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The effects of Ca^{2+} and Sr^{2+} on Ca^{2+}-sensitive biochemical changes in human erythrocytes and their membranes

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1. The Ca^{2+}-dependency of K^+ efflux, microvesiculation and breakdown of polyphosphoinositides and of ankyrin have been measured in intact human erythrocytes exposed to ionophore A23187 and HEDTA [*N'*-(2-hydroxyethyl)ethylenediamine *NNN'*-triacetate]–Ca^{2+} buffers. Half-maximal responses were observed at pCa values of 6.4, 4.1, 5.0 and 4.8 respectively. 2. The Ca^{2+} dependencies of K^+ efflux and breakdown of polyphosphoinositides and ankyrin measured in erythrocyte ghosts without addition of ionophore showed almost identical values with those seen in whole cells treated with ionophore. 3. We conclude that ionophore A23187 is able to cause rapid equilibration of extracellular and intracellular [Ca^{2+}] in intact cells and that in the presence of a suitable Ca^{2+} buffer, ionophore A23187 can be used to precisely fix the intracellular concentration of Ca^{2+} in erythrocytes. 4. The relatively high concentration of Ca^{2+} required to produce microvesiculation in intact cells may indicate that microvesiculation could be at least partly dependent on a direct interaction of Ca^{2+} with phospholipid. 5. Results obtained with Sr^{2+} paralleled those with Ca^{2+}, although higher Sr^{2+} concentrations were required to achieve the same effects as Ca^{2+}. Mg^{2+} produced none of the changes seen with Ca^{2+} or Sr^{2+}.

In the preceding paper (Allan & Thomas, 1981) we showed that in order for microvesiculation of human erythrocytes to occur the cells had (*a*) to undergo KCl efflux resulting in cell shrinkage, (*b*) to experience a rise in intracellular Ca^{2+} concentration and probably (*c*) to suffer breakdown of membrane polyphosphoinositides (phosphatidylinositol 4-phosphate + phosphatidylinositol 4,5-bisphosphate).

We decided to investigate the effect on the above changes of variation in the external [Ca^{2+}], reasoning that it might be possible to uncouple the presumed linkage between these different events if they had different Ca^{2+} dependencies. It was assumed that in the presence of ionophore A23187, the internal [Ca^{2+}] would depend on the external [Ca^{2+}] but initially no assumptions were made regarding the precise internal concentration of Ca^{2+}. However, some of the changes produced by ionophore A23187 + Ca^{2+} in whole cells could also be brought about in isolated cell membranes treated with different concentrations of Ca^{2+} in the absence of ionophore A23187, so that measurements on membranes provided an independent way of assessing the Ca^{2+} requirement for each biochemical parameter, assuming, of course, that these parameters had the same Ca^{2+}-sensitivity in isolated membranes as they did in intact cells. The results showed that there was little difference between the Ca^{2+}-sensitivities of the various biochemical changes measured in ghosts and the apparent sensitivities measured in intact cells treated with ionophore A23187 in the presence of Ca^{2+}-buffers.

Abbreviation used: HEDTA, *N'*-(2-hydroxyethyl)-ethylenediamine *NNN'*-triacetate.

Materials and methods

Packed human erythrocytes were prepared from fresh blood as described by Allan & Thomas (1981). Membranes were produced by the procedure of Allan *et al.* (1980) and were finally sedimented by centrifugation in 130mM-NaCl/200mM-4-morpholinepropanesulphonic acid/NaOH buffer, pH 7.1.

Cells (0.5ml) were incubated for 3min at 37°C in 4.5ml of the same buffer (with the addition of 10mM-glucose) in the presence of various concentrations of Ca^{2+} buffered with 10mM-HEDTA and 5μM-ionophore A23187 (Eli Lilly Co., Indianapolis, IN, U.S.A.). Membranes (0.5ml) were

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incubated similarly but without addition of ionophore. Further experiments were performed on cells and membranes that were exposed to Sr^{2+} instead of Ca^{2+}. Incubations were terminated by addition of 200–500 μl of 100 mM-EDTA.

Ca^{2+} was added in the form of $CaCl_2$ (1 M standard AnalaR solution; BDH Chemicals, Poole, Dorset, U.K.). Spectroscopic grade $SrCl_2$ (BDH), containing less than 5 p.p.m. Ca^{2+}, was used. HEDTA was obtained from Sigma Chemical Co. It was found to lose 9% of its weight on drying and a correction was made for this.

