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# SHORT COMMUNICATION

## Phospholipid labelling by [$^{32}$P]-orthophosphate and [$^{3}$H]-myo-inositol in the stimulated goldfish brain *in vivo*[1]

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THE RAPID turnover of phosphatidate (PhA) and phosphatidyl inositol (PhI) in neural tissue has been related to synaptic function by a number of observations. Increased incorporation of labelled phosphate into these lipids has been reported when cholinergic agents are added to incubated slices of brain (BROSSARD and QUASTEL, 1963; HOKIN and HOKIN, 1955), to homogenates (REDMAN and HOKIN, 1964; DURELL and SODD, 1964), and to synaptic nerve-ending preparations (SCHACHT and AGRANOFF, 1972). Stimulation of labelling of PhI and PhA has also been reported in brain slices (PUMPHREY, 1969) and in excised sympathetic ganglia (LARRABEE and LEICHT, 1965) that have been stimulated electrically. Although these findings suggest that functional activity in the intact brain could affect phospholipid turnover, there have been few studies *in vivo*. In the present paper, we report the effect of pentylenetetrazol and electrically-produced convulsions on incorporation of [$^{32}$P]-orthophosphate and [$^{3}$H]-*myo*-inositol into brain phospholipids at various times following injection of isotope.

### METHODS

Common goldfish (*Carassius auratus*) weighing approximately 10 g (brain weight, 80–100 mg) were obtained from Ozark Fisheries (Stoutland, Mo.). Isotopic compounds were injected intracranially as described previously (AGRANOFF and KLINGER, 1964). Brains were removed following spinal section and were rapidly rinsed with isotonic saline and frozen on dry-ice. They were homogenized in chloroform-methanol-HCl (HAJRA, SEGUIN and AGRANOFF, 1968) and the extracted lipids were separated by thin-layer chromatography (HOLLANDER, HALLENBECK and AGRANOFF, 1970). Radioactive lipids were located following radioautography and were subsequently scraped from the TLC plate and counted by liquid scintillation spectrometry. When [$^{3}$H] and [$^{32}$P] incorporation were measured in the same experiment, lipids were oxidized in a Packard Tri-Carb Sample Oxidizer and the recovered $^{3}$HOH was counted separately.

### RESULTS

*Time course.* Studies on the basal incorporation of $^{32}P_i$ into the aqueous HCl-soluble fraction and into various phospholipids demonstrated that about 15 per cent of the injected $^{32}P_i$ entered the brain in 2·5 min and that by 15–30 min, 30 per cent of the injected dose (5·8 $\times$ $10^6$ c.p.m.) was recovered in the brain (Fig. 1a). The initial rate of incorporation of $^{32}P_i$ into lipids was greatest for PhA, followed in descending order by PhIP, $PhIP_2$ and PhI. Significant incorporation into phosphatidyl choline (PhC) was detected only after 15 min and into phosphatidyl ethanolamine (PhE) after 30 min. [$^{3}$H]-*Myo*-inositol (Fig. 2) entered the brain less efficiently than $^{32}P_i$ and was present in the lipid fraction primarily as PhI. Some labelled PhIP and $PhIP_2$ were seen at later times. Radioactivity in the aqueous, HCl-soluble fraction was > 98 per cent free inositol.

*Effects of Pentylenetetrazol (PTZ) or Electroconvulsive shock (ECS).* Experimental animals received 1·5 $\mu$g of PTZ (Metrazol, Knoll Pharmaceuticals) intraperitoneally 5 min after injection of $^{32}P_i$ or [$^{3}$H] *myo*-inositol intracranially. Convulsions started 1–3 min after the PTZ injection and lasted 2–5 min. Incorporation of precursor into each lipid was expressed as a percentage of that for the control group (Figs. 1B and 2B). With both $^{32}P_i$ and [$^{3}$H] *myo*-inositol, no statistically significant changes in the aqueous, HCl-soluble fraction radioactivity resulted from the PTZ injection. There was, however, a marked stimulation of incorporation of $^{32}P_i$ into PhI, PhA and $PhIP_2$ ($P < 0·01$) at the first time

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*Abbreviations used*: PhA, phosphatidic acid; PhC, phosphatidyl choline, PhE, phosphatidyl ethanolamine; PhI, phosphatidyl inositol; PhIP, phosphatidyl inositol phosphate; $PhIP_2$, phosphatidyl inositol diphosphate; ECS, electroconvulsive shock; PTZ, pentylenetetrazol.

