SINGLE CHANNEL CONDUCTANCE OF THE Ca\textsubscript{v}2.2 CALCIUM CHANNEL

by

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Abstract:
Calcium ions (Ca$^{2+}$) are admitted into presynaptic nerve terminals through voltage gated calcium channels and diffuse to bind and activate the secretory vesicle discharge mechanism. Current research favors a highly ‘modal’ organization where the release sites are activated by one or a few closely apposed channels (Stanley, 1997). To fully understand the nanophysiology of transmitter release site activation, it is necessary to determine the rate of Ca$^{2+}$ flux through individual channels at normal physiological external concentrations. OBJECTIVE: To explore the relationship between Ca$\text{v}2.2$ channel conductance and external Ca$^{2+}$ across the physiological range. CONCLUSION: The conductance of the Ca$\text{v}2.2$ channel was determined across the range of 1-100 mM [Ca$^{2+}$]$_{\text{EXT}}$. With 2 mM [Ca$^{2+}$]$_{\text{EXT}}$, the conductance was determined to be 2.76 ± 0.24 pS.
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1. Introduction:

1.1. Chemical Neurotransmission:

Chemical synapses in the nervous system provide specialized communication between neural cells, or between neurons and other specialized cells, like muscles and glands. The transfer of information begins when one neuron sends an electrical signal down its axon, termed an action potential (AP). The AP is a depolarization of the axon membrane, caused by the influx of sodium ions through voltage-activated sodium channels (Hille, 2001). At the end of an AP, the electrical signal reaches the presynaptic terminal, where the depolarization triggers the opening of voltage-activated calcium channels (covered in detail below). It is the influx of calcium ions through these channels that ultimately triggers the release of neurotransmitters (Llinas et al., 1981; Augustine et al., 1985), which in turn traverse the synaptic cleft and activate excitatory or inhibitory receptors on the postsynaptic terminal of the receiving cell (see Figure 1). The entire process, from when the AP reaches the terminal and when the postsynaptic current can be measured, takes only 0.6 ms (see Figure 2) (Lisman et al., 2007).

1.1.1. Active Zone Structure

The site of vesicle release is termed the active zone, which is defined physiologically as the site of neurotransmitter release (Zhai and Bellen, 2004). It is here where vesicles come to cluster, dock and fuse to the plasma membrane (Couteaux and Pecot-Dechavassine, 1970; Heuser and Reese, 1973). The active zone itself is densely packed with proteins and other molecules, as can be seen from the electron density in electron micrographs of various presynaptic terminals (Zhai and Bellen, 2004). Just above the active zone is a specialized cytomatrix made up of proteins that can be divided
**Figure 1. Steps in the process of chemical synaptic transmission.** Details of steps from when the action potential reaches the presynaptic terminal to evoked neurotransmission. Modified from (Lisman et al., 2007).

**Figure 2. Timing of steps in transmission.** Voltage change from an action potential (top) causes calcium channels to open and whole cell calcium currents to increase (middle). The calcium ions trigger release of neurotransmitters which activate postsynaptic receptors and ultimately cause ions to flow into the postsynaptic terminal (bottom). Modified from (Lisman et al., 2007).
into three categories: classical cytoskeletal proteins, which help to form the structure of the active zone cytomatrix; scaffolding proteins, which most likely link ion channels and the fusion specific proteins to the active zone; and finally, the active zone specific proteins, which help to modulate vesicle docking, priming and fusion (Zhai and Bellen, 2004).

The plasma membrane of the active zone is where its alignment to the postsynaptic density is mediated, and where the vesicle fusion machinery and the calcium channels are found (see Figure 3) (Walrond and Reese, 1985; Broadie et al., 1995; Ellisman et al., 1976; Garcia et al., 1995; Heuser et al., 1974; Hiesinger et al., 2001). Because of the short delay between calcium influx and transmitter release, close to 0.2 ms (Parsegian, 1977; Stanley, 1997), it was known early on that the calcium channels themselves must be part of the active zone. Indeed, current understanding places the calcium channel less then 50 nm away from the vesicle release mechanism in most synapses (Stanley, 1997; Atwood and Karunanithi, 2002). The main calcium channels responsible for triggering fast exocytosis are the N and P/Q type calcium channels, and to a lesser extent the R-type channels (Wu et al., 1999; Iwasaki et al., 2000; Reid et al., 2003; Momiyama and Takahashi, 1993; Mintz et al., 1995). The entire calcium channel family is covered in more detail below.

1.1.2. Calcium Signal During Action Potential

Once the AP causes the membrane to depolarize to a potential where calcium channels activate (around – 30 mV), there is a 0.1 ms delay before the channels will allow calcium to enter the cell (Lisman et al., 2007). Initially, due to this delay, channels will begin to open near the peak of the AP (Figure 2) (Geiger and Jonas, 2000). This
Figure 3. Organization of the active zone vesicle release machinery. Fusion of vesicle with the presynaptic terminal begins when the calcium sensor activates the SNARE complex. Once activated, the SNARE complex will bring the two plasma membranes together and cause fusion, ultimately releasing the vesicle stores into the synaptic cleft. Modified from (Shahrezaei and Delaney, 2005).
results in a small current of calcium, since the electrochemical driving force for the cation at this point is small. However, as the AP becomes polarized again, the driving force increases, resulting in a large influx of calcium ions. This influx is short lived as the channels become inactivated before the AP has ended (Figure 2) (Lisman et al., 2007). Even though the calcium channel is open for only an instant, the concentration of calcium rises up to millimolar concentrations (from a resting concentration of 50 nM (Meinrenken et al., 2003)) in close proximity to the channel pore, and decreases greatly over tens of nanometers (Rios and Stern, 1997). Due to the presence of endogenous buffers, this calcium signal is contained within a very small area around the channel, and disappears quickly after the channel is closed (Meinrenken et al., 2003). This calcium ‘domain’ is covered in more detail below. Interestingly, in single-channel patch-clamp experiments looking at channel kinetics at a constant test voltage, the probability of calcium channel opening, and the mean open time, is random and exponentially distributed (Jones, 2003). One has to wonder how evolution would wind up using such a random component to be responsible for a job that requires precision and accuracy. It turns out that the AP waveform itself helps to synchronize the gating of the channel, greatly reducing the randomness of open times / calcium entry (Jones, 2003).

1.1.3. The Calcium Sensor: Synaptotagmin

After calcium has entered the cell, it must then bind to a calcium sensor for exocytosis to be triggered. Two types of exocytosis are known to occur: a fast (200 µs after calcium influx) and synchronous release (Sudhof, 2004); and a slower, asynchronous release characterized as an increase in the rate of spontaneous release which continues for more than a second after calcium influx (Barrett and Stevens, 1972;
This literature review will only focus on the former type of release. The calcium sensor requires a binding of 4-5 calcium ions in order to be activated (Dodge, Jr. and Rahamimoff, 1967; Wolfel and Schneggenburger, 2003; Schneggenburger and Neher, 2000; Bollmann et al., 2000), which current understanding identifies as the synaptic protein synaptotagmin (Sudhof, 2004). Indeed, synaptotagmin has been shown to bind five calcium ions, three on its C$_{2}$A domain (Ubach et al., 1998) and two on its C$_{2}$B domain (Fernandez et al., 2001), and with an affinity that matches that known for the calcium sensor, about 10-20 µM (Bollmann et al., 2000; Schneggenburger and Neher, 2000). Some of the most convincing evidence for this theory comes from knock-out (KO) and mutation experiments in mice. With the selective KO of synaptotagmin-1 in mouse hippocampal neurons, Geppert et al found a selective loss of fast calcium triggered exocytosis (Geppert et al., 1994). Furthermore, when Fernandez-Chacon et al created a point mutation in the C$_{2}$A domain of synaptotagmin-1, a shift in calcium dependent exocytosis was found that was identical to the calcium affinity shift in synaptotagmin-1 (Fernandez-Chacon et al., 2001). The mechanism by which synaptotagmin triggers exocytosis is believed to involve synaptotagmin’s complexes with phospholipids (essential for proper calcium affinity) and the SNARE complexes (Sudhof, 2004).

