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ACTION OF DI- AND TRI-VALENT CATIONS ON CALCIUM-ACTIVATED K^+-EFFLUX IN RAT ERYTHROCYTES

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Abstract—Isolated rat erythrocytes were labelled with [^{86}Rb] as a tracer for intracellular K^+. It was demonstrated that rat erythrocytes possess a Ca^{2+}-mediated K^+-efflux mechanism similar to that reported for human erythrocytes. This model was used to investigate the interactions of di- and tri-valent cations on potassium [^{86}Rb] permeability in intact cells. Low concentrations of Ag^{2+} and Hg^{2+} haemolysed erythrocytes and Pb^{2+} produced a selective increase in [^{86}Rb] efflux which became self-inhibitory at concentrations above 100 μM. The effects of Pb^{2+} were potentiated by A23187. Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Y^{2+} and Ba^{2+} did not initiate [^{86}Rb] efflux, even in the presence of 0.5 μM A23187 and at concentrations as high as 1 mM. All of these cations, except Ba^{2+}, were potent inhibitors of [^{86}Rb] efflux evoked by 50 μM Ca^{2+} + 0.5 μM A23187. The lanthanides Tb^{3+}, Gd^{3+}, Eu^{3+}, Sm^{3+} and La^{3+} increased [^{86}Rb] efflux at low concentrations in the presence of A23187, but were self inhibitory at higher concentrations. They also inhibited Ca^{2+}-mediated [^{86}Rb]-efflux. It is concluded that the effectiveness of a cation in activating [^{86}Rb] efflux is, in part, related to its non-hydrated crystalline ionic radius, and that the site of activation may only accommodate ionic radii between 0.95 and 1.00 A.

A number of multi-valent cations, such as Cd^{2+}, Pb^{2+} and Hg^{2+}, are significant environmental pollutants which exhibit a wide variety of cellular toxic effects under different experimental conditions. This has made it difficult to construct a satisfactory hypothesis to explain the diversity of metal-induced cellular damage at a molecular level. The importance of Ca^{2+} in mediating and regulating numerous cell functions is widely recognised [1] and the potential interference of heavy metals with the cellular actions of calcium may provide a new perspective in understanding their mode of action. Numerous reports indicate that multi-valent cations can act as calcium antagonists. For example, Cd^{2+} reduces the responses of smooth muscle to a wide variety of agonists, probably by decreasing the availability of calcium [2], and both Pb^{2+} and Cd^{2+} are powerful inhibitors of Ca^{2+}-activated acetylcholine release both from amphibian [3] and mammalian nerve muscle preparations [4]. In addition to such well documented inhibitory effects, a number of multi-valent cations have calcium-mimetic actions, particularly at low concentrations and in the absence of extracellular Ca^{2+} or Mg^{2+}. Manganese supports dopamine release from striatal synaptosomes [5], Sr^{2+} and Ba^{2+} release neurotransmitters from squid synapses [6], La^{3+} acts as a calcium surrogate at mouse motor nerve terminals [7], and Zn^{2+} generates action potentials in snail neurons [8]. When a range of concentrations are studied biphasic responses may be observed, when low concentrations of cations mimic the actions of calcium, and high concentrations act as calcium antagonists. This has been reported for the effect of Mn^{2+} on prolactin secretion [9], La^{3+} on amylase secretion [10], and lanthanides on histamine release from rat peritoneal mast cells [11, 12]. To explain these diverse effects of heavy metals Cheung [13] has hypothesised that the intracellular targets for heavy metals are the intracellular calcium-binding proteins where the metals substitute for calcium with resulting disruption of normal calcium homeostasis. This hypothesis is supported by the observations that the heavy metals Pb^{2+}, Cd^{2+} and La^{3+} bind to calmodulin and other intracellular calcium-binding proteins in a manner similar to calcium [14–16], and that low concentrations of heavy metals can stimulate [17, 18] and high concentrations inhibit [19] calmodulin-dependent activation of phosphodiesterase *in vitro*.

In a wide variety of cells, for example cardiac Purkinje fibres [20], intestinal smooth muscle cells [21], amphibia motor neurones [22] and guinea-pig hepatocytes [23], raising the intracellular calcium increases K^+-permeability. The most widely studied Ca^{2+}-sensitive K^+-permeability mechanism exists in erythrocytes [24] and this paradigm of a calcium-mediated process was chosen to investigate the ability of heavy metals to substitute for Ca^{2+} in evoking K^+ release. As part of the hypothesis being tested was that multi-valent cations with high toxicity might exert part of their effect through some interaction with calcium, it seemed appropriate to investigate a number of cations to see whether those of high toxicity differed in any fundamental way from those of lower toxicity. A preliminary account of some of these results has been represented [25].

