Phosphatidylinositol Cleavage in Lymphocytes

REQUIREMENT FOR CALCIUM IONS AT A LOW CONCENTRATION AND EFFECTS OF OTHER CATIONS

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The soluble activity in lymphocytes which converts phosphatidylinositol into 1,2-diacylglycerol and inositol phosphates requires Ca^{2+} ions. At pH 7 maximum activity occurs at $[\text{Ca}^{2+}]_{\text{free}} \approx 0.7 \mu\text{M}$ whereas at pH 5.5 the equivalent value is approx. $50 \mu\text{M}$. At $[\text{Ca}^{2+}]_{\text{free}} \approx 1 \mu\text{M}$, a concentration similar to common intracellular values, essentially all activity is confined to the peak of activity at pH 7.0. Previous reports of requirements for larger amounts of Ca^{2+} may reflect the fact that the Ca^{2+} -buffering capacity of phosphatidylinositol means that high substrate concentrations can effectively decrease $[\text{Ca}^{2+}]_{\text{free}}$. Cations which displace Ca^{2+} from association with phosphatidylinositol can, at low $[\text{Ca}^{2+}]_{\text{free}}$, enhance enzyme activity. Phosphatidylinositol breakdown in intact cells might be controlled, at least in part, by changes in intracellular $[\text{Ca}^{2+}]_{\text{free}}$.

The soluble fraction of lymphocytes, in common with that from other tissues, catalyses the breakdown of phosphatidylinositol to 1,2-diacylglycerol and a mixture of *myo*-inositol 1:2-cyclic phosphate and *myo*-inositol 1-phosphate (Allan & Michell, 1974). In view of previous reports of a Ca^{2+} requirement for such activities, our assays have included this ion as a routine. The present report describes the effects of Ca^{2+} more precisely than previously, particularly for the activity at pH 7.0. The results suggest that changes in intracellular $[\text{Ca}^{2+}]$ might have a role in controlling phosphatidylinositol cleavage (and turnover) in intact cells.

Materials and Methods

These were mainly those described in the accompanying paper (Allan & Michell, 1974). The enzyme preparations were lymphocyte soluble fractions prepared as described therein, but without addition of EGTA [ethanedioxybis(ethylamine)tetra-acetate]. The assay medium was, unless otherwise specified, the pH 7.0 system used by Allan & Michell (1974).

Effects of metal-ion removal were studied by using EDTA, which chelates many multivalent cations, EGTA (similar to EDTA except for its low Mg^{2+} affinity) and *o*-phenanthroline, which complexes transition metals but not Ca^{2+} or Mg^{2+} .

Known concentrations of free Ca^{2+} were added in the form of either Ca^{2+} -EDTA or Ca^{2+} -EGTA buffers, the values of free Ca^{2+} being calculated from the data of Raaflaub (1960): measurements were made at phosphatidylinositol concentrations of 1.2 mM and 0.12 mM.

Activity at a variety of pH values and $1 \mu\text{M}$ - Ca^{2+} was measured by addition of sufficient Ca^{2+} to assay media containing 2 mM-EDTA to give the required ion concentration at each pH.

The calculated values for $[\text{Ca}^{2+}]_{\text{free}}$ must be regarded as approximate, as we were working under non-standard conditions: our incubations were at 37°C and I approx. 0.01 mol/l, whereas the quoted values for Ca^{2+} chelation by EDTA and EGTA were for 25°C and $I = 0.1$. The combined effects of these deviations would be expected to decrease the true $[\text{Ca}^{2+}]_{\text{free}}$ values to about half those quoted. The presence of phosphatidylinositol would decrease these values further, but the extent of this further effect is difficult to assess (see the Results section).

The effects of addition of other cations were studied both in the presence of a fixed free Ca^{2+} concentration (approx. $10 \mu\text{M}$, buffered with Ca^{2+} -EDTA) and in the presence of a small final concentration of unbuffered Ca^{2+} (approx. $6 \mu\text{M}$); this Ca^{2+} , which represented that present in the sample of the soluble fraction which was used as the enzyme preparation, was estimated by atomic-absorption spectroscopy (Willis, 1961).

Results

Requirements for Ca^{2+} at pH 7.0 and 5.5

EDTA and EGTA both caused essentially complete loss of activity at pH 7.0 whereas *o*-phenanthroline had no effect (Fig. 1). Activity was restored by Ca^{2+} but not by Mg^{2+} .

