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A CALCIUM REQUIREMENT FOR THE PHOSPHATIDYLINOSITOL RESPONSE FOLLOWING ACTIVATION OF PRESYNAPTIC MUSCARINIC RECEPTORS

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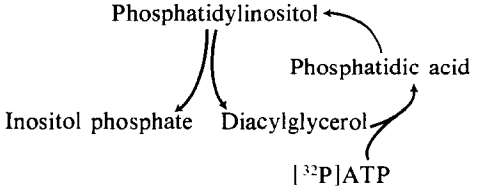
Abstract—The labelling *in vitro* by [^{32}P]phosphate of phosphatidic acid and phosphatidylinositol in synaptosomes from guinea-pig brain was studied. Acetylcholine increased the labelling and evidence is provided that pre-synaptic muscarinic receptors are involved. The increase was not seen in the presence of EGTA. Experiments with various calcium buffers indicated that concentrations of greater than 10^{-7} M free Ca^{2+} are required in the incubation medium for this phospholipid effect. A similar muscarinic effect in parotid gland is unaffected by EGTA. It is suggested that, in the parotid, postsynaptic receptors mediate increased phosphatidylinositol labelling and increased availability of Ca^{2+} for stimulus–secretion coupling. Presynaptic receptors similarly mediate increased labelling, which differs in being sensitive to EGTA and associated with decreased availability of Ca^{2+}.

Activation of α-adrenergic and muscarinic receptors in many tissues is accompanied by an increased incorporation of [^{32}P]-phosphate into phosphatidic acid and phosphatidylinositol [1]. The increased turnover of these phospholipids appears to be the consequence of a stimulation of phosphatidylinositol breakdown to diacylglycerol and inositol phosphate [2, 3] and the cycle of reactions thought to be involved is outlined below.



Direct chemical evidence for loss of phosphatidylinositol has been obtained in rat parotid gland [4], mouse pancreas [5] and guinea-pig ileum [6], using cholinergic drugs in each case.

Stimulation of [^{32}P]phosphate incorporation into phosphatidic acid and/or phosphatidylinositol is seen when synaptosomes from brain cortex are incubated with acetylcholine [7–9]. This effect is prevented by atropine [10], a result we questioned in a preliminary study [11] but now confirm. Though some postsynaptic membrane is attached to most synaptosomes, the phospholipid response must depend on presynaptic muscarinic receptors, since labelling with [^{32}P]phosphate requires ATP-generating systems present only within the nerve ending. Synaptosomal phospholipid responses to suitable agonists can therefore be exploited in the study of presynaptic receptors.

Activation if α-adrenergic and muscarinic receptors causes entry of Ca^{2+} into many tissues [1, 12]. Presynaptic α-adrenergic receptors have been shown to limit transmitter release by a negative feed-back mechanism and it has been suggested [13] that this involves restriction of the availability of Ca^{2+} for stimulus–secretion coupling. Less is known about presynaptic cholinergic receptors, though a muscarinic system in cerebral cortex depresses acetylcholine release [14], muscarinic receptors in the hypothalamus reduce release of 5-hydroxytryptamine [15], and similar striatal presynaptic receptors inhibit release of dopamine [16].

The physiological significance of the phospholipid changes following receptor activation is still not understood. Oron *et al.* [17], for instance, regard K^+ release and enhanced phospholipid turnover following activation of parotid gland receptors as two parallel but independent events. The widely distributed enzyme hydrolysing phosphatidylinositol to diacylglycerol and cyclic inositol phosphate [18, 19] is activated by Ca^{2+} *in vitro*, supporting the view that increased turnover of this phospholipid is a side effect of receptor-mediated Ca^{2+} entry. Michell *et al.* [20] on the other hand, consider phosphatidylinositol breakdown to be implicated in control of Ca^{2+} gating. If this is true of presynaptic muscarinic receptors the increase in phospholipid labelling should not depend on external Ca^{2+}. We have therefore studied the Ca^{2+} requirement in a synaptosomal system.

