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Activation of Plant Plasma Membrane Ca^{2+}-Permeable Channels by Race-Specific Fungal Elicitors[1]

Angie Gelli, Verna J. Higgins, and Eduardo Blumwald*

Department of Botany, University of Toronto, 25 Willcocks Sreet, Toronto, Ontario, Canada M5S 3B2

The response of plant cells to invading pathogens is regulated by fluctuations in cytosolic Ca^{2+} levels that are mediated by Ca^{2+}-permeable channels located at the plasma membrane of the host cell. The mechanisms by which fungal elicitors can induce Ca^{2+} uptake by the host cell were examined by the application of conventional patch-clamp techniques. Whole-cell and single-channel experiments on tomato (*Lycopersicon esculentum* L.) protoplasts revealed a race-specific fungal elicitor-induced activation of a plasma membrane Ca^{2+}-permeable channel. The presence of the fungal elicitor resulted in a greater probability of channel opening. Guanosine 5′-[β-thio]diphosphate, a GDP analog that locks heterotrimeric G-proteins into their inactivated state, abolished the channel activation induced by the fungal elicitor, whereas guanosine 5′[γ-thio]triphosphate, a nonhydrolyzable GTP analog that locks heterotrimeric G-proteins into their activated state, produced an effect similar to that observed with the fungal elicitor. Mastoparan, which stimulates GTPase activity, mimicked the effect of GTP[γ]S. The addition of HA1004 (a protein kinase inhibitor) in the presence of the elicitor totally abolished channel activity, whereas okadaic acid (a protein phosphatase inhibitor) moderately enhanced channel activity, suggesting that the activation of the channel by fungal elicitors is modulated by a heterotrimeric G-protein-dependent phosphorylation of the channel protein.

Many cellular processes, including plant responses to pathogens, are regulated by changes in cytosolic Ca^{2+} levels, where Ca^{2+} ions can serve to transduce a particular stimulus or stress that is imposed on a cell to target proteins that guide the cellular response (Bush, 1993). Changes in host-cytosolic Ca^{2+} concentrations that are due to an increased influx of Ca^{2+} across the plasma membrane in response to pathogens are well documented (Dixon et al., 1994; Ebel and Cosio, 1994). Nevertheless, an unequivocal demonstration of the involvement of plasma membrane Ca^{2+}-permeable channels mediating the transient increase of cytosolic Ca^{2+} in response to pathogen infection and the mechanisms by which pathogens induce Ca^{2+} uptake by the host cell have not been achieved.

In the interaction between tomato (*Lycopersicon esculentum* L.) and the fungus *Cladosporium fulvum*, disease resistance involves plant resistance genes (*Cf* genes), the products of which respond specifically to the products of a single avirulence (*avr*) gene in the pathogen (De Wit, 1992). The recognition of elicitors, coded by the *avr* genes, by putative receptors at the host-plasma membrane initiates a cascade of events that lead to the induction of host-defense responses. Immediately after recognition of a fungal elicitor by a receptor protein in the plasma membrane, the activation of ion fluxes and the production of H_2O_2 are the initial responses detected in plant cells, followed by transient changes in the phosphorylated state of various key proteins (Dixon et al., 1994). These proteins, including *cis*-acting elements and *trans*-acting factors, play a role in activating defense-related genes and inactivating several other non-defense-related genes (Dixon et al., 1994).

Previous work with tomato *Cf5* cell-suspension cultures showed that treatment with IFs containing the *avr5* elicitor (IF_4) led to a heterotrimeric 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., 1997).

Hyperpolarization of the electrical membrane potential difference resulted in the activation of Ca^{2+} (Ba^{2+})-permeable channels in the plasma membrane of tomato cells (Gelli and Blumwald, 1997). These Ca^{2+} (Ba^{2+})-permeable channels were active at membrane potential differences more negative than −120 mV and became much less active upon depolarizing the membrane where the electrochemical driving force for Ca^{2+} influx was reduced. At hyperpolarized membrane potential differences corresponding to the resting membrane potential differences (−121 mV in intact cells of soybean suspension cultures; Parsons and Sanders, 1989) channels remained essentially closed (20 mM extracellular Ca^{2+}; Gelli and Blumwald, 1997). Whole-cell and single-channel currents were active at membrane potential differences that were within the range of the Nernst potential for K^+ (−167 mV) and Cl^- (−158 mV), suggesting that these currents were not due to the movement of K^+ or Cl^-. Furthermore, the shift in the E_{rev} (with increasing extracellular concentrations of Ca^{2+}) toward the Nernst potential for Ca^{2+} suggested that these channels were more permeable to Ca^{2+} and Ba^{2+} than to

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* Corresponding author; e-mail blumwald@botany.utoronto.ca; fax 1–416–978–5878.

Abbreviations: avr, avirulence gene; *avr5*, *avr* from *Cf5*; *Cf5*, *Cladosporium fulvum* race 5; E_{rev}, reversal potential; IF, intracellular fluid; IF_4 (IF_5), IF from *C. fulvum* race 4 (race 5); I-V, current-voltage; P_o, probability that any one channel is open; pS, picosiemens.

