Modulation of Na^+,Ca^{2+} exchange current by EGTA calcium buffering in giant cardiac membrane patches

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Abstract

Effects of calcium buffering by EGTA were examined on sodium-calcium exchange currents (I_{NaCa}) in inside-out giant cardiac membrane patches. Free calcium concentrations (Ca^{2+}) were monitored with a calcium electrode and a fluorescent calcium indicator (Calcium Green-5N). With 1.8 μM cytoplasmic Ca^{2+}, inward I_{NaCa} increased 2-fold at -120 mV when EGTA concentration was increased from 0.1 mM to 10 mM (37°C and 140 mM extracellular sodium). Stimulation by EGTA was decreased or abolished under conditions of attenuated exchanger turnover rate: temperature < 30°C, extracellular sodium < 70 mM, and membrane potential $> +60$ mV. EGTA concentration had no effect on outward I_{NaCa} with 100 mM cytoplasmic Na^+ and 0.8 μM cytoplasmic Ca^{2+}, conditions under which the current inactivated by about 70%. EGTA (0.1–10 mM) and BAPTA (10 mM) inhibited the current by about 80% when the outward I_{NaCa} was stimulated by 2 mM cytoplasmic ATP or by phosphatidylserine. The apparent K_i for EGTA was 0.2 mM. The electroneutral calcium ionophore, A23187, activated outward I_{NaCa} even in presence of 10 mM EGTA. Our results are consistent with EGTA acting as a simple calcium buffer with no direct effect on the exchanger. At low concentrations of EGTA, inhibition of the inward I_{NaCa} is expected due to submembrane calcium depletion by the exchanger; enhancement of the outward I_{NaCa} at low EGTA concentrations is expected because submembrane calcium accumulates and activates I_{NaCa} via regulatory calcium binding sites.

Keywords: EGTA; Sodium–calcium ion exchange; Ionophore A23187; Submembrane calcium ion; Temperature; Giant patch; Cardiac membrane

1. Introduction

Several calcium chelators, including EGTA, have been reported to stimulate Na,Ca exchange activity as the calcium buffer capacity is increased and free calcium is held constant [1]. Similar effects are described for the sarcolemmal calcium pump [2,3]. Obviously, the existence of such direct effects of calcium buffers on calcium transporters would compromise the interpretation of experimental results using these buffers, and further studies were therefore essential.

Another possible interpretation of these results stems from a consideration of calcium diffusion to the exchanger at different concentrations of EGTA. The Na,Ca exchanger is able to extrude calcium efficiently when free Ca^{2+} is in the range of several micromolar. At low free Ca^{2+}, relatively small calcium transport activities may result in

significant changes of free calcium next to the membrane, especially in the absence of mobile calcium buffers (see [4] for complete references). The maximum exchanger turnover rate is suggested to be 5000 s^{-1} [9], and the apparent K_d for cytoplasmic free Ca^{2+} is about 5 μM for the calcium extrusion mode of operation. Therefore, a turnover rate of about 800 s^{-1} may be expected with 1 μM free calcium. This rate is close to the measured rates of diffusion- limited calcium binding by fast calcium chelators (1000 s^{-1} = 10^9 M^{-1} s^{-1} $*1$ μM) [5]. Thus, it appears entirely reasonable that exchange rates could become limited by diffusion of calcium to its binding sites at low concentrations of EGTA and low free calcium concentrations.

In this light, we carried out two types of experiments. First, we examined effects of EGTA-calcium buffer capacity on both the inward and outward exchange currents in giant cardiac membrane patches. Second, we examined these effects under different conditions to test whether the effects might be the result of significant submembrane calcium gradients. If cytoplasmic EGTA has direct effects

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on the exchanger, then stimulatory effects on both inward and outward currents would be expected. But if the primary cause of these effects is the influence of EGTA on submembrane Ca^{2+}, different effects on inward and outward exchange currents may be expected. It is expected that effects of EGTA related to submembrane calcium concentration changes will be greatest under conditions favoring the largest possible exchange rates. Preliminary results of this study have been published in abstract form [6].

