Biochemical and Biophysical Research Communications 402 (2010) 147–152

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Optimal metabolic regulation of the mammalian heart Na^+/Ca^{2+} exchanger requires a spacial arrangements with a PtdIns(4)-5kinase

Diego Forcato [1], Velia Posada [1,2], Luis Beaugé *, Graciela Berberián *

Laboratorio de Biofísica, Instituto de Investigación Médica "Mercedes y Martín Ferreyra" (INIMEC-CONICET), CC 389, 5000 Córdoba, Argentina

ARTICLE INFO

Article history:
Received 28 September 2010
Available online 8 October 2010

Keywords:
Na^+/Ca^{2+} exchanger
Mammalian heart
PtdIns(4)-5kinase
NCX1 metabolic regulation
NCX1–PtdIns(4)-5kinase complex

ABSTRACT

In inside-out bovine heart sarcolemmal vesicles, p-chloromercuribenzenesulfonate (PCMBS) and n-ethylmaleimide (NEM) fully inhibited MgATP up-regulation of the Na^+/Ca^{2+} exchanger (NCX1) and abolished the MgATP-dependent PtdIns-4,5P2 increase in the NCX1–PtdIns-4,5P2 complex; in addition, these compounds markedly reduced the activity of the PtdIns(4)-5kinase. After PCMBS or NEM treatment, addition of dithiothreitol (DTT) restored a large fraction of the MgATP stimulation of the exchange fluxes and almost fully restored PtdIns(4)-5kinase activity; however, in contrast to PCMBS, the effects of NEM did not seem related to the alkylation of protein SH groups. By itself DTT had no effect on the synthesis of PtdIns-4,5P2 but affected MgATP stimulation of NCX1: moderate inhibition at 1 mM MgATP and 1 μM Ca^{2+} and full inhibition at 0.25 mM MgATP and 0.2 μM Ca^{2+}. In addition, DDT prevented coimmunoprecipitation of NCX1 and PtdIns(4)-5kinase. These results indicate that, for a proper MgATP up-regulation of NCX1, the enzyme responsible for PtdIns-4,5P2 synthesis must be (i) functionally competent and (ii) set in the NCX1 microenvironment closely associated to the exchanger. This kind of supramolecular structure is needed to optimize binding of the newly synthesized PtdIns-4,5P2 to its target region in the exchanger protein.

1. Introduction

The Na^+/Ca^{2+} exchangers comprise a family of constitutive plasma membrane proteins responsible for net Ca^{2+} extrusion in most cells. They do so by exchanging one internal Ca^{2+} for three external Na^+ ions [1,2]. The mammalian heart possesses a single isoform named NCX1. In order to perform cation translocation NCX1 needs to have Ca^{2+} bound to intracellular non-transporting Ca^{2+} regulatory sites [1,2]; ionic and metabolic regulations of this system affect that binding. Intracellular protons, by themselves or in synergism with internal Na^+ (also known as Na^+_i-inactivation) inhibit the exchanger by reducing the affinity of the regulatory sites for Ca^{2+} [3,4]. These inhibitions are antagonized by MgATP through the production and subsequent binding to the exchanger of PtdIns-4,5P2 [1,2,4–9]. Actually, the degree of that regulation goes in parallel with the amount of PtdIns-4,5P2 coimmunoprecipitating with NCX1 [9,10].

Modifications of disulfide bonds affect the structure and function of several ion regulatory proteins, i.e. ion channels, pumps and transporters [11]. In NCX1, intramolecular disulfide bonds between cysteine residues have been proposed to be functionally relevant [12]. In heart microsomal vesicles and in the absence of MgATP, a combination of oxidizing (H_2O_2, glutathione disulfide) and reducing (dithiothreitol, glutathione) agents markedly stimulated NCX1. These compounds did not seem to act by themselves but required the presence of Fe^{2+} [13–15]. The molecular mechanism was initially proposed as due to a rearrangement of disulfide bonds [13]. However, in NCX1 with all its cysteines mutated, the DTT/Fe^{2+} effect remained unaltered, indicating that disulfide bonds are not involved. Furthermore, the data was consistent with being primarily due to the removal of a Na^+-dependent inactivation process [15]. On the other hand, none of those works looked into possible influences of these compounds on the metabolic regulation of the exchanger.

Here we used the thiol blockers PCMBS and NEM and the reducing agent DTT as tools to investigate the role of a PtdIns(4)-5kinase in the pathways involved in MgATP up-regulation of NCX1. The results show that thiol blockers prevent MgATP up-regulation by inhibiting the PtdIns(4)-5kinase, an effect that can be reversed by

Abbreviations: NCX1, mammalian heart Na^+/Ca^{2+} exchanger; PtdIns(4)-5kinase, phosphatidylinositol(4)-5kinase; PKA, protein kinase A; PKC, protein kinase C; PP1 and PP2A, two serine/threonine protein phosphatases; mAKAP, protein kinase A-anchoring protein; PCMBS, p-chloromercuribenzenesulfonate; NEM, n-ethylmaleimide; DTT, dithiothreitol.

* Corresponding authors. Fax: +54 351 469 5163.
E-mail addresses: lbeauge@immg.uncor.edu (L. Beaugé), graciela.berberian@gmail.com (G. Berberián).
[1] These authors contributed equally to this work.
[2] Present address: Departamento de Química Biológica (CIQUIBIC-CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina.