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MATERIALS AND METHODS

Cell preparation. Blood (10 ml) was removed from anaesthetised male rats through the abdominal vena cava using heparinized syringes. The erythrocytes were separated by low speed centrifugation and the plasma and buffy coat removed by aspiration. The cells were washed by centrifugation, twice with 10 ml 150 mM NaCl, 1 mM EGTA, 5 mM HEPES*–NaOH pH 7.1 and three times with $NaNO_3$ buffer (150 mM $NaNO_3$, 1 mM KNO_3, 0.5 mM $MgSO_4$, 5 mM HEPES pH 7.1), removing the supernatant and top layer of erythrocytes between each wash. The erythrocytes were resuspended in 25 ml $NaNO_3$ buffer and incubated for 30–40 min at 36° with 10–25 μCi [^{86}Rb] (Radiochemical Centre, Amersham) to act as a tracer for intracellular K^+. The cells were diluted to 80 ml with $NaNO_3$ buffer containing 1 mM EGTA to remove traces of extracellular Ca^{2+} resulting from the slight haemolysis that occurred during the incubation. The cells were washed once more in $NaNO_3$–EGTA and three times in $NaNO_3$ buffer without EGTA to remove both EGTA and unincorporated [^{86}Rb] before finally being resuspended in 80 ml $NaNO_3$ buffer at room temperature. Erythrocytes were used within 2 hr of collection. More than 96% of [^{86}Rb] was intracellular.

[^{86}Rb] *release.* All incubations were carried out in polystyrene tubes. Each tube contained 50 μl glass distilled water in which the appropriate cations were dissolved to give a final concentration over the range 0.1–1000 μM with or without the divalent ionophore A23187 (Sigma) at a final concentration of 0.5 μM. [^{86}Rb] release was initiated by adding 450 μl erythrocyte suspension and incubating at 36° with gentle shaking. After 5 min, 2 × 200 μl samples were removed and the erythrocytes separated over 500 μl silicone oil (bromododecone 372 g: dodecone 7 g, Aldrich Chemical Co.) at 12,000 *g* for 30 sec. Aliquots (100 μl) of the supernatants were taken for [^{86}Rb] determination by scintillation counting, and the remainder of the supernatant and oil removed by aspiration. The pellet was resuspended in 0.4 M perchloric acid, the precipitated protein removed by centrifugation, and a 50 μl aliquot of the supernatant taken for scintillation counting. [^{86}Rb] counts were corrected from a quench curve prepared by adding aliquots of perchloric acid-treated erythrocytes to a known quantity of [^{86}Rb]. No correction was made for [^{86}Rb] decay. [^{86}Rb] released is expressed as a % of the total [^{86}Rb] appearing in the supernatant.

$$\%\ [^{86}\text{Rb}]\ \text{released} = \frac{\text{cpm supernatant}}{\text{cpm (supernatant + pellet)}} \times 100$$

Haemolysis of the cells was monitored by visual inspection for haemoglobin in the supernatants, and checked at least once in all samples showing greater than 20% [^{86}Rb] release by measuring lactic dehydrogenase (Sigma Kit NO 340-U.V.) in the supernatant.

* Abbreviations used: EGTA, ethyleneglycol-bis-(β-amino ethylether)- *N,N,N,*^{1}*N*^{1}-tetra acetic acid; HEPES, *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid.

Reduction of cellular Ca^{2+} *levels.* Erythrocytes were washed as before except that during the labelling stage they were incubated with 1 μM A23187 and 2 mM EGTA in addition to [^{86}Rb] in order to reduce intracellular Ca^{2+}. To remove the A23187 at the end of the incubation 1% bovine serum albumin (Sigma) was added to the $NaNO_3$–EGTA wash buffer [26]. Control cells, incubated without 2 mM EGTA and 1 μM A23187, were washed in an identical manner.

Reagents and solutions. All solutions were made from Analar grade chemicals and were obtained from BDH except for europium (III) nitrate pentahydrate, terbium (III) nitrate pentahydrate, lanthanum chloride heptahydrate, samarium (III) chloride hexahydrate, gadolinium nitrate pentahydrate, ytterbium (III) nitrate pentahydrate (Aldrich Chemical Co). Salts were dissolved in distilled water at a concentration of 10 mM and diluted in $NaNO_3$ buffer immediated before use. Fresh solutions were prepared for each experiment. A23187 was dissolved in dimethylsulphoxide and stored at −20°. The final concentration of dimethylsulphoxide in the cell suspension was 0.5%. Control experiments demonstrated that this concentration did not affect any of the parameters studied.

RESULTS

The present experiments demonstrate that rat erythrocytes, unlike rat hepatocytes [23], possess a calcium-dependent K^+ permeability mechanism similar to the well characterised mechanism that exists in human erythrocytes and other cells. Rat erythrocytes pre-labelled with [^{86}Rb] as a tracer for intracellular K^+ only slowly release their [^{86}Rb] following incubation in Ca^{2+}-free of Ca^{2+}-containing buffers (results not shown). In the absence of added extracellular Ca^{2+} 0.5 μM A23187 did not trigger [^{86}Rb] release, but the addition of a low concentration of calcium led to rapid release of [^{86}Rb] that reached a maximum of about 800 μM Ca^{2+} and then declined slightly (Fig. 2). The calcium-dependent release was completely abolished by excess EGTA or a low concentration of Ni^{2+} or Co^{2+}. This rat erythrocyte model was used to investigate the effects of multivalent cations on the calcium-activation of [^{86}Rb] efflux.

