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Activation of Ca^{2+} Release in Isolated Sarcoplasmic Reticulum

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Summary. The relationship between Ca^{2+} release from sarcoplasmic reticulum, induced by elevated pH, tetraphenylboron (TPB^-) or chemical modification, and the change in the surface charge of the membranes as measured by the fluorescence intensity of anilinonaphthalene sulfonate (ANS) is examined. The stimulated Ca^{2+} release is inhibited by dicyclohexylcarbodiimide and external Ca^{2+}. TPB^-, but not tetraphenylarsonium (TPA^+), causes a decrease in ANS^- fluorescence, with 50% decrease occurring at about 5 μM TPB^-. The decrease in ANS^- fluorescence as well as the inhibition of Ca^{2+} accumulation induced by TPB^- are prevented by TPA^+. A linear relationship between the decrease in membrane surface potential and the extent of the Ca^{2+} released by TPB^- is obtained. Similar levels of [^{3}H]TPB^- bound to sarcoplasmic reticulum membranes were obtained regardless of whether or not the vesicles have taken up Ca^{2+}. The inhibition of Ca^{2+} accumulation and the [^{3}H]TPB^- incorporation into the membranes were correlated. Ca^{2+} release from sarcoplasmic reticulum, by pH elevation, chemical modification or by addition of NaSCN (0.2 to 0.5 M) or the Ca^{2+} ionophore ionomycin, is also accompanied by a decrease in ANS^- fluorescence intensity. However, chemical modification and elevated pH affects the surface potential much less than SCN^- or TPB^- do. These results suggest that the enhancement of Ca^{2+} release by these treatments is not due to a general effect on the membrane surface potential, but rather through the modification of a specific protein. They also suggest that membrane surface charges might play an important role in the control mechanism of Ca^{2+} release.

Key Words sarcoplasmic reticulum · Ca^{2+} release · surface charge · tetraphenylboron · ANS fluorescence

Introduction

The mechanism by which a voltage change across the surface membrane of skeletal muscle fibers initiates muscle contraction is not fully understood. The available evidence (Jobsis & O'Conner, 1966; Winegrad, 1970; Ebashi, 1976; Fabiato & Fabiato, 1977) indicates that the final stage in the coupling of excitation to contraction involves the release of Ca^{2+} ions into the myoplasm from its intracellular storage location, the sarcoplasmic reticulum. The higher [Ca^{2+}] in turn activates the contractile proteins (Ebashi, 1976). However, the step that links the voltage change across the membrane of the T-system to the release of calcium ions, via a Ca^{2+}-permeable ionic channel in the sarcoplasmic reticulum membranes, has not yet been characterized.

Studies with intact and skinned muscle fibers and isolated sarcoplasmic reticulum have yielded three main hypotheses for the mechanism of Ca^{2+} release: (a) Ca^{2+}-induced Ca^{2+} release (Endo, Tanaka & Ogawa, 1970; Ford & Podolsky, 1970; Endo, 1977), which suggests that during the depolarization of the T-system, a small amount of Ca^{2+} crosses the tubular lumen into the gap between the T-system and the sarcoplasmic reticulum, and triggers massive Ca^{2+} release from the sarcoplasmic reticulum. (b) Depolarization-induced Ca^{2+} release, which suggests that the action potential transmitted from the T-system to the sarcoplasmic reticulum "depolarizes" the membrane and permits efflux of Ca^{2+} (Endo & Nakajima, 1973; Kasai & Miyamoto, 1973; Stephenson, 1978). (c) Charge movement-induced Ca^{2+} release. Basically this hypothesis entails a mechanical model (Schneider & Chandler, 1973) in which the movement of a voltage sensor, a charged macromolecule embedded in the T-system membrane, controls the opening of a Ca^{2+} release channel in the sarcoplasmic reticulum via a connecting microfibrillar "rod." Several other mechanisms proposed for the physiological release of Ca^{2+} include: a change in membrane surface charge, and/or a pH gradient, and inositol trisphosphate (Winegrad, 1982; Shoshan, MacLennan & Wood, 1983; Volpe et al., 1985; Vergara, Tsien & Delay, 1985). However, the mechanism responsible for Ca^{2+} release from sarcoplasmic reticulum and the proteins involved in this process have remained the subject of much conjecture.

Recent studies indicate that there is more than one type of Ca^{2+} release system in the sarcoplasmic reticulum which operates via two or more types of divalent cation channels (Miyamoto & Racker,

1982; Palade, Mitchell & Fleischer, 1983; Martonosi, 1984; Taguchi & Kasai, 1984; Shoshan-Barmatz, 1986; Smith, Coronado & Meissner, 1985, 1986; Suarez-Islea et al., 1986). These channels differ in: their divalent cation conductivity and specificity, their distribution across the different structural regions of the sarcoplasmic reticulum membrane and also in their activation mechanism. One of these channels is the "ligand-gated" channel present in the heavy sarcoplasmic reticulum vesicles. This channel has a large conductance, activated by adenine nucleotides and Ca^{2+} and inhibited by Mg^{2+} and ruthenium red (Martonosi, 1984; Smith et al., 1985, 1986). The presence of a second channel with small conductance and which is present in all sarcoplasmic reticulum structural regions was demonstrated recently (Smith et al., 1986; Suarez-Islea et al., 1986). However, the control mechanism of this channel is not know yet.

We have studied the Ca^{2+} release in unfractionated native sarcoplasmic reticulum membranes and developed conditions for activation of Ca^{2+} release (Shoshan et al., 1983; Shoshan-Barmatz, 1986, 1987*a,b*; Shoshan-Barmatz, Ouziel & Chipman, 1987). Our results suggest that a positively charged, internally located, amino group(s) is involved in the control of one or more forms of the Ca^{2+} release channels.

