Phospholipid Metabolism and Adrenal Medullary Activity

I. The Effect of Acetylcholine on Tissue Uptake and Incorporation of Orthophosphate-^{32}P into Nucleotides and Phospholipids of Bovine Adrenal Medulla

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SUMMARY

Bovine adrenal medullary slices were incubated at 30° in Locke's solution containing orthophosphate-^{32}P or glycerol-1-^{14}C. ^{32}P was incorporated into all individual phospholipids, but at different rates. The highest specific activity observed was in phosphatidylinositol, followed by phosphatidic acid, phosphatidylcholine, phosphatidylserine, lysophosphatidylcholine (lysolecithin), sphingomyelin, and phosphatidylethanolamine.

Acetylcholine (10^{-5} M) in the presence of eserine (10^{-5} M) produced a 3-fold increase in catecholamine release and stimulated the incorporation of ^{32}P into phosphatidic acid (3.4-fold), phosphatidylinositol (2.7-fold), and phosphatidylcholine (1.4-fold).

The uptake of orthophosphate-^{32}P into the chromaffin tissue, as well as the specific activities and tissue levels of orthophosphate and nucleotides, were not modified upon acetylcholine stimulation.

Glycerol-1-^{14}C was incorporated into all the individual phospholipids, but, in contrast to ^{32}P incorporation, acetylcholine stimulation did not increase the incorporation of glycerol-1-^{14}C into phospholipids.

No differences were observed in the total and individual phospholipid contents between control and stimulated slices.

The time course of the ^{32}P incorporation into phosphatidic acid and phosphatidylinositol in response to acetylcholine stimulation suggested that phosphatidic acid acts as a precursor in the synthesis of phosphatidylinositol in this tissue.

The possible mechanism of the action of acetylcholine on the phospholipid turnover of the adrenal medulla is discussed.

INTRODUCTION

It has been demonstrated that acetylcholine stimulates the incorporation of ^{32}P into phospholipids of the adrenal medulla of guinea pigs (1). ^{32}P incorporation into phospholipids is also increased in tissues other than the adrenal medulla where acetylcholine seems to be the physiological transmitter (2–7). In view of the above observations, it has been postulated that phospholipids may play an active role in the secretion of amines from the adrenal medulla (1), as well as in the secretion or active transport of other substances from organs where acetylcholine exerts its action (2–4). But there is still much controversy about the correlation between acetylcholine-induced secretion and acetylcholine-stimulated ^{32}P incorporation into phospholipids. Since nothing is known of the rate of ^{32}P uptake into the adrenal medulla or

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its rate of incorporation into nucleotides, it was necessary to study these parameters of phosphorus metabolism before attempting to correlate the metabolic effect of acetylcholine on phospholipid metabolism with the acetylcholine-evoked catecholamine secretion.

This is a report of the effect of acetylcholine stimulation on the tissue uptake and incorporation of ^{32}P into nucleotides and phospholipids of the bovine adrenal medulla.

METHODS

Adult bovine adrenal glands were placed on ice after their removal in the slaughterhouse and were used within 40 min. The medullae were separated from the cortices. Three to four slices were obtained from each medulla by means of a Stadie-Riggs slicer. Each slice was incubated at 30° in a stoppered 25-ml Erlenmeyer flask which contained 3 ml of Locke's solution of the following composition: NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2.2 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 6.0 mM; and glucose, 10.0 mM. The medium had 95% O_2 + 5% CO_2 as the gas phase, which was renewed each time the flasks were opened for additions or transfers. The flasks were shaken during the incubations. ^{32}P was added to the medium as orthophosphate-^{32}P (specific activity, 10.2 mCi/μmole). When acetylcholine was present in the incubation medium, eserine was also added. The incubation times, as well as ^{32}P, glycerol-1-^{14}C, acetylcholine, and eserine concentrations are given in the legends for each table and figure. The average weight of the slices was 110.9 ± 2.4 mg. At the end of the incubation period, the slices were removed from the incubation flasks and frozen in dry ice The tissue was homogenized in 5% trichloracetic acid, and the acid-soluble and acid-insoluble materials were separated by centrifugation. Aliquots of the acid-soluble material were extracted with isobutyl alcohol, and the inorganic phosphate content was measured by the technique of Martin and Doty (8). The radioactivity was determined in a liquid scintillation counter (Nuclear-Chicago) using the following scintillation fluid: Nuclear-Chicago solubilizer, 1 ml; and 0.5% 2,5-diphenyloxazole in toluene, 14 ml. The volumes of the isobutyl alcohol aliquots varied between 100 and 200 μl. The nucleotide fraction of the acid-soluble extracts was separated by absorption on Norit A according to the technique described by Crane and Lipmann (9). The nucleotides absorbed on Norit A were hydrolyzed for 10 min at 100° in 1 N HCl. After boiling, the charcoal was removed by filtration on Millipore filters. The filtrate thus obtained was neutralized by the addition of silicate-free 1 N NaOH and assayed for phosphorus content and radioactivity as indicated above. The acid-insoluble material was extracted with chloroform–methanol (2:1) containing 0.25% HCl. The chloroform–methanol–HCl extracts were shaken with 0.2 volume of cold KCl (0.8%), and the upper and lower phases were allowed to separate. The upper, aqueous phase was removed and discarded; then the interphase was washed twice with "theoretical upper phase" solution (10). Finally, the lower phase was made up to a constant volume with methanol. Aliquots of the lower phase were taken for determinations of total lipid phosphorus and total lipid radioactivity. The remaining lower phase was evaporated to dryness under vacuum. The phospholipids were resuspended in chloroform–methanol and spotted on thin layer plates. Silica gel G plates 0.25 mm thick were used, and the individual phospholipids were separated by a two-dimensional technique. The composition of the solvents used was as follows: solvent I, chloroform–methanol–7 N ammonium hydroxide (60:35:5); solvent II, chloroform–methanol–7 N ammonium hydroxide (35:60:5) (11). The spots were detected and identified as described elsewhere (12). After chromatography, the plates were dried at room temperature, covered with a plastic film, stapled to sheets of Kodak X-ray film, and placed in film holders. After 2–4 days of exposure, the films were developed. The different phospholipid spots were scraped off the plates, and the phospholipids were eluted with solvents I and II. The phosphorus content of individual phospholipids was determined by the method indicated. Aliquots

