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Reincorporated Plasma Membrane Ca^{2+}-ATPase can Mediate B-Type Ca^{2+} Channels Observed in Native Membrane of Human Red Blood Cells

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Abstract. Recently, we reported indirect evidence that plasma membrane Ca^{2+}-ATPase (PMCA) can mediate B-type Ca^{2+} channels of cardiac myocytes. In the present study, in order to bring more direct evidence, purified PMCA from human red blood cells (RBC) was reconstituted into giant azolectin liposomes amenable to the patch-clamp technique. Purified RBC PMCA was used because it is available pure in larger quantity than cardiac PMCA. The presence of B-type Ca^{2+} channels was first investigated in native membranes of human RBC. They were detected and share the characteristics of cardiac myocytes. They spontaneously appeared in scarce short bursts of activity, they were activated by chlorpromazine (CPZ) with an EC_{50} of 149 μmole/l or 1 mmole/l vanadate, and then switched off by 10 μmole/l eosin or dose-dependently blocked by 1–5 mmole/l ATP. Independent of membrane potential, the channel gating exhibited complex patterns of many conductance levels, with three most often observed conductance levels of 22, 47 and 80 pS. The activation by vanadate suggests that these channels could play a role in the influx of extracellular Ca^{2+} involved in the vanadate-induced Gardos effect.

In PMCA-reconstituted proteoliposomes, nearly half of the ATPase activity was retained and clear "channel-like" openings of Ba^{2+}- or Ca^{2+}-conducting channels were detected. Channel activity could be spontaneously present, lasting the patch lifetime or, when previously quiescent, activity could be induced by application of 50 μmole/l CPZ only in presence of 25 U/ml calmodulin (CaM), or by application of 1 mmole/l vanadate alone. Eosin (10 μmole/l) and ATP (5 μmole/l) significantly reduced spontaneous activity. Channel gating characteristics were similar to those of RBC, with main conductance levels of 21, 40 and 72 pS. The lack of direct activation by CPZ alone might be attributed to a purification-induced modification or absence of unidentified regulatory component(s) of PMCA.

Despite a few differences in results between RBC and reincorporated PMCA, most probably attributable to the decrease in ATPase activity following the procedure of reincorporation, the present experimental conditions appear to reveal a channel-mode of the PMCA that shares many similarities with the B-type Ca^{2+} channel.

Key words: B-type Ca^{2+} channel — Red Blood Cell — Reconstituted plasma membrane calcium ATPase — Chlorpromazine — Calmodulin — Vanadate

Introduction

In a recent study (Antoine, Pinet & Coulombe, 2001), we presented indirect evidence that plasma membrane Ca^{2+}-ATPase (PMCA), in some form of "channel mode" of the pump, could mediate the activity of B-type Ca^{2+} channels observed in membranes of cardiac myocytes. This evidence came from the fact that the activity of these channels was discovered to be modulated by agents recognized to act on PMCA. Chlorpromazine (CPZ), an inhibitor of calmodulin (CaM), has been shown to markedly activate B-type Ca^{2+} channels (Lefevre et al., 1995). These CPZ-activated channels were found to be completely blocked by inhibitors of PMCA such as eosin, AlF_3 and lanthanum. Their activity was reduced, in a dose dependent manner, by calmodulin (CaM) and internal ATP (Antoine et al., 2001). Finally, the mono-

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clonal anti-PMCA antibody 5F10 was able to induce B-type channel activity.

In the present work, in order to bring more direct evidence that the B-type Ca^{2+} channel could be some form of PMCA pump, we investigated whether channel activity, similar to that of B-type Ca^{2+} channel activity, could be detected in membranes of giant azolectin liposomes reincorporating purified PMCA proteins. Giant liposomes are easily amenable to the patch-clamp technique (Berrier et al., 1996). As purified PMCA proteins from plasma membranes of cardiac myocytes were not available in large enough quantity, we used the available proteins, purified from membranes of human red blood cells (RBC). However, this constrained us to first of all test whether a channel activity similar to the B-type channel activity of cardiac cells could be demonstrated in the native membrane of human RBC. Our results show that: 1) in native membranes of RBC, as in cardiac membranes, CPZ was able to markedly activate Ba^{2+}-conducting channels, which were then blocked by eosin. The elementary conductances of these channels were very comparable to those of cardiac B-type Ca^{2+} channels; 2) Ba^{2+}- or Ca^{2+}-conducting channel activity was detected in membranes of liposomes incorporating PMCA, activity, which was markedly increased only with the concomitant presence of CaM and CPZ, and which shared the same levels of conductance observed in native RBC membranes.

Materials and Methods

Red Blood Cell Preparation

The RBC preparation was adapted from the procedure described by Leinders, van Kleef & Vijverberg, (1992). RBCs were obtained from the blood of healthy donors by fingertip puncture and were used on the same day. A drop of blood was immediately diluted in 1 ml of Tyrode's solution in a Falcon tube and stored at 4°C. Aliquots were then prepared by adding 1–2 μl of the diluted sample to 1 ml of hyposmotically modified Tyrode's solution (normal Tyrode's solution diluted in H_2O: 2v/1v) in 35-mm Petri dishes (Nunclon) to obtain a low density of swollen RBCs, loosely attached to the bottom of the dishes. Because of the small diameter of a RBC (≈8.5 μm), the modified Tyrode's solution was used in order to ensure cell swelling, facilitating the approach of the patch pipette and sealing (Hamill, 1985), For patch-clamp experiments, dishes were placed on the stage of an inverted phase-contrast microscope (400 × magnification) and RBCs were selected according to their characteristic shape.

PMCA Protein Purification

The procedure of purification of human RBC PMCA was that described by Penniston et al. (1988). PMCA proteins were diluted in EDTA and 0.05% Triton X-100 for a final concentration of 34.4 μg/ml, and then stored in liquid N_2. This preparation produces pure PMCA as judged by gel electrophoresis, sugar- and amino-acid analysis (Graf et al., 1982). The preparation was also nearly totally free of outwardly opening channels observable under the conditions used here. Such a channel was only observed once in about 950 membrane patches tested.

Gel Electrophoresis

SDS-gel electrophoresis was performed as described by Caride et al. (1996). After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

Preparation of Giant Proteoliposomes

The procedure used to prepare giant proteoliposomes was similar to that used by Berrier et al. (1992). As much as possible, all the different maneuvres were made under sterile conditions in order to avoid contamination with bacterial toxins. In brief, azolectin (L-α-phosphatidylcholine from soybean, type IV-S), was sonicated for 1–2 min at 10 mg/ml in a K^+-rich buffer solution, using a bath sonicator to yield small multilamellar liposomes. In standard experiments, 1 μg of PMCA protein was suspended in 50 μl of liposome solution containing Triton X-100 (0.1% w/v), giving a ratio (w/w) of 1 μg protein/1 mg lipid. These quantities were chosen to give, by a gross estimation, about 50 to 100 PMCA molecules under a standard membrane patch area (≈10 μm^2 following Sakmann & Neher, 1985). The estimation took into account that the lipid head surface is ≈0.7 nm^2 (Levy et al., 1990), the respective molecular weights of lipid and protein are near 1,000 and 130,000, the quantity of lipids adsorbed by Bio-Beads was around 25% (Levy et al., 1990), and that, in the case of sarcoplasmic Ca^{2+}-ATPase (SERCA), the transfer of proteins to lipid membrane was partial, leading to about 30% protein-free liposomes (Levy et al., 1992).

This suspension, to which 91 mg (dry weight) of Bio-Beads were added to adsorb the detergent, was kept overnight at 4°C, with constant agitation. The suspension of liposomes was centrifuged at 90,000 rpm for 30 min in a TL 100 Beckman centrifuge and the pellet was resuspended in 20 μl of 10 mmole/l HEPES (pH 7.0). The liposomes were then fused into giant liposomes using a cycle of dehydration-rehydration, as described by Criado & Keller, (1987). 6–7-μl drops of the giant proteoliposome suspension were deposited in wells of a 96-well culture dish (Nunclon) and dehydrated for 30 min at room temperature in a dessicator using a vacuum pump. The dehydrated film was covered with 10 μl of K^+-rich buffer solution and stored overnight at 4°C. For patch-clamp recordings, a 0.5–2 μl drop of the giant proteoliposome suspension was deposited on a Petri dish and diluted with 1.0 ml of the bath solution, which was the same as the K^+-rich buffer solution.

Measurement of PMCA ATPase Activity

ATPase activity was measured in medium containing (in mmole/l): KCl 120, TES-triethanolamine 30 (pH 7.2), $MgCl_2$ 5, ATP-Mg^{2+} 2.5, dithiothreitol 1, ouabain 0.5, EGTA 0.2, $CaCl_2$ 0.21 (giving a free Ca^{2+} concentration of 10 μM), 235 nmole/l calmodulin, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin. After an incubation of 30 min at 37°C, 250 μl of incubation medium was added to 1 ml of Fiske-Subbarow reagent. Reaction was started by addition of 0.5 μg of purified PMCA. Following an incubation of 10 min at 37°C, the absorbance reading was made at 690 nm. For the measure of activity of ATPase reincorporated into liposomes, at time-matched patch-clamp experiments, proteoliposomes were added to incubation medium supplemented with 0.5% Triton X-100, sonicated for 10–20 sec and then added to Fiske-Subbarow reagent.