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.10.005

DTT. In addition, DTT prevented spatial association between the exchanger protein and a PtdIns(4)-5kinase, required for a proper MgATP (PtdIns-4,5P2 mediated) up-regulation process.

2. Materials and methods

The techniques used in this work are described in detail elsewhere [8–10] and only the relevant points or new information will be mentioned here.

2.1. Preparation of cardiac membrane vesicles

Sarcolemmal vesicles were prepared from bovine hearts obtained immediately after killing the animals [16] and were stored (3–4 mg protein per mL) at −70 °C in the solution of 160 mM NaCl, 20 mM Mops/Tris (pH 7.4), and 0.1 mM EDTA. Total protein was measured with BSA as standard [17].

2.2. Na^+/Ca^{2+} exchange flux

The uptake of $[^{45}Ca]Ca^{2+}$ (forward mode in inside-out vesicles) was measured at 37 °C in media containing low (16 mM) or high (160 mM) NaCl, 3 mM free Mg^{2+}, 0.4 mM vanadate, 0.15 mM EGTA, 20 mM Mops/Tris (pH 7.4 at 37 °C) and variable $[Ca^{2+}]$ with and without 1 mM ATP. In some cases (see legends to figures), before the uptake began, the vesicles were pre-incubated for 15 min at 20 ± 2 °C in the absence of Na^+ gradient (high Na^+ solution) with or without sulfhydryl agents. Other details are given in the figure legends. The kinetic data were fitted to Michaelian functions by using the SCoP Program 3.5 (Scop, Simulation Resources Inc., Redlands).

2.3. Immunological quantification of PtdIns-4,5P2 bound to Na^+/Ca^{2+} exchanger

This was performed as [18] with some modification [9,10]. Aliquots of 35–45 μg total membrane protein were incubated for 20 s at 37 °C in media similar to those used for Ca^{2+} uptake. The reaction was stopped by adding denaturing and reducing Laemmli SDS-sample buffer; samples were then run in discontinuous SDS–PAGE [19] and electro-transferred to polyvinylidene difluoride (PVDF) membranes for Western blots. The primary monoclonal antibodies were against PtdIns-4,5P2 (Assay Designs Inc. and against NCX1 (Novus Biologicals, USA).

2.4. TLC separation of phospholipids from phosphorylated cardiac membrane vesicles

The separation was performed as [8]. The ^{32}P-labeled phospholipids were visualized in a phosphoimage instrument (Storm 840, Molecular Dynamics). PtdIns-4P and PtdIns-4,5P2 were identified with co-migrating commercial standards by submitting the plates to an atmosphere of saturated iodine vapor. Quantitative analysis of $[^{32}P]$phosphoinositides was performed with the Image Quant software (Molecular Dynamics). Duplicate experiments were repeated at least three times.

2.5. Coimmunoprecipitation of PtdIns(4)-5kinase with NCX1

Aliquots of 150 mg total membrane protein were incubated for 20 s at 37 °C in 0.1 mL of media used for Ca^{2+} uptake. The reaction was halted with 0.5 mL of a stop solution containing 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM vanadate, 20 mM Mops/Tris (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, and 1 mg/mL of leupeptin, 1 mg/mL of aprotinin, 1 mg/mL of pepstatin A plus 0.2 mM phenylmethanesulfonyl fluoride. After 20 min at 4 °C, primary antibodies against PtdIns(4)-5kinase (goat polyclonal antibody PtdIns Kinase I (E-12), Santa Cruz Biotech.) or NCX1 (guinea pig NCX1 antiserum [20]) were added at a final dilution of 1:500 (V:V) and the mixtures were incubated overnight at 4 °C. Then, protein A/G Plus + agarose (Santa Cruz) was added at a ratio of 0.01 mg per mg of total protein and the incubation continued for 1 h at RT. The immunoprecipitates were washed twice with the stop solution (see above) and subjected to electrophoresis and Western blotting. In the negative controls, non-immune guinea pig serum was used.

2.6. Assay of PtdIns(4)-5kinase activity

Coimmunoprecipitates with NCX1 were washed and resuspended with phosphorylation buffer (Mops/Tris 20 mM, NMG–Cl 160 mM, $MgCl_2$ 3 mM, EGTA 0.15 mM, vanadate 0.4 mM, and $CaCl_2$ 0.14 mM) and then incubated for one min at 30 °C with 50 μg of PtdIns-4P and 0.5 mM $[^{32}P]$ATP. The reaction was stopped at 0 °C by adding 350 μL of cold $CHCl_3$:MetOH:12 N HCl 500:500:3 (V:V). $[^{32}P]$PtdIns-4,5P2 was separated and quantified as described above.

2.7. Solutions

All solutions were made with deionized ultrapure water (18 MΩ). NaCl Ultrex was from J.T. Baker, Phillipsburg, USA; all other chemicals were analytical reagent grade. The ATP sodium salt (Boehringer Mannheim, Mannheim, Germany) was transformed into Tris salt by passage through an Amberlite IR-120-P column. Tris, NMG, Mops, PtdIns-4P, and PtdIns-4,5P2, were from Sigma Chemical Co. (St. Louis, USA). SDS–PAGE reagents were from Bio-Rad (California, USA); pre-stained molecular mass standards were Kaleidoscope (Bio-Rad). $[Mg^{2+}]$ was estimated by taking a dissociation constant of 0.091 mM for MgATP while $[Ca^{2+}]$ was calculated with the MaxChelator Program (http://www.stanford.edu/~cpatton/maxc.html).

