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EFFECTS OF PENTOBARBITAL AND VERATRIDINE ON PHOSPHATIDYLINOSITOL AND PHOSPHATIDATE METABOLISM IN RAT PAROTID ACINAR CELLS

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Abstract—The metabolism of phosphatidylinositol and phosphatidate was studied in isolated rat parotid cells, incubated in a physiological buffer containing [^{32}P]phosphate or [^{3}H]glycerol. Carbamylcholine and epinephrine stimulated ^{32}P incorporation into both of these phospholipids, causing their half-maximal effects at 2 and 0.8 μM respectively. The former concentration is much lower than that anticipated from binding studies. The Hill coefficients for carbamylcholine activation of ^{32}P incorporation were 0.61 ± 0.05 for phosphatidate and 0.64 ± 0.05 for phosphatidylinositol. Pentobarbital (0.58 mM) inhibited the increased ^{32}P incorporation caused by 5 μM carbamylcholine but not 100 μM carbamylcholine. Pentobarbital inhibited the incorporation of ^{3}H equally in the presence and absence of epinephrine, indicating that the effect of pentobarbital on ^{32}P incorporation is on turnover and not on *de novo* synthesis. Veratridine (200 μM) had no effect on phospholipid metabolism in the presence and absence of either carbamylcholine or epinephrine, which contrasts with our previous findings in synaptosomes [J. C. Miller and I. Leung, *Biochem. J.* **178**, 9 (1979)].

The increase in turnover of phosphatidylinositol and phosphatidate is a well established phenomenon associated with the stimulation of several receptors, including muscarinic and α_1-adrenergic receptors [1]. However, there are some important differences in the metabolism of these phospholipids in different tissues. For example, in many tissues including parotid gland [2, 3], adrenal medulla [4], lacrimal gland [5] and vas deferens [6], the incorporation of ^{32}P into phosphatidylinositol and phosphatidate is independent of the presence of calcium and is not affected by EGTA.† However, in other tissues such as synaptosomes [7, 8], pineal cells [9] and neutrophils [10], the effect is dependent on the presence of calcium. Other major differences have been observed in the relative stimulation of ^{32}P incorporation into these lipids in the changes in concentration of phosphatidylinositol and phosphatidate. Thus, in parotid gland the major increase in ^{32}P incorporation during prolonged stimulation with carbamylcholine is into phosphatidylinositol which is accompanied by a drop in phosphatidylinositol concentration [11]. In comparison, carbamylcholine causes more ^{32}P incorporation into phosphatidate than phosphatidylinositol in synaptosomes. Furthermore, loss of ^{32}P from phosphatidate and from di- and triphosphoinositide but not phosphatidylinositol can be demonstrated in pre-labeled synaptosomes [12].

In a previous report, we demonstrated that barbiturates block muscarinically stimulated ^{32}P incorporation into phosphatidylinositol and phosphatidate in synaptosomes [13] and, recently, similar results have been reported by Aly and Abdel-Latif [14]. Since barbiturates are known to affect Ca^{2+} fluxes [15, 16], it is possible that the effects we observed were, in fact, secondary to the effect of barbiturates on Ca^{2+} flux, rather than acting directly on the enzymes involved in phospholipid metabolism. To answer this question, we repeated similar experiments on the effect of pentobarbital on the stimulated ^{32}P incorporation into phosphatidylinositol and phosphatidate in isolated parotid cells. We found that pentobarbital acts in a similar way in both parotid cells and synaptosomes, indicating that it does not act indirectly through its effect on Ca^{2+} fluxes.

METHODS

The methods used for the preparation of parotid cells and for ^{32}P incorporation experiments were as previously described [17]. Briefly, rats were killed by cervical dislocation, and the parotid glands were removed, chopped, and digested with collagenase and hyaluronidase. The cells were isolated by centrifugation and incubated in a physiological salt solution buffered to pH 7.4 with 25 mM HEPES or a modified Krebs–Ringer bicarbonate [13] containing 20 μCi/ml [^{32}P]phosphate and 2% bovine serum albumin. Cell integrity was checked by trypan blue exclusion. The lipids were extracted and separated by thin-layer chromatography, and the ^{32}P incorporation into the lipids was measured by liquid scintillation spectroscopy.

