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Divalent Cation Channels Activated by Phenothiazines in Membrane of Rat Ventricular Myocytes

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Abstract. Phenothiazines (PTZ) such as chlorpromazine (CPZ) or trifluoperazine (TPZ) induced a sustained divalent cation-permeable channel activity when applied on either side of inside-out patches or on external side of cell-attached patches of adult rat ventricular myocytes. The percentage of active patches was ≈20%. In the case of CPZ, the K_d of the dose-response curve was 160 μM. CPZ-activated channels were potential-independent in the physiological range of membrane potential and were permeable to several divalent ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}). At least three levels of currents were usually detected with conductances of 23, 50 and 80 pS in symmetrical 96 mM Ba^{2+} solution and 17, 36 and 61 pS in symmetrical 96 mM Ca^{2+} solution. Saturation curves corresponding to the three main conductances determined in Ba^{2+} symmetrical solutions (tonicity compensated with choline-Cl) gave maximum conductances of 36, 81 and 116 pS (with corresponding half-saturating concentration constants of 31.5, 38 and 34.5 mM). The corresponding conductance values were estimated to 1.7, 3.3 and 5.2 pS in symmetrical 1.8 mM Ba^{2+} and to 1.1, 2.4 and 3.7 pS in symmetrical 1.8 mM Ca^{2+} (the value in normal Tyrode solution). Channels were poorly permeable to monovalent cations, such as Na, with a P_{Ba}/P_{Na} ratio of 10. A PTZ-induced channel activity similar to that described in cardiac cells was also observed in cultured rat aortic smooth muscle cells but not in cultured neuroblastoma cells.

PTZ-activated channels described in cardiac cells appear very similar to the sporadically active divalent ion permeable channels described in a previous paper (Coulombe et al., 1989). Surprisingly, when 100 μM CPZ were applied to myocytes studied in the whole-cell configuration, and maintained at a holding potential of −80 mV in the presence of 24 mM external Ca^{2+} or Ba^{2+}, no detectable macroscopic inward current could be observed, whereas the L-type Ca^{2+} current triggered by depolarizing pulses was markedly and reversibly reduced. The possible reasons are discussed.

Key words: Rat ventricular myocytes — Calcium background channels — Chlorpromazine — Trifluoperazine — W-7 — Polymixin B

Introduction

In the heart, transient systolic elevations in cytosolic free calcium concentration ($[Ca^{2+}]_i$) control many cellular functions and notably the contraction process. These cyclic changes in $[Ca^{2+}]_i$ are triggered by Ca^{2+} entry through L-type voltage-activated calcium channels which induces Ca^{2+} discharges from sarcoplasmic reticulum stores. Although, it is widely accepted that L-type Ca^{2+} channels represent the main pathway of calcium entry in active cardiac cells, the existence in quiescent cells of a small calcium influx has been recognized for a long time (Niedergerke, 1963). Such a source of calcium entry can help to maintain homeostatic $[Ca^{2+}]_i$ by operating a balance between diastolic Ca^{2+} extrusion and tonic Ca^{2+} influx. The mechanism responsible for the resting calcium influx in cardiac cells is still poorly understood.

During the last few years, several types of divalent cation permeable channels different from the L-, T-, or N-type calcium channels have been observed in cardiac membranes either in sarcolemmal vesicles from calf ventricular muscle incorporated in artificial lipid bilayers (Rosenberg, Hess & Tsien, 1988) or, more directly, in

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single adult rat ventricular myocytes (Coulombe et al., 1989). In our experiments, such background, tonically-active nickel-insensitive calcium channels were detectable in a large membrane potential range (including potentials as negative as −180 mV), i.e., far from that in which classical voltage-dependent calcium channels activate. Such a channel activity was not frequently observed (about 20% of tested patches), occurred in irregular bursts separated by long-lasting quiescent periods (several min) and exhibited complex gating behavior. Two or three conductance levels (≈20, 40 and 80 pS) were frequently detected. Similar calcium and barium permeable channels giving rise to three different types of unitary currents have been subsequently described in glomerulosa cells from adrenal gland (Durroux et al., 1992). On the other hand, the existence in lymphocytes or mast cells of a very different type of Ni^{2+}-sensitive channels with extremely low single-channel conductance (≈20 fS) and high calcium-selectivity (channels carrying Ca^{2+} about twice as well as Ba^{2+} or Sr^{2+}) has been proposed as a means for electrically nonexcitable cells of replenishing their empty calcium stores (Hoth & Penner, 1992; Zweifach & Lewis, 1993). Upon intracellular calcium store depletion such channels appear to give rise to an inward calcium current activated by a novel calcium-influx factor (Parekh, Terleau & Stühmer, 1993; Randriamampita & Tsien, 1993; *see also* Clapham, 1993).

Here we report that following application of phenothiazines, (compounds commonly used in human therapy as antipsychotic drugs), and other anticalmodulin agents we consistently observed in membrane patches of rat ventricular myocytes the development of a channel activity quite similar to that sporadically recorded in the absence of drug in the same type of cardiac membranes (Coulombe et al., 1989). We also observed a PTZ-induced activity in cultured rat aortic smooth muscle cells. A preliminary report of some of these findings has appeared elsewhere (Lefevre, Coulombe & Coraboeuf, 1994).

Materials and Methods

Preparation of Myocytes

Single ventricular cells were obtained from adult rat hearts by enzymatic dissociation as described previously (Lefevre, Coulombe & Coraboeuf, 1991). The digested ventricles were maintained in "KB medium" (Isenberg & Klöckner, 1980), the left and right ventricle were dissected into small fragments of tissue. Cells were mechanically dispersed by gently shaking small pieces of tissue in a plastic Petri dish containing K^+-rich solution and kept at room temperature before being used. The dish was then mounted on the stage of an inverted microscope. All experiments were performed at room temperature (19–24°C).

Cultured Cells

Differentiated neuroblastoma × glioma NG 108-15 cells were used according to Rouzaire-Dubois (1990). Rat-aortic smooth muscle cells were cultured according to Chamley-Campbell et al. (1979).

Current Recordings

We used the classical four patch-clamp configurations cell-attached, inside-out, outside-out and whole-cell. Patch pipettes of a resistance of 1–5 MΩ were pulled from borosilicate glass capillaries (Corning code 7740) using a two-stage vertical puller and were not fire-polished before use. The currents were recorded using a patch clamp amplifier (Axopatch 200A) and filtered through an 8-pole Bessel low pass filter 920 LPF (Frequency Devices) setting of 1 kHz for single-channel recordings and 20 kHz (−3 dB point) for macroscopic current recordings. Stimulus protocols, online data acquisition and analysis were controlled by a microcomputer (Tandon, MCS 486) and a software program (ACQUIS1) developed for us by Gerard Sadoc. In the whole-cell clamp condition, the resistance in series to the cell membrane was compensated, whereas neither cell membrane capacitive current nor leakage current was compensated. Ionic currents were digitalized at 5 kHz, using an S200 interface (Cambridge Research System, Cambridge, UK) and stored in a computer file for latter analysis. Currents were elicited by 300 msec voltage step to 0 mV from a holding potential of −80 mV, at a frequency of 0.1 Hz. For single-channel recordings, currents were stored upon digital audio tape (DTR 1200, Biologic) and for subsequent analysis data were digitalized at a rate of 5 kHz to the computer. Currents were retrieved on a HP Laserjet III (Hewlett-Packard, San Diego, CA).

