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THE MODIFICATION OF THE UNIDIRECTIONAL CALCIUM FLUXES OF SARCOPLASMIC RETICULUM VESICLES BY MONOVALENT CATION IONOPHORES

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Summary

Calcium uptake by rabbit skeletal muscle sarcoplasmic reticulum vesicles in phosphate-containing media exhibits time-dependent changes that arise from changing rates of calcium influx and efflux. The monovalent cation ionophore gramicidin, added before the start of the calcium uptake reaction, delayed the spontaneous calcium release that normally occurred after approx. 6 min in such reactions; the rate of calcium efflux was inhibited while calcium influx was little affected. Under these conditions, Ca^{2+}-activated ATPase activity could remain unaltered.

Gramicidin stimulated calcium uptake irrespective of the presence of a K^+ gradient across the vesicle membrane. Valinomycin stimulated calcium uptake in a manner similar to that for gramicidin even in an NaCl-containing medium lacking potassium. Thus, dissipation of a transmembrane K^+ gradient is unlikely to account for the effects of these ionophores on the spontaneous changes in calcium flux rates.

Addition of gramicidin to partially calcium-filled vesicles inhibited the phase of spontaneous calcium reuptake because both calcium influx and efflux were inhibited. Addition of gramicidin to partially calcium-filled vesicles in the presence of a water-soluble protein, such as bovine serum albumin, creatine kinase or pyruvate kinase, markedly stimulated calcium uptake. This stimula-

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

As used in this article. calcium uptake and calcium release refer to the rate of net gain or loss of calcium by the vesicles; calcium influx and calcium efflux refer to unidirectional calcium fluxes into and out of the vesicles, respectively.

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tory effect was due primarily to inhibition of calcium efflux, calcium influx being minimally influenced by the ionophore.

After cleavage of the 100 000 dalton ATPase to 50 000 dalton fragments, which was not associated with changes in Ca^{2+}-activated ATPase activity or initial calcium uptake rate, gramicidin increased rather than decreased calcium content when added to vesicles after the initial maximum in calcium content. Thus, the ability of monovalent cation ionophores to block calcium efflux from calcium-filled vesicles may reflect their interaction with a portion of the Ca^{2+}-activated ATPase protein.

Introduction

Sarcoplasmic reticulum vesicles exhibit spontaneous changes in their calcium content which arise from changing rates of unidirectional calcium flux into and out of the vesicles [1]. Whereas the mechanism of calcium influx has been extensively described [2], that of calcium efflux remains poorly understood. This efflux, which does not occur by complete reversal of the Ca^{2+}-activated ATPase reaction, appears to be a regulated process because it has a similar Ca^{2+} sensitivity to calcium uptake and is inhibited by elevated Mg^{2+} concentrations [3]. We have previously demonstrated that calcium release from calcium-filled vesicles is delayed by low concentrations of the divalent cation ionophore X537A [4]. Because this ionophore can also act as a monovalent cation ionophore [5] we wanted to determine whether monovalent cation ionophores had a similar ability to modify calcium release and thus indicate the mechanism of calcium efflux from sarcoplasmic reticulum vesicles. In the present report we demonstrate that monovalent cation ionophores can delay Ca^{2+} release from calcium-filled vesicles by selectively blocking calcium efflux. They appear to block calcium efflux by interacting directly with the Ca^{2+}-activated ATPase rather than by modifying the transmembrane monovalent cation gradient, which supports our hypothesis that the efflux of calcium from sarcoplasmic reticulum vesicles is mediated via the 100 000 dalton Ca^{2+}-activated ATPase.

Materials and Methods

Unless otherwise specified, experimental procedures were as described elsewhere [1]. 'Light' sarcoplasmic reticulum vesicles were used in all experiments. Vesicles were treated with trypsin in 20% sucrose/ 40 mM histidine buffer (pH 6.8) for 10 min at 25°C. Proteolysis was stopped by addition of a 2-fold excess of trypsin inhibitor (w/w). Ionophores were added in ethanol such that the final concentration of ethanol in the reaction mixture did not exceed 0.5% (v/v). These low ethanol concentrations were without significant effects on calcium uptake or Ca^{2+}-activated ATPase activity of sarcoplasmic reticulum vesicles [6]. Unless specified, the final concentration of gramicidin in the reaction mixture was 5 μg/ml. Trypsin (Type III), trypsin inhibitor (Type I) gramicidin, valinomycin, poly-DL-alanine Type I (M_r 2100) and gramicidin S were obtained from the Sigma Chemical Company. Antamanide was a generous gift of Dr. Th. Wieland, Max Planck Institute, Heidelberg, F.R.G. Alamethacin was a generous gift of Dr. G. Whitfield, Upjohn Company. Nigericin was a generous gift of Dr. R. Hammill of Eli Lilly and Co.

Results

Stimulation of calcium uptake by gramicidin

We have previously shown [7] that monovalent cation ionophores, such as gramicidin, stimulate calcium uptake by sarcoplasmic reticulum vesicles partially filled with calcium. In these experiments, calcium contents were assayed by Millipore filtration. To determine whether the observed effects of the ionophores were related to artefacts in this method, calcium contents were also assayed by separating the vesicles from reaction mixtures by centrifugation at 80 000 × *g* for 10 min [8] at varying times after the start of calcium uptake reactions carried out in the presence or absence of gramicidin. As shown in Fig. 1, the ability of gramicidin to stimulate calcium uptake cannot be ascribed to an artefact in the Millipore filtration method.

