THE ELECTROPHYSIOLOGICAL CHARACTERIZATION
OF PLANT CALCIUM CHANNELS
AND THEIR ROLE IN CALCIUM SIGNALING

by

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A thesis submitted in conformity with the requirements
for the degree Doctor of Philosophy in the
Graduate Department of Botany
University of Toronto

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Doctor of Philosophy 1997, Graduate Department of
Botany, University of Toronto

ABSTRACT

The identification and characterization of Ca$^{2+}$ channels in the vacuolar and the plasma membrane of higher plants was accomplished through the use of conventional patch clamp techniques. Intact vacuoles from sugar beet cells and were isolated and used to identify Ca$^{2+}$ currents at the whole vacuole level and outside-out patches of membrane were used for single channel analysis. Permeability and selectivity studies strongly suggested that one of the channels identified in the vacuolar membrane could efficiently and selectively release Ca$^{2+}$ from the vacuole resulting in increased levels of cytosolic Ca$^{2+}$. A second channel co-residing with the vacuolar-release channel is the SV (slow-vacuolar type) channel. Although this channel is active at non-physiological potentials, the Ca$^{2+}$ permeability of the SV channel suggests that it could be involved in the sequestration of Ca$^{2+}$ following an increase in cytosolic Ca$^{2+}$ levels. The proposed role of the SV channel in Ca$^{2+}$-induced Ca$^{2+}$-release is also discussed.

Stimulus-induced fluctuations in cytosolic Ca$^{2+}$ are mediated by Ca$^{2+}$ channels located in both the vacuolar and plasma membrane. The role of a plasma membrane Ca$^{2+}$ channel in stimulus-response coupling and the mechanisms of channel regulation were
investigated by exposing protoplasts isolated from tomato cells near-isogenic for the resistance gene Cf5 to elicitor preparations containing the avr5 gene from race 4 (IF4) of the fungus *Cladosporium fulvum*. The stimulation of Ca$^{2+}$ channels by IF4, was up-regulated by phosphorylation mediated by the action of a G protein. The concerted action of Ca$^{2+}$ channels in the vacuolar and plasma membrane in maintaining Ca$^{2+}$ homeostasis and the mechanisms by which plant cells generate and perceive a stimulus-specific Ca$^{2+}$ signal is discussed.
ACKNOWLEDGMENTS

I am very grateful to my supervisor, Dr. Eduardo Blumwald, who seven years ago took a chance and accepted a fourth year project student based solely on her enthusiasm for scientific research. Much of my enthusiasm was inspired by hearing and watching him bring membrane transport to life in his third year course. It became very clear to me early on, that this was where I belonged and this was where I remained, for my entire graduate career. Throughout my graduate studies, I benefited enormously from the degree of freedom and independence that Eduardo allows his students, from the endless stimulating discussions and from the numerous scientific meetings he enabled me to attend. This thesis is dedicated to him.

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PREFACE

This thesis is divided into 7 chapters. The first chapter (literature review) begins with an overview of the patch clamp technique in terms of the hardware involved. This is followed by a description of mathematical expressions used in the analysis of electrophysiological data. Following this is a review of recent literature on the mechanisms involved in Ca\(^{2+}\) mobilization across the plant vacuolar and plasma membrane and their role in maintaining Ca\(^{2+}\) homeostasis. The chapter ends with a description of the role of Ca\(^{2+}\) channels in Ca\(^{2+}\) cellular signaling.

The materials and methods used throughout the experimental work are discussed in chapter 2. This chapter illustrates all materials and methods from the protocols used in the isolation of vacuoles and protoplasts to the experimental conditions used in recording the electrophysiological data.

All the experimental work is presented in chapters 3, 4, 5 and 6. The identification and characterization of two Ca\(^{2+}\) channels in the vacuolar membrane of sugar beet cells is presented in chapters 3 and 4. The description of a Ca\(^{2+}\) channel in the plasma membrane of tomato cells is presented in chapter 5. Further work on this channel resulted in the identification of a potential regulatory mechanism and a proposed role of the channel in stimulus-response coupling which is presented in chapter 6. The concerted role of the diverse Ca\(^{2+}\) channels in cellular Ca\(^{2+}\) homeostasis and in cellular signaling is discussed in the general discussion in chapter 7. In addition, the mechanisms used by plant cells to generate and perceive a stimulus-specific Ca\(^{2+}\) signal are also discussed. The bibliography follows chapter 7.
This dissertation includes information from a series of papers published as original articles in refereed journals. These include:


Authorization to include information from these papers in this dissertation has been received from the publishers.

All experiments described in this dissertation were performed solely by the author except for chapter 4, which was done cooperatively with Dr. Omar Pantoja.
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LIST OF ABBREVIATIONS

A  area
a_j  activity
α  channel opening rate
ADP  adenosine-5’-diphosphate
ADP(β)S  adenosine-5’-0-(2-thio-diphosphate)
ATP  adenosine-5’-triphosphate
ATP(γ)S  adenosine-5’-0-(3-thio-triphosphate)
β  channel closing rate
c  concentration
C_f  stray capacitance
C_m  membrane capacitance
DHP  dihydropyridine
E  electrical membrane potential
ER  endoplasmic reticulum
E_N  Nernst potential
E_{Ca}  Ca^{2+} nernst potential
E_K  K^{+} nernst potential
E_{Cl}  Cl^{-} nernst potential
E_{rev}  reversal potential
F  Faraday constant (0.0965 kJmol^{-1} mV^{-1})
g or γ  conductance

$G_{\text{max}}$  maximum conductance

$\Delta G_i$  free energy

$G\Omega$  gigaohm

GDP  guanosine-5'-diphosphate

GDP(β)S  guanosine-5'-0-(2-thiodiphosphate)

GTP  guanosine-5'-triphosphate

GTP(γ)S  guanosine-5'-0-(2-thiotriphosphate)

HA 1004  $N$-(2-guanidinoethyl)-5-isoquinolinesulfonamide

i  cytoplasmic side of membrane

$i_j$  single channel currents

$I_j$  whole cell currents

KDa  kilodalton

KHz  kilohertz

$K_m$  binding constant

$\lambda$  activity coefficient

MΩ  megaohm

Mes  (2-[N-morpholine ethane sulfonic acid]

mM  millimolar

ms  millisecond

mV  millivolts

n  number of open channels

N  total number of channels in membrane
nM  nanomolar
0   extracellular side of membrane
P   permeability constant
Po  open channel probability
pA  picoampere
pF  picofarad
pS  picosiemens
R   universal gas constant (8.31 J K\(^{-1}\) mole\(^{-1}\))
R_a access resistor
R_f feedback resistor
R_m membrane resistance
R_p pipette resistance
R_s series resistance
\(\Delta \psi\) electrical membrane potential difference
SV  slow-activating type
\(\Theta\) relative conductance
\(\tau\) time constant
\(t_{\text{open}}\) channel mean open time
\(t_{\text{closed}}\) channel mean closed time
T   absolute temperature
\(\mu_j\) electrochemical potential
VK  fast-activating type
<table>
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<th>Symbol</th>
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<tr>
<td>$V_{cmd}$</td>
<td>command (clamped) potential</td>
</tr>
<tr>
<td>$V_m$</td>
<td>membrane potential</td>
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<tr>
<td>$V_c$</td>
<td>membrane potential with reference to the cytosol</td>
</tr>
<tr>
<td>$V_v$</td>
<td>membrane potential with reference to the vacuole</td>
</tr>
<tr>
<td>$z_j$</td>
<td>charge of ion</td>
</tr>
<tr>
<td>$z$</td>
<td>gating charge</td>
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1.1 AN HISTORICAL PERSPECTIVE

Voltage clamping techniques originated from an attempt to record currents through individual ion channels in biological membranes (Hille, 1992). Ion channels catalyze the transfer of water, inorganic and organic ions and uncharged solutes across a lipid membrane (Blatt & Thiel, 1993). Channels effectively reduce the free energy for transport by providing a polar environment for diffusion through the apolar lipid phase of the membrane. As such the structure of a channel consists of a proteinaceous cylinder spanning the plane of the membrane where the inner cavity constitutes a water-filled pore that communicates directly with the bulk aqueous phases on either side of the membrane (Hille, 1992).

It had become clear by the early 1970s that integral membrane proteins (ion channels) were responsible for the electrical signaling mechanisms of nerve and muscle. The specific and selective actions of certain toxins, proteases and protein-modifying agents had demonstrated that sodium and potassium channels were comprised of separate macromolecules (Hille, 1992). Early studies had demonstrated that certain proteins isolated from bacteria and some antibiotic polypeptides could induce step-like changes in membrane conductance, which were attributed to the opening and closing of channel-like structures. These distinct conductance changes were of the same order of magnitude as single-channel conductances inferred from analysis of current fluctuations in the neuromuscular junction and in the nodes of Ranvier (Conti et al., 1984).

The early work with artificial lipid bilayers had demonstrated that the electronic components available at that time were capable of handling small signals characteristic of
electrical currents in biological systems. However, background noise in all standard voltage-clamp setups was higher by a few orders of magnitude than the signals to be measured. Thus an attempt was made to isolate a small area of membrane surface (a "patch") for localized electrical measurement by placing a heat-polished measuring glass micropipette onto the surface of a voltage-clamped cell (Neher & Sakmann, 1976). Considerations for background noise led to the idea that such pipettes should allow resolution of currents in the picoampere range whenever the seal resistance (the resistance between the interior of the pipette and the reference electrode) could exceed the pipette internal resistance (Neher & Sakmann, 1976). Thus a high seal resistance was crucial because it could determine the level of background noise in the recordings but also it ensured that most of the currents originating in a small patch of membrane flowed from the pipette into the current-measurement circuitry. Single channel current recordings improved significantly when tight pipette-membrane seals, with resistances of $5 - 100 \, \text{G} \Omega$ (a "gigaseal") could be obtained when precautions were taken to keep the pipette surface clean and when suction was applied to the membrane interior (Sigworth and Neher, 1980; Hamill et al., 1981). The discovery of the gigaseal revolutionized electrophysiology because it increased resolution, decreased the variability of channel step sizes and allowed potentials to be applied across the seal for local voltage stimulation. Furthermore, the gigaseal provided mechanical stability so that patches could be excised from the parent cell and studied in isolation.
1.2 MEMBRANE CONFIGURATIONS IN CURRENT MEASUREMENTS

Following the formation of a gigaseal, several recording configurations are possible (Fig. 1.1). Initially when the pipette touches a cell (Fig. 1.1A), the pipette resistance (which is continually monitored) increases. The application of suction to the interior of the pipette draws a small portion of cell membrane into the pipette producing a sudden increase in resistance and resulting in the formation of a seal between the membrane and the pipette (Fig. 1.1B). The patch of membrane trapped within the pipette can be ruptured by further suction or a voltage pulse of several hundred millivolts to produce a tight seal whole-cell configuration (Fig. 1.1C). Patch rupture is detected as a sudden increase in the capacitative current in response to a test potential step, as the membrane capacitance of the cell is seen through the series resistance ($R_s$; equal to $R_a$ plus the pipette resistance, $R_p$) of the pipette.

A whole-cell configuration results in electrical and diffusional continuity between the pipette and the cytoplasm, most often without altering the seal resistance between the glass pipette and the membrane. The $R_a$ between the pipette microelectrode and the cytoplasm is much lower than the membrane resistance ($R_m$) of a small cell thus the electrical membrane potential of the cell is clamped to the pipette potential. By definition then, the membrane potential of the cell will be equal to the pipette potential minus the bath potential. The collective activity of ion channels in the whole-cell can then be detected by monitoring the current.

When the pipette is pulled away from the whole-cell configuration, a small piece of membrane is withdrawn from the cell which breaks off and reseals as an outside-out patch
Figure 1.1. Membrane configurations for the recording of channel currents in both whole cell and isolated membrane patch. A, A glass micropipette with a resistance of 6 to 10 MΩ, approaches a cell and makes contact. B, Applying gentle suction produces a tight seal (with resistances of 3 to 6 GΩ) between the opening of the pipette tip and the cell membrane. C, Applying further suction ruptures the membrane within the pipette tip generating a whole cell configuration such that the content of the pipette is continuous with the cytosol of the cell. D, Pulling the pipette away from the whole cell tears away a small section of membrane that reseals to the pipette forming an outside-out membrane patch. In this configuration the cytosolic-side of the patch faces the contents of the pipette. E, Briefly exposing the pipette to air from the tight seal configuration generates a membrane vesicle within the pipette tip which upon placing the pipette back into the bathing chamber, ruptures the vesicle and forms an inside-out patch of membrane. In this configuration, the cytosolic-side of the membrane faces the bathing chamber.
Further suction

Expose the tip to air

Whole cell

Inside-out patch

Pull-away

Outside-out patch
on the pipette (Fig. 1.1D). The voltage and polarity conventions are identical to the whole-cell, however, the membrane surface area is now greatly reduced. This allows the monitoring of the dynamics of one channel protein by measuring the current through a single ion channel. In addition, an isolated patch of membrane can be torn off the cell by pulling away the pipette after seal formation and briefly exposing it to the air (Fig. 1.1E). When the patch is excised from the cell-attached configuration, the resulting patch is inside-out thus the cytoplasmic side of the membrane faces the bathing solution. In this case, voltage and polarity conventions are opposite to those in out-side out patches since now the membrane potential of the patch is the bath potential minus the pipette potential.

1.3 THE MEMBRANE AS AN ELECTRICAL CIRCUIT

Ionic channels change the membrane potential ($V_m$) by catalyzing electric current so that opposite charges are lined up at the outer and inner surfaces of the membrane unable to freely cross the insulator-like lipid bilayer. The cell, the two solutions of ions and the cell membrane constitute the membrane capacitance (Hille, 1992). The charge ($q$) that accumulates in the external (and internal) fluid at the membrane surfaces is the actual source of $V_m$ and the two are related at all instants of time by the following equation:

$$q = C_m V_m$$  \hspace{1cm} (1.1)

where $C_m$ is the membrane capacitance of the cell. Equation 1.1 requires that whenever a net current flows across the membrane through a channel, $V_m$ must change and since current is defined as the rate of flow of charges then the following is true:
\[
\frac{dq}{dt} = C_m \left( \frac{dV_m}{dt} \right) = I_c
\]  

The term \( I_c \) is the current flowing onto (and off of) the capacitor plates. Thus all current flowing through a channel is accumulating on the outer (or inner) side of the membrane and there is an equal and opposite charge left behind. The high electrical capacitance of biological membranes (1.0 \( \mu \text{F/cm}^2 \)) slows down the voltage response (Fig. 1.2.) to any current by a membrane time constant (\( \tau \)) that depends on the product of the membrane capacitance and the membrane resistance (\( \tau = C_m R_m \)). In other words, if a current is applied to a membrane, either by channels elsewhere in the cell or by current from the electrode, this current first satisfies the requirement for charging the membrane capacitance, then it changes the membrane voltage. Thus when the membrane capacitance is in a circuit, the voltage is not reached immediately, instead, it is approached with the time constant (\( \tau \)). In various resting cell membranes, \( \tau \) ranges from 10 \( \mu \text{s} \) to 1 s which corresponds to resting \( R_m \) values of 10 to \( 10^6 \) \( \Omega \text{cm}^2 \) (Nobel, 1991; Hille, 1992).

1.4 VOLTAGE CLAMP PRINCIPLES

For ion channels whose opening is regulated by \( V_m \), the current flowing through them will change \( V_m \) and change the behaviour of the channels. However, when \( V_m \) is held constant membrane current is directly proportional to the fraction of all channels that are open and to the conductance of the channel. With the voltage-clamp technique
Figure 1.2. The cell membrane as an electrical circuit. A, A cell membrane behaves electrically like a circuit with a capacitor and a resistor in parallel. $V_r$ is the resting potential of the cell. B, Membrane potential ($V_m$, upper trace) and membrane current ($I_m$, lower trace) are shown. $V_\infty$ represents the change in $V_m$ (membrane potential) from $V_r$ (resting potential) to a steady state level. The time constant ($\tau$) is the time at which $V_m$ has reached 63% of its steady state level, since the voltage approaches steady state along an exponential time course: $V(t) = V_\infty(1 - \exp(-t/\tau))$. $V_m$ has nearly converged upon its steady state level after 4 time constants. $I_m$ can be divided into a capacitance current ($I_c$) and into an ionic current ($I_i$).
(Hamill, et al., 1981) the $V_m$ is held constant while the current moving through the membrane is measured (Fig. 1.3). In this technique an operational amplifier (op amp) and a large feedback resistor ($R_f$; typically 50 GΩ) are placed in a current-to-voltage converter circuit such that the op amp will vary its output to maintain $V_m$ at the command potential ($V_{cmd}$). The currents measured with a patch voltage clamp are very small, therefore, an extremely high valued $R_f$ is used to provide adequate gain and to achieve a good signal-to-noise ratio.

In a voltage-clamp experiment the pipette electrode forms a tight seal with a cell membrane, so the cytoplasm is electrically continuous with the solution in the pipette and completely isolated from the external bathing solution (i.e. a whole cell configuration). There is some resistance to current flow from the pipette to the cytoplasm ($R_a$) due to the small tip diameter of the pipette. A silver-/silver chloride electrode in the pipette solution connects it to the negative input of an op amp and $V_{cmd}$ is connected to the positive input. Since the op amp has extremely high gain, the potential at the negative input is forced to follow the potential at the positive input. In other words, the high gain of the op amp ensures that the difference between $V_{cmd}$ and the potential of the pipette is negligible. If $V_{cmd}$ is a voltage step, the potential at the negative input (and hence the pipette) is also a step. The current required to achieve this step is passed through $R_f$ and through the associated stray feedback capacitance ($C_f$) of the current-to-voltage converter. The output of the current-to-voltage converter will settle to a final value with the time constant $R_fC_f$. The high input impedance (resistance) of the op amp ensures that the op amp draws
Figure 1.3. Simplified schematic of the patch clamp current-to-voltage circuit.

A, Op amp $A_1$ and its feedback resistor $R_f$, constitute a current-to-voltage converter. The potential at the negative input of the op amp is forced to follow the $V_c$ at the positive input. The pipette current ($I_p$) is identical to the current in $R_f$. The current in $R_f$ generates a voltage across it that is measured in the differential amplifier $A_2$. $C_f$ represents the stray capacitance across $R_f$. In practice, $C_f$ consists of many resistor-capacitor components that make the frequency response of $R_f$ very complex. B, An equivalent circuit of the experimental arrangement. A clamp battery sets $V_{clamp}$ and thereby fixes $V_m$ to be equal to $V_{clamp}$, regardless of the values of the ionic conductances. An ammeter added in series to the clamp battery allows measurement of the current necessary to hold $V_m$ equal to $V_{clamp}$ without interfering with its flow. Note that $V_m$ will not equal $V_{clamp}$ unless the series resistance ($R_{external} + R_{access}$) of $I_{clamp}$ is zero.
A

\[ V_o = I_p R_f \]

B

\[ R_{\text{external}} \]

\[ g_{Ca} \]

\[ g_K \]

\[ g_{Cl} \]

\[ C_m \]

\[ I_{\text{clamp}} \]

\[ R_{\text{access}} \]

\[ V_{\text{clamp}} \]

Ammeter
essentially no current through the input terminals since such currents would disturb the measurement. The low output impedance enables the op amp to supply any amount of current (Sigworth, 1983; Simpson, 1987; Finkel, 1991).

Incoming signals must be filtered to remove high frequency noise and this is accomplished by a low pass filter. This type of filter limits the range of frequencies that can pass (bandwidth) by eliminating signals and noise above the cut-off frequency. For example channel openings that are obscured by noise in a 10 kHz bandwidth become easily distinguishable if the bandwidth is limited to 1 kHz. A commonly used filter is the 4 pole Bessel filter.

1.5 THE ELECTROCHEMICAL POTENTIAL OF IONS

The net flux of ions into or out of the cell is determined by two balancing forces: i) the ion concentrations in and outside of the cell and ii) the electrical potential across the cell membrane. In biological systems, the influence of these two forces on the ability of an ion to move across the membrane is given by the electrochemical potential of the ion ($\mu_j$). The movement of ions will be from regions of higher electrochemical potential to regions of lower electrochemical potential. The electrochemical potential of an ion is defined as:

$$\mu_j = \mu_j^* + RT \ln a_j + z_jFE$$  \hspace{1cm} (1.3)

where $\mu_j$ is the chemical potential of species $j$, $\mu_j^*$ is a constant and refers to the electrochemical potential at the standard state of species $j$, $R$ is the gas constant, $T$ is the absolute temperature, $a_j$ is the activity of species $j$, $z_j$ is the charge associated with species $j$, $F$ is the Faraday constant and $E$ is the electrical potential. It should be noted that
biological systems are considered "open" systems where the influence of gravity or volume changes on $\mu_i$ are null (Nobel, 1991; Hille, 1992).