K^+ efflux and microvesicle release from cells and loss of polyphosphoinositides, synthesis of phosphatidate and breakdown of polypeptide 2.1 (ankyrin) to polypeptide 2.3 in cells and membranes were measured as described in the preceding paper (Allan & Thomas, 1981).

Values for concentrations of free Ca^{2+} present with various ratios of total Ca^{2+} and HEDTA were deduced from the data of Raaflaub (1960). These values agreed well with independent determinations of free Ca^{2+} using a Ca^{2+}-sensitive electrode (Pye–Unicam, Cambridge, U.K.) (see Fig. 1).

The apparent stability constant for the Ca^{2+}–HEDTA complex was taken as $10^{5.66}$ at 37°C, pH 7.1 and ionic strength 0.1 (Simons, 1976*a*). Buffered Sr^{2+} solutions were prepared as for Ca^{2+}, assuming the value of $10^{4.29}$ for the apparent stability constant of the Sr^{2+}–HEDTA complex under similar conditions (Simons, 1976*b*). Above 0.1 mM-Ca^{2+} or

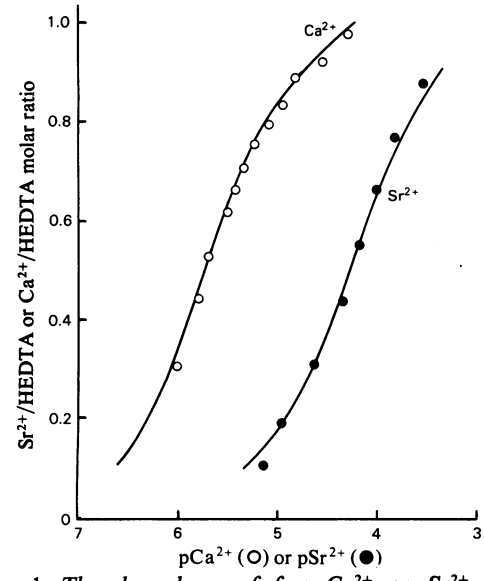

Fig. 1. *The dependence of free Ca^{2+} or Sr^{2+} concentration on the ratio of Ca^{2+}_{total}/HEDTA or Sr^{2+}_{total}/HEDTA*

Various amounts of Ca^{2+} or Sr^{2+} were added to 10 mM-HEDTA in 20 mM-4-morpholinepropanesulphonic acid/NaOH buffer, pH 7.1, and resulting free bivalent cation concentrations were calculated as described in the Materials and methods section (——). These values are compared with Ca^{2+}-electrode measurements (O and ●). Closely similar results were seen in four experiments.

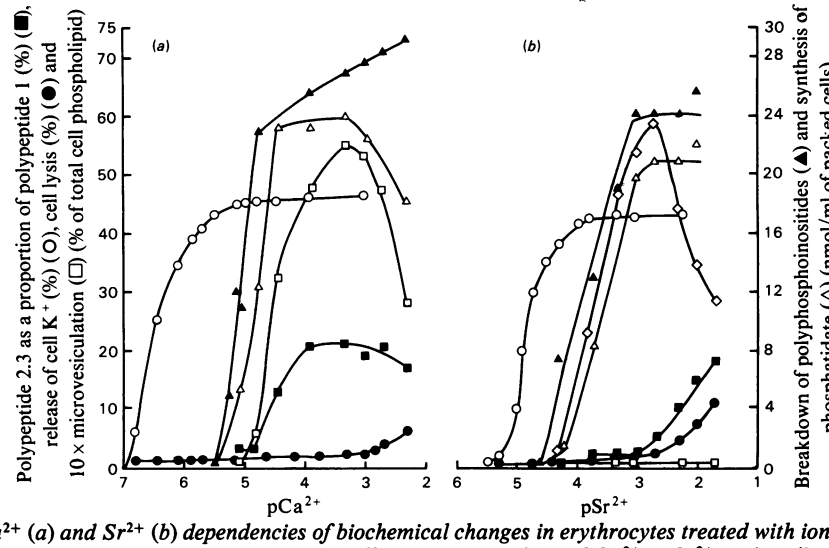

Fig. 2. *Ca^{2+} (a) and Sr^{2+} (b) dependencies of biochemical changes in erythrocytes treated with ionophore A23187*

