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Ca^{2+} DISPLACEMENT BY POLYMYXIN B FROM SARCOLEMMA ISOLATED BY 'GAS DISSECTION' FROM CULTURED NEONATAL RAT MYOCARDIAL CELLS

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Amphiphilic, cationic Polymyxin B is shown to displace Ca^{2+} from 'gas dissected' cardiac sarcolemma in a dose-dependent, saturable fashion. The Ca^{2+} displacement is only partially reversible, 57% and 63%, in the presence of 1 mM or 10 mM Ca^{2+}, respectively. Total Ca^{2+} displaced by a non-specific cationic probe, lanthanum (La^{3+}), at maximal displacing concentration (1 mM) was 0.172 ± 0.02 nmol/μg membrane protein. At 0.1 mM, Polymyxin B displaced 42% of the total La^{3+}-displaceable Ca^{2+} or 0.072 ± 0.01 nmol/μg protein. 5 mM Polymyxin displaced Ca^{2+} in amounts equal to those displaced by 1 mM La^{3+}. Pretreatment of the membranes with neuraminidase (removal of sialic acid) and protease leads to a decrease in La^{3+}-displaceable Ca^{2+} but to an increase in the fraction displaced by 0.1 mM Polymyxin from 42% to 54%. Phospholipase D (cabbage) treatment significantly increased the La^{3+}-displaceable Ca^{2+} to 0.227 ± 0.02 nmol/μg protein ($P < 0.05$), a gain of 0.055 nmol. All of this phospholipid specific increment in bound Ca^{2+} was displaced by 0.1 mM Polymyxin B. The results suggest that Polymyxin B will be useful as a probe for phospholipid Ca^{2+}-binding sites in natural membranes.

Introduction

Although the mechanism of coupling of excitation to contraction in cardiac muscle remains unclear, the necessity for extracellular Ca^{2+} has been recognized since 1883 [1]. More recent evidence indicates that the quantity of Ca^{2+} bound at the cell surface correlates directly with the contractile strength of the muscle [2–4]. In addition conditions which stimulate increased influx of Ca^{2+} via Na^{+}-Ca^{2+} exchange have been shown to lead to increased binding of Ca^{2+} at the cell surface [5]. These observations and others have stimulated many investigators to examine the molecular nature of the Ca^{2+}-binding sites in cell membranes. In erythrocyte membranes [6,7] and in cardiac sarcolemma isolated from rat [8], 80% of the bound Ca^{2+} has been reported to be associated with membrane protein. In contrast, in rat liver plasma membrane [9] and in cardiac sarcolemma isolated from rabbit [10] approx. 80% of the Ca^{2+} binding was found associated with the phospholipids, specifically negatively charged phospholipids. The importance of the phospholipid moiety of the membrane to the control of Ca^{2+} fluxes in cardiac tissue is further indicated by experiments examining the effects of phospholipases on Ca^{2+} permeability [11]. These authors found that treatment of

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cultured myocardial cells with phospholipase C results in increased membrane permeability to Ca^{2+}, K^+ and La^{3+} and also results in intramembrane particle aggregation. These studies of sarcolemmal Ca^{2+} binding and permeability indicate an important role for phospholipids in the control of Ca^{2+} movements in cardiac tissue. To study the role of the phospholipids further, a probe which binds specifically to the phospholipid moiety would be helpful. Recent studies indicate that Polymyxin B has the characteristics of such a probe.

Polymyxin B is an amphiphilic peptidolipid (M_r 1200) characterized by a heptapeptide ring and a fatty acid attached to the decapeptide through an amide bond [12]. Five of its ten amino acids are positively charged 2,4-diaminobutyric acid groups. It is hypothesized that the nonpolar residues of Polymyxin B penetrate the hydrophobic domain of the membrane with the polar groups situated at the membrane/aqueous interface, where they interact with negatively charged phospholipids. In support of this hypothesis, experiments with lipid monolayers [13], liposomes [14–16] and isolated phospholipids and membranes [17,18] all indicate that Polymyxin B binding requires the presence of anionic phospholipids.

