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The Effects of Barbiturates on the Metabolism of Phosphatidic Acid and Phosphatidylinositol in Rat Brain Synaptosomes

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Barbiturates and diphenylhydantoin inhibit the carbamoylcholine-stimulated increase in ^{32}P incorporation into phosphatidylinositol and phosphatidic acid, but have a relatively slight effect on the incorporation of ^{32}P into these lipids in the absence of carbamoylcholine and no effect on ^{32}P incorporation into phosphatidylcholine and phosphatidylethanolamine. Inhibition of the carbamoylcholine-stimulated increase was observed for pentobarbital, thiopental, phenobarbital, 5-(1,3-dimethylbutyl)-5-ethylbarbiturate, (+)- and (−)-5-ethyl-*N*-methyl-5-propylbarbiturate and diphenylhydantoin. Similar concentrations of barbiturates and diphenylhydantoin were previously reported to inhibit the K^+-stimulated Ca^{2+} influx, and therefore other agents that affect Ca^{2+} influx were tested to find whether they had any effect on ^{32}P incorporation into these lipids. K^+ (35mM) increases ^{32}P incorporation into phosphatidic acid, but to a smaller degree than 100 μM-carbamoylcholine, and its effect was inhibited by pentobarbital. Veratridine (75 μM) does not increase ^{32}P incorporation into either phosphatidic acid or phosphatidylinositol, but did inhibit the carbamoylcholine-stimulated increase in ^{32}P incorporation into phosphatidylinositol. The possible relationship between the phospholipid effect and stimulated Ca^{2+} influx is discussed.

The turnover of the polar head groups of phosphatidylinositol and its precursor, phosphatidic acid, is increased in a number of tissues by muscarinic and α-adrenergic stimulation (see Michell, 1975, for a review), and there is mounting evidence that this increase in turnover plays a role in the response to these receptors (Michell *et al.*, 1976; Miller, 1977). However, the relationship between the increased turnover and the physiological response to the receptor is not clear. It has been suggested that the probable first step in the turnover cycle, the breakdown of phosphatidylinositol, may be involved in the opening of 'Ca^{2+} gates', as Ca^{2+} is not required for the increase in turnover of the phospholipids, but is required for the physiological response to the receptor (Jafferji & Michell, 1976*a*,*b*). A number of agents that inhibit smooth-muscle contraction and Ca^{2+} influx have been tested, but these, except for phenoxybenzamine, have been shown to have no effect on the carbamoylcholine-stimulated increase in phospholipid turnover (Jafferji & Michell, 1976*a*). As these authors point out, these results do not eliminate the hypothesis, but there is very little support at present that the phospholipid turnover is indeed associated with the opening of 'Ca^{2+} gates'.

One group of compounds whose effect on phospholipid metabolism has not been studied includes barbiturates, and the chemically similar diphenylhydantoin, which have been shown to inhibit specifically the K^+-stimulated increase in Ca^{2+} influx into synaptosomes (Sohn & Ferrendelli, 1973; Blaustein & Ector, 1975) and ganglia (Blaustein, 1976). We have therefore studied the effects of these agents on the uptake of ^{32}P into phosphatidic acid and phosphatidylinositol in synaptosomes in the presence and absence of carbamoylcholine and other agents to find whether there is any correlation between the effects of these agents on the stimulated Ca^{2+} influx and phospholipid turnover. Such studies may result in further evidence on whether or not the phospholipid effect is associated with the opening of 'Ca^{2+} gates' and may yield information on the mechanism of action of barbiturates.

Methods and Materials

Pentobarbital, phenobarbital, thiopental sodium and diphenylhydantoin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., veratridine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and Gelman IPLC-SA t.l.c. plates were from Gelman Instrument Co., Ann Arbor, MI, U.S.A. 5-(1,3-Dimethylbutyl)-5-ethylbarbituric acid was a gift from Eli Lilly Co., Indianapolis, IN, U.S.A., and (+)- and (−)-5-ethyl-*N*-methyl-5-propylbarbiturate were generous gifts from Professor J. Knabe,

Department of Pharmaceutical Chemistry, University of the Saarland, 77 Saarbrücken, Germany. The source of all other chemicals and supplies has been described previously (Miller, 1977).

