J. Membrane Biol. 58, 21–33 (1981)

The Journal of
Membrane Biology

# Ion-Induced Release of Calcium from Isolated Sarcoplasmic Reticulum

Anthony H. Caswell and Neil R. Brandt
Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

**Summary.** Choline Cl addition to either longitudinal reticulum or terminal cisternae of skeletal muscle sarcoplasmic reticulum caused release of $Ca^{2+}$ which had previously accumulated in the presence of ATP. However the extent of release was considerably greater in terminal cisternae. $Ca^{2+}$ accumulation and release by terminal cisternae were also observed using chlorotetracycline as a probe for membrane-associated $Ca^{2+}$. Among a number of salts and ions tested for effectiveness in causing $Ca^{2+}$ release the order was gluconate$^-$ < cacodylate$^-$ < isethionate$^-$ = methane sulfonate$^-$ < methylsulfate$^-$ < $SCN^-$ for anions, and $K^+ = Na^+ = Li^+$ < choline$^+$ = tetramethylammonium$^+$ for cations. Valinomycin enhanced $Ca^{2+}$ accumulation in the presence of ATP both in the absence and presence of the releasing agent, choline Cl. The concentration of sucrose in the medium exerted no discernible effect on the rate or extent of $Ca^{2+}$ release from terminal cisternae. The rate of release was estimated using a stopped-flow mixing apparatus. The rapid phase of release was complete in 6 sec when choline Cl or KSCN were employed to initiate release. $Ca^{2+}$ efflux was slower when release was initiated by EGTA addition. The estimated rate of release was 4–6 nmol/mg protein/sec. The fluorescent probe, 1-anilino-8-naphthalene sulfonate was employed to estimate the influence of ions on the surface potential of terminal cisternae. A broad inverse correlation was observed between the fluorescence of the probe in the presence of various salts and their ability to induce $Ca^{2+}$ release.

A number of hypotheses have been proposed to explain the mode by which the message of excitation is transferred from the transverse tubules to terminal cisternae to initiate calcium release from sarcoplasmic reticulum in muscle. Two hypotheses, though not mutually exclusive, have introduced the concept of charge or potential transfer. One hypothesis proposes that the sarcoplasmic reticulum is depolarized during excitation. The second argues that charge transfer occurs through the junctional feet to the terminal cisternae upon stimulation of muscle. In each case the subsequent event is the opening of a channel in the sarcoplasmic reticulum which permits Ca release into the cytoplasm.

Since the original observation by Costantin and Podolsky (1967) that a drop of KCl could cause contraction of a skinned skeletal muscle fiber, the possibility that the depolarization of sarcoplasmic reticulum could be the means of initiating Ca release has had experimental support. This hypothesis has been the subject of extensive tests. Current experimental evidence in this area may be considered under three headings: (1) the ionic composition of intact muscle; (2) skinned fiber studies on induced depolarization; (3) induced depolarization of isolated sarcoplasmic reticulum.

Somlyo, Shuman and Somlyo (1977) and Somlyo et al. (1979) have demonstrated that the ionic composition of terminal cisternae from Toadfish is similar to that of the cytoplasm on a milligram dry weight basis except that in relaxed muscle the Ca in the sarcoplasmic reticulum is higher. Thus, Na, K and Cl levels do not materially differ between sarcoplasmic reticulum and cytoplasm. This has suggested that a significant membrane potential across the sarcoplasmic reticulum membrane does not exist in relaxed muscle.

The evidence on the experimental possibility of inducing depolarization of sarcoplasmic reticulum by artificially altering the ionic medium of the cytoplasmic space of skinned muscle fibers has been reviewed recently by Endo (1977) and Fabiato and Fabiato (1977). Transfer of fibers from a medium containing

0022-2631/81/0058-0021 $02.60

an apparently impermeable anion to one containing a permeable one causes an efflux of $Ca^{2+}$ from storage sites in the fiber and a concomitant contraction of the muscle. Nevertheless the rate of $Ca^{2+}$ efflux was about 100 times slower than that which is considered to occur in intact muscle during physiological contraction (Stephenson, 1978). Endo and Nakajima (1973) have argued that the ion modification has altered the potential of the sarcoplasmic reticulum rather than the transverse tubule since the same effect is observed when only a portion of the muscle is skinned and hence the transverse tubules are open to the extracellular space. Costantin and Podolsky (1967) showed that neither K methanesulfate nor K propionate induced contraction while KCl and KBr did. Endo and Nakajima (1973) have found that Li or Tris methanesulfonate induced contraction. They concluded that a depolarization of an internal membrane is responsible for the observed effects.

The evidence on $Ca^{2+}$ release from isolated sarcoplasmic reticulum induced by depolarization has proved equivocal. The strongest arguments for its existence have been those of Kasai and Miyamoto (1973, 1976*a, b*). These authors diluted sarcoplasmic reticulum vesicles from a medium containing K methanesulfonate to one containing KCl and observed a considerable loss of $Ca^{2+}$ from the sarcoplasmic reticulum. They did not observe this release when they diluted into choline Cl and were unable to induce release when they modified the cation rather than the anion. Alterations of the osmolarity of the medium altered the extent of $Ca^{2+}$ release (Kasai Miyamoto (1976*a*). Meissner and McKinley (1976) and Beeler, Russell and Martonosi (1979) have argued that the release observed was caused by transfer from an isotonic to a hypotonic environment since both $K^+$ and $Cl^-$ can cross the membrane. These authors were unable to observe a Ca release consistent with alterations of ionic media that would be expected to produce depolarization.

A second hypothesis which correlates electrical potential with Ca release has been described by Schneider and Chandler (1973). They propose that depolarization of muscle leads to movement of charge in the region of the transverse tubule-sarcoplasmic reticulum junction. This charge displacement is responsible for initiating $Ca^{2+}$ release from the sarcoplasmic reticulum. The charge movement in the region of the sarcoplasmic reticulum or in the connections of the triad junction may then serve as a trigger which is independent of the transmembrane potential of the sarcoplasmic reticulum.

In general a good agreement has been observed between the movement of charge and the contraction of muscle. Thus charge occurs with a half-maximum value on depolarization of the muscle to $-49$ mV (Schneider & Chandler, 1973), is diminished by prior depolarization (Adrian & Almers, 1976) and recovers with the same time course as that for mechanical activation (Adrian, Chandler & Rakowski, 1976). It is not clear whether stretching the fibers abolishes the charge movement although $Ca^{2+}$ release still occurs (Adrian, Caputo & Huang, 1978; Hui & Gilly, 1979).

In this paper we have sought to examine and to define conditions which induce $Ca^{2+}$ release from sarcoplasmic reticulum. We observe $Ca^{2+}$ release associated with alterations of ionic environment. This release differs by several criteria from that observed by Kasai and Miyamoto (1976*a*).

## Materials and Methods

*Preparation of Sarcoplasmic Reticulum Organelles*

Microsomes were prepared by first stunning a $3\frac{1}{2}$ lb rabbit with a blow on the neck followed by bleeding. A single back (sacrospinalis) muscle was excised and immediately homogenized in 225 ml of 250 mM sucrose, 2 mM histidine, pH 7.4, at 0 °C. Homogenization was carried out in a Waring blender for three 30-sec pulses with 30-sec intervening periods. The homogenate was centrifuged for 20 min at $10{,}000 \times g$. The supernatant was passed through cheesecloth and centrifuged for 30 min at $100{,}000 \times g$. The precipitate was resuspended using a glass-Teflon homogenizer in 60 ml of homogenizing medium and centrifuged for 30 min at $100{,}000 \times g$. The pellet was resuspended in 9 ml of homogenizing medium.

The microscomes were fractionated on a continuous sucrose density gradient from 12.5% w/w to 44% w/w which contained 0.01% $NaN_3$. Two tubes of a Sorvall TV850 vertical rotor were employed and 4 ml of microsomes were placed on each gradient. The gradients were centrifuged for a total running time of 1 hr at $160{,}000 \times g$ maximum speed. Two bands were obtained from the fractionation as described previously (Caswell, Lau & Brunschwig, 1976). Electron microscopy reveals that the light fraction contains empty vesicles whose source is predominantly the longitudinal reticulum while the presence of electron-dense matter within vesicles from the heavy fraction as well as the presence of T tubules in the conformation of intact triad junctions has led to the designation "terminal cisternae/triads". These were removed separately from the gradient and employed directly in assays or immediately frozen in the freezer compartment of a refrigerator. The total time for preparation of the organelles was between 3 and 4 hr.