In this communication we report on the involvement of surface charge in the control mechanism of the Ca^{2+} release channel(s) of sarcoplasmic reticulum. These results indicate a correlation between the activation of Ca^{2+} release and the modification of the surface charge of the membrane. The change in the surface charge probably affects a positively charged amino group on a specific protein(s), involved in the gating mechanism.

Materials and Methods

Materials

ATP, Tris, MOPS, TPA^+ and ANS were obtained from Sigma Chemical Co., TPB^- from BDH Chemicals, and acetic anhydride from Merck. Acetic anhydride was dissolved in dry ether and used immediately. The concentration of ether never exceeded 2% in any sample. $[^{45}Ca]Cl_2$ was obtained from the Radiochemical Center, Amersham. Since K^+ precipitates TPB^-, all the chemicals were used as the sodium salts. $[^3H]TPB^-$ was a generous gift from Dr. R. Kaback and was further purified by thin-layer chromatography on silica gel with chloroform/methanol/H_2O (65 : 35 : 2, vol/vol) as a solvent. TPB^- moves with an R_f of about 0.35 and was extracted from the plate with H_2O.

Preparations

Sarcoplasmic reticulum vesicles were prepared from rabbit white skeletal muscle as described by Campbell and MacLennan (1981). This preparation contains a mixture of light, heavy and intermediate vesicles. Protein concentration was determined according to Lowry et al. (1951). Chemical modification of sarcoplasmic reticulum by acetic anhydride or DCCD was carried out as described in the Table legends.

Assays

Active Ca^{2+} uptake by sarcoplasmic reticulum vesicles was determined by Millipore filtration with $[^{45}Ca]Cl_2$. The basic reaction mixture contained 30 mM Tris-maleate, pH 6.8, 100 mM NaCl, 3 mM $MgCl_2$, 3 mM ATP, 0.5 mM $CaCl_2$ (containing $[^{45}Ca]$, about 10^6 cpm/μmol), 0.5 mM EGTA, and 50 mM KP_i. The uptake was started by the addition of sarcoplasmic reticulum, 40 to 80 μg/ml. After 1 or 2 min of incubation at room temperature, 0.18-ml samples were filtered through 0.3 μm nitrocellulose filters, and washed with 5 ml of 0.15 M NaCl. Radioactivity on the filters was measured in a liquid scintillation counter. Ca^{2+} efflux from actively loaded vesicles was carried out as described in Fig. 1. For active loading, the vesicles were incubated with the reaction mixture for Ca^{2+} uptake for 15 min at room temperature. The loaded vesicles were collected by centrifugation at 100,000 $\times$ *g* for 20 min, and resuspended (at 10 mg/ml) in 20 mM Tris-maleate, pH 6.8 and 100 mM NaCl.

ANS^- Fluorescence Measurements

Fluorescence changes upon interaction of ANS^- with the sarcoplasmic reticulum were measured with a Hitachi Perkin-Elmer MPF-2A spectrofluorimeter at 22 to 23°C. Excitation was at 370 nm and emission was measured at 470 nm, using slits giving an 8-nm bandwidth and the ratio mode of the instrument.

$[^3H]TPB^-$ Binding

$[^3H]TPB^-$ binding was measured by filtration with 0.2 μm nitrocellulose filters. The radioactivity remaining on the filter was counted. Parallel samples without protein were filtered to measure nonspecific retention.

Abbreviations

EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; Mops, 3-(N-morpholino)propanesulfonic acid; ANS^-, 1-anilino-8-naphthalene sulfonate; TPB^-, tetraphenylboron; TPA^+, tetraphenyl arsonium; DCCD, N,N'-dicyclohexylcarbodiimide.

Results

Ca^{2+} release from preloaded sarcoplasmic reticulum vesicles could be induced by TPB^- but not

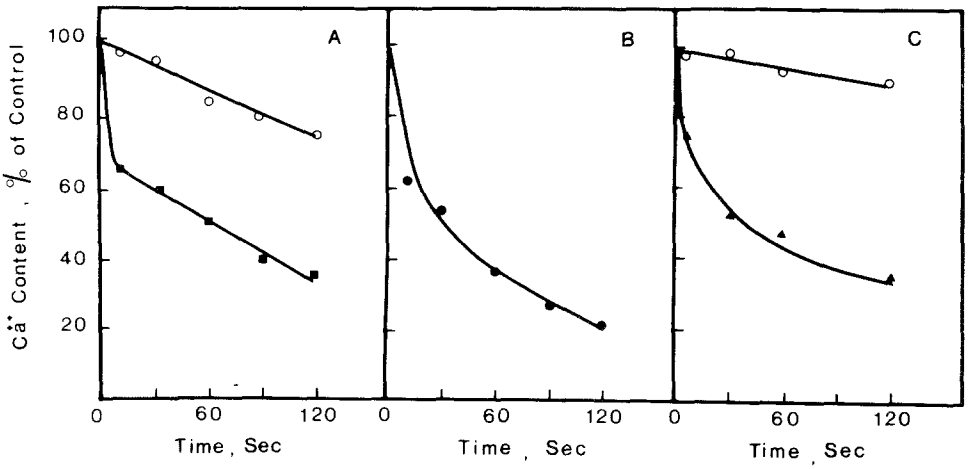

Fig. 1. Ca^{2+} release by TPB^-, elevated pH and by chemical modification of sarcoplasmic reticulum membranes. Ca^{2+} phosphate-loaded vesicles were prepared as described in Materials and Methods and acetic anhydride-modified membranes were prepared as described by Shoshan-Barmatz (1986). For (*A*) and (*C*), Ca^{2+} release was initiated by 50-fold dilution of the loaded vesicles into solution containing Tris-maleate, 20 mM, pH 6.8, NaCl, 100 mM and EGTA, 1 mM. In (*B*) (●), the vesicles were diluted into a solution where Tris-HCl replaced Tris-maleate and the pH was 8.0. At the indicated times, samples were assayed for Ca^{2+} content as described in Materials and Methods. ○ and ■, indicate Ca^{2+} release from loaded vesicles untreated or treated with 0.4 mM acetic anhydride for 2 min, respectively. The presence of 50 μM TPB^- in the release medium is indicated by ▲. Control activity (100%) was = 0.62 μmol/mg protein, for untreated or acetic anhydride-treated vesicles

