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Calcium-Activated Hydrolysis of Phosphatidyl-*myo*-inositol 4-Phosphate and Phosphatidyl-*myo*-inositol 4,5-Bisphosphate in Guinea-Pig Synaptosomes

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1. Addition of the bivalent ionophore A23187 to synaptosomes isolated from guinea-pig brain cortex and labelled with [^{32}P]phosphate *in vitro* or *in vivo* caused a marked loss of radioactivity from phosphatidyl-*myo*-inositol 4-phosphate (diphosphoinositide) and phosphatidyl-*myo*-inositol 4,5-bisphosphate (triphosphoinositide) and stimulated labelling of phosphatidate. No change occurred in the labelling of other phospholipids. 2. In conditions that minimized changes in internal Mg^{2+} concentrations, the effect of ionophore A23187 on labelling of synaptosomal di- and tri-phosphoinositide was dependent on Ca^{2+} and was apparent at Ca^{2+} concentrations in the medium as low as 10^{-5}M. 3. An increase in internal Mg^{2+} concentration stimulated incorporation of [^{32}P]phosphate into di- and tri-phosphoinositide, whereas lowering internal Mg^{2+} decreased labelling. 4. Increased labelling of phosphatidate was independent of medium Mg^{2+} concentration and apparently only partly dependent on medium Ca^{2+} concentration. 5. The loss of label from di- and tri-phosphoinositide caused by ionophore A23187 was accompanied by losses in the amounts of both lipids. 6. Addition of excess of EGTA to synaptosomes treated with ionophore A23187 in the presence of Ca^{2+} caused a rapid resynthesis of di- and tri-phosphoinositide and a further stimulation of phosphatidate labelling. 7. Addition of ionophore A23187 to synaptosomes labelled *in vivo* with [^{3}H]inositol caused a significant loss of label from di- and tri-phosphoinositide, but not from phosphatidylinositol. There was a considerable rise in labelling of inositol diphosphate, a small increase in that of inositol phosphate, but no significant production of inositol triphosphate. 8. ^{32}P-labelled di- and tri-phosphoinositides appeared to be located in the synaptosomal plasma membrane. 9. The results indicate that increased Ca^{2+} influx into synaptosomes markedly activates triphosphoinositide phosphatase and diphosphoinositide phosphodiesterase, but has little or no effect on phosphatidylinositol phosphodiesterase.

Phosphatidyl-*myo*-inositol 4-phosphate (diphosphoinositide) and phosphatidyl-*myo*-inositol 4,5-bisphosphate (triphosphoinositide) are found in a variety of mammalian tissues, in particular brain and kidney, and certain micro-organisms (Hawthorne & White, 1975). Although these phospholipids are quantitatively minor components of cell membranes, the very rapid turnover of their monoester phosphate groups (Brockerhoff & Ballou, 1962; Eichberg & Dawson, 1965; White & Larrabee, 1973) suggests they have an important role in membrane function. Brain has at least two pools of di- plus tri-phosphoinositide, a relatively stable pool associated with myelin and a very labile pool probably located in plasma membranes (Eichberg & Hauser, 1973). The presence of a rapidly turning over pool of di- plus triphosphoinositide in peripheral nerve (Sheltawy & Dawson, 1969) suggests a role in nerve conduction, and the possible involvement of the di- and triphosphoinositides in controlling membrane-bound Ca^{2+} or permeability to ions has been discussed by a number of authors (Dawson, 1969; Kai & Hawthorne, 1969; Hendrickson & Reinertsen, 1971; Michell, 1975).

Direct evidence for changes in di- and tri-phosphoinositide metabolism as a result of nervous activity has been difficult to produce. White *et al.* (1974) reported that electrical stimulation of vagus nerve for 30min increased [^{32}P]phosphate incorporation into diphosphoinositide and triphosphoinositide. White & Larrabee (1973) reported a specific decrease in the labelling of triphosphoinositide in vagus nerve after electrical stimulation for 3h, and Pumphrey (1969) found no significant changes in the labelling of di- and tri-phosphoinositides after electrical stimulation of brain slices. The evidence suggesting an effect of acetylcholine on di- and tri-phosphoinositide metabolism is similarly confusing. A number of attempts to alter the labelling of diphosphoinositide and triphosphoinositide by the addition of acetylcholine to media bathing brain slices (Palmer & Rossiter, 1965), sympathetic ganglia (Hokin, 1965) and synaptosomes

(Yagihara & Hawthorne, 1972) proved unsuccessful. By contrast Schacht & Agranoff (1972) observed a decrease in labelling of di- and tri-phosphoinositides in synaptosomes incubated with acetylcholine. More recently Abdel-Latif *et al.* (1977) reported that acetylcholine stimulated hydrolysis of triphosphoinositide in rabbit iris muscle and Tret'jak *et al.* (1977) found an increase in triphosphoinositide breakdown in crab nerve fibres incubated with very high concentrations of acetylcholine. The mechanism by which nervous activity or acetylcholine affects di- and triphosphoinositide metabolism is unknown.

Hydrolysis of di- and tri-phosphoinositides can occur by two mechanisms (Kai & Hawthorne, 1969). The action of phosphomonoesterase on triphosphoinositide yields diphosphoinositide, which may be further degraded to phosphatidylinositol. Alternatively the di- and tri-phosphoinositides may be degraded by a phosphodiesterase yielding inositol di- or tri-phosphate and diacylglycerol. The di- and triphosphoinositides are synthesized by sequential phosphorylation of phosphatidylinositol with ATP (Kai & Hawthorne, 1969). Di- and tri-phosphoinositides rapidly disappear from tissues *post mortem* (Dawson & Eichberg, 1965). The activity *in vitro* of the synthesizing enzymes is considerably less than that of the hydrolytic enzymes and this suggests a strict regulation of the latter *in vivo*. One candidate for such regulation is the intracellular free Ca^{2+} ion concentration. Ca^{2+} ions have marked effects *in vitro* on the activities of the enzymes involved in di- and tri-phosphoinositide synthesis and breakdown, though whether they cause stimulation or inhibition is often influenced by the presence of Mg^{2+} ions (Dawson & Thompson, 1964; Kai & Hawthorne, 1969). In the present paper we describe the effects on di- and tri-phosphoinositide metabolism of altering the intracellular Ca^{2+} ion concentration. The bivalent ionophore A23187 was used with synaptosomes from guinea-pig brain cortex.

