Subcellular localization of phospholipid changes in response to muscarinic stimulation of perfused bovine adrenal medulla

Nor AZILA and John N. HAWTHORNE

Department of Biochemistry, University Hospital and Medical School, Clifton Boulevard, Nottingham NG7 2UH, U.K.

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The effect of carbachol on catecholamine secretion and [^{32}P]P_i incorporation into phospholipids was studied in perfused bovine adrenal medulla. After a labelling period, the gland was stimulated with carbachol in the absence of ^{32}P . Subcellular fractions were then prepared from the medulla. Carbachol roughly halved the specific radioactivities of phosphatidylinositol and phosphatidate in microsomal, chromaffin-granule, mitochondrial and plasma-membrane fractions. With Ca^{2+} -free perfusion medium, catecholamine secretion was abolished but the phospholipid changes remained. Stimulation of secretion by KCl was not accompanied by phospholipid changes. The results are not consistent with the theory relating phosphatidylinositol hydrolysis and Ca^{2+} gating.

Stimulation of appropriate receptors in a variety of systems increases phosphatidylinositol turnover, the initial event probably being hydrolysis of the lipid (Hokin-Neaverson, 1974; Jones & Michell, 1974). Previous work (Mohd. Adnan & Hawthorne, 1981) confirmed the reports of Hokin *et al.* (1958) and Trifaró (1969) that in the presence of [^{32}P]P_i, stimulation of adrenal medulla enhanced labelling of phosphatidylinositol and phosphatidate. The most crucial of the many unresolved questions is where, in the cell, the receptor-stimulated phosphatidylinositol hydrolysis occurs. If it is involved in controlling cell-surface Ca^{2+} permeability then it must be at the plasma membrane. However, at present, data on different systems suggest different sites for this primary event. In synaptosomes (Pickard & Hawthorne, 1978) and islets of Langerhans (Clements *et al.*, 1978) the phosphatidylinositol response was reported to be in the vesicle fraction. However, Kirk *et al.* (1981), using vasopressin, obtained a generalized loss of labelled phosphatidylinositol in all of their subcellular fractions from rat hepatocytes. The present work reports the loss of radioactivity from phosphatidate and phosphatidylinositol in response to muscarinic stimulation of prelabelled perfused adrenal medulla. Losses are localized by subcellular fractionation. The main advantages of the adrenal medulla, over nervous or other secretory tissues, are that its secretory-vesicle membrane can be detected by two unique components, dopamine β -hydroxylase and cytochrome b_{561} and that plasma-membrane fractions have been prepared from it.

Ca^{2+} is increasingly being recognized as an

important factor in a wide range of intracellular processes and particularly as a second messenger in the initiation of response to various hormones and neurotransmitters (Rasmussen *et al.*, 1972; Berridge, 1975). Examples include adrenal medulla, where acetylcholine-stimulated release of catecholamine is dependent on extracellular Ca^{2+} . In many, if not all, situations where an extracellular agonist brings about a rise in intracellular Ca^{2+} as a result of a change in cell-surface permeability to Ca^{2+} , there is a concomitant increase in the rate of phosphatidylinositol turnover (Michell, 1975). Thus the effect of Ca^{2+} on secretion of catecholamine and phosphatidylinositol turnover has also been studied in the perfused medulla. Elevation of extracellular K^{+} concentration has been reported to substitute effectively for acetylcholine, even in the presence of cholinergic blocking agents (Douglas & Rubin, 1961). K^{+} -stimulated release is also dependent on extracellular Ca^{2+} (Douglas, 1975; Ishikawa & Kanno, 1978; Trifaró & Lee, 1980) and the phosphatidylinositol response under these conditions is also reported.

Materials and methods

[^{32}P]P_i (carrier-free) was obtained from Amersham International, Amersham, Bucks., U.K. Atropine sulphate and carbamoylcholine chloride (carbachol) were obtained from Sigma Chemical Co., London, U.K. Hexamethonium bromide was purchased from BDH, Poole, Dorset, U.K.

Bovine adrenal glands weighing between 10 and 14 g were used. Glands were removed within 15 min

of the death of the animal and kept on ice for about 30 min until the perfusion began.