Table 1. DCCD modification of sarcoplasmic reticulum vesicles prevents Ca^{2+} release induced by TPB^-, elevated pH and by chemical modification with acetic anhydride[a]

Ca^{2+} release stimulated by:	Ca^{2+} released, % of control	
Membrane preparation:	*Untreated*	*DCCD-treated*
TPB^{-1}	52	8
Acetic anhydride modification	61	0
pH elevation	80	22

[a] Ca^{2+} phosphate-loaded vesicles and acetic anhydride-modified membranes were prepared as described in Fig. 1. The loaded vesicles, unmodified or acetic anhydride-modified (1 mg/ml), were incubated with or without 1 mM DCCD in the presence of 20 mM MES, pH 6.1, 0.5 mM EGTA and 100 mM NaCl. After 3 min of incubation at 22°C, the vesicles were diluted 10-fold into solution containing 20 mM MOPS, pH 6.8, 100 mM NaCl, and 1 mM EGTA (control). For activation of Ca^{2+} release, 75 μM TPB^- was added to the above solution or MOPS was replaced by Tris-HCl, pH 8.0. In the case of acetic anhydride modification, Ca^{2+} release was activated by 20-fold dilution of the modified membranes into release medium at pH 6.8. One min after the exposure of the vesicles to the release medium, aliquots were assayed for Ca^{2+} content as described in Fig. 1. The results presented are percent of the Ca^{2+} content of untreated vesicles diluted into the release solution at pH 6.8 (100%).

TPA^+ (Shoshan et al., 1983), elevated pH (Shoshan et al., 1981) or chemical modification of amino groups in sarcoplasmic reticulum (Shoshan-Barmatz, 1986). These findings are summarized in Fig. 1.

Dicyclohexylcarbodiimide (DCCD), an inhibitor of Ca^{2+} release activated by external Ca^{2+} (Yamamoto & Kasai, 1982) or elevated pH (Shoshan et al., 1981) also blocks Ca^{2+} release stimulated by the various treatments (Table 1). The three treatments, in addition to stimulation of Ca^{2+} release, also cancel positive charges on the membrane. It is possible that a membrane surface charge controls a Ca^{2+} release channel(s) in the sarcoplasmic reticulum. This suggestion is examined further here, using ANS^- fluorescence intensity as a reflec-

tor of the electrostatic potential at the membrane surface (Haynes, 1974; Chiu et al., 1980).

Effect of TPB^- on the Fluorescence of ANS^- in the Presence of Sarcoplasmic Reticulum Membrane

The findings (Shoshan et al., 1983) that TPB^- and trinitrophenol (TNP^-) but not TPA^+ induce Ca^{2+} release from sarcoplasmic reticulum indicate that the sign of the charge of the penetrating compound is of more importance than other details of its structure. If the (Ca^{2+} + Mg^{2+})-ATPase functions electrogenically and a membrane potential with positive polarity inside is built up during Ca^{2+} uptake, then TPB^- may act by affecting this membrane potential. However, if a membrane potential controls the Ca^{2+} release channel, one would expect that the membrane-permeating anion, SCN^- would act in the same way as TPB^-. Contrary to this prediction we found that SCN^- at concentrations between 0.1 and 20 mM had no effect on Ca^{2+} uptake nor did it induce Ca^{2+} release (*not shown*). Thus, it is possible that TPB^- is incorporated in the membrane and thereby affects the membrane surface charge or surface potential.

Electrostatic surface potential is a potential difference between the bulk aqueous phase and the membrane surfaces, and is due to the presence of fixed electrical charges on the membrane. Chiu et al. (1980) have presented evidence that ANS^- fluorescence intensity responds to changes in the surface potential of sarcoplasmic reticulum membranes. Figure 2 demonstrates the effect of TPB^- and TPA^+ on the fluorescence of ANS^-, in the presence of sarcoplasmic reticulum, as a probe of the surface potential of this membrane. As shown, TPB^- causes a decrease of ANS^- fluorescence, while TPA^+ has little effect. The half-maximal decrease of ANS^- fluorescence was obtained at about 5 μM TPB^- and the maximal decrease (about 80%) was obtained with about 60 μM TPB^-. These concentrations are similar to those found for the stimulation of Ca^{2+} release in SR vesicles (Shoshan et al., 1983 and Fig. 1). The decrease in ANS^- fluorescence intensity, induced by TPB^-, occurs only in the presence of sarcoplasmic reticulum. TPB^- has no effect on ANS^- fluorescence in H_2O or dioxane, suggesting that the effect is caused by an interaction of TPB^- with the membrane. The decrease of ANS^- fluorescence by TPB^- is very rapid and is completed during the mixing time of the cuvette (1 to 2 sec). Because of experimental limitations these kinetics have not yet been resolved. TPB^- decreases ANS^- fluorescence in sarcoplasmic reticulum vesicles regardless of whether or not they have taken up Ca^{2+} (*not shown*).