Data Analysis

Elementary conductances were determined as previously reported (Coulombe et al., 1989). Dose-effect data points were fitted to Hill equation and elementary conductance data points to Michaelis-Menten equation by use of the nonlinear least-squares gradient-expansion algorithm of Marquardt, using the program FIT developed for personal computers by Gérard Sadoc. As already pointed out in our previous paper (Coulombe et al., 1989) ion channel open probability (P_o) was difficult to estimate because sustained channel activity showed complex gating behavior (multiple current levels of different amplitudes corresponding to channel various conductance substates). To circumvent this problem we used the method previously described. In brief, for a period of time of at least 4 min starting at the moment when the maximum channel activation was reached, mean single-channel current was computed by calculating the time integral of single-channel current over this period divided by the integral of the maximum current, which would flow through the same channels over the same period if they were permanently open.

Solutions

The K^+-rich solution in which cells were maintained before being used, contained (in mM): 135 KCl, 5 NaCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, the pH was adjusted to 7.4 with KOH. A stream of solution from one of a series of five piped outlets continuously superfused the cell from which recording was being made. The flow rate of perfusion solutions was 50–100 μl/min. For results described in Fig 1. to Fig 4, obtained in inside-out patch configuration, the control superfusion solution of the internal face of the membrane in which phenothiazines were added at appropriate concentration, contained (in mM): 60 K-aspartate, 60 Cs-aspartate, 10 KCl, 0 or 1 $BaCl_2$, 10 HEPES, 1 EGTA; pH was adjusted to 7.4 with CsOH. The pipette solution contained (in mM): 96 $BaCl_2$, 10 HEPES; pH was adjusted to 7.4 with BaOH. For whole-cell experiments, the bath solution was the same as that in which cells were maintained, with addition of 1 μM ryanodine. The superfusion solution contained (mM): 105 choline-Cl, 20 TEA-Cl, 1 $MgCl_2$, 10 or 24 $CaCl_2$, or 24 $BaCl_2$, 10 HEPES, 10 glucose, 0.001 ryanodine,

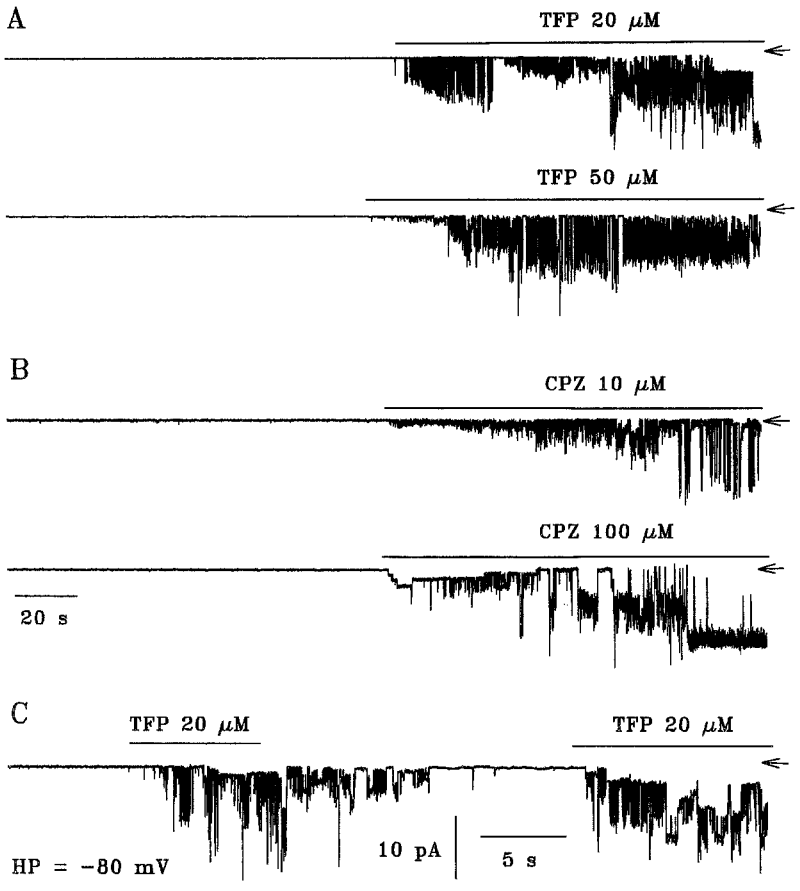

Fig. 1. Phenothiazine-induced single Ba^{2+} channel activity in previously quiescent inside-out membrane patches from rat ventricular myocytes. Trifluoperazine (TFP), in (*A*), and chlorpromazine (CPZ) in (*B*) were applied at the indicated concentrations to the internal face of the membrane patches during the periods of time indicated by horizontal bars. In (*C*) are shown the reversibility and reproducibility of the effect of two successive short exposures to TFP. Patch membrane holding potential (HP = −80 mV) was the same for all the traces. Arrows indicate zero current levels. Downward deflections of the current trace represent inwardly directed membrane currents. Vertical scale in *C* is applicable to *A* and *B*. Horizontal scale in *B* is applicable to *A*.

3 4-aminopyridine; pH was adjusted to 7.4 with (CsOH). In some experiments ryanodine was omitted and 10 mM caffeine added in order to empty the sarcoplasmic reticulum Ca pool. The pipette solution contained (mM): 105 Cs-aspartate, 20 TEA-Cl, 4 MgATP, 3 $MgCl_2$, 10 EGTA, 10 glucose, 2 KH_2PO_4, 10 HEPES; pH was adjusted to 7.4 with CsOH. Otherwise, solutions were as indicated in figure legends.

Results

Phenothiazine Induced Barium-Permeable Channel Activity

In inside-out patches, when the patch pipette contained 96 mM Ba^{2+}, addition to the internal superfusion bath medium of micromolar concentrations of phenothiazines (PTZ), induced, in a few seconds or less, single channel activity in previously quiescent inside-out membrane patches from ventricular rat myocytes. Figure 1*A* and *B* shows typical segments of single channel current recordings obtained from 4 different membrane patches, before and during application of trifluoperazine (TFP) or chlorpromazine (CPZ) when membrane patch potential was maintained at −80 mV. Such a fast-developing clearly detectable PTZ-induced activity was observed systematically in ≈20% of at least 250 membrane patches. Usually, channel activity persisted until the break of the patch membrane when PTZ perfusion was maintained. When TFP was used instead of CPZ patches tended to get broken faster and more frequently. When low concentrations of PTZs were applied to the membrane for short periods (less than 10 sec), the effect was fairly reversible and reproducible as shown in Fig. 1*C*, whereas superfusions for longer periods or with higher drug concentrations were always followed by persisting residual activity. As shown in Fig. 1, PTZ-induced activity exhibited complex gating behavior with several levels of elementary conductances. At the beginning of PTZ superfusion, channel activity was usually small then increased progressively until reaching a sustained, although somewhat irregular, high value. A similar type of sustained activity was observed in cell-attached configuration when PTZ was added to the patch pipette medium. In the absence of PTZ in the pipette solution the probability that episodic spontaneous activity of Ba^{2+}-permeable channels occurred during the first two seconds following patch formation was extremely low (<1%). In contrast, when PTZ was present in the pipette solution, sustained channel activity occurred immediately on patch formation in 4 out of 21 patches and this activity persisted when patches were excised to inside-out configuration.

