

RAPIDLY-LABELLED, ACIDIC PHOSPHOLIPIDS OF THE GOLDFISH BRAIN¹

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Abstract—Homogenates and particulate fractions of goldfish brain incorporated radioactivity from γ -[³²P]ATP selectively into acidic phospholipids during brief periods of incubation. Phosphatidate and lysophosphatidate became strongly labelled and activity was also found in phosphatidyl inositol phosphate and in phosphatidyl inositol diphosphate. When tetraphenylborate (a K⁺-complexing agent) was added, a selective stimulation of incorporation of ³²P into phosphatidate occurred. The addition of perchlorate (also known to bind K⁺) did not produce a similar stimulation, nor did the addition of K⁺ block the stimulation by tetraphenylborate. The stimulation of the labelling of phospholipids by tetraphenylborate appeared to be the result of multiple actions. Besides the evidence that it acted by stimulating the phosphoinositide phosphodiesterase of brain, data were obtained suggesting that it stimulated diglyceride kinase and blocked endogenous destruction of ATP as well. The stimulation by tetraphenylborate was blocked by addition of atropine but not of arecoline.

THE PHOSPHOINOSITIDES, although ubiquitous in animal tissues, frequently exist in trace amounts demonstrable only by tracer methods. The greater content of phosphoinositides in excitable tissues (FOLCH-PI, 1949; DAWSON and DITTMER, 1961; BROCKERHOFF and BALLOU, 1962) and the evidence from labelling experiments of rapid turnover (DAWSON, 1954; HOKIN and HOKIN, 1958; ANSELL and SPANNER, 1959; LEBARON, KISTLER and HAUSER, 1960; HÖLZL and WAGNER, 1964; KFOURY and KERR, 1964; SEIFFERT and AGRANOFF, 1965) have suggested an important physiological role for these compounds in excitable tissues. Alterations of the labelling of phosphatidyl inositol (PhI) and phosphatidic acid (PhA) have been described in a variety of neural and secretory tissues in response to the appropriate neurohumor or secretagogue (cf. reviews by: HAWTHORNE, 1960; HOKIN and HOKIN, 1960; CUTHBERT, 1967). In sympathetic ganglia increased labelling has been correlated with synaptic transmission (LARRABEE and LEICHT, 1965; LARRABEE, 1968). Recently DURELL, GARLAND and FRIEDEL (1969) have suggested that such alterations are the result of activation of phosphatidyl inositol phosphodiesterase releasing diglyceride and inositol phosphate, but the detailed mechanism of the effect of acetylcholine remains speculative. The present experiments were undertaken to clarify the nature of these alterations in phospholipid labelling. The goldfish was selected because of the current interest in this laboratory in goldfish behaviour and its metabolic consequences. Various aspects of the labelling patterns of phospholipids in the goldfish brain, the effects of acetylcholine in goldfish brain, and the stimulation by tetraphenylborate of phospholipid labelling comprise the present report.

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Abbreviations used: PhA, phosphatidic acid; PhI, phosphatidyl inositol; PhIP, phosphatidyl inositol phosphate; PhIP₂, phosphatidyl inositol diphosphate; TPB, sodium tetraphenylborate.

MATERIALS AND METHODS

Preparation of tissue. Homogenates of 25 brains (wet wt. about 80 mg each) from common goldfish (*Carassius auratus*; body wt. 8.5–11 g) were prepared with 3 vol. of 0.25 M-sucrose and were used directly or as a source of subcellular fractions. After removal of cellular debris by centrifugation at 1000 g for 10 min, a crude mitochondrial fraction was obtained by centrifugation at 20,000 g for 10 min. The post-mitochondrial supernatant fraction was either used as such or a microsomal pellet was sedimented by centrifugation of this fraction at 100,000 g for 60 min. The resulting pellet was washed once and resuspended in 0.25 M-sucrose. In other studies, after removal of cellular debris, homogenates were frozen (in a dry ice-acetone bath) and thawed three times, and the material was sedimented at 100,000 g for 60 min, washed once, resuspended in 0.25 M-sucrose, and was used directly or stored frozen (particulate fraction). For experiments with brain minces, pooled brains of goldfish in Krebs-Ringer bicarbonate buffer (UMBREIT, BURRIS and STAUFFER, 1957) were minced finely with a scissors; 100–105 mg of tissue were blotted with Whatman 54 filter paper, weighed, and suspended in 1.86 ml of the buffer. For the regional studies, brains of five fish were dissected freehand into forebrain, tectum, tegmentum, cerebellum and vagal lobes. Each regional sample was divided into an experimental and control portion by sectioning in the median sagittal plane.

Incubation and extraction. All incubations were carried out at 20°C. Homogenates or subcellular particles from 60 mg of brain were incubated, with shaking, in a final vol. of 1.2 ml containing 33 mM-glycylglycine buffer (pH 7.4), 8.3 mM-NaF, 8.3 mM-MgCl₂ and 5 μ Ci of either ³²P_i (0.08 μ mol), γ -[³²P]ATP or γ -[³³P]ATP (0.1–0.2 μ mol). In experiments designed to label PhI, 0.1 mM-CTP and 0.1 mM-*myo*-inositol were also present.

