Influence of Polymyxin B, a Probe for Anionic Phospholipids, on Calcium Binding and Calcium and Potassium Fluxes of Cultured Cardiac Cells

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SUMMARY. Polymyxin B, an amphiphilic, cationic peptidolipid, which is thought to bind to anionic phospholipids in cell membranes, is shown to interact with the cellular calcium of cultured neonatal rat myocardial cells in a dose-dependent, partially reversible manner. At concentrations of less than or equal to 0.1 mM, it has two distinct effects. First, it results in displacement of 1.4 ± 0.3 mmol Ca/kg dry weight, which is equivalent to 18.1 ± 3.4% of the total exchangeable cellular calcium. Total calcium displaced by polymyxin B and a nonspecific cationic probe, lanthanum, at its maximal displacing concentration (1 mM), was 5.9 ± 1.3 mmol/kg dry weight. Thus, the total displaceable calcium represented 76.3 ± 2.5% of the total exchangeable calcium. Second, polymyxin B (≤0.1 mM) causes a reduction in net uptake of calcium, and slows the efflux of both calcium and potassium. Concentrations of polymyxin B higher than 0.1 mM result in an initial displacement of calcium, followed by an irreversible and sustained period of enhanced net calcium uptake. Efflux of calcium is slowed at the higher polymyxin B concentrations, whereas efflux of potassium is enhanced. Cellular contractile activity and electrical activity are irreversibly altered only by the higher concentrations. The results suggest that polymyxin B is a useful probe for the role of membrane phospholipids in control of ion binding and fluxes. (Circ Res 53: 679-687, 1983)

The influence of membrane composition on membrane function has been the center of considerable interest in recent years (Bearer and Friend, 1980, 1982; Ponce-Hornos et al., 1982; Roelofsen and Van Deenen, 1973). In the field of cardiac cell function, this interest has centered on the role(s) of the glycocalyx and phospholipids in the binding of Ca and in the control of transmembranous ion fluxes (Bers et al., 1981; Frank et al., 1977; Langer and Nudd, 1980; Philipson et al., 1980). Exploration of the roles of the various classes of phospholipids has been limited by the absence of a probe which would specifically bind to, or alter, this membrane component. There is now considerable evidence to suggest that polymyxin B (PXB) is a probe that binds to membrane phospholipids of a net negative charge, or to a negative dipole component (Feingold et al., 1974; Imai et al., 1975; Teuber and Miller, 1977).

A few studies exist which have examined the effects of PXB on intact biological systems. Polymyxin B has been used as an ultrastructural probe in sperm to detect regions of high vs. low concentration of anionic phospholipid. The interaction of PXB with sperm membrane is detectable as deformations of the membrane visible by freeze-fracture electron microscopy only in the areas specialized for fusion (Bearer and Friend, 1980, 1982; Bradley et al., 1980). This study correlates membrane function (fusion potential) with membrane composition. The effects of PXB at the frog neuromuscular junction have also been studied. PXB is a very potent neuromuscular block whose effects can be partially reversed by addition of calcium (Durant and Lambert, 1981). These studies indicate that PXB influences both structural and physiological properties of the intact cell.

It is the goal of this study to determine what effect(s) PXB has on the Ca-binding properties and Ca and K flux parameters of intact cultured neonatal rat myocardial cells. This drug has previously been shown to compete successfully with Ca for Ca-
binding sites in sarcolemma isolated from these cells (Burt and Langer, 1983). It will be shown that exposure of heart cells to PXB at low concentrations (<0.1 mM) results in displacement of Ca from the cell surface, reduces net Ca and K fluxes, and diminishes spontaneous electrical activity and maximum diastolic membrane potential. At higher concentrations, PXB results in irreversible alterations of net Ca and K fluxes and cellular depolarization.

Methods

The methods used for on-line measurement of isotopic exchange have been described in detail (Frank et al., 1977). The technique involves establishing a monolayer of cultured neonatal rat myocardial cells on one surface of each of two disks composed to polystyrene combined with scintillant. The cells are derived by trypsinization of minced cardiac tissue obtained from 0- to 2-day-old rats, according to the techniques of Harary and Farley (1963), with the modifications of Blondel et al. (1971), to improve the percentage of myoblasts vs. fibroblasts in the cultures. After 2-3 days in culture, a contiguous monolayer of cells is formed which is 80-90% myoblastic and exhibits spontaneous, synchronous contractile activity.