Current-Voltage Relationships and Elementary Conductances

In Fig. 2*A* current traces recorded at various membrane potentials are shown in a typical experiment in which Ba^{2+} channel activity was induced by 20 μM CPZ applied to the internal face of an inside-out patch. The chosen recording segments correspond to periods when channel activity appeared relatively regular. In these traces several current levels are clearly distinguishable. Current-voltage relationships corresponding to the three current levels most frequently observed in 8 experiments similar to that of Fig. 2*A* are shown in Fig. 2*B*. The *I-V* curves are not linear, a characteristic that results very likely, at least in part, from asymmetrical concentrations of the main permanent ion, Ba^{2+} concentration being very low or nil on the intracellular face of the membrane. The fact that the three curves do not reach zero current values, or only at strongly positive potentials, suggests (according to the Goldman-Hodgkin-Katz theory) that the channels are much more permeable to Ba^{2+} than to K^+ or Cs^+. When calculated in the −100 to −60 mV range, elementary conductances are 23, 50 and 80 pS (Fig. 2*B*, broken lines).

Dose-Response Curve of CPZ-induced Channel Activity

Because CPZ-induced channel activation appeared to occur in a dose-dependent manner (*see* Fig. 1*B*), we tried to determine the dose-response relationship of CPZ in inside-out experiments by using cumulative rising CPZ concentrations. It appeared very difficult to use concentration of CPZ higher than 500 μM because of increasing patch membrane fragility, possibly as a result of CPZ accumulation in the intramembranar phase (Lieber et al., 1984). Figure 3*A* shows an example of current traces obtained on the same patch during successive applications of different concentrations of CPZ. Figure 3*B* shows the dose-response relationship obtained by plotting relative mean channel current calculated as described in Materials and Methods, vs. the concentration of CPZ. When the data were fitted with the following Hill equation:

$$y = 1 - 1/(1 + (S/S_d)^{n_H}) \quad (1)$$

where y is the relative mean channel current, S the concentration of CPZ (in M), S_d the apparent dissociation constant corresponding to the concentration of CPZ at which half-maximal channel activation occurred and n_H the Hill coefficient, the best fit gave the following values: $S_d = 1.6 \times 10^{-4}$ M and $n_H = 0.89$.

Voltage-dependence of CPZ-induced Channel Activity

To determine whether CPZ-activated Ba^{2+} permeable channel activity was dependent on membrane potential or not, we computed the mean patch membrane current activated by 20 μM CPZ at different membrane potentials. Figure 4 shows the result obtained for 4–6 different experiments. The slope of the straight line determined by linear least-square fit of individual data was 0.000189 (correlation coefficient 0.093) indicating that the slope is not statistically different from zero. It can therefore be concluded that channel activity induced by CPZ is voltage independent.

Permeability to Cations other than Ba^{2+}

Experiments were carried out using Ca^{2+}, Mg^{2+} or Mn^{2+} instead of Ba^{2+} in the pipette medium. Typical results obtained in 4 different experiments using 48 mM divalent cations are shown in Fig. 5. It can be seen that in each case at least two levels of conductance are observable. In fact, most often, three levels can be detected. This demonstrates that several divalent cations, including Ca^{2+}, can cross the channels activated by PTZs. Twenty successful inside-out were studied with Ca^{2+} in the pipette solution, 14 with Mg^{2+} and 5 with Mn^{2+}. All of these experiments gave results similar to those shown in Fig. 5. Elementary conductances with Ca^{2+} as current carrier were somewhat smaller than those measured with Ba^{2+} as current carrier. When Ca^{2+} was used the three main conductances calculated between −100 and −60 mV were 17, 36 and 61 pS, from 6 different patches. Using the corresponding three main conductance levels obtained in six other patches when Ba^{2+} was used as charge carrier, the ratio g_{Ca}/g_{Ba} was calculated as being 0.74, 0.72 and 0.76 respectively. When Mn^{2+} was used as charge carrier however the proportion of successful recordings was, for some unknown reason, substantially less (1/15) than the proportion of 1/5 observed when other divalent cations were used. It is possible that, although permeant, Mn^{2+} ions also exerts some inhibitory effect on PTZ-activated channels. On the other hand, when 150 mM Na^+ instead of 45 mM divalent cations were added in the pipette solution, no channel activity was observed in the presence of CPZ in 32 out of 33 patches. Only in 1 patch some channel activity of unclear origin could be recorded.

Channel Conductances as a Function of Ba^{2+} Concentration

We used symmetrical Ba^{2+} concentrations ranging from 6 to 200 mM to determine the values of the three main conductance levels at each Ba^{2+} concentration. As