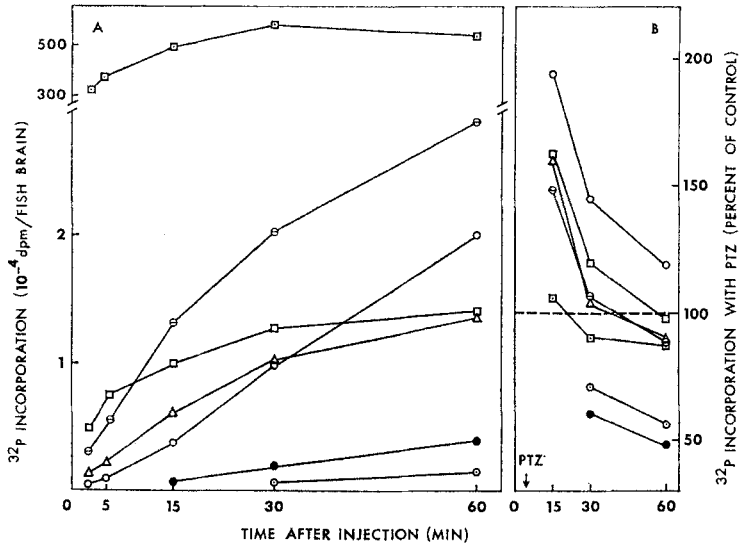


FIG. 1.—(a) Incorporation of $^{32}P_i$ into phospholipids and aqueous, HCl-soluble fraction as a function of time after the fish received 10 $\mu$l of $H_3\,^{32}PO_4$ (carrier-free, neutralized with $NaHCO_3$) and 10 $\mu$Ci (7·5 $\mu$mol) of [$^3$H]-*myo*-inositol intracranially in a volume of 10 $\mu$l. At the times indicated, fish were sacrificed and lipids were extracted as described in Methods. Each point represents the mean of 3 groups of 4 pooled fish brains.

(b) Stimulation of labelling as a function of time after PTZ treatment. Fish were treated as in controls (in *A*) but received 1·5 mg of PTZ injected in 10 $\mu$l intraperitoneally at 5 min after the intracranial injection of radioisotope. Statistical significance of the stimulation of labelling of PhA, PhI, PhIP and $PhIP_2$ at 15 min: $P < 0{\cdot}01$; at 60 min: PhA, PhIP and $PhIP_2$: N.S.; PhI: $P < 0{\cdot}025$. *Symbols*: —⊡—, aqueous HCl-soluble fraction; — □ —, PhA; — ○ —, PhI; — ⊖ —, PhIP; — △ —, $PhIP_2$; — ● —, PhC; — ⊙ —, PhE.

point measured (10 min after PTZ injection and 2–4 min after convulsions had ceased). The magnitude of this effect decreased rapidly with time, and by 60 min, it remained significant only for PhI ($P < 0{\cdot}025$). Contrary to the labelling observed in these acidic phospholipids, the incorporation of $^{32}P$

TABLE 1.—INFLUENCE OF ELECTROCONVULSIVE SHOCK ON LIPID LABELLING

| Fraction | $10^{-3}$ d.p.m. of $^{32}$P/fish brain | | % change |
|---|---|---|---|
| | Control | ECS | |
| Acid-soluble | 3780 ± 380 | 3730 ± 580 | N.S. |
| Total lipid extract | 31 ± 3·40 | 35·7 ± 7·8 | N.S. |
| PhA | 9·7 ± 0·60 | 12·3 ± 1·50 | +27* |
| PhI | 3·8 ± 0·30 | 5·0 ± 0·60 | +32* |
| PhIP | 11·2 ± 0·70 | 10·7 ± 0·90 | N.S. |
| $PhIP_2$ | 5·3 ± 0·50 | 6·1 ± 0·20 | +15† |

* $P < 0{\cdot}02$.

