INHIBITION *IN VITRO* OF INCORPORATION OF [³²P]PHOSPHATE INTO RABBIT AND HUMAN ENDOMETRIAL PHOSPHOLIPIDS

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(Received 15th January 1970, revised 6th April 1970)

Summary. Endometria from rabbits and humans were incubated with [³²P]phosphate and several physiological or synthetic amino-alcohols. Sphingomyelin, phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine were then isolated and their specific radioactivities determined. Addition of choline to the media resulted in an increased incorporation of ³²P into phosphatidyl choline; neither ethanolamine nor serine had any significant effect on any of the phospholipid classes. Aminomethylpropanol, 3-aminopropanol, triethanolamine, and dimethylaminoisopropanol in concentrations of 0.001 M to 0.1 M resulted in inhibition of ³²P incorporation into all the rabbit endometrial phospholipid classes studied; the first three compounds had a proportionately greater effect upon phosphatidyl choline, whereas dimethylaminoisopropanol had a more uniform inhibitory effect on all the phospholipids. Methyl serine added to the media had no significant effect on incorporation of ³²P into any of the phospholipid classes.

Incubation of human endometria with 0.01-M concentrations of the above amino-alcohols resulted in similar inhibitory effects on ³²P incorporation into the phospholipid classes studied.

INTRODUCTION

Alterations in endometrial phospholipid concentration have been produced by oestrogen administration to castrated rats (Davis & Alden, 1959). Synthesis of phospholipids from ³²P-labelled precursors in rat uterine subcellular particles was shown to be increased by oestradiol (Gorski & Nicolette, 1963); in another study, oestradiol also increased the synthesis of choline, ethanolamine and inositol containing phospholipids from [³²P]phosphate and [¹⁴C]acetate precursors (Aizawa & Mueller, 1961).

In rabbits, the transition from the non-pregnant to the early pregnant state was accompanied by significant decreases in the incorporation of [¹⁴C]acetate into most of the endometrial phospholipid classes (Morin & Carrion, 1968).

Linoleic acid containing species of phosphatidyl choline and phosphatidyl ethanolamine showed a relatively increased concentration in the early pregnancy endometrium.

The present experiments were conducted to study the effects *in vitro* of several metabolic antagonists of amino-alcohol components of phospholipids on the synthesis of endometrial phospholipids from [^{32}P]phosphate and to assess the potential of these or similar agents for future investigations of the significance of phospholipids in the implantation process.

MATERIALS AND METHODS

Thirty-six female, New Zealand White rabbits, 6 months old and fed a stock commercial diet (Purina) since weaning, were anaesthetized with IV sodium pentobarbital. The endometria were dissected free, placed in an isotonic Krebs-Ringer-glucose solution at pH 7.4, and after the addition of 200 μC of [^{32}P]phosphate (International Chemical and Nuclear Company, carrier free) plus either a substrate or metabolic antagonist to each, were incubated for 4 hr in air at 37.5° C in a Dubnoff metabolic shaking incubator. Substrates used were choline, ethanolamine or serine, and antagonists used were 2-amino-2-methyl-1-propanol, 3-amino-1-propanol, dimethylaminoisopropanol, triethanolamine and methyl serine (all compounds from K and K Laboratories, purity 95 to 99%). Concentrations and mixtures used are given in the tables. Four separate endometrial incubations were carried out with each substrate, each antagonist and each substrate-antagonist mixture. At the end of the incubation period, the flasks were placed on ice and, after cooling to 0° C, the contents were centrifuged, washed twice with 20 ml of isotonic phosphate buffer, pH 7.4, for 20 min each time and re-centrifuged. The tissues were then refrigerated overnight in 50 ml of isotonic phosphate buffer; the next day, the tissues were washed once more and centrifuged. Analyses of the total phospholipid content of the tissues before washing and analysis of the phospholipid content of the wash solutions revealed total phospholipid losses of less than 5% for the combined washing procedures. The total endometrial lipids were then extracted in a ground-glass homogenizer with two successive 50-ml portions of methylal/methanol, 4:1. The combined extracts were evaporated to dryness in a rotary vacuum evaporator down to 0.5 ml. Silica gel H (Brinkmann) plates, 0.5 mm thick, were prepared by the method of Skipski, Peterson & Barclay (1964). After drying and heating at 110° C for 1 hr, the plates were placed in the developing mixture and the solvents allowed to rise to the top of the plates to remove impurities. The plates were then reactivated for 1 hr at 110° C just before use. The total endometrial lipids were applied as a band across the plate and the phospholipids separated, using an ascending developing mixture of chloroform: methanol:acetic acid:water 25:15:4:2. The sphingomyelin, phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine bands were collected separately with a vacuum zone extractor. Elution of the phospholipids from the silica gel was accomplished as previously described (Skipski *et al.*, 1964). Aliquots of the eluates from each fraction were analysed for content of phosphorus (Bartlett, 1959). Other aliquots were assayed for radioactivity using a

PPO-POPOP in toluene scintillator and a Packard Model 3314 liquid scintillation spectrometer.

