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Are B-type Ca^{2+} Channels of Cardiac Myocytes Akin to the Passive Ion Channel in the Plasma Membrane Ca^{2+} Pump?

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Abstract. The present study demonstrates that B-type Ca^{2+} channels observed in rat ventricular myocytes markedly reacted to agents known to affect the ion-motive plasma membrane Ca^{2+}-ATPase (PMCA) pump. Chlorpromazine (CPZ)-activated B-type Ca^{2+} channels were completely blocked by internal application of PMCA pump inhibitors, namely La^{3+} (100 μM), eosin (10 μM) and AlF_3 (100 μM). Calmodulin (50 U/ml), the main endogenous positive regulator of PMCA, was unable to activate but significantly reduced CPZ-activated B-type channel activity. In the same manner, ATP (1 and 4 mM), the main energizing substrate of PMCA, was able to reversibly and significantly reduce this activity in a dose-dependent manner. Interestingly, anti-PMCA antibody 5F10, but not anti-Na/K ATPase antibody (used as a negative control) induced a marked Ba^{2+}-conducting channel activity that shared the same characteristics with that of CPZ-activated B-type channels. 5F10-Activated channels were mostly selective towards Ba^{2+}, mainly had three observed conductance levels (23, 47 and 85 pS), were observed with a frequency of about 1 out of 5 membrane patches and were completely blocked by 10 μM eosin. These results suggest that B-type Ca^{2+} channels are some form of the PMCA pump.

Key words: B-type Ca^{2+} channel — Eosin — AlF_3 — Lanthanum — Calmodulin — Plasma membrane Ca^{2+} ATPase (PMCA)

Introduction

The existence of divalent cation permeable channels different from L-, T- or R-type Ca^{2+} channels was first evidenced over ten years ago in the sarcoplasmic membrane of cardiac cells. Rosenberg and colleagues (1988) observed background or B-type Ca^{2+} channels in sarcolemmal vesicles from calf ventricular muscle incorporated into artificial lipid bilayers. Such channels have also been observed in native membranes of rat ventricular myocytes (Coulombe et al., 1989; Wang, Clague & Langer, 1995), of rat aortic smooth muscle cells (Lefevre et al., 1995) and more recently in human atrial myocytes (Antoine et al., 1998). B-type Ca^{2+} channels are different from other known Ca^{2+} channels in that they are independent of membrane potential, being observed over a large range of membrane potential (from −180 to +60 mV). They can be detected in nearly 20% of tested patches and are insensitive to L-type Ca^{2+} channel blockers (Ni^{2+}, Co^{2+}, Cd^{2+} and D600). In control conditions, their sparsely spontaneous activity occurs in irregular bursts separated by long-lasting quiescent periods. They are activated by anticalmodulin compounds such as chlorpromazine (CPZ), trifluoperazine and W7 (Lefevre et al., 1995). Their activity is markedly enhanced by metabolic inhibition and hydrogen peroxide in rat ventricular (Wang et al., 1995) and in human atrial myocytes (Antoine et al., 1998). They exhibit variable degrees of complex gating patterns with one-to-three most frequently observed conductance states (around 20, 50 and 80 pS in 48 mM Ba^{2+}) sometimes combining to give larger levels.

In a previous paper (Lefevre et al., 1995), we suggested that B-type Ca^{2+} channels might not be genuine ionic channels but rather result from a functioning of the plasma membrane Ca^{2+}-ATPase pump (PMCA) similar to that reported in the case of the sarcoplasmic reticulum Ca-ATPase, SERCA (de Meis, 1991; de Meis & Inesi, 1992; Wang, Tang & Eisenberg, 1992; de Meis, Wolosker & Engelenger, 1996; Du, Ashley & Lea, 1998), which is believed to operate either as a pump or as a passive cation channel. Because CPZ and H_2O_2 (Le-

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fevre et al., 1995; Wang et al., 1995; Antoine et al., 1998) activate B-type Ca^{2+} channels, it is tempting to formulate the simple hypothesis that the PMCA functions as a pump in the presence of internal calmodulin (CaM) whereas in the absence of this substance, or in the presence of a CaM inhibitor like CPZ (Prozialeck & Weiss, 1982), the Ca^{2+} transport function would be lost and the PMCA molecule may behave as a Ca^{2+} permeable channel. At least two results are in favor of this assumption. (i) CaM is known as an endogenous activator of PMCA; the CaM binding domain of the pump molecule acts as an internal restrainer of active transport in the absence of CaM (Carafoli et al., 1992). Therefore, it might be assumed that CaM inhibitors, such as CPZ, restrain the active function of PMCA, thus allowing it to shift towards the passive channel mode. (ii) H_2O_2, the most abundant reactive oxygen species normally produced by cell metabolism, has recently been reported to induce oxidative modification of a site of the CaM molecule composed of carboxyl-terminal vicinal methionines which are conserved between all calmodulins from higher plants to humans. The H_2O_2-induced alteration does not prevent the inactive CaM from binding to the autoinhibitory sequence of PMCA but prevents the native, normal, CaM from binding to this site and activating the pump (Yao et al., 1996). This suggests that the main mechanism involved in the H_2O_2-induced openings of B-type Ca^{2+} channel might be the same as that of CaM inhibitors, i.e., preventing PMCA from CaM-induced activation.

The aim of the present work was to bring further experimental support to the above assumption by exploring whether agents known to act on PMCA pump could also act on B-type Ca^{2+} channel activity. The results show that CPZ-induced B-type Ca^{2+} channel activity was markedly blocked by PMCA inhibitors eosin, AlF_3 and lanthanum, and was reduced by ATP_i and CaM. In addition, anti-PMCA antibody 5F10 clearly activated Ba^{2+}-conducting channels similar to that of B-type Ca^{2+} channels.

Materials and Methods

Preparation of Rat Ventricular Myocytes

All experiments were performed in accordance with institutional guidelines for animal care. Single ventricular cells were obtained from adult rat hearts by an enzymatic dissociation procedure. Male Wistar rats (180–200 g) were anesthetized by intraperitoneal pentobarbital sodium injection (35 mg/kg body weight). The heart was quickly excised and cannulated by the aorta and then mounted on a Langendorff column before being retrogradely perfused. During all the dissociation procedure, solutions were continuously bubbled with 100% O_2 and maintained at 37°C. A first perfusion of 5 min with Tyrode's solution (*see* composition below) containing 1.8 mM $CaCl_2$, was followed by a second perfusion of 3 min with nominally Ca^{2+}-free Tyrode's solution. The perfusion solution was then switched to a recirculating Ca^{2+}-free Tyrode's solution containing collagenase (Type A, Boehringer Mannheim, Germany), 1 g/l; BSA (Bovine Serum Albumin, Sigma, Saint Louis, MO), 5 g/l; hyaluronidase (Sigma), 0.4 g/l; pH 7.4 (NaOH) for 10–20 min. The heart was then rinsed during 3 min with Ca^{2+}-free Tyrode's solution. The digested heart was placed in a K^+-rich medium (*see* composition below) with 5 g/l BSA. The atria were removed, the left and right ventricles were dissected into small fragments of tissue ($\approx$1 mm^3) and incubated for 5 min in K^+-rich medium at 37°C containing BSA, 5 g/l. Cells were dispersed by gentle agitation of the tissue pieces and filtered. After slow centrifugation (1 min, 50 × *g*), cells were resuspended in K^+-rich medium containing BSA, 5 g/l; protease (type XIV, Sigma), 1 g/l; and incubated for 5–15 min at 37°C. Finally, they were gently centrifuged, resuspended in K^+-rich solution, plated in 35 mm plastic Petri dishes (Nunclon), and kept at +4°C at least 1 hr before being used. They were then washed with Tyrode's solution containing 1.8 mM $CaCl_2$.

