Identification and Partial Characterization of Phospholipases in Isolated Adrenocortical Cells

THE EFFECTS OF SYNACTHEN [CORTICOTROPIN-(1-24)-TETRACOSAPEPTIDE] AND CALCIUM IONS

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Phospholipase A activity was determined in homogenates and subcellular fractions of trypsin-dispersed cat adrenocortical cells. At pH 7.4 homogenate phospholipid hydrolysis was activated by added Ca^{2+} and inhibited by EGTA. Phospholipid degradation in the presence and absence of Synacthen was completely blocked by EGTA. Ca^{2+} -dependent activation of a membrane-bound phospholipase may be a critical control mechanism for regulating the molecular changes taking place during stimulation by Synacthen.

Work from our laboratory has implicated prostaglandins in the steroidogenic action of Synacthen [corticotropin-(1–24)-tetracosapeptide]. Prostaglandins are present in isolated cat adrenocortical cells (Laychock & Rubin, 1976), and the conversion of arachidonic acid into prostaglandins is enhanced by Synacthen (Laychock & Rubin, 1975) through some mechanism that requires Ca^{2+} (Laychock *et al.*, 1977). The controlling factor in prostaglandin synthesis appears to be the activation of a phospholipase that is capable of supplying non-esterified fatty acids, such as arachidonic acid, from membrane phospholipids (Kunze & Vogt, 1971). Not only does the addition of arachidonic acid augment prostaglandin synthesis in many tissues (Cohen & Jaffe, 1973; Kuehl *et al.*, 1973; Silver *et al.*, 1973), but treatment with phospholipase A increases tissue prostaglandin formation (Bartels *et al.*, 1971). More direct evidence that membrane-active agents enhance prostaglandin synthesis by stimulating phospholipase-induced phospholipid degradation derives from the findings that phospholipase is present in most cells (Vanden Bosch, 1974), and that hormones and other membrane-active substances liberate non-esterified fatty acids, including arachidonate, by enhancing phospholipase activity (Bills *et al.*, 1976; Haye *et al.*, 1976). Thus to elucidate more completely the mechanism by which Synacthen enhances adrenocortical prostaglandin synthesis and to gain additional insight into the molecular events

that antecede the steroidogenic action of Synacthen, we have examined phospholipase activity in isolated adrenocortical cells.

Materials and Methods

Synacthen was generously donated by Ciba Pharmaceuticals, Summit, NJ, U.S.A., and had a biological activity of 106 units/mg of corticotropin (Schwyzer & Kappeler, 1963). The following compounds were obtained from the sources indicated: trypsin and lima-bean trypsin inhibitor, Worthington Biochemical Corp., Freehold, NJ, U.S.A.; modified Eagle medium (no. 193G), Grand Island Biological Co., Grand Island, NY, U.S.A.; EGTA and bovine serum albumin (fatty acid-free), Sigma Chemical Co., St. Louis, MO, U.S.A.; Calcein, G. Frederick Smith Chemical Co., Columbus, OH, U.S.A.

Adrenal glands were excised from cats and the cortical cells dispersed by using a trypsin-treatment procedure previously described (Rubin & Warner, 1975). The final incubation medium was modified Eagle medium supplemented to contain 2.5 mM- Ca^{2+} , 0.2% bovine serum albumin and 0.04% lima-bean trypsin inhibitor. After trypsin treatment the cortical cells (approx. $2.5 \times 10^5/\text{ml}$) were incubated for 15 min at 37°C in 1 ml of incubation medium in the presence or absence of Synacthen (250–500 μunits). Five beakers of Synacthen-stimulated cells were pooled and five beakers of untreated cells were pooled, to give an average total of 2.5×10^6 – 5×10^6 cells per experiment. The Synacthen-treated and control cells were centrifuged at

