Calcium Currents in the A7r5 Smooth Muscle–derived Cell Line

Increase in Current and Selective Removal of Voltage-dependent Inactivation by Intracellular Trypsin

CARLOS A. OBEJERO-PAZ, STEPHEN W. JONES, and ANTONIO SCARPA

From the Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT We studied the effects of trypsin on L-type calcium current in the A7r5 smooth muscle cell line. Intracellular dialysis with trypsin increased the whole-cell current up to fivefold. The effect was concentration dependent, and was prevented by soybean trypsin inhibitor. Ensemble analysis indicated an increase in the number of functional channels, and possibly a smaller increase in the open probability, with no change in the single channel current. The shape of the current–voltage curve was unaffected. Trypsin also nearly eliminated inactivation of currents carried by Ba²⁺, but had little or no effect on the rapid inactivation process in Ca²⁺. This indicates that trypsin removes voltage-dependent but not Ca²⁺-dependent inactivation, suggesting the existence of distinct protein domains for these two mechanisms of calcium channel inactivation.

INTRODUCTION

Ever since Armstrong et al. (1973) reported that intracellular pronase removes inactivation of the sodium current of the squid giant axon, proteases have been used to characterize the kinetic mechanisms of sodium channel inactivation (Gonoi and Hille, 1987). Recently, Hoshi et al. (1990) reported that intracellular dialysis with trypsin also removes inactivation of the A-type potassium current. This might suggest that inactivation of voltage-dependent channels occurs through a common mechanism, involving an intracellular domain that is sensitive to proteases. The simplest physical picture of this inactivation process is the “ball and chain” model, initially proposed for sodium channels by Armstrong and Bezanilla (1977). Zagotta et al. (1990) demonstrated that a synthetic “ball” peptide can reconstitute inactivation of A-type potassium channels, after removal of the putative ball and chain by mutagenesis.

Address reprint requests to Dr. Obejero-Paz, Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106.
Does calcium channel inactivation occur by a process resembling inactivation of sodium and (A-type) potassium channels? The classical answer is that calcium channel inactivation is fundamentally different, depending on entry of calcium into the cell (Eckert and Chad, 1984). However, a slower voltage-dependent inactivation process clearly exists for calcium currents of invertebrate neurons (Brown et al., 1981), as well as vertebrate heart (Kass and Sanguinetti, 1984) and smooth muscle (Ganitkevich et al., 1987). We call this inactivation "voltage dependent" to be consistent with previous usage and to distinguish it from calcium- and/or current-dependent inactivation, although the voltage dependence of calcium current inactivation may result from coupling to activation kinetics, with no intrinsic voltage dependence of the microscopic inactivation process (Giannattasio et al., 1991).

In the heart, trypsin slows inactivation with Ca$^{2+}$ as the charge carrier, with little effect on inactivation with Ba$^{2+}$ (Hescheler and Trautwein, 1988). However, a brief report of trypsin effects on calcium currents in smooth muscle cells from coronary artery found no effect on inactivation with Ca$^{2+}$ (Klöckner, 1988).

The goal of this study was to characterize the effect of trypsin on calcium currents in the A7r5 smooth muscle cell line, where we have established procedures for separation of Ca$^{2+}$- and voltage-dependent inactivation processes (Giannattasio et al., 1991). We find that trypsin removes voltage-dependent but not Ca$^{2+}$-dependent inactivation in these cells. There is also a several-fold increase in the amplitude of the current, primarily due to an increase in the number of functional channels.

Preliminary reports of these results have appeared (Obejero-Paz et al., 1991a, b).

**METHODS**

Whole-cell currents were recorded at room temperature from A7r5 cells as described (Marks et al., 1990; Giannattasio et al., 1991). Currents were usually sampled at 5–10 kHz after filtering at 1–2 kHz with a 4-pole Bessel filter. Electrodes were 1.5–6 MΩ. In some experiments electrodes were coated with a silicone resin to decrease capacitance.

The extracellular solution contained (in mM): 112 NaCl, 5 CsCl, 1.2 MgCl$_2$, 10 CaCl$_2$ or BaCl$_2$ (as noted), 10 glucose, and 2.5 NaHEPES (pH 7.4). The intracellular solution contained (in mM) 120 CsCl, 4 MgCl$_2$, 5 TrisATP, 1 CsEGTA, and 2.5 CsHEPES (pH 7.2). Trypsin (type III, lot 18F-0828) and soybean trypsin inhibitor (type I-S, lot 96F-8115) were from Sigma Chemical Co. (St. Louis, MO).

