TETRAPHENYLBORATE STIMULATION OF PHOSPHOLIPID LABELING IN GOLDFISH BRAIN

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Summary

The presence of tetraphenylborate (TPB, 10^{-3} M to 10^{-4} M) in goldfish brain particulate preparations during a 5-15 min incubation with γ-^{32}P-ATP or γ-^{33}P-ATP results in marked stimulation of labeling of phosphatidate and a decrease in labeling of phosphatidyl inositol phosphate. Incorporation into phosphatidyl inositol diphosphate, a minor labeled phospholipid, is also depressed by TPB. We suggest the TPB effect is mediated by stimulation of a phosphodiesterase releasing diglyceride from phospholipid, which then reacts with labeled ATP, catalyzed by diglyceride kinase.

Sodium tetraphenylborate (TPB) forms insoluble salts with potassium, ammonium and choline ions[1]. Dilute solutions dissociate cells[2], swell mitochondria[3], uncouple oxidative phosphorylation[4] and inhibit light-induced uptake of monovalent cations by chloroplasts[5]. We report here that this unusual agent profoundly alters phospholipid labeling in goldfish brain preparations.

Methods. Goldfish brain homogenates (1:3, in 0.25 M sucrose) were used directly or as a source of microsomal fraction. Following removal of cellular debris at 1000xg for 10 min, mitochondria and other large particles were sedimented by centrifugation at 20,000xg for 10 min, and the microsomal pellet was obtained by centrifugation of the supernatant fraction at 100,000xg for 60 min.

Incubations were at 20° in 1.2 ml containing 33 mM glycylglycine buffer (pH 7.4), 8.3 mM NaF, 8.3 mM $MgCl_2$, γ-^{32}P-ATP or γ-^{33}P-ATP (5 μC, 0.1-0.2 μmoles) and homogenate or particles from 60 mg of brain. When it was desired to label phosphatidyl inositol (PhI), 0.1 mM CTP and 0.1 mM myo-inositol were added. TPB, Na (Sigma) was added in water.

The reaction was stopped with chloroform-methanol-HCl, extracted and washed as described previously[6]. Individual lipids were separated by thin layer chromatography on oxalate-silica gel H plates[7]. Plates were developed with a modified solvent, chloroform-methanol-4N ammonia (3:3:1) which gave good separation of phosphatidyl inositol diphosphate ($PhIP_2$), phosphatidyl inositol phosphate (PhIP) and PhI, but poor resolution of phosphatidate (PhA) from PhI. A second separation of the lipid on basic carbonate plates[8], developed with chloroform-methanol-glacial acetic acid-water (50:25:7:3), separated PhI from PhA while poorly resolving PhIP from $PhIP_2$. The identity of labeled lipids was confirmed by paper chromatography on formaldehyde-treated filter paper[6], and by high voltage electrophoresis of their deacylated glycerophosphates[6]. The labeled regions were located by scanning the paper or by autoradiography of the thin layer plates. The spots were then cut out or scraped and counted by liquid scintillation. ATP in the aqueous layer was measured following high voltage electrophoresis.

Results and Discussion. PhA and PhIP are the major radioactive lipids. PhI is labeled when CTP and inositol are supplied to homogenates or to mixed particles, but not with microsomes. The phosphatidate-cytidyl transferase in goldfish brain may be mitochondrial as observed in chick brain[9]. $PhIP_2$ was only slightly labeled in these experiments, and then only in whole homogenates, presumably since PhIP kinase is in the supernatant fraction[10]. Some labeling of lysophosphatidate is also seen, and there are in addition two faintly labeled unidentified spots which migrate between $PhIP_2$ and PhIP on the oxalate plates. Deletion of NaF decreases labeling to one-third.

TPB stimulates labeling of lipid, with a narrow range of optimal concentration (Fig. 1). TPB also appears to preserve ATP, which is destroyed during incubation at a much greater rate than can be accounted for by the lipid labeling. TPB conserves ATP even better at concen-

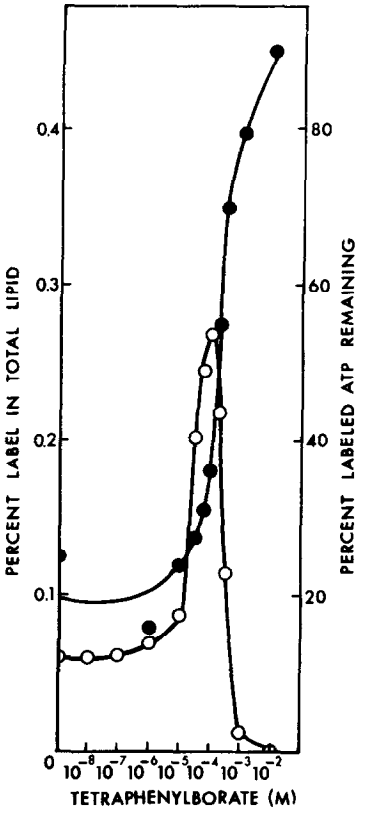

Fig. 1. Effect of TPB on lipid labeling and ATP hydrolysis. Microsomal preparations (0.65 mg protein) were incubated 15 min in varying final concentrations of TPB and 5 μC of γ-^{33}P-ATP (0.2 μmoles ATP). Lipid (○), ATP (●).

