Tetraphenylboron Causes Ca\textsuperscript{2+} Release in Isolated Sarcoplasmic Reticulum and in Skinned Muscle Fibers*

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The lipophilic anion tetraphenylboron (TPB-) but not the lipophilic cation tetraphenylarsenium (TPA+) inhibited ATP-dependent Ca\textsuperscript{2+} accumulation by isolated sarcoplasmic reticulum. TPB- did not inhibit ATP hydrolysis but did induce Ca\textsuperscript{2+} release from preloaded vesicles. It did not appear to disrupt lipid bilayers or to act as a Ca\textsuperscript{2+} ionophore since it had no effect on the Ca\textsuperscript{2+} content of phospholipid vesicles. TPB- also induced Ca\textsuperscript{2+} release from sarcoplasmic reticulum in chemically skinned muscle fibers causing tension development. In contrast to other Ca\textsuperscript{2+}-releasing agents such as caffeine, proton ionophores, or quercetin, the rise to peak tension was slow and tension was sustained, suggesting that Ca\textsuperscript{2+} release channels, once opened by TPB-, were held open as long as the compound was present in the membrane. Ca\textsuperscript{2+} uptake was re-established upon removal of TPB- or addition of TPA+. TPB- or TPA- would probably distribute within the membrane, altering surface charges on both sides of the membrane. The fact that only a negatively charged ion brought about opening of Ca\textsuperscript{2+} release channels suggests that specific surface charges control Ca\textsuperscript{2+} release channels in sarcoplasmic reticulum. Although we have not been able to prove that TPB- acts exclusively on physiologically relevant Ca\textsuperscript{2+} release channels, we have shown that TPB- does not release Ca\textsuperscript{2+} from proteoliposomes reconstituted with the Ca\textsuperscript{2+} + Mg\textsuperscript{2+} ATPase. Thus TPB- does not induce Ca\textsuperscript{2+} release through channels formed by the ATPase molecule.

The release and reuptake of Ca\textsuperscript{2+} by the sarcoplasmic reticulum are responsible for the contraction and relaxation, respectively, of skeletal and cardiac muscle (1). These processes have been studied in isolated sarcoplasmic reticulum with differing degrees of success. The molecular mechanism for Ca\textsuperscript{2+} uptake is understood in general terms (2, 3), and the initial rate of Ca\textsuperscript{2+} transport is sufficient to account for muscle relaxation (4). The process of Ca\textsuperscript{2+} release, however, is only beginning to be understood (5).

Ca\textsuperscript{2+} release under physiological conditions occurs at rates 100- to 1000-fold faster than the rate of Ca\textsuperscript{2+} uptake (6). These rates suggest that Ca\textsuperscript{2+} is released through an open channel rather than through an enzymatic process. The key questions in Ca\textsuperscript{2+} release, then, are concerned with the nature of the Ca\textsuperscript{2+} release channel and with how opening of the channel is controlled.

Control of the Ca\textsuperscript{2+} release channel has been difficult to study in partially resolved systems, perhaps because key elements in the control system are lost upon disruption of the integrated sarcotubular network. Skinned muscle fibers (7, 8) represent a partially resolved system. Ca\textsuperscript{2+} release is no longer triggered by electrical stimulation because the sarcolemna is either physically removed or chemically disrupted, although the T-system remains. Sarcoplasmic reticulum vesicles represent a still further resolved system. This membrane system is reduced to vesicular form, and the system and sarcosomal elements are largely purified away. Ca\textsuperscript{2+} release from sarcoplasmic reticulum has generally been more difficult to obtain in vesicles than in skinned fibers. The differences in the response of these two systems may be a reflection of loss of elements of the T-system during purification of sarcoplasmic reticulum or of the fact that continuity of structure is destroyed when sarcoplasmic reticulum is fragmented.

There is some evidence that sarcoplasmic reticulum vesicles are heterogeneous with regard to their ability to release Ca\textsuperscript{2+}. Weber and Herz (9) first noted that caffeine, which releases Ca\textsuperscript{2+} readily from sarcoplasmic reticulum in skinned fibers, will induce some Ca\textsuperscript{2+} release from heavy, but not from light sarcoplasmic reticulum. These observations have recently been confirmed and extended by Ohnishi (10). Campbell and Shamos (11) found that sodium dantrolene, a reagent that affects cellular Ca\textsuperscript{2+} concentrations in vivo (12), would inhibit the passive release of Ca\textsuperscript{2+} from heavy vesicles, which are