Synaptosomes were prepared from rat brain by homogenization followed by differential and sucrose-gradient centrifugation, as described previously (Miller, 1977). Phenobarbital, (+)- and (−)-5-ethyl-*N*-methyl-5-propylbarbiturate and diphenylhydantoin were dissolved in NaOH, neutralized with HCl, and other components added so that the final composition of the solution was that of a modified Krebs–Ringer buffer (0.118 M-NaCl, 4.7 mM-KCl, 0.75 mM-$CaCl_2$, 1.18 mM-KH_2PO_4, 1.18 mM-$MgSO_4$, 24.8 mM-$NaHCO_3$, 10 mM-glucose and 10 mM-sodium succinate), adjusted to pH 7.4 with O_2/CO_2 (19:1, v/v). Veratridine solutions were made up in a similar manner, except that it was dissolved in HCl and neutralized with NaOH. All other agents were dissolved directly in the modified Krebs–Ringer buffer.

The synaptosomes (0.5–1.0 mg of protein determined by the method of Lowry *et al.*, 1951) were incubated for 1 h at 30°C in 1 ml of the modified Krebs–Ringer buffer containing 20–30 μCi of $[^{32}P]P_i$ and the reaction was stopped by the addition of the solvents for the extraction of the phospholipids (Miller, 1977) or by rapid filtration on Whatman GF/F glass-fibre filters with a Millipore 1225 Sampling Manifold. The filters were transferred to test tubes, and the phospholipids extracted from them by the addition of 4.75 ml of chloroform/methanol/0.9% NaCl (5:10:4, by vol.). The phospholipid extraction was then continued as described previously (Miller, 1977) and the phospholipids were separated by t.l.c. on Gelman IPLC-SA plates that had been dipped in 5 mM-Na_2CO_3 and air-dried, before development in chloroform/methanol/acetic acid/water (250:125:40:2, by vol.) (Geison *et al.*, 1976). The lipid bands were stained with I_2, identified by comparison with standards, cut out and placed in scintillation vials, and the uptake of ^{32}P was measured with a Packard Tri-Carb liquid-scintillation spectrometer.

Results

Effect of pentobarbital on ^{32}P uptake into phospholipids

The incorporation of ^{32}P was measured for the two major phospholipids, phosphatidylcholine and phosphatidylethanolamine, as well as the two that are affected by muscarinic stimulation, phosphatidic acid and phosphatidylinositol. The incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol was significantly inhibited by 0.6 mM-pentobarbital, and the carbamoylcholine-stimulated increase into phosphatidic acid was significantly decreased (Table 1). No effects were observed in the incorporation of ^{32}P into either phosphatidylcholine or phosphatidylethanolamine, indicating that the effect of pentobarbital is not a generalized decrease in phospholipid metabolism. All subsequent experiments were limited to the effects on phosphatidic acid and phosphatidylinositol metabolism.

Effect of some anaesthetic barbiturates

In all cases the carbamoylcholine-stimulated increase in ^{32}P incorporation was more strongly inhibited by the barbiturates than was ^{32}P incorporation in the absence of carbamoylcholine (Table 2). The carbamoylcholine-stimulated increase was totally inhibited by 1 mM-pentobarbital, and the percentage increase was decreased to one-half by 1 mM-phenobarbital and to one-third by 0.6 mM-thiopental.

We have also studied the effects of a pair of optical isomers, (+)- and (−)-5-ethyl-*N*-methyl-5-propylbarbiturate. These barbiturates have been shown to have marked differences in their biological effects. For example, the sleep time in rats induced by (−)-5-ethyl-*N*-methyl-5-propylbarbiturate is 6 times that induced by the (+)-isomer (Knabe & Franz, 1975)

Table 1. *Effect of barbiturates and carbamoylcholine on the metabolism of some phospholipids*

Synaptosomes were incubated in a modified Krebs–Ringer buffer as described in the Methods and Materials section and ^{32}P incorporation into the phospholipids was measured. The results are expressed as mean ± S.D. ($n = 4$). The statistical significance of the effect of pentobarbital alone was estimated by Student's t test: *$P<0.05$, **$P<0.01$, ***$P<0.001$. The statistical significance of pentobarbital on the carbamoylcholine-stimulated increase was estimated by 2×2 factorial variance analysis: †$P<0.05$, ††$P<0.01$, †††$P<0.001$.