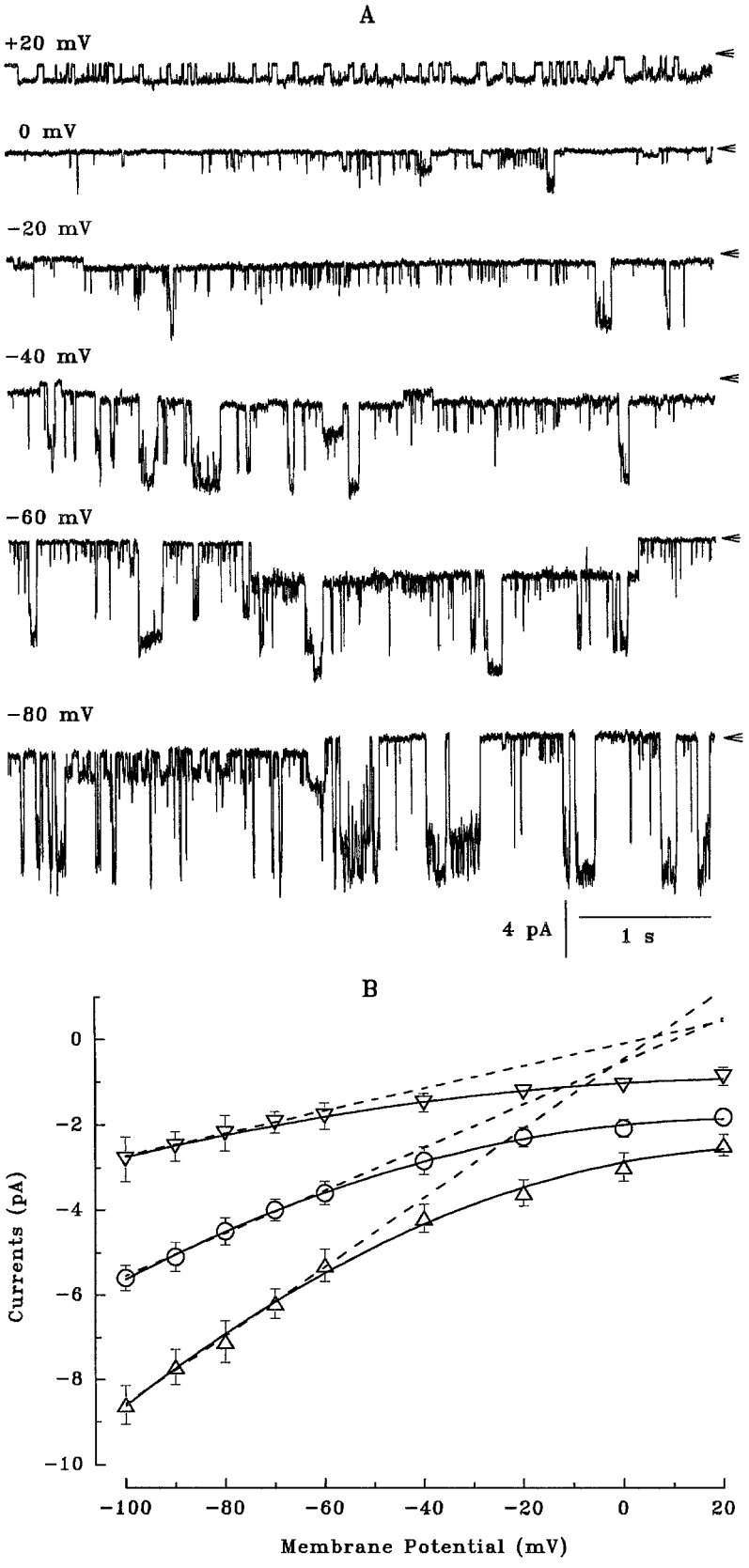

Fig. 2. CPZ-induced single Ba^{2+} channel activity at different holding potentials in inside-out membrane patch. (*A*) Representative records of channel openings induced by 20 μM CPZ, showing multiple current levels. Membrane potentials are indicated above each recording. Arrows indicate zero current levels. (*B*) Single-channel current amplitudes as a function of membrane potential for three of the most frequently observed current levels, (mean values ± SD (bar), 8 experiments). The broken lines represent least-squares fits of the data for membrane potentials more negative than −60 mV, gave slope conductances of 26, 50 and 80 pS. In the *I-V* relationships, the points are connected by an arbitrary polynomial relation with no theoretical significance.

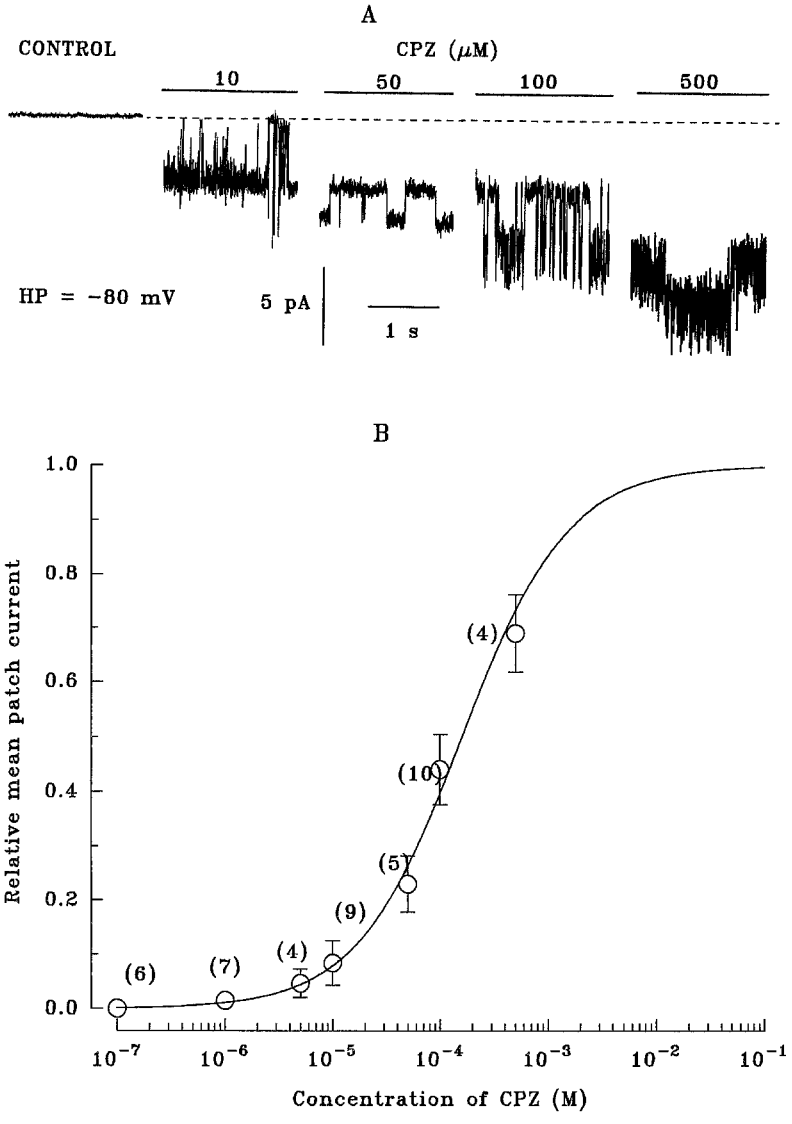

Fig. 3. Effect of increasing CPZ concentration on Ba^{2+} channel activity. (*A*) Segments of steady-state recordings of barium currents obtained for successive exposures of the internal face of an inside-out patch to increasing CPZ concentrations as indicated above each trace. The control recording was obtained in the absence of CPZ. Other traces were taken at the time when the maximum channel activation was reached. Broken line represents zero current level. (*B*) Concentration-response relationship of the relative mean patch current activated by CPZ. The curve was fitted to the mean values (open circle) according to the Eq. (1) with a S_d of 160 μM and a slope factor n_H of 0.89. Numbers in parenthesis and bars denote numbers of measurements obtained from different patches and corresponding SEM values respectively.

shown in Fig. 6 the relations were clearly nonlinear indicating that Ba^{2+} current tends to saturate with increasing permeant ion concentration. For each of the main channel states, the maximum conductances were 36, 81 and 116 pS whereas the corresponding half-saturating concentration constants were 31.5, 38 and 34.5 mM. From the results shown in Fig. 6 the main conductance states extrapolated to a Ba^{2+} concentration of 1.8 mM are 1.7, 3.3 and 5.2 pS whereas in the presence of 1.8 mM Ca (i.e., the Ca^{2+} concentration in normal Tyrode solution) the corresponding conductances would be 1.1, 2.4 and 3.7 pS.