The concentration of ionized calcium required to

restore activity at a phosphatidylinositol concentration of 1.2 mM was determined by using Ca^{2+} -EDTA and Ca^{2+} -EGTA buffers (Fig. 2). At pH 7.0 maximum activity was seen at $[\text{Ca}^{2+}]_{\text{free}} \approx 10 \mu\text{M}$ (EDTA buffer)

Fig. 1. Effects of Ca^{2+} and Mg^{2+} and of chelators on phosphatidylinositol-cleaving activity

The standard assay conditions were used, except that 0.4 mM- CaCl_2 was replaced by cations and/or chelators at the concentrations indicated.

or $\approx 2 \mu\text{M}$ (EGTA buffer). As phosphatidylinositol could itself influence $[\text{Ca}^{2+}]_{\text{free}}$ similar experiments were done at a phosphatidylinositol concentration of 0.12 mM; these yielded maximum activity at lower values of $[\text{Ca}^{2+}]_{\text{free}}$ (approx. $0.7 \mu\text{M}$ in Ca^{2+} -EDTA and approx. $0.3 \mu\text{M}$ in Ca^{2+} -EGTA). Since it has been reported previously that in Tris buffers the values obtained by using EGTA may be as much as 6 times too low (Ogawa, 1968) it is likely that the values obtained with the EDTA buffers are the more accurate. Thus it seems that in a situation where the Ca^{2+} -binding influence of phosphatidylinositol could be neglected there would be little activity at $[\text{Ca}^{2+}]_{\text{free}} \leq 0.01 \mu\text{M}$ and activity would be close to maximum at $[\text{Ca}^{2+}]_{\text{free}} \geq 1 \mu\text{M}$. At pH 5.5 and 0.12 mM-phosphatidylinositol the $[\text{Ca}^{2+}]_{\text{free}}$ required for full expression of the enzyme activity was approx. $50 \mu\text{M}$; at this concentration the activity was of similar magnitude to that at pH 7.0 and $[\text{Ca}^{2+}]_{\text{free}} \approx 0.7 \mu\text{M}$.

As would be expected from these observations, at $[\text{Ca}^{2+}]_{\text{free}} \approx 1 \mu\text{M}$ only the peak of activity at pH 7.0 was observed (Fig. 3).

Inhibition by excess of phosphatidylinositol

When comparison was made of the usual assay system (total $\text{Ca}^{2+} = 0.4 \text{ mM}$, phosphatidylinositol = 1.2 mM) with assays at other phosphatidylinositol concentrations, the observed activity was found to

Fig. 2. Effects of variations in $[\text{Ca}^{2+}]_{\text{free}}$ on phosphatidylinositol-cleaving activity

The $[\text{Ca}^{2+}]_{\text{free}}$ concentration in each incubation was maintained by the presence of Ca^{2+} -EDTA (●, ■) or Ca^{2+} -EGTA (○) buffers; these contained 2 mM-EDTA and 2 mM-EGTA and sufficient Ca^{2+} to give the required concentration (Raafaub, 1960; and see also the Materials and Methods section). Assays were conducted at pH 7.0 (●, ○) and at pH 5.5 (■), at substrate concentrations of (a) 1.2 mM and (b) 0.12 mM.

be lower at either lower or higher phosphatidylinositol concentrations (Fig. 4). The decrease at high concentrations seemed likely to be due to the removal of Ca²⁺ from free solution by the added phosphatidylinositol. This was confirmed when it was found that addition of extra Ca²⁺ restored the lost activity. At various concentrations of substrate a similar high activity was seen at a Ca²⁺ (total)/phosphatidylinositol ratio of approx. 0.3 (Fig. 4). Since, from the data obtained by using ion buffers, approx. 0.5–1.0 μ M-free Ca²⁺ seems to be the concen-

Fig. 3. Variation of phosphatidylinositol-cleaving activity with pH in the presence of a low concentration (1 μ M) of free Ca²⁺

The incubation media contained 2 mM-EDTA and sufficient CaCl₂ to give, at each pH, a free Ca²⁺ concentration of approx. 1 μ M (Raaflaub, 1960; and see also the Materials and Methods section).

Fig. 4. Influence of phosphatidylinositol concentration on phosphatidylinositol-cleaving activity

The points depicted on the curve were derived from assays in which 0.4 mM-Ca²⁺ was used. The other data come from assays at higher Ca²⁺ concentrations; ○, 0.8 mM-Ca²⁺; ■, 2 mM-Ca²⁺; □, 4 mM-Ca²⁺.

Fig. 5. Effects of chlorpromazine, cinchocaine and KCl on phosphatidylinositol cleavage at pH 7.1

Increasing concentrations of cations were added to incubations in which either (a) the free Ca²⁺ concentration was buffered to approx. 10 μ M with EDTA (Raaflaub, 1960; and see also the Materials and Methods section), or (b) the total Ca²⁺ concentration (unbuffered) was approx. 6 μ M. These data can be considered in conjunction with those in Fig. 6 of the preceding paper (Allan & Michell, 1974), in which these cations were added to assays containing a total Ca²⁺ concentration of 0.4 mM. ○, KCl; Δ, chlorpromazine; ●, cinchocaine.

tration needed for activity close to the maximum this seems likely to be the concentration present at the Ca²⁺/substrate ratio of 0.3. From these values an approximate apparent association constant (K_A) for the interaction of Ca²⁺ with negatively charged phosphatidylinositol under the conditions of our assay ($I \approx 0.01$ mol/l) can be calculated; the value obtained is of the order of 10^6 M⁻¹. This falls between

the values calculated previously of 1×10^7 – 4×10^7 at $I \approx 0$ (Hauser *et al.*, 1969) and 1×10^3 – 2×10^3 at $I \approx 0.1$ mol/l (Abrahamson *et al.*, 1968). Possible reasons for the wide differences between the two values have been discussed further by Hauser *et al.* (1969).