2. Materials and methods

2.1. Outward and inward Na,Ca exchange current recording

Outward and inward Na,Ca exchange currents were monitored in giant cardiac membrane patches (3–7 pF) from guinea pig myocytes. The methods, conditions, and solutions were the same as described previously [7,8]. Voltage clamp experiments were performed with Axopatch® 1C and 200A amplifiers. Data was stored and analyzed on IBM-compatible computers.

The current–voltage relations of the inward I_{NaCa} for each patch were determined by subtracting current–voltage relations with and without cytoplasmic free calcium. The mean of the relations were determined in each experiment and then averaged for several experiments.

2.2. Solutions used to study the inward current

Cytoplasmic solutions contained 120 mM CsOH, 20 mM tetraethylammonium hydroxide, 20 mM Hepes, and 0.5 mM $MgCl_2$. In addition, 'high EGTA' solution contained 10 mM EGTA and 8.5 mM $CaCO_3$ (1.8 μM Ca^{2+}). 'Low EGTA' solution contained 9.9 mM sucrose, 100 μM EGTA and 85 μM $CaCO_3$ (1.8 μM Ca^{2+}). Control solution contained 10 mM EGTA and no Ca^{2+}. In all solutions, pH was adjusted to 7.0 with 2-(N-morpholino)ethanesulfonic acid (Mes).

The pipette (extracellular) solution contained 10 mM EGTA, 120 mM NaOH, 4 mM $MgCl_2$, 20 mM tetraethylammonium hydroxide, 20 mM CsOH, 10 mM Hepes, 200 μM ouabain, 2 μM verapamil, 2.38 mM $CaCl_2$, and pH was adjusted to 7.0 with Mes. In experiments with low extracellular sodium, NaOH was replaced by N-methyl-D-glucamine (NMG).

2.3. Solutions used to study the outward current

Cytoplasmic solutions contained 100 mM CsCl or NaCl, 18 mM CsCl, 18 mM tetraethylammonium hydroxide, 25 mM Hepes, and 1 mM $MgCl_2$. In addition, 'high EGTA' solution contained 10 mM EGTA and 7 mM $CaCO_3$ (0.75 μM Ca^{2+}). 'Low EGTA' solution contained 9.9 mM

sucrose, 100 μM EGTA and 70 μM $CaCO_3$ (0.75 μM Ca^{2+}). Control solutions contained 10 mM EGTA and no Ca^{2+}. In all solutions, the pH was adjusted to 7.0 with NMG or Mes.

The pipette (extracellular) solution contained 20 μM EGTA, 4 mM $CaCl_2$, 100 mM NMG, 10 mM LiCl or KCl, 2 mM $MgCl_2$, 20 mM tetraethylammonium hydroxide, 20 mM CsOH, 10 mM Hepes, 200 μM ouabain, 2 μM verapamil, and pH was adjusted to 7.0 with Mes.

2.4. Measurements of free Ca^{2+} in experimental solutions

The free calcium concentrations of experimental solutions were verified to be within a few percent of calculated values by both a calcium electrode and Calcium Green-5N. The calcium electrode was from Orion Research (Model 93-20). Calcium Green-5N was from Molecular Probes. Fluorescence measurements, carried out with an excitation wave length of 480 nm and an emission wavelength of 540 nm, were more reliable for the range of Ca^{2+} concentrations of interest here. To vary the EGTA buffer capacity, calcium-containing solutions were prepared with a high (20–40 mM) EGTA concentration. The added calcium concentration was calculated, and the resulting free calcium concentrations of solutions were verified by the above methods. The concentrated EGTA-calcium solution was diluted with the same solution lacking EGTA and calcium. Both the optical and electrical methods indicated no change of the free calcium concentration with EGTA-calcium dilution to within a few percent. All solutions were prewarmed and agitated before use.