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4300g for 10min at 4°C. The supernatant was decanted and the cells resuspended in a sufficient volume of hypo-osmotic 10mM-Tris/HCl buffer, pH7.4, to achieve a cell density of approx. 3.0×10^6 cells/ml. The osmotically ruptured cells were homogenized at 4°C by ten passes at low speed on a motor-driven stirrer by using silicone-treated glassware and a Teflon pestle. Samples of this homogenate were used immediately to determine phospholipase activity. Subcellular fractions of the refrigerated homogenate were prepared by centrifuging a sample for 10min at 4°C and 19000g. Portions of the decanted supernatant were used to measure phospholipase activity. The 19000g pellet was resuspended in a volume of 10mM-Tris/HCl, pH7.14, equal to the original sample of whole homogenate; samples of the suspension were used to determine enzyme activity.

Phospholipase A activity was examined by using [^{14}C]oleate-labelled phospholipids (Patriarca *et al.*, 1972) of autoclaved *Escherichia coli* as substrate as previously described. More than 95% of the incorporated label was in phospholipid, and the distribution of the label closely corresponded to the chemical composition of *E. coli* phospholipid: phosphatidylethanolamine, 50–65%; phosphatidylglycerol, 15–20%; cardiolipin, 10–15%. Radio-labelled *E. coli* were autoclaved for 15min at 120°C and 2.7kg/cm². As demonstrated by snake-venom (*Crotalus adamanteus*) hydrolysis, more than 95% of the [^{14}C]oleate was in the 2-acyl position of the phospholipids. Less than 4% of the total label was associated with neutral lipid. Since phospholipase A2 specifically hydrolyses the fatty acid linkage in the C-2 position of phospholipid, most of the labelled fatty acid released in the present experiments was presumed to be a measure of phospholipase A2 activity. The release of [^{14}C]oleate was measured in reaction mixtures that contained 4×10^8 radio-labelled autoclaved *E. coli* cells (representing 5000c.p.m. and 8nmol of phospholipid), 100mM of the appropriate buffer, various cofactors (see the Results section) and 100–300µg of protein in a final volume of 500µl. The reaction mixture was incubated for 60–120min at 37°C in a shaking water bath, and the reaction was stopped by the addition of 3ml of methanol/chloroform (2:1, v/v). Lipids were extracted overnight (Bligh & Dyer, 1951) and ^{14}C -labelled non-esterified fatty acid was separated from phospholipid by t.l.c. (Franson *et al.*, 1974); both fatty acid and phospholipid were scraped off the plate and counted for radioactivity by scintillation spectrometry. Samples of homogenate were also analysed for protein (Lowry *et al.*, 1951) and rates of hydrolysis were expressed as the percentage of total oleate released/h per mg of homogenate protein, or as a percentage of unstimulated (control) rates of hydrolysis. Statistical

significance of differences in phospholipase activity was tested by paired *t*-test analysis to minimize variability due to differences between experiments.

Fluorometric analysis of total cortical-cell calcium by using Calcein (Jaanus & Rubin, 1971) was carried out on 200µl samples of cortical-cell homogenate suspended in 100mM-Tris/HCl, pH7.4, containing no added Ca^{2+} . Duplicate calcium determinations on samples from two different preparations yielded values of 0.3 and 0.5mM (mean 0.4).

Results

Initial experiments carried out with homogenates of dispersed cortical cells defined two peaks of enzyme activity at pH4.5 and 7.4–8.0 (Fig. 1). The hydrolysis of phospholipid occurring at pH7.4 increased in a linear manner with time for at least 90min and with the amount of tissue homogenate. Hydrolysis at pH7.4 was a linear function of the Ca^{2+} concentration up to 10mM, and 20mM caused no further enhancement of hydrolysis (Fig. 2). Since, in the absence of any added Ca^{2+} , the average Ca^{2+} concentration in the crude homogenate was 0.4mM (see the Materials and Methods section), EGTA (0.4mM) was added to the medium to chelate the residual Ca^{2+} . The addition of EGTA decreased

