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MECHANISM OF CARDIAC Na^+–Ca^{2+} EXCHANGE CURRENT STIMULATION BY MgATP: POSSIBLE INVOLVEMENT OF AMINOPHOSPHOLIPID TRANSLOCASE

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SUMMARY

1. The sensitivity of outward Na^+–Ca^{2+} exchange current to charged amphiphiles and phospholipids was tested in giant excised inside-out membrane patches from guinea-pig and rabbit myocytes.

2. Screening of membrane surface potentials with dimethonium (10 mM), spermine (200 μM) and spermidine (100 μM) was without effect, while the positively charged ionic detergents hexadecyltrimethylammonium and dodecyltrimethylammonium strongly inhibited steady-state outward exchange current (0·1–10 μM).

3. Interventions expected to increase negative surface charge included treatment of the cytoplasmic surface with phospholipase D, application of dodecylsulphate (1–10 μM), application of the short-chain phosphatidylserine derivative, dicapryl phosphatidylserine (C_{10}PS), and inclusion of 1–3% phosphatidylserine in the hydrocarbon mixture used to coat electrodes. Each intervention strongly stimulated Na^+–Ca^{2+} exchange current in a similar way to MgATP, reducing the fractional decay of outward exchange current (inactivation) during application of high cytoplasmic sodium.

4. The MgATP-stimulated exchange current was inhibited with a K_i of approximately 1 μM by pentalysine, which is known to associate with phosphatidylserine head groups. After 'deregulation' of the exchanger by chymotrypsin, pentalysine was without effect.

5. Inclusion in the pipette of 0·2 mM-pyridyldithioethylamine (an oxidizing inhibitor of aminophospholipid translocase) abolished stimulation of outward exchange current by MgATP without inhibiting basal outward exchange current or sodium pump current.

6. Application to the cytoplasmic side of 1·5 mM-diamide, which reportedly decreases membrane phospholipid asymmetry, apparently reversed the effect of MgATP. After treatment with diamide and subsequently with dithiothreitol, Na^+–Ca^{2+} exchange current was again stimulated by MgATP. Diamide was without effect when secondary exchange regulation had been previously removed by chymotrypsin.

7. Potassium current carried by the surface potential-sensitive ionophore, nonactin, was stimulated by MgATP when extracellular surface charge had been

neutralized. The effect was largest (40–90%) when low ionic strength cytoplasmic solutions were employed, consistent with an increase of negative membrane charge on the cytoplasmic side during MgATP application.

8. Potassium current carried by nonactin was inhibited by MgATP when cytoplasmic surface charge had been neutralized and extracellular solutions of low ionic strength were employed, consistent with a decrease of negative membrane charge on the extracellular side.

9. These results indicate that the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current could involve changes of charged membrane lipids, that the effect probably involves a transmembrane, oxidation-sensitive protein, that pentalysine-sensitive sites are involved, that phosphatidylserine mimics the effect of MgATP, and that the effect extends to a simple surface potential-sensitive ionophore. All results are consistent with the activation by MgATP of an aminophospholipid translocase as the underlying mechanism.

INTRODUCTION

The preceding paper (Collins, Somlyo & Hilgemann, 1992) characterized the stimulatory effect of MgATP on outward Na^+–Ca^{2+} exchange current in giant excised patches from cardiac myocytes and concluded that the involvement of a protein kinase in the effect is unlikely. The range of possible alternative mechanisms is of course broad. One striking characteristic of the MgATP-dependent stimulation, which may serve as a guide-post for identification of the mechanism, is an ATP concentration dependence in the range of several millimolar. In this paper, we present evidence for the possible involvement of a phospholipid translocase.

A possibility that acid phospholipids might regulate Na^+–Ca^{2+} exchange arises from findings that cardiac Na^+–Ca^{2+} exchange activity in vesicles is strongly affected by interventions which change lipid charge (for review see Reeves & Philipson, 1989). In cardiac sarcolemmal vesicles and in reconstitution experiments it has been shown that (1) phosphatidylserine (PS) strongly stimulates exchange activity (Vemuri & Philipson, 1988), (2) anionic amphiphiles stimulate Na^+–Ca^{2+} exchange activity (Philipson, 1984), (3) generation of phosphatidate by phospholipase D stimulates activity (Philipson & Nishimoto, 1984), and (4) cationic amphiphiles inhibit exchange activity (Philipson, 1984).

An asymmetric distribution of aminophospholipids, in particular of PS, is a basic property of most biological membranes and is thought to be of general structural and functional importance. Cell shape, cytoskeleton attachment, protein anchoring, endocytosis, neutrotransmitter release, and cell–cell interactions are all potentially strongly influenced by PS asymmetry (for reviews see Mato, 1990; Devaux, 1991). Phospholipid asymmetry is likely to be altered or lost in cardiac vesicles and is absent in membranes used for reconstitution, which until the last few years were the only systems available to study details of cardiac Na^+–Ca^{2+} exchange function. Seigneuret & Devaux (1984) described a system in human erythrocytes which specifically translocates the aminophospholipids PS and phosphatidylethanolamine to the inner leaflet of the plasma membrane with millimolar dependence on MgATP. Vanadate was reported to inhibit this process in red cells, as monitored via the spin-label technique, but recent studies using fluorescent PS analogues suggest that the

translocation mechanism can be insensitive to vanadate from the cytoplasmic side (A. J. Schroit & J. Connor, personal communication). Although multiple factors may be involved (Connor & Schroit, 1990), this enyme is currently the only suggested active mechanism known for maintenance of phospholipid asymmetry. Rates of phospholipid randomization are unknown for most cell types other than erythrocytes, but could well be expected to occur in ATP-depleted cardiac cells during storage for more than 8 h.

In the light of the existing literature, we hypothesized that PS translocation could be involved in the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current (Hilgemann & Collins, 1990). Specifically, our hypothesis states that, upon MgATP application, an increase in negatively charged phospholipids occurs at the cytoplasmic side of the membrane, presumably via activation of the translocase mentioned above, and that PS interaction with a regulatory (anchoring) region of the cytoplasmic domain of the exchanger disinhibits ion transport function.

This paper presents several initial tests of the working hypothesis. First, we attempted to change the charge of membrane lipids at the cytoplasmic surface with phospholipase D and ionic detergents. Second, we tested whether PS could mimic the effects of MgATP. Third, agents which are known to bind to PS head groups, such as pentalysine, were tested since they would be expected to inhibit or reverse the MgATP effect by disrupting the interaction between the exchanger and membrane PS. Fourth, we attempted to affect the PS translocation mechanism using an extracellular oxidizing agent, pyridyldithioethylamine (PDA), which is known to inhibit translocase activity (Connor & Schroit, 1988). Fifth, the sulphydryl-oxidizing reagent diamide, which cross-links spectrin molecules, was examined as this agent is known to accelerate PS randomization in ATP-depleted erythrocyte membrane (Haest, Plasa, Kamp & Deuticke, 1978; Middelkoop, Van der Hoek, Bevers, Comfurius, Slotboom, Op den Kamp, Lubin, Zwaal & Roelofsen, 1989).

Finally, we directly tested whether a change of membrane charge may accompany the stimulatory effect of MgATP. To do so, the potassium conductance generated by the peptide antibiotic, nonactin, was examined in giant patches. Nonactin conductance is known to be sensitive to membrane surface potential (McLaughlin, Szabo, Eisenman, & Ciani, 1970), and it has been used to quantify membrane surface charge asymmetry (Hall & Latorre, 1976; Hall, 1981).

METHODS

The giant cardiac membrane patch method was used with guinea-pig, rabbit and mouse myocytes as described in the accompanying article (Collins *et al.* 1992). Solution compositions for outward exchange current measurements were modified, as follows, from the preceding article. The total chloride concentrations were reduced to 20 mM in both the cytoplasmic (superfusion) and the extracellular (pipette) solutions by replacing chloride with 2-(*N*-morpholino)ethanesulphonic acid (MES). These substitutions had no evident effect on experimental results and eliminated the possibility of significant chloride current in experimental results.

For experimental results on nonactin-mediated potassium current, solution changes from the standard solutions are given with the results. When phospholipids were included in the hydrocarbon electrode coat, 5–25% glycerol (by weight) was added to the hydrocarbon mixture and phosopholipids were added immediately before experimentation. The addition of glycerol to the hydrocarbon coat increased success rates of high resistance sealing with cardiac myocytes and had no apparent effect on experimental results obtained.