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trations that are extremely inhibitory for lipid labeling, as discussed below. The increased incorporation of radioactivity is due to increased labeling of PhA. PhIP and $PhIP_2$ are actually decreased (Fig. 2). The labeling is presumed to be due to the action of diglyceride kinase[11]. The increase in labeled PhA may be due to 1) preservation of ATP, 2) stimulation of diglyceride kinase, 3) increase in precursor diglyceride or 4) stimulation of phosphatidate phosphohydrolase, which, by acting on a large pool of endogenous PhA, could produce diglyceride and yet increase labeling of PhA.

TPB prevents hydrolysis of ATP, but its effect is greatest at

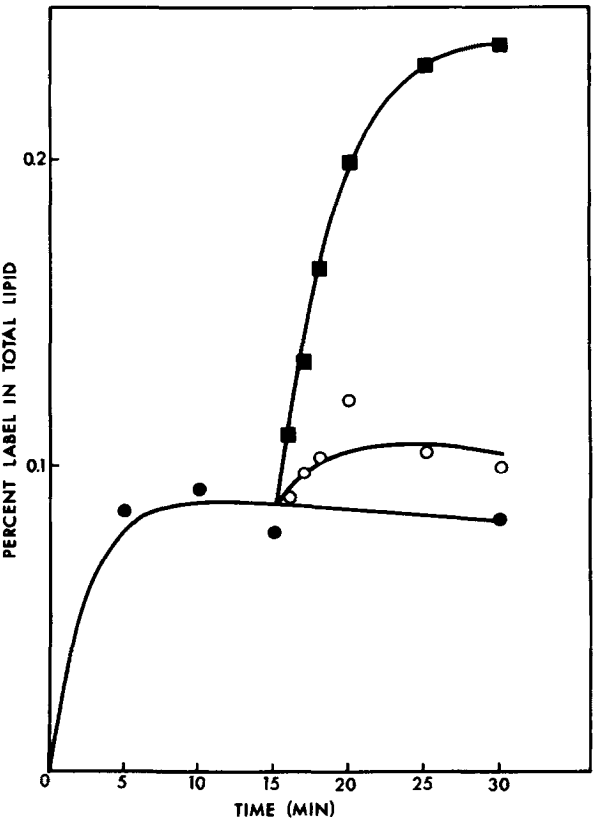

Fig. 2. Effect of delayed addition of supplementary ATP with and without TPB. Microsomes (0.69 mg protein) were incubated with 5 μC (0.1 μmole) of γ-^{33}P-ATP for 30 min (●). To other incubation mixtures, 5 μC of the ATP (○), or 5 μC of the ATP and 0.12 μmole of TPB (■) were added at 15 min.

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concentrations inhibitory for lipid labeling. Also, in other experiments, we have found that time courses of lipid labeling show early marked stimulation by TPB at a time when little difference in ATP concentration is evident. From Fig. 3 it can further be seen that ATP alone does not increase phospholipid labeling. TPB does not therefore appear to exert its effect on lipid labeling via the demonstrated effect on ATP levels.

If stimulation of diglyceride kinase accounted for the TPB stimulation, we would anticipate more rapid initial labeling of phosphatidate with TPB, but little change in the final level (unless precursor ATP or