Cells were treated with ionophore A23187 and buffered concentrations of Ca^{2+} or Sr^{2+} as described in the Materials and methods section. Incubations were carried out for 5 min at 37°C with 5 μM-ionophore A23187, except in the case of microvesiculation induced by Sr^{2+}, where incubation was for 15 min with 10 μM-ionophore A23187. ●, Cell lysis; O, total cell K^+ released; ▲, breakdown of polyphosphoinositides; △, synthesis of phosphatidate; ■, polypeptide 2.3 as a proportion of polypeptide 1 (ankyrin breakdown); □, total cell lipid released as microvesicles; ◇, total cell lipid released as microvesicles after incubation with Sr^{2+} and 10 μM-ionophore A23187 for 15 min. The data from (*a*) and (*b*) were obtained from two separate erythrocyte samples. Similar results were seen with six further samples of cells.

1 mM-Sr^{2+}, unbuffered bivalent cation was added. All Ca^{2+} and Sr^{2+} buffer solutions were carefully adjusted to pH 7.1 if necessary.

Results

Fig. 2(*a*) shows the influence of variation of the external [Ca^{2+}] on the various biochemical changes undergone by intact erythrocytes in the presence of ionophore A23187. The results of several experiments are combined in Table 1, which shows the mean values for the [Ca^{2+}] giving half-maximal changes in each of the measured parameters. K^+ efflux was obviously more sensitive to Ca^{2+} than were any of the other changes; it occurred at [Ca^{2+}] 20–50-fold less than breakdown of polyphosphoinositides or ankyrin. As expected from their close metabolic connection (Allan & Thomas 1981), breakdown of polyphosphoinositides and synthesis of phosphatidate showed similar Ca^{2+}-dependencies, resembling that observed previously for phosphatidate synthesis (Allan *et al.*, 1976*a*). Microvesiculation required relatively higher [Ca^{2+}], reaching a peak near 1 mM-Ca^{2+}, but declining at still higher [Ca^{2+}]. It seems possible that this inhibitory factor may have led to an underestimate of the maximal microvesiculation and hence of the [Ca^{2+}] required for half-maximal microvesiculation. At 20 mM-Ca^{2+}, microvesiculation was very low and was obscured by increasing cell lysis.

Fig. 2(*b*) and Table 1 demonstrate that similar results were obtained when cells were treated with Sr^{2+} instead of Ca^{2+} in the presence of ionophore A23187. It was clear, however, that 5–100-fold higher Sr^{2+} concentrations were required to achieve the same effects as those obtained with Ca^{2+}. To obtain significant microvesiculation with Sr^{2+} it was necessary to incubate with a higher concentration of ionophore A23187 and for longer periods than was necessary with Ca^{2+} (Fig. 2*b*). Mg^{2+} substituted for Ca^{2+} in the above experiments produced none of the

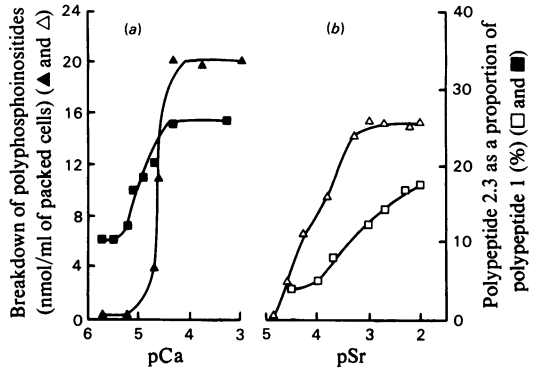

Fig. 3. *Ca^{2+} (a) and Sr^{2+} (b) dependencies of polyphosphoinositides and ankyrin breakdown in erythrocyte membranes*

Membranes were incubated with Ca^{2+} and Sr^{2+} buffers as described in the Materials and methods section and measurements were made of polyphosphoinositides (▲, △) and polypeptide 2.3 (■, □) content in the treated membrane. Essentially similar results were seen in three further experiments.