In experiments involving preparation of "heavy terminal cisternae" the method of Lau, Caswell and Brunschwig (1977) was employed to separate transverse tubules from terminal cisternae. Electron microscopy reveals that this preparation is highly enriched in vesicles containing electron-dense material. Terminal cisternae/triads were sedimented by centrifugation, resuspended in 5 ml of 250 mM sucrose, 2 mM histidine, pH 7.4, and passed through a French press at 5,000 p.s.i. The eluate was centrifuged for 2 hr in a TV850 vertical rotor at $160{,}000 \times g$. The band at approximately 40% w/w sucrose was withdrawn and employed directly.

*Calcium Accumulation and Release*

Millipore filtration with 0.22 μ filters was employed. The incubation medium was 150 mM sucrose, 15 mM histidine sulfate, 5 mM

NaCl, 3 mM $MgSO_4$, 100 μM $CaCl_2$, 161 μM Tris EGTA, 100 mM K cacodylate, 0.25 mg/ml creatine phosphokinase (Sigma Co.), 1 μC $^{45}CaCl_2$ (ICN Chemical Co.), pH 6.8. Vesicles (150 μg protein/ml) were incubated at 37 °C in the medium for 12 min before addition of ATP and creatine phosphate. Aliquots (0.5 ml) were withdrawn and filtered by vacuum. The filters were placed in a scintillation cocktail containing Triton X100 and counted 24 hr later. The addition of salts in this and subsequent experiments was carried out from 2 M stock solutions to give final concentrations of 0.1 M unless otherwise stated.

In experiments using the fluorescent chelate probe, chlorotetracycline, the conditions of fluorescence assay were similar to those described previously (Caswell & Warren, 1972). The incubation medium was that described above except that K cacodylate was not routinely added and 10 μM chlorotetracycline was present. A vibrating stirrer was present in the cuvette in a Perkin-Elmer MPF3L spectrofluorometer. Excitation wavelength was 390 nm and emission wavelength 530 nm. The fluorometer was thermostatted at 37 °C. The vesicles and medium were preincubated for 6.5 min before addition of ATP. A fluorescence artifact associated with binding of ATP to chlorotetracycline was subtracted from the fluorescence trace at the time of ATP and creatine phosphate addition in order to indicate $Ca^{2+}$ accumulation and release.

In stopped-flow experiments an Aminco-Morrow Stopped-flow apparatus (cat. no. N-8409) was employed thermostatted at 37 °C. The mixing chamber gives a 1:1 mix from the two syringes. Both syringes contained 150 mM sucrose, 15 mM histidine sulfate, 5 mM NaCl, 3 mM $MgSO_4$, 50 μM $CaCl_2$, 72 μM Tris EGTA, 0.1 M K gluconate, 10 μM chlorotetracycline, 2 mM Tris ATP, 2.5 mM creatine phosphate, 0.25 mg/ml creatine phosphokinase; pH was 6.8 and temperature 37 °C. Syringe 1 contained in addition to the above medium 0.2 M of the salt which was employed to initiate release except that the EGTA concentration was 5 mM. Syringe 2 contained 175 μg protein/ml of terminal cisternae/triads which were added to initiate Ca accumulation. Mixing was initiated at various times after addition of vesicles to the medium of syringe 2. In the early times after ATP addition the Ca release was superimposed on the fluorescence rise caused by active Ca accumulation. The traces which are shown were therefore obtained when the fluorescence had reached a steady level. Traces were recorded both on an oscillograph and on a paper chart recorder. The latter was employed for following the long-term fluorescence signal.

*Ca-Stimulated Phosphatase*

Two methods were employed. 3-O-methylfluorescein phosphatase was assayed according to the method of Hill, Summer and Waters (1968) as follows: the medium contained 150 mM sucrose, 15 mM histidine sulfate, 5 mM NaCl, 3 mM $MgSO_4$, 72 μM Tris EGTA, 60 mM K gluconate, 20 μM 3-O-methylfluorescein phosphate, 28 μg protein/ml of terminal cisternae/triads. The reaction was followed in a Perkin Elmer fluorometer at excitation wavelength of 470 nm and emission of 510 nm. The phosphatase was calculated from the rate of appearance of fluorescence in comparison with standard aliquots of 3-O-methylfluorescein. Ca-stimulated phosphatase was initiated by addition of 50 μM $CaCl_2$. The basal phosphatase was calculated by subtracting the slope in the absence of $CaCl_2$ from that in its presence.

Ca-stimulated ATPase was assayed by monitoring continuously the output from a pH-meter to detect the acidification of the medium induced by ATP hydrolysis. The medium contained 100 mM sucrose, 5 mM histidine sulfate, 5 mM NaCl, 3 mM $MgSO_4$, 50 μM $CaCl_2$, 72 μM EGTA, 0.1 M K gluconate, 165 μg protein/ml of terminal cisternae/triads; pH was 6.8 and temperature 37 °C. The reaction was initiated by addition of 3.3 mM ATP. The control activity was determined in the presence of 1.6 mM Tris EGTA and subtracted from that in 72 μM EGTA.

*1-Anilinonaphthalene-8-sulfonate (ANS) Fluorescence*

The medium contained 150 mM sucrose, 15 mM histidine sulfate, 5 mM NaCl, 3 mM $MgSO_4$, 100 μM $CaCl_2$, 161 μM Tris EGTA, 100 mM K gluconate, 0.25 mg/ml creatine phosphokinase, 15 μM ANS and 0.165 mg protein/ml terminal cisternae/triads; pH was 6.8 and temperature 37 °C. The fluorescence conditions were the same as with chlorotetracycline except that the excitation wavelength was 370 nm and the emission wavelength 450 nm. The salts were injected into the chamber with a syringe so that a continuous record could be obtained during salt addition.

## Results

### *Calcium Release Induced by Choline Cl*

Figure 1 shows active $Ca^{2+}$ uptake and release by microsomes from skeletal muscle which have been subfractionated into longitudinal reticulum, terminal cisternae/triads and heavy terminal cisternae. Phosphate potential was maintained with an ATP regenerating system containing creatine phosphokinase and creatine phosphate. The ionic strength of the incubation medium has been maintained with K cacodylate. After 3 min of incubation in ATP, 0.1 M choline Cl has been added to a portion of the incubation mixture (dashed line) while the rest has not been treated (solid line). The upper trace is of longitudinal reticulum and shows that choline Cl induces a slight but significant release of $Ca^{2+}$ which is greater than that of the control. However, a spontaneous release occurs after a few minutes so that both treated and control vesicles release the major portion of the accumulated $Ca^{2+}$. This spontaneous release has been observed before (Huxtable & Bressler, 1974; Sorenson & de Meis, 1977). The middle curve shows a similar incubation of terminal cisternae/triads. The $Ca^{2+}$ uptake is maintained for a longer period after ATP addition than in the case of the longitudinal reticulum. Upon addition of choline Cl, a large release of $Ca^{2+}$ occurs such that approximately 50% of the $Ca^{2+}$ which had accumulated after ATP addition is now released. A large part of this release appears to occur within 30 sec of addition of salt. Since the terminal cisternae/triad band contains transverse tubules as well as terminal cisternae we tested the effect of removal of the transverse tubules on this release process. This was achieved by passing the vesicle preparation through a French press and centrifuging the eluate on a continuous sucrose density gradient. The appearance of a free T tubule band above the terminal cisternae indicates the separation of the organelles. The French press treatment has been demonstrated previously to cause complete disruption of the junction (Lau et al., 1977). The $Ca^{2+}$ accumulation induced by ATP and release by choline Cl in heavy terminal

