Phospholipid Turnover in *Torpedo marmorata* Electric Organ During Discharge *in vivo*

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One electric organ of anaesthetized *Torpedo marmorata* was stimulated through electrodes placed on the electric lobe of the brain. Nerves to the other electric organ were cut to provide an unstimulated control. Glucose 6- ^{32}P phosphate was injected into each organ 16 h before electrical stimulation. After stimulation for 10 min at 5 Hz, the organs were removed, homogenized and centrifuged on a density gradient for the preparation of subcellular fractions. Stimulation increased the incorporation of ^{32}P into phosphatidate, phosphatidylinositol and phosphatidylcholine. The increased phosphatidate labelling, but not that of the other two lipids, was seen in fractions rich in synaptic vesicles. Stimulation had no effect on ATP labelling. The phosphatidate content of most fractions fell slightly after stimulation, but amounts of other phospholipids were not affected.

There is a change in the rate of metabolism or turnover of certain phospholipids when the tissue in which they occur is stimulated by drugs, hormones, transmitter substances, electrical pulses or other means. With some exceptions the effect appears to be confined to phosphatidylinositol and its precursor, phosphatidic acid. Usually, the diacylglycerol backbone of both molecules is conserved. Since the first observation of a phospholipid effect (Hokin & Hokin, 1953), a large number of tissues have been shown to exhibit such a response to stimulation (for reviews, see Hawthorne, 1973; Michell, 1975), but the phenomenon has eluded satisfactory explanation.

In nervous tissue the effect appears to be connected with synaptic transmission rather than neuroconduction (Hokin & Hokin, 1958; Larrabee *et al.*, 1963). An enhanced turnover of phosphatidic acid and phosphatidylinositol can be induced in synaptosomes prepared from guinea-pig or rat brain by stimulating with acetylcholine (Schacht & Agranoff, 1972; Yagihara & Hawthorne, 1972; Widlund & Heilbronn, 1974), K^+ ions (Hawthorne & Bleasdale, 1975) or electrical pulses (Bleasdale & Hawthorne, 1975). In electrical stimulation of synaptosomes, the enhanced phosphatidate turnover is synchronous with release of transmitter substance, and both enhanced turnover and release require Ca^{2+} (De Belle-roche & Bradford, 1972; Hawthorne & Bleasdale, 1975). This suggests that an increased metabolism of

phosphatidate accompanies the presynaptic events of synaptic transmission. At least part of the increased incorporation of ^{32}P into this phospholipid during release of acetylcholine from synaptosomes in response to electrical stimulation is associated with synaptic vesicles (Bleasdale & Hawthorne, 1975). Its role here may be concerned with maintaining the balance between vesicular and cytoplasmic acetylcholine during transmission (Widlund & Heilbronn, 1974) or else with the release process itself.

However, a precise characterization of the relationship between synaptic transmission and the phospholipid effect in brain is hampered by the presence of several different transmitter substances, many cell types and the consequent impurity of derived subcellular fractions. Some of these difficulties may be overcome by studying less complex synaptic tissues. The electric organs of *Torpedo* and other electric fish are examples of such tissues and consist of predominantly two cell types, the electroplaque cells and their innervating cholinergic neurons. The present study was undertaken to determine whether a phospholipid effect accompanied discharge of the electric organ of *T. marmorata in vivo* and if so, to ascertain its subcellular location.

Experimental

Materials

The sources of most of the chemicals used here have been previously described (Yagihara *et al.*, 1973; Widlund & Heilbronn, 1974) and any others were of

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commercially available analytical grade. Hexokinase (EC 2.7.1.1), Type F-300 from baker's yeast, was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., as were apyrase (EC 3.6.1.5), grade 1 from potato, and desiccated firefly lanterns.

Fish

The fish used in these experiments were adults of the species *Torpedo marmorata*, kindly supplied by the Station de Biologie Marine, Arcachon, France. Fish were transported by air and then maintained in aquaria for about 10 days before use.

Preparation of fish for experiments *in vivo*

Before all experimental operations, fish were anaesthetized with tricaine (*m*-ethoxycarbonyl-anilinomethanesulphonic acid; MS 222; Sandoz Ltd., Basle, Switzerland) at a concentration of about 0.3 g/litre. The minimum effective dose varied between fish and therefore the anaesthetic was added slowly to sea water containing the fish, taking care not to provoke discharge of the electric organs. Anaesthesia was maintained by superfusing the gills with sea water containing MS 222 (0.1 g/litre) at 20°C. Experiments *in vitro* showed that MS 222 had no effect on phospholipid metabolism in the electric organ (J. E. Bleasdale, unpublished work).