Perfusion of adrenal glands

Connective tissue and fat around the glands were removed and incisions (two per gland) made in the cortical tissue, without damaging the medulla, so that perfusion fluid could flow out. The glands were cannulated and perfused, in a retrograde manner via the central vein opening, with Locke's solution containing $4.2 \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i/\text{ml}$ at a flow rate of $4.0 \pm 0.2 \text{ ml/min}$ for 1 h. The Locke's solution was composed of 154 mM-NaCl, 5.6 mM-KCl, 2.2 mM- CaCl_2 , 1.0 mM- MgCl_2 , 6.0 mM- NaHCO_3 and 10.0 mM-glucose. The perfusion-fluid reservoir was maintained at 30°C and continuously gassed with O_2/CO_2 (19:1). The glands were then washed with non-radioactive Locke's solution for 25 min before stimulation for 2 min. As perfusion continued, the stimulant, 15 mM-carbachol or 56 mM-KCl (saline for control), was injected in a volume of 1 ml in a series of four injections, each separated by 30 s, into the perfusion fluid just before it entered the gland. The level of catecholamine release was estimated from the perfusate. Collection of perfusate (2 min period) was started after 15 min of washing and continued until the perfusion was terminated, when the glands were placed on ice. The medulla was then fractionated immediately.

In conditions where the glands were stimulated with carbachol in the absence of extracellular Ca^{2+} , the 1 h prelabelling was done in the presence of Ca^{2+} after which Ca^{2+} -free Locke's solution containing 0.1 mM-EGTA was used. When the glands were stimulated with K^+ the perfusion fluid contained 0.2 mM-hexamethonium and 0.1 mM-atropine to block nicotinic and muscarinic cholinergic receptors.

Fractionation of adrenal medulla

Fractionation schemes are shown in Figs. 1(a) and 1(b). The fractionation is a modification of the combined methods of Smith & Winkler (1967) and Wilson & Kirshner (1976). Cortical tissue and as much connective tissue as possible were removed, the medulla chopped finely with scissors and placed in ice-cold 0.3 M-sucrose/10 mM-Tris/HCl buffer (pH 7.0), referred to as ST buffer. It was then homogenized at 4°C in a Potter-Elvehjem homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) having a glass mortar and Teflon pestle to give a 1:8 (w/v) homogenate. The homogenate was then filtered through two layers of cheesecloth. The filtrate was centrifuged at low speed (1200 g for 10 min) to remove unbroken cells and nuclei. This pellet was resuspended and re-centrifuged at the same speed. The low-speed supernatants were combined and centrifuged at 12000 g for 20 min.

The 'fluffy layer' that lay loosely on top of the sediment was decanted along with the supernatant (S_2). The sediment (large-granule fraction) was resuspended in ice-cold ST buffer but care was taken to exclude erythrocytes that may be present at the bottom of the sediment. The suspension was then centrifuged again at 12000 g for 20 min. The sediment (washed large-granule fraction) was resuspended in ST buffer (1 ml/g of tissue). A portion (4.5 ml) of the suspension was layered on 12 ml of ice-cold 1.6 M-sucrose. After centrifugation at 66000 g for 100 min several layers could be distinguished, as shown in Fig. 1(a).

The pellet from the low-speed centrifugation was resuspended in the combined supernatants S_2 and S_3 , as indicated in Fig. 1(a), and centrifuged at 800 g for 10 min. The pellet obtained was resuspended in ST buffer and re-centrifuged at 800 g (10 min). The supernatants S_4 and S_5 (Fig. 1b) were combined and centrifuged at 26000 g (20 min). The supernatant obtained was centrifuged at 65000 g for 1 h to give the microsomal fraction. The 26000 g pellet was resuspended in ST buffer, layered over 1.4 M-sucrose and centrifuged at 65000 g for 90 min. The interfacial material obtained was diluted 1:2 with 10 mM-Tris/HCl buffer (pH 7.4), layered over 1.0 M-sucrose containing 1 mM-dithiothreitol and centrifuged at 65000 g for 2 h. The material obtained from the interface was taken as the crude plasma-membrane fraction.

Lipid extraction and analysis

The membrane fractions were freeze-dried and lipid extracted by the method (a) of Yagihara *et al.* (1973), except that the trichloroacetic acid treatment was omitted and neutral solvents were used. Cholesterol was estimated by g.l.c. (Miettinen *et al.*, 1965) with 5α -cholestane as internal standard. Phospholipids were separated by two-dimensional t.l.c. on silica gel H spread 0.4 mm thick as a slurry in 3% magnesium acetate. Plates (20 cm square) were divided into four equal squares by scoring lines across them and the lipid mixture was applied at the four corners. By suitable inversion of the plates in their tanks after the solvent had run half-way, four separations could then be made on each plate. Chromatograms were developed twice in the first dimension with chloroform/methanol/33% NH_3 (65:25:5, by vol.) and dried in a cool air stream for 1 h. The second-dimension solvent (once only) was chloroform/acetone/methanol/glacial acetic acid/water (6:8:2:2:1, by vol.). Lipids were detected by exposure to I_2 vapour. Dragendorff reagent and ninhydrin were sprayed to confirm the identity of choline- and ethanolamine-/serine-phospholipids respectively.

