Biochem. J. (1981) **198**, 133–140
Printed in Great Britain

The polyphosphoinositide phosphodiesterase of erythrocyte membranes

C. Peter DOWNES and Robert H. MICHELL
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

(*Received 12 March 1981/Accepted 8 April 1981*)

1. A new assay procedure has been devised for measurement of the Ca^{2+}-activated polyphosphoinositide phosphodiesterase (phosphatidylinositol polyphosphate phosphodiesterase) activity of erythrocyte ghosts. The ghosts are prepared from cells previously incubated with [^{32}P]P_i. They are incubated under appropriate conditions for activation of the phosphodiesterase and the released ^{32}P-labelled inositol bisphosphate and inositol trisphosphate are separated by anion-exchange chromatography on small columns of Dowex-1 (formate form). When necessary, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate can be deacylated and the released phosphodiesters separated on the same columns. 2. The release of both inositol bisphosphate and inositol trisphosphate was rapid in human ghosts, with half of the labelled membrane-bound phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate broken down in only a few minutes in the presence of 0.5 mM-Ca^{2+}. For both esters, optimum rates of release were seen at pH 6.8–6.9. Mg^{2+} did not provoke release of either ester. 3. Ca^{2+} provoked rapid polyphosphoinositide breakdown in rabbit erythrocyte ghosts and a slower breakdown in rat ghosts. Erythrocyte ghosts from pig or ox showed no release of inositol phosphates when exposed to Ca^{2+}. 4. In the presence of Mg^{2+}, the inositol trisphosphate released from phosphatidylinositol 4,5-bisphosphate was rapidly converted into inositol bisphosphate by phosphomonoesterase activity. 5. Neomycin, an aminoglycoside antibiotic that interacts with polyphosphoinositides, inhibited the breakdown of both phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, with the latter process being appreciably more sensitive to the drug. Phenylmethanesulphonyl fluoride, an inhibitor of serine esterases that is said to inhibit phosphatidylinositol phosphodiesterase, had no effect on the activity of the erythrocyte polyphosphoinositide phosphodiesterase. 6. These observations are consistent with the notion that human, and probably rabbit and rat, erythrocyte membranes possess a single polyphosphoinositide phosphodiesterase that is activated by Ca^{2+} and that attacks phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate with equal facility. Inhibition of this activity by neomycin seems likely to be due to interactions between neomycin and the polyphosphoinositides, with the greater inhibition of phosphatidylinositol 4,5-bisphosphate breakdown consistent with the greater affinity of the drug for this lipid. In addition, erythrocyte membranes possess Mg^{2+}-dependent phosphomonoesterase that converts inositol 1,4,5-triphosphate into inositol bisphosphate.

The polyphosphoinositides (PtdIns4*P* and PtdIns4,5P_2) are quantitatively minor constituents of the lipids of most tissues (Hawthorne & Michell, 1966). In general, it seems likely that they are concentrated at the plasma membrane (Michell, 1975). Although present in very small quantities, they usually show rapid metabolic turnover, at least so far as the monoester phosphate groups attached to the inositol ring are concerned. It is thought that rapid metabolic turnover of the monoester phosphate groups of polyphosphoinositides is caused mainly by enzyme-catalysed cycles of phosphorylation and dephosphorylation of the inositol ring. However, mammalian tissues contain phosphodiesterases capable of attacking these lipids, and

Abbreviations used: PtdIns4*P*, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4-phosphate; PtdIns4,5P_2, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4,5-bisphosphate.

0306-3283/81/070133-08$01.50/1

recent studies have suggested that control of phosphodiesterase activity might play an important role in determining the overall balance between synthesis and degradation of these lipids. In particular, it has been discovered that in situations that lead to increased availability of Ca^{2+} at the inner face of the plasma membrane there is often an increase in the degradation of pre-existing polyphosphoinositides. This is true of erythrocytes from some species (Lang *et al.*, 1977; Allan & Michell, 1977, 1978, 1979*a*), the smooth muscle of the iris (Akhtar & Abdel-Latif, 1978*a*,*b*; Abdel-Latif *et al.*, 1978) and synaptosomes (Griffin & Hawthorne, 1978; Griffin *et al.*, 1979, 1980), and in all of these tissues there is evidence to suggest that it is polyphosphoinositide phosphodiesterase activity, rather than monoesterase activity, that is activated by an increase in cytosolic $[Ca^{2+}]$.