Effects of ions which displace Ca^{2+} from phosphatidylinositol

In the presence of 0.4 mM- CaCl_2 and 1.2 mM-phosphatidylinositol a variety of cations decreased the activity observed at pH 7.0 (Figs. 5 and 6 of Allan & Michell, 1974), probably by decreasing the negative charge of the substrate. If, however, these cations were added to incubations in which the only Ca^{2+} (approx. $6 \mu\text{M}$ total concentration) was that present in the enzyme preparations, then activation was observed at low concentrations, followed by inhibition at higher concentrations (Fig. 5b). It seemed likely that this pattern was a composite one which resulted from a combination of (a) activation owing to Ca^{2+} release from binding to phosphatidylinositol at low cation concentrations, and (b) inhibition at high cation concentrations. This interpretation is supported by the further observation that in the presence of a free Ca^{2+} concentration of approx. $10 \mu\text{M}$, buffered with EDTA, the stimulatory portion of the response was not seen, but the inhibition still occurred (Fig. 5a).

Discussion

It is clear from the observations reported here and in the accompanying paper (Allan & Michell, 1974) that lymphocyte soluble fractions contain an enzyme(s) which catalyses phosphatidylinositol cleavage at a pH and free Ca^{2+} concentration similar to those thought to prevail in the cytosol of many cells. The activity seen at pH 5.5 and much higher Ca^{2+} concentrations, although studied in some detail in several tissues (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Friedel *et al.*, 1969; Keough & Thompson, 1972; Jungalwala *et al.*, 1971; Dawson *et al.*, 1971), seems unlikely to be of significance under physiological conditions. Both activities could be properties of the same enzyme, with activity at pH 5.5 representing an interesting but physiologically irrelevant facet of its character. If this proves to be so then a pH 7.0 activity similar to that described here should be found wherever at pH 5.5 activity has previously been demonstrated. If, as seems likely, the Ca^{2+} requirement is determined by Ca^{2+} binding to the enzyme then the approximately 50-fold change in Ca^{2+} affinity seen between pH 5.5 and pH 7.0 may reflect a change in ionization of the ligand group or groups involved; the nature of the ligand(s) is not yet known. The alternative view, suggested by Jungalwala *et al.* (1971), is that Ca^{2+} is needed in a form

bound to the substrate, with inhibition by chlorpromazine being due to displacement of this Ca^{2+} . This now seems unlikely in view of the low Ca^{2+} /substrate ratios needed for maximum activity when a Ca^{2+} buffer is used.

The effects of cations on activity appear to be ascribable to three phenomena. First, there is the specific requirement for a low concentration of Ca^{2+} ions, the precise extent of which has not been apparent in earlier experiments because Ca^{2+} buffers have not been used. Secondly, there are stimulatory effects of a variety of cations at low concentrations; these appear to be due to the displacement into free solution of Ca^{2+} ions bound to phosphatidylinositol, the result being enzyme activation. Finally, at higher cation concentrations and pH 7.0, but not at pH 5.5, there is inhibition of activity; this seems likely to be due to a decrease in the negative charge on the substrate leading to a decrease in enzyme activity (Allan & Michell, 1974). The relative effects of different cations in the latter two situations are consistent with their known affinities for phosphatidylinositol and their abilities to displace bound Ca^{2+} from phosphatidylinositol (Hauser & Dawson, 1967, 1968); K^+ and Na^+ are relatively ineffective, bivalent ions are much more effective and amphiphilic cations (cinchocaine, a local anaesthetic, and chlorpromazine) are very effective. Concentrations of NaCl and KCl which mimic the intracellular ionic strength partially inhibit phosphatidylinositol cleavage, even when the $[\text{Ca}^{2+}]_{\text{free}}$ is buffered to a fairly stable value (Fig. 5a). It is clear, however, that even under these conditions a considerable proportion of the total activity remains expressed provided that sufficient Ca^{2+} is available to activate the enzyme.