1.1.4. **SNARE Complex: Vesicle Fusion**

The SNARE complex is a group of proteins that are responsible for holding the vesicle to the presynaptic terminal (Sudhof, 2004), and for ultimately fusing the two together (Figure 3) (Rizo, 2003; Jahn et al., 2003), resulting in the release of neurotransmitters to the synaptic cleft. The complex is made up of specialized proteins
that span the plasma or vesicle membranes. The vesicle spanning SNARE protein (v-SNARE) is called synaptobrevin, or VAMP, which stands for ‘vesicle-associated membrane protein’ (Sudhof, 2004). The plasma spanning proteins (t-SNARE) are syntaxin-1 and SNAP-25 (synaptosome-associated protein of 25,000 daltons) (Sollner et al., 1993). These proteins are molecularly related by a homologous 70-residue sequence named the SNARE motif (Sudhof, 2004). The mechanism by which the SNARE complex works to fuse the vesicle with the plasma membrane involves the orchestrated bundling of these three proteins. Once synaptotagmin binds calcium, it displaces a SNARE-bound-protein called complexin (Giraudo et al., 2006; Tang et al., 2006; Schaub et al., 2006; Zimmerberg et al., 2006). Once removed, the SNARE proteins pull the plasma and vesicle membranes closer and closer together, eventually creating an unstable intermediate that will either progress into full blown fusion, or regress to a docked state (Sudhof, 2004).
1.2. Nanodomain vs. Microdomain Gating:

How far exactly is the calcium channel from the calcium sensor? And how many channels does it take to gate release? It turns out that answering these questions is much more difficult than one may first suppose. Yet, since the answers may have large implications towards better understanding the synaptic system, and potential insights into psychiatric disorders or neural pathology, many researchers have forged ahead.

The experimental approaches that have given the most insight into this issue include the use of imaging, electrophysiological and biochemical techniques. Examples of electrophysiological experiments include patch clamp recordings with caged exogenous buffers (EGTA and BAPTA), modified AP waveforms, and calcium channel antagonist application. Exogenous calcium buffer use was first pioneered by Adler et al. in 1991 (Adler et al., 1991). EGTA and BAPTA, the most commonly used buffers, have very similar affinities for calcium, but BAPTA has significantly faster binding kinetics (Meinrenken et al., 2003). Due to this difference, EGTA has been shown to be unsuccessful at sequestering calcium near the pore. Hence, if a very intimate channel to sensor relationship exists within a synapse that gates evoked transmission, EGTA application will have little to no effect (Neher, 1998). Modified AP waveforms (Borst and Sakmann, 1999) and channel antagonists (Shahrezaei et al., 2006) can be used to reduce the number of calcium channels that open for a given stimulus, which in turn give insights into how many channels are required to gate release. Freeze-fracture experiments have been used to directly quantify the number and distance of putative calcium channels to docked vesicles (Heuser et al., 1979). Finally, pull-down assays may
give insights into whether or not channels can be so close to the vesicle release apparatus that they are actually physically linked (Stanley, 1997).

1.2.1. Calcium Domain:

The exact shape and characteristics of a calcium domain will depend on multiple factors. As already discussed, the main limiting factor preventing calcium ions from diffusing freely throughout the cell is the many fast acting endogenous buffers (Meinrenken et al., 2003). These buffers are important for proper cell maintenance, since too much calcium can be toxic (Stanley, 1997). These buffers account for 97.5% of sequestered calcium once spatial and chemical equilibriums have been reached (Meinrenken et al., 2003). It has been calculated that, if it were not for buffers, the calcium concentration in the cell would reach upwards of 12 µM within milliseconds, as opposed to the actual 400 nM (Helmchen et al., 1997). Another major factor influencing the shape of the calcium concentration topography is the physical geometry of the active zone. For example, the synaptic vesicle can act as a diffusion barrier to calcium, which may prevent calcium from diffusing away, thus increasing the calcium concentration around the calcium sensor. This example alone can account for a potential 13-fold difference in calcium concentrations the sensor may be exposed to (from ~15 µM to ~200 µM) (Shahrezaei and Delaney, 2004). The calcium channels themselves can also have a large impact. Since the N-, P/Q-, and R-type channels have different properties that can lead to different calcium influxes, including: probability of opening; inactivation; mean open time; and conductances (which I will come to later in my thesis), it is easy to see how synapses may behave differently, depending on the channels that are present in the active zone. The last factor I will touch on is the number of calcium channels that are
required to open in order to gate vesicle release, which will be covered in the next two sections.

1.2.2. Microdomain:

An active zone that is gated by a microdomain will occur when multiple calcium channels open simultaneously and their respective calcium domains overlap (Figure 4). This overlapping of calcium concentrations will in effect create a larger domain, such that the concentration of calcium farther from the calcium clusters will be larger than if a single channel were to have opened on its own. Since the domain is only limited to the number of calcium channels available, the distance that the overlapping calcium channels need to be with respect to the calcium sensor is much less restricted (Simon and Llinas, 1985; Fogelson and Zucker, 1985). Furthermore, because the microdomain requires multiple calcium channels to function properly, this type of gating can also be seen as having a high ‘channel cooperativity’ (Bertram et al., 1999).

One of the most well characterized synapses, and the best example of a microdomain gated chemical synapse, is the young (P8-10) calyx of Held of rat (for a comprehensive review, see (Meinrenken et al., 2003)). The calyx of Held is a rather large synapse (~15 µm across) in the mammalian auditory CNS, kind of like a hand holding a baseball, with the hand representing the calyx and the ball the postsynaptic cell (Meinrenken et al., 2003). Due to its large size, experiments that are practically impossible on other small synapses can be performed, including simultaneous recordings of pre- and postsynaptic currents. The microdomain of the calyx of Held is created from a cluster of calcium channels, whose distance from the vesicles ranges between 30 to 300 nm (Meinrenken et al., 2002).
Figure 4. Relationship between calcium channels and vesicle release machinery. Microdomain gated synapse (left) requires the simultaneous opening of multiple calcium channels clustered ~100 nm from calcium sensor. Nanodomain gated synapse (right) requires a more intimate relationship between one or a couple channels with the calcium sensor, on the order of 25 nm. Modified from (Stanley, 1997).
Inside a cluster, it has been calculated that ten or more channels are needed to open in order to gate phasic release (Meinrenken et al., 2002).

Evoked transmission is not developmentally static, however. It turns out that the calyx of Held is a great model for microdomain/overlapping/channel cooperativity only up to a certain age, after which the channel cooperativity decreases (Fedchyshyn and Wang, 2005; Iwasaki and Takahashi, 1998). This may also have something to do with the parallel shift in calcium channel expression, moving from a 50% P/Q-, 25 % N-, and 25% R-type (Wu et al., 1999) to an almost pure P/Q-type gated active zone (Iwasaki et al., 2000). I have, however, not found any evidence to directly relate these two occurrences.

1.2.3. Nanodomain:

The nanodomain, unlike the microdomain, is formed from one or a few calcium channels (Figure 4). This domain has a very steep calcium concentration gradient around the pore (Fogelson and Zucker, 1985; Simon and Llinas, 1985), and thus limits how far the calcium sensor can be in order for it to be gated by a threshold of calcium (10-20 µM) (Llinas et al., 1992; Adler et al., 1991; Yamada and Zucker, 1992; Bollmann et al., 2000). Since only a single or a few channels gate the calcium sensor, these AZs are said to have low channel cooperativity.

There are a number of synapses that have been shown to be gated by nanodomains or with low channel cooperativity. Perhaps one of the most famous synapses, not only in terms of this small subject matter but also because it was the first synapse to be studied in depth, is that of the squid giant axon. It is believed to have a channel to sensor distance in the nanometer range (Neher, 1998) and low channel
cooperativity (Llinas et al., 1981). Low channel cooperativity has also been reported in mouse motor nerve terminals and frog amygdala synapses (Quastel et al., 1992; Mulligan et al., 2001). Using freeze-fracture experiments, the frog neuromuscular junction has been reported to have an intermembrane protein (putative calcium channel, (Pumplin et al., 1981)) to vesicle distance of 10-30 nm (Harlow et al., 2001). Furthermore, it has been calculated that fusion occurs from one or two channels in this synapse (Shahrezaei et al., 2006) with more evidence of low channel cooperativity coming from indirect electrophysiology experiments (Yoshikami et al., 1989; Augustine et al., 1991) and calcium imaging (Wachman et al., 2004). A recent paper has found evidence for a channel to sensor distance of 10-20 nm in parvalbumin-containing basket cells in rat hippocampal neurons (2 months) (Bucurenciu et al., 2008). The first and only synapse to have direct evidence of a single calcium channel gating evoked transmission is the chick ciliary calyx (Stanley, 1993). This synapse is believed to have a channel to sensor distance of 25 nm (Stanley, 1997).