In previous studies, polyphosphoinositide breakdown has been assayed, after chromatographic separation of the extracted lipids, either by direct measurements of the decreased concentrations or radioactivities of the polyphosphoinositides or by assaying the released diacylglycerol. Both of these practical approaches are relatively time-consuming and have therefore limited the amount of available information about the membrane-bound polyphosphoinositide phosphodiesterase(s). An alternative is to measure the release from externally added polyphosphoinositides of water-soluble phosphates (e.g. Thompson & Dawson, 1964*a*,*b*,*c*), but in such assays the substrates are usually presented to the enzyme(s) in a physical state quite different from that which normally exists in the plasma membrane. To circumvent some of these problems, we developed a simplified radiochemical procedure for measuring rates of attack on erythrocyte membrane polyphosphoinositides by the phosphodiesterase(s) present in the same membrane. By using this assay, we have examined the characteristics of these reactions in some detail. Some of these results have been presented in preliminary form (Downes & Michell, 1980).

Materials and methods

Human blood was obtained from the Blood Transfusion Centre and used within 7 days of donation. Ox, pig and rat blood samples were obtained from freshly killed animals, and rabbit blood was withdrawn from the ear vein of New Zealand White rabbits. Acid citrate/dextrose was present in each sample as anticoagulant.

Preparation of ^{32}P-labelled membranes

Blood samples were centrifuged at 2500*g* for 5min and the plasma and leucocytes were carefully removed by aspiration. The sedimented erythrocytes were then washed three times in 154mM-NaCl buffered at pH7.2 with 1.5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]. The washed erythrocytes were labelled with $[^{32}P]P_i$ (30μCi/ml of packed cells) in a low phosphate medium under conditions designed to maximize their polyphosphoinositide content (Shukla *et al.*, 1979). Haemoglobin-free ghosts were then isolated by lysis and washing in 20mM-Tris/HCl/1mM-EDTA, pH7.4, with a final wash in EDTA-free buffer.

Polyphosphoinositide phosphodiesterase assay

Samples (0.5ml) of packed erythrocyte membranes (2–2.5mg of membrane protein) were incubated in a final volume of 1ml of EDTA-free buffer at 37°C. The polyphosphoinositide phosphodiesterase was activated by the addition of $CaCl_2$ to a concentration of 0.5mM. The reactions were stopped by the addition of 1ml of ice-cold 10% trichloroacetic acid and the samples were placed on ice for 10min. After brief centrifugation 1ml portions of the supernatants were removed, neutralized with NaOH and diluted to 5ml with water. They were then loaded on to anion-exchange columns [1ml of Dowex-1 (X10; 200–400 mesh; formate form)]. The radioactive phosphate esters were eluted sequentially using eluents based on those described by Ellis *et al.* (1963), namely 0.1M-formic acid/0.2M-ammonium formate (for P_i), 0.1M-formic acid/0.4M-ammonium formate (for inositol bisphosphate) and 0.1M-formic acid/1.0M-ammonium formate (for inositol trisphosphate). In routine assays, 15ml fractions were collected and their radioactivities were determined by Čerenkov counting. Zero-time controls were included in all experiments and all values were corrected for the radioactivities found in the inositol phosphate fractions from these control incubations.

Determination of pH optimum

pH was varied in the range 5.0–9.0 using 10mM-Tris/10mM-imidazole buffers. The presence of 10mM-imidazole did not affect the activity of the enzyme at pH7.4.

Determination of radioactivity incorporated into PtdIns4P and PtdIns4,5P_2

When necessary, polyphosphoinositides were extracted from erythrocyte ghosts as described by Shukla *et al.* (1979). The extracts were dried under vacuum and the phospholipids were deacylated by mild alkaline hydrolysis using the procedure of Ellis *et al.* (1963). Briefly, the lipids were dissolved in 1ml of chloroform to which was added 0.2ml of methanol and 0.2ml of 1.0M-NaOH in methanol/water (19:1, v/v). After 20min at room temperature, chloroform (1ml), methanol (0.6ml) and water (0.6ml) were added and the samples were

mixed vigorously. The two phases were separated by centrifugation and 1 ml of the upper aqueous phase containing the deacylated phospholipids was removed. This was diluted to 5 ml and made 5 mM with respect to sodium tetraborate. The samples were loaded on to Dowex-1 formate columns (as used for separation of inositol phosphates), which were eluted with 20 ml of 0.005 M-disodium tetraborate/0.18 M-ammonium formate. After this treatment the only ^{32}P-labelled deacylated lipids that remained on the columns were glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate (derived from PtdIns4*P* and PtdIns4,5P_2 respectively). These were eluted sequentially with 0.1 M-formic acid/0.3 M-ammonium formate (for glycerophosphoinositol 4-phosphate) and 0.1 M-formic acid/0.75 M-ammonium formate (for glycerophosphoinositol 4,5-bisphosphate). Radioactivity was determined by Čerenkov counting.