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1 The abbreviations used are: T, transverse tubular; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TPB-, tetraphenylboron; TPA+, tetraphenylarsenium; TNP+, trinitrophenol.
believed to originate in the junctional regions of sarcoplasmic reticulum (13). Also, Miyamoto and Racker (14) found a more rapid ruthenium red-sensitive $Ca^{2+}$ release in heavy fractions of sarcoplasmic reticulum than in lighter fractions. $Ca^{2+}$ is not released from partially disrupted sarcoplasmic systems by electrical depolarization, but other stimuli for $Ca^{2+}$ release have been found and theories for control of $Ca^{2+}$ release have been developed. The theory of $Ca^{2+}$-induced $Ca^{2+}$ release (15, 16) developed from studies of skinned fibers. It was proposed that a small amount of $Ca^{2+}$, entering the cell during depolarization of surface and T system membranes, could trigger the release of $Ca^{2+}$ from the sarcoplasmic reticulum or surface charge (28). We now report that tetraphenylboron but not tetraphenylarsonium can stimulate $Ca^{2+}$ potential or surface charge (28). We now report that tetraphenylboron but not tetraphenylarsonium can stimulate $Ca^{2+}$ potential or surface charge (28). We now report that tetraphenylboron but not tetraphenylarsonium can stimulate $Ca^{2+}$ potential or surface charge (28).

The membrane is so permeable to $Na^+$, $K^+$, and $H^+$ that it is offset a transmembrane pH gradient of the magnitude measured across the membrane (22, 23), could release $Ca^{2+}$. These studies suggested that a pH gradient (or a membrane potential) across the sarcoplasmic reticulum membrane may be a controlling element for $Ca^{2+}$ release. These conclusions may, however, have to be re-evaluated in light of a recent finding that the T system in skinched fibers might have the capacity to form a membrane potential (27). If so, then the effect of $H^+$ ionophores or of elevated pH might be on the T system that is present in the skinched fibers. We have asked whether a proton gradient and/or an associated membrane potential could be a controlling influence on $Ca^{2+}$ uptake (24). This evidence signifies the negative logarithm of free $Ca^{2+}$ in molar units). $Ca^{2+}$ and $Ca^{2+}$ release solution was the wash solution (signified by $PM$) according to Avron (29). Conditions for $Ca^{2+}$ uptake were as described in Ref. 35 and were assayed by the Millipore filtration method (36).

**Skinned Fiber Studies**

**Preparation**—Skinned fibers were prepared from rabbit psoas muscle as described previously (6). Assays—In a typical experiment protocol fibers were exposed to a subjunctional level of free $Ca^{2+}$ to permit the sarcoplasmic reticulum to accumulate $Ca^{2+}$. The fibers were then washed twice with wash solution (signified by $w$ in the relevant figures) to remove EGTA and added $Ca^{2+}$. TPA$^+$ or TPB$^-$ was then added to the wash solution and initiation of tension was recorded. The composition of the $Ca^{2+}$-load solution was $170$ mM Na propionate, $2.5$ mM Mg propionate, $2.5$ mM ATP, $10$ mM imidazole, $pH 7.0, 5$ mM EGTA, $pCa 6.0$ (where $pCa$ signifies the negative logarithm of free $Ca^{2+}$ in molar units). $Ca^{2+}$ and EGTA were omitted from the wash solution and Na propionate was increased to $185$ mM to maintain ionic strength. Other solution components were the same. The $Ca^{2+}$ release solution was the wash solution with the addition of TPA$^+$ or TPB$^-$, or $Ca^{2+}$, and EGTA buffers was started. The concentration of free $Ca^{2+}$ in the $Ca^{2+}$/EGTA buffers was started with a computer program for solving the multiple equilibrium equation for $Ca^{2+}$, $Mg^{2+}$, $EGTA$, and ATP. The following apparent dissociation constants (m$^{-1}$) at $pH 7.0$ were used: $Mg^{2+}$/EGTA, 40; $Ca^{2+}$/EGTA, 1.92 $\times$ 10$^{5}$; Mg/ATP $1 \times 10^{5}$; $Ca^{2+}$/ATP $5 \times 10^{5}$ (57). All experiments were conducted at $23 \pm 1^\circ C$, pH $7.0 \pm 0.2$, and ionic strength $200$ nm.

**RESULTS**

*Effect of TPB$^-$ on Fragmented Sarcoplasmic Reticulum*—Fig. 1 shows that the lipophilic anion TPB$^-$ was a very effective inhibitor of $Ca^{2+}$ accumulation by isolated sarcoplasmic reticulum vesicles. The inhibition was apparent both in the absence of phosphate (A) and in the presence of $20$ mM phosphate added as a permeant anion to support high levels of $Ca^{2+}$ accumulation (B). Half-maximal inhibition of $Ca^{2+}$ accumulation was observed at about $7 \mu M TPB^-$ in the absence of phosphate and at about $15 \mu M TPB^-$ in the presence of $20$ mM phosphate.