Single-Channel Recording

All experiments were conducted at room temperature. Standard patch-clamp technique was used to record single-channel activity (Hamill et al., 1981). Patch pipettes were pulled from borosilicate capillaries (Corning Kovar Sealing 7052, WPI, FL) using a three-stage horizontal puller (DMZ-Universal Puller, Zeitz Instrument, Germany) and were fire-polished before use. The resistance of the pipette was 10–15 MΩ. Application of the pipette to the surface of a proteoliposome or a RBC and gentle suction generally resulted in the formation of a GΩ seal. The patch was then excised to inside-out configuration and unitary currents were recorded. When necessary, inside-out configuration was obtained by passage to the liquid-air interface, which led to disruption of the vesicle at the tip of the patch pipette. Although the inherent transmembrane potential of a human RBC is around −10 mV (Gedde & Huestis, 1997), membrane potential was varied in the range of −80 to +20 mV to increase the resolution for determination of channel conductances. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and filtered through an 8-pole Bessel low-pass filter (920LPF, Frequency Devices) setting of 1 kHz (−3dB point). They were digitized at 10 kHz using Acquis1 software (Bio-Logic, Claix, France). In the case of proteoliposomes, even if it was not known whether or not the reconstituted PMCA proteins had the same orientation as in the native RBC membrane, we applied the same convention for membrane potential as for the RBC membrane. Membrane potential was given in terms of potential of bath solution, assigning zero-potential level to the pipette medium. When control patch-clamp experiments were conducted on protein-free azolectin liposomes, all the membrane patches were electrically silent over at least 20-min running tests.

Data Analysis

Elementary conductances were determined and mean patch current was calculated as previously reported (Coulombe et al., 1989). 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. The K^+-rich buffer solution was (in mmole/l): KCl 100, HEPES 10, pH was adjusted to 7.0 with KOH. Tyrode's solution contained (in mmole/l): NaCl 135, KCl 4, $MgCl_2$, $CaCl_2$ 1.8, NaH_2PO_4 1, pyruvic acid 2.5, glucose 10, HEPES 10; pH was adjusted to 7.4 with NaOH. For all experiments, the pipette solution contained (in mmole/l): $BaCl_2$ 48, HEPES 10; pH was adjusted to 7.4 with CsOH. The superfusion control solution and bath solution contained (in mmole/l): K-aspartate 128; KCl 2; $BaCl_2$ 1; EGTA 5; HEPES 10; glucose 10; pH was adjusted to 7.4 with KOH. Stock solutions of chlorpromazine (CPZ) 10 mmole/l, and eosin 2 mmole/l, were prepared in ethanol. In experiments where CPZ or eosin was used, ethanol was added at appropriate concentration to the control superfusion solution. ATP refers to ATP-magnesium salt. A stream of solution from one of a series of piped outlets continuously superfused the bath side of membrane patch from which a recording was being made. The flow rate of perfusion solutions was 50–100 μl/min. In the present study, even if Ca^{2+} could be used as charge carrier to record channel activity similar to that of B-type Ca^{2+} channels (Lefevre et al., 1995), Ba^{2+} was used as 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), and this property appeared to be also true for B-type Ca^{2+} channels (*see* Lefevre et al., 1995). The use of Ba^{2+} made it easier to distinguish subconductance levels when they occurred. We also wanted to avoid activation of Ca^{2+}-activated K^+ or Cl^- channels by Ca^{2+} flowing through these channels. As Ba^{2+} is a blocker of practically all known K^+ channels (Hille, 1992), of Na^+ and Cl^- channels, it is a useful ion to study Ca^{2+} channels. Eosin refers to eosin Y, vanadate to Na-orthovanadate. Chemicals were purchased from Sigma Chemical (St Louis, MO).

Results

Ba^{2+}-Conducting Channels in Membrane of Human Red Blood Cell

Spontaneous Activity

In order to test for the presence of channel activity similar to that of B-type Ca^{2+} channels observed in cardiac myocytes, inside-out patch recordings were performed from membranes of human RBCs. When the patch pipette contained 48 mmole/l Ba^{2+} and the patch membrane potential was continuously held at −80 mV (no voltage pulses being applied) spontaneous inwardly directed currents corresponding to channel openings could be recorded (Fig. 1) similar to those observed in sarcolemmal membranes of rat ventricular myocytes (Coulombe et al., 1989; Wang, Clague & Langer, 1995). Ba^{2+} ion was used as charge carrier for reasons stated in Materials and Methods. These Ba^{2+}-conducting channels exhibit complex activity with bursts of intense activity (mean duration of 11.3 ± 12.5 sec, $n = 31$) followed by long-lasting quiescent periods of 54 to 364 sec (203 ± 147 sec, $n = 15$). Current traces of Fig. 1 also illustrate the complex behavior of current levels observed during such bursts of activity. Complex gating patterns could be recorded as exemplified by the lower current traces: the gating was variable (*compare* the two lower traces in Fig. 1*A* and *B*) and sometimes also changed with time at a given membrane potential. The lower trace in Fig. 1*A* shows at least three amplitudes of single-channel current corresponding to at least three substate conductances assumed to originate from the same channel (because cascade closures of three different levels appear to be initiated by an unique opening, *see* also Fig. 2*A*, lower trace). The spontaneous activity occurred in 30% of tested membrane patches (17 out of 59).

Effect of CPZ, Eosin and ATP

The next step was to test whether CPZ was able to activate Ba^{2+}-conducting channels as observed for B-type Ca^{2+} channels in cardiac myocytes. Addition to the cytosolic superfusion solution of 50 μmole/l CPZ induced, in a few seconds, single-channel openings in previously quiescent inside-out membrane patches from RBC. Figures 2*A* and 3*A* show representative current traces obtained before and during application

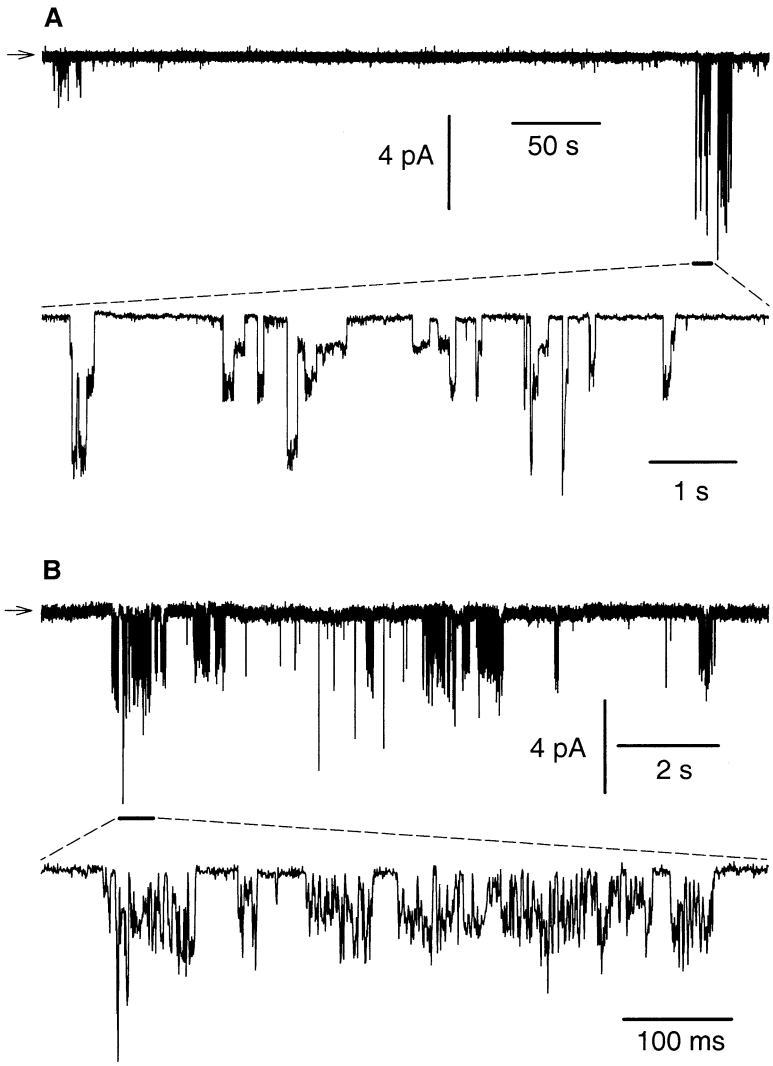

Fig. 1. Ba^{2+}-permeable channels can be observed in membrane patches from human red blood cells (RBC). Upper traces are representative inside-out recordings of spontaneous bursts of activity of Ba^{2+}-conducting channels showing (*A*) the sporadic bursting nature of the channel activity; (*B*) an example of gating of these channels during a short burst of activity, obtained during application of control superfusion solution to the cytosolic face of the membrane patches. Lower traces are expanded time-scale extracts illustrating the complexity and the diversity of gating of channel activity similar to that of B-type Ca^{2+} channels recorded in cardiac myocytes. In this and other figures, arrows indicate zero-current levels (closed level), and downward deflections are inwardly directed membrane currents. Membrane patch holding potential (HP) was −80 mV for all traces. Recording media were as described in Materials and Methods.

of CPZ to the cytosolic face of membrane patches when potential was maintained at −80 mV. Such a fast-developing clearly detectable CPZ-induced activity was observed in 45 out of 92 membrane patches. If CPZ application was maintained, channel activity persisted until the rupture of the patch membrane. At the beginning of CPZ superfusion, channel activity was rather small (Fig. 2*A* and 3*A*), then increased progressively until it reached a sustained, although somewhat irregular, large intensity. As illustrated in Fig. *2A* and 3*A*, CPZ-induced activity exhibited complex gating behavior with several levels of elementary conductances (*see* the lower current traces extracted from the upper current traces of Fig. 2*A* and 3*A*). The lower trace in Fig. 3*A* clearly shows at least three amplitudes of channel current levels corresponding to at least three substate conductances, as observed for the spontaneous channel activity.

We then determined the dose-response relationship of CPZ by using cumulative rising CPZ concentrations. Application of concentrations higher than 500 μmole/l was very difficult because of increasing patch membrane fragility, possibly as a result of CPZ accumulation in the intramembranar phase (Lieber et al., 1984). Figure 2*B* shows the dose-response relationship obtained by plotting relative mean single-channel current (*see* Materials and Methods) versus the concentration of CPZ. When the data were fitted with the following Hill equation:

$$y/y_{\max} = 1/[1 + (EC_{50}/EC)^{n_H}] \quad (1)$$

where $y/y_{\max}$ is the relative mean patch current, EC the concentration of CPZ (in mole/l), EC_{50} the apparent association 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 (± SD): EC_{50} = 149 ± 78 μmole/l and n_H = 0.76 ± 0.22.

