The control by Ca^{2+} of the polyphosphoinositide phosphodiesterase and the Ca^{2+} -pump ATPase in human erythrocytes

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1. Both the Ca^{2+} -pump ATPase and the polyphosphoinositide phosphodiesterase of the erythrocyte membrane can, when assayed under appropriate conditions, be activated by Ca^{2+} in the micromolar range. We have therefore compared the mechanisms and affinities for Ca^{2+} activation of the two enzymes in human erythrocyte membranes, to see whether the polyphosphoinositide phosphodiesterase would be active in normal healthy erythrocytes. 2. At physiological ionic strength and in the presence of calmodulin, the Ca^{2+} -pump ATPase was activated by Ca^{2+} in a highly co-operative manner, with half-maximal activation occurring at about $0.3\ \mu\text{M}\text{-Ca}^{2+}$. At an optimal Ca^{2+} concentration, calmodulin stimulated the Ca^{2+} -sensitive ATPase activity about 10-fold. 3. Ca^{2+} activated the polyphosphoinositide phosphodiesterase in a non-co-operative manner. The Ca^{2+} requirements for breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were identical, which supports our previous conclusion that Ca^{2+} activates a single polyphosphoinositide phosphodiesterase that degrades both lipids with equal facility. Added calmodulin did not affect the activity of the polyphosphoinositide phosphodiesterase. 4. At low ionic strength in the absence of Mg^{2+} , half-maximal activation of the phosphodiesterase was at about $3\ \mu\text{M}\text{-Ca}^{2+}$. The presence of $1\ \text{mM}\text{-Mg}^{2+}$ shifted the Ca^{2+} activation curve to the right, as did elevation of the ionic strength. When the Ca^{2+} -pump ATPase and the polyphosphoinositide phosphodiesterase were assayed in the same incubations and under conditions of intracellular ionic strength and Mg^{2+} concentration, the ATPase was fully activated at $3\ \mu\text{M}\text{-Ca}^{2+}$, whereas no polyphosphoinositide phosphodiesterase activity was detected below $100\ \mu\text{M}\text{-Ca}^{2+}$. 5. The Ca^{2+} -pump ATPase of the erythrocyte membrane normally maintains the Ca^{2+} concentration of healthy erythrocytes below approx. $0.1\ \mu\text{M}$. It therefore seems unlikely that the polyphosphoinositide phosphodiesterase of the erythrocyte membrane ever expresses its activity in a healthy erythrocyte.

In a previous paper (Downes & Michell, 1981a) we described a simple new assay procedure that facilitated the characterization of the polyphosphoinositide phosphodiesterase of human erythrocyte membranes. This enzyme, which has a specific requirement for Ca^{2+} , degrades $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ to 1,2-diacylglycerol with the release of the appropriate inositol phosphates. In mature erythrocytes this would be an irreversible process, since these cells cannot synthesize phospholipids *de novo* (Hokin & Hokin, 1963; Scherphof & Van

Deenen, 1966; Shohet & Nathan, 1970). Despite this, past observations on the Ca^{2+} -stimulated accumulation of diacylglycerol and the breakdown of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ in human erythrocytes and erythrocyte ghosts have suggested that the polyphosphoinositide phosphodiesterase might be sensitive to concentrations of Ca^{2+} in the micromolar or sub-micromolar range (Allan & Michell, 1976, 1978; Allan *et al.*, 1976; Allan & Thomas, 1981).

There have been a variety of reports concerning the Ca^{2+} affinity of the Ca^{2+} -pump ATPase of the erythrocyte membrane, the enzyme that is responsible for maintaining the low physiological intracellular Ca^{2+} concentration; the most recent reports

Abbreviations used: $\text{PtdIns}4\text{P}$, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4-phosphate; $\text{PtdIns}(4,5)\text{P}_2$, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4,5-bisphosphate.

tend to agree that it is half-maximally activated by about $0.3\text{--}0.4\text{ }\mu\text{M-Ca}^{2+}$ (Muallem & Karlish, 1980; Klinger *et al.*, 1980; Downes & Michell, 1981*b,c*). It was therefore difficult to understand how the intracellular Ca^{2+} concentration in a healthy human erythrocyte could be maintained throughout its long lifespan at a sufficiently low level for the polyphosphoinositides of the cell not to be degraded. To resolve this apparent paradox, we have directly compared the Ca^{2+} -activation characteristics of the two enzymes in the same type of erythrocyte ghost preparation.

