STC antisera with principal cells of the white sucker CS is due to the presence of STC resulting from the expression of white sucker STC mRNA.
Figure 1. The sequencing strategy employed for white sucker STC cDNA amplification products. The direction of sequencing is indicated by the lines with arrows.
217 bp internal control for R.A.C.E.
Figure 2. The cDNA encoding white sucker [C. commersoni] STC is 1563 nucleotides in length. The start codon [bp 106-108], the stop codon [bp 862-864], and the polyadenylation signal [bp 1514-1519] are highlighted. A 756 open reading frame [underline] encodes a 252 amino acid primary translation product.
Figure 3. Alignment of the deduced STC amino acid sequences from white sucker, coho salmon [Wagner et al., 1992], Australian eel [Butkus et al., 1987], and human [Chang et al., 1995]. The consensus sequence demonstrates the degree of similarity between the predicted proteins which is particularly significant between F_{37}-M_{193}. Dashes have been inserted to optimize the sequence alignment. The location of the degenerate primers [DEG1 and DEG2] and the gene specific primers [GSP1 and GSP2] used for PCR are indicated. The N-linked glycosylation consensus sequence [\{\}, potential dibasic cleavage sites [asterisk] and several potential phosphorylation consensus sites [underline] are indicated. Note the spatial conservation of the 11 cysteine residues.
HUMAN STC  MLQRQSA----V LILVVISASA TERRBQGOSV SFREKRFVAAQ SMHANVRCLN 47
EEL STC    MLNLRSL-IL TTVLVTHA-- --YEEDESEHPF SYTCSRSAAS GSFVVRACLH 46
SALMON STC MLNLAGCNV FLVLOTHA-- --YTDFFIEA SYTCSRSAAS GSFVVRACLH 47
SUCKER STC MLNSSG-LL LTVLVTHA-- --YTFEEHPF QYRHGRSAAS GSFVVRACLH 46
Consensus  ML...SG-. .LTVTV-A-- --.H.---- .SYR.AFPA. SF .DVVRACLH 50

** GSP2 **
HUMAN STC  GALQVGCDFP ACLEMCPCDP DONGDICRP CYSLGKPSFQ GSYJVRSLK 97
EEL STC    GALQVGCDFP ACLEMCPCDP DONGDICRP CYSLGKPSFQ GSYJVRSLK 96
SALMON STC GALAVGRCDFP ACLEMCPCDP DONGDICQLF PQTAATVDPQ GSYJVRSLK 96
SUCKER STC GALQVGCDFP ACLEMCPCDP DONGDICRP CYSLGKPSFQ GSYJVRSLK 96
Consensus  GALQVGC-.F ACLEMCPCDP DONLIC...F .H.AA.F.PQ GSYJVRSLK 100

** DEG1 **
HUMAN STC  CIQAVTFSKY PLAIRCSTP QMIXAVQHGR CYSLGKPSI ARSHFREYRH 147
EEL STC    CIQAVTFSKY PLAIRCSTP QMIXAVQHGR CYSLGKPSI ARSHFREYRH 146
SALMON STC CIQAVTFSKY PQTIRACSTP QMIXAVQHGR CYSLGKPSI ARSHFRAYN 147
SUCKER STC CIQAVTFSKY PQTIRACSTP QMIXAVQHGR CYSLGKPSI ARSHFRAYN 146
Consensus  CIQAVTFSKY P. TIRAC.S.P Q.ML. HVGGR CYSLGKPSI A.RSHFRAYN 150

** DEG2 **
HUMAN STC  VQVFVPFLPS YTFELVLGSL LGCSDFYTVF IGGASLKRQH PHEASPLIS- 196
EEL STC    VQVFVPFLPS YTFELVLGSL LGCSDFYTVF PHEASPLISQ PHEASPLISL- 195
SALMON STC VQVFVPFLPS YTFELVLGSL LGCSDFYTVF PHEASPLISQ PHEATPLISL- 196
SUCKER STC VQVFVPFLPS YTFELVLGSL LGCSDFYTVF PHEASPLISQ PHEATPLISL- 196
Consensus  VQVFVPFLPS YTFELVLGSL L.CSDFYTVF .PHEASPLISQ PHEATPLISL- 200

HUMAN STC  LQTHDCL--- -AQTHFRAAF KEVTFR-- QKSLAVLSEL ROEHRGSPK-- 238
EEL STC    LQTHDCL--- -AQTHFRAAF KEVTFR-- QKSLAVLSEL ROEHRGSPKPC 241
SALMON STC LQTHDCL--- -AQTHFRAAF KEVTFR-- QKSLAVLSEL ROEHRGSPKPC 241
SUCKER STC LQTHDCL--- -AQTHFRAAF KEVTFR-- QKSLAVLSEL ROEHRGSPKPC 242
Consensus  LQ.K.CP.. ............. ........... F.P.K.P... G.O..PT.L-- 250

HUMAN STC  --IX------R- ---TEISSA 247
EEL STC    --IX------R- ---TEISSA 247
SALMON STC --AR------KRS VEALSVER 256
SUCKER STC --AR------TR- ---SIEVSS 252
Consensus  --AR------R. ............ 269
**Figure 4.** Northern blot analysis confirms that a 1.6 kb mRNA encodes white sucker STC in the CS [lane 1]. No hybridization signal is apparent in either kidney [lane 2], gut [lane 3], or muscle [lane 4] total RNA suggesting that expression of the STC gene is confined to the CS. Each lane was loaded with 15 µg of total RNA from the various tissues, probed under high stringency conditions, and exposed to X-ray film for 1 hour at room temperature.
CHAPTER 7
CHAPTER 7

Tissue-specific stanniocalcins in the silver arawana [Oseoglossiformes] as revealed through partial clones of stanniocalcin cDNAs

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ABSTRACT

Molecular biological protocols demonstrated that the reason for cross-reactivity of salmon stanniocalcin [STC] antisera with the corpuscles of Stannius [CS] and certain kidney cells of the Osteoglossiformes, Osteoglossum bicirrhosum, is due to the expression of STC mRNA. PCR generated approximately a 500 bp CS-specific and a 300 bp kidney-specific, partial STC cDNA amplification products. Northern blot analysis revealed that the corpuscular form of STC is translated from a 3.3 kb message, whereas the renal form is translated from a substantially smaller, 0.8 kb mRNA. Southern blot analysis of arawana genomic DNA demonstrated a tissue-specific expression of the hormone and was suggestive of a duplication event of the STC gene during teleost evolution.
Introduction

Stanniocalcin [STC], a homodimeric glycoprotein hormone implicated in calcium and phosphate regulation [Wagner et al., 1991; Wagner et al., 1986, 1988; Lafeber et al., 1988], is produced by the corpuscles of Stannius [CS]. STC has been recently identified in mammalian genomes [Chang et al., 1995, 1996; Wagner et al., 1995; Olsen et al., 1996]. Interestingly, its role in mineral metabolism appears to have also been conserved [Lu et al., 1994; Olsen et al., 1996; Wagner et al., 1997]. Whereas mammalian STC gene expression occurs in variety of tissues, the synthesis of piscine STC is confined to the CS [Chang et al., 1995]. Several studies have demonstrated that certain cells of the mammalian kidney is a likely source of immunoreactive STC [Wagner et al., 1995; De Niu et al., 1998]. To date, no extracorpuscular sources of STC have been characterized in the fishes but STC-like immunoreactivity has recently been noted in the kidney tubules of several species of osteoglossomorpha, ancient teleosts [chapter 4].

Previous researches into the CS suggested that they are embryologically derived from the pronephric and/or mesonephric ducts [Garrett, 1942; Kaneko et al., 1992]. Since the bowfin [Amia calva], the most ancient extant species known to possess these glands, has several hundred CS many of which have an intimate association with distal renal tubules [Youson et al., 1976] it has been suggested that this type of arrangement reflects a more ancestral organization compared to a reduction in number and a more posterior distribution of this endocrine tissue in the more modern bony fishes, the teleosts [Bouchot, 1953; Youson et al., 1976].

STC occurs as a glycosylated, disulfide-linked homodimer [Wagner et al., 1986, 1988, 1992; Lafeber et al., 1988; Butkus et al., 1987; Olsen et al., 1996; Chang et al., 1995, 1996]. Despite the considerable similarity in the primary structure of piscine and mammalian STC, variability is also evident. Previous research into the forms of STC in species of ancient lineage
suggest that the hormone has undergone considerable and rapid evolutionary change during the evolution of the teleosts among the Neopterygii [Marra et al., 1992, 1994, 1995, unpublished data; Wagner et al., 1992; 1995].

Wagner et al. [1992] were the first to suggest the possibility of different forms of circulating and bioactive forms of STC. Recent evidence is supportive that multiple forms of STC may indeed be present [Wagner et al., 1998]. However, at present it is difficult to say whether these STC isoforms represent differential posttranscriptional processing of the STC message or the expression of more than one STC gene-related product.

As noted above, our earlier examinations of STC in certain members of the Osteoglossomorpha, demonstrated that in addition to the CS parenchyma, certain cells of the distal renal tubules were also reactive to antibodies generated against salmonid forms of the hormone [Marra et al., unpublished data]. Furthermore, the silver arawana showed a "higher" teleost distribution of the CS [a pair of glands more posteriorly distributed], but a STC with a molecular weight similar to that of the more ancient non-teleost, the gar [Marra et al., 1995]. The present report provides evidence that the STC-like immunoreactivity observed in a member of the Osteoglossiformes, both corpuscular and extracorpusrucular, is due to the translation of mRNAs encoding STC. Furthermore, our results suggest that the STC primary transcript is differentially processed or that a gene duplication event has occurred during the evolution of this hormone.

**Materials and Methods**

**Animals and tissue collection**

Adult and juvenile silver arawana [Osteoglossum bicirrhosum] were purchased from a commercial supplier [Toronto, Canada] and transported to the freshwater facilities at the University of Toronto at Scarborough. After a period of acclimatization in tanks containing 18°
C, dechlorinated, aerated water, the specimens were sacrificed by an overdose of tricaine methanesulfonate. The CS, kidneys and various control tissues [gut, brain, and muscle] from the specimens were harvested frozen in liquid nitrogen and stored at \(-70^\circ\) C until their use for molecular biology.

