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EFFECT OF PENTOBARBITAL ON REGIONAL BRAIN PHOSPHOLIPID SYNTHESIS*†

REIKO NATSUKI, ROBERT J. HITZEMANN, BARBARA A. HITZEMANN and HORACE H. LOH‡

Langley Porter Neuropsychiatric Institute, and Departments of Pharmacology and Psychiatry, University of California, San Francisco, CA 94143, U.S.A.

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Abstract—Rats were given 45 mg/kg i.p. sodium pentobarbital 15 min prior to the intraventricular injection of 200 μCi [^{32}P]phosphoric acid and 50 μCi [^{3}H]glycerol. The animals were sacrificed 1 hr later, subcellular fractions were prepared from four subcortical brain regions and phospholipids were extracted. Pentobarbital significantly increased the ratio of [^{3}H]- and [^{32}P]-triphosphatidylinositol (TPI) to diphosphatidylinositol (DPI) in the microsomal but not synaptosomal fractions. The possible relationship of this change to nicotinic receptor activity is discussed. Pentobarbital specifically decreased ^{32}Pi but not [^{3}H]glycerol incorporation into synaptosomal phosphatidylinositol (PI). Thus, pentobarbital induced the opposite of the "neurotransmitter effect" on PI turnover. Pentobarbital either decreased or had no effect on the incorporation of ^{32}Pi and [^{3}H]glycerol into phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

An investigation of the effects of pentobarbital on phospholipid turnover in discrete regions and subcellular fractions of the rat brain seems warranted for several reasons. First, knowledge of the effects of pentobarbital on phosphatidylinositol (PI) synthesis and turnover should help to reinforce the developing theories concerning the relationship between this membrane component and nerve function. Larrabee *et al.* [1] have observed that stimulation of the sympathetic ganglia increases the incorporation of ^{32}Pi into PI. Similarly, several groups have found that the incubation of brain tissue with acetylcholine (Ach), norepinephrine (NE) or dopamine (DA) increases the incorporation of ^{32}Pi into PI [2–10]. Since barbiturates are known to decrease neurotransmitter release [11], we predict that pentobarbital might have especially potent effects on PI synthesis and metabolism.

Second, knowledge of the effects of pentobarbital on polyphosphoinositide synthesis and turnover should help clarify the relationship between these interesting membrane components and nervous activity. It has been suggested that the interconversion of triphosphatidylinositol (TPI) and diphosphatidylinositol (DPI) may regulate membrane bound Ca^{2+} levels in nervous tissue [12–14]. In support of this hypothesis, Buckley and Hawthorne [15] found an increase in high affinity Ca^{2+} binding to erythrocyte membranes when PI was converted to DPI and TPI. However, the relative contributions of DPI and TPI to the increase in high affinity binding was not determined. It has been difficult to show a change in TPI and/or DPI turnover that could be specifically associated with a change in nervous activity [16, 17]. White *et al.* [18] were able, under some special conditions, to demonstrate that stimulation of the vagus nerve caused increased incorporation of ^{32}Pi into TPI and DPI. These data agreed with the earlier findings of Schacht and Agranoff [19] that pentylenemetrazol or electroconvulsive shocks increased TPI and DPI labeling by ^{32}Pi in the goldfish brain. Recently, we reported [20] in a preliminary communication that morphine and pentobarbital, two drugs which decrease ACh release, and mecamylamine, a nicotinic receptor blocker, increased the ratio of [^{32}P]TPI to [^{32}P]DPI in midbrain microsomes. In contrast, eserine markedly decreased the [^{32}P]TPI to [^{32}P]DPI ratio. These data may suggest that the conversion of TPI to DPI is important in nicotinic receptor activation [21]. In the present study, we have extended our preliminary observations on the effects of pentobarbital on the TPI–DPI interconversion by investigating the pentobarbital effects in four brain regions and two subcellular fractions. Based on the report of Salvaterra *et al.* [22] showing the highest density of brain nicotinic receptors in the microsomal fraction, we predict that pentobarbital will show a more significant effect on the TPI to DPI ratio in the microsomal as compared to the synaptosomal fraction.

Third, previously we observed [23] that chronic pentobarbital treatment had only minor effects on phospholipid synthesis in rat brain subcortical microsomes and synaptic plasma membranes. The possibility is now suggested that pentobarbital, like morphine [24, 25], may have marked acute effects on phospholipid synthesis and that tolerance develops to these acute effects. Thus, knowledge of the acute pentobarbital effects on phospholipid synthesis and turnover may provide insight into the mechanisms of central pentobarbital tolerance and dependence.