For charged ions in aqueous solutions, the thermodynamic effective concentration (activity; $a$) of the ion can differ greatly from the actual concentration ($c$). In an ideal solution of infinite dilution, ions interact only with water but in solutions of finite concentrations ions experience weak ionic attractions that become stronger with increasing concentrations. Thus ion-ion interactions reduce the chemical potential of ions since each ion is less available or less free at finite concentrations than at infinite dilution. Thus ionic solutions are said to have ionic activities, ($\lambda$), that are smaller than the ionic concentrations, ($c$) and the activity of an ion will equal its concentration only in solutions that are extremely dilute. Activity coefficients ($\lambda$) of ions can be estimated from the following form of the Debye-Hückel equation:

$$\ln \lambda_{\pm} \equiv \frac{1.17z_+ z_- \sqrt{0.5 \sum c_j z_j^2}}{32 + \sqrt{0.5 \sum c_j z_j^2}}$$  \hspace{1cm} (1.4)

where $z_+$ is the charge number of the cation, $z_-$ that of the anion and $c$ is its concentration. The activity coefficient of a particular ionic species depends on all the ions in the solution as indicated by the ionic strength terms ($\sum c_j z_j^2$).
When ions of some species \( j \) are in equilibrium across a membrane, its electrochemical potential outside (\( \varphi \)) is equal to that inside (\( i \)) thus \( \mu_j^0 = \mu_j^i \). Then the electrical potential difference \( E' - E^0 \) across the membrane at equilibrium is given by:

\[
E^j_N = E^i - E^0 = \frac{RT}{zF} \ln \frac{a_j^0}{a_j^i} = 2.3 \frac{RT}{zF} \log \frac{a_j^0}{a_j^i} \tag{1.5}
\]

then at 25 °C, the equation becomes:

\[
E^j_N = \frac{59}{z} \log \frac{a_j^0}{a_j^i} \tag{1.6}
\]

The electrical potential difference \( E^j_N \) is referred to as the Nernst potential and it describes the relationship of the internal and external activities of an ionic species \( j \) with the electrical potential difference across a membrane. At equilibrium, a ten-fold difference in activity of a monovalent ion across a membrane is energetically equivalent to a 59 mV difference in the electrical potential. Therefore a fairly small electrical potential drop can balance a large difference in ion activity across a membrane. This equation is true for all biologically relevant ions such as \( \text{K}^+ \), \( \text{Ca}^{2+} \), \( \text{Na}^+ \) or \( \text{Cl}^- \). These equations imply that: (i) if the pores in a membrane are permeable to only one of these ions, then the membrane potential will change to the Nernst potential for that same ion and (ii) if the membrane potential is maintained at the Nernst potential, then there will be no net flux of ions through the ion selective pores (Nobel, 1991; Hille, 1992).
1.6 MEMBRANE DIFFUSION POTENTIALS AND PERMEABILITY RATIOS

Passive fluxes of ions are caused by gradients in the chemical potentials of the various ions and they lead to an electrical potential difference (diffusion potential) across a membrane. For many plant cells, the total ionic flux consists mainly of movements of $K^+$, $Na^+$ and $Cl^-$ since these ions have fairly high concentrations in and outside plant cells. A flux density of an ion of species $j$ depends on the product of its concentration ($c$) and its mobility ($u$):

$$J_j = -u_j c_j \frac{d\mu_j}{dx} \quad (1.7)$$

thus ions with high local concentrations or moving easily across the membrane will tend to be major contributors to the total ionic flux density. If it is assumed that ions cross the membrane independently without interacting with each other and that the electric field in the membrane is constant (i.e. the membrane potential drops linearly across the membrane) then a single expression can describe the diffusion potential across a membrane when the ionic flux density is due to the movements of $K^+$, $Na^+$ and $Cl^-$:

$$E_M = \frac{RT}{F} \ln \left( \frac{P_K c_K^O + P_{Na} c_{Na}^O + P_{Cl} c_{Cl}^O}{P_K c_K^i + P_{Na} c_{Na}^i + P_{Cl} c_{Cl}^i} \right) \quad (1.8)$$

where $P$ is the permeability coefficient for each ion. The Goldman-Hodgkin-Katz (GHK) voltage or constant field equation gives the electrical potential difference arising from the different tendencies of $K^+$, $Na^+$ and $Cl^-$ to diffuse across a membrane to regions of lower
electrochemical potential. The GHK equation can also be written in terms of the current carried by any ion, $S$:

$$I_s = P_s z^2 \frac{EF}{RT} \left[ [S]_i - [S]_o \exp \left( \frac{-zEF}{RT} \right) \right]$$

(1.9)

If the $K^+$, $Na^+$ and $Cl^-$ are the main permeant ions then in the GHK voltage equation shown above, $E$ is referred to the reversal potential ($E_{rev}$) or the zero-current potential where no net current flows. If only one ion is permeant then $E_{rev}$ becomes the Nernst potential ($E_N$) for that ion but, with several permeant ions $E_{rev}$ is a weighted mean of all the Nernst potentials. The GHK equations are used in the determination of membrane permeability to ions because they define absolute ionic permeability in terms of flux measurements and ionic permeability ratios in terms of zero-current potentials. However when determining channel selectivity, changes in zero-current potentials are used rather than absolute zero-current potentials since cells may not have equilibrated with recording solutions during the measurement of zero-current potentials. When other ions such as $Ca^{2+}$ cross a membrane in appreciable amounts, they will also make a contribution to the membrane potential. Thus permeability ratios, $P_{Ca^{2+}}/P_{K^+}$, $P_{Cl^-}/P_{K^+}$, $P_{Ca^{2+}}/P_{Cl^-}$, for solutions containing a mixture of monovalent and divalent ions (Allen & Sanders, 1994) can be calculated from the GHK constant field equations such that:

$$\frac{P_{Ca^{2+}}}{P_{K^+}} = \left( \frac{Q^-_{Cl^-}Q^{A}_{K^+} - Q^{B}_{Cl^-}Q^{A}_{K^+}}{Q^{A}_{Cl^-}Q^{A}_{Ca} - Q^{A}_{Cl^-}Q^{B}_{Ca}} \right)$$

(1.10)
where $Q$ is defined as:

$$
\frac{P}{Cl^-} = -\frac{P}{Ca^2+}Q_C^B - \frac{Q_K^B}{Q_C^B} \tag{1.11}
$$

$$
z^2 \frac{EF^2 [S]_i - [S]_o \exp\left(\frac{-zEF}{RT}\right)}{RT} \left(1 - \exp\left(\frac{-zEF}{RT}\right)\right) \tag{1.12}
$$

and where $[S]$ is the activity of the ion on the cytoplasmic ($i$) and extracellular ($o$) side of the cell membrane, $A$ and $B$ are solutions with distinct ion concentrations and $P$, $z$, $F$, $T$, $R$ and $E$ have the same meaning as previously described.

1.7 CURRENT-VOLTAGE RELATIONS OF ION CHANNELS

Channel activity in whole-cell measurements reflects the ensemble behavior of channels distributed over the entire membrane surface. Thus whole-cell records provide an automatic statistical summation in time of the current passing through hundreds or even thousands of discrete channels in a single cell. Whole-cell ($I_w$) and single-channel ($i_j$) currents are related simply as:

$$
I_w = P_o N_i \sum_j \gamma_j (V - E_j) \tag{1.13}
$$
where $N_j$ is the number of channels of type $j$ and $P_0$ is the channel open probability ($0 < P_0 < 1$). The single-channel current, $i_j$, is expanded by Ohm’s law such that:

$$i_j = \gamma_j (V - E_j)$$

(1.14)

where $V$ is the membrane voltage and $E_j$ is the prevailing ionic equilibrium potential. The single channel conductance, $\gamma_j$, reflects the inherent permeability of the channel pore and the availability (activity) of permeant ions (Hille, 1992). The ability of channels to “gate” or regulate ion flux is reflected in $P_0$ and may be a function of time and membrane voltage. Both $\gamma_j$ and $N_j$ are essentially constant over the time scales typical of voltage clamp measurements (1-10,000 ms). Thus whereas single channel recordings provide a means to discriminate between permeation and gating, whole-cell measurements may not distinguish between changes in $\gamma_j$, $N_j$ and $P_0$.

Currents recorded under voltage clamp are a function of time and they may be activated by various means, such as a step in voltage, or by ligand or ion binding (Hamill et al., 1981). A current may also deactivate following a step in voltage, usually of opposing sign to that which previously activated the current. Finally, some currents inactivate with time even when the voltage is held constant or in the continued presence of the activating factor or agonist. Current-voltage measurements are used to gain information about ionic channels. For example, two current-voltage relations of equal slope but different zero-current potentials could suggest the presence of different channels with different ionic selectivities or the same channel exposed to different concentrations of the permeant ions at the two sides of membrane. Non-linear current-voltage relations
suggest that the membrane conductance is changing with voltage, a property known as rectification. Inward rectification of a current-voltage relation implies that the channel is open at negative membrane potential differences but closed at positive potentials.

The voltage dependence of channel opening (activation) is steep and sigmoidal as determined in voltage clamp studies where membrane potential is the only parameter varied. It was originally proposed that the voltage dependence is due to voltage-sensitive components, which may be gating charges or dipoles, that are intrinsic to the channel protein and function as the voltage sensor of the channel. These voltage sensors are most probably located within the membrane since most of the drop in membrane potential is over the interior of the membrane which is a region of low dielectric constant and high hydrophobicity (Jan & Jan, 1989). The number of charges within a channel molecule that have to be translocated across the membrane as the channel opens (gating charge) can be estimated by examining the voltage-dependence of channel activation. To determine the voltage-dependency of channel gating, the relative conductance of channel currents can be plotted as a function of the clamped electrical membrane potential difference and fit with the following Boltzman expression:

$$\ln \left[ \frac{1 - \theta(V)}{\theta(V)} \right] = \frac{\Delta G_i}{RT} + \frac{zEF}{RT}$$

(1.15)

where the relative conductance (\(\theta\)) is defined as the ratio \(G/G_{max}\). The total free energy associated with the transition from the closed to the open state of a channel within an electric field is defined by two variables: the gating charge (\(z\)) and the internal free energy
of opening ($\Delta G_r$) associated with the two conformational states (open and closed) of the channel protein in the electric field (Labarca et al., 1980).

The opening and closing of ion channels generate membrane currents that vary in a step-like manner with time between (in most cases) two values, fully open and fully closed (Neher & Sakmann, 1976; Hamill et al., 1981). The manner in which the channel varies between its open time and its closed time depends on the underlying channel-gating mechanisms, thus analysis of records of single-channel currents can describe the mechanisms by which channels open and close. A simple reaction mechanism for an ion channel can be described as a transition between a single closed state of the ion channel and a single open state:

$$\text{closed} \xrightarrow{\beta} \text{open} \xleftarrow{\alpha}$$

where $\alpha$ describes the closing rate of the open channel and $\beta$ describes the opening rate of the closed channel. Because the closing and opening of an ion channel is a random process then the rate constants must be interpreted in a probabilistic way. Thus the length of time for which the system stays in a particular state such as the open state, can be described by a probability density function (pdf):

$$f(t) = \alpha \exp(-\alpha t) \quad (1.17)$$

when $t$ is $\geq 0$. This pdf is described as an exponential distribution with a mean of $1/\alpha$ which is a simple exponentially decaying curve. The closed time has the following pdf:

$$f(t) = \beta \exp(-\beta t) \quad (1.18)$$
Thus the length of time for which each channel stays open or closed is described by an exponential distribution, which ensures that the total current through a large number of channels will decay along an exponential time course (Colquhoun & Hawkes, 1983). In general then, the lifetime in any single state (open or closed) is exponentially distributed such that the mean lifetime is equal to the inverse of the sum of transition states that lead away from that state.

The evaluation of channel kinetics is often complicated by the presence of more than one channel in the patch of membrane. Thus the method of Labarca et al., (1980) can be used under these circumstances to make estimates of mean open and closed times of one channel. The method assumes that a patch of membrane contains a group of \( N \) identical, independent channels, with the kinetic scheme discussed above (one open and one closed state) and with the value of \( N \) determined from the number of current levels in single channel recordings. In this case, \( k_- \) is the rate of closing for the open channel (equivalent to \( \alpha \)) and \( k_+ \) is the opening rate of the closed channel (equivalent to \( \beta \)). The group of channels can have \( N + 1 \) states, denoted by \((n, N - 1)\) for \( n = 0 \) to \( N \), where \( n \) channels are in the open state and \( N - n \) are in the closed state. The group of channels leave the state \((n, N - n)\) when any of the open channels close or when any of the closed channels open. Because the rates of channel closing and channel opening are independent, the rate at which the group of channels leave the state \((n, N - n)\) is given by:

\[
k_n = nk_- + (N - n)k_+ \quad (1.19)
\]

Each rate constant, \( k_n \), can be obtained from fitting exponentials to the histograms of the time intervals (dwell time histograms) in which exactly \( n \) channels are open. The values of
$k_n$ obtained for $n = 1, 2, \text{ or } 3$, are then plotted as a function of $n$. The slope and the intercept ($n = 0$) of the plot give estimates of $(k_- - k_+)$ and $Nk_+$, respectively. The mean lifetime of the open state is given by $1/k_-$. and the mean lifetime of the closed state is given by $1/k_+$. 

The validity of this method depends on the following assumptions: (i) the distribution of dwell times in each state is exponential, (ii) the reciprocal time constants (rates) of these exponentials vary linearly with the number of states, (iii) the number of channels $N$ can be determined, (iv) the channels have identical kinetics, (v) the channels are independent and (vi) each channel has only one open and one closed state (Labarca et al., 1980).

The probability that any one channel is open (open channel probability; $P_o$) can be calculated from the mean open and mean closed times:

$$
\frac{t_{\text{open}}}{t_{\text{open}} + t_{\text{closed}}} \quad (1.20)
$$

(Palmer & Frindt, 1986). As well amplitude histograms of channels can be used to determine $P_o$.

$$
P_o = \frac{\sum nA_n}{N \sum_{n=0}^{N} A_n} \quad (1.21)
$$
where \( n \) is the number of conducting channels, \( N \) is the total number of channels in the patch of membrane and \( A_n \) is the area under the peaks of the amplitude histogram corresponding to \( n = 0, 1, 2 \) etc. (Labarca et al., 1980) (Plant et al., 1994). The estimation of \( P_O \) was subject to the assumed value of \( n \).

All ion channels are proteinaceous aqueous pores that can pass at least 1 pA (picoampere) of current which corresponds to a turnover of \( 1 \times 10^6 \) ions per second. Despite being highly permeable (i.e. high rates of transport), ion channels can be selective. Some channels can discriminate between \( \text{Na}^+ \), \( \text{K}^+ \) and \( \text{Ca}^{2+} \) so that the permeability to one species of ion is 5-1000 (Tsien, 1987; Thiel & Blatt, 1994) times greater than the permeability to the others, despite the similar size of the ions. This is accomplished by the selective binding of the ions to the channel protein as they move through. Binding sites are evident in most voltage-sensitive cation channels because the amount of current flowing through a channel saturates in a concentration-dependent manner (Jan & Jan, 1989). In this respect, the transport is distinct from simple diffusion across the lipid bilayer where the flux increases linearly as the concentration of substrate increases.

1.8 PRIMARY AND SECONDARY TRANSPORT IN PLANT CELLS

In plants, a few transport systems are actively energized by the hydrolysis of ATP or other high energy phosphates (i.e. GTP, PPI). ATP-energized \( \text{H}^+ \) transport generates a difference in pH (\( \Delta \text{pH} \)) and electrical charge (\( \Delta \Psi \)) between the different compartments in the cell. The resulting \( \text{H}^+ \) electrochemical potential difference (or proton motive force) drives the secondary transport of other solutes by providing potential energy such that:
Thus the proton motive force (pmf) in mV at 25 °C is defined as:

$$\Delta \mu_{H^+} = F\Delta E + 2.3RT \log \frac{H^+_{i}}{H^+_{o}}$$  \hspace{1cm} (1.22)$$

One of the most important functions supported by the pmf is the accumulation of nutrients, ions and metabolites via secondary transport processes. Secondary active transport refers to the movement of solutes coupled to the flux of protons moving down their electrochemical gradient. Thus solutes, such as Ca\(^{2+}\), which have a sharp electrochemical gradient directed into the cytosol, will be transported out of the cytosol via the calcium-proton antiport (Schumaker & Sze, 1986; Blumwald & Poole, 1986).

1.9 MECHANISMS INVOLVED IN MAINTAINING RESTING LEVELS OF Ca\(^{2+}\)

Homeostasis of cellular Ca\(^{2+}\) is maintained by the active extrusion of Ca\(^{2+}\) from the cytosol to the extracellular space (Ca\(^{2+}\)-ATPase) (Rasi-Caldogno et al., 1987) or by the sequestration of Ca\(^{2+}\) into the vacuole (nH\(^+\)/Ca\(^{2+}\) antiporter) (Blumwald & Poole, 1986) and/or the endoplasmic reticulum (Ca\(^{2+}\)-ATPase) (Buckout, 1983). Ca\(^{2+}\)-ATPases have not been identified in vacuolar membranes. The Ca\(^{2+}\)-ATPases are strictly ATP or GTP dependent, inhibited by vanadate, insensitive to protonophores, stimulated by calmodulin
and they demonstrate a \( \text{Ca}^{2+} \)-dependent formation of a phosphorylated intermediate (Bush, 1995). Kinetic analysis of ATPases in many cells has yielded estimates of \( K_m \) for \( \text{Ca}^{2+} \) of 0.07 \( \mu \text{M} \) (Rasi-Caldogno et al., 1987) and 12 \( \mu \text{M} \) (Liss et al., 1991). Calcium transport activities with these characteristics have been shown in virtually all membrane preparations from plant cells (reviewed by Bush, 1995). Calcium transport into the ER is driven by a primary \( \text{Ca}^{2+} \)-ATPase which shows similar properties to those of the \( \text{Ca}^{2+} \)-ATPase of plasma membrane (Briskin, 1990). During signal transduction the \( \text{Ca}^{2+} \)-ATPases are believed to function primarily to restore cytosolic \( \text{Ca}^{2+} \) to resting values.

The second major class of efflux transporters are the \( \text{Ca}^{2+}/\text{nH}^+ \) antiporters found in the vacuolar membrane. Because they are secondary transporters they do not directly require ATP for transport. Instead, the antiporters use either one of the components of the pmf, \( \Delta \text{pH} \) (Blumwald & Poole, 1986) or \( \Delta \Psi \) (Blackford et al., 1990) to drive \( \text{Ca}^{2+} \) uptake. Kinetic analyses of the antiporter activity in membrane preparations estimated the \( K_m \) for \( \text{Ca}^{2+} \) to be 10 to 67 \( \mu \text{M} \) (Blumwald & Poole, 1986) which is at least an order of magnitude higher than that for the \( \text{Ca}^{2+} \)-ATPases. Thus, although the antiporter has a high capacity for \( \text{Ca}^{2+} \) transport, it has a low affinity for \( \text{Ca}^{2+} \). However, calmodulin has been suggested to increase the affinity of the antiporter for \( \text{Ca}^{2+} \) in sugar beet tap roots by about a 1000-fold (Bush, 1995). Based on the apparent electrogeniciry of transport, the stoichiometry of \( \text{H}^+ \) exchange for \( \text{Ca}^{2+} \) has been proposed to be two (Blumwald & Poole, 1986) or greater (Blackford et al., 1990). This stoichiometry is required for the maintenance of the gradients that are normally established across the vacuolar membrane.
Figure 1.4. Schematic diagram of primary and secondary Ca\(^{2+}\) transporters in the vacuolar and plasma membrane of a higher plant cell. Ca\(^{2+}\) efflux transporters such as primary Ca\(^{2+}\)-ATPases (at the plasma membrane and the ER) and secondary nH\(^+\)/Ca\(^{2+}\) antiporters (at the vacuolar membrane) pump Ca\(^{2+}\) out of the cell (into the extracellular space) and into intracellular compartments such as the vacuole and the ER. The vacuolar nH\(^+\)/Ca\(^{2+}\) antiporter is energized by proton electrochemical gradients established by the primary H\(^+\) pumps in the plasma membrane (H\(^+\)-ATPase) and the vacuole (H\(^+\)-ATPase and PPI-ase). Calmodulin (CaM) regulates Ca\(^{2+}\)-ATPases in one or several types of membranes and may also regulate nH\(^+\)/Ca\(^{2+}\) antiporter.
and it also supports the sole function of the antiporter in Ca\textsuperscript{2+} accumulation into the vacuole. In other words, the antiporter could not normally run in reverse and serve as a pathway for Ca\textsuperscript{2+} efflux (out of the vacuole) into the cytosol. Thus the antiporter functions to dampen large changes in cytosolic Ca\textsuperscript{2+} and to maintain the Ca\textsuperscript{2+} stored in the vacuole.

Calcium release into the cytosol from either the internal or apoplastic Ca\textsuperscript{2+} pools is achieved through the opening of channels permeable to divalent cations down a steep electrochemical gradient. The speed of Ca\textsuperscript{2+} influx through channels, together with sensors in the channel that mediate the transition from the open to the closed state, permit fine control over the kinetic and spatial properties of Ca\textsuperscript{2+} influx.

1.10 CALCIUM CHANNELS AT THE PLASMA MEMBRANE

Initial indirect evidence for the existence of Ca\textsuperscript{2+} channels at the plasma membrane of plant cells came through the use of pharmacological agents that blocked or promoted the opening of voltage-gated Ca\textsuperscript{2+} channels in animal cells by binding to specific regions of the \( \alpha \)1 subunit of the channel protein (Tsien & Tsien, 1990). These agents include dihydropyridines such as nifedipine and nitrendipine, phenylalkamines such as verapamil and bepridil and benzothiazepines such as diltiazem. In radiometric studies, verapamil blocked Ca\textsuperscript{2+} influx into protoplasts from cultured cells of carrot (Graziana et al., 1988) and Amaranthus (Rengel & Elliot, 1992). A cytokinin-induced Ca\textsuperscript{2+} uptake in protoplasts from moss was blocked by verapamil, nifedipine and diltiazem with half maximal inhibition between 0.1 to 0.5 \( \mu \text{M} \) (Schumaker & Gizinski, 1993).
unknown membrane origin was partially purified from maize coleoptiles (Harvey et al., 1989) and shown to retain Ca$^{2+}$-channel activity when incorporated into lipid bilayers (Tester & Harvey, 1989). The verapamil derivative LU 49888 was used to purify a 75-kDa protein located primarily in the plasma membrane of carrot cell-suspensions (Thuleau et al., 1990) which upon reconstitution into liposomes formed Ca$^{2+}$-permeable channels (Thuleau et al., 1993).

Direct measurements of voltage-gated Ca$^{2+}$ channels has been achieved with the patch clamp technique. The earliest electrophysiological evidence for the operation of Ca$^{2+}$ permeable channels in the plasma membrane came from studies in charophyte algae (Hayama et al., 1979; Lunevsky et al., 1983). Many of the studies on Ca$^{2+}$ permeation through the plasma membrane of higher plants have shown the activation of Ca$^{2+}$ permeable channels at depolarized electrical membrane potential differences (Fairely-Grenot & Assmann, 1992; White, 1994; Huang et al., 1994; Thuleau et al., 1994; Marshall et al., 1994; Piñeros & Tester, 1995) including ABA-activated nonspecific Ca$^{2+}$ permeable channels (Schroeder & Hagiwara, 1990). These depolarization-gated channels have a bell-shaped current-voltage relationship, with channel opening upon depolarization and subsequent increase in Ca$^{2+}$ current to a maximum upon further successive depolarizations. The Ca$^{2+}$ current then decreases upon further depolarization as the electrochemical driving force for Ca$^{2+}$ influx diminishes. The activation of these channels at membrane potential differences positive to the normal range of resting potentials (-80 to -120 mV) (Fairley et al., 1991) suggests that these channels may respond to stimuli that
induce membrane depolarizations by increasing cytosolic Ca\textsuperscript{2+} that subsequently initiates various signal transduction processes.

However, Ca\textsuperscript{2+} channels in the plasma membrane can also be activated by hyperpolarizing (more negative than the resting potentials) the electrical membrane potential difference (Stoeckel & Takeda, 1995; Gelli & Blumwald, 1997). These channels are voltage-dependently gated with a different current-voltage relation than that of the putative depolarization-activated Ca\textsuperscript{2+} channels. In addition, nonspecific cation channels, also activated by the hyperpolarization of the membrane potential, were suggested as providing a pathway for Ca\textsuperscript{2+} influx (Fairley et al., 1991; Cosgrove & Hedrich, 1991). However, Ca\textsuperscript{2+} can trigger many cellular responses which implies that its influx must be tightly regulated. Because of the nonspecific nature of these channels, they may simply be functioning as a “leak” mechanism in restoring normal resting potentials. The existence of hyperpolarization- and depolarization-activated Ca\textsuperscript{2+}-permeable channels in the plasma membrane may be a required feature of plant cells that allows them to efficiently couple the perception of stimuli at the plasma membrane to fluctuations in cytosolic Ca\textsuperscript{2+} concentrations.

Calcium influx can also occur via plasma membrane stretch-activated channels. These channels are sensitive to mechanical manipulation of the plasma membrane such as changes in turgor (Cosgrove & Hedrich, 1991) and temperature (Ding & Pickard, 1993). The channels are activated by changes in tension across the channel protein suggesting that they may be linked to the cytoskeleton through connections with actin filaments. Thus a mechanosensitive Ca\textsuperscript{2+} channel has been suggested to be a possible link in transduction
chains leading to stomatal closure (Cosgrove & Hedrich, 1991). Nevertheless, for many plant cells, especially guard cells and other cells that regulate volume rather than turgor, it is difficult to see how primary control or gating of channel activities could be mediated by shearing forces with any degree of homeostatic efficiency. Perhaps in these instances mechanosensitivity acts in a preconditioning step where the channel is poised for activation.