Results

Validation of the phosphodiesterase assay

The principle used for separating inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate, the water-soluble products of phosphodiesterase attack on PtdIns4*P* and PtdIns4,5P_2, on anion-exchange columns arises from the work of Hawthorne and his colleagues (Ellis *et al.*, 1963). We have, however, devised simplified conditions in which batchwise elution of small resin columns that have been loaded with the neutralized trichloroacetic acid-soluble products of our enzyme incubation gives rapid separation of P_i, inositol bisphosphate and inositol trisphosphate. Such a separation is illustrated in Fig. 1. Recovery of radioactivity from such columns normally exceeded 90% of that loaded, and each fraction, if diluted and rechromatographed on a second column, could again be eluted quantitatively in the same fraction. The identities and homogeneity of the inositol bisphosphate and inositol trisphosphate were confirmed by paper chromatography and high-voltage electrophoresis followed by autoradiography, using the methods employed previously (Allan & Michell, 1978). Although a small amount of [^{32}P]P_i is released from such ghosts during incubation, this is not substantially influenced by Ca^{2+} (Allan & Michell, 1978).

Similar experiments were done to validate the separation conditions chosen for resolution of glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate (results not shown).

General characteristics

On addition of 0.5 mM-Ca^{2+} to ghosts incubated at 37°C and pH 7.4, there was rapid liberation of

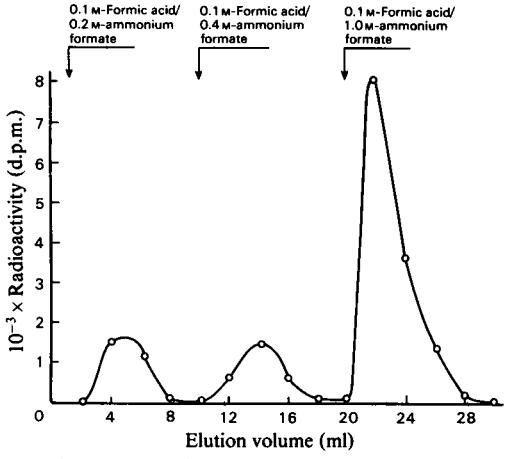

Fig. 1. *Separation of radioactive inositol phosphates on Dowex-1 (formate form)*

The trichloroacetic acid-soluble fraction was obtained from ^{32}P-labelled erythrocyte ghosts that had been treated with 0.5 mM-$CaCl_2$ for 10 min at 37°C. The sample was loaded on to a Dowex-1 column and eluted as described in the Materials and methods section. Fractions (2 ml) were collected and their radioactivities were determined by Čerenkov counting. Several similar columns gave essentially identical elution profiles.

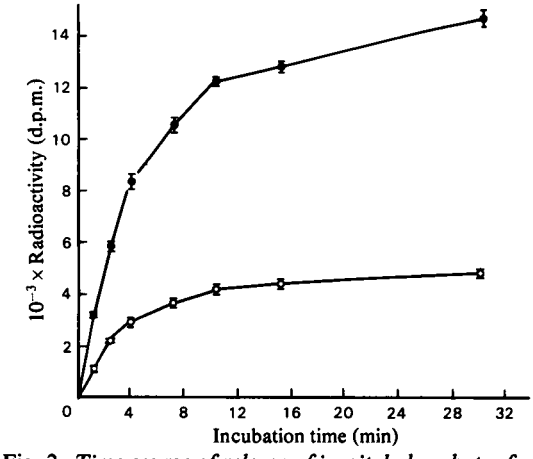

Fig. 2. *Time course of release of inositol phosphates from human erythrocyte ghosts*

^{32}P-labelled erythrocyte ghosts were incubated at 37°C in the presence of 0.5 mM-$CaCl_2$ for the times indicated. Inositol bisphosphate (○) and inositol trisphosphate (●) release were measured as described in the text. Each point is the mean ± S.E.M. for five replicates in a single experiment. Very similar results were obtained in several experiments with different blood samples. There was no detectable release of either phosphate ester in the absence of Ca^{2+}, provided that EDTA or EGTA was present.

inositol bisphosphate and inositol trisphosphate from membrane-bound polyphosphoinositides (Fig. 2). For both esters, release was almost complete within about 15 min and half-maximal release took about 3–4 min; these values are close to published values for the appearance of diacylglycerol under similar incubation conditions (Fig. 2 of Allan & Michell, 1976). During incubations long enough for the release of inositol phosphates to reach a maximum, about two-thirds of the PtdIns4*P* and PtdIns4,5P_2 of the ghosts were broken down. The reason for the stability of the remainder is still unknown.