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METHODS AND MATERIALS

Materials. Carrier free [^{32}P]phosphoric acid (^{32}Pi) and [2-^{3}H]glycerol (sp. act. 10 Ci/m-mole) were obtained from New England Nuclear Corp., Boston, MA.

Experimental. Male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing 180–220 g were implanted with a cannulae guide over the lateral ventricle and then allowed 4–5 days to recover from surgery before beginning the experiment. At zero time the animals were given i.p. 45 mg/kg of sodium-pentobarbital or saline. Fifteen min later the animals were given intraventricularly 200 μCi ^{32}Pi and 50 μCi [^{3}H]glycerol dissolved in 20 μl of a modified Krebs–Ringer bicarbonate buffer in which the sodium diphosphate was omitted. One hr later the animals were sacrificed, and the brains rapidly removed, dissected and immediately homogenized in 9 vol. of 0.32 M sucrose plus 20 mM Tris, pH 9.5, at 4°. The alkaline-homogenizing medium decreases the hydrolysis of the polyphosphoinositides [26]. Microsomes and nerve ending particles were prepared by standard centrifugation techniques [27, 28]. A portion of the total homogenate was mixed with 2 vol. of 10% trichloroacetic acid (TCA), centrifuged and an aliquot of the supernatant was counted for total non-precipitable ^{32}P and ^{3}H.

Phospholipid analysis. Phospholipids were extracted from the microsomes as described by Eichberg and Dawson [29]. The chloroform–methanol (1:1) soluble phospholipids, namely, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were separated on Silica gel-G thin-layer chromatographic (TLC) plates using a solvent system of chloroform–methanol–water–acetic acid (conc.) (25:15:2:4, v/v). The phospholipids were identified, eluted and specific activities determined as described elsewhere [27]. Diphosphatidylinositol (DPI) and triphosphatidylinositol (TPI), the chloroform–methanol–HCl (conc.) (2:1:0.01, v/v) soluble phospholipids, were separated on Silica gel-H TLC plates using a solvent system of chloroform–methanol–4 N ammonium hydroxide (9:7:2, v/v) [30]. The phospholipids were located by iodine vapor, the plates were scraped and the DPI and TPI were extracted from the Silica gel using the developing solvent. Lipid phosphorus levels were analyzed as described by Bartlett [31]. DPI and TPI data are presented as cpm/μmole of total acidic lipid P applied to the plate. Statistical analysis of the data was performed using the Student's *t*-test (two tailed).

RESULTS

Effect of pentobarbital on microsomal and synaptosomal TPI and DPI synthesis. The data in Fig. 1 illustrate that pentobarbital either decreased or had no effect on the incorporation of [^{3}H]glycerol into DPI and TPI depending on the brain region considered. The decrease in [^{3}H]glycerol incorporation into DPI in the brainstem and neostriatum was markedly greater than the decrease in incorporation into TPI. This differential effect of pentobarbital on [^{3}H]TPI and [^{3}H]DPI levels is further shown in Table 1. In all four brain regions, pentobarbital significantly increased the ratio of [^{3}H]TPI to [^{3}H]DPI. The data in Fig. 2 illustrate that pentobarbital had no effect on ^{32}Pi incorporation into TPI in the brainstem, midbrain and neostriatum but significantly increased incorporation 170 per cent in the diencephalon. In contrast, pentobarbital significantly decreased ^{32}Pi incorporation into DPI in all brain regions but the diencephalon. As with the [^{3}H]polyphosphoinositides, the ratio of [^{32}P]TPI to [^{32}P]DPI was significantly increased in all brain regions after pentobarbital treatment (Table 1).