Phospholipid spots were scraped off and digested in 72% HClO_4 for phosphorus estimation by the

Fig. 1. Subcellular fractionation of adrenal medulla

method of Bartlett (1959). Samples of the HClO_4 digest after dilution with water were mixed with 8 vol. of Fiso-fluor 1 (Fisons, Loughborough, Leics., U.K.) for liquid-scintillation counting of ^{32}P .

Marker assays for the fractions

Catecholamines were measured by the method of Renzini *et al.* (1970). Dopamine β -hydroxylase (EC 1.14.17.1) was assayed by the method of Nagatsu & Udenfriend (1972) with slight modification adapted from Belpaire & Laduron (1968). Arylesterase (EC 3.1.1.2) and glucose 6-phosphatase (EC 3.1.3.9) were determined by the method of Shephard & Hübscher (1969) and De Duve *et al.* (1955) respectively. Succinate dehydrogenase (EC 1.3.99.1) was measured by the method of Pennington (1961). Cytochrome *c* oxidase (EC 1.9.3.1) was assayed by a modification (Cooper & Hawthorne, 1975) of the method of Wharton & Tzagoloff (1967). Acid phosphatase (EC 3.1.3.2) was assayed by a modification (Cooper & Hawthorne, 1975) of the fluoride-sensitive hydrolysis of glycerol 2-phosphate (Hübscher & West, 1965). Lactate dehydrogenase (EC 1.1.1.27) was measured by the method of Bergmeyer *et al.* (1963). Acetylcholinesterase (EC 3.1.1.7) was assayed by the method described by Hawkins & Knittle (1972). Ca^{2+} -dependent ATPase (EC 3.6.1.3) was measured by the method of Goz (1967).

Electron microscopy

Subfractions were fixed by resuspension in a solution of 2% (w/v) formaldehyde plus 2% (w/v) glutaraldehyde in 0.02 M-phosphate buffer (pH 6.9). After overnight fixation they were washed with six changes of 0.02 M-phosphate buffer containing 10% (w/v) sucrose at 4 h intervals. They were then post-fixed in 1% OsO_4 and dehydrated with graded concentrations of ethanol. The dehydrated samples were then embedded in Araldite epoxy resin. Tissue sections were mounted on copper grids and stained with uranyl acetate.

Results

Analysis of fractions

Tables 1(a) and 1(b) illustrate the subcellular protein and marker distributions as a percentage of the homogenate. Cross-contamination of the membrane fractions was relatively low as shown by their enzyme marker activities. The chromaffin-granule fraction showed a 4-fold increase in dopamine β -hydroxylase activity and 5-fold of catecholamine. Less than 2% of mitochondrial and 3% of microsomal marker were present in this fraction. Plasma-membrane contamination was shown by the activi-

ties of acetylcholinesterase (5.5%) and Ca^{2+} -dependent ATPase (2.8%). The electron micrograph (Plate 1d), however, shows that these structures are not present in the chromaffin-granule fraction. The plasma-membrane fraction gave approx. 11-fold increases in acetylcholinesterase and Ca^{2+} -dependent ATPase. Contamination of this fraction by chromaffin granules, mitochondria and microsomes, as shown by their marker enzyme activities, was very low if not negligible. Mitochondrial markers, succinate dehydrogenase and cytochrome *c* oxidase showed parallel distribution with 9-fold and 10-fold enrichment respectively in the mitochondrial fraction. Approx. 6% of granule marker and 6–9% of microsomal marker can be detected. The electron micrograph (Plate 1c) confirms the presence of small granules in the mitochondrial fraction. Table 2 gives relative specific activities of marker enzymes in the subcellular fractions studied in more detail below.