For on-line measurement of isotopic exchange, the disks are assembled into a flow cell so that the cells face an internal compartment. This compartment is perfused through four entry ports which direct the perfusate over the surfaces of the cells. The perfusate exits through a single larger port at the top, center of the flow cell. The assembled flow cell is inserted into a modified Beta-Mate II spectrometer wherein the photomultiplier tubes have been arranged such that each flow cell is within 4-5 mm of the scintillant-containing disks. The standard perfusate contained (in mmol/liter): NaCl, 133; KCl, 3.6; CaCl2, 1.0; MgCl2, 0.3; glucose, 16; and either N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer, 3.0 (pH 7.2), or NaH2PO4, 10 mM (pH 7.15 or 7.35), as indicated. For isotopic studies, 45Ca (ICN Corp) was added at 1.0 μCi/ml and 42K (New England Nuclear) was added at 1.0 μCi/ml (concomitant adjustment of KCl in the solution to maintain 3.6 mM).

The standard protocol for isotopic exchange studies was as follows (Burt and Langer, 1982). After the assembled flow cell with attached cells is inserted into the spectrometer, the cells were perfused at 10 ml/min for 30 minutes to preequilibrate the cells to the solution. For studies employing 45Ca, the isotope is then introduced into the perfusate and a continuous on-line monitor of 45Ca associated with the cells and in the perfusate is obtained. At this flow rate, the time required for equilibration of the flow cell with the isotope is approximately 10 minutes. Due to the relatively low energy of the β-emission, the 45Ca associated with the cells can be counted at an efficiency of 35%, whereas that in the perfusate is counted at an efficiency of less than 1%. The last intervention in most experiments was the introduction of 1 mM La+++ into the 45Ca-containing perfusate. La+++ displaces 45Ca from the cell surface and prevents further influx or efflux of 45Ca from the cells (Langer and Frank, 1972). At the completion of a study, the discs are removed from the flow cell and washed with isotope-free La+++ containing perfusate seven times (5-ml aliquots) to remove unbound isotope. The cells then are scraped onto a preweighed piece of Millipore filter paper and dried overnight. After

the cell dry weight has been determined (usually 1-4 mg), the paper is placed in a scintillation vial, cocktail (Aquasol, New England Nuclear) is added, and the quantity of 45Ca remaining with the tissue determined after the sample has digested for 24 hours. The amount of 45Ca displaced by La+++ (and other agents) and the amount remaining in the tissue represent the total exchangeable 45Ca. The procedures for determining whether or not the cells in each portion of an experiment were in steady state with respect to 45Ca exchange, and whether the mean values of 45Ca associated with the cells in each portion are significantly different from one another, have been discussed in detail (Burt and Langer, 1982).

The efflux of 45Ca can also be studied. The cells are treated as above except that after exposure to the isotope, the flow cell is perfused with 45Ca-free perfusate at 25 ml/min. At this rate of perfusion, the exchange time (1/2) of the flow cell is 9-12 seconds. During the washout period, the 45Ca observed in the flow-cell is cell-associated 45Ca, since the perfusate is isotope-free.

Due to the high energy emission of 42K, the uptake of this isotope by the cells cannot be distinguished from isotope in the perfusate by the scintillant disk, flow-cell technique. As a result, only efflux studies were performed. The scintillation disks with cells were assembled into the flow cell and the chamber filled with 42K-containing perfusate. After 1 hour, the flow-cell was inserted into the spectrometer and perfused with isotope-free perfusate at 25 ml/min. A continuous record of the quantity of 42K remaining in the cells over time was obtained. 42K data were corrected for isotope decay and plotted on semilog paper.

The scintillation disk, flow-cell technique allows one to make a continuous record of the 45Ca associated with the cells during uptake and the 45Ca or 42K associated with the cells during washout. In most experiments, the cellular response to control conditions and interventions are obtained with the same culture.

Standard techniques (Burt, 1982) were used for intracellular recording. Cells were visualized through phase contrast optics at 100X. Electrodes (35-60 MΩ, filled with 2.7 M KCl) were lowered into the cell, by means of a Narashige M0103 hydraulic drive micromanipulator. Potentials were amplified and displayed on an oscilloscope and stored on tape. Impalement was considered acceptable when a clean drop to a stable (1 minute or longer) maximum diastolic potential was observed.

Where appropriate, data are expressed as the mean ± SEM.