In contrast to TPB$^-$, the lipophilic cation TPA$^+$ was not an effective inhibitor of $Ca^{2+}$ accumulation by sarcoplasmic reticulum, either in the presence or absence of inorganic phosphate (Fig. 1). Table 1 shows that TPA$^+$ and TPB$^-$ were antagonistic. If TPA$^+$ were added first to isolated vesicles, followed 1 min later by the addition of TPB$^-$ and by ATP to start the uptake reaction, TPA$^+$ prevented the TPB$^-$-induced inhibition of $Ca^{2+}$ uptake.
Since Ca\(^{2+}\) transport is mediated by the Ca\(^{2+}\) + Mg\(^{2+}\) ATPase, we examined the effect of TPB\(^{-}\) on ATPase activity. Fig. 2 shows that TPB\(^{-}\) was not an inhibitor of the Ca\(^{2+}\)-dependent ATPase. Indeed, the ATPase activity was slightly stimulated in the presence of TPB\(^{-}\). Stimulation of ATPase activity has previously been observed when ATP hydrolysis was uncoupled from Ca\(^{2+}\) accumulation by detergent (32) or other permeabilizing agents such as diethyl ether (39) or EGTA (39).

![Fig. 1. Tetraphenylboron but not tetraphenylarsonium inhibits Ca\(^{2+}\) uptake by sarcoplasmic reticulum in the presence and absence of P. Ca\(^{2+}\) uptake by sarcoplasmic reticulum vesicles was assayed as described under "Experimental Procedures." Sarco-
plasmic reticulum (88 pg/ml) was incubated for 2 min at room temperature in a solution containing 20 mM Tris-maleate, pH 6.8, 100 mM NaCl, 5 mM MgCl\(_2\), 5 mM ATP, 0.5 mM EGTA, 0.5 mM CaCl\(_2\) (3.0 \times 10\(^6\) cpn/μmol), and the indicated concentration of TPB\(^{-}\) or TPA\(^{+}\). In B, the assay medium also contained 20 mM P.](http://www.jbc.org/)

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![Fig. 2. Tetraphenylboron inhibits Ca\(^{2+}\) uptake but not ATPase activity. Conditions for Ca\(^{2+}\) uptake (A) and ATPase activity (B) were as described in Fig. 1A, except that, for the assay of ATPase activity, unlabeled CaCl\(_2\) was used and [γ\(^{32}\)P]ATP was added to a specific activity of 6 \times 10\(^7\) cpn/μmol. Ca\(^{2+}\)-ATPase activity was assayed as described under "Experimental Procedures." TPB\(^{-}\) concentration was 26 μM and sarcoplasmic reticulum vesicles were 67 μg/μl.](http://www.jbc.org/)

![Fig. 3. Ca\(^{2+}\) release induced by TPB\(^{-}\). Ca\(^{2+}\) release was initiated by the addition of TPB\(^{-}\) or TPA\(^{+}\), 2 min after initiation of Ca\(^{2+}\) uptake by the sarcoplasmic reticulum vesicles (87 μg/ml). At the indicated times, samples were assayed for Ca\(^{2+}\) content as described under "Experimental Procedures." Conditions for Ca\(^{2+}\) uptake were as described in Fig. 1A. Control activity (100%), 152 nmol of Ca\(^{2+}\)/mg of protein. ○—○ and ■—■ indicate the presence of TPB\(^{-}\) at 26 and 92 μM, respectively; ○—○ indicates the presence of 92 μM TPA\(^{+}\).](http://www.jbc.org/)
release from calcium phosphate-loaded vesicles (Fig. 4). Again, both the rate and the extent of Ca\(^{2+}\) release were dependent on the TPB\(^-\) concentration. Since our preparation contained both light and heavy vesicles, we did not distinguish in this experiment whether release was from the light or the heavy fraction.

Since it was important to establish whether TPB\(^-\) was causing Ca\(^{2+}\) release through an ionophoric activity or through an effect on the integrity of phospholipid bilayers, we tested its effect on the content of Ca\(^{2+}\)-loaded phospholipid (asolectin) vesicles. Fig. 5 shows that TPB\(^-\) at 50 \(\mu\)M or even 100 \(\mu\)M did not cause a significant increase in the Ca\(^{2+}\) permeability of these phospholipid vesicles. By contrast, the Ca\(^{2+}\) ionophore A23187 caused a rapid release of Ca\(^{2+}\) from an aliquot of the same phospholipid vesicles.