3. Results

3.1. EGTA increases inward Na^+, Ca^{2+} exchange current in giant cardiac membrane patches only at high exchange rates

Fig. 1 describes the effect of EGTA buffer capacity on current–voltage relations of the inward exchange current with 120 mM extracellular sodium and 1.8 μM cytoplasmic free calcium at 37°C. This concentration of free calcium activated approx. 15% of the maximal inward exchange current under these conditions. The results shown are average current–voltage relations for I_{NaCa}. Standard errors of the mean are given as dotted lines. Reduction of EGTA concentration from 10 mM (filled circles) to 0.1 mM (open circles) decreased the inward exchange current by about 29% at 0 mV and 43% at − 120 mV. In panel A, results are normalized to the current with 10 mM EGTA at 0 mV in all patches; in panel B, results are scaled to the current magnitude at 0 mV in each data set. While the decrease of EGTA concentration from 10 mM to 0.1 mM decreases I_{NaCa} at all membrane potentials (Fig. 1A), the fractional decrease was larger at more negative membrane

potentials, where the exchange rate was higher. Accordingly, the current–voltage relation becomes flatter (i.e., less steep) in the negative potential range with low EGTA concentration. This would be expected if diffusion of calcium to the membrane became rate-limiting at low EGTA concentrations. The relative slope of the current–voltage relation with 0.1 mM EGTA was 2-fold less than with 10 mM EGTA (Fig. 1B), and it was similar to the relations obtained with low cytoplasmic free calcium concentrations.

If calcium diffusion to the membrane becomes rate-limiting for activated exchange current at low EGTA concentrations, then factors which reduce the exchange rate should markedly attenuate inhibition by 0.1 mM EGTA with respect to 10 mM EGTA. Since sodium-calcium exchange is sensitive to temperature and membrane potential changes, the current–voltage relations were examined at different temperatures with different EGTA buffer capacities at 1.8 μM free calcium. As shown in Fig. 2A, reduction of temperature from 38°C to 30°C with 0.1 mM EGTA decreased the current by $19 \pm 4\%$ at negative potentials. The effect of reducing temperature was about
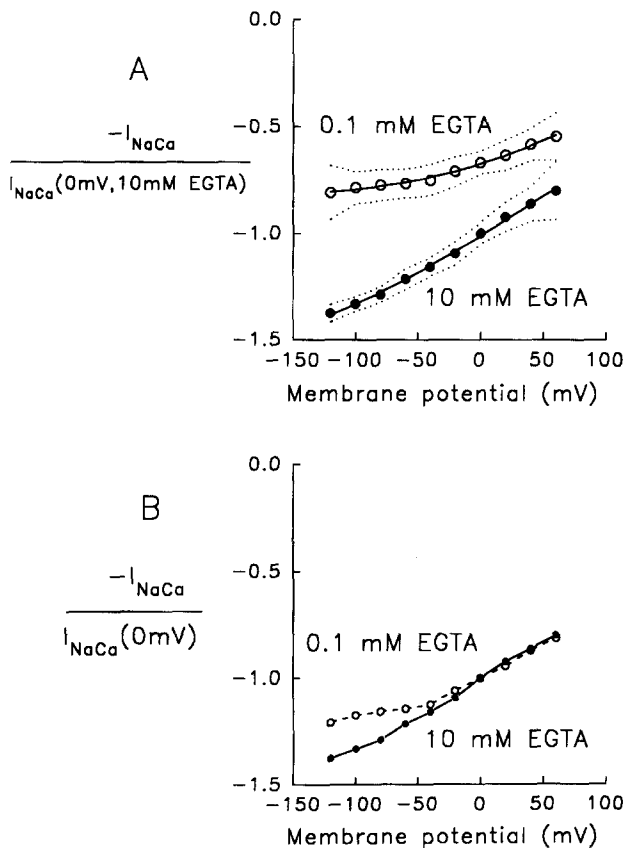

Fig. 2. Effect of temperature on current–voltage relation of inward I_{NaCa} at 0.1 and 10 mM EGTA (1.8 μM Ca^{2+}). (A) Decrease of temperature from 38 to 30 to 25°C has larger effect at 10 mM EGTA than at 0.1 mM. Results are from a single patch. (B) Fractional effect of increasing the EGTA concentration from 0.1 mM to 10 mM on current–voltage relation of inward I_{NaCa}.