Verification of purity and activity of veratridine.

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† Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetra-acetic acid; and HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid.

Veratridine was found to be chromatographically pure by thin-layer chromatography in ethanol–glacial acetic acid (96:4, v/v), and 100 μM was found to be physiologically active in a lobster nerve preparation. It was dissolved as previously described [13].

Equilibrium dialysis of pentobarbital. Bovine serum albumin was included in these experiments to maintain the integrity of the cells. However, bovine serum albumin is known to adsorb barbiturates and, therefore, we used equilibrium dialysis to estimate the concentration of free pentobarbital. Pentobarbital was dissolved in the physiological buffer containing 2% bovine serum albumin, and a trace of [^{14}C]pentobarbital was added. Two dilutions of this were made, and all were dialyzed overnight at 4° in 10 vol. of buffer containing no bovine serum albumin. The ratio of the counts inside and outside the dialysis bag was used to estimate the concentration of free pentobarbital.

RESULTS

Concentration dependence of the carbamylcholine and epinephrine effects. The concentration of carbamylcholine required for 50% of the maximum stimulation of ^{32}P incorporation (EC_{50}) was 2 μM (Fig. 1). This is about one-tenth that anticipated from the K_d measured in muscarinic binding studies [18]. Conversely, the EC_{50} for epinephrine was 0.8 μM (Fig. 1) which is comparable to the K_d obtained from binding studies [19]. Results similar to these have been published by Weiss and Putney [20] who have suggested that there are spare muscarinic receptors for methacholine in parotid cells. The slope of the response curve for carbamylcholine was low; the Hill plots for carbamylcholine and epinephrine are shown in Fig. 2. The Hill coefficient for carbamylcholine was 0.61 ± 0.05 for phosphatidate and 0.64 ± 0.05 for phosphatidylinositol, indicating more than one binding site activating phospholipid turnover. Similarly, multiple binding sites were observed in muscarinic binding studies. The Hill coefficients for epinephrine were closer to 1 (1.01 ± 0.12 for phosphatidate and 0.86 ± 0.06 for phosphatidylinositol).

The maximum effect of epinephrine was smaller than that for carbamylcholine, and the effects were not additive (Table 1). This indicates that the same populations of lipids were involved and that there were both carbamylcholine and epinephrine receptors on the same cells. Similar differences in the degree of stimulation between methacholine and epinephrine have been reported previously [20].

Effect of pentobarbital. In previous studies with synaptosomes, we had demonstrated that pentobarbital inhibits the ^{32}P incorporation that is stimulated by 100 μM carbamylcholine [13]. When parotid cells were incubated with this concentration of carbamylcholine, however, we were unable to demonstrate any inhibition by pentobarbital (Table 2). However, pentobarbital markedly inhibited ^{32}P incorporation stimulated by 5 μM carbamylcholine (Table 2). As demonstrated above, the EC_{50} for carbamylcholine in parotid cells was much lower than that observed in synaptosomes [21]. This suggests that the pentobarbital effect may be overcome by stimulation of spare receptors or by a competitive effect.