[^{86}Rb] *release in the absence of* 0.5 μM A23187

Iron, manganese, cadmium and the lanthanides, except for gadolinium and lanthanum, caused cell agglutination and 15–20% [^{86}Rb] release without haemolysis at concentrations above 500–800 μM. The effect was variable from experiment to experiment both in the amount of cell clumping and [^{86}Rb] released, and is assumed to be a non-specific effect upon the cell surface. Neither agglutination nor [^{86}Rb] release was observed in any experiment at a cation concentration below 500 μM.

Action of Ag^+ *and* Hg^{2+}

Both Ag^+ and Hg^{2+} produced a concentration dependent haemolysis of rat erythrocytes in the absence of chloride ions. Ag^+ was more potent than Hg^{2+}, with visible haemolysis occurring at con-

centrations as low as 5 μM, so it was not possible to demonstrate any selective effect of Ag^+ on [^{86}Rb] efflux independent of haemolysis. At concentrations below 100 μM, Hg^{2+} had a selective effect on [^{86}Rb] release, but at higher concentrations, [^{86}Rb] release was associated with haemolysis. There was no evidence that the effect of either cation was potentiated by 0.5 μM A23187.

Action of Pb^{2+}

Lead did not produce haemolysis over the concentration range 1–1000 μM; and the [^{86}Rb] efflux elicited by increasing concentrations of lead is shown in Fig. 1. An analysis of the dose–response curve shows that [^{86}Rb] release slowly increased with lead concentration to reach a maximum of 40% cellular [^{86}Rb] at an external lead concentration of 100–200 μM. Above this [^{86}Rb] efflux was reduced, indicating a self-inhibition with increasing concentrations of lead. It is unlikely that the latter inhibition was due to blockage of K^+-efflux, or the anion (NO_{3-}) transport that accompanies [^{86}Rb] efflux, as valinomycin still released [^{86}Rb] at lead concentrations as high as 1 mM. In the presence of 0.5 μM A23187 the action of lead in triggering [^{86}Rb] efflux was significantly potentiated (Fig. 1), so that approximately 80% of [^{86}Rb] was released at an external lead concentration of 50–100 μM, and as maximum enhancement of [^{86}Rb] efflux occurred at lower lead concentrations, the dose–response curve was displaced to the left. A23187 may also enhance the self-inhibitory effect of lead at concentrations above 100 μM, but this was difficult to determine in the presence of enhanced [^{86}Rb] efflux. The [^{86}Rb] efflux evoked by 50 μM Ca^{2+} and 0.5 μM A23187 was not potentiated by a low concentration of lead and, at concentrations above 800 μM, lead inhibited calcium-mediated [^{86}Rb] efflux (Table 1).

Action of Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+} *and* Mn^{2+}

These elements belong to the transition series of elements and are neighbours in Period IV of the periodic table. These ions did not release [^{86}Rb] in the presence or absence of 0.5 μM A23187, except at concentrations that caused agglutination of the cell suspension. This effect was absent for Ni^{2+}, Co^{2+} and Zn^{2+} over the concentration range 1–1000 μM. Mn^{2+} (800–1000 μM) caused agglutination without releasing [^{86}Rb], Fe^{2+} (800–1000 μM) also caused agglutination and released approximately 50% of the total [^{86}Rb] and 10–15% of the total lactic dehydrogenase. These effects of Fe^{2+} were potentiated by 0.5 μM A23187. Cu^{2+} was without effect until a concentration of 800 μM, when it produced complete haemolysis both in the presence and absence of 0.5 μM A23187. At low concentrations all the ions in the group were inhibitors of [^{86}Rb] release evoked by 50 μM Ca^{2+} + 0.5 μM A23187 (Table 1). The order of potency was $Ni^{2+} > Co^{2+} = Mn^{2+}$ (= Zn^{2+}) > $Fe^{2+} > Cu^{2+}$ and the inhibitory effect of the ions was surmountable by increasing the external calcium concentration to 100 μM. Zn^{2+} was a power-