† $P < 0{\cdot}05$,

Fish were injected intracranially with 10 $\mu$Ci of carrier-free $^{32}P_i$, and experimental animals received ECS 10 min later (10 mA, 60 Hz, 1 s). Control and shocked fish were sacrificed at 5 min thereafter and lipids were extracted as described in Methods. Values represent the means ±S.D. of radioactivity for 16 fish, analysed in groups of 4.

N.S. = No significant change.

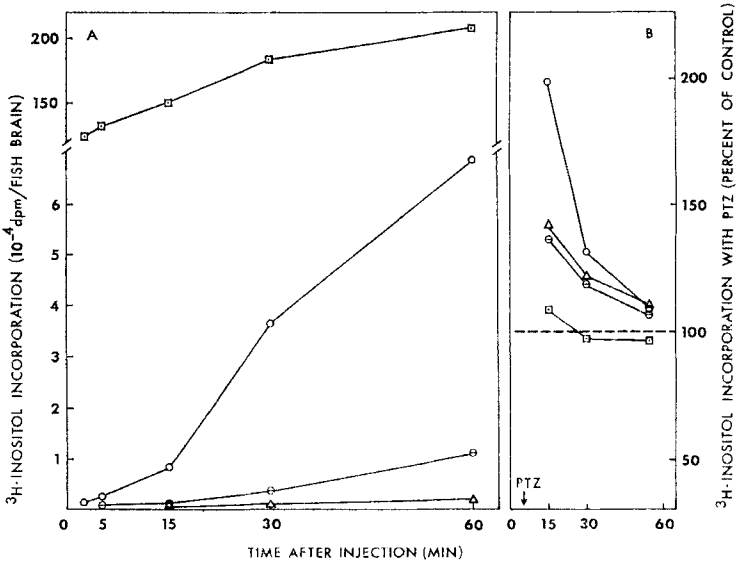


FIG. 2.—(a) Incorporation of [$^3$H[-*myo*-inositol into phospholipids and aqueous, HCl-soluble fraction as a function of time after incranial injection of radioisotope. See legend to Fig. 1 for details and key to symbols.

(b) Stimulation of labelling as a function of time after PTZ treatment; see legend to Fig. 1. Statistical significance of stimulation of labelling of PhI at 15 min: $P < 0{\cdot}01$. PhIP and PhIP$_2$: N.S.

into PhC and PhE was decreased by the PTZ treatment. Stimulation by PTZ of incorporation of [$^3$H] *myo*-inositol into PhI paralleled that seen with $^{32}P_i$, while labelling of PhIP or PhIP$_2$ appeared much less marked.

Electrically-induced convulsions (DAVIS and HIRTZEL, 1970) also altered the lipid labelling pattern (Table 1). As with PTZ, no significant change in the total radioactivity of the aqueous, HCl-soluble fraction was detected, and the effects on lipid labelling, while smaller than those seen with PTZ, were qualitatively similar.