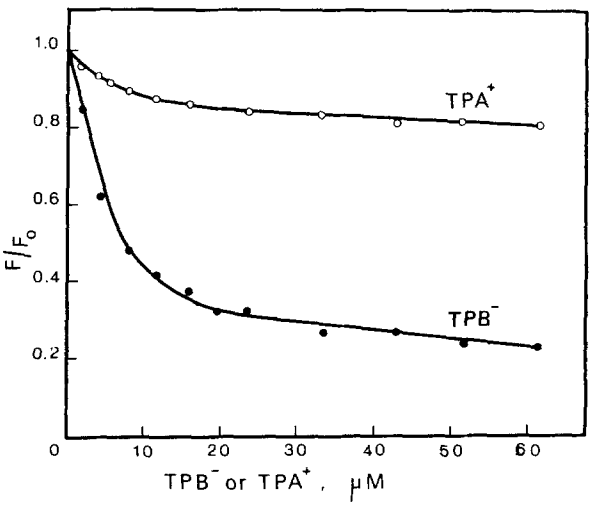

Fig. 2. TPB^- but not TPA^+ causes decreases of ANS^- fluorescence intensity. ANS^- fluorescence was measured as described in Materials and Methods. The reaction mixture for the ANS^- fluorescence assay contained 20 mM MOPS, pH 6.7, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $CaCl_2$, 2×10^{-5} M ANS^- and 33 μg/ml sarcoplasmic reticulum. The titration curve of the ANS^- fluorescence decrease by TPB^- or TPA^+ was obtained by adding 5-μl aliquots of TPA^+ or TPB^- solutions of 10^{-3} M, 2×10^{-3} M, successively, to give the indicated final concentrations. F_o and F indicate the fluorescence intensity before and after the addition of TPA^+ or TPB^-.

Effect of TPA^+ on the TPB^- Induced Decrease of ANS^- Fluorescence and on TPB^--Induced Ca^{2+} Release

If the negatively charged TPB^- when dissolved in the membrane affects the membrane surface charge, then the positively charged TPA^+ should antagonize TPB^- activity due to its solubility in the membrane and the opposite charge. Figure 3 shows the effect of TPA^+ on the decrease of ANS^- fluorescence caused by TPB^-. TPA^+ prevents the decrease of ANS^- fluorescence by TPB^-, when the ratio TPB^-/TPA^+ was about 2 or less. At higher ratios, nevertheless, a decrease of ANS^- fluorescence by TPB^- is still obtained. Moreover, when TPB^- was present at relatively low concentration, TPA^+ causes an increase in ANS^- fluorescence intensity. This result may indicate that there is an increase in the affinity and/or capacity for ANS^- because of an increased number of positive charges in the membrane. The ability of TPA^+ to increase ANS^- fluorescence only in the presence of low concentrations of TPB^- may be due to an increase of

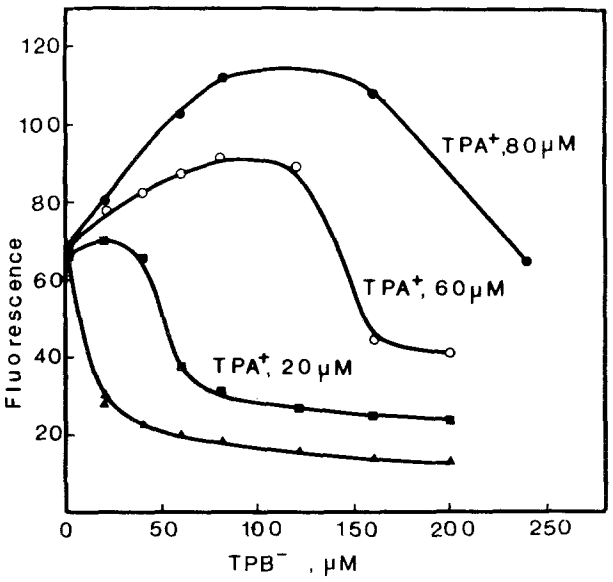

Fig. 3. Effect of TPA^+ on the decrease of ANS^- fluorescence by TPB^-. The experimental conditions were as described in Fig. 2, except that TPA^+, at the indicated concentrations, was included in the assay medium and TPB^- was added to give the final concentration indicated. In the ordinator the fluorescence is given in arbitrary units

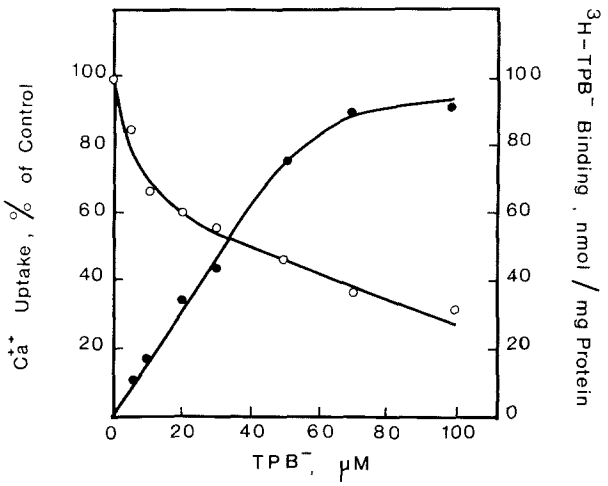

Fig. 4. Correlation between the inhibition of Ca^{2+} accumulation and $[^3H]TPB^-$ incorporation into sarcoplasmic reticulum membranes. Ca^{2+} accumulation (○) and $[^3H]TPB^-$ binding (●) were assayed in similar media (Fig. 1), except that $[^{45}Ca]CaCl_2$ (containing 1.2×10^6 cpm/μmol) or $[^3H]TPB^-$ (containing 1×10^3 cpm/nmol) were used, respectively. Sarcoplasmic reticulum membranes were present at 0.5 mg/ml. $[^3H]TPB^-$ binding was carried out as described in Materials and Methods. Before addition of the membranes two samples of 0.18 ml were filtered through the nitrocellulose filters to measure nonspecific binding. After the addition of the membranes and incubation at 22°C for 2 min, 0.18-ml duplicate samples were also filtered. Ca^{2+} accumulation in the control (100%) was 0.841 μmol/mg protein/2 min

the availability of hydrophobic regions for which TPA^+ has higher affinity. Similar results have been reported in mitochondria, where it has been suggested that TPB^- acts as a membrane carrier for TPA^+ (Grinius et al., 1970). The inhibition of calcium accumulation by TPB^- was almost completely prevented by TPA^+ (Shoshan et al., 1983).