K^+ or Cl^-. The single-channel conductance suggested that Ba^{2+} (11 pS) and Ca^{2+} (4 pS) permeated the channel pore slowly; however, the apparent K_d values (Ca^{2+}, 4.7 mM; Ba^{2+}, 7.4 mM) suggested that both ions interacted strongly with the channel pore. Moreover, the zero-current potentials suggested a comparable selectivity between Ba^{2+} and Ca^{2+} but a 20- to 25-fold selectivity over K^+ and a 10- to 13-fold selectivity over Cl^- (Gelli and Blumwald, 1997). Thus, a hyperpolarization-activated influx of Ca^{2+} into the host cell could provide a pathway for the elevation of cytosolic free Ca^{2+} concentrations that mediates the induction of several biochemical pathways that are part of the plant defense response (Ebel and Cosio, 1994).

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; Lohse and Hedrich, 1992; Ullrich and Novacky, 1991). 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. pathogen infection) perceived at the plasma membrane. This could involve the regulation of ion channels through the association with heterotrimeric G-proteins. Heterotrimeric 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 and Assmann, 1991). Of the heterotrimeric G-proteins that have been distinguished so far (elongation factors, tubulins, membrane-bound, heterotrimeric G-proteins, and small G-proteins), the latter has been implicated in mediating the defense responses of host cells to fungal elicitors (Sano and Ohashi, 1995).

An indirect pathway in heterotrimeric 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; Allen and Sanders, 1994b; Thiel and Blatt, 1994; Armstrong et al., 1995; Schmidt 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 chemosensory transduction of fungal signals (Grosskopf et al., 1990; Conrath et al., 1991; Felix et al., 1991; Xing et al., 1996). 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 and Boudet, 1987), and the opening and closing of stomata (Luan et al., 1993; Thiel and 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., 1990).

Here we present evidence for an elicitor-induced pathway for the influx of Ca^{2+} into the host cell, a prerequisite for the activation of the many biochemical processes that make up the defense response of the host cell. This IF_4-induced influx of Ca^{2+} is mediated through the activation of a host-plasma membrane, Ca^{2+}-permeable channel, which is modulated by a heterotrimeric G-protein-dependent activation of a protein kinase.

MATERIALS AND METHODS

Preparation of IFs

IFs from tomato (*Lycopersicon esculentum* L.) leaf tissue that was infected with *Cladosporium fulvum* were prepared according to the method of De Wit and Spikman (1982). The *Cf5* incompatible race 4 or compatible race 5 was inoculated onto cv Bonny Best (no known *Cf* genes). Control IF was obtained from uninoculated plants that were incubated under the same conditions as the inoculated plants. The IFs were precipitated with acetone (90%) and the pellet was then freeze-dried and resuspended in distilled water to give the original volume and stored overnight at −20°C. The preparations were centrifuged at 1650*g* for 20 min, the supernatant was discarded, and the pellet was resuspended in distilled water, boiled for 5 min, and centrifuged at 1650*g* for 20 min (although boiling causes some loss of activity of the *avr5* elicitors, it effectively inactivates many enzymes in the IF and precipitates considerable contaminating protein). The supernatant contains specific elicitors for each of the *Cf* genes on which the specific race of *C. fulvum* is avirulent (i.e. causes hypersensitive response). Generally, a ratio of IF:water of 1:32 (v/v) was used and contained between 0.10 and 0.35 μg protein/μL.

Plant Material

Cell suspensions of tomato were grown in 500-mL Erlenmeyer flasks containing 90 mL of Murashige-Skoog medium (Murashige and Skoog, 1962). The flasks were kept in the dark at 23°C on a rotary shaker (120 rpm) and subcultured weekly. Cells were 3 to 5 d old in all of the experiments. Protoplasts were prepared from 3- to 5-d-old cultured cells by digestion for 1.5 h in 2.0% cellulase (Seishin, Chuo-ku, Tokyo, Japan), 0.5% BSA, and 0.1% pectolyase (Seishin). The digested cells were filtered through Miracloth (Calbiochem) 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 $CaCl_2$, 15 mM Tris-Mes, 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 average (±SD) diameter of the isolated protoplasts under the above conditions was 28 ± 5 μm.

Measurements of Whole-Cell Currents in Isolated Protoplasts

Experiments were performed using conventional whole-cell and single-channel patch-clamp techniques (Hamil et al., 1981). Protoplasts were kept in a glass bathing chamber that contained $BaCl_2$ or $CaCl_2$ (as described in the legends to figures), 0.1 mM potassium glutamate, 1 mM $MgSO_4$, 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 and List, New York, NY) from borosilicate glass capillaries (Kimax-51, VWR Scientific, Boston, MA) coated with silicone (Sigmacote, Sigma) and fire polished, had a tip resistance of 5 to 10 MΩ when filled with 100 mM potassium glutamate, 1 mM $MgSO_4$, 0.05 mM $CaCl_2$ plus 0.1 mM 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid to give a final free Ca^{2+} 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 a 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 done at 23°C in a voltage-clamp mode using an amplifier (model 3900, Dagan, Minneapolis, MN) digitized on-line (TL-1 DMA Interface, Axon Instruments, Foster City, CA), stored on a 386-based 33-MHz computer and acquired and analyzed with pClamp 6.0.2 software (Axon). Voltage-pulse protocols were applied during data acquisition as described in the legends to the figures. Tail-current experiments were performed by activating inward currents with −160-mV pulses for 2.4 s followed by a step up in voltage to −60 mV. This protocol was repeated 15 times with a subsequent increase in the deactivating pulse of 10 mV. Whole-cell currents were filtered at 500 Hz with a four-pole Bessel filter (DAGAN) contained in the Dagan amplifier. All membrane potentials reported here have been corrected for liquid junction potentials (Barry and Lynch, 1991). The mean steady-state, whole-cell currents were measured at 4.8 s from the onset of the test voltage potential. Whole-cell currents were plotted against the respective membrane voltages.

