Effect of Stimulation and Acetylcholine on ^{32}P and ^{14}C Incorporation into Phospholipids of Eel Electroplax

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Abstract ☐ Incorporation of ^{32}P and ^{14}C into phosphatidylinositol and phosphatidylcholine was increased when electroplax of the Sachs organ of the electric eel was exposed to 10^{-4} M concentrations of acetylcholine or physostigmine or when the cells were stimulated. In contrast, there was no significant effect on the specific activities of sphingomyelin, phosphatidylserine, phosphatidylethanolamine, or phosphatidic acid. The concentration of total phospholipids was unaltered. These results support the proposals that the neurotransmitter action of acetylcholine is associated with an increased turnover of phospholipids.

Keyphrases ☐ Acetylcholine—effects on ^{32}P and ^{14}C incorporation into eel electroplax phospholipids ☐ Electroplax, eel—stimulation and acetylcholine effects on ^{32}P and ^{14}C incorporation ☐ Radiolabeled phosphorus and carbon—effects of stimulation and acetylcholine on incorporation into eel electroplax phospholipids ☐ Stimulation—effects on ^{32}P and ^{14}C incorporation into eel electroplax phospholipids

Various functions have been proposed for phospholipids in bioelectrically excitable tissues including their possible involvement in the control of membrane resistance and capacitance, in the genesis of bioelectricity, as receptors for various drugs, and in active transport (1–7). Earlier studies from this laboratory, however, showed that axonal conduction and normal permeability were maintained in the squid giant axon even after extensive splitting of its phospholipids by phospholipases A and C (8–10). It was of interest to extend these studies to a synaptic-containing preparation since some investigators related the action of synaptic transmitters to an interaction with membrane phospholipids (6, 11–14). The isolated single electroplax used in the present study has acetylcholine as the neurotransmitter at the thousands of synapses present on each cell, and the conducting membrane can be stimulated either directly or indirectly *via* the nerves. The phospholipid composition of this preparation was recently reported (15) from this laboratory, and the postsynaptic and action potentials were noted to be blocked when more than one-third of the phosphatidylcholine of the cell was hydrolyzed by phospholipase A. As a further test of the possible involvement of phospholipids in the bioelectrical functioning of the electroplax, the incorporation of ^{32}P and ^{14}C into the phospholipids of control, stimulated, and acetylcholine- and physostigmine-treated electric tissue was measured.

MATERIALS AND METHODS

Cells from the Sachs organ were incubated in 7 ml. of eel Ringer's solution (pH 7) of the following composition (mM): NaCl, 160; KCl, 5; CaCl$_2$, 2; MgCl$_2$, 2; NaH$_2$PO$_4$, 0.3; Na$_2$HPO$_4$, 1.2; and glucose, 10. In addition, each milliliter of the solution contained 25 μc. of ^{32}P-H$_3$PO$_4$[1] (>99% radiochemical purity) and 2.5 μc. of U-^{14}C-

D-glucose[1] (>95% radiochemical purity, U = uniformly labeled). For the stimulation experiments, slices of Sachs organ, 1 cell thick and 15–30 cells long (1–2 g.), were incubated in this solution for 1 hr., after which the rows were stimulated with supramaximal voltages 5–10 times/sec. for 2 hr. with a 10-min. rest period every 20 min. In control experiments, the action potentials decreased no more than 20% during this 2-hr. period. Extracellular silver–silver chloride electrodes inserted into each end of the row were used for stimulating and recording electrical activity. Depending on the direction of the current, the innervated (conducting) membrane of the cell was either stimulated directly or indirectly *via* the nerve endings. Control slices were incubated for 3 hr. without stimulation.

In other experiments, 30 single isolated cells (~2 g.) from the Sachs organ were exposed for 6 hr. to eel Ringer's solution containing acetylcholine and physostigmine (10^{-4} M of each) or physostigmine (10^{-4} M) in addition to the radioactive compounds. This concentration of physostigmine decreases the action potential very slowly, taking several hours to block conduction, whereas acetylcholine plus physostigmine blocks electrical activity within 1 or 2 min. Control cells were exposed for the same length of time to a similar solution not containing acetylcholine or physostigmine. After incubation, lipid extracts of the tissues were prepared using chloroform–methanol (1:3, followed by 2:1) and were spotted on a two-dimensional thin-layer chromatogram used for the separation of the individual phospholipids (8–10, 15). Each phospholipid spot was visualized with iodine vapors, scraped off of the chromatogram, eluted with 4 ml. of chloroform–methanol (1:1), and divided into two equal samples. Both samples were evaporated to dryness, after which phosphorus was determined in one sample (16) and 10 ml. of scintillator fluid[2] was added to the other sample. This latter sample was counted in a scintillation spectrometer[3] with the windows set to count ^{14}C and ^{32}P simultaneously. An automatic external standard was used to monitor counting efficiency, and all samples were corrected for background, for radioactive decay of ^{32}P, and for the approximately 15% of the ^{32}P counts that appeared in the ^{14}C channel. A small portion (5%) of the original lipid extract was used for the determination of total lipid phosphorus. Elution of phospholipids from silica gel with chloroform–methanol may not completely extract the phospholipids (17–19); therefore, it is essential that all results be reported as specific activities, that is, counts per minute per micromole of phosphorus.