3. Results and discussion

3.1. Thiol blocking reagents and the extravesicular Ca^{2+} activation of the Na^+-gradient-dependent Ca^{2+} uptake (forward mode). Relations with MgATP and PtdIns-4,5P2

Fig. 1A illustrates the effects of 100 μM PCMBS on the extravesicular Ca^{2+} activation (up to 10 μM) of the forward Na^+/Ca^{2+} exchange flux with (filled symbols) and without (open symbols) 1 mM MgATP. The lines through the points are the best simultaneous fits to Michaelian functions. In the absence of PCMBS the nucleotide activates the exchange by increasing the apparent affinity for Ca^{2+} without changes in the V_{max} (see also [8]). In the presence of PCMBS there is no MgATP up-regulation of the exchanger, the K_m for Ca^{2+} is identical to the control without MgATP and the V_{max} is reduced to half. The actual numbers of the kinetic constants are given in the legend to Fig. 1. Regarding PCMBS inhibition of MgATP up-regulation, dose–response curves made at 1 μM Ca^{2+} (not shown) indicated it is about 80–90% at 10 μM PCMBS and full at 100 μM.

We have already reported that in bovine cardiac sarcolemmal vesicles MgATP up-regulation of Na^+/Ca^{2+} exchange is directly related to the PtdIns-4,5P2 that bound to the exchanger protein regardless of the overall membrane content of this compound [9,10]. In line with these observations Fig. 1B shows that, at 1 μM Ca^{2+} and 100 μM PCMBS, MgATP fails to increase the PtdIns-4,5P2 bound to NCX1. In addition, Fig. 1C demonstrates that