**RNA extraction and cDNA library construction**

Total RNA was isolated from frozen arowana CS and kidney tissue using the guanidinium thiocyanate/ cesium chloride method. Arowana CS and kidney Poly(A)+ RNA was isolated from the total cellular RNA using the Oligotex mRNA isolation kit [Qiagen, Chatsworth, USA] according to protocol. The cDNA libraries were constructed using the Marathon PCR cDNA amplification kit [Clontech, Palo Alto, USA] following the suggested protocol. Briefly, Poly(A)+ RNA [1\(\mu\)g] was reverse transcribed using MMLV reverse transcriptase and converted to double stranded cDNA by standard methods. The oligo dT-primed CS and kidney cDNAs were blunt-ended using T4 DNA polymerase and ligated to partially double stranded synthetic adapters. The adaptor-ligated *O. bicirrhosum* CS and kidney cDNAs were appropriately diluted in tricine-EDTA buffer and subjected to standard PCR protocols. All PCR amplification products were subcloned by blunt-ended cloning using the *Srf I* restriction endonuclease into pCR-script Amp SK(+) vectors and propagated in *Epicurian Coli* XL1-Blue MRF+ Kan supercompetent cells [Stratagene, La Jolla, USA]. Positive clones were selected by blue-white colour screening and antibiotic resistance, characterized by endonuclease digestion, and sequenced.
PCR protocol

Arawana genomic DNA was used as a template in a standard PCR protocol in order to obtain STC sequence information for use in R.A.C.E. [rapid amplification of cDNA ends] protocols. The genomic DNA was subjected to PCR using degenerate 30-mer primers corresponding to conserved regions of salmon, eel, and human STC [Fig. 4]. The PCR reaction parameters (100 µl volume): 25 mM each dNTP, 1 µg of each upstream and downstream degenerate primer, 0.32 mM MgCl₂, 1 pg CS and kidney cDNA template, 10X PCR reaction buffer [Pharmacia], 1 unit Taq polymerase [Pharmacia], were subjected to 30 cycles consisting of a 20 second 93° C denaturation step, 1 min 48° C annealing step, and a 2 min 72° C extension step on a Perkin-Elmer 2400 thermal cycler. Arawana CS and kidney cDNA libraries were then subjected to 5' R.A.C.E. reactions as described in chapter 6 using "touchdown PCR". The adaptor primer 5'CCATCCTAATACGACTCACTATAGGC3' [AP1] was supplied by Clontech [Palo Alto, USA] and the inner antisense primer 5'CCGACACCATCCTCTGGAA GGTGGAGC' [GSP1] for 5' R.A.C.E. was determined after sequence analysis of the genomic PCR amplification products. Amplification products were resolved on a 1.3% agarose-ethidium bromide gel and viewed with an ultraviolet transilluminator. All PCR products were sequenced on an ABI 373A automated DNA sequencer at the York University Biotechnology Centre.

Northern Blot Analysis

RNA [approximately 15 µg] from each of arawana CS, kidney, gut [intestine], heart, and brain was suspended in 10 µl of deionized formamide for 15 min at room temperature. To each suspension 5 µl of 37% formaldehyde and 5 µl of 1x MOPS buffer, pH 7.0, containing 8 mM sodium acetate and 1 mM EDTA were added. The RNA was incubated at 65° C for 15 min,
electrophoresed on a 1.0% agarose gel containing 1x MOPS, and transferred to a Hybond-N membrane. A cDNA for arawana CS STC and kidney STC were labeled with $^{32}$P[dCTP (Amersham, Mississauga, ON) by random priming. The membrane was hybridized for 16 h at 42$^\circ$ C and washed twice at 42$^\circ$ C in 2x SSPE containing 0.1% SDS for 15 min followed by a washing at 50$^\circ$ C in 1x SSPE containing 0.1% SDS for 10 min. A final washing occurred at 55$^\circ$ C in 0.1x SSPE containing 0.1% SDS for 10 min. After the final washing the membrane was exposed to X-ray film. The membrane was then stripped using a solution of 5 mM TRIS [pH 8.0] and 2 mM EDTA containing Denhardt's solution [0.1 X] at 65$^\circ$ C for 3.5 hr. The membrane was then re-probed using the radioactively labeled arawana kidney STC amplification product and exposed to X-ray film.

**Southern blot analysis**

Isolation of arawana genomic DNA was performed using a procedure modified from Blin and Stafford [1976]. Briefly, excised brain tissue was placed in liquid nitrogen and crushed to a powder by mortar and pestle. Following the evaporation of the liquid nitrogen, 10 volumes of extraction buffer [10 mM TRIS-Cl, pH 8.0, 0.1 M EDTA, pH 8.0, 20 $\mu$g/ml pancreatic RNAase, 0.5% SDS] was added to the powdered tissue and incubated at 37$^\circ$ C for 1 hr. Proteinase K was added to the solution to a final concentration of 100 $\mu$g/ml and incubated at 50$^\circ$ C for 3 hr with gentle agitation. The DNA was extracted [X 3] with an equal volume of phenol equilibrated with 0.5 M TRIS-Cl [pH 8.0]. After the third extraction the arawana genomic DNA was precipitated with 0.2 volumes of 10 M ammonium acetate and 2 volumes of ethanol, washed with 70% ethanol, and resuspended with 10 mM TRIS-EDTA [pH 8.0]. Aliquots of DNA [30 $\mu$g] were digested with PstI. The restriction digests were then resolved by electrophoresis in 0.6% agarose gels containing ethidium bromide and blotted onto nitrocellulose by capillary action in 10x...
SSPE. DNA was crosslinked to the membrane by UV light. This procedure was repeated for a second southern blot.

The membranes were incubated at 67°C for 12 hr in prehybridization buffer [6x SSPE, 5x Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA]. Prehybridization buffer was then replaced by hybridization buffer [6x SSPE, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA] with an α-32P dCTP, random prime-labelled arawana CS STC probe. Hybridization continued overnight at 67°C. Membranes were washed twice in 2x SSPE, 0.1% SDS for 15 min at 42°C, followed by a stringent wash in 0.1% SSPE, in 0.1% SDS at 60°C for 20 min and at again at 68°C for 30 min. Hybridized DNA was detected by autoradiography after a 2 wk exposure at -70°C. This procedure was repeated with a similar Southern blot and probed with a radioactively labeled arawana kidney STC amplification fragment.

Results

Due to the greater availability of silver arawana tissue, our previous characterization of putative O. bicirrhosum STC, and since the immunolabelling procedures clearly demonstrated the existence of an extracorpuscular STC-like molecule, we chose to examine STC in this species using molecular technology. In the initial PCR screening of arawana genomic DNA the degenerate primers yielded a 400 bp amplification product. This product was larger than was expected, based on the positioning of the primers. Genebank searches revealed that much of the sequence was highly homologous to the known piscine [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995] and mammalian [Chang et al., 1995, 1996; Olsen et al., 1996] forms of STC with a sequence identity of 77% at the nucleotide level, and the percentage of amino acid sequence identity ranging from 72%-81%. The regions of the arawana-amplified product which
had a high degree of identity with STC cDNA sequences in eel, salmon, and mammals, were inferred to be part of the gene exon and the rest to represent intronic sequence.

From this sequence information, gene specific primers were generated in order to subject the arawana CS and kidney cDNA libraries to R.A.C.E. reactions under high stringency conditions. The 5' R.A.C.E. reaction, using the arawana CS cDNA as a template yielded an amplification product that was approximately 500 bp in length. Genebank searches revealed a high degree of sequence similarity between the putative arawana CS STC cDNA and the core portion [F85 – M128] of piscine and mammalian STC at both the nucleotide and the amino acid level [Fig. 4]. The nucleotide and deduced amino acid identities of this amplified fragment ranged between 70% - 76%. Interestingly, when individual regions were compared separately the sequence prior to F85 in the 5' end of the molecule was divergent to that of known STCs with only a 55% similarity noted. However, after this region the molecule was clearly recognizable as STC, sharing up to 90% similarity with piscine and mammalian forms of the hormone. Despite the presence of a conserved dibasic R120-R121 site, similar to that reported for other STCs [Butkus et al., 1987; Wagner et al., 1992; Chang et al., 1995, 1996; Olsen et al., 1996], an obvious N-linked glycosylation consensus sequence was conspicuously missing. In addition, multiple potential phosphorylation sites characterize this fragment of arawana CS STC [Fig. 4]. The deduced amino acid sequences of the arawana CS and kidney forms of STC were aligned with those of coho salmon [Wagner et al., 1992] and human [Chang et al., 1995] using the ClustalW Multiple Sequence Alignment program at the Baylor College of Medicine [Fig. 4].

Similarly, a 5' R.A.C.E. reaction using arawana kidney cDNA generated an amplification product of approximately 300 bp in length. Genebank searches revealed that this amplified nucleotide sequence shared a high degree of identity with piscine and mammalian STC ranging between 70% - 76%. The deduced amino acid sequence of the putative arawana kidney STC
compared to some other known STCs is demonstrated in figure 4. The similarities at the nucleotide and amino acid levels confirm that this PCR product was amplified from a mRNA in the kidney of the arawana which encodes STC and corresponds to the 5' end of the molecule. In fact, an analysis of similarity using Geneworks [V. 6.0] suggests that this 5' fragment of the arawana kidney form of STC is more closely related to human STC than to the known piscine forms of STC. Despite the fact that this amplification product is a partial sequence many of the salient features of STC are represented. A dibasic site at R149 – R150 represents a potential location for proteolytic cleavage. Most of the cysteine residues appear to be spatially conserved and at least two N-linked glycosylation sites are present [Fig. 4]. Similar to the arawana CS form and to white sucker STC [chapter 6] numerous phosphorylation sites are present [Fig. 4].