MATERIALS AND METHODS

Preparation of synaptosomes. The method was based on that used by Gray and Whittaker [21]. Guinea-pigs (weighing 300–500 g) were lightly anaesthetized with diethyl ether and killed by decapitation. Brains were

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removed, chilled in ice-cold 0.32 M sucrose and the cerebellum and some white matter discarded. All subsequent operations were carried out at 0–4° and sucrose solutions were adjusted to pH 7.4 with Tris. Forebrains were homogenized in 10 vol. 0.32 M sucrose using eight up-and-down strokes of a Potter–Elvehjem Teflon–glass homogenizer with a radial clearance of 0.15–0.18 mm rotating at 800 rev./min. The homogenate was centrifuged at 1000 *g* for 10 min and the resulting pellet washed twice. The supernatants from the low-speed centrifugations were combined and centrifuged at 10.000 *g* for 20 min. The pellet (the crude mitochondrial fraction) was washed once by resuspending in 0.32 M sucrose and centrifuging at 20.000 *g* for 20 min, resuspended in 0.32 M sucrose and layered on a discontinuous sucrose gradient consisting of equal volumes of 0.8 M or 1.0 M sucrose over 1.2 M sucrose. Gradients were centrifuged at 95.000 *g* for 90 min in either an MSE 6 × 15 ml or 3 × 23 ml swing-out rotor. Synaptosomes sedimenting to the 0.8 M or 1.0 M/1.2 M sucrose interface were removed, diluted 3–4 fold with incubation medium (see below), centrifuged at 20.000 *g* for 10 min and then resuspended in incubation medium.

Incubations. The incubation medium contained, unless otherwise stated, 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgCl_2$, 1.6 mM cytidine, 1.6 mM *myo*-inositol, 10 mM glucose and 26 mM Tris–HCl or 26 mM $NaHCO_3$; pH 7.4. Synaptosomes were incubated at a protein concentration of 2–3 mg/ml at 37° in air or O_2:CO_2 (95:5, by vol.).

Labelling, extraction and separation of lipids. Synaptosomes were incubated in medium containing 25–100 μCi of [^{32}P]phosphate/ml. Incubations were stopped by addition of an equal volume of 20 per cent (w/v) trichloroacetic acid. After 10 min at room temperature samples were centrifuged at 1000 *g* for 5 min and the pellet washed once with 5 per cent trichloroacetic acid and once with water. The water-washed pellet was extracted with 4 ml chloroform/methanol/conc. HCl (200:100:1, by vol.) and the extract washed once with 1 ml 0.1 M HCl and twice with its "synthetic upper phase". The lower phase was evaporated to dryness under nitrogen, the residue redissolved in chloroform/methanol (90:10, by vol.) and stored at −20°. Phosphatidic acid and phosphatidylinositol were separated by two-dimensional t.l.c. on silica gel H. Thin-layer plates were activated for 1 hr at 110° before use and chromatograms developed with chloroform/methanol/35 per cent NH_3/H_2O (120:70:2:8, by vol.) in the first dimension and chloroform/methanol/glacial acetic acid/water (80:40:7.2:1.4, by vol.) in the second dimension. Lipids were located by exposure to iodine vapour.

Measurement of free Ca^{2+} *concentration.* Free Ca^{2+} concentration in the range 10^{-7} M to 10^{-4} M was measured using the metallochromic indicator dye Arsenazo III [22]. Absorbance of the dye at 654 nm in the incubation media was compared with the absorbance of the same concentration of dye in 26 mM Tris–HCl, pH 7.4 containing various known concentrations of Ca^{2+}. Buffer alone contained less than ~10^{-7} M Ca^{2+} as determined by flame photometry.

Solutions containing low concentrations of free Ca^{2+} were obtained by using calcium–EDTA buffers [23].

Determination of bound ^{45}Ca *and externally bound* Ca^{2+}. Synaptosomes were pre-incubated for 20 min before addition of 5 μCi ^{45}Ca (sp. activity 522 μCi/μmol) per ml incubation. 0.1 ml aliquots were removed at intervals, diluted 50-fold with ice-cold Ca^{2+}- and Mg^{2+}-free incubation medium, filtered through Millipore filters (25 mm diameter, 0.45 μm pore diameter) which were washed three times with 2 ml ice-cold medium. Filtration and washing took less than 30 sec.

As reported elsewhere [24] addition of ^{45}Ca to synaptosomes results in a very rapid initial uptake of ^{45}Ca (complete in 2–3 min) followed by a much slower and prolonged increase in ^{45}Ca binding. The ^{45}Ca bound in the early rapid phase was readily displaced when 1 mM La^{3+} was included in the washing medium [25] and this appears to confirm that the initial binding of ^{45}Ca is the result of equilibration with Ca^{2+} bound superficially. The later increase in ^{45}Ca binding presumably reflects ^{45}Ca entry into synaptosomes.