were also added to scintillation vials for determination of the ^{32}P radioactivity. Corrections for 100% efficiency were made in all samples. Aliquots of the incubation medium taken at different intervals of time were treated with perchloric acid (0.4 N) and centrifuged at 150,000 $g \times$ min. Catecholamines were determined in the resultant supernatant fluids by the trihydroxyindole fluorometric method (13).

Acetylcholine and eserine were obtained from Calbiochem. Norit A was purchased from Fisher Scientific Company, and 2,5-diphenyloxazole was obtained from New England Nuclear Corporation. Orthophosphate-^{32}P was purchased from Charles Frost Company. Glycerol-1-^{14}C was obtained from Nuclear-Chicago, Inc.

RESULTS

Incorporation of orthophosphate-^{32}P into the phospholipids of the adrenal medulla as a function of time. Bovine adrenal medullary slices were incubated with orthophos-

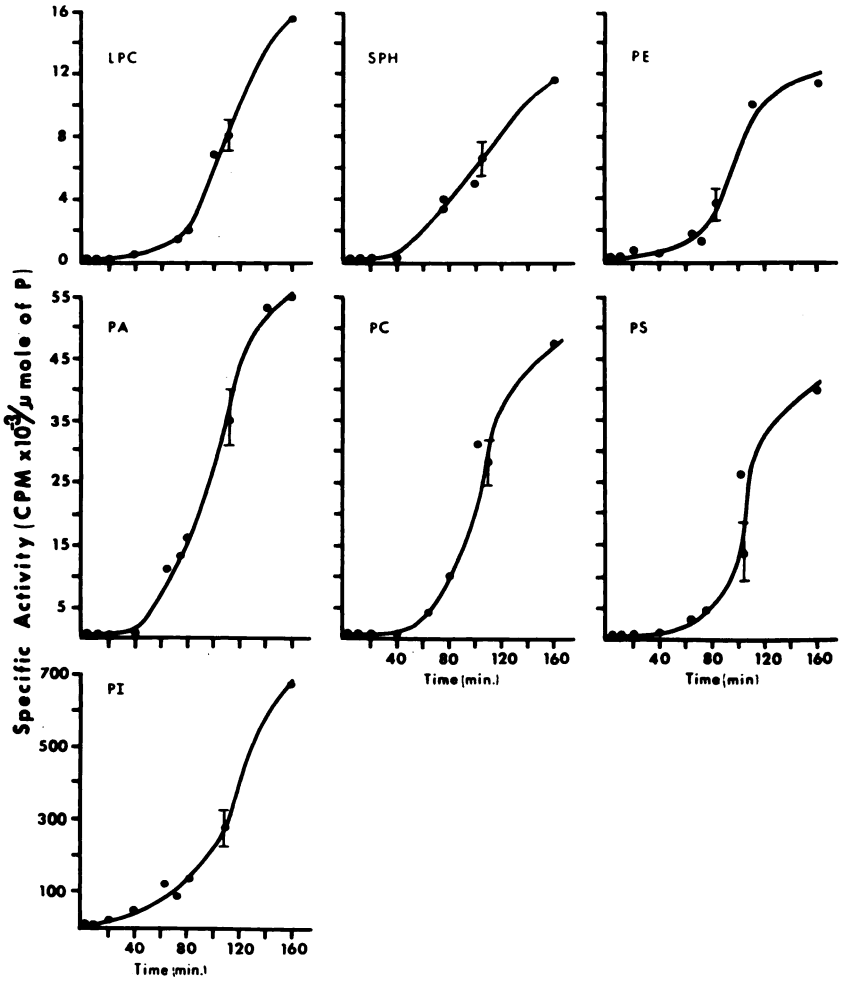

FIG. 1. *Specific activity time-curves of individual phospholipids of the adrenal medulla*