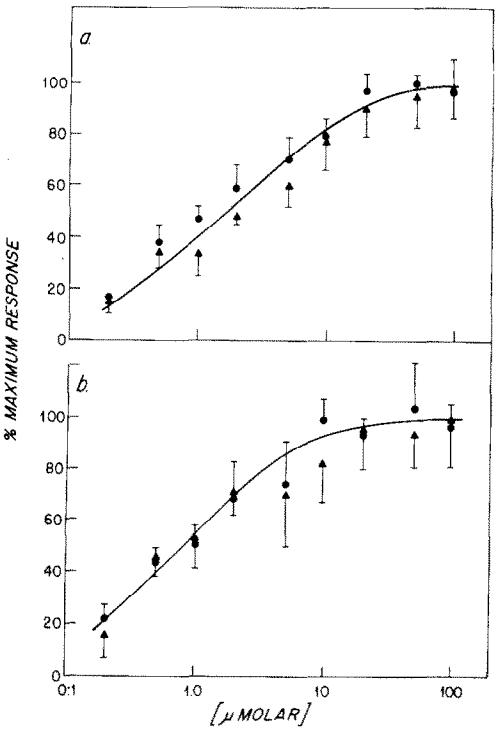

Fig. 1. Concentration dependence of the carbamylcholine and epinephrine effects. Preparations of parotid acinar cells were preincubated for 20 min in a physiological salt solution buffered with HEPES (see Methods) containing 20 μCi/ml [^{32}P]phosphate before the addition of (a) carbamylcholine or (b) epinephrine. In panel a, the combined results from three cell preparations are shown, two of which were incubated with carbamylcholine for 30 min and one for 10 min. The maximum increases in ^{32}P incorporation in these preparations were 727, 435 and 468%, respectively, for phosphatidate and 1341, 953 and 603% for phosphatidylinositol. In each preparation, the carbamylcholine concentrations were repeated in triplicate; each point represents the mean and standard deviation where N = 6–9. Key: (▲) phosphatidate, and (●) phosphatidylinositol. In panel b, one cell preparation was incubated fo 10 min with epinephrine. The maximum increases in ^{32}P incorporation were 408 and 642% into phosphatidate and phosphatidylinositol respectively. Each point represents the mean and standard deviation where N = 3. Key: (▲) phosphatidate, and (●) phosphatidylinositol.

Effect of epinephrine and pentobarbital on [^{3}H]glycerol incorporation into phospholipids. As we observed an inhibitory effect of 0.58 mM pentobarbital on ^{32}P incorporation in the absence of carbamylcholine (Table 2), we wished to establish whether this effect was on head group turnover or *de novo* synthesis. To do this, we treated parotid cells with pentobarbital in the presence of [^{3}H]glycerol. Pentobarbital inhibited ^{3}H incorporation into the phospholipids (Table 3) to a degree similar to the inhibition of ^{32}P incorporation by pentobarbital alone (Table 2). This indicates that the inhibition of ^{32}P incorporation in the absence of agonist was an inhibition of *de novo* synthesis.

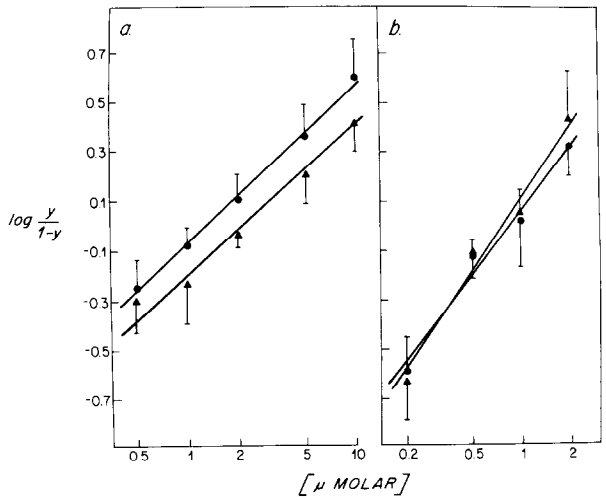

Fig. 2. Hill plots of the carbamylcholine and epinephrine effects. The data from Fig. 1 were analyzed further. (a) Carbamylcholine. The Hill coefficient for phosphatidylinositol (●) was 0.64 ± 0.05 and for phosphatidate (▲) 0.61 ± 0.05. (b) Epinephrine. The Hill coefficient for phosphatidylinositol (●) was 0.86 ± 0.06 and for phosphatidate (▲) 1.01 ± 0.12.