Northern and Southern blot analysis

Northern blot analysis demonstrated that a 3.3 kb mRNA from the CS encodes a CS form of STC in the arawana [Fig. 5A]. This O. bicirrhosum STC message approximates the size of that observed in the eel [3.5 kb] and is larger than the 1.6 kb message observed in the white sucker [chapter 6] and the 2.0 kb salmonid STC mRNA hybridization signal [Wagner et al., 1992]. When the arawana kidney STC 5' cDNA was used as a probe, two substantially smaller [0.8 and 0.6 kb] hybridization signals were observed [Fig. 5B]. The expression of the STC gene(s) is unique to the CS and kidney of the arawana since no hybridization signal was observed when either the CS or the kidney STC amplification products were used to probe the message pools of control tissues [Fig. 5]. These results confirm the immunocytochemical studies which localized STC in the secretory granules of the CS and suggested STC synthesis [Marra et al., 1995]. These results indicate that either the STC primary transcript is processed in a tissue specific manner or that STC may be one in a family of similar genes.
Southern blots, performed on arawana genomic DNA digested with PstI, were probed with the arawana CS 5' R.A.C.E. or kidney 5' R.A.C.E. amplification products [Fig. 6]. The results demonstrated that the CS probe hybridized to various DNA fragments ranged in size from 700 – 1200 bp [Fig. 6A] whereas the kidney STC probe hybridized uniquely to a 1300 bp DNA fragment. These results suggest that there may be more than one copy of the STC gene in the arawana. Alternatively, the multiple hybridization signals observed may be indicative of gene duplication events during the evolution of these ancient bony fishes.

Discussion

This study is the first to examine the spatial expression of the STC gene in a representative of the most ancient group of Teleostei, the Osteoglossiformes. These results provide an explanation for the cross-immunoreactivity of salmon STC antisera with arawana CS and certain cells of the kidneys. This immunological response is due to the presence of at least two forms of the hormone from the expression of tissue specific STC mRNAs. We have cloned and sequenced partial cDNAs, one from an arawana CS cDNA library and the other from an arawana kidney cDNA library, which represent the N-terminus of two STC related molecules. Wagner et al. [1998] demonstrated the presence of two forms of STC in the Atlantic salmon and Verbost et al. [1993] gave strong evidence for two bioactive principles in the CS of both tilapia and rainbow trout. This present study provides the first molecular evidence that STC may encompass a family of related proteins as suggested by Wagner [1994].

The STC-like fragment that we cloned from the arawana CS bears considerable homology to the mid portion of other reported STCs [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995; Chang et al., 1995, 1996; Olsen et al., 1996]. However, much of the N-terminus is divergent and maintains approximately a 50% similarity to the STC forms reported in referenced

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studies. In addition, the N-linked glycosylation consensus sequence which characterizes the reported STCs is absent in this arawana CS STC that we isolated. Interestingly, one of the STC forms reported by Wagner et al., [1998] was differentially glycosylated and had a different subunit structure. Our previous results suggested that on the basis of periodic acid-Schiff positive staining of the CS, arawana STC may indeed be glycosylated [Marra et al., 1995]. In addition, despite the presence of a conserved amino acid sequence and a dibasic cleavage site in the core of our arawana CS STC amplification product, the spatially conserved cysteine residues, which also characterize other reported STCs [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995; Chang et al., 1995, 1996; Olsen et al., 1996], are lacking. This observation suggests that not only is the primary structure of this product substantially altered but also that the tertiary structure must be significantly different from previously reported STCs. Collectively, these results suggest that we have identified a different form of STC in the CS of the arawana as compared to those described in other vertebrates [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995; Chang et al., 1995, 1996; Olsen et al., 1996].

The R.A.C.E. product amplified using the arawana kidney cDNA library as a template is clearly recognizable as STC. Despite the fact that we have characterized only the N-terminal end of the molecule many of the salient features which characterize STC are present. Firstly, the deduced amino acid sequence of our clone shares almost a 90% similarity with the N-terminal of previously reported STCs [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996]. Furthermore, within this primary structure an N-linked glycosylation sequence, an Arg-Arg dibasic pair, and spatially conserved cysteine residues which all characterize this hormone in other species have been conserved in this arawana kidney STC molecule. We have noted the presence of phosphorylation consensus patterns in STC from the white sucker [chapter 6]. The CS form of arawana STC contains at least 1 cAMP
phosphorylation site [R₁₂₀ – S₁₂₃], 1 casein kinase phosphorylation site [T₆₅–E₆₇], and 3 protein kinase C phosphorylation sites [S₅–R₇ , S₁₀₃–R₁₀₅ , T₁₁₂–K₁₁₄]. Similarly, arawana kidney STC possesses 1 cAMP phosphorylation site [R₁₂₀ – S₁₂₃], 1 casein kinase phosphorylation site [S₆₉–D₇₂], and at least 5 protein kinase C phosphorylation sites [S₃₃–R₃₅ , T₃₆–R₃₈ , S₆₉–R₇₁ , S₁₀₃–R₁₀₅ , T₁₁₂–K₁₁₄]. At present there is no evidence that STC undergoes phosphorylation events, however, the presence of these consensus patterns suggests that such a post-translational event may in deed be important in the biological activity of this hormone.

It appears clear that from these results and those of Wagner et al. [1991, 1992, 1998] on several salmonid specimens that more than one form of STC is likely to be present in some piscine vertebrates. These heterogeneous forms of STC could arise from either the post-transcriptional modification of the STC primary transcript, in a tissue specific manner, or by the expression of various STC-related genes. Wagner et al. [1992] were the first to suggest that there may be more than one copy of the STC gene. However, given the tetraploid nature of the salmonids this would be expected. Our Southern blot analysis of the arawana genome, a species not suggested to have a tetraploid karyotype, demonstrates that there may be more than one STC or STC-related gene.

In summary, the immunoreactive profile observed in the CS and certain kidney cells of the arawana [Marra et al., 1995, unpublished data] is most likely due to the tissue-specific expression of two different STC-related mRNAs. It remains to be determined if the CS of these specimens also synthesize and secrete an STC molecule that has the salient features previously described for this hormone in other species; such as N-linked glycosylation, spatially conserved cysteine residues, and a conserved N-terminal [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996]. It also remains to be determined if this extracorpuscular STC profile is unique to this order or is more wide spread in
the bony fishes. In light of previous ontogenetic studies on the CS [Garrett, 1942; Kaneko et al., 1992], it is an intriguing possibility that the arawana kidney form of STC may represent the evolutionary fore-runner of the more modern forms of this hormone, for example, the form found in the mammalian kidney. Our present analysis does indicate that the arawana kidney form of STC is more closely related to human STC than to the piscine forms of this hormone. STC gene expression was previously believed to occur exclusively in the CS of the bony fishes. However, recent evidence from mammals refutes this suggestion as STC has been shown to be expressed in a wide variety of tissues [Chang et al., 1995, 1996; Wagner et al., 1995; Olsen et al., 1996]. It is possible that other vertebrate tissues express STC at different developmental stages or under specific physiological or pathological states [Sterba et al., 1993]. The elucidation of the most ancient forms of STC might contribute to an explanation of both the origin of this class of protein hormone and the nature of this hormone in higher vertebrates. The renal-specific expression of STC in humans is likely of ancient rather than modern origins.
Figure 1. Alignment of the deduced STC amino acid sequences from arawana CS STC [ASC-STC], arawana kidney STC [AK-STC], coho salmon [Wagner et al., 1992], and human [Chang et al., 1995]. The consensus sequence demonstrates the degree of similarity between the predicted proteins. Dashes have been inserted to optimize the sequence alignment. The location of the gene specific primers [underline] used for PCR are indicated. The N-linked glycosylation consensus sequence [bold], potential dibasic cleavage sites [ ] and several potential phosphorylation consensus sites [double underline] are indicated.
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**Figure 2a, b.** a Northern blot analysis confirms that a 3.3 kb mRNA encodes arawana CS STC [lane 1]. No hybridization signal is apparent in either kidney [lane 2], gut [lane 3], muscle [lane 4], brain [lane 5], or heart [lane 6] total RNA suggesting that expression of the arawana CS STC gene is confined to the CS. Each lane was loaded with 15 µg of total RNA from the various tissues, probed under high stringency conditions, and exposed to X-ray film for 1 hour at room temperature. b A relatively smaller message(s) are evident in the kidney RNA pool when probed with the kidney STC fragment [lane 2]. The message appears to be specific for kidney tissue since no hybridization signals were observed in the RNA isolated from the CS [lane 1], gut [lane 3], muscle [lane 4], brain [lane 5], or heart [lane 6].
Figure 3. Samples of PstI digested arawana genomic DNA [30 μg/lane] were subjected to Southern blot analysis. The blot was first probed with the arawana CS STC 5’ R.A.C.E. fragment [A] followed by the arawana kidney STC 5’ R.A.C.E. fragment [B] under high stringency conditions. The arawana CS probe hybridized to 3 DNA fragments of 700, 950, and 1200 bp. The arawana kidney amplified fragment hybridized to a single 1300 bp fragment of DNA.
CHAPTER 8
CHAPTER 8

Stanniocalcin in the basal Actinopterygii

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We describe the isolation and sequence of a partial cDNA clone that is complimentary to a mRNA that encodes stanniocalcin [STC] in the corpuscles of Stannius of the gar, Lepisosteus osseus. PCR paradigms were used to screen cDNA libraries, generated from the mRNA isolated from the corpuscles of Stannius [CS] of the gar and mRNA isolated from the kidneys and CS of Amia calca, for STC. This is the first report of a partial cDNA clone of STC from an animal with the most ancient lineage thus far studied. Sequence analysis of the cDNA revealed that gar STC appears to have many of the salient features described for the forms of the hormone from more recently derived vertebrates. However, gar STC lacks an N-linked glycosylation site in the core of the molecule. The N-terminal of gar STC shares a considerable degree of sequence similarity with teleostean and mammalian forms of stanniocalcin. Despite the presence of conserved epitopes in bowfin STC permitting immunoreactivity with antisera to teleost STC, we were unable to isolate a clone from a kidney cDNA library of this species that demonstrated similarity to known forms of the hormone. However, several amplification products generated from the A. calva cDNA did reveal small areas of similarity which could account for the STC-like immunoreactivity observed in the CS of this species.
Introduction

For many years the phylogenetic relationship of three extant groups of Neopterygii of Class Actinopterygii, the Amiidae, the Lepisosteidae, and the Teleostei, has been controversial. Huxley [1861] was the first to support the monophyletic assemblage of the Amiidae and Lepisosteidae into the Holostei. It was not until more than one hundred years later that Patterson [1973] delivered evidence for an amiid-teleost monophyletic group known as Halecostomi. A little over a decade ago it was proposed that the Lepisosteidae may be a sister group of the Teleostei. Evidence supports all three of these proposed arrangements. Despite recent scientific advances, the controversy continues to persist. A revisitation of the holostean/halecostome issue, employed more recent morphological and molecular data, and indicated that the lepisosteid-amid-teleost problem is still an unresolved issue [Olsen and McCune, 1991; Patterson, 1994; Gardiner et al., 1996].