Relationship Between the Inhibition of Ca^{2+} Accumulation and $[^3H]TPB^-$ Binding to Sarcoplasmic Reticulum

The correlation between the inhibition of Ca^{2+} accumulation, as a reflector of Ca^{2+} release, and $[^3H]TPB^-$ incorporation into the sarcoplasmic reticulum membranes is shown in Fig. 4. A similar concentration of TPB^- is required for 50% inhibition of Ca^{2+} accumulation or for the half-maximal binding. Under the conditions used, the maximal binding (B_{max}) of $[^3H]TPB^-$ to the membrane is about 150 nmol/mg protein and half-maximal binding (K_b) is obtained at about 50 μM TPB^- from the double reciprocal plot (*not shown*). The same $[^3H]TPB^-$ binding profile (B_{max} and K_b) was obtained whether the binding was measured in the presence or absence of ATP and $CaCl_2$ or in the presence of the Ca^{2+} ionophore A23187.

Relationship Between the Inhibition of Ca^{2+} Accumulation and the Change in Surface Potential Caused by TPB^-

Surface potential and surface charge are interconnected according to the Gouy-Chapman equation. Therefore, changes of surface potential reflect, in fact, changes of surface charge density produced, for example, by changing pH or adding lipophilic ions such as TPB^-. A change in the surface potential can be measured using a charged amphiphatic fluorescent molecule such as ANS^- (Chiu et al., 1980). Assuming that the binding constant of the dye to the membrane (K_a') depends on the membrane surface potential according to the equation (Nalecz et al., 1980):

$$K_a' = K_a^o \exp \frac{Z\varepsilon\psi}{KT} \quad (1)$$

where K_a^o is the binding constant to uncharged membrane, ψ is surface potential, Z is an integer denoting the number of elementary charge of ANS^- (-1), ε is the electron charge, K is the Boltzmann

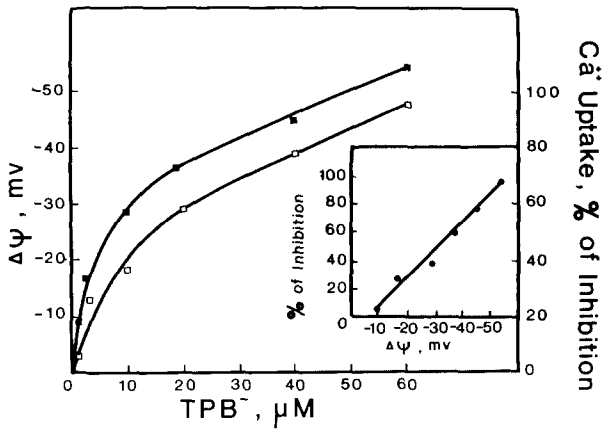

Fig. 5. Relationship between the inhibition of Ca^{2+} accumulation and the change in surface potential caused by TPB^-. Ca^{2+} accumulation (□) was measured in the presence or absence of the indicated concentrations of TPB^-. The change of the surface potential (■) was calculated from the binding constant (K'_a) of ANS^- to the sarcoplasmic reticulum membranes according to the formula (Nalecz et al., 1980):

$$\Delta\psi = \frac{KT}{-Z\varepsilon}\frac{K'_a}{K''_a}$$

where K'_a and K''_a are the binding constants of ANS^-, in the absence or the presence of TPB^-, respectively (*see* text). The binding constants of ANS^- to the membrane were obtained from the double-reciprocal plots of ANS^- fluorescence intensity as a function of ANS^- concentration in the presence or absence of TPB^- (*not shown*)

constant, and T the absolute temperature. Hence, for two different surface potential values (ψ' and ψ'', where $\psi'' - \psi' = \Delta\psi$),

$$\frac{K'_a}{K''_a} = \exp\frac{Z\varepsilon\Delta\psi}{KT} \quad (2)$$

and

$$\Delta\psi = \frac{KT}{Z\varepsilon}\ln\frac{K'_a}{K''_a}. \quad (3)$$

Figure 5 shows an experiment where the effect of TPB^- on the surface potential of the membrane and on Ca^{2+} accumulation is compared. The values of $\Delta\psi$ are calculated from Eq. (3), where the binding constants of ANS^- to the membrane were determined without and with different concentrations of TPB^-. The degree of inhibition of Ca^{2+} accumulation, as a reflector of the balance between the influx of Ca^{2+} by the Ca^{2+} pump and the efflux of Ca^{2+} induced by TPB^-, seems to be quantitatively related to the change in the surface potential caused by TPB^- (Fig. 5 inset).

Table 2. Correlation between the effect of NaCl and NaSCN on ANS^- fluorescence and their ability to stimulate Ca^{2+} release[a]

Salt added	Concentration (mM)	Fluorescence relative units	Ca^{2+} released, % of vesicles Ca^{2+} content
None	—	13	—
NaCl	100	29	9
NaCl	300	36	4
NaCl	500	40	12
NaSCN	100	19	—
NaCl + NaSCN	100 + 100	20	—
NaCl + NaSCN	100 + 200	17	37
NaCl + NaSCN	100 + 400	15	85

[a] ANS^- fluorescence measurements were as in Fig. 2 except that NaCl, $MgCl_2$ and $CaCl_2$ were omitted from the reaction mixture and the indicated salts were added. Ca^{2+} release was assayed as in Fig. 1. Ca^{2+} content of calcium phosphate-loaded vesicles (100%) was 0.360 μmol/mg protein. The numbers in the Table indicate percent of Ca^{2+} released 1 min after 50-fold dilution of the loaded vesicles in 20 mM Tris-maleate, pH 6.8, 1 mM EGTA and the indicated salts.