Table 1. Combined effects of carbamylcholine and epinephrine*

	^{32}P incorporation (cpm ± S.D.)	
	Phosphatidate	Phosphatidylinositol
Control	300 ± 18	547 ± 56
30 μM Epinephrine	890 ± 44	1858 ± 223
100 μM Carbamylcholine	1333 ± 96	3849 ± 236
30 μM Epinephrine and 100 μM carbamylcholine	1193 ± 152	3212 ± 221

* The incorporation of ^{32}P was measured in phosphatidylinositol and phosphatidate after their extraction from parotid cells that had been incubated for 30 min at 37° in a modified Krebs–Ringer bicarbonate buffer, pH 7.4, containing 2% bovine serum albumin, as described in Methods. The samples were repeated in triplicate.

Table 2. Effect of pentobarbital on carbamylcholine-stimulated ^{32}P incorporation*

	^{32}P incorporation (% of relevant control)	
	Phosphatidate	Phosphatidylinositol
Control	100 ± 11	100 ± 16
0.33 mM Pentobarbital	111 ± 7	111 ± 11
0.58 mM Pentobarbital	81 ± 9	77 ± 10
5 μM Carbamylcholine	461 ± 82	1356 ± 197
5 μM Carbamylcholine + 0.33 mM pentobarbital	254 ± 39†	937 ± 90‡
100 μM Carbamylcholine	491 ± 72	1441 ± 33
100 μM Carbamylcholine + 0.33 mM pentobarbital	466 ± 41	1371 ± 109
100 μM Carbamylcholine + 0.58 mM pentobarbital	420 ± 49	1477 ± 214

* Parotid acinar cells were incubated for 30 min in a physiological salt solution buffered with HEPES. Bovine serum albumin adsorbs pentobarbital; the concentrations above are the estimated free pentobarbital concentrations as determined by equilibrium dialysis. The total concentrations were 0.6 and 1.0 mM respectively. The results are expressed as the percent increase in ^{32}P incorporation over that in the absence of carbamylcholine and are shown as mean ± S.D. (N = 3). The significance of the effect of pentobarbital was estimated by 2 × 2 factorial variance analysis.

† $P < 0.02$. ‡ $P < 0.01$.

Table 3. Effect of epinephrine and pentobarbital on [^{3}H]glycerol incorporation into phospholipids*

	[^{3}H]Gylcerol incorporation (% of control)		
	Phosphatidylcholine	Phosphatidylinositol	Phosphatidate
Control	100 ± 13	100 ± 14	100 ± 4
0.33 mM Pentobarbital	88 ± 3	81 ± 19	81 ± 19
0.58 mM Pentobarbital	63 ± 3†	45 ± 17‡	45 ± 8†
30 μM epinephrine	75 ± 9‡	96 ± 6	86 ± 15
0.33 mM Pentobarbital and 30 μM epinephrine	72 ± 9‡	99 ± 19	80 ± 22
0.58 mM Pentobarbital and 30 μM epinephrine	52 ± 7†	53 ± 10†	44 ± 6†

* Parotid acinar cells were incubated under the same conditions as in Table 1, except that the buffer contained 20 μCi/ml of [^{3}H]glycerol. The concentration of pentobarbital shown is the free concentration estimated by equilibrium dialysis. The significance of the difference from the control was estimated by Student's *t*-test (N = 3).
† $P < 0.01$.
‡ $P < 0.05$.

It is interesting to note that epinephrine inhibited the ^{3}H incorporation into phosphatidylcholine. This may explain the apparent stimulation of ^{3}H incorporation into phosphatidylinositol relative to other lipids reported previously [22].

Effect of veratridine. Veratridine had no effect on incorporation of ^{32}P into either phosphatidate or phosphatidylinositol in parotid cells (Table 4). This is in contrast to the effect demonstrated in synaptosomes [13], where ^{32}P incorporation into phosphatidylinositol was inhibited by veratridine in the presence of carbamylcholine.