Fig. 1. Effect of EGTA (0.1 mM and 10 mM) on the current–voltage relation of inward I_{NaCa} at constant free calcium (1.8 μM). (A) The average data ($n = 4$) from different patches were normalized to the value of the current at 0 mV and 10 mM EGTA. (B) The data for 0.1 μM EGTA were normalized to the value of the current at 0 mV and 0.1 mM EGTA.
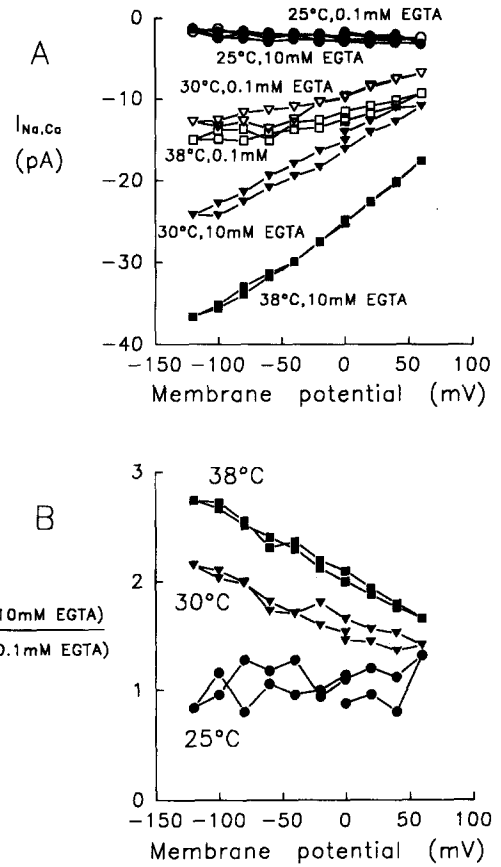
$34 \pm 6\%$, however, with 10 mM EGTA. At still lower temperatures (25°C), the inward exchange current decreased to a small magnitude which was almost independent of membrane potential, and EGTA concentration was without effect. Presumably, a voltage-independent process in the exchange cycle becomes rate-limiting in the inward current exchange cycle at low temperatures.

In Fig. 2B, the inward exchange current with 10 mM EGTA is plotted as a fraction of current with 0.1 mM EGTA. The effect of increasing EGTA was strongest at 38°C and negative potentials (i.e., the condition with the highest exchanger turnover rate). The fractional stimulation of current by increasing EGTA was smaller at more depolarized potentials and at lower temperatures.

Another possibility to reduce exchanger turnover, independent of calcium concentration, is to reduce the concentration of extracellular sodium. Similar to results at low temperatures, EGTA concentration changes were almost without effect with 40 mM extracellular sodium (two observations; no results presented for brevity).

In principle, the inward exchange current might be limited by calcium binding rates, per se, when free calcium

is low, even in the presence of high calcium buffer capacity. An expected result would be that the current–voltage relations become less steep at low cytoplasmic free calcium. Fig. 3 shows that this was indeed the case under our present experimental conditions at 37°C. It is noted that other explanations for this effect have also been suggested [9].

Panel A shows the current–voltage relations at 15.5, 3, 1.8 an 0.7 μM free calcium. The results have been normalized to the current at +60 mV in panel B. The inward I_{NaCa} current–voltage relationship became progressively less steep as cytoplasmic calcium was reduced, and it became completely flat at the lowest free calcium concentrations. This is somewhat different from results for the chymotrypsin-deregulated exchanger, whereby the inward exchange current usually remains somewhat voltage-dependent at low Ca^{2+} [10].