Materials and Methods

Preparation of synaptosomes

The method was based on that used by Gray & Whittaker (1962). Guinea pigs (300–500g) were lightly anaesthetized with diethyl ether and killed by decapitation. Their brains were removed, chilled in ice-cold 0.32M-sucrose and the cerebellum and some white matter discarded. All subsequent operations were carried out at 0–4°C and sucrose solutions were adjusted to pH7.4 with Tris. Forebrains were homogenized in 10vol. of 0.32M-sucrose by using eight up-and-down strokes of a Potter–Elvehjem Teflon/glass homogenizer with a radial clearance of 0.15–0.18mm rotating at 800rev./min. The homogenate was centrifuged at 1000*g* for 10min and the resulting pellet washed twice by resuspending in 0.32M-sucrose and centrifuging at 1000*g* for 10min. The supernatants from the low-speed centrifugations were combined and centrifuged at 10000*g* for 20min. The pellet (crude mitochondrial fraction) was washed once by resuspending in 0.32M-sucrose and centrifuging at 20000*g* for 20min, resuspended in 0.32M-sucrose and layered on a discontinuous sucrose gradient consisting of equal volumes of 1.0M- and 1.2M-sucrose. Gradients were centrifuged at 95000*g* for 90min in either an MSE 6×15ml or 3×23ml swing-out rotor. Synaptosomes sedimenting to the 1.0M/1.2M-sucrose interface were removed, diluted 3–4-fold with incubation medium (see below), centrifuged at 20000*g* for 10min and then resuspended in incubation medium at a protein concentration of approx. 5mg/ml.

Incubations

The incubation medium was based on that used by Bradford (1969) and contained, unless otherwise stated, 124mM-NaCl, 5mM-KCl, 1.2mM-KH_2PO_4, 1.3mM-$MgCl_2$, 26mM-$NaHCO_3$, pH7.4, 0.75mM-$CaCl_2$, 1.6mM-cytidine, 1.6mM-*myo*-inositol and 10mM-glucose. Synaptosomes were incubated at 37°C in air. When subsynaptosomal fractions were to be prepared incubations were terminated by dilution with ice-cold medium and transfer to an ice/water bath. Otherwise incubations were terminated by addition of an equal volume of 20% (w/v) trichloroacetic acid. After 10min at room temperature samples were centrifuged at 1000*g* for 5min and the pellets washed once with 5% trichloroacetic acid containing 1mM-EDTA and once with water.

Labelling of synaptosomes

Synaptosomes were labelled *in vitro* by incubation in media containing 50–100μCi of [^{32}P]phosphate/ml. Synaptosomes labelled *in vivo* were isolated from guinea pigs that had received intracerebral injections of either 100μCi of [^{32}P]phosphate (sp. radioactivity 160μCi/μmol) (Pickard & Hawthorne, 1978) or 40μCi of *myo*-[2-^{3}H]inositol (sp. radioactivity 5μCi/μmol, in iso-osmotic saline) 2h before death.

Extraction and separation of lipids

The water-washed trichloroacetic acid pellet was extracted twice with 1.5ml of chloroform/methanol/conc. HCl (100:100:1, by vol.) and once with 1ml of chloroform/methanol/conc. HCl (200:100:1, by vol.). The extracts were combined, 1.5ml of chloroform was added and the whole washed once with 1.5ml of 0.1M-HCl and twice with its 'synthetic upper phase'. After the last wash the lower phase was evaporated to dryness under N_2, the lipid residue

redissolved in chloroform/methanol (90:10, v/v) and stored at −20°C. Di- and tri-phosphoinositide, phosphatidic acid and a combined phosphatidylinositol and phosphatidylserine fraction were separated by two-dimensional t.l.c. on silica gel H. Thin-layer plates were made from a slurry of silica gel H in 1% potassium oxalate and activated for 1h at 110°C before use. Chromatograms were developed with chloroform/methanol/35% NH_3/water (45:35:2:8, by vol.) in the first dimension and chloroform/methanol/glacial acetic acid/water (80:40:7.4:1.2, by vol.) in the second dimension, plates being air-dried for 1h between solvents. Lipids were located by exposure of plates to I_2 vapour. Di- and tri-phosphoinositides constitute only a small proportion of the total synaptosomal phospholipids. Consequently in experiments where only radioactivity of lipids was determined a non-radioactive crude diphosphoinositide fraction from ox brain (Folch, 1949) was added to samples as carrier (1–2μg of lipid phosphorus per plate). Where amounts of phospholipids were to be determined, up to 5μmol of synaptosomal phospholipid had to be applied to each plate for these minor components to be visible and for accurate phosphate analysis. Di- and tri-phosphoinositide were identified by co-chromatography with standards prepared from crude ox brain diphosphoinositide fraction by DEAE-cellulose column chromatography (Hendrickson & Ballou, 1964). Phosphatidic acid was identified by co-chromatography with a standard prepared by the action of phospholipase D on phosphatidylcholine (Kates & Sastry, 1969). Phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidylcholine were separated by two-dimensional t.l.c. on silica gel H as described by Anderson *et al.* (1970).

Isolation of nucleotides

The supernatant and washings of the trichloroacetic acid-precipitated synaptosomal pellet were combined and mixed for 30min at room temperature with 50mg of purified activated charcoal (Norit A). The charcoal was sedimented by centrifugation at 1000*g* for 5min and washed twice with 4ml of water. Nucleotides were extracted with aq. 10% pyridine as described by Burnstock *et al.* (1970) and the extract was applied directly to a column of anion-exchange resin (Dowex 1X8, Cl^- form, 100–200 mesh; 4cm × 0.5cm). Columns were eluted with 20mM-NH_4Cl in 20mM-HCl to remove mono- and di-phosphates and 250mM-HCl to remove triphosphates as described by Glynn & Chappell (1964) for the isolation of ATP. Nucleotide recovery was calculated from the recovery of 0.1μCi of [8-^{3}H]ATP (sp. radioactivity 23μCi/μmol) added to the trichloroacetic acid-precipitated synaptosomal suspensions and was normally 40–50%. Nucleoside triphosphates were determined from total inorganic phosphate after digestion for 1h at 180° C in 72% (w/v) perchloric acid.