Channel Activity in the Simultaneous Presence of External Divalent and Monovalent Ions

These experiments were performed in outside-out configuration. Although previous experiments have shown that Na^+ ions do not easily permeate PTZ-activated channels, this does not exclude that these ions when applied externally might exert some modulating influence on the current carried by divalent ions. Therefore we measured channel activity induced by 20 μM CPZ in the simultaneous presence of 24 mM Ba^{2+} (or Ca^{2+}) and 100 mM Na^+ in the superfusion solution of the external face of the membrane. Figure 7 shows typical results obtained in these conditions. It can be seen that current amplitudes measured at a membrane potential of −80 mV are not markedly different from those measured in the absence of external sodium except that substates seem somewhat smaller in the presence than in the absence of sodium when compared with the large conductance level as indicated by the amplitude histograms shown in the right part of Fig. 7. When 50 mM Na^+ were included in the pipette solution and the external medium contained 100 mM Ba^{2+} and 50 mM Na^+, both inward and tiny outward channel currents were observed with a reversal

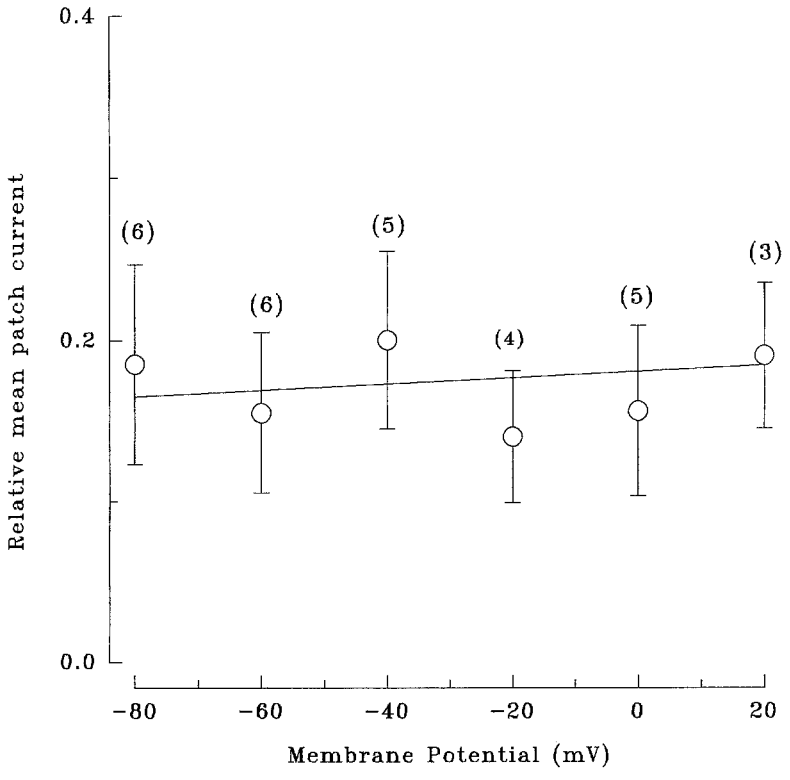

Fig. 4. Voltage-independence of CPZ-induced Ba^{2+} channel activity. Relationship between relative mean patch current activated by 20 μM CPZ and membrane potential. Open circles and error bars show mean and SEM values obtained from different inside-out patches (patch number in parenthesis).

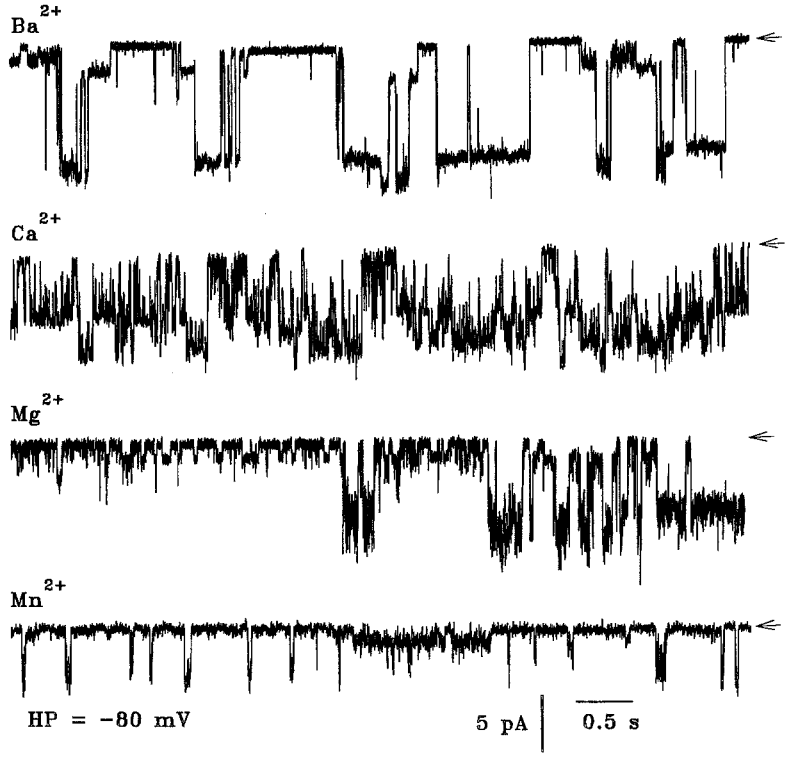

Fig. 5. Representative recordings of steady-state CPZ-induced single-channel activity with different divalent cations used as the charge carrier. Current traces recorded from different inside-out membrane patches. The charge carrier used is indicated above each current trace. HP was −80 mV. In all these experiments, the bath solution contained (in mM): 60 K-aspartate, 60 Cs-aspartate, 10 KCl, 10 HEPES, 1 EGTA, 1 EDTA, 20 μM CPZ; pH was adjusted to 7.4 with CsOH. The pipette contained (in mM): 48 of one of the following salts, as required: $BaCl_2$, or $CaCl_2$ or $MgCl_2$, or $MnCl_2$ and 10 HEPES; pH was adjusted to 7.4 with $Ba(OH)_2$, or $Ca(OH)_2$, or N-methyl D-glucamine.

potential around +35 mV (n = 4). From these experiments a ratio $P_{Ba}/P_{Na} \approx 10$ can be obtained using the equation derived from constant field theory and the assumption of zero surface potential difference (Rosenberg et al., 1988).

Effect of other Internal Substances or Ions

Because phenothiazines are potent calmodulin antagonists, we tested the effects of W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide) a phenothiazine un-