Phospholipase D was purchased from Calbiochem (USA). Sodium dodecylsulphate (SDS),

dodecyltrimethylammonium bromide (DDTMA), hexadecyltrimethylammonium bromide (HDTMA), spermine, diamide and nonactin were purchased from Sigma (USA). Bovine brain phosphatidylserine (PS), bovine heart phosphatidylcholine (PC), and dicapryl phosphatidylserine (C_{10}PS), as well as the other short-chain PS derivatives tested, were purchased from Avanti Polar Lipids (USA). Pentalysine was from Peptides International (USA). PDA was a gift of Dr Alan J. Schroit (Houston).

RESULTS

Phospholipase D stimulates outward Na^+–Ca^{2+} exchange current

Phospholipase D is an enzyme which hydrolyses the bond between the phosphate moiety and the head-group of membrane phospholipids, thereby increasing negative charge density in the form of phosphatidate. Stimulation of Na^+–Ca^{2+} exchange current by phospholipase D applied to the cytoplasmic side of the membrane is shown in Fig. 1. The results are from a guinea-pig cardiac patch. At time zero the cytoplasmic surface of the patch was being superfused with a solution containing 100 mM-caesium and no sodium. Ten seconds later the caesium was rapidly replaced with sodium, and a mainly transient outward Na^+–Ca^{2+} exchange current was activated, typical for patches from contracted myocytes. At 60 s, 0·5 mg (22·5 units) per ml phospholipase D was added to the superfusion solutions, and after a delay of 2 min the exchange current began to increase. At this point washout of the phospholipase D was begun slowly, since experience had shown that prolonged treatment disrupted patches. Switching back from sodium to caesium at 260 s confirmed that the current increase was not due to leak current, and the next sodium substitution at 275 s revealed that a transient component of the current was still present. During two further rapid switches from caesium to sodium both the peak and the steady-state currents continued to increase. The protracted rise of current after removal of phospholipase D was a very consistent result and presumably represents a long-term association of this phospholipase with the membrane. Removal of calcium in the continued presence of sodium at 440 s indicated that the exchange current induced by phospholipase D was largely calcium dependent. In other patches, relatively large fractions of the current were found to be calcium independent after phospholipase D treatment. More detailed observations during extended treatments then revealed that the cytoplasmic calcium dependence was progressively and completely lost after the maximum stimulatory effect was obtained in the presence of 1 μM-free cytoplasmic calcium (two observations not shown). Note that the phospholipase D treatment reduces the relative magnitude of current decay during application of sodium, similar to the stimulatory effect of MgATP.

Exchange current modulation by ionic detergents

A second way to change surface charges was to use detergents known to alter surface potential (see McLaughlin, 1977). As shown in Fig. 2, positively charged detergents were found to be potent inhibitors of Na^+–Ca^{2+} exchange current from the cytoplasmic side. HDTMA virtually abolished the exchange current at a concentration of 5 μM without disruption of the patch and with no apparent reversal upon removal of the detergent (five observations). Figure 2*A* illustrates the effect of 1 μM-HDTMA with respect to the MgATP effect. Curve 1 is the current transient obtained before MgATP, curve 2 is after MgATP, and curve 3 is after 1 μM-HDTMA.

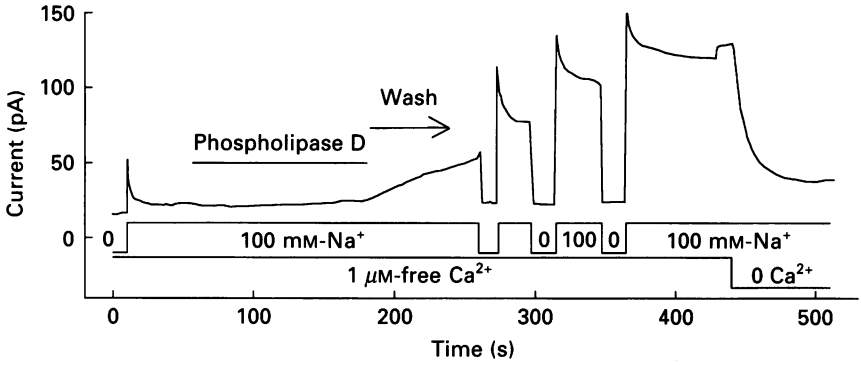

Fig. 1. Stimulation of Na^+–Ca^{2+} exchange current by phospholipase D. The steps below the figure indicate the concentrations of sodium and calcium in the superfusion solution. Near the beginning of the figure an outward exchange current transient was activated by rapidly replacing the 100 mM-caesium in the superfusion solution with 100 mM-sodium. The bar above the current record indicates the presence of 0·5 mg (22·5 units)/ml phospholipase D in the superfusion solution. The delay between application of phospholipase D and the onset of current increase is typical, as some delay was observed in all twelve patches in which this enzyme stimulated the exchange current. Washout of phospholipase D was started immediately upon observation of an increase of current because previous experience had shown that longer phospholipase D treatment consistently resulted in a disruption of patches. Towards the end of the record (440 s) calcium was removed from the superfusion solution and the current declined by 90%. Patch from guinea-pig myocyte.

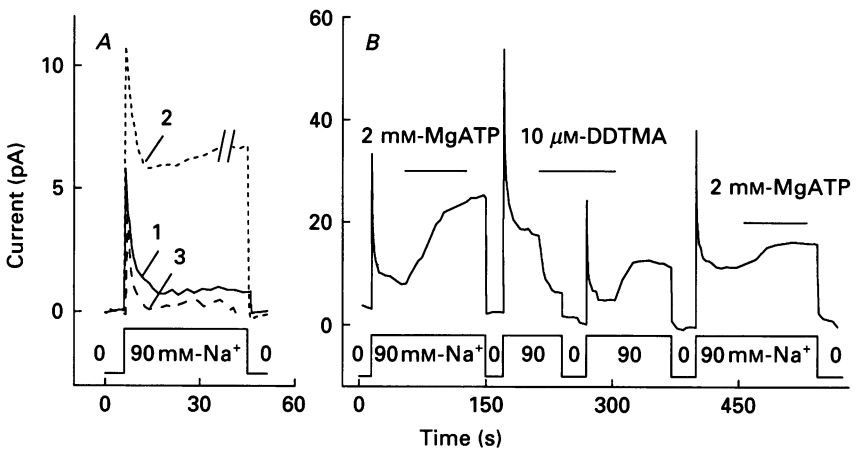

Fig. 2. Apparent reversal of the MgATP effect by positively charged detergents. *A*, superimposed current transients from the same patch, numbered in chronological order. Curve 1, before MgATP; curve 2, after 2 mM-MgATP; curve 3, after 1 μM-HDTMA. The break in trace 2 indicates that the 90 mM-Na^+ step lasted longer (140 s) in the original record. *B*, stimulation of exchange current by MgATP followed by inhibition by DDTMA. The current recovered somewhat upon DDTMA removal and showed a limited response to a second MgATP treatment. Patches from guinea-pig myocytes.

In this patch, MgATP approximately doubled the peak exchange current and induced a steady-state exchange current of about 50% of the peak. As shown in Fig. 2*B*, the effects of DDTMA were qualitatively similar to those of HDTMA, although

more detergent was required and the current recovered partially upon removal of the detergent. The effect of positively charged detergents was a pseudo-reversal of the MgATP effect, whereby the steady-state current was greatly reduced and the peak current was reduced by about 50%. It appears of interest that in three patches

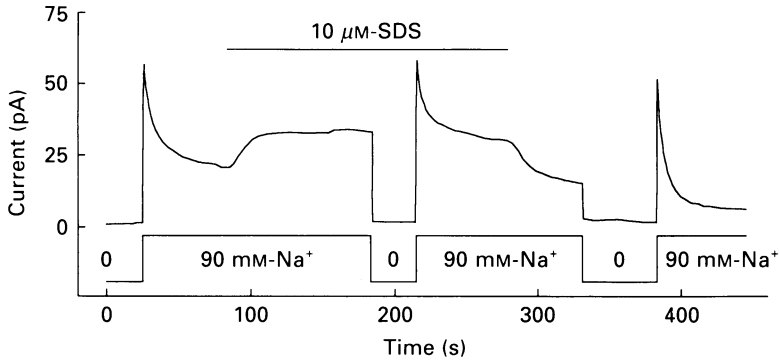

Fig. 3. Stimulation of Na^+–Ca^{2+} exchange current by 10 μM-SDS. The bar indicates the time during which the superfusion solution contained SDS. Patch from guinea-pig myocyte. Similar results were obtained in a total of seven patches.

treated with chymotrypsin (which removes regulation by MgATP, sodium and calcium; Hilgemann, 1990) DDTMA still reduced the exchange current by 40–50% but did not induce current transients during sodium application.