Single-Channel Recordings and Data Analysis

Experiments were performed with the classical inside-out patch-clamp configuration. Only rod-shaped adherent cells with clear striations, sharp edges, without granulation and showing no spontaneous contractile activity, were chosen. Patch pipetes were pulled from borosilicate glass capillaries (Corning code 7740, Corning Glass, or Corning Kovar Sealing code 7052, WPI, FL) using a horizontal puller (DMZ-Universal Puller, Zeitz Instrument, Germany) and fire-polished before use. The pipette resistance was 5–10 MΩ. The currents were recorded using a patch-clamp amplifier (Axopatch 200A, Axon Instruments, CA) and filtered through an eight-pole Bessel low pass filter 920LPF (Frequency devices) setting of 1 or 2 kHz, and stored upon digital audio tape (DTR 1200, Bio-Logic, France). Data were digitized at 5 or 10 kHz with a Digidata 1200 (Axon Instruments) using Acquis1 software (Bio-Logic, France), and stored in a computer file for latter analysis. Currents were retrieved on a HP Laserjet 4000 N (Hewlett-Packard, CA). All experiments were conducted at room temperature (20–24°C). Elementary conductance were determined as previously reported (Coulombe et al., 1989). Channel activity (mean patch current) was calculated by integrating current flow during the channel openings and dividing the integral by the total sampling time. When appropriate, data are given as mean ± SD (standard deviation) of *n* determinations.

Solutions

Before use, all the solutions were filter-sterilized using 0.2 μm filtration membranes. Tyrode's solution contained (in mM): NaCl, 135; KCl, 4; Na-pyruvate, 2.5; $MgCl_2$, 2; NaH_2PO_4 1; HEPES, 10; glucose, 20; pH was adjusted to 7.4 with NaOH. The K^+-rich medium in which cells were maintained before use, contained (in mM): L-glutamic acid monopotassium salt, 70; KCl, 25; KH_2PO_4, 10; $MgCl_2$, 3; EGTA, 0.5; HEPES, 10; glucose, 20; taurine, 10; pH was adjusted to 7.4 with KOH. A stream of solution from one of a series of five piped outlets continuously superfused the membrane patch from which recording was being made. The flow rate of superfusion was 50–100 μl/min. For all experiments, the superfusion solution contained (in mM): K-aspartate, 128; KCl, 2; $BaCl_2$, 1; EGTA, 5; HEPES, 10; glucose, 10; pH was adjusted to 7.2 with KOH. In experiments where chlorpromazine (CPZ) or eosin was used, ethanol was added at the appropriate concentration to the control superfusion solution. PMCA antibody (IgG2A clone 5F10, from Affinity Bioreagents (ABR), CO), and Na^+/K^+-ATPase antibody (IgG1 clone M8-P1-A3, ABR) were used at a dilution of 1/500 and 1/100 respectively, in accordance with specifications of

the distributor. As antibodies were delivered in a solution of sodium azide (NaN_3) for their conservation, NaN_3 was added at the appropriate concentration to the control and CPZ superfusion solutions in experiments in which antibodies were used. Calmodulin (CaM) was used at a concentration of 50 U/ml. For experiments in which CaM was used, 250 μM Ca^{2+} was added to all the superfusion solutions. In all experiments, CPZ was used at a concentration of 50 μM, according to the IC_{50} of 160 μM obtained for B-Type channel activation by CPZ (Lefevre et al., 1995). For all experiments, the pipette solution contained (in mM): $BaCl_2$, 48; HEPES, 10; pH was adjusted to 7.4 with CsOH. Otherwise, solutions were as indicated in figure legends. In spite of the fact that Ca^{2+} can be used as charge carrier in order to record B-type Ca^{2+} channels (Lefevre et al., 1995), Ba^{2+} was used as the charge carrier because it is generally admitted that Ba^{2+} ions are more permeable than Ca^{2+} ions through several types of Ca^{2+}-channel (L- and T-type), this property appeared to be also true for B-type Ca^{2+} channel (*see* Lefevre et al., 1995). The use of Ba^{2+} made it easier to distinguish subconductance levels when they occurred. In our previous studies we also wanted to avoid putative activation of Ca^{2+}-activated K^+ or Cl^- channels, and to avoid possible inactivation or modulation of B-type channels by Ca^{2+} flowing through these channels. As Ba^{2+} is a blocker of practically all known K^+ channels (Hille, 1992) and of some other channel types, it is a useful tool to study Ca^{2+} channels (especially to establish *I-V* curves when K^+ ions are used on one side of the membrane). Eosin refers to eosin Y. Chemicals were purchased from Sigma Chemical (St. Louis, MO).

Results

Effect of PMCA Pump Inhibitors on B-Type Ca^{2+} Channels

As previously described in quiescent inside-out membrane patches from rat ventricular and human atrial myocytes, application to the internal face of the membrane of micromolar concentration of CPZ, markedly induced, in a few seconds, B-type Ca^{2+} channel activity in patches held at a potential of −80 mV (Lefevre et al., 1995; Antoine et al., 1998). In spite of the fact that Ca^{2+} can be used as charge carrier in order to record B-type Ca^{2+} channels (Lefevre et al., 1995; Antoine et al., 1998), in the present study, Ba^{2+} was used for reasons stated in the Materials and Methods. Figure 1 shows typical segments of single-channel current recordings obtained from 3 different membrane patches illustrating such an activation and the inhibition of this activity by eosin, lanthanum and aluminum fluoride. Eosin, by interacting with the nucleotide binding site, has been shown to be a potent inhibitor of PMCA in red blood cell inside-out vesicles ($IC_{50} \approx 0.3$ μM; Gatto & Milanick, 1993), in bovine sarcolemmal cardiac vesicles ($IC_{50} \approx 1$ μM; Gatto et al., 1995) and in rat ventricular myocytes (Choi & Eisner, 1999*b*). Figure 1*A* exemplifies that application of 10 μM eosin to the internal face of the patch membrane completely and irreversibly blocked CPZ-activated B-type Ca^{2+} channels. This blocking effect was reproduced in 10 other membrane patches. The lower traces in Fig. 1*A* show at least three amplitudes of single-channel current corresponding to the most commonly observed conductance levels for B-type Ca^{2+} channel (Lefevre et al., 1995; Antoine et al., 1998). Lanthanum is a well-known typical and effective inhibitor of the P class of ion-motive ATPases to which the PMCA pump belongs (for review *see* Carafoli, 1991, 1994). In rat aortic myocytes, La^{3+} (0.006–0.25 mM) reversibly inhibits the ATPase activity of PMCA, without affecting the Na^+–Ca^{2+} exchange activity (Shimizu, Borin & Blaustein, 1997). Figure 1*B* shows a typical effect of application of 100 μM La^{3+} to the internal face of a membrane patch. La^{3+} markedly blocked CPZ-induced B-type Ca^{2+} channel activity. This result was confirmed in 8 other measurements. The effect of La^{3+} reversed more slowly with longer durations of application. Interestingly, when tested with patch pipettes containing 100 μM La^{3+}, in four inside-out membrane patches, CPZ was able to induce normal B-type channel activity, indicating that La^{3+} acted only on the internal face of the cell membrane. As La^{3+} is also known to be a potent blocker of Ca^{2+} channels (Herrington et al., 1996), we chose to test another type of Ca^{2+}-ATPase inhibitor, AlF_3. Aluminofluorides affect the activity of enzymatic systems such as G-proteins and Ca^{2+}-ATPase. Among numerous aluminofluoride complexes, AlF_4^-, AlF_3, $Al(OH)F_3^{2-}$ have been shown to inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Troullier, Girardet & Dupont, 1992). They inhibit SERCA by acting as an analogue of inorganic phosphate, binding to the E_2 form of the enzyme and preventing its return to the E_1 conformation (Wang et al., 1992). Rao (1990) has shown that AlF_3 is a powerful noncompetitive inhibitor of synaptosomal Ca^{2+}-ATPase. Figure 1*C* shows a current trace exemplifying the effect of application of 100 μM AlF_3 to the cytosolic face of an inside-out membrane patch exhibiting CPZ-induced B-type channel activity. The activity was completely and irreversibly inhibited in a few seconds or less. The same result was obtained in three other experiments. As AlF_3 is a well known activator of G-proteins, we used internal application of GTPγS (100 μM) in order to test for a putative G-protein-gating of B-type channels. In four inside-out membrane patches showing a marked activation of B-type channels by CPZ, no evidence of block was observed after addition of GTPγS. In the same way, in membrane patches responding to CPZ, application of GTPγS prior to activation by CPZ had failed to induce any channel activity ($n = 4$, *not shown*).