RESULTS

All treatments caused a significant increase in ^{32}P and ^{14}C incorporation into phosphatidylinositol and phosphatidylcholine (Tables I and II). No significant changes were seen in the specific activities of sphingomyelin, phosphatidylserine, phosphatidylethanolamine, or phosphatidic acid. Although there appeared to be a tendency toward an increase with phosphatidic acid, these results are probably the least reliable because of the very small amounts of phosphatidic acid present. In the initial experiments, it appeared that a large incorporation of ^{14}C into sphingomyelin was occurring, which would have been most unusual considering the known metabolic stability of this phospholipid in other membrane systems. Later it was found that this radioactivity was associated with a non-phosphorus-containing compound, which plated out on the sphingomyelin spot and adjacent area. By careful separation of supernates and phases during each step of the lipid extraction, especially at the stage of washing with pure solvent upper phase, it was possible to decrease markedly the amount of this contaminant, although the sphingomyelin values still probably represent maximum values.

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[1] New England Nuclear Corp., Boston, Mass.

[2] Aquasol, New England Nuclear Corp., Boston, Mass.
[3] Packard model 3375.

Table I—[32]P Incorporation into Phospholipids of the Sachs Organ of the Electric Eel[a]

Tissue Condition	c.p.m. (hundreds)/μmole P[b]					
	SM	PC	PI	PS	PE	PA
Single cells						
Control	3.1 ± 0.8	4.5 ± 0.2	43 ± 6	11 ± 4	9.7 ± 1.0	19 ± 7
ACh + Physo	2.2 ± 0.6	8.7 ± 0.4**	164 ± 9**	18 ± 6	9.2 ± 1.1	31 ± 3
Physo	3.7 ± 0.2	5.8 ± 0.5*	83 ± 4**	18 ± 4	10.3 ± 1.0	26 ± 6
Rows of cells						
Control	4.0 ± 1.1	5.2 ± 0.7	76 ± 10	4.3 ± 1.2	13 ± 5	13 ± 2
DS	3.0 ± 0.5	21 ± 3**	190 ± 19**	3.8 ± 0.8	14 ± 4	19 ± 2
IS	3.0 ± 0.7	29 ± 2**	217 ± 17**	2.4 ± 0.7	15 ± 3	13 ± 2

[a] SM = sphingomyelin, PC = phosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine, PE = phosphatidylethanolamine, PA = phosphatidic acid, ACh = 10^{-4} M acetylcholine, Physo = 10^{-4} M physostigmine, IS = indirect stimulation, and DS = direct stimulation. Single cells were exposed to 25 μc. ^{32}P-H_3PO_4/ml. incubation mixture for 6 hr., while rows of cells were exposed for 3 hr. [b] Each value (mean ± standard error) is based upon three experiments. ** = $p < 0.01$ compared to controls and * = $p < 0.05$ compared to controls.

Table II—[14]C Incorporation into Phospholipids of the Sachs Organ of the Electric Eel[a]

Tissue Condition	c.p.m. (hundreds)/μmole P[b]					
	SM	PC	PI	PS	PE	PA
Single cells						
Control	17 ± 4	46 ± 4	130 ± 20	33 ± 2	42 ± 5	76 ± 45
ACh + Physo	21 ± 5	132 ± 11**	840 ± 50**	41 ± 9	31 ± 8	162 ± 50
Physo	24 ± 5	61 ± 5*	370 ± 90*	50 ± 10	28 ± 6	98 ± 30
Rows of cells						
Control	10 ± 3	24 ± 10	110 ± 20	14 ± 3	5 ± 2	44 ± 14
DS	7 ± 2	202 ± 23**	520 ± 40**	21 ± 7	7 ± 1	93 ± 23
IS	10 ± 3	111 ± 19**	320 ± 10**	14 ± 3	2 ± 1	89 ± 21

[a] Single cells were exposed to 2.5 μc. U-^{14}C-glucose/ml. of incubation mixture for 6 hr., while rows of cells were exposed for 3 hr. Abbreviations are given in Table I. [b] Each value (mean ± standard error) is based upon three experiments. ** = $p < 0.01$ compared to controls, and • = $p < 0.05$ compared to controls.

In four experiments, single cells were exposed to 2.5 μc. N-methyl-^{14}C-choline[1]/ml. of incubation mixture. More than 90% of the radioactivity incorporated into the phospholipids was found in phosphatidylcholine, a small amount was found in sphingomyelin, and phosphatidylethanolamine and phosphatidylserine remained nonradioactive. This is in agreement with earlier findings that choline is incorporated as a unit into lecithin (20–23).