In this paper the influence of Polymyxin B on the Ca^{2+}-binding capacity of sarcolemma isolated from cultured neonatal rat myocardium is examined. It will be shown that Polymyxin B displaces Ca^{2+} from the membrane in a dose-dependent fashion. This displaceable Ca^{2+} is quantified and compared to the quantity of Ca^{2+} displaced by the potent, but non-specific, La^{3+}. Finally the influence of Phospholipase D on the quantity of Ca^{2+} bound by the membrane, and on the quantity of Ca^{2+} displaced by Polymyxin B relative to that displaced by La^{3+} is examined.

Materials and Methods

Preparation of cells and sarcolemmal membranes

Synchronously beating confluent monolayers of neonatal rat myocardial cells were established in culture according to the techniques of Harary and Farley [19] with the modification of Blondel et al. [20] to ensure a high percentage of myoblasts (80–90% after 2–4 days in culture [21]). The cells were grown on scintillant-containing polystyrene disks treated for improved adhesion of the cells (Falcon, Div. of Becton, Oxnard, CA). Sarcolemmal membranes were prepared according to the technique developed by Langer et al. [22]. The scintillation disk with monolayer attached is removed from the incubation medium, excess fluid removed from the surface (cells remain moist) and the disk placed on a platform within a stainless steel chamber. The disk is held in place by 3 pins at its circumference. The top of the chamber is placed in position and clamped in place. Entering through the top of the chamber and extending downward into the chamber is the 'dissection valve' which is 8 mm in diameter. The distal portion of this valve is of conical shape and flat on the bottom such that upon elevation of the platform within the chamber it sits flush on the center of the scintillator disk. This dissection valve is in series with an inlet valve attached to a tank of compressed nitrogen (N_2) gas (maintained at 1800–2200 lb/inch^2).

The dimensions and configuration of the dissection valve outlet are of critical importance for the separation of sarcolemma from other cell constituents. The internal valve cylinder and external wall are beveled at the valve exit (radius of curvature 1.55 mm) so that upon opening the inlet valve N_2 exits in a stream parallel to the surface of the monolayer. If the gas exits downward onto the disk the cells are swept from the surface, and if it exits even marginally upward the cells remain intact. The circumferential valve opening is also critical. It is set at 0.3 mm for optimal results. This opening, with an input pressure between 1800 and 2200 lb/inch^2, gives a gas velocity optimal for membrane dissection.

When the input valve is rapidly opened (< 1 s) the high velocity N_2 stream exits through the dissection valve and travels radially over the surface of the culture. It appears that the upper surface of the cells is sheared open, the cellular material blown out and sarcolemma left in a fenestrated planar and, in some areas, wrinkled or rolled form.

Previous analysis [22] has indicated the purity of the preparation. Activity (nmol P_i/mg per h) increased in the membrane preparation as compared to cell homogenate for the following enzymes: Mg^{2+}-ATPase, 1.7 ± 0.3 to 11.6 ± 2.3;

(Na^+-K^+)-ATPase, 1.0 ± 0.3 to 15.2 ± 3.8; 5′-AMPase, 2.2 ± 1.2 to 24.6 ± 4.0. Activity of succinate dehydrogenase (nmol *p*-iodonitrotetrazolium violet/mg per h) fell from 0.9 ± 0.2 to non-detectable and protein decreased by over 100-fold. Therefore sarcolemmal enzyme markers increased 7–15-fold in specific activity, no residual mitochondrial activity was detectable and total protein decreased to less than 1% of the homogenate value.

To document further the small intracellular residuum after 'gas dissection' intact cells grown on the scintillator disks were labeled with $^{42}K^+$. Extracellular $^{42}K^+$ was washed out leaving only intracellular $^{42}K^+$. The level of cellular $^{42}K^+$ activity was recorded using the scintillator disk-flow technique as described below [23]. The disks then were placed in the chamber and membranes made by gas dissection. The disks, with attached membranes, were reinserted in the flow cell and residual $^{42}K^+$ activity recorded. The residual activity, representing retained intracellular material, in six preparations was less than 1%.

The technique permits instantaneous preparation of membranes from living cells in a form amenable to binding studies with exposure only to inert N_2 in the preparative process. The membranes remain active enzymatically and there is evidence for little cellular contamination.