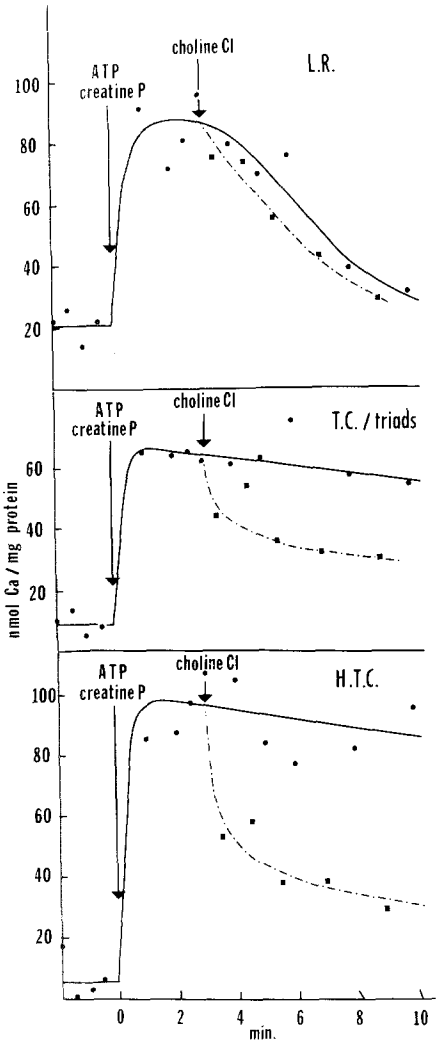


**Fig. 1.** $Ca^{2+}$ release from subfractions of sarcoplasmic reticulum initiated by addition of choline Cl. The incubation medium was that described in Materials and Methods. After 12 min incubation of the vesicles in the medium, $Ca^{2+}$ accumulation was initiated by addition of 2.5 mM Tris ATP, 3.6 mM creatine phosphate. Three min subsequently a portion of the suspension was withdrawn and 0.1 M choline Cl added. Each point represents a single fraction 0.5 ml of which was filtered through a 0.22 μm Millipore filter. ●—● represents vesicles which have not been treated with choline Cl while ■ ·—·—· ■ represents choline Cl-treated vesicles. The three traces were carried out respectively on longitudinal reticulum, terminal cisternae/triads and heavy terminal cisternae prepared as described in Materials and Methods

cisternae are demonstrated in the bottom trace of Fig. 1. $Ca^{2+}$ accumulation into the terminal cisternae occurs after the French press treatment upon addition of ATP. Choline Cl initiates a large release of $Ca^{2+}$ from the vesicles so that less than 50% of the accumulated $Ca^{2+}$ remains in the vesicles 3 min after the salt has been added. Thus neither transverse tubules nor intact triads are required for $Ca^{2+}$ release induced by choline Cl.

In the experiments to be described subsequently $Ca^{2+}$ uptake and release have been monitored using the fluorescent chelate probe, chlorotetracycline. This probe fluoresces from $Ca^{2+}$ or $Mg^{2+}$ which is attached by electrostatic attraction to the membrane surface. Previous experiments have shown that accumulation of $Ca^{2+}$ is associated with enhanced $Ca^{2+}$ binding to the inside of sarcoplasmic reticulum and enhanced fluorescence of chlorotetracycline (Caswell & Warren, 1972). The fluorescence is linear with $Ca^{2+}$ uptake into sarcoplasmic reticulum. This method of monitoring $Ca^{2+}$ movements was employed because the probe is fluorescing from internal sites and is therefore little affected by large alterations of the external environment. Also fluorescence is not materially influenced by light-scattering changes which may occur in the vesicles on addition of salts. The technique is sensitive and, although it does not respond instantaneously to $Ca^{2+}$ accumulation, it responds rapidly to $Ca^{2+}$ release (Caswell & Pressman, 1972; Caswell, 1979).

Figure 2 shows the $Ca^{2+}$ uptake and release in terminal cisternae/triads monitored with the fluorescent chelate probe. In Fig. 2*A* no $K^+$ has been added to the medium. ATP and creatine phosphate induce an enhanced fluorescence associated with $Ca^{2+}$ uptake. Subsequent administration of 0.1 M choline Cl causes a rapid decline in fluorescence associated with $Ca^{2+}$ release. In Fig. 2*B* the medium contains K cacodylate and is therefore comparable to the experiments of Fig. 1. Choline Cl again causes a decline in fluorescence, albeit slower than that of Fig. 2*A*. Thus the direct observation of $Ca^{2+}$ release by radioisotope assay matches the similar observation using the fluorescent chelate probe. The fluorescence after choline Cl administration is still above the basal level indicating that only a portion of the $Ca^{2+}$ is released which is also in accord with Fig. 1. When choline Cl is administered in Fig. 1 or Fig. 2*B*, it is likely that a rapid influx of KCl occurs into the terminal cisternae since sarcoplasmic reticulum is permeable to both these ions (Meissner & McKinley, 1976; Kometani & Kasai, 1978). It is possible that this internal KCl could cause $Ca^{2+}$ release perhaps by displacing $Ca^{2+}$ bound to calsequestrin. However in Fig. 2*A* no $K^+$ is present and $Na^+$ is present only in low concentration. Nevertheless the $Ca^{2+}$ release is as great or slightly greater than that when $K^+$ is present and is materially more rapid. Hence no correlation is observed between $Ca^{2+}$ release and the expected ingress of salt into the vesicle. In Fig. 2*C* choline Cl has been added before ATP. Subsequent addition of ATP causes a low degree of $Ca^{2+}$ accumulation indicating that neither the time nor the order of addition of choline Cl is important. In Fig. 2*D* K cacody-

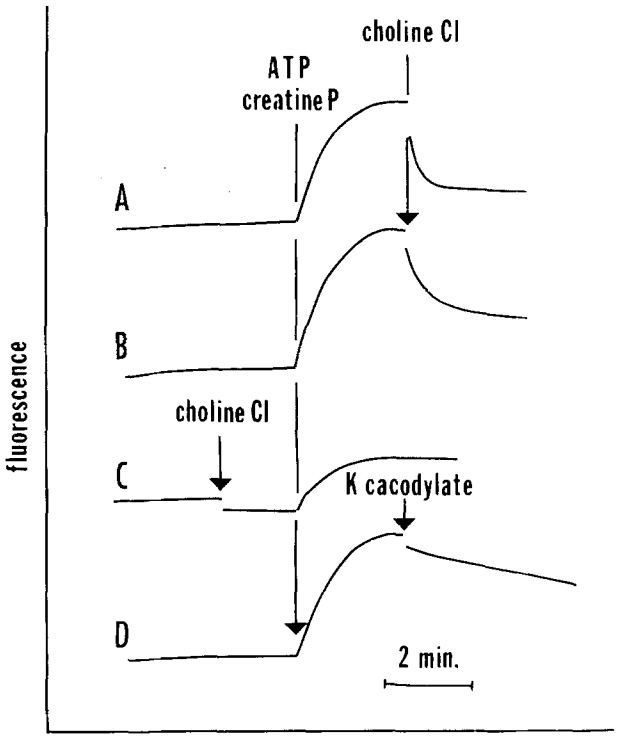


**Fig. 2.** Fluorescence visualization of $Ca^{2+}$ release induced by choline Cl. The medium was that described in Materials and Methods except that in traces *B-D* 0.1 M K cacodylate was present. The medium contained terminal cisternae/triads at a concentration of 100 μg protein/ml. The incubation conditions were similar to those of Fig. 1 except that preincubation before ATP and creatine phosphate addition was 6.5 min. Choline Cl and K cacodylate were added to final concentrations of 0.1 M. The gaps in the traces reflect the time when the fluorometer light was switched from the photomultiplier while the addition was made. A fluorescence artifact associated with binding of chlorotetracycline to ATP has been subtracted from the traces

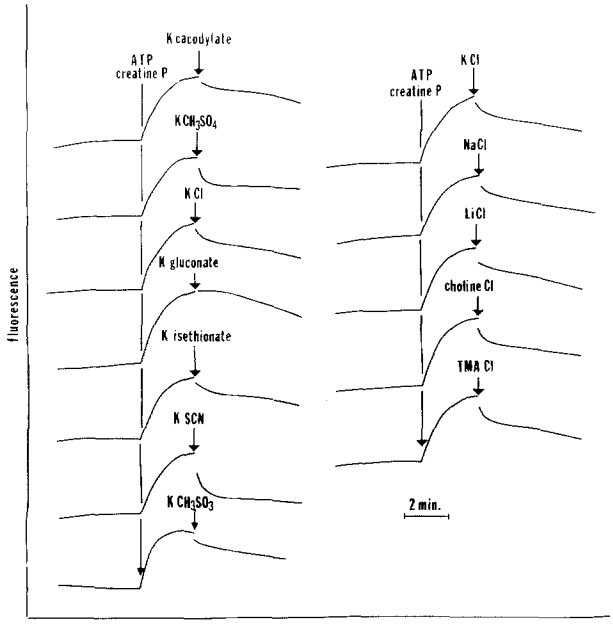


**Fig. 3.** Influence of different ions on $Ca^{2+}$ release observed fluorometrically. The incubation conditions were identical to those of Fig. 2 except that 0.12 M K cacodylate was present in the incubation medium. 0.1 M of the specified salts was added as indicated

late has been added instead of choline Cl as a control. The degree of $Ca^{2+}$ release here is low indicating that choline Cl did not exert a nonspecific action through increasing the ionic strength.