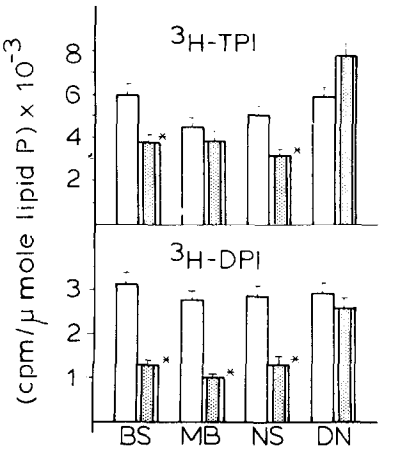

Fig. 1. Effect of pentobarbital on [^{3}H]glycerol incorporation into microsomal TPI and DPI. Rats were given i.p. 45 mg/kg of sodium-pentobarbital 15 min prior to the injection of 200 μCi [^{32}P]phosphoric acid and 50 μCi [^{3}H]glycerol. One hr later the animals were sacrificed and four subcortical regions, the pons-medulla or brainstem (BS), the midbrain (MB), the neostriatum (NS) and the diencephalon (DN), were dissected. Microsomes were prepared from these regions, phospholipids were extracted and their specific activities determined. N was 10–12 pairs of animals/brain region. In this figure, data are expressed as the mean ± S.E. (cpm/μmole of lipid P applied to the TLC plate) x 10^{-3}. Key: (□) control; (▨) pentobarbital; and (*) significantly different from control, $P < 0.05$.

The effects of pentobarbital on the incorporation of ^{32}Pi and [^{3}H]glycerol into the synaptosomal frac-

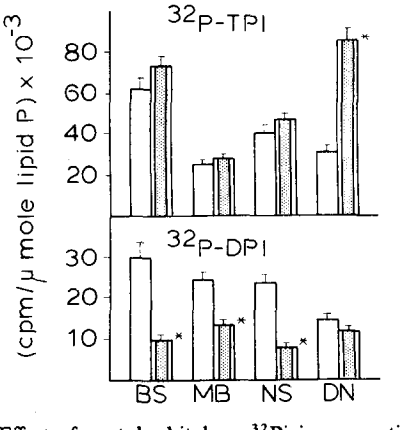

Fig. 2. Effect of pentobarbital on ^{32}Pi incorporation into microsomal TPI and DPI. Details are given in the legend to Fig. 1. Key: (□) control; (▨) pentobarbital; and (*) significantly different from control, $P < 0.05$.

Table 1. Effect of pentobarbital on the ratio of labeled TPI to DPI*

Group	[^{3}H]TPI / [^{3}H]DPI	[^{32}P]TPI / [^{32}P]DPI
Brainstem		
Control	1.8 ± 0.2	2.1 ± 0.2
Pentobarbital	2.9 ± 0.2†	7.6 ± 0.8†
Midbrain		
Control	1.6 ± 0.1	1.0 ± 0.1
Pentobarbital	3.9 ± 0.5†	2.2 ± 0.2†
Neostriatum		
Control	1.8 ± 0.1	1.7 ± 0.2
Pentobarbital	2.5 ± 0.2†	6.0 ± 0.7†
Diencephalon		
Control	2.0 ± 0.2	2.1 ± 0.3
Pentobarbital	3.0 ± 0.2†	7.3 ± 0.9†

* Rats were given 45 mg/kg of sodium-pentobarbital i.p. 15 min prior to the intraventricular injection of 200 μCi ^{32}Pi and 50 μCi [^{3}H]glycerol. One hr later the animals were sacrificed, brains dissected and microsomes were prepared. TPI and DPI were extracted from the microsomes and their specific activities determined. N = 10–12 pairs of animals/group. Data are expressed as the ratio of [^{3}H]TPI to [^{3}H]DPI and [^{32}P]TPI to [^{32}P]DPI.
† Significantly different from control, $P < 0.05$.

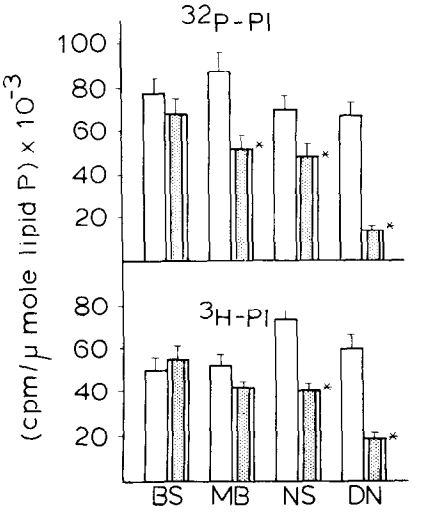

Fig. 3. Effect of pentobarbital on ^{32}Pi and [^{3}H]glycerol incorporation into microsomal PI. Details are given in the legend to Fig. 1. Data in this figure and Figs. 4–6 are now expressed as mean ± S.E. (cpm/μmole of lipid P) × 10^{-3}. Key: (□) control; (▨) pentobarbital; and (*) significantly different from control, $P < 0.05$.

tion were examined in both the neostriatum and the midbrain. The data in Table 2 show that the only significant pentobarbital effect was to inhibit the incorporation of [^{3}H]glycerol into both DPI and TPI in the neostriatum. No significant changes in the ratio of labeled TPI to DPI were observed in either brain region.