Results

Polymyxin B has a dose-dependent effect on the binding and net flux of 45Ca by the cultured cells. In HEPES-buffered solutions, as seen in Figure 1a, exposure to PXB at concentrations of up to and including 0.1 mM, results in a dose-dependent displacement of 45Ca from the cells. Exposure to PXB at concentrations exceeding 0.1 mM, results in an initial small displacement followed by a net uptake of 45Ca. In HEPES-buffered solutions, 80% of the total exchangeable 45Ca is La+++ displaceable. These responses suggest a dramatic effect of PXB on baseline parameters of Ca binding (≤0.1 mM) and Ca fluxes (<0.1 mM).
A similar dose dependency is seen when the buffer is changed to 10 mM PO₄⁻⁻. In this solution, the cells exhibit a continuous net uptake of ⁴⁵Ca dependent on buffer pH. The additional slower phase of ⁴⁵Ca uptake with phosphate as compared to HEPES buffer (compare control uptake patterns in Fig. 1, a and b) is attributed to activation of intracellular components of ⁴⁵Ca exchange (Langer and Nudd, 1980; Ponce-Hornos et al., 1982). At pH 7.15, mitochondrial Ca exchange is activated, whereas, at pH 7.35, mitochondrial as well as other intracellular pools, most likely sarcoplasmic reticulum, are activated. As seen in Figure 1b, exposure of the cells to 0.05 mM PXB results in a displacement of ⁴⁵Ca and a slowing of the net uptake pattern. Upon exposure to 0.1 mM PXB, the net uptake of ⁴⁵Ca is increased. Finally, exposure to 0.5 mM PXB leads to a transient slowing of ⁴⁵Ca uptake rate, followed by a dramatic increase in the net uptake. In four experiments similar to that seen in Figure 1b, the cells exhibited an increased rate of ⁴⁵Ca uptake upon exposure to 0.1 mM PXB. In each of these experiments, the cells were exposed to a lower concentration of PXB prior to exposure to the 0.1 mM PXB. When cells are exposed directly to 0.1 mM PXB, as shown in Figure 2b, the PXB results in an
initial displacement of $^{45}$Ca and a sustained reduction in the rate of $^{45}$Ca uptake. Exposure of cells directly to PXB concentrations greater than 0.1 mM always led to an initial displacement, followed by an enhanced $^{45}$Ca uptake rate as compared to control. These observations are consistent with a threshold effect of PXB on membrane function. It is interesting to note that these characteristic effects of PXB on Ca exchange in $\text{PO}_4^{3-}$-buffered solutions occur regardless of solution pH (7.15 or 7.35), in spite of the different bases for Ca compartmentalization and, hence, patterns, under these conditions.

The influence of PXB on $^{45}$Ca flux is, in part, reversible. As seen in Figure 2a, the steady state uptake of $^{45}$Ca by cells in HEPES-buffered saline before and after exposure to 0.05 mM PXB is not dramatically different. Note the presence of a blank in this experiment. PXB has no effect on the blank, indicating that it does not alter $^{45}$Ca counting efficiency. Similarly, the rate of net uptake of $^{45}$Ca by cells in 10 mM $\text{PO}_4^{3-}$-buffered saline recovers after exposure to 0.1 mM PXB (Fig. 2b). In contrast, after exposure to concentrations greater than 0.1 mM PXB, in either HEPES- or $\text{PO}_4^{3-}$-buffered solutions, $^{45}$Ca binding and uptake are irreversibly altered.

The quantity of $^{45}$Ca displaced by PXB, compared...
with that displaced by \( \text{La}^{+++} \), a nonspecific competitor for Ca-binding sites, was determined by means of the protocol shown in Figure 3. Cells were exposed to standard perfusate containing \(^{45}\text{Ca}\) until steady state uptake had been established. The cells were next exposed to \( 0.1 \text{ mM} \) final concentration of the standard, \(^{45}\text{Ca}\)-containing perfusate until a new steady state had occurred. At this time, \( 1 \text{ mM} \text{La}^{+++} \) was introduced and a final steady state attained. The quantity of \(^{45}\text{Ca}\) displaced by PXB and the total \(^{45}\text{Ca}\) displaced by PXB and \( \text{La}^{+++} \) were determined and expressed as a function of the total exchangeable \(^{45}\text{Ca}\) (displaced \(^{45}\text{Ca}\) plus \(^{45}\text{Ca}\) remaining in the cells following displacement). PXB displaced \( 1.4 \pm 0.3 \text{ mmol Ca/kg dry weight} \) or \( 18.1 \pm 3.4\% \) of the total exchangeable \(^{45}\text{Ca}\). \( \text{La}^{+++} \) displaced an additional \( 4.6 \pm 1.1 \text{ mmol Ca/kg} \). Displaceable \(^{45}\text{Ca}\) represented \( 76 \pm 2.5\% \) of the total exchangeable \(^{45}\text{Ca}\).

