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Proteolytic activation can produce a phosphatidylinositol phosphodiesterase highly sensitive to Ca^{2+}

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The phosphatidylinositol phosphodiesterase of rat brain shows little activity under conditions likely to pertain *in vivo* (neutral pH and micromolar Ca^{2+} concentrations). A short incubation of a brain supernatant with trypsin, or a longer pre-incubation of the supernatant alone, produce new forms of the enzyme, which are active under such conditions. A possible role of receptor-linked proteinases in initiating phosphatidylinositol catabolism is discussed.

An increased rate of turnover of phosphoinositides accompanies the stimulation of many tissues, and this may fulfil an important role in cell activation and function (for review, see Hokin, 1968; Michell, 1975; Irvine *et al.*, 1982). It is now generally accepted that the turnover of phosphoinositides is regulated primarily through the activity of the phospholipases that degrade them. Phosphatidylinositol, the principal mammalian inositide (White, 1973), is predominantly hydrolysed by a Ca^{2+}-dependent phosphodiesterase (phospholipase C-like) activity that is found in the cytoplasm of most (probably all) mammalian cells (Dawson, 1959; Kemp *et al.*, 1961; Shukla, 1982). Notwithstanding a possible contribution to increased phosphatidylinositol turnover from other metabolic routes (for discussion, see Irvine *et al.*, 1982; Michell, 1982; Irvine, 1982), it is therefore likely that the control of the activity of this enzyme is central to the regulation of phosphatidylinositol turnover. Recent studies from this laboratory have suggested that a likely regulatory factor on phosphatidylinositol phosphodiesterase is the physicochemical form of its membrane-bound substrate (Irvine *et al.*, 1979, 1982; Dawson *et al.*, 1980; Hirasawa *et al.*, 1981*a*). Here we provide evidence for an additional (or alternative) control mechanism. We show that phosphatidylinositol phosphodiesterase exists in brain in a relatively inactive form and that proteolytic cleavage can convert this into a form which is more likely to be active *in vivo*.

Materials and methods

Materials

Trypsin and crude soya-bean trypsin inhibitor were purchased from Sigma.

Chromatofocusing of rat brain supernatant

The preparation of a rat brain homogenate and its fractionation on a chromatofocusing column (Pharmacia Fine Chemicals) were carried out exactly as described by Hirasawa *et al.* (1982).

Enzyme assay

[^{32}P]Phosphatidylinositol was prepared as described by Irvine *et al.* (1978), and the assay procedure was as in Irvine *et al.* (1979). If the water-soluble products were to be analysed, the method described by Hirasawa *et al.* (1982) was followed. Ca^{2+} concentrations below 1 mM were controlled by Ca^{2+}–EDTA buffers (Hirasawa *et al.*, 1981*b*).

Results and discussion

Our initial observation of the phenomenon described here was that brief trypsin treatment of a cytosolic brain supernatant increased the activity of phosphatidylinositol phosphodiesterase (K. Hirasawa, R. F. Irvine & R. M. C. Dawson, unpublished work) and furthermore that pre-incubation of such a brain supernatant by itself could also cause some activation. To investigate this effect in more detail, we turned to the separation of the different forms of the enzyme by column chromatofocusing. This is a technique that elutes proteins as discrete fractions from a column, on the basis of their isoelectric points (Pharmacia Fine Chemicals). Fig. 1(*a*) shows a typical analysis of a membrane-free brain supernatant by this technique and corresponds to data on the heterogeneity of phosphatidylinositol phosphodiesterase (Hirasawa *et al.*, 1982). Trypsin treatment of the enzyme (Fig. 1*b*) changed the pattern noticeably. The forms (pre-