Incorporation of ^{32}P into electric-organ phospholipids

Various means of labelling electric-organ phospholipids were investigated first, and included superfusion of electric organ *in situ* with media containing $^{32}\text{P}_i$, direct injection of $^{32}\text{P}_i$ into the organs and injection of $^{32}\text{P}_i$ (with or without carrier) into the bloodstream. The compact structure of the electric organ and its mucilaginous covering present a considerable diffusion barrier to directly injected $^{32}\text{P}_i$. It was therefore necessary to present the radioisotope in a form which, once it had overcome the diffusion barrier, was more readily taken up by the cells than orthophosphate. It was found that glucose 6- ^{32}P phosphate directly injected into the organs gave at least a fourfold better labelling than $^{32}\text{P}_i$, and this technique was used in all the experiments described below. Glucose 6- ^{32}P phosphate was prepared as follows. [γ - ^{32}P]ATP was prepared as described by Glynn & Chappell (1964), but after incubation, instead of separating [^{32}P]ATP from the mixture, 30 μmol of D-glucose and approx. 10 units of yeast hexokinase were added. The mixture was incubated for a further 30 min at 26°C. After this time CaCl_2 was added to a final concentration of 1 mM and the mixture cooled to 4°C. No attempt was made to isolate glucose 6- ^{32}P phosphate, and the reaction mixture as such was injected directly into the electric organs. Fish were anaesthetized as described above and each organ received five 1 ml (approx. 0.8 mCi total) portions of radioisotope, injected in a predetermined pattern

over the organ and to a depth of about 1.5 cm. A preliminary time-course experiment showed that the specific radioactivities of all lipids were still increasing 24 h after injection. In subsequent experiments a labelling period of 16 h was allowed. During this period fish were not anaesthetized.

Selective stimulation of one electric organ

At 16 h after injection of the radioisotope, each fish was again anaesthetized and the brain exposed. The nerves from one lateral-line (electric) lobe of the brain to its corresponding electric organ were severed inside the cranial cavity, taking care not to rupture blood vessels. The nerves to the other electric organ were left intact. The electric lobes were now stimulated electrically for 10 min by using the following parameters: frequency 5 Hz, voltage amplitude 10–15 V, delay 4 ms, duration 1.5 ms. The anaesthetized fish was kept alive during this period by circulating sea water over the gills and dorsal surface of the body (Plate 1). Simultaneous recordings of potential across each organ were made by placing platinum electrodes on the dorsal and ventral skin covering each organ. In the inactive organ, maximum recorded potentials did not exceed 50 mV and were constant throughout the stimulation period. In the discharging organ potentials of 20–30 V were initially recorded and these decayed to 2–5 V after 10 min of stimulation.

Preparation of subcellular fractions

Immediately after cessation of stimulation, samples of each organ (approx. 50 g) were excised and cleaned of skin, nerves and blood. The anaesthetized fish was then killed by destroying the brain. All further procedures were carried out at 4°C. Each sample was homogenized by using an Ultra-Turrax homogenizer (Jenke und Kunkel KG, Staufen im Breisgau, Germany) operating at 14000 rev./min for 90 s. Homogenates were diluted with ice-cold 0.4 M-NaCl (0.6 ml/g of tissue) and filtered through cheesecloth (1 mm gauge). The filtrates were now re-homogenized in either an Aldridge (Aldridge *et al.*, 1960) or a Potter-Elvehjem homogenizer (radial clearance 0.15 mm, 840 rev./min, 12 strokes) and then centrifuged (10000 g_{av} , for 30 min). The pellet (P_1) was discarded and the supernatant (S_1) fractionated further as shown in Scheme 1. This method is based on the work of Israël *et al.* (1968, 1970). The NaCl in the density gradient is added to maintain iso-osmoticity with elasmobranch plasma.

Characterization of subcellular fractions

The following enzyme and chemical assays were used to examine the composition of subcellular fractions. Acetylcholinesterase (EC 3.1.1.7) was assayed as described by Ellman *et al.* (1961); this enzyme is concentrated in the innervated electroplaque plasma membrane (cf. Couceiro *et al.*, 1955). Rotenone-

EXPLANATION OF PLATE I

(a) General view of apparatus for stimulation of electric organ in anaesthetized *Torpedo marmorata*. (b) Close-up view of exposed electric lobe of brain, showing stimulating electrodes in position.

EXPLANATION OF PLATE 2

Electron micrographs of fractions containing synaptic vesicles

(a) Fraction 4; (b) fraction 5; (c) fraction 6. In each case the bar represents 500 nm. The preparation of specimens followed the method of Kanaseki & Kodota (1969). The smaller, more uniform, structures are the synaptic vesicles (SV). Contamination with other membranes is evident, particularly in fraction 4.