Minces were incubated with 5.5 μ mol of inositol, 1.3 μ mol of glycerol, 11 μ mol of glucose, 5 μ Ci of ³²P_i and 1.86 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) in a total volume of 2 ml. In this case the final specific activity of the added ³²P_i was 2.26 μ Ci/ μ mol. Results are expressed either as the percentage of the initially added radioactivity that was incorporated into lipid or as nmols or pmols of lipid formed. In the latter instance, the amount of lipid formed was calculated from the specific activity of the γ -[³²P]ATP added. When ³²P_i was used, specific activity was calculated on the basis of the presence of 0.01 μ mol of P_i/mg brains and in experiments with Krebs-Ringer bicarbonate buffer, for the P_i present. Incubations were terminated with chloroform-methanol-6N-HCl (10:20:1, by vol.) and were extracted and washed as described previously (HAJRA, SEGUIN and AGRANOFF, 1968). Lipids were separated by TLC on oxalate-silica gel H plates (GONZALEZ-SASTRE and FOLCH-PI, 1968) developed with chloroform-methanol-4N-ammonia (3:3:1, by vol.). This effected good separations of phosphatidyl inositol diphosphate (PhIP₂), phosphatidyl inositol phosphate (PhIP) and PhI, but PhA and PhI were poorly resolved. Separation of the lipid on basic carbonate plates (SKIPSKI, PETERSON and BARCLAY, 1962) developed with chloroform-methanol-glacial acetic acid-water (50:25:7:3, by vol.), separated PhI from PhA, while poorly resolving PhIP from PhIP₂. By using both the oxalate and carbonate plates, each class of labelled lipids could be resolved and quantitated. The identity of the labelled lipids was confirmed by paper chromatography on formaldehyde-treated filter paper (HAJRA *et al.*, 1968) and by high-voltage electrophoresis (SEIFFERT and AGRANOFF, 1965) of their deacylated glycerophosphates (HÜBSCHER, HAWTHORNE and KEMP, 1960). The labelled regions were located by radioscanning or by autoradiography. The located spots were then cut out or scraped off and counted in 10 ml of toluene containing 5% (v/v) ethanol, 0.5% (w/v) PPO and 0.03% (w/v) dimethyl POPOP by liquid scintillation spectrometry. In all experiments reported, duplicate incubation tubes were analysed. Variability was generally less than 10 per cent, although considerable variation was found from preparation to preparation of goldfish brain as seen below.

ATP and P_i in the aqueous layer following lipid extraction of incubation mixtures were measured after high-voltage electrophoresis (HAJRA *et al.*, 1968). Protein was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) and phosphate by the method of CHEN, TORIBARA and WARNER (1956).

Materials. To obtain diglyceride, crude lecithin was prepared from eggs (HANAHAN, TURNER and JAYKO, 1951) and incubated at 37°C with *Cl. perfringens* phospholipase C (EC 3.1.4.3) (Sigma Chemical Co., St. Louis, Mo.; Schwartz Biochemicals, Brooklyn, N.Y.) in the presence of 0.15 M-tris buffer (pH 7.4) and 0.01 M-CaCl₂. The product was purified by solvent fractionation (LONG and MAGUIRE, 1954). For purposes of incubation, diglyceride was pipetted in chloroform-methanol (2:1, v/v), detergent was added, and the solvent was evaporated. The diglyceride-detergent mixture was emulsified in the incubation medium by sonication prior to the addition of enzyme. The compounds γ -[³²P]ATP and γ -[³³P]ATP were prepared enzymically (GLYNN and CHAPPELL, 1964). During the course of this work ³²P_i became commercially available and ³²P_i, ³³P_i and [³H]inositol were obtained from New England Nuclear Corp. (Boston, Mass.). Cytidinediphosphoryl[1,2-¹⁴C]choline was purchased from TracerLab (Boston, Mass.). Sodium tetraphenylborate, acetylcholine chloride, eserine (all from Sigma Chemical Co.), atropine (Pierce Chemicals, Rockford, Ill.), arecoline (Nutritional Biochemicals, Cleveland, Ohio) and carbamylcholine (Merck-Sharp and Dohme, West Point,

Pa.) were used as aqueous solutions. Arlacel 20, Arlex, Tween 20, Tween 40, Tween 80, Renex 698 and Myrj 52 (Atlas Chemical Co., Wilmington, Del.), Triton X-100 and Triton CF-54 (Rohm and Haas, Philadelphia, Pa.), Nonex (Union Carbide Corp., N.Y.) and Cutscum (Fisher Scientific, Pittsburg, Pa.) were added in benzene. Sodium dodecyl sulphate (Matheson, Coleman and Bell, Cincinnati, Ohio) and sodium deoxycholate (Mann Biochemicals, N.Y.) were added as aqueous solutions. Hexokinase (Boehringer and Söhne, Mannheim, Germany) was dialysed to remove $(\text{NH}_4)_2\text{SO}_4$.

RESULTS

Effect of cholinergic agents on labelling of lipids

Brain minces. The presence of 3×10^{-4} M- eserine and acetylcholine (10^{-3} M- 10^{-8} M) during a 2 h incubation invariably produced a 10-50 per cent increase in total lipid radioactive phosphorus. Eserine alone was also stimulatory, and there was no apparent concentration optimum for ACh. Carbamylcholine, added in the absence of eserine, produced a comparable stimulation, and although a clear concentration gradient could not be obtained, all the concentrations used (from 10^{-3} to 10^{-8} M) were stimulatory. The concentration of 5×10^{-5} M-carbamylcholine was selected for

TABLE 1.—EFFECT OF CARBAMYLCHOLINE ON INCORPORATION OF $^{32}\text{P}_i$ INTO PHOSPHOLIPIDS IN VARIOUS REGIONS OF GOLDFISH BRAIN

Region		Forebrain	Tectum	Tegmentum	Cerebellum	Vagal lobes
I	Wet wt./brain (mg)	11.4	15.3	17.3	13.4	31.4
	Control (nmols of ^{32}P lipid formed)	4.75	9.2	11.7	6.1	13.5
	+ Carbamylcholine 5×10^{-5} M (nmols of ^{32}P lipid formed)	5.5	10.0	13.1	9.6	15.9
	% Stimulation	15.7	8.7	11.5	57.4	18.1
II	Control (nmols of ^{32}P lipid formed)	4.1	7.55	9.5	5.35	11.6
	+ Carbamylcholine 5×10^{-5} M (nmols of ^{32}P lipid formed)	5.65	8.6	11.9	8.65	15.9
	% Stimulation	38.5	13.3	25.3	61.4	37.1

Five fish brains were subdivided by dissection into the designated regions and each sample was hemisected. Minces of half of each pooled region were incubated for 2 h in 2 ml of Krebs-Ringer bicarbonate buffer in the presence or absence of 5×10^{-5} M-carbamylcholine and $^{32}\text{P}_i$ as described in Methods.

further studies. Labelled lipid/mg of brain (Table 1) was relatively high in tegmentum and vagal lobes. Stimulation of labelling by carbamylcholine was greatest in cerebellum (60 per cent) followed by forebrain and vagal lobes (16-39 per cent), with the smallest effect in the tectum. When carbamylcholine was added to minced cerebellar preparations, increased labelling occurred during the course of a 4 h incubation. Stimulation of incorporation by carbamylcholine was greatest in PhI, with significant effects in PhA and PhIP as well, but relatively small increases in PhIP₂ and phosphatidyl choline (Fig. 1). When eserine and acetylcholine (10^{-4} M) were added to cerebellar preparations, labelling of lipids by radiophosphorus was stimulated by 14 to 35 per cent and the effect was blocked by pre incubation with 10^{-6} M-atropine. Atropine alone did not inhibit labelling at concentrations as high as 10^{-4} M.