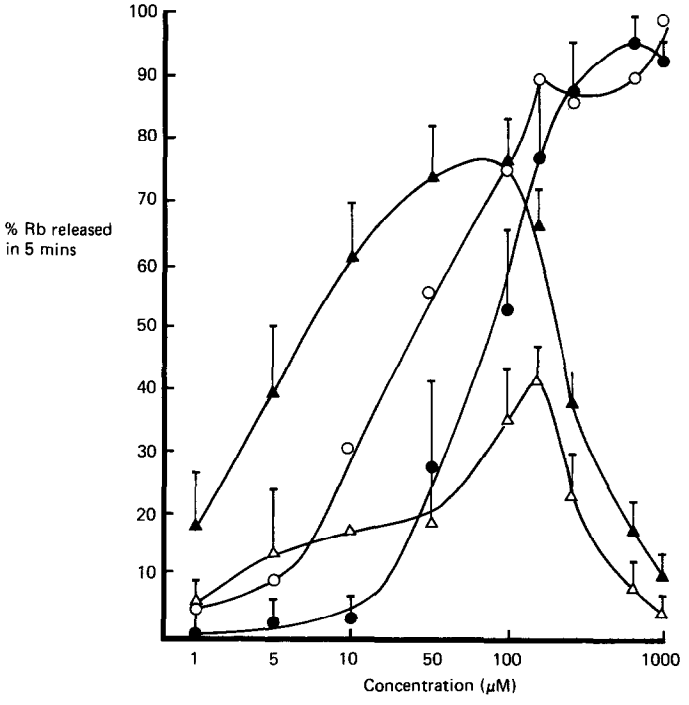

Fig. 1. Action of Ag^+, Hg^{2+}, Pb^{2+} and Pb^{2+} + A23187 on [^{86}Rb] release from rat erythrocytes. ○ Ag, ● Hg^{2+}, □ Pb^{2+} and ■ Pb^{2+} + 0.5 μM A23187. Results are expressed as the % of [^{86}Rb] appearing in the supernatant after 5 min incubation. Each point represents mean ± SEM of at least four determinations except for Ag which is the mean from two experiments. 0.5 μM A23187 did not increase % [^{86}Rb] efflux occurring with Ag^+ or Hg^{2+} so these results are not displayed. Ag^+ produced a concentration-dependent haemolysis over the whole concentration range and Hg^{2+} haemolysis above 100 μM.

Table 1. Effect of period 4 elements on [^{86}Rb] release evoked by 50 μM Ca^{2+} + 0.5 μM A23187

	% [^{86}Rb] release					
	Cu^{2+}	Fe^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}	Ni^{2+}
50 μM Ca^{2+} + A23187						
Cation (conc μM)	100	100	100	100	100	100
0.1	89 (2)	90 (2)	79 (2)	123 ± 6 (4)	47 ± 8 (4)	22 ± 2 (4)
1.0	55	87	48	187 ± 16	7 ± 2	—
5.0	55	89	9	169 ± 2	5 ± 2	—
10.0	50	89	3	117 ± 10	6 ± 2	—
50	30	77	—	10 ± 2	4 ± 2	—
100	32	54	—	6 ± 2	—	—
200	65	17	10	6 ± 1	—	—
400	40	19	5	—	—	—
800	248	61	19	—	—	—
1000	225	40	28	—	—	—

Release (mean ± SEM) is expressed as a % of the release evoked by 50 μM Ca^{2+} ± 0.5 μM A23187 (Absolute release 31 ± 4%, N = 6). Blank spaces indicate the concentrations of cations which completely inhibit [^{86}Rb] efflux. Values for Cu^{2+}, Fe^{2+}, and Mn^{2+} are averages of two experiments in duplicate.

ful inhibitor of calcium-dependent [^{86}Rb] efflux at concentrations above 50 μM, but at lower concentrations it significantly potentiated calcium-dependent [^{86}Rb] efflux (Table 1).

Action of lanthanides

The lanthanides ytterbium (Yb^{3+}), terbium (Tb^{3+}), europium (Eu^{3+}), lanthanum (La^{3+}), samarium (Sm^{3+}) and gadolinium (Gd^{3+}) exhibited complex effects on the release of [^{86}Rb] from rat erythrocytes. In the absence of A23187, low concentrations were without effect, but at concentrations above 400 μM, Yb^{3+}, Tb^{3+}, Eu^{3+} and Sm^{3+}, but not Gd^{3+} or La^{3+}, caused cell agglutination and the release of approximately 20% of the intracellular [^{86}Rb] without concomitant release of lactic dehydrogenase. In the presence of 0.5 μM A23187 all the lanthanides tested except Yb^{3+} caused efflux of [^{86}Rb]. These effects are shown in Fig. 2. At low concentrations (1–10 μM), the lanthanides Sm^{3+}, Eu^{3+}, Gd^{3+} and Tb^{3+} were more potent than calcium in triggering [^{86}Rb] release but with increasing con-