The corpuscles of Stannius [CS] are endocrine organs intimately associated with the kidneys of only the Neopterygii among the Grade Pisces including Amia calva [the bowfin] and Lepisosteus osseous [the gar] and the Teleostei. In the more modern ray-finned fishes, [actinopterygii], this endocrine tissue has been shown to be involved in the maintenance of calcium [So and Fenwick, 1979; Wagner et al., 1986, 1993; Lafeber et al., 1988; Sundell et al., 1992] and phosphate [Lu et al., 1994] homeostasis through the synthesis and secretion of a glycoprotein hormone referred to as stanniocalcin [STC]. Previously, we have demonstrated the presence of STC-like immunoreactivity in the CS of the bowfin [Marra et al., 1992], the gar [Marra et al., 1994], and certain members of the Osteoglossiformes [Marra et al., 1995, unpublished data]. However, despite the fact that we have demonstrated the expression of two STC related genes in Osteoglossum bicirrhosum [chapter 7], to date no evidence exists at the
molecular level that the STC gene is present or is expressed in either the bowfin or the gar, extant fishes with the most ancient lineages known to possess the CS.

Considerable variation exists in terms of number, distribution, and organization of the CS among teleosts and the bowfin and gar. Whereas the bowfin with several hundred glands represents a more ancestral organization of CS [Youson et al., 1976], the 5-7 medially located CS in the kidney of the gar appears to be intermediate to the teleost state [Bhattacharrya et al., 1982; Marra et al., 1994]. In the more modern teleosts the anatomical distribution of the CS is characterized by a reduction in the number and a more posterior location of the glands. In the bowfin many CS maintain an intimate association with the distal renal tubules [Youson et al., 1976; Marra et al., 1992] from which they are believed to arise [Garrett, 1942].

The nature of STC itself appears to vary only slightly between species and has some salient features which characterize it as a unique hormone. In its native state STC occurs as a labile dimeric molecule [Wagner et al., 1986, 1992, 1994; Butkus et al., 1987; Lafeber et al., 1988]. The molecular weight of the STC molecule varies between species. In the salmon the monomer migrates with an apparent molecular weight of 26 kDa [Wagner et al., 1988] whereas in trout and eels the respective values are 28 and 30 kDa [Flik et al., 1989]. Periodic acid-Schiff staining and concanavalin A affinity demonstrate the glycosylated nature of the STC molecule. The dimeric and glycosylated nature of STC along with a well conserved primary and tertiary structure have been validated by molecular biological protocols in numerous species including mammals [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996; chapter 6]. These results suggest that STC has had a long evolutionary history among the vertebrates.

In contrast to STC in the more modern vertebrates, comparatively little is known about the more ancient forms of this hormone. Our previous examinations of bowfin and gar STC
revealed that, whereas the bowfin [Marra et al., 1992] has one of the smallest putative dimeric hormones [45 kDa], the gar [Marra et al., 1994] has one of the largest [68 kDa]. Interestingly, the gar and arawana demonstrated identical immunoreactive profiles during Western blot analysis [Marra et al., 1995]. Furthermore, we have demonstrated that both the bowfin and the gar putative STCs are likely to be glycosylated, however, neither molecule demonstrated affinity to the concanavalin A suggesting a change in the glycomoiety of STC during the evolution of fishes [Marra et al., 1992, 1994] or a remarkably different conformation of the molecules compared to teleost STC. In addition, immunolabelling techniques demonstrated that antibodies generated against teleost forms of STC are capable of recognizing both bowfin and gar STC [Marra et al., 1992, 1994]. However, attempts to obtain a hybridization signal using salmon STC cDNA fragments as probes during Northern blot analyses of bowfin CS mRNA failed even under the lowest conditions of stringency [Wagner et al., 1992]. Collectively, the above suggests that, despite the preservation of certain epitopes, STC has undergone considerable evolutionary pressure. It is also not unreasonable to assume that there may be more than one form of STC. Recently, we demonstrated that the CS of the arawana produce one form of STC which bares a considerable similarity to the mid-portion of hormones described for other species, however, this form of STC is not glycosylated and its N-terminal demonstrates a divergence in primary structure when compared to other STCs [chapter 7]. This suggestion of multiple forms of the hormone is further supported by the observations of Wagner et al. [1998], who demonstrated the presence of two forms of STC in the Atlantic salmon, and earlier reports of multiple STC-like immunoreactive bands in CS extracts of coho salmon [Wagner et al., 1992].

To date, most comparative investigations of STC have focused on the relatively more modern bony fishes and vertebrates. An examination of the forms of STC from vertebrates of ancient lineage would be useful for not only understanding the nature of this hormone but also in
the interpretation of evolutionary trends in this endocrine system in general. As a first step
towards understanding the early history of the STC gene, in this report we employed biochemical
and molecular biological protocols to examine what may be the most ancient forms of this
hormone in the bowfin [A. calva] and the gar [L. osseus].

Material and Methods

Animals and tissue collection

Adult bowfin, Amia calva, were trap-netted in Lake Ontario, Bay of Quinte [Hay Bay],
Napanee [Lennox and Addington County], Canada, in September and October, 1997. Adult gar,
Lepisosteus osseus, were trap-netted in Lake Ontario, East Lake, Picton, Canada, in September
and October, 1997. The specimens, varying in length from 50-100 cm for the gar and 30-75 cm
in length for the bowfin, were transported to the freshwater facilities at the University of Toronto
at Scarborough. The specimens were maintained in a large fiberglass tanks containing flow-
through, dechlorinated, aerated tap water with the temperature maintained at 10°C. After a
period of acclimation [3 days] the specimens were sacrificed by an overdose of tricaine
methanesulfonate. Following a mid-ventral, cloacal-pericardial incision and after removal of the
viscera, the retroperitoneal kidneys were exposed. The kidneys of the gar were removed and
examined under a dissecting microscope for the characteristic white corpuscles [Youson et al.
1976; Bhattacharyya et al., 1982]. The kidneys of the bowfin were excised in their entirety.
Various tissues [muscle, heart, brain] were also removed from each specimen to be used as
controls during Northern blot analysis. The tissues of each specimen were quickly and carefully
excised and immediately frozen in liquid nitrogen, and stored at -70°C until further use.
RNA extraction and cDNA library construction

Total RNA was isolated from frozen gar and bowfin tissues using the guanidinium thiocyanate/cesium chloride method. Gar CS Poly(A)$^+$ RNA was isolated from the total cellular RNA using the Oligotex mRNA isolation kit [Qiagen, Chatsworth, CA] according to protocol. Bowfin CS are embedded within the substance of the kidney and are difficult to excise quickly. Therefore, in order to reduce the possibility of RNA degradation, total kidney and RNA was isolated from the bowfin as above. The cDNA libraries were constructed using the Marathon PCR cDNA amplification kit [Clontech, Palo Alto, CA] following the suggested protocol. The gar CS and bowfin CS+kidney [hereafter referred to as kidney] cDNA libraries were screened for STC by PCR. All PCR amplification products were subcloned by blunt-ended cloning using the SrfI restriction endonuclease into pCR-script Amp SK(+) vectors and propagated in Epicurian Coli XL1-Blue MRF$^+$ Kan supercompetent cells [Stratagene, La Jolla, CA]. Positive clones were selected by blue-white colour screening and antibiotic resistance, characterized by endonuclease digestion, and sequenced on a Pharmacia ALF automated sequencer at the Hospital for Sick Children Biotechnology Service Centre.

PCR protocol

The gar CS and bowfin kidney cDNA libraries were subjected to PCR using degenerate primers corresponding to conserved regions of salmon, eel, and human STC in combination with each other or with vector-arm primers [Fig. 1]. In addition, the bowfin kidney cDNA library was subjected to PCR using a degenerate upstream primer deduced from the N-terminal amino acid sequence purified by HPLC. The PCR reaction parameters [100 µl volume]: 25 mM each dNTP, 1µg of each upstream and downstream degenerate primer, 0.32 mM MgCl$_2$, 1 pg CS cDNA template, 10X PCR reaction buffer [Pharmacia], 1 unit Taq polymerase [Pharmacia], were
subjected to 30 cycles consisting of a 20 second 93°C denaturation step, 1 min 48°C annealing step, and a 2 min 72°C extension step on a Perkin-Elmer 2400 thermal cycler [Perkin-Elmer]. Amplification products were resolved on a 1.3% agarose-ethidium bromide gel and viewed with an ultraviolet transilluminator. The deduced amino acid sequences were aligned [Fig. 1] with those of coho salmon [Wagner et al., 1992], human [Chang et al., 1995] and arawana [chapter 7] using the ClustalW Multiple Sequence Alignment program at the Baylor College of Medicine.

Northern Blot Analysis

RNA [15 μg] from each of gar CS, kidney, and muscle was suspended in 10 μl of deionized formamide for 15 min at room temperature. RNA [15 μg] from bowfin posterior kidney [containing CS RNA], anterior kidney [without CS RNA], muscle, and brain were similarly suspended. To each suspension 5 μl of 37% formaldehyde and 5 μl of 1x MOPS buffer, pH 7.0, containing 8 mM sodium acetate and 1 mM EDTA were added. The RNA was incubated at 65°C for 15 min, electrophoresed on a 1.0% agarose gel containing 1x MOPS, and transferred to a Hybond-N membrane. A cDNA for STC was labeled with [32P]dCTP by random priming. Membranes were hybridized for 16 h at 42°C and washed twice at 42°C in 2x SSPE containing 0.1% SDS for 15 min followed by a washing at 50°C in 1x SSPE containing 0.1% SDS for 10 min. A final washing occurred at 55°C in 0.1x SSPE containing 0.1% SDS for 10 min. After the final washing the membrane was exposed to X-ray film. The gar Northern was probed with a putative gar STC amplification product. Previously [chapter 7] we had suggested that the arawana kidney form of STC may represent an evolutionary forerunner of the more modern forms of the hormone. Therefore, a cross-species hybridization was attempted on the bowfin Northern using a radiolabelled arawana kidney STC fragment.