Fig. 1. pH-dependence of phospholipase activity of isolated adrenocortical-cell homogenates

Isolated cortical cells prepared by trypsin digestion were homogenized in 10mM-Tris/HCl, pH7.4, and protein samples (264µg) were added to a total reaction volume of 500µl, which contained 4×10^8 ^{14}C -labelled *E. coli* cells, 6mM- Ca^{2+} and 100mM of the appropriate buffer (pH3–5.5, sodium acetate; pH6.5, Tris/maleate; pH7.4, Tris/HCl; pH8–10, Tris base). Incubations were carried out at 37°C for 2h. Phospholipase activity was determined from the amount of ^{14}C -labelled non-esterified fatty acid released from [^{14}C]oleate-labelled *E. coli* and was calculated as a percentage of the total lipid radioactivity in *E. coli*. All values were corrected for non-enzymic hydrolysis, which did not exceed 2% over the entire pH range, and are expressed as percentage of hydrolysis/mg of protein.

Table 1. Effect of Synacthen on phospholipase A activity of cortical-cell homogenate and its dependence on Ca²⁺

Cortical cells were incubated with or without Synacthen (250–500 μ units) for 15 min and were homogenised in 10 mM-Tris/HCl, pH 7.4. Samples (100–200 μ l) of the homogenate were added to the standard assay reaction mixture and were incubated at 37°C for 2 h in the presence and absence of CaCl₂ or EGTA. Results are means $\pm$ S.E.M.

Addition		Hydrolysis (%/h per mg of protein)		Percentage of control
EGTA (mM)	CaCl ₂ (mM)	Control	+Synacthen	
—	—	5.8 $\pm$ 2.6	6.5 $\pm$ 2.8	127 $\pm$ 10*
—	3	7.6 $\pm$ 2.6	9.8 $\pm$ 3.2	117 $\pm$ 3*
0.4	—	<0.18 $\pm$ 0.06	<0.18 $\pm$ 0.02	—

* Average relative increase in phospholipase activity induced by Synacthen obtained from seven different experiments. Paired *t*-test analysis gives *P* < 0.05 for differences between phospholipase activity in Synacthen-treated and in unstimulated cells.

enzyme activity to below detectable values (Table 1), which in four separate experiments represented less than 8.2 $\pm$ 1.1% (mean $\pm$ S.E.M.) of values obtained in the absence of EGTA. This marked inhibitory effect of EGTA was readily reversed by the addition of 3 mM-Ca²⁺. Mg²⁺ did not enhance the hydrolysing activity of the homogenate. In contrast with the Ca²⁺-dependent phospholipase activity at pH 7.4, activity at pH 4.5 was inhibited by Ca²⁺, but not by EGTA. Thus, in one experiment, at pH 4.5 10 mM-Ca²⁺ decreased hydrolysis by 53% and the addition of EGTA enhanced enzyme activity by 6%; in this same experiment at pH 7.5, 10 mM-Ca²⁺ increased activity by 100% and EGTA decreased it by 94%. Non-esterified fatty acid release was stimulated by incubating cortical cells with Synacthen (250–500 μ units) for 15 min before hypo-osmotic lysis and homogenization of tissue. The effect of Synacthen on phospholipid hydrolysis by homogenate at pH 7.4 is shown in Table 1. In the absence of added Ca²⁺, Synacthen evoked a significant increase in enzyme activity of 27%. Although added Ca²⁺ augmented basal enzyme activity (Fig. 2), increasing Ca²⁺ concentrations failed to enhance further the stimulant effect of Synacthen. Thus, in the presence of 3 mM-Ca²⁺, Synacthen augmented hydrolysis by 17% (Table 1). When EGTA was added, Synacthen was unable to enhance fatty acid accumulation to detectable concentrations (Table 1). At pH 7.4 an enriched particulate fraction manifested lower basal phospholipid-splitting activity (8.1 $\pm$ 2.2% hydrolysis/ml) than corresponding samples of the whole homogenate (14.4 $\pm$ 2.2% hydrolysis/ml). In this particulate fraction of cells exposed to Synacthen, phospho-