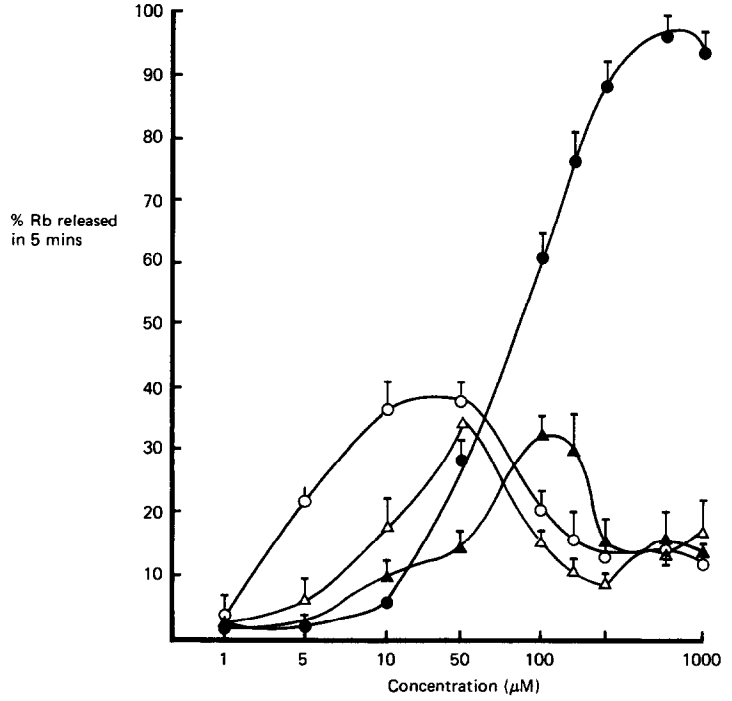

Fig. 2. Action of Ca^{2+} and the lanthanides on [^{86}Rb] release in the presence of 0.5 μM A23187: ●, Ca^{2+}; ▲, La^{3+}; ○, Sm^{3+}; △, Tb^{3+}; = Gd^{3+} = Eu^{3+}. Results are mean ± SEM of at least four experiments. The curves for Tb^{3+}, Gd^{3+} and Eu^{3+} are almost identical and only the points for Tb^{3+} are shown.

Table 2. Effect of lanthanides on [^{86}Rb] efflux evoked by 50 μM Ca^{2+} + 0.5 μM A23187

	% [^{86}Rb] release					
	Yb^{3+}	Tb^{3+}	Gd^{3+}	Eu^{3+}	Sm^{3+}	La^{3+}
50 μM Ca^{2+} + A23187 +lanthanide (μM)	100	100	100	100	100	100
1	91 ± 6	89 ± 6	97 ± 2	84	87	122
5	99 ± 6	102 ± 25	97 ± 14	100	74	136
10	101 ± 10	112 ± 36	80 ± 13	88	68	133
50	31 ± 14	64 ± 22	63 ± 2	63	34	91
100	12 ± 4	47 ± 16	37 ± 2	43	39	63
200	15 ± 6	36 ± 17	13 ± 3	4	27	39
400	35 ± 14	37 ± 17	10 ± 2	4	36	18
800	81 ± 35	47 ± 13	7 ± 4	62	74	6
1000	81 ± 42	61 ± 14	4 ± 5	68	72	10

Release (mean ± SEM) is expressed as a % of the total release induced by 50 μM Ca^{2+} + 0.5 μM A23187 [Absolute release 29 ± 4%, N = 18]. For Eu^{3+}, Sm^{3+}, and La^{3+} values are averages of duplicate values from two experiments.

centrations of the cations more complex responses were observed. The ability of the lanthanides to release [^{86}Rb] reached a maximum at 50 μM when approximately 40% of the intracellular [^{86}Rb] release declined sharply so that the slope of the concentration–response curve resembled that of Pb^{2+}. La^{3+} was less potent than the other lanthanides tested with a maximum effect on [^{86}Rb] release occurring at a concentration of 100–200 μM (Fig. 2). With Ca^{2+} a maximum effect on [^{86}Rb] release was observed at about 800 μM, with a small decline in the response obtained at 1 mM. The plateau effects on the residual [^{86}Rb] release seen at lanthanide concentrations above 400 μM were associated with cell agglutination and were similar to the effects observed in the absence of A23187. Because of the nature of the dose–response curves, with the maximum [^{86}Rb] release occurring at different concentrations, it is difficult to establish a satisfactory order of potency for the series of lanthanides. However, if it is assumed that the most potent elements will cause maximal [^{86}Rb] release at lower concentrations, then the order of potency would be $Sm^{3+} > Tb^{3+} = Gd^{3+} = Eu^{3+} = (Ca^{2+}) > La^{3+}$, with Yb^{3+} inactive. In addition to Ca^{2+}-mimetic actions, higher concentrations of the lanthanides were Ca^{2+}-antagonists when tested for their ability to inhibit [^{86}Rb] release evoked by 50 μM Ca^{2+} + 0.5 μM A23187 (Table 2). Eu^{3+}, Yb^{3+}, Tb^{3+} and Sm^{3+} were potent inhibitors at concentrations between 100 and 200 μM, but above 400 μM their inhibitory potency decreased as cell agglutination became apparent. Gd^{3+} and La^{3+} which did not cause agglutination showed a concentration-dependent inhibition of Ca^{2+}-evoked [^{86}Rb] release over the range 10–1000 μM, although low concentrations of La^{3+} behaved like Zn^{2+} in initially potentiating [^{86}Rb] release. Potentiation of Ca^{2+}-evoked [^{86}Rb] was not observed for the other lanthanides even at concentrations that were Ca^{2+}-mimetic, but the inhibitory effects of all the lanthanides were reduced by increasing the external Ca^{2+} concentration to 100 μM.