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Scheme 1. Fractionation of *Torpedo* electric organ

sensitive and rotenone-insensitive NADH-cytochrome *c* oxidoreductase (EC 1.6.99.3) were determined by the method of Duncan & Mackler (1966), as markers for mitochondria and endoplasmic reticulum respectively. Occluded (apyrase-resistant) ATP was assayed by the firefly luciferin-luciferase method (Stanley & Williams, 1969) as modified by Dowdall *et al.* (1974) after release of ATP from fractions by treatment with 0.1 vol. of 0.25 M-HClO₄. Occluded ATP can be considered a synaptic-vesicle marker (Dowdall *et al.*, 1974). Acetylcholine was assayed as a synaptic-vesicle marker by using the leech dorsal-muscle bioassay as described by Szerb (1961) or the guinea-pig ileum bioassay (Blaber & Cuthbert, 1961). No eserine was present and thus acetylcholine in the cytosol was hydrolysed by acetylcholinesterase.

Extraction and analysis of lipids

Lipids were extracted from acid-precipitated sub-cellular fractions by using neutral chloroform/methanol mixtures. These methods and the subsequent chromatography were described previously (Yagihara *et al.*, 1973). Lipid spots on thin-layer chromatograms were removed and digested in 72% (w/v) HClO₄ (0.7 ml, 180°C for 60 min). The volume of each digest was made up to 5 ml with water and samples were then taken for ³²P determinations and total P assay (Yagihara *et al.*, 1973). In calculating lipid specific radioactivities, account was taken of the possibility of unequal labelling of precursors in the two organs as a result of the injection procedure. Values were corrected with respect to specific radioactivities of either phosphatidylethanolamine or

ATP in the whole homogenate, both of which remained unaffected by electrical stimulation (Tables 3 and 5). Corrections were never more than 25%, and were usually much less.

Other methods

ATP specific radioactivity in whole homogenates was determined as described elsewhere (Bleasdale & Hawthorne, 1975). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Phosphate was determined as described by Bartlett (1959), but half-quantities were used.

Results

Distribution of enzyme and chemical markers

Since earlier work had indicated that at least part of the phospholipid effect associated with synaptic tissue was localized to synaptic vesicles (Yagihara *et al.*, 1973; Bleasdale & Hawthorne, 1975), the fractionation procedure used in the present study was devised primarily to yield a fraction enriched in synaptic vesicles.

Both acetylcholine and ATP were previously shown to be within synaptic vesicles derived from *Torpedo* electric organ (Heilbronn, 1973; Whittaker *et al.*, 1972; Dowdall *et al.*, 1974). In the present work, determinations of occluded ATP and acetylcholine, as well as electron microscopy (Plate 2), indicate that synaptic vesicles are present in fractions 3–6 (Scheme 1), but that the fractions are increasingly contamin-

ated with other membranes (Table 1). Fraction 4, in agreement with Israël *et al.* (1970), contained much the highest total amount of bound acetylcholine, though the peak concentration of ATP was in fraction 5. This is probably due to overloading of the gradient. Experiments with lower concentrations of tissue show acetylcholine and ATP peaks in fraction 4. Fraction 6 in particular appears to be enriched in both plasma membrane and endoplasmic reticulum when compared with the whole tissue. These data imply that much of the phospholipid-metabolizing capacity of fraction 6 (below) can be ascribed to microsomal elements rather than synaptic vesicles. Most mitochondria are discarded along with fraction P₁, and the rotenone-sensitive NADH-cytochrome *c* oxidoreductase activity found in fractions 1–8 was not associated with whole mitochondria (shown by electron microscopy; A. Hedberg, G. Toresson & J. E. Bleasdale, unpublished work). Presumably, the harsh homogenization required to release synaptic vesicles also disrupts some mitochondria.

Phospholipid composition of subcellular fractions

Table 2 shows the phospholipid composition of electric-organ subcellular fractions. Only the lipids shown were analysed. These values were obtained by measuring total phosphorus in all spots on two-dimensional thin-layer chromatograms, except the origin and two unidentified lipids (*R_F* in the first direction, 0.25, 0.27; *R_F* in the second direction, 0.05, 0.05), which together accounted for 2–5% of total lipid P. Since phosphatidylserine and phosphatidyl-

Table 1. Distribution of enzyme and chemical markers among density-gradient fractions

Values given are the means of the numbers of experiments indicated in parentheses $\pm$ S.D. Rotenone-sensitive and rotenone-insensitive NADH-cytochrome *c* oxidoreductase activities are assayed in the same sample and calculated from the difference in reaction rate before and after adding rotenone. Acetylcholine, ATP and enzyme values are for resting organ.