Discussion

In isolated phospholipid and membrane preparations, polymyxin B has been shown to bind only to membranes containing anionic phospholipids or phosphatidyl ethanolamine, a phospholipid with a negative dipole (Feingold et al., 1974; Imai et al., 1975; Storm et al., 1977; Teuber and Miller, 1977). As such, PXB could prove to be a useful probe of the role of anionic phospholipids in membrane function. The purpose of this paper is to examine this possibility in cultured cardiac cells. That PXB interacts with natural membranes (Bearer and Friend, 1980; Feingold et al., 1974; Teuber and Miller, 1977) and isolated cardiac membranes has recently been demonstrated (Burt and Langer, 1983). With cardiac membranes PXB was shown to displace \( \text{Ca}^{++} \) from the membrane in a dose-dependent manner. Furthermore, convincing evidence was provided that, rather than binding to the protein or saccharide moieties of the membrane, PXB (at \( 0.1 \text{ mM} \)) interacts preferentially with the lipid moiety, specifically the anionic phospholipids. Since the anionic phospholipids are the major site of Ca binding in cardiac tissue (Philipson et al., 1980), and since Ca binding is directly related to the contractility of this tissue (Bers and Langer, 1979; Bers et al., 1981), PXB may be a useful probe for study of the role of this component in the control of transsarcolemmal Ca
and K movements and Ca binding. The results of the present study support this possibility. Polymyxin B binds to and displaces Ca from the intact cell in a dose-dependent manner, just as it did from the membranes isolated from these cells (Burt and Langer, 1983). However, in the intact cells, there is a threshold concentration (0.1 mM) below which displacement of Ca occurs and a slowing of uptake is observed, and above which, after an initial period of Ca displacement, enhanced Ca uptake is seen. At the threshold concentration, PXB displaced 18% of the total displaceable Ca from the cells. This is in
contrast to the isolated membranes of these cells where the same concentration of PXB displaced 42% of the displaceable Ca. This difference is not surprising, in view of the fact that, with the isolated membrane preparation, PXB had access to both the intracellular and extracellular faces of the membrane (Langer et al., 1978). Anionic phospholipids are generally found in higher concentrations in the inner leaflet of the membrane (Bretscher, 1973; Thompson, 1978), which would account for the higher percent displacement by PXB in this system compared to the intact cells where PXB is restricted to

![Graph showing the influence of polymyxin B on 42K efflux.](image-url)

**Figure 5.** Influence of polymyxin B on 42K efflux. In both experiments, the cells were labeled for 1 hour prior to washout in HEPES-buffered saline containing 1 μC/ml of 42K. The washout solution was of the same composition minus the isotope. After a steady efflux rate was observed, 0.1 mM PXB (part a) or 1 mM PXB (part b) was introduced. Note that in part a, the PXB (0.1 mM) caused an immediate slowing of efflux. In contrast, 1 mM PXB, in part b, resulted in an initial period of slowed efflux, followed by a period of increased efflux rate.
FIGURE 6. Electrical activity of cells before (part a), during (part b), and after (part c) exposure to 0.1 mM polymyxin B. Prior to PXB exposure, the cells exhibit rhythmic electrical and contractile activity. At 141 beats/min, the maximum diastolic potential was −72 mV and amplitude was 86 mV. During exposure to PXB, the cells exhibit “sluggish,” synchronous beats, irregularly. The resting potential was −40 mV, and amplitude was 60 mV. A small (4–5 mV) hyperpolarizing after potential is discernible. In part c, standard perfusate has been replaced. The cells (after 10 minutes) again exhibit rhythmic electrical and contractile activity. The beat rate does not recover fully, only to 70 beats/min, and maximum diastolic potential recovers partially to −64 mV.

The influence of PXB on Ca fluxes is of interest. The observed slowing of Ca uptake (≤0.1 mM PXB) is consistent with observations correlating the quantity of Ca bound to the membrane with cell contractility and the magnitude of Ca influx (Bers and Langer, 1979; Bers et al., 1981). Control of Ca binding and flux resides in both the glyocalyx and the lipid bilayer, whereas control of K permeability resides only at the bilayer (Langer et al., 1981). In the present study, the influence of the bilayer on control of Ca and K fluxes is examined with a probe thought to interact rather specifically with the anionic phospholipids (and phosphatidyl ethanolamine) of the membrane. As stated above, PXB (≤0.1 mM) slows Ca uptake. This response could be due to a decreased influx or increased efflux or both. As shown in Figure 4, PXB application (0.1 mM) results in an immediate slowing of Ca efflux. The slowed efflux is in the face of a reduction in net Ca uptake in the case of 0.1 mM PXB. In 0.1 mM PXB, contractile activity of the cells was infrequent and sluggish when present, and contracture is not evident. Although it is impossible to say with certainty how PXB influences influx, it seems likely that at concentrations less than or equal to 0.1 mM, influx is slowed as is efflux. This would account for the diminished contractile activity. At 1.0 mM PXB, influx of Ca is enhanced and efflux diminished. The result might be cellular Ca overload which is consistent with the observed cell deterioration. The lack of a difference of the PO₄³⁻-buffered solution's pH on the Ca uptake pattern is further support for the hypothesis that PXB exerts a direct effect at the cell surface, rather than at intracellular sites.