Action of Ba^{2+}, Sr^{2+} *and* Cd^{2+}

Ba^{2+} and Sr^{2+} were inactive in releasing [^{86}Rb] in the absence or presence of A23187, nor did they inhibit [^{86}Rb] efflux initiated by 50 μM Ca^{2+} and 0.5 μM A23187 at concentrations up to 1000 μM. Cd^{2+} was weaker than the lanthanides in releasing [^{86}Rb] in the presence of A23187, and showed a similar biphasic response with a maximum release of 21 ± 6% (N = 4) occurring at a Cd^{2+} concentration of 400 μM. Cd^{2+} was also a relatively potent inhibitor of Ca^{2+} + A23187 mediated [^{86}Rb] efflux with a maximum inhibition of 60 ± 10% at 100 μM. The inhibitory effect did not increase at higher concentrations and cell agglutination occurred above 800 μM Cd^{2+}.

Ca^{2+} *depleted cells*

In cells depleted of calcium by incubation with EGTA and A23187, there was a slight increase in Ca^{2+} and A23187-induced [^{86}Rb] efflux, a marked increase in the sensitivity to Cd^{2+} (Fig. 3), but no increase in the sensitivity to low concentrations of Pb^{2+}, Gd^{3+}, Tb^{3+}, Sm^{3+} or Eu^{3+}.

DISCUSSION

In recent years it has become apparent that a large number of excitable and non-excitable cells possess a K^+-permeability mechanism which is controlled, or gated, by the concentration of ionized calcium in the cytoplasm, so that increases in cystolic Ca^{2+} lead to increased K^+-efflux [24]. The mechanism is apparently lacking in rat hepatocytes [23], but the present experiments demonstrate that rat erythrocytes possess a Ca^{2+}-activated K^+-permeability mechanism similar to that in human erythrocytes. The rat erythrocyte has been used here as a model to investigate the comparative effects of multi-valent cations on Ca^{2+}-mediated K^+-efflux.

Any experimental approach to comparative studies on the effects of cations and calcium is beset by

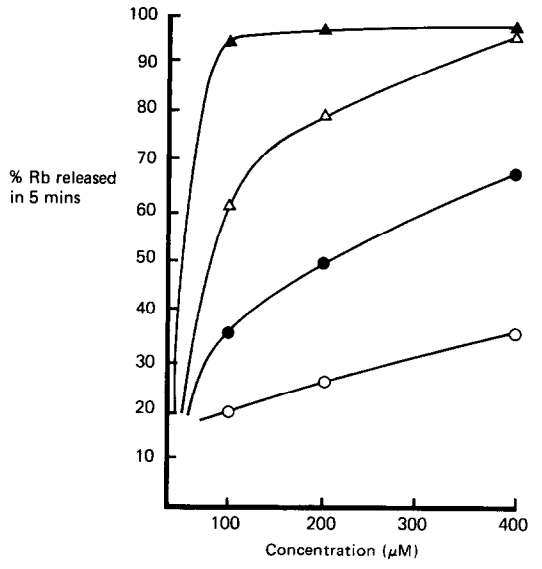

Fig. 3. The action of Ca^{2+} and Cd^{2+}, in the presence of 0.5 μM A23187 on [^{86}Rb] efflux from Ca^{2+}-depleted erythrocytes: △, Ca^{2+}; ○, Cd^{2+}; filled symbols represent depleted cells. [^{86}Rb] release was determined after 5 min incubation.

a number of problems, which may influence the interpretation of the results. In the present experiments many of the cations tested did not release [^{86}Rb], except in the presence of A23187, and although often erroneously termed a calcium-ionophore, A23187 facilitates the membrane penetration of a wide variety of di- and tri-valent cations in exchange for H^+ [27]. In intact cells A23187 has been reported to facilitate the intracellular penetration of cobalt [28], the lanthanides, terbium, praseodymium and lanthanum [12], manganese [9], and vanadium [29], suggesting that the [^{86}Rb] efflux from rat erythrocytes occurring in the presence of A23187 results from facilitated penetration of the cations through the erythrocyte membrane. We attempted to overcome these problems of ion penetration by attempting to incorporate heavy metals into erythrocyte ghosts by the method of Simons [30] but met with only limited success. Additional problems of interpretation occur because the system is not Ca^{2+}-free. Calcium is present as a contaminant in the analytical grade chemicals used in the preparation of physiological solutions and is also present in the erythrocytes themselves. It may be unclear therefore, to what extent the permeability change induced by di- and tri-valent cations, especially in the presence of A23187, is a consequence of increased Ca^{2+}-influx from traces of calcium contaminating the medium, or to the liberation of calcium from intracellular binding sites. In the present experiments neither of these possibilities can be excluded but several observations make them unlikely.

(1) All experiments contained a control incubation in which the erythrocytes in the nominally Ca^{2+}-free buffer were incubated with 0.5 μM A23187. Under those conditions [^{86}Rb] release was 3–6% of the total, which was not further reduced by the inclusion of 100 μM EGTA in the incubation buffer and was identical to the "release" occurring in the absence of A23187. It probably represents [^{86}Rb] carry over from the labelling procedure and leakage from damaged cells, suggesting either that the contaminating Ca^{2+}-level was too low to trigger [^{86}Rb] efflux or that the haematocrit was sufficiently high to sequester trace levels of calcium. It can be seen from Fig. 2, that at concentrations between 10–50 μM, Ca^{2+} was required in the medium before significant [^{86}Rb] efflux occurred.