Measurement of Single-Channel Currents in Isolated Protoplasts

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

Permeability ratios for solutions containing a mixture of monovalent and divalent ions were calculated as described by Allen and Sanders (1994a) and Gelli and Blumwald (1997). With this method the calculations of permeability ratios, where all three are ion permeable, necessitate the measurement of at least two values of the E_{rev} in two different solutions followed by the use of the zero-current equations for the two conditions to calculate relative permeabilities. Activities of the ions were derived by using activity coefficients estimated from the Debye and Hückel equation (Nobel, 1991). P_os were calculated with amplitude histograms obtained from single-channel recordings as:

$$P_o = \frac{\sum_{n=0}^{N} n \times A_n}{N \sum_{n=0}^{N} A_n}$$

where N is the number of channels in the patch, P_o is the probability that any one channel is open, and A_n is the area under the peaks with the n channels open (Labarca et al., 1980; Palmer and Frindt, 1986; Plant et al., 1994). The estimation of P_o was subject to the assumed value of n, which was estimated by the number of channels present in the single-channel records.

Chemical Reagents

GTP[γ]S, GDP[β]S, ATP[γ]S, ADP[β]S and okadaic acid, HA1004, staurosporine, and mastoparan were obtained from Calbiochem.

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 the Ca^{2+} currents, Ba^{2+} is the preferred cation, since Ba^{2+} blocks K^+ channels in the plasma membrane of both animal (Armstrong and Taylor, 1980) and plant cells (Wenger et al., 1994) that could potentially interfere with the recording of Ca^{2+} currents. Also, Ba^{2+} is much less likely to activate Ca^{2+}-dependent K^+ channels, and it 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 and 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 2 times greater than what would be obtained in the presence of 20 mM extracellular Ca^{2+} (Gelli and Blumwald, 1997).

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 in-

crements of 20 mV from a holding potential of −60 mV (Fig. 1, A–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 and Blumwald, 1997). At these higher concentrations, whole-cell currents were larger and they achieved a saturation of a cord conductance at −140 mV. The whole-cell I-V relationship exhibited characteristics similar to the product from P_o 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 and Blumwald, 1997). Figure 1A shows whole-cell currents recorded in the presence of 20 mM Ba^{2+} (on the extracellular side of membrane). The whole-cell inward currents were a result of Ba^{2+} moving into the cytosol in response to negative membrane potentials (Fig. 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 the 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 their direction at 20 mV in the presence of 20 mM Ba^{2+} (Fig. 1D) and at 10 mV in the presence of 5 mM Ba^{2+} in tail-current experiments (not shown). This shift in the E_{rev} toward $E_{Ba^{2+}}$ with increasing concentrations of Ba^{2+} suggested that Ba^{2+} was the main contributor to the whole-cell currents. Given the discrepancy between E_{rev} and $E_{Ba^{2+}}$, a weak permeability of glutamate through the channel cannot be ruled out. Using these E_{rev}s for the calculation of permeability ratios, a P_{Ca}/P_K of about 20, a P_{Cl}/P_K of about 2, and a P_{Ca}/P_{Cl} of about 10 were obtained.

The Ba^{2+} currents were time-dependent and inward-rectifying, i.e. 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. 1E). The nonlinear I-V relationship of the time-dependent, inward currents was evident. The addition of race 4 IF to the extracellular side of the membrane (IF_4, containing the *avr5* elicitor) resulted in a significant increase in the magnitude of the time-dependent inward currents (Fig. 1B), but no increase in the instantaneous current was observed. This increase in the inward current was observed within 1 to 3 min of adding the IF_4 (within the time required for the diffusion of IF_4). The I-V plot revealed an IF_4-induced 2-fold increase in the inward currents (Fig. 1E). This effect was race-specific, since the addition of race 5 IF, lacking the *avr5* elicitor (Fig. 1, C and E) or control IF (Fig. 1E) did not affect the time-dependent, inward currents.