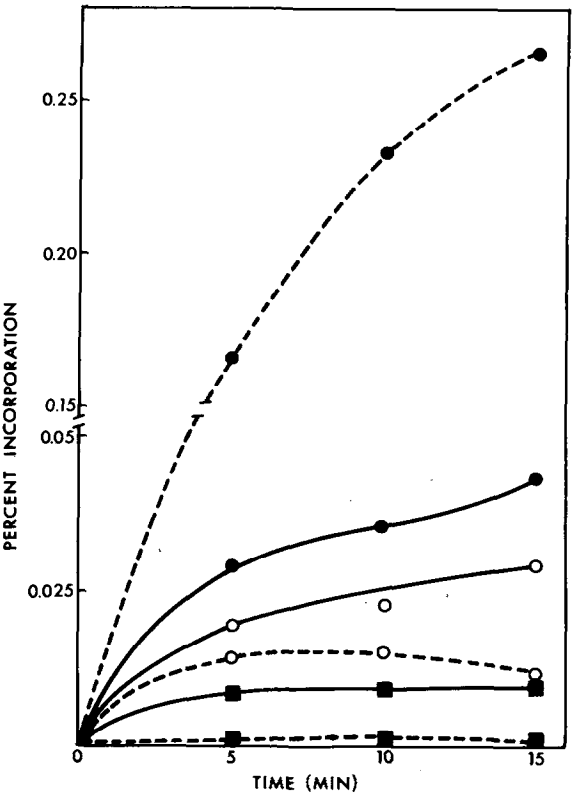

Fig. 3. Time course of labeling of individual lipids in the presence or absence of 10^{-3} M TPB. Homogenates were incubated with 5 μC of γ-^{32}P-ATP (0.2 μmole) for the times indicated. Following extraction, lipids were separated by thin layer chromatography and located by autoradiography on X-Ray film for 12-48 hrs. Radioactive spots were counted as described. PhA (●), PhIP (○), $PhIP_2$ (■), solid lines - controls, dashed lines - TPB.

diglyceride was being consumed by a competing reaction). Fig. 2 shows that the highest level of labeling observed in control experiments did not approach that seen with TPB.

Sequential addition of labeled ATP (with or without TPB) after preincubation without TPB, indicated that endogenous diglyceride is limiting and its increased availability can explain the TPB effect. Increased labeling in the presence of TPB and leveling off following a new addition of ATP support this possibility. Fig. 2 suggests that PhIP could be a source of diglyceride. This possibility was tested in

experiments with homogenates in which lipids were prelabeled and remaining ATP was trapped by hexokinase and glucose (Table I). TPB markedly decreases the amounts of prelabeled PhIP and $PhIP_2$ with slight decrease in PhI. By contrast, PhA is unchanged. TPB may activate a phosphoinositide phosphodiesterase and thus increase the available diglyceride. Such an enzyme is known[12]. Diglyceride could come from more prevalent but unlabeled brain phospholipids, such as phosphatidyl ethanolamine, phosphatidyl choline, etc. This is unlikely, since phospho-

Table I

Effect of tetraphenylborate on prelabeled lipids of goldfish brain homogenates

Exp	Time (Min)	Treatment	PhA (pmoles)	PhI (pmoles)	PhIP (pmoles)	$PhIP_2$ (pmoles)
1	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, -2 Min (pre)	284.9	12.4	6.2	3.7
2	30	†	81.3	84.0	102.9	23.1
	30	+TPB, -2 Min (pre)	237.6	31.5	15.3	3.0
	30	-CTP, -Inositol†	197.1	37.5	134.7	18.6
	30	-CTP, -Inositol +TPB, -2 Min (pre)	364.8	25.8	22.5	2.7

Yields are based on the specific activity of the added ATP (5 μC, 0.1 μmole).
*Hexokinase, 60 μg Boehringer-Mannheim, dialyzed to remove $(NH_4)_2SO_4$ and glucose, 40 μmoles.
†0.1 ml H_2O added instead of TPB.
**0.12 μmole TPB in 0.1 ml.

lipase C is probably absent from animal tissues[13].

The results in Fig. 2 as well as those in Table I indicate that TPB does not stimulate phosphatidate phosphohydrolase. Furthermore, the presence of magnesium and fluoride should completely inhibit this activity[14].

The mechanism of the TPB effect may not be related to its reaction with potassium. Other biological effects of TPB are also regarded as independent of potassium-binding[4]. We have not completely ruled out the possible activation of diglyceride kinase by TPB. Stimulation of the kinase or of a phosphodiesterase by TPB could result from reaction with guanidinium groups[1].

Of heuristic value is the possibility that TPB may be a cholinergic agent. Acetylcholine has long been implicated in stimulation of phosphatidate and PhI labeling by phosphate[15,16]. Available diglyceride may mediate this effect in avian salt gland and in guinea pig nerve endings[17]. ^{3}H Inositol-prelabeled inositides may release inositol phosphates after incubation with acetylcholine[17], suggesting that degradation of inositol phosphatide by phosphodiesterase to produce diglyceride is related to the acetylcholine effect. We interpret this data to suggest that PhIP can be the major source of the water-soluble inositol phosphates and diglyceride.

The similarities of action on lipid labeling of TPB and acetylcholine may be of interest in view of the recent report that TPB depolarizes neuromuscular junctions and causes a twitch in phrenic nerve diaphragm preparations[18].

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