Table 3 gives the phospholipid composition and cholesterol content of the selected membrane fractions. The plasma-membrane fraction contained slightly more sphingomyelin and phosphatidylserine and negligible lysophosphatidylethanolamine compared with other fractions. The cholesterol/phospholipid molar ratio is highest in this fraction. When compared with the chromaffin-granule fraction, plasma membranes had more phosphatidylcholine and sphingomyelin and less phosphatidylethanolamine, lysophosphatidylethanolamine and lysophosphatidylcholine.

Effect of secretagogues on phospholipid labelling

As shown in Fig. 2 carbachol provoked catecholamine secretion from the perfused adrenal medulla, the quantity secreted being much less during the second stimulation. The secretion was accompanied by a reduction in the specific radioactivity of phosphatidylinositol and phosphatidate (Table 4). Other phospholipids were unaffected. In the experiments of Tables 4–7 perfusion was stopped after 30 min (stimulation twice) or after 16 min (stimulation once) and the glands were quickly cooled for fractionation. Times refer to Fig. 2. The loss of label was observed in all the subcellular fractions studied and was significantly greater in the chromaffin granule and microsomal fractions, compared with those of the plasma membrane, when the glands were stimulated twice. However, after only one period of stimulation, the loss was much the same in each of the fractions. Stimulation had no significant effect on the content of phosphatidate or phosphatidylinositol in the various fractions (Table 5) though because of the small quantities available for t.l.c. (0.2–1.2 μg of lipid phosphorus in the spots corresponding to these lipids) changes of 20% or less would not be detected.

Stimulation of the adrenal once, with carbachol, in

EXPLANATION OF PLATE 1

Electron micrographs of subcellular fractions from adrenal medulla

(a) Plasma membrane; (b) microsomal; (c) mitochondrial; (d) chromaffin granule. Magnification 21 000 $\times$ in each case.

Table 1. *Relative distribution of protein and markers in subfractions of adrenal medulla*

Each result is the mean $\pm$ s.d. of values from four preparations. The values represent total activity of fractions as a percentage of total activity found in the homogenate (taken as 100%). Abbreviations: N, nuclear pellet; PFS, particle-free supernatant; M_p, microsomal pellet; S_A, supernatant from gradient of Fig. 1(a); Mt, mitochondrial fraction; L, lysosomal fraction; G, chromaffin-granule fraction; P_B, pellet from gradient I, Fig. 1(b); P_C, pellet from gradient II, Fig. 1(b); S_B, pooled supernatants from gradients I and II; PM, plasma membrane fraction. Homogenate values are given as: *nmol of substrate converted/mg of protein per min, † μ mol of substrate converted/mg of protein per min. Catecholamine is given as μ mol/mg of protein.

(a)

Fraction	Protein	Acid phosphatase	Lactate dehydrogenase	Dopamine β -hydroxylase	Catecholamine
N	22.4 $\pm$ 3.7	6.7 $\pm$ 3.8	3.9 $\pm$ 1.2	4.7 $\pm$ 1.8	4.4 $\pm$ 1.5
PFS	35.3 $\pm$ 1.9	31.8 $\pm$ 1.3	77.2 $\pm$ 12.1	17.7 $\pm$ 8.9	21.2 $\pm$ 6.4
M _p	5.5 $\pm$ 1.1	10.5 $\pm$ 1.4	2.5 $\pm$ 1.0	3.9 $\pm$ 2.5	3.3 $\pm$ 0.5
S _A	0.6 $\pm$ 0.1	0.3 $\pm$ 0.2	0.4 $\pm$ 0.1	0.7 $\pm$ 0.1	0.8 $\pm$ 0.2
Mt	7.7 $\pm$ 0.5	5.6 $\pm$ 3.0	1.0 $\pm$ 0.6	5.4 $\pm$ 1.7	4.6 $\pm$ 1.3
L	7.0 $\pm$ 2.6	32.2 $\pm$ 6.3	1.4 $\pm$ 0.7	12.6 $\pm$ 4.2	13.3 $\pm$ 6.1
G	9.2 $\pm$ 2.0	0.9 $\pm$ 0.7	—	35.0 $\pm$ 10.9	45.1 $\pm$ 10.1
P _B	2.8 $\pm$ 0.6	0.8 $\pm$ 0.7	—	6.2 $\pm$ 2.5	7.6 $\pm$ 3.4
P _C	1.9 $\pm$ 0.8	1.3 $\pm$ 0.7	—	1.9 $\pm$ 0.8	1.1 $\pm$ 1.0
S _B	4.2 $\pm$ 3.0	3.3 $\pm$ 0.9	4.2 $\pm$ 0.9	2.1 $\pm$ 0.5	1.3 $\pm$ 1.7
PM	1.8 $\pm$ 0.4	—	0.4 $\pm$ 0.3	0.5 $\pm$ 0.2	—
Recovery (%)	98.4 $\pm$ 3.7	93.4 $\pm$ 8.5	91.0 $\pm$ 8.0	90.7 $\pm$ 10.9	102.7 $\pm$ 11.4
Homogenate values	—	2.2 $\pm$ 0.5*	146.9 $\pm$ 15.0*	105.3 $\pm$ 15.9*	0.46 $\pm$ 0.07