Addition of gramicidin to vesicles immediately prior to the start of a calcium uptake reaction had no significant effect on the initial rate of calcium uptake (Fig. 2A), but delayed the onset of the phase of calcium release which began at 6—8 min [1,7]. As a result, gramicidin prolonged the initial phase of calcium uptake and increased the maximum calcium content of the vesicles. Similar results were obtained when vesicles were incubated for 1 h with ionophore before the calcium uptake reaction was initiated by addition of 5 mM MgATP (Fig. 2A). Calcium uptake was also stimulated by gramicidin when vesicles were preincubated for 24 h in media containing no added KCl, 120 mM KCl, or

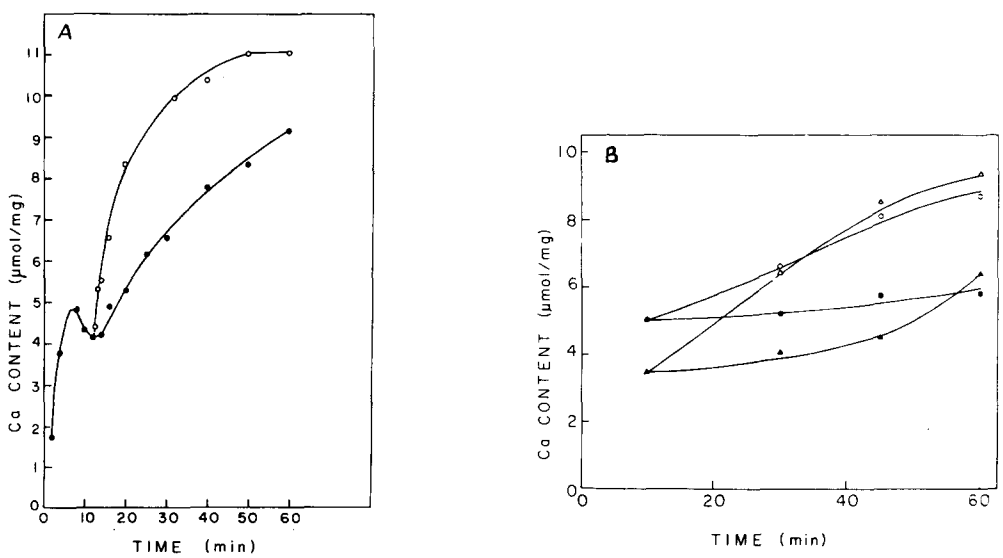


Fig. 1. Effect of gramicidin on calcium uptake of vesicles partially filled with Ca^{2+}, assayed by two methods. A. Calcium uptake assayed by Millipore filtration in 120 mM KCl, 40 mM histidine buffer (pH 6.8), 50 mM Tris phosphate, 5 mM MgATP, 5 mM phospho*enol*pyruvate, 72 μM $CaCl_2$ and 0.15 mg/ml pyruvate kinase. Reactions were started by addition of vesicles (6 μg/ml). Gramicidin was added 12 min after initiation of calcium uptake. B. Ca^{2+} uptake assayed by both Millipore filtration and ultracentrifugation. Reaction mixture as A except that $CaCl_2$ concentration was 60 μM. Calcium contents were assayed either after centrifugation at 80 000 × *g* for 10 min (▲,△) or by Millipore filtration (●,○). Control (●,▲); gramicidin (○,△).

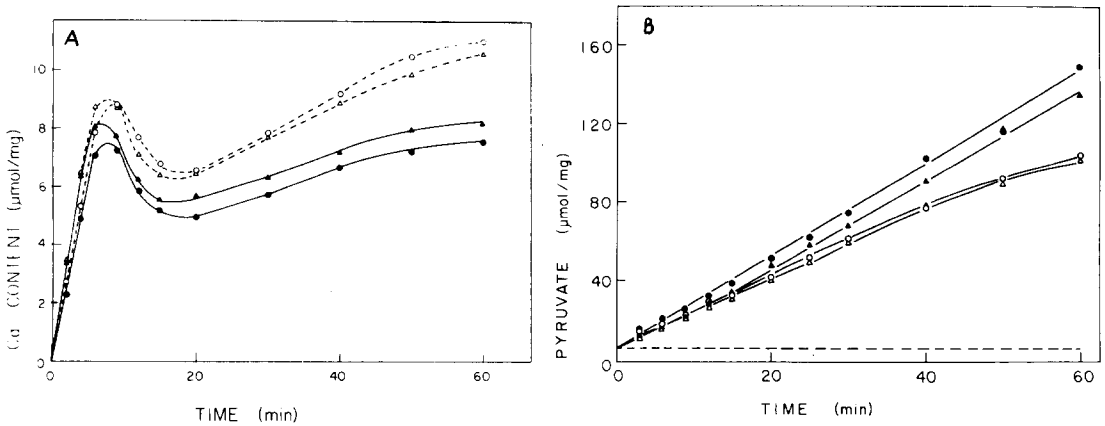


Fig. 2. Effect of preincubation of vesicles with gramicidin on Ca^{2+} uptake and Ca^{2+}-activated ATPase activities. A, calcium uptake assayed as described in Fig. 1A. B, Ca^{2+}-activated ATPase activity determined as described in 'Materials and Methods'. Reactions started by addition of vesicles to a complete reaction mixture (●). Reactions started by addition of vesicles to a mixture containing gramicidin (○). Vesicles incubated 1 h in a medium lacking MgATP and reaction started by addition of MgATP (▲); vesicles preincubated 1 h in mixture containing gramicidin and reaction started by addition of MgATP (△); as (●) but 1 mM EGTA substituted for $CaCl_2$ in assay medium (— — —).