Technique for measurement of $^{45}Ca^{2+}$ binding

Binding of $^{45}Ca^{2+}$ was detected by the scintillation-disk flow cell technique [11,23]. Briefly, two disks with membranes on one surface of each disk, are assembled into a flow cell such that the disk surfaces with the membranes attached form the walls of a perfusion chamber or flow cell (6 ml volume). The flow cell is then inserted into the well of a modified Beta-Mate II spectrometer (Beckman Instruments) such that the disks are directly apposed to the photomultiplier tubes. The $^{45}Ca^{2+}$ bound to the membrane layer counts with an efficiency of 39.7% derived as follows: Initially the efficiency of the system was calculated for $^{45}Ca^{2+}$-labeling of whole cells bound to the scintillator disks [23]. This efficiency is, however, too low for application to the much thinner gas-dissected membranes since the thicker cells block more of the low energy $^{45}Ca^{2+}$ beta emission than the thinner (approx. 100 Å thick) membrane. The efficiency for counting of $^{45}Ca^{2+}$ bound to the membrane layer was measured by placement of known isotopic activity directly on the disk, covering the dried sample by a layer of clear cellulose tape and counting under perfusion conditions identical to those used for counting $^{45}Ca^{2+}$ uptake by the membranes. The directly applied isotope was found to count with an efficiency of 39.7% which is 1.15-times the counting efficiency previously derived for $^{45}Ca^{2+}$ in whole cells in the system used for the present study.

With the membranes on the disks $^{45}Ca^{2+}$ uptake can be followed on-line continuously over a period of hours with each pair of membranes acting as its own control as described previously for whole cell monolayers [21,23].

All experiments were performed at 22–24°C. The perfusate had the following composition unless otherwise specified (in mM): NaCl, 133; KCl, 3.6; $CaCl_2$, 1.0; $MgCl_2$, 0.3; glucose, 16.0; and *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) buffer, 3.0. The pH was adjusted to 7.2. Solutions containing $^{45}Ca^{2+}$ (ICN, Irvine, CA) had a specific activity of 1 μCi/ml. Phospholipase D (cabbage) was obtained from Boehringer-Mannheim. Polymyxin B, protease (type IV) and $LaCl_3$ were obtained from Sigma. Neuraminidase (*Clostridium perfringens*) was obtained from Worthington Biochemicals. All other chemicals were of reagent grade quality. All solutions were made with deionized water.

The typical experimental protocol involved preparation of the membranes and insertion of the disks with membranes attached into the flow cell as described above. The membranes were then washed for 30 min at 10 ml/min with standard perfusate. They were then exposed to standard perfusate containing $^{45}Ca^{2+}$ for 25–30 min. When this step was performed at the normal perfusion rate of 10 ml/min the exchange time of the flow cell (equilibrium time) was 10 min and the total exposure 30 min. If the perfusion rate was increased to 25 ml/min for the first five minutes of exposure to $^{45}Ca^{2+}$, the exchange time of the flow cell was reduced and total exposure (remainder at 10 ml/min) was 25 min. Both procedures resulted in equivalent $^{45}Ca^{2+}$ binding. The initial exposure to $^{45}Ca^{2+}$ established the control level of Ca^{2+}-

binding to the membranes.

For experiments in which the membranes were treated with enzyme the flow cell was drained and filled with the experimental buffer without enzyme. After a 30-min incubation at 37°C the flow cell was again drained, washed at 10 ml/min for 10 min and reexposed to $^{45}C^{2+}$ in standard perfusate as described above. The quantity of $^{45}Ca^{2+}$-binding established during this period can be compared to the control period to determine the effects, if any, of the experimental buffer on $^{45}Ca^{2+}$-binding. The flow cell was then drained once more and filled with experimental buffer with enzyme. After incubation at 37°C for 30 min and a 10-min wash period at 10 ml/min, the membranes were again exposed to $^{45}Ca^{2+}$ in standard perfusate. Any change in the quantity of $^{45}Ca^{2+}$-binding as compared to the control level could then be attributed to the effect of the enzymatic treatment. After 25–30 min in the standard perfusate, 0.1 mM Polymyxin B in standard perfusate (with $^{45}Ca^{2+}$) was introduced. After 25 min 1 mM La^{3+} in standard perfusate (with $^{45}Ca^{2+}$) was introduced for 20 min. Changes in the $^{45}Ca^{2+}$-binding which occur during these last two interventions are attributable to the presence of Polymyxin B or La^{3+}, respectively. At this point the flow cell was disassembled and the membranes removed from the disks by gently scraping with a rubber policeman in the presence of two 0.05-ml aliquots of 1 M NaOH followed by two 0.05-ml aliquots of 1 M HCl. The membranes were assayed for total protein using the technique of Lowry et al. [24] or for lipid-associated phosphate as described below. For Phospholipase D experiments the experimental buffer contained 0.08 M sodium acetate and 0.02 M $CaCl_2$, pH 5.6.