The amount of externally bound Ca^{2+} was calculated from the total Ca^{2+} present in the medium and the proportion of added ^{45}Ca bound superficially. The latter was determined by measuring the ^{45}Ca bound 10, 20 and 30 min after ^{45}Ca addition and extrapolating the resulting curve (giving the rate of ^{45}Ca entry) to the time of addition of ^{45}Ca.

Determination of radioactivity. ^{32}P was determined by liquid scintillation counting in a scintillation fluid prepared by mixing toluene containing 6 g 2,5-diphenyloxazole and 120 mg 1,4-*bis*[2-(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. ^{45}Ca trapped on Millipore filters was determined after suspending the dried filter in scintillation fluid containing no Triton or water.

Materials. Acetylcholine, eserine sulphate and atropine sulphate were obtained from Sigma, London. Arsenazo III was bought from Aldrich Chemical Co., Milwaukee, U.S.A. and Tubocurarine from E. R. Squibb and Sons, Princeton, U.S.A. Radioactive materials were obtained from the Radiochemical Centre, Amersham. Sodium [^{32}P]phosphate (PBS.2P) was in isotonic solution, specific radioactivity 3–7 mCi per mg P and $^{45}CaCl_2$ in aqueous solution (CES.3) had a specific radioactivity of 11.6 mCi per mg Ca.

RESULTS

Previous studies [8, 9] have shown that the incorporation of radioactivity from [^{32}P] phosphate into synaptosomal phosphatidic acid and phosphatidylinositol is approximately linear with time up to 1 hr in the presence or absence of acetylcholine and that the effect of acetylcholine in stimulating labelling is maximal at 10^{-4} M [8]. Atropine, but not tubocurarine, blocks the stimulation of labelling (Table 1) and this confirms that the changes in phospholipid turnover are related to the activation of muscarinic receptors.

Table 2 summarises the calcium requirement of the system. Incubation in a medium without added Ca^{2+} had no effect on the stimulation by 10^{-4} M acetylcholine of phospholipid ^{32}P labelling even though the total Ca^{2+} concentration in the medium after incubation was shown by flame photometry to be below 10^{-5} M. Addition of 0.5 mM EGTA abolished the stimulation of labelling but this was restored when excess Ca^{2+} was

Table 1. Muscarinic nature of the stimulation of synaptosomal phospholipid labelling by acetylcholine

Drugs (final concn 10^{-4} M)	% increase in ^{32}P labelling Phosphatidic acid	Phosphatidylinositol
Acetylcholine, eserine	54.7 ± 17.7(7)	53.3 ± 31.0(7)
Acetylcholine, eserine, atropine	8.2 ± 6.5(4)*	1.0 ± 8.1(4)†
Acetylcholine, eserine, tubocurarine	41.0 ± 9.0(4)	31.5 ± 6.2(4)

Synaptosomes were incubated for 45 min in the presence or absence of the drugs indicated. Figures in the Table, based on control incubations without drugs, are the means ± S.D. of the number of experiments indicated in parenthesis. Only the results with atropine differ significantly (Student's *t* test) from incubations with acetylcholine/eserine alone: * $P < 0.001$; † $P < 0.01$. Mean values for the incorporation of radioactivity into phosphatidic acid and phosphatidylinositol in control incubations were 335 and 1915 c.p.m. respectively.

present with the EGTA. These results indicate that Ca^{2+} ions are required for the phospholipid changes caused by activation of presynaptic muscarinic receptors.

The calcium requirement was investigated more closely using calcium buffers. The stimulation of ^{32}P labelling by 10^{-4} M acetylcholine was reduced or abolished by calcium–EDTA buffers providing free Ca^{2+} in the concentration range 10^{-9} M–10^{-7} M (Fig. 1). Calcium–citrate buffers providing free Ca^{2+} concentrations of 10^{-7} M–10^{-5} M had little or no effect on the stimulation of phosphatidylinositol labelling at a free Ca^{2+} concentration of approximately 10^{-7} M. In the absence of EDTA or citrate (and Mg^{2+}), the free Ca^{2+} concentration in the medium was considerably less than the total Ca^{2+} concentration, presumably because of the buffering effect of phosphate in the medium. Under these conditions, the stimulation of both phosphatidic acid and phosphatidylinositol labelling was partly dependent on free Ca^{2+} concentration in the range 10^{-5} M–10^{-6} M. Lowering free Ca^{2+} concentration had little effect on phosphatidic acid labelling in the absence of acetylcholine, though phosphatidylinositol labelling was reduced in media containing Ca–EDTA buffers. We conclude that a free Ca^{2+} concentration in the medium greater than 10^{-7} M is required for the phospholipid effect.