If cytoplasmic free Ca^{2+} concentration next to the membrane changes significantly as a result of exchange activity, it should be possible to study the kinetics of the depletion and replenishment during appropriate voltage pulse protocols. Fig. 4 describes results for inward exchange current with 1.8 μM free cytoplasmic calcium during such a protocol. Voltage was first stepped to a
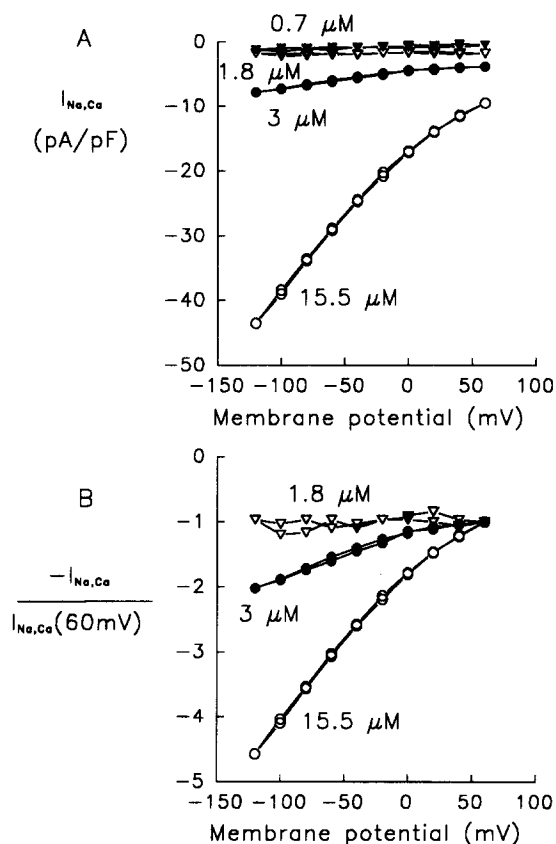

Fig. 4. Effect of EGTA on transients of the inward I_{NaCa} during 2 ms voltage pulses (1.8 μM free calcium).

Fig. 3. Effect of free cytoplasmic calcium concentration (Ca^{2+}) on inward I_{NaCa} at constant concentration of EGTA (10 mM). (A) Effect of variation of Ca^{2+} from 0.7 μM to 15.5 μM on current density. (B) Results of (A) have been normalized to the value of I_{NaCa} occurring at 60 mV.
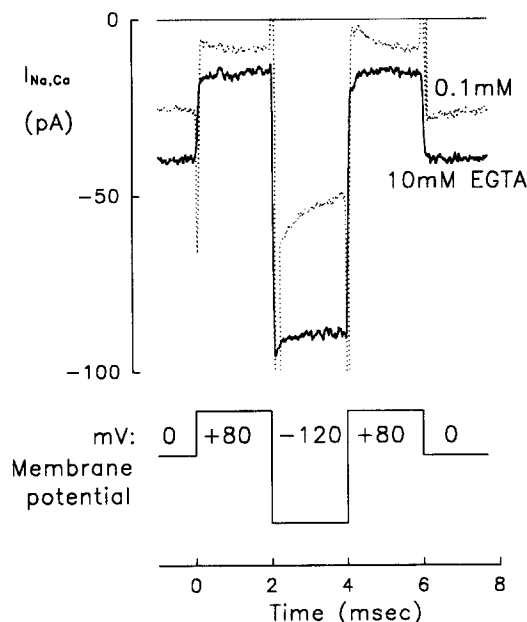
positive potential (+80 mV) to decrease exchange rate and thereby reduce any preexisting depletion. Voltage was then stepped to −120 mV to activate exchange current and thereby favor depletion of cytoplasmic calcium. The results are subtractions of records in the presence of cytoplasmic calcium from results in the absence of cytoplasmic calcium. Results with 10 mM EGTA are given as a solid line; results with 0.1 mM EGTA are given as a dotted line. There were no transient current components with 10 mM EGTA. Transients with a time constant of about 1 ms appear at 0.1 mM EGTA.