*Ion Specificity for Ca Release*

Figure 3 shows the influence on $Ca^{2+}$ release of addition of a wide range of salts. The traces on the left side of the Figure represent variation of anions in which the cation added is $K^+$. The medium contains 0.12 M K cacodylate. Addition of K gluconate has no effect on $Ca^{2+}$ accumulation. K cacodylate, K isethionate and K methanesulfonate ($CH_3SO_3$) have a small effect in initiating release. KCl and K methylsulfate ($CH_3SO_4$) initiate a larger release and KSCN causes the largest extent of $Ca^{2+}$ release. The order of anions in inducing release is fairly close to the known or expected permeability of anions. Thus gluconate is known to be highly impermeant and cacodylate and isethionate are expected to have low permeability. Methanesulfonate is known to have a lower permeability than $Cl^-$ (Kometani & Kasai, 1978). $SCN^-$ is moderately lipid soluble and may have a fairly high permeability, although this has not been tested in sarcoplasmic reticulum. The order of ability to induce $Ca^{2+}$ release appears to correlate well with the expected permeability of anions. However alternative interpretations are possible including a dependence on lipid solubility or molecular size of the anions. Therefore the factor which determines $Ca^{2+}$ release may be membrane permeability of the anion or its propensity to bind to a cationic site in the membrane.

On the right side of Fig. 3 the cation has been varied while the anion is $Cl^-$. K, Na and Li chlorides all induce a similar degree of release. Choline and tetramethylammonium (TMA) chloride both induce a greater release than the others. The order of cations in inducing $Ca^{2+}$ release appears to correlate inversely with their permeabilities. The sarcoplasmic reticulum is permeant to $K^+$, $Na^+$ and $Li^+$, but impermeant to choline and tetramethylammonium (Kometani & Kasai, 1978). The relatively small influence of altering cations may be attributed in part to the prior presence of $K^+$ in the medium. These data differ from those of Kasai and Miyamoto (1976*a*, *b*) in that choline Cl was ineffective in releasing $Ca^{2+}$ in their experiments.

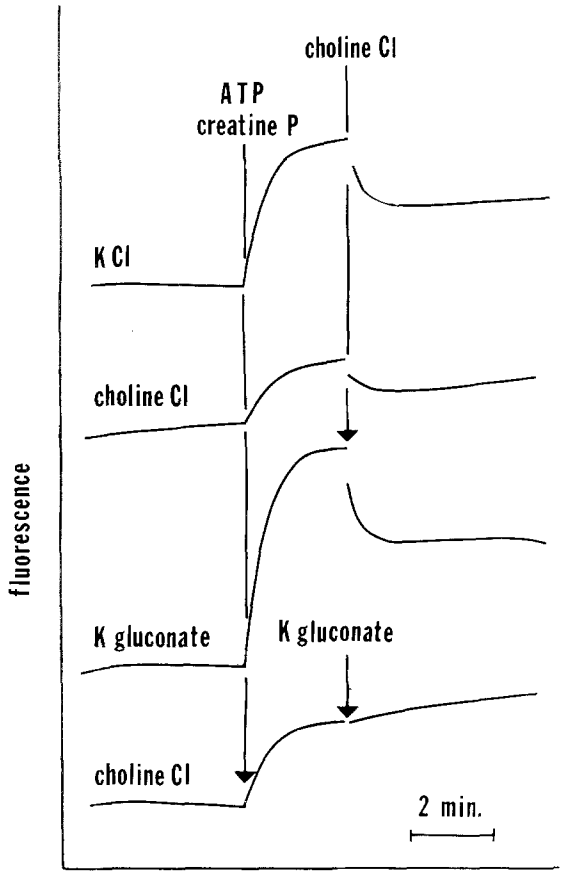


**Fig. 4.** Influence of different ions on $Ca^{2+}$ uptake and release. The incubation conditions were the same as those of Fig. 3 except that no K cacodylate was present in the incubation medium. 0.1 M of the salt indicated was present in the incubation medium or was added at the arrow as indicated

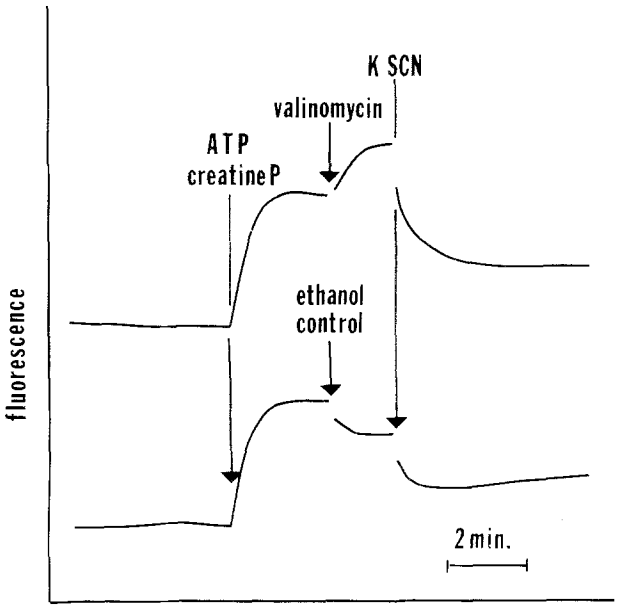


**Fig. 5.** Influence of valinomycin on $Ca^{2+}$ uptake and release. The incubation conditions were the same as those of Fig. 3 except that 0.1 M K gluconate was present in the medium. Final concentration of valinomycin was 1 μM and of ethanol was 1%. Final concentration of KSCN was 0.1 M. Terminal cisternae/triad concentration was 78 μg protein/ml

Figure 4 shows the effects on $Ca^{2+}$ release of altering the ionic milieu in different ways. If choline Cl is added to a medium which contains 0.1 M KCl then release is seen (top trace). A small further release occurs when choline Cl is added to a medium which already contains choline Cl. The release on addition of choline Cl is greater when the original medium contained K gluconate (third curve). The fluorescent traces indicate that the amount of $Ca^{2+}$ remaining after release is in the order choline Cl < KCl < K gluconate in the original medium. In the bottom trace of Fig. 4K gluconate has been added to a medium which contained choline Cl. A small but significant enhancement of uptake is observed.

The experiments shown in Fig. 5 were designed to test the influence of ion permeability on $Ca^{2+}$ accumulation and release. Kometani and Kasai (1978) have determined that addition of $5 \times 10^{-7}$ M valinomycin significantly enhances $K^+$ permeability in sarcoplasmic reticulum. The medium in Fig. 5 contains K gluconate. The penetration of $K^+$ into the vesicles in the presence or absence of valinomycin will be primarily limited by the slow permeation of the gluconate anion. Therefore the influence of valinomycin will be expected to be a consequence of enhancing $K^+$ permeability without substantially influencing the ion gradient. The data of Fig. 5 show that valinomycin enhances accumulation of $Ca^{2+}$. Subsequent addition of KSCN causes a large $Ca^{2+}$ release although the final level of $Ca^{2+}$ is higher than in the control. The control shows that the ethanol vehicle causes a slight release of $Ca^{2+}$ so that valinomycin may be exerting a greater effect in enhancing $Ca^{2+}$ uptake than the upper trace reflects. The data of Fig. 5 are consistent with the interpretation of Fig. 3 that high cation permeability is associated with a diminished extent of $Ca^{2+}$ release. It is, however, also possible that valinomycin is exerting effects by forming a $K^+$-valinomycin complex which dissolves within the membrane bilayer and therefore influences the surface potential of the membrane (Haynes, 1972).