Fraction	No. of expts.	Protein (% of that recovered)		ATP (nmol/mg of protein) (3)	Acetylcholine* (nmol/mg of protein) (3)	Acetylcholinesterase (ΔE_{412} /mg of protein) (3)	NADH-cytochrome <i>c</i> oxidoreductase (ΔE_{550} /mg of protein)	
		Resting organ (5)	Discharging organ (2)				Rotenone-sensitive (3)	Rotenone-insensitive (3)
1		64.9 $\pm$ 3.1	59.5 $\pm$ 5.7	0.12 $\pm$ 0.02	0.27 $\pm$ 0.19	20.3 $\pm$ 1.4	0.08 $\pm$ 0.01	0.01 $\pm$ 0.001
2		13.4 $\pm$ 2.1	19.3 $\pm$ 4.9	0.36 $\pm$ 0.2	0.10 $\pm$ 0.03	22.2 $\pm$ 1.2	0.08 $\pm$ 0.02	0.13 $\pm$ 0.03
3		5.1 $\pm$ 0.5	4.3 $\pm$ 1.1	2.20 $\pm$ 1.5	12.38 $\pm$ 3.31	42.3 $\pm$ 2.7	0.16 $\pm$ 0.06	0.34 $\pm$ 0.06
4		5.2 $\pm$ 1.1	6.0 $\pm$ 0.8	4.35 $\pm$ 2.3	36.27 $\pm$ 13.71	47.3 $\pm$ 3.1	0.30 $\pm$ 0.04	0.54 $\pm$ 0.23
5		2.0 $\pm$ 1.0	1.6 $\pm$ 0.9	9.71 $\pm$ 4.1	19.79 $\pm$ 6.70	47.3 $\pm$ 3.0	0.72 $\pm$ 0.05	0.96 $\pm$ 0.05
6		1.9 $\pm$ 0.7	2.0 $\pm$ 1.0	6.15 $\pm$ 3.0	7.65 $\pm$ 3.81	96.3 $\pm$ 4.0	1.08 $\pm$ 0.02	1.57 $\pm$ 0.10
7		1.1 $\pm$ 0.7	1.0 $\pm$ 0.8	2.89 $\pm$ 2.0	2.44 $\pm$ 1.74	33.5 $\pm$ 2.5	0.93 $\pm$ 0.40	1.52 $\pm$ 0.3
8		6.3 $\pm$ 1.4	6.4 $\pm$ 1.9	0.40 $\pm$ 0.1	0.94 $\pm$ 0.68	23.4 $\pm$ 3.5	0.03 $\pm$ 0.02	0.99 $\pm$ 0.4
Homogenate		—	—	0.61 $\pm$ 0.04	112 $\pm$ 177	49.9 $\pm$ 2.4	0.63 $\pm$ 0.07	0.80 $\pm$ 0.21
Recovery (% of fraction S ₁)			87 $\pm$ 11	91 $\pm$ 7		65 $\pm$ 15	110 $\pm$ 9	76 $\pm$ 8

* No eserine present.

Table 2. *Phospholipid composition of electric-organ subcellular fractions and the effect of organ discharge*

R, resting organ; D, discharging organ. Values given are percentages of recovered lipid P and are the means of three experiments. Recoveries were usually less than 100% because the origin on the plates was not analysed (where highly polar lipids such as the polyphosphoinositides remain) and two unidentified lipids were likewise excluded from calculations. Most of the changes due to discharge were within the s.d. of the means (see the text). Only those for phosphatidic acid are recorded, since discharge was accompanied by a loss of this lipid from most fractions. Abbreviations: LPC, lysophosphatidylcholine; Sm, sphingomyelin; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid.

Fraction	LPC R	Sm R	PC R	LPE R	PS+PI R	PE R	PG+CL R	PA	
								R	D
Homogenate	8.0	14.4	26.3	16.3	15.0	18.0	0.9	1.2	0.9
S ₁	7.9	15.0	25.1	16.6	15.3	18.5	0.8	1.0	0.7
P ₁	8.9	15.7	24.6	14.5	17.0	17.4	0.8	1.4	1.2
1	6.3	12.6	37.2	9.8	10.8	20.9	0.8	1.5	1.4
2	6.8	14.6	35.7	10.9	9.8	19.9	1.0	1.2	1.0
3	7.5	14.5	33.5	13.1	10.3	18.4	1.1	1.6	1.3
4	6.5	15.8	31.3	13.6	12.6	18.5	0.4	1.3	1.3
5	5.6	13.5	34.1	13.7	13.1	17.6	0.8	1.7	1.4
6	6.0	12.8	32.6	14.6	13.0	17.9	1.2	1.8	1.2
7	7.4	14.8	30.9	14.0	12.5	18.5	0.9	1.0	0.9
8	8.0	15.0	24.3	17.6	15.7	17.9	0.6	0.9	0.9

inositol could not always be completely resolved, they were analysed together as a routine. In three experiments, where separation was complete, the two lipids were present in a mean phosphatidylserine/phosphatidylinositol molar ratio of 8:5.5. For the same reasons, phosphatidylglycerol and cardiolipin were analysed together. These two lipids were present in approximately equal amounts (two experiments).