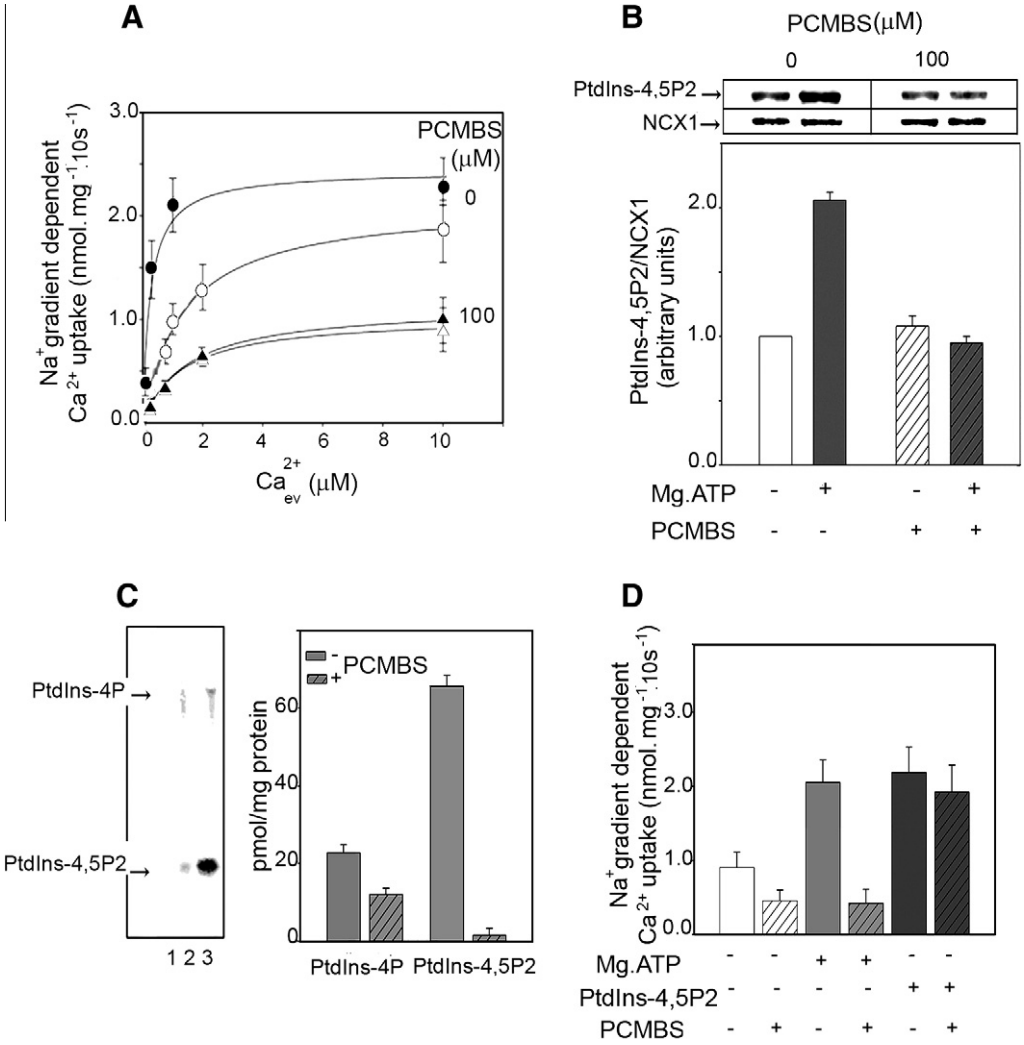

Fig. 1. Effects of PCMBS on metabolic regulation of mammalian heart Na^+/Ca^{2+} exchanger, PtdIns-4,5P2 synthesis and PtdIns-4,5P2 binding to the exchanger. (A) Extravesicular (intracellular) Ca^{2+} activation of the Na^+-gradient-dependent Ca^{2+} uptake. Open symbols correspond to experiments without and filled symbols with MgATP. Circles represent experiments without PCMBS and triangles with 100 μM PCMBS. The lines are the best fit to Michaelian functions. The fitting parameters were (a) controls without MgATP: K_m 1.34 ± 0.04 μM and V_{max} 2.14 ± 0.08 nmol/mg s, (b) controls with MgATP: K_m 0.23 ± 0.01 μM and V_{max} 2.04 ± 0.10 nmol/mg s, (c) PCMBS-treated without MgATP: K_m 1.48 ± 0.03 μM and V_{max} 1.09 ± 0.04 nmol/mg s, and (d) PCMBS-treated with MgATP: K_m 1.39 ± 0.03 μM and V_{max} 1.13 ± 0.02 nmol/mg s. (B) MgATP-dependent binding of PtdIns-4,5P2 to the Na^+/Ca^{2+} exchanger. Top: Immunoblot co-migration detection of PtdIns-4,5P2 and Na^+/Ca^{2+} exchanger protein. The 140-kDa molecular mass standards are indicated by the arrows. Bottom: Bar graph (mean ± SE) of PtdIns-4,5P2 bound to the exchanger from three independent immunoblots normalized as described in Section 2. Note that 100 μM PCMBS prevents the MgATP stimulated PtdIns-4,5P2 binding to NCX1. (C) Net production of phosphoinositides by microsomal vesicles. Left: Phosphoimage of one-dimensional TLC separation of *de novo* [^{32}P]phosphoinositides synthesized by vesicles incubated in the low [Na^+]–Ca^{2+} uptake media with 0.5 mM [^{32}P]-γ-ATP. The PtdIns-4P and PtdIns-4,5P2 standards are indicated. Lane 1: negative control run without vesicles; Lane 2: vesicles incubated and pre-treated with 100 μM PCMBS; Lane 3: untreated vesicles. Right: PtdIns-4P and PtdIns-4,5P2 *de novo* synthesized from three independent experiments (means ± SEM). Note that 100 μM PCMBS decreases by 50% the production of PtdIns-4P and abolishes that of PtdIns-4,5P2. (D) Na^+-gradient-dependent Ca^{2+} uptake at 1 μM $Ca^{2+}_{e.v.}$. The data represent means ± SEM of three different experiments. Note that 100 μM PCMBS reduced to a half the basal Ca^{2+} uptake, prevented MgATP stimulation but had no effect on the PtdIns-4,5P2 stimulation of the exchanger flux.

PCMBS reduces to a half the net synthesis of PtdIns-4,P and completely abolishes that of PtdIns-4,5P2. This concurs with the idea that the actual, or at least the main, mechanism for inhibition involves an impairment of PtdIns(4)-5kinase activity. This is indeed corroborated in the complementary experiments on exchange fluxes illustrated in Fig. 1D. While PCMBS prevents MgATP up-regulation of NCX1, the activation seen following preincubation of the vesicles with PtdIns-4,5P2 remains practically intact. In other words, PCMBS affects the pathways of PtdIns-4,5P2 production but leaves unaltered the interaction between the exchanger and PtdIns-4,5P2.

To establish if the results just described depend on alkylation of thiol groups and are not due to another unspecific effect of PCMBS, we studied N-ethylmaleimide (NEM), a chemically different compound that is also an alkylating agent, though it is irreversible [21,22]. The outcome, illustrated in Fig. 2A, was that at 100–200 μM concentration NEM also prevented MgATP up-regulation of NCX1 and this concurred with a failure to increase the amount of PtdIns-4,5P2 bound to exchanger (Fig. 2B). On the other hand, the only difference with PCMBS was that NEM did not modify the V_{max} of the basal non-up-regulated fluxes.

3.2. Effects of dithiothreitol on MgATP regulation of NCX1 in the presence and absence of thiol-reactive reagents

If the blocking of thiol groups is involved in the results described above, dithiothreitol (DTT) should reverse the effects of PCMBS but not those seen with NEM. In the experiments