The reduction of current subsequent to its stimulation by hyperpolarization, and the rebound of current upon depolarization, are qualitatively as expected for Ca^{2+} depletion developed with 0.1 mM EGTA, but not with 10 mM EGTA. These results are qualitatively predictable from the exchange current magnitudes and the given diffusion distances.

Calcium diffusion supporting the steady state exchange current through the membrane can be approximated by the equation:

$$\frac{I}{S} = zFD\frac{\partial([Ca^{2+}]_i)}{\partial x}$$

where

$I = 10^{-11}$ A	exchange current through the patch,
$S = 10^{-10}$ m^2	approximated surface area of the patch membrane,
$z = 1$	charge moved per exchange cycle,
$D = 8 \cdot 10^{-10}$ m^2/s	diffusion coefficient,
$F = 96$ kC/mol	Faraday constant,
$\partial([Ca^{2+}]_i)/\partial x$	calcium concentration gradient.

With high exchange activity (Fig. 2) the gradient will be about 2 μM/μm. It is our routine observation that membrane rises up in the pipette tip by a distance approximately equal to the pipette diameter [11]. For 10 μm, the depletion can be expected to be equal to 20 μM total calcium concentration. That is 10-fold larger than the free calcium concentration in the present experiments (2 μM). Although exact predictions will depend on calcium buffer kinetics and diffusion rates, the least possible effect can be calculated assuming an instantaneous calcium buffer. For example, a 20 μM change of the total calcium corresponds to a change of free calcium of more than 50% with 100 μM EGTA and 70 to 90 μM total calcium (i.e., free calcium concentrations of 1–3 μM). Thus, a stimulating effect due to increasing the EGTA concentration appears inevitable for the inward exchange current under our experimental conditions.

3.2. Effect of cytoplasmic EGTA and submembrane cytoplasmic Ca^{2+} on outward I_{NaCa}

In general, an agent which stimulates or inhibits the inward exchange current would be expected to modulate the outward exchange current in the same sense. This is the case, for example, for blocking ions (e.g., Ni^{2+}), dichlorobenzamil, and ATP [12,13]. As described in Fig. 5, this is not the case for EGTA.

Fig. 5 describes typical results obtained with the outward exchange current when the EGTA buffer capacity was changed and the bulk free calcium remained constant 0.75 μM. The outward exchange current was activated by applying 100 mM cytoplasmic sodium, substituted for cesium in the presence of 4 mM calcium on the extracellular (pipette) side. Under these conditions, the outward exchange current inactivated by about 70% to a relatively low steady state value. Results shown before 0 (Panel A) and after (Panel C) lowering EGTA concentration from 10 mM to 0.1 mM (Panel B). There is no detectable effect of EGTA under these conditions.

For the outward current, cytoplasmic calcium concentrations would be expected to increase (not decrease) near the cytoplasmic membrane surface as the current is progressively activated. The outward exchange current is secondarily activated by a rise in cytoplasmic free calcium
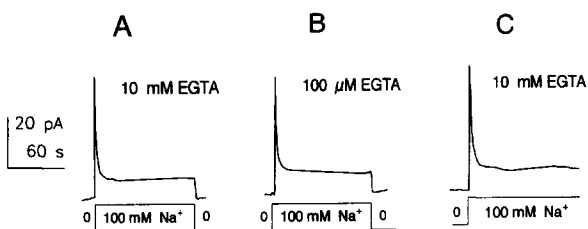
Fig. 5. Lack of effect of EGTA buffering on outward I_{NaCa}. The concentration of free calcium is the same (0.75 μM) at the different concentrations of EGTA (10 or 0.1 mM).
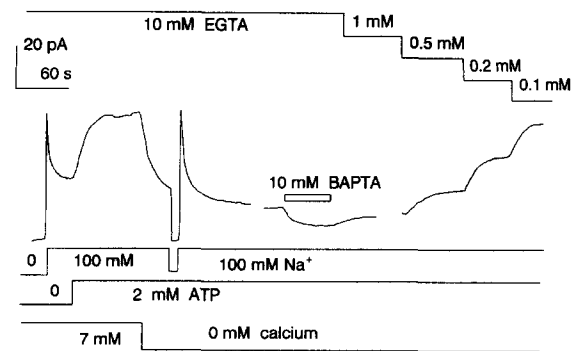
Fig. 6. Inhibition of the ATP-stimulated outward I_{NaCa} by EGTA and BAPTA buffering of cytoplasmic solution in the absence of Ca^{2+} in bulk cytoplasmic solution. See text for details.