It has been shown that eosin, by interacting with the nucleotide binding site, inhibits PMCA in RBC inside-out vesicles (Gatto & Milanick, 1993), and also

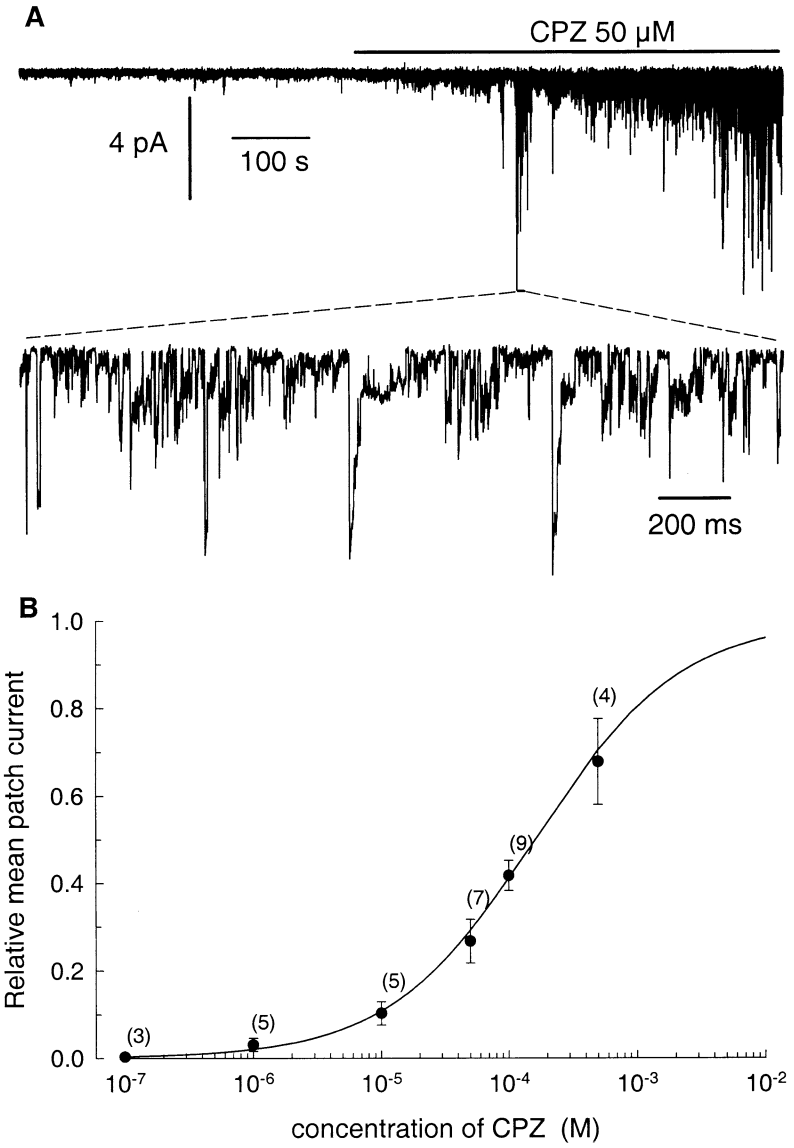

Fig. 2. Chlorpromazine (CPZ) markedly induced Ba^{2+}-conducting channel activity in membrane patches from human RBC (*A*) in a dose-dependent manner (*B*). Upper trace is a representative recording obtained from a previously quiescent inside-out membrane patch in control conditions and during application of CPZ (*A*). In this and other figures, agents were applied at the indicated concentrations to the cytosolic face of the membrane patches, during the periods of time indicated by horizontal bars above the current traces. Lower trace is an expanded time-scale extract showing that the complexity of gating of CPZ-induced channel activity was similar to that of spontaneously observed activity. HP was −80 mV. (*B*) Concentration-response relationship of the relative mean patch current activated by CPZ. The curve was fitted to the raw data according to Equation (1) with an EC_{50} of 149 µmole/l and a slope factor n_H of 0.76. Numbers in parenthesis and bars denote numbers of measurements obtained from different patches and corresponding SEM values, respectively.

inhibits B-type Ca^{2+} channels in cardiac myocytes (Antoine et al., 2001). The recording of Fig. 3*A* shows that addition to internal medium of 10 µmole/l eosin completely blocked CPZ-activated Ba^{2+}-conducting channels. This result was reproduced in 5 experiments. It is worth noting that in 3 other membrane patches showing spontaneous activity (without CPZ), eosin, at the same concentration, was able to reduce these patches to silence until they broke. This observation gives a strong indication that the CPZ-induced activity and the spontaneous activity are generated by the same type of channel. Moreover, internal application of ATP reversibly reduced, in a dose-dependent manner, the CPZ-induced channel activity (Fig. 3*B* and *C*). The effects of CPZ, eosin and ATP were very comparable to those observed for these reagents on B-type Ca^{2+} channel activity in cardiac myocytes (Antoine et al., 2001).

Current-Voltage Relationships, Elementary Conductances and Voltage-Independence

To go further in the verification that Ba^{2+}-conducting channels of RBC may correspond to B-type Ca^{2+} channels of cardiac myocytes, we established their current-voltage relationships. Fig. 4*A* shows current recordings at different membrane potentials, in a representative experiment in which Ba^{2+}-conducting channels were activated by 50 µmole/l CPZ applied to the cytosolic face of an inside-out patch. Three of the current levels were most frequent, as underlined by the dashed horizontal lines at −80 mV. Current-voltage relationships corresponding to these current levels, computed from 3–5 experiments similar to that of Fig. 4*A*, are shown in Fig. 4*B*. The *I–V* curves are not linear, a characteristic resulting very likely, at least in part, from asymmetrical concentrations of the main

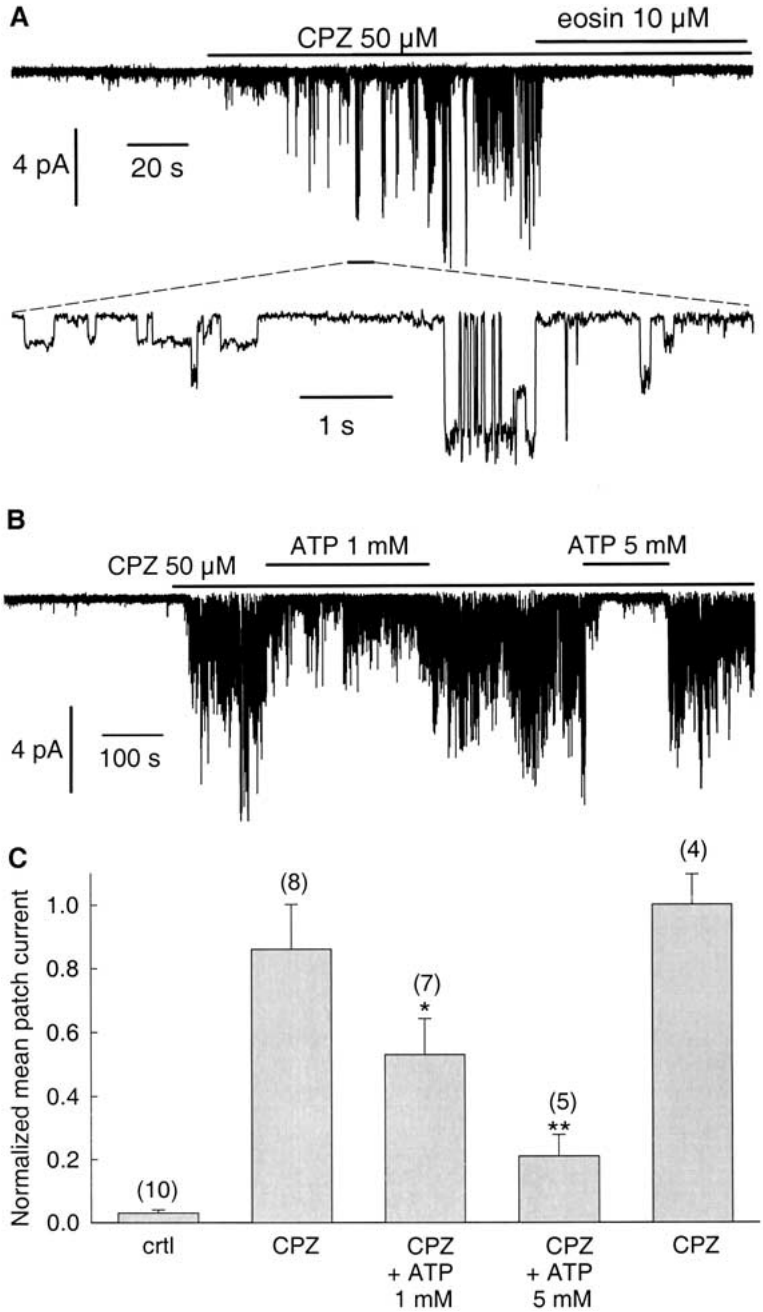

Fig. 3. CPZ-induced channel activity was blocked by eosin and ATP. Representative recordings obtained from previously quiescent inside-out membrane patches in control conditions, during application of CPZ and when eosin (*A*) or ATP (*B*) were added to the superfusion medium. Lower trace is an expanded time-scale extract showing the three most frequently observed levels of conductance of CPZ-induced channel activity. HP was −80 mV. (*C*) Bar graph showing the significant reduction in relative mean patch current caused by increased concentration of ATP to CPZ-induced B-type channel activity. This graph is a summary of many experiments conducted as shown in panel *B*. Mean patch currents, from experiments similar to that presented in *B*, were normalized with respect to the mean current calculated under CPZ activation (washout). Numbers in parenthesis and bars denote numbers of measurements obtained from different patches and corresponding SD values respectively; $^{*}P < 0.01$ and $^{**}P < 0.005$ versus CPZ alone; Student's *t*-test.

permeant ion, Ba^{2+}, the concentration of this ion being nominally of 1 mmole/l on the internal face of the membrane. In our experimental conditions, the calculated Nernst equilibrium potential for Ba^{2+} was +47 mV. The fact that the three curves tend to intercept zero-current values only at highly 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 22, 47 and 80 pS (Fig. 3*B*). These elementary conductances were comparable to those of 26, 50 and 80 pS obtained for CPZ-induced cardiac B-type Ca^{2+} channels (Lefevre et al., 1995). To determine whether CPZ-activated channels were dependent on membrane potential or not, the normalized mean single-channel current activated by 50 μmole/l CPZ was computed at different membrane potentials. Figure 4*C* shows the result obtained. A linear regression of all individual data gave a slope of -4.3×10^{-4}, with a correlation coefficient r of 0.021, indicating that the slope is not statistically different from zero. Thus, it can be concluded that CPZ-induced channel activity is voltage-independent.