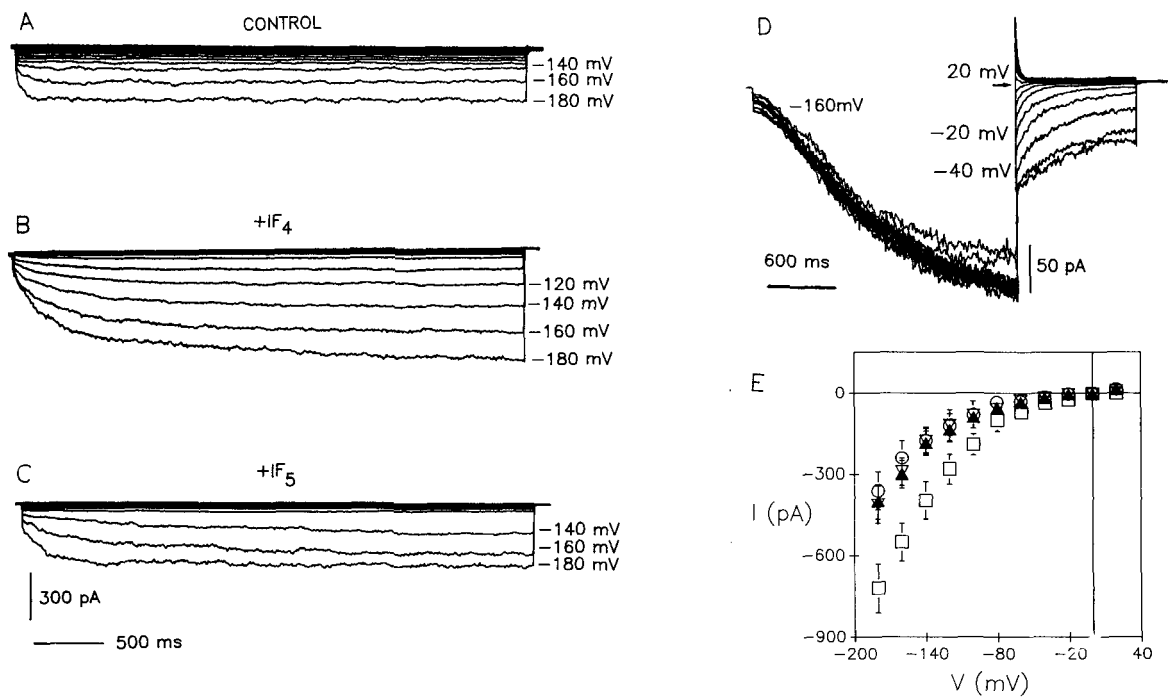

Figure 1. Inwardly rectifying Ba^{2+} currents stimulated by IF_4 (containing the *avr5* fungal elicitor). A, Voltage-dependent currents were activated by membrane potentials more negative than −100 mV when protoplasts were bathed in 20 mM $BaCl_2$ in the absence of IF_4. The time and amplitude scales shown refer to A, B, and C. B, The presence of IF_4 increased the magnitude of the steady-state, voltage-dependent currents. Currents reached a saturable maximum within 5 s from the onset of the voltage pulse. C, With protoplasts exposed to IF_5 (not containing the *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, I-V plot from protoplasts in A (○), B (□), C (▽), and control IF (▲; IFs from noninfected 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 means ± SD (n = 12).

Hyperpolarization-Induced Currents at the Single-Channel Level

Single-channel data revealed some inherent properties of the hyperpolarization-induced, Ca^{2+} (Ba^{2+})-permeable channels previously characterized (Gelli and Blumwald, 1997). In this report results obtained in single-channel experiments were corroborated with those obtained in whole-cell studies. Figure 2 shows some original single-channel recordings from isolated patches of membrane that were exposed to 50 mM Ca^{2+} on the extracellular side of the plasma membrane. These recordings revealed voltage-dependent channel activity (Fig. 2A) as previously reported (Gelli and Blumwald, 1997). Channel activity was detected at membrane potential differences that corresponded to the E_{rev}s of K^+ (−167 mV) and Cl^- (−158 mV), suggesting that the downward deflections corresponded to the movement of Ca^{2+}. Channel events became more frequent with increasingly negative membrane voltages (Fig. 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. 2B). At a membrane potential difference of −180 mV, the mean P_o was 0.10 ± 0.03. The P_o of the Ca^{2+}-permeable channels was dependent on the membrane voltage as shown by the sigmoid-type relationship (Fig. 2C). The P_o began to increase between −150 and −160 mV and reached its highest value at −190 mV. Significant P_o values were achieved by single channels beginning at −140 mV. Further characterization of the channels revealed a sensitivity to nifedipine and La^{3+} (Gelli and Blumwald, 1997). Channel currents were completely blocked by 10 μM La^{3+} (extracellular side of membrane) in a voltage-dependent manner and 50% inhibited by 100 μM nifedipine (extracellular side of membrane).

These hyperpolarization-induced, Ca^{2+}-permeable channels were more permeable to Ca^{2+} (Fig. 2D) than to K^+ or Cl^-. The E_{rev}s previously measured (14, 30, and 42 mV; Gelli and Blumwald, 1997) approached the equilibrium potential for Ca^{2+} (154 mV) as the concentration of extracellular Ca^{2+} (or Ba^{2+}) increased from 5 to 20 to 50 mM