Membrane samples were assayed for lipid-associated phosphate using a modification of the procedures developed by Stewart [25]. Membrane lipid was first extracted using the techniques of Bligh and Dyer [26] scaled down for the small sample size of 0.2 ml. A 0.5-ml sample of lipid extract (in chloroform) is evaporated to dryness. The samples are then digested in 0.5 ml of perchloric acid (70%) and a drop of nitric acid for 3 h at 180°C. After cooling, 2 ml of 1 M Hepes is added to each sample. One ml of this digest mixture is then removed and 1.5 ml of Stewart Color Reagent [25] added. 30 min later the absorbance of the sample is determined at 640 nm. This procedure gives a reproducible linear response for inorganic phosphate standards and for phosphate extracted and digested from phosphatidylcholine standards in the range of 5–50 ± 3 (S.D.) nmol total phosphate.

Data analysis

$^{45}Ca^{2+}$-binding levels were determined by taking the mean of the counts/min after equilibration of the flow cell (achievement of asymptotic count levels). In all cases the standard error of the mean was less than 150 cpm at count levels in the 10^5 cpm range. All data in the tables and text are expressed as the mean ± S.E. Student's *t*-test for significance difference of two means were performed. *P* values of less than 0.05 were interpreted as significant.

Results

Polymyxin B displaces $^{45}Ca^{2+}$ from membranes in a dose-dependent manner. In Fig. 1a it can be seen that the displacement resulting from exposure to 0.1 mM polymyxin B is less than that resulting from exposure to 1 mM Polymyxin B. However, the additional displacement caused by 1 mM Polymyxin B was half that caused by the initial exposure to 0.1 mM despite a 10-fold increase in concentration. This suggests that Ca^{2+} displacement by Polymyxin B is a saturable phenomenon. The saturating concentration of Polymyxin B is 5 mM, which causes a further displacement of Ca^{2+} from membranes previously exposed to 1 mM Polymyxin B as shown in Fig. 1b. It is interesting to note that 1 mM La^{3+} (maximal displacing concentration of this cation) produces no further displacement after application of 5 mM Polymyxin B. In experiments testing the effects of Polymyxin B on intact cells, 0.1 mM Polymyxin B was found to be the threshold concentration for irreversible changes in Ca^{2+} permeability. For this reason, in the remaining experiments to be discussed, 0.1 mM Polymyxin B is used almost exclusively.

Displacement of $^{45}Ca^{2+}$ by Polymyxin B is partially reversible (57%) by simply removing the Polymyxin B from the perfusate (see Fig. 2a). Since Ca^{2+} has been reported to successfully compete for some classes of Polymyxin B binding sites