(b)

Fraction	Succinate dehydrogenase	Cytochrome <i>c</i> oxidase	Glucose 6-phosphatase	Arylesterase	Acetylcholin-esterase	Ca ²⁺ -dependent ATPase
N	13.4 $\pm$ 6.4	4.5 $\pm$ 3.3	8.6 $\pm$ 5.1	4.5 $\pm$ 0.9	6.7 $\pm$ 2.2	15.5 $\pm$ 2.8
PFS	—	—	35.3 $\pm$ 7.2	49.4 $\pm$ 8.0	24.7 $\pm$ 6.4	21.2 $\pm$ 0.9
M _p	2.8 $\pm$ 0.6	4.4 $\pm$ 3.0	28.6 $\pm$ 4.6	26.4 $\pm$ 2.3	12.1 $\pm$ 5.0	12.1 $\pm$ 4.6
S _A	—	—	0.6 $\pm$ 0.6	0.4 $\pm$ 0.3	0.5 $\pm$ 0.3	0.2 $\pm$ 0.1
Mt	66.5 $\pm$ 8.3	77.8 $\pm$ 10.3	5.4 $\pm$ 1.2	8.5 $\pm$ 1.8	12.3 $\pm$ 1.1	11.6 $\pm$ 3.1
L	7.7 $\pm$ 2.9	6.3 $\pm$ 4.2	1.4 $\pm$ 1.1	1.4 $\pm$ 0.8	4.9 $\pm$ 0.3	2.1 $\pm$ 0.4
G	1.4 $\pm$ 1.0	0.9 $\pm$ 0.7	2.8 $\pm$ 0.4	1.8 $\pm$ 0.9	5.5 $\pm$ 2.1	2.8 $\pm$ 1.5
P _B	2.0 $\pm$ 1.0	1.1 $\pm$ 0.6	3.4 $\pm$ 1.9	1.7 $\pm$ 0.9	2.8 $\pm$ 1.4	1.4 $\pm$ 1.0
P _C	5.4 $\pm$ 5.2	5.8 $\pm$ 0.7	2.5 $\pm$ 1.2	1.9 $\pm$ 1.6	2.5 $\pm$ 1.5	1.5 $\pm$ 1.2
S _B	0.2 $\pm$ 0.1	—	2.1 $\pm$ 0.7	3.8 $\pm$ 2.9	10.9 $\pm$ 8.9	4.2 $\pm$ 1.2
PM	—	—	0.9 $\pm$ 0.3	0.7 $\pm$ 0.6	19.6 $\pm$ 2.1	20.3 $\pm$ 1.0
Recovery (%)	99.4 $\pm$ 12.2	100.8 $\pm$ 10.1	91.6 $\pm$ 7.1	100.5 $\pm$ 9.6	102.5 $\pm$ 9.6	92.9 $\pm$ 8.5
Homogenate values	36.2 $\pm$ 1.9*	126.8 $\pm$ 28.3†	10.6 $\pm$ 1.8†	19.2 $\pm$ 2.3†	23.5 $\pm$ 2.7*	74.4 $\pm$ 11.1*

Table 2. *Relative specific activities of marker enzymes in subcellular fractions of adrenal medulla*

Results are means from four fractionations and represent the specific activity or content in a particular fraction divided by the homogenate specific activity or content. Homogenate protein content was 147.9 mg.