Materials and methods

Human blood was obtained from the Blood Transfusion Centre within 7 days of donation, and erythrocyte ghosts isolated in 2.5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/ 1 mM-EDTA , pH 7.0, as described previously (Downes & Michell, 1981*b*).

$\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent ATPase assays were done as previously described (Downes & Michell, 1981*b*). For assay of the polyphosphoinositide phosphodiesterase, washed erythrocytes were first labelled with [^{32}P]P_i (Downes & Michell, 1981*a*) before preparation of Hepes ghosts (Downes & Michell, 1981*b,c*). The ^{32}P -labelled erythrocyte ghosts were isolated in $2.5\text{ mM-Hepes}/1\text{ mM-EGTA}$ and were given a final wash in $20\text{ mM-Hepes}/1\text{ mM-EGTA}$, pH 7.0, since the activity of the polyphosphoinositide phosphodiesterase was diminished at very low ionic strengths (C. P. Downes, unpublished work). The polyphosphoinositide phosphodiesterase was usually assayed, exactly as described by Downes & Michell (1981*a*), by measuring the radioactivities of the released inositol bisphosphate and inositol trisphosphate, after their separation on small columns of Dowex-1 (formate form). However, the inositol phosphates did not bind quantitatively to the resin columns at ionic strengths greater than 0.1. For experiments using such incubation media, the polyphosphoinositide phosphodiesterase activity was determined by measuring the total Ca^{2+} -stimulated release of radioactively labelled organic phosphates from the labelled ghosts (for incubation conditions, see the legend to Fig. 3). Reactions were stopped by addition of an equal volume of 1 M-HClO_4 . After centrifugation, a sample of the acid-soluble fraction was removed and made 0.5% (w/v) with respect to ammonium molybdate. Isobutyl alcohol/toluene (1:1, v/v; 2 vol.) was added and the two phases were mixed vigorously. The radioactivity of a portion of the lower aqueous phase, containing only organic phosphates, was determined by Čerenkov counting. Previous experiments have shown that the only radioactive organic phosphates released from Ca^{2+} -stimulated ^{32}P -

labelled erythrocyte ghosts are inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate (Allan & Michell, 1978; Downes & Michell, 1981*a*).

Ionized Ca^{2+} concentrations in the range $100\text{ nM--}10\text{ }\mu\text{M}$ were controlled by using $\text{Ca}^{2+}\text{-EGTA}$ buffers calculated using a $K_{\text{Ca}^{2+}\text{-EGTA}}$ value of 11.0, as recommended by Owen (1976). For concentrations greater than $10\text{ }\mu\text{M}$, Ca^{2+} was added in excess of the EGTA. The free Ca^{2+} concentrations achieved were checked using a Ca^{2+} -specific electrode, and they were not appreciably changed by the presence of erythrocyte ghosts.

Calmodulin was isolated from bovine brain by the method of Grand *et al.* (1979).

Results

Our previous work (Downes & Michell, 1981*b,c*) has demonstrated that the $2.5\text{ mM-Hepes}/1\text{ mM-EGTA}$ ghosts used in these studies are freely permeable to Ca^{2+} , ATP and calmodulin. Briefly, the sensitivity of the Ca^{2+} -pump ATPase to these components is not affected by treatment with saponin, and the sensitivity of the ATPase to Ca^{2+} in these ghosts is identical with that of the membranes of saponin-permeabilized erythrocytes. We therefore believe that under appropriate incubation conditions the pattern of activation of the Ca^{2+} -pump ATPase in these ghosts, and probably also of other enzymes dependent on Ca^{2+} and/or calmodulin, can be a reasonably accurate reflection of the pattern of their activation in the intact erythrocyte.