Effect of Internal ATP

PMCA, as a member of the P-class of ion-motive ATPases, uses the energy of ATP hydrolysis in order to extrude intracellular Ca^{2+}; it was therefore of interest to test the effect of ATP on CPZ-induced B-type Ca^{2+} chan-

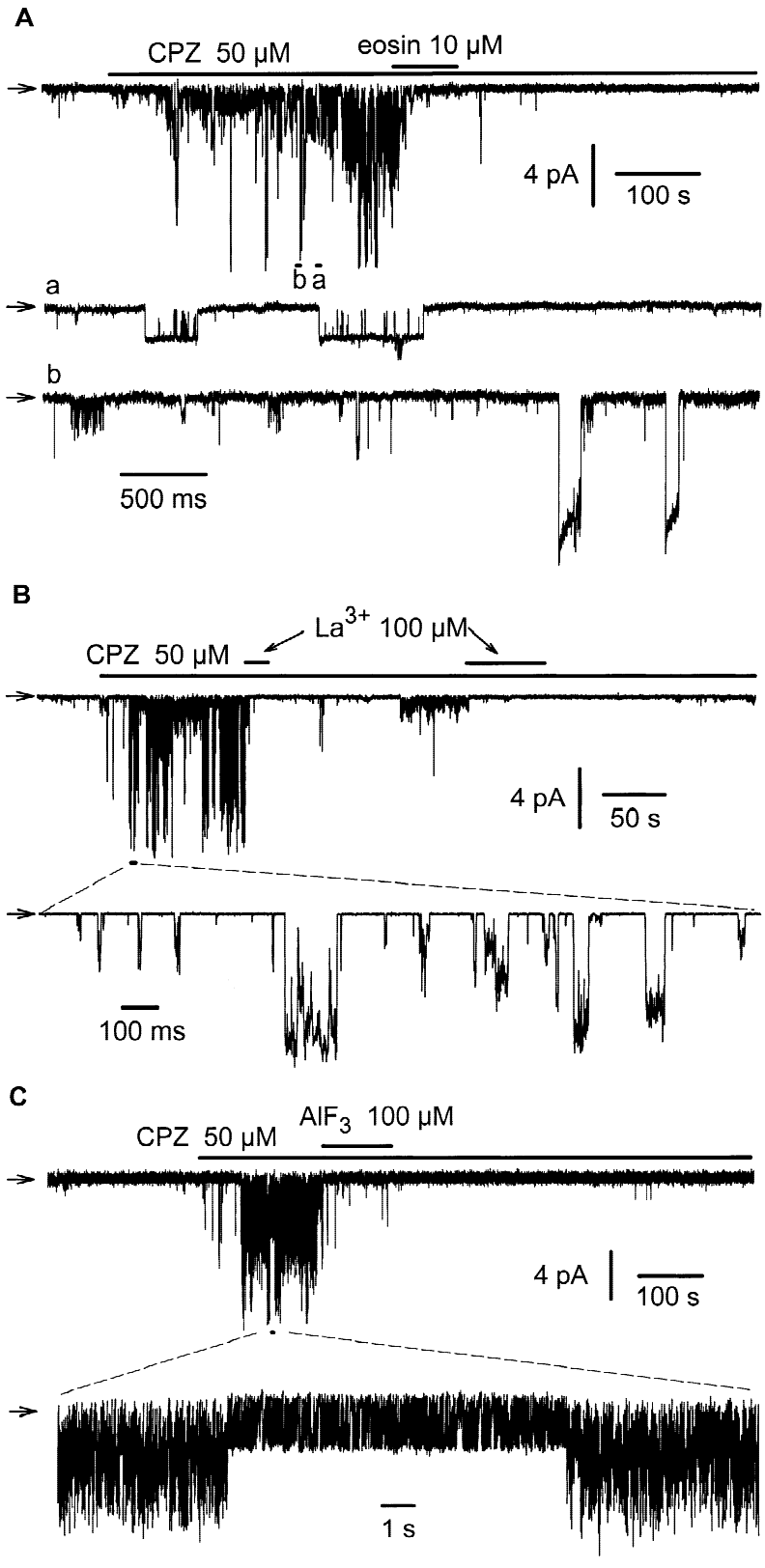

Fig. 1. Inhibition of chlorpromazine (CPZ)-induced B-type Ca^{2+} channel activity by eosin, lanthanum and aluminum fluoride. Upper traces: representative inside-out recordings illustrating that CPZ-activated B-type Ca^{2+} channel activity from previously quiescent membrane patches, was completely inhibited by eosin (*A*), lanthanum (La^{3+}) (*B*) and aluminum fluoride (AlF_3) (*C*). In this and other figures, agents were applied at indicated concentrations to the cytosolic face of the membrane patches as indicated by horizontal bars above the current traces. Membrane patch holding potential (HP) was −80 mV for all traces. In *B* is also shown the partial reversibility of a short exposure to 100 μM La^{3+}. Lower traces: expanded time-scale extracts showing the complexity and the diversity usually observed for the gating of CPZ-induced B-type Ca^{2+} channels. Arrows indicate zero current level. Recording media were as described in Materials and Methods.