Homogenates and subcellular particles. Numerous attempts were made to demonstrate stimulation of lipid labelling by acetyl- and carbamylcholine in broken cell preparations from whole brains under various conditions. Only a small degree of

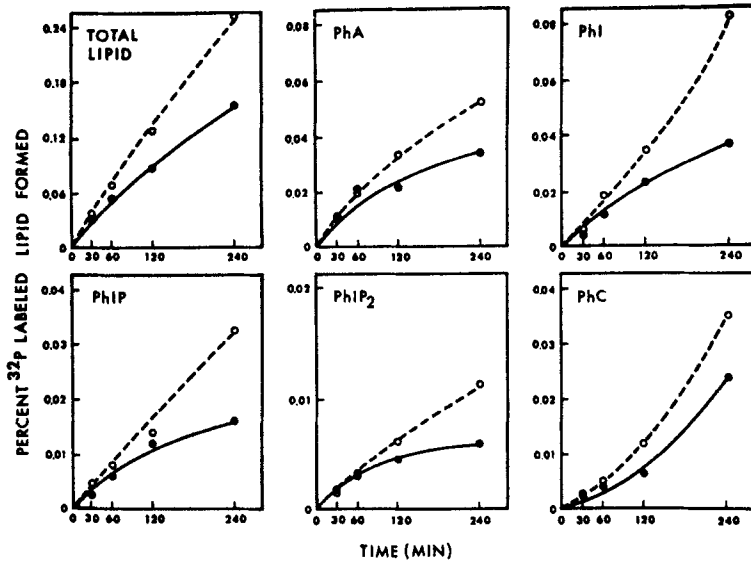


FIG. 1.—Labelling of individual lipids of goldfish brain in the presence or absence of 5×10^{-5} M-carbamylcholine as a function of time of incubation. Minced cerebellar hemispheres (33.5 mg) from five brains were incubated with $5 \mu\text{Ci}$ of $^{32}\text{P}_i$ ($1.03 \mu\text{mols}$) for the times indicated. Following extraction, a portion of the total lipid was counted. Lipids were then separated by TLC and located by autoradiography. Solid lines, controls; dashed lines, + carbamylcholine. PhC = phosphatidyl choline.

stimulation was occasionally found and examination of individual lipids indicated that none was more strikingly stimulated in the presence or absence of fluoride or acetylcholine than the total extract.

Composition of incubation medium

At pH 7.4, glycylglycine was less inhibitory to labelling than Krebs-Ringer bicarbonate, tris or triethanolamine buffers and was used in further experiments even though lower, more poorly buffered concentrations of glycylglycine yielded considerably higher incorporation. The addition of NaF produced a three-fold increase of $^{32}\text{P}_i$ incorporation into lipids in homogenates, while the addition of KF was slightly more stimulatory. CaCl_2 (2.1×10^{-4} M) was markedly inhibitory. Addition of potassium glutamate (5×10^{-4} M) or glutathione (5×10^{-4} M) was ineffective, and nicotinamide (10^{-3} M) was inhibitory. Additions of various nucleotides were without effect, except for AMP, which at 10^{-4} M enhanced incorporation of $^{32}\text{P}_i$ by about 10 per cent. Increasing the osmolarity of the medium with sucrose, KCl or NaCl proved to be inhibitory. These studies led to the choice of the standard incubation mixture already described in Methods. As previously observed in mitochondrial labelling systems (GALLIARD, MICHELL and HAWTHORNE, 1965; COLODZIN and KENNEDY, 1965; HAJRA *et al.*, 1968; KAI, SALWAY and HAWTHORNE, 1968), γ - ^{32}P ATP was a much better precursor of rapidly-labelled lipid than $^{32}\text{P}_i$. In a standard incubation mixture γ - ^{32}P ATP was somewhat superior to $^{32}\text{P}_i$, but after freezing and thawing, only γ - ^{32}P ATP could serve as a precursor, an observation in agreement with that reported by HAJRA *et al.* (1968).

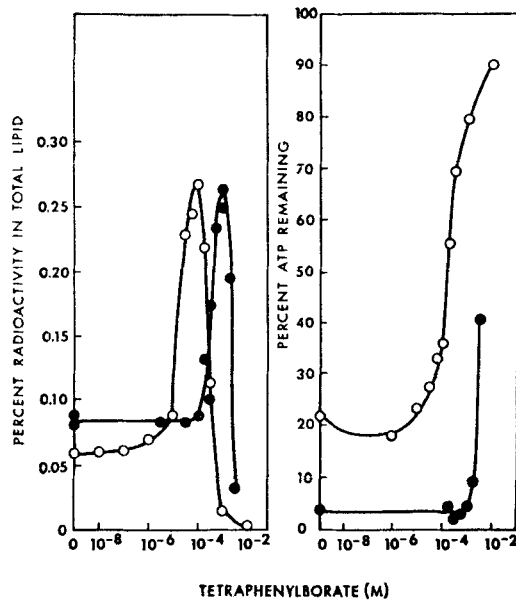


FIG. 2.—Effect of TPB on labelling of lipids and on hydrolysis of ATP in homogenates (●) (6.0 mg protein) or microsomal preparations (○) (0.32 mg protein) from goldfish brain. Samples were incubated for 15 min with varying final concentrations of TPB and 5 μ Ci of γ - 32 P]ATP (0.2 μ mol). (a) Percentage of added radioactivity incorporated into lipid; (b) Percentage of added radioactivity remaining in ATP.