800 mM KCl after which calcium uptake was assayed in media containing 120 mM KCl.

Gramicidin did not significantly affect the maximum calcium content of sarcoplasmic reticulum vesicles in the absence of a calcium precipitating anion.

Effects of gramicidin on Ca^{2+}-activated ATPase activity

We have previously shown [1] that lowering the substrate concentration, which slows the initial rate of calcium uptake, delays the initial maximum in calcium content but does not abolish the complex time-dependent changes in calcium content. Ca^{2+}-activated ATPase activity was not inhibited when gramicidin was added to vesicles prior to initiation of calcium uptake with 0.5 mM MgATP. Gramicidin delayed the onset of the phase of calcium release in a similar manner to that observed in 5 mM MgATP (Fig. 2A).

In the presence of 5 mM MgATP, gramicidin had little effect on initial Ca^{2+}-activated ATPase activity when added to vesicles prior to initiation of calcium uptake (Fig. 2B). However, after approx. 5 min, this activity became inhibited. The inhibitory effect of gramicidin on Ca^{2+}-activated ATPase activity in 5 mM MgATP was not due to an artefact arising from inhibition of the pyruvate kinase activity. Thus the conversion of phospho*enol*pyruvate to pyruvate, following addition of 0.1 mM ADP to a vesicle-free reaction mixture containing the pyruvate kinase ATP-generating system, was rapid being complete within 15 s in both the presence and absence of gramicidin.

To determine whether a Ca^{2+} gradient across the vesicle membrane was required for the inhibition of Ca^{2+}-activated ATPase activity by gramicidin in 5 mM MgATP, studies were carried out with a particulate ATPase preparation purified by the method of Meissner and Fleischer [9]. The ATPase activity of this preparation, which was incapable of maintaining a calcium gradient, was modified by gramicidin in a manner similar to that seen with intact vesicles, i.e.

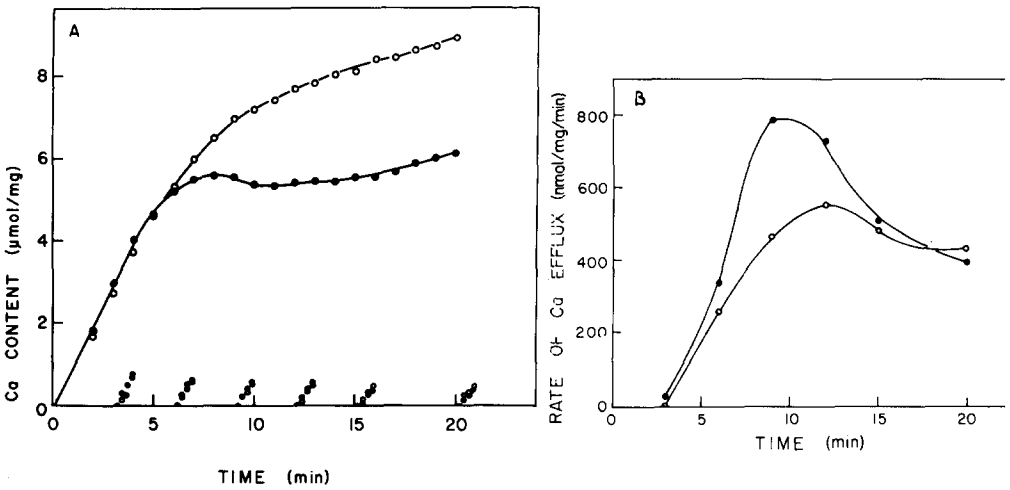


Fig. 3. A. Modification of unidirectional calcium fluxes by gramicidin, calcium uptake by vesicles in the presence (○) and absence (●) of gramicidin. The rate of calcium influx (see Ref. 1 for methods) is plotted along the abscissa. Reaction mixtures contained 0.12 M KCl, 40 mM histidine buffer (pH 6.8), 50 mM Tris phosphate, 5 mM MgATP and 72 μM $CaCl_2$. Reactions were started by the addition of vesicles (6 μg/ml). B. Calcium efflux rates calculated in the presence (○) and absence (●) of gramicidin.

Ca^{2+}-activated ATPase activity was inhibited in 5 mM MgATP, but not in 0.5 mM MgATP. Preincubation for 1 h with gramicidin did not change this effect of different MgATP concentrations. Thus a Ca^{2+} gradient did not appear to be required for these effects of gramicidin on Ca^{2+}-activated ATPase activity. Furthermore, the stimulation of calcium uptake by gramicidin was not necessarily accompanied by inhibition of ATP hydrolysis.