	^{32}P incorporation (c.p.m./mg of protein)			
	Control	0.1 mM-Carbamoylcholine	0.6 mM-Pentobarbital	0.1 mM-Carbamoylcholine + 0.6 mM-pentobarbital
Phosphatidylcholine	482 ± 67	452 ± 84	505 ± 109	444 ± 68
Phosphatidylinositol	1554 ± 208	2735 ± 318	1210 ± 108*	2117 ± 364
Phosphatidylethanolamine	259 ± 40	292 ± 72	261 ± 44	290 ± 39
Phosphatidic acid	4211 ± 124	7486 ± 305	3165 ± 182***	4811 ± 700††

Table 2. *Effects of anaesthetic barbiturates on ^{32}P incorporation into phosphatidic acid and phosphatidylinositol*
The statistical significance of the effect of barbiturate alone and that of the effect of barbiturate on the carbamoylcholine-stimulated increase in ^{32}P incorporation were estimated as in Table 1. The results are expressed as means ± S.D. ($n = 4$ in all experiments, except that with pentobarbital, where $n = 3$). The effects of (+)- and (−)-5-ethyl-*N*-methyl-5-propylbarbiturate were not significantly different by Student's *t* test, except for the effects on phosphatidylinositol in the presence of carbamoylcholine, where $P<0.02$.

	Barbiturate concn. (mM)	^{32}P incorporation (% of control): Control	0.1 mM-Carbamoylcholine	Barbiturate	Barbiturate + 0.1 mM-carbamoylcholine
Pentobarbital					
Phosphatidic acid	1	100±6	220±9	81±12	91±4†††
Phosphatidylinositol	1	100±17	226±6	77±25	94±5†††
Phenobarbital					
Phosphatidic acid	0.5	100±9	190±24	122±22	177±17
	1.0			97±11	88±15†††
Phosphatidylinositol	0.5	100±4	171±39	86±10	116±24
	1.0			78±13*	97±7†
Thiopental					
Phosphatidic acid	0.2	110±14	195±15	99±16	178±13
	0.6			92±9	121±7††
Phosphatidylinositol	0.2	100±19	177±31	99±21	154±13
	0.6			65±7*	103±14
(+)-5-Ethyl-*N*-methyl-5-propylbarbiturate					
Phosphatidic acid	0.5	100±12	176±6	89±4	119±17††
Phosphatidylinositol	0.5	100±11	193±8	79±11*	136±12†
(−)-5-Ethyl-*N*-methyl-5-propylbarbiturate					
Phosphatidic acid	0.5	as (+)-isomer		72±14**	101±8††
Phosphatidylinositol	0.5			72±3***	111±4†††

Table 3. *Effect of the convulsant barbiturate, 5-(1,3-dimethylbutyyl)-5-ethylbarbiturate, and the anti-convulsant, diphenylhydantoin, on ^{32}P incorporation into phosphatidic acid and phosphatidylinositol*
Experimental conditions and statistical methods were as in Table 1.

	Drug concn. (mM)	^{32}P incorporation (% of control): Control	0.1 mM-Carbamoylcholine	Drug	Drug + 0.1 mM-carbamoylcholine
5-(1,3-Dimethylbutyl)-5-ethylbarbiturate					
Phosphatidic acid	0.1	100±4	178±9	144±5**	249±13
	0.3			116±3**	172±6†
Phosphatidylinositol	0.1	100±7	186±12	102±12	156±10
	0.3			64±6***	103±8†
Diphenylhydantoin					
Phosphatidic acid	0.2	100±1	204±6	111±6**	146±13†††
	0.5			70±4***	86±2†††
Phosphatidylinositol	0.2	100±4	147±12	81±6**	122±13
	0.5			74±5***	78±4††

and the anaesthetic dose in tadpoles for the (−)-isomer is half that of the (+)-isomer (L. Chang & K. W. Miller, personal communication). We found that the difference in effect of these isomers was not great enough to be statistically significant, except for the effect on phosphatidylinositol in the presence of carbamoylcholine, where (−)-5-ethyl-*N*-methyl-5-propylbarbiturate was the more potent inhibitor (Table 2).