Effects of sodium tetraphenylborate (TPB)

The addition of TPB caused a dramatic increase in the labelling of lipids of homogenates of goldfish brain by γ - 32 P]ATP, with a sharp optimum for TPB at 10^{-3} M (Fig. 2). A sharp optimum (10^{-4} M) was also seen in labelling experiments with microsomal preparations. In each case the TPB to protein ratio was approximately the same, about 0.25 μ mol of TPB/mg of protein. Clearly TPB also preserved ATP, particularly at concentrations which inhibited labelling of lipids. At 10^{-3} M, TPB stimulated labelling of lipids from γ - 32 P]ATP for all intervals of incubation of homogenates, mitochondria or post-mitochondrial supernatant fraction (Fig. 3). The cytosol (100,000 *g* supernatant) fraction was not labelled. Although labelling of mitochondrial lipids was markedly stimulated, mitochondria were not studied further because of the anticipated difficulty in assessing the role of endogenous ATP production, particularly in the presence of TPB, a known inhibitor of oxidative phosphorylation (UTSUMI and PACKER, 1967).

Identification of labelled lipids. The increased labelling of lipids in homogenates from γ - 32 P]ATP during a 15 min incubation with 10^{-3} M TPB was entirely due to a dramatic increase in labelling of PhA and an actual decrease in labelling of PhIP and PhIP₂ (Fig. 4). In other experiments in which CTP and inositol were added to homogenates (see Table 5, below), PhI was labelled, and this labelling was also decreased by addition of TPB. In order to investigate further the nature of the TPB stimulation, microsomes were pre incubated with 10^{-4} M-TPB for 2 min. In samples taken shortly after addition of γ - 32 P]ATP (Fig. 5), incorporation into lipids was

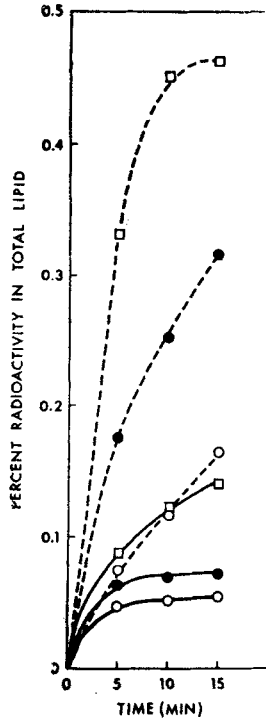


FIG. 3.—Labelling of lipids in various preparations from goldfish brain as a function of time of incubation. Homogenates (\square) (6.0 mg protein), mitochondria (\bullet) (1.9 mg protein) and the post-mitochondrial supernatant (\circ) (2.1 mg protein) were incubated with $5 \mu\text{Ci}$ of $\gamma\text{-}^{32}\text{P}\text{ATP}$ ($0.2 \mu\text{mol}$) for the times indicated. Solid lines, controls; dashed lines, +TPB, 10^{-3} M.

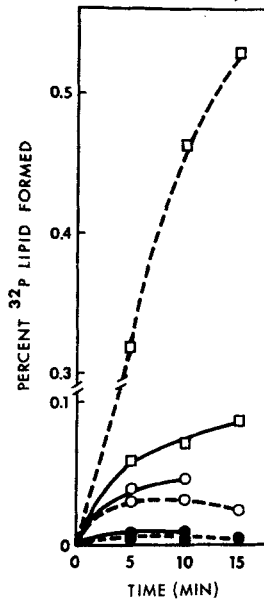


FIG. 4.—Labelling of individual lipids in the presence or absence of TPB as a function of time of incubation. Homogenates of goldfish brain were incubated with $5 \mu\text{Ci}$ of $\gamma\text{-}^{32}\text{P}\text{ATP}$ ($0.2 \mu\text{mol}$) for the times indicated. PhA (\square), PhIP (\circ), PhIP₂ (\bullet); solid lines, controls; dashed lines, +TPB, 10^{-3} M.

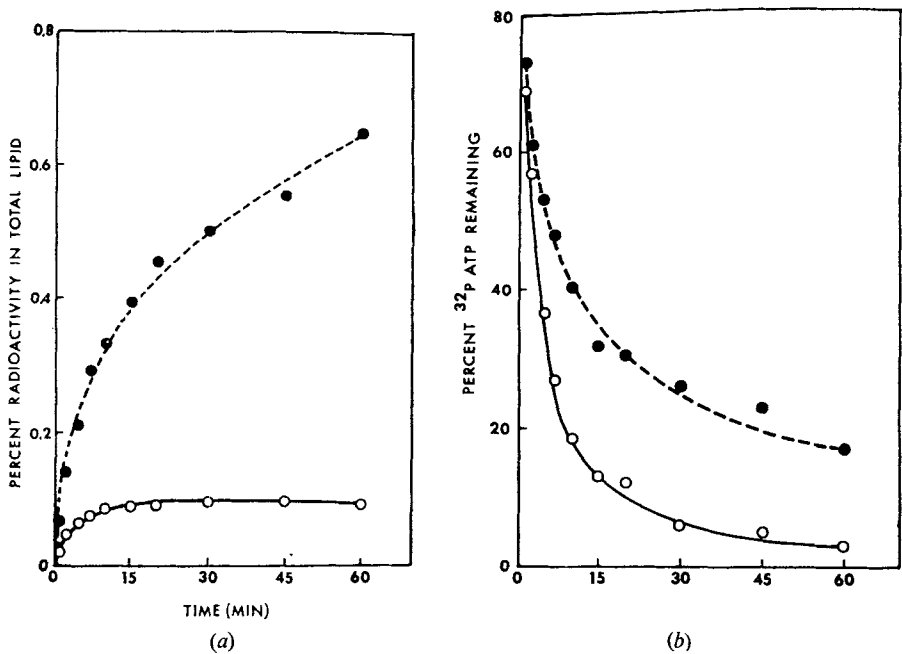
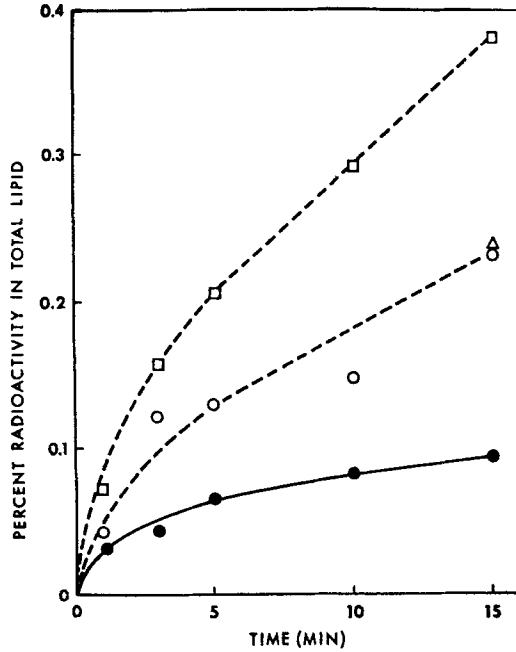