Synaptosomal membranes have a high capacity to bind Ca^{2+} and its removal might be expected to destabilise their structure. The amount of Ca^{2+} bound externally is dependent partly on free Ca^{2+} concentration in the medium and partly on the Ca^{2+} buffer present (Fig. 2). There is no clear relationship between the amount of bound Ca^{2+} and the magnitude of the effect of acetylcholine on phospholipid labelling. Certainly depletion of bound Ca^{2+} in Ca–EDTA buffers would not appear to be responsible for the absence of a response to acetylcholine since similarly low levels of Ca^{2+} were bound to synaptosomes incubated in citrate or phosphate buffers without abolition of the effect.

DISCUSSION

Increased labelling of phosphatidylinositol and phosphatidate with ^{32}P (the phosphatidylinositol effect) in response to muscarinic activation has been known for many years but neither the detailed biochemical changes nor the physiological role are yet understood. The primary biochemical response to receptor activation is variously considered to be conversion of phosphatidylinositol to diacylglycerol [4, 20] or phosphatidate [26], hydrolysis of phosphatidate to diacylglycerol [10] or reaction with CMP to produce CDP–diacylglycerol [26]. At present there is better evidence for the first of these alternatives, conversion of

Table 2. Calcium requirement of the muscarinic phospholipid response

Modifications of medium	% increase in ^{32}P labelling Phosphatidic acid	Phosphatidylinositol
None (0.75 mM Ca^{2+})	54.7 ± 17.7(7)	53.3 ± 31(7)
No Ca^{2+}	71.0 (2)	66.0 (2)
No Ca^{2+}, 0.5 mM EGTA	0.0 ± 15.2(4)*	0.7 ± 20.3(4)†
1.25 mM Ca^{2+}, 0.5 mM EGTA	58 (1)	83 (1)

Synaptosomes were incubated for 45 min and results are expressed as in Table 1. * Differs significantly from result with 0.75 mM Ca^{2+} at $P < 0.001$; † differs significantly from result with 0.75 mM Ca^{2+} at $P < 0.02$ (Student's *t* test). Figures represent means with S.D. where appropriate and numbers of experiments are given in parenthesis.

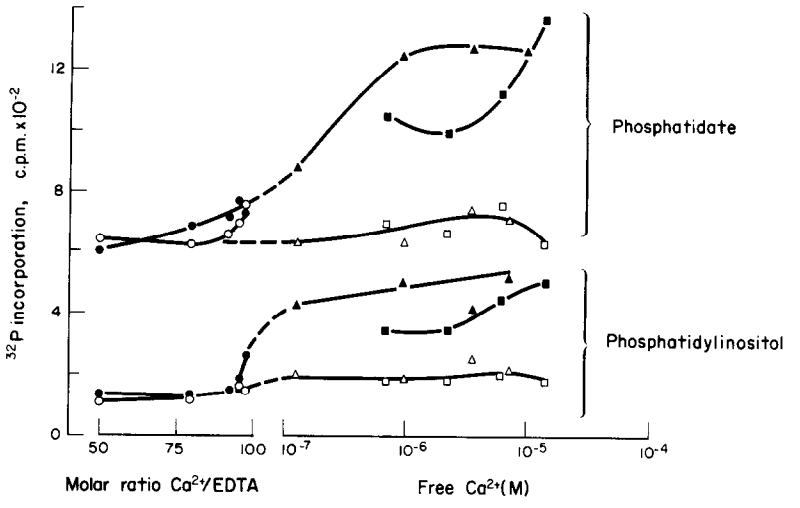

Fig. 1. Effect of medium free Ca^{2+} concentration on stimulation of synaptosomal phospholipid labelling by acetylcholine. Synaptosomes were incubated for 1 hr in incubation medium containing 50 μCi [^{32}P]phosphate/ml, no Mg^{2+} and varying amounts of Ca^{2+} buffered with either 5 mM EDTA (○, ●), 5 mM citrate (△, ▲) or by the 1.2 mM phosphate in the medium (□, ■). Open symbols, control; closed symbols, + 10^{-4} M acetylcholine (+ 10^{-4} M eserine). Free Ca^{2+} concentration in the range 10^{-5}—10^{-7} M was measured using the metallochromic indicator dye Arsenazo III as described in the Materials and Methods section. The Ca^{2+}–EDTA buffers produced free Ca^{2+} concentrations below 10^{-7} M, in contrast to the data of Raaflaub [23] which predicted free Ca^{2+} concentrations in the range 10^{-8}–10^{-6} M.