As a complement to the phospholipase D result, the negatively charged detergent SDS also stimulated Na^+–Ca^{2+} exchange current in excised patches (ten experiments). As shown in Fig. 3, steady-state exchange current in excised patches was stimulated by SDS from the cytoplasmic side. Outward exchange current was activated with 90 mM-sodium, and at 80 s 10 μM-SDS was superfused, resulting in a 40% increase of the steady-state exchange current. During this period a syringe was placed so as to apply SDS as rapidly as possible through the caesium-containing line. Accordingly, the patch was exposed to SDS-free solution for not more than 4 s immediately following the switch to caesium.

The peak current observed upon switching from caesium to sodium at 215 s was not changed at all in the presence of SDS (i.e. 57 pA greater than the caesium leak both before and in SDS). Removal of SDS at 280 s revealed that its stimulatory effect was reversible, and that the exchange current had decayed during the SDS treatment. Concentration dependence of the SDS effect was roughly linear up to 10 μM. In every patch examined under control conditions, the stimulatory effect of 10 μM-SDS was at least as large as in the case presented here, and in some cases steady-state current increased to nearly the peak current magnitude. While patches were consistently stable with 10 μM, patch disruption occurred with concentrations of 30 μM-SDS or more.

As an attempt to test whether the effect of SDS was restricted to patches in which the exchange current displays secondary regulation, SDS was applied to three patches which had been treated with chymotrypsin. These experiments were

performed with submaximal sodium concentrations in the superfusion solution to be certain that the exchange current was not maximally activated. In two of these patches SDS was without effect, while in the third patch SDS stimulated the current only slightly.

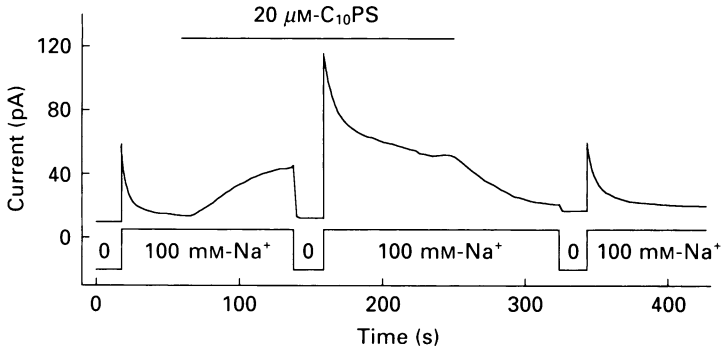

Fig. 4. Stimulation of Na^+–Ca^{2+} exchange current by dicapryl phosphatidylserine (C_{10}PS); patch from guinea-pig myocyte. C_{10}PS (20 μM) was applied to the cytoplasmic surface of the patch as indicated by the bar above the current record. In patches from guinea-pig myocytes, 20 μM-C_{10}PS stimulated Na^+–Ca^{2+} exchange current in a total of four patches, while 10 μM-C_{10}PS had no effect in one patch. C_{10}PS (20–30 μM) had no effect on Na^+–Ca^{2+} exchange current in five patches from rabbit myocytes. C_{10}PS was added to the superfusion solution in the form of a 2 mM sonicated stock solution in dimethyl sulphoxide (DMSO). Superfusion solution with 20 μM-C_{10}PS therefore also contained 1 % DMSO. In control experiments 1 % DMSO had no effect on Na^+–Ca^{2+} exchange current (four patches from guinea-pig myocytes).

Membrane charge screening is without effect on exchange current

The action of charged amphiphiles on Na^+–Ca^{2+} exchange current in excised sarcolemmal patches could conceivably be caused by changes in membrane surface potential *per se* and therefore would be expected to depend on the ionic strength of solutions. To test this possibility, the effects of non-amphiphilic agents which screen surface charge were investigated. The polycation spermine had no effect on the exchange current after stimulation by MgATP, at a concentration of 200 μM (one observation). Similarly, no effects were noted with 100 μM-spermidine (one observation), nor did the divalent cation dimethonium (10–20 mM; McLaughlin, Eng, Vaio, Wilson & McLaughlin, 1983) inhibit exchange current either before or after application of MgATP (two observations).

Phosphatidylserine stimulates outward Na^+–Ca^{2+} exchange current

The above results indicate the need for a hydrophobic moiety on a charged molecule if the molecule is to have an influence on Na^+–Ca^{2+} exchange activity. For such a species to be a putative physiological regulator of exchange function, it would have to be normally present in the vicinity of the exchanger. A leading candidate would be PS, since it is the most abundant negatively charged phospholipid in the sarcolemmal membrane (e.g. Mato, 1990). As a first test of whether PS stimulates the Na^+–Ca^{2+} exchange current, it was applied directly to excised patches in the form of

soluble or semisoluble PS analogues. Both dicaprylyl PS (eight carbons) and dicaproyl PS (six carbons) inhibited reversibly the Na^+–Ca^{2+} exchange current in a concentration range of 5–50 μM and disrupted patches at higher concentrations. As shown in Fig. 4, the PS analogue dicapryl PS (with acyl chains at the *sn*-1 and *sn*-2

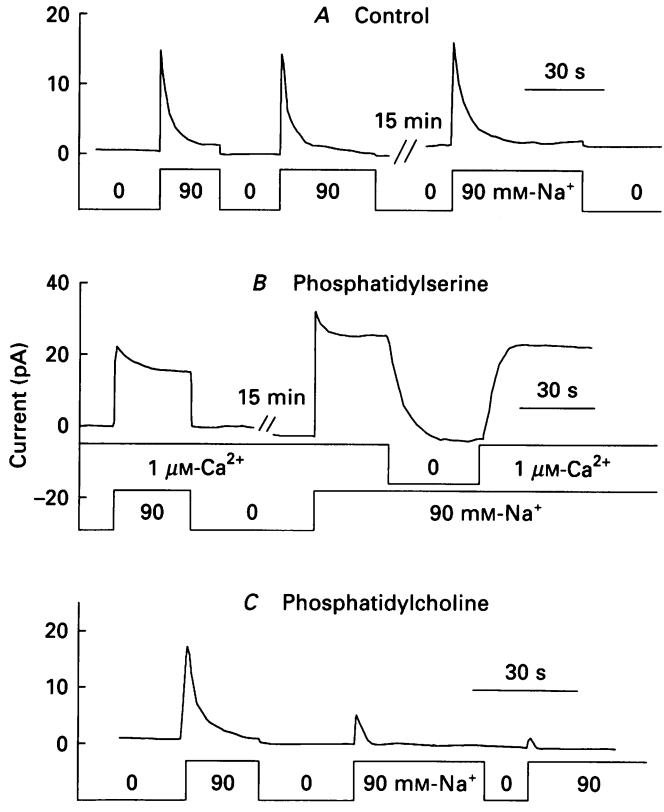

Fig. 5. Modulation of Na^+–Ca^{2+} exchange by purified phospholipids. Outward Na^+–Ca^{2+} exchange current transients; electrode tips treated with standard coating mixture containing 25% (by weight) glycerol (*A*), 25% glycerol plus 2% purified bovine brain phosphatidylserine (*B*), 25% glycerol plus 2% purified bovine heart phosphatidylcholine (*C*). Patches from rabbit myocytes.

positions both containing ten carbon atoms) had a strong stimulatory effect in the concentration range of 10–30 μM (four observations in patches from guinea-pig myocytes). Longer chain PS analogues and PS itself formed micelles and had no effect when applied to patches in this way.