If ghosts were incubated in the presence of either 1 mM-EGTA or -EDTA, without the addition of Ca^{2+}, there was no appreciable appearance of inositol phosphates. Similarly, ghosts incubated with 1 mM-$MgCl_2$ failed to release any inositol phosphates. It therefore appears, as concluded previously from studies of diacylglycerol production (Allan & Michell, 1976), that this phosphodiesterase is activated specifically by Ca^{2+} ions. However, inclusion of Mg^{2+} together with Ca^{2+} gave an unexpected result; inositol trisphosphate initially accumulated at almost the usual rate, but it then began to disappear (Fig. 3*a*). This suggested that the erythrocyte membrane preparations contained an Mg^{2+}-dependent phosphomonoesterase that was active against inositol trisphosphate. Support for this view came from experiments in which inositol trisphosphate accumulated in the presence of Ca^{2+} and then disappeared when the Ca^{2+} was chelated and Mg^{2+} added (Fig. 3*b*). Subsequent experiments with isolated inositol bisphosphate and inositol trisphosphate used separately as substrates (C. P.

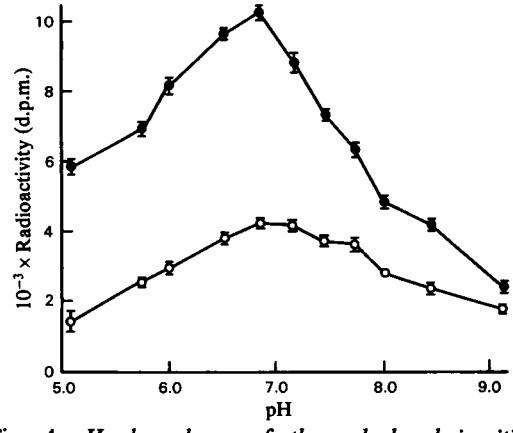

Fig. 4. *pH dependence of the polyphosphoinositide phosphodiesterase*

pH was varied as described in the Materials and methods section. Inositol bisphosphate release (○) and inositol trisphosphate release (●) were measured after incubation with 0.5 mM-Ca^{2+} for 2 min at 37°C. Each point is the mean ± S.E.M. for four replicates in a single experiment.

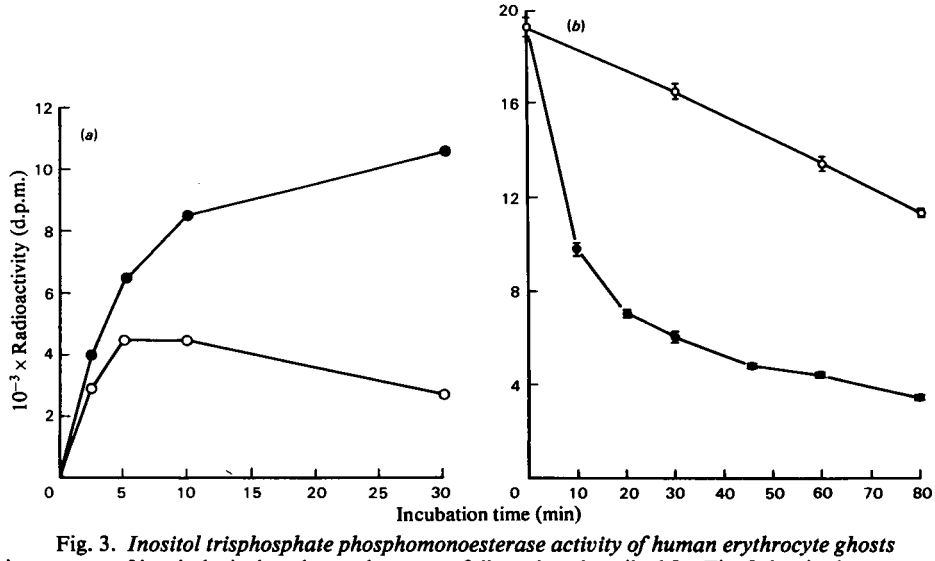

Fig. 3. *Inositol trisphosphate phosphomonoesterase activity of human erythrocyte ghosts*

(*a*) The time course of inositol trisphosphate release was followed as described for Fig. 2, but in the presence of either 0.5 mM-Ca^{2+} (●) or 0.5 mM-Ca^{2+} + 1.0 mM-$MgCl_2$ (○). (*b*) Erythrocyte ghosts were incubated with 0.5 mM-$CaCl_2$ for 15 min at 37°C to liberate inositol trisphosphate. EGTA was then added to a concentration of 1 mM to inhibit the polyphosphoinositide phosphodiesterase. The samples were then incubated at 37°C for the times shown in the presence (●) or absence (○) of 1 mM-$MgCl_2$.

Downes & C. Holmquist, unpublished work) have only shown Mg^{2+}-dependent breakdown of inositol trisphosphate. These observations suggest that the erythrocyte possesses a specific inositol trisphosphate phosphomonoesterase.

Release of inositol bis- and tris-phosphates both showed broad and similar pH–activity profiles, with maximum activity around pH 6.8–6.9 (Fig. 4).