Because these characteristics of Ba^{2+}-permeable channels of RBC closely resemble those of cardiac B-type Ca^{2+} channels, we could reasonably conclude

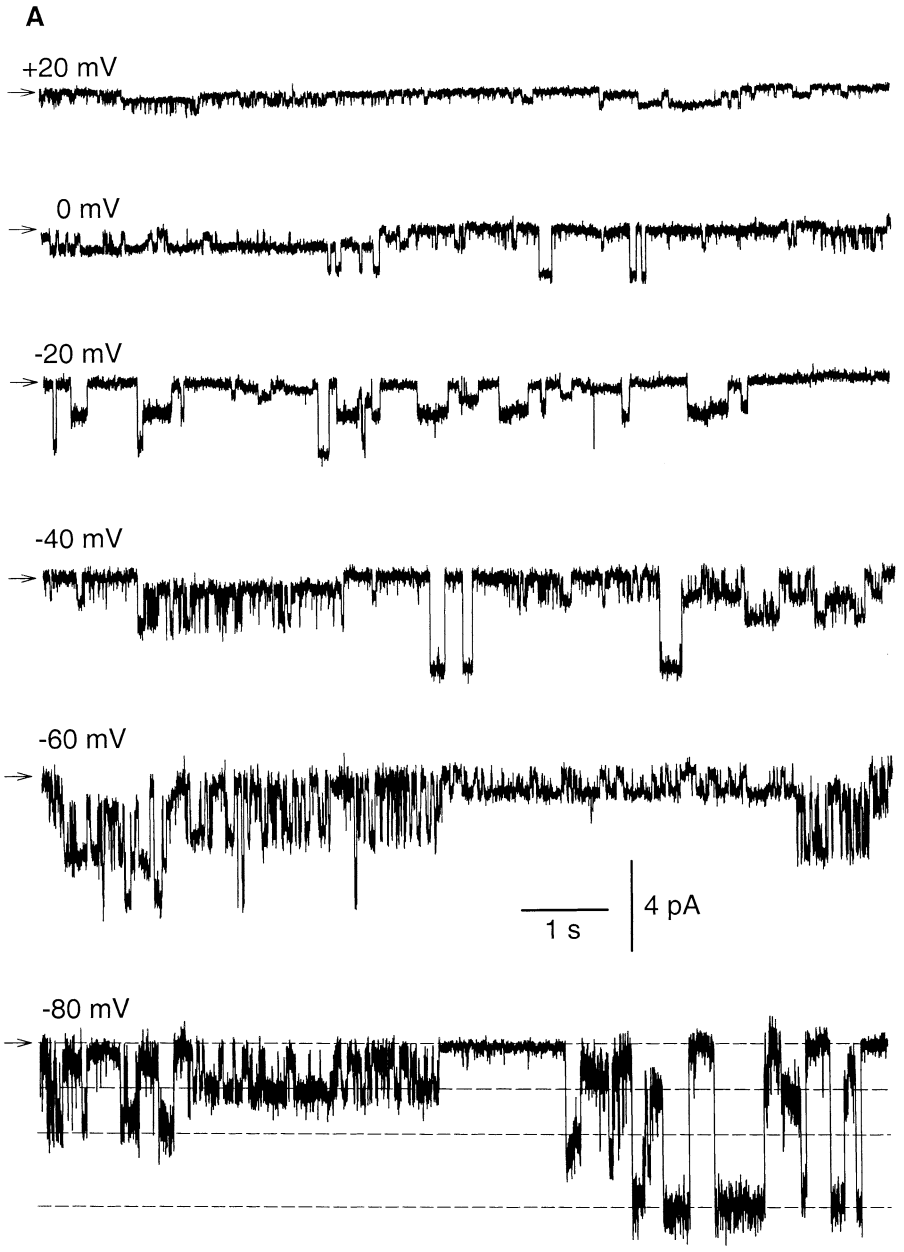

Fig. 4. Determination of single-channel current–voltage relationships and voltage independence of CPZ-activated Ba^{2+}-conducting channels in membrane of human RBC. (*A*) Representative recordings of single-channel openings induced by application of 50 μmole/l CPZ to the cytosolic face of an inside-out membrane patch, showing multiple current levels. Membrane potentials are indicated above each trace.

that they belong to the same class of channels. We then proceeded to experiment with purified PMCA proteins incorporated into giant liposomes.

Purified PMCA Reincorporated in Giant Liposomes

The purified PMCA used for these experiments was free of detectable amounts of contaminating proteins, as is shown in Fig. 5*A*. The PMCA used for this gel analysis was from the same preparation used for the experiments reported here. The PMCA protein was reincorporated into azolectin liposomes obtained by sonication. These proteoliposomes were then enlarged, by a cycle of dehydration-rehydration, into giant liposomes amenable to patch clamp. In order to test whether the ATPase activity was retained upon the procedure of reincorporation, we measured the activity (*see* Materials and Methods) of the original and the reincorporated enzyme at the time-matched patch-clamp experiments. We observed a decrease of ATPase activity from 17.8 ± 3.1 μmole/(mg min), (n = 3), a value in accordance with Graf et al. (1982), to 9.7 ± 3.9 μmole / (mg min), (n = 6). A part of the 45% lowering in activity can also be attributable to the presence of a higher level of Triton X-100 in the assay of the giant liposomes. As the remaining activity was still quite appreciable, we proceeded to patch-clamp experimentation. With 48 mmole/l Ba^{2+}

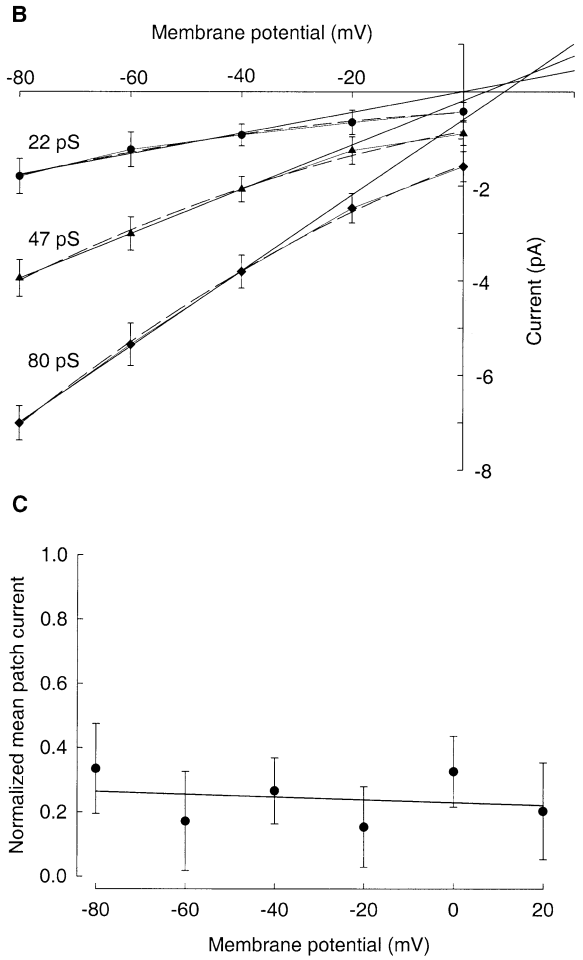

Fig. 4. *Continued.* (*B*) Single-channel current amplitudes as a function of membrane potential for three of the most frequently and easily detected current levels, as underlined by horizontal dashed lines on the −80 mV trace in Fig. 4*A*, from 3 different membrane patches. Regression lines are least-squares fits to the data for membrane potentials more negative than −40 mV, yielding slope conductance values noted above each respective line. In the *I–V* relationships, the symbols are fitted by an arbitrary polynomial curve (dashed lines) with no theoretical significance. (*C*) Relationship between relative mean patch current activated by 50 μmole/l CPZ and membrane potential, from 3–5 different inside-out membrane patches.

in the pipette medium, excised "inside-out-like" membrane patches displayed well-defined Ba^{2+}-conducting channel openings when membrane potential was maintained at negative values. Three main categories of responses were routinely obtained from membrane patches of PMCA-enriched proteoliposomes. In the first category, recordings were completely devoid of activity, exhibiting flat current traces over a period of time (at least 20 to 30 min), and they remained unresponsive to all agents tested. At least one third of all patches were found in this category. In the second category, patches exhibited spontaneous and almost permanent activity. This activity consisted of Ba^{2+}-conducting channel-like openings observed in about 1/3 of patches (193 out of 579). In the last category, they stayed quiescent until CPZ and CaM were applied together or when challenged with vanadate alone (*see* below).