The effect of TPB\(^-\) on Ca\(^{2+}\) release from sarcoplasmic reticulum vesicles was only slightly pH dependent over the range from 6.1 to 7.8 (Fig. 6). In this pH interval, Ca\(^{2+}\) uptake in the absence of TPB\(^-\) was optimal at about pH 6.5. When 46 \(\mu\)M TPB\(^-\) was added, Ca\(^{2+}\) was released after 2 min in all cases, although slightly more Ca\(^{2+}\) remained in the vesicles in the pH range 6.1-6.3 than in the range 6.5-7.8.

**Effect of TPB\(^-\) on Skinned Muscle Fibers**—The sarcoplasmic reticulum in skinned muscle fibers is morphologically intact although connections (feet) between the sarcoplasmic reticulum and the transverse tubular system are disrupted (40). The sarcoplasmic reticulum was loaded with Ca\(^{2+}\) in a solution containing subtension concentrations of Ca\(^{2+}\) (pCa 6.6). The loading solution was then replaced with a solution containing the lipophilic ions, and Ca\(^{2+}\) release from the sarcoplasmic reticulum was monitored by measurement of the tension generated by the contractile apparatus in the presence of the released Ca\(^{2+}\).

TPB\(^-\), but not TPA\(^+\), caused tension following a period of Ca\(^{2+}\) loading in skinned fibers (Fig. 7). The characteristics of Ca\(^{2+}\) release differed significantly from Ca\(^{2+}\) release induced by caffeine, quercetin, or H\(^+\) ionophore, all of which gave a rapid transient tension. The release by TPB\(^-\) was relatively slow in developing. Tension was transient at 5 \(\mu\)M TPB\(^-\) but did not go back to base-line. At higher concentrations tension was sustained.

The upper tracing in Fig. 8 shows that TPA\(^+\) can block TPB\(^-\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum. If the bathing solution containing TPA\(^+\) and TPB\(^-\) were
replaced by one containing only TPB- then Ca\textsuperscript{2+} was released and a sustained tension was observed. These observations suggest that TPA\textsuperscript{+} inhibition of TPB\textsuperscript{-}-induced Ca\textsuperscript{2+} release is reversible. The fiber in the lower tracing of Fig. 8 was first exposed to 5 mM EGTA and 2% Brij-58, a nonionic detergent that abolishes the ability of the sarcoplasmic reticulum to accumulate net quantities of Ca\textsuperscript{2+} (8). Following the Ca\textsuperscript{2+}-loading procedure, 50 \mu M TPB\textsuperscript{-} did not elicit a tension in the detergent-treated fiber. Mg\textsuperscript{2+}, on the other hand, which can displace passively bound Ca\textsuperscript{2+} and elicit force generation in detergent-treated fibers (8), did cause a tension that was abolished by EGTA. These data show that TPB\textsuperscript{-} did not stimulate tension either by an indirect effect on the contractile proteins or through release of passively bound Ca\textsuperscript{2+} but rather through stimulation of the sarcoplasmic reticulum to release actively accumulated Ca\textsuperscript{2+}.

Because of its ability to release Ca\textsuperscript{2+} from the sarcoplasmic reticulum, it would be predicted that TPB\textsuperscript{-} would also inhibit net Ca\textsuperscript{2+} uptake. Fig. 9 shows that TPB\textsuperscript{-}, when present in the Ca\textsuperscript{2+}-loading solution, reduced net Ca\textsuperscript{2+} uptake sufficiently to reduce or abolish tensions normally elicited by 15 \mu M TPB\textsuperscript{+}.

Fractionation of the Ca\textsuperscript{2+} Release System—TPB\textsuperscript{-} clearly releases Ca\textsuperscript{2+} from sarcoplasmic reticulum vesicles. It is not obvious whether it does so by a specific interaction with the physiologically relevant Ca\textsuperscript{2+} release channels or by interaction with other channels such as a channel for Ca\textsuperscript{2+} uptake, for anion uptake, or for monovalent cations. In order to obtain some insight into the specificity of TPB\textsuperscript{-} for Ca\textsuperscript{2+} release channels, we have purified the ATPase from sarcoplasmic reticulum membranes and examined the ability of vesicles reconstituted with the partially purified and fully purified ATPase to retain, in the presence of TPB\textsuperscript{-}, Ca\textsuperscript{2+} accumulated by ATP-dependent Ca\textsuperscript{2+} uptake.