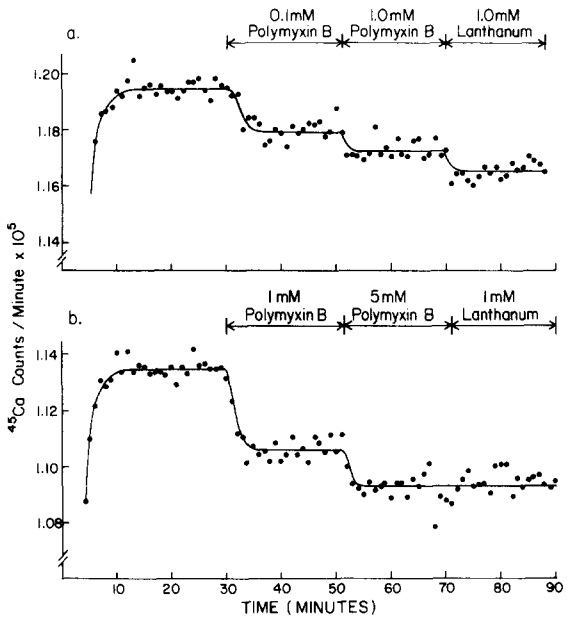

Fig. 1. Dose-dependent displacement of $^{45}Ca^{2+}$ from membranes by Polymyxin B. In (a) following perfusion with control solution (with $^{45}Ca^{2+}$), 0.1 mM Polymyxin B, 1 mM Polymyxin B and 1 mM La^{3+} were introduced successively. Introduction of 0.1 mM Polymyxin B results in a large displacement of $^{45}Ca^{2+}$ while 1 mM Polymyxin leads to a smaller displacement in spite of the 10-fold increase in concentration. Note that 1 mM La^{3+} leads to a further displacement of $^{45}Ca^{2+}$. In (b) following the control solution, 1 mM Polymyxin B, 5 mM Polymyxin B and 1 mM La^{3+} all in standard perfusate were introduced successively. The increase from 1 mM to 5 mM Polymyxin B results in a further displacement of $^{45}Ca^{2+}$ from the membranes. Note that 1 mM La^{3+} no longer leads to increased displacement. Flow rate in both experiments was 10 ml/min. The actual quantities of $^{45}Ca^{2+}$ displaced in the two experiments are not directly comparable due to a different quantity of membrane in the two experiments.

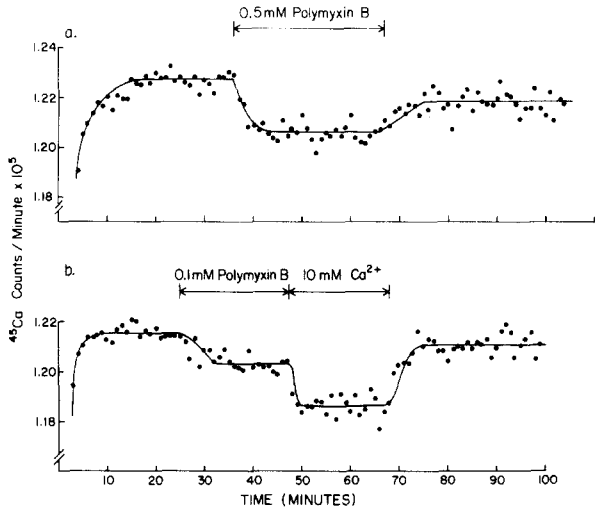

Fig. 2. Partial reversal of the ^{45}Ca displacement by Polymyxin B. In (a) following perfusion with the standard solution and 0.5 mM Polymyxin B the membranes were reexposed to standard solution (Polymyxin B free). Note the partial recovery (57%) of $^{45}Ca^{2+}$ binding. Flow rate in (a) was 10 ml/min throughout. In (b) the membranes were perfused successively with control solution, 0.1 mM Polymyxin B in control solution, 10 mM Ca^{2+} in control solution and finally control solution again. Note the partial recovery (63%) of $^{45}Ca^{2+}$ binding. $^{45}Ca^{2+}$ concentration in all solutions was constant, although the specific activity dropped when the carrier Ca^{2+} concentration was increased from 1 mM to 10 mM. Flow rate was 25 ml/min for the initial 5 min of perfusion and 10 ml/min thereafter.

[9,13,27] the capacity of an increased concentration of Ca^{2+} to restore control $^{45}Ca^{2+}$-binding levels was tested. As seen in Fig. 2b, using 0.1 mM Polymyxin B, membranes perfused with 10 mM Ca^{2+} prior to the return to standard perfusate show a return to 63% of the $^{45}Ca^{2+}$-binding observed during the control period.