Adrenal medullary slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 160 min. Slices were removed from the medium at different intervals of time, and the specific activities of the individual phospholipids were determined as described in METHODS. Each point is the average of three or four individual values. LPC, lysophosphatidylcholine; SPH, sphingomyelin; PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

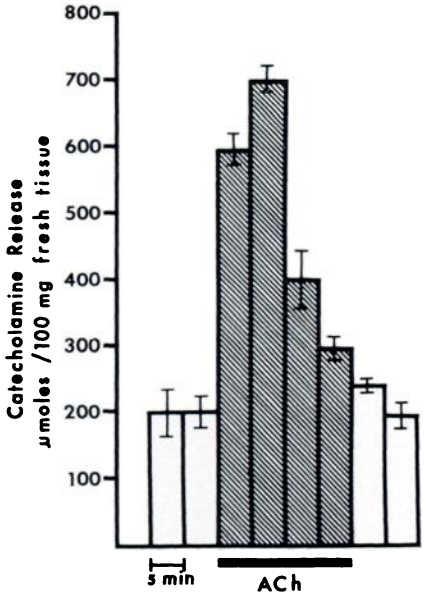

FIG. 2. *Release of catecholamines from bovine adrenal medullary slices in the presence of acetylcholine (ACh) (10^{-5} M) plus eserine (10^{-5} M)*

The slices were incubated *in vitro* for 60 min prior to the addition of acetylcholine plus eserine and were incubated for 20 min after this addition. After the stimulation period, which is indicated by the horizontal bar, the slices were placed in a medium devoid of acetylcholine and eserine.

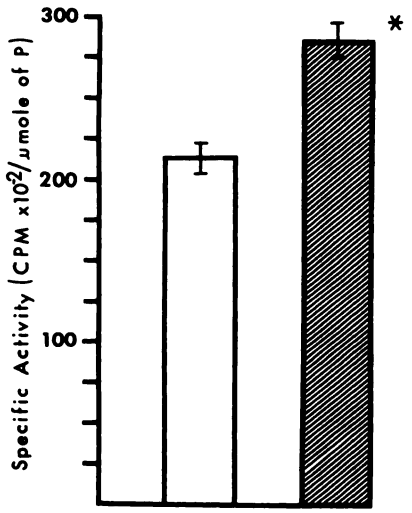

FIG. 3. *Specific activity of total lipid extract*

Adrenal medullary slices were incubated with orthophosphate-^{32}P (10 μCi/ml) for 60 min before the addition to the incubation medium of acetylcholine (10^{-5} M) plus eserine (10^{-5} M), and for 20 min after this addition. The open bar represents the mean ± standard error of results obtained with six control slices, and the shaded one, the mean ± standard error of six stimulated slices. The asterisk denotes $p < 0.001$.

phate-^{32}P (50 μCi/ml) for 160 min. Slices were removed from the incubation medium at different intervals of time, and the specific activities of the individual phospholipids were determined as indicated in METHODS.

It can be seen in Fig. 1 that there were no appreciable changes in the specific activities of any of the phosphatides during the first 40 min of incubation, but the specific activity of all phospholipids rose sharply after this period of time. Phosphatidylinositol showed the highest specific activity, followed by phosphatidic acid, phosphatidylcholine, phosphatidylserine, lysophosphatidylcholine, sphingomyelin, and phosphatidylethanolamine.

Increased ^{32}P incorporation into phospholipids of the adrenal medulla during stimulation by acetylcholine. Slices were incubated with orthophosphate-^{32}P for 60 min; then acetylcholine (10^{-5} M) in the presence of eserine (10^{-5} M) was added to half the vessels, and the incubation was continued 20 min longer. A 2.5–3-fold stimulation of catecholamine release was obtained under these experimental conditions (Fig. 2). Acetylcholine stimulation produced a significant increase in the specific activity of the total lipid extract (Fig. 3). The increased ^{32}P incorporation due to acetylcholine was not observed in all the individual phospholipids. Acetylcholine stimulated the incorporation of ^{32}P into phosphatidic acid, phosphatidylinositol, phosphatidylcholine, and the phosphorus-containing spot that remained at the origin of the chromatograms (Table 1). There was an increase in the specific activities of the same phospholipids (Table 2). The changes observed after stimulation by acetylcholine were not due to changes in the total or individual phospholipid contents, as depicted in Table 3. The relatively large content of lysolecithin, which confirms previous findings, can also be seen in this table (12, 14, 15). The lysolecithin was

TABLE 1

^{32}P radioactivity in phospholipids in the presence and absence of acetylcholine plus eserine

Adrenal medullary slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 60 min before the addition to the incubation medium of acetylcholine (10^{-5} M) plus eserine (10^{-5} M) and for 20 min after this addition. The radioactivity shown accounted for 97.3 ± 5.0% of the total radioactivity in the lipid extracts.