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Isolation of bowfin STC

Bowfin STC was prepared from a modified procedure described by Sundell et al. [1992] and Yamashita et al. [1995]. One bowfin kidney weighing approximately 20 g was homogenized and extracted in 20 ml of cold homogenization buffer [35% ethanol – 10% ammonium acetate, pH 8.0; containing 5 mM EDTA and 1.5 mM phenylsulfonylflouride] at 4°C overnight. The bowfin kidney homogenate was centrifuged at 10000 X g at 4°C overnight and the supernatant collected. The extract was precipitated using 500 ml of ice-cold 100% ethanol at 4°C overnight. The precipitate was collected by centrifugation and resuspended in 5 ml of solublization buffer [12% stacking gel buffer, 1% SDS and 10% glycerol]. The samples were then resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS-PAGE] at 100 V for 18 hr on large slab gels. After Comassie blue staining, resolved proteins in the 40-50 kDa size range were excised and electroeluted at 350 V at 4°C overnight. The isolated proteins were dialyzed against 8 L of deionized water over a period of 48 hr and lyophilized. After resuspension in phosphate buffered saline [PBS], bowfin STC was purified by rpHPLC on a C-18 column [Waters, Toronto, ON] with a linear gradient of 20-60% acetonitrile in 0.1% TFA for 30 min. STC was monitored by Western blot analysis [as described by Marra et al., 1994, 1995] using anti-chum salmon STC serum during purification. The N-terminal of the purified STC immunoreactive fraction was analyzed and sequenced on a Porton Gas-phase microsequencer, model 2090, at the Hospital for Sick Children Biotechnology Service Centre, Peptide Sequence Analysis Facility.

Results

Bowfin STC amino acid sequence

The alcoholic extraction of bowfin kidney, containing the CS, resolved by SDS-PAGE and purified by HPLC yielded two immunoreactive bands of 22 and 24 kDa. The presumptive
bowfin STC was eluted as a single peak on rpHPLC at 22 min [flow rate of 1 ml / min] corresponding to 50% acetonitrile in 0.1% TFA. We assume that the two STC-like immunoreactive bands represent a reduced form of the STC dimer. The amino acid sequence of this presumptive bowfin immunoreactive isolate, KTYERCELART, has no sequence similarity to any known STCs reported to date [Wagner et al., 1986, 1988, 1992; Lafeber et al., 1988; Butkus et al., 1987; Chang et al., 1995, 1996; Marra et al., unpublished data].

cDNA sequence analysis

In the initial screening of the gar CS cDNA library the PCR yielded a 365 bp amplification product. Genebank searches revealed that much of the sequence was highly homologous to the known piscine [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995] and mammalian [Chang et al., 1995, 1996; Olsen et al., 1996] forms of STC at the nucleotide and at the amino acid level with the percentage of sequence identity ranging from 72%-81%. The deduced amino acid sequence of the putative L. osseus STC compared to some other known STCs is demonstrated in figure 1. The similarities at the nucleotide and amino acid levels confirm that this PCR product was amplified from a mRNA in the CS of the gar which encodes STC. Although this amplification product is a partial sequence, corresponding to the 5' end of other reported STC molecules, many of the salient features of STC are represented [Fig. 1]. A dibasic site a R_{113} – R_{114} represents a potential location for proteolytic cleavage and the cysteine residues within this portion of the molecule appear to be spatially conserved. Interestingly, a single N-linked glycosylation consensus sequence [N_{20} – R_{23}] occurs in what may be the leader sequence of gar STC, whether this site is actually glycosylated remains to be determined. Further, the conserved glycosylation site [NST] observed in other species [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996;
Marra et al 1998a, b] is not present in gar STC and may explain our previous lectin chromatographic results [Marra et al., 1994]. Similar to the arawana and white sucker STC [chapter 6, 7] numerous potential phosphorylation sites are present [Fig. 1].

When the bowfin kidney-CS cDNA library was used as a template for PCR no combination of primers was able to amplify a product that demonstrated a strong similarity to any previously described STC molecules. Any similarity to STC was reserved to the extreme ends of our PCR products and most likely represents the amplification of the primers [Fig. 2]. However, employing an upstream primer [#1, see Fig. 1] and a downstream vector-arm primer, we generated an amplification product containing a deduced 16 amino acid string which demonstrated a 50% similarity to an area of the eel STC C-terminus. Further, the N-terminal of this amplification product, corresponding to the area of the molecule encompassed by primer #1, consisted of a 13 deduced amino acid string that has a 61% sequence similarity to a corresponding area of eel STC [Fig. 2].

Northern and Southern blot analysis

Northern blot analysis demonstrated that approximately a 3.7 kb mRNA from the CS encodes STC in the gar [Fig. 3]. The gar STC message approximates the 3.5 kb size of that observed in the eel [Butkus et al., 1987] and the 3.3 kb message reported for the arawana [chapter 7] and is larger than the 1.6 kb message observed in the white sucker [chapter 6] and the 2.0 kb salmonid STC mRNA hybridization signal [Wagner et al., 1992]. The expression of the STC gene is confined to the CS of the gar since no hybridization signal was observed when the CS STC amplification product was used to probe the message pools of heart and brain tissues [Fig. 3]. These results support our previous immunohistochemistry studies which localize STC
gene expression to the CS of *L. osseus* [Marra et al., 1994]. In contrast, we were unable to detect any hybridization signal in tissues of the bowfin even at the lowest conditions of stringency.

**Discussion**

In this report we have cloned a partial cDNA from mRNA in the CS of the gar, *Lepisosteus osseus*, which encodes what may be one of the most ancient forms of STC reported to date. This study was intended to compliment our previous work on the CS of the bowfin and gar and the characterization of immunoreactive STC in these specimens and to further our understanding of STC from an evolutionary perspective [Marra et al., 1992, 1994]. Originally, we reported that the then known chemical profiles of bowfin and gar STC were suggestive of evolutionary pressures placed upon the hormones of these organisms of ancient lineage [Marra et al., 1992, 1994; Wagner et al., 1992]. For instance, previous studies have shown that STC is a homodimeric glycoprotein [Wagner et al., 1986, 1992; Laseber et al., 1988; Flik et al., 1989; Sundell et al., 1992] and its glycosylated nature has enabled purification by concanavalin A-sepharose. However, lectin chromatographic studies indicated that neither bowfin or gar STC is capable of binding concanavalin A [Marra et al., 1992, 1994]. On the basis of our previous results with light microscopy and periodic acid-Schiff it appears that both bowfin and gar STC are glycosylated. The absence of an N-linked glycosylation consensus sequence in the gar STC fragment supports our previous hypothesis that it’s carbohydrate moiety may differ from that in teleosts. In light of a recent report of a form of Atlantic salmon STC which does not have an affinity for concanavalin A [Wagner et al., 1998], the possibility of multiple forms of STC in the gar and bowfin is certainly a possibility. Further, similar to white sucker [chapter 6] and arawana [chapter 7] STC, the primary structure of the gar hormone demonstrated numerous phosphorylation consensus sites. Phosphorylation may provide a level of control for STC similar
to that described for other peptide hormones [Eipper and Mains, 1982]. To date, no evidence exists for such post-translational phosphorylation events in STC, but, the possibility of such a biological control mechanism is a definite probability based on the number of sites present.

The molecular biology results demonstrate that the reason for cross-reactivity of salmon STC antisera with gar CS is due to the presence of the hormone resulting from the expression of STC mRNA. Northern blot analysis demonstrated that the expression of the STC gene is confined to the CS of the gar. The deduced amino acid sequence of gar STC indicates that it possesses the relatively invariant “protein core” common to all reported STCs to date. Within this protein core the presence of an Arg-Arg cleavage site and the spatial conservation of the cysteine residues suggests a structural and, therefore, a probable functional conservation of important domains in gar STC. In fact, a comparison of the protein core of gar STC [R45 - Y130] with those of arawana kidney, salmon, eel, and mammalian demonstrates a high sequence identity approaching 80 percent. Therefore, it appears that STC has been well conserved during lepisosteid-teleost evolution both at the protein and nucleotide levels. Since the N-terminal portion of gar STC is similar to the bioactive portions of the teleost molecules [Butkus et al., 1989; Milliken et al., 1990; Verbost et al., 1993], it would be reasonable to assume that gar STC carries out the same role in mineral metabolism as its teleosteans counterparts. However, an alternate role for this hormone in the Lepisosteidae can not be ruled out until comparable physiological studies are conducted.

From an evolutionary perspective our present results are intriguing. Our previous morphological and immunohistochemical data [Marra et al., 1992] demonstrate the presence of STC-like immunoreactivity in the CS of the bowfin. However, to date we have been unable to corroborate these results with molecular data. Immunological techniques suggest that there are similarities between bowfin, gar, and teleost hormones based on their common cross-reactivity to salmon STC antiserum. Our inability to screen for a recognizable form of STC in the bowfin
implies that in spite of the fact that one or more epitopes of the protein have been preserved, the nucleotide sequence encoding *A. calva* STC may show considerable variation from the teleost and gar mRNA. This view is further supported by previous research [Wagner et al., 1992] and our own inability to obtain a hybridization signal with various STC fragments during cross-species Northern blot analysis. This result is consistent with the high degree of variation of bowfin insulin [Conlon et al., 1991] and growth hormone [Rubin et al., 1995] when their primary structures are compared to those of gar or the teleosts. The small areas of homology that we describe could account for the STC-like immunoreactivity observed in the CS of the bowfin. Our results demonstrate that gar STC is similar to salmon, human and arowana kidney STC. Interestingly, the arowana, a member of the Osteoglossiforme order, is one of the most ancient teleosts. The present results indicate that *Amia calva* may represents a nodal point in STC evolution. This animal is one of the most ancient extant actinopterygians and its STC is different from that of all other ray-finned fishes. Whereas, our previous morphological observations [Marra et al., 1992, 1994, chapter 4] could support an amiid-teleost CS relationship, our present molecular results do not corroborate this hypothesis. However, Gardiner et al. [1996] noted that in the actinopterygians the rate of morphological and molecular changes do not always appear to be correlated. It must also be appreciated, however, that our inability to screen for STC by PCR using degenerate primers in the bowfin may be due to a problem of sensitivity rather than to the absence of a recognizable STC molecule. Due to our inability of generating a "pure" bowfin CS cDNA library, high background is a technical problem difficult to overcome.