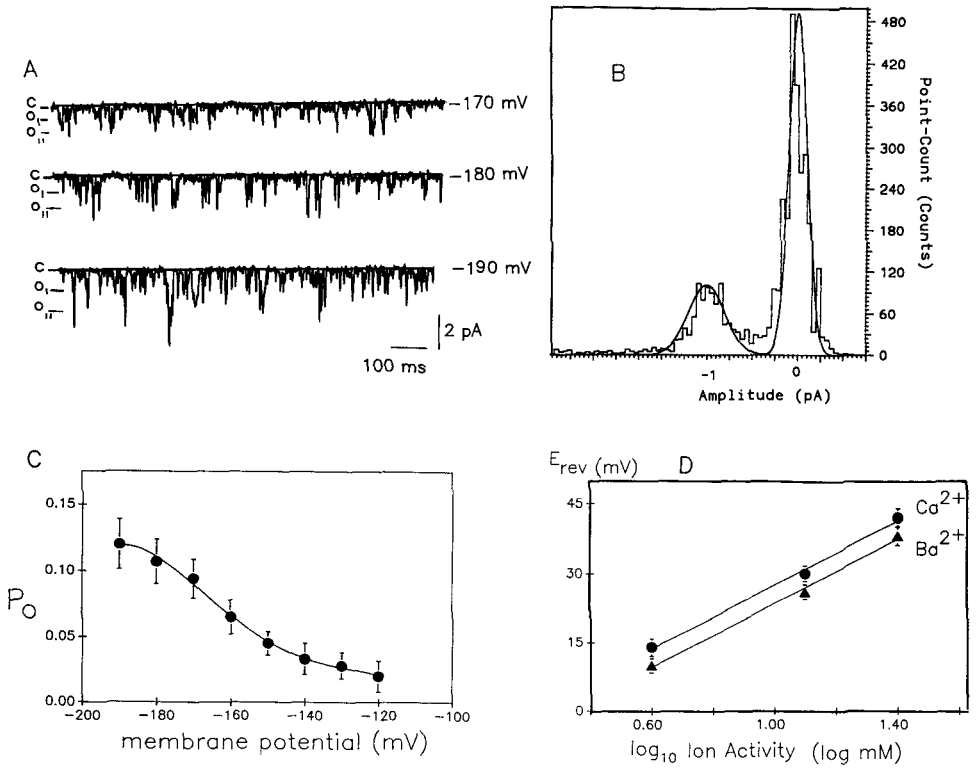

Figure 2. Some inherent properties of the hyperpolarization-induced, Ca^{2+}/Ba^{2+}-permeable channels. A, Voltage-dependent, single-channel currents with Ca^{2+} as the charge carrier. Single-channel recordings from isolated patches of membrane from protoplasts exposed to 50 mM Ca^{2+} (extracellular side of membrane) and polarized from membrane voltages of −170 to −190 mV. Downward deflections indicate one or two channels open and correspond to the movement of Ca^{2+} into the cytosol. c, Closed state; o, open state. B, An amplitude histogram of single-channel currents recorded at −180 mV under conditions similar to those 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 P_o for this patch of membrane was 0.09. C, The P_o of the channel is voltage-dependent. The channel P_o increased as the membrane voltage was made more negative and it reached its highest value at −190 mV. Values are means ± SD (n = 8). D, The measured E_{rev} varied linearly with the log of the extracellular Ca^{2+} concentration. Channel currents were measured over a range of extracellular Ca^{2+} or Ba^{2+} concentrations (5, 20, and 50 mM; activities: 3.9, 13.3, and 26.1 mM) and the corresponding E_{rev} were determined from a least-squares fit of the channel I-V plot. Data points are means ± SD (n = 5).

(activities: 3.9, 13.3, and 26.1 mM), respectively. The measured E_{rev}s varied linearly with the log of the extracellular Ca^{2+} concentration (Fig. 2D). Based on the E_{rev}s, permeability ratios ($P_{Ca^{2+}}/P_{K^+}$ and $P_{Ca^{2+}}/P_{Cl^-}$) (Gelli and Blumwald, 1997) revealed that these channels were about 25 times more permeable to Ca^{2+} than to K^+ and about 13 times more permeable to Ca^{2+} than to Cl^-. Similar permeability ratios were obtained with Ba^{2+} as the charge carrier (Fig. 2D). Although the permeability ratios were inferred by linear extrapolations to zero-current potentials away from the measured range of potentials, the extrapolations appeared reasonable and compatible with the relative currents through the channel.

An IF_4-Induced Increase in Single-Channel Activity

The effect of the fungal elicitor on the time-dependent inward currents was corroborated in single-channel experiments (Fig. 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. Figure 3A shows an original recording of single channels that were 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. 3B), as shown by the increase in channel events (downward deflections, Fig. 3B) and by the increase in P_o with respect to time (Fig. 3C). The mean P_o increased from 0.09 to 0.3 after the addition of IF_4 (Table I). The single-channel conductance (11 pS) did not significantly change in the presence of IF_4; thus, single-channel current levels remained unchanged. Single-channel activities were not affected by the addition of either IF_5 or control IF (not shown), suggesting that the changes in the single-channel kinetics were race-specific.

Heterotrimeric G-Proteins Mediate the Stimulatory Effect of IF_4

We tested the possible involvement of heterotrimeric G-proteins in the elicitor-induced activation of the plasma membrane Ca^{2+}-permeable channels by using nonhydrolyzable guanidine nucleotide analogs. In studies of heterotrimeric G-protein regulation of ion channels in animal (Brown, 1991) and plant cells (Fairley-Grenot and Assmann, 1991), 100 μM to 1 mM GTP and GDP analogs were normally used. Here we used 300 μM GTP[γ]S, an analog