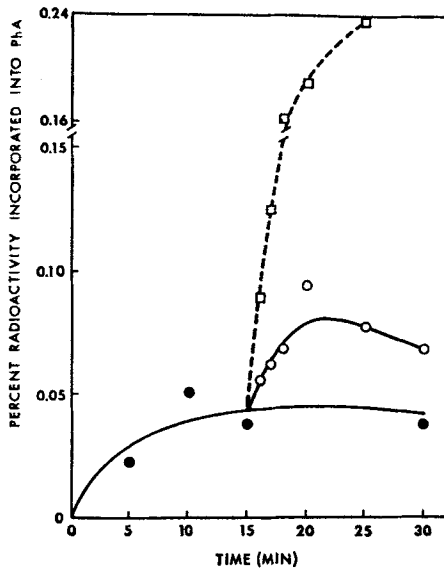
FIG. 5.—Labelling of lipids and hydrolysis of ATP in the presence or absence of TPB as a function of time of incubation. Microsomal preparations (0.32 mg protein) were incubated with 5 μCi of γ - ^{32}P ATP (0.1 μmol) for the times indicated. (a) Total lipids. (b) ATP and P_i were separated by electrophoresis at 4000 V on Whatman No. 1 paper, located by autoradiography and counted. Solid lines, control; dashed lines, +TPB.

stimulated, although little difference was yet evident in the amount of ATP remaining (Fig. 5). Labelling in the incubated controls appeared to have equilibrated by 10 min. If the TPB was added simultaneously with ATP with no preincubation, there was a smaller degree of stimulation (Fig. 6a). If after a 15 min incubation, TPB was added together with the supplemental γ - ^{32}P ATP, a rapid rise in the labelling of PhA occurred (Fig. 6b) without evidence of a lag period, which might have been expected had endogenous diglyceride been consumed during the first 15 min. Findings on total lipids were similar (HOLLANDER *et al.*, 1969), as were those in experiments with the particulate fraction incubated under similar conditions.

Effects of detergents, ions, and atropine. Further studies with the particulate preparation tested the possibility that TPB acted by release of endogenous diglyceride. Varying degrees of inhibition of the labelling of lipids were seen following addition to incubation mixtures of the nonionic detergents: preincubation mixture, 0.001–0.5 mg of Tween 20 or 0.25–0.5 mg of Tween 40, Tween 80, Triton X-100, Triton CF-54, Nonex, Renex 698 or Myrj 52. Arlex (0.25–0.5 mg) had no effect on lipid labelling but decreased the effect of TPB, while Arlacel 20 (0.05–0.5 mg) and Cutscum (0.05–0.5 mg) had no effect on either labelling or the TPB stimulation. An anionic detergent, sodium deoxycholate (2.5×10^{-3} M) markedly stimulated labelling and its effect was abolished by TPB. This inhibitory interaction of TPB and deoxycholate occurred over a wide range of concentrations for each substance. Sodium dodecyl



(a)



(b)

FIG. 6.—(a) Comparison of the effects of TPB present during pre incubation with those of TPB added simultaneously with ATP. Microsomal preparations from goldfish brain (0.37 mg protein) were pre incubated for 10 min in the presence or absence of 10⁻⁴ M-TPB and then incubated with 5 μCi of γ -[³²P]ATP (0.2 mol) for the times indicated. Solid line, control (●); dotted line, + TPB: (○) 10⁻⁴ M-TPB added with ATP; (□) 10⁻⁴ M-TPB added during pre incubation; (Δ) 10⁻⁴ M-TPB added with ATP without preincubation. (b) Effect of delayed addition of supplementary ATP with and without TPB. Microsomes (0.34 mg protein) were incubated with 5 μCi of γ -[³²P]ATP (0.1 μmol) (●). An additional 5 μCi of the ATP (○) or 5 μCi of the ATP and 0.12 μmol of TPB (□) were added at 15 min.

TABLE 2.—INFLUENCE OF ATROPINE AND ARECOLINE ON THE TETRAPHENYLBORATE ENHANCEMENT OF THE LABELLING OF PHOSPHOLIPIDS IN PARTICULATE PREPARATIONS FROM GOLDFISH BRAIN

Experiment	Preincubation treatment	% of radioactivity incorporated
I	—	0.055
	Atropine at -5 min	0.056
	Atropine at -5 min, TPB at -2 min	0.059
	TPB at -5 min, atropine at -2 min	0.230
	TPB at -5 min	0.242
II	—	0.101
	Arecoline at -5 min	0.086
	Arecoline at -5 min, TPB at -2 min	0.269
	TPB at -5 min, arecoline at -2 min	0.310
	TPB at -5 min	0.308

Particulate preparations (1.5 mg) were preincubated for 5 min and then incubated with 5 μ Ci of γ -[32 P]ATP (0.1 μ mol). Atropine (10^{-4} M), arecoline (10^{-4} M), or TPB (5×10^{-4} M) were added at the times indicated. In other experiments, TPB at -10 min and at -2 min had effects on labelling similar to those of TPB at -5 min above.

sulphate (10^{-3} M) produced a profound inhibition of labelling. Following preincubation of microsomal preparations with 10^{-4} M-TPB, the addition of 3 equivalents of KCl did not reverse the TPB-mediated increase of lipid labelling during the 60 min following the addition of γ -[32 P]ATP. The addition of an equimolar amount of choline chloride was also without effect. At 10^{-3} M, a known potassium complexing agent, sodium perchlorate, had no effect on labelling of lipids in homogenates, but higher concentrations inhibited labelling. Because of the reported cholinergic action of TPB (see Discussion below), the possibility that atropine might block the enhancement by TPB of lipid labelling was examined. The TPB effect was completely blocked by 10^{-4} M-atropine, but a similar concentration of arecoline had no effect (Table 2).