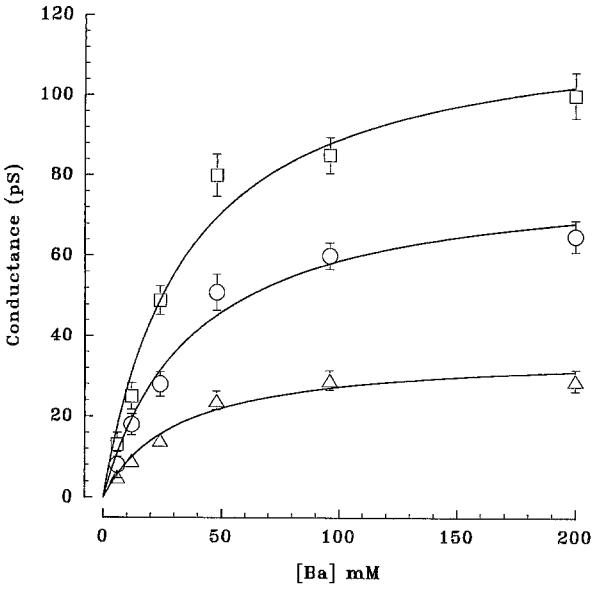

Fig. 6. Dependence of the three main conductances of CPZ-activated channels on the concentration of Ba^{2+} in symmetrical solutions. Channel conductances, obtained from the slopes of *I-V* plots in symmetrical solutions, is plotted as a function of Ba^{2+} concentration on both sides of the patch membrane. The smooth curve was drawn according to the following Michaelis-Menten equation $\gamma = \gamma_{max}/(1 + K_{Ba}/[Ba])$ where γ is the channel conductance, [Ba] the barium ion concentration, γ_{max} the maximal channel conductance and K_{Ba} the half-saturating concentration. The values of γ_{max} and K_{Ba} for the three conductance levels were 35, 69 and 110 pS and 31.5, 38 and 34.5 mM, respectively, as determined by a nonlinear least-squares fit of the data (*see* Materials and Methods). Channel activity was obtained in the presence of 6, 12, 24, 48, 96 and 200 mM $BaCl_2$ (with tonicity compensated to 300 mOsm with choline-Cl, except for 200 mM $BaCl_2$ solution) on both sides of the patch membrane. Each solution contained 10 mM HEPES and pH was adjusted to 7.4 with CsOH.

related calmodulin inhibitor. In 3 different inside-out patches out of 16, superfusion of internal face of the membrane with 10 μM W-7 induced a channel activity similar to that elicited by phenothiazines (*not illustrated*). We also examined the effects of several other substances or ions applied to the internal face of the membrane and the results were as follows: 20 μM polymixin B and 50 μM dodecyltrimethylammonium (DDTMA), two cationic amphipaths, induced Ba^{2+} permeable channel activity in 2 out of 11 and in 1 out of 6 inside-out patches respectively. On the other hand 1 μM ADP-ribose, 1 mM Ca^{2+}, 1 to 10 mM Mg^{2+}, 2 mM MgATP did not induced any detectable activity (at least 15 patches in each case), whereas, on channels previously activated by 20 μM CPZ, 1 mM vanadate, 20 μM ruthenium red, 10 μM ryanodine or 60 mM Na^+ (substituted for 60 mM K^+), had no blocking effect on channel activity (at least 5 patches in each case).

Effect on Whole-Cell Current

We tested the possibility that the PTZ-activated channels may induce currents detectable in whole cell recording configuration. Figure 8 shows records of currents obtained with a pulse protocol eliciting the high threshold Ca^{2+} current, i_{CaL}, at 10 sec-pulse intervals. In Fig. 8*A* it can be seen that successive additions of 20 and 100 μM CPZ, failed to induce any clearly noticeable inward current when the cell was maintained at a holding potential of −80 mV, in spite of the fact that the external medium contained 10 mM Ca^{2+}. The same result was observed in 21 other cells, and also when Ba^{2+} was used instead of Ca^{2+} (4 cells) or Na^+ was replaced by choline in order to block Na-Ca exchange current (6 cells). Similarly, no detectable change in holding current during CPZ superfusion was observed when $[Ca]_o$ was increased up to 24 mM (10 cells). On the other hand Fig. 8*B* shows that i_{CaL} was clearly and reversibly inhibited by CPZ in a dose-dependent manner. We also tested the possibility that the divalent cation permeable channels activated by PTZ at the single channel level might belong to the recently described class of channels activated by depletion of intracellular calcium stores (Clapham, 1993). We therefore applied simultaneously to the cell 10 mM caffeine and 100 μM CPZ, or 100 μM CPZ when pipette contained 5 μM thapsigargin, but no detectable macroscopic current developed at the holding potential in 5 and 4 different experiments, respectively.

Investigation in other Cell Types

Experiments were conducted in other cell types to detect the possible existence of PTZ-activated channels. We chose cultured neuroblastoma cells because PTZ are used as neuroleptic drugs and cultured rat aortic smooth muscle cells because, like cardiac cells, they are contractile cells belonging to the cardiovascular system in which large calcium movements are required for normal activity. In neuroblastoma cells, a spontaneous episodic single channel activity very similar to that described by Coyne et al., (1987) was observed, with Ba^{2+} as charge carrier. However, application of 20 or 100 μM CPZ to the internal face of excised inside-out patches did not leave to clear activation of this activity. In contrast, in cultured aortic smooth muscle cells we were able to observe Ba^{2+} permeable channel currents activated by 20 and 50 μM CPZ, similar to those described in this paper for cardiac myocytes. This activity was dependent on the age of the culture. Before 4 to 5 days of culture spontaneous episodic channel activity was rarely observed. At 6 to 8 days, activity was more frequently observed both in cell attached and inside-out patch configurations and, at this time, the activity was clearly increased by CPZ. The characteristics of channel activation by CPZ was similar to that observed in cardiac cells. A complete description of these results will be reported elsewhere.

Discussion

The main result reported in the present paper is that two phenothiazines (trifluoperazine, TFP, and chlorproma-

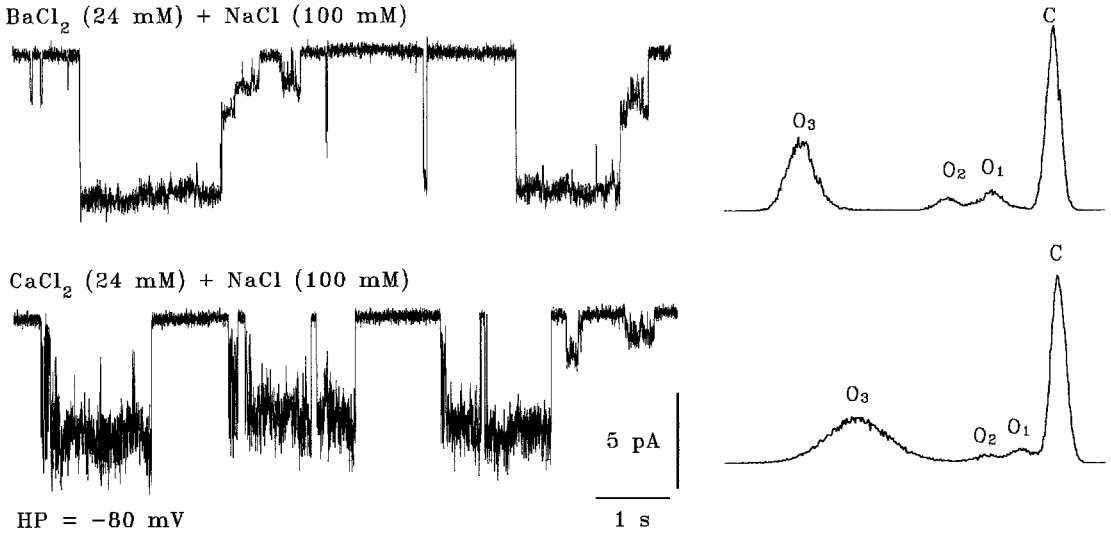

Fig. 7. CPZ-activated channel current in the simultaneous presence of Ba and Na, or Ca and Na, in outside-out patch configuration (left). Ion concentrations are indicated above each trace as part of superfusion media (applied to the external face of the membrane) completed with 10 mM HEPES and adjusted to pH 7.4 with $Ba(OH)_2$ or $Ca(OH)_2$. The pipette (internal) solution contained (in mM): 100 K-aspartate, 25 Cs-aspartate, 2 $BaCl_2$, 2 $MgCl_2$, 1 EGTA, 20 μM CPZ, 10 HEPES; pH was adjusted to 7.4 with CsOH. Amplitude histograms (right) show that the three main levels of activity (O_1, O_2, O_3) exist in the presence of external Na. C: closed level.