In order to quantify the amount of $^{45}Ca^{2+}$ displaced by Polymyxin B and/or La^{3+} a series of experiments similar to those shown in Fig. 3 was performed. Application of 1 mM La^{3+} (Fig. 3a), a concentration sufficient to observe maximal Ca^{2+} displacement and inhibition of fluxes, electrical and contractile activity [28–31], resulted in a displacement of 0.158 ± 0.02 nmol $Ca^{2+}/\mu g$ membrane protein ($n = 5$). If the membranes were first exposed to 0.1 mM Polymyxin B and then 1 mM La^{3+} as in Fig. 3b, the total Ca^{2+} displacement was 0.187 ± 0.03 nmol $Ca^{2+}/\mu g$ membrane protein. These figures are not significantly different ($P > 0.1$), so the data were pooled to yield total displaceable Ca^{2+} of 0.172 ± 0.02 nmol $Ca^{2+}/\mu g$ membrane protein ($n = 5$). Polymyxin B (0.1 mM) displaced 0.072 ± 0.01 nmol $Ca^{2+}/\mu g$ membrane protein ($n = 5$). This represents 42% of the total displaceable Ca^{2+}.

In order to test whether Polymyxin B displaces Ca^{2+} from non-phospholipid sites such as sialic acid or protein, membranes were pretreated with neuraminidase and protease. In order to assess the activity of the protease (Protease, Sigma, Type IV) membranes were scraped from half of each of four disks and assayed for protein. The remaining half

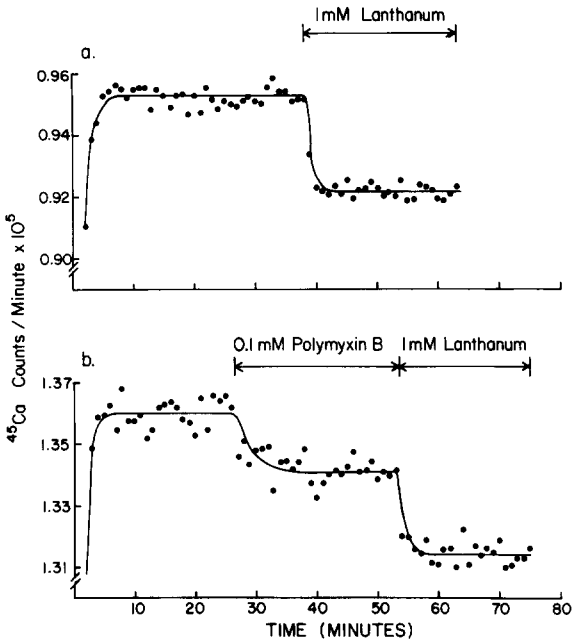

Fig. 3. Displacement of $^{45}Ca^{2+}$ by Polymyxin B and La^{3+}. In (a) membranes are first exposed to standard perfusate with ^{45}Ca, followed by 1 mM La^{3+} in standard perfusate. The decrease in detected $^{45}Ca^{2+}$ represents displacement of $^{45}Ca^{2+}$ from membrane binding sites by La^{3+}. In (b) membranes are exposed to standard perfusate, followed by 0.1 mM Polymyxin B in standard perfusate and then 1 mM La^{3+} in standard perfusate. Both Polymyxin B and lanthanum result in displacement of $^{45}Ca^{2+}$ from the membrane. Although the actual quantities of $^{45}Ca^{2+}$ displaced in these two experiments are not directly comparable (due to different amounts of membrane present and different isotope decay time), the corrected data (per μg membrane protein) indicate that the total displaced $^{45}Ca^{2+}$ is comparable with the two procedures.

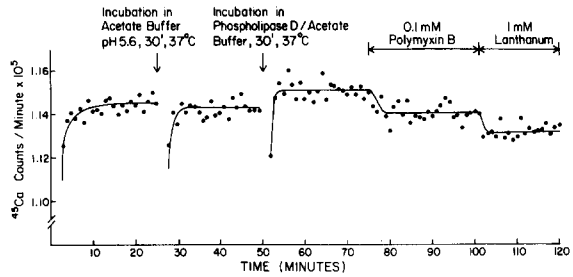

Fig. 4. Influence of phospholipase D on $^{45}Ca^{2+}$ binding and $^{45}Ca^{2+}$ displacement by Polymyxin B and La^{3+}. The membranes were first exposed to standard perfusate with $^{45}Ca^{2+}$ to establish control levels of $^{45}Ca^{2+}$-binding. They were then exposed to the experimental buffer (0.08 M sodium acetate, 0.02 M $CaCl_2$, pH 5.6) for 30 min at 37°C. Upon reexposure to the standard perfusate the $^{45}Ca^{2+}$-binding level was unchanged which indicates that the experimental buffer incubation has no significant effect on $^{45}Ca^{2+}$ binding. The membranes were then treated with 0.1 U/ml Phospholipase D in the experimental buffer for 30 min at 37°C. Upon reintroduction of standard perfusate with $^{45}Ca^{2+}$ the $^{45}Ca^{2+}$-binding level was significantly higher than in the control period. Perfusion with Polymyxin B followed by La^{3+} led to $^{45}Ca^{2+}$ displacement as before; however, it appears as if Polymyxin B displaces more than 50% of the total displaceable $^{45}Ca^{2+}$.