Addition of lipids extracted from TPB-stimulated preparations. If the TPB effect were mediated via the release of diglyceride from endogenous lipids, addition of lipids extracted from such incubations might stimulate labelling of lipids by ATP in the absence of TPB. Particulate preparations from goldfish brain in 10 times the usual amount were incubated with 5×10^{-4} M-TPB in the absence of ATP. In the control samples, TPB was added at the end of incubation just prior to extraction. Lipids were extracted from the two incubated samples and were chromatographed on a silica gel TLC plate with light petroleum-ether (70:30 v/v). The area corresponding to that of authentic diglyceride was scraped off and formed into a small column from which the lipid was eluted with chloroform-methanol (2:1 v/v). When a portion (20%) of the lipids purified from each incubation was then incubated with the particulate fraction in the presence or absence of TPB, labelling of lipids was markedly increased only when TPB was present during the final incubation (Table 3). The relatively marginal effect of the added purified lipid might reflect destruction during purification of the small amount of diglyceride released in the preparatory incubation or lesser accessibility for labelling of the added diglyceride than of that produced in membranes, e.g. at a site near diglyceride kinase. When diglyceride produced enzymically from egg lecithin was added (Table 3), labelling of lipids from ATP was enhanced in the samples incubated without TPB, but still not to the degree seen in the presence

TABLE 3.—THE EFFECT OF ADDITION OF EXOGENEOUS DIGLYCERIDE TO THE PARTICULATE FRACTION OF GOLDFISH BRAIN INCUBATED IN THE PRESENCE OR ABSENCE OF TETRAPHENYLBORATE

Added lipid	% of radioactivity incorporated into lipid	
	—TPB	+TPB
None	0.019	0.164
Incubated and extracted particulates	0.020	0.186
TPB-incubated and extracted particulates	0.026	0.195
Diglyceride (μg)		
1	0.018	0.166
5	0.018	0.253
10	0.022	0.209
50	0.024	0.298
100	0.039	0.423
200	0.026	0.481
400	0.033	0.327

See text for details and discussion. Lipids to be added were extracted in the usual way, with omission of HCl. The purified lipid was emulsified with 0.5 mg of Cutscum and incubated with particulate preparations (1.5 mg protein) for 15 min with 5 μCi of γ -[^{32}P]ATP (0.2 μmol).

TABLE 4.—EFFECTS OF PREINCUBATION OF THE PARTICULATE FRACTION FROM GOLDFISH BRAIN WITH PHOSPHOLIPASE C

Particulate preparation	Addition	% of radioactivity incorporated into lipid	
		—TPB	+TPB
No preincubation	—	0.066	0.247
Preincubated	—	0.052	—
	Phospholipase C	0.146	0.287
	Sodium dodecyl sulphate	0.027	0.007
Pre incubated with phospholipase C	—	0.269	0.602
	Phospholipase C	0.257	—
	Sodium dodecyl sulphate	0.415	0.792

A particulate preparation (10-fold; 8.6 mg of protein) was preincubated 20 min in the presence or absence of phospholipase C (1.0 mg) in a total vol. of 12 ml. The phospholipase C was then removed by centrifuging at 100,00 g for 60 min and the pellet was washed once and resuspended in 2.5 ml of 0.25 M-sucrose. The washed particulate fraction (0.25 ml) was then incubated for 15 min with 5 μCi of γ -[^{32}P]ATP (0.1 μmol) in the presence or absence of 5×10^{-4} M-TPB, 10^{-8} M-sodium dodecyl sulphate or 1.0 mg of phospholipase C in the usual 1.2 ml volume.

of TPB. The combined addition of diglyceride and TPB produced the highest degree of labelling of lipids.

Addition of phospholipase C. Since diglyceride obtained from egg lecithin stimulated the preparation, a further attempt was made to produce diglyceride *in situ* by adding phospholipase C to the preparation during a 20 min pre incubation (Table 4). The use of a particulate preparation permitted sedimentation of the particles and removal of the soluble phospholipase C after the pre incubation. After this pretreatment, the phospholipids were labelled from ATP to the same extent as that seen with TPB. Enhanced labelling also occurred when phospholipase C was added during incubation of previously untreated particulate preparations. Sodium dodecyl sulphate, which inhibited both the labelling of lipids and the effect of TPB, paradoxically stimulated the labelling of lipids in the presence or absence of TPB if the preparation had first been treated with phospholipase C.

TABLE 5.—EFFECT OF TETRAPHENYLBORATE ON PRELABELLED LIPIDS IN PREPARATIONS FROM GOLDFISH BRAIN

Preparation	Incubation time	Conditions	pmols of [³² P]lipid formed			
			PhA	PhI	PhIP	PhIP ₂
Particulates	10	—	60.6	28.8	63.4	10.4
	15	H + G* at +10 min	61.6	28.4	65.2	11.6
	30	H + G at +10 min†	73.4	39.2	58.4	13.4
	30	H + G at +10 min +TPB‡ at +15 min	80.4	37.6	45.6	12.2
	30	— †	79.8	43.2	58.4	12.6
	30	+TPB‡ at -2 min	1152.0	80.4	43.8	10.4
Homogenate	10	—	79.5	35.6	59.3	21.9
	15	H + G at +10 min	77.3	28.5	43.1	16.5
	30	H + G at +10 min	86.7	36.1	43.9	15.4
	30	H + G at +10 min +TPB§ at +15 min	73.5	22.1	14.0	4.6
	30	—	124.5	45.8	71.7	20.8
	30	+TPB§ at -2 min	284.9	12.4	6.2	3.7

Yields are based on the specific activity of the added ATP (10 μ Ci; 0.2 μ mol, for particulates; 5 μ Ci; 0.1 μ mol, for homogenates). Particulate preparations contained 1.5 mg of protein; homogenates, 6.0 mg of protein. ATP was added to the particulate fraction in divided doses at 0 and 5 min.