Isolation of [^{3}H]inositol phosphates

The supernatants and washings of the trichloroacetic acid-precipitated pellets were combined, extracted four times with diethyl ether and applied to similar Dowex columns, which were then eluted with water to remove inositol, 30mM-HCl to remove inositol phosphate, 90mM-HCl to remove inositol diphosphate and 500mM-HCl to remove inositol triphosphate. The identity of fractions was confirmed by comparison of their elution characteristics with those of ^{32}P-labelled inositol phosphates prepared by acid hydrolysis (Dawson & Dittmer, 1961) of di- and tri-phosphoinositide isolated from synaptosomes labelled *in vitro* with [^{32}P]phosphate.

Preparation of subsynaptosomal fractions

The method was a modification of that used by Whittaker *et al.* (1964). Synaptosomes were sedimented by centrifugation at 20000*g* for 10min and resuspended at a protein concentration of 4–5mg/ml in 5mM-Tris/HCl, pH9.5, containing 1mM-EDTA. After stirring for 30min at 0°C the lysed synaptosomes were layered on a discontinuous density gradient made from 2ml portions of 0.4M-, 0.6M-, 0.8M-, 1.0M- and 1.2M-sucrose, each containing 25mM-Tris/HCl, pH9.5. Gradients were centrifuged at 95000*g* for 90min in an MSE 6×15ml swing-out rotor. Fractions were designated *O*, *D*, *E*, *F*, *G*, *H* and *I* after the original authors. Part of each fraction was retained for protein and marker-enzyme assays, the remainder was precipitated by addition of an equal volume of 20% trichloroacetic acid and lipid extracted as described above.

Determination of radioactivity

^{32}P and ^{3}H were determined by liquid-scintillation counting in a scintillation fluid prepared by mixing toluene containing 6g of 2,5-diphenyloxazole and 120mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre with Triton X-100 and water in the proportions 6:3:1 (by vol.). Scrapings from thin-layer plates were added directly to scintillation fluid. Studies with ^{32}P-labelled phospholipids indicated that all phospholipid was eluted from the silica gel by the Triton/toluene/water mixture. Aqueous samples were added to toluene/Triton X-100 mixtures to give the same final proportions as above.

Other determinations

The following enzymes were used to characterize subsynaptosomal fractions: cholinesterase (EC

3.1.1.8), lactate dehydrogenase (EC 1.1.1.27), succinate dehydrogenase (EC 1.3.99.1), arylesterase (EC 3.1.1.2) and ($Na^+ + K^+$)-dependent adenosine triphosphatase (EC 3.6.1.3). Cholinesterase, lactate dehydrogenase and succinate dehydrogenase assays are described elsewhere (Yagihara *et al.*, 1973). Arylesterase was assayed by the method of Shephard & Hübscher (1969). Adenosine triphosphatase activity was assayed in 30mM-Tris/HCl, pH7.1, containing 7.5mM-$MgCl_2$, 120mM-NaCl, 20mM-KCl and 5mM-ATP. A 1ml mixture, containing 10–100μg of protein, was incubated at 37°C for 10min. The assay was terminated by addition of an equal volume of 10% (w/v) trichloroacetic acid and inorganic phosphate determined by the method of Chen *et al.* (1956). ($Na^+ + K^+$)-dependent adenosine triphosphatase was taken as the difference in activity in the presence and absence of 1mM-ouabain.

Inorganic phosphate was determined by the method of Bartlett (1959) and protein by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Materials

Ionophore A23187 (E. Lilly and Co., Indianapolis, IN, U.S.A.) was dissolved in ethanol at a concentration of 1mg/ml and stored at −20°C.

Results

Effect of ionophore A23187 on synaptosomal phospholipids

The phospholipids most readily labelled by incubating synaptosomes in medium containing [^{32}P]-phosphate were phosphatidic acid, diphosphoinositide and triphosphoinositide (Table 1). Of the other lipids only phosphatidylinositol incorporated appreciable radioactivity. Addition of ionophore A23187 (in ethanol final concentration 10μg/ml; see the Materials and Methods section) to synaptosomes incubated in medium containing 0.75mM-$CaCl_2$ and 1.3mM-$MgCl_2$ caused a marked decrease in the radioactivity of di- and triphosphoinositide and an increase in the radioactivity of phosphatidic acid. No changes occurred on the addition of ethanol alone. Ionophore A23187 caused no changes in the radioactivity of phosphatidylinositol or any other phospholipid, though for the latter only a large change would have been statistically significant. In synaptosomes isolated from animals labelled with [^{32}P]phosphate *in vivo* (Table 2) the most radioactive phospholipid was phosphatidylinositol. Considerable radioactivity was observed in other phospholipids, though phosphatidic acid, diphosphoinositide and triphosphoinositide were less well labelled than *in vitro*. This difference in labelling pattern may reflect a particular importance of phosphatidylinositol in synaptic function *in vivo* (Pickard & Hawthorne, 1978), whereas the relatively low radioactivity of phosphatidic acid and di- and tri-phosphoinositide probably reflects their rapid turnover and the loss of label during isolation and incubation of the synaptosomes in unlabelled medium. Addition of ionophore A23187 caused a decrease in labelling of di- and tri-phosphoinositide, an increase in the labelling of phosphatidic acid, but no changes in the radioactivity of any other phospholipid (Table 2).

Ionophore A23187 has a marked selectivity towards bivalent cations (Pfeiffer *et al.*, 1974), and only in their absence has ionophore A23187 been shown to affect membrane permeability to uni-

Table 1. *Effect of ionophore A23187 on the radioactivity in phospholipids of synaptosomes labelled with [^{32}P]phosphate in vitro*

Synaptosomes isolated from guinea-pig cortex were incubated for 30min at 37°C in a medium based on that of Bradford (1969) and containing 0.75mM-$CaCl_2$, 1.3mM-$MgCl_2$ and 100μCi of [^{32}P]phosphate/ml. Ionophore was added after 20min to a final concentration of 10μg/ml. The phospholipids were isolated and their radioactivity was determined as described in the Materials and Methods section. The results are the means±S.D. for five experiments with the same synaptosomal preparation. Values that are statistically significant (Student's *t* test) compared with controls are shown by: *$P<0.001$.