As an alternative method for manipulating the phospholipid composition of the patch membrane, purified phospholipids were mixed into the electrode tip coating material, together with approximately 25% glycerol (see Methods and preceding article), under the assumption that phospholipid exchange between the hydrocarbon coat and the patch membrane might occur. Typical results obtained using this procedure are shown in Figs 5 (patches from rabbit myocytes) and 6 (patches from guinea-pig myocytes). Figure 5*A* shows typical results using the coating mixture

with added glycerol and no added phospholipids. The Na^+–Ca^{2+} exchange current was 90% transient and was stable for more than 15 min. In contrast, when purified brain PS was included in the coating mixture (2% by weight) the transient fraction was almost eliminated (Fig. 5*B*). Removal of calcium from the cytoplasmic side

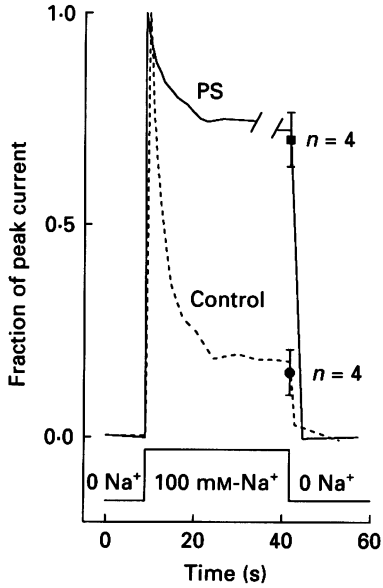

Fig. 6. Stimulation of Na^+–Ca^{2+} exchange current by purified phosphatidylserine. Electrode tips were treated with standard coating mixture containing 25% (by weight) glycerol or 25% glycerol plus 2% purified bovine brain PS. The outward Na^+–Ca^{2+} exchange current transients shown are representative of four records without (dashed line) and four records with (continuous line) PS, normalized to the peak current value and superimposed. Symbols and error bars represent mean ± S.E.M. normalized steady-state current. Values for patches with PS are highly significantly different from values for patches without PS ($P < 0{\cdot}001$; Student's t test). Patches from guinea-pig myocytes.

showed that secondary regulation by internal calcium was still present, and therefore that modulatory factors had not been destroyed or lost as with chymotrypsin treatment. Four of five other patches tested with PS-containing electrode coat from the same batch of myocytes gave very similar results, while the fifth patch showed the usual strong inactivation phase on application of sodium. Comparing results of these six patches to results from forty patches without added PS, the fractional inactivation of exchange current on application of cytoplasmic sodium was highly significantly reduced ($P < 0{\cdot}01$).

Figure 5*C* is a current record obtained when 2% purified PC from cardiac muscle, instead of PS, was added to the same hydrocarbon mixture for electrode coating (patch from same batch of myocytes). The Na^+–Ca^{2+} exchange current was mostly transient in all cases, as under control conditions, and in contrast to control conditions the current ran down rapidly, and within less than 5 min could no longer be activated. The great majority of patches made with PC-containing hydrocarbon

mixtures disrupted spontaneously, and records longer than 10 min were not obtained from this myocyte batch (four patches). The current records in Fig. 6 are representative transients from guinea-pig myocyte patches with and without purified PS in the electrode tip coating. Out of five patches made with electrodes

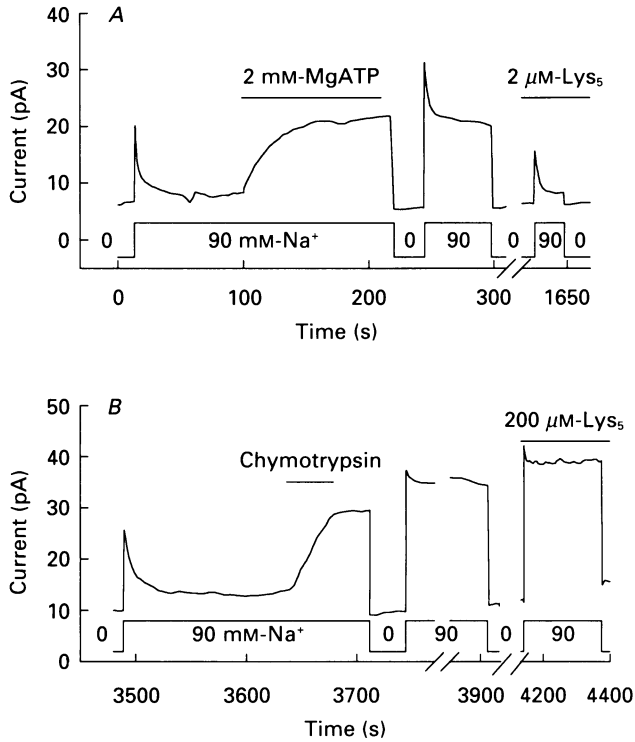

Fig. 7. Effect of pentalysine on Na^+–Ca^{2+} exchange current after stimulation by MgATP and after chymotrypsin treatment. Similar effects of pentalysine were observed in ten patches. *A*, inhibition of exchange current by pentalysine after stimulation by MgATP. Between 300 and 1600 s the current ran down slowly and was twice restimulated with MgATP. Just prior to pentalysine treatment the instantaneous peak current was 26 pA and the steady-state current was 12·5 pA (without leak subtraction). Addition of 2 μM-pentalysine (Lys_5; indicated by the bar above the current record) to the superfusion solution inhibited the peak current by 50% and the steady-state current by 60%. *B*, ineffectiveness of pentalysine after chymotrypsin treatment. From the same patch as in *A*. Chymotrypsin (3 mg/ml) in the superfusion solution increased the steady-state current and almost eliminated the transient component. The current continued to increase slowly between 3900 and 4400 s in the presence of 8, 20 and then 200 μM-pentalysine. This increase was not due to pentalysine, since the current continued to increase slowly after pentalysine removal.

treated with PS-containing coating material, four patches displayed markedly reduced inactivation compared to control patches. Statistical analysis (Student's *t* test) revealed that the steady-state Na^+–Ca^{2+} exchange current, expressed as a fraction of the peak current activated upon switching to 100 mM-cytoplasmic sodium, was significantly greater ($P < 0·05$) when PS was present. When the single PS patch

without reduced inactivation was omitted from the statistical analysis on the basis of a Q test, the difference was highly significant ($P < 0{\cdot}001$). This result is represented in Fig. 6.

Pentalysine is an inhibitor of $[Ca^{2+}]_i$*-dependent outward exchange current*

Pentalysine was predicted to be a good inhibitor of the outward current on the basis that it binds to acidic phospholipids (Kim, Mosior, Chung, Wu & McLaughlin,

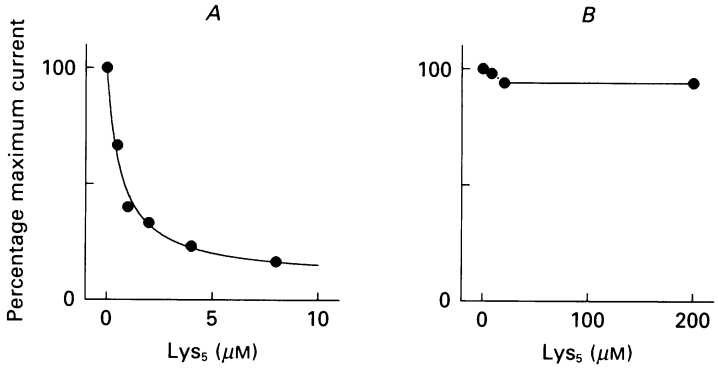

Fig. 8. Dependence of Na^+–Ca^{2+} exchange current inhibition on pentalysine (Lys_5) concentration. All data points are from the same patch as in Fig. 5. Current is expressed as the fraction of the steady-state current in the absence of pentalysine. *A*, after MgATP. Half-maximal inhibition is at 0·68 μM. *B*, after chymotrypsin treatment.

1991) and therefore should prevent interactions of the exchanger with acidic phospholipids. As shown in Fig. 7, the MgATP-stimulated exchange current was rapidly and reversibly inhibited by pentalysine in micromolar concentrations. In Fig. 7*A* the current was first stimulated by MgATP, and it remained elevated after MgATP removal. Application of 2 μM-pentalysine to the patch inhibited exchange current to about the same degree. Both onset and recovery of the effect were 80% complete in < 20 s (not shown). Figure 7*B* is from the same patch as in Fig. 7*A* after a concentration–response relation for pentalysine had been obtained. As shown, the exchange current was strongly stimulated, as usual, by treatment of the patch with 1 mg/ml chymotrypsin. In marked contrast to the effect on the MgATP-stimulated current, up to 2 mM-pentalysine has virtually no effect on the exchange current after deregulation by chymotrypsin. Figure 8 shows the concentration–steady-state exchange current relationship for pentalysine before (panel *A*) and after (panel *B*) the chymotrypsin treatment.

A further striking feature of the effect of pentalysine was that it consistently inhibited only the calcium-sensitive component of the exchange current, as determined by removing calcium from the cytoplasmic solution (see for instance last part of Fig. 1). In Fig. 9, data are presented from patches in which both inhibition of outward current by 50 μM-pentalysine and removal of cytoplasmic calcium were tested. It can be seen from this figure that the pentalysine-sensitive fraction of the exchange current in general correlates well with the calcium-dependent fraction.