Table 1. *Concentrations of free Ca^{2+} and Sr^{2+} that cause half-maximal Ca^{2+}-sensitive changes in intact erythrocytes and membranes*

Concentrations of Ca^{2+} and Sr^{2+} giving half-maximal changes were determined in several experiments (numbers in parentheses) of the same type as those shown in Fig. 2. Values are means ± S.D. of pCa or pSr.

	pCa or pSr giving half-maximal change of:				
	K^+ efflux	Polyphosphoinositide breakdown	Phosphatidate synthesis	Increase in polypetide 2.3	Microvesiculation
(*a*) Ca^{2+}					
Cells + ionophore A23187	6.4 ± 0.1 (4)	5.0 ± 0.3 (3)	4.8 ± 0.2 (5)	4.8 ± 0.1 (3)	4.1 ± 0.3 (4)
Membranes (no ionophore A23187)	6.7–6.3 (Porzig, 1975) 6.4 (Romero, 1976) 6.4 (Simons, 1976*a*,*b*)	4.8 ± 0.3 (3)	—	4.9 ± 0.2 (3)	—
(*b*) Sr^{2+}					
Cells + ionophore A23187	5.0 ± 0.3 (3)	3.7 ± 0.3 (3)	3.5 ± 0.4 (3)	2.4 ± 0.2 (4)	<1.5 (3)*
Membranes (no ionophore A23187	5.3 (Simons, 1976*b*)	3.9 ± 0.2 (3)	—	3.2 ± 0.2 (4)	—

* After 5 min with 5 μM-ionophore A23187 no significant microvesiculation was seen with Sr^{2+} at concentrations up to 20 mM. However, when cells were incubated for 15 min with 10 μM-ionophore, half-maximal microvesiculation was observed at pSr 3.7 ± 0.3 (3).

changes achieved with Ca^{2+} or Sr^{2+} (results not shown).

Experiments in which erythrocyte membranes were treated with Ca^{2+} or Sr^{2+} without ionophore A23187 mimicked some of the changes seen in intact cells treated with ionophore (Figs. 3*a* and 3*b*; Table 1). It was clear that with Ca^{2+} the ion requirement for both polyphosphoinositide and ankyrin breakdown was almost the same in membranes as it was in intact cells. This applied also to polyphosphoinositide breakdown induced by Sr^{2+}, but not apparently to Sr^{2+}-stimulated ankyrin breakdown, where intact cells required higher Sr^{2+} concentrations than membranes to produce the same increase in polypeptide 2.3 (Table 1).

It is interesting to note that large increases in some of the measured parameters occurred over a relatively narrow range of Ca^{2+} concentrations, e.g. polyphosphoinositide breakdown in cells (Fig. 2*a*) or ghosts (Fig. 3*a*) where increases from 10% to 90% of maximal response occurred over only a 3-fold range of $[Ca^{2+}]$. This could indicate a co-operative interaction of Ca^{2+}.

Discussion

Although, as shown in the preceding paper (Allan & Thomas, 1981), KCl efflux resulting in cell shrinkage is essential in order for Ca^{2+}-induced microvesiculation to occur, it is clear from the present work that K^+ efflux can occur at much lower $[Ca^{2+}]$ than those required for microvesiculation (Fig. 2*a*). This observation confirms the conclusion that KCl efflux itself is not sufficient to produce microvesiculation (Allan & Thomas, 1981). A similar argument applies to the relationship between breakdown of polyphosphoinositides and microvesiculation and it seems clear from Fig. 2(*a*) that even a combination of K^+ efflux and polyphosphoinositide breakdown (e.g. at pCa 5.0) allows little microvesiculation. This is also true for Sr^{2+}, where under conditions producing maximum K^+ release, polyphosphoinositide breakdown and phosphatidate synthesis (e.g. pSr 3 in the standard incubation conditions) no microvesiculation occurred at all (Fig. 1). Microvesiculation only occurred with Sr^{2+} in the presence of higher concentrations of ionophore A23187 and with longer incubation periods (Fig. 2, Table 1). The reason for this contrast between the effects of Ca^{2+} and Sr^{2+} on microvesiculation is not clear.

Microvesiculation requires comparatively high $[Ca^{2+}]$, suggesting that the molecular site that binds Ca^{2+} and controls the microvesiculation process has a relatively low affinity for this ion. At these concentrations Ca^{2+} may act via a direct physical interaction with negatively-charged membrane phospholipids. The apparent association constant for the interaction of Ca^{2+} with negatively charged lipids is of the order of $10^4 M^{-1}$ (Hendrickson & Fullington, 1965; Abramson *et al.*, 1968; Barton, 1968) and in the presence of Ca^{2+} these lipids are known to undergo alterations in their physical structure that may be similar to the changes undergone during fusion of biological membranes (Papahadjopolous *et al.*, 1974, 1976). Thus the fusion event implicit in microvesiculation may depend on titration of negatively-charged lipids with Ca^{2+}. It may be significant that the gross intracellular concentrations of phosphatidylserine (the major anionic lipid of human erythrocytes) is about 1 mM, which is similar to the concentration of Ca^{2+} that induces maximum microvesiculation in the presence of ionophore A23187. This hypothesis could be consistent with the ideas of Cullis & Hope (1978), who have suggested that the interaction of Ca^{2+} with negatively-charged lipids might precipitate a change in membrane phosphatidylethanolamine from a bilayer configuration to an inverted micellar structure known as 'hexagonal H_{II}', which increases the likelihood of membrane fusion. Such a process could be particularly significant for the erythrocyte membrane, whose internal lipid leaflet consists largely of phosphatidylserine and phosphatidylethanolamine.