1.2.4. Implications of Synapses Gated by Nano or Microdomains:

Evolution may have come upon the microdomain and nanodomain gated synapses for different specialized functions. For one, the microdomain gated synapses will have to rely on the proper coordination of many channels (Roberts, 1994). Relying on many channels, however, can give a synapse many options. For example, a synapse can change the degree of channel cooperativity by 'choosing' among the many permutations of different calcium channels expressed in different numbers (Mintz et al., 1995; Reid et al., 1997; Wu et al., 1999). Despite the fact that many channels are required to open simultaneously, the main consensus seems to be that an overlapping domain would
provide greater reliability and temporal fidelity (Stanley, 1997; Roberts et al., 1990; Simon and Llinas, 1985; Zucker and Fogelson, 1986; Takahashi et al., 1996). The synapse gated by a nanodomain, on the other hand, behaves completely differently. Take for instance the fact that the nanodomain is wholly reliant on the simple on/off or open-closed fluctuations of a single (or a few) channel(s) (Simon and Llinas, 1985; Roberts, 1994). This can lead to increased efficacy, and temporal precision (Bucurenciu et al., 2008). Another point to consider is that, since the nanodomain is exceptionally close to the calcium sensor, the diffusional component of synaptic delay is minimized (Meinrenken et al., 2002). Interestingly, it has also been reported that the nanodomain may increase the ratio of synchronous to asynchronous release (Hefft and Jonas, 2005; Matsui and Jahr, 2004).

Finally, one final important implication I would like to consider regarding the nanodomain is the possibility that, due to their physical intimacy, a physical tether may connect the calcium channel and the secretory machinery (Stanley, 1997; Spafford and Zamponi, 2003; Berkefeld et al., 2006). This tether, if it exists, could have large ramifications in terms of neuronal pathology and mental health. For instance, it is easy to imagine how a genetic mutation of this protein tether could modify the channel-sensor distance, modifying the synapse and ultimately the mental phenotype. Evidence for this tethering has come from pull-down assays that have associated the N- and P/Q-type channels with key synaptic proteins (see below). Indeed, since these synaptic proteins have all been found to bind to a specialized location on the presynaptic calcium channels, termed the synprint region (see below), functional experiments have been performed to test this connection. Although it has been found that injecting a competitive synprint
region in rat neurons can reduce excitatory postsynaptic potentials by 50% (Mochida et al., 1996), evidence from invertebrates has led some to believe this may be due simply to channel modulation, and not tethering (Spafford and Zamponi, 2003).
1.3. Voltage-Gated Calcium Channels:

It has been said that the very presence of voltage gated calcium channels defines a
cell as being excitable (Hille, 2001). However, it should be noted that these channels
have recently been found in low quantities in cells not typically seen as excitable (e.g.
immune cells) (Cahalan et al., 2001). Voltage gated calcium channels were first
identified in crustacean muscles in 1953, where experimenters who were examining APs
decided to leave sodium out of the bath solution (Fatt and Katz, 1953). They were
probably shocked when they found that they were still able to elicit an AP. Eventually it
was determined that a new type of voltage gated ion channel was responsible, and that
calcium influx was depolarizing the membrane (Fatt and Ginsborg, 1958).

Voltage gated calcium channels, from here on referred to simply as calcium
channels, along with the potassium and sodium channels, form a superfamily of voltage-
activated ion channels (Hille, 2001). As the name suggests, this superfamily represents a
set of membrane spanning, pore forming proteins that open according to voltage changes
and selectively allow the passage of certain ions across their respective electrochemical
gradients. The similarity between these channels can most easily be seen when one
considers that the mutation of only three amino acids in a sodium channel can convert it
to a functional calcium channel (Catterall et al., 2005).

1.3.1. The α1 Subunit:

The main constituent of the calcium channel is a 190-250 kDa membrane
spanning protein, termed the α1 subunit (Figure 5a) (Catterall et al., 2005). This subunit
not only contains the calcium selective pore, but is also responsible for its voltage
activation, gating properties, and contains most of the sites of regulation by toxins and
Figure 5. **Voltage gated calcium channel overview.** A) The composition of the calcium channel, made up of the pore forming α1 subunit and one of each ancillary subunits: β, α2-δ and possibly γ. B) Structure of the α1 subunit. The dark shaded transmembrane alpha helices represent the positively charged S4 regions. C) A phylogenetic analysis of HVA and LVA calcium channels. Abbreviations: MYA, million years ago. Modified from (Spafford and Zamponi, 2003).
second messengers (Catterall et al., 2005). The α1 subunit is made up of a single protein strand, which can be divided into four homologous transmembrane domains (termed I through IV). The transmembrane domains are connected by cytoplasmic linkers, with the I and IV domains containing the cytoplasmic N- and C- terminals, respectively. Each domain contains six membrane-spanning alpha helices: S1-S6, with an external re-entrant loop between S5 and S6 (Figure 5b). It is this set of four ‘P-loops’ that are responsible for the calcium selectivity (discussed below) (Heinemann et al., 1992; Yang et al., 1993).

In total there are ten distinct genes that code for the calcium channel α1 subunit in mammals (Jones, 2003). These genes can be divided into the subfamilies CaV1, CaV2 and CaV3. It is interesting to note that all three of these classes of calcium channels have been found in life forms as far down the evolutionary ladder as the nematode Caenorhabditis elegans (Schafer and Kenyon, 1995). The subfamilies have an amino acid inter-family sequence similarity of less than 40%, but can be more than 70% similar within a subfamily (Figure 5c) (Catterall et al., 2005). The α1 subunits that are encoded from the genes are further subdivided into two biophysically distinct groups: those that are activated at low voltages and those activated at high voltages. The low-voltage activated (LVA) channels are comprised of the CaV3 family (CaV3.1 / α1G; CaV3.2 / α1H; and CaV3.3 / α1I), also known as T-type channels (Hille, 2001). T-type channels generally participate in the generation of repetitive electrical activity (Jones, 2003). They can be blocked with the addition of one of the following to the bath solution: Kurtoxin, a toxin found in the venom of the scorpion Parabuthus transvaalicus; or nickel, with CaV3.2 being the most sensitive (Kisilevsky and Zamponi, 2008). The high-voltage activated (HVA) family is divided into two distinct pharmacological groups: the
dihydropiridine sensitive Ca\textsubscript{v}1 family (Ca\textsubscript{v}1.1 / \alpha\textsubscript{1S}; Ca\textsubscript{v}1.2 / \alpha\textsubscript{1C}; Ca\textsubscript{v}1.3 / \alpha\textsubscript{1D}; and Ca\textsubscript{v}1.4 / \alpha\textsubscript{1F}), or L-type channels; and the Ca\textsubscript{v}2 family (Hille, 2001). L-type channels are predominant in muscle and endocrine cells, where they are involved in contraction and secretion (Catterall et al., 2005). As already mentioned, L-type channels are sensitive to dihydropyridines, which can not only block L-type currents (nifedipine), but also enhance them ((-)BayK8644) (Hille, 2001). The Ca\textsubscript{v}2 channel family is made up of the following \alpha1 subunits: Ca\textsubscript{v}2.1 / \alpha1A, or P/Q-type channel; Ca\textsubscript{v}2.2 / \alpha1B, or N-type channel, which is the main focus of my thesis; and finally the Ca\textsubscript{v}2.3 / \alpha1E, or R-type channel. Ca\textsubscript{v}2.1 and 2.2 channels are particularly important for evoked neuronal synaptic transmission (Dunlap et al., 1995), with the former more often linked to excitatory transmission, and the latter to inhibitory (Potier et al., 1993). Ca\textsubscript{v}2.1 channels are selectively blocked by \omega-Agatoxin IVA, which is extracted from the North American funnel spider \textit{Agelenopsis aperta} (Adams et al., 1993). Ca\textsubscript{v}2.2 channels are selectively blocked by \omega-Conotoxins, specifically \omega-Conotoxin-GVIA, which is extracted from the cone-snail \textit{Conus greographus} (Adams et al., 1993; Feng et al., 2003; Olivera et al., 1984; Reynolds et al., 1986). The key to \omega-Conotoxin GVIA’s ability to block N-type currents is its ability to dock to the channel’s outer region (specifically the S5 and P region of repeat III), thus blocking ion entry (Ellinor et al., 1994). Ca\textsubscript{v}2.3 channels are also involved in neurotransmission, albeit to a lesser extent. Although initially named for its resistance to the other known calcium channel blockers (Randall and Tsien, 1995), R-type channel currents have subsequently been found to be blocked by SNX-482, a toxin isolated from the tarantula \textit{Hysterocrates gigas} (Bourinet et al., 2001; Newcomb et al., 1998).
1.3.2. Calcium Channel Subunits:

Along with the $\alpha_1$ subunit, calcium channels are usually expressed with other subunits, which include $\beta$, $\alpha_2/\delta$, and sometimes $\gamma$ (Figure 5a) (Jones, 2003). In mammals, a standard calcium channel will contain the $\alpha_1$, $\beta$, and $\alpha_2/\delta$ subunits in 1:1:1 stoichiometries (Catterall, 2000). $\beta$ subunits are encoded by four distinct genes, termed $\beta_1$ through $\beta_4$ (Dolphin, 2003). They are cytoplasmic and bind to the alpha interaction domain (AID) located on the $\alpha_1$ I-II linker (Pragnell et al., 1994). The main function of the $\beta$ subunit is as a chaperone molecule, enhancing $\alpha_1$ expression in the plasma membrane (Herlitze et al., 2003). However, it should be noted that the $\beta$ subunit expression can also regulate channel assembly, activation and inactivation (Dolphin, 2003; Catterall, 2000). $\alpha_2/\delta$ subunits are also encoded by four distinct genes (Kisilevsky and Zamponi, 2008). Although named as though it were two subunits, the $\alpha_2$ and $\delta$ parts are encoded by the same gene, but are post-translationally cleaved, and later reattached with a disulfide bond (De Jongh et al., 1990; Klugbauer et al., 2003). The $\alpha_2/\delta$ subunit is expressed in the plasma membrane with the small alpha helical $\delta$ unit in the membrane and the $\alpha_2$ unit on the outside of the cell (Klugbauer et al., 2003). $\alpha_2/\delta$, like $\beta$ subunits, can also regulate channel trafficking, membrane targeting, activation and inactivation (Catterall, 2000). The $\gamma$ subunit is a four alpha helical transmembrane spanning protein, with cytoplasmic N- and C- termini (Arikkath et al., 2003). Although it is known that skeletal muscle calcium channels co-purify with $\gamma$ subunits, it remains unclear if other novel $\gamma$-like cloned subunits are tightly associated with calcium channels, or are even proper calcium channel subunits to begin with (Kisilevsky and Zamponi, 2008).

1.3.3. Interaction with Secondary Proteins:
Calcium channels also interact with various secondary proteins that act by altering the channel’s functional properties. These secondary proteins include G-proteins, protein kinase C (PKC), calmodulin, and certain presynaptic proteins. G-protein coupled receptor (GPCR) activation can lead to calcium channel current inhibition (Beech et al., 1992; Bernheim et al., 1991; Caulfield et al., 1994; Dunlap and Fischbach, 1981; Golard and Siegelbaum, 1993; Ikeda, 1992; Ikeda and Schofield, 1989; Lipscombe et al., 1989; Mintz and Bean, 1993; Shapiro and Hille, 1993; Zhu and Ikeda, 1993). This inhibition can manifest itself as both a voltage dependent (VD) and a voltage independent (VI) component (Tedford and Zamponi, 2006). The VD component is caused by the G$_{\beta\gamma}$ subunit interacting with a cytoplasmic site on the $\alpha_1$-subunit, thought to be located on the I-II linker and the N-terminal (Figure 5b) (Agler et al., 2005). This interaction leads to a type of channel inhibition that can be easily relieved with strong depolarization or a train of AP pulses (thus making it voltage dependent) (Bean, 1989; Hille, 1994; Shapiro and Hille, 1993; Zamponi and Snutch, 1998a; Zamponi and Snutch, 1998b). PKC activation can lead to interference of the N-type channels ability to undergo VD inhibition (Barrett and Rittenhouse, 2000; Swartz, 1993; Swartz et al., 1993). VI inhibition is a slower process, and has been shown to involve, in some cases, the removal of calcium channels from the plasma membrane (Kisilevsky and Zamponi, 2008). Calmodulin represents an important calcium activated feedback mechanism, which regulates channel activity (Halling et al., 2005). Calmodulin is thought to be associated with the $\alpha_1$ C-terminus at a conserved region containing pre-IQ and IQ motifs (Kisilevsky and Zamponi, 2008). Calmodulin modulation can work by enhancing (calcium dependent facilitation (CDF)) or inhibiting (calcium dependent inhibition (CDI)) calcium currents. L-type channels
usually undergo CDI from local calcium signals, while the other channels usually experience CDI from global calcium signals (Liang et al., 2003). The P/Q channel is generally believed to be the only channel that can undergo CDF, which is activated by a local calcium signal (Chaudhuri et al., 2004). N and P/Q channels importantly have been shown to interact with and be regulated by presynaptic proteins (Kisilevsky and Zamponi, 2008). Syntaxin 1, SNAP-25, Rim (N-type only), and synaptotagmin 1 (P/Q type only) have all been shown to interact with these channels (Coppola et al., 2001; Jarvis and Zamponi, 2001; Sheng et al., 1994; Sheng et al., 1997; Charvin et al., 1997). One site of action on the α1 subunit for synaptic protein interaction occurs on the I-II linker, appropriately termed the synprint site (Figure 5b) (Sheng et al., 1994). Deletion or substitution of the synprint site can lead to loss of presynaptic localization of these channels (Mochida et al., 2003; Harkins et al., 2004), which has led to speculation that one of the functions of the synprint site may be to aid synaptic vesicles locate sources of calcium entry (Zamponi, 2003).

1.3.4. Activation and Inactivation of the Channel:

Activation and inactivation are important properties of the calcium channel that help to determine the timing and amount of calcium that enters the cell. Inactivation comes in two forms, a calcium dependent process and a voltage dependent one (Budde et al., 2002; Eckert and Chad, 1984; Hering et al., 2000; Stotz and Zamponi, 2001). The voltage dependent mechanism is believed to be a result of a variety of mechanism (Jones, 2003), while the calcium dependent process is calmodulin related.

The mechanism of voltage activation is thought to involve the four S4 segments (Figure 5b) (Jones, 2003). These S4 segments contain positive Arginine and Lysine
residues, generally spaced in every third position along the helix (Jones, 1998). It is this positive charge that is believed to be responsible for the channel’s ability to sense voltage changes. Thus, the channel can be considered to be an allosteric protein, where four separate voltage sensors regulate the pore (Jones, 2003). If calcium channels behave in a similar manner as voltage dependent potassium channels from bacteria do, then the activation of the channel comes from an outward movement of a ‘voltage-sensor paddle’, which is composed of the S4 and S3 segments (Jiang et al., 2003). The movement of the paddle in turn pulls on the S4-S5 linker, which itself pulls on the cytoplasmic gate of the channel, thus allowing ions to flow through (Jiang et al., 2003).

1.3.5. **P-loop:**

The selectivity that the calcium channel displays for calcium over all other ions is quite astounding. Consider, for instance, how monovalent ions are present at 100 fold higher concentrations than calcium (Tsien et al., 1987), yet the channel shows a greater than 1000 permeability ratio between calcium and sodium or potassium (Jones, 1998). The calcium channel’s ability to select for calcium is contained in the P-loops mentioned earlier (Heinemann et al., 1992; Yang et al., 1993). The four P-loops made up from the four domains each contain a highly conserved negative amino acid. In HVA channels, these residues are all glutamate, and in LVA channels, they are two glutamates and two aspartates (Jones, 1998). These charges are so crucial that a mutation at even one of the residues can eliminate the high affinity binding of calcium (Catterall et al., 2005). The exact mechanism by which these residues enable the channel to select calcium will be covered in detail in the next section.
1.4. Transport Rates of Calcium Channels:

The transport rate of an ion through a channel is an important property that will determine the amount of ions that will pass through in the short amount of time that the channel is open. It should be obvious by this point that this property is a key element in determining the calcium domain that is so crucial for phasic release of neurotransmitters. Transport rates, or conductances, are measured, usually at the molecular level, using the single-channel variant of the patch-clamp method (Figure 6). This technique allows for a direct voltage and solution control of a single ion channel, with current measurements on the picoampere scale. There are three single-channel patch-clamp variations, each with their own benefits (Figure 6). The on-cell method allows for control of the channel’s external environment while maintaining the channel’s natural environment in the cell’s plasma membrane and internal cytoplasm. The inside-out patch is obtained when the channel and its surrounding membrane is removed from the cell, allowing for control of the environment that would usually be the cytoplasm of the cell. Finally, the outside-out patch is similar to the inside-out patch but the channel becomes flipped, allowing for more direct access to the environment that would usually be the external part of the cell.