Spontaneous Activity

With the same experimental conditions as above, representative current recordings, presented in Fig. 5, exemplified the activity observed. As in membranes of RBC, channel openings occurred spontaneously but with many more longer bursts of intense activity giving rise to an almost sustained activity. This channel activity was comparable to that of RBC and cardiac myocytes. The channel gating was complex, variable from patch to patch (compare Fig. 5*B*, traces at −20 and −40 mV) and displayed many amplitudes of current level with three predominant levels (Fig. 5*B*, trace at -60 mV). Experiments were also carried out using Ca^{2+} instead of Ba^{2+} as charge carrier. Figure 5*C* shows an example of a current trace presenting the characteristics observed when Ca^{2+} was present in the pipette.

Current–Voltage Relationships and Elementary Conductances

As in RBC and cardiac myocytes, many current levels were observed in recordings from proteoliposomes. Among them, three predominant most frequently appearing levels could be easily detected. The amplitude histogram in Fig. 6*A*, computed from a 4-min recording, confirms this observation. Figure 6*B* shows the current-voltage relationships obtained by plotting the amplitude of the three most often observed unitary current levels as a function of membrane potential. Measurements were made from recordings of spontaneously observed channel activity similar to those of Fig. 5. Probably because of the large dispersion of current amplitudes (which may be a consequence of the multiple orientations of the proteins in the membrane) a Goldman-type rectification was not clearly resolved. Nevertheless, a linear fit of data obtained in the range of tested membrane potentials gave elementary conductances of 21, 40 and 72 pS, fairly similar to those computed for RBC.

Effects of Eosin and ATP

The action of eosin and ATP on spontaneous activity of membrane patches from proteoliposomes was assessed. Figure 7 shows that eosin (10 μmole/l) and ATP (5 mmole/l) significantly and reversibly decreased channel activity. However, consequent residual mean channel activity remained under eosin and ATP—respectively, 0.10 ± 0.04 ($n = 6$) and 0.18 ± 0.09 ($n = 8$)—whereas in RBC, channel activity was, respectively, completely blocked and decreased to

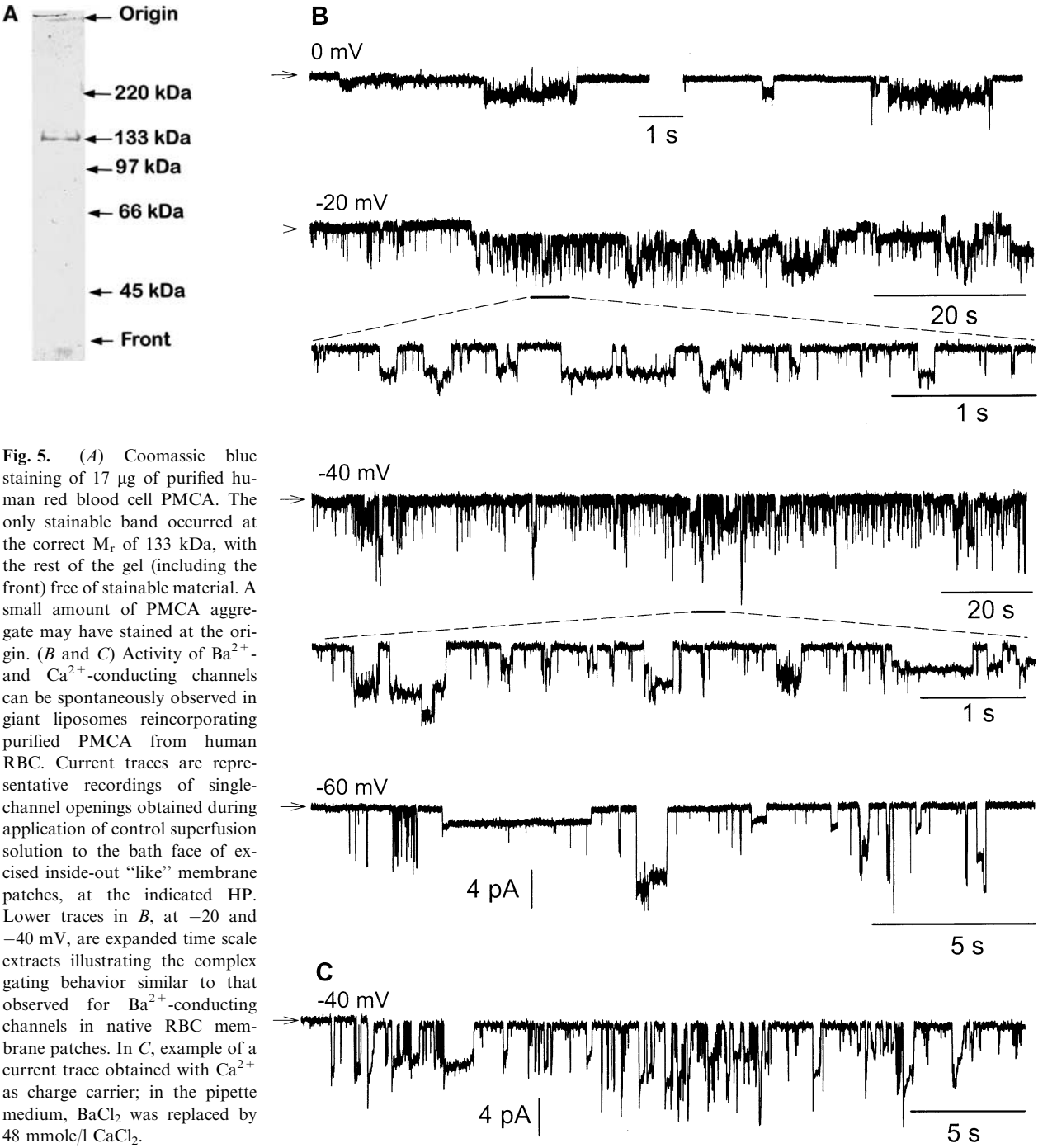

Fig. 5. (*A*) Coomassie blue staining of 17 μg of purified human red blood cell PMCA. The only stainable band occurred at the correct M_r of 133 kDa, with the rest of the gel (including the front) free of stainable material. A small amount of PMCA aggregate may have stained at the origin. (*B* and *C*) Activity of Ba^{2+}- and Ca^{2+}-conducting channels can be spontaneously observed in giant liposomes reincorporating purified PMCA from human RBC. Current traces are representative recordings of single-channel openings obtained during application of control superfusion solution to the bath face of excised inside-out "like" membrane patches, at the indicated HP. Lower traces in *B*, at −20 and −40 mV, are expanded time scale extracts illustrating the complex gating behavior similar to that observed for Ba^{2+}-conducting channels in native RBC membrane patches. In *C*, example of a current trace obtained with Ca^{2+} as charge carrier; in the pipette medium, $BaCl_2$ was replaced by 48 mmole/l $CaCl_2$.

0.11 ± 0.06 (n = 5). Explanations for this difference could be that some of the PMCA proteins were reincorporated inside-out and/or some of them were inactivated, as reflected in the lowering of ATPase activity after the long reincorporation and patch-clamp procedure.

Effect of CPZ and CaM

As CPZ markedly activated Ba^{2+}-conducting channels in cardiac myocytes and in RBC, we then tested the application of 50 μmole/l CPZ to the bath face of previously quiescent inside-out membrane patches from proteoliposomes. Results are illustrated in Fig. 8*A*, where no detectable channel activity was induced by CPZ alone. In a total of 58 quiescent membrane patches, application of CPZ alone invariably failed to induce any channel activity. Because one of the main differences with native RBC patch membranes could be the absence, in proteoliposomes, of CaM linked to PMCA, we added CaM to the superfusion medium. Interestingly, as also

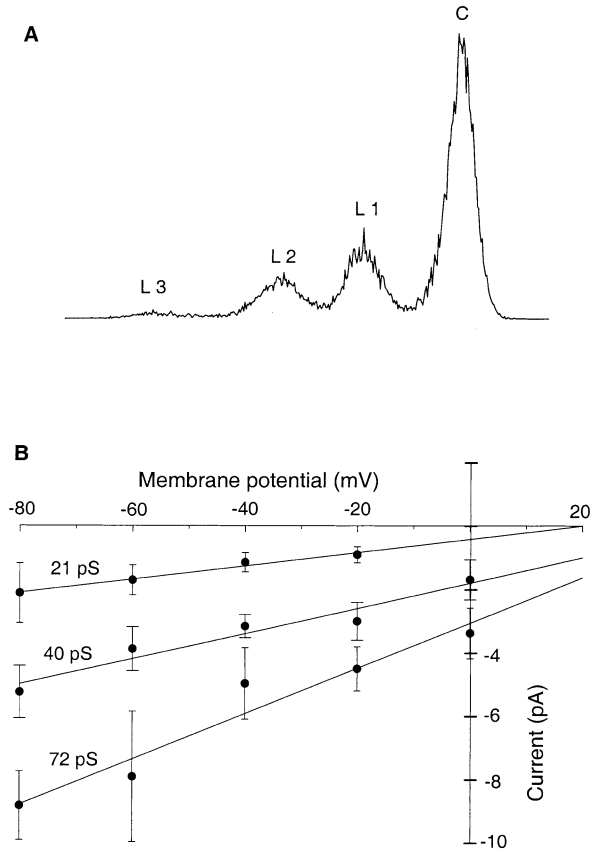

Fig. 6. Determination of the three main conductance levels of spontaneously active Ba^{2+}-conducting channels. (*A*) Current amplitude histogram, computed from a 4-min record, shows the three detected frequently observed current amplitudes (L_1, L_2, L_3); *C* is the closed state. (*B*) Current-potential relationships for the three conductance levels, determined from recordings of Fig. 5*B* and others (5–10 measurements from 10 different membrane patches) obtained in the same experimental conditions. Regression lines are least-square fits to the data, yielding elementary conductance values noted above each respective line.

shown by the recording in Fig. 8*A*, co-application of CaM and CPZ markedly activated Ba^{2+}-conducting channels. The presence of CaM, the main endogenous regulator of PMCA, thus appeared necessary to obtain the activating effect of CPZ, although there remained the possibility that CaM itself might be able to activate the channels. Figure 8*B* shows that this was not the case, since application of CaM to the bath face of a previously quiescent excised membrane patch was unable to activate any channels, whereas further addition of CPZ to the superfusion medium succeeded to induce Ba^{2+}-conducting channel activity. This activation by co-application of CaM and CPZ was observed in only four out of 69 membrane patches. The low success rate could be explained by the fact that 1) probably many quiescent membrane patches were devoid of purified proteins; 2) the effect of CaM is highly dependent on protein orientation (which is unknown in our conditions), since CaM needs to reach its site; 3) the way the aggregation of purified PMCA occurs is probably also of great importance (*see* Discussion). The CaM-CPZ-induced channel openings presented gating properties very similar to those observed for CPZ-induced channel activity in native membranes of RBC.