Pentobarbital did not significantly affect the levels of non-precipitable ^{3}H or ^{32}P in any of the brain regions studied (data not shown). No attempt was made to measure actual precursor specific activity.

Effect of pentobarbital on microsomal and synaptosomal PI synthesis. The data in Fig. 3 illustrate that pentobarbital depressed the incorporation of both ^{32}Pi and [^{3}H]glycerol into microsomal PI in the neostriatum and diencephalon. In the midbrain, only the incorporation of ^{32}Pi was affected. Pentobarbital had no effect on either ^{32}Pi or [^{3}H]glycerol incorporation in the brainstem. Pentobarbital significantly inhibited ^{32}Pi but not [^{3}H]glycerol incorporation into synaptosomal PI in both the neostriatum and midbrain (Table 3). It may be important to note that while the specific activities of microsomal [^{3}H]PI and

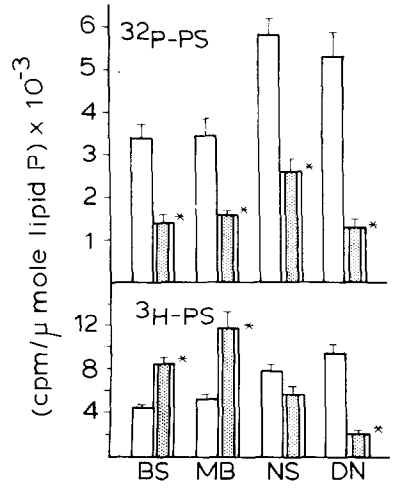

Fig. 4. Effect of pentobarbital on ^{32}Pi and [^{3}H]glycerol incorporation into microsomal PS. Details are given in the legends to Figs. 1 and 3. Key: (□) control; (▨) pentobarbital; and (*) significantly different from control, $P < 0.05$.

Table 2. Effect of pentobarbital on the incorporation of ^{32}Pi and [^{3}H]glycerol into synaptosomal TPI and DPI*

Group	[^{3}H]DPI (cpm/μmole lipid P) × 10^{-2}	[^{3}H]TPI (cpm/μmole lipid P) × 10^{-2}	[^{3}H]TPI / [^{3}H]DPI	[^{32}P]DPI (cpm/μmole lipid P) × 10^{-3}	[^{32}P]TPI (cpm/μmole lipid P) × 10^{-3}	[^{32}P]TPI / [^{32}P]DPI
Midbrain						
Control	5.1 ± 0.5	4.4 ± 0.4	0.9 ± 0.1	7.1 ± 0.8	18.2 ± 2.1	2.6 ± 0.3
Pentobarbital	3.9 ± 0.3	3.7 ± 0.5	0.9 ± 0.1	6.7 ± 0.9	21.6 ± 2.6	3.2 ± 0.3
Neostriatum						
Control	4.0 ± 0.4	4.0 ± 0.3	1.0 ± 0.1	9.6 ± 0.8	19.6 ± 1.6	2.0 ± 0.2
Pentobarbital	1.8 ± 0.2†	2.2 ± 0.2†	1.2 ± 0.1	7.7 ± 0.7	16.4 ± 1.7	2.1 ± 0.2

* Experimental details are given in the legends to Table 1 and Fig. 1. Synaptosomes were prepared from the midbrain and neostriatum, TPI and DPI were extracted and their specific activities determined. N = 6–8 pairs of animals/group.
† Significantly different from control, $P < 0.05$.