In summary, we have cloned a partial cDNA which is complimentary to a mRNA encoding gar STC. This is the first report of a STC-like gene in a sub-teleost in Neopterygii. Expression of the gene is confined to the CS. However, we were unable to detect a STC-like message in the tissues of the bowfin, one of the most ancient extant ray-finned fishes. We must
state that based on our previous results with the bowfin, this inability may be technical rather than evolutionary. Since gar STC is more similar to that of the basal teleosts, the result is consistent with Olsen’s [1984] suggestion that the gar are more closely related to the more modern bony fishes than bowfin. The deduced amino acid sequence of gar STC is similar enough to teleost STC that antibodies generated against the salmon form of the hormone are able to recognize it during immunolabelling protocols [Marra et al., 1994]. However, the lack of a N-linked glycosylation site supports our previous suggestion that STC has been modified during the evolution of actinopterygian fishes.
Figure 1. Alignment of the deduced STC amino acid sequence from gar, coho salmon [Wagner et al., 1992], arawana kidney [chapter 7], and human [Chang et al., 1995]. The degree of similarity between the predicted proteins is particularly significant between R_{45}-Y_{130}. The location of the upstream [Dg 1] and downstream [Dg 2] degenerate primers used for PCR are indicated. Note the absence of a N-linked glycosylation site in the protein core of gar STC compared to the conserved site in the other specimens [bold]. Interestingly, such a site [———] is present in the putative leader sequence of gar STC. A potential dibasic cleavage site [{} and several potential phosphorylation consensus sites [double underline] are indicated.
Figure 2. Deduced amino acid sequence of a PCR fragment, amplified from the bowfin kidney-CS cDNA library using the degenerate upstream primer #1 and a vector arm primer, compared to eel [Butkus et al., 1987] STC. Two areas of similarity between D_{60}-I_{72} and W_{222}-T_{237} of eel STC and the bowfin amplification product were identified by BLAST. These similarities could account for the STC-like immunoreactivity observed in the CS of the bowfin. Note the absence of the N-linked glycosylation site as compared to that observed in the eel [bold].
E-SCC  DNSTCNDGMHRI...........WGPPCSRSTPTCAPGT
+N TC+TDGM+ I W PC+ CAP T
B-SCC  ENRTCDTDGMHEI...........WFQPCTGCVLPCAPET
Figure 3. Northern blot analysis confirms that the STC gene is expressed in the CS of *Lepisosteus osseus*. A 3.7 kb mRNA encodes gar STC [*lane 1*]. No hybridization signal was apparent in either heart [*lane 2*] or brain [*lane 3*] when the respective RNA pools were probed with the gar STC fragment under high stringency conditions.
PART III

GENERAL DISCUSSION
The focus of this study was to reconstruct the lines of evolution within the CS-STC endocrine complex and perhaps help confirm and complete the understanding of the phylogenetic relationships between the more ancient actinopterygian fishes. Since the phylogenetic distribution of endocrine tissues and their hormones among the vertebrates reflects the process of evolution, I attempted to carry out such a study by examining and comparing the morphology of this endocrine tissue and the structure of its secreted hormone(s) in different species. My choice to examine these CS and STC archetypes stems from a basic scientific need, the lack of an evolutionary perspective on STC, and that these most ancient, extant, fishes have long been considered to be an evolutionary bridge connecting the actinopterygian fishes and the cartilagenous fishes. Fossil evidence suggests that the holostean fishes, represented to this day by two extant genera [Lepisosteus and Amia], inhabited the Mesozoic oceans and gave rise to the teleost fishes [Young, 1981; Romer and Parsons, 1986]. Given the phylogenetic position of the holosteans within the subclass Actinopterygii, this study was based on the assumption that the analysis of the CS and the characterization of STC in these animals would provide critical information relevant to understanding the radiation of this endocrine system in the ray-finned fishes and in more contemporary vertebrates.

Morphology and Distribution of the CS

The light and electron microscopic observations of the CS in the bowfin and gar are consistent with other structural studies on this endocrine tissue [Ogawa 1967; Krishnamurthy and Bern 1969; Tomasulo et al. 1970; Carpenter et al. 1973; Youson et al. 1976; Wendelaar Bonga et al. 1977; Bhattacharyya and Butler 1978; Bhattacharyya et al. 1982]. However, the topographic and fine-structural variation of the CS endocrine tissue in certain members of the Osteoglossiformes and at least one member of Cypriniformes is suggestive of innovations in CS
organization unique among the vertebrates. Whereas both type-1 and type-2 cells are present in the gar [similar to other freshwater and euryhaline fish], *Amia calva* appears to be an exception as only type-1 cells are present in this species [Youson and Butler 1976], a condition consistent with the suggestion of a marine origin for this species [de Smet, 1962]. Embryologically, the CS originate from cells of the pronephric, mesonephric, and in some cases the opisthohemric ducts [Garrett, 1942; de Smet, 1962]. We and others have demonstrated that many of the several hundred, encapsulated, CS in the bowfin remain in close association with the kidney cells from which they arise [Garrett, 1942; de Smet, 1962; Youson et al., 1976; Marra et al., 1992]. In contrast, the 5-7 CS of the gar aggregate into larger, encapsulated, ovoid bodies localized to the more anterior positions of the kidneys [Bhattacharyya et al., 1982; Marra et al., 1994]. Whether such an aggregation is functionally and evolutionarily significant or is a result of fortuitous ontogenic events remains unknown. However, such aggregations of endocrine tissues are commonly seen as one ascends the phylogenetic scale [Pang and Schreibman, 1986]. Therefore, the distribution of the gar CS suggests a more recent phylogenetic organization whereas that of the bowfin represents a more primitive tendency among the vertebrates. This notion is supported by evidence that links the distribution of the adrenocortical tissue in an animal to its position on the taxonomic scale [Chester Jones et al., 1962; Youson, 1985].

Also, among the vertebrates, there appears to be an evolutionary trend toward a more intimate association of various endocrine cells as one ascends the evolutionary scale, often reflecting a functional significance [Bentley, 1998]. For example, the vertebrate pituitary is a conglomerate of various cell types and the degree of association of the individual cells and their relationships to neural and vascular tissues can provide information about the evolutionary changes that have occurred in this gland. Lagios [1970] clearly points out that the morphology of the hypothalamo-hypophyseal complex does not support paleontologic evidence [Patterson,
[1982; Lauder and Liem, 1983] that the bowfin is a form transitional to the teleosts. This is especially interesting in light of the phylogenetic relationship that is considered to exist between the bowfin and the more modern teleosts [Patterson, 1982; Lauder and Liem, 1983]. In fact, immunochemical studies focused on the cells producing growth hormone suggest a chondrostean-amiid rather than an amiid-telostean relationship [Hayashida and Lagios, 1969]. A further example is the intimate association of adrenocortical and chromaffin tissue [Pang and Schreibman, 1985]. In this light, it is interesting to note that Youson and Butler [1976] demonstrated that the fine structure of the bowfin adrenocortical cells is more similar to that of the sturgeon than to either the cyclostomes and the more modern teleosts. Along this line of reasoning, then, the aggregation of the type-1 and type-2 cells into larger bodies in the gar may be indicative of a more modern evolutionary trend in CS organization. However, it is important to realize that this character state may also simply reflect the embryogenesis of the CS in the gar. In contrast, in the bowfin, the diffuse distribution of the CS which originate from several generations of kidneys may be representative of a more primitive state and may reflect a pattern present in ancestral fish. Interestingly, the more modern CS distribution pattern is characterized by a reduction in CS number, as in the gar, but a more posterior localization of this endocrine tissue in relation to the kidneys. Therefore, the CS distribution pattern observed in the gar may represent an intermediate between that of the bowfin and that of the more modern bony fishes.

Although Amia and Lepisosteus are the only surviving genera in the superorder Holostei, these genera diverged and are evolutionarily separated by millions of years [Young, 1981] and are even further removed from the Teleostei [Nelson, 1969; 1994]. Considering their disparate evolutionary histories, it would be reasonable to assume that the CS in the genera making up the bony fishes would also diverge. Therefore, it is possible that the morphologic hallmarks separating the Holostei and the Teleostei may not be paralleled by similar progressions of CS.
organization. On the other hand, given the common embryological origin of the CS, similarities in structure, function, and their association with the kidneys should not be unexpected.

In our examinations of the CS in members of the Osteoglossiformes STC-like immunoreactivity was observed in the CS parenchyma of each specimen examined. In addition, STC-like immunolabelling was also observed in certain kidney cells of the distal renal tubules. To date, this extraparenchymal staining pattern has only been observed in certain members of the Osteoglossomorpha and may be unique to this more ancient group of bony fishes. Given these observations, the distribution of the CS in the bowfin, and the taxonomic position attributed to the bony-tongued fishes, it would be compelling to suggest a close phylogenetic relatedness between these two genera. However, our biochemical and molecular observations do not support this view. It is difficult to suggest any definitive explanation for this varied pattern of distribution of the CS in the Neopterygii. Considering that the morphological and physiological diversity [and plasticity] of the endocrine systems are directly related to the success of a species to their ever changing ecological habitats, it should not be surprising that different species exhibit, not only considerable interspecific structural and functional diversities, that reflect adaptations to different environments, but also considerable similarities. Therefore, any similarities may reflect a reutilization of a successful pattern from a limited genetic pool, and as such, any similarities may arise by convergent evolution. In other words, any comparable degree of organization has been attained independently and in parallel. In contrast any differences in tissue distribution may reflect a species-specific specialization.

Based on our morphologic and fine structural observations and those of previous researchers [Ogawa 1967; Krishnamurthy and Bern 1969; Tomasulo et al. 1970; Carpenter et al. 1973; Youson et al. 1976; Wendelaar Bonga et al. 1977; Bhattacharyya and Butler 1978; Bhattacharyya et al. 1982; Marra et al., 1992, 1994, 1995] it is my contention that the distribution
of the CS in the bowfin reflects on an earlier actinopterygian pattern whereas that in the gar may reflect an intermediate trend in the evolution of this endocrine tissue from the Holostei to the Teleostei. Other comparisons of actinopterygian endocrine tissues have also shown the gar to be more teleost-like in character, whereas the bowfin represents a more ancient archetype [Conlon et al., 1991; Rubin and Dores, 1996; Dores et al., 1996]. The biochemical and molecular observations of the present study further support this hypothesis.