1.4.1. Measuring Conductance:

Determining the conductance of a channel can be tricky, but for calcium channels it can border on down right impossible. The reason that calcium channels are special is because of the exceptionally small amount of calcium that is inside the cell, and the huge difference of concentrations between the two sides of the membrane (Hille, 2001). Usually a channel’s conductance can be calculated from its chord conductance, which
Figure 6. Variations on the patch clamp method. The different types of patch-clamp methods and how they are obtained. This thesis employed the on-cell method pictured at the top. Taken from Wikimedia Commons, http://upload.wikimedia.org/wikipedia/commons/1/1f/Patchmodes.svg
is calculated from Ohm’s law (Jones, 1998). Ohm's law can be written as the following: \( g_{Ca} = I / (E - E_{Ca}) \). Here \( g_{Ca} \) is the chord conductance of calcium; \( I \) is the current; \( E \) is the potential and \( E_{Ca} \) is calcium’s equilibrium potential. An ion’s equilibrium potential is reached when the electrochemical gradient of the ion is zero. Usually this potential can be measured as a ‘reversal potential’, which is the voltage at which there is no net flow of ions in both directions, or when the observed current equals zero. The Nernst equation predicts that calcium’s reversal potential should be +124 mV (100 nM [Ca]_i and 2 mM [Ca]_o (Hille, 2001)), but when measured from whole cell or single channel recordings, is closer to +60 mV. The reason for this large voltage difference is because of the outward movement of monovalent ions through the channels (in an unperfused cell, this monovalent current will most likely come from potassium ions, since they are \( 10^6 \) times more prevalent in the cell than calcium) (Hille). However, even when this monovalent current is eliminated, the tiny amount of calcium on the inside of the cell is too small to be measured as outward current, hence making the equilibrium potential almost impossible to measure. Even if the equilibrium potential could be measured, however, there still remains an even larger obstacle. Goldman (Goldman, 1943) and Hodgkin & Katz (Hodgkin and Katz, 1949) have shown that any ion with an unequal concentration on the two sides of a membrane (calcium has a 20,000:1 ratio) will exhibit non-linear I-E relations, thus making it non-Ohmic to begin with (Hille, 2001).

How then do scientists measure conductance? It turns out that calcium ions will exhibit linear and ohmic behaviours at hyperpolarized potentials, along the lines of < -30 mV (Church and Stanley, 1996; Bittner and Hanck, 2008; Rubart et al., 1996; Yue and Marban, 1990). It is this 'slope conductance' that is measured instead of the 'chord
Another problem that plagues electrophysiologists is the signal to noise ratio encountered when performing single channel measurements. Many researchers will end up using barium instead of calcium, since it often gives the largest currents in HVA calcium channels, and block potassium currents at the same time (Hille, 2001). Furthermore, barium will most often be used in high concentrations, usually 100 mM, 50 times the concentration of $[\text{Ca}]_{\text{Ext}}$ in physiological systems. Due to some technological advancements though, a couple of calcium channel conductances have been measured using calcium and at low concentrations. See Table 1 for an overview of the current conductance levels measured for the calcium channel family.

1.4.2. Selectivity and Rate Theory:

The calcium channel pore is highly selective for divalent cations, yet also produces high conductance rates for these ions. This behavior comes from the fact that the channel contains multiple binding sites within the pore, where ions must pass in single file (Almers and McCleskey, 1984; Hess and Tsien, 1984; Friel and Tsien, 1989; Nonner et al., 2000; Nonner and Eisenberg, 1998; Corry et al., 2001). The EEEE locus, as already mentioned, is the most important location for binding (Hille, 2001). It can bind a single divalent cation with high affinity, or two divalent ions with low affinity (Ellinor et al., 1995; Yang et al., 1993; Kim et al., 1993; Mikala et al., 1993; Sather et al., 1994; Ellinor et al., 1995; Tang et al., 1993; Yatani et al., 1994; Bahinski et al., 1997; Heinemann et al., 1992). These interaction sites come from the eight carboxyl groups (two from each glutamate), four of which form the central high affinity site, with the other two binding locations formed from the other four carboxyl groups (two for each site) (from Wang et al. (Wang et al., 2005), based on structure models of Lipkind and
Fozzard (Lipkind and Fozzard, 2001), step models of Dang and McCleskey (Dang and McCleskey, 1998), and the experimental findings of Kuo and Hess (Kuo and Hess, 1993). According to the Eyring rate theory, these binding sites act as energy wells, which are in turn separated by energy barriers (Lauger, 1973). In order to traverse the channel pore, ions must be able to overcome these energy barriers. Due to a divalent ion’s strong positive charge, it will be able bind to the highly negative pore with high affinity (deep energy well). This is why, for example, the channel can select for calcium over sodium, even though they are nearly identical in size (2.00 vs 2.04 Å, resp). Sodium ions simply do not have enough positive charge to neutralize the pore without over crowding it (Wang et al., 2005). After an appropriate ion has become bound to a low energy well, it will only leave once it has enough energy to overcome the next energy barrier. This is hypothesized to come from an appropriate second ion entering the pore, binding to another site, and pushing the first ion out with electrostatic repulsion (Hess and Tsien, 1984; Almers and McCleskey, 1984). It is this negative cooperativity between binding sites that creates the high conductance of the channel (Kim et al., 1993; Yang et al., 1993; Ellinor et al., 1995).

If an ion has an extremely high affinity for the binding site, the energy well may be so deep that the electrostatic repulsion from an incoming ion will not have enough energy to kick the high affinity ion out of the pore. This is the case for nickel, cadmium, cobalt, manganese, magnesium and lanthanum, which can block calcium channels at 10 μM to 20 mM concentrations (however, it should be noted that in some channels, CaV1.1 for instance, some of these ions are permeable) (Hille, 2001). Indeed, this is the very reason that when divalent ions are removed from the external solution, calcium channels
<table>
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<td>Xenopus oocytes</td>
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<td>12.5</td>
<td>-50</td>
<td>100 Ba</td>
<td>(Bourinet et al., 1996)</td>
</tr>
<tr>
<td>Xenopus oocytes</td>
<td>(\text{CaV}2.3)</td>
<td>12</td>
<td>-50</td>
<td>100 Ca</td>
<td></td>
</tr>
<tr>
<td>HEK293</td>
<td>(\text{CaV}3.1)</td>
<td>7.5</td>
<td>-50</td>
<td>115 Ba</td>
<td>(Perez-Reyes et al., 1998)</td>
</tr>
<tr>
<td>Xenopus oocytes</td>
<td>(\text{CaV}3.2)</td>
<td>9</td>
<td>-40</td>
<td>110 Ba</td>
<td>(Williams et al., 1999)</td>
</tr>
<tr>
<td>Xenopus oocytes</td>
<td>(\text{CaV}3.3)</td>
<td>11</td>
<td>-70</td>
<td>115 Ba</td>
<td>(Lee et al., 1999)</td>
</tr>
<tr>
<td>Guinea-pig CA3 neurons</td>
<td>(\text{CaV}3.X)</td>
<td>7</td>
<td>-50</td>
<td>90 Ba</td>
<td>(Mogul and Fox, 1991)</td>
</tr>
<tr>
<td>Rat motoneuron</td>
<td>(\text{CaV}3.X)</td>
<td>7</td>
<td>-30</td>
<td>110 Ba</td>
<td>(Umemiya and Berger, 1995)</td>
</tr>
<tr>
<td>Chick sensory neurons</td>
<td>(\text{CaV}3.X)</td>
<td>8</td>
<td>-15</td>
<td>110 Ba</td>
<td>(Nowycky et al., 1985)</td>
</tr>
<tr>
<td>Mouse sensory neurons</td>
<td>(\text{CaV}3.X)</td>
<td>7.2</td>
<td>NA</td>
<td>60 Ba</td>
<td>(Kostyuk et al., 1988)</td>
</tr>
<tr>
<td>Rat retinal ganglion cells</td>
<td>(\text{CaV}3.X)</td>
<td>8</td>
<td>-30</td>
<td>96-110 Ba</td>
<td>(Karschin and Lipton, 1989)</td>
</tr>
<tr>
<td>Rat CA1 pyramidal cells</td>
<td>(\text{CaV}3.X)</td>
<td>9</td>
<td>NA</td>
<td>20 Ba</td>
<td>(Magee and Johnston, 1995)</td>
</tr>
<tr>
<td>Rat and chick dorsal root ganglia neurons</td>
<td>(\text{CaV}3.X)</td>
<td>5.2</td>
<td>-60</td>
<td>50 Ca</td>
<td>(Carbone and Lux, 1987)</td>
</tr>
</tbody>
</table>

* NR: not reported.
will allow monovalent ions through. The monovalent ions are no longer blocked by a tight binding divalent ion. Furthermore, this explains why the addition of calcium in the micromolar range can block this current (Kostyuk et al., 1983; Almers et al., 1984a; Hess et al., 1986; Coronado and Affolter, 1986b; Coronado and Affolter, 1986a), since once a calcium ion is bound to the pore, it will require another calcium ion to remove it.