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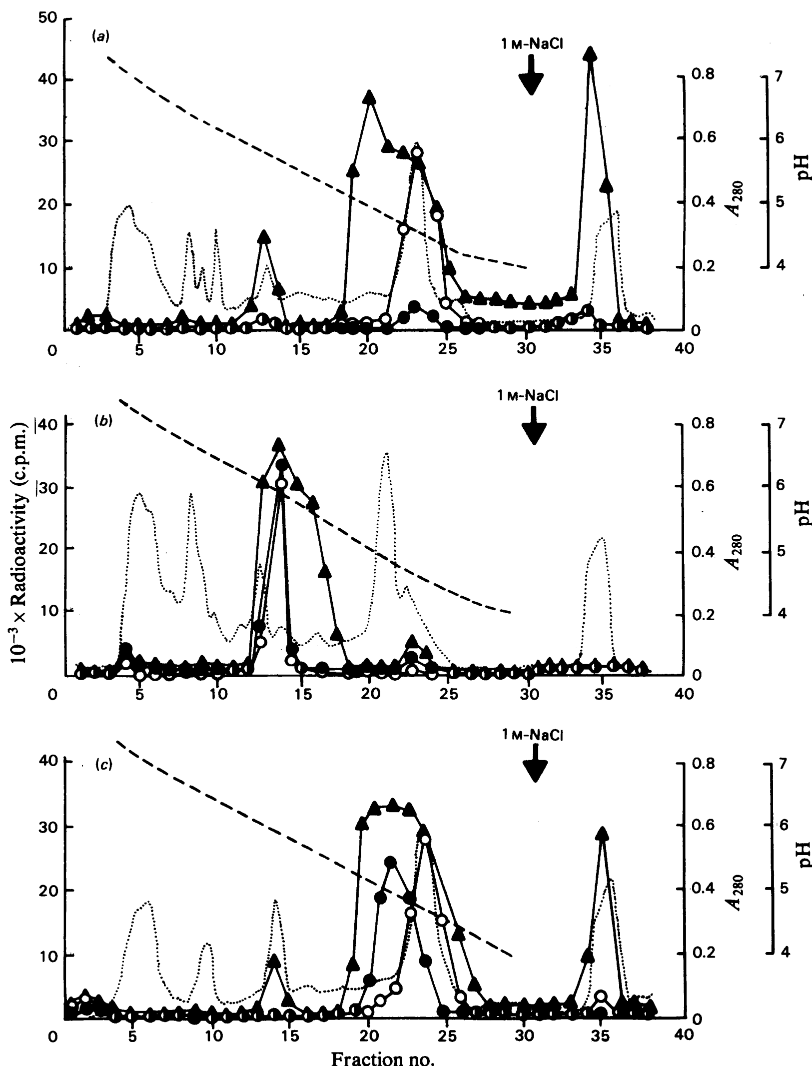

Fig. 1. *Chromatofocusing of rat brain phosphatidylinositol phosphodiesterase*
(*a*) shows the fractionation of a brain supernatant applied directly to the chromatofucusing column (see the Materials and methods section). (*b*) and (*c*) are the results of chromatofocusing the same supernatant, except, for (*b*) the supernatant was treated with 0.75mg of trypsin (Sigma) for 20min at 37°C followed by soya-bean trypsin inhibitor, and, for (*c*), the supernatant was pre-incubated at 37°C for 1h. The A_{280} (· · · · ·) and pH (– – –) of the fractions were determined and their phosphatidylinositol phosphodiesterase activity was assayed at pH 5.5, 1mM-Ca^{2+} (▲), pH 7.25, 1mM-Ca^{2+} (○), and pH 7.25, 1μM-Ca^{2+} (●). Fractions at the top of all peaks were checked for liberation of products, and in all cases phosphoinositol and cyclic phosphoinositol were the sole water-soluble products liberated.

sumably isoenzymes; Hirasawa *et al.*, 1982) active at pH 5.5 and 1mM-Ca^{2+} and also those active at pH 7.25 and 1mM-Ca^{2+} are shifted to a higher isoelectric point. More importantly, whereas in the control sample (Fig. 1*a*) there is little activity under conditions similar to those *in vivo* (1μM-Ca^{2+} at pH 7.25), in the trypsin-treated sample there is a clear peak of such activity, which coincides with the 1mM-Ca^{2+}, pH 7.25 peak (fraction 14, pI 6.1). The Ca^{2+} activation curve of this peak (fraction 14 of Fig. 1*b*) is shown in Fig. 2 and contrasts markedly with the corresponding fraction from a control brain supernatant assayed at the same pH. Addition of soya-bean trypsin inhibitor to the incubation abolished the effect of trypsin treatment, as did prior heat treatment of the trypsin.