Effect of chemically similar non-anaesthetics

We wished to compare the results obtained with the anaesthetics described above with those of chemically similar compounds that are not anaesthetics to

ascertain whether these results have any relevance to anaesthesia. 5-(1,3-Dimethylbutyl)-5-ethylbarbiturate is a convulsant barbiturate, but was found to have a similar effect to other barbiturates on the K^+-stimulated increase in Ca^{2+} uptake (Blaustein & Ector, 1975). We found that 0.3mM-5-(1,3-dimethylbutyl)-5-ethylbarbiturate inhibited the carbamoylcholine-stimulated increase in ^{32}P incorporation into both phosphatidic acid and phosphatidylinositol (Table 3). However, 0.1 mM-5-(1,3-dimethylbutyl)-5-ethylbarbiturate increased ^{32}P incorporation into phosphatidic acid both in the presence and absence of carbamoylcholine and a concentration of 0.3 mM increased ^{32}P incorporation into phosphatidic acid in the absence of carbamoylcholine. No such increase was observed in the incorporation of ^{32}P into phosphatidylinositol.

Diphenylhydantoin is chemically similar to the barbiturates, and like the barbiturates inhibits the K^+-stimulated increase in Ca^{2+} influx (Sohn & Ferrendelli, 1973). However, it is an anti-convulsant, not an anaesthetic. We found that diphenylhydantoin (0.2 and 0.5mM) inhibited the increase in ^{32}P incorporation caused by carbamoylcholine (Table 3). In the absence of carbamoylcholine 0.2mM-diphenylhydantoin stimulated ^{32}P incorporation into phosphatidic acid, but inhibited ^{32}P incorporation into phosphatidylinositol, whereas 0.5mM-diphenylhydantoin inhibited ^{32}P uptake into both phosphatidic acid and phosphatidylinositol.

Effects of agents that increase Ca^{2+} influx

K^+ causes an increase in Ca^{2+} influx into synaptosomes (Blaustein & Ector, 1975) and has been shown previously to cause an increase in ^{32}P uptake into phosphatidylinositol and phosphatidic acid in synaptosomes (Hawthorne & Bleasdale, 1975). However, 35mM-K^+, which was reported to cause optimal stimulation of ^{32}P uptake (Hawthorne & Bleasdale, 1975), consistently caused a smaller increase in ^{32}P uptake into phosphatidic acid than 100μM-carbamoylcholine (Table 4). This was confirmed by comparison of the two agents on one preparation of synaptosomes (results not shown). We also confirmed that the stimulation of ^{32}P incorporation was greater by 35mM- than by 70mM-K^+. The K^+-stimulated increase in ^{32}P incorporation was inhibited by pentobarbital.

Veratridine has also been found to increase the K^+-stimulated Ca^{2+} influx into synaptosomes (Blaustein & Ector, 1975). However, veratridine did not stimulate the uptake of ^{32}P into phosphatidic acid or phosphatidylinositol (Table 4), although 75μM-veratridine did inhibit the carbamoylcholine-stimulated increase in ^{32}P uptake into phosphatidylinositol.

Table 4. *Effect of K^+ and veratridine on ^{32}P incorporation into phosphatidic acid and phosphatidylinositol*

The effect of high concentrations of K^+ was compared with a control that contained 5mM-K^+. The statistical significance of the effects was estimated as in Table 1.

	^{32}P incorporation (% of control)	
	Phosphatidic acid	Phosphatidylinositol
Control	100±5	100±9
35mM-K^+	149±5***	84±8
1 mM-Pentobarbital	60±7***	46±10***
35mM-K^++1 mM-pentobarbital	61±2†††	37±1
Control	100±6	100±17
0.1 mM-Carbamoylcholine	160±6	204±12
75μM-Veratridine	117±12	89±13
75μM-Veratridine+0.1 mM-carbamoylcholine	182±24	122±10††

Discussion

We have found that barbiturates and related compounds inhibit the carbamoylcholine-stimulated increase in ^{32}P uptake into phosphatidic acid and phosphatidylinositol. The question that arises is whether these effects may be related to the opening of 'Ca^{2+} gates', as has been suggested by Jafferji & Michell (1976*a*,*b*), on the basis of the observation that the increased turnover of phosphatidic acid and phosphatidylinositol does not require the presence of Ca^{2+}, although the physiological responses to the muscarinic receptor do require this ion.