1.4.3. Evidence for the Multi-Ionic Pore

The multi-ionic single-file nature of the calcium channel is revealed in other ways as well. One of the more peculiar observations, appropriately dubbed the anomalous mole fraction effect (AMFE), is found when a mixture of divalent cations is used as the external solution (Hille, 2001). For example, the conductance of a mixture of external calcium and barium of a certain concentration will be lower than if there was only barium or calcium alone at the same concentration (Almers and McCleskey, 1984; Hess and Tsien, 1984). The reason that this occurs is the following. Once calcium is bound to the pore, it will lie deep in an energy-well and will not want to budge until another calcium ion enters the channel and occupies a nearby binding site. Thus, the first calcium ion will effectively block ions, while not adding anything to the current itself (Yue and Marban, 1990). Another example of the multi-ionic nature of the pore is the conductance’s dependence on concentration. If a channel were a simple single-channel pore, then the transport rate would be expected to saturate at a high enough concentration and behave under Michaelis-Menten kinetics (which is most often used to describe the saturating behaviour of most enzymes) (Lauger, 1973; Bell and Miller, 1984). At limited concentration ranges, on the order of one or two orders of magnitude of change, the conductance may appear to operate in this way (Hille, 2001). However, with a large
enough concentration range covered, the conductance dependence will obey multi-ion saturation kinetics. Indeed, it has been shown that an n-site model can produce up to n rising phases in the conductance dependence (Hille and Schwarz, 1978).

1.4.4. Barium vs. Calcium

Why then do most calcium channels have a higher transport rate for barium over calcium? One explanation that has been suggested involves the size difference between the two. Although they can both neutralize the negative charges in the pore equally, barium, which is 36% larger than calcium (2.72 vs 2.00 Å, respectively), may produce a larger steric hindrance (Wang et al., 2005). This would create a low energy well for the ion, and would result in a faster exit rate. This may not be the whole story, unfortunately. For instance, Wang et al. (Wang et al., 2005) found that a single mutation of a non-negative residue in the pore loop of domain III could change the whole cell $g_{Ba}/g_{Ca}$ from 2.2 to 0.95, which was further shown to be a result of a specific decrease in barium conductance. It turns out that additional amino acids, termed the divalent cation selection (DCS) and located above the selectivity filter, may help determine the channel’s selectivity and permeability properties (Cens et al., 2007).
2. Aims of Thesis and Rationale:

In order to fully understand evoked neurotransmission, direct measurements of central steps along the release path must be made. These steps include: the behaviour of endogenous buffers, calcium sensor mechanics, active zone topography and calcium channel biophysics. To this day, the ‘calcium domain’ – i.e. the 3D spatial concentration map of calcium that is created almost instantaneously when a channel opens – remains at best an approximation. One reason for this is due to presynaptic type calcium channel transport rates at physiological external calcium levels remaining unquantified. Instead, researchers have relied upon the conductance measurements of the non-presynaptic type calcium channel, Ca\(_V\)1.X (Meinrenken et al., 2003), whose conductance has been studied with calcium concentrations ranging from 1 to 110 mM (Church and Stanley, 1996).

For my thesis, I chose to directly measure the conductance of the presynaptic type Ca\(_V\)2.2 calcium channel at 2 mM calcium and in a native system (chick dorsal root ganglia (DRG) neurons) using non-standard single channel recordings. This conductance value can by predicted by multiplying the 110 mM barium conductance ratio of Ca\(_V\)1 (26 pS) (Church and Stanley, 1996) and Ca\(_V\)2.2 (15.5 pS) (Lipscombe et al., 1988), by the known 2 mM calcium conductance of Ca\(_V\)1 (2.6 pS) (Church and Stanley, 1996):

\[
\left( \frac{g_{Ba100Ca_V\ 2.2}}{g_{Ba100Ca_V\ 1.X}} \right) \times g_{Ca2Ca_V\ 1.X} = g_{Ca2Ca_V\ 2.2}
\]

Thus, we hypothesized the conductance of the Ca\(_V\)2.2 calcium channel at 2 mM calcium to be 1.55 pS.
3. Methods:

3.1. Cells:

Acute dorsal root ganglia neurons from E15 chicks were used due to their natural expression of virtually only the \( \text{Ca}_{2.2} \) calcium channel (Chan and Stanley, 2003). The basic ganglia digestion has been reported before (Stanley and Goping, 1991), but briefly involves enzymatic digestion using collagenase, hyaluronidase, trypsin inhibitor and dyspase in MEM solution and incubated at 37°C with 8% CO\(_2\) and 92% O\(_2\) for an hour. After digestion, ganglia were triturated, plated, and placed back in the incubator for another hour. Cells were then washed twice with fresh MEM, incubated for another hour, then transferred to another incubator at room temperature, again with 8% CO\(_2\).

3.2. Electrophysiology:

On-cell single channel patch clamp recordings were obtained at room temperature using a Nikon inverted microscope and a PlanApo 60/1.40 oil objective. Patch pipette solutions were (in mM, unless otherwise stated): BaCl\(_2\) or CaCl\(_2\) 1-100; HEPES (Na) 10; TEA-Cl 30; TTX 0.3 µM; 4-AP 2; Ni(ClO\(_4\))\(_2\)•6H\(_2\)O 0.1; Nifedipine 2 µM; R-ros covitine 100 µM. Bath solutions were (in mM): KAsp 140; EGTA (Cs) 5; HEPES (Cs) 10; MgCl\(_2\) 1. Osmolarity was adjusted with N-Methyl-D-Glucamine Chloride salt. Bath solution osmolarity was adjusted to 309 mOsm and that of the patch pipette to 314 mOsm. A very high concentration of potassium was used in the bath solution in order to collapse the resting potential, which allowed for greater control over the membrane potential at the location of the patch. Besides using chick DRG neurons, \( \text{Ca}_{2.2} \) channel currents were further isolated by including various blockers for other types of channels in the patch pipette solution. These include nickel to block low voltage activating \( \text{Ca}_{3.X} \) / T-type
channels; the dihydropyridine CaV1.2 / L-type channel antagonist nifedipine; potassium blockers 4-AP and TEA-Cl; and finally TTX to block sodium channels.

Online filtering was done at 10 kHz with a sampling rate of 20 μs, while offline low Bessel filtering was done at 1 kHz using pClamp 9.2 software.

3.3. Noise reduction:

Noise reduction was accomplished by using pure quartz glass (Sutter P-2000 Laser Based puller); low bath volume to reduce capacitance between the glass and solutions; and finally an Axon 200B with Cooled Head Stage to reduce electrical noise. Noise was successfully reduced to approximately 0.17 pA I\textsubscript{RMS}.

3.4. Voltage Protocols:

Currents were elicited using up to four different voltage protocols (Figure 7): a simple ramp protocol at a rate of 1 mV/ms from the holding potential (-80 mV) to +80 mV; a depolarized-repolarizing ramp protocol, involving a 20 ms step to the calcium channel reversal potential, +60 mV, and then ramped down at 1 mV/ms to the holding potential; a simple 200 ms depolarizing step at various potentials; and finally the same step protocol but following a 20 ms initial step to +60 mV. The first two protocols were used early on in the experiment since I was unaware of another way of activating the channels. However, when I discovered the last two protocols mentioned above, with the initial step to +60 mV in order to activate the channels but not illicit current (since +60 mV is around calcium’s reversal potential), almost all recordings were made with them.
Figure 7. **Voltage protocols used to elicit currents.** From top to bottom: ramp protocol, 160 ms; step protocol to various potentials (-30 mV pictured), 200 ms; depolarized-hyperpolarizing ramp, 160 ms; and step protocol preceded by depolarization to reversal potential (+60 mV). Note, all plus and minus symbols are reversed for the actual protocols since the patch pipette was on the surface of the cell and therefore on the outer side of the channel.
4. Results:

4.1. Enhancing Channel Open Times:

One reason CaV2.2 single channel recordings have been so difficult to record in the past is due to the channel’s transient open times. (R)-roscovitine, a cyclic-kinase inhibitor, has recently been implicated in enhancing whole cell calcium currents through its ability to slow deactivation kinetics of CaV2.2 channels (Cho and Meriney, 2006). Furthermore, I had seen some evidence that (R)-roscovitine's ability to prolong the channel's mean open time occurs without affecting the channel's conductance (Pilato et al., 2008). Therefore, I decided to use 100 µM (R)-roscovitine alternatively in the patch pipette in order to obtain longer and cleaner channel openings, allowing for more accurate measurements. Initial tests with 100 mM barium in the patch pipette did give promising improvements in mean channel open times (Figure 8). Since the aim of this study was not to look at (R)-roscovitine’s ability to enhance mean channel open times, I did not pursue further analysis of this phenomenon.

4.2. Conductance Measurements with High External Calcium:

Current amplitude levels were determined by eye using the computer program Clampfit, included in the pClamp 9.2 suite (Figure 9A). Cursors were aligned with the closed and open current levels, and the difference in current was recorded. This method was used instead of the standard all-points histogram analysis since channel openings were rare. However, when it was possible to do a histogram analysis (Figure 9A), amplitude levels were found to be indistinguishable (Figure 9B).