Effects of possible inhibitors

In previous studies in which Ca^{2+}-stimulated polyphosphoinositide breakdown was examined in synaptosomes (Griffin & Hawthorne, 1978; Griffin *et al.*, 1979, 1980), inhibition was observed after addition of neomycin, a cationic aminoglycoside antibiotic. In principle, such inhibition might be caused by a decrease in the rate of Ca^{2+} entry, by interaction between neomycin and the anionic polyphosphoinositides or by direct inhibition of the phosphodiesterase; both of the former explanations have previously been offered (Schacht *et al.*, 1977, 1978; Griffin *et al.*, 1980). In our experiments we used relatively permeable erythrocyte ghosts that were prepared in the presence of EDTA (Bramley & Coleman, 1972), and hence the drug should have had direct access both to the polyphosphoinositides and to the phosphodiesterase. Under these conditions it still brought about inhibition of the phosphodiesterase activity, with the breakdown of PtdIns4,5P_2 appreciably more susceptible to inhibition than the breakdown of PtdIns4P (Fig. 5).

It has recently been reported that phenylmethanesulphonyl fluoride, a serine esterase inhibitor, is an effective inhibitor of platelet phosphatidylinositol phosphodiesterase at submillimolar concentrations (Walenga *et al.*, 1980). When tested at a concentration of 1 mM against the polyphosphoinositide phosphodiesterase of human erythrocyte membranes, it had no effect (results not shown).

Species distribution

In a standard 10 min incubation with 0.5 mM-Ca^{2+}, human erythrocyte ghosts degraded about half of their polyphosphoinositides. When ghosts prepared from ^{32}P-labelled erythrocytes from other species were tested, we found that the polyphos-

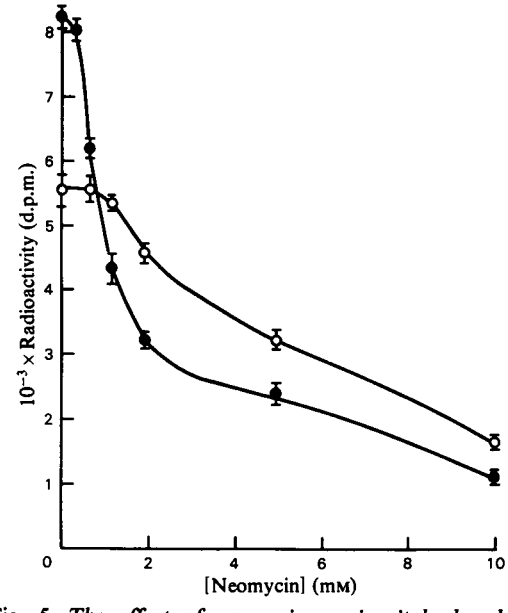

Fig. 5. *The effect of neomycin on inositol phosphate release from erythrocyte ghosts*

Membranes were incubated with 0.5 mM-Ca^{2+} and the indicated concentrations of neomycin. Inositol bisphosphate release (○) and inositol triphosphate release (●) were measured after incubation for 2 min at 37°C. Results are means ± S.E.M. of four replicate assays in a single experiment, one of three that gave similar results.

Table 1. *Species distribution of the polyphosphoinositide phosphodiesterase*

^{32}P-labelled erythrocyte ghosts were prepared from samples of human, rabbit, rat, pig and ox blood. They were incubated for 10 min at 37°C in the presence of either 0.5 mM-$CaCl_2$ or 1 mM-EDTA. Radioactivities of the released inositol phosphates and of the remaining polyphosphoinositides were determined as described in the Materials and methods section. Each value is the mean result from two separate blood samples (triplicate determinations on each sample) and represents the radioactivity in the phospholipids from 0.5 ml of packed ghosts or the radioactivity in the inositol phosphates released from 0.5 ml of packed ghosts after incubation with Ca^{2+}. Abbreviation used: N.D., none detected.

	^{32}P radioactivity (d.p.m.)					
	PtdIns4P			PtdIns4,5P_2		
Species	EDTA	+Ca^{2+}	Released inositol bisphosphate	EDTA	+Ca^{2+}	Released inositol trisphosphate
Human	3900	2050	2800	27500	15000	15500
Rabbit	2100	740	1800	10700	3600	9200
Rat	660	610	260	9050	7550	1430
Ox	710	660	N.D.	8370	8170	N.D.
Pig	720	670	N.D.	10300	9400	N.D.

phoinositides of rabbit membranes were degraded at least as fast as those of human membranes, that rat membranes degraded polyphosphoinositides at a slower, but measurable rate, and that pig and ox ghosts were essentially inactive (Table 1). During these experiments, we also observed marked differences amongst the different species in the relative rates at which their erythrocytes incorporated ^{32}P into polyphosphoinositides. Under identical incubation conditions, human erythrocytes incorporated about three times as much ^{32}P as any of the other species. In addition, the amount of ^{32}P that went into PtdIns4*P* in rat, ox and pig was less than 10% of that in PtdIns4,5P_2, meaning that the most reliable estimates of polyphosphoinositide breakdown in these species were given by PtdIns4,5P_2.