Under these conditions, calcium currents appear well isolated within 3 min of dialysis (see Marks et al., 1990). The holding potential was usually −60 mV. In some batches of cells, a low threshold (T-type) current was evident, which was avoided by changing the holding potential to −40 mV (as noted).

**Ensemble Analysis**

Ensemble analysis was based on the method of Sigworth (1980a, b). Brief voltage steps were given to +10 mV from a holding potential of −40 mV every 2 s in cells dialyzed with trypsin. Currents were filtered at 2–5 kHz and sampled at 25 kHz, with digital Gaussian filtering at 1.5 kHz. Linear leakage and capacitative currents were subtracted. Since calcium currents are small with respect to the capacitative currents in this preparation, this procedure is particularly important. Data were rejected if residual capacitative transients caused large variances at the beginning of the depolarization, so the number of currents averaged varied. To minimize the effects of drift in current amplitude (due to rundown or the action of trypsin), currents were
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Scaled to have the same integral (Fenwick et al., 1982) and then averaged point-by-point. Cells where currents showed a strong initial rundown were not used for ensemble analysis. The variance and mean current of each ensemble were smoothed by a three-point running average (Bean et al., 1984) before final analysis.

The relation between the variance ($s^2$) and mean current ($I$) is parabolic:

\[ s^2 = iI - I^2/N \]  

(1)

where $i$ is the single channel current and $N$ is the number of functional channels (Sigworth, 1980a). We found it easier to assess agreement with theory using a linearized form (Kimitsuki et al., 1990):

\[ s^2/I = i - 1/N \]  

(2)

The parameters $i$ and $N$ were fit by weighted least-squares analysis. Data at small $I$ tended to deviate from the line, presumably due to variance contributed by capacity transients, and were not included in the fit.

The channel open probability ($p$) at the time of maximal current ($I_{\text{max}}$) was calculated from:

\[ p = I_{\text{max}}/(iN) \]  

(3)

R E S U L T S

**Trypsin Increases Whole-Cell Calcium Currents**

Intracellular dialysis with trypsin produced a slow increase in the amplitude of calcium current, requiring several minutes (Fig. 1A). The shape of the current-voltage curve (Fig. 1B) was not greatly affected, indicative of a true increase in calcium current rather than reduction of a countervailing outward current, for example. In four cells with 50 $\mu$g/ml trypsin, with 10 mM Ca$^{2+}$ as the charge carrier, the peak currents increased by 2.1 $\pm$ 0.2 (mean $\pm$ SEM) at peak effect (12–32 min).

A total of 60 cells were tested with trypsin (generally 50–100 $\mu$g/ml). 19 cells were rejected because of an excessive increase in leakage current, an increase in outward current, or (two cells) the presence of T current. 40 of the 41 remaining cells showed an increase in current during dialysis with trypsin, although the increase in current was often preceded or followed by rundown (see Fig. 4D). 10 of those cells were rejected because of slow clamp, which may have resulted from “plugging” of the pipette with trypsin.

In principle, dialysis of trypsin into the cell should follow an exponential time course, depending on the molecular weight of trypsin, the series resistance, and the cell volume (Pusch and Neher, 1988). We calculate that the intracellular trypsin concentration should reach 25% of the concentration in the pipette within 3 min (for a molecular weight of 24,000, with $R_s = 4$ M$\Omega$ and 30 pF cell capacitance). In practice, the time course of the trypsin effect varied from cell to cell, and there was usually a lag of several minutes before the onset of the effect. This could be due to obstacles to diffusion near the tip of a trypsin-containing pipette, or to intracellular trypsin inhibitors (Waxman and Krebs, 1978). The lag is useful, as currents recorded at early times during trypsin dialysis were indistinguishable from currents in control cells (Marks et al., 1990; Giannattasio et al., 1991), providing an internal control for each cell.