*Modification of Incubation Conditions*

Figure 6 shows the effects of altering the osmotic pressure on $Ca^{2+}$ release. The sucrose concentration was varied from 50 to 250 mM. Within the limits of experimental variation alteration of sucrose concentration causes no change in the extent of $Ca^{2+}$ accumulation

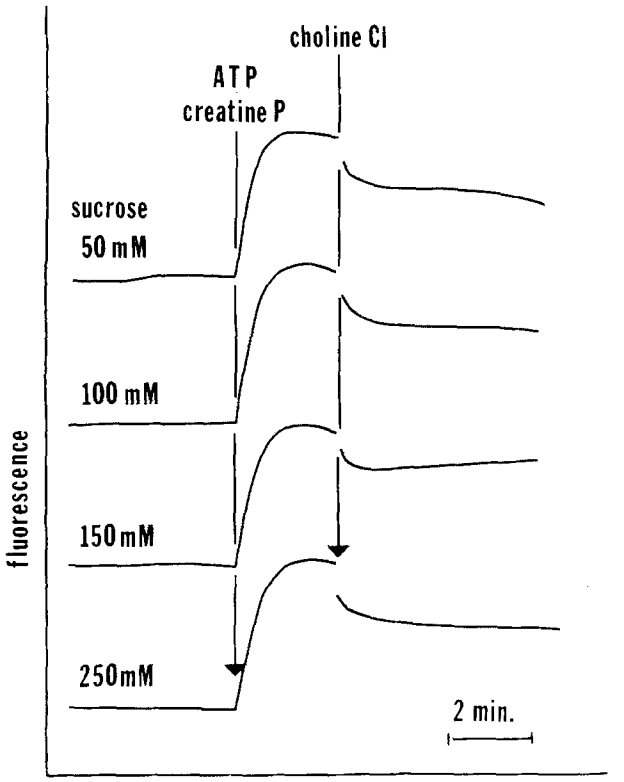


**Fig. 6.** Effect of osmotic pressure on $Ca^{2+}$ release. Incubation conditions were the same as Fig. 3 except that 0.1 M K gluconate was present, concentration of terminal cisternae/triads was 78 μg protein/ml and sucrose concentration was as described in the Figure

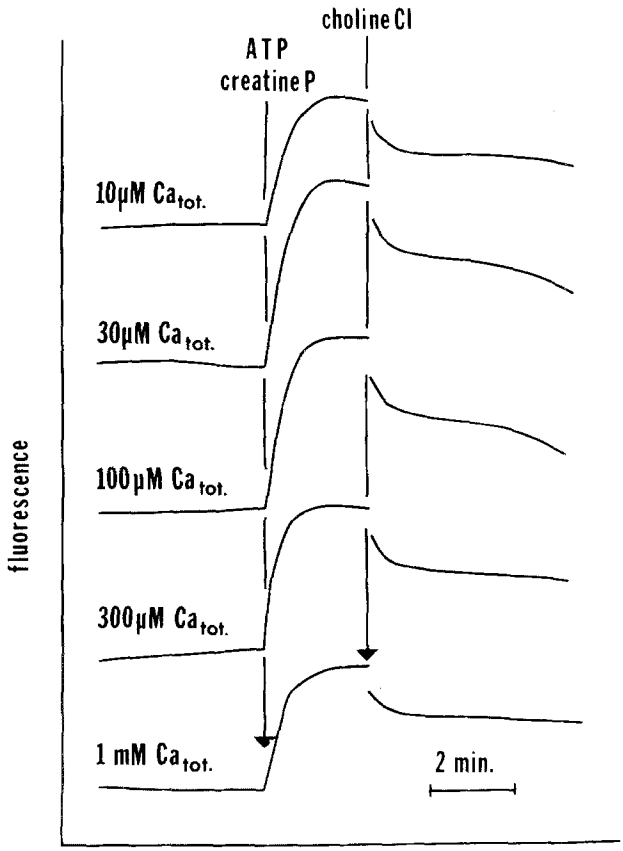


**Fig. 7.** Effect of total external Ca concentration on $Ca^{2+}$ release. Incubation conditions were the same as Fig. 3 except that 0.1 M K gluconate was present and concentration of terminal cisternae/triads was 78 μg protein/ml. Total Ca concentration was as described in the Figure and Tris EGTA was added at various concentrations to give a free $Ca^{2+}$ activity of 10 μM. The concentration of EGTA to achieve this was estimated from the affinity constant of CaEGTA described by Kim and Padilla (1978) as $1.26 \times 10^{-5}$ at pH 6.8

or the release induced by addition of choline Cl. This experiment suggests that the $Ca^{2+}$ release demonstrated in this report is not a consequence of an altered tonicity in the medium.

Figure 7 represents an examination of whether the induction of $Ca^{2+}$ release by choline Cl could occur through a mechanism in which a small pulse of $Ca^{2+}$ release is amplified in a cascade process through Ca-induced $Ca^{2+}$ release. If a cascade process exists, then increasing Ca buffering should prevent the cascade. To test this the free Ca concentration has been held at a fixed value while the amount of total Ca available for buffering has been varied. This has been carried out by varying $Ca^{2+}$ and EGTA in order to obtain a free $[Ca^{2+}]$ of 10 μM. The Figure shows that variation of the buffering Ca from 10 μM to 1 mM has no discernible influence on the rate and extent of $Ca^{2+}$ release induced by choline Cl. Therefore in this experiment no amplifying role occurs through changes in external $[Ca^{2+}]$ causing feedback enhancement of $Ca^{2+}$ release.

We have also used 100 μM $CaCl_2$ and varying concentrations of EGTA so that free $Ca^{2+}$ levels have been varied between 100 and 2 μM. At the low levels of free $Ca^{2+}$ the extent of $Ca^{2+}$ accumulation has diminished by a factor of 2. The extent of release on addition of choline Cl has also diminished to the same extent. This indicates that within experimental observation the degree of $Ca^{2+}$-loading does not play a significant role in opening Ca channels.

### *$Ca^{2+}$-Stimulated Phosphatase*

In conditions in which $Ca^{2+}$ release is observed in the presence of active $Ca^{2+}$ accumulation the final Ca concentration in the organelles will be a function not only of the passive permeability but also of the

**Table 1.** Ca-stimulated 3-O-methylfluorescein phosphatase

| Salt added | % Activity ± SD |
|---|---|
| None | 100 |
| K cacodylate | 40 ± 12 |
| K methylsulfate | 107 ± 32 |
| KCl | 70 ± 7 |
| K gluconate | 50 ± 7 |
| K isethionate | 74 ± 7 |
| KSCN | 258 ± 76 |
| K methanesulfonate | 79 ± 3 |
| NaCl | 43 ± 2 |
| LiCl | 63 ± 6 |
| Choline Cl | 71 ± 3 |
| Tetramethylammonium Cl | 91 ± 7 |

Assay conditions were described in Materials and Methods. In each case the reaction was followed for 2 min and found to be linear. The results were obtained from three experiments.

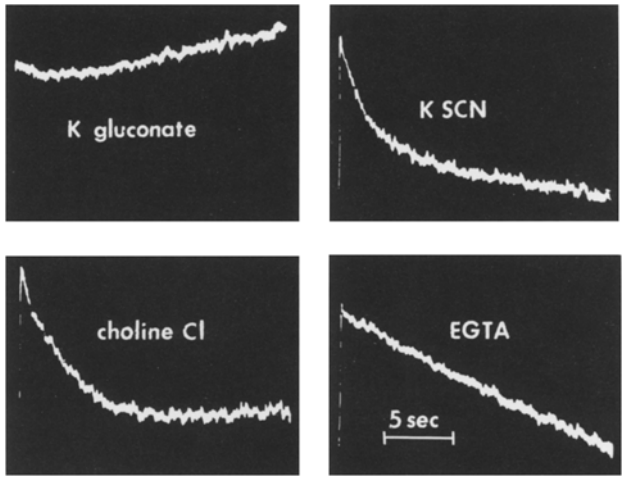


**Fig. 8.** Stopped flow fluorometric recording of $Ca^{2+}$ release from terminal cisternae/triads. The conditions of the assay were as described in Materials and Methods. The oscilloscope trace was triggered at the termination of flow. The amplifier time constant was 50 msec. Each trace represents the influence of 0.1 M of the salt indicated on the fluorescence of chlorotetracycline in the presence of terminal cisternae/triads except that the EGTA concentration was 2.5 mM

rate of $Ca^{2+}$ accumulation. In order to test the influence of the different salts on the Ca pump, the rate of phosphate release from a high-energy phosphate donor was assayed. The fluorescence increase on breakdown of 3-O-methylfluorescein phosphate was assayed in the presence and absence of $Ca^{2+}$. We have previously shown that this is a property of the Ca pump of sarcoplasmic reticulum (Brandt, Caswell & Brunschwig, 1980). Table 1 shows that different salts have very different influences on the rate of phosphate production, but that there is no correlation between $Ca^{2+}$-stimulated phosphatase activity and ability to induce $Ca^{2+}$ release. KSCN and K methylsulfate stimulate phosphatase while choline Cl and tetramethylammonium Cl inhibit phosphatase although these salts all initiate extensive $Ca^{2+}$ release. Similarly K gluconate and NaCl both inhibit phosphatase to a similar extent while only NaCl initiates release. It appears therefore that direct effects on the Ca pump may be responsible only for minor changes in the $Ca^{2+}$ content of terminal cisternae. ATPase was also assayed directly using a pH electrode to assay for ATP breakdown since hydrolysis is associated with acidification of the medium. The data were similar in that KSCN stimulated ATPase while choline Cl caused a slight inhibition.