The influence of PXB on K fluxes also proves to be of interest. Again, PXB has a dose-dependent response. At or below the threshold concentration (≤0.1 mM), PXB slows K efflux, whereas, at concentrations exceeding threshold (>0.1 mM), it enhances K efflux. These effects are consistent with the observed electrical effects. Namely, at the threshold concentration, maximum diastolic potential is more positive, consistent with a decrease in K permeability. Similarly, at 1 mM PXB, the cells depolarize and do not recover their normal electrical activity upon removal of the PXB. This observation is consistent with irreversible membrane damage. It is interesting to note that, in preliminary studies, 0.1 mM PXB does not result in the dramatic alteration of membrane structure shown by Friend and colleagues (Bearer and Friend, 1980, 1982) to be evidence of PXB binding to the membrane. In contrast, 2 mM PXB, which results in an immediate increase in net Ca uptake, does result in the characteristic structural deformations (Joy S. Frank, personal observations).

The concentration-dependent effects of PXB on transsarcolemmal ion fluxes suggest dual modes of action of the drug on the membrane. It is interesting to correlate the physiological responses of the cells to PXB with the mechanism by which PXB is thought to interact with the membrane. As stated in the introduction, PXB is thought to insert its lipophilic tail into the lipid bilayer and associate its positively charged head-group with negatively charged phospholipids (Feingold et al., 1974; Imai et al., 1975; Storm et al., 1977; Teuber and Miller, 1977). Two effects would be predicted to occur as a result of this interaction. First, insertion of the lipophilic tail should result in expansion of the membrane leaflet (El Mashak and Tocanne, 1980; Gall and Trudell, 1980). This expansion could be the basis for the apparent buckling of the membrane observed by freeze-fracture EM at high PXB doses (Bearer and Friend, 1980, 1982; Bradley et al., 1980).
the association of the positively charged headgroup with negatively charged phospholipids should lead to some observable competition for Ca**-binding sites (Burt and Langer, 1983; El Mashak and Tocanne, 1980; Sis and Galla, 1981). These two mechanisms of action could underly the observed cell responses. At low concentrations (<0.1 mM), some membrane expansion must occur; however, the predominant observable response is a reduction of Ca binding and a slowing of transsarcolemmal ion fluxes. At concentrations exceeding the threshold value, the initial response is still the same; however, as exposure time is increased, the integrity of the membrane as a permeability barrier appears to be lost, with the net effect of irreversible increased Ca influx, K efflux, and cell depolarization. These responses could reflect the "point of no return" in expansion of the membrane and, hence, loss of permeability integrity.

In summary, polymyxin B binding (0.1 mM) leads to displacement of Ca from the membrane and a reduction of transsarcolemmal Ca and K fluxes. These effects are probably mediated through interaction of PXB with anionic phospholipids in the external leaflet of the membrane. The results are consistent with an important role for the anionic phospholipids in the control of Ca binding and transsarcolemmal Ca and K fluxes, and with the capacity of PXB to serve as a probe in further studies designed to explore that role.

We express our appreciation to Ginny Childs and Heidi Horwitz for their assistance with the illustrations and typing of this manuscript.

This research was supported by U.S. Public Health Service Grants HL1135-S and HL28559-01, American Heart Association, Greater Los Angeles Affiliate 652-F2, and the Castera Foundation.

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Received March 29, 1983; accepted for publication September 9, 1983.

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INDEX TERMS: Polymyxin B • Cardiac heart cells • Anionic phospholipids • Ca binding • Ca and K fluxes


Burt JM (1982) Electrical and contractile consequences of Na* or Ca** gradient reduction in cultured heart cells. J Mol Cell Cardiol 14: 99-110


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Circ Res. 1983;53:679-687
doi: 10.1161/01.RES.53.5.679

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