(2) Cells collected and washed in buffers containing 1 mM EGTA to remove loosely bound Ca^{2+} were more sensitive to A23187 and cations, suggesting that the metals did not act by displacing Ca^{2+} from the erythrocyte membrane.

(3) It is possible that cations displace Ca^{2+} from intracellular sites. The observations that (i) [^{86}Rb] efflux occurring with low concentrations of lanthanides was greater than the efflux observed with similar concentrations of Ca^{2+} (Fig. 2) and (ii) that lanthanides retained their ability to stimulate [^{86}Rb] efflux in cells in which the Ca^{2+} content had been reduced by prior incubation with A23187 and EGTA argue against this possibility.

In the absence of A23187, only Ag^+, Hg^{2+} and Pb^{2+} released [^{86}Rb], suggesting that the erythrocyte membrane was normally impermeable to the majority of cations tested. The release of [^{86}Rb] induced by Ag^+ was the result of haemolysis. This direct haemolytic effect of Ag^+ on rat erythrocytes confirms its well documented haemolytic action on human erythrocytes [31], and the marked sensitivity of rat erythrocytes to Ag^+ probably results from the absence of Cl^- in the media which would precipitate Ag^+. Similar observations were made with Hg^{2+}, but at higher concentrations. The failure of A23187 to potentiate either the [^{86}Rb]-releasing action or the haemolysis induced by Ag^+ and Hg^{2+} may indicate that these cations are not transported by A23187, or that they act on the membrane and this action is not influenced by the presence of the ionophore. It has been suggested that both Ag^+ and Hg^{2+} may increase membrane permeability by interacting with sulphhydryl groups within the membrane [32].

The ability of Pb^{2+} to release K^+ from human erythrocytes is well documented [33, 34]. The present experiments confirm that this also occurs in rat erythrocytes and that Pb^{2+} has a biphasic response. Low concentrations of Pb^{2+} release [^{86}Rb] in a concentration-dependent manner, but at concentrations above 100 μM a self inhibitory effect was observed. Pb^{2+}-activated [^{86}Rb]-efflux was significantly potentiated by low concentrations of A23187 demonstrating that not only does Pb^{2+} penetrate the erythrocyte through the anion carriers [34], but under the appropriate conditions it is translocated by A23187. The potentiation by ionophore was most marked on the stimulation of [^{86}Rb] efflux, suggesting that the ionophore delivers more Pb^{2+} to its site of action before it is sequestered by intracellular binding sites. While this work was in progress Shields *et al.* [26] reported a similar biphasic affect of Pb^{2+} on human erythrocytes and the potentiation of the response by A23187 and from results obtained by application of the patch-clamp technique to the

erythrocyte membrane, suggested that Pb^{2+} activated the K^+ efflux mechanism by interacting with a site that is probably identical to the Ca^{2+}-activated site.

The ability of Ca^{2+} to promote K^+-efflux in the presence of A23187 is shared to a varying extent by the lanthanides (Fig. 2), which, although less potent, gave bell-shaped dose–response curves similar to that obtained with Pb^{2+}. It is well documented that lanthanum and other metals are capable of modifying Ca^{2+}-activated processes, and it has been suggested that the ability of metals to interact with Ca^{2+}-binding sites is related to the size of the non-hydrated ionic radius [11, 35–37]. A plot of % ([^{86}Rb] release versus crystalline ionic radius is shown in Fig. 4. It is difficult to establish an absolute potency sequence for this series of metals, as the apparent potency could be influenced by their binding and transport by A23187, and their affinity for intracellular buffering systems controlling the free ion concentration within the cell. In Fig. 4 a concentration of 50 μM has been chosen for comparison, as it is the standard concentration of Ca^{2+} used throughout the experiments, and it is close to the peak concentration for the majority of calcium-mimetic cations. Ag^+ and Hg^{2+} have been included for the sake of completeness, although their mode of action may differ fundamentally from the other cations tested.

Ca^{2+} has a non-hydrated crystalline ionic radius of 0.99 A. Cations with an ionic radius of less than 0.90 A were inactive in initiating [^{86}Rb] efflux, and are all inhibitors of Ca^{2+}-activated [^{86}Rb] efflux. The lanthanides Tb^{3+}, Gd^{3+}, Eu^{3+} and Sm^{2+} have ionic radii close to that of Ca^{2+}, and at 50 μM concentration were almost equi-active with Ca^{2+} in releasing [^{86}Rb], although La^{3+}, the first element in the series, and Yb^{3+} the penultimate element, were less active, as was Sr^{2+}. Ba^{2+}, with an ionic radius of 1.35 A, was inactive. The odd elements in this series are Cd^{2+} and Pb^{2+}. Cd^{2+} has an ionic radius (0.97) close to that of Ca^{2+}, suggesting that it should have strong Ca^{2+}-mimetic actions and yet at 50 μM concentration it was almost inactive in initiating [^{86}Rb] efflux. The lack of potency may be more apparent than real as the peak effect of Cd^{2+} was not reached until a concentration of about 200 μM (Fig. 3), although at that level it was still significantly less potent than Ca^{2+}. Cadmium is also reported to be less active than Ca^{2+} in activating the K^+-current in molluscan neurones [36]. Pb^{2+} was the most potent element tested and with an ionic radius of 1.20 and on the basis of ionic radius, it is less potent than Ca^{2+}. With the exception of Pb^{2+} and Cd^{2+}, the effectiveness of multivalent cations in activating [^{86}Rb] efflux from rat erythrocytes is related to their ionic radius, and as the data in Fig. 4 indicates, the site of activation may only accommodate those ions with radii between 0.95–1.05 A.