[14]. Therefore a reduction of cytoplasmic EGTA should cause an increase in the submembrane calcium concentration and stimulate the current. As shown in Fig. 6, a decrease of the EGTA buffer capacity indeed stimulates the outward exchange current when the current is activated by cytoplasmic ATP. In this experiment, the current was stimulated by 2 mM cytoplasmic ATP to approximately the magnitude obtained on application of sodium before inactivation.

As indicated beneath the record in Fig. 6, when cytoplasmic calcium was removed, the current declined to a steady state level. The presence of a cytoplasmic-independent outward current in the presence of ATP is typical [7]. Subsequent addition of 10 mM BAPTA in the presence of 10 mM EGTA decreased the residual current by about 25%. This is consistent with the idea that 10 mM EGTA is not sufficient for total reduction of submembrane calcium concentration in this condition of increased calcium flux. Removal of BAPTA and reduction of EGTA concentration to 1, 0.5, 0.2 and 0.1 mM stimulated the exchange current up to the maximum value observed in the presence of 0.75 μM free cytoplasmic Ca^{2+}. The stimulation, rather than inhibition, of outward current is consistent with in increase of submembrane free calcium as EGTA the buffer capacity is reduced, and the outward current is activated via a calcium regulatory site of the exchanger [14]. The K_d for cytoplasmic free calcium in giant patches is about 0.5 μM in the presence of 2 mM ATP [7]. Accordingly, an increase of total calcium concentration of about 0.1 mM next to the membrane (0.3 μM free Ca^{2+}) would explain the stimulatory effects of reducing EGTA during this experiment. Thus, activation of the outward exchange current by reduction of EGTA concentration is produced by intracellular submembrane calcium which is transported to the cytoplasmic submembrane space by the exchanger itself.

Based on this hypothesis, it should be possible to activate the outward exchange current by generating a transmembrane calcium flux by other means as well. The electroneutral calcium ionophore, A23187, was tested. In the absence of exchange current, application of A23187 (1
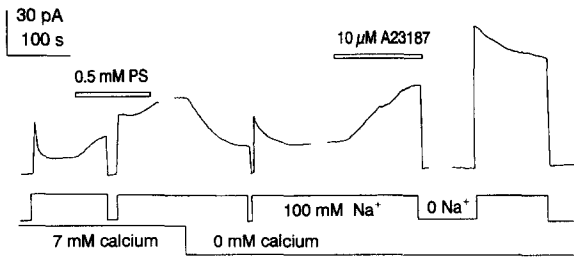
Fig. 7. Stimulation of outward I_{NaCa} by electroneutral calcium ionophore, A23187, in absence of cytoplasmic Ca^{2+} with 10 mM EGTA.