* Hexokinase, 60 μ g, and glucose, 40 μ moles.

† 0.1 ml of H₂O added instead of TPB.

‡ 0.6 μ mol of TPB in 0.1 ml.

§ 1.2 μ mol of TPB in 0.1 ml.

Addition of TPB to labelled lipids. Particulate preparations or homogenates of goldfish brain were preincubated with γ -[³²P]ATP for 10 min and the residual ATP was then destroyed by addition to each tube of glucose and hexokinase (Table 5). Upon addition of TPB and continued incubation, labelled PhA remained unchanged but radioactive PhIP and PhIP₂ underwent accelerated degradation, especially in homogenates. When preparations were incubated as in Table 5 but with 0.23 μ Ci of [³H]inositol (0.12 μ mol) plus ATP and varying amounts of TPB, incorporation of inositol was progressively inhibited with about 50 per cent reduction of incorporation at 2.5×10^{-4} M and 75 per cent inhibition at 10^{-3} M-TPB. In the presence of TPB, the incorporation into lipids of radioactivity from cytidinediphosphoryl[1,2-¹⁴C]-choline (0.1 μ Ci; 0.011 μ mol) was increased by 5 to 10 per cent. For the latter experiments, the particulate preparation was preincubated with TPB for 10 min, and an equimolar amount of unlabelled choline was added just before the addition of the labelled CDP-choline to prevent possible precipitation of the radioactive precursor with TPB.

DISCUSSION

The molecular mechanisms by which the neurohumoral actions of acetylcholine are mediated are poorly understood. One of the best known biochemical correlates of the action of acetylcholine is the stimulation of labelling of phospholipids in tissue slices (HOKIN and HOKIN, 1953), with PhA and PhI preferentially affected (CUTHBERT,

1967). This phenomenon is general for slices from neural and secretory tissues in response to the appropriate neurohumor or hormone (HAWTHORNE, 1960; HOKIN and HOKIN, 1960; CUTHBERT, 1967) and is generally believed to result from the enzymic release of diglyceride (DURELL *et al.*, 1969). The enhanced labelling of PhI in cat sympathetic ganglia (but not in nerve trunks) during perfusion with acetylcholine and during physiological or electrical preganglionic stimulation, contrasted with the absence of such enhancement in antidromically stimulated or curarized ganglia, provides further evidence for a relationship between the stimulation of lipid labelling and the physiological action of acetylcholine (LARRABEE and LEICHT, 1965; LARRABEE, 1968).

Whatever the physiological role of acetylcholine may be in the central nervous system, its effect on labelling of brain lipids is pronounced, and brain is the only tissue in which this stimulation has been seen in subcellular preparations (HOKIN and HOKIN, 1958; DURELL and SODD, 1964). In our experiments, carbamylcholine stimulated labelling of lipids in minces of goldfish brain and the highest enhancement was found in cerebellum, in contrast to the relative distribution among regions of the guinea pig brain (HOKIN, HOKIN and SHELPS, 1960). This may be a species difference (SILVER, 1967). In other species, subcellular fractions were less dramatically stimulated by acetylcholine or by carbamylcholine (HOKIN and HOKIN, 1958; REDMAN and HOKIN, 1964; DURELL and SODD, 1964). We found a marginal effect with these agents, but the use of an unusual cholinergic drug, TPB, resulted in striking effects in all subcellular fractions examined. From our pharmacological evidence, a possible cholinergic basis for its effect on lipid labelling is suggested.

Sodium TPB was initially introduced as an analytical reagent because it forms insoluble salts with potassium, ammonium and choline ions but does not precipitate with sodium, lithium or divalent cations (FLASCHKA and BARNARD, 1960). TPB has been used to precipitate catecholamines for chromatographic analysis (HAUPTMANN and WINTER, 1966). Dilute solutions of TPB dissociate cells (RAPPAPORT, 1966), cause mitochondria to swell (HARRIS and LEONE, 1966), uncouple oxidative phosphorylation (UTSUMI and PACKER, 1967), inhibit light-induced uptake of monovalent cations by chloroplasts (HORTON and PACKER, 1968), and inhibit Mg^{2+} -activated ATPase in the thyroid gland (STANBURY and WICKEN, 1969). Moreover TPB depolarizes neuromuscular junctions and causes a twitch in phrenic nerve-diaphragm preparations (GUIDERI, SEIFTER and SEIFTER, 1968). We propose that TPB produces a specific enhancement of the activity of phosphoinositide phosphodiesterase. It also prevents ATP hydrolysis and probably stimulates diglyceride kinase. The mechanism of the TPB effect may be unrelated to its reaction with potassium. Other biological effects of TPB appear to be independent of potassium-binding (UTSUMI and PACKER, 1967; STANBURY and WICKEN, 1969). The TPB effect reported here cannot be reproduced by the K^+ -complexing agent, sodium perchlorate, and the addition of K^+ has no effect when added to a preparation preincubated with TPB.