### *Rate of $Ca^{2+}$ Release*

Since the first phase of release occurred within the time of resolution for mixing in the fluorometer, we assayed the rate of $Ca^{2+}$ release in a stopped-flow apparatus. Some representative traces are shown in Fig. 8. Both syringes contained identical media except that the releasing salt was placed in one syringe and the vesicles in the other. Therefore little or no fluorescence change would be expected to accompany mixing other than that associated with $Ca^{2+}$ movements. The control consisted of mixing K gluconate with the vesicles. Very little fluorescence change of chlorotetracycline was observed. The slow increase of fluorescence could be a reflection of a slow futher influx of $Ca^{2+}$ or of chlorotetracycline in the presence of ATP, since these experiments were carried out approximately 2 min after addition of ATP and creatine phosphate before the plateau had been reached. Addition of either choline Cl or KSCN caused an initial phase of release which was essentially complete within 6 sec. The complexity of the curves precluded obtaining effective time constants, but approximately half the $Ca^{2+}$ has been released within 2 sec. By comparison the influence on Ca release of inhibiting the Ca pump was examined. The Ca pump was inhibited completely by addition of 2.5 mM EGTA. A steady decline of fluorescence indicates $Ca^{2+}$ release. The total release with EGTA was considerably greater than with choline Cl or KSCN (not shown). Nevertheless the initial rate of release was considerably slower. This provides further evidence that the $Ca^{2+}$ release is not associated primarily with a loss of active $Ca^{2+}$ uptake, but with an increased passive permeability of $Ca^{2+}$. From Fig. 1 approximately 30 nmol $Ca^{2+}$/mg protein is released by choline Cl in the terminal cisternae/triad band and approximately 50 nmol/mg is released by pure heavy terminal cisternae. About half of the $Ca^{2+}$ is released in the early rapid phase (Fig. 2). Hence

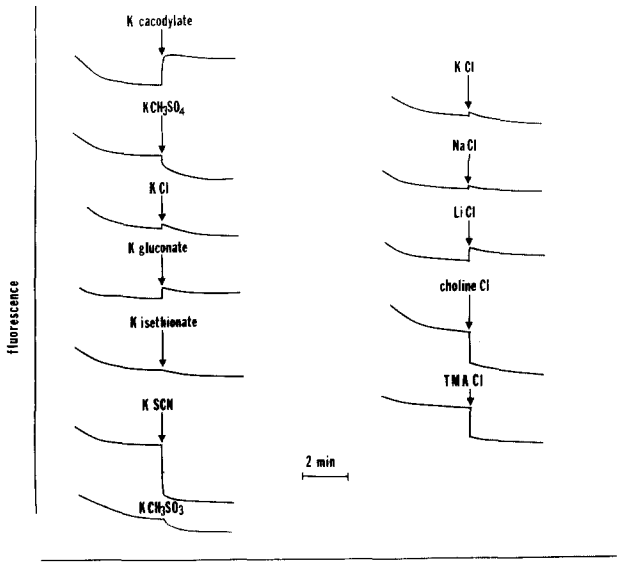


**Fig. 9.** ANS fluorescence changes associated with salt addition to terminal cisternae/triads. The conditions were as described in Materials and Methods. Final salt concentration was 0.1 M

about 8 nmol is released in 2 sec or about 12 nmol from the heavy terminal cisternae if conditions are similar. This represents only an order of magnitude calculation.

*Surface Potential Determination*

The introduction of fluorescent probes which respond to alterations of membrane potential suggested their employment in studying the influence of salts on sarcoplasmic reticulum $Ca^{2+}$ movements. We found that the potential sensing probe, 3,3′-dipentyl-2,2′-oxacarbocyanine, gave slow and small responses to the addition of salts to terminal cisternae/triads. We therefore employed 1-anilinonaphthalene-8-sulfonate (ANS) as a probe since this anionic fluorophore binds to membranes and responds to alterations of surface potential or electrical field with a change of fluorescence (Vanderkooi & Martonosi, 1969; Chiu, Mouring, Watson & Haynes, 1980). Figure 9 shows the fluorescence changes of ANS in terminal cisternae/triads when different salts have been administered in the absence of energized $Ca^{2+}$ accumulation. Some salts such as K gluconate and K cacodylate cause a rapid increase in fluorescence which is essentially complete in the mixing time of the apparatus. Other salts including choline Cl, tetramethylammonium Cl and KSCN cause a rapid loss of fluorescence while NaCl, KCl, K methanesulfonate and K isethionate initiate little response. The degree of correspondence between the ability of salts to induce $Ca^{2+}$ release from terminal cisternae/triads and their effects on ANS fluorescence is high. Thus salts which are ineffective in initiating release cause a fluorescence increase while salts which are effective cause a fluorescence decrease. The major exception is LiCl which causes an increase in fluorescence of ANS but is moderately effective in causing $Ca^{2+}$ release. Also K cacodylate gives a greater fluorescence increase than K gluconate while K cacodylate causes more $Ca^{2+}$ release than K gluconate. Nevertheless these exceptions represent small differences in a framework of overall high correlation.

The interpretation of the responses of ANS as with most other fluorescent probes of biological microenvironments is not easy. The rate of change of fluorescence when salts have been added is greater than the reported rate of transmembrane transport of ANS ($t_{1/2}=8$ sec, Chiu et al., 1980). Therefore the response represents either the movement of ANS into or off the outer surface of the membrane or a change in microenvironment of the bound ANS which changes the quantum yield of fluorescence. Since the overall lipid charge on the membrane is negative, anions would not be expected to bind effectively to lipid groups on the membrane. Nevertheless different anions give widely different fluorescence responses. Thus cacodylate causes a fluorescence increase while $SCN^-$ causes fluorescence decrease and $Cl^-$ has little effect. Therefore the immediate local effects of salts do not correlate well with their expected binding properties unless specific positively charged groups are also present on the membrane. An alternative interpretation of the data of Fig. 9 is that the salts cause the generation of an electrical potential gradient across the membrane which is manifested as a local electric field in the vicinity of the probe. However when terminal cisternae/triads have been incubated in a hypotonic medium such that they have lost their capacity to accumulate $Ca^{2+}$ they still show similar responses to salt additions (not shown). This indicates that the production of a transmembrane potential is not essential to the ANS response to salts.

## Discussion

*Sources of $Ca^{2+}$ Release Induced by Ionic Perturbation*

The data presented in this paper indicate that specific alterations in the ionic environment in which the terminal cisternae are suspended induce a rapid $Ca^{2+}$ release from the vesicles. We feel that those ionic manipulations induce a change in either the membrane potential or surface charge which is related to the release of actively sequestered $Ca^{2+}$. This interpretation should be considered with care in order

to eliminate alternative interpretations, particularly in view of the fact that earlier claims that $Ca^{2+}$ release is induced by changes of membrane potential have been questioned (Meissner & McKinley, 1976; Beeler et al., 1979).

The employment of alteration of ionic composition or permeability is the current practicable way of altering the membrane potential in vesicles which are not accessible to electrodes. However this procedure may alter the environment of the vesicles in other ways. We have considered the following possibilities:

1. The alteration of ion alters the osmolarity of the solution to cause either hypertonicity or hypotonicity. Hypertonicity may cause $Ca^{2+}$ release by reducing the internal fluid volume, concentrating the $Ca^{2+}$, so that the pump is unable to sustain the $Ca^{2+}$ gradient and release occurs. Hypotonicity may cause swelling of the vesicle and rupture of the membrane. In our experiments hypotonicity is avoided by adding concentrated salt to the vesicles rather than diluting the vesicles into the salt solution as has been employed by others. Also Fig. 6 shows that varying the osmotic pressure by manipulation of the sucrose concentration in the suspension has no discernible effect on the extent or rate of $Ca^{2+}$ release induced by choline Cl. The addition of salt may cause hypertonicity in some experiments but not in all cases in which $Ca^{2+}$ release has been observed. Thus when choline Cl is added to vesicles in a medium of K gluconate, it may be expected that no osmotic pressure change occurs since $K^+$ and $Cl^-$ are permeable. In the original medium the impermeability of gluconate will prevent entry of either gluconate$^-$ or $K^+$ while, after choline Cl addition, $K^+$ and $Cl^-$ may penetrate the vesicle while choline gluconate maintains the osmotic pressure. It could be argued that a transient hypertonicity occurred, but the release of $Ca^{2+}$ is sustained. Furthermore the absence of $Ca^{2+}$ release induced by K gluconate addition does not support the conclusion that hypertonicity is responsible for $Ca^{2+}$ efflux from the vesicles.