Calcium channels in the plasma membrane transduce external signals into fluctuations in free cytosolic Ca\(^{2+}\). Stimulus-specificity is most likely encoded through a multiplicity of Ca\(^{2+}\) pathways each with its own gating properties. Most Ca\(^{2+}\)-permeable channels exhibit both voltage- and ligand-sensitivities and in a few cases some depend on two or more ligands. Some of the factors that control channel gating are: voltage, cytosolic Ca\(^{2+}\), vacuolar Ca\(^{2+}\), plant growth regulators, secondary messenger metabolites (i.e. inositol 1,4,5-triphosphate (IP\(_3\)) and cyclic adenosine 5'-diphosphoribose (cADR)), G proteins, protein kinases and protein phosphatases. The factors controlling channel gating are many and this does not seem unreasonable given the capacity of these proteins to move Ca\(^{2+}\) at potentially useful but equally dangerous rates. Elevation of Ca\(^{2+}\) throughout the cytosol would be toxic to the cell. Some key aspects of these gating characteristics are unique to plants (i.e. auxin, abscisic acid) however in other respects certain gating patterns are similar to those seen in animal cells. Channel sensitivities to Ca\(^{2+}\), IP\(_3\), nucleotide triphosphates, protein kinases and protein phosphatases suggest that some mechanisms of control and signaling are indeed common to both plant and animal cells.
1.11 Calcium Channels at the Vacuolar Membrane

Vacuoles of mature plant cells accumulate Ca$^{2+}$ to millimolar levels creating a large internal pool of Ca$^{2+}$ that can act as a source for the Ca$^{2+}$ signal. Multiple pathways for the release of Ca$^{2+}$ from the vacuole have been identified, these include channels sensitive to voltage (both positive and negative), IP$_3$ and cADPR.

Calcium mobilization from the vacuole can be induced by IP$_3$ (Alexandre et al., 1991). IP$_3$ is one of two second messenger products of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) hydrolysis by phospholipase C (PLC). Binding of IP$_3$ to putative ryanodine receptors in the vacuolar membrane triggered the release of Ca$^{2+}$ (Cote & Crain, 1994). Ryanodine receptors have been described in animal cells as ion channels that mediate intracellular Ca$^{2+}$ mobilization (Berridge, 1993). An isoform of a ryanodine receptor (RYR2) can be activated by the nicotinamide adenine dinucleotide (NAD$^+$) metabolite, cADPribose, which induces the mobilization of vacuolar Ca$^{2+}$ (Allen et al., 1995). The presence of two types of vacuolar ligand-gated Ca$^{2+}$ mobilization pathways, each of which could act coordinately with voltage-gated Ca$^{2+}$ release channels in the same membrane, could enhance the possibilities of generating a Ca$^{2+}$ signal specific for each stimulus. The dependency of channel gating on voltage is the most common. One class of voltage-gated channels are low conductance channels active at membrane potentials that are negative (cytosolic side) and serve to specifically release vacuolar Ca$^{2+}$ in response to stimuli (Johannes et al., 1992, Gelli & Blumwald, 1993). These channels are active at negative vacuolar membrane potential differences (-40 mV to -100 mV; cytosolic side).
Figure 1.5. Ca\(^{2+}\)-permeable channels at the plasma membrane and vacuolar membrane of a higher plant cell. A schematic representation of Ca\(^{2+}\)-permeable channels that function solely in the movement of Ca\(^{2+}\). Rapid influx of Ca\(^{2+}\) into the cytosol occurs via the opening of vacuolar Ca\(^{2+}\) channels at negative membrane potentials (cytosolic side). Some of these channels are voltage-activated while others require IP\(_3\) and cADR. A vacuolar Ca\(^{2+}\)-permeable channel (SV-type) active at positive membrane potentials (cytosolic side) may function in a Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) mechanism; alternatively this channel could function to move Ca\(^{2+}\) into the vacuole (see text).

Ca\(^{2+}\)-permeable channels in the plasma membrane allow the influx of Ca\(^{2+}\) from the extracellular space. Among these types of channels are ones activated by hyperpolarized- or depolarized- membrane potential differences. (+) denotes activation, (-) denotes inhibition, Δψ (+) denotes depolarized membrane potential differences and Δψ (-) denotes hyperpolarized membrane potential differences. The effects of different nucleotides and modes of regulation are explained in the text.
physiological values of pH and resting levels of cytosolic Ca$^{2+}$ concentrations. The channels were sensitive to nifedipine and verapamil (Ca$^{2+}$ channel antagonists) and Bay K 8644 (Ca$^{2+}$ channel agonist) but insensitive to IP$_3$.

Activation of Ca$^{2+}$ release channels from vacuoles and ER and the release of K$^+$ from VK (fast-activating type) channels would polarize the vacuolar membrane potential difference to about +100 mV (cytoplasmic side). Under conditions of elevated cytosolic Ca$^{2+}$ such a positive shift in vacuolar membrane potential could activate slow-vacuolar (SV) type channels (Hedrich & Neher, 1987). The SV type of channel is activated by increased cytosolic Ca$^{2+}$ levels and is weakly selective for Ca$^{2+}$ ($P_{Ca}/P_K = 3$). Thus, following a positive-going vacuolar membrane potential difference and an elevation in cytosolic Ca$^{2+}$, SV channels could function in the sequestration of cytosolic Ca$^{2+}$ into the vacuole (Pantoja, Gelli & Blumwald, 1992). However due to the prevailing physiological gradient of Ca$^{2+}$ across the vacuole, the SV channels could also potentially provide a pathway for Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) (Ward & Schroeder, 1994).

1.12 THE ROLE OF CALCIUM CHANNELS IN CELL SIGNALING

Changes in the electrical potential difference across the plasma membrane of higher plants have been shown to be among the most rapid physiological responses induced by abiotic (Assmann et al., 1985; Serrano et al., 1988; Felle, 1988) and biotic stresses (Ullrich & Novacky, 1991; Lohse & Hedrich, 1992). The hyperpolarization of the electrical plasma membrane potential difference has been identified as an early response of plant cells to blue and red light (Shimazaki et al., 1980; Assmann et al., 1985; Serrano et al., 1988),
auxins (Felle, 1988), the fungal toxin fusicoccin (Marre, 1985) and race specific fungal elicitors (Vera-Estrella et al., 1994). Other physiological stimuli such as Rhizobium lipochitooligosaccharide signal molecules (Ehrhardt et al., 1992) and ABA (Kasamo, 1981) induce a depolarization of the plasma membrane potential.

The sensitivity of the electrical membrane potential to different stimuli suggests that the electrogenic influx of $\text{Ca}^{2+}$ across the plasma membrane could serve for the transduction of signals perceived at the plasma membrane. The influx of $\text{Ca}^{2+}$ via voltage-gated channels produces a transient increase in cytosolic $\text{Ca}^{2+}$ that is able to dictate and specify a response to a particular stimuli. Increases in cytosolic $\text{Ca}^{2+}$ activity, seemingly due to fluxes through $\text{Ca}^{2+}$-permeable channels, are involved in cellular responses to plant growth regulators, such as ABA (Gilroy et al., 1991), cytokinins (Hahm & Sanders, 1991), auxins (Gehring et al., 1990) and gibberellic acid (GA) (Bush & Jones, 1988). Many of these studies used calmodulin ($\text{Ca}^{2+}$-binding protein) -binding inhibitors, channel blockers and other inhibitors of $\text{Ca}^{2+}$ transport to demonstrate that these stimuli were transduced through elevations of cytosolic free $\text{Ca}^{2+}$ concentrations. Elevations of cytosolic $\text{Ca}^{2+}$ have been quantified based on aequorin fluorescence, a $\text{Ca}^{2+}$-dependent luminescent nontoxic protein that resides in the cytosol and can be potentially expressed in any viable cell of transgenic plants (Knight et al., 1991). The use of this procedure clearly demonstrated that temperature, touch, wind, salination, wounding and fungal elicitors increased cytosolic levels of $\text{Ca}^{2+}$ (Knight et al., 1991). Interestingly, calmodulin gene expression was also greatly increased by touch (Braam & Davies, 1990).
Cells must be able to distinguish between the similarly sized Ca\(^{2+}\) changes that are induced by different stimuli. One possible mechanism may involve the concerted action of Ca\(^{2+}\) changes and the action of several target proteins activated by the stimulus-receptor complex. Among these target proteins are G proteins which have the ability to undergo conformational changes in response to the binding of GTP or GDP affecting the ability of these proteins to interact with other downstream proteins. The heterotrimeric G protein is the group of proteins most often associated with transmembrane signal transduction (Verhey & Lomax, 1993; Xing et al., 1996b). In its resting state, the heterotrimeric G protein exists as a \(\alpha\beta\gamma\) heterotrimer composed of three subunits; the \(\alpha\)-subunit which binds and exchanges guanine nucleotides and the \(\beta\) and \(\gamma\) subunits which anchor the complex to the membrane. Upon activation of the receptor by an agonist, the receptor binds to the \(\alpha\)-subunit, promoting the release of pre-bound GDP and the binding of GTP. Thus the \(\alpha\)-subunits cycle between an inactive GDP-bound and an active GTP-bound form. The activated GTP-bound \(\alpha\)-subunit dissociates from the \(\beta\gamma\)-complex and associates with cellular effector proteins. These proteins are the catalytically active compounds responsible for the amplification of the initial signal as long as the \(\alpha\)-subunit stays bound to them. Termination of the active state occurs as a result of the intrinsic GTPase activity of the \(\alpha\)-subunit, which results in the release of the terminal phosphate from bound GTP (Brown, 1991).

Among the effector proteins that initiate the eventual physiological responses and are downstream of the G proteins are protein kinases and protein phosphatases. Protein kinases are enzymes which transfer a phosphoryl group from ATP, or more rarely from
GTP, to a serine, threonine or tyrosine residue of a protein substrate and thereby alter the activity of the substrate. Conversely, protein phosphatases remove the phosphoryl group from the protein substrate (Roberts & Harmon, 1992). Recent findings strongly suggest that Ca^{2+}/calmodulin-(CDPKs), Ca^{2+}/phospholipid-dependent kinases and Ca^{2+}-dependent phosphatases are key elements in Ca^{2+} based signaling pathways since they control the phosphorylation state of key effector proteins (Roberts & Harmon, 1992). Both the receptor-like protein kinases and the CDPKs are the kinases most strongly associated with the plasma membrane. The activity of the CDPKs is greatly stimulated (20- to 100-fold) by micromolar concentrations of free Ca^{2+}. Therefore, it appears that these enzymes are capable of sensing intracellular changes in the free Ca^{2+} concentrations and of transmitting the message through the action of their protein kinase activities. Branching of transduction pathways that produce more than one kind of signal, occurs as a result of these key effector protein kinases, suggesting that a combination of signals may be required for a particular cellular response. Moreover, different signaling pathways may interact or “crosstalk” through protein-protein interactions resulting in a complex modulation of responses.

Another, equally plausible mechanism that allows plant cells to distinguish between different stimuli-induced Ca^{2+} changes may be the differences in the spatial and temporal organization of cytosolic Ca^{2+} fluctuations. In this mechanism the timing, speed and location of the Ca^{2+} change encode for specificity. The relatively high concentrations of Ca^{2+} inside the ER, the vacuole and the extracellular space, enable each of these compartments to serve as a source of stimulus-induced increase in cytosolic Ca^{2+}. Thus
Ca$^{2+}$ influx localized at particular membranes, along with low diffusion rates for Ca$^{2+}$ in the cytosol, make localized gradients in Ca$^{2+}$ possible. The existence of such gradients in tip-growing cells have been previously known and recently have been shown to be essential for tip growth in pollen tubes (Miller et al., 1992; Malho et al., 1995). A similar gradient has been reported in response to GA in barley aleurone protoplasts (Gilroy & Jones, 1992). Localized changes in cytosolic Ca$^{2+}$ may be particularly important for sustained responses such as those induced by GA or tip growth since prolonged elevations in Ca$^{2+}$ throughout the cell would impair normal cellular processes (Bush, 1995). Although in most cases the spatial organization of cytosolic Ca$^{2+}$ changes is not clear, it appears that the source of Ca$^{2+}$ differs between stimuli, supporting a role for the different spatial organization of calcium gradients in the cell.
OBJECTIVES

Calcium release into the cytosol from either internal or apoplastic Ca\(^{2+}\) pools is believed to occur through the opening of channels permeable to and selective for divalent cations (Ca\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\)). These channels are located at the vacuolar and plasma membranes and most probably the ER. A substantial amount of indirect evidence has strongly suggested the presence of Ca\(^{2+}\) channels, however, a direct demonstration of Ca\(^{2+}\) channels and a detailed characterization of the kinetics of channel activity has been limited. In addition, the mechanisms of Ca\(^{2+}\) channel activation by which stimulus-induced elevations in cytosolic Ca\(^{2+}\) occur and the nature of the up- and down-regulation of these mechanisms is not completely understood.

Thus, the aims of this thesis were to: 1) identify Ca\(^{2+}\) channels at the vacuolar and plasma membranes of tomato and sugar beet cells through the application of conventional electrophysiological techniques. 2) characterize these channels on the basis of their voltage-dependence, permeability, selectivity and sensitivity to Ca\(^{2+}\) channel agonists and antagonists. 3) examine the possible involvement of the plasma membrane Ca\(^{2+}\) channel in transducing the presence of specific fungal elicitors into an increase in cytosolic Ca\(^{2+}\). 4) investigate the mechanisms by which the plasma membrane Ca\(^{2+}\) channel is regulated in response to fungal elicitation.
2.1 PLANT MATERIAL

Cell suspensions of sugar beet (*Beta vulgaris* L.) (Blumwald and Poole, 1987) and tomato (*Lycopersicon esculentum* L.) (Vera-Estrella et al., 1992) were grown in 500 ml Erlenmeyer flasks containing 90 ml of Murashige and Skoog medium. The flasks were kept in the dark at 23°C on a rotary shaker (120 rpm) and subcultured weekly.

2.2 ISOLATION OF INTACT PROTOPLASTS AND VACUOLES

Protoplasts were prepared from 3 to 5 day old cultured cells by digestion for 1.5 hours in a digestion buffer (0.4 M sorbitol, 0.5 mM CaCl2, 0.3 M Mes/KOH pH 5.5 with 2.0% cellulase (Seishin Corporation, Chuo-ku, Tokyo Japan), 0.5% BSA and 0.1% pectolyase (Seishin Corporation, Chuo-ku, Tokyo Japan)). Digestion of cells was carried out in the dark at 30 °C on a rotary shaker. The digested cells were filtered through Miracloth to remove all cell debris and the filtrate containing the protoplasts was centrifuged at 200 g for 5 min. The protoplasts were resuspended in 10 ml of wash buffer (0.4 M sorbitol, 0.2 mM CaCl2, 15 mM Tris-Mes buffer pH 5.5) and collected after centrifugation at 200 g for 5 min. This washing step was repeated twice. The protoplasts were loaded on top of a 0/25% discontinuous Percoll gradient and centrifuged at 250 g for 20 min. Purified protoplasts were collected at the 0/25% Percoll interface. The protoplasts were resuspended in wash buffer and centrifuged at 200 g for 5 min. This washing step was repeated twice.

The incubation of one volume of purified protoplasts (0.4 M sorbitol, 0.2 mM CaCl2, 15 mM Tris Mes buffer pH 5.5) with 4 volumes of lysis buffer (2 mM EDTA, 2
mM EGTA, 50 mM KCl, 15 mM Tris pH 8.0) for 10 min at 4 °C resulted in the lysis of the protoplasts and the subsequent release of intact vacuoles to the lysis buffer. The lysis of the protoplasts and the release of the vacuoles was monitored by following the accumulation of Neutral Red in the vacuolar milieu under the light microscope. The lysis was stopped and the vacuoles stabilized by the addition of an equal volume of 0.7 M sorbitol, 50 mM KCl, 3 % Ficoll and 15 mM Tris-Mes buffer pH 8.0 (stabilization buffer). The buffer containing the vacuoles was filtered through Miracloth to remove protoplast debris and the vacuoles were collected by flotation. A Ficoll gradient was generated by layering 3 ml of isolation buffer (0.4 M sorbitol, 50 mM KCl, 5 mM Tris-Mes buffer pH 8.0) on top of the vacuole suspension followed by centrifugation at 800 rpm for 25 min at 4 °C. Vacuoles floated and were recovered at the top of the gradient and at the 0/3 % Ficoll interface. For the isolation of tomato vacuoles 5 ml of vacuole suspension was layered on top of a 3 % and a 1.5 % Ficoll gradient followed by centrifugation at 800 rpm for 20 min. Pure vacuoles were collected from the 1.5 /0 % Ficoll interface.

2.3 PREPARATION OF INTERCELLULAR FLUIDS

Intercellular fluids (IF) from tomato leaf tissue infected with *Cladosporium fulvum* were prepared according to De Wit and Spikman (1982). The *Cf5* incompatible race 4 or compatible race 5 were inoculated onto the cultivar Bonny Best (no known *Cf* genes). Control IF was obtained from uninoculated plants incubated under the same conditions as the inoculated plants. The IFs were precipitated with acetone (90 %), the pellet was then freeze-dried and resuspended in distilled water to give the original volume and stored at -
20 °C overnight. The preparations were centrifuged at 1,650 g for 20 min, the supernatant discarded and the pellet resuspended in distilled water and boiled for 5 min followed by centrifugation at 1,650 g for 20 min (while boiling causes some loss of activity of the avr5 elicitors, it effectively inactivates many enzymes in the IF and precipitates considerable contaminating protein). The supernatants contain specific elicitors for each of the Cf genes on which the specific race of C. fulvum is avirulent (i.e. causes cell necrosis referred to as the hypersensitive response). Generally a ratio of IF to water of 1/32 was used and contained between 0.10 - 0.35 μg protein/μl.

2.4 CHEMICAL REAGENTS

GTP[γ]S, GDP[β]S, ATP[γ]S and ADP[β]S were purchased as lithium salts from Calbiochem (CA, USA). Okadaic acid, HA1004, staurosporine and mastoparan were also obtained from Calbiochem. All dihydropyridines (Bay K 8644, nifedipine) and phenylalkamines (verapamil) were purchased from Fluka. The Ca$^{2+}$ chelator [bis-(o-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid]-tetrosodium salt (BAPTA) was purchased from Calbiochem (CA, USA).

2.5 MICROPIPETTE FABRICATION

Several factors promoted gigaseal formation including Millipore-filtered solutions, positive pressure within the pipette during the approach to the cell, a clean cell surface and the micropipette glass. Glass pipettes for electrophysiological recordings were made in three steps: glass pipettes were pulled, pipette shanks were coated with Sylgard or
Sigmacote (Sigma, MO, USA) and pipette tips were fire polished. Glass pipettes were pulled in two stages using a vertical microelectrode puller. In the first pull the glass capillary was thinned over a length of 7 to 10 mm to obtain a minimum diameter of 200 μm. The capillary was then recentered within the heating coil and in the second pull the thin part broke resulting in two pipettes. Using a fixed pulling length and fixed settings for the two stages it was possible to obtain a large number of pipettes with similar tapering and tip openings (1-5 μm). The pipette glass wall at the pipette tip was polished (smoothed out) using a microforge shortly after coating the pipette. The heat was supplied by a V-shaped platinum-iridium filament bearing a glass ball. The filament was heated to a dull red glow and a stream of air was directed towards the glass ball, restricting the heat to its immediate vicinity. The tip of the pipette was brought to within 10-20 μm of the glass ball for a few seconds in order to smooth out the pipette tip. Finally pipette shanks within 50 μm were coated with Sylgard or Sigmacote in order to reduce the pipette-bath capacitance and to form a hydrophobic surface. Although coating the pipette tip was not required for gigaseal formation it served to reduce background noise. Thus pipettes had resistance values in the range of 6-10 MΩ and opening tip diameters between 0.5 and 1 μm.

2.6 MEASUREMENTS OF WHOLE-CELL CURRENTS IN INTACT PROTOPLASTS.

Experiments were performed using conventional whole-cell and single channel patch clamp techniques (Hamill et al., 1981). Protoplasts were kept in a glass chamber containing 5, 10, 20, 35 or 50 mM BaCl₂ (or CaCl₂) as described in the figure legends, 0.1
mM potassium glutamate, 1 mM MgSO₄, 5 mM Tris-Mes buffer (pH 6.1) and sorbitol to give a final osmolarity of 450 mosmol. Glass pipettes pulled with a vertical puller (Adams & List, New York, USA) from borosilicate glass capillaries (Kimax-51, VWR, Boston, MA USA) coated with silicone (Sigmacote) and fire polished, had a tip resistance of 5-10 MΩ when filled with 100 mM potassium glutamate, 1 mM MgSO₄, 3 mM MgATP (freshly added to the pipette solution), 0.05 mM CaCl₂ + 0.10 mM BAPTA to give a final free Ca²⁺ concentration of 100 nM (Tsien, 1980), 5 mM Tris-Mes buffer (pH 7.3) and sorbitol with a final osmolarity of 475 mosmol.

The whole cell configuration was initially obtained by forming a gigaseal with resistance of 6 to 8 GΩ in the cell-attached mode followed by the application of further suction to rupture the plasma membrane within the pipette tip. Whole-cell experiments were performed at 23 °C in voltage-clamp mode using Dagan 3900 amplifier (Dagan Corporation, MA USA) digitized on line (TL-1 DMA Interface; Axon Instruments), stored on a 386 based 33 MHz computer and acquired and analyzed with pClamp 6.0.2 software (Axon Instruments, CA USA). Voltage-pulse protocols were applied during data acquisition as described in the legends to figures. All membrane potentials reported here have been corrected for liquid junction potentials (Barry & Lynch, 1991). Whole-cell currents were filtered at 500 Hz with a four-pole Bessel filter contained in the Dagan amplifier. In the whole cell configuration, the bathing solution represents the extracellular side of the plasma membrane and the cytosolic side of the vacuolar membrane. The pipette solution represents the cytosolic side of the plasma membrane and the lumenal side
(intravacuolar) of the vacuolar membrane. This convention holds true for outside-out patches of both vacuolar and plasma membranes as well.

2.7 MEASUREMENTS OF SINGLE CHANNEL CURRENTS

For single channel measurements, outside-out patches of plasma membrane were obtained after the whole-cell mode by quickly pulling the pipette away from the protoplasts. Both the bathing and the pipette solutions used in single channel measurements were the same as those used in whole-cell measurements. Single channel currents were measured with the same equipment and computer software as that used in whole-cell measurements. Data were filtered with a four-pole Bessel filter at 200 Hz, digitized at 2KHz and stored on disk.