Discussion

Until 1978, it was assumed that polyphosphoinositide breakdown in erythrocytes was a result only of phosphomonoesterase action. This seemed reasonable in view of the fact that erythrocytes cannot synthesize these lipids *de novo*, so that phosphodiesterase attack would inevitably cause an irreversible loss of inositol lipids from the cell. However, Allan & Michell (1978) then demonstrated that human erythrocytes contain an active polyphosphoinositide phosphodiesterase, and they suggested that activation of this enzyme by Ca^{2+} could probably explain previous reports of Ca^{2+}-stimulated polyphosphoinositide breakdown in intact human erythrocytes (Lang *et al.*, 1977) and of Ca^{2+}-stimulated diacylglycerol generation both in human erythrocytes (Allan & Michell, 1975; Allan *et al.*, 1976) and in human erythrocyte ghosts (Allan & Michell, 1976). In the erythrocyte, it appeared that either accumulation of diacylglycerol or disappearance of polyphosphoinositides was likely to be an essential event in Ca^{2+}-stimulated echinocytosis and membrane vesiculation (Allan & Michell, 1975, 1977, 1979*a*; Allan *et al.*, 1978). Recent studies (D. Allan & P. Thomas, personal communication) make it more likely that the breakdown of polyphosphoinositides, rather than accumulation of diacylglycerol, is essential to the vesiculation response, and it also seems possible that Ca^{2+}-stimulated polyphosphoinositide breakdown in other tissues might be implicated in Ca^{2+}-controlled membrane fusion events such as exocytosis (Allan & Michell, 1979*b*).

In addition to the studies on erythrocytes, it has become clear in the last few years that Ca^{2+}-stimulated polyphosphoinositide breakdown also occurs, probably by the phosphodiesterase route, in iris smooth muscle (Akhtar & Abdel-Latif, 1978*a*,*b*, 1980), in synaptosomes exposed to either veratridine or the ionophore A23187 in the presence of Ca^{2+} (Griffin & Hawthorne, 1978; Griffin *et al.*, 1979, 1980) and in K^+-depolarized *Paramecium* (Kaneshiro & Rhoads, 1979). Although few in number, these cell-types are very varied. In addition, Ca^{2+}-dependent polyphosphoinositide phosphodiesterase activity has been reported in several other types of cell [liver (Hawthorne & Kemp, 1964); cerebral cortex (Thompson & Dawson, 1964*a*; Keough & Thompson, 1972); kidney (Tou *et al.*, 1973); *Crithidia fasciculata* (Palmer, 1973)] and polyphosphoinositide breakdown might be a very widespread facet of cellular responsiveness to changes in cytosol Ca^{2+} concentration. With this in mind, we selected the Ca^{2+}-sensitive polyphosphoinositide phosphodiesterase as a possible exemplar of this group of enzymes that could be easily studied.

Assay of the phosphodiesterase

The assay procedure that we have developed has both advantages and disadvantages. On the credit side, it is possible in 2 days, using labelled ghosts from a single batch of erythrocytes, to separately measure the released inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate in samples from about 50 enzyme incubations. Deacylation of the extracted membrane lipids can be used to generate phosphodiesters that, separated on the same small ion-exchange resin columns, provide an independent route by which to assay polyphosphoinositide concentrations in control and Ca^{2+}-treated ghosts. The phosphodiesterase is acting on its natural substrate in something close to its natural environment, and the problems inherent in isolating lipid substrates and in devising physiologically meaningful methods for presentation of the substrates are obviated. On the debit side, kinetic studies of the enzyme activity have to be done under the predetermined conditions that prevail in the isolated membranes. We have not yet attempted to devise conditions, maybe by using detergents, that would persuade this membrane-bound phosphodiesterase to act either on externally added substrate or on the labelled polyphosphoinositides of membranes that lack the phosphodiesterase (e.g. pig or ox erythrocyte membranes).