2. $Ca^{2+}$ release may occur through the penetration of salts into the interior of the vesicle. The salt or ion may then either displace $Ca^{2+}$ directly from binding sites or act through an intermediate to initiate $Ca^{2+}$ release. For large scale penetration of salts to occur both ions of the pair must be able to penetrate the vesicle. For example, when choline Cl is added to a suspension containing K gluconate, it may be expected that KCl penetrates the vesicle. $K^+$ uptake will be slight in the absence of $Cl^-$. The KCl may then cause $Ca^{2+}$ release from within the vesicle. Experiments which preclude this process include those of Fig. 2 in which sarcoplasmic reticulum has been incubated in a medium of low ionic strength. Addition of choline Cl can be expected to produce little movement of either choline or Cl into the vesicle since the permeability of choline is low ($\tau = 360$ sec, Kometani & Kasai, 1978) compared with the rate of $Ca^{2+}$ release. Nevertheless the $Ca^{2+}$ released is as large as that in which the medium contained K gluconate and apparently faster.

3. Specific receptor mediated binding of the salt or ion may occur which initiated $Ca^{2+}$ release. We have employed a wide range of salts with considerable variation of chemical structure. Although they give rise to a variable degree of release, we cannot detect any aspect of chemical structure which could be considered to confer specificity of binding. We find that maximal $Ca^{2+}$ release requires high concentrations of salts as is indicated for choline Cl in Fig. 4. This is not in accordance with current understanding of binding of an agonist to a specific receptor.

We consider that the most probable interpretation of our results is that salt administration is directly influencing either the electrical potential across the membrane or the charge of groups located on or near the surface of the membrane. The distinction between these two mechanisms is significant but not easy to assess in practice. The membrane potential will be determined by the permeability, charge and concentration of the ions on both sides of the membrane according to the Goldman equation (1943). The permeabilities of some ions have been estimated, but not those of all which we have employed. The estimates give permeability orders for cations of $K^+ > Na^+ > Li^+ >> \text{choline}^+$ and for anions of $Cl^- > \text{methanesulfonate}^- >> \text{gluconate}^-$ (Meissner & McKinley, 1976; Kometani & Kasai, 1978). This is in approximate accord with the effects of salts in causing $Ca^{2+}$ release. The more permeable anions and less permeable cations caused higher $Ca^{2+}$ release. If the permeability of $K^+$ is artificially raised by administration of valinomycin, then $Ca^{2+}$ uptake is enhanced and release is slightly depressed. This is in good accord with the deduction that membrane potential controls $Ca^{2+}$ permeability. On the other hand Beeler et al. (1979) have argued that membrane potentials created by altering the external ionic environment are rapidly depleted as the ions cross the membrane and their gradients diminish. This would be expected particularly if both ions are highly permeable such as KCl or KSCN and hence after the initial $Ca^{2+}$ release, there should be a reaccumulation of $Ca^{2+}$. However a further factor should be considered. In our experiments the initial incubation conditions have been in K gluconate or K cacodylate (except in Fig. 2*A*). Since the anion has low permeability it is likely, though not demonstrated, that the initial

conditions include a positive potential inside and it is possible that depletion of this potential rather than the generation of a negative potential is responsible for $Ca^{2+}$ release.

The alternative interpretation of our data is that the salts or ions are attracted by electrostatic force to a portion or component of the membrane surface. Factors which could influence this binding include lipid solubility of the ions and their charge. If the release of $Ca^{2+}$ is triggered by an alteration of surface membrane charge, then both cations and anions will exert an influence depending on their charge and lipid solubility. The order of lipid or membrane solubility of the salts which we have employed is not known and hence we are unable to provide effective correlation of the effect of salts on $Ca^{2+}$ release with their effects on surface charge. The influence of salts on the fluorescence of ANS shows an excellent agreement with their effects on $Ca^{2+}$ release. The fact that the ANS repsonse is also manifested in vesicles which have been osmotically shocked implies that this response is one of surface charge rather than membrane potential. Thus it appears possible that local or general surface charges may influence passive $Ca^{2+}$ permeability.

*Comparison with Other Experiments*

Kasai and Miyamoto (1973, 1976*a*, *b*) originally reported that dilution of sarcoplasmic reticulum vesicles from a medium of K methanesulfonate into one of KCl caused $Ca^{2+}$ release. This observation has been confirmed by Meissner and McKinley (1976), Beeler et al. (1979) and Ohnishi (1979). Meissner and McKinley and Beeler et al. attribute the release in part to osmotic effects caused by the inability of KCl to sustain an osmotic pressure and in part by an influence of KCl on the rate of $Ca^{2+}$ accumulation. Kasai and Miyamoto (1976*b*) have responded that, although an influence of osmotic pressure on $Ca^{2+}$ release is observed, nevertheless release induced by immersion in an environment of low ionic strength is smaller in magnitude than that observed when dilution into KCl is employed. They also demonstrated that release of $K^+$, $Na^+$, sucrose or inulin was no greater when they diluted the vesicles into KCl than into K methanesulfonate.

Meissner and McKinley (1976) and Beeler et al. (1979) comment on the apparent lack of consistency in the effect of ions in inducing release of $Ca^{2+}$. Thus dilution of vesicles into a medium containing choline Cl causes no $Ca^{2+}$ release (Kasai & Miyamoto, 1973). Meissner and McKinley (1976) found that dilution of vesicles from choline Cl to K gluconate had a similar influence on $Ca^{2+}$ release to that induced by dilution from K gluconate to choline Cl. McKinley and Meissner (1978) and Beeler et al. (1979) have employed oxacarbocyanine dyes to monitor apparent alterations of membrane potential induced by altering the ionic milieu. Beeler et al. (1979) found that in some conditions Ca release could be induced by ions which caused only slight change in fluorescence of the dye while in other conditions little Ca release was observed when a large fluorescence change of the dye was observed.

Despite the superficial resemblance between our data and those of Kasai and Miyamoto (1976*a*, *b*), there are a number of differences both in the experimental protocol and significantly in the experimental observations. In the other experiments on ion-induced release of $Ca^{2+}$ prolonged incubation in a salt solution has presumably resulted in an even distribution of ions on either side of the membrane. Thus the initial membrane potential of the vesicles is presumably low. Dilution of the vesicles into a salt solution containing a permeant anion may induce a negative potential inside. In our experiments a short incubation in K gluconate or K cacodylate may cause an initial positive potential inside, since the anion is impermeant. Subsequent addition of a salt containing a permeant anion may cause a partial depolarization. In excitable membranes the initial electrical potential is critical in determining the extent of inducible excitation.

Our experimental observations differ from those of Kasai and Miyamoto (1976*b*) in that the $Ca^{2+}$ release is not significantly affected by osmotic pressure and is carried out in conditions in which no drop in osmotic pressure occurs. We also find a number of differences in the ions which induce response. Thus unlike the observations of Kasai and Miyamoto (1973) and Beeler et al. (1979), choline Cl is able to induce a $Ca^{2+}$ release which is greater than that with KCl. Our data are more consistent in general with an influence of anion permeability or lipid solubility on $Ca^{2+}$ release than those cited above. We conclude that, although our data are susceptible to more than one interpretation, ours is more clearly linked to a specific ionic mechanism that those described previously.

*Physiological Significance*

We have observed an ion-dependent release of $Ca^{2+}$ from sarcoplasmic reticulum. It is unlikely that excitation-contraction coupling involves gross alterations of ionic composition in the cytoplasm. However the

ionic alteration may be mimicing a localized alteration such as an influence on membrane potential or surface charge. Our experiments determining the rate of $Ca^{2+}$ release indicate that this is much slower than that expected in excitation-contraction coupling. However, if our data are compared with those in which skinned fibers are induced to release $Ca^{2+}$, the rates are broadly similar. Stephenson (1978) has calculated the stimulated $Ca^{2+}$ release from skinned fibers to be 4.2 pmol·cm$^{-2}$ of sarcoplasmic reticulum area·sec$^{-1}$. If the lipid-to-protein ratio of sarcoplasmic reticulum averages 0.75 μmol/mg protein (Lau et al., 1979) and the membrane is 50 Å thick, then the release rate is 9.3 nmol/mg protein/sec. This compares with a value of 4–6 nmol/mg protein/sec obtained in our experiments on isolated terminal cisternae. No circumstances have been described in which artificial alteration of ionic environment in skeletal muscle causes $Ca^{2+}$ release which is as rapid as that which occurs *in vivo*. However this may reflect either our inability to effect a sufficiently fast alteration of environment or that the extent of perturbation is not as great as that which occurs under physiological stimuli. Our finding that release of $Ca^{2+}$ is greater in terminal cisternae than in longitudinal reticulum is consistent with the report of Winegrad (1965) that the terminal cisternae are the site of physiological release.