Table 3. Effect of pentobarbital on the incorporation of ^{32}Pi and [^{3}H]glycerol into synaptosomal PI*

Group	[^{3}H]PI (cpm/μmole lipid P) × 10^{-3}	[^{32}P]PI (cpm/μmole lipid P) × 10^{-3}	[^{32}P]PI / [^{3}H]PI
Midbrain			
Control	8.0 ± 0.9	70.4 ± 6.2	8.8 ± 0.9
Pentobarbital	10.5 ± 1.0	34.4 ± 4.1†	3.2 ± 0.4†
Neostriatum			
Control	6.8 ± 0.5	78.4 ± 7.1	11.5 ± 1.0
Pentobarbital	5.5 ± 0.6	43.1 ± 3.9†	7.8 ± 0.7†

* Experimental details are given in the legends to Table 1 and Fig. 1. Synaptosomes were prepared from the midbrain and neostriatum, PI was extracted and its specific activity determined. N = 6–8 pairs of animals/group.
† Significantly different from control, $P < 0.05$.

[^{32}P]PI were approximately equal, the specific activity of synaptosomal [^{32}P]PI was eight to twelve times that of [^{3}H]PI.

Effect of pentobarbital in microsomal PS synthesis. Pentobarbital significantly decreased ^{32}Pi incorporation into PS in all brain regions studied (Fig. 4). In contrast, pentobarbital decreased [^{3}H]glycerol incorporation only in the diencephalon and significantly increased the incorporation of this label in both the brainstem and midbrain.

Effect of pentobarbital on microsomal PC and PE synthesis. Since PC and PE are synthesized via a similar metabolic route, we have compared the effects of pentobarbital on the incorporation of ^{32}Pi and [^{3}H]glycerol into these two phospholipids. While pentobarbital markedly decreased ^{32}Pi incorporation into PC in all brain regions studied, the incorporation of ^{32}Pi into PE was significantly decreased only in the diencephalon (Figs. 5 and 6). The effect of pentobarbital on [^{3}H]glycerol incorporation was similar for both phospholipids in that a significant decrease in [^{3}H]PC and [^{3}H]PE specific activity was found in the neostriatum.

DISCUSSION

One attractive hypothesis concerning the function of neuronal polyphosphoinositides is that the inter-

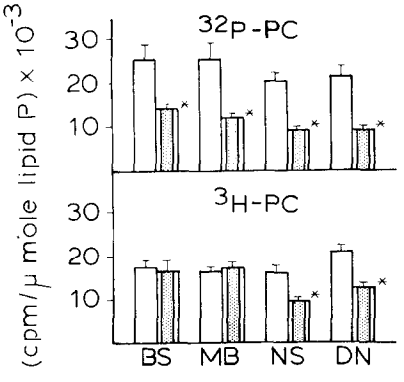

Fig. 5. Effect of pentobarbital on ^{32}Pi and [^{3}H]glycerol incorporation into microsomal PC. Details are given in the legends to Figs. 1 and 3. Key: (□) control; (▨) pentobarbital; and (*) significantly different from control, $P < 0.05$.

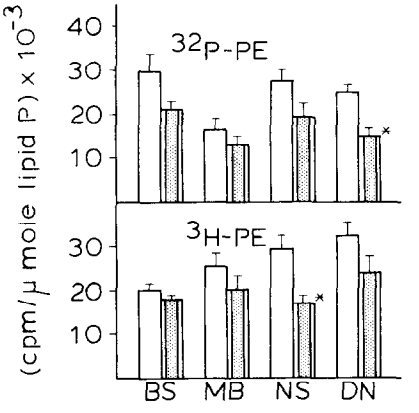

Fig. 6. Effect of pentobarbital on ^{32}Pi and [^{3}H]glycerol incorporation into microsomal PE. Details are given in the legends to Figs. 1 and 3. Key: (□) control; (▨) pentobarbital; and (*) significantly different from control, $P < 0.05$.

conversion between TPI and DPI regulates the amount of membrane bound Ca^{2+}. Since Ca^{2+} is less avidly bound to DPI than TPI [14], the conversion of TPI to DPI could result in the release of membrane bound Ca^{2+}, a phenomenon which is known to be associated with the development of the action potential [32]. In addition, some evidence suggests that the conversion of TPI to DPI is associated with nicotinic receptor activation [21]. We have observed in a preliminary study [20] that morphine and pentobarbital, two drugs which decrease ACh release, and mecamylamine, a nicotinic receptor blocking agent, increased the ratio of [^{32}P]TPI to [^{32}P]DPI in rat midbrain microsomes under experimental conditions of drug and isotope administration similar to those described in this paper. In contrast, eserine markedly decreased the [^{32}P]TPI to [^{32}P]DPI ratio. Recently, we have found (unpublished observations) that atropine has no effect on the [^{32}P]TPI to [^{32}P]DPI ratio. The results of the present study show that the pentobarbital effect is not limited to the midbrain but occurs in three other subcortical brain regions as well. This widespread nature of the pentobarbital effect is consistent with the ubiquitous distribution of nicotinic receptors in the rat brain [22]. Furthermore, our observation that the drug-induced change in the