	Microsomal	Mitochondrial	Chromaffin granule	Plasma membrane
Succinate dehydrogenase	0.88	9.22	0.28	—
Cytochrome <i>c</i> oxidase	1.01	10.34	0.13	—
Acetylcholinesterase	2.09	1.54	0.54	11.29
Ca ²⁺ -dependent ATPase	2.19	1.73	0.40	11.62
Arylesterase	4.84	1.08	0.23	0.29
Glucose 6-phosphatase	5.16	0.63	0.25	0.36
Acid phosphatase	1.77	0.68	—	—
Lactate dehydrogenase	0.38	0.20	—	—
Dopamine β -hydroxylase	0.77	0.79	3.97	0.46
Catecholamine	0.50	0.52	5.04	—
Protein content (mg)	8.2	18.1	10.6	3.2

Table 3. *Phospholipid composition and cholesterol content of subcellular fractions from bovine adrenal medulla*
Abbreviations used: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; PA, phosphatidic acid; n.d., none detected. Units for cholesterol and total phospholipid are $\mu\text{mol}/\text{mg}$ of protein. Results are means $\pm$ S.D. from five preparations.

Phospholipids	Phospholipid content (% of total)			
	Chromaffin granule	Plasma membrane	Microsomal	Mitochondrial
PC	24.3 $\pm$ 2.7	35.4 $\pm$ 2.8	38.1 $\pm$ 4.3	40.3 $\pm$ 2.8
LPC	12.8 $\pm$ 1.5	4.2 $\pm$ 1.6	3.8 $\pm$ 1.0	4.5 $\pm$ 1.1
PE	31.8 $\pm$ 1.5	27.7 $\pm$ 1.9	28.7 $\pm$ 4.1	34.7 $\pm$ 1.6
LPE	3.0 $\pm$ 1.2	n.d.	1.5 $\pm$ 0.7	2.3 $\pm$ 1.0
PS	8.3 $\pm$ 0.8	9.4 $\pm$ 1.0	6.9 $\pm$ 1.5	5.4 $\pm$ 1.0
PI	4.7 $\pm$ 0.5	3.5 $\pm$ 1.0	6.2 $\pm$ 1.6	5.8 $\pm$ 1.1
SM	12.0 $\pm$ 1.9	14.5 $\pm$ 0.4	9.0 $\pm$ 2.2	4.8 $\pm$ 0.8
PA	1.6 $\pm$ 0.7	1.7 $\pm$ 0.9	1.6 $\pm$ 2.5	1.8 $\pm$ 0.4
Recovery (%)	98.5 $\pm$ 0.8	96.4 $\pm$ 3.2	95.8 $\pm$ 2.5	97.5 $\pm$ 5.5
Cholesterol	0.36 $\pm$ 0.09	0.19 $\pm$ 0.2	0.12 $\pm$ 0.04	0.11 $\pm$ 0.06
Total phospholipid	0.61 $\pm$ 0.14	0.27 $\pm$ 0.09	0.29 $\pm$ 0.08	0.39 $\pm$ 0.11
Cholesterol/phospholipid (molar ratio)	0.59 $\pm$ 0.16	0.70 $\pm$ 0.12	0.41 $\pm$ 0.02	0.28 $\pm$ 0.02

Table 4. *Effect of carbachol on ^{32}P specific radioactivity of adrenal phospholipids*

Values are means $\pm$ S.D. of eight results from two perfusions in each case. Values significantly different from plasma-membrane result are indicated as follows: * $P < 0.001$; † $P < 0.01$.

Stimulation ...	Decrease in specific radioactivity (%)			
	Phosphatidylinositol		Phosphatidate	
	Twice	Once	Twice	Once
Homogenate	47.4 $\pm$ 23.7	55.7 $\pm$ 21.8	47.6 $\pm$ 11.8	51.7 $\pm$ 17.1
Chromaffin granule	64.9 $\pm$ 12.9*	45.2 $\pm$ 13.7	61.7 $\pm$ 21.0†	41.4 $\pm$ 16.7
Plasma membrane	40.8 $\pm$ 11.5	45.3 $\pm$ 16.5	35.0 $\pm$ 16.1	43.5 $\pm$ 12.4
Microsomal	59.2 $\pm$ 15.3†	53.3 $\pm$ 14.4	64.1 $\pm$ 12.6*	57.1 $\pm$ 17.2
Mitochondrial	50.0 $\pm$ 8.9	48.7 $\pm$ 13.4	46.3 $\pm$ 13.4	44.9 $\pm$ 12.0

Table 5. *Phosphatidylinositol and phosphatidate concentrations in subcellular fractions from control and carbachol-stimulated adrenal medulla*

Values are means $\pm$ S.D. from three perfusion experiments and represent nmol of phospholipid/mg of protein. Glands were stimulated twice with carbachol, as in Fig. 2.