Effect of gramicidin on unidirectional calcium fluxes

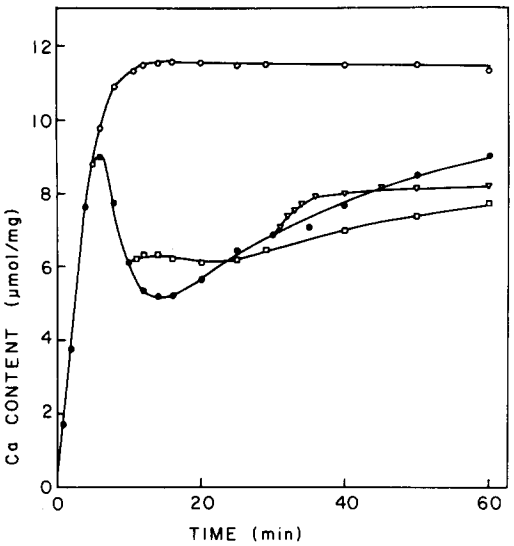
The stimulation of calcium uptake by gramicidin shown in Fig. 2A could reflect increasing calcium influx, decreasing calcium efflux, or both. The presence of gramicidin prior to the initiation of the calcium uptake had little effect on calcium influx determined at various times after the start of the reaction (Fig. 3A), so that the prolongation of the initial phase of calcium uptake by gramicidin can be attributed almost entirely to inhibition of calcium efflux (Fig. 3B). Similarly, the stimulation of calcium uptake seen after addition of

TABLE I

EFFECTS OF GRAMICIDIN ON UNIDIRECTIONAL CALCIUM Ca^{2+} FLUXES

Vesicles were partially filled with Ca^{2+} for 12 min at which time gramicidin (5 μg/ml final concentration) was added as shown in Fig. 4. Ca^{2+} influx was measured as described in Methods soon after this time (13–15 min) and Ca^{2+} influx and efflux rates were determined in the presence and absence of gramicidin. *P* represents the significance of the differences between the Ca^{2+} flux rates of control and gramicidin-containing vesicles. $n = 6$; n.s., not significant.

	Ca^{2+} influx	Ca^{2+} efflux
Change in rate	−9%	−88%
S.E.	14%	13%
	n.s.	$P < 0.01$



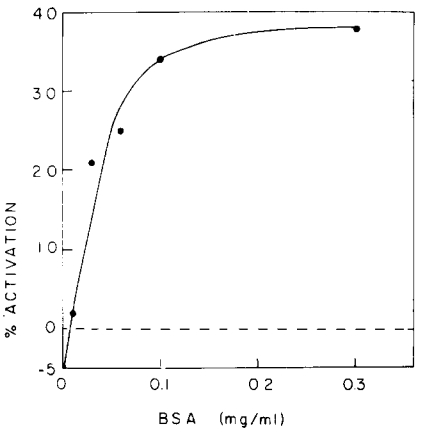


Fig. 4. Response of partially calcium-filled vesicles to gramicidin added at different times after initiation of calcium uptake. Reactions were started by addition of vesicles (6 μg/ml) to a reaction mixture as described (●) in Fig. 3. Gramicidin was added to reaction mixtures 4 (○), 10 (□) and 30 min (▽) after initiation of calcium uptake.

Fig. 5. Concentration-dependence of the ability of bovine serum albumin to modify the effect of gramicidin on calcium uptake. Reaction mixtures as described in Fig. 3 contained varying concentrations of bovine serum albumin (BSA). Reactions were started by addition of vesicles (6 μg/ml) and gramicidin was added at 12 min. The calcium contents of the control and gramicidin containing vesicles were compared after a further 18 min of calcium uptake and the percent stimulation of calcium uptake by gramicidin determined.

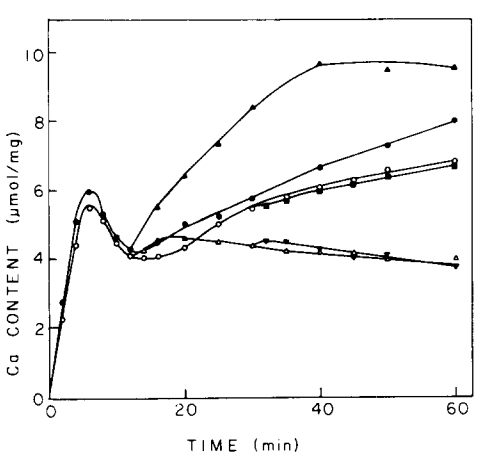


Fig. 6. Effects of water-soluble protein on calcium uptake by vesicles in the presence of gramicidin. Calcium uptake was assayed in reaction mixtures similar to those described in Fig. 3 except for the omission of water-soluble protein (○); or inclusion of 0.15 g/l creatine kinase (●). Gramicidin was added to the creatine kinase-containing reaction mixture at 12 min (▲), creatine kinase was added to the reaction mixture lacking this protein at 30 min (■); or gramicidin was added to the latter at 12 min (△) after which creatine kinase was added at 30 min (▲).