In such ghosts, only about one-fifth of the maximum ATPase activity is expressed in the absence of added calmodulin, even in the presence of 0.1 mM-Ca^{2+} . However, even in the absence of calmodulin about half the expressed activity is seen at concentrations of Ca^{2+} as low as $1\text{ }\mu\text{M}$. Calmodulin both increases the maximum activity (about 5-fold) and confers much greater Ca^{2+} sensitivity on the ATPase; activation is highly co-operative and is half-maximal at about $0.3\text{ }\mu\text{M-Ca}^{2+}$ (Downes & Michell, 1981*b,c*). Since Ca^{2+} leaks into healthy erythrocytes only slowly (Schatzmann, 1975) and the Ca^{2+} -pump ATPase activity is high, it seems likely that these characteristics of the pump ensure that Ca^{2+} concentrations in healthy erythrocytes normally remain at or below about 100 nM .

Effects of Ca^{2+} and calmodulin on the polyphosphoinositide phosphodiesterase

When the polyphosphoinositide phosphodiesterase was assayed at low ionic strength in the absence of added Mg^{2+} , as has been done in the past (Allan & Michell, 1978; Downes & Michell, 1981*a*), there was appreciable activation at about $1\text{ }\mu\text{M-Ca}^{2+}$. However, the increase in phosphodiesterase activity

with increasing Ca²⁺ concentration was more gradual than with the Ca²⁺-pump ATPase (Fig. 1). Release of inositol bisphosphate and of inositol trisphosphate occurred in parallel over the entire range of Ca²⁺ concentrations (see the ratios given in Fig. 1), an observation that is in accord with our previous suggestion that the same phosphodiesterase attacks both PtdIns4P and PtdIns(4,5)P₂ (Downes & Michell, 1981a). The stimulation of phosphodiesterase activity by Ca²⁺, and also the changes in apparent Ca²⁺ affinity caused by Mg²⁺ and elevated ionic strength (see below), might, at least in principle, be caused by a stimulatory interaction between the phosphodiesterase and Ca²⁺, by the Ca²⁺ salts of PtdIns4P and PtdIns(4,5)P₂ being the preferred substrates or by some combination of these two factors. PtdIns(4,5)P₂ has a substantially higher affinity for Ca²⁺ than does PtdIns4P (Hendrickson & Reinertsen, 1971) but the phosphodiesterase shows no such discrimination; this suggests to us that the major influence of Ca²⁺ may be exerted on the phosphodiesterase rather than on its substrates.

Addition of calmodulin had no influence on the activity of the polyphosphoinositide phosphodiesterase (Fig. 2); the concentration of calmodulin used would have been more than adequate to fully activate the Ca²⁺-pump ATPase (Downes & Michell, 1981b,c). The data were obtained at a Ca²⁺ concentration of

Fig. 1. Erythrocyte membrane polyphosphoinositide phosphodiesterase: activation by Ca²⁺ under conditions of low ionic strength in the absence of Mg²⁺

The polyphosphoinositide phosphodiesterase activity was assayed by measuring the release of the individual ³²P-labelled inositol bisphosphate and inositol trisphosphate. The values plotted are the sums of the two phosphate esters, and the numbers alongside each point give the ratio (d.p.m. in inositol trisphosphate)/(d.p.m. in inositol bisphosphate). Each point is the mean of triplicate incubations in a single experiment, one of three that gave similar results.

Fig. 2. Erythrocyte membrane polyphosphoinositide phosphodiesterase: lack of effect of calmodulin

Assays were done as for Fig. 1, both without added calmodulin (○) and in the presence of 600 nM calmodulin (●). Each point is the mean of duplicate assays in a single experiment. A variety of experiments were undertaken (at various Ca²⁺ and calmodulin concentrations, and with various types of erythrocyte ghost preparation) and in none of these did calmodulin have any appreciable effect on the polyphosphoinositide phosphodiesterase activity.