Since chord conductances could not be properly measured (see above), slope conductances were used to obtain transport rates. Due to the non-linear, non-Ohmic
Figure 8. Effects on single channel open times using (R)-roscovitine. Examples of single channel currents recorded without (left) and with (right) (R)-roscovitine. Note the reduction in transient openings and the increase in long channel openings. Recordings were made with 100 mM barium in the patch pipette, and using a step protocol from holding potential (-80 mV) to -20 mV.
Figure 9. Determining the conductance with 100 mM $[Ca]_{Ext}$. A) Example of how current amplitudes at various test potentials were determined by eye using Clampfit 9.2. Respective histogram analysis of elicited single channel currents shown on right. B) Sample of ramp traces with overlay of amplitudes determined from step protocols. Note the similarity in current-voltage relationships obtained from the different voltage protocols. Unfilled circles represent current amplitudes determined from traces in A, filled squares represent mean current amplitudes determined by eye. Mean single channel conductance was determined to be 11.70 pS ($n = 6$).
dependence of current on voltages higher than -30 mV, this slope was measured, when possible, at more hyperpolarized levels, between -80 mV and -30 mV (Figure 9b). Slope conductances were measured by either finding the slope to currents elicited by ramp protocols or by doing a line of best fit to the mean current amplitudes determined from step protocols.

Initially it was believed that the ramp protocols would be particularly significant in measuring conductances since they allow for the recording of current amplitudes from different voltage levels in a single trace. However, in most cases these protocols would only elicit channel openings at potentials more positive than -30 mV. Although the depolarized-repolarizing ramp did seem to elicit current at more hyperpolarized levels, it was the step protocols that were most successful at obtaining currents at voltages less than -30 mV. When channel openings were obtained at the same voltages using different voltage protocols, current levels were found to be identical.

With 100 mM calcium in the patch pipette, an activation potential of around -30 mV and a reversal potential around +60 mV was found. Conductance values ranged from as low as 8.5 to as high as 16.5 pS, with a mean conductance of 11.7 ± 2.9 pS (n = 6) (note: conductances are given in means plus or minus standard errors).

4.3. \( \omega \)-Conotoxin-GVIA:

Next, in order to determine whether or not currents were coming from \( \text{Ca}_v\)2.2 channels, I obtained recordings with patch pipette solutions that contained 2 \( \mu \)M \( \omega \)-conotoxin-GVIA, a strong calcium channel antagonist. With conotoxin in the patch pipette, it was rare that any channel currents could be discerned. When currents were detected, they were only found in a few out of many sweeps. This \( \omega \)-conotoxin-GVIA
Figure 10. Use of $\omega$-Conotoxin-GVIA. Comparison of ramp traces with (top) and without (bottom) the toxin. The rare currents recorded with conotoxin were smaller than the more prevalent currents recorded without conotoxin. Recordings obtained with 100 mM [Ca]$_{\text{Ext}}$. Mean conductance level for conotoxin resistant channel was 4.80 pS (n = 3).
resistant current was smaller than the currents measured without conotoxin (Figure 10). Since the larger currents were never detected with conotoxin in the patch pipette, they were identified as coming from Ca\textsubscript{V}2.2 currents. The Ca\textsubscript{V}2.2-resistant currents had a mean conductance of 4.8 pS with a range of 4.2 to 6.4 pS (n = 3). No attempt was made to determine if the smaller currents were from another channel or were a result of a conotoxin resistant sub-conducting state of Ca\textsubscript{V}2.2 channels. *

4.4. Conductance Measurements with Lower External Calcium:

Naturally I went on to measure conductances with lower concentrations of calcium; in mM and decreasing order they were: 30, 10, 4, 3, 2, 1.5, and 1 (Figure 11). Even at concentration levels as low as 1 mM, a clear conductance value could be determined. Rarely, two distinct current levels could be detected in a single patch (Figure 12). The two current levels did not appear to be distinct conductance states of the same channel since unambiguous transitions from one to the other were not observed. On the other hand, if the currents were from two different channels, a third current level would be expected if the channels were open simultaneously. This was not observed as well. Thus, in order to test if they came from different channels, ω-conotoxin-GV1A was once again used. Conductances from ω-conotoxin-GV1A resistant currents were small and matched the conductances of the smaller current levels detected without conotoxin. At lower concentrations (< 3mM), however, the conotoxin resistant conductances were very similar in size with the ‘larger’ conotoxin-sensitive currents. As before, the larger conducting currents were identified as coming from Ca\textsubscript{V}2.2 channels (Figure 13). A non-linear decrease in conductance was found with decreasing external calcium concentrations. This relationship, as per previously published studies
Figure 11. Cav2.2 conductances obtained with lower [Ca]_{Ext} concentrations. From top to bottom, recordings were obtained using 30, 10 and 2 mM calcium. Mean conductances were 9.77 (n = 3), 6.43 (n = 8), and 2.76 pS (n = 4), respectively.
Figure 12. Two conductance states in a single patch. These two current levels were detected using step protocols with 10 mM calcium in the patch pipette. The conductances measured were 5.5 and 2.5 pS.
Figure 13. **Conductance dependence on calcium concentration.** Top graph shows all conductances found over the calcium concentration range of 1 to 100 mM. Bottom graph shows the larger conducting channel identified as belonging to the Cav2.2 channel. Data points fit to a Langmuir isotherm:

\[ g = \frac{g_{\text{max}}}{1 + K_D/[\text{Ca}_{\text{Ext}}]} \]
Yue and Marban, 1990), was fit with a Langmuir isotherm (Michaelis-Menten kinetics) (Figure 13). The fit gave an $r^2$ value of 0.87 and a $K_D$ value of $7.4 \pm 1.2$ mM. At 2 mM calcium, which is closest to the true physiological value, $\text{Ca}_V2.2$ conductance was found to be $2.76 \pm 0.24$ pS ($n = 4$).

4.5. *Conductance Measurements with High External Barium:*

I next moved on to measuring conductances with barium as the external divalent ion (Figure 14). Barium is known for having a higher conductance than calcium, and at high concentrations gives great signal to noise ratios. Single channel recordings of the calcium channel families with 100-110 mM barium have been performed many times before (see Table 1), with a distinct conductance being identified for each family. With 100 mM $[\text{Ba}^{2+}]_{\text{Ext}}$, conductances were found to vary greatly over a large range, from 9.4 to 22.0 pS ($n = 10$). Unfortunately, two distinct levels were not found in a single patch, which could potentially help to divide the conductances into two groups, as was found to be the case with calcium.

When I moved on to measuring currents with lower $[\text{Ba}^{2+}]_{\text{Ext}}$ concentrations, however, in very rare instances two discernable amplitudes were found in a single patch (Figure 15). As with calcium, the larger conductance was identified as belonging to $\text{Ca}_V2.2$ channels and the lower conductance was left unidentified. Thus, having identified the current level of interest, I found the $\text{Ca}_V2.2$ channel to have a mean conductance of $19.81 \pm 2.07$ pS ($n = 6$) in 100 mM external barium. This value agrees well with previous literature findings (see Table).

4.6. *Conductance Measurements with Lower External Barium:*

I next went on to measure barium conductances with lower external barium
Figure 14. *Ca*\textsubscript{v}2.2 current recordings and conductance at 100 mM barium. Selected traces on left show current amplitude and channel openings at various test potentials. Ramp with overlay of current amplitudes taken from step protocols (right) taken from same cell as on the left. Mean channel conductance for 100 mM [Ba]\textsubscript{Ext} was 19.81 pS (n = 6).
Figure 15. Two conductance states in a single patch. These two current levels were taken from a ramp protocol with 10 mM Barium in the patch pipette. The upper trace gave a conductance of 5.9 pS and the lower trace gave a conductance of 11.6 pS.
Figure 16. Cav2.2 conductances obtained with lower [Ba]_{ext} concentrations. From top to bottom, recordings were obtained using 30, 10 and 2 mM barium. Mean conductances were 14.00 (n = 3), 10.93 (n = 2), and 4.02 pS (n = 2), respectively.
concentrations (Figure 16). The full range of concentrations used was (in mM and decreasing order): 30, 10, 3, and 2. Again, the use of the rare patch recordings that had two distinct current levels was used to divide the conductances into two groups (except at the 2 and 3 mM concentrations). The larger conducting current was identified as coming from CaV2.2 channels (Figure 17).