Stanniocalcin Distribution

This is the first study to examine the CS in the ancient Neopterygii using immunological techniques [see table. 1a for summary]. Our results suggest that there are similarities between STC from the more ancient and the more recent vertebrates. This conclusion is based on their common cross-reactivity to salmon STC antiserum. However, it is also apparent that in the bowfin and gar, there are major differences in the structure of the carbohydrate moieties and possibly in the size of the protein core of their STCs compared to those of the more evolutionarily recent species [Marra et al., 1992, 1994; Wagner et al., 1986, 1992]. Furthermore, the STC-like immunoreactive profile observed in the Osteoglossomorpha is unique among the vertebrates. We have identified STC-like immunoreactivity in the CS and renal cells of certain members of the Osteoglossomorpha subdivision. This immunoreactive profile is most likely due to the tissue-specific expression of two different STC-related mRNAs. The diffuse distribution of kidney STC in the nephric tissue of the arowana, butterfly fish, and the featherfin knife fish [all members of the Osteoglossomorpha] parallels the human renal STC-like immunoreactive profile [Chang et al., 1995; Olsen et al., 1996]. Hence, the presence of STC in mammalian kidney may be a consequence of both the early origins of this hormone and the embryological origins of the CS in bony fishes. Whereas our immunolabelling results imply an STC distribution and character
profile dissimilar to that observed in more recent species, and since immunological cross reactions can be indicative of chemical relationships between hormones [Bentley, 1998], our observations suggest that STC has undergone considerable evolutionary change yet maintains one or more common structural features recognized by STC antibodies. Because such observations can only be suggestive and, in an effort to construct a more ordered story, I examined the primary structure of STC in the representative specimens.

*The Stanniocalcin Molecule*

The usefulness of phylogenetic information gained from examining the amino acid sequences of protein hormones has been previously reviewed [Dores et al., 1996]. The value of such primary information varies depending on the size of the molecule and the degree of conservation [Dores et al., 1996]. Our examinations into the primary structures of various STCs [see table. 1b for summary] demonstrate that despite the large number of invariant and conserved amino acid sequences, useful for identifying functionally important and related regions of a molecule, this hormone is large enough to contain a greater number of variable and, therefore, phylogenetically informative sites for analysis [Dores et al., 1996].

The sequence analysis of the cDNA encoding white sucker STC revealed that the mRNA has an open reading frame of 756 bp encoding a 252 amino acid, a primary translation product containing an odd number of Cys residues, an N-linked glycosylation site, and several potential post-translational cleavage and phosphorylation sites. Therefore, white sucker STC shares a considerable degree of sequence similarity with eel, salmon, and human stanniocalcin. However, the deduced amino acid sequence of white sucker STC is intermediate to those of the coho salmon [Wagner et al., 1992] and the Australian eel [Butkus et al., 1987] with respect to evolutionary relatedness, which is consistent with the presently accepted taxonomic position of
the Cypriniforme order [Nelson, 1994]. This conservation of the STC amino acid sequences and its structural organization, especially between such evolutionarily distant species as human and white sucker, suggest that they may have shared a common STC ancestor.

Molecular biological protocols carried out in arawana demonstrated that cross-reactivity of salmon STC antiserum to the CS and kidney cells can be accounted for by the presence of mRNAs coding for STCs. Whether the same holds true for the other members of the Osteoglossomorpha remains to be determined, but based on our observations of a similar STC immunoreactive profile in the featherfin and the butterfly fish the likelihood of a similar STC mRNA is possible. PCR, using arawana CS and kidney cDNA libraries as target templates, generated two tissue-specific, partial STC cDNA amplification products that we refer to as CS-STC and kidney-STC, respectively. Nucleotide sequencing of the two related cDNAs indicated that the corresponding protein sequences are unique, suggesting STC gene duplication and divergence has occurred during the evolution of this fish. This proposal is supported by the Southern blot analysis of the arawana genomic DNA.

The adaptive significance of the renal-STC is not clear at present. The morphology of these STC-immunoreactive kidney cells was similar to that of “renal mitochondria-rich cells” suggesting a role in mineral metabolism. If one considers the long phylogenetic history between the piscine and the mammalian vertebrates, the structure of STC has been remarkably well conserved reflecting little flexibility in the primary structure of the functional domains. Although there is no factual evidence, it may also be possible that the role of STC as a regulator of renal phosphate movement [Lu et al., 1994] has also been conserved. This would not be unreasonable considering the phosphate deficient environment fishes once inhabited and, to this day, continue to populate. Contemporary vertebrates use STC for calcium or phosphate regulation [Lu et al., 1994]. Therefore it is not unreasonable to suggest that the ancestral hormone may have been
utilized for similar purposes. Therefore, although the ancestral function can only be speculated it may have involved the regulation of ion metabolism. However other physiological roles cannot be ruled out. Given the distribution of kidney-STC in these extant species, some sort of paracrine role is suggested, although, an endocrine or autocrine role for the kidney-specific form of STC can not be dismissed. It is, of course, also possible that these observations may simply represent an evolved specialization of the osteoglossomorph kidney that has permitted them to adapt to changing environments.

The STC-like fragment that we cloned from the arawana CS bears considerable homology to the mid portion of other reported STCs [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995; Chang et al., 1995, 1996; Olsen et al., 1996]. However, many of the salient features that characterize piscine and mammalian [STC1] such as the N-linked glycosylation consensus sequence, dibasic cleavage sites, and the spatially conserved cysteine residues are missing [Chang et al., 1998]. These observations imply that not only is the primary structure of this product substantially altered but also that the tertiary structure must be significantly different from previously reported STCs. Collectively, these results infer that we have identified a different form of STC in the CS of the arawana as compared to those described in other vertebrates [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995; Chang et al., 1995, 1996; Olsen et al., 1996]. This supports the proposition of different forms of the STC piscine hormone that are differentially glycosylated and variable in subunit structure [Wagner et al., 1998].

The fact that Western blotting demonstrates gar and arawana STC to be similar in size is significant considering the controversy regarding their presumptive evolutionary relationship. These results correspond with the paleontological assumption that Osteoglossiformes diverged early among teleosts [Colbert and Morales, 1991]. However, it remains to be determined if the
CS of the arawana also synthesize and secrete an STC molecule that more closely resembles those previously described, having such features as N-linked glycosylation, spatially conserved cysteine residues, and a conserved N-terminal primary structure which characterize STCs in general [Butkus et al., 1987; Wagner et al., 1992; Yamashita et al., 1995 Chang et al., 1995, 1996; Olsen et al., 1996]. The foregoing observations suggest several possibilities. Firstly, that the STC gene is identical in all vertebrates but subject to post-transcriptional changes. Secondly, that duplication of the STC gene has occurred followed by an evolutionary divergence and the assumption of diverse biological roles for the translated peptides. This supposition that one type of hormone is well conserved and the other is varied is supported by similar observations in somatotropin, prolactin, and calcitonin [Rand-Weaver et al., 1993; Suzuki et al., 1998].

This is the first report of an ancient neopterygian form of STC that we isolated from a cDNA library of the CS of the gar which, in more modern vertebrates, is known to affect calcium and phosphate homeostasis. This portion of the study compliments our previous work on the CS of the bowfin and gar and the characterization of immunoreactive STC in these specimens and helps to further our understanding of STC from an evolutionary perspective [Marra et al., 1992, 1994]. In light of recent data reporting the presence of multiple forms of STC in various genera [Chang and Reddel, 1998; Ishibashi et al., 1998; Wagner et al., 1998], the possibility of multiple forms of this hormone in the Actinopterygii is also certainly a possibility. Our findings suggest that STC has been well conserved during lepisosteid-teleost evolution both at the protein and nucleotide levels. Using molecular biological protocols we were able to clone a partial cDNA that is complimentary to a mRNA encoding gar STC. The deduced amino acid sequence of gar STC shows a high degree of similarity to previously characterized forms of the hormone [Butkus et al., 1987; Wagner et al., 1992; Chang et al., 1996]. However, the absence of a conserved glycosylation site supports our previous suggestion that STC has been modified during the
evolution of actinopterygian fishes [Marra et al., 1992, 1994]. However, we were unable to
detect a STC-like message in the tissues of the bowfin, one of the most ancient extant ray-finned
fishes, despite the immunoreactivity of their CS to teleost STC antisera. The results of this study
suggest that gar STC is more similar to that of the basal teleosts, arawana kidney-STC, and
mammalian [STC1], a suggestion consistent with Olsen's [1984] supposition that Lepisosteus is
more closely related to the more modern bony fishes than is Amia.

The presence of STC in members of a broad spectrum of orders of bony fishes,
mammals, and STC-like immunoreactivity in the plasma of elasmobranch fish and amphibians
[Wagner et al., 1995], lends support to the hypothesis that the STC gene was present in ancestral
vertebrates. However, the apparent absence of a recognizable form of STC in the bowfin is
confusing. The immunolabelling protocols carried out in the present study demonstrate that STC
has undergone evolutionary stress, but that conserved epitopes recognized by the various
polyclonal antisera must still remain [Marra et al., 1992, 1994]. It is important to note that the
isolated bowfin RNA, utilized for Northern analysis or cDNA library construction, was
characterized as to be of "good" quality. This was confirmed by ethidium bromide staining of
the total RNA isolated from bowfin kidney containing CS and resolved in agarose-formaldehyde
gels [Appendix A]. Further, after generating the bowfin kidney-CS cDNA library, a mass digest
using the recommended endonucleases confirmed that the cloned transcripts were a good
representation of the encoded information in the tissue mRNA. Ethidium bromide staining of the
complementary DNA, digested from the bowfin kidney-CS cDNA library, demonstrates insert
sizes ranging from 200 bp to 6 kb [Appendix A]. This suggests that a recognizable STC message,
if present, should be detectable using accepted molecular paradigms. Therefore, apart from a lack
of experimental sensitivity, our negative molecular results in the bowfin may reflect very low
levels of the STC message or an extreme dissimilarity of the primary structure of Amiiforme
STC. The latter suggestion is supported by previous research [Wagner et al., 1992] and our own inability to obtain a hybridization signal with various STC fragments during cross-species Northern blot analysis. Furthermore, it is also possible that in the bowfin the STC gene may be developmentally regulated and active only in early development. Technically, the anatomical distribution of the bowfin CS negates the ability to isolate high quality CS RNA which is free from contaminating kidney tissue message. A molecular approach utilizing a subtractive hybridization from bowfin renal tissue containing CS and that devoid of the glands would ameliorate these technical difficulties. These negative data could be interpreted to mean that the STC gene appeared during the divergence of *Lepisosteus*. However, the possibility that putative bowfin STC lacks the structural determinants necessary to be recognized by heterologous molecular probes must be considered. These results also strongly suggest that during the evolution of STC there must have been strong selection pressure which changed or eliminated this molecule during the radiation of the holosteans.