Permeability ratios for solutions containing a mixture of monovalent and divalent ions were calculated from the constant field equations proposed by Goldman-Hodgkin-Katz, as previously described in the literature review. Activities of ions were derived by using activity coefficients estimated from the Debye & Hückel equation (Nobel, 1991). For the measurement of whole cell and single channel currents Ba\(^{2+}\) was used as a Ca\(^{2+}\) analogue for several reasons: (i) Ba\(^{2+}\) blocks potassium channels in the plasma membrane of both animal (Armstrong & Taylor, 1980) and plant (Wegner et al., 1994) cells that could potentially mask Ca\(^{2+}\) currents; (ii) Ba\(^{2+}\) enhances the resolution of Ca\(^{2+}\) channels since the magnitude of the single channel currents are larger with Ba\(^{2+}\) as the charge carrier (Gelli & Blumwald, 1993).
The open and closed time constants were determined from exponential fittings of open and closed time histograms using the pClamp software. Histogram fits were determined to be good only if the standard errors of time constants were less than 10 % and if the criteria set by the pClamp software (i.e. goodness of fit ≈ 2.0) was met. Mean open and closed times and P₀ values for patches of membrane that had more than one channel were determined according to the method of Labarca et al., (1980) as described in the literature review.

2.8 MEASUREMENTS OF WHOLE-CELL AND SINGLE CHANNEL CURRENTS FROM VACUOLES.

In recording ionic currents from whole vacuoles and isolated patches of vacuolar membrane similar whole-cell and single channel patch clamp techniques were used (Hamill et al., 1981). For electrophysiological measurements in endomembranes the vacuolar lumen is considered electrically equivalent to the extracellular space (Bertl et al., 1992). This implies that the potential difference across the vacuolar membrane (tonoplast) is calculated as the electrical potential of the cytoplasm minus that of the vacuole. Negative or inward currents across the tonoplast correspond to cation flow into the cytoplasm from the vacuole or anion flow out of the cytoplasm into the vacuole and are plotted downward in all figures. Conversely, positive charges leaving the cytoplasm, or negative charges entering the cytoplasm are represented as positive or outward currents and are plotted upward in all figures.
Vacuoles were maintained in a recording chamber (cytoplasmic side of vacuole) in 100 mM KCl, 3 mM MgATP, 2 mM MgCl₂, 5 mM Tris/Mes pH 7.5, 1 mM CaCl₂ and 1.1 mM BAPTA to give a final concentration 100 nM free Ca²⁺. The pipette filling solution (lumen of vacuole) consisted of either BaCl₂, SrCl₂ or CaCl₂ at the concentrations indicated in the figure legends, 2 mM MgCl₂ and 5 mM Tris-Mes pH 6.0. Both the bathing and the pipette solutions were adjusted to 450 milliosmolar with D-sorbitol.
3.1 INTRODUCTION

Homeostasis of cellular Ca\(^{2+}\) is maintained by the active extrusion of Ca\(^{2+}\) from the cytosol to the extracellular space (Rasi-Caldogno et al., 1987) or by the sequestration of Ca\(^{2+}\) into the vacuole (Blumwald & Poole, 1986). Thus, both the vacuole and the extracellular space can act as sources for the elevation of cytosolic Ca\(^{2+}\) in response to stimuli. The vacuole of a mature plant cell is the main storage compartment of intracellular Ca\(^{2+}\) where free Ca\(^{2+}\) concentrations are within 1 to 5 mM. Release of vacuolar Ca\(^{2+}\) results in an elevation of free cytosolic Ca\(^{2+}\) concentration from steady state levels of 100-150 nM to greater than 300 nM (Bush, 1995). A vacuolar Ca\(^{2+}\) release channel would provide a specific pathway for the elevation of cytosolic Ca\(^{2+}\) when required by the cell for signal transduction mechanisms. Here, electrophysiological techniques were employed in order to identify voltage-dependent channels permeable to and selective for divalent cations (Ca\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\)).

It should be noted that in this thesis vacuolar membrane potentials are considered according to the proposed convention for electrical measurements in endomembranes (Bertl et al., 1992). According to this convention the vacuolar lumen is considered to be equivalent to the extracellular space. Thus for plant vacuoles, the vacuolar membrane voltage is considered to be the difference between the voltage at the cytosolic side minus the voltage at the vacuolar side. Therefore, when positive voltage differences are set across the tonoplast, these voltage differences will be considered negative tonoplast potentials, making the tonoplast electrically equivalent to the cell plasma membrane.
3.2 RESULTS

3.2.1 CALCIUM RELEASE FROM ISOLATED VACUOLES

Whole vacuolar currents were recorded with BaCl$_2$, SrCl$_2$ or CaCl$_2$ (10 mM) in the pipette (inside the vacuole) and 100 mM KCl in the recording chamber (cytoplasm) while cytosolic Ca$^{2+}$ (100 nM) and the pH gradient (1.5 units) were maintained at physiological levels. Figure 3.1 shows the activation of currents from the vacuole into the cytoplasm (inward currents) in response to voltage pulses applied to the membrane ranging from a holding potential of +20 mV to ±100 mV in increments of 20 mV. Negative membrane potential differences elicited large time-dependent currents in the presence of BaCl$_2$ (Fig. 3.1A), whereas positive voltage pulses resulted in small instantaneous currents. The same protocol was applied to vacuoles exposed to SrCl$_2$ (Fig. 3.1B) and CaCl$_2$ (Fig. 3.1C) in the pipette. Again negative membrane potential differences elicited inward currents, however, they were of smaller magnitude than those recorded in the presence of BaCl$_2$. The currents recorded with the various divalent ions were plotted against their respective voltages (Fig. 3.1D). The current-voltage relationship demonstrated that these currents were voltage-dependent, and of the three divalent ions used, Ba$^{2+}$ induced currents with the largest magnitude, followed by Sr$^{2+}$ and Ca$^{2+}$.

An estimate of the selectivity of the channels was obtained by tail current experiments (Fig. 3.2). In this particular tail current experiment, currents were activated by voltage pulses of -100 mV for 6.5 sec and deactivated by stepping up the voltage to -80 mV. This protocol was repeated 15 times with a subsequent increase in the deactivating pulse of 10 mV. Inward currents reversed ($E_{rev}$, equivalent to the zero-current potential)
Figure 3.1. Voltage-dependent currents from whole vacuoles of sugar beet cells exposed to BaCl₂, SrCl₂ and CaCl₂. A, Voltage clamp recordings were obtained by applying voltage pulses of 100 mV to -100 mV in steps of 20 mV from a holding potential of 20 mV. B, With the lumenal side of the vacuolar membrane (pipette) exposed to 10 mM BaCl₂, negative voltage pulses (Vₘ=Vₑ - Vₚ) induced time-dependent currents that reached a maximum current level in 3 s. The time-dependent currents were not activated at positive membrane potentials. C, Time-dependent currents were induced by negative membrane potentials in the presence of SrCl₂; however, current levels were smaller in magnitude. Again, positive potentials did not elicit time-dependent currents. D, In the presence of CaCl₂, negative membrane potentials induced currents of a much smaller magnitude. E, Current levels measured at 4.8 s from the onset of the voltage pulses (0 to -100 mV) were plotted against their respective voltages. Current levels were obtained from experiments performed in the presence of BaCl₂ (Δ), SrCl₂ (●) and CaCl₂ (O). Experimental data are the mean ±SE of nine vacuoles.
Figure 3.2. Tail current recordings determined the reversal potential of the time-dependent currents. An estimate of current specificity was obtained through tail current experiments. A, Currents were activated by voltage pulses of -100 mV for 6.5 s and deactivated by stepping up the voltage to -80 mV. This protocol was repeated 15 times with a subsequent increase in the deactivating pulse of 10 mV. Tail currents reversed direction ($E_{rev}$) between 20 and 30 mV. B, Negative voltage pulses of -100 mV induced time-dependent inward currents. These currents were subsequently deactivated as the voltage pulses were stepped up to less negative potentials in increments of 10 mV. These experiments were performed with whole vacuoles exposed to 10 mM BaCl$_2$ (lumenal side of vacuolar membrane) and 100 mM KCl (cytosolic side of vacuolar membrane). Similar results were observed in at least five vacuoles.
at 30 mV with vacuoles exposed to 10 mM Ba\textsuperscript{2+} in the pipette and 100 mM KCl in the recording chamber, indicating a permeability ratio, $P_{\text{Ba}^{2+}}/P_{\text{K}^+}$ of 20.

3.2.2 SINGLE CHANNEL RECORDINGS

Single channel activity was recorded in isolated outside-out patches (lumen of vacuole exposed to the pipette solution) with 10 mM BaCl\textsubscript{2}, SrCl\textsubscript{2}, or CaCl\textsubscript{2} in the pipette and 100 mM KCl in the recording chamber (cytoplasmic side of vacuolar membrane). Single channel currents were plotted against their respective membrane voltages (Fig. 3.3A). The current-voltage plot demonstrated that the current levels increased as the membrane potential was made more negative and that the currents varied linearly with decreasing membrane voltages. A single channel conductance of 14, 10 and 6 picosiemens (pS) was obtained with 10 mM Ba\textsuperscript{2+}, Sr\textsuperscript{2+} and Ca\textsuperscript{2+} as the charge carrier, respectively.

The selectivity of these currents was determined from single channel recordings obtained under the same conditions as explained above. From the current-voltage graph, the zero-current potentials (arrows on Fig. 3.3A) appeared at 40 mV, 45 mV and 55 mV for Ca\textsuperscript{2+}, Sr\textsuperscript{2+} and Ba\textsuperscript{2+}, respectively thus suggesting that these channels were 20 to 23 times more selective for the divalent cations than for K\textsuperscript{+}. Single channel currents were also recorded in the presence of increasing concentrations of Ba\textsuperscript{2+}, Sr\textsuperscript{2+} and Ca\textsuperscript{2+} (10, 20, 40 and 80 mM) and plotted against the activity of the divalent cations (Fig. 3.3B). The channel currents displayed an apparent saturation with increasing activity of divalent cations. These current values were used to generate an Eadie-Hofstee plot (Fig. 3.3B, inset) from which $K_d$ values of 13.3, 18.1 and 24.3 mM and maximum currents of 3.7, 3.4
Figure 3.3. The current-voltage relationship of single-channel records with Ba\(^{2+}\), Sr\(^{2+}\) and Ca\(^{2+}\) as the charge carriers. Isolated patches of vacuolar membrane (outside-out) were exposed to 100 mM KCl and 100 nM free Ca\(^{2+}\) (cytoplasmic side) with 10 mM BaCl\(_2\) (Δ), SrCl\(_2\) (●) and CaCl\(_2\) (○) (lumenal side). A, Single channel activity was recorded and plotted against the respective voltage. The linear relationship between the single channel current and the voltage is evident. Current levels were largest with Ba\(^{2+}\) as the charge carrier, as was observed in the whole vacuole configuration. Least-squares analysis gave zero-current potentials of 40 mV, 45 mV and 55 mV (arrows) for 10 mM CaCl\(_2\), SrCl\(_2\) and BaCl\(_2\), respectively. B, The dependence of single channel currents (I\(_{\text{m}}\)) recorded at -60 mV were plotted against the activity of the different divalent cations. Channel currents saturated with increasing concentrations of the cations. Inset, An Eadie-Hofstee plot of the experimental data gave K\(_d\) values of 13.3 mM, 18.1 mM and 24.3 mM and single channel maximum currents of 3.7 pA, 3.4 pA and 3.1 pA for Ba\(^{2+}\), Sr\(^{2+}\) and Ca\(^{2+}\), respectively. C, The P\(_o\) of the channels is voltage-dependent. The P\(_o\) increased between 0 and -60 mV and reached a maximum at -70 mV to -80 mV. Experimental data are the mean ± SE for five vacuoles.
and 3.1 pA for Ba\(^{2+}\), Sr\(^{2+}\) and Ca\(^{2+}\), respectively, were obtained. The single-channel open probability (P\(O\)) was dependent on the vacuolar membrane potential difference and it reached a maximum at -70 mV to -80 mV.

3.2.3 PHARMACOLOGICAL CHARACTERIZATION OF THE VACUOLAR Ca\(^{2+}\)-RELEASE CHANNEL.

The effects of the Ca\(^{2+}\) channel antagonists nifedipine (a dihydropyridine) and an agonist Bay K 8644 (a dihydropyridine) on the single channel kinetics were studied. Currents recorded in the presence of Ba\(^{2+}\) from an outside-out patch of membrane clamped at membrane voltage of -80 mV in the presence and absence of nifedipine are shown in Figure 3.4. Prior to the addition of nifedipine, the single channel current recordings from controls displayed large channel activity with at least two channels in the isolated patches (Fig. 3.4A) where downward deflections corresponded to the movement of Ca\(^{2+}\) from the vacuole into the cytosol. After the addition of 100 μM nifedipine to the lumenal side of the vacuolar membrane, channel currents were inhibited, as shown by the shortened open times and the reduced number of channel openings (Fig. 3.4C). The inhibition of channel activity is further demonstrated in the amplitude histograms obtained in the absence (Fig. 3.4B) and presence (Fig. 3.4D) of nifedipine. The absence of the peaks corresponding to the simultaneous opening of one and two channels is evident when comparing the two amplitude histograms.

Figure 3.5 shows the effect of an agonist, Bay K 8644, on the vacuolar Ca\(^{2+}\) currents. Current recordings obtained in the presence of 100 μM Bay K 8644 in the
Figure 3.4. A reduction in single channel activity by the 1,4-DHP antagonist, nifedipine.

A, An original recording of single channel currents from an outside-out patch of vacuolar membrane exposed to 10 mM BaCl₂ (lumenal side) and 100 mM KCl and 100 nM free Ca²⁺ (cytosolic side). This trace obtained at -80 mV shows the activation of at least three channels, as indicated by the different current levels (O₁, O₂, O₃). Similar results were observed in at least eight vacuoles. B, Amplitude distribution histograms from single channel records similar to those shown. The largest peak of the histogram occurs at 0 pA and represents all channels in the closed state. The additional peaks correspond to one (approximately -2.0 pA) and two (approximately -4.0 pA) channels opening simultaneously. C, A reduction in single channel activity by nifedipine. A single channel recording was obtained at -80 mV from an outside-out patch with the lumenal side of the vacuolar membrane exposed to 100 μM nifedipine. Channel activity is inhibited as demonstrated by the reduced number of events and the shortened open times. Similar results were observed in at least five vacuoles. D, Amplitude distribution histograms from single channel records similar to that shown in C. Inhibition of channel activity is demonstrated by the absence of the peaks representing the simultaneous opening of one and two channels.
pipette demonstrated an increase in the frequency of channel opening while the current levels remained unchanged (Fig. 3.5B). Dwell time distribution histograms were generated from single channel recordings obtained in the absence (Fig. 3.5A) and presence of Bay K 8644 (Fig. 3.5C). The values for the mean open and closed times of these channels were calculated as previously described. The similarities in the channel mean open time (23.6 ± 3.5 ms versus 25.2 ± 4.4 ms) and mean closed time (1388 ± 25 ms versus 1212 ± 32 ms) before and after the addition of the agonist suggest that Bay K 8644 did not significantly affect the time that the channel spent in the open and closed states. The histograms of the open and closed states were fitted with one exponential, suggesting that only one conductive and one nonconductive state were detected in the absence of the agonist. However, two exponentials were required when fitting the histograms of currents recorded in the presence of Bay K 8644. This suggested that the agonist induced the activation of another conducting and nonconducting state of the channel. These results suggested that the increase in the frequency of channel openings elicited by Bay K 8644 was due to a mechanism other than the prolongation of the open times.
Figure 3.5. The effects of Bay K 8644 (a 1,4-DHP agonist) on the single channel kinetics. A, A single channel record obtained from an outside-out patch of vacuolar membrane exposed to 10 mM BaCl₂ (lumenal side) and 100 mM KCl and 100 nM free Ca²⁺ (cytosolic side). Channel activity was recorded at a membrane potential of -80 mV. The trace shows the presence of at least two channels in the patch (O₁, O₂). B, The distribution of dwell times for the channel in the open and closed state from single channel recordings similar to those shown in A. The histograms for the open and the closed times were best fit with a single exponential. Rate constants for the open and closed times were 32.14 ms⁻¹ and 588.24 ms⁻¹, respectively. The mean open and mean closed times are 23.6 ± 3.5 ms and 1388 ± 25 ms, respectively. C, Single channel recording obtained from an outside-out patch clamped at -80 mV and exposed to 100 μM Bay K 8644. D, Dwell time distribution histograms were constructed from single channel recordings similar to those shown in C. Histograms for both the open and closed times were best fit with two exponentials with rate constants of 6.43 ms⁻¹ and 32.14 ms⁻¹ (open times) and 8.22 ms⁻¹ and 428.74 ms⁻¹ (closed times). The mean open and closed times are 25.2 ± 4.4 ms and 1217 ± 32 ms, respectively.
3.3 DISCUSSION

Voltage patch-clamp experiments in intact vacuoles and isolated vacuolar membrane patches were employed to study the release of Ca$^{2+}$ from vacuoles into the cytosol in sugar beet cell (*Beta vulgaris* L.) and tomato cell (*Lycopersicon esculentum* L.) suspension cultures. The evidence provided in this chapter, suggests that the currents recorded in both the whole vacuole and the isolated membrane patch moved through voltage-dependent Ca$^{2+}$ channels. Channels allowing the movement of Ca$^{2+}$ out of the vacuole were identified at physiological conditions of pH and at resting levels of free cytosolic Ca$^{2+}$. These channels were voltage-dependent, active only at negative membrane potential differences and permeable to divalent cations (Ca$^{2+}$, Ba$^{2+}$, Sr$^{2+}$). However, measurement of single channel currents revealed that slope conductance was largest and the zero-current potential was slightly more positive when Ba$^{2+}$ was the charge carrier, suggesting a slightly higher selectivity for and permeability to Ba$^{2+}$. The saturation of single channel currents with increasing concentrations of divalent ions suggested the presence of an ion binding site within the channel. These channels were IP$_3$-insensitive suggesting that vacuoles contain multiple Ca$^{2+}$ release channels (see chapter 1)

The channels displayed sensitivity to a class of drugs known as dihydropyridines that act on Ca$^{2+}$ channels in animal cells. Experiments where the lumenal side (extracellular side of the channel) of the vacuolar membrane was exposed to Bay K 8644, (an agonist), demonstrated that the mean open time of the channels was not affected while the frequency of opening increased. This was reflected by the difference between the mean closed times of the channel (before and after the addition of Bay K 8644). In addition, Bay
K 8644 activated a different conducting state of the channel as well as an additional closed state, suggested by the presence of additional rate constants in the dwell time histograms for both the open and closed state of the channel. These results suggest that Bay K 8644 may be activating channels not normally open at resting conditions or it may be enhancing the availability of each Ca$^{2+}$ channel.

The sensitivity of the channels to DHPs was further supported by experiments performed with nifedipine. Experiments revealed an inhibition of channel activity when nifedipine, an antagonist, was present on the extracellular side of the vacuolar membrane. The single channel current was not completely blocked since some brief openings were still observed. This suggested that nifedipine was not blocking the pore of the channel but rather it may have bound somewhere on the outer surface of the channel protein. Channel activity was also inhibited by verapamil, La$^{3+}$ and cytosolic Ca$^{2+}$ concentrations higher than 1μM. Permeability and selectivity studies strongly suggested that these channels could efficiently and selectively release Ca$^{2+}$ from the vacuole at hyperpolarized (with reference to the cytosol) membrane potential differences. An elevation of the cytoplasmic free Ca$^{2+}$ concentration to levels above 100 nM leads to Ca$^{2+}$-activated responses (Bush, 1995). Fluctuations in Ca$^{2+}$ concentration can be the result of Ca$^{2+}$ release from intracellular Ca$^{2+}$ pools such as those from vacuoles. The channel described here provides a pathway for the release of vacuolar Ca$^{2+}$ into the cytosol.

The increase in cytosolic Ca$^{2+}$ could act as a positive feedback mechanism for Ca$^{2+}$-activated Ca$^{2+}$ release from other intracellular storage pools (Ward & Schroeder, 1991) that could trigger the initiation of signal transduction processes. Moreover, the
The efflux of Ca$^{2+}$ from the vacuole (resting membrane potential -20 mV on cytosol side) into the cytosol will depolarize (toward positive membrane potentials) the vacuolar membrane potential toward the Ca$^{2+}$ equilibrium potential (100 mV). The depolarization of the vacuolar membrane could then serve as a negative feedback mechanism to attenuate the signal transduced by Ca$^{2+}$. Thus, depolarization of the vacuolar membrane with the increase in Ca$^{2+}$ concentration in the vicinity of the vacuole will allow the efflux of K$^-$ into the cytosol through Ca$^{2+}$-activated K$^+$ channels (FV-type channels) (Hedrich and Neher, 1987) and the influx of Ca$^{2+}$ through Ca$^{2+}$ channels activated at positive vacuolar membrane potentials (Pantoja et al., 1992). The influx of positively charged ions into the vacuole will result in the polarization of the vacuolar potential to resting levels. Return of the remaining cytosolic Ca$^{2+}$ to steady state levels would be achieved by the plasma membrane Ca$^{2+}$-ATPase (Rasi-Coldogno et al., 1987) and the vacuolar Ca$^{2+}$/H$^+$ antiport (Blumwald and Poole, 1986; Schumaker and Sze, 1987; Blackford et al., 1990). A description of the mechanisms regulating channel activity and the comparison among the different Ca$^{2+}$ release channels is discussed in chapter 7.
4.1 INTRODUCTION

The controlled regulation of cytosolic Ca\(^{2+}\) involves the active extrusion of Ca\(^{2+}\) into the extracellular space and the ER via Ca\(^{2+}\)-ATPases and the sequestration of Ca\(^{2+}\) into the vacuole via the H\(^+\)/Ca\(^{2+}\) antiport. However, Ca\(^{2+}\)-permeable channels at the vacuolar membrane, active at positive (with reference to the cytosol) membrane potential differences could provide a pathway for the sequestration of Ca\(^{2+}\) following cytosolic Ca\(^{2+}\) fluctuations. For example, activation of vacuolar Ca\(^{2+}\) release channels (chapter 3) would result in the elevation of cytosolic Ca\(^{2+}\) and in the depolarization (towards more positive potentials) of the vacuolar membrane potentials. Moreover, the depolarization of the vacuolar membrane potential along with the increase in Ca\(^{2+}\) concentration in the vicinity of the vacuole, could allow the efflux of K\(^+\) into the cytosol through Ca\(^{2+}\)-activated K\(^-\) channels (Hedrich & Neher, 1987) which would contribute to the depolarization of the vacuolar membrane potential. Under these conditions, the electrochemical potential for Ca\(^{2+}\) in the cytosol, through the generation of localized micro-gradients of Ca\(^{2+}\), could potentially be larger than that in the vacuole. Thus, the cell could then remove cytosolic Ca\(^{2+}\) through the opening of vacuolar channels active at positive membrane potentials whereby Ca\(^{2+}\) could move into the vacuole down its electrochemical gradient. This mechanism could provide a rapid and efficient pathway for Ca\(^{2+}\) sequestration since channels can move 10\(^6\) - 10\(^8\) ion s\(^{-1}\), whereas antiports and pumps have a much slower rate of turnover, on the order of 10\(^3\) - 10\(^4\) ion s\(^{-1}\).
It had been previously demonstrated that vacuoles from a variety of plant species, contained channels active at positive vacuolar membrane potentials that were weakly selective for monovalent cations (Hedrich & Neher, 1987; Tester, 1990). These channels, referred to as the slow-vacuolar type (SV), have a unitary conductance between 50 to 80 pS, they are strongly outward rectifying (i.e. active only at positive membrane potentials) and they function only in the presence of high cytosolic Ca\(^{2+}\) concentrations (greater than 1\(\mu\)M). Here, electrophysiological techniques were used in order to determine whether vacuolar membranes of sugar beet and tomato cell suspension cultures, contained channels permeable to divalent cations, active at positive vacuolar membrane potentials (cytosolic side).
4.2 RESULTS

4.2.1 WHOLE VACUOLAR CURRENTS

Currents carried by $\text{Ba}^{2+}$ (used as a $\text{Ca}^{2+}$ analog) showed a strong outward rectification, that is, the magnitude of the currents elicited by positive polarization of the vacuole were larger than those elicited by negative polarization (Fig. 4.1A,B). Whole vacuole outward currents (out of the cytosol) reached steady state 2 to 3 s after the onset of the voltage pulse. When $\text{BaCl}_2$ was substituted by $\text{KCl}$ on the cytosolic side of the vacuoles and the concentration of cytosolic $\text{Ca}^{2+}$ was less than 0.1 $\mu$M, voltage pulses between -100 to 100 mV elicited only small instantaneous currents (Fig. 4.1C). Thus the outward rectification obtained with $\text{BaCl}_2$ was the result of $\text{Ba}^{2+}$ moving into the cytosol and was not due to the movement of vacuolar $\text{Cl}^-$ in the opposite direction. With vacuoles exposed to symmetrical $\text{BaCl}_2$ solutions, outward currents were not observed, suggesting that intravacuolar $\text{Ba}^{2+}$ may have blocked these currents. Negative membrane potential differences elicited only small inward currents under both conditions. The magnitude of the outward currents, however, increased as the membrane potential was made more positive (Fig. 4.2A). The results from experiments with the two different $\text{Ba}^{2+}$ concentrations (Fig. 4.2A) indicated that the currents may have reached saturation. The currents elicited in vacuoles exposed to the 100 mM KCl bathing solution were of smaller magnitude than those with $\text{Ba}^{2+}$ and varied linearly in the range of voltage studied.