The observation that incubation of brain supernatant alone could produce a similar activation to a short trypsin treatment prompted us to investigate the effect of such pre-incubation (at 37°C for 60 min) on the elution pattern of the phosphatidylinositol phosphodiesterase. The results of such an experiment are shown in Fig. 1(*c*). Again, a peak of activity is observed at 1 μM-Ca^{2+} at pH 7.25 where there was none before. In this instance it does not coincide with the peak of 1 mM-Ca^{2+} and pH 7.25, as neither the latter nor the activities at pH 5.5 have shifted significantly (compare Figs. 1*a* and 1*c*). The Ca^{2+} activation curve of fraction 21 (pI 5.0) of Fig. 1(*c*) is shown in Fig. 2; this altered form of the enzyme is completely inhibited by 1 mM-Ca^{2+}. Thus, pre-incubation of a brain supernatant at 37°C has a similar effect (in principle, if not in detail) to a short trypsin treatment, namely an appearance of a new form of the enzyme that is more sensitive to Ca^{2+} by two to three orders of magnitude at neutral pH. The difference in pattern between Figs. 1(*b*) and 1(*c*) (which was highly reproducible) indicates that the endogenous proteinase activity on the brain supernatant is not exactly the same as trypsin, and addition of trypsin inhibitor to the pre-incubation did not prevent the conversion of phosphatidylinositol phosphodiesterase (results not shown). Several neutral proteinases are found in brain (Uzman *et al.*, 1962; Riekkinen & Rinne, 1968; Barrett, 1977) but we do not yet know which is involved in this phenomenon.

The pH optimum of the activated form of enzyme (fraction 14, pI 6.1, of Fig. 1*b* or fraction 21, pI 5.0, of Fig. 1*c*) is 7.0–7.25 if assayed with 1 μM-Ca^{2+} Fig. 3), so that this form of the enzyme might be expected to be active under physiological conditions. Exact quantitative predictions of activity *in vivo* are complicated by the presence of other cations (e.g. K^+ and Mg^{2+}) that can compete with Ca^{2+}-binding sites, for example on inositides (Hauser & Dawson, 1967) or proteins, and so alter the Ca^{2+} dependence of inositide-cleaving enzymes (Downes & Michell, 1982). Suffice to say, however, that as free Ca^{2+} is at a very low level in cells (see Metcalfe *et al.*, 1980) the 2–3-fold shift in the order of the Ca^{2+} dependency of phosphatidylinositol phosphodiesterase (Fig. 2) is likely to have a profound effect on its activity *in vivo*.

It is noteworthy that both in its pH optimum of 7.0–7.25 at 1 μM-Ca^{2+} (Fig. 3), and in its Ca^{2+} dependency (Fig. 2), the activated phosphatidylinositol phosphodiesterase of brain closely resembles the activity studied in pig lymphocytes by Allan & Michell (1974). Either lymphocytes possess sig-