If the turnover of phosphatidic acid and phosphatidylinositol is involved in the opening of 'Ca^{2+} gates', then it would be expected that transmitters would increase Ca^{2+} influx. There is indirect evidence of this in the giant synapse of the squid, where postsynaptic influx of Ca^{2+} is induced by presynaptic electrical stimulation (Kusano *et al.*, 1975), and a small increase in Ca^{2+} influx caused by carbamoylcholine has been demonstrated directly in the parotid gland (Putney, 1976). It has also been demonstrated that in mouse fibroblast-cell cultures carbamoylcholine causes an Na^+-dependent increase in Ca^{2+} influx into myotubes (Stallcup & Cohn, 1976).

Depolarizing concentrations of K^+ cause an increase in Ca^{2+} influx, and it might be expected that similar conditions would cause an increase in the turnover of phosphatidylinositol and phosphatidic acid. Such an increase has been demonstrated previously in synaptosomes (Hawthorne & Bleasdale, 1975), although we found that the increase caused by 35mM-K^+, which had been reported as optimal, caused considerably less stimulation than 100μM-carbamoylcholine. We found that pentobarbital blocked the K^+-stimulated increase in ^{32}P incorpor-

ation, indicating that barbiturates block a step common to both K^+- and carbamoylcholine-stimulated phospholipid metabolism. Veratridine is also a depolarizing agent that causes an increase in Ca^{2+} influx in synaptosomes (Blaustein & Ector, 1975). However, veratridine did not significantly increase the incorporation of ^{32}P into phosphatidic acid or phosphatidylinositol (Table 4), but did partially prevent the carbamoylcholine effect of phosphatidylinositol. The difference between K^+ and veratridine may be that the latter is thought to act directly on opening Na^+ channels, which results in a subsequent Ca^{2+} influx, and as the effect of veratridine is blocked by tetrodotoxin, but not by antagonists (Catterall & Nirenberg, 1973), it is thought to have no effect on receptors.

The results of the experiments described above indicate that it is possible that the breakdown of phosphatidylinositol may indeed be associated with the opening of 'Ca^{2+} gates', and this is supported by the similarity between the range of concentrations of pentobarbital used here to inhibit the stimulation of phosphatidic acid and phosphatidylinositol turnover by 0.1 mM-carbamoylcholine and the ID_{50} for pentobarbital (0.47 mM), reported by Blaustein & Ector (1975) for the K^+-stimulated increase in Ca^{2+} influx.

It is of interest to consider whether these effects are related to the mechanism of anaesthesia, or any other known physiological effect of these compounds. First, we may compare the concentrations of the barbiturates used here with the anaesthetic doses of these agents. The anaesthetic dose of pentobarbital in tadpoles at 25°C is 0.16 mM (Lee-son *et al.*, 1975), which is considerably lower than that which inhibits ^{32}P incorporation (Table 1). However, the stimulated ^{32}P incorporation was comparatively sensitive to phenobarbital, and was almost completely inhibited by 1 mM (Table 2), which is about one-third of the anaesthetic dose in tadpoles (Lee-son *et al.*, 1975). Furthermore, no significant inhibition was observed with 0.2 mM-thiopental, which is 6 times the anaesthetic dose in tadpoles. The increased ^{32}P uptake was also relatively insensitive to (+)- and (−)-5-ethyl-*N*-methyl-5-propylbarbiturate. Moreover, diphenylhydantoin, which is not an anaesthetic, also specifically inhibits the carbamoylcholine-stimulated increase in ^{32}P uptake. This lack of correlation suggests that this effect is not involved in the mechanism of action of anaesthesia. Other recent evidence in agreement with this conclusion is that the fast nicotinic excitatory postsynaptic potential in frog ganglia is blocked by low concentrations of pentobarbital, but the slow muscarinic inhibitory postsynaptic potential is not affected until the concentration is raised approx. 10-fold (Nicoll, 1978).

If the effects of barbiturates on the stimulated increase of ^{32}P uptake are not involved in the mechanism of action of anaesthesia, it is possible that the phospholipid effect may be associated with the anti-convulsant properties of barbiturates and diphenylhydantoin. The relatively potent effect of phenobarbital suggests that this may be so, but it would have to be assumed that 5-(1,3-dimethylbutyl)-5-ethylbarbiturate has both convulsant and anti-convulsant properties. As 5-(1,3-dimethylbutyl)-5-ethylbarbiturate caused an increase in ^{32}P uptake at low concentrations, and inhibited it at higher concentrations, our results indicate that this may be possible.

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