0.5 mM, but calmodulin was still without effect on the phosphodiesterase when it was assayed at a suboptimal Ca²⁺ concentration (10 μM; results not shown).

Trifluoperazine, a cationic amphiphilic neuroleptic drug, has been widely used as a putative calmodulin antagonist; it half-maximally inhibits the calmodulin-dependent cyclic nucleotide phosphodiesterase of brain at approx. 10 μM (Levin & Weiss, 1979). Although it did inhibit the erythrocyte polyphosphoinositide phosphodiesterase, half-maximal inhibition required approx. 170 μM-trifluoperazine (results not shown).

Direct comparison of polyphosphoinositide phosphodiesterase and Ca²⁺-pump ATPase

The results discussed this far suggest that the polyphosphoinositide phosphodiesterase can be fully activated in the absence of calmodulin, but only at concentrations of Ca²⁺ rather unlikely to be attained in the healthy erythrocyte. However, all of these phosphodiesterase assays were done under Mg²⁺-free conditions of low ionic strength quite unlike those prevailing within cells. When we determined the Ca²⁺ activation curve of the phosphodiesterase in the presence of 1 mM-Mg²⁺, we found that it was displaced to the right of that shown in Fig. 2 by approximately an order of magnitude (results not

Fig. 3. Simultaneous assay of Ca^{2+} -pump ATPase and of polyphosphoinositide phosphodiesterase in the same incubations: activation by Ca^{2+} under conditions of ionic strength, Mg^{2+} and ATP concentration approximating to those in the cell

Incubations (1.5 ml) contained 1.5–2 mg of membrane protein, 30 mM-Hepes, 30 mM-KCl, 80 mM-NaCl, 0.1 mM-ouabain, 1 mM-EGTA, to which various concentrations of Ca^{2+} were added before adjustment of the pH to 7.0: some incubations also contained 1 mM- Mg^{2+} (△), whilst the remainder contained 4 mM- Mg^{2+} , 3 mM-ATP and 280 nM-calmodulin (▲, ●). After 5 min at 37°C, 0.75 ml of each incubation was removed to a separate tube containing 0.75 ml of 1 M- HClO_4 (for analysis of radioactivity in released organic phosphates; see the text). Trichloroacetic acid (20%, w/v; 0.75 ml) was added to the remaining 0.75 ml of each incubation and ATPase was assayed as the release of P_i . Each point is the mean of triplicate incubations in a single experiment, one of three that gave similar results. The symbols represent Ca^{2+} -ATPase activity (●) and polyphosphoinositide phosphodiesterase activity [either in the presence of ATP and 280 nM-calmodulin (▲) or in their absence (△)].

shown). If the ionic strength was raised, the apparent affinity of the phosphodiesterase for Ca^{2+} was further reduced. We therefore decided to undertake simultaneous assays of the polyphosphoinositide phosphodiesterase and of the Ca^{2+} -pump ATPase under the conditions of approximately physiological ionic strength, Mg^{2+} concentration and ATP concentration that were routinely used for the ATPase assays. The intention was to make a direct comparison of the relative Ca^{2+} sensitivities of the two activities under conditions relatively similar to those prevailing in the intact cell.