Patches from rabbit myocytes were an exception, in that pentalysine was often almost without effect under control conditions (□ in Fig. 9). Also, patches from guinea-pig myocytes with fast reversal of the MgATP effect were relatively insensitive to pentalysine (not shown). The exchange current in patches from both

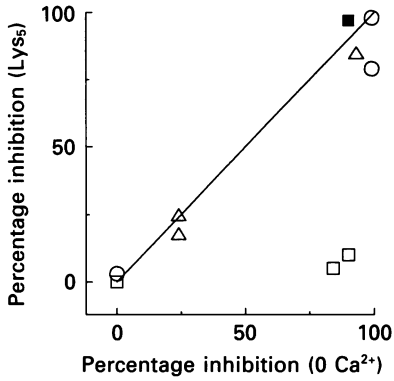

Fig. 9. Correlation between pentalysine (Lys_5) sensitivity and internal calcium dependence of Na^+–Ca^{2+} exchange current. The vertical axis represents the steady-state current inhibited by 50 μM-pentalysine (except filled square with 200 μM; PS added to electrode coat). Results are expressed as a percentage of the steady-state current in the absence of pentalysine. The horizontal axis represents the percentage of the steady-state current which was inhibited by removal of calcium (1 μM) from the superfusion solution. The two data points nearest the origin are from patches treated with chymotrypsin. The two triangles in the lower left part of the figure are from patches treated with MgATP. The open data points near the upper right corner are from untreated patches. ○, guinea-pig, △, mouse; □, rabbit.

guinea-pig and rabbit myocytes (■ in Fig. 9) with PS included in the electrode coat could be completely inhibited by pentalysine, albeit with a relatively low affinity (K_i, the concentration giving half-maximal inhibition, 10–60 μM). Similarly, the efficacy of pentalysine to inhibit the phospholipase D-stimulated current was low (K_i in the range of 100 to several hundred micromolar, two observations).

Vanadate does not inhibit the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current

Experiments on the vanadate sensitivity of the MgATP effect were carried out as a first possible test for aminophospholipid translocase involvement in the MgATP mechanism, since the erythrocyte enzyme was reported to be a vandate-inhibited ATPase, based on experiments with spin-labelled phospholipid analogues (Seigneuret & Devaux, 1984). Large stimulations of the outward exchange current by MgATP were obtained in seven patches pre-incubated with 10 μM- to 1 mM-vanadate on the cytoplasmic side (not shown, patches with both fast and slow reversal of the MgATP effect), apparently contradicting an involvement of the enzyme. Vanadate also had no evident effect on the reversal of the MgATP effect when it was applied after MgATP (two observations with 25 μM-vanadate; not shown).

Inhibition of outward Na^+–Ca^{2+} exchange current by PDA

The agent pyridylthioethylamine (PDA) is a relatively specific oxidizing agent for the translocase and has been shown to inhibit translocase activity (Connor & Schroit, 1988). The inhibition by extracellular PDA of MgATP-induced stimulation of

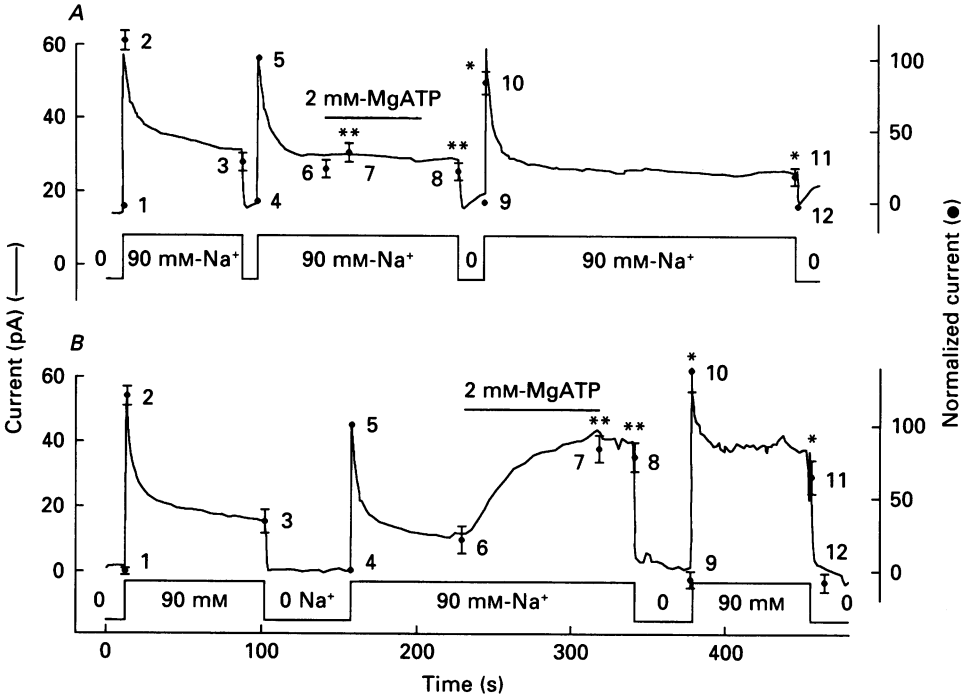

Fig. 10. Inhibition of the MgATP effect by external PDA. The current traces are from single representative patches with (*A*) or without (*B*) 0·2 mM-PDA in the electrode solution. The points numbered 1–12 represent the means±S.E.M. of the normalized current at that point in the experimental protocol (*A*, eight patches; *B*, eleven patches). Current was normalized as a percentage of the leak-subtracted pre-ATP peak (point 5−point 4). For points without error bars, the S.E.M. is within the range covered by the point. Asterisks indicate that the normalized current at that point was significantly smaller $*P < 0{\cdot}01$; $**P < 0{\cdot}001$) with PDA in the electrode. Point 7 represents the maximum steady-state current reached during MgATP application. The following absolute current magnitudes were not significantly different (PDA *vs.* no PDA): the leak current in Cs^+ (point 4; PDA: mean = 8·9 pA, S.E.M. = 2·4 pA; no PDA: mean = 6·9 pA, S.E.M. = 1·9 pA); the pre-ATP peak (point 5; PDA: mean = 42·1 pA, S.E.M. = 10·3 pA; no PDA: mean = 52·0 pA, S.E.M. = 9·2 pA); the pre-ATP steady state (point 6; PDA: mean = 18·4 pA, S.E.M. = 5·4 pA; no PDA: mean = 22·3 pA, S.E.M. = 8·2 pA). The absolute magnitude of the MgATP-stimulated current (point 7−point 6) was significantly smaller with PDA (PDA: mean = 3·1 pA, S.E.M. = 1·1 pA; no PDA: mean = 24·2 pA, S.E.M. = 5·3 pA; $P < 0{\cdot}01$).

outward exchange current is presented in Fig. 10. The experimental records illustrate the protocol followed in a randomized series of experiments in which the MgATP effect was examined in the presence (eight patches; top traces) or absence (eleven