Phospholipid	Control	Acetylcholine + eserine	p
	(cpm × 10^{-3})/g fresh medulla		
Lysophosphatidylcholine	36 ± 6[a] (7)[b]	40 ± 7 (10)	
Sphingomyelin	56 ± 7 (6)	61 ± 8 (10)	
Phosphatidylserine	30 ± 6 (7)	32 ± 4 (10)	
Phosphatidylcholine	340 ± 44 (6)	493 ± 62 (9)	<0.001
Phosphatidylinositol	1,465 ± 173 (7)	3,835 ± 224 (10)	<0.001
Phosphatidylethanolamine	26 ± 5 (7)	30 ± 5 (9)	
Phosphatidic acid	25 ± 6 (7)	92 ± 10 (10)	<0.005
Origin	7,375 ± 87 (7)	12,272 ± 200 (9)	<0.05

[a] Mean ± standard error.

[b] The number of experiments is indicated in parentheses.

especially concentrated in the chromaffin granule membrane (12, 15).

Uptake of orthophosphate-^{32}P and specific activity of nucleotide in the presence and absence of acetylcholine. To determine whether the action of acetylcholine in increasing the ^{32}P labeling of the phospholipids was due to its effect on the turnover of phosphorus in these lipids rather than to secondary changes in the specific activity of precursors, tissue slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 100 min in the presence and absence of acetylcholine (10^{-5} M) plus eserine (10^{-5} M). Figure 4 shows that the time course of the uptake of orthophosphate-^{32}P into the medullary tissue was not modified by acetylcholine stimulation. It can also be seen that after some of the slices had been transferred to a nonradioactive medium there was a parallel drop in the ^{32}P content of the tissue in both control and acetylcholine-stimulated slices. The first and faster rate of ^{32}P uptake and the earlier and rapid loss after the slices had been placed in a nonradioactive medium might have been due to diffusion into and out of the extracellular space of the chromaffin

TABLE 2

Specific activity of individual phospholipids in the presence and absence of acetylcholine plus eserine

Adrenal medullary slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 60 min before the addition of acetylcholine (10^{-5} M) plus eserine (10^{-5} M) to the incubation medium, and for 20 min after this addition. The radioactivity shown accounted for 97.3 ± 5.0% of the total radioactivity in the lipid extracts.

Phospholipid	Control	Acetylcholine + eserine
	(cpm × 10^{-3})/μg P	
Lysophosphatidylcholine	37 ± 6.5[a] (7)[b]	40 ± 6.1 (7)
Sphingomyelin	68 ± 8.3 (7)	65 ± 5.4 (7)
Phosphatidylserine	164 ± 13 (7)	154 ± 12 (7)
Phosphatidylcholine	140 ± 15 (7)	202 ± 21 (7)
Phosphatidylinositol	1,004 ± 98 (7)	2,709 ± 108 (7)
Phosphatidylethanolamine	22 ± 3.5 (7)	25 ± 4.0 (7)
Phosphatidic acid	124 ± 12 (7)	422 ± 28 (7)
Origin	9,728 ± 115 (7)	14,979 ± 244 (7)

[a] Mean ± standard error.

[b] The number of experiments is indicated in parentheses.

TABLE 3

Levels of individual phospholipids in the presence and absence of acetylcholine plus eserine

Adrenal medullary slices were incubated for 60 min before the addition of acetylcholine (10^{-5} M) plus eserine (10^{-5} M) to the incubation medium, and for 20 min after this addition.

Phospholipid	Control	Acetylcholine + eserine
	μg P/g fresh tissue	
Total lipid extract	775 ± 23[a] (16)[b]	784 ± 20 (14)
Lysophosphatidylcholine	90 ± 8.0 (7)	89 ± 9.0 (7)
Sphingomyelin	79 ± 4.9 (7)	85 ± 7.8 (7)
Phosphatidylserine	18 ± 2.5 (7)	20 ± 4.9 (7)
Phosphatidylcholine	237 ± 11 (7)	234 ± 12 (7)
Phosphatidylinositol	140 ± 7.8 (7)	136 ± 10 (7)
Phosphatidylethanolamine	117 ± 9.1 (7)	115 ± 8.3 (7)
Phosphatidic acid	19 ± 3.7 (7)	21 ± 5.2 (7)
Origin	73 ± 7.4 (7)	78 ± 8.6 (7)

[a] Mean ± standard error.