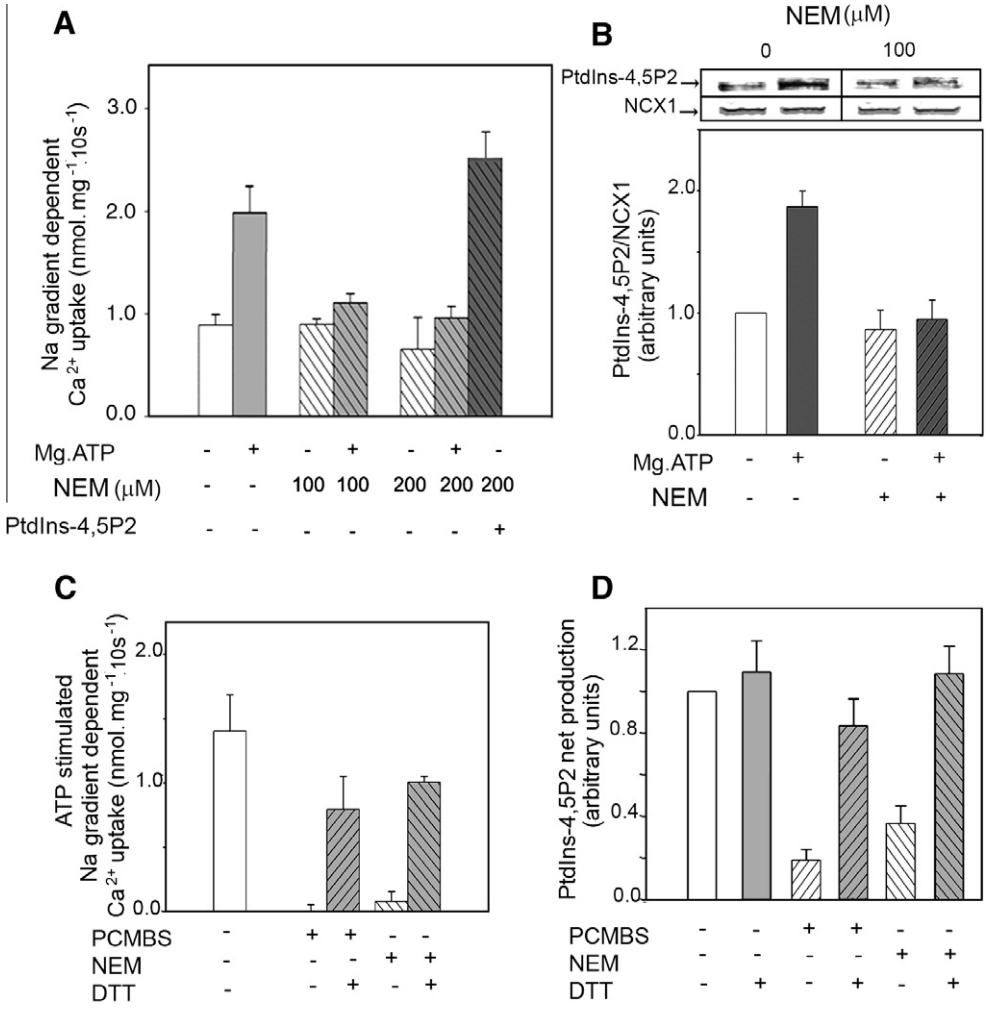

Fig. 2. NEM and PCMBS effects on MgATP stimulation of a Na^+-gradient-dependent Ca^{2+} uptake, PtdIns-4,5P2 binding to the exchanger and PtdIns(4)-5kinase activity in bovine heart sarcolemmal vesicles in the presence and absence of DTT. (A) Na^+-gradient-dependent Ca^{2+} uptake at 1 μM $Ca^{2+}_{e.v.}$. The data represent means ± SEM of three different experiments. Note that, at the concentrations used here, NEM did not modify the basal Ca^{2+} uptake but prevented MgATP stimulation. (B) MgATP-dependent binding of PtdIns-4,5P2 to the Na^+/Ca^{2+} exchanger. Top: Immunoblot detection of PtdIns-4,5P2 and Na^+/Ca^{2+} exchanger protein. The 140-kDa molecular mass standards are indicated by the arrows. Bottom: Bar graph (mean ± SE) of PtdIns-4,5P2 bound to the exchanger from three independent immunoblots normalized as described in Section 2. Note that 100 μM NEM prevents MgATP stimulation of PtdIns-4,5P2 binding to NCX1. (C) DTT antagonizes PCMBS and NEM inhibition of the MgATP stimulated Na^+-gradient-dependent Ca^{2+} uptake. Vesicles were pre-incubated with or without 100 μM PCMBS, 100 μM NEM and/or 1 mM DTT, alone or in combination. The presence of ligands is indicated at the bottom of the figure with a (+) sign. The values correspond to the mean ± SEM from three different experiments. Note: (i) the complete inhibition of the MgATP up-regulation by PCMBS and NEM and (ii) DTT reverses that inhibition although reversal is not complete. (D) DTT overcomes PCMBS or NEM inhibition of PtdIns(4)-5kinase activity of the enzyme coimmunoprecipitated with NCX1. *De novo* [^{32}P]-PtdIns-4,5P2 synthesis was assayed in the low [Na^+]–Ca^{2+} uptake media with 0.5 mM [^{32}P]-γ-ATP with or without 1 mM DTT. The source of PtdIns(4)-5kinase was the coimmunoprecipitate with NCX1 antibody from vesicles pre-treated with and without 100 μM PCMPS or 100 μM NEM. The bars represent means ± SEM of triplicate determinations expressed relative to the activity in control vesicles. Note that DTT, which was added after coimmunoprecipitation, has no effect in controls while it reverses both PCMBS and NEM inhibition.

illustrated in Fig. 2C, MgATP stimulation of Na^+-gradient-dependent Ca^{2+} was followed in membrane vesicles pre-treated with 100 μM PCMBS or 100 μM NEM, washed and then exposed to 1 mM dithiothreitol (DTT) in the uptake solutions. As expected, the addition of DTT counteracted PCMBS but, surprisingly, also NEM. The recovery depended on the compound but it never reached 100%: it was about 60% with PCMBS and around 70% with NEM, a difference which is statistically not significant.