Calculations from the Boltzmann plot (Fig. 4.2B) indicated that the $\text{Ba}^{2+}$ currents were sensitive to the electric field ($z=1.6$) across the vacuole. These results also suggested
Figure 4.1. Voltage-dependent Ba\(^{2+}\) currents in the whole vacuole. A, B, With vacuoles exposed to a bathing solution containing 30 mM BaCl\(_2\), pulses to -100 mV elicited only small instantaneous outward currents, whereas polarization of the vacuole to potentials more positive than 40 mV evoked large and time-dependent outward currents (out of the cytosol and into the vacuole). C, Replacing BaCl\(_2\) with 100 mM KCl in the bath solution eliminated the large time-dependent outward currents.
Figure 4.2. Current-voltage relationship from whole vacuole experiments. A, Outward currents recorded with 30 mM (O) or 50 mM (●) BaCl₂ in the bath were of similar magnitude at all positive potentials. With 100 mM KCl in the bath (Δ), the instantaneous currents showed a linear relation between 100 mV to -100 mV. B, Boltzmann plot of the currents recorded with 30 mM BaCl₂ in the bath. The line is a least squares fit of slope (z) 1.6 and y-intercept of ΔGᵣ=-2.05 kcal mol⁻¹. All data are the mean ± SD of five measurements. Error bars are smaller than data points.
that the vacuolar \( \text{Ba}^{2+} \)-permeable channels require less energy to change from the closed to the open state (\( \Delta G_i = 2.05 \text{ kcal mol}^{-1} \)), as compared to \( \text{Ca}^{2+} \)-permeable channels in the plasma membrane (\( \Delta G_i = 2.81 \text{ kcal mol}^{-1} \)) (Gelli & Blumwald, 1997). Also a positive value for \( \Delta G_i \) suggested that the opening of the channel was not a spontaneous process.

An estimate of the selectivity of the channels was obtained from the zero-current potentials measured by the tail-currents method (Fig. 4.3A). Outward currents, with a zero-current potential of -10 mV with 30 mM \( \text{Ba}^{2+} \) in the cytosolic side and 100 mM \( K^+ \) in the vacuole, were 2.3 times more permeable to \( \text{Ba}^{2+} \) than to \( K^+ \) (Fig. 4.3B). Whole-vacuolar currents inactivated within 10 min, thus preventing further characterization of the outward currents (Fig. 4.3C). The magnitude of the currents elicited by the activating voltage pulse to +100 mV decreased as the experiment proceeded, reaching about one-half the initial value within 5 min. This current decrease could not be accounted for by a partial resealing of the pipette, because at the end of every experiment the magnitude of the vacuolar capacitance remained unchanged.

### 4.2.2 ISOLATED PATCHES OF VACUOLAR MEMBRANE

With outside-out patches of vacuolar membrane, it was possible to resolve outward currents at the single channel level (Fig. 4.4). The magnitude of the current moving through a single channel increased as the membrane potential difference across the outside-out patch was made more positive. Clamping the patch of membrane to negative
Figure 4.3. Reversal potential and inactivation of the Ba\(^{2+}\) currents. A, Outward currents were activated by 100 mV pulses for 5 s and deactivated by stepping down the voltage to 80 mV. This protocol was repeated 15 times with the deactivation pulse increased by 10 mV for each following pulse. B, The outward currents reversed direction at -10 mV. For clarity, only the responses to deactivating pulses between 60 and -30 mV are shown. C, Inactivation of the Ba\(^{2+}\) currents during the recording of the reversal of the outward currents. The magnitude of the currents elicited by the activating voltage pulses decreased continuously from the first pulse (upper trace) to the last one (bottom trace). The duration of the experiment was 3.75 min. Bathing solution in both (B) and (C) contained 30 mM \(\text{BaCl}_2\).
potentials failed to stimulate the opening of single channels. These results indicate that ion channels are the unitary entities for the macroscopic currents recorded in the whole vacuole experiments. The $P_O$ of the channels was dependent on the vacuolar membrane potential difference as shown by the increase in the number of channels opening simultaneously and also by an apparent increase in the mean open time (Fig. 4.3A). In contrast to the whole-vacuole currents, the single channel currents did not inactivate with time and in some cases it was possible to record these currents over a period of 20 min. Single channel currents displayed sensitivity to verapamil, in that, only brief openings were observed with no apparent reduction in the magnitude of the single channel currents. This suggested that the inhibitor may have acted on the gating of the channel (reduced mean open time) rather than plugging the pore of the channel.

The single channel current-voltage relationship revealed that the outward rectification, also observed in the whole vacuole, was a result of the inability of the channels to allow the movement of ions in the inward direction (into the cytosol) (Fig. 4.5A). The current-voltage relationship was approximately ohmic between 0 and 70 mV at any activity of cytosolic $Ba^{2+}$, with a slope conductance of about 40 pS for 100 mM $Ba^{2+}$ and a permeability ratio ($P_{Ba^{2+}}/P_{K^+}$) of 5 to 7. Between 10 and 100 mM of cytosolic $Ba^{2+}$, the extrapolated zero-current potential values moved toward the equilibrium potential for $Ba^{2+}$ (Fig. 4.5A, arrows), further indicating that the outward rectifying currents were carried by $Ba^{2+}$. The magnitude of the single channel current showed a tendency to saturate with increasing concentrations of cytosolic $Ba^{2+}$ in accordance with Michaelis-
Menten kinetics (Fig. 4.5B). The saturation of the single channel currents correlated well with the whole-vacuole experiments (see Fig. 4.2A).
Figure 4.4. **Single channel recordings of Ba$^{2+}$ currents.** Outside-out patches of vacuolar membranes were continuously polarized to a range of voltages. A, Step-like events were recorded only when the patches of vacuolar membrane were held at potentials between 0 and 70 mV, as indicated by the opening (o) and closing (c) of single channels. The presence of more than one channel in the patches was indicated by the occurrence of simultaneous openings ($O_1, O_2, ..., O_n$). B, Addition of 100 μM verapamil to the bath inhibited the channel activity as indicated by the brief openings and the absence of simultaneous events. (A) and (B) are recordings from the same patch exposed to 50 mM BaCl$_2$ in the bathing solution and 100 mM KCl in the pipette. Similar results were obtained with four other outside-out patches. C, The open probability of the channels increased between 0 and 50 mV, reaching a maximum at 50 to 60 mV. Data from five different patches of vacuolar membrane (mean ± SD).
**Figure 4.5.** The current-voltage relationship of single channel recordings with Ba\textsuperscript{2+} as the charge carrier. A, With 100 mM KCl in the recording pipette and 10 mM (■), 30 mM (O), 50 mM (●), or 100 mM (□) BaCl\textsubscript{2} in the bath, the magnitude of the single channel currents varied linearly with the membrane potential in outside-out patches of membrane. Least squares analysis gave a zero-current potential of 3 mV, -26 mV, -29 mV and -33 mV (arrows) for 10 mM, 30 mM, 50 mM and 100 mM Ba\textsuperscript{2+}, respectively. B, The dependence of the single channel current amplitude on the activity (α) of Ba\textsuperscript{2+}. The single channel current values were measured at 60 mV and plotted against α of cytosolic Ba\textsuperscript{2+}. The magnitude of the single channel currents showed an apparent saturation at 100 mM Ba\textsuperscript{2+}. (Inset) An Eadie-Hofstee plot of the single channel currents gave an apparent K\textsubscript{d} = 16.2 mM and a single channel current maximum of 3.4 pA. Data are the mean ± SD of five patches. Error bars are smaller than data points.
4.3 DISCUSSION

$\mathrm{Ba}^{2+}$ currents were detected in whole vacuoles and isolated patches of vacuolar membrane that corresponded to the movement of $\mathrm{Ba}^{2+}$ from the cytosol into the vacuole at depolarized membrane potential differences (with respect to the cytosol) (Pantoja, Gelli & Blumwald, 1992). These channels had a single channel conductance of 40 pS and an open channel $P_o$ dependent on positive vacuolar membrane potentials. These results suggested that vacuolar membranes have channels that can effectively mediate the movement of $\mathrm{Ca}^{2+}$ into the vacuole in a voltage-dependent manner, following elevations in cytosolic $\mathrm{Ca}^{2+}$ levels.

Activation of the vacuolar outward rectifying $\mathrm{Ca}^{2+}$ channels may function as an alternative mechanism for the regulation of cytosolic $\mathrm{Ca}^{2+}$ through a negative feedback mechanism. Under conditions that may induce the production of intracellular IP$_3$ (i.e. hormone or light) activation of a $\mathrm{Ca}^{2+}$-release channel would increase cytosolic $\mathrm{Ca}^{2+}$ levels with a concomitant polarization of the vacuolar membrane to 100 mV, close to the equilibrium potential for $\mathrm{Ca}^{2+}$. As a consequence, a higher cytoplasmic electrochemical potential for $\mathrm{Ca}^{2+}$ could be established, activating the vacuolar channel for the removal or sequestration of cytosolic $\mathrm{Ca}^{2+}$. Regulation of the remaining cytosolic $\mathrm{Ca}^{2+}$ would be achieved by the plasma membrane $\mathrm{Ca}^{2+}$-ATPase (Rasi-Caldogno et al., 1987) and the vacuolar $\mathrm{Ca}^{2+}/\mathrm{H}^+$ (Blumwald & Poole, 1986).

The single channel conductance, the voltage-dependence, the activation and inactivation kinetics of the outward rectifying channels and their non-selective nature ($P_{\mathrm{Ba}}/P_K \approx 2.3$) suggested that these channels could potentially be the SV type channels.
previously described (Hedrich & Neher, 1987). SV-type channels were characterized as nonselective Ca\(^{2+}\)-activated voltage-dependent (positive membrane potentials) channels in the vacuolar membrane. Although the Ca\(^{2+}\)/Ba\(^{2+}\) permeability of these channels had not been addressed, the results presented in this chapter would suggest that SV type channels are permeable to Ba\(^{2+}\). This finding led to the proposed role of the SV type channel in a Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) mechanism (Ward & Schroeder, 1994).

However, the question remains as to how these channels function *in vivo* when the normal range of resting membrane potentials in vacuoles is about -20 mV and the opening range of these channels appears to be restricted to positive potentials. It has been suggested that the Ca\(^{2+}\)-dependent opening of highly K\(^+\)-selective channels into the cytosol, might enforce a positive swing in the membrane potential toward E\(_K\). This would generate an indirect pathway for the opening of the SV channel that would be active at membrane potentials closer to E\(_K\) (0 to 60 mV) where the driving force for K\(^+\) is greatly reduced. In addition, the relatively high K\(^+\) permeability through the SV channel maintains the membrane potential sufficiently displaced from E\(_{Ca}\) (≈ 100 mV) so that the driving force for Ca\(^{2+}\) flux through the channel is large (Ward & Schroeder, 1994). The implication of the SV channel co-residing with other voltage-dependent Ca\(^{2+}\) channels in the vacuolar membrane is discussed in chapter 7.
5.1 INTRODUCTION

Changes in the electrical potential difference across the plasma membrane of higher plants have been shown to be among the most rapid alterations induced by abiotic (Assmann, et al., 1985; Serrano, Zeiger & Hagiwara, 1988) and biotic stresses (Felle, 1988; Ullrich & Novacky, 1991; Lohse & Hedrich, 1992). The sensitivity of the electrical membrane potential to different stimuli suggests that the electrogenic exchange of ions across the plasma membrane could serve for the transduction of signals (i.e. fungal elicitors) perceived at the plasma membrane. The hyperpolarization of the electrical plasma membrane potential difference has been identified as an early response of plant cells to blue and red light (Shimazaki et al., 1980; Assmann et al., 1985; Serrano et al., 1988), auxins (Felle, 1988), the fungal toxin fusicoacin (Marre, 1985) and specific fungal elicitors (Vera-Estrella et al., 1994a).

The treatment of tomato cell suspension cultures with specific fungal elicitors led to a guanine nucleotide binding protein (G-protein)-mediated dephosphorylation of the host plasma membrane H⁺-ATPase. This resulted in the stimulation of the H⁺-pump activity with the concomitant hyperpolarization of the electrical potential difference across the plasma membrane and the acidification of the extracellular milieu (Vera-Estrella et al., 1994a; Xing et al., 1996a). Thus a hyperpolarization-activated influx of Ca²⁺ into the host cell could provide a pathway for the elevation of cytosolic free Ca²⁺ concentrations that would subsequently mediate the induction of several biochemical pathways that are part of the plant defense response (Ebel & Cosio, 1994). Many of the biochemical responses associated with the plant defense mechanisms are inhibited by the depletion of extracellular
Ca$^{2+}$ or stimulated in the presence of ionophores that allowed the entry of Ca$^{2+}$ into the cells (Ebel & Cosio, 1994; Smith, 1996) suggesting that fluctuations in cytosolic Ca$^{2+}$ are required for an effective defense response by the cell.

The earliest electrophysiological evidence for the operation of Ca$^{2+}$-permeable channels in the plasma membrane came from studies done in charophyte algae (Hayama et al., 1979; Lunevsky et al., 1983). Most of the studies on Ca$^{2+}$ permeation through the plasma membrane of higher plants have shown the activation of Ca$^{2+}$-permeable channels at depolarized electrical membrane potential differences (Fairely-Grenot & Assmann, 1992; White, 1994; Thuleau et al., 1994; Marshall et al., 1994; Piñeros & Tester, 1995) including an ABA-activated nonspecific Ca$^{2+}$-permeable channel (Schroeder & Hagiwara, 1990). Plasma membrane stretch-activated channels have also been reported as pathways for the influx of Ca$^{2+}$ (Cosgrove & Hedrich, 1991; Ding & Pickard, 1993).

Recently, hyperpolarization-activated Ca$^{2+}$ influx has been also reported (Stoeckel & Takeda, 1995). Although a full characterization of these currents has yet to be done, it appears that these channels are different from the putative depolarization-activated Ca$^{2+}$ channels. Other reports have described hyperpolarization-activated nonselective cation channels that could also potentially provide a pathway for the influx of Ca$^{2+}$ (Fairley, Laver & Walker, 1991; Cosgrove & Hedrich, 1991). The existence of hyperpolarization- and depolarization-activated Ca$^{2+}$-permeable channels in the plasma membrane may be a required feature that allows plant cells to efficiently couple the perception of stimuli at the plasma membrane to fluctuations in cytosolic Ca$^{2+}$ concentrations.
The hyperpolarization-activated influx of $\text{Ca}^{2+}$ into tomato cells was examined by the application of conventional patch clamp techniques. In this chapter we present evidence for $\text{Ca}^{2+}$ influx at hyperpolarized membrane potentials in the plasma membrane of tomato cells. These channels are voltage-dependently gated, selective for $\text{Ca}^{2+}$ over $K^+$ and $\text{Cl}^-$, sensitive to inhibitors including $\text{La}^{3+}$ and nifedipine and they provide a mechanism for the influx of $\text{Ca}^{2+}$ into tomato cells.
5.2 RESULTS

5.2.1 HYPERPOLARIZATION-INDUCED INFLUX OF Ba\(^{2+}\) AND Ca\(^{2+}\).

Whole cell currents were measured when the electrical potential difference across the plasma membrane of tomato protoplasts was clamped from -180 mV to 40 mV in increments of 20 mV from a holding potential of -60 mV. Only membrane voltages more negative than -120 mV (hyperpolarized membrane potentials) elicited large inward currents with Ba\(^{2+}\) as the charge carrier (Fig. 5.1A). Current activation was fast and was exponential with time. The voltage activation of the Ba\(^{2+}\) currents was time-dependent and the magnitude of the currents was largest at -180 mV. These currents were active at membrane voltages that were within the range of the equilibrium potentials for K\(^+\) (-167 mV) and Cl\(^-\) (-158 mV) suggesting that these currents were not due to the movement of K\(^+\) or of Cl\(^-\).

Similarly, replacing Ba\(^{2+}\) with Ca\(^{2+}\) on the extracellular side of the protoplasts resulted in inward currents elicited by hyperpolarized membrane voltages. These currents were time- and voltage-dependent. With Ca\(^{2+}\) as the charge carrier, current activation was faster and the magnitude of the currents smaller (Fig 5.1B). Activation of Ca\(^{2+}\) currents also followed an exponential time course. The non-linear current-voltage relationship (Fig. 5.1C) revealed inward currents that were strongly voltage-dependent with maximum current activation only at hyperpolarized membrane potentials.

Whole cell currents were also measured during a 1.2 s voltage ramp. Whole cells were exposed to 50 mM Ca\(^{2+}\) while the membrane voltage was clamped from -200 mV to
100 mV for 1.2 sec (Fig. 5.1 D). As observed in Figs. 5.1 A & B, inward currents were largest at negative membrane potentials that were within the range of the reversal potentials for K\(^+\) and Cl\(^-\) suggesting that the voltage-activated currents were due to the movement of Ca\(^{2+}\) into the cell. The Ba\(^{2+}\) inward currents reversed direction at 20 mV in the presence of 20 mM Ba\(^{2+}\) and at 10 mV in the presence of 5 mM Ba\(^{2+}\) in tail current experiments. These reversal potentials suggested that all three ions (K\(^-\), Cl\(^-\) and Ba\(^{2+}\)) were permeable. However, the change in the reversal potential toward the Nernst potential for Ba\(^{2+}\) (154 mV) indicated that the channel was more permeable to Ba\(^{2+}\) than to K\(^+\) or Cl\(^-\).

Using these reversal potentials for the calculation of permeability ratios, a \(P_{\text{Ca}^{2+}}/P_{\text{K}^{+}}\) of about 20, a \(P_{\text{Ca}^{2+}}/P_{\text{Cl}^{-}}\) of about 10 and a \(P_{\text{Cl}^{-}}/P_{\text{K}^{+}}\) of about 2 were obtained. Similar results were obtained with Ca\(^{2+}\) as the charge carrier (not shown).

To examine the voltage-dependency of channel gating, a Boltzmann plot (Fig. 5.2) was constructed from whole cell recordings similar to those shown in Fig. 1. The gating charge \((z)\), corresponding to the slope of the fit, revealed that both Ba\(^{2+}\) \((z=1.09)\) and Ca\(^{2+}\) \((z=1.09)\) currents were sensitive to the electric field across the plasma membrane, comparable to that of K\(^-\) currents in the plasma membrane of xylem parenchyma cells \((z=1.16 \pm 0.22)\) (Wegner et al., 1992). The free energy (Gibbs free energy = \(\Delta G_i\)) associated with the transition of the channel from the closed to the open state in the plasma membrane was slightly greater for Ba\(^{2+}\) \((\Delta G_i = 2.92 \text{ kcal mol}^{-1})\) than for Ca\(^{2+}\) \((\Delta G_i = 2.81 \text{ kcal mol}^{-1})\).
Figure 5.1. Voltage-dependent Ba\(^{2+}\) and Ca\(^{2+}\) inward currents of tomato protoplasts. Inward currents across protoplasts were measured by clamping the membrane potential to -180 mV for a duration of 5 sec followed by a 20 mV increment from -180 mV to 40 mV from a holding potential of -60 mV. A, Protoplasts bathed in 50 mM Ba\(^{2+}\) (100 nM free cytoplasmic calcium (pipette interior)) displayed time-dependent inward currents in response to negative membrane voltages. Largest current levels corresponded to the largest negative membrane voltage. B, Replacing the protoplast's bathing solution of 50 mM Ba\(^{2+}\) with 50 mM Ca\(^{2+}\) revealed a similar type of voltage-dependent current, however, the time-dependence of voltage-activation appeared to be faster with Ca\(^{2+}\) than with Ba\(^{2+}\). C, Current-voltage plot from protoplasts in A (■) and B (●). Current levels measured at 4.8 sec from the onset of the imposed voltage as described above were plotted against their respective voltages. Values are Mean ± S.D. (n=12). D, Whole cell currents measured in the presence of 50 mM Ca\(^{2+}\) during a 1.2 sec voltage ramp ranging from -200 mV to 100 mV. Similar results were observed in at least 12 other protoplasts.
membrane potential (mV)

whole cell current (pA)

D
**Figure 5.2.** A Boltzman plot of the whole cell currents. Currents were recorded in 50 mM of either Ba\(^{2+}\) (●) or Ca\(^{2+}\) ( ○) in the bathing solution and with 100 nM cytoplasmic free calcium in the pipette. The relative conductance (θ) is defined as the ratio \(G/G_{\text{max}}\). The gating charge (z) of the channel is represented by the slope of the least-squares fit line. The free-energy (kcal/mol) associated with the transition of the channel from the closed to the open state was determined from the Y-intercept (ΔG\(_i\)) of the plot. Values are Mean ± SD (n=12). Error bars are smaller than symbol size.
membrane potential (mV)

\[ \frac{[\theta/e - (1 - \theta)]}{\theta} \]
5.2.2 INHIBITION OF INWARD CURRENTS BY NIFEDIPINE.