This was readily achieved by incubating ^{32}P -labelled ghosts together with unlabelled ATP. However, the high ionic strength of the incubation media and the presence in the incubation of Mg^{2+} , which activates an inositol trisphosphate phosphomonoesterase present in human erythrocyte membranes (Downes & Michell, 1981a; C. P. Downes & R. H. Michell, unpublished work), necessitated a change of experimental design. First, the released inositol trisphosphate was continually being broken down to inositol bisphosphate and P_i as it was produced. Secondly, the elevated ionic strength precluded the efficient adsorption of labelled inositol bisphosphate on to our resin columns. To circumvent these problems, we simply assayed the Ca^{2+} -stimulated accumulation of total labelled organic phosphates [i.e. mostly inositol bisphosphate, derived both directly from $\text{PtdIns}4\text{P}$ and indirectly from $\text{PtdIns}(4,5)\text{P}_2$]. Although this method mainly assays the

release of the less heavily labelled 4-phosphate of these two lipids (much of the 5-phosphate is lost as $[\text{}^{32}\text{P}]\text{P}_i$), it should provide a valid but indirect measurement of the Ca^{2+} -sensitivity of the polyphosphoinositide phosphodiesterase.

The results of this type of experiment are shown in Fig. 3. As mentioned above, the Ca^{2+} -pump ATPase was co-operatively activated by Ca^{2+} and was fully active at approx. $3\text{ }\mu\text{M-Ca}^{2+}$. At much higher concentrations of Ca^{2+} it was inhibited, as has previously been reported (e.g. Gopinath & Vincenzi, 1978; Klinger *et al.*, 1980). By contrast, there was no detectable activation of the phosphodiesterase at Ca^{2+} concentrations below $100\text{ }\mu\text{M}$, and phosphodiesterase activity appeared still to be rising at 3.3 mM-Ca^{2+} , the highest concentration that was tested. The activation pattern of the phosphodiesterase was unchanged when ATP and calmodulin were omitted from the assay, thus demonstrating that the observed decrease in Ca^{2+} -sensitivity was due solely to the effects of raised ionic strength and Mg^{2+} .

Discussion

An early observation by Allan & Michell (1976) suggested that the Ca^{2+} -stimulated production of 1,2-diacylglycerol in human erythrocyte ghosts was half-maximally activated at approx. 100 nM-Ca^{2+} , which is probably a similar concentration to that prevailing in healthy erythrocytes. Later studies

indicated that this production of diacylglycerol was due to the activation of a Ca²⁺-dependent and membrane-bound polyphosphoinositide phosphodiesterase that is present in the erythrocytes of man and of some other mammals (Allan & Michell, 1977, 1978; Downes & Michell, 1981a). If a polyphosphoinositide phosphodiesterase of such high Ca²⁺-sensitivity were normally to be present in erythrocytes then it would be very surprising to find appreciable concentrations of PtdIns4P and PtdIns(4,5)P₂ persisting in the membranes of circulating mature erythrocytes. The observations presented here, and confirmed independently in recent studies by Allan & Thomas (1981), indicate that the polyphosphoinositide phosphodiesterase is much less sensitive to Ca²⁺ than had been indicated by the results of Allan & Michell (1976); no reason for this discrepancy has been revealed by either study. Moreover, the polyphosphoinositide phosphodiesterase has not previously been assayed under ionic conditions similar to those in the cell and we now find that under such conditions activation by Ca²⁺ only occurs at concentrations greater than 0.1 mM.

Ca²⁺ and calmodulin, the potential activators of the Ca²⁺-pump ATPase and polyphosphoinositide phosphodiesterase that we have studied, exert control over plasma-membrane enzymes through interactions at the cytoplasmic surface of the membrane, so it was important that our experiments should be done with freely permeable erythrocyte ghosts. For this purpose we chose the 2.5 mM-Hepes/1 mM-EGTA-prepared ghosts that we have previously employed for studies of the kinetics of the Ca²⁺-pump ATPase (Downes & Michell, 1981b,c). It is clear that in these ghosts the sites at which Ca²⁺ and calmodulin activate the ATPase are freely accessible; it was assumed that the same freedom of access should apply to any sites through which added ligands might modify the activity of the phosphodiesterase.