In brain, phosphoinositide phosphodiesterase (THOMPSON and DAWSON, 1964) cleaves PhIP and PhIP₂ to diglyceride, inositol diphosphate and inositol triphosphate, respectively. Initially this enzyme was reported not to act on PhI but recent studies (THOMPSON, 1967) suggest that it may do so by releasing inositol monophosphate and diglyceride. Release of inositol monophosphate from PhI has been reported in guinea pig brain preparation (FRIEDEL, BROWN and DURELL, 1967). Specific brain enzymes act on phosphoinositides and phosphatidate to release diglyceride, whereas

degradation of other phospholipids is mediated primarily via deacylation (ANSELL and HAWTHORNE, 1964). In animal tissues, enzymic activity that liberates diglyceride from other phospholipids (phospholipase C, lecithinase; BARKHOLZ, ROITMAN and GATT, 1966) is probably relatively minor. The release of diglyceride from the inositides has another unique aspect. Like other phospholipids, phosphoinositides have a characteristic fatty acid distribution with saturation of the fatty acid in the 1' position of glycerol and unsaturation at the 2' position, but additional specificity in phosphoinositides exists since the 1' position is occupied predominantly by stearate and the 2' position contains over 60 per cent arachidonate (SVENNERHOLM, 1968). Thus phosphodiesterase may play a key role in physiological processes that mediate the availability of the essential fatty acid, arachidonate, in brain. The enzyme is activated by cationic 'amphipathic' substances, such as cetyltrimethylammonium bromide, and inhibited by anionic detergents, including sodium dodecyl sulphate (THOMPSON and DAWSON, 1964). TPB is not a conventional detergent but it is anionic and might be expected to inhibit rather than to stimulate the phosphodiesterase. Therefore TPB does not appear to produce its effect via a detergent action. Predictably from the proposed mechanism of the TPB stimulation, preincubation of preparations from goldfish brain with sodium dodecyl sulphate profoundly inhibits lipid labelling in the presence or in the absence of TPB (Table 4). When diglyceride is furnished, sodium dodecyl sulphate is no longer inhibitory, and a significant increase in labelling is seen.

Labelling of PhA by γ -[^{32}P]ATP is presumably mediated via diglyceride kinase (HOKIN and HOKIN, 1959). Possible mechanisms of action of TPB include: (1) a blocking of the endogeneous destruction of ATP, (2) a stimulation of diglyceride kinase, (3) an increase in available diglyceride from inositides (hydrolysis in the large endogeneous pool of PhA itself by PhA phosphohydrolase could also produce diglyceride and increase labelling) or (4) a combination of the above possibilities. TPB delayed hydrolysis of ATP in our preparations (Fig. 5b) but this alone could not explain the stimulation of lipid labelling. The effect of TPB was evident within 1 min, before substantial differences in the concentration of ATP were evident. There was no lag period in the stimulation by TPB (Fig. 5a). Preservation of ATP by TPB was greatest at concentrations inhibitory for labelling of lipids (Fig. 2). The addition of supplemental ATP alone to microsomal fractions produced only minimal increases in labelling of PhA (Fig. 6b). Therefore TPB does not appear to affect labelling of lipids solely via its effect on levels of ATP. By contrast, sequential addition of labelled ATP and TPB after preincubation without TPB (Fig. 6b) did markedly increase the labelling of PhA, a finding suggesting either that endogenous diglyceride was limiting and TPB increased its availability, or that TPB preserved ATP and stimulated diglyceride kinase as well. It is possible that TPB reacted slowly with an enzyme catalysing a rate limiting step, for example, diglyceride kinase, a phosphodiesterase, an acylase leading to diglyceride synthesis or inhibition of PhA phosphohydrolase. The decreased label in the inositides in the presence of TPB in contrast to the stimulation of PhA, suggests that the former could be a source of diglyceride.

If stimulation of diglyceride kinase alone accounted for the TPB effect, we would have anticipated rapid initial labelling of PhA and little change in its final level (unless ATP or diglyceride were being consumed by competing reactions). The maximum level of labelling observed in control incubations did not approach that seen with TPB (Figs. 3-5). Since degradation of most of the added ATP was indeed

occurring by unrelated reactions and this was inhibited by TPB (Fig. 5b), stimulation of diglyceride kinase could not be ruled out. The stability of prelabelled PhA (Table 5) indicates that TPB does not stimulate PhA phosphohydrolase. Furthermore, the presence of magnesium and fluoride should completely inhibit this enzyme (COLEMAN and HÜBSCHER, 1962). The stimulation by TPB of the labelling of PhA is thus attributable to multiple actions including the release of diglyceride from the inositides through the action of phosphoinositide phosphodiesterase and a probable enhancement of diglyceride kinase activity. Conservation of ATP, particularly at longer incubation times, also plays a role.

The differences in labelling of lipids in homogenates and in particulate preparations treated with TPB after trapping of ATP are compatible with present knowledge of subcellular distribution of enzymes in the guinea pig brain. PhIP kinase, phosphoinositide phosphodiesterase, PhIP₂ monoesterase, PhIP monoesterase, and PhI phosphodiesterase are primarily in the cytosol, while diglyceride kinase, PhI kinase and CDP-diglyceride:PhA transferase are particulate (HARWOOD and HAWTHORNE, 1969). Our present experiments with preparations of goldfish brain are consistent, since some phosphoinositide phosphodiesterase activity persisted in the particulate after fractionation, although as expected, larger effects on labelling of phosphoinositides are seen in homogenates.

The proposed effect of TPB on phosphoinositide phosphodiesterase might be the consequence of TPB binding guanidinium groups (FLASCHKA and BARNARD, 1960) either on the phosphodiesterase itself or on other membrane proteins. In contrast to other cholinergic agents the dramatic effects of TPB on subcellular fractions may be attributable to secondary effects of TPB on ATP and on diglyceride kinase which would tend to amplify its effect on the phosphoinositide phosphodiesterase. It is less likely that these other effects are related to its cholinergic action. In the guinea pig, acetylcholine does not affect diglyceride kinase (HOKIN and HOKIN, 1959) and in a number of studies it does not spare ATP. The striking block of the TPB effect by atropine might be interpreted as evidence for an interaction of TPB with a cholinergic receptor or alternatively as precipitation of the tertiary amine with TPB. No visible precipitate is seen upon mixing TPB and atropine at 10^{-4} M and further, a control experiment was performed with arecoline, another tertiary amine which shares the methylpiperidine structure with atropine but which differs from atropine pharmacologically in being a parasympathomimetic agent while atropine is parasympatholytic. The cholinergic action of TPB previously reported (GUIDERI *et al.*, 1968) and these effects of drugs on labelling of lipids indicate a possible cholinergic role for TPB.

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