[b] The number of experiments is indicated in parentheses.

tissue. Nucleotide specific activity was measured under the same experimental conditions as described above, and time course curves similar to those for orthophosphate-^{32}P were obtained (Fig. 5). Again, stimulation with acetylcholine did not produce any significant differences. After some of the slices were removed from the radioactive medium to a nonradioactive one, the nucleotide specific activity did not drop as rapidly as the orthophosphate radioactivity. The nucleotide radioactivity remained

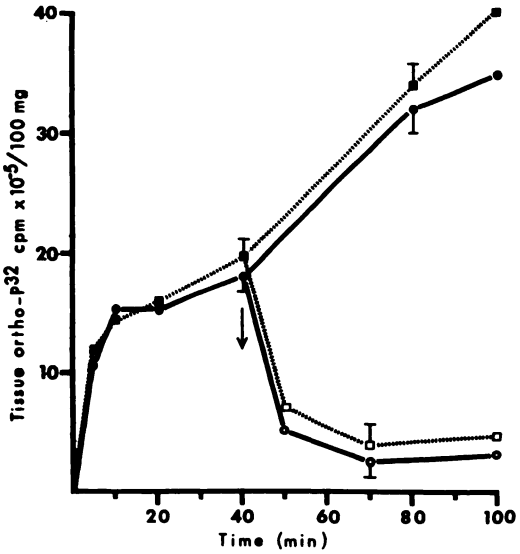

FIG. 4. *Orthophosphate-^{32}P uptake into adrenal medullary slices and loss after transfer to a nonradioactive medium*

The slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 100 min in the presence (■) or absence (●) of acetylcholine (10^{-5} M) plus eserine (10^{-5} M). After 40 min of incubation, half the slices were transferred to a nonradioactive medium without (○) or with (□) acetylcholine. The arrow indicates the time of transfer of the slices.

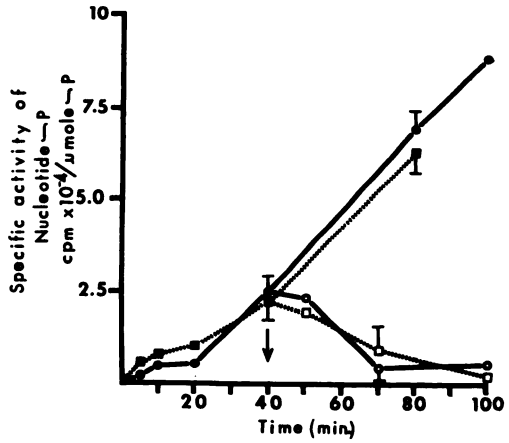

FIG. 5. *Specific activity of the nucleotide phosphorus of adrenal medullary slices before and after transfer to a nonradioactive medium*

The slices were incubated with orthophosphate-^{32}P (50 μCi/ml) in the presence (■) or absence (●) of acetylcholine (10^{-5} M) plus eserine (10^{-5} M). After 40 min of incubation, half the slices were transferred to a nonradioactive medium without (○) or with (□) acetylcholine. The arrow indicates the time of transfer of the slices.

TABLE 4

^{32}P radioactivity in phosphatidic acid and 10-min acid-hydrolyzable phosphate esters in the presence and absence of acetylcholine

Adrenal medullary slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 60 min before the addition of acetylcholine (10^{-5} M) plus eserine (10^{-5} M) to the medium, and for 20 min after this addition.

Substance	Control	Acetylcholine + eserine
	cpm/100 mg fresh medulla	
Phosphatidic acid	257 ± 50[a] (7)[b]	679 ± 63 (8)
10-min acid-hydrolyzable phosphate esters	800,300 ± 985 (5)	799,600 ± 575 (5)

[a] Mean ± standard error.

[b] The number of experiments is indicated in parentheses.

relatively constant during the first 10–15 min after the slices were transferred. These experiments were performed under conditions in which acetylcholine was present in the incubation medium throughout. Therefore, it was desirable to determine the uptake of orthophosphate-^{32}P and the specific activity of nucleotide phosphorus under the same experimental conditions in which ^{32}P incorporation into phospholipids was measured. Table 4 shows that following acetylcholine stimulation there was an increase in the radioactivity of phosphatidic acid, although the radioactivity of the 10-min-hydrolyzable phosphate esters remained unchanged. Total nucleotide and inorganic phosphorus concentrations, as well as their specific activities, were unaffected by acetylcholine stimulation (Table 5).