was then treated for 30 min at 37°C with protease (500 U/ml). These halves were then assayed for protein. This procedure assumes equal distribution of membrane across the surface of the disk which is not always true. Despite this difficulty, the protease reduced protein content by 12.4%. Previous experiments using the same concentration of neuraminidase (0.5 U/ml) reduced sialic acid in membrane vesicles by 57% [10].

Membranes were first exposed to $^{45}Ca^{2+}$ in standard perfusate to establish a control $^{45}Ca^{2+}$-binding level. The membranes were then treated successively with neuraminidase and protease for 30 min each at 37°C and relabeled with $^{45}Ca^{2+}$. In each experiment the post-treatment $^{45}Ca^{2+}$-binding level was less than the pretreatment level, an indication of a loss of, presumably, non-phospholipid binding sites. The membranes were then exposed to $^{45}Ca^{2+}$-containing perfusate with 0.1

TABLE I

	Total displaceable Ca^{2+} (nmol/μg protein)	Polymyxin-displaceable Ca^{2+} (nmol/μg protein)	% of total displaced by Polymyxin B
Control	$0.172 \pm 0.02 (n = 10)$	$0.072 \pm 0.01 (n = 5)$	42
Phospholipase D	$0.227 \pm 0.02 (n = 10)$ [a]	$0.141 \pm 0.02 (n = 4)$ [a]	62

[a] Significantly different from control.

mM Polymyxin and finally with 1 mM La^{3+} as in Fig. 3b. The Polymyxin-displaceable fraction (relative to La^{3+} displacement) increased from 42 ± 3% in control membranes to 54 ± 7% in the enzyme-treated membranes.

Treatment of membranes with phospholipase D, as shown in Fig. 4, resulted in an increase in the total amount of $^{45}Ca^{2+}$ bound to the membrane as well as an increase in total La^{3+}-displaceable Ca^{2+} (0.228 ± 0.02 nmol $Ca^{2+}/\mu g$ protein, $n = 10$, $P < 0.05$) and Polymyxin-displaceable Ca^{2+} (0.142 ± 0.02 nmol $Ca^{2+}/\mu g$ protein, $n = 4$, $P < 0.05$). After phospholipase D treatment, Polymyxin displaced 125% (0.069 nmol) of the La^{3+}-displaceable increment (0.055 nmol). Phospholipase D exposure increased the fraction of Ca^{2+} displaced by Polymyxin relative to La^{3+}, from 42% to 62% (see Table I).

Discussion

The purpose of the present study is to examine the specificity of the amphiphilic, positively charged Polymyxin B for displacement of Ca^{2+} bound to myocardial cell membrane phospholipids. In lipid monolayers it has been shown that Polymyxin is only adsorbed to monolayers containing negatively charged lipids and that the adsorption is quantitatively related to the charge density of the lipid layers [13]. Therefore interaction of the drug with anionic phospholipids and competition at these sites for bound Ca^{2+} can be assumed to be present in natural membranes. Its possible selectivity for lipid sites over proteins or polysaccharides in natural membranes could be based on the penetration of its hydrophobic portion into lipid domains and the interaction of the charged groups with the lipid anionic groups at the aqueous interface [12]. The results of the present study support this possibility.

First it is necessary to document that the uniquely-prepared gas dissected membranes used in the present study are characteristic of natural membranes with respect to their constitution. Tibbits et al. [32] has isolated sarcolemma of high purity from adult rat heart. The phospholipid phosphate content was 0.76 nmol/μg membrane protein in these vesicles. In five experiments analysis of the lipid/protein ratio in the gas dissected membranes of this study gave an identical ratio. This ratio is typical of rat sarcolemma and is 1/2 to 2/3 that found for rabbit sarcolemma [10]. Therefore the gas-dissected membrane preparation used in this study appears to be representative of rat heart sarcolemma.