The question has been debated for several years whether $Ca^{2+}$ release may be effected by a wave of depolarization of the sarcoplasmic reticulum. Ostensible support for this hypothesis provided by alteration of the ion environment of skinned muscle fibers (Endo & Nakajima, 1973) has been countered by the observations of Somlyo et al. (1977, 1979) that no substantive gradient either of $K^+$ of $Cl^-$ exists across sarcoplasmic reticulum at rest or during contraction. Unless $K^+$ and $Cl^-$ are impermeant in their charged form across the sarcoplasmic reticulum or unless there are large scale binding sites for the ions in the vesicle, these experiments preclude a material potential existing in the muscle at rest. However, a potential gradient could be formed during excitation as $Ca^{2+}$ permeability increases. We have emphasized in this paper that modification of the ionic environment could influence either the surface charges or the potential of the membrane. This may arise either as a consequence of imposing a transmembrane potential or by direct interaction of the ions at the membrane surface. If the observations described in this paper represent the opening of $Ca^{2+}$ channels which occurs *in vivo*, then we believe it more probable that alteration of surface charge distribution is the mechanism through which this occurs.

This work was supported by U.S. Public Health Service research grant 1 R01 AM-21601, by U.S. Public Health Service training grant 1 T32 HLB-07188 and by the Florida Heart Association.

## References

Adrian, R.H., Almers, W. 1976. Charge movement in the membrane of striated muscle. *J. Physiol.* **254**:339

Adrian, R.H., Caputo, C., Huang, C.L.-H. 1978. Effect of stretch on intramembrane charge movement in striated muscle. *J. Physiol.* **284**:151P

Adrian, R.H., Chandler, W.K., Rakowski, R.F. 1976. Charge movement and mechanical repriming in skeletal muscle. *J. Physiol.* **254**:361

Beeler, T., Russell, J.T., Martonosi, A. 1979. Optical probe responses on sarcoplasmic reticulum: Oxacarbocyanines as probes of membrane potential. *Eur. J. Biochem.* **95**:579

Brandt, N.R., Caswell, A.H., Brunschwig, J.-P. 1980. An ATP energized $Ca^{2+}$ pump in isolated T tubules of skeletal muscle. *J. Biol. Chem.* **255**:6290

Caswell, A.H. 1979. Methods of measuring intracellular calcium. *Int. Rev. Cytol.* **56**:145

Caswell, A.H., Lau, Y.H., Brunschwig, J.-P. 1976. Ouabain binding vesicles from skeletal muscle. *Arch. Biochem. Biophys.* **176**:417

Caswell, A.H., Pressman, B.C. 1972. Kinetics of transport if divalent cations across sarcoplasmic reticulum induced by ionophores. *Biochem. Biophys. Res. Commun.* **49**:292

Caswell, A.H., Warren, S. 1972. Observation of calcium uptake by isolated sarcoplasmic reticulum employing a fluorescent chelate probe. *Biochem. Biophys. Res. Commun.* **46**:1757

Chiu, V.C.K., Mouring, D., Watson, B.D., Haynes, D.H. 1980. Measurement of surface potential and surface charge densities of sarcoplasmic reticulum membranes. *J. Membrane Biol.* **56**:121

Costantin, L.L., Podolsky, R.J. 1967. Depolarization of the internal membrane system in the activated frog skeletal muscle. *J. Gen. Physiol.* **50**:1101

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**:71

Endo, M., Nakajima, Y. 1973. Release of calcium induced by "depolarization" of the sarcoplasmic reticulum membrane. *Nature, New Biol.* **246**:216

Fabiato, A., Fabiato, F. 1977. Calcium release from the sarcoplasmic reticulum. *Circ. Res.* **40**:119

Goldman, D.E. 1943. Potential, impedance and rectification in membranes. *J. Gen. Physiol.* **27**:37

Haynes, D.H. 1972. Detection of ionophore-cation complexes on phospholipid membranes. *Biochim. Biophys. Acta* **255**:406

Hill, H.D., Summer, G.K., Waters, M.D. 1968. An automated fluorometric assay for alkaline phosphatase using 3-O-methylfluorescein phosphate. *Anal. Biochem.* **24**:9

Hui, C.S., Gilly, W.F. 1979. Mechanical activation and voltage dependent charge movement in stretched muscle fibres. *Nature* **281**:223

Huxtable, R., Bressler, R. 1974. The effect of deuterium ion concentration on the properties of sarcoplasmic reticulum. *J. Membrane Biol.* **17**:189

Kasai, M., Miyamoto, H. 1973. Depolarization induced calcium release from sarcoplasmic reticulum membrane fragments by changing ionic environment. *FEBS Lett.* **34**:299

Kasai, H., Miyamoto, H. 1976*a*. Depolarization-induced calcium release from sarcoplasmic reticulum fragments. I. Release of calcium taken up upon using ATP. *J. Biochem.* **79**:1053

Kasai, M., Miyamoto, H. 1976*b*. Depolarization-induced calcium release. II. Release of calcium incorporated without ATP. *J. Biochem.* **79**:1067

Kim, Y.S., Padilla, G.M. 1978. Determination of free Ca ion concentrations with an ion selective electrode in the presence of chelating agents in comparison with calculated values. *Anal. Biochem.* **89**:521

Kometani, T., Kasai, M. 1978. Ionic permeability of sarcoplasmic reticulum vesicles measured by light scattering method. *J. Membrane Biol.* **41**:295

Lau, Y.H., Caswell, A.H., Brunschwig, J.-P. 1977. Isolation of transverse tubules fractionation of triad junctions of skeletal muscle. *J. Biol. Chem.* **252**:5565

Lau, Y.H., Caswell, A.H., Brunschwig, J.-P., Baerwald, R.J., Garcia, M. 1979. Lipid analysis and freeze fracture studies on isolated transverse tubules and sarcoplasmic reticulum subfractions of skeletal muscle. *J. Biol. Chem.* **254**:540

McKinley, D., Meissner, G. 1978. Evidence for a $K^+$, $Na^+$ permeable channel in sarcoplasmic reticulum. *J. Membrane Biol.* **44**:159

Meissner, G., McKinley, D. 1976. Permeability of sarcoplasmic reticulum membrane. The effect of changed ionic environments on $Ca^{2+}$ release. *J. Membrane Biol.* **30**:79

Ohnishi, S.T. 1979. A method for studying the depolarization-induced calcium ion release from fragmented sarcoplasmic reticulum. *Biochim. Biophys. Acta* **587**:121

Schneider, M.F., Chandler, W.K. 1973. Voltage dependent charge movement in skeletal muscle: A possible step in excitation-contraction coupling. *Nature* **242**:244

Somlyo, A.V., Gonzales, H., Shuman, H., McCleland, G., Somlyo, A.P. 1979. Changes in sarcoplasmic reticulum composition in activated frog skeletal muscle. *Biophys. J.* **25**:A25

Somlyo, A.V., Shuman, H., Somlyo, A.P. 1977. Elemental distribution in striated muscle and the effects of hypertonicity. Electron probe analysis of cryo sections. *J. Cell Biol.* **74**:828

Sorenson, M.M., Meis, L. de 1977. Effects of anions, pH and magnesium on calcium accumulation and release by sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* **465**:210

Stephenson, E.W. 1978. Properties of chloride-stimulated $^{45}Ca$ flux in skinned muscle fibers. *J. Gen. Physiol.* **71**:411

Vanderkooi, J., Martonosi, A. 1969. Sarcoplasmic reticulum. VII. Use of 8-anilino-1-naphthalene sulfonate as conformation probe on biological membranes. *Arch. Biochem. Biophys.* **133**:153

Winegrad, S. 1965. The location of muscle calcium with respect to the myofibrils. *J. Gen. Physiol.* **48**:997

Received 7 March 1980; revised 20 August 1980