	^{32}P incorporated (c.p.m./μmol of total synaptosomal phospholipid)	
	Control	+Ionophore A23187
Triphosphoinositide	5568±524	1912±381*
Diphosphoinositide	4049±224	1908±269*
Phosphatidic acid	2885±159	4605±293*
Phosphatidylethanolamine	128±31	105±15
Phosphatidylinositol	776±43	719±77
Phosphatidylserine	123±30	114±16
Lysophosphatidylethanolamine	60±33	48±26
Phosphatidylcholine	55±25	50±12

Table 2. *Effect of ionophore A23187 on radioactivity in phospholipids of synaptosomes labelled with [^{32}P]phosphate in vivo*

Synaptosomes were isolated from cerebral cortices of guinea pigs that had received an intracerebral injection of 100 μCi of [^{32}P]phosphate 2h before death. Conditions of incubation were the same as described in Table 1, except that the incubation medium contained no ^{32}P. The results are the means ± S.D. for five experiments with the same synaptosomal preparation. Values that are statistically significant (Student's *t* test) from controls are shown by: *$P<0.002$; **$P<0.001$.

	^{32}P incorporated (c.p.m./μmol of total synaptosomal phospholipid)	
	Control	+Ionophore A23187
Triphosphoinositide	1697 ± 194	1199 ± 146*
Diphosphoinositide	1085 ± 27	819 ± 53**
Phosphatidic acid	980 ± 52	1212 ± 53**
Phosphatidylethanolamine	698 ± 46	699 ± 38
Phosphatidylinositol	4978 ± 158	4990 ± 219
Phosphatidylserine	95 ± 29	92 ± 19
Lysophosphatidylethanolamine	530 ± 189	491 ± 219
Phosphatidylcholine	1951 ± 86	1949 ± 133

valent cations (Reed & Lardy, 1972; Flatman & Lew, 1977*a*). The effect of ionophore A23187 on labelling of synaptosomal di- and tri-phosphoinositides was dependent on the medium concentration of both Mg^{2+} and Ca^{2+}. Addition of ionophore A23187 to synaptosomes incubated in media with Mg^{2+} concentrations below 0.7mM (and no added Ca^{2+}) caused a loss of label from di- and tri-phosphoinositide (Fig. 1). Addition of ionophore A23187 to synaptosomes incubated in media with Mg^{2+} concentrations greater than 1.3mM caused an increase in di- and tri-phosphoinositide labelling. Addition of ionophore A23187 to synaptosomes incubated in media containing even low Ca^{2+} concentrations caused a marked loss of radioactivity from di- and tri-phosphoinositide, and this increased with increasing Ca^{2+} concentration (Fig. 2). The loss of radioactivity from synaptosomal di- and tri-phosphoinositides described in Tables 1 and 2 is therefore a result of Ca^{2+} influx rather than a change in intrasynaptosomal Mg^{2+} concentration. Ionophore A23187 has similar affinities for Ca^{2+} and Mg^{2+} (Pfeiffer *et al.*, 1974), and the rate of transport of Ca^{2+} into the synaptosome must depend on the ratio of Ca^{2+} to Mg^{2+} as well as the absolute Ca^{2+} concentration. That the ionophore has such a marked effect even at low Ca^{2+} concentrations and high Mg^{2+}/Ca^{2+} ratios (43:1 at a Ca^{2+} concentration of 0.03mM; Fig. 2) emphasizes the sensitivity of synaptosomal di- and tri-phosphoinositide metabolism to changes in intracellular Ca^{2+} concentration.

The effect of ionophore A23187 on labelling of synaptosomal phosphatidic acid was independent of medium Mg^{2+} concentration (Fig. 1) and apparently only partly dependent on medium Ca^{2+} concentration (Fig. 2). However, originally Ca^{2+}-free medium in which synaptosomes had been incubated was shown by flame photometry to contain approx. 10 μM-Ca^{2+}, presumably because of leakage from damaged synaptosomes and active outward Ca^{2+} transport. The increase in phosphatidic acid labelling observed on addition of ionophore A23187 to synaptosomes incubated in media containing no added Ca^{2+} could therefore still be a result of Ca^{2+} influx.

The loss of label from di- and tri-phosphoinositide caused by ionophore A23187 occurred rapidly and was reversed by chelation of medium Ca^{2+} with EGTA (Fig. 3). Studies with ^{45}Ca (Hawthorne & Griffin, 1978) showed that Ca^{2+} influx into synaptosomes caused by ionophore A23187 is very rapid, occurring largely within 20s of addition of ionophore. Ca^{2+} efflux on addition of excess of EGTA to the medium is equally rapid. The effect of ionophore A23187 on the labelling of phosphatidic acid is evident immediately after addition of ionophore (Fig. 3), and subsequent addition of excess of EGTA caused a further increase in phosphatidic acid labelling.

The effects of ionophore-mediated Ca^{2+} influx and efflux on the radioactivity and amounts of synaptosomal phospholipids and nucleoside triphosphates in a number of experiments are summarized in Table 3. Addition of ionophore A23187 produced an average 66% loss of radioactivity from triphosphoinositide and 56% loss from diphosphoinositide. The effect on the amounts of both phospholipids was somewhat less, an average 41% loss of triphosphoinositide and 43% loss of diphosphoinositide. Decrease of elevated intracellular Ca^{2+} concentrations by addition of EGTA caused a considerable increase in radioactivity of di- and tri-phosphoinositide and a return of the amounts of both phospholipids to control values. The increases in the radioactivity of phosphatidic acid on addition of ionophore A23187 and EGTA were not accompanied by changes in the amounts of phosphatidic acid. Addition of ionophore A23187

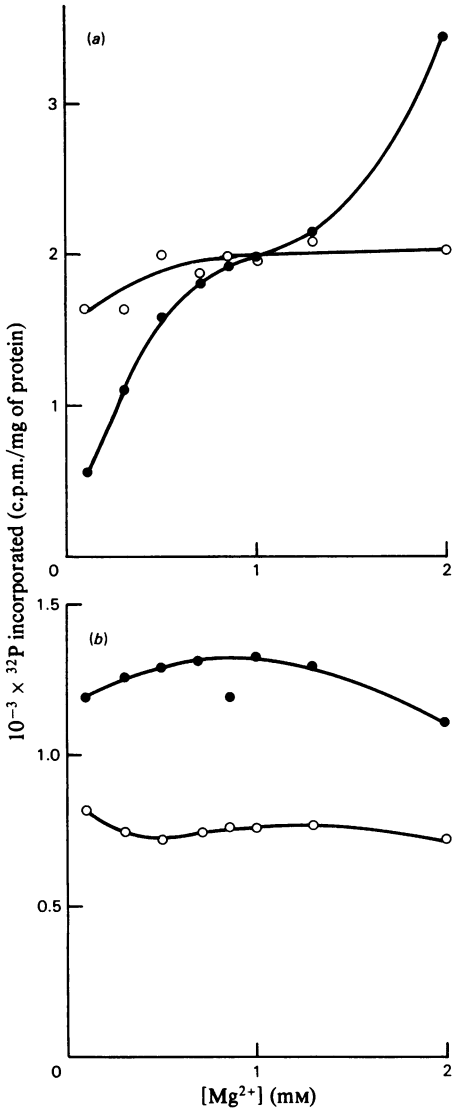

Fig. 1. *Effect of ionophore A23187 on the radioactivity of (a) triphosphoinositide and (b) phosphatidic acid in synaptosomes labelled with [^{32}P]phosphate in vitro: dependence on medium Mg^{2+} concentration*