Studies similar to those in the Sachs organ were also carried out on rows of cells from the Main organ, but the results were more variable and the ^{32}P incorporation was very low.

Total lipid phosphorus values of the tissues were not affected by stimulation or exposure to acetylcholine (Table III). The values for the Sachs organ are slightly higher and the values for Main organ are slightly lower than those previously found (15). The absolute values will, however, vary considerably, depending on the area of the Sachs and Main organs used. The closer to the head end of the eel the tissue samples are taken from, the more tightly packed are the cells and, therefore, the higher are the values for phosphorus per unit weight.

DISCUSSION

All experimental conditions studied caused depolarization of the innervated membrane of the electroplax. It is, however, unlikely that the results are due to an increased penetration and, therefore, an increase in the specific activity of ^{32}P-H_3PO_4 or U-^{14}C-glucose within the cell. If that were the case, one would expect to see increased specific activity of ^{32}P and ^{14}C in all phospholipids, whereas only an increased incorporation into phosphatidylcholine and phosphatidylinositol was found.

Acetylcholine, physostigmine, and stimulation cause an influx of sodium into the cell and an efflux of potassium, which may then be followed by an increase in active transport of sodium. The effects of these treatments on phospholipid metabolism may be associated with ionic changes occurring as a result of depolarization. Depending on conditions, ionic alterations may either decrease or increase ^{32}P incorporation into brain phospholipids (24, 25). The application of acetylcholine and physostigmine to a wide variety of tissues increases incorporation into both phosphatidic acid and phosphatidylinositol and may be due to a stimulation of active transport processes (26–31). In the electric organ, however, it has been reported (32) that phospholipids do not act as intermediates in the transport adenosine triphosphatase system.

The Main organ of the electric eel showed very low ^{32}P incorporation, probably because of the permeability barriers to the penetration of phosphorus in this tissue. The cells are much closer together in the Main organ than in the Sachs organ, so that it is not possible to dissect out single cells. By comparing the results shown in Tables I and II, one can see that even in the Sachs organ the incorporation of ^{14}C is much greater than the incorporation of ^{32}P, even though the specific activity of ^{32}P used in the incubation mixture was much higher than that of ^{14}C. Phosphorus does not readily penetrate through neuronal membranes (33–36), which could explain these findings.

Table III—Total Lipid Phosphorus Values for Sachs and Main Electric Organ[a]

Treatment	Sachs Organ		Main Organ
	Single Cells	Rows of Cells	Rows of Cells
Control	0.80 ± 0.05 (3)	1.33 ± 0.04 (3)	2.85 ± 0.15 (12)
ACh + Physo	0.78 ± 0.02 (3)	—	2.80 ± 0.19 (3)
Physo	0.73 ± 0.06 (3)	—	2.72 ± 0.19 (3)
DS	—	1.26 ± 0.05 (3)	2.91 ± 0.16 (6)
IS	—	1.20 ± 0.04 (3)	—

[a] All results are presented as micromoles per gram wet weight (means ± standard errors), with numbers of experiments shown in parentheses. ACh = 10^{-4} M acetylcholine, Physo = 10^{-4} M physostigmine, DS = direct stimulation, and IS = indirect stimulation.

The results with phosphatidylinositol resemble those seen by Larrabee and coworkers (13, 14) on the sympathetic ganglia. An increased incorporation of ^{32}P and ^{14}C into the phospholipids of the electroplax was observed irrespective of whether one stimulated the conducting membrane directly or via the nerve endings, which would indicate a site of action not exclusively localized to the synaptic region. In contrast, the increased incorporation observed previously (13, 14) appeared to be due exclusively to the transmitter, acetylcholine, acting on the postsynaptic cell. Acetylcholine acts only at the synapses of the electroplax; however, depolarization of these thousands of synapses causes a short-circuiting and depolarization of the entire conducting membrane.

The increased turnover of phosphatidylinositol in the electroplax, as a result of stimulation or the action of acetylcholine, is in agreement with suggestions that both excitation and the action of acetylcholine are mediated via an increase in phosphatidylinositol of polyphosphoinositide metabolism (6, 12). The increased turnover of phosphatidylcholine is of special interest in relationship to the proposal of Watkins (11) that acetylcholine, due to its structural similarity to the polar head group of phosphatidylcholine, may compete with this phospholipid for binding to a receptor protein. In fact, DeRobertis (37) suggested that the acetylcholine receptor of the electric organ is a proteolipid. If acetylcholine displaces phosphatidylcholine from a receptor proteolipid, the phospholipid may be more readily available to metabolic enzymes, thereby giving rise to the increased ^{32}P and ^{14}C incorporation values found in this study.

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ACKNOWLEDGMENTS AND ADDRESSES