The effect of trypsin was dose dependent (Fig. 1C), although 100 $\mu$g/ml trypsin...
eventually caused an irreversible increase in leakage current, suggestive of a generalized deleterious effect on the cell. In two cells dialyzed with 100 µg/ml trypsin plus 80 µg/ml soybean trypsin inhibitor, the current showed only a slow rundown. Control cells showed variable degrees of rundown rather than spontaneous increases in current (Marks et al., 1990), and the rate and extent of inactivation were stable for tens of minutes in control cells (Giannattasio et al., 1991).

A rapid inactivation process is still obvious after prolonged dialysis with trypsin (Fig. 1 A), even with a double-pulse protocol (Fig. 2 A). Inactivation during 60-ms depolarizations to +20 mV was 54 ± 3% initially, and 42 ± 6% after 12–32 min of dialysis (n = 5, 50 µg/ml trypsin). On this time scale, inactivation is predominantly Ca\(^{2+}\) dependent, and can be fit by a single exponential plus a constant (Giannattasio et al., 1991). Trypsin had no effect on the time constant of inactivation (Fig. 2 B). Inactivation follows calcium entry rather than increasing monotonically with voltage both at early times and after trypsin (Fig. 2, C and D). However, after trypsin, more calcium entry was required to produce the same amount of inactivation (Fig. 2 D), as discussed below.

During longer depolarizations, control currents also show a slower voltage-dependent inactivation process, so a fit to the time course of a current requires the sum of two exponentials plus a constant (noninactivating) component (Giannattasio et al., 1991). This is initially true for trypsin-dialyzed cells, but at later times only one
exponential (plus a constant) is required (Fig. 3 A). At voltages where Ca\(^{2+}\)-
dependent inactivation normally dominates, trypsin had no effect on the more rapid
time constant for inactivation (Fig. 3 B).

Fig. 3 demonstrates that rapid Ca\(^{2+}\)-dependent inactivation survives trypsin treat-
ment, but the slower voltage-dependent inactivation seems to be absent. This can be
tested more directly with Ba\(^{2+}\) as the charge carrier, as Ba\(^{2+}\) produces little or no
Ca\(^{2+}\)-dependent inactivation in these (Giannattasio et al., 1991) and other cells
(Eckert and Chad, 1984).

Trypsin Removes Voltage-dependent Inactivation

Before trypsin action, inactivation is much slower with Ba\(^{2+}\) than with Ca\(^{2+}\) (Fig. 4 A),
and inactivation increases monotonically with voltage (Fig. 4 B). Trypsin steadily
reduces the amount of inactivation to near zero from an initial maximum of ~ 50% at
positive voltages (Fig. 4, B and C).
FIGURE 3. Effects of trypsin on calcium current inactivation during longer depolarizations in 10 mM Ca$^{2+}$. (A) Currents during 330-ms depolarizations, with 30-ms postpulses. The smooth curves superimposed on the currents during the prepulses are a two-exponential fit to the current at 2.3 min and a one-exponential fit at 30 min. (B) Time constants of inactivation measured from two-exponential fits at 2.3 min (□, △) and one-exponential fits at 30 min (■). Data in this figure are from cell c0310, $R_{p}$ 2.9 MΩ, whole-cell capacitance 27.1 pF.

FIGURE 4. Effects of 60 μg/ml trypsin on inactivation with 10 mM Ba$^{2+}$. (A) Currents at +10 mV in response to 800-ms prepulses and 40-ms postpulses, recorded at the times indicated. (B) Voltage dependence of inactivation, shown as in Fig. 2 C, measured at 3 (■), 11 (○), 22 (○), and 31 min (▲). (C) Time course of removal of inactivation by trypsin. Values are the postpulse/prepulse current ratio from the protocol of A. (D) Time course of the peak current measured during prepulses to +10 mV. Currents were normalized to that recorded ~4 min after establishment of the whole-cell configuration. Data in this figure are from cell e0509, $R_{p}$ 6.4 MΩ, whole-cell capacitance 21.4 pF, holding potential −40 mV.
As in Ca\textsuperscript{2+}, the peak amplitude of the current tends to increase during dialysis with trypsin, although this effect is often superimposed on rundown (Fig. 4 D). In 10 cells where long depolarizations were given rarely to limit use-dependent rundown, currents with Ba\textsuperscript{2+} increased by a factor of 5.6 ± 0.9 during trypsin dialysis, with maximal effect at 18–31 min. In these 10 cells, trypsin reduced inactivation from 49 ± 5 to 8 ± 3% (measured as the ratio of postpulse current to prepulse current, each measured 30 ms after the start of the voltage step, from the protocol of Fig. 3 A, with 330-ms prepulses). This indicates near complete removal of voltage-dependent inactivation on this time scale. After allowing for rundown, the two effects of trypsin (to increase peak current and to remove inactivation) developed with roughly the same time course (Fig. 4, C and D).