The fact that NEM inhibition of MgATP up-regulation in NCX1 can be reversed by DTT implies that the effect of this compound does not involve alkylation of SH groups; this is supported by the observation that, in contrast to PCMBS, NEM lacks an effect on the basal exchange fluxes (Figs. 1 and 2). It has already been shown that, at concentrations up to 200 μM or lower, NEM activates ryanodine receptors by a reversible interaction not related to alkylation [23]. In that case reversibility was detected either by addition of DTT or by simple washing. In our experiments washing was not effective but DTT was; that may just mean that the binding of NEM is stronger in our preparation. On the other hand, as already suggested [23] NEM seems to produce DTT alkylation; it is not unlikely that this would facilitate its detachment from the non-SH inhibitory site. Also, in hamster thin ascending of Henle's loop the stimulation of Cl-induced by NEM was reversible [24]. At any rate, as happened with the two aforementioned observations, the actual site and mechanism of NEM action remains elusive.

Looking for the possible common mechanism, in follow-up experiments we studied the effects of PCMBS, NEM and DTT on the activity of the PtdIns(4)-5kinase that coimmunoprecipitates with NCX1 (see [25]). Before immunoprecipitation, the microsomes were pre-treated with 100 μM PCMBS or 100 μM NEM; control vesicles were exposed to none of these ligands. Assays of net [^{32}P]PtdIns-4,5P2 production from γ-[^{32}P]ATP were performed in

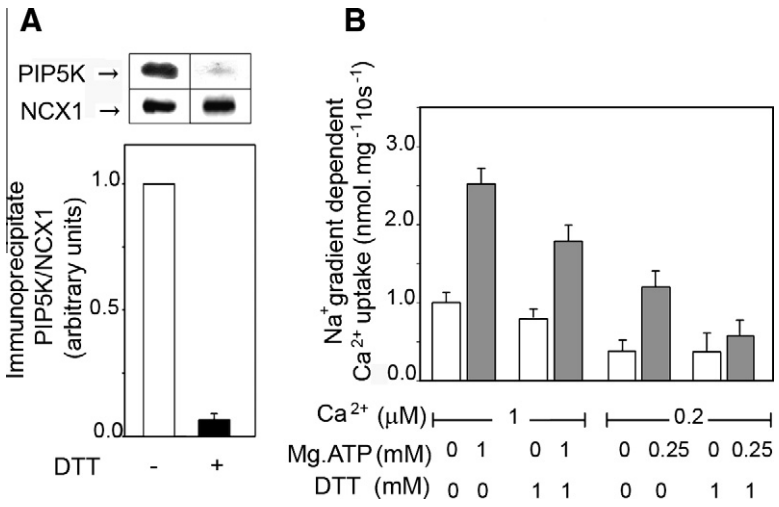

Fig. 3. Effects of DTT on the PtdIns(4)-5kinase/NCX1 complex and MgATP stimulation of a Na^+-gradient-dependent Ca^{2+} uptake in bovine heart sarcolemmal vesicles. (A) DTT prevents the coimmunoprecipitation between PtdIns(4)-5kinase and NCX1. Microsomal vesicles were pre-incubated for 15 min at RT with and without 1 mM DTT. Top: Immunoblot detection of PtdIns(4)-5kinase and NCX1. Bottom: Quantification of three different experiments (mean ± SEM). (B) DTT impairs MgATP up-regulation the Na^+/Ca^{2+} exchanger. Na^+-gradient-dependent Ca^{2+} uptake was measured at two different Ca^{2+} and MgATP concentrations in microsomal vesicles previously incubated with and without 1 mM DTT. Note that pre-treatment with 1 mM DTT produced a moderate inhibition of MgATP up-regulation at 1 μM Ca^{2+} and 1 mM MgATP ($P < 0.05$) but abolished stimulation at 0.2 μM Ca^{2+} and 0.25 mM MgATP. Each bar represents the means ± SEM of three different experiments.

the absence and presence of 1 mM DTT (Fig. 2D). Net PtdIns-4,5P2 production was amply, though not fully, inhibited by the two SH blockers; and the reversal seen with DTT was practically complete with both. In the control group, DTT had no effect on the net PtdIns-4,5P2 synthesis.

The fact that DTT almost fully antagonizes PCMBS and NEM inhibition of PtdIns(4)-5kinase activity but reverses the inhibition of the exchange fluxes to a lesser extent may reflect effects of DTT other than that of just protecting PtdIns-4,5P2 synthesis. One possibility could be that it acts on the spatial distribution of the exchanger and the synthesizing enzyme, an arrangement required for MgATP up-regulation of NCX1. A role of PtdIns(4)-5kinases on PtdIns-4,5P2 location where the enzyme is located in close vicinity to the target has already been suggested (see [26]). If this explanation is correct, it may be reflected in coimmunoprecipitation and transport assays. And this is what was we actually found. Fig. 3A indicates that when 1 mM DTT was present in the coimmunoprecipitation step, the amount of PtdIns(4)-5kinase associated to the NCX1 protein practically did not exist. It is important to point out that in this case an extra amount of the kinase was detected in the supernatant, over the usual mark found in the control samples (not shown here).