The organic inhibitor nifedipine, known for its effectiveness in blocking L-type calcium channels in animal cells (Catteral & Striessnig, 1992; Hille, 1993) and Ca$^{2+}$-permeable channels in plant cells (Gelli & Blumwald, 1993; Allen & Sanders, 1994) was used here in an attempt to further characterize the inward currents. The half-blocking concentrations reported are within the range of 0.5 to 100 µM, depending on the type of cell (Hille, 1992). Inward currents were reduced by about 50% when 100 µM of nifedipine was added to the extracellular side (bathing chamber) of the protoplasts (Fig. 5.3). The external Ba$^{2+}$ concentration was maintained at 50 mM. Whole-cell currents were recorded by applying voltage pulses from -180 mV in increments of 20 to 40 mV from a holding potential of -60 mV before (Fig. 5.3A) and after (Fig. 5.3B) the addition of nifedipine. The presence of nifedipine reduced whole cell currents and also slowed down current activation (Fig. 5.3B). The non-linear current-voltage relationship (Fig. 5.3C) clearly demonstrated the reduction in whole cell currents by nifedipine. The block of whole cell currents was not strongly voltage-dependent as was shown by plotting the fraction of current inhibited by nifedipine as a function of the applied membrane voltage (Fig. 5.3D).
Figure 5.3. Inhibition of Ba$^{2+}$ currents by nifedipine. A, Inward currents recorded from protoplasts exposed to 50 mM Ba$^{2+}$. B, Voltage-dependent currents were approximately 50% inhibited when protoplasts were exposed to 100 μM nifedipine. This inhibition was observed irrespective of the divalent cation used in the bathing solution. C, Current-voltage plot from protoplasts in A (■) and B (●). Current levels measured at 4.8 sec from the onset of the imposed voltage as described in Fig. 1 were plotted against their respective voltages. Values are Mean ± S.D. (n=12). D, The fraction of current (measured from whole-cell recordings as shown in A & B) inhibited by nifedipine was plotted as a function of membrane voltage. Values are Mean ± S.D. (n=12).
5.2.3 VOLTAGE-DEPENDENT Ca\(^{2+}\) CURRENTS AT THE SINGLE CHANNEL LEVEL.

Single channel currents were measured from outside-out patches of plasma membrane exposed to various concentrations of Ca\(^{2+}\) and Ba\(^{2+}\) (bathing solution) while polarizing the plasma membrane to membrane potentials from -120 mV to -190 mV. Single channel recordings from isolated patches exposed to 50 mM Ca\(^{2+}\) revealed voltage-dependent channel activity (Fig. 5.4 A) in that channel events became more frequent with increasingly negative membrane voltages. Channel activity was detected at membrane potentials corresponding to the reversal potentials of K\(^+\) (-167 mV) and Cl\(^-\) (-158 mV) suggesting that the downward deflections corresponded to the movement of Ca\(^{2+}\). Channel recordings at potentials greater than -200 mV were difficult to obtain since the isolated patch of membrane became very unstable at these large potential differences.

The current-voltage relationship constructed from single channel recordings similar to those shown in figure 5.4 A was ohmic (Fig. 5.4 B) for all three concentrations of extracellular Ca\(^{2+}\) (5 mM, 20 mM and 50 mM) (activities: 3.9 mM, 13.3 mM and 26.1 mM). Least squares analysis yielded reversal potentials of 14 mV, 30 mV and 42 mV for channel currents recorded in the presence of 5 mM, 20 mM and 50 mM Ca\(^{2+}\), respectively. The reversal potential of the single channel currents shifted toward the equilibrium potential for Ca\(^{2+}\) (\(\approx +133\) mV with 5 mM (extracellular) and 100 nM (cytoplasmic)) with increasing extracellular Ca\(^{2+}\) concentrations (Fig. 5.4 B). Based on the reversal potentials, permeability ratios (\(P_{Ca^{2+}}/P_{K^+}\) and \(P_{Ca^{2+}}/P_{Cl^-}\)) revealed that these channels are between 20 to 25 times more permeable to Ca\(^{2+}\) than to K\(^+\) and between 10 to 13 times more permeable
Figure. 5.4. Voltage-dependent single channel currents with Ca$^{2+}$ as the charge carrier. 

A, Single channel recordings of outside-out patches of membrane from protoplasts exposed to 50 mM Ca$^{2+}$ and polarized from membrane voltages of -150 mV to -190 mV. Downward deflections indicate 1 or 2 channels open and correspond to the movement of Ca$^{2+}$ into the cytosol. B, Current-voltage plots from single channel recordings similar to those shown in (A). Channel currents were measured over a range of extracellular Ca$^{2+}$ concentrations: 5 mM (○), 20 mM (■) and 50 mM (▲) (activities: 3.9 mM, 13.3 mM and 26.1 mM). Single channel currents (I_{SC}) reversed (E_{rev}) direction at 14 mV, 30 mV and 42 mV (indicated by arrows) as determined from a least-squares fit. Data points are Mean ± S.D. (n=8). Error bars are smaller than size of symbols. C, The open probability (P_{o}) of the channel is voltage-dependent. The channel P_{o} increased as the membrane voltage was made more negative and it reached a maximum at -190 mV.
to Ca\(^{2+}\) than to Cl\(^{-}\). The open probability (P\(_{o}\)) of the Ca\(^{2+}\)-permeable channels was dependent on the membrane potential difference (Fig. 5.4C) as shown by the sigmoidal-type of relationship. The P\(_{o}\) began to increase between -150 mV and -160 mV and reached a maximum at -190 mV.

Single channel currents, recorded in the presence of increasing concentrations of extracellular Ca\(^{2+}\) and Ba\(^{2+}\), (from 5 mM to 50 mM) were plotted against the activity of the divalent cations (Fig. 5.5 A). Current amplitudes were obtained from single channel records similar to those shown in Fig. 5.4. The currents displayed an apparent saturation with increasing activity of Ca\(^{2+}\) and Ba\(^{2+}\). These current amplitudes were used to generate an Eadie-Hofstee plot (Fig. 5.5 B) from which \(K_d\) values of 4.7 mM and 7.4 mM and maximum current values of 1.1 pA and 2.9 pA for Ca\(^{2+}\) and Ba\(^{2+}\), respectively, were obtained. Single channel currents, recorded in the presence of 20 mM Ca\(^{2+}\) or 20 mM Ba\(^{2+}\), resulted in two different levels of current amplitude when the membrane potential difference was maintained at -180 mV (Fig. 5.6). The unitary conductance decreased from 11 pS to 4 pS when Ba\(^{2+}\) was replaced by Ca\(^{2+}\). The current-voltage relationship was ohmic for both divalent cations (Fig. 5.6 C). Least squares analysis resulted in reversal potentials of 26 mV (Ba\(^{2+}\)) and 30 mV (Ca\(^{2+}\)) suggesting that these channels are similarly permeable to Ba\(^{2+}\) and Ca\(^{2+}\) (P\(_{Ba}\)\(^{2+}\) \(\approx\) P\(_{Ca}\)\(^{2+}\)). Substituting these reversal potentials into the Goldman, Hodgkin and Katz equations, permeability ratios P\(_{Ca}\)\(^{2+}\)/P\(_{K}\)\(^{-}\) = 20 and P\(_{Ca}\)\(^{2+}\)/P\(_{Cl}\)\(^{-}\) = 7 were obtained, suggesting that these channels are more permeable to Ca\(^{2+}\) and Ba\(^{2+}\) ions than to K\(^{+}\) or Cl\(^{-}\) ions.
Figure 5.5. The dependence of single channel currents on the activity of Ba$^{2+}$ and Ca$^{2+}$.

Channel currents were measured from single channel recordings similar to those shown in fig. 5.3A. A, Single channel currents ($I_{\infty}$) recorded at -180 mV were plotted against divalent ion activity. Channel currents saturated with increasing activities of the divalent ions. B, An Eadie-Hofstee plot of experimental data in (A) gave $K_d$ values of 4.7 mM and 7.4 mM and single channel maximum currents of 1.1 and 2.9 for Ca$^{2+}$ and Ba$^{2+}$ respectively. Values are Mean ± S.D. (n=8).
A

Ion Activity (mM)

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B

Isc (pA)/Ion Activity (mM)

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Ba$^{2+}$

Ca$^{2+}$
Figure 5.6. Single channel activity with Ba\(^{2+}\) and Ca\(^{2+}\) as the charge carrier. Channel recordings were obtained while polarizing the membrane to -180 mV in the presence of 20 mM Ba\(^{2+}\) or Ca\(^{2+}\). A, With Ba\(^{2+}\) as the charge carrier, single channel current amplitude was -2.0 pA at -180 mV. At least two channels were present in the patch of membrane. B, With Ca\(^{2+}\) as the charge carrier, single channel current amplitude was much smaller (-0.8 pA at -180 mV) and fewer channel events were observed. At least two channels were present in the patch of membrane. C, Current-voltage relationship of single channels is linear for both Ba\(^{2+}\) and Ca\(^{2+}\). Largest current amplitudes were observed at increasingly negative membrane voltages. Least-squares analysis gave reversal potentials (E\text{rev}\) of 26 mV and 30 mV (indicated by arrows) for Ba\(^{2+}\) and Ca\(^{2+}\), respectively. Values are Mean ± S.D. (n=8). Error bars are smaller than the data points.
5.2.4 INHIBITION OF SINGLE CHANNEL CURRENTS BY La$^{3+}$.

Among the transition heavy metals, Al$^{3+}$, Gd$^{3+}$, La$^{3+}$ and Zn$^{2+}$ have been shown to block Ca$^{2+}$-permeable channels in both animal cells (Hille, 1992) and plant cells (Johannes, Brosnan & Sanders, 1992; Gelli & Blumwald, 1993). The concentrations used to effectively block these channels have ranged from 0.5 to 20 mM (Hille, 1993). Here, single channel currents, recorded in the presence of 50 mM Ca$^{2+}$ while clamping the membrane voltage from -160 mV to -180 mV (Fig. 5.7A), were blocked by exposing the patch of membrane to 10 μM of La$^{3+}$ (Fig. 5.7C). Similar results were observed in the presence of Ba$^{2+}$ (data not shown). The frequency of channel events (Fig. 5.7A) was greatly reduced by La$^{3+}$ (Fig. 5.7C). The inhibition of channel activity was further demonstrated by the amplitude histograms constructed from single channel recordings similar to those shown in the absence (Fig. 5.7B) and the presence of La$^{3+}$(Fig. 5.7D). The absence of the peak (amplitude = -1 pA) (Fig. 5.7D) corresponding to the opening of one channel is evident upon comparison (Fig. 5.7D).

Single channel recordings obtained in the presence of 50 mM Ca$^{2+}$ while clamping the membrane voltage to -180 mV, were used in constructing open (Fig. 5.8A) and closed time histograms (Fig. 5.8B). Both histograms were fitted with one exponential suggesting the presence of one closed (or one open) state of the channel (Fig. 5.8). The time constants were 3.8 ms (open times) 7.1 ms (closed times). The mean open and mean closed times of the channel were voltage-dependent (Fig. 5.8C). The mean closed times decreased while the mean open times increased with increasingly negative membrane voltages.
Figure 5.7. Inhibition of channel activity by La$^{3+}$.  A, Voltage-dependent single channel currents recorded in the presence of 50 mM Ca$^{2+}$. The number of channel events increased as the membrane voltage became increasingly negative. At least two channels were present in the patch of membrane. B, Amplitude distribution histogram from single channel recordings similar to those shown in A. The largest peak (0 pA) represents the channel in the closed state. The additional peak at -1 pA corresponds to the opening of one channel. C, Inhibition of single channel activity recorded in the presence of 10 μM La$^{3+}$ on the extracellular side of the membrane. Channel activity was significantly reduced as demonstrated by the reduced number of channel events. D, Amplitude distribution histogram from single channel recordings similar to those shown in C. Inhibition of channel activity is demonstrated by the absence of the peak corresponding to the opening of one channel. Similar results were observed in 6 other protoplasts.
Figure 5.8. Dwell time distribution histograms of the Ba\textsuperscript{2+} and Ca\textsuperscript{2+}-permeable channel. Open and closed time histograms were constructed from single channel currents recorded at -180 mV similar to those shown in Figure 5.4, 5.6 and 5.7. A, Open and closed time histograms from single channel currents recorded in the presence of Ca\textsuperscript{2+} were fitted with one exponential with the following time constants: 3.8 ms (open times) and 7.1 ms (closed times). Events shorter than 1 ms were omitted from the fit. C, The mean open (■) and mean closed (○) times were determined as previously described and plotted against the applied membrane voltages. Both the mean open and mean closed times varied linearly with membrane voltage. Values are Mean ± S.D. (n=8).
5.3 DISCUSSION

5.3.1 THE VOLTAGE-DEPENDENCY OF HYPERPOLARIZATION-ACTIVATED Ca\(^{2+}\) CURRENTS.

The large electrochemical potential gradient (200 to 300 mV) for Ca\(^{2+}\) ions across the plasma membrane, directed into the cytosol, allows for signal amplification by transducing an imposed signal into a controlled increase of cytosolic calcium (Bush, 1995). The localized signal perception at the plasma membrane can result in the hyperpolarization of the membrane potential from the normal range of resting potentials (Shimazaki et al., 1980; Assmann et al., 1985; Marre, 1985; Serrano et al., 1988; Felle, 1988). Plasma membrane hyperpolarization has been inferred through the activation of the tomato plasma membrane H\(^{+}\)-ATPase by fungal elicitors (Vera-Estrella et al., 1994a).

Previous work with tomato Cf5 cell suspension cultures showed that treatment with intercellular fluids containing the \textit{avr5} fungal elicitor led to a guanine nucleotide binding protein (G-protein)-mediated dephosphorylation of the host-plasma membrane H\(^{+}\)-ATPase. This dephosphorylation resulted in the stimulation of the H\(^{+}\)-pump activity with the concomitant hyperpolarization of the electrical potential difference across the plasma membrane and the acidification of the extracellular milieu (Vera-Estrella et al., 1994a). Thus the activation of Ca\(^{2+}\)-permeable channels at hyperpolarized membrane potentials could provide a pathway for the influx of Ca\(^{2+}\) into the host cells in response to the presence of an avirulent pathogen. The subsequent elevation of cytosolic free calcium concentration could activate signal transduction pathways linking the perception of signals (i.e. fungal elicitors) at the host plasma membrane to the induction of a series of
biochemical responses (defense responses) that are essential to the survival of the affected cell (Xing et al., 1996a).

Although most of the studies on Ca²⁺ permeation through the plant plasma membrane have shown the activation of Ca²⁺-permeable channels at depolarized potentials (Schroeder & Hagiwara, 1990; Fairely-Grenot & Assmann, 1992; White, 1994; Thuleau et al., 1994; Marshall et al., 1994; Piñeros & Tester, 1995), few studies have shown Ca²⁺ influx at hyperpolarized potentials (Stoeckel & Takeda, 1995). Stoeckel and Takeda (1995) demonstrated that the delayed outward-rectifying K⁺ currents in pulvinar protoplasts were reduced by increased cytosolic Ca²⁺ levels that correlated with Ca²⁺ influx at hyperpolarized potentials. The hyperpolarization-associated rundown in the K⁺ outward current was reversibly blocked by La³⁺ (2 mM) and Gd³⁺ (0.5 to 2.0 mM) and it was reversed by lowering external Ca²⁺ or polarizing the membrane to less negative membrane potentials. These results suggested the operation of Ca²⁺-permeable channels at hyperpolarized membrane potentials. Although a full characterization of these channels has yet to be done, it is likely that the Ca²⁺ influx reported by Stoeckel and Takeda (1995) and those described here, are different from the putative depolarization-activated Ca²⁺ influx previously reported. The existence of hyperpolarization- and depolarization-activated Ca²⁺-permeable channels may allow plant cells to efficiently couple the perception of stimuli at the plasma membrane to fluctuations in cytosolic Ca²⁺ concentrations.

The Ca²⁺-permeable channels reported here are active at electrical membrane potential differences more negative than -120 mV (-120 mV to -190 mV) and become
much less active upon depolarizing the membrane where the electrochemical driving force for Ca\(^{2+}\) influx is reduced. Thus the influx of Ca\(^{2+}\) into the cytosol is a voltage-dependent process. Furthermore, the Boltzmann relationship for the whole cell currents revealed that channel openings (the transition from the closed state to the open state) are associated with the movement of a gating charge \((z = 1.09)\) suggesting that the currents are sensitive to the electric field across the membrane, thus they are voltage-dependently gated. It is very unlikely that the inward rectifying Ca\(^{2+}\) currents are due to a Goldman-style non-linear diffusion through an open channel that could arise when a steep gradient of ions exists across the membrane (Hille, 1992).

5.3.2 SELECTIVITY AND INHIBITION OF THE Ca\(^{2+}\)-PERMEABLE CHANNELS.

The single channel conductance suggested that Ba\(^{2+}\) (11 pS) and Ca\(^{2+}\) (4 pS) permeated the channel pore slowly, however the apparent permeabilities and the apparent \(K_d\) values suggested that both ions interacted strongly with the channel pore. Reversal potentials of the single channel currents suggested a comparable selectivity between Ba\(^{2+}\) and Ca\(^{2+}\) although the apparent \(K_d\) values suggested a somewhat higher binding affinity for Ca\(^{2+}\) (4.7 mM) over Ba\(^{2+}\) (7.7 mM). The channels reported here were about 24 times more permeable to Ca\(^{2+}\) than to K\(^{+}\) under ionic conditions of 100 mM K\(^{+}\) and 100 nM Ca\(^{2+}\) in the cytosol and 0.1 mM K\(^{+}\) and 5 mM Ca\(^{2+}\) in the extracellular space. This value of selectivity is comparable to that reported for the Ca\(^{2+}\) channel in the vacuolar membrane (Johannes et al., 1992; this thesis chapter 3) and it falls in the range (3 - 26) of the
selectivity ratios reported for the depolarization-induced Ca\(^{2+}\) channels (White, 1994; Thuleau et al., 1994; Piñeros & Tester, 1995).

Further characterization of the hyperpolarization-activated channels was attempted by examining the effect of channel blockers on whole cell and single channel currents. A series of organic (1,4-dihydropyridines including nifedipine) and inorganic (transition metals including La\(^{3+}\)) blockers have been used in the characterization of calcium channels in animal and plant systems. However, the response of Ca\(^{2+}\) channels to these blockers is variable when applied at high concentrations because of nonspecific effects (Hille, 1992) (Terry, Findlay & Tyerman, 1992) (Wegner et al., 1994). In several studies, La\(^{3+}\) and dihydropyridines have shown a moderate inhibition of outward-rectifying K\(^+\) channels in the range of 10 \(\mu\)M to 100 \(\mu\)M (Terry et al., 1992) and a stronger inhibition of inward-rectifying K\(^+\) channels at concentrations greater than 1 mM (Wegner et al., 1994). In Chara, La\(^{3+}\) was effective in blocking action potentials irreversibly but also blocked K\(^+\) and Cl\(^-\) channels (Tyerman, Findlay & Paterson, 1986; Smith, Walker & Smith, 1987). Although these studies suggested that these blockers may not be highly specific for Ca\(^{2+}\) channels, more specific type of blockers have yet to be developed. In this study, Ca\(^{2+}\)-permeable channels were significantly inhibited by 10 \(\mu\)M La\(^{3+}\) and 50% inhibited by 100 \(\mu\)M nifedipine. The block by La\(^{3+}\) appeared to be voltage-dependent since fewer channel events were observed as the electrical membrane potential difference became more negative, suggesting that La\(^{3+}\) was bound to the channel within the electric field of the membrane. The inhibition of whole cell currents by nifedipine was not voltage-dependent.
5.3.3 THE PHYSIOLOGICAL ROLE OF HYPERPOLARIZATION-ACTIVATED Ca\textsuperscript{2+}-PERMEABLE CHANNELS.

Although not fully characterized, a few reports have described hyperpolarization-activated cation channels that could provide a pathway for the influx of Ca\textsuperscript{2+} (Terry, Tyerman & Findlay, 1991; Cosgrove & Hedrich, 1991; Fairley et al., 1991; Stoeckel & Takeda, 1995). The single channel conductance ranged from 10 pS (Cosgrove & Hedrich, 1991) to 20 pS (Fairley et al., 1991), channels were weakly selective for Ca\textsuperscript{2+} and K\textsuperscript{+} (P\textsubscript{Ca\textsuperscript{2+}}/P\textsubscript{K\textsuperscript{+}} ≈ 1) (Cosgrove & Hedrich, 1991) and Cl\textsuperscript{-} (P\textsubscript{K\textsuperscript{+}}/P\textsubscript{Cl\textsuperscript{-}} ≈ 0.43 (Fairley et al., 1991) and channels were inhibited by 1 mM La\textsuperscript{3+} (Stoeckel & Takeda, 1995).

The tomato plasma membrane Ca\textsuperscript{2+}-permeable channels have a characteristically small conductance (4 pS for Ca\textsuperscript{2+} and 11 pS for Ba\textsuperscript{2+}) with a higher selectivity for divalent cations (P\textsubscript{Ca\textsuperscript{2+}}/P\textsubscript{K\textsuperscript{+}} ≈ 20-25 and P\textsubscript{Ca\textsuperscript{2+}}/P\textsubscript{Cl\textsuperscript{-}} ≈ 10-13). It is unlikely that these hyperpolarization-activated currents are mediated through the K\textsuperscript{+} inward rectifier (Schroeder et al., 1987). Although Ca\textsuperscript{2+} ions can permeate the K\textsuperscript{+} inward rectifier in guard cells (Fairley-Grenot & Assmann, 1992) and in xylem parenchyma cells (Wegner et al., 1994), these K\textsuperscript{+} channels are strongly inhibited by Ba\textsuperscript{2+} ions (K\textsubscript{i} of 1.3 mM) (Wegner et al., 1994). It has been suggested that Ba\textsuperscript{2+} ions can permeate K\textsuperscript{+} channels, but so sluggishly that they effectively block the pore (Armstrong & Taylor, 1980). Tomato plasma membrane channels were permeated by Ba\textsuperscript{2+} with a comparable permeability to that observed with Ca\textsuperscript{2+}, suggesting that the channels mediating the hyperpolarization-induced Ca\textsuperscript{2+} currents are different from the K\textsuperscript{+} inward rectifier.
In conclusion, the hyperpolarization-activated plasma membrane currents described in this report provide a mechanism for the influx of Ca\textsuperscript{2+} into tomato cells. The hyperpolarization of the electrical potential difference across the plasma membrane during pathogen attack would activate these channels allowing the influx of Ca\textsuperscript{2+} into the host cell. The subsequent elevation of cytosolic Ca\textsuperscript{2+} serves to activate signal transduction pathways that link the perception of fungal elicitors at the plasma membrane to the induction of plant defense responses (Xing et al., 1996a). The hyperpolarization-induced increase in cytosolic Ca\textsuperscript{2+} concentration is currently being quantified and mechanisms of Ca\textsuperscript{2+} influx regulation are being investigated.
6.1 INTRODUCTION

By tightly regulating or gating the high flux capacity of Ca\textsuperscript{2+} channels, cells can effectively modulate cytosolic Ca\textsuperscript{2+} concentrations (intracellular signaling) and thereby prevent a lethal situation that could ensue if Ca\textsuperscript{2+} channels remained opened continuously. Strong evidence to date supports the role of Ca\textsuperscript{2+}-based signal transduction pathways in stimulus-response coupling (Bush, 1995). Moreover, the sensitivity of the electrical membrane potential to different stimuli suggests that the electrogenic exchange of ions across the plasma membrane could serve for the transduction of many signals perceived at the plasma membrane. Ca\textsuperscript{2+} channels are the focus for multiple control pathways which implies that in the realm of signal crosstalk some degree of redundancy in channel behavior exists (Blatt and Thiel, 1993). For example, channels may exhibit both voltage- and ligand-sensitivities.