DISCUSSION

In this project, we set out to establish whether or not the effect of barbiturates on the muscarinically stimulated turnover of phosphatidylinositol and phosphatidate in synaptosomes was secondary to their effect on Ca^{2+} fluxes. We report here that pentobarbital did inhibit the muscarinically stimulated ^{32}P incorporation in parotid cells, but that the inhibition was overcome by high concentrations of carbamylcholine.

There is one notable difference in the phospholipid effect in parotid cells and in synaptosomes which may help to explain the differences we have observed in these two preparations. The dose–response curve for the muscarinically stimulated ^{32}P incorporation into phosphatidate and phosphatidylinositol was lower than that of ligand binding for parotid cells (Fig. 1, [20]), whereas it is similar to that of ligand binding in synaptosomes [21]. Weiss and Putney [20] suggested that this may be explained by the presence of "spare" receptors for the phospholipid effect in parotid cells; this was based on the similarity between the dose–response curves for ^{32}P incorporation and K^+ release, and the demonstration of spare receptors for K^+ release in this tissue [23]. In contrast, there appear to be no "spare" adrenergic receptors, as the stimulation of ^{32}P incorporation and K^+ release was similar to receptor binding (Fig. 1, [20]).

The effect of pentobarbital in parotid cells was overcome when the cells were stimulated with 100 μM carbamylcholine (Table 2). However, inhibition persists when synaptosomes are stimulated with the same concentration of carbamylcholine [13]. Therefore, if we assume that there is a common mechanism, the most likely explanation of this is that the inhibition is overcome by the stimulation of the spare receptors. This, in turn, suggests that pentobarbital may act by binding to the receptor, or by

Table 4. Effect of veratridine on carbamylcholine and epinephrine stimulation of ^{32}P incorporation*

	^{32}P incorporation (% of control)	
	Phosphatidate	Phosphatidylinositol
Control	100 ± 2	100 ± 15
200 μM Veratridine	99 ± 27	110 ± 11
30 μM Epinephrine	236 ± 39	538 ± 31
200 μM Veratridine and 30 μM epinephrine	246 ± 65	570 ± 108
100 μM Carbamylcholine	322 ± 54	775 ± 66
200 μM Veratridine and 100 μM carbamylcholine	320 ± 71	847 ± 18

* Parotid acinar cells were incubated under the same conditions as those in Table 1.

preventing the formation of an activated receptor–effector complex.

The maximum stimulation of ^{32}P incorporation into phosphatidylinositol and phosphatidate was considerably greater for carbamylcholine than for epinephrine. This difference cannot be explained by the difference in numbers of receptors on the cell, which have been estimated as 23,000 muscarinic sites/cell [24] and 15,000 sites/cell [19], particularly if there is a 10-fold excess of muscarinic receptors. One possible explanation is that there is a limited amount of an enzyme which becomes tightly coupled to form a muscarinic receptor–carbamylcholine–enzyme complex, whereas an α-adrenergic receptor–epinephrine–enzyme complex is not so tightly coupled, resulting in a shorter time of stimulated activity and thus less rapid phospholipid turnover.

Another difference in the phospholipid effect in synaptosomes and parotid cells was demonstrated by veratridine. This agent had no effect on the ^{32}P incorporation into phosphatidylinositol or phosphatidate in parotid cells (Table 3), although in synaptosomes inhibition of carbamylcholine-stimulated ^{32}P incorporation in phosphatidylinositol (but not phosphatidate) was observed. Veratridine stimulates the entry of Ca^{2+} into cells and, on the assumption that this occurred under the conditions here, suggests that Ca^{2+} influx does not have any effect on phospholipid metabolism in parotid cells. Similar conclusions have been drawn from previous studies in which changes in the ionic composition of the external solution had no effect on the phospholipid effect in parotid tissue fragments [3].

The results discussed above confirm that there are differences between the phospholipid effects in parotid cells and in synaptosomes, in their sensitivities to carbamylcholine and to veratridine. However, it seems likely that pentobarbital acts in a similar manner in both of these tissues and that the effect of barbiturates in synaptosomes is not secondary to Ca^{2+} fluxes.

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