to 20 μM) had no detectable effect on membrane current under the conditions of these experiments (4 mM extracellular calcium, cytoplasmic calcium < 1 μM). In the experiment shown in Fig. 7, the exchange current was pre-stimulated by application of phosphatidylserine (PS) vesicles at a apparent concentration of 0.5 mM. Similar to results with ATP [7], the half-maximal cytoplasmic calcium concentration needed to activate the current was reduced from about 2 μM to about 0.5 μM with PS application (data not shown). After activation by PS vesicles, cytoplasmic A23187 (10 μM) strongly stimulated the outward exchange current, even in absence of cytoplasmic calcium at 10 mM EGTA. The stimulatory effect of A23187 did not reverse on its removal, presumably because A23187 dissociates only very slowly, or not at all, from the membrane. The submembrane total calcium concentration presumably reaches 1 to several millimolar to activate the current in the presence of 10 mM EGTA.

4. Discussion

In this article we have examined effects of EGTA buffer capacity on sodium-calcium exchange current in giant cardiac membrane patches. We have no evidence that EGTA buffering has a direct effect on the cardiac Na,Ca exchanger. Rather, all effects of changing EGTA buffer capacity on inward and outward exchange currents are logically accounted for by the existence of significant and predictable submembrane calcium concentration changes.

A wide range of arguments support our conclusion. First, reduction of EGTA buffer capacity resulted in a flattening of the inward current–voltage relationship at negative membrane potentials. This is expected if calcium diffusion, a voltage-independent process, becomes rate-limiting at negative potentials at low EGTA concentrations. Second, the apparent stimulatory effect of EGTA was reduced (or abolished) when the exchange rate was limited by decreasing temperature or by reducing extracellular sodium concentration. When the exchange rate is low, calcium diffusion presumably does not become limiting for exchange current. Third, we have isolated current transients for the inward exchange current, at low EGTA concentrations, which were consistent with calcium depletion occurring at negative potentials. These current tran-

sients were abolished by higher EGTA buffer capacity. We expect that these results can be explained well without assuming the existence of a restricted or 'fuzzy' submembrane space [15]. However, more detailed kinetic simulations would be required for verification. Fourth, the expected magnitudes of calcium depletion in our experiments can be calculated, and they are appropriate to explain the effects of lowering the EGTA concentration. Fifth, an increase of the EGTA buffer capacity did not stimulate, but rather inhibited the outward exchange current. Since the inhibitory effect of EGTA buffering depends on the activation status of the outward current, the results are readily explained by an accumulation of submembrane calcium with activation of the outward exchange current via cytoplasmic regulatory sites.

The EGTA concentrations used in our experiments (100 μM and 10 mM) are higher than those used in the vesicle experiments of Trosper and Philipson (0, 22, 43 and 88 μM EGTA) [1]. Nevertheless, we argue that the physical basis of EGTA effects are the same. The fact that increasing free Ca^{2+} concentration up to 40 μM eliminates the stimulating effect of EGTA [1] is consistent with a calcium depletion occurring in the absence of EGTA with 5 μM free Ca^{2+}

It is possible that the rate of calcium binding will limit exchanger rates at low cytoplasmic free calcium concentrations, even in the presence of high buffer concentrations. That inward currents become less voltage-dependent at low free calcium concentrations is in principle consistent with this possibility. In fact, the current becomes entirely voltage-independent at low free calcium concentrations when the exchanger is not 'deregulated' with chymotrypsin. This is not the case after chymotrypsin treatment, whereby some voltage-dependence remains at lowest free calcium concentrations [1]. An interesting possibility for future work would be that calcium diffusion up to the exchanger transport sites might be modified by structural/electrostatic properties of the cytoplasmic loop of the exchanger.

In conclusion, the described effects of changing EGTA calcium buffer capacity on Na,Ca exchange current in giant cardiac membrane patches are accounted for by the opposite influences of exchanger activity and EGTA concentration on the submembrane calcium concentration.

Acknowledgements

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[1] There is a discrepancy in our published results on this point, in that a complete loss of voltage dependence of the inward current at low cytoplasmic free calcium concentrations was first described in chymotrypsin-treated patches. It is now clear that the chymotrypsin treatment in those experiments was incomplete.

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