Effects of Vanadate on PMCA-enriched Proteoliposomes and on RBC

To further establish a relationship between the B-type channel activity and PMCA, we tried another agent known to interact with PMCA. Although not selective among them, Na-orthovanadate (vanadate) is the classic and widely used potent inhibitor of all P-type ion-motive ATPases, a class to which the PMCA pump belongs (Carafoli, 1991; Carafoli, 1994). Present at physiological pH as $[VO_3(OH)]^{2-}$, a penta-coordinate stereo analogue of phosphate, vanadate has been found to inhibit the RBC Ca^{2+} pump. Our first approach was to test vanadate as a channel blocker in spontaneously active patches. It appeared clearly that channel activity was not decreased by 1 mmole/l vanadate, but rather enhanced. Figure 9*A* shows that vanadate, when applied to the membrane side facing the bath, reversibly induced Ba^{2+}-conducting channel openings in an excised quiescent membrane patch from a proteoliposome. The expanded trace visible in the lower part of Fig. 9*A* gives an example of the complex gating observed in the presence of vanadate, fairly comparable with that of B-type Ca^{2+} channels. The three most frequently observed levels of conductance were again easily detectable. This result was reproduced in five other experiments. It was then of interest to return back to RBC and test whether vanadate had the same effect. This was indeed the case: Fig. 9*B* shows a typical recording illustrating that, when vanadate was applied to the cytosolic face of a previously moderately active membrane patch, a clear increase in activity occurred. Again, the lower expanded trace of Fig. 9*B* shows the complexity of channel gating and its similitude to that observed in membranes of proteoliposomes. Three other membrane patches provided the same result, giving a mean relative patch current of 0.44 ± 0.12. The vanadate-induced channel activity appeared dose-dependent, since lower concentrations of vanadate (1 and 10 μmole/l) also induced a channel activity for which the mean relative patch current was, respectively, 0.30 ± 0.16 (n = 3) and 0.37 ± 0.10 (n = 4). For these experiments, superfusion medium was supplemented with, in mmole/l, EGTA 0.001, Ca^{2+} 0.93, Ba^{2+} 0, Mg^{2+} 2. In the case of RBCs, unlike proteoliposomes, the action of vanadate was not reversible.

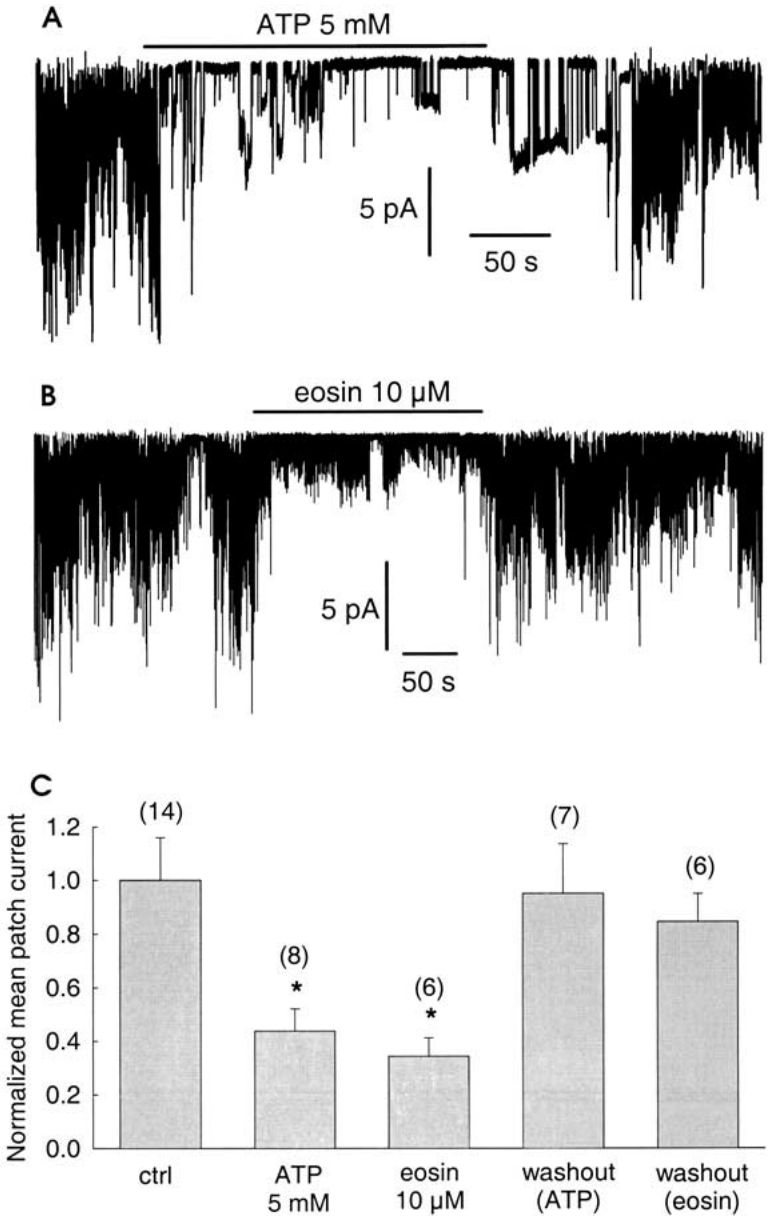

Fig. 7. ATP and eosin can markedly and reversibly reduce spontaneous channel activity of reincorporated PMCA. (*A*) and (*B*) Typical recordings of channel activity obtained before, during application of ATP or eosin to the cytosolic face of inside-out membrane patches, and during washout. HP was −40 mV. (*C*) Bar graphs summarizing the significant and reversible decrease in relative mean patch current. Mean patch currents, from experiments similar to that presented above, were normalized with respect to the mean current calculated in control. Numbers in parenthesis and bars denote numbers of measurements obtained from different patches and corresponding SEM values, respectively; $^{*}P < 0.01$ versus control; paired *t*-test.

Discussion

Two main new results are reported in the present paper. The first result is that B-type Ca^{2+}-channel activity can be detected in human RBCs. B-type Ca^{2+} channels observed in RBCs share the following characteristics with those previously reported in rat ventricular myocytes (Coulombe et al., 1989; Wang et al., 1995; Lefevre et al., 1995; Antoine et al., 2001) and in human atrial myocytes (Antoine et al., 1998). (i) Their activity was spontaneously observed in short bursts, with a low frequency of occurrence. (ii) They were readily activated by CPZ and then blocked by eosin and ATP. (iii) The spontaneous and CPZ-induced activities occurred over a large range of steady-state applied potentials (−80 to +20 mV). (iv) Their gating pattern was complex with at least three main current levels. The second result is that purified PMCA from human RBCs, reconstituted in liposomes, can give rise to Ba^{2+}-conducting channel activity. Gating and conductance levels were very similar to those of B-type channels of native RBCs. With reconstituted PMCA, channel activity was induced only by co-application of CPZ-CaM. Spontaneous activity was significantly reduced by eosin and ATP. Interestingly, orthovanadate was able to enhance channel activity in both native RBCs and in PMCA-enriched proteoliposomes.

B-Type Ca^{2+} Channel in RBCs

It was not in the scope of the present work to study the rationale of the presence of calcium channels in membranes of RBCs. There is an abundant literature