Fraction	Phosphatidylinositol		Phosphatidate	
	Control	Carbachol	Control	Carbachol
Chromaffin granule	27.2 $\pm$ 3.2	25.1 $\pm$ 4.7	13.1 $\pm$ 1.3	11.6 $\pm$ 1.3
Plasma membrane	55.2 $\pm$ 8.9	61.1 $\pm$ 10.0	22.8 $\pm$ 5.4	24.1 $\pm$ 7.4
Microsomal	41.9 $\pm$ 3.9	45.2 $\pm$ 7.1	12.9 $\pm$ 3.7	13.4 $\pm$ 4.1
Mitochondrial	38.0 $\pm$ 8.9	43.5 $\pm$ 15.3	9.1 $\pm$ 3.0	10.2 $\pm$ 5.5

the absence of Ca^{2+} , produced similar phospholipid effects (Table 6), but there was no stimulation of catecholamine secretion. The changes in Table 6 are not significantly different from those obtained when glands were stimulated once with carbachol in the presence of Ca^{2+} (Table 4). When 56 mM-KCl was used instead of carbachol as a stimulant, secretion was not accompanied by any change in phos-

pholipid labelling. The secretory response to the KCl was similar to the carbachol response in Fig. 2.

Table 7 gives the specific radioactivities of certain phospholipids in homogenate and subcellular fractions from the control glands. Standard deviations are not given. They averaged 25%, since there was considerable variation in the uptake of ^{32}P from one perfusion to the next.

Fig. 2. Effect of carbachol on catecholamine secretion by perfused medulla

Each point represents a mean value from three experiments and the bar represents s.d. The shaded blocks beneath the graph indicate periods of treatment with carbachol (O) or saline (●) as control.

Table 6. Effect of carbachol on phospholipid labelling in the absence of extracellular Ca^{2+}

The perfusion experiment was similar to that of Fig. 2, but with only one period of stimulation. The perfusion medium contained no Ca^{2+} salts and 0.1 mM-EGTA. Each value is the mean of results from three perfusions $\pm$ s.d.

Fraction	Decrease in specific radioactivity (%)	
	Phosphatidylinositol	Phosphatidate
Homogenate	38.8 $\pm$ 9.0	33.2 $\pm$ 9.9
Chromaffin granule	37.5 $\pm$ 6.3	49.3 $\pm$ 15.7
Plasma membrane	36.2 $\pm$ 6.3	42.5 $\pm$ 16.4
Microsomal	32.6 $\pm$ 7.8	48.2 $\pm$ 15.2
Mitochondrial	38.3 $\pm$ 9.7	41.6 $\pm$ 21.8

Discussion

We have previously shown (Mohd. Adnan & Hawthorne, 1981) that the phosphatidylinositol response in bovine adrenal medulla is associated with muscarinic and not nicotinic receptors. This has recently been confirmed by Fisher *et al.* (1981) with chromaffin-cell cultures. The present study indicates that KCl depolarization, which presumably allows Ca^{2+} entry through voltage-dependent channels, causes catecholamine secretion without a concomitant phosphatidylinositol response, implying that the latter occurs only when the cholinergic receptors are stimulated. Stimulation with carbachol in a Ca^{2+} -free medium produced the opposite effect, loss of label without catecholamine secretion. Both results are consistent with Michell's theory that phosphatidylinositol hydrolysis is associated with the control of cell-surface Ca^{2+} gates (Michell *et al.*, 1977), but also with the proposal below that muscarinic receptors do not induce secretion in the bovine medulla. However, the generalized losses of labelled phosphatidylinositol from all the subcellular fractions analysed in this tissue (Table 4) and in their own work with rat hepatocytes (Kirk *et al.*, 1981) lend no support to the theory. The suggestion that an exchange protein (Demel *et al.*, 1977) redistributes phosphatidylinositol so that all membranes share the loss of labelled lipid could explain the results. The synthesis of liver phosphatidylinositol from CDP-diacylglycerol takes place on membranes of the endoplasmic reticulum, with some contribution from Golgi membranes (Williamson & Morré, 1976). Plasma membrane and mitochondria lack synthetic ability. In brain, also, endoplasmic reticulum is the major site of synthesis (Bishop & Strickland, 1970) and it is reasonable to assume the same for adrenal medulla. Both phosphatidylinositol and phosphatidate are highly labelled in the microsomal fraction (Table 7). If receptor activation causes loss of phosphatidylinositol from plasma membrane and the loss is made good by transfer from endoplasmic reticulum, the major loss of labelled phosphatidylinositol should be from the microsomal fraction, with a much smaller loss from plasma

Table 7. Specific radioactivity of phospholipids in subcellular fractions from adrenal medulla

Values are derived from the control series illustrated in Fig. 2 and indicate ^{32}P labelling. Means from 3 experiments are given.