In a second experimental series, samples of mid-proliferative human endometria were obtained immediately after hysterectomy for non-neoplastic disorders in women of 30 to 40 years of age. Four samples each were incubated with 200 μC [^{32}P]phosphate and either no substrate, choline, aminomethylpropanol 3-aminopropanol, triethanolamine, dimethylaminoisopropanol or methyl serine. Incubation conditions, lipid extraction, phospholipid isolation, and quantification and liquid scintillation counting were carried out as described for the rabbit experiments.

The probabilities that apparent differences in the data were due to chance were calculated by the *t* test.

RESULTS

The amounts of the individual phospholipids in rabbit and human endometrium were essentially the same as reported previously (Morin & Carrion, 1968). None of the substrates or metabolic antagonists added to the incubation mixture had any significant effects on the amounts of the individual phospholipids.

TABLE 1
SPECIFIC ACTIVITIES OF SOME RABBIT ENDOMETRIAL PHOSPHOLIPIDS AFTER INCUBATION WITH [^{32}P]PHOSPHATE AND AMINO-ALCOHOLS*

Substrate	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
None	7,240 $\pm$ 2,480	40,160 $\pm$ 6,820	22,870 $\pm$ 4,750	16,240 $\pm$ 2,700
Choline 0.01 M	7,160 $\pm$ 1,010	58,570 $\pm$ 5,520	23,290 $\pm$ 3,400	14,340 $\pm$ 2,840
Aminomethylpropanol				
0.001 M	6,980 $\pm$ 2,460	25,620 $\pm$ 5,310	18,960 $\pm$ 2,310	12,590 $\pm$ 2,480
0.01 M	2,210 $\pm$ 913	11,970 $\pm$ 2,450	8,990 $\pm$ 1,840	3,310 $\pm$ 880
0.1 M	903 $\pm$ 104	1,210 $\pm$ 236	2,210 $\pm$ 775	1,450 $\pm$ 561
Triethanolamine				
0.001 M	3,970 $\pm$ 615	27,820 $\pm$ 3,100	15,570 $\pm$ 1,200	14,830 $\pm$ 2,110
0.01 M	2,420 $\pm$ 599	6,060 $\pm$ 709	6,800 $\pm$ 1,790	6,620 $\pm$ 1,420
0.1 M	1,480 $\pm$ 206	570 $\pm$ 237	2,570 $\pm$ 752	1,650 $\pm$ 480
Dimethylamino- isopropanol				
0.001 M	6,800 $\pm$ 1,040	37,350 $\pm$ 4,650	13,050 $\pm$ 1,590	15,910 $\pm$ 3,130
0.01 M	2,940 $\pm$ 755	16,360 $\pm$ 2,210	9,430 $\pm$ 2,050	7,540 $\pm$ 1,540
0.1 M	1,180 $\pm$ 390	3,920 $\pm$ 1,800	3,808 $\pm$ 632	2,220 $\pm$ 710
3-Aminopropanol				
0.001 M	5,710 $\pm$ 752	23,380 $\pm$ 3,630	14,060 $\pm$ 1,850	12,590 $\pm$ 1,350
0.01 M	3,650 $\pm$ 710	6,080 $\pm$ 847	4,700 $\pm$ 980	7,440 $\pm$ 1,750
0.1 M	1,490 $\pm$ 440	750 $\pm$ 433	2,760 $\pm$ 549	2,570 $\pm$ 434
Methylserine				
0.001 M	7,640 $\pm$ 1,930	41,580 $\pm$ 6,100	22,380 $\pm$ 3,250	19,210 $\pm$ 2,880
0.01 M	8,770 $\pm$ 1,640	37,070 $\pm$ 4,310	25,860 $\pm$ 4,500	12,220 $\pm$ 2,110
0.1 M	7,080 $\pm$ 1,340	35,380 $\pm$ 7,670	20,900 $\pm$ 3,740	16,890 $\pm$ 3,700

* d/min/mg phospholipid $\pm$ S.D.

The results of incubation of the rabbit endometria with ^{32}P and the substrates or antagonists are indicated in Table 1. Addition of 0.01 mmol/ml of choline to the media resulted in a slight increase in the specific activity of phosphatidyl

choline. Addition of greater amounts of choline to the media had no further stimulatory effect. Ethanolamine and serine in concentrations of 0.01 and 0.1 M produced no alterations in the final specific activities of any of the phospholipids studied.

All the metabolic antagonists, except methyl serine, produced inhibition of incorporation of [^{32}P]phosphate into all the phospholipid classes studied; this inhibition was significant at concentrations of 0.01 M of antagonist and was most marked at the 0.1-M concentration levels. Aminomethylpropanol, triethanolamine and 3-aminopropanol seemed to have a proportionately greater effect upon phosphatidyl choline than upon the other phospholipids. Dimethylaminoisopropanol resulted in a more uniform inhibition of all the phospholipid classes studied.