The chief argument against the proposition that Ca^{2+} acts to produce microvesiculation of erythrocytes through such interactions with negatively-charged phospholipids is that microvesiculation seems to be very ion-specific. Mg^{2+} will not induce microvesiculation even though it does interact with negatively-charged lipids in a similar way to Ca^{2+} (Papahadjopolous *et al.*, 1976). It therefore cannot be excluded that interaction of Ca^{2+} or Sr^{2+} with a specific structural protein may be crucial to the vesiculation process. It is interesting in this connection to note recent work by Mombers *et al.* (1980) that shows that spectrin interacts specifically with negatively-charged lipids and that Ca^{2+} promotes the extrusion of spectrin from a phosphatidylserine monolayer. Such a process occurring in the intact erythrocyte treated with Ca^{2+} and ionophore A23187 could explain the observed exclusion of spectrin from microvesicles budding from the membrane bilayer (Allan *et al.*, 1976*b*).

The Ca^{2+} requirement we have observed for K^+ efflux agrees well with the Ca^{2+} sensitivities of K^+ efflux measured in intact cells that had been ATP-depleted (Ferreira & Lew, 1976). These authors suggest that K^+ efflux in fresh cells shows a lower and variable affinity for Ca^{2+} (see Lew & Ferreira, 1978), but we have been unable to confirm this with the fresh cells that we have used. Lew & Ferreira (1978) do see a high-affinity pattern for Ca^{2+} under conditions where the Ca^{2+} pumping mechanism cannot keep pace with Ca^{2+} entry, e.g. after ATP depletion, Mg^{2+} depletion, cell aging or

at high concentrations of ionophore A23187. The conditions that we have employed include the use of relatively high ionophore A23187 and Ca^{2+} buffer concentrations, which would tend to swamp the effect of the Ca^{2+} pumping mechanism, and thus to reveal only a single high-affinity requirement for Ca^{2+} in this system. Possibly the apparent low-affinity pattern seen by Lew & Ferreira is a consequence of a non-equilibrium situation caused by an active Ca^{2+}-pump opposing the ionophore A23187-induced Ca^{2+} influx.

The Ca^{2+} requirement for breakdown of polyphosphoinositide and synthesis of phosphatidate in intact cells in the present experiments is consistent with that measured previously (Allan *et al.*, 1976*b*), but there is an obvious discrepancy between the present results for isolated membranes as compared with those reported previously (Allan & Michell, 1976), where half-maximal production of diacylglycerol was observed at concentrations of free Ca^{2+} below $1\mu M$. The reason for this discrepancy is unknown, but the present data (themselves) agree broadly with results reported by Downes & Michell (1981), although these authors show a less co-operative dose–response relationship than we have observed. In general our dose–response curves appear to be rather co-operative (Fig. 2). This could be due to an involvement of calmodulin in the case of the intact cells, but this interpretation is less viable in the experiments with well-washed ghosts (Fig. 3).

It is noteworthy that in Table 1 there is a close correspondence between the Ca^{2+}-sensitivities of the various parameters measured in intact cells treated with ionophore A23187 and in isolated cell membranes exposed to Ca^{2+} (and to a lesser extent, Sr^{2+}) without ionophore. This result would only be expected if under the conditions of these experiments ionophore A23187 were able to rapidly produce equilibration of the external and internal Ca^{2+} concentration. Such an interpretation is supported by recent work of Reichstein & Rothstein (1981). It would strongly suggest that ionophore A23187 in the presence of Ca^{2+} buffer could fix the Ca^{2+} concentration within erythrocytes and this would make it possible to investigate the effect of precise concentrations of Ca^{2+} on other kinds of biological activity in erythrocytes besides the ones considered in the present work. The possibility of extending this procedure to other cell types seems less likely since nucleated cells should have more effective forms of internal Ca^{2+}-buffering (e.g. mitochondria) than do erythrocytes.

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