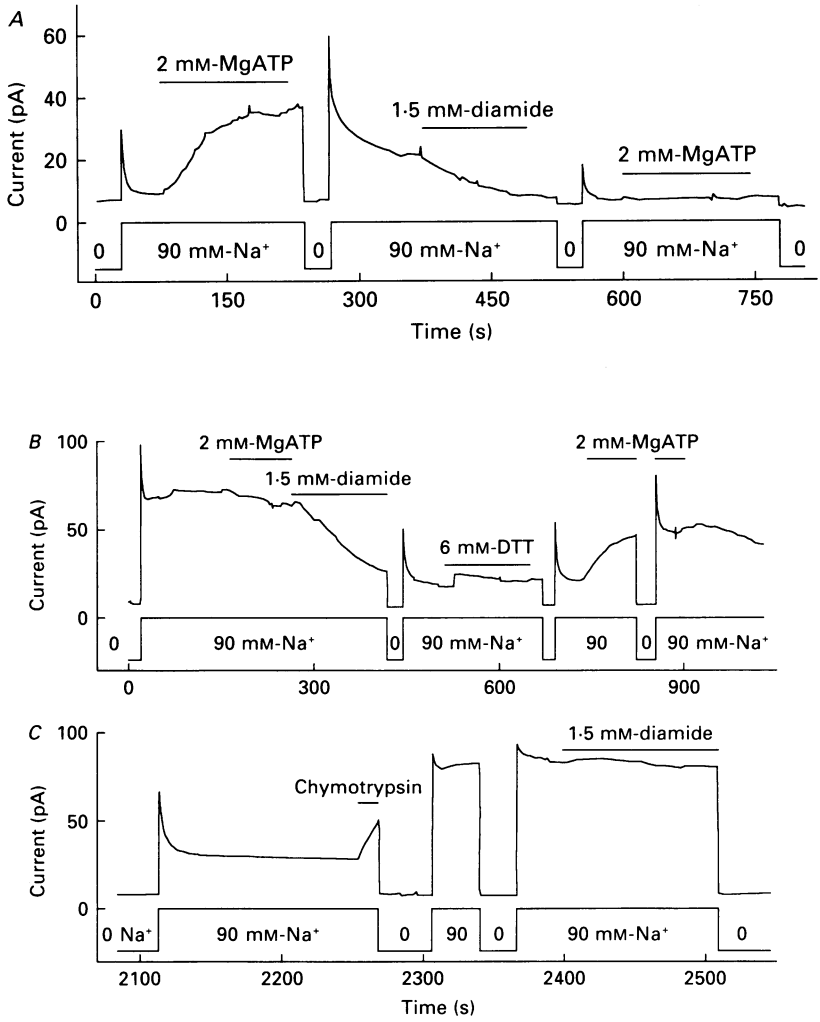

Fig. 11. Inhibition of Na^+–Ca^{2+} exchange current by diamide and removal of diamide sensitivity by chymotrypsin. Similar effects of diamide were observed in three patches. *A*, stimulation of Na^+–Ca^{2+} exchange current by MgATP, followed by diamide treatment. The current decreased rapidly during superfusion of the patch with diamide-containing solution. Application of MgATP subsequent to diamide treatment failed to stimulate the current. *B*, inhibition of exchange current by diamide and subsequent stimulation by MgATP following DTT treatment. At the beginning of the record the current was already in a stimulated state as indicated by the relatively small transient component and the absence of further stimulation by MgATP. Addition of diamide to the superfusion solution resulted in a rapid decrease of the current. DTT had no effect on the current *per se*, but the current did respond subsequently to MgATP. *C*, no effect of diamide after chymotrypsin treatment. From the same patch as in *B*. Chymotrypsin (1 mg/ml) was added to the superfusion solution as indicated by the bar.

patches; bottom traces) of 0·2 mM-PDA in the patch electrode. The points in Fig. 10 represent the data from all patches with (Fig. 10*A*) and without (Fig. 10*B*) external PDA, normalized so that the magnitude of the leak-subtracted peak current before

MgATP (point 5 minus point 4) equals 100. Error bars give the standard error of the means of the experiments. The presence of PDA did not significantly change the absolute magnitude of the pre-ATP peak current (point 5 in Fig. 10), or the absolute magnitude of the leak current (point 4 in Fig. 10). However, the magnitude of the MgATP-stimulated increase of steady-state current (point 7 minus point 6 in Fig. 10) was reduced to a few per cent of control ($P < 0{\cdot}01$) when PDA was present in the electrode solution. Expressed as a percentage of the peak current magnitude obtained on second application of sodium (point 5), the steady-state current before MgATP was not significantly changed, whereas the steady-state current after MgATP was highly significantly reduced by PDA ($P < 0{\cdot}001$ for points 7 and 8; $P < 0{\cdot}01$ for points 10 and 11).

It should be noted that a previous series of similar experiments was executed in which the electrode contained only 20 μM-ouabain and 10 mM-KCl. Those results also indicated a highly significant reduction of the MgATP effect. However, a small reversible 'stimulatory effect' of MgATP equilibrated within the time course of solution switches. It was completely inhibited by 20 μM-vanadate, and it was absent in subsequent experiments in which the extracellular ouabain was raised to 250 μM. It was thereafter verified that PDA and *N*-ethylmaleimide do not inhibit sodium pump current when included in the pipette at concentrations which abolish the stimulation of exchange current by MgATP. When *N*-ethylmaleimide was tested from the cytoplasmic side (0·5 mM), it rapidly inhibited sodium pump current (not shown for brevity), indicating that these agents cannot reach cytoplasmic sites of action when included in the pipette. *N*-ethylmaleimide, like PDA, abolished the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange when included in the pipette at a concentration of 0·5 mM (two observations on patches from myocyte batches with highly reproducible MgATP effects).

The cross-linking agent diamide apparently reverses the MgATP effect

Figure 11 presents cytoplasmic effects of the cross-linking oxidizer, diamide. In Fig. 11*A*, 1·5 mM-diamide was applied after application and removal of 2 mM-MgATP. Current run-down after MgATP was relatively rapid in this patch, and diamide increased the rate of run-down by about 2-fold, whereby the steady-state current reached pre-ATP levels after about 2·5 mins. In two other experiments, it was found that higher diamide concentrations did not inhibit the current more rapidly or to a greater extent than described here. A subsequent application of MgATP was in each case without effect. In Fig. 11*B* the exchange current was already in a stimulated state, and MgATP application at the onset of the record was without effect. Application of 1·5 mM-diamide rapidly reduced steady-state exchange current to about 20% of control. After the inhibition by diamide and subsequent application of dithiothreitol, to reverse oxidation caused by diamide, a second MgATP application resulted in stimulation of the exchange current. In this experiment, as well as three others, it was remarkable that dithiothreitol did not increase the exchange current after inhibition by diamide unless MgATP was reapplied. This supports the notion that diamide is in some way reversing the MgATP action and not simply inhibiting the exchange current. The records in Fig. 11*C* are a continuation of this same experiment after the diamide/ATP sequence had been repeated one more time. As indicated by the horizontal bar in Fig. 11*C*, 1 mg/ml chymotrypsin was applied to remove secondary exchange regulation. As usual, the current was stimulated and current transients upon application of cytoplasmic sodium were largely eliminated. After chymotrypsin, application of

diamide was without effect on the exchange current. This finding suggests that diamide is indeed acting on the exchange current through the secondary modulatory mechanisms susceptible to destruction by chymotrypsin.

In an attempt to manipulate specifically the PS head group and thereby test whether PS was essential for the MgATP effect, we treated excised patches with a preparation of a PS-specific decarboxylase isolated by the method of Kanfer & Kennedy (1964). This preparation immediately destabilized patches, presumably as a result of detergents necessary to solubilize the enzyme and support activity.

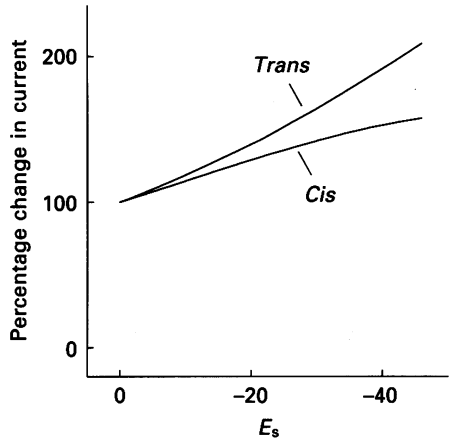

Fig. 12. Simulated effects of negative surface potential (E_s) on the current carried by a simple monovalent ion transporter with hypothetical nonactin-like properties. For theory, see McLaughlin & Eisenberg (1975) and Hall & Latorre (1976). Note that a surface potential of −50 mV on either membrane side roughly doubles the current, although the effect may be larger on one side or the other.

Nonactin-carried potassium current is modulated by MgATP, as expected for PS translocation

The use of nonactin to test for membrane surface charge changes during MgATP application is described in Figs 12–15. Model calculations of the effect of surface potential on a simple transporter are presented in Fig. 12 to point out the theoretical basis of the experiments. Potassium current carried by the ionophore, nonactin, is sensitive to membrane surface potential on both sides (for background, see McLaughlin, 1977; for details, see McLaughlin *et al.* 1970; McLaughlin & Eisenberg, 1975; Hall & Latorre, 1976). The simulated results show the expected effects of adding surface potential on each membrane side for the case of a 10:1 ion gradient at 0 mV. Addition of a 40 mV surface potential would increase the current by about 45% on the *cis* side (from which ions are transported) and by about 60% on the *trans* side (to which ions are transported).