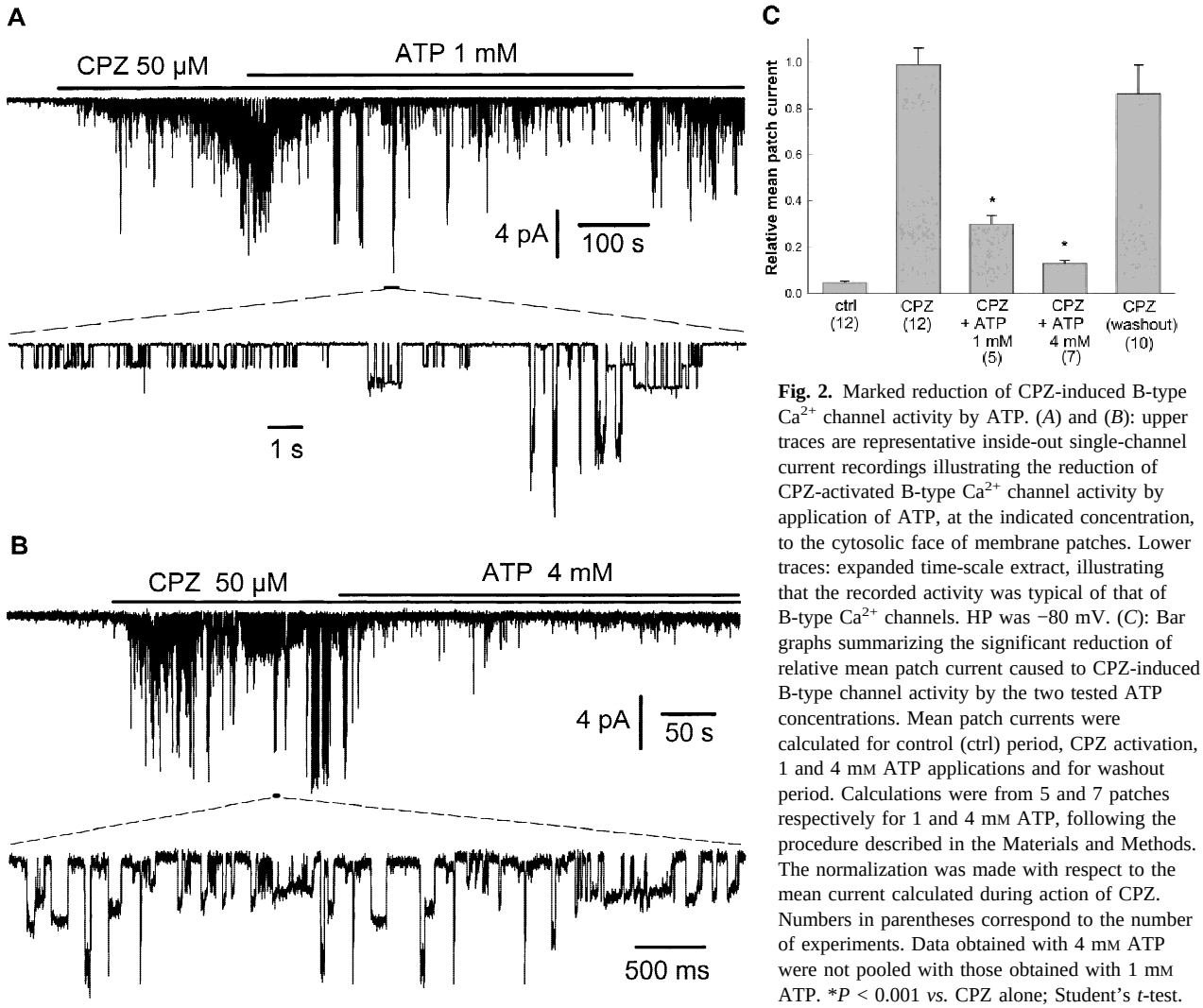

Fig. 2. Marked reduction of CPZ-induced B-type Ca^{2+} channel activity by ATP. (*A*) and (*B*): upper traces are representative inside-out single-channel current recordings illustrating the reduction of CPZ-activated B-type Ca^{2+} channel activity by application of ATP, at the indicated concentration, to the cytosolic face of membrane patches. Lower traces: expanded time-scale extract, illustrating that the recorded activity was typical of that of B-type Ca^{2+} channels. HP was −80 mV. (*C*): Bar graphs summarizing the significant reduction of relative mean patch current caused to CPZ-induced B-type channel activity by the two tested ATP concentrations. Mean patch currents were calculated for control (ctrl) period, CPZ activation, 1 and 4 mM ATP applications and for washout period. Calculations were from 5 and 7 patches respectively for 1 and 4 mM ATP, following the procedure described in the Materials and Methods. The normalization was made with respect to the mean current calculated during action of CPZ. Numbers in parentheses correspond to the number of experiments. Data obtained with 4 mM ATP were not pooled with those obtained with 1 mM ATP. $*P < 0.001$ *vs.* CPZ alone; Student's *t*-test.

nel activity. As illustrated in Fig. 2, application of 1 and 4 mM ATP to the internal face of membrane patches clearly reduced CPZ-induced channel activity in a dose-dependent manner. The reduction of activity was incomplete, since a residual activity persisted even after long-lasting exposures to ATP (more than 10 min). The upper current trace of Fig. 2*A* also exemplifies the reversibility of this inhibitory effect. The lower traces are expanded time-scale extracts showing the complex gating usually observed for CPZ-induced B-type Ca^{2+} channels, even in the residual activity during application of ATP (Fig. 2*A*, lower trace). Figure 2*C* presents bar graphs summarizing the results obtained from 5 and 7 cumulated experiments, respectively with 1 and 4 mM ATP, which confirmed the significant reduction of channel activity by ATP, in a dose-dependent manner. The mean current activity was computerized in control, during CPZ activation and after addition of ATP and subsequently during the washout of ATP. The normalization was made, respectively, to the mean current activity calculated during CPZ activation.

Effect of Calmodulin

PMCA pump activity is directly regulated by the binding of calmodulin (CaM), in the presence of Ca^{2+}, to a highly basic C-terminal region (autoinhibitory domain) (Carafoli, 1997; Guerini, 1998; Penniston & Enyedi, 1998). In the absence of Ca^{2+}-CaM complex, the PMCA is autoinhibited. CaM stimulates the pump by increasing both Ca^{2+} affinity and maximum velocity (Enyedi & Penniston, 1993). Thus, it appeared interesting to test the effect of applications of a high concentration of CaM (50 U/ml) simultaneously with 250 µM Ca^{2+} to the internal face of membrane patches. Figure 3*A* shows that application of CaM to the internal face of a previously quiescent inside-out membrane patch was unable to ac-

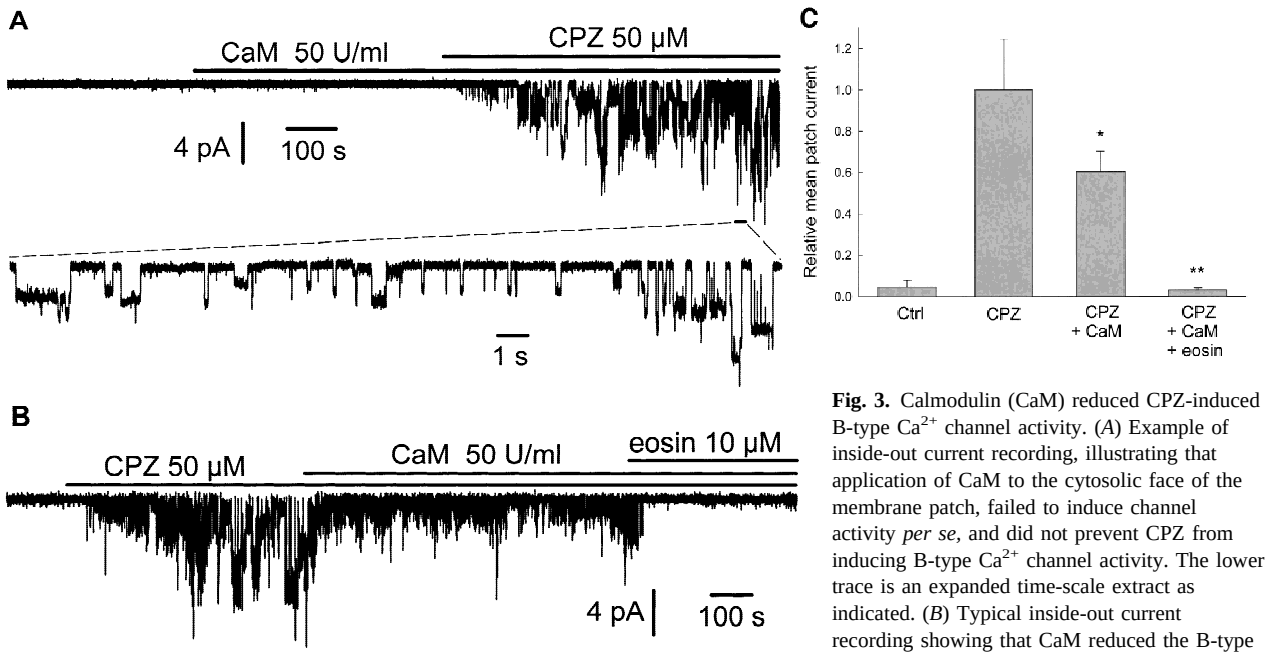

Fig. 3. Calmodulin (CaM) reduced CPZ-induced B-type Ca^{2+} channel activity. (*A*) Example of inside-out current recording, illustrating that application of CaM to the cytosolic face of the membrane patch, failed to induce channel activity *per se*, and did not prevent CPZ from inducing B-type Ca^{2+} channel activity. The lower trace is an expanded time-scale extract as indicated. (*B*) Typical inside-out current recording showing that CaM reduced the B-type Ca^{2+} channel activity previously induced by CPZ, and that eosin application completely blocked this activity. Recording media were as described in Materials and Methods, and all the internal superfusion media including control medium were supplemented with 250 μM Ca^{2+}. Holding potential was −80 mV. (*C*) Bar graphs showing the significant reduction in relative mean patch current caused by CaM to CPZ-induced B-type channel activity. Mean patch currents were calculated from 5 experiments similar to that presented in *B* and were normalized with respect to the mean current calculated under CPZ activation. Mean patch current was calculated following the procedure presented in Materials and Methods. $*P < 0.01$ and $**P \ll 0.001$ *vs.* CPZ alone; paired *t*-test.

tivate B-type Ca^{2+} channels, whereas addition of CPZ to the superfusion medium succeeded to activate B-type Ca^{2+} channels. This absence of activation was observed in 4 other experiments. The CPZ-induced channel activity observed in the presence of CaM appeared to be of a lower level than that usually observed under CPZ-activation (*see* Fig. 1). Figure 3*B* shows a sample of current trace recorded when CaM was added to the superfusion medium after activation of B-type Ca^{2+} channel by CPZ, illustrating the observation that less pronounced channel openings occurred in the presence of CaM and that, as expected, 10 μM eosin completely inhibited channel activity. The bar graphs of Fig. 3*C* summarize data pooled from 4 experiments, revealing significant differences in normalized mean patch current activity observed in control, during CPZ action and after addition of CaM and subsequently of eosin to the superfusion medium. The normalization was made with respect to the mean current activity calculated during CPZ activation.