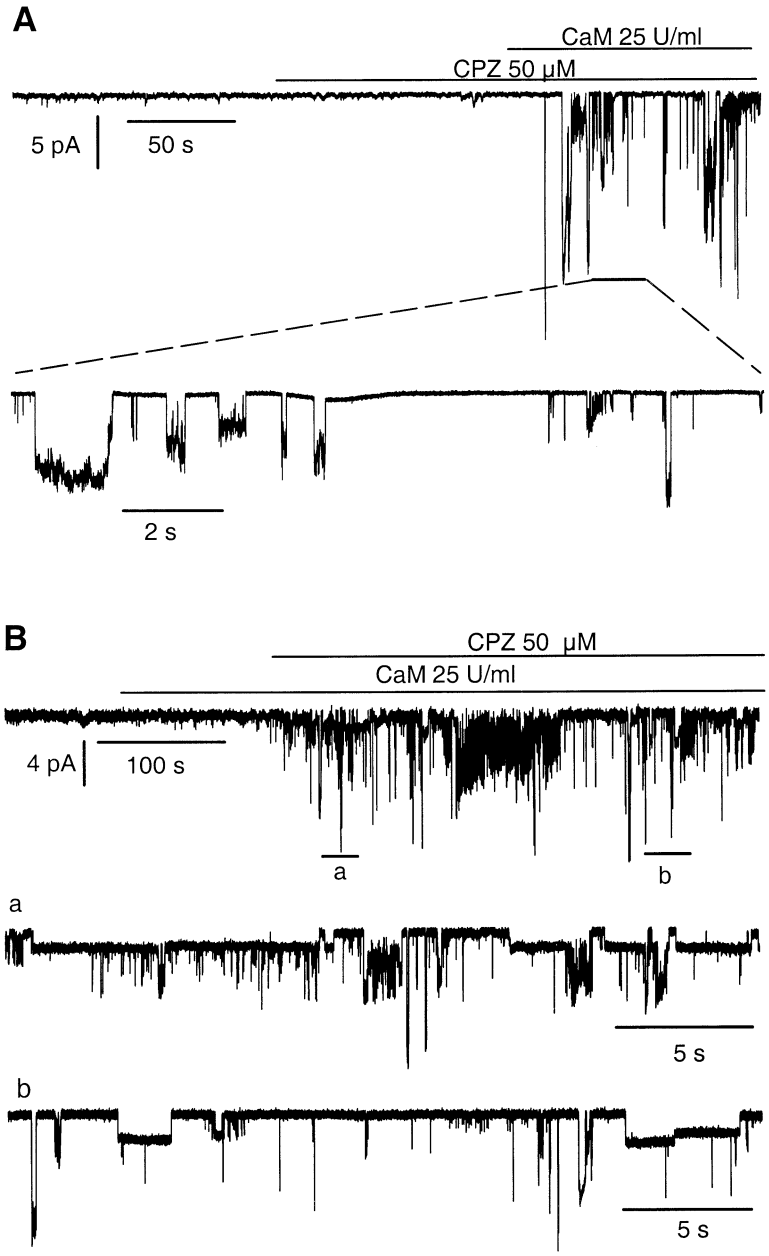

Fig. 8. Ba^{2+}-conducting channels can be activated only by concomitant application of CPZ and calmodulin (CaM), to liposomes reincorporating purified PMCA. (*A* and *B*) Upper traces: representative recordings illustrating that application of CPZ or CaM alone failed to induce any channel activity, whereas a marked activation of Ba^{2+}-conducting channels occurred upon simultaneous application of CaM and CPZ to the bath face of previously quiescent inside-out "like" excised membrane patches. 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 Ba^{2+}-conducting channels in native RBC membranes. HP was −40 mV.

reporting that the calcium influx through RBC membranes, measured during various conditions and by different techniques, might be mediated by voltage-independent (Miller et al., 1996), or sometimes by voltage-dependent calcium channels (Yang, Andrews & Low, 2000). However, few reports deal with direct patch-clamp recordings of calcium-channel activity in this type of cell. For instance, in cell-attached patches from human erythroblasts, Cheung et al., (1997) detected a 3.2-pS voltage-independent Ca^{2+} channel whose open probability was increased by erythropoietin. In the present study, we bring evidence of the existence of Ca^{2+}-permeable channels that could also be responsible for a calcium influx pathway in RBC. These channels share most of the characteristics of B-type Ca^{2+} channels observed in cardiac myocytes: (i) in our "control" recording inside-out patch conditions, they are spontaneously observed during very scarce episodic bursts of activity; (ii) they are activated by CPZ in a dose-dependent manner with an EC_{50} of 149 µmole/l very similar to the 160 ρmole/l reported value in rat cardiac myocytes (Lefevre et al., 1995); (iii) when activated by CPZ they are switched off by 10 µmole/l eosin and reversibly blocked, in a dose-dependent manner, by ATP; (iv) also when activated by CPZ, three main conductance levels of 22, 47 and 80 pS were observed, highly comparable to those of 26, 50 and 80 pS obtained for CPZ-induced

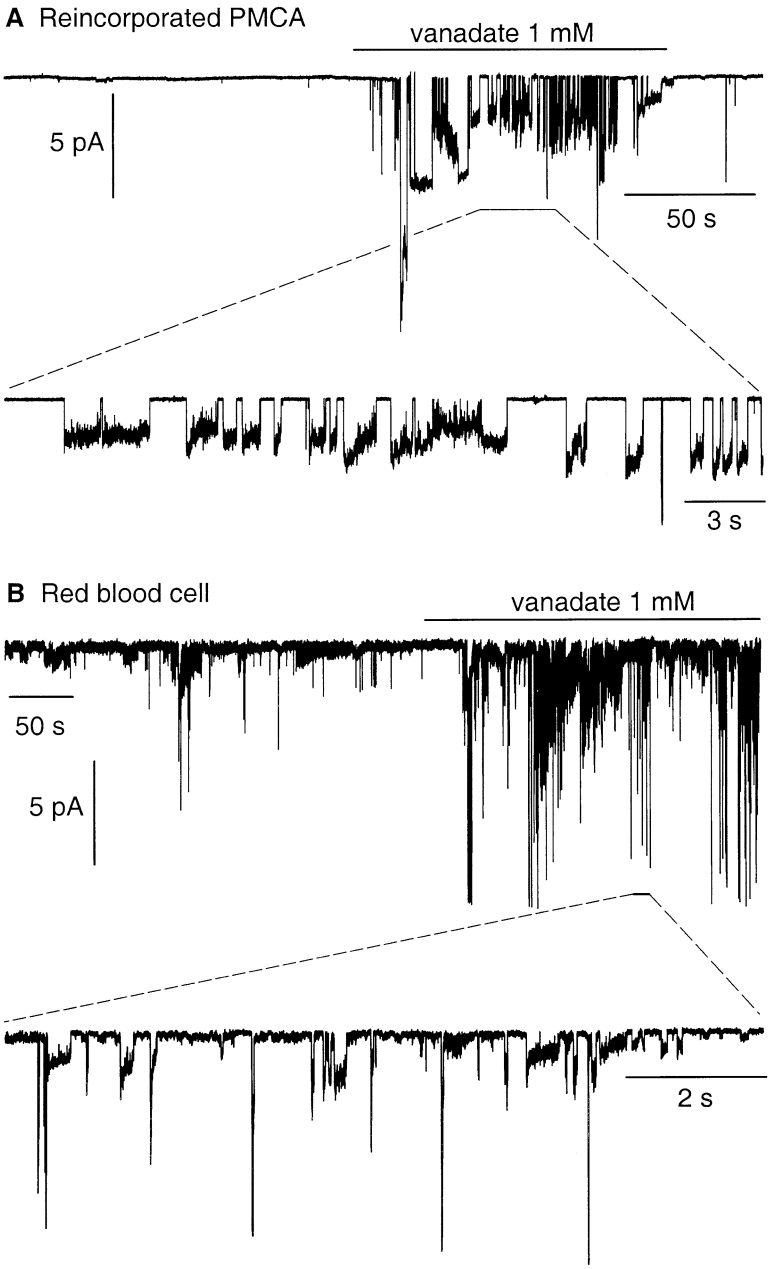

Fig. 9. Vanadate markedly increased Ba^{2+}-conducting channel activity in membrane of RBC (*B*) and induced a similar type of activity in liposomes reincorporating PMCA (*A*). Upper traces are representative recordings of single-channel openings obtained before and during application of Na^{+}-orthovanadate to the cytosolic face (*B*) or to the bath face (*A*) of inside-out membrane patches. Lower traces are expanded time-scale extracts from upper traces and illustrate the observed complex gating behavior displaying different conductance levels. HP was −80 mV.

cardiac B-type Ca^{2+} channels for the three most frequently observed conductance levels (Lefevre et al., 1995); (v) they are independent of membrane potential. It can thus be reasonably concluded that B-type Ca^{2+} channels are present in RBC. However, caution is required prior to attributing a physiological role to these channels. Interestingly, Raess & Keenan (1996) observed a passive calcium influx in ATP-depleted human RBC, which was insensitive to ryanodine and L-type Ca^{2+} inhibitors, but blocked by PMCA inhibitors. These authors suggested that this Ca^{2+} influx was mediated by PMCA, since the influx was blocked when the ATPase was inhibited. In cardiac cells, addition of cytosolic ATP markedly reduced B-type Ca^{2+} channel openings (Antoine et al., 2001), indicating that the Ca^{2+} influx mediated by these channels, in a physiological situation, is of very low intensity, as for RBC. In the present inside-out recordings, channel activity also occurred in the absence of cytosolic ATP and was blocked by ATP and the PMCA inhibitor eosin, indicating that B-type channels can be good candidates to mediate calcium influx in ATP-depleted RBCs.

The present results were obtained from excised inside-out patches from RBCs maintained in hypotonic medium to render feasible patch-clamp experiments. Swelling may activate mechano-gated channels. Stretch-induced membrane deformation is

the basal test in study of mechanosensitive channels. Membrane deformation resulting from uneven partitioning of amphipath between membrane monolayers is also the cause of the activation of mechanosensitive channels induced by amphipathic compounds such as CPZ (Martinac, Adler & Kung, 1990). That CPZ-induced channel activation reported in the present study results from the same mechanism is, however, unlikely because negative or positive pressures, up to 150 mmHg, applied to the patch pipette failed to trigger channel openings. The same result was reported in cardiac myocytes (Lefevre et al., 1995).