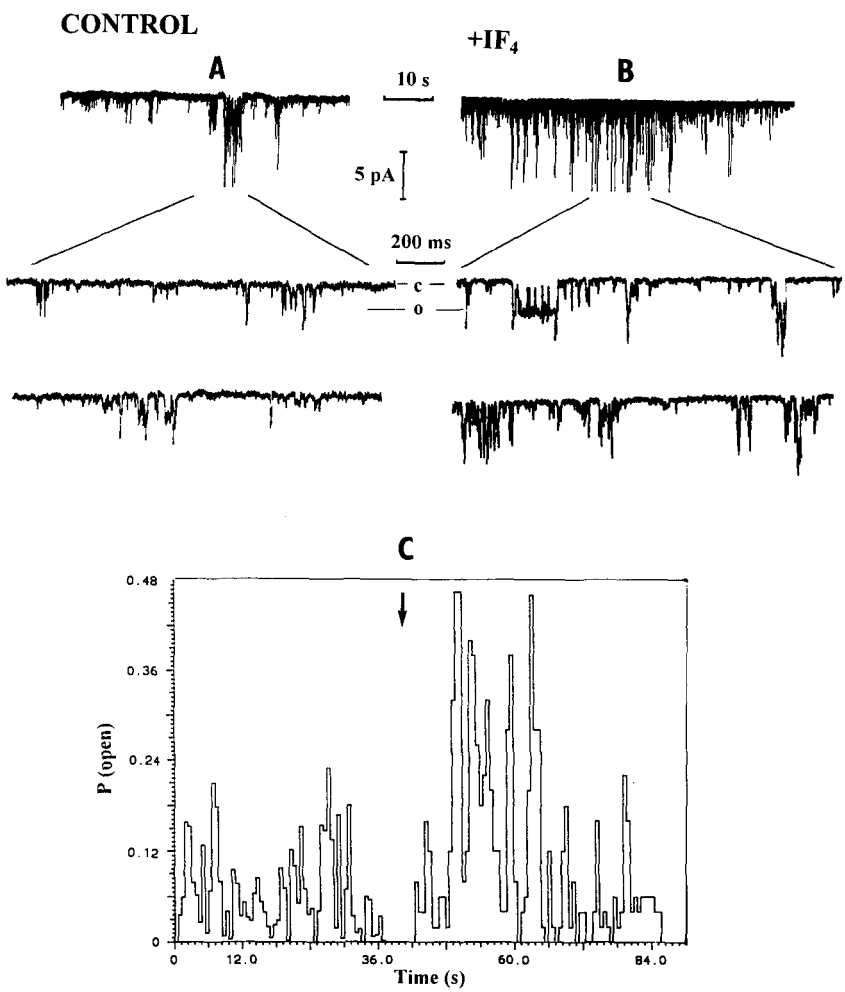

Figure 3. Ba^{2+}-permeable channels activated by IF_4. A, Outside-out patches of plasma membrane maintained at membrane potentials of −180 mV revealed voltage-dependent channels. Downward deflections (with a single-channel amplitude of −2 pA) from the closed (c) state to the open (o) state corresponded to Ba^{2+} moving into the cytosol of tomato protoplasts in response to the hyperpolarization of the membrane potential. B, IF_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 that was exposed to 20 mM Ba^{2+} in the absence (A) and presence (B) of IF_4. Similar results were obtained in eight other patches. C, A time course of channel P_o of single-channel recordings in A and B. P_o increased from 0.08 ± 0.02 to 0.31 ± 0.03 after the addition of IF_4. The arrow indicates the time at which IF_4 was added to the bath.

Table I. *The effects of IF_4, heterotrimeric G-protein regulators, okadaic acid, and HA1004 on single-channel kinetics*

The concentrations used in 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 Ba^{2+}. Values are means ± SD (n = 6).

Treatment	P_o
Control	0.10 ± 0.04
+IF_4	0.32 ± 0.04
+GTP[γ]S	0.30 ± 0.03
+Mastoparan	0.32 ± 0.04
+GDP[β]S	0.11 ± 0.06
+Okadaic acid	0.13 ± 0.05
+HA1004	0.08 ± 0.02
+IF_4 + GTP[γ]S	0.31 ± 0.06
+IF_4 + Mastoparan	0.34 ± 0.05
+IF_4 + GDP[β]S	0.10 ± 0.04
+IF_4 + Okadaic acid	0.46 ± 0.03
+IF_4 + HA1004	0.07 ± 0.02

that locks heterotrimeric 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[γ]S increased the magnitude of the time-dependent, inwardly rectifying currents as shown in the I-V relationship (Fig. 4A). However, in the presence of GDP[β]S, an analog that locks heterotrimeric 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. 4A).

Further support for the involvement of a heterotrimeric 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 independently of the agonist-receptor interaction, thus mimicking the effect of heterotrimeric 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, which was similar to that observed in the presence of GTP[γ]S (Fig. 4A). Moreover, when control nucleotides ATP[γ]S and ADP[β]S were tested on the whole-cell currents, no significant effects were observed (results not shown), providing further support for the specificity of the GTP[γ]S effect.

The effects of the guanosine nucleotide analogs were studied in the presence of IF_4. Similar increases in the magnitude of the whole-cell currents were observed with GTP[γ]S and mastoparan in the presence of IF_4 (Fig. 4B). Only the currents activated by the voltage pulses of −180 mV (measured at 4.8 s from the onset of the voltage pulse) are shown in Figure 4B. A comparison between currents recorded in the absence and presence of IF_4 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 IF_4 (Fig. 4B). The inactivation of the heterotrimeric G-protein by the addition of GDP[β]S totally abolished the elicitor-induced channel activation (Fig. 4B), further supporting the involvement of a heterotrimeric G-protein in the modulation of the IF_4 effect.

Single-channel experiments corroborated 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 IF_4 (Fig. 4C). This is demonstrated by the increase in the frequency of channel events in the original single-channel records (Fig. 4C) and in the reported P_o values (Table I). 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 in the P_o values, were comparable to those obtained in the presence of the elicitor. Single-channel experiments revealed that GTP[γ]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.

The IF_4 Effect Is Modulated by Phospho/Dephosphorylation Events

The involvement of membrane-associated effectors in the heterotrimeric G-protein-mediated, IF_4-induced stimulation of inward currents was examined by using okadaic acid, an inhibitor of phosphatases 1A and 2B. Channel activity that was measured in the presence of IF_4 (Figs. 3B and 4C), GTP[γ]S, or mastoparan (Fig. 4C) was moderately enhanced by the addition of okadaic acid (0.1 μM to the cytoplasmic side of the plasma membrane; Fig. 5B). This was reflected by an increase in P_o to values larger than those observed with IF_4 (Table I). However, okadaic acid had no significant effect in the absence of IF_4, GTP[γ]S, or mastoparan (Table I). This suggested that channel stimulation by IF_4 was a specific response to the fungal elicitor and that this response was mediated by a heterotrimeric G-protein.