At 2 mM external barium, mean conductance was found to be $4.02 \pm 1.50$ pS ($n = 2$). Similarly with calcium, conductance was found to decrease non-linearly with lower patch electrode barium concentrations (Figure 18). The Langmuir isotherm fit to the conductance dependence gave an $r^2$ value of 0.92 and a $K_D$ value of $12.0 \pm 3.0$ mM.
Figure 17. Identifying the lower conducting channel with barium. Top graph shows all conductances found over the barium concentration range of 2 to 100 mM. Red circles represent lower conducting currents found in conjunction with a larger current in the same patch. Bottom graph shows how the lower conducting currents that weren’t found together with a larger conducting current were identified.
Figure 18. Conductance dependence on barium concentration. Larger conducting channel identified as belonging to the Ca\textsubscript{v}2.2 channel from Figure 15 fit to Langmuir isotherm.
5. Discussion:

This thesis presents the first quantification of the conductance of a Ca\textsubscript{V}2.2 channel in a native system at physiologically relevant calcium concentrations (1-4 mM). In E15 chick dorsal root ganglia neurons, using the on-cell single channel patch clamp technique at room temperature, the mean conductance of the Ca\textsubscript{V}2.2 channel in 2mM external calcium concentration is 2.76 ± 0.24 pS (n = 4); 1.22 pS more than my hypothesized value.

5.1. Identifying the Channel:

The following evidence was used to identify the single channel currents observed as belonging to the Ca\textsubscript{V}2.2 channel: chick dorsal root ganglia neurons express, in terms of calcium channels, express virtually only the Ca\textsubscript{V}2.2 family (90% of whole cell current blocked with \(\omega\)-Conotoxin-GVIA application; Chan and Stanley, 2003); agreement with previously published studies which used 100-110 mM barium as the divalent charge carrier (see Table 1); activation at -30 mV and reversal at +60 mV; and the absence of current when in the presence of \(\omega\)-Conotoxin-GVIA. The channel, however, exhibited two separate conductance levels, similar to findings of Ca\textsubscript{V}1 from Church and Stanley (Church and Stanley, 1996). Whereas Church and Stanley only observed two conductance levels with calcium as the charge carrier, I observed both levels using either barium or calcium. The smaller conducting current was, in both cases, rare, small, and resistant to \(\omega\)-Conotoxin-GVIA (but was only tested with calcium). Although this small conductance was quantified, its channel identity remains unknown. Due to its resistance to the well-known Ca\textsubscript{V}2.2 antagonist \(\omega\)-Conotoxin-GVIA (Adams et al., 1993; Feng et al., 2003; Olivera et al., 1984; Reynolds et al., 1986), this current could either come from
another calcium channel, or perhaps from an as yet unidentified conotoxin-resistant low-conducting alternative open configuration of Ca\textsubscript{V}2.2. When the two currents were found in the same trace, no unambiguous transition between the two levels were found, which would be indicative of a shift from one open-configuration to another. Finally, since nickel was used to block T-type currents, and it is known that the Ca\textsubscript{V}3.1 and Ca\textsubscript{V}3.3 channels are not very sensitive to this block (Kisilevsky and Zamponi, 2008), the lowconducting current is most likely from one of these channels.

5.2. Conductance Dependence on Divalent Ion Concentration:

Attempts to fit the Ca\textsubscript{V}2.2 conductance dependence on divalent cation concentrations using the Michaelis-Menten equation were moderately successful for both barium and calcium. This agrees well with similar findings from the Ca\textsubscript{V}1.X channel (Church and Stanley, 1996). These results suggest that the channel exhibits behaviour similar to a single-ion binding mechanism with either barium or calcium as the charge carrier. The K\textsubscript{D} value that is obtained can be interpreted as being the dissociation constant between the channel pore and the divalent ion. Thus, since the pore has a higher K\textsubscript{D} value with barium than with calcium, it should kick out barium faster and have a higher conductance; which is exactly what is found.

It should be noted that in 1990, Marban and Yue gave evidence that modeling the Ca\textsubscript{V}1.X conductance dependence on barium concentrations using a single-ion pore mechanism was unsuccessful (Yue and Marban, 1990). Instead, using the Eyring rate theory, they were able to show that the dependence was best modeled with a multi-ion pore occupancy mechanism. Specifically, they found a three-ion binding pore best fit their results. As discussed in the introduction, current understanding of the calcium
channels favours a multi-ion binding pore. My findings, however, as has been argued before by Almers and McCleskey, does not argue against the multi-ion pore mechanism for the Ca\textsubscript{v}2.2 channel, only that it can be successfully \textit{modeled} as behaving like a single-ion pore in the concentration range that was used (Almers et al., 1984b). Indeed, Bertil Hille wrote “in experiments with limited concentration changes of only one or two orders of magnitude, the conductance might appear to obey the predictions of independence or of simple one-ion saturation” (Hille, 2001).

5.3. \textit{Similar Conductance with Ca\textsubscript{v}1.X Channel with Calcium:}

One surprising finding that came from this study was the fact that the conductance of Ca\textsubscript{v}2.2 with external calcium across a range of 1 to 100 mM is almost identical to that of the L-type channel, Ca\textsubscript{v}1.X (Figure 19). This is significant since many scientists using studies with barium as the charge carrier have thought of differences in conductance values as biophysical identifiers of the channels themselves (Catterall et al., 2005). Could the other HVA channels (Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.3) also have identical conductances with calcium? Some supporting evidence of this phenomenon comes from a 1996 study by Bourinet \textit{et al} (Bourinet et al., 1996). They found that, with 100 mM calcium as charge carrier, the Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 channels had almost identical conductances (approximately 9.8 pS). Thus, researchers in the future should be cautious when using conductance values (at least with calcium as the charge carrier) in order to distinguish channel types. It remains to be seen if this holds true across the whole 1 to 100 mM calcium range. It is interesting to think of how, although the high voltage activated channels have evolved for different functions for different areas of different
Figure 19. Side-by-side comparison of CaV1.X (L-type) and CaV2.2 (N-type) conductance dependence on concentration (barium and calcium). Note the difference in barium conductances contrasted with the similarity in calcium conductances between the two calcium channels. Graph on left is taken from Church and Stanley (Church and Stanley, 1996).
cells, evolution may not have deviated from a single conductance value. It would appear that in terms of ways of controlling calcium levels, the cell has evolved what seems to be a confusing and complex array of buffers, secondary subunits, kinases, g-proteins, and more, instead of changing the pore kinetics themselves.

5.4. Implications for Evoked Transmitter Release:

With the CaV2.2 conductance level at physiological calcium determined, better models of the calcium domain around a calcium channel after an action potential may be prepared. Two points, however, should be made. For one, it may be that the current domain gating models put forward have already been using this exact value, since they were using the conductance determined for CaV1.X channels (see Meinrenken et al., 2003). Thus, although this experiment has resulted in a precise value, it may not make a difference in how researchers are modeling the calcium domain. Secondly, the conductance of the calcium channel is not the only value needed to determine the exact nature of the calcium channel/release mechanism relationship. There still remain some pieces of the puzzle for which we have only approximations of their possible true values. Examples include: active zone geometry; endogenous buffer kinetics and affinities; and calcium sensor kinetics and affinities. With these considerations in mind, however, one can distinguish between a model that uses a single calcium channel domain to trigger exocytosis, and one that uses overlapping domains. Thus, any model that requires a calcium flux higher than 775 ions/ms (at -30 mV) must use overlapping calcium domains to gate vesicle release.
6. Future Directions:

One of the major claims of this study is the fact that the conductance of a presynaptic type calcium channel has been found at *physiologically* relevant calcium concentrations. However, there are a couple of things that remain unphysiological about the study that prevent direct generalizations to presynaptic calcium channel biophysics. For one, the stimulus given to the channel was artificial and far from physiological. In order to obtain true presynaptic calcium channel behaviour (including the probability of opening, and the channel’s open time) a stimulus more similar to an action potential should be examined. A second point to consider is the fact that although the channel was examined in its natural environment (on-cell single channel recordings do not directly alter the cell’s internal machinery or environment), this environment was in the plasma membrane of the soma of the cell, not at the presynaptic terminal. Although *prima facie* the conductance at the single channel level should not differ between the two environments, it remains to be tested if synaptic proteins can alter this property significantly. Furthermore, the $\alpha_1$ subunit of $\text{Ca}_\text{V}2.2$ channels expressed at the presynaptic terminal will not necessarily be identical to the ones expressed on the neuronal membrane.

Future experiments should also focus on using some of the techniques discussed in this paper to determine the proper conductance of the rest of the presynaptic type calcium channels: the $\text{Ca}_\text{V}2.1 / \text{P/Q-type}$ and the $\text{Ca}_\text{V}2.3 / \text{R-type}$. The $\text{Ca}_\text{V}2.1$ channel is of considerable significance since, at least in the mammalian calyx of Held, this channel is responsible for at least 50% of the channels gating presynaptic release in neonates, and is present almost exclusively in mature rats (Meinrenken et al., 2003). Indeed, some
initial experiments of my own with CaV2.1 channels were attempted, but left out of this thesis (this was left out since it was incomplete and a complete, proper analysis of the CaV2.1 channel was going to be another project unto itself), give promising reason to believe that at physiologically relevant external calcium concentrations, the conductance value may turn out to be identical to the CaV1.X and CaV2.2 channels.


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