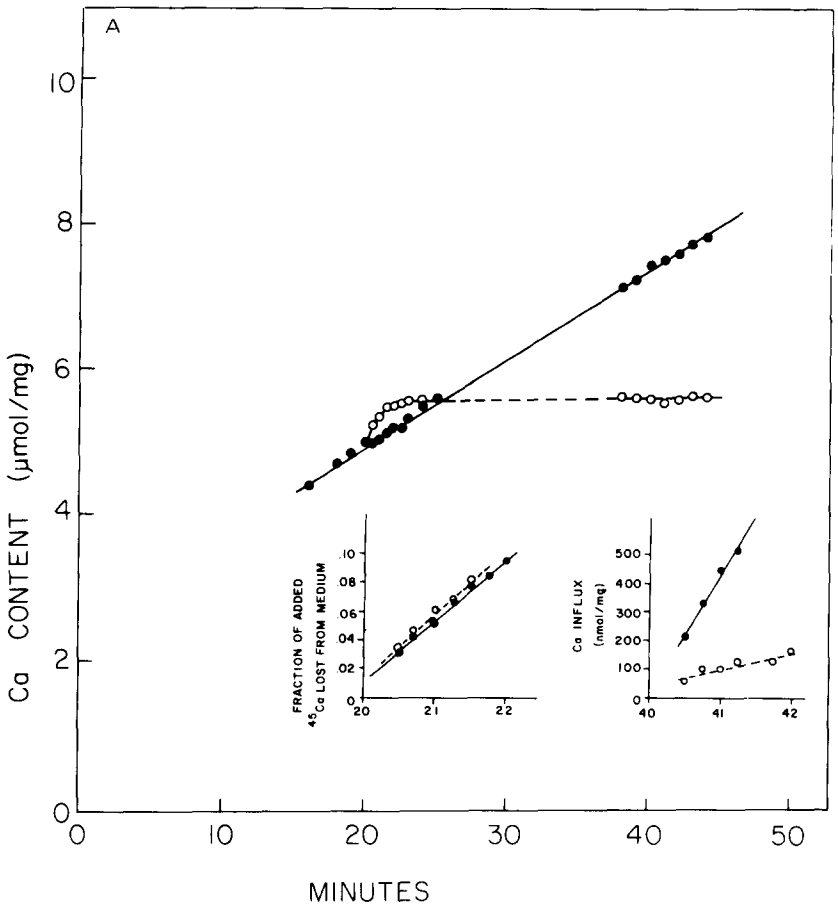


Fig. 7A.

gramicidin at 12 min to partially calcium-filled vesicles [7] resulted from an 88 ± 13% inhibition of calcium efflux; calcium influx was inhibited only 9 ± 14% (Table I).

Effect of soluble proteins on modification of calcium uptake by gramicidin

Addition of gramicidin to partially calcium-filled vesicles, 10 or 30 min after initiation of calcium uptake in a reaction mixture lacking the ATP regenerating components pyruvate kinase and phospho*enol*pyruvate, stopped the phase of calcium release and inhibited the phase of spontaneous calcium reuptake (Fig. 4). However, when gramicidin was added at any time before the initial maximum in calcium content at 6—8 min, the phase of calcium release was delayed such that the maximum calcium content of the vesicles was increased (Fig. 4).

Calcium uptake was stimulated when gramicidin was added 10 or 30 min after initiation of calcium uptake when a water soluble protein such as bovine serum albumin was included in the reaction mixture. Calcium uptake in a reaction similar to that described in Fig. 1A was stimulated maximally by gramicidin when the bovine serum albumin concentration exceeded 0.1 g/l (Fig. 5). To

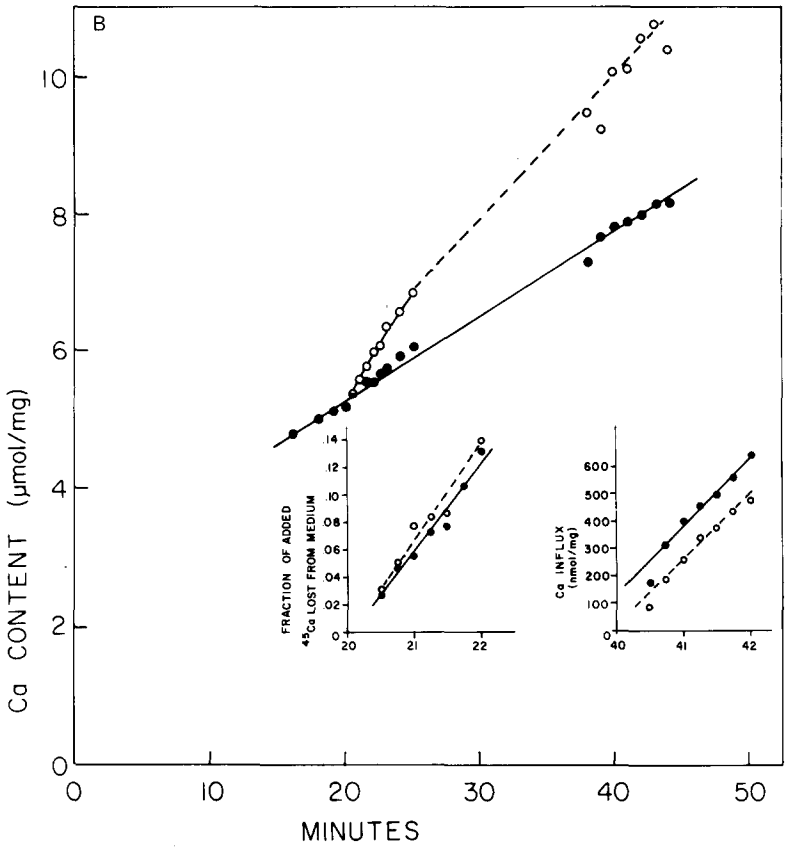


Fig. 7. Modification of calcium fluxes by gramicidin in the presence and absence of a water-soluble protein. Vesicles were partially filled with calcium as described in Fig. 3 (●) in the absence (A) or presence (B) of bovine serum albumin (0.15 mg/ml) and gramicidin (5 μg/ml) was added at 20 min (○). Calcium influx was determined as described in Methods. This flux measurement at 20 min could not be determined because calcium uptake rates were non-linear (especially in A). The rate of ^{45}Ca loss from the medium at this time is an accurate measure of calcium influx because initially, the calcium contents of control and gramicidin containing reactions are identical.

determine the order of addition of water-soluble protein and ionophore that stimulated calcium uptake maximally, the times of addition of gramicidin and the water-soluble protein creatine kinase were varied (Fig. 6). Gramicidin stimulated calcium uptake only when creatine kinase had been present prior to addition of ionophore.