Maximum activity of the Ca²⁺-pump ATPase requires the presence of Na⁺, K⁺, calmodulin, MgATP²⁻ (as substrate) and Ca²⁺ (see Scharff, 1981, for review); assays are therefore routinely done in the presence of 80 mM-Na⁺ and 30 mM-K⁺. By contrast, previous studies of the Ca²⁺-activated polyphosphoinositide phosphodiesterase were done at relatively low ionic strength (20 mM-Hepes) in the absence of Mg²⁺ (Allan & Michell, 1976; Downes & Michell, 1981a). The studies reported here have revealed that the apparent affinity of the phosphodiesterase for Ca²⁺ is markedly reduced either when Mg²⁺ is present or when the ionic strength is raised by the inclusion of either NaCl or KCl; at 0.5 mM-Ca²⁺, both 0.12 M-NaCl and 0.12 M-KCl reduce the phosphodiesterase activity by about 30% (results not shown). When both Mg²⁺ concentration

and ionic strength were close to physiological values (Fig. 3) then the phosphodiesterase remained inactive up to 100 μ M-Ca²⁺, i.e. up to a Ca²⁺ concentration that is probably about 1000-fold higher than ever occurs in healthy cells. It is apparent from Fig. 3 that the concentrations of Ca²⁺ that activated the phosphodiesterase also inhibited the Ca²⁺-pump ATPase. This revives the possibility, previously mooted by Buckley & Hawthorne (1972), that the activity of the Ca²⁺-pump ATPase may be modulated by changes in polyphosphoinositide concentrations. However, this must for the moment remain an open question, since we have not undertaken experiments specifically designed to address it, and the experiments of Buckley & Hawthorne (1972) were done before the essential role of calmodulin in the control of this enzyme was appreciated.

The lack of effect of calmodulin on phosphodiesterase action both at low ionic strength (Fig. 2) and at high ionic strength in the presence of Mg²⁺ (Fig. 3) suggests that the activation of this enzyme by Ca²⁺ does not involve calmodulin. This is also in accord with its low apparent affinity for Ca²⁺; calmodulin-dependent enzymes are usually activated by micromolar concentrations of Ca²⁺ (e.g. the Ca²⁺-pump ATPase in the present paper). Although the inhibition by trifluoperazine might be interpreted as an indication of the involvement of calmodulin, the concentration needed for effective inhibition was relatively high. Amphiphilic cationic drugs of this type interact strongly with anionic lipids, including polyphosphoinositides (Dawson & Hauser, 1970), and they would therefore be expected to modify the availability of such lipids to enzymes that metabolize them; see, for example, the effects of chlorpromazine on phosphatidylinositol phosphodiesterase (Allan & Michell, 1974). On balance, therefore, we consider it very improbable that calmodulin has any role in the control of the polyphosphoinositide phosphodiesterase.

Given the likelihood that the polyphosphoinositide phosphodiesterase will never be activated by Ca²⁺ in the healthy erythrocyte, then its existence in these cells is puzzling. We suggest that two possible explanations are initially worth considering. The first, previously suggested by Allan & Michell (1979), is that the phosphodiesterase might become activated when Ca²⁺ homeostasis starts to fail in senescent erythrocytes, and that the consequent degradation of the polyphosphoinositides might play some part in the membrane modifications leading to cell destruction. A second alternative arises from our recent studies that suggest that the initiating event in receptor-stimulated inositol lipid turnover might, at least in vasopressin-stimulated hepatocytes, be the phosphodiesterase-catalysed breakdown of PtdIns(4,5)P₂ (Kirk *et al.*, 1981;

Michell *et al.*, 1981). If this proves correct, then the key enzyme in such responses would probably be a plasma-membrane polyphosphoinositide phosphodiesterase whose activity was controlled through activation of hormone receptors. It seems possible that the plasma-membrane phosphoinositide phosphodiesterase of the erythrocyte might be a relic of such a receptor-coupled system that was active during erythropoiesis, maybe in rather the same way that mature erythrocytes retain a substantial complement of the guanyl nucleotide-binding protein that is essential to the coupling between adenylate cyclase and receptors (Kaslow *et al.*, 1979).

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