If the inhibition of phosphatases 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 μM HA1004 (a protein kinase inhibitor) in the presence of IF_4; however, this reduction in channel activity was not observed in the absence of IF_4 (Table I). The effect of HA1004 in the presence of IF_4 was evident from the significant reduction in channel events as seen in the original single-channel record (Fig. 5C). A similar response was observed when staurosporine (a less specific protein kinase inhibitor) was added (not shown). Thus, both protein kinase inhibitors eliminated the IF_4-induced stimulation in channel activity. These results strongly suggest that the IF_4-induced channel activation may be mediated by the action of a protein kinase that stimulates channel activity by inducing the phosphorylation of the channel protein.

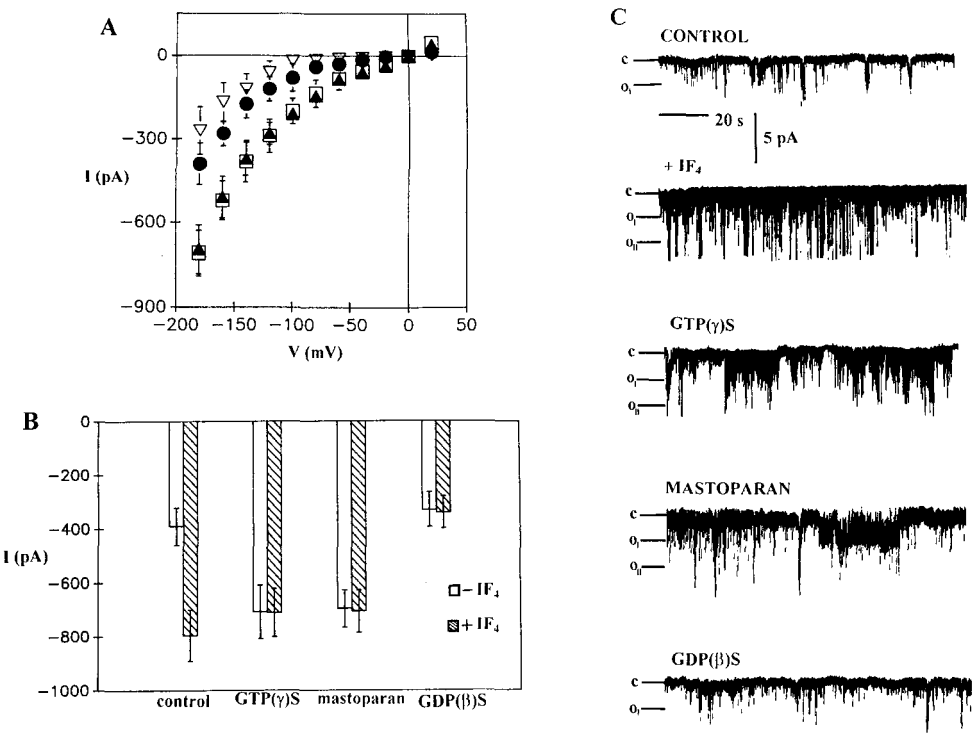

Figure 4. Activation of Ba^{2+} currents by IF_4 is mediated by heterotrimeric G-proteins. Whole protoplast currents were activated by a voltage protocol similar to that in Figure 1. A, I-V plot from whole-cell recording similar to that in Figure 1A (●) and from similar whole-cell recordings from protoplasts exposed to GTP[γ]S (□), mastoparan (▲), and GDP[β]S (▽). Both GTP[γ]S and mastoparan increased the magnitude of the e protoplast inward current by 2-fold in contrast to the effect of GDP[β]S. These plots were constructed as explained in Figure 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[γ]S, mastoparan, and GDP[β]S in the absence (white bar) and presence (hatched bar) of IF_4. GTP[γ]S and mastoparan increased voltage-dependent inward currents to magnitudes similar to those induced by IF_4. C, Single-channel recordings were made from isolated outside-out patches of membrane polarized to −180 mV. Downward deflections correspond to the opening of at least two channels (O_I, O_{II}). GTP[γ]S, mastoparan, and IF_4 activated single channels as shown by the significant increase in channel events in contrast to GDP[β]S. Values are means ± SD (n = 8).

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 gene in the plant that responds specifically to the product of a single *avr* gene in the pathogen (De Wit, 1992). The *avr* genes have been shown to code directly or indirectly for elicitor molecules, which most likely bind to receptors located at the plasma membrane of the host plant cells. In this study we used tomato cells near isogenic for the resistance gene *Cf5* and IFs containing the *avr5* gene product from race 4 or containing no *avr5* gene product from race 5 to demonstrate a race-specific effect of the fungal elicitor on the host-plasma membrane Ca^{2+}-permeable channels. There is substantial evidence demonstrating that unpurified or partially purified IFs exhibit the required specificity. De Wit and Spikman (1982) initially showed the necrotic effect of IF_4 on *Cf5* plants. Vera-Estrella et al. (1992) showed that similar IF_4s, 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, 1994b), the induction of a protein kinase cascade mediating the phospho/dephosphorylation of the plasma membrane H^+-ATPase (Xing et al., 1996), and the activation of a stimulatory type of heterotrimeric G-proteins (Xing et al., 1997) have also been shown to be specifically induced on *Cf5* tomato cells by IF_4.