Time course of ^{32}P incorporation into phosphatidic acid and phosphatidylinositol in response to stimulation by acetylcholine. Since it had been shown that phosphatidic acid could serve as a precursor of phosphatidylinositol in other tissues, and that acetylcholine stimulates the conversion of phosphatidic acid to phosphatidylinositol (4), it was desirable to study the time course of ^{32}P incorporation into these two phosphatides during acetylcholine stimulation. Figure 6 shows that the radioactivity of phosphatidic acid remained unchanged during the first 10 min of exposure to acetylcholine and then rose over the next 15 min, after which it showed a small decrease with time. The phosphatidylinositol radioactivity was constant for 10 min, then increased markedly over the next 30 min.

Effect of acetylcholine on the incorporation of glycerol-1-^{14}C into phospholipids of the adrenal medulla. It is known that L-α-glycerophosphate is involved in one of the pathways leading to the formation of phosphatidic acid (2 acyl-CoA + L-α-glycerophosphate → phosphatidic acid + CoA) (16, 17). If this is the pathway by which

TABLE 5

Levels and specific activities of orthophosphate and nucleotide phosphorus in the presence and absence of acetylcholine

Adrenal medullary slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 60 min before the addition of acetylcholine (10^{-5} M) plus eserine (10^{-5} M) to the medium, and for 20 min after this addition.

Substance	Specific activity		Tissue level	
	Control	Acetylcholine + eserine	Control	Acetylcholine + eserine
	(cpm × 10^{-3})/μmole P		*μg/100 mg fresh medulla*	
Orthophosphate	3,532 ± 47.5[a] (4)[b]	3,555 ± 33.4 (4)	49.5 ± 4.6 (13)	49.7 ± 4.5 (12)
Nucleotide phosphorus	1,598 ± 22.4 (4)	1,595 ± 11.8 (5)	15.7 ± 2.2 (12)	15.1 ± 2.0 (13)

[a] Mean ± standard error.

[b] The number of experiments is indicated in parentheses.

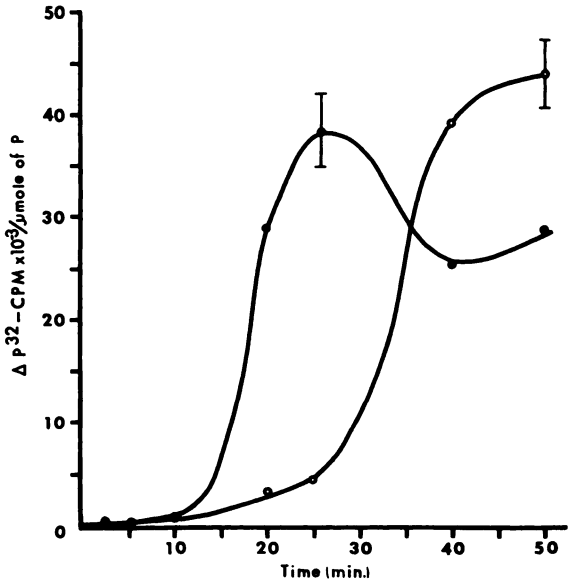

FIG. 6. *Time course of increase in ^{32}P incorporation into phosphatidic acid* (●) *and phosphatidylinositol* (○) *after stimulation by acetylcholine*

Adrenal medullary slices were incubated with orthophosphate-^{32}P (50 μCi/ml) for 60 min before the addition of acetylcholine (10^{-5} M) plus eserine (10^{-5} M) to the incubation medium. The differences in the specific activities between stimulated and control slices are plotted. Each point is the average of three or four individual experiments.

acetylcholine exerts its action of increasing the formation of phosphatidic acid, it might be expected that the incorporation of glycerol-1-^{14}C into phosphatidic acid would be stimulated in response to stimulation by acetylcholine. Table 6 shows that this was not the case. Acetylcholine stimulation did not increase the incorporation of glycerol-1-^{14}C into phospholipids of the adrenal medulla. The absence of such an effect suggests that acetylcholine stimulates the formation of a fraction of phosphatidic

TABLE 6

Effect of acetylcholine on the incorporation of glycerol-1-^{14}C into phospholipids of adrenal medulla

Adrenal medullary slices were incubated with glycerol-1-^{14}C (2.5 μmoles/ml; specific activity, 1 μCi/μmole) for 60 min before the addition of acetylcholine (10^{-5} M) plus eserine (10^{-5} M) to the incubation medium, and for 20 min after this addition.