The different subcellular fractions do not vary greatly in phospholipid composition. Fraction 6, which of the density-gradient fractions has the highest specific activity of rotenone-sensitive NADH-cytochrome *c* oxidoreductase, also has the most cardiolipin. The fractions richest in synaptic vesicles are relatively richer in phosphatidate than is the homogenate. A more-pronounced enrichment of phosphatidate in a synaptic-vesicle fraction from guinea-pig cerebral cortex has been noted (Yagihara *et al.*, 1973). The similar phospholipid compositions of all fractions does not argue against at least a partial resolution of membranes, since synaptic vesicles and plasma membrane from rat brain have been reported to have similar phospholipid compositions (Breckenridge *et al.*, 1973).

When phospholipid analyses were performed on subcellular fractions from discharging organ (Table 2), the changes observed were on the whole small and were nearly all within the standard deviation. Further experiments are required to determine if any of the small changes are significant. The only consistent change in all fractions appeared to be a decrease in phosphatidate after organ discharge.

Effects of organ discharge on incorporation of ³²P into phospholipids

After labelling electric-organ phospholipids with ³²P for 16h, one organ was selectively discharged for 10min before samples of each organ were taken for subcellular fractionation. Phospholipids were extracted from each fraction and chromatographed as described above. The incorporation of ³²P into each lipid was measured and expressed as either c.p.m./nmol of lipid (phosphatidate and phosphatidylinositol+phosphatidylserine) or c.p.m./μmol of total lipid P (other lipids). These data are shown in Tables 3 and 4.

The changes in phospholipid turnover induced by organ discharge, which are shown in Table 3, tend to be small and variable. Most of the variability may be due to experimental error, since these lipids incorporated little ³²P. The increases in total lipid specific radioactivity on discharge can be attributed largely to enhanced labelling of phosphatidate, phosphatidylinositol+phosphatidylserine and phosphatidylcholine (see below). The failure of the other lipids to respond to stimulation agrees with earlier work on other synaptic tissues (for a review, see Hawthorne, 1973). Two exceptions in the present work may be lysophosphatidylcholine and phosphatidylglycerol/cardiolipin. Here, large increases in ³²P incorporation after discharge were detected in fraction 5. In analogous experiments, no such effects on the turnover of these lipids could be detected in a synaptic-vesicle fraction prepared from synaptosomes stimulated either electrically (Bleasdale & Hawthorne, 1975) or

Table 3. Effect of electrical discharge on the turnover of electric-organ phospholipids: subcellular distribution

R, resting organ; D, discharging organ. Values in the first two columns for each lipid are ^{32}P radioactivities (c.p.m.) in a particular lipid/ μmol of total lipid P. Specific radioactivities could not be calculated because of the poor incorporation of ^{32}P into these phospholipids. Each value is the mean of the number of experiments shown in parentheses. The third column for each lipid shows percentage changes, $\pm\text{s.d.}$. Abbreviations for phospholipids are as given in the legend to Table 2.

Fraction	Total phospholipid			LPC			Sm			LPE			PE			PG+CL		
	No. of expts. ... (6)			(3)			(3)			(3)			(3)			(3)		
	R	D	Change (%)	R	D	Change (%)	R	D	Change (%)	R	D	Change (%)	R	D	Change (%)	R	D	Change (%)
Homogenate	480	562	17 $\pm$ 7	308	307	-0.3 $\pm$ 10	55	46	-16 $\pm$ 21	502	476	-5 $\pm$ 19	299	275	-8 $\pm$ 12	240	343	43 $\pm$ 26
S ₁	542	721	33 $\pm$ 20	293	238	-19 $\pm$ 25	61	76	25 $\pm$ 15	478	639	34 $\pm$ 33	320	332	4 $\pm$ 17	221	247	12 $\pm$ 30
P ₁	466	485	4 $\pm$ 15	326	453	39 $\pm$ 41	53	22	-58 $\pm$ 47	512	374	-27 $\pm$ 22	290	223	-23 $\pm$ 42	252	249	-1 $\pm$ 49
1	437	551	26 $\pm$ 20	314	218	-31 $\pm$ 19	60	24	-59 $\pm$ 34	532	577	9 $\pm$ 30	281	535	90 $\pm$ 40	216	223	3 $\pm$ 17
2	451	433	-4 $\pm$ 19	277	493	78 $\pm$ 65	52	70	34 $\pm$ 28	501	897	79 $\pm$ 28	284	344	21 $\pm$ 34	211	325	54 $\pm$ 19
3	461	572	24 $\pm$ 11	293	343	17 $\pm$ 28	47	57	20 $\pm$ 10	447	349	-22 $\pm$ 17	323	351	9 $\pm$ 18	247	416	68 $\pm$ 41
4	490	681	39 $\pm$ 19	302	372	23 $\pm$ 32	59	128	118 $\pm$ 87	546	728	33 $\pm$ 40	317	461	45 $\pm$ 16	250	141	-44 $\pm$ 38
5	538	448	39 $\pm$ 12	323	1292	300 $\pm$ 83	61	91	49 $\pm$ 18	578	473	-18 $\pm$ 38	329	593	80 $\pm$ 40	215	693	222 $\pm$ 81
6	576	778	35 $\pm$ 10	339	500	47 $\pm$ 46	49	95	93 $\pm$ 34	602	585	-3 $\pm$ 15	330	369	12 $\pm$ 35	262	278	6 $\pm$ 40
7	557	774	39 $\pm$ 30	370	730	97 $\pm$ 43	63	89	41 $\pm$ 29	552	363	-34 $\pm$ 22	324	389	20 $\pm$ 19	269	143	-47 $\pm$ 51
8	499	639	28 $\pm$ 10	290	305	5 $\pm$ 20	57	85	49 $\pm$ 31	507	414	-18 $\pm$ 19	305	274	-10 $\pm$ 29	223	224	0.5 $\pm$ 28