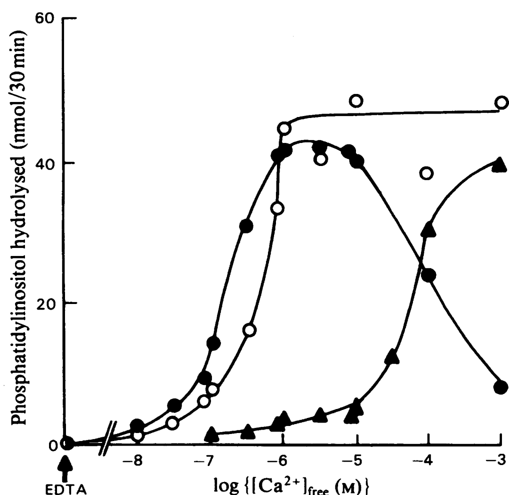

Fig. 2. *Ca^{2+} dependency of phosphatidylinositol phosphodiesterase*
The fractions assayed are: ▲, fraction 24 (pI 4.5) from Fig. 1(*a*); ○, fraction 14 (pI 6.1) from Fig. 1(*b*); ●, fraction 21 (pI 5.0) from Fig. 1(*c*). The fractions were assayed at pH 7.25. Similar results were obtained in an identical experiment. For the Ca^{2+} dependency of the original supernatant, see Hirasawa *et al.* (1981*b*).

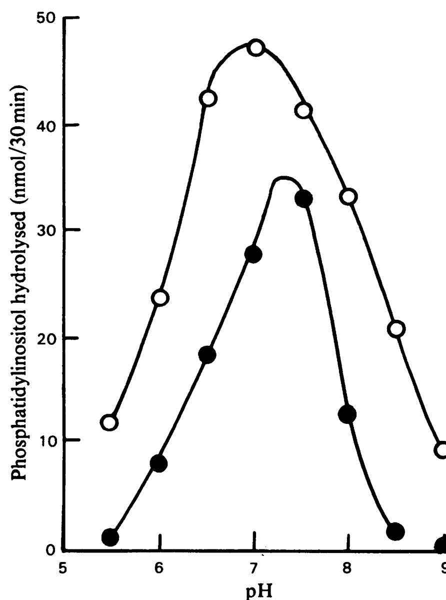

Fig. 3. *Effect of pH on phosphatidylinositol phosphodiesterase*
The assays were performed at 1 μM free Ca^{2+}–EDTA buffer (Raaflaub, 1956). Symbols: ○, fraction 14 (pI 6.1) from Fig. 1(*b*); ●, fraction 21 (pI 5.0) from Fig. 1(*c*). Similar results were obtained in two identical experiments. For the pH dependence of the original brain homogenate at 1 μM-Ca^{2+}, see Hirasawa *et al.* (1981*b*).

nificant amounts of this form of enzyme *in vivo*, or it is formed during isolation of the lymphocytes (or during their homogenization). If the former is true, and resting lymphocytes already possess the Ca^{2+}-sensitive form, then the control of phosphatidylinositol phosphodiesterase on stimulation must presumably lie in the sort of mechanism described elsewhere (Irvine *et al.*, 1979, 1982; Dawson *et al.*, 1980; Hirasawa *et al.*, 1981*a*), or by means as yet unknown. Alternatively, if the Ca^{2+}-sensitive form of the enzyme described here is not found in resting tissues, then our results raise the interesting possibility that a receptor-stimulated proteolytic conversion of phosphatidylinositol phosphodiesterase to a more Ca^{2+}-sensitive form may be an important step in the stimulation of phosphatidylinositol hydrolysis. Limited proteolysis can increase the sensitivity to Ca^{2+} of other enzymes, including some that require calmodulin (Meijer & Guerrier, 1982), though we have found no effect of calmodulin on any form of phosphatidylinositol phosphodiesterase (K. Hirasawa, R. F. Irvine & R. M. C. Dawson), unpublished work); receptor-stimulated proteinases could provide a common link between such activities.

This is the first direct demonstration of an intracellular phospholipase that can have its activity altered by proteolytic cleavage (Van den Bosch, 1980). Whether or not any other phospholipases, including those catabolizing the polyphosphoinositides (Dawson & Thompson, 1964; Michell, 1982), will exhibit parallel properties if analysed by similar techniques will be an interesting area of exploration in the search for cell control mechanisms.

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