Phospholipid	Control	Acetylcholine + eserine
	(cpm × 10^{-2})/g fresh medulla	
Total lipid extract	588 ± 6.9[a] (6)[b]	595 ± 6.5 (6)
Lysophosphatidylcholine	3.8 ± 1.3 (5)	4.0 ± 0.39 (6)
Sphingomyelin	10.8 ± 1.0 (5)	10.9 ± 2.0 (6)
Phosphatidylserine	5.8 ± 0.70 (5)	6.1 ± 1.2 (6)
Phosphatidylcholine	164 ± 17 (5)	167 ± 12 (6)
Phosphatidylinositol	275 ± 21 (5)	279 ± 23 (6)
Phosphatidylethanolamine	62.1 ± 4.2 (5)	59.9 ± 5.0 (6)
Phosphatidic acid	37.1 ± 3.8 (5)	36.6 ± 4.8 (6)
Origin	29.2 ± 6.0 (5)	30.9 ± 2.3 (6)

[a] Mean ± standard error.

[b] The number of experiments is indicated in parentheses.

acid in which the phosphate is not derived from L-α-glycerophosphate, and that there is no formation *de novo* of the diglyceride moiety. Furthermore, the results indicate that the glycerol-1-^{14}C was incorporated in a pool of phosphatidic acid which did not show any response to stimulation by acetylcholine.

DISCUSSION

Incorporation of orthophosphate-^{32}P into individual phospholipids in the control slices was observed only after 35–40 min of incubation in the presence of the radioisotope. This delay was probably due to a slow diffusion (10–15 min) of ^{32}P into the extracellular space (Fig. 4) and to slow labeling of the nucleotide pool of the chromaffin tissue. Nucleotide became significantly labeled after 25–30 min of incubation (Fig. 5). In perfusion experiments,[1] the phospholipids of the adrenal medulla became labeled after 5 min of perfusion with Locke's solution containing ^{32}P.

The greatest incorporation of the isotope occurred into phosphatidylinositol, and this observation is in agreement with findings in other organs (18–21). In contrast to other tissues, the specific activity of phosphatidylserine was higher than that of phosphatidylethanolamine. This difference could probably be explained by the observation of Borkenhagen *et al.* (22), who showed that phosphatidylserine is converted into phosphatidylethanolamine in liver homogenates.

The data presented in this paper, showing that upon acetylcholine stimulation there was an increased incorporation of ^{32}P into phospholipids of the bovine adrenal medulla, particularly into phosphatidic acid and phosphatidylinositol, corroborate the early observation of Hokin *et al.* (1) in guinea pig adrenal medulla. Furthermore, the present results show that this increased ^{32}P labeling of phospholipids was due to an increase in the turnover of both phosphatides, rather than to increased uptake of ^{32}P into tissue (Fig. 4 and Table 5) or to increased specific activity of the nucleotide pool (Fig. 5 and Tables 4 and 5).

The increases in ^{32}P incorporation into phosphatidic acid and phosphatidylinositol were 240% and 170%, respectively, after acetylcholine stimulation. Since no major changes were observed in the radioactivity of the other phospholipids (except for a 44% increase in phosphatidylcholine and a 54% increase in the unidentified phosphorus-containing spot remaining at the origin of the chromatograms), it can be concluded that the action of the transmitter is exerted on the rate of formation of these two phosphatides. The results indicate that a metabolic pool of phosphatidic acid was formed in response to the neurotransmitter (Fig. 6). Because so much evidence has accumulated in favor of the role of phosphatidic acid as an intermediary for the synthesis of phosphatides (3, 4, 17), and especially phosphatidylinositol (3, 4), it seems likely that phosphatidic acid was a precursor for the increased synthesis of phosphatidylinositol in this tissue during acetylcholine stimulation. The results presented above on the time course of ^{32}P incorporation into phosphatidylinositol in response to acetylcholine stimulation (Fig. 6) agree with the kinetics of the precursor-product relationship (23). The increase in phosphatidic acid specific activity was initially more rapid than that of phosphatidylinositol. Although the net specific activity of phosphatidic acid was not significantly different from the specific activity of phosphatidylinositol, phosphatidic acid cannot be excluded as a precursor of phosphatidylinositol, since these studies were carried out with whole tissue, and it is possible that different pools of these two phosphatides exist, with different rates of turnover. Acetylcholine perhaps stimulates the formation of a particular pool of phosphatidic acid, which is a precursor of a specific phosphatidylinositol pool. Preliminary experiments carried out in this laboratory seem to indicate that the higher ^{32}P incorporation in response to acetylcholine stimulation occurs at the level of the microsome fraction. This result is not surprising, since it has been demonstrated that

[1] J. M. Trifaró and A. M. Poisner, unpublished observations.

chromaffin cells are very rich in endoplasmic reticulum after stimulation by cold (24). Cold stress has been shown to release catecholamines from the adrenal medulla (25).

It has been pointed out that in other tissues acetylcholine produces its effects of increasing the phosphatidic acid turnover through activation of the diglyceride kinase, with formation of phosphatidic acid from previously formed diglyceride (4, 26). Therefore, the possibility exists that acetylcholine exerts its effect through the same mechanism, since the experiments with glycerol-1-^{14}C showed no increase in the incorporation of this molecule into phospholipids upon stimulation with acetylcholine (Table 6).