zine, CPZ) were able to activate divalent cation permeable channels in a dose-dependent manner when applied at micromolar concentrations (≥5 μM) on either side of inside-out patches or on external side of cell-attached patches of rat ventricular cardiac membranes. Plasmatic concentrations of CPZ ranging between 0.15 and 2 μM have been reported in humans (Baldessarini, 1980). Channel activation was fully reversible only for short exposures of low phenothiazine (PTZ) concentrations. PTZ-induced channel activity was voltage independent. Channels were permeable to several divalent ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}) and at least three levels of currents were usually detected. The presence of large concentrations (100 mM) of Na^+ in the external medium did not prevent the development of PTZ-activated channels; however when external medium contained only NaCl (in the presence of 2 mM Mg^{2+}), no channel activity was induced by PTZ. On the other hand, when the external solution contained both Ba^{2+} and Na^+ ions and the internal solution contained a relatively large Na^+ concentration (50 mM) with no divalent cations (known to block Na^+ permeation through i_{CaL} channel, Hess & Tsien, 1984) small outward currents carried by Na ions could be recorded through PTZ-activated channels, thus allowing an estimation of the P_{Ba}/P_{Na} ratio. This ratio ($P_{Ba}/P_{Na} \approx 10$) is similar to that of B channels from bovine cardiac tissue studied in planar lipid bilayers (Rosenberg et al., 1988).

PTZ-activated channels appear very similar to the sporadically active divalent ion permeable channels described in a previous paper (Coulombe et al., 1989). The activity of these channels occurred spontaneously in irregular bursts separated by quiescent periods of several minutes and could be recorded in a very large potential range (−180 to +50 mV). These channels exhibited a complex gating behavior with at least 3 conductance levels close to those described in the present paper. They were permeable to Ba^{2+} and Ca^{2+} but, apparently, neither to Na^+ nor K^+. Similar to the channels described here they were insensitive to Co^{2+}, Ni^{2+} or Cd^{2+} ions. Although such a similarity does not prove identity it is tempting to propose that the background type of divalent ion permeable channels normally present but poorly active in cardiac membranes can be consistently activated by application of substances including several calmodulin inhibitors and amphiphiles. Although the nature of the channels described in the present paper is unknown, they can hardly be considered as resulting from some abnormal membrane fragility since phenothiazines—and other structurally dissimilar calmodulin inhibitors—have been shown to be able to prevent the formation of sarcolemmal defects in rat and mouse cultured myocardial cells (Scott et al., 1986).

Because PTZs are known to exert calmodulin inhibition (Prozialeck & Weiss, 1982) although being not entirely specific calmodulin inhibitors (Marshak, Watson & Van Eldik, 1981) and because a structurally dissimilar calmodulin inhibitor, W-7, activated the same type of channels as PTZs did, it is a priori tempting to speculate that this effect occurred via a calmodulin-dependent indirect or direct mechanism. An attractive possibility would be that the channels studied here would be Ca^{2+}-calmodulin regulated channels (in the present case Ca^{2+}-calmodulin inhibited channels) as those discovered in different species and tissues (Saimi & Kung, 1994) including the ryanodine-receptor Ca^{2+}-release channel

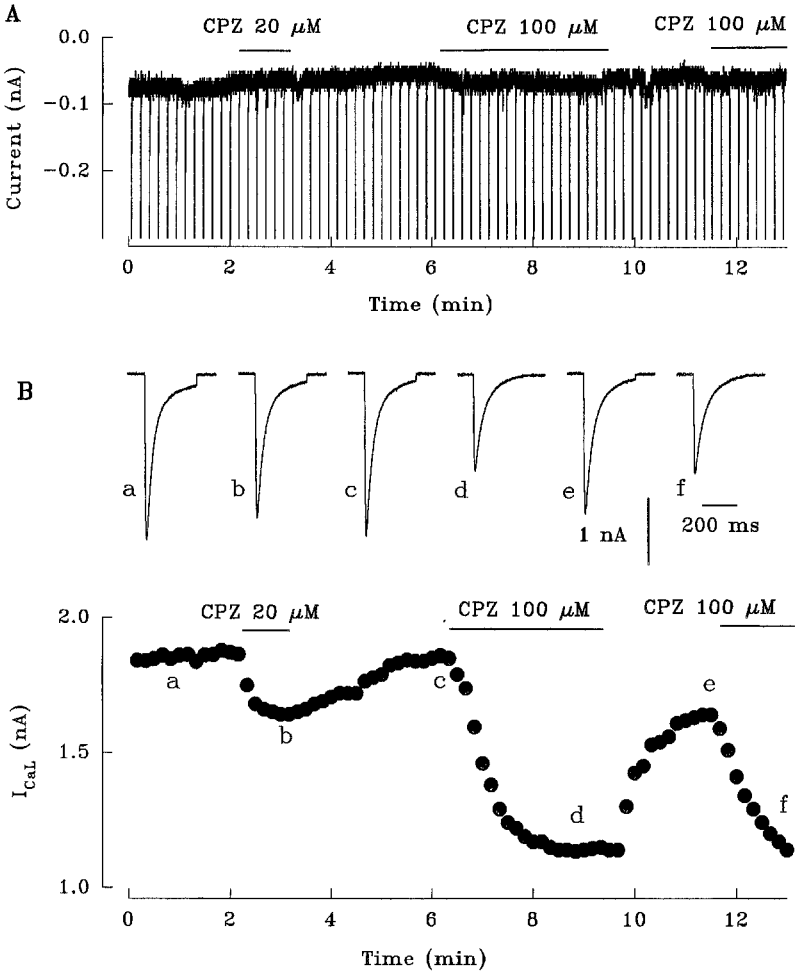

Fig. 8. Effect of successive applications of different concentrations of CPZ on the holding current (*A*) and the high threshold voltage-activated calcium current, i_{CaL} (*B*). (*A*) Continuous current recording (holding potential −80 mV); successive vertical lines are part of i_{CaL} elicited as indicated in Materials and Methods. (*B*) Upper panel, representative recording samples of i_{CaL}, before, during and after the applications of CPZ as indicated by the corresponding letters on the lower panel which shows the peak amplitude of i_{CaL}, as a function of time. Whole-cell recording configuration.

(Smith, Rousseau & Meissner 1989). This possibility can however be questioned for several reasons. Firstly, the K_m of channel activation by CPZ found here (160 μM, Fig. 3) is much higher than that determined in the case of direct PTZ inhibition of calmodulin, although several different sites with different affinities exist on calmodulin. For example, in the case of TFP a K_{d1} of 1.9 μM and a K_{d2} of 18 μM have been found (Zimmer & Hofmann, 1987). Secondly, other substances, such as polymyxin B or DDTMA which are not calmodulin inhibitors also activate the channel. Thirdly, channels directly regulated by Ca^{2+}-calmodulin need both Ca^{2+} and calmodulin to be activated or inhibited (Saimi & Kung, 1994), i.e., the complete absence of internal calcium mimics the effect of calmodulin inhibitors. This was not the case in the present work, since suppression of Ca_i and/or addition of EGTA did not induce channel activation (*not illustrated*).