The spontaneous channel openings were observed in 30% of membrane patches from RBCs, whereas they were seen in 20% of rat ventricular or human atrial myocytes. Assuming that the channel activity is mediated by PMCA, this difference may arise (i) from a higher density of PMCA in RBCs, (ii) from a singular localization of PMCA in cardiac myocytes or (iii) from differences in PMCA isoforms expressed in these two cell types. The number of PMCA molecules has been reported to be between 700 and 4500 per RBC (Carafoli, 1991; Rega & Garrahan, 1975; Graf, Filoteo & Penniston, 1980; Jarrett & Kyte, 1979). The RBC surface being around 163 μm^2 (Eylar et al., 1962), the number of PMCA proteins potentially present in a patch area ($\approx$10 μm^2) is in the range of 40–300 PMCA, assuming a uniform distribution. As far as we know, no data are available concerning cardiac myocytes. In stereociliar hair cells, Yamoah et al., (1998) estimated a density of 2000 PMCA proteins per μm^2 of membrane. Such a high density would be surprising for cardiac cells. But even though the cardiac PMCA density might be higher than in RBC, a lower frequency of observation in cardiac cells could also be explained by a difference in localization of clusters of PMCA. Close to 100% of cardiac PMCA are localized to caveolae (Hammes et al., 1998). The patch pipette may have a higher probability to reach a cluster of PMCA molecules in RBC than a caveola in a cardiac cell. However, the low incidence of detected activity in RBC might originate from the swelling of RBC, which may be interfering with the typical homogeneous distribution of PMCA throughout the membrane. The last possible explanation, concerning this difference in frequency of observation between RBCs and cardiac cells, is the tissue distribution for the different PMCA isoforms (Strehler & Zacharias, 2001). Four genes code for PMCA, and alternative splicing gives rise to about 15 isoforms. The four main isoforms are PMCA1, PMCA2, PMCA3 and PMCA4. PMCA2 and 3 are mainly present in the nervous system, whereas PMCA1 and 4 are ubiquitous. PMCA4 is less expressed than PMCA1 in all cell types except in RBCs and other cells derived from hematopoietic stem cells, where it is the most abundant isoform (Paszty et al., 1998; Stauffer, Guerini & Carafoli, 1995). To give some credibility to this explanation, it would be necessary to compare the data from two experiments in which an equal quantity of PMCA1 or PMCA4 had been reconstituted, and observe that PMCA4 mediates channel activity more frequently than PMCA1.

Reincorporated PMCA

It has been reported, in the case of the reincorporation of purified SERCA proteins, that their transfer to lipid membranes occurred only partially, leading to 30% of protein-free liposomes (Rigaud, Pitard & Levy, 1995). Taking into account that a similar process is likely to occur with PMCA reincorporation, we then have an explanation for the category of at least one third of all tested membrane patches that were totally devoid of any activity. This means that two categories of membrane patches that contained purified PMCA were obtained. The first category exhibited spontaneous activity, and in the second, the activity needed to be triggered. Both categories occurred with about the same frequency. An explanation for the existence of these two categories can be that the purified PMCA appeared in different forms. That such forms exist has been reported by Penniston et al. (1988). They observed that appropriate pooled fractions of the purification, observed by SDS-polyacrylamide gel electrophoresis, were mainly constituted of a single 138-kDa peptide containing the Ca^{2+}-dependent ATPase activity, sometimes accompanied by a 124-kDa band corresponding to a proteolytic product of the ATPase (probably a fragment deprived of the CaM-binding domain (Vorherr et al., 1991) and a frequently observed band between 250 and 300 kDa corresponding to the dimer of purified PMCA).

Spontaneous Recorded Activity

The frequency of spontaneous channel activity was higher in proteoliposomes (1/2) than in RBCs (1/3), even if the quantity of purified PMCA proteins (*see* Materials and Methods) was estimated in order to obtain a density of PMCA comparable to that of RBC. An explanation would be that the quantity of PMCA used was very likely underestimated.

The quasi-sustained channel activity occurring during the whole patch lifetime was a marked difference in comparison with the scarce bursts of activity observed in RBC. This difference may come from: (i) the difference in lipid environment of reconstituted PMCA. Azolectin used to make liposomes is rich in phosphatidylserine, an acidic phospholipid. Acidic phospholipids are known to enhance ATP hydrolysis of PMCA (Carafoli, 1991); (ii) the absence of cholesterol in the proteoliposomes

used. It has been shown that cholesterol directly interacts with PMCA by inhibiting its previously CaM-induced ATPase activity (Ortega & Mas-Oliva, 1986); (iii) the fact that the protein environments of PMCA surely differ. Indeed, in their natural environment, PMCA are associated with other proteins such as the MAGUK family proteins (Kim et al., 1998); (iv) the presence, following the reincorporation procedure, of a fraction of PMCA proteins that were functionally impaired. Even though purified PMCA proteins conserved, in vitro, a high ATPase activity (Penniston et al., 1988), the integrity of the "pumping" or transporting properties of some of them could have been somewhat altered by the reincorporation procedure, as shown by the decrease in ATPase activity following this procedure.

CPZ CaM-induced Activity

The fact that, in reconstituted PMCA, CPZ was able to induce channel openings only in the presence of CaM was unexpected. Only the CPZ-CaM complex seems to be able to induce a channel mode of reconstituted PMCA pump, perhaps because of the depletion of substrates or cofactors whose presence was unknown. Such substrates or cofactors might remain associated with the membrane derived directly from an intact cell, while they would certainly be removed during the extensive purification process, which produces reconstituted pump.

Possible Relationship of Channel Activity to PMCA Conformation

It is possible to interpret these and previous results in terms of the E_1-E_2 equilibria involved in the enzyme's cycle, which is widely accepted to be responsible for the transport of Ca^{2+}. The enzyme must go through the whole cycle in order to split ATP and transport Ca^{2+}. Many other activities observed in this enzyme are due to one or another part of the cycle, and they occur without the enzyme's going through the whole cycle (Rossi, Garrahan & Rega, 1986), Since VO_4^-, is an inhibitor of the whole enzyme cycle but stimulates the channel, the channel must also possess an enzyme property involving only part of the cycle. From this point of view, the effects of VO_4^-, and those previously observed in cardiac myocytes for La^{3+} (Antoine et al., 2001) make sense. La^{3+} stops the pump cycle just downstream of E_1P, so it will favor the buildup of the E_1 state. VO_4^- emulates the transition state complex of PO_4^- with the enzyme, and favors the E_2 state (Barrabin, Garrahan & Rega, 1980). Therefore, we would assign the channel activity as an action of the E_2 state, which has the Ca^{2+} sites poised to release Ca^{2+} into the intracellular space. This would explain the stimulation of the channels by VO_4^-, and their inhibition by La^{3+}.

It is somewhat harder to interpret the reported inhibition of the channels by AlF_3 (Antoine et al., 2001) in terms of this theory. Because of the similarity in structure between AlF_4^-, VO_4^- and PO_4^-, AlF_4^-, like VO_4^-, is believed to replace PO_4^-, in its complex with the pump. However, investigations on the sarco(endo)plasmic reticulum Ca^{2+} pump (SERCA) have shown significant differences between the two inhibitors. In the case of VO_4^-, it stabilizes the E_2 state of the enzyme and competes with ADP for its binding site (Pick, 1982). AlF_4^-, on the other hand, forms a stable complex with ADP, Ca^{2+} and the pump, which is an inactive dead end (Troullier, Girardet & Dupont, 1992). If the E_2 state is responsible for the channel activity, the inhibitory action of AlF_4^- could be explained if small amounts of ADP and Ca^{2+} were present. They may have been present associated with the membrane of the myocytes where the addition of AlF_3 was tested (Antoine et al., 2001).

The actions of chlorpromazine are hard to interpret because of its multiple effects on PMCA and membranes. It not only binds to calmodulin but also dissolves in membranes (Lieber et al., 1984). It partitions into red cell membranes with a partition coefficient of about 2000, and will keep on entering the membrane until it exceeds the amount of lipid present. So the action of chlorpromazine on the channel might be due in part or entirely to its going into the membrane instead of binding to calmodulin.

The effects of ATP and eosin on the E_1-E_2 distribution of PMCA are not known, so their effect on channels cannot yet be interpreted in terms of this theory. Even though the E_1-E_2 effects of eosin and ATP are not known, their inhibition of the channel activity certainly points to PMCA as the molecule responsible for the channel activity.

Effect of Na^+-Orthovanadate

Vanadate markedly enhanced Ba^{2+}-conducting channel activity in RBC and induced channel openings in PMCA-enriched liposomes. The vanadate-activated current recorded in both preparations (Fig. 9), looks very similar to the above CPZ-enhanced and CPZ-CaM-induced channel activity. Vanadate is specific for P-type ATPases, and, as already mentioned, stabilizes the E_2 state. It is well known that vanadate, in addition to being a potent inhibitor of PMCA in RBC, gives rise to a Ca^{2+} influx mediated by a Ca^{2+}-activated K^+ channel, the so-called Gardos $SK_{(Ca)}$ channel (Latorre et al., 1989). Varecka, Peterajova & Pisova (1998) brought evidence that this Ca^{2+}-influx occurs *via* a pathway homologous to L-type Ca^{2+} channels. Taken together, these observations on the action of vanadate

may lead to the suggestion that at least a part of the vanadate-dependent Ca^{2+}-influx pathway originates from the vanadate-induced passive Ca^{2+}-channel mode in the PMCA pump.

Conductance Levels

As for cardiac cells, three main conductance levels were most often easily detected in RBC and proteoliposome experiments, with nearly the same values in the same recording conditions: 23, 47 and 85 pS for cardiac myocytes (Antoine et al., 2001), 22, 47, 80 pS for RBCs and 21, 40, 72 pS for proteoliposomes. These conductance levels seem to correspond to the most stable and probable conductance states (Antoine et al., 2001) because (i) multiple lower and larger conductance levels were also observable but with considerably lower frequency; (ii) cooperative aggregation of lower levels (*see* Fig. 1*A*) or disaggregation of larger levels (*see* Fig. 2*A*) very often subsequently passed through the three most stable states. We have already discussed (Antoine et al., 2001) how these most probable conductance states could be generated by a(n) (de)assembling of many units of the lowest conductance level, each of them being putatively mediated by one PMCA.

Taken together, despite some discrepancies in results between RBC and reincorporated PMCA, most probably attributable to the lowering of the ATPase activity consequently to reincorporation, the results of the present study bring direct evidence of a passive channel-mode of the PMCA, which shares many similarities with the B-type Ca^{2+} channel. Our study also shows the existence in RBCs of B-type Ca^{2+} channels, activatable by vanadate, which might account for an influx of Ca^{2+} into the cell involved in the vanadate-induced Gardos effect.

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