Effects of Ca^{2+} and Mg^{2+}

As realized previously (see Allan & Michell, 1974, 1978), a dependence on Ca^{2+} ions appear to be a universal characteristic of polyphosphoinositide phosphodiesterases. However, there has been some ambiguity in the interpretation of these data, in that some workers also appeared to find activation by other cations, both bivalent (e.g. Mg^{2+}) and amphiphilic (e.g. cetyltrimethylammonium) (Thompson & Dawson, 1964*a*,*b*,*c*; Tou *et al.*, 1973). It was suggested by Allan & Michell (1974) that all of these effects are, in fact, explicable in terms of a specific

requirement for a small concentration of Ca^{2+}; we proposed that the other 'activating' ions simply displaced bound Ca^{2+} and hence made it available to activate the phosphodiesterase(s). The present results with Mg^{2+} add a further element to any consideration of the previously published data. Not only is Mg^{2+} alone completely inactive as a stimulator of the polyphosphoinositide phosphodiesterase, but it also activates an inositol triphosphate phosphomonoesterase, hence decreasing the accumulation of inositol triphosphate during incubations with Ca^{2+}. The activity of any such enzyme in previous studies would therefore have tended to decrease and enhance, respectively, the recorded activities of polyphosphoinositide phosphodiesterase and polyphosphoinositide phosphomonoesterase. The apparent effect on the former activity would probably have been more modest, since two-thirds of the organic phosphate released from PtdIns4,5P_2 and all of that released from phosphatidylinositol 4-phosphate would still have been detected as phosphodiesterase product by the usual assays of total released organic phosphate.

A second ambiguity introduced by this phosphomonoesterase would appear in the analyses of Ca^{2+}-stimulated polyphosphoinositide breakdown in intact cells, where intracellular Mg^{2+} will always be available. In the studies by Griffin & Hawthorne (1978) on Ca^{2+}-treated synaptosomes, a major observation was that PtdIns4,5P_2 breakdown occurred without any substantial accumulation of inositol trisphosphate. Although the authors interpreted this in terms of a Ca^{2+}-stimulated PtdIns4,5P_2-specific phosphomonoesterase, they also recognized the possibility that they might be observing the sequential action of a phosphodiesterase on PtdIns4,5P_2 and a phosphomonoesterase on inositol 1,4,5-trisphosphate; it now seems likely that the latter was the correct explanation. As the authors realized, the presence of an inositol trisphosphate phosphomonoesterase probably explains the very low recoveries of inositol trisphosphate that were recorded in a recent search for the products of Ca^{2+}-stimulated PtdIns4,5P_2 breakdown in iris smooth muscle (Akhtar & Abdel-Latif, 1980).

Species distribution

The original identification of polyphosphoinositides as a source of the diacylglycerol liberated in response to intracellular Ca^{2+} was made with human and rabbit erythrocytes (Allan & Michell, 1978). However, it remained possible that polyphosphoinositides were not the major source of this diacylglycerol. One way to resolve this question was to compare the species distributions of the two processes. In a previous study, Allan & Michell (1977) showed that diacylglycerol and phosphatidate were produced in response to Ca^{2+} in the erythrocytes of rat, rabbit, human and guinea pig, but not in those of sheep, ox or pig. We have therefore made quantitative measurements of the rates of Ca^{2+}-stimulated polyphosphoinositide breakdown by the phosphodiesterase route in erythrocyte ghosts from five of these species. In human and rabbit, as found before by Allan & Michell (1978), we observed rapid polyphosphoinositide breakdown. In the rat, the process was much slower but still easily measured. No inositol bisphosphate or inositol trisphosphate was released from the polyphosphoinositides of pig and ox ghosts in the presence of Ca^{2+}. A similar observation on the stability of the polyphosphoinositides of pig ghosts was previously made by Buckley & Hawthorne (1972), who demonstrated that all of the inositol lipids of these membranes could be converted into polyphosphoinositides and that these were then stable during incubation in the presence of Ca^{2+}. In none of the three species in which polyphosphoinositide breakdown occurred was there any indication of selective or more rapid attack on either PtdIns4*P* or PtdIns4,5P_2. It therefore appears likely that in all of these species the polyphosphoinositides are indeed the major source of diacylglycerol and phosphatidate in Ca^{2+}-treated erythrocytes.

One polyphosphoinositide phosphodiesterase or two?

In most previous studies, relatively little attempt has been made to determine whether PtdIns4*P* and PtdIns4,5P_2 are attacked by the same phosphodiesterase, mainly because of the difficulty of separately following the breakdown of each lipid. With our new assay procedure, this is now routine and the results are in general accord with the notion that erythrocyte membranes possess a single polyphosphoinositide phosphodiesterase that attacks PtdIns4*P* and PtdIns4,5P_2 with equal facility. To summarize the evidence in favour of this view, (*a*) the time courses of attack on both lipids in human ghosts are the same (Fig. 2), as are the pH optima (Fig. 4) and the amounts of Ca^{2+} needed for activation (P. Downes, unpublished work) and (*b*) parallel rates of attack on the two lipids are also seen in rabbit and rat erythrocyte membranes.

The human erythrocyte is a convenient cell in which to study Ca^{2+}-stimulated polyphosphoinositide breakdown, since its polyphosphoinositides can be labelled to high specific radioactivity without most other lipids becoming labelled and its plasma membranes are easily isolated. However, there are no natural stimuli that are known to elevate the cytosol Ca^{2+} concentration in the mature erythrocyte. It will be interesting to see whether a similar phosphodiesterase also acts on plasma-membrane polyphosphoinositides in other cells or whether the polyphosphoinositide metabolism of such cells will present a more complex picture.