phosphatidylinositol to diacylglycerol and inositol 1,2-cyclic phosphate, than for the others. It has been suggested that this reaction may have a physiological function in calcium gating [20]. Activation of post-synaptic muscarinic receptors generally increases Ca^{2+} influx, making this ion available for stimulus–secretion coupling. Pre-synaptic muscarinic receptors, on the other hand, inhibit secretion of transmitter and probably do so by restricting Ca^{2+} influx [13, 27]. A phosphatidylinositol effect is associated with both types of receptor and we are now in a position to compare the Ca^{2+} requirements.

Activation of post-synaptic muscarinic receptors in rat parotid gland produces a phosphatidylinositol effect which is seen equally well whether the medium contained 2.5 mM Ca^{2+} or no calcium salts and 0.2 mM EGTA [28]. Activation of the pre-synaptic muscarinic receptors of our synaptosomes produces a similar phosphatidylinositol effect which is abolished by EGTA (Table 2). More detailed study suggests that the external free Ca^{2+} concentration must be greater than 10^{-7} M for the phospholipid effect. Since intracellular free Ca^{2+} concentrations are of this order, it seems possible that Ca^{2+} influx is involved in the synaptosomal phosphatidylinositol changes. In experiments under slightly different conditions (60 min incubation using 'light' synaptosomes from rat brain with 30 μM acetylcholine/100 μM eserine), Miller [29] found that omission of Ca^{2+} from her medium reduced the acetylcholine-induced increase in labelling from 58 to 36 per cent (phosphatidate) and from 21 to 3 per cent (phosphatidylinositol). No chelating agent such as EGTA was used.

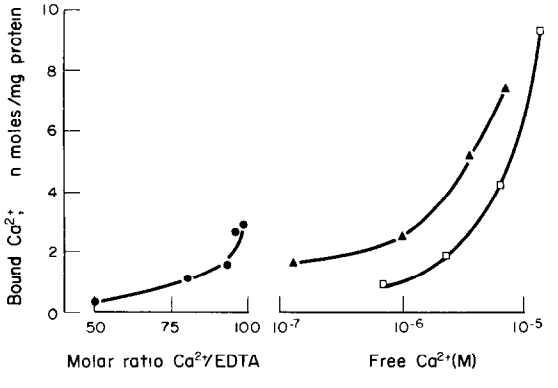

Fig. 2. Effect of medium free Ca^{2+} concentration on amount of Ca^{2+} bound externally to synaptosomes. Free Ca^{2+} concentration in the medium was varied as described in the legend to Fig. 1. Externally bound Ca^{2+} was determined as described in the Materials and Methods section. Ca–EDTA buffers, ●; Ca–citrate buffers, ▲; Ca–phosphate buffers, □.

The generalization of Michell *et al.* [20] that hydrolysis of this lipid is concerned with the opening of calcium gates in response to muscarinic activation appears too sweeping. If we accept the views that post-synaptic muscarinic receptors make more Ca^{2+} available for stimulus–secretion coupling while pre-synaptic receptors reduce availability of Ca^{2+}, it would seem that hydrolysis of phosphatidylinositol opens gates in one system and closes them in the other.

Oron *et al.* [17] suggested that phosphatidylinositol is not directly involved in stimulus–secretion coupling. While this view may be incorrect, the present evidence at least makes it difficult to see how metabolism of this lipid controls calcium availability. Without attempting an explanation, we suggest in conclusion that there are two different muscarinic phosphatidylinositol responses. In the first (parotid, postsynaptic), an EGTA-

insensitive phospholipid effect is associated with increased Ca^{2+} availability. In the second (synaptosomal, presynaptic) an EGTA-sensitive phospholipid effect is associated with decreased availability of Ca^{2+}.

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