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-
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 re-incorporating 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 hypotonically modified Tyrode’s solution (normal Tyrode’s solution diluted in H$_2$O: 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 x 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 Pennistom 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 Coo-massie 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 manoeuvres were made under sterile conditions in order to avoid contamination with bacterial toxins. In brief, azolectin (t-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 a 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 Criaud & 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 μl 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 (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 mmole/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 re-incorporated 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.