Fig. 2. Ca²⁺-dependence of phospholipase activity at pH 7.4. Various concentrations of CaCl₂ were added to the standard reaction mixture containing 100 μ l samples of homogenate and were incubated for 2 h at 37°C. Fatty acid release in the presence of various amounts of added Ca²⁺ is expressed as a percentage of the hydrolysis in the absence of added CaCl₂. Each point represents the mean ($\pm$ S.E.M.) obtained from four different observations. The average value obtained with 20 mM-CaCl₂ was obtained from two observations.

lipase activity was 166 $\pm$ 17% of that of corresponding unstimulated controls (*P* < 0.05) (*n* = 4). By contrast, activity in the supernatant fraction of cells treated with Synacthen was only 79 $\pm$ 17% of corresponding controls. The addition of EGTA abolished phospholipid-splitting activity in the enriched particulate fraction whether or not the cells had been exposed to Synacthen.

Discussion

The present investigation has demonstrated the existence of a group of phospholipid-splitting enzymes in cat adrenocortical cells. Although both specific 1-acyl and 2-acyl hydrolases are found in most mammalian tissues (Brockerhoff & Jensen, 1974), the fact that the substrate used in these experiments was labelled in the 2-position strongly suggests that phospholipase A2 activity is responsible for the observed fatty acid accumulation. However, it is possible that hydrolysis proceeds through a sequence of reactions involving lysophospholipases and phospholipase A1, which are acyl hydrolases, acting at the C-1 position of phosphatides. The cortical phospholipase, which had maximal activity at pH 4.5, was inhibited by Ca²⁺, suggesting that this activity is associated with the lysosomes of the adrenal cortex. The phospholipase with maximal activity at pH 7.4 had an absolute requirement for Ca²⁺. This particular enzyme is probably not of microsomal origin, since myocardial microsomal phospholipase, for example,

which is also optimally active at physiological pH, is minimally inhibited by Ca^{2+} -chelating agents (Weglicki *et al.*, 1971). Mitochondrial phospholipases, on the other hand, are Ca^{2+} -dependent, but have a pH optimum of 8.0–9.5 (Weglicki *et al.*, 1971; Waite & Sisson, 1971). Since maximal phospholipid-splitting activity was observed between pH 7 and 8 and was Ca^{2+} -dependent, this enzyme may be derived from plasmalemma. In this context, membrane-associated phospholipase A2 with similar characteristics has been demonstrated in rabbit polymorphonuclear leucocytes (Franson *et al.*, 1974).

The finding of a greater stimulant effect of Synacthen in the enriched particulate fraction than in the whole homogenate confirms that the phospholipase stimulated by Synacthen is associated with a membranous fraction. However, it is not clear whether Synacthen augments phospholipid-splitting activity by increasing the activity of the enzyme or by changing its properties so that a greater proportion of the enzyme sediments with this fraction. Moreover, it is not clear whether Synacthen expresses its effect by directly stimulating a hormone-sensitive phospholipase or by producing an increase in the Ca^{2+} concentration at the enzyme site. In this regard, Synacthen is capable of causing an intracellular translocation of cortical cell Ca^{2+} (Jaanus & Rubin, 1971). Despite these uncertainties, the fact that in the particulate fraction both basal and Synacthen-stimulated phospholipid-splitting activity were completely suppressed by EGTA underlines the critical nature of calcium's role in this system; it also provides an explanation for the previously described decrease in prostaglandin synthesis caused by Ca^{2+} deprivation (Laychock *et al.*, 1977). In conclusion, the results of the present experiments reveal that phospholipase activity may be a critical control mechanism for regulating the molecular changes taking place during Synacthen stimulation. Further studies are needed to elucidate the precise cellular location for this enzyme.

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