Synaptosomes were incubated for 30min at 37°C in media containing 100μCi of [^{32}P]phosphate/ml, no Ca^{2+} ions and varying $MgCl_2$ concentration. Ionophore A23187 was added after 20min to give a concentration of 10μg/ml. Symbols: ○, control, no additions; ●, ionophore A23187 added. The effect on diphosphoinositide labelling was very similar to that for triphosphoinositide.

caused a marked decrease in the amount and radioactivity of nucleoside triphosphates that was only partially reversed by subsequent addition of EGTA.

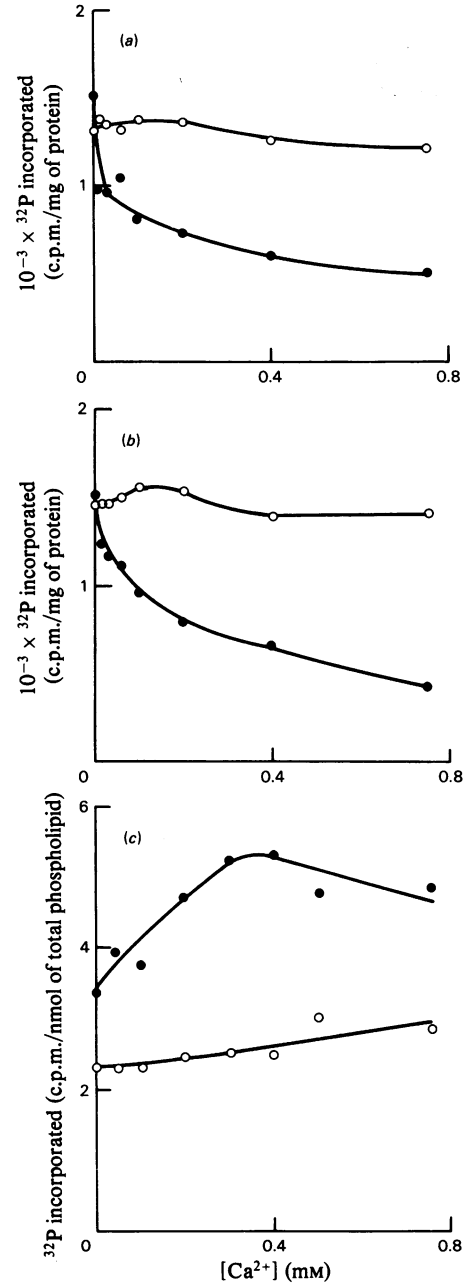

Fig. 2. *Effect of ionophore A23187 on the radioactivity of (a) diphosphoinositide, (b) triphosphoinositide and (c) phosphatidic acid in synaptosomes labelled with [^{32}P]-phosphate in vitro: dependence on medium Ca^{2+} concentration*

Synaptosomes were incubated for 30min in media containing 100μCi of [^{32}P]phosphate/ml, 0.8mM-$MgCl_2$ and varying $CaCl_2$ concentration. Ionophore A23187 was added after 20min to give a concentration of 10μg/ml. Symbols: ○, control, no additions; ●, ionophore A23187 added.

Mechanism of Ca^{2+}-dependent di- and tri-phosphoinositide hydrolysis

Hydrolysis of di- and tri-phosphoinositide could be due to the activation of monoesterase or diesterase (Thompson & Dawson, 1964; Dawson & Thompson, 1964). Activation of diesterases would lead to production of diacylglycerol and water-soluble inositol di- and tri-phosphates. A small increase in amount of synaptosomal diacylglycerol is not readily detectable, and the identification of radioactive inositol phosphates in synaptosomes labelled with [^{32}P]phosphate is complicated by the presence of other highly labelled di- and tri-phosphates. Use was therefore made of synaptosomes isolated from brains labelled *in vivo* with *myo*-[2-^{3}H]inositol. Most of the lipid-soluble ^{3}H in these synaptosomes was in phosphatidylinositol, though both di- and tri-phosphoinositide contained appreciable radioactivity (Table 4). Addition of ionophore A23187 in the presence of Ca^{2+} caused a loss of label from di- and tri-phosphoinositide, but no significant loss from phosphatidylinositol. Analysis of the water-soluble products by ion-exchange chromatography showed a marked increase in [^{3}H]inositol diphosphate, a small increase in [^{3}H]inositol phosphate, but no significant production of [^{3}H]inositol triphosphate. In three similar experiments the increase in inositol diphosphate labelling accounted for $71 \pm 15\%$ (mean $\pm$S.D.) of the radioactivity lost from di- and tri-phosphoinositide.

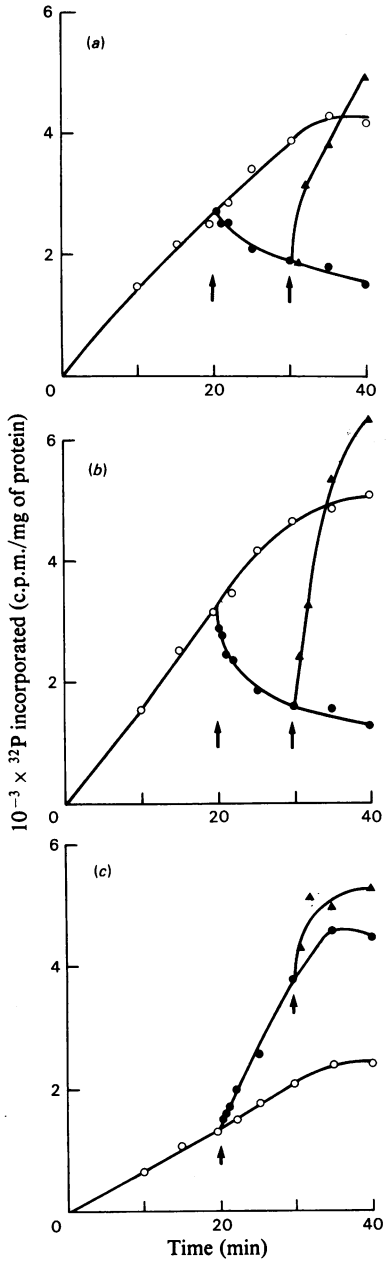

Fig. 3. *Effect of ionophore A23187 on radioactivity in (a) diphosphoinositide, (b) triphosphoinositide and (c) phosphatidic acid of synaptosomes labelled with [^{32}P]phosphate in vitro: time course and influence of EGTA*