Another interesting possibility would be that background calcium channels are not genuine ionic channels but result from some alteration of active transport systems. This has been suggested by several works showing that Ca^{2+} release from vesicles derived from the sarcoplasmic reticulum can be dissociated from pump function, suggesting that the Ca-ATPase can operate either as a pump or as a Ca^{2+} channel (deMeiss, 1991; deMeiss & Inesi, 1992). Such channels were only weakly selective for Ca^{2+} ions, with a permeability ratio Ca^{2+} to K^+ of 3.4. Interestingly, it has been shown that the efflux of Ca^{2+} from loaded skeletal muscle sarcoplasmic reticulum vesicles was greatly increased by phenothiazines, such as trifluoperazine and chlorpromazine (deMeiss, 1991). This might be an indirect indication that in our experiments, TFP-activated divalent ion permeable channels might be a form of the sarcolemmal cardiac Ca-ATPase. However, in sarcoplasmic reticulum vesicle experiments the PTZ-increased efflux of Ca was reduced by extravesicular Mg^{2+} and Ca^{2+} whereas in our experiments channel activity was modified neither by 1 mM intracellular Ca^{2+} nor by 1–10 mM intracellular Mg^{2+}. In addition, the channel activity studied in blebs of skeletal muscle sarcoplasmic reticulum and supposed to result from Ca^{2+} pump alteration was markedly reduced by 1 mM vanadate (Wang, Tang & Eisenberg, 1992) whereas this substance produced no effect in our experiments. Although such discrepancies might result from differences in sarcoplasmic and sarcolemmal CaATPase properties, it is nevertheless not possible in the present state of our investiga-

tions to conclude in favor of, or against the hypothesis that channel activity described in the present paper results from Ca-ATPase uncoupling.

The results obtained at the single-channel level prompted us to search for the development of some macroscopic current induced by PTZ but, surprisingly, such a current was never observed in 51 different whole cell recordings, in which the external Ca^{2+} concentration was either 10 or 24 mM. The reason why PTZ-activated channels did not give rise to detectable macroscopic currents in the whole cell recording configuration is not clear. At least two main possibilities can be suggested. The first possibility is that PTZ-induced currents are too small to be clearly observable in whole cell conditions. Using pipettes between 3 to 5 MΩ resistance the patch area is about 5 μm^2 (Sakmann & Neher, 1983). Since only one patch out of five is active, the current crossing each active patch can be considered as corresponding to a membrane area of ≈25 μm^2. In the present work, the maximum PTZ-induced current crossing each active patch at −80 mV in the presence of 96 mM Ba^{2+} is about 7 pA (Fig. 2*B*) and the mean current (about 20% of the maximal value, Fig. 4) is therefore 1.4 pA. This value is reduced by factors of ≈2 at 24 mM Ba_o and ≈4 at 10 mM Ba_o (i.e., to 0.7 pA and 0.35 pA, respectively, Fig. 6) and would be reduced to ≈0.48 and 0.24 pA in the presence of 24 and 10 mM Ca^{2+}, respectively. In the case of a cell with a membrane area of 3500 μm^2 the macroscopic currents induced by 20μM PTZ in the presence of 10 mM and 24 mM external Ca^{2+} can therefore be estimated to 34 pA and 68 pA, i.e., only about twice and four times the thickness of the base line in Fig. 8, respectively. This suggests that the macroscopic current induced by phenothiazines is very likely a small current, but nevertheless it should have been detected at the higher external concentration used in whole-cell recording configuration, except if some undetected factor nonefficient after patch formation, can counteract the PTZ-induced channel activation. The second possibility is that the channels studied in the present work are not activatable by PTZ in normal conditions whereas they can be activated after membrane suction and patch formation as a result of membrane deformation, cytoskeleton disruption or other inhibitory submembrane system disorganization. Suction-induced membrane deformation has become the basal test in the study of stretch sensitive channels. Membrane deformation resulting from uneven partitioning of amphipath between membrane monolayers is also the cause of the activation of bacteria mechanosensitive channels induced by amphipathic compounds such as CPZ or trinitrophenol (Martinac et al., 1990). That PTZ-induced channel activation reported in the present work results from the same mechanism is however unlikely because negative pressures higher than 100 mbar applied to the patch pipette failed to trigger channel openings. Although it is indeed possible that PTZ cannot activate Ca^{2+} permeable channels in normal conditions, the opposite possibility is however supported by the observation that 10–100 μM TFP substantially increases free cytosolic calcium in plant protoplats in vivo (Gilroy, Hughes & Trewavas, 1987), an effect attributed to the calmodulin inhibitory effect of TFP because other anticalmodulins such as W-5 or W-7 exerted the same effect.

If it is assumed that a PTZ-activated current occurs in intact cardiac cells and that its amplitude, at normal external Ca^{2+} concentrations, is weak (for example 10 pA) the quantity of Ca^{2+} entering the cell through background Ca^{2+} channels during each cycle can be much less than that entering the cell during each peak of current through voltage gated Ca^{2+} channels. Because our results confirm that PTZs and calmodulin antagonists sizeably depress i_{CaL} (Klöckner & Isenberg, 1987; our Fig. 8), the global effect of PTZ may very well be a decrease rather than an increase in cell calcium load. This can explain at least in part why PTZs can suppress transient depolarizations in isoproterenol and strophantidin-treated cardiac cells (Kremers et al., 1985) and exert a protective action against calcium overload in heart failure (Schaffer et al., 1983), but not why cationic amphiphiles prevent calcium leak induced by ATP depletion in the presence of Ca^{2+} channel blockers (Clague Harvey & Langer, 1993). Interestingly, CPZ has been reported to inhibit several ionic currents in addition to i_{CaL}, specially potassium currents, and these inhibitory effects have been reported to be responsible for cardiac arrhythmias resulting from the use of this antipsychotic drug (*for references see* Kon, Krause & Gögelein, 1994).

The fact that a PTZ-induced Ba^{2+} permeable channel activity similar to that found in cardiac cells was also observed in aortic smooth muscle cells, but not in neuroblastoma cells, extends the interest of our observations and suggests that the channels described here may be involved in calcium movements related to the control of contractility in cells belonging to the cardiovascular system. Although we ignore presently if these calcium permeable channels are of physiological significance, it is tempting to hypothesize that they might be the equivalent in cardiovascular contractile cells of the very small calcium permeable channels activated in nonexcitable cells by intracellular calcium store depletion via some still unknown diffusible factor (*for review see* Fasolato, Innocenti & Pozzan, 1994). It remains to search for some cellular mechanism or substance capable of controlling their activity.

Note added in proof: While this manuscript was being reviewed, an increase in calcium background channel activity by metabolic inhibition or hydrogen peroxide in membrane of adult rat ventricular myocytes was reported (Wang, S-Y, Clague, J.R., Langer, G.A. 1995. J. Mol. Cell. Cardiol. **27,** 211-222).

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