Considering what appears to be a long phylogenetic history from the ancient bony fishes to the mammals, the structure of STC has been remarkably well conserved, probably reflecting its physiological importance. Gene duplication appears to be a recurring theme in the evolution of proteins and STC may not be an exception [Ohno, 1970; Ohta, 1991]. Gene duplication permits the two copies to accumulate mutations based on selection pressure [Bentley, 1998]. As long as one gene copy adequately performs its designed function, the second is free to drift, take on novel functions and enhance the fitness of the organism [Danielson and Dores, 1998]. Gar STC, arawana kidney STC, eel and salmon STC [Butkus et al., 1987; Wagner et al., 1992], and mammalian STC1 [Chang et al, 1996] appear to be the most closely related forms of the hormone. Furthermore, arawana corpuscular STC and mammalian STC 2 [Chang and Reddel, 1998; Ishibashi et al., 1998] appear to have accumulated changes to the basic STC plan during
the evolution of the vertebrates. Duplication of the STC gene during osteoglossomorph radiation may have produced the progenitors of two major lines of hormones. Although speculative, our results are suggestive of such a supposition. However, the relatively few STC sequences known in the narrow range of taxa contributes to a lack of phylogenetic resolution. With the available data, it is my contention that gar STC appears to occupy a primeval position on the STC evolutionary tree and each of the aforementioned may have arisen from this ancestral molecule. With the present evidence, several STC divergences can be predicted.

The presence of STC-like immunoreactive molecules in elasmobranch fishes [Wagner et al., 1998] and the lack of an identifiable STC gene in the bowfin suggests that STC may have been lost during some point in the radiation of the fishes. Based on the presence of at least two distinguishable STCs in arawana and two related but variable mammalian forms of the hormone, divergence from the ancestral molecule may have occurred. Therefore, it appears that the "teleostean-type" and "mammalian-type" of STC may have arisen in an ancestor shared with the holosteans. The gar hormone, therefore, appears to be in the main vertebrate phyletic line. Such a suggestion is not unreasonable and parallels the evolution of growth hormone, a molecule also known to influence mineral homeostasis in fishes [Rubin and Dores, 1996]. During vertebrate evolution there must have been strong selection pressure on STC which changed this molecule. The characterization of bowfin STC would help to clarify this hypothesis.

The course of evolutionary events responsible for the diversity of STC lineages can only be speculated upon. Perhaps these animals faced special [or at least different] mineralostatic and osmotic problems not faced by their ancestors so that the solutions to them were the evolution of hormones based on the available genetic material. The apparent loss of STC, or at least its modification to a yet unrecognizable form, in the bowfin suggests that during their evolution this hormone was not needed, silenced, or modified to meet their particular environmental demands.
Whatever the circumstances, their survival to this day suggests that their available endocrine repertoire allowed the species to appropriately adapt.

An interesting hypothesis is that the CS are predecessors of the juxtaglomerular apparatus or perhaps being an early component of the renin-angiotensin system. Interestingly, granulated juxtaglomerular cells have not been identified in the bowfin and members of the Chondrostei but are present in the gar and several teleosts [Ogawa et al., 1972; Wilson, 1984]. Furthermore, renin-like activity has been localized to the CS by previous investigators and corpuscular extracts have been demonstrated to produce angiotensin-like peptides [Chester-Jones et al., 1966; Sokabe et al., 1968; Ogawa and Oguri, 1978]. These angiotensin-like and renin-like CS products were demonstrated to have effects on mineral movement, including calcium [Ogawa and Sakobe, 1982]. It is well established that the renin-angiotensin system is involved in osmoregulation, ionoregulation as well as the control of blood circulation [Wilson, 1984]. In light of the consistent reports of the effects of corpuscular extracts on cardiovascular system in eels [Butler and Oudit, 1995, 1996], perhaps in the more ancestral vertebrates the strategy used to address ionic and osmotic physiological disturbances was a redistribution of blood to tissues capable of ameliorating the problem by employing the cardiovascular system. A hypothesis supported by the observation that injections of CS extracts redistributes blood to the branchial epithelium [Butler and Oudit, 1995]. It may be possible that during piscine radiation into marine environments, calcium imbalances arose as an additional threat and alternative survival strategies were selected for. Namely, the evolution of STC as a calcitropic agent. Therefore, in ancestral vertebrates STC may have served several roles and evolved as demands changed. Our results suggest that this functional diversity arose as a gene duplication event. This modification on the basic plan of an already existing gene has been played upon many times during vertebrate evolution. The radiation of prolactin and somatostatin are only two examples [Martin and
Faulkner, 1996; Sinha, 1995]. However, the possibility that the diversity arises from other
mechanisms such as exon shuffling [Doolittle, 1985], posttranscriptional editing [Hajduk et al.,
1993] or posttranslational modifications can not be discounted.

The examination of gill arch anatomy persuaded Nelson [1969] to establish Holostei as a
clade. Based on fossil evidence, Patterson [1973] strongly supported the monophyly of an amiid-
teleost group, known as Halecostomi. Patterson determined that amiids were more closely related
to teleosts than to lepisosteids, thus invalidating Holostei as a natural group and forming the
basis for Halecostomi [Patterson, 1973]. This work strongly influenced subsequent research on
1983; Arratia and Schultze, 1990; Gardiner et al., 1996]. Based on morphological and
mitochondrial DNA data, the phylogenetic validity of Holostei however, is still strongly sup-
ported [Normark et al., 1991; Jollie, 1984]. In a more recent examination of additional
morphological and molecular data, Patterson concluded that the halecostome/holostean
controversy must remain unresolved until more sophisticated morphological and more extensive
molecular sequence data are available [Patterson, 1994]. Interestingly, Gardiner et al. [1996] also
supported a monophyletic Holostei with molecular data, but their morphological data appeared to
support the Halecostomi. Olsen and McCune (1991) also consider the lepisosteid-amiid-teloste problem to be unresolved. The apparent conflict between morphological and molecular data
surrounding the Holostei/Halecostomi controversy appears not to be uncommon [Olsen and
McCune, 1991; Gardiner et al., 1996].

Conclusions

The detection of STC in one of the most ancient bony fishes dates the origin of STC and
STC-like products at least to some point in the Mesozoic era. Since modern teleosts are believed
to have evolved from an ancestor that gave rise to the holosteans, the pattern of CS-STC
distribution reflects on an earlier actinopterygean pattern. Further, the gar CS-STC distribution
pattern may signify an intermediate pattern in the evolutionary trend to that observed in the early
teleosts. The strongest supported phylogenies for the basal bony fishes place *Amia* as either the
sister group to *Lepisosteus*, forming the Holostei, or as the sister group to Teleostei, forming
Halecostomi. Assuming that one of these two phylogenies is correct, then either Holostei or
Halecostomi must be a monophyletic group, while the other must be a non-monophyletic group.

With respect to the CS and STC, the morphological data of this study is equivocal in support of a
Holostei or Halecomorphi monophyly [Fig. 1a,b], whereas the molecular data suggests that
lepisostid STC is more similar to that seen in vertebrates of more modern lineage than to that in
the only surviving amiid, the bowfin [Fig. 1c].

I sincerely hope that my work has provided some insights into the understanding of the
corpuscles of Stannius and stanniocalcin in the ancient bony fishes. It has raised many questions
which I believe are worth pursuing and provided some fundamental insights with regard to the
phylogenetics of this endocrine tissue.
Figure 1. Phylogeny of the CS and STC of the main groups of Neopterygi. Morphology of the CS suggest a Holostei or Halecostomi relationship [a, b], whereas molecular and biochemical characteristics of gar STC suggest that it is more similar to that observed in the more modern bony fishes [c].
### Summary

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>STCir</th>
<th>STC (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. calva</em></td>
<td>CS</td>
<td>+</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>kidney</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>L. osseus</em></td>
<td>CS</td>
<td>+</td>
<td>68</td>
</tr>
<tr>
<td><em>O. bicirrhosum</em></td>
<td>CS</td>
<td>+</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>+</td>
<td>28-32</td>
</tr>
<tr>
<td><em>P. buchholzi</em></td>
<td>CS</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>C. chitala</em></td>
<td>CS</td>
<td>+</td>
<td>18-26</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>G. petersii</em></td>
<td>CS</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

### Table 1. Summary of STC immunoreactive profile in various species of Neopterygi [a] and whether this study was able to detect a STC-like message in the CS or kidneys of the representative specimens [b].

<table>
<thead>
<tr>
<th>Species</th>
<th>CS STC</th>
<th>K-STC</th>
<th>Message size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. calva</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. osseus</em></td>
<td>+</td>
<td>-</td>
<td>3.7</td>
</tr>
<tr>
<td><em>O. bicirrhosum</em></td>
<td>+</td>
<td>+</td>
<td>3.3 (CS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8, 0.6 (k)</td>
</tr>
<tr>
<td><em>C. commersoni</em></td>
<td>+</td>
<td>-</td>
<td>1.6</td>
</tr>
</tbody>
</table>

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APPENDIX A
Total bowfin kidney and CS RNA was isolated using the guanidinium thiocyanate/cesium chloride method. Bowfin CS Poly(A)+ RNA was isolated from the total cellular RNA using the Oligotex mRNA isolation kit [Qiagen] according to the manufacturer’s protocol. Total bowfin RNA was visualized on a 1% agarose gel containing ethidium bromide and formaldehyde [a]. A cDNA library was constructed using the Zap Express cDNA synthesis kit [Stratagene] following the suggested protocol. Briefly, Poly(A)+ RNA [5μg] was reverse transcribed using MMLV reverse transcriptase and converted to double stranded cDNA by standard methods. The oligo dT-primed CS cDNAs were blunt-ended using T4 DNA polymerase and ligated to partially double stranded synthetic adaptors and directionally inserted into the Zap Express lambda vector. In order to assay the cDNA quality, which is suggestive of mRNA presence, 1μg of the bowfin kidney-CS cDNA was digested at 37°C using Xho I and Eco RI. Visualized on a 0.5% agarose gel containing ethidium bromide, the bowfin kidney-CS cDNA library contains cloned fragments ranging in size from 0.2 kb to 6 kb [b].
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