The different responses to gramicidin observed in the presence or absence of pyruvate kinase (Figs. 1A and 4) did not reflect an effect of ADP, which, in the absence of pyruvate kinase, would accumulate in the reaction mixture. Instead, these different responses appeared to be due to an non-enzymatic effect of this water-soluble protein because similar results were obtained when bovine serum albumin, creatine kinase (Fig. 6) or a variety of other proteins were substituted for pyruvate kinase in experiments such as shown in Figs. 1A and 2A (in the absence of gramicidin, these proteins had no effect on calcium uptake).

The inhibition of calcium reuptake by gramicidin observed in the absence of water-soluble protein (Fig. 4) arises from an inhibition of both calcium influx

and efflux (Fig. 7A). When gramicidin was added to partially calcium-filled vesicles 20 min after the start of a reaction (Fig. 7A), calcium content transiently increased due to an inhibition of calcium efflux. This transient increase was followed by a period when calcium content remained constant so that the calcium reuptake phase was inhibited. Both calcium influx and efflux were inhibited during this plateau phase in calcium content (e.g. at 40 min in Fig. 7A). In the presence of a water-soluble protein such as bovine serum albumin (Fig. 7B), calcium reuptake was stimulated due to slowing of calcium efflux without inhibition of calcium influx.

Effect of gramicidin on Ca^{2+} content of Trypsin-treated vesicles

To determine whether the inhibition of calcium efflux by gramicidin observed in Fig. 7A was sensitive to modification of the Ca^{2+}-activated ATPase polypeptide structure, we investigated the effect of gramicidin on the calcium content of trypsin-treated vesicles. Under appropriate conditions this protease cleaves the 100 000 dalton ATPase to two 50 000 dalton fragments with no loss of calcium uptake or Ca^{2+}-activated ATPase activity [10]. Gramicidin delayed the calcium release phase of both control and trypsin-treated vesicles when added before the initial maximum in calcium content (Fig. 8). However, addition of gramicidin after this maximum increased the calcium content of trypsin-treated vesicles (Fig. 8B) whereas, as shown previously (Fig. 4), the calcium reuptake phase of control vesicles was inhibited (Fig. 8A).

Effect of KCl on the modification of Ca^{2+} uptake by ionophores

In reactions carried out in the absence of added KCl, calcium uptake was stimulated when gramicidin was present prior to initiation of the reaction (Fig. 9); bovine serum albumin did not modify this stimulation of calcium uptake. The K^+ concentration in these reaction mixtures, determined by flame photometry, was less than 5 μM. Addition of gramicidin to vesicles after the initial maximum in calcium content in the absence of added KCl was similar to that observed in the presence of KCl (Fig. 4, i.e. calcium release occurred in the absence of bovine serum albumin and calcium uptake was stimulated when the water-soluble protein was present). Although the rate of calcium uptake in 800 mM KCl was considerably slower than that in 120 mM KCl [1], the effect of water-soluble proteins on the stimulation of calcium uptake by gramicidin was the same as that observed in the absence or presence of 120 mM KCl.

Addition of either gramicidin or valinomycin to partially calcium-filled vesicles in the presence of 100 mM NaCl and pyruvate kinase also stimulated calcium uptake (Fig. 10).

Effect of other ionophores on Ca^{2+} uptake

Significant differences between the effects of different ionophores and poly-DL-alanine, an ionophore-like compound, on partially calcium-filled vesicles were observed. Calcium uptake was assayed in 0.12 mM KCl/40 mM histidine buffer, pH 6.8, 50 mM Tris phosphate, 5 mM MgATP, 72 μM $CaCl_2$, 0.15 μg/ml pyruvate kinase and 5 mM phospho*enol*pyruvate. Reactions were started by addition of vesicles (6 μg/ml) and ionophores (5 μg/ml final concentration)

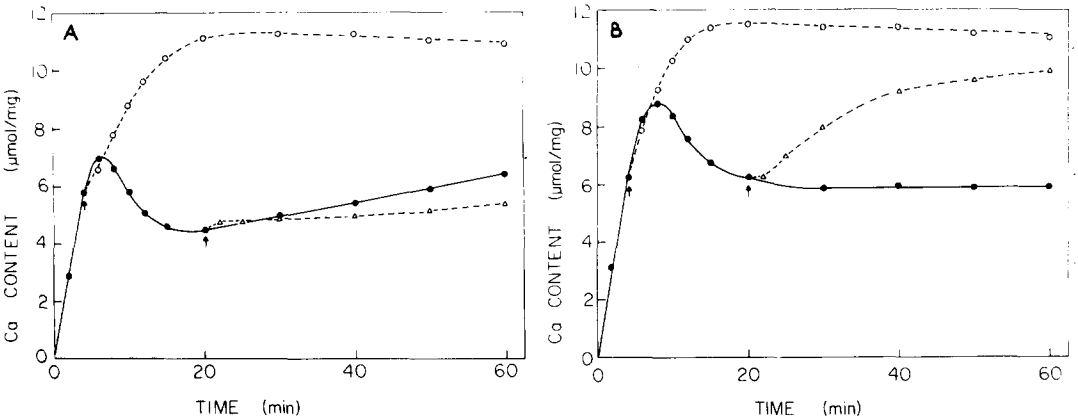


Fig. 8. Effect of gramicidin on calcium content of trypsin-treated vesicles. Gramicidin (5 μg/ml) was added at 4 (○) and 20 (△) min to control (A,●) and trypsin-treated (B,●) vesicles partially filled with calcium as described in Fig. 3. Vesicles (1 mg/ml) were treated with trypsin (17 μg) as described in Methods and following addition of trypsin inhibitor (40 μg) vesicles were used immediately in calcium uptake reactions.