The experiments were designed to test the hypothesis that negative surface charge might be translocated from the extracellular side to the cytoplasmic side. In preparatory experiments, appropriate nonactin concentrations were determined. Further, it was verified from previous work (Hilgemann, 1989) that 100 mM-cytoplasmic potassium causes almost no endogenous channel current with the

solutions employed, and that MgATP induced almost no potassium current with the solutions routinely employed. Figure 13 presents an example of experiments performed to test for a change of cytoplasmic surface charge, taking care to neutralize extracellular surface charge with divalent cations and to enhance

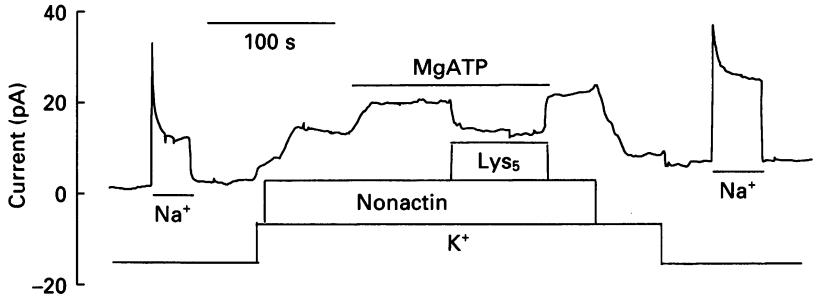

Fig. 13. Long-term stimulation by MgATP of outward potassium current carried by nonactin in a patch with slow reversal of the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current. Rabbit patch. Extracellular surface charge is neutralized by 5 mM-calcium and 10 mM-magnesium. Outward exchange current was first activated with the usual solutions. Then, as indicated, cytoplasmic solution was switched to one with potassium (45 mM) and nonactin (15 μM). The nonactin-induced current is increased by about 40% on addition of 2 mM-MgATP. Pentalysine (0·1 mM) reversibly inhibits a fraction of total current comparable to the stimulatory effect of MgATP which is maintained. After washout of nonactin and return to normal solutions, activation of outward Na^+–Ca^{2+} exchange current reveals that it is stimulated in comparison to the initial response, as expected for a long-term stimulatory effect of MgATP. See text for details.

cytoplasmic surface potential with a low ionic strength solution. In the case of Fig. 13 with a rabbit myocyte patch, the usual pipette solution was employed with 8 mM-added magnesium. Outward exchange current was activated with the usual cytoplasmic solutions in the first part of the record. The cytoplasmic solution was then replaced by one with 45 mM-potassium (complete composition: 45 mM-K-MES, 125 mM-sucrose, 20 mM-HEPES, 10 mM-EGTA, and 25 mM-TEA, 10 as TEA-MES; pH 7·0), and 15 μM-nonactin was applied.

Nonactin induced a 12 pA outward current in this patch, and on application of 2 mM-ATP the current increased by another 8 pA. As indicated, 0·1 mM-pentalysine was superfused to neutralize the surface potential. Pentalysine inhibited a current component very similar in magnitude to the effect of MgATP, and from other results it is known that 0·1 mM exerts a maximal effect. Both MgATP and pentalysine were then washed out, and the current remained stimulated from the pre-MgATP level. Next, nonactin was washed out, and finally, the usual cytoplasmic solutions were applied to induce an outward Na^+–Ca^{2+} exchange current transient. The exchange current was stimulated in comparison to the initial transient with a much larger effect on the steady-current than the peak current, as expected for a long-lasting effect of MgATP. In two similar experiments, 45 mM-potassium was included in the pipette, and current–voltage relations were determined over an 80 mV range (not shown). The nonactin current reversed as expected at 0 mV, and MgATP increased roughly equally both inward and outward currents by 20–35%.

Nonactin currents were studied in other patches from rabbit myocyte batches with rapid reversal of the MgATP effect to test whether effects of MgATP on nonactin current correlate phenomenologically with the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current. In three experiments from rabbit cell batches with fast

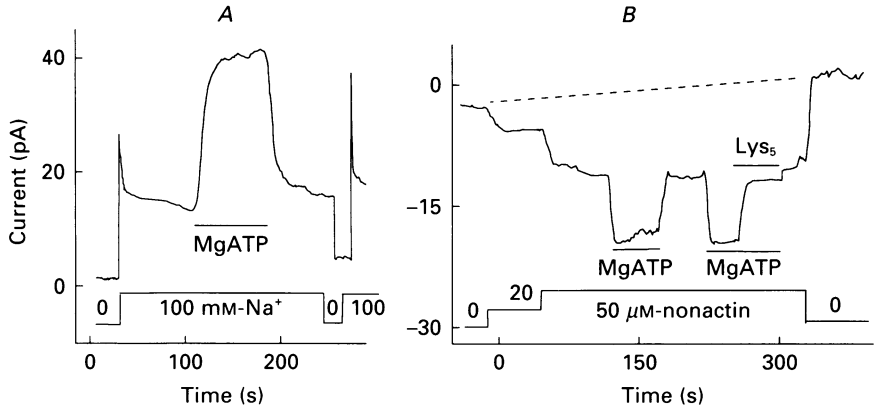

Fig. 14. Rapidly reversing stimulation by MgATP of inward potassium current carried by nonactin in a patch from cell batch with rapidly reversing stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current. Rabbit patch. Extracellular surface charge is neutralized by 10 mM-magnesium and 5 mM-calcium. *A*, typical stimulatory effect of MgATP on outward Na^+–Ca^{2+} exchange current with rapid decay in this cell batch (ten observations). *B*, inward nonactin current with 80 mM-extracellular potassium. Stimulatory effect of MgATP decays similarly rapidly to the effect of Na^+–Ca^{2+} exchange current. See text for details.

reversals, rapidly reversing stimulation of the nonactin current by MgATP was also found under the conditions of Fig. 13. As shown in Fig. 14, similar observations were made for inward nonactin current. Panel *A* of the figure shows the MgATP effect for a patch from the same cell batch with very rapid reversal (half-time of decay t_{50}, 3–4 s; six observations). Panel *B* shows results obtained for inward nonactin current, whereby the pipette contains 80 mM-potassium (usual pipette solution with 80 mM-K-MES + 20 mM-TEA-MES replacing 100 mM-NMG-MES, 8 mM-additional $MgCl_2$; usual cytoplasmic solution with 200 mM-sucrose replacing 100 mM-Cs-MES). First 20 and then 50 μM-nonactin was applied. Nonactin equilibration was very rapid, and a steady-state inward current of −12 pA was induced. On application of 5 mM-MgATP, the current increased to −21 pA, and the effect reversed within seconds on removal of MgATP. As indicated, MgATP was again applied, followed by 50 μM-pentalysine together with MgATP. As with the outward current, pentalysine inhibited the inward current by nearly the same amount as it was stimulated by MgATP. Pentalysine and MgATP, and finally nonactin, were then washed out. A 3 pA shift of baseline current was apparent from the starting current level.

To test for a change of extracellular surface potential during cytoplasmic application of MgATP, surface charges on the cytoplasmic side were neutralized with pentalysine and low ionic strength solutions were used in the pipette (complete composition: 0·12 mM-$CaCl_2$, 20 mM-KCl, 250 mM-sucrose, 10 mM-HEPES, 3 mM-

NMG and 0·3 mM-ouabain; pH 7·0). As with the previous experiments, it was advantageous to use patches with rapid decay of the MgATP effect in order to study multiple responses.

Figure 15 shows an example with measurement of both exchange current and outward nonactin current in the same patch. Outward Na^+–Ca^{2+} exchange current

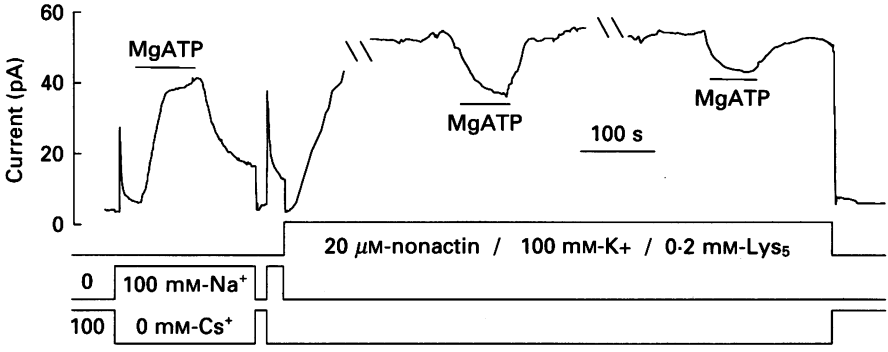

Fig. 15. Rapidly reversing inhibition by MgATP of outward potassium current carried by nonactin in a patch from cell batch with rapidly reversing stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current. Rabbit patch. Cytoplasmic surface charge is neutralized during application of nonactin by 0·2 mM-pentalysine. In the first part of the record, outward exchange current is examined with rapidly reversible MgATP effect. The outward nonactin current is reversibly inhibited by MgATP with similar kinetics. See text for details.

was first activated with the usual cytoplasmic solutions, and a MgATP response was induced. Reversal of the MgATP effect was complete for the most part within 20 s, although a slow component of decay was apparent during the following outward Na^+–Ca^{2+} exchange current transient. On switching the cytoplasmic solution to one with 20 μM-nonactin, 0·2 mM-pentalysine, and 100 mM-caesium replaced by 100 mM-potassium, a K^+-nonactin current of 49 pA developed. On application of 2 mM-MgATP, the nonactin-induced current was reduced over the course of 40 s by just 30%. On removal of MgATP the effect reverses over a similar time course. The response was repeated 7 times in this patch, and the last response is shown in the figure. The response was on average a 26 ± 8% (S.D.) reduction of the current. The effect of MgATP on nonactin current declined with time, similar to the decline noted in the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current (Collins *et al.* 1992). The inhibitory effect of MgATP under these conditions was absolutely dependent on the presence of pentalysine (two similar observations). Also, it was absolutely dependent on the presence of low ionic strength solutions in the pipette (four observations).