Synaptosomes were incubated for 40min at 37°C in medium containing 0.75mM-$CaCl_2$, 1.3mM-$MgCl_2$ and 100μCi of [^{32}P]phosphate/ml. Ionophore A23187 was added after 20min to a concentration of 10μg/ml and EGTA after a further 10min to a concentration of 1mM. Both these additions are indicated by arrows. Symbols: ○, control, no additions; ●, ionophore A23187 added; ▲, ionophore A23187 and EGTA added.

Subsynaptosomal distribution of ^{32}P-labelled di- and tri-phosphoinositides

The subcellular distribution of labelled phospholipids was investigated in synaptosomes that had been incubated for 30min in medium containing [^{32}P]phosphate. Subsynaptosomal fractions were prepared after osmotic rupture by fractionation on a discontinuous sucrose gradient. In preliminary work synaptosomes were osmotically ruptured in 5mM-Tris/HCl, pH8.1 (Cotman & Mathews, 1971) and the sucrose solutions of the density gradient adjusted to pH7.4 with Tris. Under these conditions the overall recovery of radioactive di- and tri-phosphoinositide was only 40–45%. In studies on the subcellular distribution of di- and tri-phosphoinositides in rat brain Eichberg & Hauser (1973) observed that recovery of di- and tri-phosphoinositides was significantly greater when fractionation was carried

Table 3. *Effects of ionophore A23187 and EGTA on radioactivities and amounts of triphosphoinositide, diphosphoinositide, phosphatidic acid and nucleoside triphosphates in synaptosomes labelled with [^{32}P]phosphate in vitro*

The experimental procedure is described in the legend to Fig. 3. Incorporation of radioactivity (c.p.m./mg of protein) and amounts of phospholipid and nucleotides are expressed relative to those in synaptosomes incubated for 30min with no additions. The mean values for the amounts of triphosphoinositide, diphosphoinositide and phosphatidic acid in these synaptosomes were 1.3, 2.6 and 5.4nmol/μmol of total phospholipid respectively. The mean value for their nucleoside triphosphate content was 4.5nmol/mg of protein. Results are the mean±S.D. for the number of experiments indicated in parentheses, each experiment being with a different synaptosomal preparation. Values that are significantly different (Student's *t* test for paired samples) from appropriate controls are shown by: *$P<0.01$; **$P<0.001$.

		Relative incorporation of ^{32}P into:			
	Incubation time (min)	Triphospho-inositide	Diphospho-inositide	Phosphatidic acid	Nucleoside triphosphates
Control	30	1.00	1.00	1.00	1.00
+Ionophore A23187	30	0.34±0.09 (11)**	0.44±0.23 (11)**	1.55±0.34 (9)*	0.41±0.02 (4)**
Control	40	1.24±0.12 (7)	1.22±0.05 (7)	1.33±0.12 (6)	1.27±0.13 (4)
+Ionophore A23187+EGTA	40	1.50±0.28 (7)	1.82±0.33 (6)**	2.87±0.84 (6)**	0.81±0.09 (4)**
		Relative amounts			
Control	30	1.00	1.00	1.00	1.00
+Ionophore A23187	30	0.59±0.08 (6)**	0.57±0.18 (5)*	0.94±0.08 (4)	0.55±0.06 (3)*
Control	40	1.00±0.06 (5)	0.96±0.12 (4)	0.97±0.11 (4)	0.87±0.10 (3)
+Ionophore A23187+EGTA	40	0.97±0.16 (5)	1.10±0.19 (4)	0.96±0.25 (4)	0.61±0.08 (3)

Table 4. *Effect of ionophore A23187 on the radioactivity of inositol phospholipids and water-soluble inositol phosphates in synaptosomes labelled with [^3H]inositol in vivo*

Synaptosomes were isolated from the cerebral cortices of six guinea pigs that had received intracerebral injections of 40μCi of *myo*-[2-^{3}H]inositol 2h before death and incubated at 37°C for 25min in medium containing 0.75mM-$CaCl_2$ and 1.3mM-$MgCl_2$. Ionophore A23187 was added to a concentration of 10μg/ml after 20min. The results are the means±S.D. for four experiments with the same synaptosomal preparation. Each incubation contained approx. 10mg of synaptosomal protein. Values that are statistically significant (Student's *t* test) from controls are shown by: *$P<0.05$; **$P<0.01$.

	^3H incorporated (d.p.m.)		
	Triphosphoinositide	Diphosphoinositide	Phosphatidylinositol
Control	1762±374	1977±259	21150±765
+Ionophore A23187	824±69**	1413±143**	20687±1331
	Inositol triphosphate	Inositol diphosphate	Inositol phosphate
Control	41±29	836±143	636±95
+Ionophore A23187	79±18	1619±124**	780±24*

out in sucrose solutions at pH9.5, apparently because of inhibition of enzymic hydrolysis. The loss of radioactive di- and tri-phosphoinositides from osmotically ruptured pre-labelled synaptosomes was also inhibited by high pH, and therefore in subsequent studies osmotic rupture and fractionation of the subsynaptosomal fractions were carried out at pH9.5 as described in the Materials and Methods section. The use of high pH had little or no effect on the subsynaptosomal distribution of marker enzymes or radioactive phospholipids but did increase the recovery of radioactive di- and tri-phosphoinositide to 65–75%.