Effect of Anti-PMCA Antibody Clone 5F10 Specific Activation

Another experimental approach to establish the existence of a link between the PMCA pump and B-type Ca^{2+} channels, was to investigate the effect on B-type channel activity of a specific antibody raised against the PMCA protein. We chose to test the commercially available monoclonal anti-PMCA antibody 5F10 because it recognizes an epitope between amino acids 719–738 of human erythrocyte PMCA, which is highly conserved in all PMCA isoforms (Caride et al., 1996). This epitope is located in the highly conserved hinge region which is on the cytosolic loop of PMCA between the putative transmembrane domains M4 and M5 (Adamo, Caride & Penniston, 1992). This large loop contains important regulatory sites such as an ATP binding site, an aspartic acid phosphorylation site and the receptor site for the inhibitory calmodulin binding domain (Carafoli, 1997; Guerini, 1998; Penniston & Enyedi, 1998). As it was difficult to predict the putative effect, if any, of clone 5F10 anti-PMCA antibody on B-type Ca^{2+} channel activity, the antibody was first evaluated as a possible inhibitor of CPZ-induced channel activity. In 6 inside-out patches exhibiting CPZ-induced channel activity, addition of anti-PMCA 5F10 to the medium that superfused the cytosolic face of the membrane patch, held at a potential of −80 mV, produced no appreciable change in channel activity (*not shown*). In a second series of experiments, we investigated whether 5F10 might be endowed *per se* with a potency for activating B-type channels. Figure 4*A* presents a current trace obtained in an inside-out membrane patch before and during application of 5F10 to the cytosolic face of the membrane patch, at a holding potential of −80 mV, showing that 5F10 mark-

edly activated Ba^{2+} conducting channels. To eliminate the possibility that the 5F10-induced channel activity might be due to a nonspecific action of the antibody, we used the anti Na^+/K^+-ATPase clone M8-P1-A3 antibody as a negative control. Clone M8-P1-A3 is an antibody that has been raised against a cytosolic loop of the Na/K pump protein (Malik, Jamieson & Ball, 1993). As illustrated by the upper current trace of Fig. 4*B*, application of M8-P1-A3 antibody to the medium superfusing the cytosolic face of the membrane patch failed to induce any single-channel activity, whereas addition of 5F10 antibody to the medium, induced marked channel activity. This result was confirmed in a total of 3 experiments.

The channels evoked by antibody 5F10 shared many characteristics with CPZ-activated B-type Ca^{2+} channels. Their frequency of observation, 5 out of 26 tested patches, was roughly comparable to the reported frequency of 20% for CPZ-induced channels, in the same preparation (Lefevre et al., 1995). Figure 4*C* shows that these channels were blocked by cytosolic application of 10 μM eosin ($n = 3$). In addition, CPZ failed to induce channel activity in patches to which prior application of 5F10 had not succeeded to induce any channel activity. The lower current traces of Fig. 4*A, B* and *C* show that 5F10-induced channels exhibited a complex gating behavior with different conductance levels similar to those observed for CPZ-induced activity.

Current-Voltage Relationships and Elementary Conductances of 5F10-Activated Channels

In Fig. 5*A*, current traces recorded at different membrane potentials are shown in a typical experiment in which Ba^{2+} conducting channels were induced by clone 5F10 applied to the cytosolic face of an inside-out patch. These current traces correspond to periods of recording during which channel activity appeared regular and in which several current levels are clearly distinguishable. Three of the current levels were most frequent as shown by the dashed horizontal lines at −80 mV. Current-voltage relationships corresponding to the three current levels most frequently observed in 2 experiments similar to that of Fig. 5*A* are shown in Fig. 5*B*. The *I-V* curves are not linear, a characteristic that results very likely, at least in part, from asymmetrical concentrations of the main permeant ion, Ba^{2+}, the concentration of this ion being nominally of 1 mM on the internal face of the membrane. In our experimental conditions, the calculated Nernst equilibrium potential for Ba^{2+}, the main permeant ion, was +47 mV. The fact that the three curves tend to approach zero current values, 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^+. When calculated in the −80 to −40 mV range, the slopes of the three straight lines gave elementary conductances of 23, 47 and 85 pS (Fig. 5*B*). These conductance values are comparable to those of 26, 50 and 80 pS obtained for CPZ-induced B-type Ca^{2+} channels for the three mostly observed conductance levels (Lefevre et al., 1995).

Discussion

The main new result reported in the present work is that agents that are known to act on plasma membrane Ca^{2+}-ATPase pump, are able to markedly modify background (B-type) Ca^{2+} channel activity in rat ventricular myocytes. Lanthanum, eosin and aluminofluoride AlF_3, potent inhibitors of PMCA, were able to completely block CPZ-activated B-type channel activity. Both internal ATP, the main energizing substrate of PMCA and calmodulin, the regulator of the pump function of PMCA, significantly reduced this channel activity. The monoclonal antibody 5F10, raised against PMCA, was able to induce sustained Ba^{2+} conducting channels sharing the characteristics of CPZ-activated B-type Ca^{2+} channels.

Inhibition of B-Type Ca^{2+} Channels

Specificity of inhibition of PMCA by eosin over other ion transport mechanisms has been assessed by different groups. Intracellular eosin, at concentrations ⩽20 μM, inhibits PMCA, but does not inhibit Na^+–Ca^{2+} exchange in ventricular cardiac myocytes (Gatto et al., 1995; Choi & Eisner, 1999*b*). Intracellular application of 20 μM carboxyeosine (IC_{50} of 0.02 μM for inhibition of PMCA), an analogue of eosin (IC_{50} of 0.04 μM), induced a decrease of 18% of I_{CaL} in rat ventricular myocytes (Choi & Eisner, 1999*a*). In the same way, eosin inhibits Na^+–K^+ pump with a potency lower than that for PMCA (Skou & Esmann, 1981).

There are few studies documenting a blocking effect of eosin on ionic conductances. Photochemical modification of externally applied eosin has been shown to block I_{Na} of squid giant axon (Oxford, Pooler & Narahashi, 1977) and of lobster axon (Pooler & Valenzeno, 1979). External application of eosin blocked NMDA channels in hippocampal neurons (Sobolevsky, Yelshansky & Khodorov, 2000) and mitochondrial inner membrane anion channels (Powers, Smith & Beavis, 1994). In the present study, 10 μM eosin completely blocked CPZ-induced B-type channels by acting on the cytosolic side of the cell membrane. This indicates that the mechanism underlying this blocking effect appears different from that of the above reported channel inhibitions by eosin. The fact that eosin inhibits PMCA and B-type channels by acting on the internal side of the cell membrane may suggest a common site of inhibition. How-