DISCUSSION

The specific hypothesis has been examined in this article that an aminophospholipid translocase may mediate the stimulatory effect of MgATP on outward Na^+–Ca^{2+} exchange current with PS acting as a second messenger. It has been demonstrated that the exchanger in cardiac giant patches is highly sensitive to its

charged lipid environment, and the results of several lines of experimentation are consistent with the working hypothesis. Accordingly, this work represents a new experimental approach to understanding the control of membrane phospholipid asymmetry and ultimately its role in the function of membrane-associated enzymes and transporters, cytoskeleton–membrane interactions, neurotransmitter release and cell–cell interactions.

Sensitivity of cardiac Na^+–Ca^{2+} exchange to lipid environment

The stimulation of exchange current by phospholipase D described here verifies with the new giant patch model that negatively charged phospholipids are potentially important modulators of Na^+–Ca^{2+} exchange (Reeves & Philipson, 1989). The prolonged action of phospholipase D may be a reflection of tight association with the membrane in a way similar to that described for phospholipase A_2 (Scott, White, Otwinowski, Yuan, Gelb & Sigler, 1990). It is notable that phospholipase D, positively charged detergents, negatively charged detergents and PS all act with a similarity to MgATP in that they affect the steady-state current more than peak current during activation by cytoplasmic sodium. Since the effects of these agents cannot be screened with polycations, in agreement with previous studies (Bers, Philipson, & Peskoff, 1985), the underlying mechanism must be a direct interaction of the charged groups with the exchanger. The common site of action can logically be suggested to be the lipid–protein interface, and the cationic region of the cytoplasmic loop of the exchanger (Nicoll, Longoni & Philipson, 1990), corresponding to the exchanger inhibitory peptide ('XIP'; Li, Nicoll, Collins, Hilgemann, Filoteo, Penniston, Weiss, Tomich & Philipson, 1991) sequence, is an attractive candidate for the inhibitory domain.

Philipson (1984) has suggested that the hydrophobic portions of amphiphiles (i.e. chain length and bond types) are critical for their ability to alter exchange activity. In the present work, HDTMA (sixteen carbon chain) shows a simple difference to cationic detergents with shorter tails in that HDTMA is irreversible on the time scale of our experiments, whereas shorter chain detergents reverse quickly. The slower dissociation of longer chain detergents is logically indicative of a tighter association with the membrane, and on this basis alone, a higher affinity action of HDTMA than SDS or DDTMA (twelve carbon chain) can be expected.

Our work with PS and PS analogues indicates more complex specificities in the acyl chains of negatively charged amphiphiles to stimulate the exchanger. Acyl chains of less than 10 carbon length not only do not allow stimulation of exchange current, but are inhibitory. This dependence on side chain length is consistent with our hypothesis that an anchoring function of the amphiphile is important in the stimulatory effect. With the technique of exchanging phospholipid from the electrode coat in giant patches, it should now be possible to establish detailed structure–function relationships in phospholipid–transporter interactions.

Sensitivity of cardiac Na^+–Ca^{2+} exchange to pentalysine

Since the specificity of charged amphiphiles for affecting the exchanger from the cytoplasmic face appears very limited, it was speculated in the working hypothesis that the most abundant physiologically occurring charged amphiphile would be a

physiological modulator of exchange function. As such, PS was the first candidate. The concentration dependence of the inhibition of MgATP-stimulated outward exchange current by pentalysine (K_i, 1–3 μM; Fig. 8) corresponds well to its association constant with membranes containing about 20% negatively charged lipids (Kim *et al.* 1991). The observed variability of the inhibitory effect under different conditions can well be expected, since binding to acidic phospholipids is a steep function of their density in a membrane (ibid.). Although pentalysine is by nature a non-specific agent, it generally blocks the fraction of current sensitive to cytoplasmic calcium (Fig. 9).

It is an important question, therefore, whether pentalysine has access to the actual calcium binding sites involved in secondary activation, and further whether PS itself might be a constituent of the binding sites. Interest in this possibility appears justified by the enigmatic nature of the secondary activation process, shifting its apparent calcium affinity by two to three orders of magnitude (Collins *et al.* 1992). According to the working hypothesis, MgATP could increase the probability of calcium binding to regulatory sites by generating more cytoplasmic binding sites. A functional as well as structural importance of PS–protein–calcium interactions at the cytoplasmic face of cells has long been inferred in relation to local anaesthetic action (see Bradford & Marinetti, 1981). Protein kinase C (Wise, Glass, Chou, Raynor, Katoh, Schatzman, Turner, Kibler & Kuo, 1982) and the annexin family of regulatory proteins (Hueber, Schneider, Mayr, Roemisch & Paques, 1990) are well-studied examples of regulatory proteins interacting both with acid phospholipids and calcium. In the case of annexins, the acid head group of phospholipids may indeed become part of regulatory calcium binding sites.

Does MgATP act via an aminophospholipid translocase?

A first simple test for the possible involvement of an aminophospholipid translocase in the stimulatory effect of MgATP was to examine sensitivity to orthovanadate (Seigneuret & Devaux, 1984). As mentioned in the Introduction, recent studies in resealed erythrocyte ghosts indicate that cytoplasmic vanadate sensitivity is not an invariable property of the translocase (A. J. Schroit & J. Connor, personal communication). Those same studies reveal no saturation of translocase activity with increasing MgATP concentration up to 4 mM, confirming previous observations (Seigneuret & Devaux, 1984) and in accord with our findings on the MgATP dependence of the stimulatory effect of MgATP on outward Na^+–Ca^{2+} exchange current (Collins *et al.* 1992).

Two tests of the working hypothesis have involved the use of oxidizing agents. In each case, the experimental results conformed to specific predictions, although these agents are highly non-specific. In the case of PDA, the prediction was made that it should inhibit the MgATP effect from the extracellular side. The exchanger itself was not inhibited by the method of PDA application, nor was the Na^+–K^+ pump, while stimulation of exchange current by MgATP was abolished. Regardless of whether a translocase is involved, this result suggests that a transmembrane protein is essential for the MgATP mechanism.

In the case of diamide, it was predicted that this agent would inhibit outward exchange current after application of MgATP, and that application of reducing

agents would not reverse this inhibition without reapplication of MgATP. Furthermore, it was predicted that inhibitory effects, specifically related to the MgATP effect, would be absent after removal of secondary exchange regulation by chymotrypsin. All of these predictions were verified (Fig. 11). Regardless of whether an aminophospholipid translocase is indeed involved, the results demonstrate that oxidative cross-linking of cytoplasmic membrane-associated protein can in some way reverse the effect of MgATP on sodium–calcium exchange.

Finally, the measurement of nonactin current has provided evidence that application of MgATP in the excised patches may result in both an increase of cytoplasmic surface charge and a decrease of extracellular surface charge. With extracellular surface charge neutralized (Figs 13 and 14), the measurements indicate an increase of cytoplasmic surface potential by up to 35 mV. With the cytoplasmic solutions employed, this would correspond to an increase of cytoplasmic surface charge density by about 180000 per μm^2. Results with cytoplasmic surface charge neutralized (Fig. 15) project to a charge reduction on the extracellular side in reasonable agreement, about 70000 per μm^2. The validity of these experiments is supported by the fact that they were successful only when surface potential was neutralized on one side and enhanced on the other side with low ionic strength solution. The application of additional methods to quantify changes of surface charge will of course be essential to validate these results.

Identification of the cause of variability of reversal rates of the MgATP effect can be expected to provide important further evidence about the MgATP mechanism. Possible explanations for a variability of PS randomization rates in our experiments range from the formation of non-bilayer structures at membrane edges of patches or at glass–membrane interfaces as sites of randomization, to a variable disruption of cytoskeleton-membrane interactions, to the regulation of a hypothetical PS-randomizing protein.

In summary, the present work provides a range of independent predictive experimental tests of a working hypothesis that an aminophospholipid 'flippase' could be involved in the stimulatory effect of MgATP on Na^+–Ca^{2+} exchange current. Remarkably, each of these tests has led to affirmative experimental results, while each of several other tests for an involvement of protein phosphorylation were negative (Collins *et al.* 1992). Until the variability of reversal rates of the MgATP effect is better understood, the possible involvement of two separate mechanisms in the MgATP effect cannot be eliminated. Clearly, further work is needed to test the working hypothesis in a more definitive way, and alternatives (e.g. an involvement of lipid kinases and/or other ATP-binding proteins associated) still need to be tested experimentally.

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