Trypsin has little effect on the peak current–voltage relations in Ba\textsuperscript{2+} (Fig. 5 A). Interestingly, in a cell where trypsin clearly acted to remove inactivation (Fig. 5 B), calcium current was still sensitive to a dihydropyridine (DHP) calcium channel antagonist (Fig. 5 C).

**Ensemble Analysis of the Effect of Trypsin**

The results reported above indicate that trypsin increases the amount of calcium current, in addition to affecting inactivation. It has often been stated that there are three ways in which a current could be increased: an increase in the number of functional channels, an increase in the probability that the channel is open, and an increase in the single channel current (\(I = Npi\)). In principle, these possibilities can be distinguished by ensemble analysis (Sigworth, 1980a, b).

Fig. 6 A illustrates averaged currents and variances, at an early time and at full trypsin effect. The variance during depolarization is clearly increased over the...
baseline variance (left), indicating detectable noise due to calcium channel gating. Similarly, the increase in current with trypsin was associated with a large increase in variance.

Ideally, a plot of the variance/current ratio vs. current should be a straight line,

### Table I

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<thead>
<tr>
<th>Effect of Trypsin on Activation Kinetics</th>
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Values are means ± SE, $n = 4$ (ensemble analysis) or $n = 5$ (current-voltage curves). All cells were dialyzed with 60 μg/ml trypsin, with 10 mM extracellular Ba²⁺. Control measurements were taken during the lag period before trypsin action, and “trypsin” indicates measurements at the point of maximal trypsin effect. Currents for ensemble analysis were measured at +10 mV. For an operational description of activation kinetics, current-voltage curves were fit by nonlinear least-squares analysis, assuming activation kinetics described by the Boltzmann equation, and a linear single channel current-voltage relation:

$$I(V) = G_{\text{max}}(V - V_{1/2})/[1 + \exp(-(V - V_{1/2})/k)]$$
where the y intercept equals \( i \) and the x intercept equals \( N_i \) (see Methods).

Unfortunately, the data (Fig. 6 B) deviate from a linear relationship in the critical region, early during the depolarization, where the current is small. This is presumably due to errors in subtraction of the capacity transient (see Methods). From inspection of Fig. 6 B, it is clear that the slopes (and x intercepts) are not well defined by the most reliable data, where the currents are larger. One extreme interpretation, that the slope is not distinguishable from zero, would yield \( i \approx 0.1 \) pA both initially and after trypsin action, despite a fivefold increase in macroscopic current. It seems clear that large changes in \( i \) (greater than twofold) can be ruled out. Changes in \( N \) and \( p \) were more difficult to separate, but our best estimates of the parameters suggest a substantial increase in \( N \), possibly with a smaller increase in \( p \) (Table I).

An increase in \( N \) should increase the maximal calcium conductance, while an increase in \( p \) might result from a shift in the voltage dependence of channel opening. Analysis of current–voltage curves (Table I) shows a more than twofold increase in conductance with trypsin, with no detectable effect on the slope of the activation curve, half-maximal activation voltage, or extrapolated reversal potential. This is additional evidence that the increase in current with trypsin is primarily due to an increase in \( N \).

DISCUSSION

Trypsin increases the L-type calcium current up to fivefold in the A7r5 cell line. Trypsin also removes voltage- but not Ca\(^{2+}\)-dependent inactivation.

Increase in Whole-Cell Current by Trypsin

Ensemble analysis suggests that the main effect of trypsin is to increase the number of functional channels (\( N \)), possibly with a smaller effect on the probability of being open (\( p \)). There was no detectable change in the shape of the current–voltage curve, again suggesting that the main effect is on \( N \).