The distributions of marker enzymes, protein and labelled phospholipids among the subsynaptosomal fractions, designated *O*, *D*, *E*, *F*, *G*, *H* and *I* by Whittaker *et al.* (1964), are shown in Table 5. The presence of most of the lactate dehydrogenase activity in fraction *O* with only low activity in other fractions indicates effective rupture of the synaptosomes in the hypo-osmotic buffer. The localization of radioactive phospholipids in the subsynaptosomal membranes is complicated by the contamination of the original synaptosomal preparation with other membranes. Because of the necessity to synthesize [^{32}P]ATP from [^{32}P]phosphate, phospholipid synthesis probably occurs only in intact synaptosomes, whereas contributions to marker-enzyme activity would be made by both synaptosomal and non-synaptosomal membranes.

The distribution of radioactive di- and tri-phosphoinositide follows most closely that of (Na^++K^+)-

Table 5. *Distribution of marker enzymes and radioactive phospholipids in fractions prepared from synaptosomes labelled with [^{32}P]phosphate in vitro*

Synaptosomes were incubated for 30min at 37°C in medium containing 50–100μCi of [^{32}P]phosphate/ml. After centrifugation and resuspension for 30min in 5mM-Tris/HCl, pH9.5, containing 1mM-EDTA the synaptosomal membranes were fractionated on a discontinuous sucrose density gradient as described in the Materials and Methods section. Fractions are those designated by Whittaker *et al.* (1964). Mean values for the specific activities of the marker enzymes were as follows: lactate dehydrogenase, 0.42μmol of NADH oxidized/min per mg of protein; succinate dehydrogenase, 0.046μmol of formazan formed/min per mg of protein; arylesterase, 0.10μmol of indoxyl acetate hydrolysed/min per mg of protein; cholinesterase, 0.10μmol of acetylthiocholine hydrolysed/min per mg of protein; (Na^++K^+)-dependent adenosine triphosphatase, 0.20μmol of substrate hydrolysed/min per mg of protein. Specific activity of phospholipids is defined as c.p.m./mg of protein. Specific activities of marker enzymes and phospholipids in the different fractions are expressed relative to those in osmotically shocked synaptosomes kept at 0°C until completion of the fractionation. Recovery of phospholipid radioactivity is expressed relative to the radioactivity in synaptosomes after labelling, but before osmotic shock. The results are the means for the numbers of experiments indicated, each experiment being run in duplicate.

	No. of experiments	Fraction ...	Relative specific activity *O*	*D*	*E*	*F*	*G*	*H*	*I*	Recovery (%)
Lactate dehydrogenase	2		2.73	1.00	0.49	0.20	0.24	0.14	0.01	90
Succinate dehydrogenase	2		0.00	0.00	0.00	0.06	0.58	1.06	2.79	96
Arylesterase	3		0.96	0.81	1.49	2.20	1.41	0.90	0.34	101
Cholinesterase	3		0.19	0.67	2.97	3.57	1.88	1.35	0.34	114
(Na^++K^+)-dependent adenosine triphosphatase	3		0.04	0.15	0.58	1.78	2.35	1.39	0.21	86
Triphosphoinositide	3		0.02	0.15	1.03	1.95	1.80	1.00	0.19	72
Diphosphoinositide	3		0.03	0.25	1.15	2.16	1.54	0.94	0.53	66
Phosphatidic acid	3		0.18	1.04	1.03	1.03	0.97	0.94	0.97	61
Phosphatidylinositol+ phosphatidylserine	3		0.16	0.65	1.00	0.95	1.00	0.80	1.37	71
Protein (% recovered)	3		24	11	5	7	20	14	22	103

dependent adenosine triphosphatase, suggesting their location in the synaptosomal plasma membrane. The low relative specific radioactivities of di- and triphosphoinositide in fraction *I*, shown by the distribution of succinate dehydrogenase to be enriched in mitochondria, suggests their absence from these organelles. Similarly the low relative specific radioactivities of di- and tri-phosphoinositide in fraction *D* suggests their absence from synaptic vesicles (Whittaker *et al.*, 1964). The radioactive di- and triphosphoinositide present in fractions *D* and *I* can probably be accounted for by the contamination of these fractions with plasma membrane, as indicated by their (Na^++K^+)-dependent adenosine triphosphatase activity. The distribution of arylesterase, a marker for brain endoplasmic reticulum (Bosmann, 1972), was very similar to that of (Na^++K^+)-dependent adenosine triphosphatase, and it is difficult to exclude the possibility that some radioactive di- and tri-phosphoinositide is located in microsomal membranes. However, the presence of appreciable arylesterase activity in fraction *D*, which contains very little radioactive di- and tri-phosphoinositide, would argue against this.

Discussion

The concentration of free Ca^{2+} in neurons is low (<1μM) and the cells have several mechanisms for maintaining this ion at a low concentration (Baker, 1972). In the present paper we demonstrate that increasing the intracellular concentration of Ca^{2+} in guinea-pig synaptosomes by using ionophore A23187 causes a rapid but reversible loss of diphosphoinositide and triphosphoinositide from the synaptosomal plasma membrane.

A major product of Ca^{2+}-dependent hydrolysis of these phospholipids in ionophore A23187-treated synaptosomes is inositol diphosphate (Table 4) and this indicates the involvement of diphosphoinositide phosphodiesterase. No significant production of inositol triphosphate was detected and this suggests that hydrolysis of triphosphoinositide occurred through the action of triphosphoinositide phosphatase. The alternative explanation for the lack of detectable inositol triphosphate, that inositol triphosphate is produced but then rapidly hydrolysed to inositol diphosphate, seems unlikely, as it implies the existence of a phosphatase that is very active towards inositol triphosphate, but not towards inositol diphosphate.

The inositol diphosphate produced by treatment of synaptosomes with ionophore A23187 in the presence of Ca^{2+} does not account for all the di- and triphosphoinositide hydrolysed. Either some diphosphoinositide is hydrolysed by the phosphatase to phosphatidylinositol or part of the inositol diphos-

phate produced by the phosphodiesterase is subsequently hydrolysed to inositol monophosphate and free inositol.

Addition of ionophore A23187 to synaptosomes incubated with Ca^{2+} causes a rapid decrease in the amount and radioactivity of synaptosomal nucleoside triphosphates (Table 3), and this is probably a result of activation of plasma membrane and mitochondrial Ca^{2+}-dependent adenosine triphosphatases. Whereas amounts and radioactivities of nucleoside triphosphate, diphosphoinositide and triphosphoinositide show similar falls on ionophore-mediated Ca^{2+} influx, subsequent removal of accumulated intrasynaptosomal Ca^{2+} by chelation with EGTA caused large increases in the amounts and radioactivities of the di- and tri-phosphoinositide, but only small increases in the amount and radioactivity of nucleoside triphosphate. This suggests that the effect of Ca^{2+} on di- and tri-phosphoinositide metabolism is not simply mediated via changes in ATP concentration. Both phosphatidylinositol kinase (Kai *et al.*, 1966) and diphosphoinositide kinase (Kai *et al.*, 1968) will be more active in the absence of Ca^{2+}.