The activation of Ca^{2+} (Ba^{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.

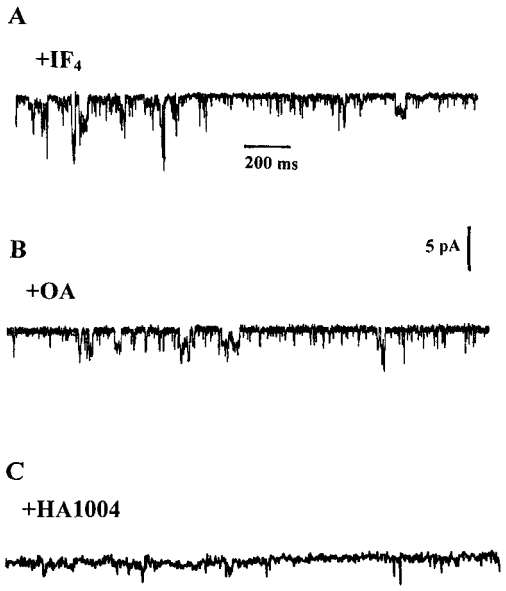

Figure 5. Channel activation by IF_4 involves the phosphorylation of Ca^{2+}-permeable channels. A, Single-channel recordings of outside-out patches of membrane from protoplasts (in the presence of 20 mM extracellular Ba^{2+}) exposed to IF_4 and polarized to −180 mV. IF_4 activated single channels as demonstrated by the increase in channel events. B, With 0.1 μM okadaic acid (inhibitor of protein phosphatase 1A and 2B) in the pipette and IF_4 in the bath, channel activity induced by IF_4 was moderately enhanced as shown by the frequency of channel opening. C, Channel activity induced by IF_4 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 six other different patches.

Although most of the studies of Ca^{2+} permeation through the plant plasma membrane have shown the activation of Ca^{2+}-permeable channels at depolarized membrane potentials (Huang et al., 1994; Thuleau et al., 1994; Piñeros and Tester, 1995), few studies have shown Ca^{2+} influx at hyperpolarized potentials (Stoeckel and Takeda, 1995; Gelli and Blumwald, 1997). Stoeckel and Takeda (1995) showed that the delayed outward-rectifying K^+ currents in protoplasts from leaves of *Mimosa pudica* L. were reduced by increased cytosolic Ca^{2+} levels that correlated with the Ca^{2+} influx at hyperpolarized potentials. This hyperpolarization-associated rundown in the K^+ outward current was reversibly blocked by La^{3+} (2 mM) and Gd^{3+} (0.5–2.0 mM), and it was reversed by decreasing external Ca^{2+} or depolarizing the membrane to less negative membrane potentials. As the driving force for passive Ca^{2+} entry increased with hyperpolarization, together with the blocking actions of La^{3+} and Gd^{3+}, the authors suggested that the Ca^{2+} influx, activated by hyperpolarization, was associated with the rundown in the K^+ outward current. The Ca^{2+} influx reported by Stoeckel and Takeda (1995) and those reported here (Gelli and Blumwald, 1997) 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 (Schwacke and Hager, 1992). Direct measurements of external Ca^{2+} with ion-selective electrodes and of Ca^{2+} fluxes using radiometric techniques (Dixon et al., 1994) revealed a large and transient Ca^{2+} influx with a concomitant acidification of the extracellular media, suggesting a correlation between the hyperpolarization of the host cell plasma membrane, the fungal elicitor activity, and Ca^{2+} influx. 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 heterotrimeric G-protein. Inward currents were activated by GTP[γ]S or mastoparan and inactivated by GDP[β]S in the absence or presence of IF_4, suggesting a heterotrimeric G-protein mediation of the IF_4-induced channel activity. Recently, the regulation of a guard cell ion channel to a receptor-activated heterotrimeric G-protein was demonstrated (Armstrong and Blatt, 1995). It is interesting that these heterotrimeric G-protein-linked receptors are activated by agents such as proteases, peptides, and small molecules, which bear some similarity to the purified fungal elicitors (Ebel and Cosio, 1994). In whole-cell experiments 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_o. Single-channel experiments revealed that IF_4 altered the P_o 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 has been shown in the regulation of plasma membrane K^+ channels (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, 1994b). Here the strong inactivation of the IF_4-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 IF_4 on the Ca^{2+}-permeable channels. Thus, protein kinases mediate the up-regulation of the Ca^{2+}-permeable channels, leading to increased cytosolic Ca^{2+} levels in response to the fungal elicitor.

In conclusion, our results show that the incompatible race-specific elicitors from *C. fulvum* activated tomato plasma membrane Ca^+-permeable channels. The activa-

tion of the channels provides a pathway for the influx of Ca^{2+} into the cells, resulting in increased cytosolic Ca^{2+} concentrations. The increase in cytosolic Ca^{2+} would initially activate plant defense responses that would limit or prevent cell death (Vera-Estrella et al., 1992, 1994a, 1994b; Degousse et al., 1994). Subsequently, cytosolic Ca^{2+} would contribute to the activation of a protein kinase cascade that allows the rephosphorylation of the plasma membrane H^+-ATPase by Ca^{2+}-dependent protein kinases, resulting in the restoration of normal cellular functions (Xing et al., 1996).

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