Ensemble analysis (Sigworth, 1980a) depends on the assumptions that channels (a) gate identically, (b) gate independently, (c) and have only two conductance states, fully closed and fully open. We assume that channels are initially identical, as currents closely resemble those in control cells, and there was a delay of several minutes in most cells before any trypsin effect was apparent. Identical gating is also a plausible assumption at late times, when no voltage-dependent inactivation was observed, suggesting that the action of trypsin was complete. Similarities in kinetics at the single channel and whole-cell levels argue for independent gating of channels, although this is difficult to test rigorously. We have observed clear examples of substates in single channel recordings from these cells (Marks, T. N., unpublished observations), but that is not seen for most channels, so substates probably contribute a small fraction of the total macroscopic current.

It is not likely that the increase in current results primarily from the removal of voltage-dependent inactivation. With ensemble analysis, any process that is rapid with respect to the interval between depolarizations (here, 2 s) would appear as a change in \( p \), and any process with a mean duration much longer than 2 s would appear as a change in \( N \) (Sigworth, 1980b). Since the time constant of inactivation is \(< 1 \) s at \(-40\) mV (Giannattasio et al., 1991), changes in resting inactivation would predominantly...
affect \( p \) rather than \( N \). Also, the point of half-maximal inactivation is positive to \(-30\) mV (Giannattasio et al., 1991; Marks et al., 1990), so removal of resting inactivation should cause less than a twofold increase in availability of channels at our holding potentials of \(-40\) to \(-60\) mV. Voltage-dependent inactivation is slow with respect to activation (Yue et al., 1990; Giannattasio et al., 1991), so few channels would be expected to inactivate before they open, in contrast to sodium channels (Gonoi and Hille, 1987). Trypsin might act by removing a separate slow inactivation process (Schouten and Morad, 1989), but it is not known whether slow inactivation occurs in smooth muscle cells.

An increase in \( N \) is consistent with the results of Klöckner (1988) on coronary artery, where trypsin decreased the number of null sweeps in single channel records. It is possible that trypsin shifts channels away from "mode 0" gating (Hess et al., 1984), which would appear as a decrease in the number of null sweeps in single channel recording, or an increase in the amplitude of whole-cell currents.

Our estimate of \( i \) in 10 mM Ba\(^{2+}\) (0.16 pA; Table I) agrees well with the value of 0.13 pA calculated by ensemble analysis in cardiac cells (Bean et al., 1984), but is lower than the value of 0.28 pA (calculated by extrapolation of single channel data from Inoue et al., 1989) for calcium channels in smooth muscle from rabbit ileum. Calcium channels in A7r5 cells rapidly flicker between open and closed states during a burst (Marks and Jones, 1991), which could cause ensemble analysis to underestimate the single channel current.

Selective Removal of Voltage-dependent Inactivation by Trypsin

Trypsin selectively removes a slow voltage-dependent inactivation process (Fig. 4), preserving the more rapid Ca\(^{2+}\)-dependent inactivation (Figs. 1–3). This strongly supports the interpretation that the voltage- and Ca\(^{2+}\)-dependent inactivated states are distinct (Yatani et al., 1983; Gutnick et al., 1989; Giannattasio et al., 1991).

Does trypsin affect Ca\(^{2+}\)-dependent inactivation at all? The extent of inactivation is reduced during long pulses in Ca\(^{2+}\) (Fig. 3A), but this effect can be explained by removal of the slower voltage-dependent inactivation. It is interesting that more Ca\(^{2+}\) entry is required to produce an equivalent amount of inactivation after trypsin (Fig. 2D). That could be interpreted as a lowered sensitivity of inactivation to Ca\(^{2+}\). However, if Ca\(^{2+}\) acts locally on a channel-by-channel basis (Yue et al., 1990; Giannattasio et al., 1991), an increase in the number of functional channels would produce a greater rise in bulk cytoplasmic free Ca\(^{2+}\), but that should not affect inactivation. Our interpretation is supported by the lack of effect of trypsin on the rapid time constant of inactivation (Figs. 2B and 3B). Thus, our data are consistent with a selective removal of voltage-dependent inactivation by trypsin.