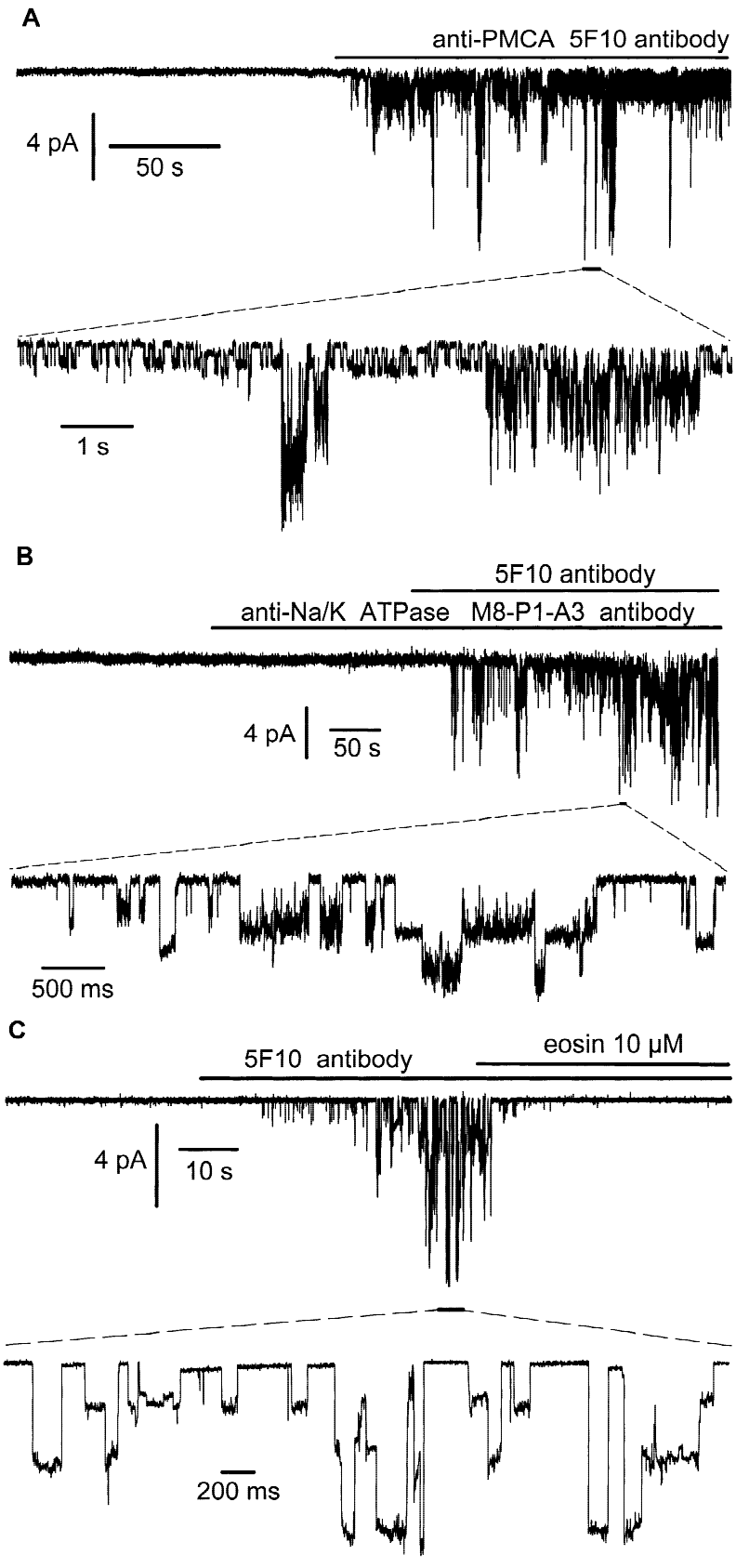

Fig. 4. Ba^{2+} conducting channel activity similar to that of B-type Ca^{2+} channels, induced by the anti-plasma membrane calcium ATPase (PMCA) antibody but not by the anti-Na^+/K^+ ATPase antibody. (*A* and *B*) Upper traces: representative inside-out recordings illustrating the marked activation of Ba^{2+} conducting channels by application of a specific monoclonal anti-PMCA antibody (clone 5F10) to the cytosolic face of previously quiescent membrane patches. In *B* it is shown that cytosolic application of a specific monoclonal anti-Na^+/K^+-ATPase antibody (clone M8-P1-A3), used as a negative control, failed to induce Ba^{2+} conducting channel activity, whereas addition to the superfusion medium of the anti-PMCA antibody 5F10 succeeded to induce channel activity as in *A*. (*C*) Eosin blocked 5F10-induced channel activity. Lower traces are expanded time-scale extracts as indicated, showing that the induced Ba^{2+} conducting channel activity presents gating characteristics similar to that of CPZ-induced B-type Ca^{2+} channels. HP was −80 mV. Solutions were as described in Material and Methods; the appropriate concentration of NaN_3 (vehicle) was added to the control perfusion solutions.

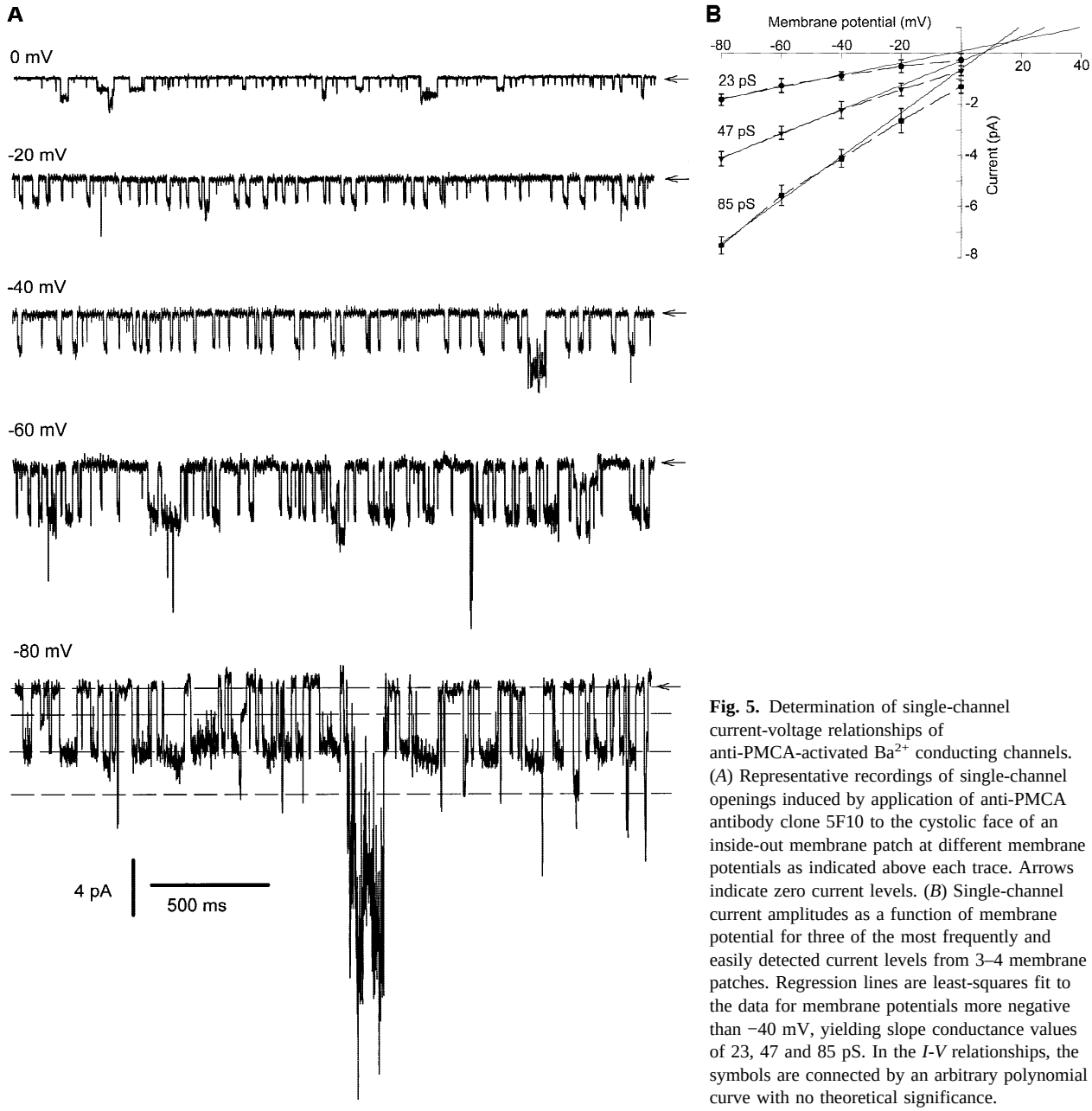

Fig. 5. Determination of single-channel current-voltage relationships of anti-PMCA-activated Ba^{2+} conducting channels. (*A*) Representative recordings of single-channel openings induced by application of anti-PMCA antibody clone 5F10 to the cystolic face of an inside-out membrane patch at different membrane potentials as indicated above each trace. Arrows indicate zero current levels. (*B*) Single-channel current amplitudes as a function of membrane potential for three of the most frequently and easily detected current levels from 3–4 membrane patches. Regression lines are least-squares fit to the data for membrane potentials more negative than −40 mV, yielding slope conductance values of 23, 47 and 85 pS. In the *I-V* relationships, the symbols are connected by an arbitrary polynomial curve with no theoretical significance.

ever, B-type channels are irreversibly blocked by eosin whereas the inhibition of cardiac PMCA by eosin has been reported to be reversible (Gatto et al., 1995). An explanation could be that the concentration of eosin used, in order to achieve a clear marked block of CPZ-activated B-type channels, was rather high in comparison with the IC_{50} of 1 μM reported for cardiac PMCA inhibition by eosin (Gatto et al., 1995).