Effect of NaCl and NaSCN on Surface Charge and Their Activity in Stimulating Ca^{2+} Release

The surface charge of a membrane is a function of ionic strength of the suspending medium. It has been shown (Chiu et al., 1980) that KCl acts to screen the net negative surface charge of sarcoplasmic reticulum membranes, and therefore to increase the ANS^- binding. Table 2 shows that the binding of ANS^- is increased by NaCl up to 0.5 M and decreased by NaSCN. SCN^-, a membrane-permeable anion, appears to act as a screen of positive surface charges, which are not readily accessible to Cl^-. Therefore, it is expected to stimulate Ca^{2+} release from the membranes as we have obtained with TPB^- which also increases the net negative charge of the membrane. Indeed, as shown in Table 2, NaSCN at high concentrations, but not NaCl, stimulates Ca^{2+} release from calcium phosphate-loaded vesicles. Quantitative analysis of the data in Table 2 is completed because the high NaSCN concentrations necessary for the stimulation of Ca^{2+} release, exert two different effects on ANS^- fluorescence. One is an enhancement of ANS^- fluorescence by Na^+, which screens the net negative charges in the membrane, and the second is a decrease in ANS^- fluorescence because of SCN^- incorporation into the membrane (as does TPB^-). Nevertheless, the results support the correlation between the stimulation of Ca^{2+} release and the increase in membrane negative charges. The results are in agreement with Caswell and Brandt's (1981) observation that salts

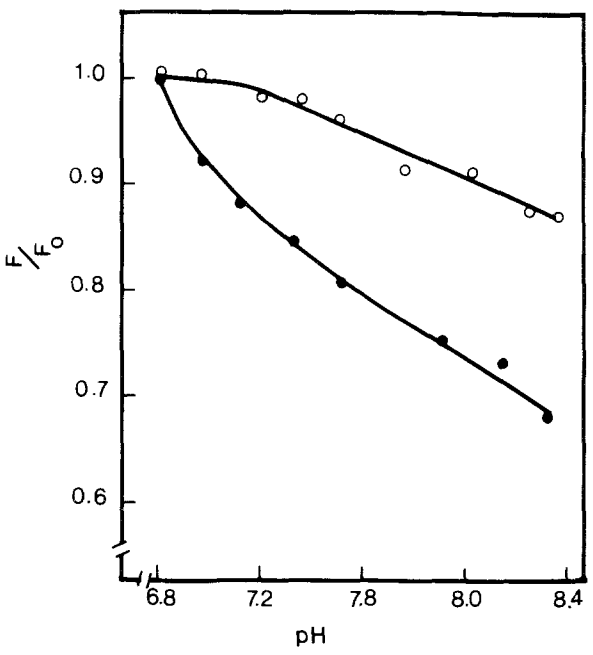

Fig. 6. pH elevation decreases the fluorescence intensity of ANS^- only in the presence of ATP. Conditions for ANS^- fluorescence measurements were as described in Fig. 2 (○—○), or in the presence of 5 mM ATP (●—●). The pH was increased to the indicated pH by the addition of a few microliters of 1 or 2 M Tris-base, 2 min after the sarcoplasmic reticulum (33 μg/ml) was added to the assay medium. The fluorescence intensity was recorded as soon as it stabilized before and after the pH elevation and these intensities were used in the calculation of the ratio F/F_o

which were ineffective in initiating Ca^{2+} release caused an increase in ANS^- fluorescence while salts which were effective caused a fluorescence decrease. Also, SCN^- at high concentration (0.2 M) was also found to trigger Ca^{2+} release in skinned muscle fibers (Caswell, Brandt & Brunschwig, 1980).

Relationship Between Ca^{2+} Release by pH Elevation and the Decrease of ANS^- Fluorescence Intensity

Several investigators reported (Winegrad, 1982; Martonosi, 1984) that the calcium taken up by isolated sarcoplasmic reticulum is rapidly released upon a sudden increase in pH. However, if the Ca^{2+} release that is induced by TPB^- or by an increase in pH share a common mechanism, it is to be expected that the Ca^{2+} release induced by an increase in pH will be accompanied by a decrease in ANS^- fluorescence intensity. In Fig. 6 it is shown that a decrease of ANS^- fluorescence occurs when the medium pH is increased. It is also clear that the decrease of ANS^- fluorescence by a pH shift is enhanced by ATP. The ATP effect could be due to stimulation of Ca^{2+} uptake or a result of ATP interaction with the Ca^{2+} release system (Martonosi, 1984; Shoshan-Barmatz, 1987*a,b*). The extent of the decrease of ANS^- fluorescence induced by the pH shift is smaller than that obtained with TPB^- (*see* Fig. 2). This result suggests that higher pH's may stimulate Ca^{2+} release through a specific effect on one or more membrane proteins rather than through a general effect on the membrane surface potential.

Effect of Mono- and Divalent-Cation Ionophores on ANS^- Fluorescence

It has been shown (Shoshan et al., 1981; Volpe & Stephenson, 1986) that H^+ ionophores such as gramicidin, nigericin, and carbonyl cyanide-*p*-trichloromethoxyphenylhydrazone (CCCP) cause a rapid, transient tension in skinned muscle fibers. However, attempts to detect Ca^{2+} release by H^+ ionophores in isolated sarcoplasmic reticulum have not been successful.

A change of the electrical conductivity of the membrane by the addition of a cation conductor, gramicidin, or a H^+/cation exchanger, nigericin, were not accompanied by changes in ANS^- fluorescence intensity (*not shown*). Thus, ionophores that did not induce Ca^{2+} release from isolated sarcoplasmic reticulum had no effect on ANS^- fluorescence. This is in agreement with the suggestion that these compounds act on the T-system membrane (MacLennan, Shoshan & Wood, 1982; Volpe & Stephenson, 1986).