The basic assumptions made here are that coimmunoprecipitation implies close spatial interaction whereas failure to coimmunoprecipitate indicates a more lax, or even absence of interaction, and that this arrangement has functional implications likely derived from the necessity that the newly formed PtdIns-4,5P2 accumulates in a region near its binding site in NCX1. If these assumptions are correct, treatment with DTT should influence the ability of MgATP to up-regulate NCX1. Based on this consideration we explored the effects of MgATP, with and without DTT, in two conditions: (i) non-saturating but close to K_m (1 μM) [Ca^{2+}] and a [MgATP] two- to threefold above its K_m (1 mM) and (ii) [Ca^{2+}] much lower, about one seventh its K_m (0.25 μM) and [MgATP] around or below its apparent affinity (0.2 mM) (see [8]). The results of these experiments, depicted in Fig. 3B, clearly show that the expectations were borne out. DTT moderately inhibits MgATP activation at relatively high [Ca^{2+}] and [MgATP] (statistically significant with $P < 0.05$) and completely prevents up-regulation at much more limiting concentrations of these ligands. The intimate mechanism of this DTT effect is not clear but it may take place by affecting thiol groups of the exchanger, the enzyme, or both, needed for close intermolecular interaction.

A close NCX1–PtdIns(4)-5kinase association in relation to their function is new and important information, but by no means surprising. A well-known feature of several proteins, particularly those related to signaling processes, is that they localize in discrete domains of the plasma membrane enriched in cholesterol and sphingolipids known as rafts or caveolae. Particular examples are L-type Ca^{2+} channels, Na^+ channels and the Na^+/Ca^{2+} exchanger (see [27–30]). These proteins co-exist with proper kinases and phosphatases, conforming an appropriate spatial microenvironment that favors their interactions. In mammalian heart plasma membrane, a macromolecular complex has been found between NCX1 and the catalytic and regulatory subunits of protein kinase A (PKA), protein kinase C (PKC), PP1 and PP2A, two serine/threonine protein phosphatases and also the protein kinase A-anchoring protein, mAKAP [30]. In mouse hippocampus neurons an association of the PIP5Kγ661 isoform of PIP(4)-5kinases with the endocytotic machinery is crucial for activation of endocytosis by locally generating PtdIsl-4,5P2 [31]. Likewise, the function of PtdIns-4,5P2 as a modulator of the Na^+/Ca^{2+} exchanger depends on a delicate spatial and temporal balance of its synthesis and degradation, related, among other things, to a close association between the responsible enzyme and the exchanger protein.

In summary, the experiments described here indicate that there is a close association between the NCX1 isoform of the Na^+/Ca^{2+} exchanger and the enzyme PtdIns(4)-5kinase. Furthermore, for a proper MgATP up-regulation of NCX1, the enzyme responsible for PtdIns-4,5P2 synthesis must fulfill two requirements: (i) be functionally competent and (ii) be closely associated to NCX1. This kind of supramolecular structure is needed to optimize binding of the newly synthesized PtdIns-4,5P2 to its target region in the exchanger protein.

Acknowledgments

The authors thank Myriam Siravegna for her technical skills and Frigorífico Juarez Beltran for providing bovine hearts. This work was supported by Agencia Nacional de Promoción Científica y

Tecnológica (FONCYT PICT-05, 38073) and Consejo Nacional de Ciencia y Técnica (CONICET, PIP GI11220090100063). G.B. and L.B. are established investigators of CONICET, D.F. and V.P. were Fellows of FONCYT and CONICET, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.005.