The sequence of activation of [^{86}Rb] efflux by the various cations is similar to that for the activation of calmodulin *in vitro*, thus Mg^{2+}, Ni^{2+}, Co^{2+} and Ba^{2+} do not activate calmodulin but La^{3+}, Sm^{3+}, Tb^{3+}, Cd^{2+} and Pb^{2+} are effective activators [13, 17, 37]. Erythrocytes contain an outwardly directed Ca^{2+}-pump stimulated by calmodulin [38] and the possibility exists that [^{86}Rb] efflux mediated by the calcium-mimetic cations occurs through modulation of the calmodulin-dependent Ca^{2+}-ATPase. However, if low concentrations of Ca^{2+}-mimetic cations were to activate calmodulin in intact erythrocytes, they would be expected to enhance the efficiency of the Ca^{2+}-pump, which in turn would decrease [^{86}Rb] efflux by reducing the cellular concentration of Ca^{2+}. Similarly if inhibitory cations were direct inhibitors of the Ca^{2+}-pump, then the inhibition of active transport out of the cell could lead to a build-up of Ca^{2+} inside the cell which in turn would activate the [^{86}Rb] efflux mechanism. If this were the case, then one might expect to see significant enhancement of the effects of Ca^{2+} and A23187 on [^{86}Rb] efflux in the

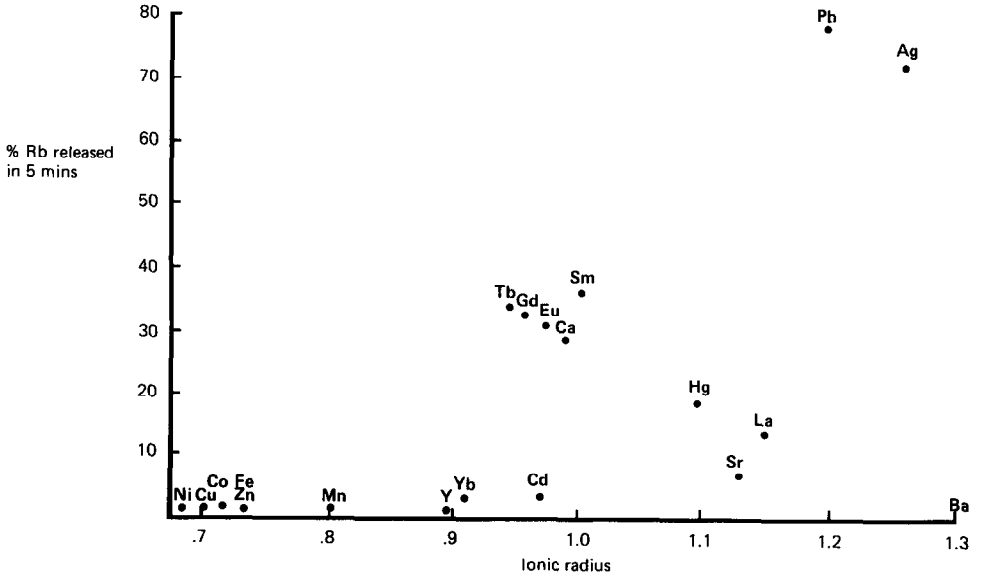

Fig. 4. The relationship between crystalline ionic radius and [^{86}Rb] release at 50 μM concentrations of the cations in the presence of 0.5 μM A23187. Ionic radii are taken from [42].

presence of low concentrations of cations, low enough to constrict the pump. This did not happen (Tables 1 and 2), except in the case of Zn^{2+}, which apparently enhanced Ca^{2+} uptake through normal influx pathways [39]. This suggests that neither calmodulin activation nor inhibition explains the observed results. This leaves the possibility that Ca^{2+}-mimetic cations such as the lanthanides act directly upon the [^{86}Rb] efflux mechanism, perhaps by first acting at the high affinity Ca^{2+}-binding site postulated by Blum and Hoffman [40] to stimulate K^+-efflux and at a higher concentrations they might either block this site or interact with a secondary low affinity inhibitory site [41]. The present experiments do not permit us to distinguish between these possibilities but the inhibitory effects of all the cations tested appear to be the result of some interaction with Ca^{2+}, as the reduction in [^{86}Rb] efflux could be surmounted by increasing the extracellular Ca^{2+}-concentration.

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