Table 4. Significance of the changes in phospholipid ^{32}P labelling accompanying electric-organ discharge

Values are mean percentage changes ($\pm\text{s.d.}$) observed on stimulating organ discharge *in vivo*, for the number of experiments shown. *P* values were calculated by a two-tailed Student-*t* test. R, resting; D, discharging. Abbreviations for phospholipids are as given in Table 2.

Fraction	Phosphatidate			Phosphatidylinositol + phosphatidylserine			Phosphatidylcholine		
	Change (%)			Change (%)			Change (%)		
	(c.p.m./nmol of PA)	R	D	(c.p.m./nmol of PS+PI)	R	D	(c.p.m./ μmol of total lipid P)	R	D
Homogenate	145	183	27 $\pm$ 18	12.0	15.4	136	175	29 $\pm$ 46	>0.2
S ₁	167	211	26 $\pm$ 27	13.1	14.3	156	199	28 $\pm$ 4	<0.05
P ₁	143	183	28 $\pm$ 41	11.8	14.3	129	143	11 $\pm$ 37	>0.4
1	116	131	12 $\pm$ 18	11.4	20.1	124	165	33 $\pm$ 27	>0.1
2	132	171	30 $\pm$ 48	11.5	9.0	143	284	99 $\pm$ 34	<0.05
3	135	137	2 $\pm$ 34	11.3	19.2	150	203	35 $\pm$ 28	>0.1
4	146	212	45 $\pm$ 23	12.6	14.2	144	201	40 $\pm$ 40	>0.05 <0.1
5	161	262	63 $\pm$ 47	13.0	9.6	165	215	31 $\pm$ 38	>0.2
6	203	321	58 $\pm$ 32	15.6	15.1	175	329	88 $\pm$ 12	<0.005
7	162	193	19 $\pm$ 18	18.0	38.1	188	463	146 $\pm$ 75	>0.05 <0.1
8	142	159	12 $\pm$ 8	13.2	10.8	129	148	15 $\pm$ 37	>0.3

with acetylcholine (Yagihara *et al.*, 1973; Widlund & Heilbronn, 1974). Further investigation of these two effects was made difficult by the relatively low yield of fraction 5 and poor basal incorporation into these lipids.

The specific-radioactivity changes recorded in Table 4 for phosphatidate and phosphatidylinositol+phosphatidylserine, however, could be measured more accurately because of better ^{32}P incorporation. Phosphatidate and phosphatidylinositol+phosphatidylserine together accounted for over 65% of total ^{32}P -labelling of phospholipids. By far the most readily labelled lipid was phosphatidate. During organ discharge the specific radioactivity of whole-tissue phosphatidate rose by 27%, and much of this increase appears to be localized in fractions 4, 5 and 6, which are richest in ATP (Table 1). The statistical significance of these changes is shown in Table 4. Organ discharge also increased labelling of phosphatidylinositol (+phosphatidylserine), but these changes were more variable. The fraction showing greatest stimulation was fraction 7, which was relatively rich in endoplasmic reticulum. The data suggest that the subcellular sites of the phosphatidate effect and the effect on turnover of phosphatidylinositol (+phosphatidylserine) are not the same.

There appears to be little doubt that the latter effect is in fact on phosphatidylinositol turnover and not on that of phosphatidylserine. Although phosphatidylinositol formed only 41% (three experiments) of the lipid P in this combined lipid fraction, it accounted for 78% of the ^{32}P incorporation (two experiments). In a single experiment, phosphatidylserine turnover, measured separately, appeared to be unaffected by discharge. The combination of the two phospholipids for analysis means that the effect on phosphatidylinositol turnover has been underestimated.