The absence of any significant effects of ionophore A23187 on di- and tri-phosphoinositide labelling in synaptosomes incubated in media containing no added Ca^{2+}, and Mg^{2+} concentrations between 0.7 and 1.3mM (Fig. 1), suggests that the intracellular free Mg^{2+} concentration lies within this range. This agrees quite closely with the value for free Mg^{2+} concentration in human erythrocytes (approx. 0.4mM) estimated by Flatman & Lew (1977*b*). The activities of phosphatidylinositol kinase and diphosphoinositide kinase *in vitro* are very dependent on medium Mg^{2+} concentration (Kai *et al.*, 1966, 1968), and this dependence probably accounts for the stimulation of synaptosomal di- and tri-phosphoinositide labelling on raising intracellular Mg^{2+} concentration and the decrease in labelling on decreasing it.

Synaptosomes contain an active diacylglycerol kinase (Lapetina & Hawthorne, 1971). It is probable that at least that part of the increase in phosphatidate labelling observed in ionophore-treated synaptosomes to be dependent on medium Ca^{2+} concentration (Fig. 2) is a result of phosphorylation of the diacylglycerol produced by phosphodiesterase hydrolysis of the di- and tri-phosphoinositides. No significant changes in the radioactivity of phosphatidylinositol were observed on addition of ionophore A23187 to synaptosomes labelled *in vivo* with [^{32}P]phosphate or [^{3}H]inositol or *in vitro* with [^{32}P]phosphate. The only evidence that increased intracellular Ca^{2+} concentration stimulated phosphatidylinositol hydrolysis is a very small increase in inositol phosphate labelling in ionophore-treated synaptosomes labelled with [^{3}H]inositol (Table 4), but there was no significant loss of radioactivity from phosphatidylinositol itself. It seems unlikely therefore that the increase in phosphatidylinositol turnover observed on repeated electrical stimulation of synaptosomes (Pickard & Hawthorne, 1978) is caused by increased intracellular Ca^{2+} concentrations activating phosphatidylinositol phosphodiesterase.

Ca^{2+}-stimulated hydrolysis of di- and tri-phosphoinositide has been reported in osmotically ruptured rabbit erythrocytes (Garrett *et al.*, 1976) and in intact human erythrocytes treated with ionophore A23187 (Lang *et al.*, 1977). Allan & Michell (1978) have demonstrated that Ca^{2+}-dependent hydrolysis of di- and tri-phosphoinositide in human erythrocyte 'ghosts' occurs by activation of phosphodiesterases rather than phosphomonoesterases. However, as triphosphoinositide phosphatase has been shown to be at least partially soluble (Harwood & Hawthorne, 1969), di- and tri-phosphoinositide breakdown in intact erythrocytes may involve both phosphomonoesterase and phosphodiesterase.

Buckley & Hawthorne (1972) found that increasing the concentrations of di- and tri-phosphoinositide in pig erythrocyte membranes increased their ability to bind Ca^{2+} and stimulated Ca^{2+}-dependent adenosine triphosphatase. This suggested that changes in the proportions of phosphatidylinositol and di- and tri-phosphoinositide in the plasma membrane, involving phosphomonoesterase and kinase activities, could be involved in the regulation of intracellular Ca^{2+} concentration. In contrast it is difficult to see how phosphodiesterase cleavage of di- and triphosphoinositide can be physiologically relevant in human erythrocytes. They are unable to synthesize phosphatidylinositol, and, although di- and triphosphoinositide could be resynthesized at the expense of existing phosphatidylinositol, any substantial activation of phosphodiesterase would rapidly lead to depletion of all three phospholipids. Significant Ca^{2+}-activated phosphodiesterase cleavage of erythroctye di- and tri-phosphoinositide probably occurs only at the end of the useful life of these cells.

Synaptosomes, on the other hand, can synthesize phosphatidylinositol from diacylglycerol via phosphatidate. Ca^{2+}-dependent phosphodiesterase breakdown of synaptosomal di- and tri-phosphoinositide can thus be physiologically relevant. The rapid resynthesis observed on removal of accumulated intrasynaptosomal Ca^{2+} (Fig. 3) probably occurs by phosphorylation of existing phosphatidylinositol.

Ca^{2+}-dependent breakdown of triphosphoinositide has been observed in iris smooth muscle incubated with acetylcholine, and this was blocked by atropine but not by D-tubocurarine (Abdel-Latif *et al.*, 1977; Akhtar & Abdel-Latif, 1977). Activation of muscarinic receptors in smooth muscle is thought to cause an increase in intracellular Ca^{2+} concentration

(Hurwitz & Suria, 1971) and this could account for the increase in triphosphoinositide hydrolysis. The increased triphosphoinositide breakdown observed in crab nerve fibres incubated with 20mM-acetylcholine (Tret'jak *et al.*, 1977) did not involve muscarinic or nicotinic cholinergic receptors, but was accompanied by an increase in Ca^{2+} efflux. This was interpreted as being the result of an increase in intracellular free Ca^{2+} concentration caused by release of Ca^{2+} previously bound by triphosphoinositide. An alternative explanation is that the high concentration of acetylcholine caused an increase in Ca^{2+} permeability and that the subsequent increase in intracellular Ca^{2+} concentration stimulated triphosphoinositide hydrolysis.

A transient increase in intracellular Ca^{2+} concentrations is thought to occur in nerve fibres during passage of the action potential and more prolonged increases in intracellular Ca^{2+} concentrations have been recorded in nerve fibres subjected to repetitive stimulation (Baker, 1972). A stimulation of mono- or di-esterases by Ca^{2+} could explain the increases in diphosphoinositide and/or triphosphoinositide turnover observed in a number of studies on repeated electrical stimulation of nerves (Birnberger *et al.*, 1971; White & Larrabee, 1973; White *et al.*, 1974; Tret'jak *et al.*, 1977). An increase in intracellular Ca^{2+} concentration in the presynaptic nerve terminal is necessary for transmitter release and the present results indicate that this could be accompanied by hydrolysis of plasma membrane di- and tri-phosphoinositide.

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