External La^{3+} is a potent blocker of many types of Ca^{2+} channels (Hille, 1992). This block seems to occur simply by plugging the channel as shown for the Ca^{2+} release-activated channel (Aussel et al., 1996). In the present study, this is not the case, since we observed that only internal application of La^{3+} reversibly blocked CPZ-activated B-type channels. This observation implies the existence of a binding site for La^{3+} on the cytosolic part of the B-type channel protein. P-type ATPases, to which the PMCA belongs, form a phosphorylated intermediate during the pump cycle. In contrast with other P-type pumps, La^{3+} inhibits PMCA by increasing the steady-state level of the phosphorylated intermediate. It has been postulated that the competitive binding of La^{3+} on the Mg^{2+} phosphorylation site inhibits the hydrolysis of this phosphorylated intermediate (Ca-

rafoli, 1994; Herscher & Rega, 1996). This site is presumably located at the inner side of the membrane. However, the internal La^{3+} concentrations that completely block the Ca^{2+} transport of PMCA only inhibit the Ca^{2+}-dependent ATPase activity by 50%, suggesting that the fraction of ATP hydrolysis that is La^{3+} insensitive is not related to Ca^{2+} transport (Carafoli, 1991). From our results obtained with La^{3+} on CPZ-activated B-type channels, we can infer that PMCA and B-type Ca^{2+} channels may possess a similar inner membrane site which, when occupied by La^{3+}, prevents the translocation of Ca^{2+} from the inside to the outside of the membrane.

Aluminum fluoride complexes (AlF_3, AlF_4^- etc.) have a variety of biological actions such as activation of GTP binding proteins and inhibition of P-type ATPases (Chabre, 1990). $A1F_4^-$ has been shown to activate G protein-gated muscarinic atrial K^+ channels (Yatani & Brown, 1991), stretch-activated cationic channels in smooth muscle (Hisada, Singer & Walsh, 1993), epidermal growth factor-activated Ca^{2+} permeable channels in A431 cells (Kuryshev et al., 1993), L-type Ca^{2+} channels in rabbit femoral arteries (Ratz & Blackmore, 1990) and Cl^- and K^+ channels in T84 cells (Hwang et al., 1994). In the present study, the possibility that B-type Ca^{2+} channels are gated by G proteins could be excluded because (i) application of internal GTPγS was unable to activate B-type channels or inhibit their activation by CPZ; (ii) the blocking effect of AlF_3 on B-type channels was irreversible, an observation that is in contradistinction with other studies where activation of G protein-gated channels by AlF_3 has been found to be reversible (Yatani & Brown, 1991). Aluminofluorides, by mimicking inorganic phosphate, inhibit P-type pumps by binding to the phosphate-binding site of the ATPase (Troullier et al., 1992). Additional experiments are required to understand the mechanism of block of B-type channels by AlF_3, and to verify whether it has some similitude with that of P-type pumps or not. But if so, this would mean that the uncoupling of the ATPase from the PMCA is not necessary to observe a channel form of PMCA.

ATP and Calmodulin

ATP and calmodulin, respectively the main energizing substrate and the primary activator-regulator of PMCA ATPase significantly reduced CPZ-activated B-type channels. A straightforward explanation, if we admit that PMCA could, under CPZ, act as a channel, would be that in presence of high concentrations of ATP or CaM, the PMCA molecule can then switch to the pump mode or just reboot the pump, ceasing to act as a passive channel.

The matter of the affinity of ATP for the PMCA is complex (Carafoli, 1991), since there are two ATP binding sites. The high-affinity site is the catalytic site and has a Michaelis constant (K_m) of 1–2 μM. The low-affinity site which is assumed to play a role in the decomposition of the phosphorylated intermediate ($E_1 \sim P$ in the conventional formalism) which undergoes a conformational transition to a state called $E_2 \sim P$, has a K_m of 145–180 μM. The latter K_m values are within a range of concentration slightly lower than the ATP concentration (1 mM) for which we observed a significant reduction of CPZ-enhanced B-type channel activity.

In the absence of CaM, the PMCA is autoinhibited by a region of the C-terminal cytosolic unit of the molecule, containing the CaM-binding domain (Penniston & Enyedi, 1998). Binding of CaM activates PMCA by relieving this autoinhibition. It is tempting to argue that, in its autoinhibited state, the PMCA may act as a divalent cation-permeable channel. The anti-CaM action of CPZ could then promote the channel mode of PMCA. However, in the absence of CaM, PMCA, in its natural habitat, is surrounded by enough acidic phospholipids to be partially activated (Niggli et al., 1981; Carafoli, 1997). Being an amphipathic molecule, another role of CPZ would be to accumulate in the membrane (Martinac, Adler & Kung, 1990) and then, by interfering with acidic phospholipids, to relieve or strongly attenuate their enhancing effect on PMCA, increasing the probability for PMCA to switch to the channel mode. Addition of CaM would then be able to partially reactivate the pump mode of PMCA and to reduce the activity of the channel mode.

Effect of 5F10

The marked and clear activating effect of monoclonal anti-PMCA antibody 5F10 was surprising and unexpected. 5F10 recognizes a region highly specialized in the regulation of PMCA molecule, containing one ATP binding site and the receptor site for the autoinhibitory CaM-binding domain. 5F10 has been shown to recognize all known isoforms of the human PMCA and to react with many tested rat tissues, in particular the heart (Caride et al., 1996). It is difficult at the present time to figure out how 5F10 exerts its activatory effect, be it by displacing, removing or competing with CaM still present on its site. The binding of CaM to PMCA is slow and the dissociation seems to be even slower because of the high affinity of CaM for PMCA (Penniston & Enyedi, 1998). As stated in the results section, 5F10-induced channel activity shares all the main characteristics of CPZ-activated B-type channels. Thus, the activating effect of 5F10 brings strong experimental evidence in favor of the hypothesis of a link between B-type Ca^{2+} channels and PMCA.

B-Type Channel Substrates and the Oligomeric State of PMCA

The ion channel of P-type ion pumps is believed to be quite narrow (MacLennan, Rice & Green, 1997), and recent 2.6 Å resolution of the crystal structure of SERCA shows that it is barely wide enough to accommodate two ions (Toyoshima et al., 2000). This does not necessarily mean that the passive ionic conductance of this channel should be small because the conductance depends also on the speed at which ions go through the channel. In blebs of sarcoplasmic reticulum (SR) from skeletal muscle, calcium conducting channels with three conductance levels (37, 50 and 70 pS) have been observed and attributed to some form of passive ion channel in the SR calcium pump (Wang et al., 1992; Du et al., 1998). In the case of B-type Ca^{2+} channels, three conductance levels were frequently observed (Coulombe et al., 1989; Lefevre et al., 1995; Antoine et al., 1998) which are also of quite large values (around 20, 45 and 80 pS). But the dynamics of occurrence of these conductance levels is still not well understood. The different current extracts from the same recording presented in Fig. 6 exemplify the way these conductances occurred. In this recording the current transitions were particularly well defined. The lowest detectable unit level of conductance was 5.8 pS (Fig. 6*A*). From cooperative transitions of this unit conductance a large spectrum of conductances occurred. The three most probable or most often occupied levels of conductance were 4, 8 and 14 times the amplitude of the unit transition, leading to the most frequently observed conductances of 23, 46 and 81 pS (Fig. 2*C*). This was clearly demonstrated by the amplitude histogram (Fig. 6*E*) showing three levels of activity in concordance with these conductances. As already reported (Lefevre et al., 1995; Antoine et al., 1998), rare large conductances were also observed (Fig. 6*D*). The pattern of transitions prevailing was that unitary transitions cooperated to give small transitions of 2–4 levels (Fig. 6*A*), the small ones combine to give intermediate transitions of 5–8 levels (Fig. 6*B*) and the intermediate ones combine to give larger transitions around 12–15 levels (Fig. 6*C*). A combination between small, intermediate and large transition was also observed. Staircase openings through different conductance levels followed by a unique closure step (Fig. 6*B* and *C*) were frequently observed. Staircase closures preceded by large unique opening step (Fig. 6*D*) were very rarely observed.