Our results clearly differ from those of Hescheler and Trautwein (1988) on cardiac calcium currents, where trypsin slowed inactivation with Ca\(^{2+}\) but not Ba\(^{2+}\) as the charge carrier, presumably reflecting selective removal of Ca\(^{2+}\)-dependent inactivation. It is possible that this reflects a real difference between calcium channels in cardiac muscle and smooth muscle, which are known to be molecularly distinct (Koch et al., 1990), as Klöckner (1988) saw no effect of trypsin on inactivation of calcium channels in coronary artery with Ca\(^{2+}\) as the charge carrier. It should also be noted that the concentrations of trypsin used here are 10–20 times lower than those
generally used by Hescheler and Trautwein (1988). One consistent finding in these studies is that trypsin increases the amplitude of calcium current.

Our observation that DHP antagonists remain effective in the absence of voltage-dependent inactivation (Fig. 5 B) agrees with the report of McCarthy and Cohen (1989).

**Possible Implications for Calcium Channel Structure**

It is striking that such a crude treatment as application of intracellular trypsin has a relatively specific effect, removal of voltage-dependent inactivation, on a variety of voltage-dependent ion channels (Armstrong et al., 1973; Hoshi et al., 1990). One attractive interpretation of this result is that ancestral voltage-dependent ion channels inactivated by a mechanism involving a trypsin-sensitive domain within the channel protein, and that this domain has been conserved in at least some present-day calcium, potassium, and sodium channels. Trypsin sensitivity is consistent with the idea that inactivation results from the movement of an exposed cytoplasmic domain, as in the ball and chain model proposed for inactivation of sodium and potassium channels (Armstrong and Bezanilla, 1977; Hoshi et al., 1990). Molecular data implicate cytoplasmic domains in inactivation of sodium and potassium channels (Stühmer et al., 1989; Vassilev et al., 1989; Hoshi et al., 1990), although mutations in sodium channels produce results not explained by simple forms of the ball and chain model (Moorman et al., 1990).

Voltage-dependent inactivation of L-type calcium current is ~100-fold slower than inactivation of sodium current, and generally slower than inactivation of A-type potassium channels (Rudy, 1988), so it is not obvious that the processes involved need be the same. However, quantitative differences in inactivation rates need not imply fundamentally different mechanisms. For example, in the ball and chain model, a decrease in the rate of binding of the ball to the mouth of the channel would produce both slower and less complete inactivation, which could explain the differences in inactivation between sodium and calcium channels. Mutations (Hoshi et al., 1990; Moorman et al., 1990) and naturally occurring variants (Rudy, 1988; Jan and Jan, 1989) can produce large, graded changes in inactivation rates.

There are other common features of inactivation among channel types, which could be considered evidence for a common mechanism. For several diverse channels, the voltage dependence of macroscopic inactivation can be explained by kinetic coupling of voltage-insensitive microscopic inactivation to the voltage-dependent activation process (Armstrong and Bezanilla, 1977; Aldrich et al., 1983; Cota and Armstrong, 1989; Chen and Hess, 1990; Zagotta and Aldrich, 1990; Giannattasio et al., 1991). Another general feature is a clear voltage dependence for recovery from macroscopic inactivation (Hodgkin and Huxley, 1952; Iverson et al., 1988; Gutnick et al., 1989; Giannattasio et al., 1991).

On the other hand, it is clear that multiple inactivation mechanisms do exist. Many channels have a slow inactivation process in the second-to-minute time range which is not affected by proteases, at least for sodium channels (Rudy, 1978). As noted above, trypsin does not seem to affect voltage-dependent inactivation of L-type calcium currents in cardiac cells, but does remove Ca^{2+}-dependent inactivation (Hescheler and Trautwein, 1988), in contrast to our results in smooth muscle. Trypsin-resistant
inactivation could reflect a completely distinct inactivation mechanism, or loss of trypsin sensitivity by divergent evolution.

We cannot rule out the alternative explanation, that trypsin-sensitive inactivation mechanisms have appeared independently several times during evolution, but this seems less parsimonious. At the least, our results strongly support the interpretation that voltage- and Ca\(^{2+}\)-dependent inactivation of calcium current are physically distinct processes. Further work is necessary to determine the site(s) of trypsin action, and to establish whether the structures affected in sodium, potassium, and calcium channels are truly homologous.

We thank Dr. Bartolo Giannattasio for helpful discussions, and Dr. Shinsaku Maruta for advice on proteases.

This work was supported by National Institutes of Health grant HL-41206 and an American Heart Association postdoctoral fellowship to C. A. Obejer-o-Paz.

*Original version received 19 February 1991 and accepted version received 7 June 1991.*

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