Second it is expected that a phospholipid-specific competition for Ca^{2+} binding should be capable of displacing most of the Ca^{2+} bound to the membrane. This is because over 80% of Ca^{2+} binding can be attributed to phospholipid in sarcolemmal vesicles extracted from rabbit heart [2]. Maximum Ca^{2+} displacement, relative to La^{3+} displacement, is achieved with 5 mM Polymyxin (Fig. 1b) and equaled 0.172 nmol/μg protein in the present study. Bers and Langer [28] using sarcolemmal vesicles extracted from neonatal rat, found bound Ca^{2+} to be approx. 0.150 nmol/μg protein by Scatchard analysis. The present study then indicates that 5 mM Polymyxin is capable of displacing virtually all of Ca^{2+} bound to the sarcolemma in equilibrium with 1.0 mM $[Ca^{2+}]_o$. Under control conditions 0.1 mM Polymyxin B displaces 42% of the total La^{3+}-displaceable Ca^{2+}. At this concentration Polymyxin B is not displacing all of the Ca^{2+} thought to be bound to the phospholipid moiety (80% of the total), i.e. 0.1 mM Polymyxin B is significantly less than the saturating concentration. Therefore it may be expected to preferentially displace from anionic phospholipid at the lower concentration.

Third, removal of other potential Ca^{2+}-binding sites such as protein and sialic acid should increase the proportion of Ca^{2+} displaced by Polymyxin B if it is phospholipid-specific. Neuraminidase and protease treatment of the membranes produced an increase of 28% (42 to 54%) in the fraction of Ca^{2+} displaced by 0.1 mM Polymyxin relative to that displaced by 1 mM La^{3+}.

Fourth, in support of the phospholipid specificity of the drug, are the results with Phospholipase D (Table I). This enzyme cleaves the phospholipid molecule at the phosphodiester linkage and converts neutral (and negatively charged) phospholipids to phosphatidic acid [33]. Therefore it would be expected to increase the proportion of anionic phospholipid and the amount of Ca^{2+} bound to the sarcolemma. Fig. 4 shows that such is the case. In 10 preparations La^{3+}-displaceable Ca^{2+} in-

creased significantly from 0.172 to 0.227 nmol/μg protein (+32%) after phospholipase D treatment (Table I). Polymyxin-displaceable Ca^{2+} increased from 0.072 to 0.141 nmol/μg protein (+96%). Therefore all (and slightly more) of the increase in bound Ca^{2+} induced by phospholipase D (0.055 nmol by La^{3+}-displacement) was displaced by Polymyxin B (0.069 nmol). These results confirm the proposal that Polymyxin has an affinity for anionic phospholipid and displaces Ca^{2+} from these molecules. However, these results, by themselves, do not prove an exclusive affinity of the drug.

As shown in Table I, 0.1 mM Polymyxin displaces 0.072 nmol Ca^{2+}/μg protein. If it is assumed that 2 mol of phospholipid bind 1 mol of Ca^{2+} then 0.144 nmol of anionic phospholipid are participating in Ca^{2+} binding under the conditions of the experiment (1 mM $[Ca^{2+}]_o$, 0.1 mM Polymyxin). Total phospholipid was measured at 0.76 nmol/μg protein in the present study and by Tibbits et al. [32] in sarcolemmal vesicles from adult rat. Thus, under the specific conditions used, about 19% of the phospholipid would be involved in Ca^{2+} binding and, therefore, presumably anionic. This value is high relative to the percent anionic phospholipid found in sarcolemmal vesicles from rat [32] and rabbit [10]. It should be noted however, that binding of Ca^{2+} and Polymyxin B does occur, though at considerably reduced levels, to the phosphate group of 'neutral' phospholipids as well [13,34] and this could account for the increase as derived from Polymyxin Ca^{2+} displacement.

In conclusion, the present results suggest that Polymyxin B is quite specific for displacement of Ca^{2+} from anionic phospholipid in natural membranes. Further studies on other natural membrane systems is indicated to explore further the specificity of this potentially useful probe.

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