were added 12 min after initiation of calcium uptake. In the presence of 0.15 g/l pyruvate kinase under these conditions, X537A and A23187 induced calcium release, as did alamethacin and gramicidin S. The monovalent cation

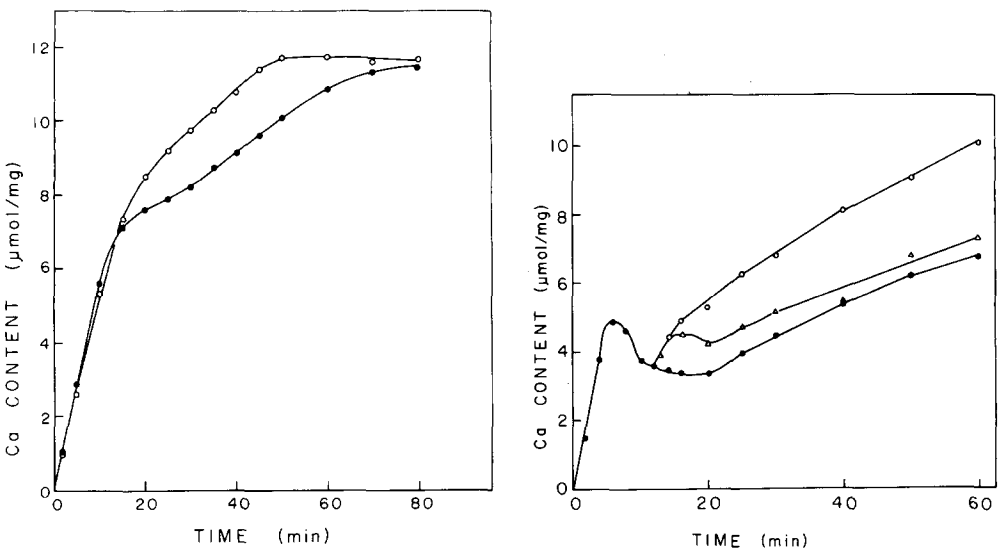


Fig. 9. Effect of gramicidin on calcium uptake by vesicles in the absence of added KCl. Calcium uptake was assayed in 40 mM histidine buffer (pH 6.8), 50 mM Tris phosphate, 5 mM MgATP and 72 μM $CaCl_2$ and reactions in the absence (●) or presence (○) of gramicidin were started by addition of vesicles (6 μg/ml).

Fig. 10. Effects of valinomycin and gramicidin on calcium uptake of vesicles in presence of NaCl. Calcium uptake was assayed in 0.1 M NaCl, 40 mM histidine buffer (pH 6.8), 50 mM Tris phosphate, 5 mM MgATP, 0.15 g/l creatine kinase and 72 μM $CaCl_2$. Reactions were started by addition of vesicles (6 μg/ml) (●). Gramicidin (○) or valinomycin (5 μg/ml (△) were added 12 min after initiation of calcium uptake.

ionophore antamanide and poly-DL-alanine were without effects, whereas gramicidin, valinomycin and nigericin all increased calcium content.

Discussion

The present study confirms and extends our previous finding [7] that monovalent cation ionophores, such as gramicidin, can stimulate calcium uptake in sarcoplasmic reticulum vesicles. Other investigators, however, have indicated that monovalent cation ionophores have no effect [11—13], or in the case of gramicidin, can inhibit calcium uptake [14]. These discrepancies appear to reflect differences in the composition of the reaction mixtures, whether maximum calcium content or rate of calcium uptake was measured [14], whether calcium binding or calcium uptake was measured [11—13], of whether soluble protein was present in the reaction mixtures. Ca^{2+} concentration was not a major factor in determining whether gramicidin stimulated calcium uptake by the vesicles as stimulation of calcium uptake by gramicidin was observed over a wide range of external Ca^{2+}. Although the present study confirms a previous report by Martonosi et al. [14] that gramicidin can inhibit Ca^{2+}-activated ase activity in 5 mM MgATP (Fig. 2B), our results with 0.5 mM MgATP demonstrate that stimulation of calcium uptake need not be accompanied by inhibition of Ca^{2+}-activated ATPase activity.

There is now considerable evidence that the calcium permeability of sarcoplasmic reticulum membranes is not constant. For example, changes in internal and external Ca^{2+} modify calcium efflux rates [3,16], and the time-dependent changes in calcium content shown in Fig. 1 are due to spontaneous changes in both calcium influx and efflux rates [1]. Gramicidin, added before the start of the calcium uptake reaction, delays the onset of spontaneous calcium efflux but has little effect on calcium influx rates measured at different times during the calcium uptake reaction (Fig. 3). The finding that gramicidin mainly influences calcium efflux rate is in accord with the observation that the ionophore had no effect on the initial calcium uptake rate because calcium efflux rate is near zero during the initial uptake phase of the reaction (Fig. 2A) [1]. Gramicidin added at the start of the reaction inhibited the calcium efflux that occurs after 6 min in the control vesicles, thereby prolonging the initial phase of calcium uptake and increasing the maximum calcium content (Fig. 3A). After 15 min, when calcium efflux from both control and ionophore-containing vesicles decreased spontaneously to similar levels, calcium reuptake rates became equal (Fig. 3A). Gramicidin, when added to partially calcium-filled vesicles in the presence of a water-soluble protein at a time when calcium efflux is rapid, also inhibited calcium efflux (Fig. 7B). Under these conditions, as when the ionophore was added prior to the start of the reaction, calcium influx was not significantly affected.