## DISCUSSION

Incorporation of $^{32}P_i$ into brain phospholipids has been reported to decrease in mice subjected to PTZ or ECS (TORDA, 1954), whereas studies in the rat (ANSELL and DOHMEN, 1957; RAMARAO, HAUSER and LEBARON, 1964) indicate that there are no changes under these conditions. HAYASHI *et al.* (1962) have reported increased labelling of several phospholipid fractions which include PhA and PhI and decreased labelling of a fraction containing PhE and PhIP$_2$ in rabbit brain following electrically-induced convulsions. MARGOLIS and HELLER (1956) found increased incorporation of [$^3$H]-inositol into PhI of rat brain after administering cholinergic agents, but did not report the effects on the labelling of PhIP or PhIP$_2$. In the present experiments, we reasoned that short incubation periods should be examined, since a single spurt in labelling may become undetectable in the presence of a significant constant basal rate of labelling. Goldfish seemed to be advantageous for such studies since the injection of $^{32}P_c$ over the brain permits a more rapid, direct entry of radioactivity than that *via* the blood. The intracranial route would appear to have an advantage over intracerebral injections, particularly over a short period of time, since incorporation is more uniform. The relatively slow metabolic rate of poikilotherms is also useful since it provides some assurance that pre-equilibrium kinetics can be established and that post-mortem changes, known to occur in PhIP and PhIP$_2$, will be minimal. The relative degree of labelling of specific phospholipids at early times is different in the fish than that reported in the chick (KOZAK and WELLS, 1969) and in rat brain (FRIEDEL and SCHANBERG, 1971), perhaps representing a species difference. We have observed that when minces or homogenates of goldfish brain are incubated with $^{32}P_i$, the degree of PhA labelling surpasses that of the inositides. In guinea pig brain homogenate and nerve-ending preparations incubated with

$^{32}P_i$, we have observed, however, that PhIP and $PhIP_2$ are more highly labelled than PhA at early times (SCHACHT and AGRANOFF, 1972; HOLLANDER *et al.*, 1970). In each instance, the phosphomonoester is presumed to be equilibrating with a pool of [$^{32}$P]ATP *via* a combination of kinases and hydrolases, while PhI becomes labelled more slowly and reflects its synthesis, as can also be seen from [$^3$H]-inositol incorporation.

The significance of the convulsant-related changes in relation to the *in vitro* effects of cholinergic agents is not clear. Although cholinergic mechanisms have been invoked to explain the action of PTZ (HAHN, 1960; GIARMAN and PEPEU, 1964), we think it more likely that events related to increased brain activity mediate the action of PTZ and ECS on lipid labelling. For example, there is a suggestive decrease in radioactivity in the aqueous, HCl-soluble fraction of brain in the PTZ-treated animals by 30 min (Fig. 1b). This may reflect efflux of precursor from the brain as a result of increased cerebral blood flow (INGVAR and LASSEN, 1962) which in turn could indicate increased metabolism. The parallel stimulation of the incorporation of $^{32}P_i$ and [$^3$H]-inositol into PhI argues against the possibility that changes in specific radioactivity of ATP alone could account for the observed effects. In four separate experiments to directly compare effects of cholinergic agents *in vivo* with their actions *in vitro*, 3 μg of carbamyl choline or 60–100 μg of eserine were injected intracranially into each of 4 goldfish 5 min following the intracranial injection of $^{32}P_i$, and the fish were killed after 15 min of *in vivo* labelling. In each instance PhI labelling in the drug-treated fish was twice that of saline-injected animals, while PhA, PhIP and $PhIP_2$ were increased about 1·5-fold. As with PTZ and ECS, there was no significant effect on the aqueous, HCl-soluble radioactivity.

It has been proposed (HOKIN and HOKIN, 1964; DURELL, GARLAND and FRIEDEL, 1969) that the rapid $^{32}$P-labelling of phospholipids reflects a cyclic reaction sequence in which a cholinergic drug stimulates cleavage of endogenous phospholipid to yield diglyceride, which is then reincorporated *via* diglyceride kinase and the CDP-diglyceride pathway (AGRANOFF, BRADLEY and BRADY, 1958; PAULUS and KENNEDY, 1960). Both PhI and PhIP + $PhIP_2$ (GARLAND and DURELL, 1971) have been suggested as sources of the diglyceride, although many questions have been raised regarding these possibilities (SCHACHT and AGRANOFF, 1972). Whether the inositide turnover reflects synaptic events (LARRABEE and LEICHT, 1965), axonal permeability (KAI and HAWTHORNE, 1969) or other aspects of brain metabolism, it is clear that *in vivo* effects similar to those seen *in vitro* can be elicited and that the magnitude of the changes is large when measurements are made at times prior to equilibration.

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