Increases in cytosolic free Ca\textsuperscript{2+} have been observed in several cell types in response to diverse stimuli such as plant hormones, mechanical stimuli, high and low temperatures, salt stress, exposure to oxidative agents, red light, self incompatibility factors and fungal elicitors (Bush, 1995). Many reported observations have highlighted the importance of Ca\textsuperscript{2+} in disease resistance (Smith, 1996). In the specific interaction between tomato and the fungus Cladosporium fulvum, disease resistance involves a plant resistance gene (Cf genes) whose products respond specifically to the products of a single avirulence (avr) gene in the pathogen. The recognition of elicitors, coded by the avr genes, by putative receptors at the host-plasma membrane induces ion fluxes, the production of H\textsubscript{2}O\textsubscript{2} and the initiation of a hypersensitive response (HR) in the host cells. This is followed by transient
changes in the phosphorylated state of various key proteins including cis-acting elements and trans-acting factors (Dixon et al., 1994). The HR response is considered as a rapid, programmed death of a limited number of host cells at the site of fungal penetration. It serves to prevent the fungus from spreading to other host cells. This process is accompanied by a cascade of events including transcriptional activation of various defense genes of the plant. The combination of the rapid collapse of the host cells in the vicinity of the invading pathogen and the activation of various defense responses surrounding the site of penetration prevents further spread of the pathogen throughout the plant tissue (De Wit, 1996). It has been suggested that resistance gene products in the host cells could be either membrane-bound receptors able to perceive the fungal elicitors or possibly key proteins involved in the early steps of the signaling pathway downstream of the receptor (Staskawicz et al., 1995).

Previous work with tomato cell suspension cultures (Cf5 cells) showed that treatment with intercellular fluids (IF) containing the avr5 elicitor (IF4) led to a G-protein-mediated dephosphorylation of the host plasma membrane H+-ATPase. This resulted in the stimulation of the H+-pump activity with the concomitant hyperpolarization of the electrical potential difference across the plasma membrane and the acidification of the extracellular milieu (Vera-Estrella et al., 1994a; Xing et al., 1996a). Thus a hyperpolarization-activated influx of Ca²⁺ into the host cell could provide a pathway for the elevation of cytosolic free Ca²⁺ concentrations mediating the induction of several biochemical pathways that are part of the plant defense response (Ebel and Cosio, 1994). Many of the biochemical responses associated with the plant defense mechanisms were
inhibited by the depletion of extracellular Ca\(^{2+}\) or the addition of Ca\(^{2+}\) channel blockers such as La\(^{3+}\), nifedipine and verapamil. These same responses were stimulated in the presence of ionophores that allowed the entry of Ca\(^{2+}\) into the cells (Schwacke and Hager, 1992; Smith, 1996) suggesting that fluctuations in cytosolic Ca\(^{2+}\) are required for an effective defense response by the cell.

Changes in the electrical potential difference across the plasma membrane of higher plants have been shown to be among the most rapid alterations induced by abiotic (Assmann et al., 1985; Serrano et al., 1988) and biotic stresses (Felle, 1988; Ullrich and Novacky, 1992; Lohse and Hedrich, 1992). The sensitivity of the electrical membrane potential to different stimuli suggests that Ca\(^{2+}\) influx across the plasma membrane could serve for the transduction of signals (i.e. fungal elicitation) perceived at the plasma membrane. This could involve the regulation of Ca\(^{2+}\)-permeable channels through the association with G proteins. G proteins have been implicated in various physiological processes in plants including ionic homeostasis (Brown, 1991), secondary messenger regulation (Allan et al., 1989) and ion channel regulation (Fairley-Grenot & Assmann, 1991). Of the G proteins so far distinguished, the membrane-bound trimeric G protein has been identified and demonstrated to be involved in mediating the activity of the H\(^{+}\)-ATPase in response to fungal elicitors (Xing et al., 1996b).

An indirect pathway in G protein-ion channel coupling involves the activation of intermediate membrane-associated effectors including protein kinases and phosphatases (Brown, 1991). Modulation of channel activity by phospho-dephosphorylation events in signal transduction pathways has been well established in animal systems and recent
evidence suggests that similar mechanisms are present in plant systems (Luan et al., 1993; Thiel & Blatt, 1994; Allen & Sanders, 1994b; Schmidt et al., 1995; Armstrong et al., 1995). Evidence for changes in the phosphorylated state of specific proteins in plant defense signaling pathways has been increasing, suggesting that both phosphatases and kinases can act as regulators of specific proteins in the transduction of fungal signals (Grosskopf et al., 1990; Felix et al., 1991; Conrath et al., 1991; Xing et al., 1996a). In addition, the involvement of phosho-dephosphorylation events has also been implicated in the up- and down-regulation of various processes including blue-light responses, photosynthesis, hormone signaling (Ranjeva & Boudet, 1987) and opening and closing of stomata (Luan et al., 1993; Thiel & Blatt, 1994; Armstrong et al., 1995; Schmidt et al., 1995). Compelling evidence has demonstrated that some of the fungal elicitor-induced defense responses can be effectively suppressed with protein kinase inhibitors (Grosskopf et al., 1990; Conrath et al., 1991) or activated by phosphatase inhibitors (Felix et al., 1991) strongly suggesting that phospho-dephosphorylation events are essential in the up- and down-regulation of plant defense responses (Dietrich et al., 1991).

The sensitivity of the plasma membrane Ca\(^{2+}\) channel in Cf5 tomato cells to specific fungal elicitors from *Cladosporium fulvum* provided a system in which to investigate the regulating mechanisms of the channel and its role in stimulus-response coupling. Thus, electrophysiological techniques were used in both the whole cell and the isolated patch of plasma membrane in order to investigate the mechanisms of channel regulation in response to fungal elicitation.
6.2 RESULTS

To examine the effect of race-specific fungal elicitors on host-plasma membrane Ca\(^{2+}\)-permeable channels, currents carried by Ba\(^{2+}\) were recorded in Cf5 tomato protoplasts. When measuring Ca\(^{2+}\) currents, Ba\(^{2+}\) is the preferred cation since Ba\(^{2+}\) blocks K\(^+\) channels in the plasma membrane of both animal (Armstrong & Taylor, 1980) and plant cells (Wegner et al., 1994) that could potentially interfere in the recording of Ca\(^{2+}\) currents. Also Ba\(^{2+}\) is much less likely to activate Ca\(^{2+}\)-dependent K\(^+\) channels and Ba\(^{2+}\) enhances the resolution of Ca\(^{2+}\) channels in that, the magnitude of the single channel currents is larger with Ba\(^{2+}\) as the charge carrier (Gelli & Blumwald, 1993). Whole and single channel currents recorded here were obtained in the presence of 20 mM extracellular Ba\(^{2+}\), thus current levels are about two times greater than what would be obtained in the presence of 20 mM extracellular Ca\(^{2+}\) (Gelli & Blumwald, 1997).

6.2.1 AN IF\(_4\)-INDUCED INCREASE IN WHOLE-CELL CURRENTS

Whole cell currents were measured when the electrical potential difference across the plasma membrane of tomato protoplasts was clamped from -180 mV to 20 mV in increments of 20 mV from a holding potential of -60 mV (Fig. 6.1A, B, C). Whole cell currents were previously recorded in the presence of 50 mM Ba\(^{2+}\) (or Ca\(^{2+}\)) on the extracellular side of the membrane (Gelli & Blumwald, 1997). The whole cell current-voltage (I-V) relation exhibited similar characteristics as the product from P\(_0\) and the single channel I-V curve suggesting that the single channels previously described were the unitary transporters underlying the time-dependent inward currents (Gelli & Blumwald,
1997). Figure 6.1A shows whole cell currents recorded in the presence of 20 mM Ba\(^{2+}\) (on the extracellular side of the membrane). The whole cell inward currents were a result of Ba\(^{2+}\) moving into the cytosol in response to negative membrane potentials (Fig. 6.1A). Currents reached a maximum steady state level within 5 s from the onset of the voltage pulse. These currents were active at membrane voltages that were within the range of equilibrium potentials for K\(^+\) (-167 mV) and Cl\(^-\) (-158 mV) suggesting that these currents were not due to the movement of K\(^+\) or Cl\(^-\). The Ba\(^{2+}\) inward currents reversed direction \((E_{\text{rev}})\) at 20 mV in the presence of 20 mM Ba\(^{2+}\) (Fig. 6.1D) and at 10 mV in the presence of 5 mM Ba\(^{2+}\) in tail current experiments. This shift in the \(E_{\text{rev}}\) toward \(E_{\text{Ba}^{2+}}\) (154 mV) with increasing concentrations of Ba\(^{2+}\) suggested that Ba\(^{2+}\) was the main contributor to the whole cell currents. Given the discrepancy between \(E_{\text{rev}}\) and \(E_{\text{Ba}^{2+}}\), a weak permeability of glutamate through the channel cannot be ruled out. Using these reversal potentials for the calculation of permeability ratios a \(P_{\text{Ca}}/P_{\text{K}}\) of about 20, a \(P_{\text{Cl}}/P_{\text{K}}\) of about 2 and a \(P_{\text{Ca}}/P_{\text{Cl}}\) of about 10 were obtained.

The Ba\(^{2+}\) currents were time-dependent and strongly inward rectifying, that is, whole cell currents were induced only by membrane potentials more negative than -100 mV. Current levels measured at 4.8 s from the onset of the voltage pulse were plotted against the respective voltages (Fig. 6.1E). The non-linear current-voltage relationship of the time-dependent inward currents was evident. The addition of race 4 IF (IF\(_{4}\), containing the \(avr5\) elicitor) resulted in a significant increase in the magnitude of the time-dependent
Figure 6.1. **Inwardly rectifying Ba\(^{2+}\) currents stimulated by IF\(_4\) (containing the \textit{avr5} fungal elicitor).** A, Voltage- and time-dependent currents were activated by membrane potentials more negative than -100 mV when protoplasts were bathed in 20 mM BaCl\(_2\) in the absence of IF\(_4\). The voltage protocol and the time and amplitude scales shown refer to A, B and C. B, The presence of IF\(_4\) increased the magnitude of voltage and time-dependent currents. Currents reached a saturable maximum within 5 s from the onset of the voltage pulse. C, With protoplasts exposed to IF\(_3\) (not containing the \textit{avr5} fungal elicitor) the magnitude of the voltage- and time-dependent currents was similar to those in A. D, Tail currents of whole protoplasts with Ba\(^{2+}\) as charge carrier, reversed direction at 20 mV. E, Current-voltage plot from protoplasts in A (o), B (\(\square\)), C (\(\triangledown\)) and control IF (\(\blacktriangle\)) (intercellular fluids from non-infected plants). Current levels were measured at 4.8 s from the onset of the voltage pulse and plotted against the respective voltages. Steady state whole-cell currents were largest with protoplasts exposed to IF\(_4\). Values are Mean ± S.D. (n=12).
inward currents (Fig. 6.1B). The current-voltage plot revealed an IF₄- induced 2-fold increase in the inward currents (Fig. 6.1E). This effect was race-specific since the addition of race 5 IF (IF₅, lacking the avr5 elicitor) (Figs. 6.1C, E) or control IF (IF₀) (Fig. 6.1E) did not affect the steady state time-dependent inward currents.

6.2.2 HYPERPOLARIZATION-INDUCED CURRENTS AT THE SINGLE CHANNEL LEVEL

Single channel data revealed some inherent properties of the hyperpolarization-induced Ca²⁺(Ba²⁺) -permeable channels previously characterized (Gelli & Blumwald, 1997). In this report, results obtained in single channel experiments were corroborated with those obtained in whole cell studies. Figure 6.2 shows some original single channel recordings from isolated patches of membrane exposed to 50 mM Ca²⁺ on the extracellular side of the plasma membrane. These recordings revealed voltage-dependent channel activity (Fig. 6.2A) as previously reported (Gelli & Blumwald, 1997). Channel activity was detected at membrane potential differences corresponding to the reversal potentials of K⁺ (-167 mV) and Cl⁻ (-158 mV) suggesting that the downward deflections corresponded to the movement of Ca²⁺. Channel events became more frequent with increasingly negative membrane voltages (Fig. 6.2A). An amplitude histogram of single channels recorded at -180 mV revealed at least one open channel in the patch of membrane with an amplitude of -1 pA (Fig. 6.2B). At a membrane potential difference of -180 mV, the mean Pₒ was 0.10.
Figure 6.2. Some inherent properties of the hyperpolarization-induced Ca²⁺/Ba²⁺-permeable channels. A, Voltage-dependent single channel currents with Ca²⁺ as the charge carrier. Single channel recordings from isolated patches of membrane from protoplasts exposed to 50 mM Ca²⁺ (extracellular side of membrane) and polarized from membrane voltages of -170 mV to -190 mV. Downward deflections indicate 1 or 2 channels open and correspond to the movement of Ca²⁺ into the cytosol. B, An amplitude histogram of single channel currents recorded at -180 mV under similar conditions as explained in (A). The peak at 0 pA corresponds to the closed state of the channel. The additional peak at -1 pA corresponds to the opening of one channel. The Po for this patch of membrane was 0.09. C, The open probability (Po) of the channel is voltage-dependent. The channel Po increased as the membrane voltage was made more negative and it reached a maximum at -190 mV. Values are Mean ± S.D. (n=8). D, The measured E₉, varied linearly with the log of the extracellular Ca²⁺ activity. Channel currents were measured over a range of extracellular Ca²⁺ or Ba²⁺ concentrations (5 mM, 20 mM and 50 mM; activities: 3.9 mM, 13.3 mM and 26.1 mM) and the corresponding E₉ were determined from a least-squares fit of the channel current-voltage plot. Data points are Mean ± S.D. (n=5).
membrane potential (mV)

C

$P_0$

$-200 \quad -180 \quad -160 \quad -140 \quad -120 \quad -100$

$0.15$

$0.10$

$0.05$

$0.00$

$\text{membrane potential (mV)}$

$E_{\text{rev}}$ (mV)

$log_{10}$ ion activity (log mM)

D

$\text{Co}^{2+}$

$\text{Ba}^{2+}$

$0$

$15$

$30$

$45$

$0.60$

$1.00$

$1.40$
± 0.03. The $P_o$ of the $Ca^{2+}$-permeable channels was dependent on the membrane voltage as shown by the sigmoidal-type relationship (Fig. 6.2C). The $P_o$ increased between -150 mV and -160 mV and reached a maximum value at -190 mV. Significant $P_o$ values were achieved by single channels beginning at -140 mV. The hyperpolarization-induced $Ca^{2+}$-permeable channels were more permeable to $Ca^{2+}$ than to $K^+$ or to $Cl^-$ as previously reported (Gelli & Blumwald, 1997, chapter 5). The measured reversal potentials varied linearly with the log of the extracellular-$Ca^{2+}$ activities (Fig. 6.2D).

6.2.3 AN IF$_4$-INDUCED INCREASE IN SINGLE CHANNEL ACTIVITY

The effect of the fungal elicitor on the time-dependent inward currents observed in whole cells was also observed in single channel experiments (Fig. 6.3). Prior to the addition of IF$_4$, voltage-dependent single channel activity, with $Ba^{2+}$ as the charge carrier, was observed at negative membrane potentials (less than -100 mV). Figure 6.3A shows an original recording of single channels active at -180 mV. Downward deflections corresponded to the movement of $Ba^{2+}$ ions into the cytosol. The addition of IF$_4$ to the extracellular side of isolated patches of tomato plasma membrane resulted in increased channel activity (Fig. 6.3B) as shown by the increase in channel events (downward deflections, Fig. 6.3B) and by the increase in $P_o$ with respect to time (Fig. 6.3C). The mean $P_o$ increased from 0.09 ± 0.02 to 0.31± 0.03 after the addition of IF$_4$ (Table VI.1). The single channel conductance (11 pS) did not significantly change in the presence of IF$_4$, thus single channel current levels
Figure 6.3. Ba\textsuperscript{2+}-permeable channels activated by IF\textsubscript{4}. A, Outside-out patches of plasma membrane maintained at membrane potentials of -180 mV revealed voltage-dependent channels. Downward deflections from the closed (c) state to the open (o) state corresponded to Ba\textsuperscript{2+} moving into the cytosol to tomato protoplasts in response to hyperpolarization of the membrane potential. B, IF\textsubscript{4} increased channel activity indicated by the greater number of channel events. A and B represent original single channel records from the same patch of plasma membrane exposed to 20 mM Ba\textsuperscript{2+} in the absence (A) and presence (B) of IF\textsubscript{4}. Similar results were obtained in eight other patches. C, A time course of channel open probability (P\textsubscript{o}) of single channel recordings in A and B. P\textsubscript{o} increased from 0.09 ± 0.02 to 0.31 ± 0.03 after the addition of IF\textsubscript{4}. The arrow indicates the time at which IF\textsubscript{4} was added to the bath.
CONTROL

A

10 s

5 pA

200 ms

+IF₄

B

C

P (open)

0 12 24 36 48 60 72 84 96 108 120 132 144 156 180

Time (s)
remained unchanged. Single channel activities were not affected by the addition of either IF$_5$ or control IF suggesting that the changes in the single channel kinetics were race-specific.

6.2.4 G-PROTEINS MEDIATE THE STIMULATORY EFFECT OF IF$_4$.

We tested the possible involvement of G proteins in the elicitor-induced activation of the plasma membrane Ca$^{2+}$-permeable channels by using nonhydrolyzable guanidine nucleotide analogues. In studies on G-protein regulation of ion channels in animal (Brown, 1991) and plant cells (Fairley-Grenot and Assmann, 1991), 100 µM to 1 mM of GTP and GDP analogues were normally used. Here we employed 300 µM of GTP$[\gamma]$S, an analogue that locks G-proteins in a GTP-bound active form (Gilman, 1987), on the cytoplasmic side (pipette) of a whole cell and on the cytoplasmic side (bathing chamber) of an inside-out patch of plasma membrane. In whole cell experiments, in the absence of IF$_4$, GTP$[\gamma]$S increased the magnitude of the time-dependent inwardly rectifying currents as shown in the current-voltage relationship (Fig. 6.4A). However in the presence of GDP$[\beta]$S, an analogue that locks G-proteins in a GDP-bound inactive form (Gilman, 1987), whole cell measurements revealed a decrease in the magnitude of the time-dependent inward currents (Fig. 6.4A).

Further support for the involvement of a G-protein in the stimulation of voltage-activated Ba$^{2+}$ currents came from the response of the whole-cell currents to mastoparan. Mastoparan stimulates GTPase activity by allowing the exchange of GDP for GTP
independent of the agonist-receptor interaction, thus mimicking the effect of G-proteins (Armstrong and Blatt, 1995). The addition of mastoparan (0.5 μM) to the cytoplasmic side of the plasma membrane enhanced the time-dependent currents similar to that observed in the presence of GTP[γ]S (Fig. 6.4A). Moreover, when control nucleotides, ATP[γ]S and ADP[β]S, were tested on the whole cell currents, no significant effects were observed, providing further support for the specificity of the GTP[γ]S effect.

The effects of the guanosine nucleotide analogues were studied in the presence of IF₄. Similar increases in the magnitude of the whole cell currents were observed with GTP[γ]S and mastoparan in the presence of IF₄ (Fig. 6.4B). Only the currents activated by voltage pulses of -180 mV (measured at 4.8 s from the onset of the voltage pulse) are shown in figure 6.4B. A comparison between currents recorded in the absence and presence of IF₄ clearly demonstrated that GTP[γ]S or mastoparan alone could stimulate whole cell currents and the maximum current levels acquired were comparable to those measured in the presence of IF₄ (Fig. 6.4B). The inactivation of the G-protein, by the addition of GDP[β]S, totally abolished the elicitor-induced channel activation (Fig. 6.4B) further supporting the involvement of a G-protein in the modulation of the IF₄ effect.

Single channel experiments confirmed the findings in whole cells. GTP[γ]S and mastoparan both increased the activity of single channels (measured at -180 mV) in the absence of IF₄. (Fig. 6.4C). This is demonstrated by the increase in frequency of channel events in the original single channel recordings (Fig. 6.4C) and in the reported Pₒ values (Table VI.1). The single channel conductance was unchanged in the presence of GTP[γ]S and mastoparan. The increase in channel events as seen in the single channel records and
Figure 6.4. Activation of Ba\(^{2+}\) currents by IF\(_4\) is mediated by G-proteins. Whole protoplast currents were activated by a voltage protocol similar to that in figure 6.1. A, Current-voltage plot from whole cell recording similar to that in figure 6.1A (O) and from similar whole cell recordings from protoplasts exposed to GTP\([\gamma]\)S (□), mastoparan (▲) and GDP\([\beta]\)S (V). Both GTP\([\gamma]\)S and mastoparan increased the magnitude of whole protoplast inward current by 2-fold in contrast to the effect of GDP\([\beta]\)S. These plots were constructed as explained in figure 6.1. B, Maximum current levels were measured at 4.8 s from the onset of an imposed membrane potential of -180 mV from whole cell recordings of protoplasts exposed to GTP\([\gamma]\)S, mastoparan and GDP\([\beta]\)S in the absence (white bar) and presence (hatched bar) of IF\(_4\). GTP\([\gamma]\)S and mastoparan increased voltage-dependent inward currents to magnitudes similar to those induced by IF\(_4\). C, Single channel recordings from isolated outside-out patches of membrane polarized to -180 mV. Downward deflections correspond to the opening of at least two channels (O\(_1\), O\(_2\)). GTP\([\gamma]\)S, mastoparan and IF\(_4\) activated single channels as shown by the significant increase in channel events in contrast to GDP\([\beta]\)S. Values are Mean ±S.D. (n=8).
in the $P_o$ values were comparable to those obtained in the presence of the elicitor. Single channel experiments revealed that GTP[$\gamma$]S or mastoparan stimulated channel activity independently of IF$_4$ and that the single channel kinetics ($P_o$ and conductance) behaved similarly to those obtained in the presence of the fungal elicitor.

6.2.5 THE IF$_4$ EFFECT IS MODULATED BY PHOSPHORYLATION/DEPHOSPHORYLATION EVENTS

The involvement of membrane-associated effectors in the G-protein-mediated IF$_4$-induced stimulation of inward currents was examined by employing okadaic acid, an inhibitor of phosphatases 1A and 2B. Channel activity measured in the presence of IF$_4$ (Fig. 6.5A), GTP[$\gamma$]S or mastoparan (Fig. 6.4C) was moderately enhanced by the addition of okadaic acid (0.1 $\mu$M to the cytoplasmic side of the plasma membrane) (Fig. 6.5B) (Table VI.1) (Cohen et al., 1990). However, okadaic acid had no significant effect in the absence of IF$_4$, GTP[$\gamma$]S or mastoparan (Table VI.1). This suggested that channel stimulation by IF$_4$ was a specific response to the fungal elicitor and that this response was mediated by a G-protein.