A speculative explanation for the dynamics of conductance levels observed for B-type Ca^{2+} channels, if we postulate that these channels are the passive channel in PMCA, would be that aggregation of PMCA could give rise to the multiplicity of conductance states observed. The oligomeric state of PMCA and SERCA is important for their enzymatic function (Reddy et al., 1999). Aggregation inhibits Ca^{2+}-ATPase enzymatic activity. Amphipathic peptides such as melittin (Voss et al., 1991) and C28R2 (Reddy et al., 1999) aggregate SERCA and inhibit its activity. We can imagine that the amphipath CPZ could act in the same manner, by favoring or modifying aggregation of PMCA, inhibiting the pump function and promoting the passive channel mode, thus giving rise to B-type channel activity. Dimers or small oligomers could promote the smallest detected unit conductance level of 5.8 pS and the dynamics of oligomerization could proceed to the achievement of three most stable aggregates corresponding to the most frequently observed large conductance levels. Such dynamics could explain the diversity in patterns of current recordings observed (compare lower traces of Fig. 1*C* and *D*, 2*A* and *B* and 4*C*) and the complex gating behavior generally observed for B-type Ca^{2+} channels (Coulombe et al., 1989; Lefevre et al., 1995; Antoine et al., 1998). This complexity is generally not encountered in genuine ion channels.

Another Interpretation

Although the hypothesis that the PMCA pump, in some circumstances, could act as a Ca^{2+} conducting channel is plausible and attractive, other interpretations cannot be ruled out. For instance, isoform 4b of PMCA possesses a PDZ domain in its C-terminal tail. This isoform can tightly anchor other proteins that possess the same domain and then form clusters of multiprotein complexes (Kim et al., 1998). This type of complex exists in cerebellar Purkinje cells, where PMCA is colocalized with P-type Ca^{2+} channels (Hillman et al., 1996). PMCA4B isoform is present in cardiac cells (Penniston & Enyedi, 1998). The marked effect of PMCA modulatory agents on B-type Ca^{2+} channels could alternatively be explained by the possibility that in rat ventricular myocytes, PMCA could be co-aggregated with B-type channels and act as a kind of controller of these channels.

While the present results bring more indirect evidences in favor of the hypothesis that the plasma membrane Ca^{2+}-ATPase mediates B-type Ca^{2+} channels, this proposal clearly requires more experimental investigations to become a fully demonstrated one. For instance, it would be particularly interesting to investigate whether channel activity, similar to that of B-type Ca^{2+} channel activity, can be detected in giant liposomes reincorporating purified PMCA proteins. Because the PMCA is assumed to be an obligatory component of eukaryotic plasma membranes (Carafoli, 1997) its putative dual mode of activity might play a role in the control of resting cell Ca^{2+} concentration in physiological conditions, in addition of being a source of cellular Ca^{2+} overload in pathophysiological conditions e.g., in the "calcium para-

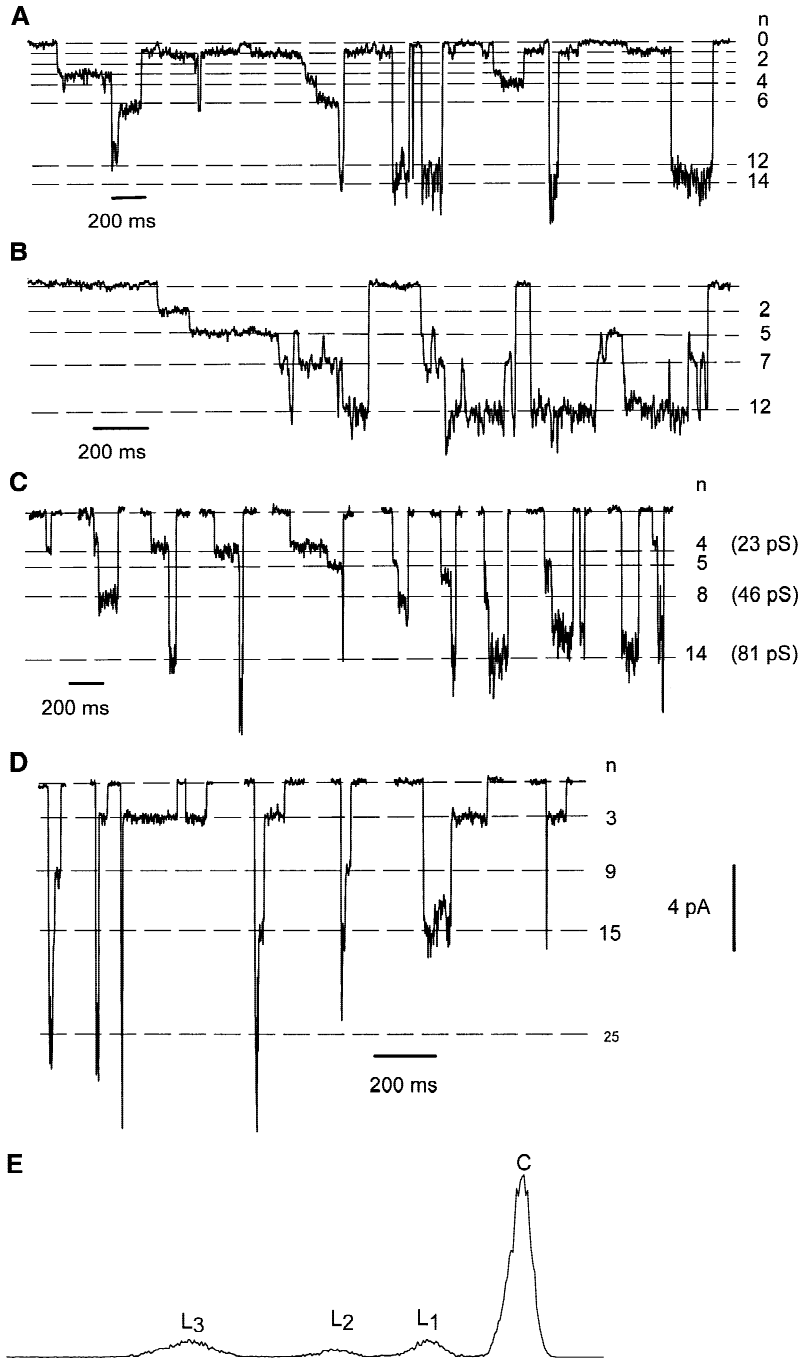

Fig. 6. Selected extracts of CPZ-activated B-type Ca^{2+} channel activity from the same current recording. These extracts (*A, B, C* and *D*) illustrate how the three most probable and frequently observed conductance levels (*E*) are generated from the cooperative transitions of a small unit conductance level. In this particular recording, the transitions between the different levels of conductance were very well resolved in time allowing to easily discriminate between the different patterns of transition and to detect a large spectrum of conductance levels from which the most probable and thus most frequently observed levels clearly emerge. The inside-out membrane patch was excised from a myocyte which had been exposed to 1 μM CPZ for 10 min. 50 μM Gd^{3+} was added to the standard pipette medium. HP was −80 mV.

dox" in the heart. Moreover, the ability of PMCA to co-aggregate with different receptors and channels, brings further scope for regulation of local Ca^{2+} signalization in microdomains at the plasma membrane. However, even if it could be demonstrated directly that, under certain experimental conditions, the PMCA mediates passive Ca^{2+} fluxes, this does not mean that this activity would serve a physiological function. It is possible that

the enzyme does have a pathway for transmembrane Ca^{2+} fluxes, but this pathway operates in passive mode only under the influence of certain perturbations such as calmodulin dissociation or antibody binding.

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