Ca^{2+} efflux from sarcoplasmic reticulum is also induced by Ca^{2+} ionophores such as A23187, X537A and ionomycin (Pfeiffer, Taylor & Larday, 1978; Beeler, Jona & Martonosi, 1979*a*). Although the mechanism by which the Ca^{2+} ionophores cause Ca^{2+} release is well established (Pfeiffer et al., 1978), we tested the effect of ionomycin on ANS^- fluorescence. In Fig. 7, a decrease in ANS^- fluorescence intensity as a function of ionomycin concentration is given. The half-maximal decrease of ANS fluorescence is obtained at about 5.4 μM or 2.7 μM of ionomycin in the absence or the presence of ATP, respectively. The maximal decrease of ANS^- fluorescence (about 45%) is obtained with about 13 μM ionomycin. The effect of other Ca^{2+} ionophores on ANS^- fluorescence intensity has not been investigated because of their fluorescence properties. These results may indicate an interaction of the ionophores with the membranes as suggested previously (Pick & Racker, 1979; Diamond et al., 1980) and, therefore, they influence the surface potential of the membrane (Haynes, 1972).

Table 3. ANS^- fluorescence in acetic anhydride-modified sarcoplasmic reticulum membranes[a]

Membrane preparations	Fluorescence (relative units)	Ca^{2+} accumulation (μmol/mg protein/min)
Control	82	1.40
Acetic anhydride-modified (0.08 mM)	74	0.48
Acetic anhydride-modified (0.16 mM)	67	0.10

[a] Acetic anhydride-modified sarcoplasmic reticulum membranes were obtained as described in Fig. 1. Conditions for ANS^- fluorescence measurements and Ca^{2+} accumulation were as described in Materials and Methods.

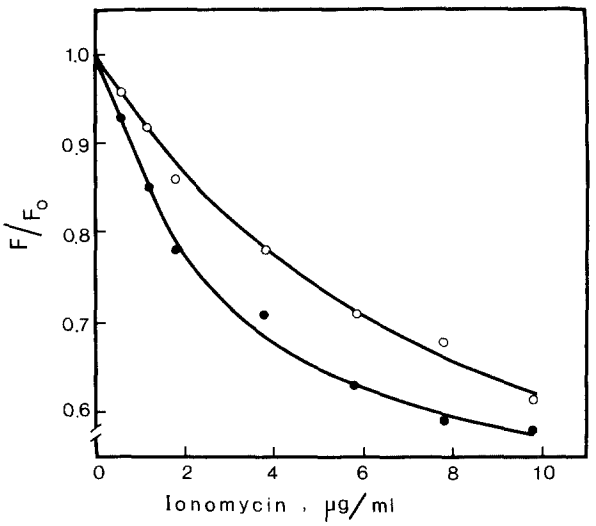

Fig. 7. Ionomycin decreases ANS^- fluorescence in the presence of sarcoplasmic reticulum. Reaction mixtures for ANS^- fluorescence measurements were as described in Fig. 2 (○—○), or in the presence of 5 mM ATP (●—●). Ionomycin to the final concentrations indicated was added to the assay medium 2 min after the addition of sarcoplasmic reticulum (33 μg/ml). The fluorescence intensity was recorded before and after the addition of ionomycin

Chemical Modification of Membrane Surface Charge in Sarcoplasmic Reticulum

The Ca^{2+} release induced by chemical modification of sarcoplasmic reticulum membranes by acetic or maleic anhydride (Shoshan-Barmatz, 1986) could be a direct result of the modification of a specific amino-acid side chain or chains or of the removal of positive charges from the membrane. Table 3 shows the ANS^- fluorescence intensity obtained in the presence of untreated or anhydride-treated membranes. The ANS^- fluorescence intensity in the modified membranes is about 82% of that obtained in the control membranes, although Ca^{2+} accumulation is inhibited by about 93%. In contrast, the ANS^- fluorescence intensity decreased dramatically to 20% when TPB^- inhibited about 90% of the Ca^{2+} accumulation by the membranes (Fig. 2). However, the mechanisms by which TPB^- or chemical modification affected the surface charge in the membrane are different. Whereas TPB^- is incorporated into the membrane, thereby adding negative charges to the membrane, acetic anhydride treatment modifies amino groups and thus results in the removal of positive charge from the membrane. The final result, however, is an increase in the net negative charge of the membrane by both treatments. The results in Table 3 suggest the involvement of specific surface charges and not a general effect of surface charge density in the control of Ca^{2+} release.

Discussion

In spite of large amounts of experimental data concerning the Ca^{2+} release in sarcoplasmic reticulum, the principles or the mechanism by which the Ca^{2+} release occurs remains unclear. Ca^{2+} release through a nucleotide-activated Ca^{2+}-gated Ca^{2+} channel, present selectively in the junctional terminal cisternae, has been demonstrated (Smith et al., 1985, 1986). However, Ca^{2+} release was also activated by several different methods, and in other structural regions of the sarcoplasmic reticulum (Shoshan et al., 1981, 1983; Palade et al., 1983; Martonosi, 1984; Taguchi & Kasai, 1984; Shoshan-Barmatz, 1986, 1987*a*,*b*; Shoshan-Barmatz et al., 1987).

Our findings on a rapid Ca^{2+} release induced by TPB^-, high pH or chemical modification of sarcoplasmic reticulum which is blocked with DCCD (Table 1) or Ca^{2+} (Shoshan et al., 1981) offer a new approach for the study of Ca^{2+} release in sarcoplasmic reticulum of both isolated vesicles and skinned muscle fibers.

If the Ca^{2+} release induced by TPB^-, high pH, and chemical modification occur through one of the Ca^{2+} release channels, it is reasonable to assume

that the opening and closing of this channel is controlled by either a membrane potential difference or by the membrane surface charge. Our results show that SCN^- (up to 50 mM) has no effect on Ca^{2+} accumulation. Thus the participation of a membrane potential is not supported by the freely permeable anion SCN^- which was expected to act similarly to TPB^-. Moreover, if a transmembrane potential with positive polarity inside is built up during Ca^{2+} uptake (Akerman & Wolff, 1979; Dupont, 1979), TPB^- should decrease its extent while high external pH should increase it. However, both TPB^- and high pH induced Ca^{2+} release (Fig. 1). It is possible, therefore, that both TPB^- and high pH act via the membrane surface charge potential.