TABLE 2

SPECIFIC ACTIVITIES OF SOME HUMAN ENDOMETRIAL PHOSPHOLIPIDS AFTER INCUBATION WITH [^{32}P]PHOSPHATE AND SEVERAL AMINO-ALCOHOLS*

Substrate	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
None	10,100 $\pm$ 1,830	52,760 $\pm$ 4,710	36,840 $\pm$ 2,150	31,130 $\pm$ 4,720
Choline 0.1 M	12,360 $\pm$ 1,300	69,780 $\pm$ 5,720	32,390 $\pm$ 4,750	37,720 $\pm$ 3,870
Aminomethylpropanol 0.01 M	2,070 $\pm$ 765	5,650 $\pm$ 1,880	13,060 $\pm$ 1,870	15,010 $\pm$ 2,450
Triethanolamine 0.01 M	3,390 $\pm$ 871	6,980 $\pm$ 1,220	15,310 $\pm$ 2,770	13,650 $\pm$ 1,970
Dimethylamino- isopropanol 0.01 M	5,720 $\pm$ 842	12,010 $\pm$ 2,320	11,270 $\pm$ 2,070	14,020 $\pm$ 2,010
3-Aminopropanol 0.01 M	2,980 $\pm$ 389	5,490 $\pm$ 1,640	17,700 $\pm$ 1,340	13,010 $\pm$ 2,370
Methylserine 0.01 M	14,560 $\pm$ 1,500	56,070 $\pm$ 8,670	34,110 $\pm$ 1,580	32,370 $\pm$ 4,100

* d/min/mg phospholipid $\pm$ S.D.

The results of incubation of human endometria with the substrates or antagonists at 0.01-M concentration are indicated in Table 2. As with the rabbit endometria, addition of choline stimulated the incorporation of [^{32}P]phosphate into phosphatidyl choline. Aminomethylpropanol, triethanolamine and dimethylaminoisopropanol significantly inhibited incorporation of ^{32}P into all the phospholipid classes but most markedly into phosphatidyl choline. Dimethylaminoisopropanol resulted in a more uniform inhibition of incorporation into all the phospholipids studied, whereas methyl serine had no significant effects.

DISCUSSION

In previous studies (Wells, 1954, 1956a, b; Wells & Remy, 1961) aminomethylpropanol was shown to inhibit total liver and kidney phospholipid synthesis *in vivo*. The aminomethylpropanol inhibited both the synthesis and oxidation of choline and also inhibited the utilization of choline for phospholipid formation.

To investigate the mechanism of action of aminomethylpropanol in the present rabbit endometrial experiments *in vitro*, concentration mixtures of 0.001 M-aminomethylpropanol (AMeP) + 0.1 M-choline, 0.005 M-AMeP + 0.1 M-choline, 0.01 M-AMeP + 0.1 M-choline and 0.1 M-AMeP + 0.1 M-choline were used in incubation experiments similar to those already described. Ratios of choline:AMeP of 1:1 produced no reversal of inhibition and resulted in ^{32}P specific activities similar to those indicated in Table 1 for 0.1 M-AMeP alone. A ratio of choline:AMeP of 50:1 resulted essentially in complete reversal of inhibition of incorporation of ^{32}P into all four phospholipids studied. It seems possible, therefore, that aminomethylpropanol acts primarily to inhibit the formation of phosphoryl choline. If constant proportions of each type of phospholipid are required for membrane structure, then a deficiency of phosphatidyl choline could block the utilization of the other phospholipids as membrane components. The accumulation of end products at the site of synthesis, therefore, may have secondarily inhibited formation of these other phospholipids.

Serine has been found to be directly incorporated in the biosynthesis of phosphatidyl serine (Ansell & Hawthorne, 1964), but in the present experiments there was no evidence that the analogue, methyl serine, inhibited this incorporation. It is possible that the methyl serine itself was incorporated and formed a phospholipid with a migration value identical to the R_F of phosphatidyl serine in the chromatography system used. Further study will be necessary to explore this possibility.

Additional experiments similar to the above were carried out to compare endometria from rabbit uterine horns in which a plastic coiled intra-uterine device (IUD) had been in place for the previous 6 weeks with the control non-IUD horn endometria. No significant differences in ^{32}P incorporation into any of the phospholipid classes were induced by the IUDs. Similarly, when four human endometria from uteri in which plastic coiled IUDs had been in place for 3 to 12 months were incubated and compared to the control endometria, no differences in incorporation of ^{32}P could be attributed to the presence of the IUDs.

The demonstration in this study of significant alterations of endometrial phospholipid synthesis *in vitro* by several agents warrants further exploration of these and similar compounds in animal experimentation *in vivo*.

ACKNOWLEDGMENT

This investigation was supported by a grant from the Ford Foundation.

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