We thank the Medical Research Council for financial support.

References

Abdel-Latif, A. A., Akhtar, R. A. & Smith, J. P. (1978) in *Cyclitols and Phosphoinositides* (Eisenberg, F. & Wells, W. W., eds.), pp. 121–143, Academic Press, New York

Akhtar, R. A. & Abdel-Latif, A. A. (1978*a*) *Biochim. Biophys. Acta* **527**, 159–170

Akhtar, R. A. & Abdel-Latif, A. A. (1978*b*) *J. Pharmacol. Exp. Ther.* **204**, 655–668

Akhtar, R. A. & Abdel-Latif, A. A. (1980) *Biochem. J.* **192**, 783–791

Allan, D. & Michell, R. H. (1974) *Biochem. J.* **142**, 599–604

Allan, D. & Michell, R. H. (1975) *Nature (London)* **258**, 348–349

Allan, D. & Michell, R. H. (1976) *Biochim. Biophys. Acta* **455**, 824–830

Allan, D. & Michell, R. H. (1977) *Biochem. J.* **166**, 495–499

Allan, D. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **508**, 277–286

Allan, D. & Michell, R. H. (1979*a*) in *Normal and Abnormal Red Cell Membranes* (Lux, S. E., Marchesi, V. T. & Fox, C. F., eds.), pp. 523–529, Alan Liss, New York

Allan, D. & Michell, R. H. (1979*b*) *Symp. Soc. Exp. Biol.* **33**, 323–336

Allan, D., Watts, R. & Michell, R. H. (1976) *Biochem. J.* **156**, 225–232

Allan, D., Thomas, P. & Michell, R. H. (1978) *Nature (London)* **276**, 289–290

Bramley, T. A. & Coleman, R. (1972) *Biochim. Biophys. Acta* **290**, 219–228

Buckley, J. T. & Hawthorne, J. N. (1972) *J. Biol. Chem.* **247**, 7218–7223

Downes, P. & Michell, R. H. (1980) *Biochem. Soc. Trans.* **8**, 127

Ellis, R. B., Galliard, T. & Hawthorne, J. N. (1963) *Biochem. J.* **88**, 125–131

Griffin, H. D. & Hawthorne, J. N. (1978) *Biochem. J.* **176**, 541–552

Griffin, H. D., Sykes, M. & Hawthorne, J. N. (1979) *Biochem. Soc. Trans.* **7**, 348–357

Griffin, H. D., Sykes, M. & Hawthorne, J. N. (1980) *J. Neurochem.* **34**, 750–752

Hawthorne, J. N. & Kemp, P. (1964) *Adv. Lipid Res.* **2**, 127–166

Hawthorne, J. N. & Michell, R. H. (1966) in *Cyclitols and Phosphoinositides* (Kindl, H., ed.), pp. 49–55, Pergamon Press, Oxford

Kaneshiro, E. S. & Rhoads, D. E. (1979) *Abstr. Int. Cong. Biochem. 11th*, 578

Keough, K. M. W. & Thompson, W. (1972) *Biochim. Biophys. Acta* **270**, 324–336

Lang, V., Pryhitka, G. & Buckley, J. T. (1977) *Can. J. Biochem.* **55**, 1007–1012

Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147

Palmer, F. B. St. C. (1973) *Biochim. Biophys. Acta* **326**, 194–200

Schacht, J., Lodhi, S. & Weiner, N. D. (1977) in *Membrane Toxicity* (Miller, M. W. & Shamoo, A. E., eds.), pp. 191–208, Plenum Press, New York

Schacht, J., Weiner, N. D. & Lodhi, S. (1978) in *Cyclitols and Phosphoinositides* (Wells, W. W. & Eisenberg, F., eds.), pp. 153–165, Academic Press, New York

Shukla, S. D., Coleman, R., Finean, J. B. & Michell, R. H. (1979) *Biochem. J.* **179**, 441–444

Thompson, W. & Dawson, R. M. C. (1964*a*) *Biochem. J.* **91**, 233–236

Thompson, W. & Dawson, R. M. C. (1964*b*) *Biochem. J.* **91**, 237–243

Thompson, W. & Dawson, R. M. C. (1964*c*) *Biochem. J.* **91**, 244–250

Tou, J. S., Hurst, M. W., Baricos, W. H. & Huggins, C. G. (1973) *Arch. Biochem. Biophys.* **154**, 593–600

Walenga, R., Vanderhoek, J. Y. & Feinstein, M. B. (1980) *J. Biol. Chem.* **255**, 6024–6027