The role of surface charge in channel gating mechanism has been discussed recently in a review by Blank (1987). ANS^- fluorescence in membranes appears to be very sensitive to electrostatic interaction, and it has been shown that the binding of ANS^- is directly influenced by the electrostatic potential at the membrane surface (Haynes, 1974; Chiu et al., 1980). As we have shown, TPB^- but not TPA^+ or SCN^- (up to 50 mM) causes a decrease in ANS^- fluorescence only in the presence of sarcoplasmic reticulum. This decrease was obtained under all the conditions tested and it was *not* necessary that Ca^{2+} uptake be carried out by the vesicles. Since ANS^- fluorescence responds to surface charge and surface potential we suggest that the decrease in ANS^- fluorescence caused by TPB^- is a reflection of a change in the surface charge of the membrane. This is also supported by our findings (Fig. 3) showing that the cation TPA^+ reverses the decrease of ANS^- fluorescence by TPB^- and prevents the inhibition of Ca^{2+} accumulation by TPB^- (Shoshan et al., 1983).

The binding of $[^3H]TPB^-$ to sarcoplasmic reticulum membranes correlated well with the inhibition of Ca^{2+} accumulation by TPB^- (Fig. 4). The binding of $[^3H]TPB^-$ was not affected by the action of the Ca^{2+} pump and neither was the decrease in ANS^- fluorescence by TPB^- suggesting that both the binding of TPB^- to the membrane and its effect on ANS^- fluorescence are due to its interaction with the membrane and not with any membrane potential which might build up upon calcium uptake (Akerman & Wolff, 1979; Dupont, 1979).

The relationship between the decrease in surface potential (becoming more negative) and the stimulation of Ca^{2+} release by TPB^- (Fig. 5) indicates that TPB^- opens the Ca^{2+} release channel through its effect on surface potential. This suggestion is further supported by the comparison between the respective effect of NaCl and NaSCN on ANS^- fluorescence and stimulation of Ca^{2+} release. While NaCl, which screens the net surface negative charge, did not stimulate Ca^{2+} release, NaSCN, which is able to increase the net negative charge on the membrane as does TPB^-, induces massive calcium release. This is in agreement with Caswell and Brandt's (1981) results.

A surface potential of about -10 mV has been measured for the outside and inside surfaces of the sarcoplasmic reticulum (Chiu et al., 1980). Surface potential is a result of surface charges which arise out of the ionization of surface groups such as the head groups of phospholipids or amino or carboxylic groups of membrane proteins. Baskin (1972) showed that the sarcoplasmic reticulum has an excess of negative charge density which has been estimated by Arrio et al. (1984) as 2900 negative charges per vesicles. This negative charge could be increased not only by lipophilic anions but also by increasing the pH, or by chemical modification of the positively charged amino groups. Our results (Table 3 and Fig. 6) show that both high pH and chemical modification of the sarcoplasmic reticulum membrane with acetic anhydride, cause a rapid release of Ca^{2+}, with a concomitant small decrease in the membrane surface potential. This may suggest that their effect is not through a general modification of the membrane surface potential, but rather to a more specific one, such as deprotonation of a highly reactive, positively charged amino group located in the Ca^{2+} release channel (Shoshan-Barmatz, 1986). Thus, the surface potential per se is of secondary importance.

However, it is possible that the activation of Ca^{2+} release by the different methods is not a direct effect of the removal of positive charge(s) from an amino group(s) but is probably a result of conformational change produced by the modification of a positively charged amino group(s). This suggestion is also supported by our recent finding that trypsin treatment of sarcoplasmic reticulum vesicles induced Ca^{2+} efflux by cleavage of a protein, which is probably part of the Ca^{2+} release channel, rather than the Ca^{2+}-ATPase (Shoshan-Barmatz et al., 1987).

We assume that one or more type of the Ca^{2+} release channels present in SR membranes is controlled by a positively charged amino group(s) located in the gate. Because of this group, the Ca^{2+} release channel could be sensitive to changes in local potential (surface charge), so that the gate is closed when a net positive charge is found at the inner or outer surface of the channel, and is open when the channel has no charge or a net negative charge. This could be obtained by TPB^- (Shoshan et al., 1983; Taguchi & Kasai, 1984; Palade, 1987), which would contribute to the membrane negative

charge or would neutralize positively charged sites within the channel.

The model also predicts that the gate would be opened by decreasing the hydrogen ion concentration in the medium or by chemical modification. Both would affect the nature of the positively charged amino group(s) involved in the gating of the Ca^{2+} release channel, and both are inducers of Ca^{2+} release from sarcoplasmic reticulum. Moreover, recently (Shoshan-Barmatz, 1987*b*) activation of Ca^{2+} release from sarcoplasmic reticulum vesicles was obtained under specific conditions that led to protein phosphorylation by ATP, which means addition of negative charge to a protein. These findings are in agreement with the suggestion (Blank, 1987) that channel gating may be due to any mechanism that leads to changes in the surface charge such as the binding of ligand or intramolecular charge movements. The involvement of surface charge distribution in the mechanism for the opening of Ca^{2+} release channels has also been suggested by Caswell and Brandt (1981).

The relationship between changes in surface charge and the activation of Ca^{2+} release presented in this study, do not resolve the issue regarding the physiological mechanism for Ca^{2+} release at the level of the sarcoplasmic reticulum, although, such mechanisms have been suggested (Caswell & Brandt, 1981; Miyamoto & Racker, 1982). Nevertheless, these results establish the effect of modification of surface charge on Ca^{2+} release channel(s), which may be relevant to Ca^{2+} release mechanisms in skeletal muscle.

Obviously, the isolation of the Ca^{2+} release channel(s) and its reconstitution into liposomes would be an important step in evaluating the control mechanisms of the Ca^{2+} release channel(s) in sarcoplasmic reticulum.

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