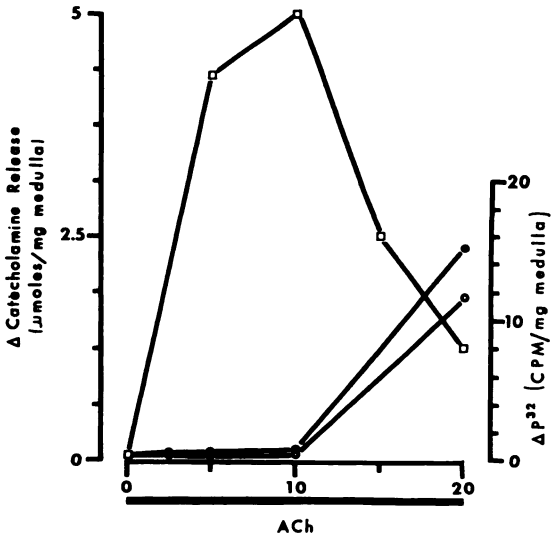

FIG. 7. *Increment in catecholamine release* (□) *and in* ^{32}P *incorporation into phosphatidic acid* (●) *and phosphatidylinositol* (○) *during stimulation with acetylcholine (ACh)*

Adrenal medullary slices were incubated for 60 min prior to the addition of acetylcholine (10^{-5} M) plus eserine (10^{-6} M). Half the slices were incubated with orthophosphate-^{32}P (50 μCi/ml) during this period. Acetylcholine plus eserine was added to half the orthophosphate-^{32}P-incubated preparations and to half the nonradioactive preparations. At the end of the incubation period (80 min), aliquots from the nonradioactive incubation media were assayed for catecholamine content. In the slices incubated in the orthophosphate-^{32}P-containing media, the radioactivity of phosphatidic acid and phosphatidylinositol was determined as described in METHODS. Each point is the average of three or four individual experiments.

It was also observed that acetylcholine stimulates the labeling of the phosphorus-containing spot which remained at the origin of the chromatograms. This spot has not yet been identified, but it is possible that it contains polyinositide phosphatides.

The total and individual phospholipid content remained unchanged during acetylcholine stimulation, as indicated in Table 3. This result was expected, since it has been shown that when the adrenal medulla is stimulated, catecholamines are released from the gland along with other normal constituents of the chromaffin granules (27), leaving the granule membrane (formed mainly by phospholipids and cholesterol) within the cell (28–31). No changes in the phospholipid and cholesterol contents of the adrenal medulla or their different subcellular fractions were detected under those circumstances (28, 30).

As far as is known, there is only one publication on the action of acetylcholine on ^{32}P incorporation into phospholipids of the adrenal medulla (1). In this early report, the authors suggested that since the increased phospholipid turnover in response to acetylcholine stimulation was similar to that found when protein secretion from exocrine glands is stimulated (2), the hypothesis that phosphatidic acid and phosphatidylinositol play an important role in the secretory process may also be applicable to catecholamine release from the adrenal medulla. The results presented in this paper neither support nor refute this hypothesis. But if the time courses of acetylcholine-evoked catecholamine release and acetylcholine-stimulated ^{32}P incorporation into phosphatidic acid and phosphatidylinositol are plotted together, it can be observed (Fig. 7) that catecholamine release reaches a maximum after 10 min; in contrast, ^{32}P incorporation into these phosphatides became apparent only after this interval of time. This effect appears to occur after secretion, and may be due to formation of new membranes. Owing to this delay in the effects of adrenal stimulation

on the labeling of these two phosphatides, it would also be difficult to subscribe to the theory that this effect is related to cell depolarization, as suggested for other tissues (32). Further studies will be necessary to answer this question.

Another interesting hypothesis that relates phospholipids to the secretory process is that advanced on the basis of the presence in the adrenal medulla (33) and in its subcellular fractions (34) of phospholipase A, together with large amounts of lysolecithin in the chromaffin granules (12, 15). It was suggested that activation of a phospholipase A, which would catalyze the conversion of phosphatidylcholine to lysophosphatidylcholine, might be involved in the release of catecholamines from the adrenal medulla (35). Lysophosphatidylcholine, which is concentrated mainly in the chromaffin granule membrane and has lytic properties (35), could produce a transient opening or increase in the permeability of the plasma membrane when the granules are in contact with it. This would permit the escape of the contents of the granules from the cell. Under the present experimental conditions, no differences in the contents and specific activities of phosphatidylcholine and lysophosphatidylcholine were found between control and acetylcholine-stimulated adrenal slices.

The possible relationship between increased phospholipid turnover and catecholamine secretion during adrenal medullary stimulation will be discussed further in a subsequent paper.

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