Fraction	$10^{-2} \times$ Specific radioactivity (c.p.m./ μ mol of lipid phosphorus)		
	Phosphatidylinositol	Phosphatidate	Phosphatidylcholine
Homogenate	884	463	11.7
Chromaffin granule	905	664	5.5
Plasma membrane	1076	495	9.1
Microsomal	1444	928	15.3
Mitochondrial	1480	662	6.7

membrane. Table 4 shows a different pattern of losses. After two periods of stimulation it is true that plasma membrane loses significantly less phosphatidylinositol and phosphatidate than the other subcellular fractions, but the chromaffin-granule fraction loses as much as the microsomal fraction. After only one period of stimulation the losses are much the same in all fractions.

Since phosphatidate changes accompany the phosphatidylinositol response to activation of cell-surface receptors, it is usually assumed that the changes are due to conversion of phosphatidate into CDP-diacylglycerol for resynthesis of phosphatidylinositol. Endoplasmic reticulum and mitochondria are the sites of CDP-diacylglycerol synthesis (Davidson & Stanacev, 1974). Exchange proteins specific for phosphatidate have not been described. The situation in stimulated tissues will be complicated by synthesis of phosphatidate from the diacylglycerol released by phospholipase C hydrolysis of phosphatidylinositol. The necessary diacylglycerol kinase has a major location in plasma membranes of brain (Lapetina & Hawthorne, 1971). Receptor-induced hydrolysis of plasma-membrane phosphatidylinositol might therefore be expected to cause increased phosphatidate synthesis in that membrane and loss of phosphatidate from microsomal and mitochondrial membranes where CDP-diacylglycerol is synthesized. The significantly smaller loss of labelled phosphatidate (Table 4) from the plasma-membrane fraction on double stimulation of adrenal medulla bears this out to some extent. Nevertheless, it is difficult to argue from either these or the phosphatidylinositol results that the plasma membrane is the site of the lipid response to carbachol. A small change in plasma-membrane phosphatidylinositol specific to the gating response could be masked by larger changes in other membranes related to secretion. It is possible that a plasma-membrane change would be seen immediately after carbachol administration and experiments to test this are in progress.

Muscarinic activation under the conditions of Fig. 2 caused no significant changes in the concentrations of phosphatidylinositol or phosphatidate in the subcellular fractions studied (Table 5), presumably because there was sufficient time for resynthesis in our experiments. The roughly 50% reduction in specific radioactivity caused by carbachol (Table 4) seems most likely to be due to loss of small highly labelled pools of lipid. If the various membrane fractions have uniformly labelled phosphatidylinositol, for example, half of the total would have to be replaced by non-radioactive lipid during the 22 min after the first stimulation (Fig. 2). Studies of turnover time in other tissues (Ansell & Hawthorne, 1964) suggest that replacement is much slower than this and, in any case, the newly syn-

thesized phosphatidylinositol would have some radioactivity under our conditions.

Bovine adrenal medulla may present a more serious problem for the proposed role of phosphatidylinositol in Ca^{2+} gating. The phosphatidylinositol changes are associated with muscarinic receptors (Mohd. Adnan & Hawthorne, 1981) but in chromaffin-cell cultures these receptors do not promote catecholamine secretion (Fisher *et al.*, 1981). Their function seems to be to reduce the secretory response to nicotinic activation (Derome *et al.*, 1981). As would be expected, activation of the muscarinic receptors caused no influx of Ca^{2+} (Fisher *et al.*, 1981). Some inconsistencies still call for explanation, however. In our previous work with bovine adrenal medulla slices (Mohd. Adnan & Hawthorne, 1981) a secretory response to muscarinic activation was seen. This difference between slices and chromaffin-cell cultures requires further investigation. Nevertheless, in cultured cells, the Ca^{2+} required for catecholamine secretion enters in response to nicotinic receptor activation, whereas the phosphatidylinositol effect is associated with inhibitory muscarinic receptors, at least with bovine adrenal medulla. It seems clear that the Ca^{2+} gating theory (Michell *et al.*, 1977) is untenable for these cells.

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