labeled TPI to DPI ratio was limited to the microsomal fraction is consistent with data indicating that the highest subcellular density of nicotinic receptors in the rat brain is found in the microsomal fraction [22]. Overall, the data from this and our previous study [20], as well as data in preparation, suggest that there is a relationship between the level of nicotinic receptor activity and the ratio of labeled TPI to DPI. At present it is not clear what is the physiological significance of this change. However, the data may indicate that changes in polyphosphoinositide metabolism are involved in nicotinic receptor activation.

The mechanism(s) by which nerve stimulation and cationic neurotransmitters stimulate the incorporation of ^{32}Pi into PI have been extensively examined. Michell [33] has concluded that the "neurotransmitter effect" is caused by the enhanced breakdown of PI to the D-1,2-diglyceride, which is then reutilized for synthesis to PI. As a consequence of this scheme, cationic neurotransmitters increase the incorporation of ^{32}Pi but not [^{3}H]glycerol into PI [33]. Thus, we predicted that pentobarbital, which is known to depress neurotransmitter release [11], would specifically decrease the incorporation of ^{32}Pi over [^{3}H]glycerol into PI. Careful examination of the data presented in Fig. 3 reveals that, in all brain regions except the neostriatum, the ratio of [^{32}P]PI to [^{3}H]PI was decreased, but a significant ($P < 0.01$) change in this ratio was found only in the diencephalon.

In both of the synaptosomal fractions examined, pentobarbital specifically decreased the incorporation of ^{32}Pi and not [^{3}H]glycerol into PI. Furthermore, pentobarbital has also been found to specifically decrease ^{32}Pi over [^{3}H]glycerol incorporation into synaptosomes prepared from the diencephalon and brainstem (unpublished observations). Thus, the pentobarbital effect on PI turnover shows some subcellular specificity, with the most significant changes being observed in the synaptosomal fraction. These data are consistent with the proposed roles of PI in neurohumoral transmission [33]. The data in the present study also suggest that the factors regulating PI turnover in the microsomes are somewhat different from those regulating synaptosomal turnover. The ratio of [^{32}P]PI to [^{3}H]PI is markedly higher in the synaptosomal than in the microsomal fraction, probably indicating that the initial incorporation of ^{32}Pi into synaptosomal PI occurs via a mechanism unrelated to microsomal PI synthesis. Overall, the pentobarbital data on PI turnover demonstrate the usefulness of a pharmacological manipulation to study the relationship between a membrane component, neurotransmitters and nerve activity.

In a previous report [23], it was observed that chronic pentobarbital treatment did not markedly affect the incorporation of ^{32}Pi into PC and PE. The data in the present study clearly demonstrate that pentobarbital markedly depresses ^{32}Pi incorporation into PC with a less marked effect on incorporation into PE. Overall, the data from this and our previous study would suggest that, as tolerance develops, the inhibitory effect on PC synthesis disappears. Interestingly, some evidence suggests that tolerance may develop to some but not all the inhibitory effects of pentobarbital on synaptosomal PI turnover. When synaptic plasma membranes (SPM) were prepared from two populations of subcortical synaptosomes, the incorporation of ^{32}Pi into PI of SPM isolated from light synaptosomes was still markedly depressed in the chronic pentobarbital animals, while the incorporation of ^{32}Pi into PI of SPM derived from heavy synaptosomes was significantly increased [34]. In the context of this discussion, it may be of more than passing interest to note that the heavy synaptosomes are enriched in NE-containing nerve terminals, while the light synaptosomes are enriched in γ-aminobutyric acid (GABA)-containing nerve terminals.

In conclusion, the data in the present study indicate that pentobarbital affects brain phospholipid synthesis in a way that is consistent with the proposed roles of the phospholipids in nervous activity. The marked effects of pentobarbital on the incorporation of ^{32}Pi and [^{3}H]glycerol into the phosphoinositides further illustrate the probable importance of these interesting membrane components in nerve function.

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