Opinions are at present divided on whether the activity which cleaves phosphatidylinositol is likely to be the same as (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Keough & Thompson, 1972) or different from (Thompson & Dawson, 1964; Tou *et al.*, 1973; Palmer, 1973) that which attacks diphosphoinositide and triphosphoinositide. It is of interest, therefore, that the effects of chelators and ions which we have observed are in many ways similar to effects reported from studies of enzymes from several sources, each of which attacked the glycerol-phosphate linkage in one or more of the phosphoinositides. Whenever appropriate assays were reported activity was unaffected by *o*-phenanthroline (Thompson & Dawson, 1964; Palmer, 1973) but inhibited by EDTA (Kemp *et al.*, 1961; Thompson & Dawson, 1964; Atherton & Hawthorne, 1968; Palmer, 1973; Tou *et al.*, 1973) and, more important, by EGTA (Lapetina & Michell, 1973b; Palmer, 1973). Ca^{2+} has generally been reported to activate (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Keough & Thompson, 1972; Tou *et al.*, 1973; Lapetina & Michell, 1973b). Further, in at least two cases other

than the present study, re-activation by Ca²⁺ has been observed even in the presence of a molar excess of EDTA or EGTA, i.e. when [Ca²⁺]_{free} was buffered to a very low value (Kemp *et al.*, 1961; Palmer, 1973). Amphiphilic cations (Thompson & Dawson, 1964; Keough & Thompson, 1972; Palmer, 1973), bivalent cations other than Ca²⁺ (Kemp *et al.*, 1961; Thompson & Dawson, 1964; Atherton & Hawthorne, 1968; Tou *et al.*, 1973) and NaCl or KCl (Thompson & Dawson, 1964; Keough & Thompson, 1972; Palmer, 1973) have all shown activation effects which appear compatible with a role in releasing traces of bound Ca²⁺ from binding to phosphoinositides. Further, excess of either bivalent cations or of an amphiphilic cation has on several previous occasions been shown to lead to a decrease in activity (Kemp *et al.*, 1961; Thompson & Dawson, 1964; Keough & Thompson, 1972; Tou *et al.*, 1973); the only exceptions have been when activity was measured at a high Ca²⁺ concentration either at the lower (approx. 5.5) pH optimum (Atherton & Hawthorne, 1968; Allan & Michell, 1974) or at pH 7.0 in the presence of an amphiphilic anion (Lapetina & Michell, 1973*b*). In brain the activities against phosphatidylinositol and against triphosphoinositide are both localized in part in the plasma membrane (Keough & Thompson, 1970; Lapetina & Michell, 1973*b*). There are thus great resemblances in the properties of the inositide-cleaving enzyme activities from several sources. In particular, all appear likely to be dependent on the presence of a very low concentration of Ca²⁺: it is not possible from the available data to decide finally whether or not the activities which cleave the different inositides are properties of the same enzyme species.

The range of Ca²⁺ concentrations over which activation occurs with the lymphocyte soluble enzyme *in vitro* is much the same as that in which Ca²⁺ exerts intracellular control of a variety of activities such as contractility, phosphorylase activation and cyclic AMP breakdown (see, for example, Rasmussen *et al.*, 1972). Many stimulated cells, including lymphocytes, show both enhanced phosphatidylinositol turnover (Lapetina & Michell, 1973*a*) and changes in the rates of movement of Ca²⁺ across their surfaces and between intracellular compartments (Allwood *et al.*, 1971; Whitney & Sutherland, 1972; Rasmussen *et al.*, 1972). It therefore seems possible that the cytosol Ca²⁺ concentration may be one of the factors which controls the rate of phosphatidylinositol turnover. However, other controls probably also apply, since (a) phosphatidylinositol turnover in pancreas and adrenal medulla can be stimulated by acetylcholine in the absence of extracellular Ca²⁺ (Hokin, 1966; Trifaró, 1969), and (b) prolonged treatment of muscle with EDTA is needed before its phosphatidylinositol turnover decreases appreciably (Lennon & Steinberg, 1973). Further, a very approximate calculation suggests

that in quiescent lymphocytes the observed rate of phosphatidylinositol turnover would be accounted for by phosphatidylinositol cleavage at about 0.01 % of the optimum rate; such stringent control seems unlikely to be achieved by Ca²⁺ alone. Another observation which, at first sight, might suggest a key role for Ca²⁺ is that reagents which displace bound Ca²⁺ from membranes and phospholipids (e.g. cinchocaine, chlorpromazine, propranolol) evoke increased ³²P_i incorporation into phosphatidylinositol in nerve (Salway & Hughes, 1972), pineal (Eichberg *et al.*, 1973), lymphocytes and parotid (D. Allan, L. M. Jones & R. H. Michell, unpublished work). However, as Eichberg *et al.* (1973) noted, the pattern of the response differs from that elicited by physiological stimuli; it now seems likely that these agents enhance phosphatidylinositol synthesis *de novo* rather than stimulating its turnover (D. Allan & R. H. Michell, unpublished work).

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