If the inhibition of phosphatase by okadaic acid resulted in a moderate enhancement of the effect of IF$_4$ on the Ca$^{2+}$-permeable channel, then protein kinase inhibitors would be expected to eliminate the stimulatory effect of IF$_4$. Single channel activity was mostly eliminated by the addition of 10 $\mu$M of HA1004 (a protein kinase inhibitor) (Asano & Hidaka, 1984) in the presence of IF$_4$, however, this reduction in channel activity was not observed in the absence of IF$_4$ (Table VI.1). The effect of
HA1004 in the presence of IF₄ was evident from the significant reduction in channel events as seen in the original single channel recording (Fig. 6.5C). A similar response was observed when staurosporine (a less specific protein kinase inhibitor) (Barford, 1991) was added. Thus both protein kinase inhibitors eliminated the IF₄-induced stimulation in channel activity. These results would strongly suggest that the IF₄-induced channel activation may be mediated by the action of a protein kinase that stimulates channel activity by promoting the phosphorylation of the channel protein.
**Figure 6.5.** Channel activation by IF₄ involves the phosphorylation of Ca^{2+}-permeable channels. A, Single channel recordings of outside-out patches of membrane from protoplasts bathed in 20 mM Ba^{2+} and exposed to IF₄ and polarized to -180 mV. IF₄ activated single channels as demonstrated by the increase in channel events. B, With 0.1 μM okadaic acid (inhibitor of protein phosphatase 1A and 2B) in the pipette, channel activity induced by IF₄ was moderately enhanced as shown by the frequency of channel opening. C, Channel activity induced by IF₄ was significantly reduced with 10 μM HA1004 (protein kinase inhibitor) in the pipette. Inhibition of channel activity is evident by the reduced frequency of channel events. Similar results were obtained in 6 other different protoplasts.
A
+IF$_4$

B
+OA

C
+HA1004
Table VI.1 The effects of IF₄, G protein regulators, Okadaic acid and HA 1004 on single channel kinetics.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pₒ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>+IF₄</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>+GTP[γ]S</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>+Mastoparan</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>+GDP[β]S</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>+Okadaic acid</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>+HA1004</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

<p>| | |</p>
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<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>+IF₄ + GTP[γ]S</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>+IF₄ + Mastoparan</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>+IF₄ + GDP[β]S</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>+IF₄ + OA</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>+IF₄ + HA1004</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>

The concentrations used in the different treatments are listed in the text. Data were obtained from amplitude histograms of single channel recordings maintained at -180 mV in the presence of 20 mM Ba²⁺. Values are Mean ± S.D. (n=6). Pₒ: open channel probability; OA: Okadaic acid.
6.3 DISCUSSION

The interaction between the leaf mold pathogen *C. fulvum* and tomato has been extensively investigated (De Wit, 1992). It is now well documented that plant disease resistance involves a resistance (R) gene in the plant that responds specifically to the product of a single avirulence (avr) (non-virulence) gene in the pathogen (De Wit, 1992). The *avr* genes have been shown to code directly or indirectly (i.e. post-translational modification) for elicitor molecules, that most likely bind to receptors located at the plasma membrane of the host plant cells. In the current study, tomato cells near isogenic for the resistance gene *Cf5* and intercellular fluids containing the *avr5* gene product from race 4 of *C. fulvum*, were used to investigate the potential involvement of a Ca\(^{2+}\) channel in transducing the presence of fungal elicitors into elevated cytosolic Ca\(^{2+}\) levels. The race-specificity of the IF\(_4\) effect was confirmed by using intercellular fluids from race 5 of *C. fulvum* which did not contain an *avr5* gene product. There is substantial evidence demonstrating that unpurified or partially purified intercellular fluids exhibit the required specificity. De Wit and Spikman (1982) initially showed the necrotic effect of race 4 IF on *Cf5* plants. Vera-Estrella et al., (1992) showed that similar race 4 IFs, but not IFs from races virulent on *Cf5* plants, caused an oxidative burst and lipid peroxidation on application. These changes, together with the β-glucanase and chitinase increases reported by Degousse et al., (1994) are all considered to be strongly associated with the plant defense response. The increase in the host plasma membrane H\(^+-\)ATPase activity, changes in the plasma membrane redox activities (Vera-Estrella et al., 1994a,b) and the induction of a protein kinase cascade mediating the phospho/dephosphorylation of the plasma
membrane H+-ATPase (Xing et al., 1996a,b) have also been shown to be specifically induced on Cf5 tomato cells by race 4 IF.

The activation of Ba\(^{2+}\) and Ca\(^{2+}\)-permeable channels by the *avr5* elicitor at hyperpolarized membrane potentials provides a pathway for the influx of Ca\(^{2+}\) into the host cells in response to pathogen infection. The subsequent elevation of cytosolic free Ca\(^{2+}\) concentration could potentially activate signal transduction pathways linking the perception of elicitors at the membrane to the induction of various biochemical responses that are essential to the survival of the infected plant.

Although most of the studies on Ca\(^{2+}\) permeation through the plant plasma membrane have shown the activation of Ca\(^{2+}\)-permeable channels at depolarized membrane potentials (Thuleau et al., 1994; Huang et al., 1994; Piñeros & Tester, 1995), few studies have shown Ca\(^{2+}\) influx at hyperpolarized potentials (Stoeckel & Takeda, 1995; Gelli & Blumwald, 1997, chapter 5). Stoeckel & Takeda (1995) suggested that Ca\(^{2+}\) influx, activated by hyperpolarized membrane potential differences, was associated with the rundown in the K\(^+\) outward current. This conclusion was based on electrophysiological data that showed Ca\(^{2+}\) influx was stimulated by hyperpolarization of the membrane and blocked by La\(^{3+}\) and Gd\(^{3+}\). The Ca\(^{2+}\) influx reported by Stoeckel & Takeda (1995) and those reported here (Gelli & Blumwald, 1997, chapter 5) are different from the putative depolarization-activated Ca\(^{2+}\) influx previously reported. It would appear that the ability of plant cells to activate Ca\(^{2+}\)-permeable channels at both depolarized and hyperpolarized plasma membrane potentials is a required feature that allows plant cells to efficiently couple the perception of stimuli at the plasma membrane to fluctuations in cytosolic Ca\(^{2+}\).
concentrations Many of the biochemical responses associated with the plant defense mechanisms were inhibited by the depletion of extracellular Ca$^{2+}$ or stimulated in the presence of ionophores that allowed the entry of Ca$^{2+}$ into the cells (Sckwacke and Hager, 1992). The activation of Ca$^{2+}$-permeable channels by IF$_4$ provides a direct demonstration of a pathway by which cytosolic free Ca$^{2+}$ can increase to levels that could initiate various plant defense responses.

The effect of IF$_4$ on the Ca$^{2+}$-permeable channels was mediated by a G-protein. Inward currents were activated by GTP[y]S or mastoparan and inactivated by GDP[β]S in the absence, or presence, of IF$_4$ suggesting a G-protein mediation of the IF$_4$-induced channel activity. Recently, the regulation of a guard cell ion channel by a receptor-activated G-protein has been demonstrated (Armstrong and Blatt, 1995). Interestingly these G-protein-linked receptors are activated by agents such as proteases, peptides and small molecules which bear some similarity to the purified fungal elicitor (Ebel and Cosio, 1994). In whole cell experiments of the current study, it was difficult to determine whether the IF$_4$-induced activation of the time-dependent inward currents was due to changes in channel conductance or in $P_0$. Single channel experiments revealed that IF$_4$ altered the $P_0$ of the channels but not the channel conductance.

Protein phospho/dephosphorylation is an important component of signal transduction cascades (Ranjeva and Boudet, 1987). Evidence supporting the modulation of ion channel activity by kinases and phosphatases have been shown in the regulation of plasma membrane K$^+$ channels (Luan et al., 1994; Thiel and Blatt, 1994; Armstrong et al., 1995) and anion channels (Schmidt et al., 1995) from guard cells and in Ca$^{2+}$-permeable
channels in guard cell vacuoles (Allen and Sanders, 1994). Here, the strong inactivation of the IF₄-induced channel currents by protein kinase inhibitors (HA1004 and staurosporine) and the moderate enhancement by the protein phosphatase inhibitor (okadaic acid) suggest that protein kinases play a role in the race specific stimulatory effect of IF₄ on the Ca²⁺-permeable channels. Thus protein kinases mediate the up-regulation of the Ca²⁺-permeable channels leading to increased cytosolic Ca²⁺ levels in response to the fungal elicitor.

In conclusion, these results revealed that incompatible race-specific elicitors from C. fulvum activated tomato plasma membrane Ca²⁺-permeable channels. The activation of the channels provides a pathway for the influx of Ca²⁺ into the cells resulting in increased cytosolic Ca²⁺ concentrations. The increase in cytosolic Ca²⁺ initially activates plant defense responses (Smith, 1996). Subsequently, cytosolic Ca²⁺ would contribute to the activation of a protein kinase cascade that allows the rephosphorylation of the plasma membrane H⁺-ATPase by Ca²⁺-dependent protein kinases resulting in the restoration of normal cellular functions (Xing et al., 1996a,b).
7.1.1 THE CELLULAR Ca^{2+} POOLS

Responses to developmental and environmental cues occur by stimulus-response coupling. In most cases stimuli induce transient or localized elevations in cytosolic Ca^{2+} that couple the stimuli to cellular responses through effector proteins such as G-proteins, protein kinases and phosphatases. In plant cells, the source for Ca^{2+} (i.e. intracellular or extracellular pools) is uncertain but is likely to be diverse and possibly stimulus specific. For example, fungal elicitors (Knight et al., 1991) elevated cytosolic Ca^{2+} by stimulating Ca^{2+} uptake from the extracellular space. Red-light, by contrast, promoted Ca^{2+} mobilization from intracellular stores (Chae et al., 1990). Erhardt et al., (1996), showed that rhizobia-induced nodulation events stimulated Ca^{2+} release from pools near the cell nucleus suggesting the involvement of internal pools. The vacuole is also a major Ca^{2+} pool. Fluorescence ratio imaging indicated that the vacuole acts as the major internal Ca^{2+} source (Gilroy et al., 1991) by providing several different pathways that result in increased levels of cytosolic Ca^{2+}. The diversity of signal transduction pathways in which the elevation of cytosolic Ca^{2+} is an integral step suggests that there must be a high degree of control of the amplitude, duration and spatial arrangement of the Ca^{2+} signal in the cytosol. Thus in order for stimulus-specific responses to occur, multiple pathways for the release of Ca^{2+} from the vacuolar pool and for the influx of Ca^{2+} from the extracellular space must exist.
7.1.2 Ca\textsuperscript{2+} MOBILIZATION FROM THE VACUOLE VIA MULTIPLE PATHWAYS

Vacuoles constitute an excellent source for the sustained elevation of cytosolic Ca\textsuperscript{2+} because they often account for 90% of the intracellular volume of a mature plant cell and they accumulate free Ca\textsuperscript{2+} to 1 mM or higher. The resultant driving force for the flux of Ca\textsuperscript{2+} into the cytosol is in excess of 100 mV, given the resting values of cytosolic Ca\textsuperscript{2+} of 100 - 200 nM and a membrane potential of -20 mV (cytosol negative). The Ca\textsuperscript{2+} release channels identified so far at the vacuolar membrane provide potential pathways for the mobilization of Ca\textsuperscript{2+} in response to stimuli. Although the capacity of IP\textsubscript{3} and cADR to mobilize Ca\textsuperscript{2+} from the vacuole has been demonstrated (Alexandre et al., 1991; Allen et al., 1995), the particular stimulus-response pathways mediated by these metabolites have not yet been demonstrated. The question of why individual vacuoles should contain more than one voltage-gated Ca\textsuperscript{2+} release channel (chapter 3; Johannes et al., 1992; Allen & Sanders, 1994) that would essentially perform the same function \textit{in vivo} must be addressed. The distinct Ca\textsuperscript{2+} release channels might be required if they are part of signal transduction pathways arising from different primary signals that subsequently converge on the same terminal response, that is the elevation of cytosolic Ca\textsuperscript{2+}. If this is the case, then differences in the regulatory factors controlling channel opening (i.e. voltage, lumenal and cytosolic Ca\textsuperscript{2+}, pH, cytosolic phosphatases) could communicate the incoming signal. Alternatively, if the channels were sensitive to the same or similar primary signals, their presence might instead represent the diverse role of Ca\textsuperscript{2+} in signaling. In this case, the cell will have the capacity to vary the amplitude and/or the duration of a Ca\textsuperscript{2+} signal.
The activation of the Ca\textsuperscript{2+} release channels \textit{in vivo} could activate the vacuolar inward Ca\textsuperscript{2+} channel (chapter 4) through both the elevation in cytosolic Ca\textsuperscript{2+} and the depolarization (cytosol side) of the vacuolar membrane potential difference. Thus \textit{in vivo}, this channel operates at membrane voltages closer to $E_K$ (0 to 60 mV; dependent on lumenal $K^+$ activity) where the driving force for $K^+$ movement is greatly reduced. Given the $P_{Ca}/P_{K}$ of 3:1, the Ca\textsuperscript{2+} selectivity of this channel was lower than that of the Ca\textsuperscript{2+} release channels where the $P_{Ca}/P_{K}$ varied between 6:1 to 100:1 (chapter 3; Johannes et al., 1992; Allen & Sanders, 1994, 1995) thus the relatively high $K^+$ permeability through this channel helps to maintain the membrane potential sufficiently displaced from $E_{Ca}$ (approximately 100 mV) so that the driving force for Ca\textsuperscript{2+} flux through the channel is large. This argument has led to the proposed role of this channel in mediating Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release from the vacuole, despite the molar excess of $K^+$ over Ca\textsuperscript{2+} in the vacuolar lumen (Ward & Schroeder, 1994; Allen & Sanders, 1995). Thus the operation and interactions of the Ca\textsuperscript{2+}-release channels offer the cell the possibility of generating stimulus-specific spatio-temporal patterning of cytosolic Ca\textsuperscript{2+} signals. The multiple pathways available to the cell for the mobilization of vacuolar Ca\textsuperscript{2+} suggests that tight regulation of Ca\textsuperscript{2+} release channels is crucial since plant vacuoles are essentially an inexhaustible supply of Ca\textsuperscript{2+}.

In addition to rapid Ca\textsuperscript{2+} release channels and accumulation mechanisms, a Ca\textsuperscript{2+} buffering system is essential to any Ca\textsuperscript{2+} storage organelle capable of rapid equilibrium with the cytosol. The vacuole and the ER are the sites most involved in controlling intracellular Ca\textsuperscript{2+} concentration. Although, uncertainties about the magnitude of the
membrane potential across the ER have made it difficult to reliably estimate $\Delta\mu_{Ca^2+}$, it has been suggested that ER luminal $Ca^{2+}$ levels are higher than 50 $\mu$M (Bush, 1995). Thus, whereas the vacuole acts as a $Ca^{2+}$ pool linked to proton homeostasis in the cell, the ER may provide a storage site for $Ca^{2+}$ independent of pH changes. Recently, a $Ca^{2+}$-binding protein, calreticulin, has been localized to ER membranes (Opas et al., 1996). Calreticulin possess a single high affinity $Ca^{2+}$-binding site (1 mole of $Ca^{2+}$/mole of protein) and a number of low affinity $Ca^{2+}$-binding sites (25-50 moles of $Ca^{2+}$/mole of protein) and is believed to function in $Ca^{2+}$ storage and $Ca^{2+}$ buffering. In addition, a group of $Ca^{2+}$-binding polypeptides (55 & 58 kDa) have been localized to the ER and to the vacuole, however, two other polypeptides (42 & 64 kDa) were localized to vacuoles only (Randall, 1992). These vacuole-associated $Ca^{2+}$-binding proteins, which are thought to play a role in $Ca^{2+}$ buffering, concurrently with the $Ca^{2+}$ transport systems previously described (chapter 1), could mediate the vacuolar component of cellular $Ca^{2+}$ homeostasis.

7.1.3 A STIMULUS-SPECIFIC $Ca^{2+}$ SIGNAL

The apoplast (intercellular space) is another source of $Ca^{2+}$ where for land plants, $Ca^{2+}$ exists at concentrations of 0.1 mM to 1.0 mM (Bush, 1995). Electrical potential differences across the plasma membrane ($\Delta\Psi$) are -100 to -120 mV and the resultant $\Delta\mu_{Ca^{2+}}$ is 20 to 35 kJ mol$^{-1}$. External stimuli that alter $\Delta\Psi$ or the cell wall pH could mobilize bound $Ca^{2+}$ from the cell wall and significantly increase $\Delta\mu_{Ca^{2+}}$. $Ca^{2+}$ is a major structural constituent of the cell wall where it is bound to polygalacturonic acid (pectic acid). It’s generally believed that the concentration of free $Ca^{2+}$ in the cell wall depends on
the pH (Gilroy et al., 1993). Light (Assmann et al., 1985), auxin (Felle, 1988) and fungal elicitors (Vera-Estrella et al., 1994) (chapter 6) for example, hyperpolarize the plasma membrane and cause cell wall acidification that could increase $\Delta \mu_{Ca^{2+}}$ by 10 to 15 kJ mol$^{-1}$ and alter $Ca^{2+}$ fluxes across the membrane.

An increase in cytosolic $Ca^{2+}$ would be rapidly attenuated by the activation of the $Ca^{2+}$-ATPase and the $Ca^{2+}$/H$^+$-antiport that together would function to extrude $Ca^{2+}$ into the extracellular space and into the vacuole, respectively. However, cytosolic $Ca^{2+}$ levels would have to remain high long enough to activate key effector proteins. This would imply that the activity of the $Ca^{2+}$-permeable channel would have to be up-regulated. For example, the enhanced activity of the $Ca^{2+}$ channel induced by phosphorylation could be a mechanism for the up-regulation of $Ca^{2+}$ influx in response to the fungal elicitors (chapter 6). This appears to be in agreement with work by Knight et al., (1991) who demonstrated that the kinetics of the cytosolic $Ca^{2+}$ transients were very short for cold and touch but significantly longer for fungal elicitors. Moreover, several reports have shown that if the influx of $Ca^{2+}$ is interrupted by the addition of an inhibitor (i.e. verapamil or La$^{3+}$) any time before 40 - 50 min have elapsed following the addition of elicitor, the particular defense response was only partly expressed (Smith, 1996). It appeared that for the cell response to be fully expressed, the influx of $Ca^{2+}$ had to be sustained suggesting that $Ca^{2+}$ influx acts as more than a simple trigger.

Given the apparent ubiquity of cytosolic-$Ca^{2+}$ elevations to a diverse array of stimuli, the question of specificity arises. One possibility for generating specificity could be the inherent nature of the $Ca^{2+}$ signal itself. For example, $Ca^{2+}$ elevation could occur in
localized sub-regions of the cell that are close to the primary and secondary effector proteins required to generate a particular response. Such a mechanism would require the localization of either plasma membrane Ca\(^{2+}\) channels or endomembrane Ca\(^{2+}\) release channels to a particular region overlying the effector proteins. In the case of the plasma membrane Ca\(^{2+}\) channels, this might depend on receptor clustering. However, the situation with the Ca\(^{2+}\) release sites on endomembranes might be somewhat more complex as it would require the participation of several other second messengers. The temporal and the spatial aspects of the Ca\(^{2+}\) signal may also be important in defining specificity. In guard cells for example, ABA generates Ca\(^{2+}\) elevation in only certain areas of the cell, however the correlation of these localized Ca\(^{2+}\)-rich spots with ion channel distribution has not been demonstrated (McAinsh et al., 1992). There is good evidence that the interaction of Ca\(^{2+}\) influx across the plasma membrane with Ca\(^{2+}\) release from intracellular stores is capable of generating a pattern of oscillations in cytosolic Ca\(^{2+}\) (McAinsh et al., 1995). Thus oscillations and localized increases in cytosolic Ca\(^{2+}\) in combination with the probable uneven distribution of key effector proteins within the cytosol, suggest there could be great range for generating specificity.

Clearly, there could be specificity built into signal transduction pathways at the level of the receptor, however, in the case of the Cf9 receptor the avr9 elicitor polypeptide binds equally to the plasma membranes of both Cf9 resistant and Cf0 susceptible tomato cells (De Wit, 1996). These results would suggest that in some of the tomato-Cladosporium fulvum interactions, the specificity may exist in the activation of the signal transduction cascade(s) rather than in the binding of the fungal elicitors to the plant
receptors. This specific activation of signaling pathways may reside at the level of the heterotrimeric G proteins and how the G protein subunits interact with specific effector proteins such as protein kinases or protein phosphatases. Specificity may also be achieved through compartmentation whereby a targeting moiety, or domain inherent to protein kinases and protein phosphatases, restricts subcellular localization and thus enhances specificity (Faux & Scott, 1996). Although this model may be premature for plant cells, in animal cells, serine/threonine phosphatases and kinases have been shown to be associated with targeting subunits. It is believed that subcellular targeting of key effector enzymes through targeting moieties contributes to the organization and specificity of signaling pathways.

In summary, the evidence conferring how plant cells encode for specificity of the Ca\(^{2+}\) signal is as yet fragmentary, nevertheless it is apparent that, when compared to animal cells, plant cells will rely on the interaction of diverse signaling pathways and diverse forms of the stimulus-induced signal. This in combination with the differential localization of receptors and key effector proteins suggests that plant cells have the potential and the opportunity for encoding specificity of the Ca\(^{2+}\) signal.

7.1.4 FUTURE PROSPECTS

There is no doubt that in plant cells the role of Ca\(^{2+}\) as a second messenger will be linked to other not as yet identified stimuli. This process will be helped by the use of transgenic aequorin and by improved Ca\(^{2+}\) indicators that will make the quantification and the identification of the temporal-spatial distribution of fluctuations in cytosolic Ca\(^{2+}\)
easier. The nature of the Ca\textsuperscript{2+} spatial gradients will be further understood by focusing on the Ca\textsuperscript{2+}-release channels in endomembranes, in particular the ER. Intracellular Ca\textsuperscript{2+}-binding proteins including calmodulin will most likely play a role in Ca\textsuperscript{2+} homeostasis and should therefore also be investigated.

Detailed information about molecular mechanisms of Ca\textsuperscript{2+} transport and the interactions between Ca\textsuperscript{2+} channels and key effector proteins in signaling transduction pathways is needed. Thus isolation of genes encoding for Ca\textsuperscript{2+} channels, and for other signal transduction components, followed by over-expression studies in heterologous systems will be important for defining direct or indirect interactions (through other secondary messengers) between channels and signaling proteins. The genes encoding for regulatory proteins that modulate or interact with Ca\textsuperscript{2+} channels can be identified through new molecular biological techniques such as two-hybrid systems and interactive cloning. Over-expression studies will also describe the activity of the encoded protein in biochemical terms and serve as a preliminary step in the full molecular characterization of the protein either by NMR or X-ray crystallography.