The ability of gramicidin to modify calcium influx is not due to the dissipation of a transmembrane monovalent cation because gramicidin had qualitatively the same effect on calcium uptake in the absence of added KCl (total K^+ less than 5 μM), as it did in 120 mM KCl (Fig. 9). Furthermore, preincubation of vesicles with gramicidin for 1 h prior to initiation of calcium uptake, which would have equilibrated K^+ across the membrane, resulted in the same stimula-

tion of maximum calcium content as was seen when gramicidin was added to the vesicles immediately prior to initiation of calcium uptake (Fig. 2). Valinomycin stimulated calcium uptake when reactions were carried out in media containing 100 mM NaCl (total K^+ less than 5 μM) even though it is a poor Na^+ ionophore, having a K^+ : Na^+ selectivity ratio of 17 000 : 1 [5] (Fig. 10). Thus calcium efflux could be modified by this ionophore without any change in K^+ distribution across the vesicle membrane, i.e. ionophore-induced changes in calcium efflux could occur independently of changing K^+ distribution across the vesicle membrane.

The gramicidin-induced modification in sarcoplasmic reticulum calcium fluxes is not the same at all phases of a calcium uptake reaction. Thus, whereas water-soluble proteins have only minimal effects on calcium uptake in the absence of gramicidin, their inclusion prior to addition of gramicidin modifies the response of calcium content to ionophore. In the absence of water-soluble protein, the sensitivity of calcium influx to gramicidin changes at 6—8 min (Fig. 7), which indicates that the calcium pump Ca^{2+}-ATPase undergoes a change after the first maximum in calcium content. The mechanism by which water-soluble proteins modify the action of gramicidin on sarcoplasmic reticulum membranes is not clear. However, it is unlikely to involve neutralization of sarcoplasmic reticulum membrane surface charge as high concentrations of KCl, which are known to reduce markedly electrostatic interactions of several proteins [16,17], do not qualitatively modify the response of calcium content to gramicidin. Furthermore, it is unlikely that bovine serum albumin modifies these calcium fluxes by binding gramicidin because water soluble proteins such as pyruvate kinase and creatine kinase, that do not bind appreciable amounts of fatty acids and other non-polar compounds, can substitute for bovine serum albumin.

In experiments using trypsin-cleaved vesicles, in which trypsin-cleavage of the Ca^{2+}-activated ATPase to 50 000 dalton fragments does not modify the initial rate of calcium uptake (Fig. 8B), the calcium reuptake phase may be slightly reduced.

Gramicidin, which inhibits the calcium reuptake phase in untreated vesicles in the absence of water-soluble proteins (Fig. 8A), stimulates calcium uptake when added to trypsin-cleaved vesicles (Fig. 8B). This change in the response to gramicidin after trypsin treatment of the vesicles suggests that the ionophore modifies calcium flux rates by interacting directly with the Ca^{2+}-activated ATPase rather than with the phospholipid bilayer.

That gramicidin was unable to modify calcium content of vesicles in the absence of 50 mM phosphate may be due to differences in the way that calcium flux rates change in these different experimental conditions. We did not observe a phase of calcium release in the absence of 50 mM phosphate (data not shown), and it is apparent in Fig. 9 that the effect of gramicidin on vesicle calcium content in 50 mM phosphate was significantly reduced when there was only a small phase of calcium release. Calcium flux rates might be modified by gramicidin in the absence of 50 mM phosphate, but because of the low vesicle calcium content and the relatively high external calcium concentration under these conditions, we could not determine calcium flux rates with protocols similar to those used to measure these rates in 50 mM phosphate.

The mechanism responsible for calcium efflux from sarcoplasmic reticulum vesicles remains poorly understood. It is unlikely that the calcium efflux described in the present report is associated with complete reversal of the calcium pump ATPase reaction because the rate of ATP hydrolysis remains constant throughout the period when calcium efflux rate increases and then decreases markedly (Ref. 1 and Fig. 2B). Our thesis that calcium efflux involves the calcium pump ATPase protein is supported by the observations of Jilka et al. [18] and Shamoo and MacLennan [19], who showed that the calcium permeability of membranes formed from either exogenous or vesicle phospholipids was markedly increased by the presence of this protein in these bilayer membranes. A role for the calcium pump ATPase in mediating calcium efflux is also suggested by our previous findings of a number of similarities between calcium efflux and calcium uptake via the calcium pump [3].

The present findings indicate that calcium efflux from sarcoplasmic reticulum vesicles is a regulated process; is subject to complex time-dependent changes [1]; is associated with the Ca^{2+}-activated ATPase protein and can be inhibited by certain monovalent cation ionophores such as gramicidin. The finding that not all monovalent cation ionophores modify this process indicates that movements of monovalent cations themselves are not responsible for these effects of the ionophores. That calcium efflux can be inhibited without a change in calcium influx (Fig. 3) indicates calcium influx and efflux are controlled independently in the sarcoplasmic reticulum membrane.

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