Although phosphatidylcholine in resting tissue was not particularly well labelled (Table 4), the effects on turnover produced by organ discharge were sufficiently large to be measured with some certainty. Pronounced stimulation of phosphatidylcholine turnover was detected in fractions 2, 6 and 7. Two of these changes were statistically significant, whereas the third and largest effect, occurring in fraction 7, had marginal significance. The subcellular location of increased phosphatidylcholine labelling resembled that of the phosphatidylinositol effect more closely than that of enhanced phosphatidate turnover.

Effect of organ discharge on precursor specific radioactivities

Although ^{32}P was administered to fish as glucose 6- ^{32}P]phosphate, it is ^{32}P ATP that eventually provides the radioisotope entering phospholipid metabolism. It was therefore decided to see if the major effects on phospholipid turnover induced by

Table 5. *Effects of organ discharge on amounts and specific radioactivity of ATP*

Mean results $\pm$ s.d. are shown, with the number of experiments in parentheses. N.D., Not determined.

	ATP	
	Concentration (nmol/mg of protein)	Specific radioactivity (c.p.m./nmol of P)
Homogenate		
Resting organ	0.61 ± 9.04 (3)	443 ± 115 (3)
Discharging organ	0.33 (1)	525 ± 131 (3)
Fraction 5		
Resting organ	9.71 ± 4.1 (3)	N.D.
Discharging organ	3.1 (1)	N.D.

organ discharge were secondary to changes in precursor specific radioactivity. Table 5 suggests that this is not the case, and is in keeping with other studies of the phospholipid effect where this parameter has been measured (Michell, 1975; Bleasdale & Hawthorne, 1975). No statistical significance could be attached to the small (<20%) increase in ATP specific radioactivity of homogenates following discharge, but such a change could explain the slightly enhanced labelling of total phospholipid in most subcellular fractions (Table 3).

Discussion

Early studies of the phospholipid effect in nervous tissue all indicated that the phenomenon was likely to be concerned with synaptic transmission rather than neuroconduction (Hokin & Hokin, 1959; Krnjević & Phillis, 1963; Larrabee *et al.*, 1963). Since then, various workers have observed that synaptosomes exhibit a pronounced phospholipid effect in response to a variety of stimuli (Schacht & Agranoff, 1972; Widlund & Heilbronn, 1974; Hawthorne & Bleasdale, 1975). These reports are compatible with a presynaptic role for the phospholipid effect in synaptic transmission, a view which is strengthened by the finding that at least part of the effect is confined to synaptic vesicles (Yagihara *et al.*, 1973; Bleasdale & Hawthorne, 1975). However, the heterogeneity of mammalian synaptosome preparations, and the unphysiological nature of the stimuli used in earlier studies, restricts their relevance to synaptic transmission *in vivo*.

In the work reported here, only the length of discharge (10min) may be questioned as unphysiological. The tissue itself is ideally suited for studying cholinergic synaptic transmission. The bilateral electric organs of *Torpedo* are believed to have developed embryologically from muscle. In *T. marmorata* they may constitute 20% or more of the total body weight. They consist of vertical stacks of hexagonally shaped cells (electroplaques), each of

which is richly innervated on its ventral side. The cholinergic nerves providing the innervation arise from cell bodies in the lateral-line (electric) lobes of the brain and pass in four large bundles between the gill arches to each organ (Fritsch, 1890). Since the nerves go to only one surface of each electroplaque, the asymmetric depolarization that they produce leads to a potential difference across a single electroplaque which when summed over a vertical column of such cells may reach as much as 50 V in *T. marmorata* (Grundfest, 1957). On the basis of previous work with synaptosomes, it is assumed that any effects on phospholipid turnover in the electric organ are occurring in the nerve endings rather than the electroplaques. Since the results of Zimmerman & Dowdall (1975) have indicated that it is possible to separate synaptosomes from electric organ of neonatal *Torpedo*, this assumption could be tested.

Electric-organ phospholipids

The amounts of each phospholipid present (Table 2) are in the range found for other synaptic tissues (cf. Eichberg *et al.*, 1964; Rouser & Yamamoto, 1969; Yagihara *et al.*, 1973; Breckenridge *et al.*, 1973), with the possible exception of sphingomyelin and the lyso compounds. The unexpectedly high sphingomyelin content warrants confirmation of identity on the two-dimensional thin-layer chromatograms. The R_F values in both dimensions are those of an authentic sphingomyelin sample, and the spot is Dragendorff-positive (Kates, 1972). The possibility remains, however, that the spot may contain another contaminating lipid. Bartels & Rosenberg (1972) analysed for phospholipid the main and Sachs electric organs of the eel *Electrophorus electricus*. Here, phosphatidylcholine plus phosphatidylethanolamine accounted for about 80% of total lipid P. In the present study these lipids form only approx. 50% of recovered lipid P, but together with their lyso derivatives this value becomes about 72%. With electric tissue from *Electrophorus* only small amounts of lyso-lipids were found (Bartels & Rosenberg, 1972). Baker *et al.* (1975) found only 0.4% lysophosphatidylcholine in *Torpedo* synaptic vesicles. It seems likely that lyso compounds are formed from plasmalogens during the fractionation or acid extraction procedures in the present work.