References

[1] M.P. Blaustein, W.J. Lederer, Sodium/calcium exchange: its physiological implication, Physiol. Rev. 79 (1999) 763–854.
[2] R. DiPolo, L. Beaugé, Sodium/calcium exchanger: influence of metabolic regulation on ion carrier interactions, Physiol. Rev. 86 (2006) 155–203.
[3] A.E. Doering, W.J. Lederer, The action of Na^+ as a cofactor in the inhibition by cytoplasmic protons of the cardiac Na^+–Ca^{2+} exchanger in the guinea-pig, J. Physiol. 480 (1994) 9–20.
[4] D.W. Hilgemann, S. Matsuoka, G.A. Nagel, A. Collins, Steady-state and dynamic properties of cardiac sodium–calcium exchange: sodium-dependent inactivation, J. Gen. Physiol. 100 (1992) 905–932.
[5] S. Luciani, S. Bova, G. Cargnelli, F. Cusinato, P. Debetto, Modulation of sodium–calcium exchange by lipids, Ann. N. Y. Acad. Sci. 639 (1991) 156–165.
[6] S. Luciani, M. Antonini, S. Bova, G. Cargnelli, F. Cusinato, P. Debetto, L. Trevisi, R. Varotto, Inhibition of cardiac sarcolemmal sodium–calcium exchanger by glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4-5-bisphosphate, Biochem. Biophys. Res. Commun. 206 (1995) 674–680.
[7] D.W. Hilgemann, R. Ball, Regulation of cardiac Na^+/Ca^{2+} exchange and KATP potassium channels by PIP2, Science 273 (1996) 956–959.
[8] G. Berberián, C. Hidalgo, R. DiPolo, L. Beaugé, ATP stimulation of Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles, Am. J. Physiol. 274 (1998) C724–C733.
[9] V. Posada, L. Beaugé, G. Berberián, Maximal $Ca^{2+}{}_i$ stimulation of cardiac Na^+/Ca^{2+} exchange requires simultaneous alkalinization and binding of the PtdIns-4,5P2 to the exchanger, Biol. Chem. 388 (2007) 281–288.
[10] C. Asteggiano, G. Berberián, L. Beaugé, Phosphatidyl inositol-4,5-bisphosphate bound to bovine cardiac Na^+/Ca^{2+} exchanger displays a MgATP regulation similar to that of the exchange fluxes, Eur. J. Biochem. 268 (2001) 437–442.
[11] A.V. Zima, L.A. Blatter, Redox regulation of cardiac calcium channels and transporters, Cardiovasc. Res. 71 (2006) 310–321.
[12] D. Nicoll, M. Ottolia, L. Lu, Y. Lu, K. Philipson, A new topological model of the cardiac sarcolemmal Na^+–Ca^{2+} exchanger, J. Biol. Chem. 274 (1999) 910–917.
[13] J.P. Reeves, C.A. Bailey, C.C. Hale, Redox modification of sodium–calcium exchange activity in cardiac sarcolemmal vesicles, J. Biol. Chem. 261 (1986) 4948–4955.
[14] Z.Q. Shi, A.J. Davison, G.F. Tibbis, Effects of active oxygen generated by DTT/Fe^{2+} on cardiac Na^+/Ca^{2+} exchange and membrane permeability to Ca^{2+}, J. Mol. Cell. Cardiol. 21 (1989) 1009–1016.
[15] L. Santacruz-Toloza, M. Ottolia, D.A. Nicoll, K.D. Philipson, Functional analysis of disulfide bond in the Na^+/Ca^{2+} exchanger, J. Biol. Chem. 275 (2000) 182–188.
[16] J.P. Reeves, The sarcolemmal sodium–calcium exchange system, Curr. Top. Membr. Transp. 25 (1985) 77–119.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurements with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[18] K. Fukami, T. Endo, M. Imanura, M. Takenawa, α-Actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine, J. Biol. Chem. 269 (1994) 1518–1522.
[19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[20] J.F. Aceto, M. Condrescu, C. Kroupis, H. Nelson, D. Nicoll, K.D. Philipson, J.P. Reeves, Cloning and expression of the bovine cardiac Na^+/Ca^{2+} exchanger, Arch. Biochem. Biophys. 298 (1992) 553–560.
[21] R.E. Abbott, D. Schachter, Impermeant maleimides: oriented probes of erythrocyte membrane proteins, J. Biol. Chem. 251 (1976) 7176–7183.
[22] J. Van Steveninck, R.I. Weed, A. Rothstein, Localization of erythrocyte membrane sulphydryl groups essential for glucose transport, J. Gen. Physiol. 48 (1965) 617–632.
[23] E.V. Menshikova, E. Cheong, G. Salama, Low N-ethylmaleimide concentrations activate ryanodine receptors by a reversible interaction, not an alkylation of critical thiols, J. Biol. Chem. 275 (2000) 36775–36780.
[24] M. Imai, Y. Kondo, Ch. Koseki, K. Yoshitomi, Dual effect of N-ethylmaleimide on Cl^- transport across the thin ascending limb of Henle's loop, Pflugers Arch. 411 (1988) 520–528.
[25] G. Berberián, D. Forcato, L. Beaugé, Key role of a PtdIns-4,5-P2 micro domain in ionic regulation of the mammalian heart Na^+/Ca^{2+} exchanger, Cell Calcium 45 (2009) 546–553.
[26] R.L. Doughman, A.J. Firestone, R.A. Anderson, Phosphatidylinositol phosphate kinases put PI(4,5)P2 in its place, J. Membr. Biol. 194 (2003) 77–89.
[27] R.C. Balijepalli, T.J. Kamp, Caveolae, ion channels and cardiac arrhythmias, Prog. Biophys. Mol. Biol. 98 (2008) 149–160.
[28] B. Pani, B.B. Singh, Lipid rafts/caveolae as microdomains of calcium signalling, Cell Calcium 456 (2009) 25–33.
[29] M. García-Marcos, J.P. Dehaye, A. Marino, Studying the role of plasma membrana microdomains in the modulation of P2XR-mediated signalling, FEBS J. 276 (2009) 330–340.
[30] D.H. Schulze, M. Muqhal, W.J. Lederer, A.M. Ruknudin, Sodium/calcium exchanger (NCX1) macromolecular complex, J. Biol. Chem. 278 (2003) 28849–28855.
[31] Y. Kanaho, A. Nakano-Kobayashi, T. Yokozeki, Novel activation and physiological function of PIP5Kγ661, Adv. Enzyme Regulat. 48 (2008) 88–96.