Fig. 10 shows that the purified ATPase upon reconstitution into asolectin liposomes had the ability to accumulate Ca\textsuperscript{2+}. The addition of TPB\textsuperscript{-} did not inhibit this Ca\textsuperscript{2+} uptake (Fig. 10A) but rather caused a slight stimulation. On the other hand, when the liposomes were reconstituted with the partially purified ATPase, TPB\textsuperscript{-} inhibited Ca\textsuperscript{2+} uptake. Although the experiments reported in Fig. 10, A and B, were carried out with phosphate in the lumen of the reconstituted vesicles, the presence of phosphate does not affect TPB\textsuperscript{-}-induced Ca\textsuperscript{2+} release (Fig. 1).

These results suggest that the Ca\textsuperscript{2+}-release induced by TPB\textsuperscript{-} involved channels that were still present in the partially purified ATPase (R\textsubscript{2}), but which were absent from the fully purified ATPase molecule. They also show that TPB\textsuperscript{-} was not acting to convert the Ca\textsuperscript{2+} uptake channel in the ATPase into a Ca\textsuperscript{2+} release channel.

**DISCUSSION**

The objective of this study was to find out whether lipophilic ions, which move within biological membranes in response to membrane potential or surface charge would induce Ca\textsuperscript{2+} release from sarcoplasmic reticulum. We found that the anions, TPB\textsuperscript{-} and TNP\textsuperscript{-}, were effective in inducing Ca\textsuperscript{2+} release from fragmented sarcoplasmic reticulum; TPB\textsuperscript{-} also induced a sustained, reversible Ca\textsuperscript{2+} release from the morphologically intact sarcoplasmic reticulum of skinned muscle fibers. These observations would suggest that TPB\textsuperscript{-} moved within the sarcoplasmic reticulum membrane, achieving a distribution of charge that affected Ca\textsuperscript{2+} release channels in such a way as to assure that they would remain open.

It seems clear that TPB\textsuperscript{-} acts directly on the sarcoplasmic reticulum, since Ca\textsuperscript{2+} release from isolated membranes was unambiguous. Therefore, we would predict that it has a different mechanism of action from these other releasing agents...
such as caffeine, quercetin, H+ ionophores, Ca2+, or Cl− which produce transient Ca2+ release from the morphologically intact sarcoplasmic reticulum of skinned fibers or from heavy fractions of sarcoplasmic reticulum which probably contain elements of the T system that are involved in the control of the Ca2+ release channel in the sarcoplasmic reticulum (41).

A key question concerning the mechanism of action of TPB− is whether it brings about Ca2+ release by an effect on the physiologically relevant Ca2+ release channels. Since TPB− did not increase the Ca2+ permeability of phospholipid vesicles, we believe that it did not have Ca2+ ionophoric activity and that it did not disrupt the integrity of phospholipid bilayers. We have been able to rule out the possibility that it acted through the channel for Ca2+ uptake, which is present in the Ca2+ ATPase, but we have not ruled out the possibility that it acts on other channels, for example, those for anion uptake or for H+, Na+, or K+, by making them cation selective. Obviously, the isolation of any or all of these ion channels from the sarcoplasmic reticulum would be an important step in working out not only the mechanism of TPB−-induced Ca2+ release but also the mechanism of the physiologically relevant Ca2+ release.

If TPB− were operating through the relevant Ca2+ channels, it would suggest several characteristics of the channel. The first prediction would be that the Ca2+ release channel present in all or most of the vesicles formed by fragmentation of sarcoplasmic reticulum. The second prediction would be that the opening and closing of the channel would be controlled by membrane surface charge. Chiu et al. (43) have discussed the possible mechanisms of triggering of Ca2+ release by surface potential perturbations. Since TPB− would move within membranes and orient itself with respect to surrounding charges it would greatly increase the negative charge at the membrane surfaces. When surface charge becomes more negative, the Ca2+ release channels would be held in an open position. This interpretation is supported by the fact that the effect of TPB− is antagonized by TPA− which, upon orientation in the membrane, would increase the positive membrane surface charge, tending to close the Ca2+ release channel. An hypothesis of how TPB− might act would be that it moves to the inner surface of the sarcoplasmic reticulum in response to a fixed positive charge that blocks access of internal Ca2+ to the Ca2+ release channel. The orientation of TPB− near this internal site and throughout the Ca2+ release channel would permit the channels to remain open and Ca2+ to flow outward, down its concentration gradient.

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Tetraphenylboron causes Ca2+ release in isolated sarcoplasmic reticulum and in skinned muscle fibers.
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