Phospholipid labelling

The experiments described in the present paper show that during discharge of *Torpedo* electric organ, there is an enhanced turnover of certain phospholipids. With the exception of phosphatidate, the total amount of such phospholipid is unaffected by discharge. The decrease in phosphatidate observed during discharge resembles a similar decrease in synaptosomal phosphatidate after stimulation with acetylcholine (Yagihara *et al.*, 1973; Schacht & Agranoff,

1974). The magnitude of the phospholipid effect in electric tissue is of the same order as that for mammalian brain, and much smaller than that for other tissues such as pancreas (Hokin, 1968). The increased incorporation of ^{32}P into phosphatidylinositol during discharge is somewhat higher than the enhancement observed with synaptosomes. However, the greatest difference between the present work and previous work with synaptosomes is the large increase in phosphatidylcholine turnover evident here but totally absent in synaptosomes (Widlund & Heilbronn, 1974; Bleasdale & Hawthorne, 1975). Work with electric tissue *in vitro* also revealed a substantially increased phosphatidylcholine labelling on stimulation with either acetylcholine or electrical pulses (Rosenberg, 1973). It is difficult to decide whether this effect is due to enhanced turnover or net synthesis, since basal labelling is so poor that the synthesis of only a little new and highly labelled phosphatidylcholine would greatly change the specific radioactivity without apparently changing the pool size. The raised diacylglycerol concentration accompanying the phospholipid effect in other tissues as a result of increased phosphatidate hydrolysis (Schacht & Agranoff, 1974) or phosphatidylinositol hydrolysis (Hokin-Neaverson, 1974; Jones & Michell, 1974) may be sufficient to stimulate phosphatidylcholine synthesis. It has already been suggested that increased cell permeability may result from stimulation of a phospholipase hydrolysing phosphatidylcholine (Heilbronn, 1972). If after hydrolysis, lysophosphatidylcholine is not recycled, but is itself hydrolysed, the replacement phosphatidylcholine synthesized after the permeability change should be labelled with ^{32}P . In such a mechanism, enhanced phosphatidylcholine turnover differs from that of phosphatidate and phosphatidylinositol, as the diacylglycerol backbone is not conserved. Rosenberg (1973) has indeed shown that electrical stimulation of isolated columns of electroplaques from *Electrophorus* increases incorporation of ^{14}C from glucose into phosphatidylcholine much more than into other lipids. Abdel-Latif *et al.* (1976) showed that higher (1–10 mM) concentrations of cholinergic or adrenergic transmitters increased the ^{32}P labelling of phosphatidylcholine in iris muscle.

Subcellular localization of phospholipid changes

The most pronounced changes in phosphatidate labelling on stimulation of electric organ were seen in fractions 4, 5 and 6 (Table 4). These fractions all contain synaptic vesicles (Plate 2) and have the highest concentrations of bound ATP (Table 1). Fraction 4 has the highest concentration of bound acetylcholine. The phosphatidate results are consistent with our previous suggestion that stimulated labelling of this phospholipid occurs in the synaptic-vesicle membrane, but they do not provide proof, since other

membranes are present. Vesicles prepared by zonal centrifugation (Dowdall *et al.*, 1974) can be much purer, as indicated by their acetylcholine content (680–1200 nmol/mg of protein). Zonal methods were not used in the present work because of possible radioactive contamination.

Although the increased labelling of phosphatidate which accompanies stimulation of electric organ seems to be associated with synaptic-vesicle fractions, the corresponding phosphatidylinositol labelling is most obvious in fraction 7. This fraction is rich in endoplasmic reticulum, to judge from rotenone-insensitive NADH-cytochrome *c* oxidoreductase (Table 1). Fraction 6, however, which is also enriched in this enzyme, showed no phosphatidylinositol effect. Phosphatidylinositol synthesis is likely to occur on the endoplasmic reticulum, and in our earlier study with brain the increased labelling of this lipid was seen in a fraction richer in such membranes than in synaptic vesicles (Yagihara *et al.*, 1973). The most marked increases in phosphatidylcholine labelling were seen in fractions 6 and 7, suggesting that synthesis of this lipid is also associated with endoplasmic reticulum.

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