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CONTROL OF SALIVARY PHOSPHOLIPID CONTENT AND COMPOSITION

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The mediation of phospholipid secretion in rat sublingual salivary gland cells maintained in the presence of [^{3}H]choline was investigated. The secretion of [^{3}H]choline-containing phospholipids was enhanced by β-adrenergic agonist, isoproterenol, to a greater extent than the cholinergic agoinst carbachol. A 2.9-fold increase in phospholipid secretion occurred with isoproterenol, while carbachol evoked only about 1.3-fold increase. In contrast to carbachol, the enhanced phospholipid secretion due to isoproterenol was accompanied by an increase in cAMP concentration. The secretion of phospholipids was also stimulated by dibutyryl-cAMP and the protein kinase C activator, phorbol myristate acetate, but not by 4α-phorbol 12, 13-didecanoate which does not activate protein kinase C. Furthermore, the effects of dibutyryl-cAMP and phorbol myristate acetate were additive. The phospholipids secreted in response to isoproterenol exhibited a 52% decrease in lysophosphatidylcholine, while those secreted in response to carbachol showed a 23% lower content of phosphatidylcholine, and were enriched in lysophosphatidylcholine (2.8-fold) and sphingomyelin (1.4-fold). The results suggest that salivary phospholipid secretion remains mainly under β-adrenergic control, while the phospholipid makeup is under cholinergic regulation.

K e y w o r d s: Salivary phospholipids, secretion, adrenergic, cholinergic, mediation

INTRODUCTION

Lipids in saliva are recognized of importance to a variety of processes occurring in the oral cavity that determine the ability of soft tissue to preserve its integrity and the tooth to resist caries and periodontal disease (1—4), yet the factors controlling their levels in secretions of salivary glands are not well explored (5—7). Generally, the control of salivary secretion is derived through sympathetic and parasympathetic innervation (8—11), and the epithelial cells of salivary glands possess receptors capable of responding to signals from

either system. The acinar cells in vitro respond to stimulation by β-adrenergic agonists with increase in mucin and amylase secretion (12—14), while the α-adrenergic and cholinergic receptor stimulation has less significant effect on salivary mucin release (15—17). The involvement of cholinergic and adrenergic mediators in the secretion of pulmonary surfactant phospholipids have also been reported (18, 19).

As salivary glands have a considerable capacity for the rapid synthesis of phospholipids (20), which exert an impact on the functional properties of saliva, (5), we examined the phospholipid secretory responses of sublingual salivary gland cells to adrenergic and cholinergic mediators.

EXPERIMENTAL PROCEDURES

Materials

Male Sprague-Dawley rats (150—200g) used for salivary gland isolation were purchased from Charles River Lab., Wilmington, MA. Tissue culture medium components were supplied by Grand Island Biological Co., Grand Island, NY, and [methyl-^{3}H] choline chloride (80Ci/mmol) by New England Nuclear, Boston, MA. Carbamylcholine chloride (carbachol), atropine sulfate, l-isoproterenol bitartrate, alprenolol, N^6O^2-dibutyryl-cAMP, 3, isobutyl-1-methylxantine (IBMX), 4α-phorbol-12, 13-didecanoate (4α-PDD), phorbol-12-myristate-13-acetate (PMA), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (Sph) were from Sigma Chemical Co., St. Louis, MO. Radio-immunoassay for cAMP [^{125}I] assay system was obtained from Amersham, Arlington Heights, IL, and Silica Gel 60 thin-layer plates from Merck.

Cell isolation

The dissected sublingual salivary glands were trimmed of fat and connective tissue, cut into small slices and minced by passage through a 50 mesh metal grid. The minced tissue was suspended in 50 vol. of ice-cold Dulbecco's modified Eagles' minimal essential medium (DMEM), containing gentamycin (100μg/ml), fungizone (100μg/ml), penicillin (1000U/ml), streptomycin (100μg/ml), and albumin (2mg/ml). The cells were gently dispersed with a glass homogenizer and were settled by centrifugation at 300xg for 5 min. Following three consecutive rinses with DMEM, the cells were resuspended in the medium to a concentration of 2×10^7 cell/ml.

Cell incubation

One milliliter aliquots of the cell suspensions were transferred to DMEM in culture dishes containing [^{3}H]choline (20μCi/ml), and incubated under 95% O_2 — 5% CO_2 atmosphere in a tissue culture incubator. Following 2h incubation, the cells were centrifuged at 300xg for 5 min, washed three times with DMEM containing 2.5mg/ml albumin to remove free [^{3}H]choline, and resuspended in a fresh DMEM. After 10 min equilibration period, 10μl of the appropriate agonists, antagonists or, as control, their diluents were added and incubated at 37^{0}C for up to 1h. Antagonists were added immediately prior to the addition of agonists and in the experiments with isoproterenol, sodium ascorbate (1mM) was included as an antioxidant (18). At the end of incubation period, the cells were centrifuged at 300xg for 5 min and washed three times with fresh medium. The medium and washes were combined, and used for the isolation of [^{3}H]choline labeled secreted phospholipids, and for the determination of lactate dehydrogenase (21), while the pelleted cells were subjected to the assay of cAMP (22) and analysis of cell associated [^{3}H]choline-containing phospholipids.

Cell preparations before and during the experimentation were assessed for viability and cellular integrity using trypan blue uptake assay and the determination of lactate dehydrogenase released into the medium (18, 21).

Phospholipid analysis

Extraction of lipids from the obtained medium and cell pellet samples was performed with chloroform-methanol (23). For this, samples were mixed with 4 volumes of chloroform-methanol (2:1, v/v), and unlabeled standards (1μg each) of phosphatidylcholine, lysophosphatidylcholine and sphingomyelin were added. The mixture was vortexed with equal volume of chloroform-water and set aside for phase separation. The chloroform phase was recovered, dried under stream of nitrogen, the lipids contained in the residue dissolved in a small volume of chloroform-methanol (4:1, v/v), applied to silicic acid column (0.5 x 5cm), and the phospholipids were eluted with methanol (23). In each experiment, an aliquot of concentrated phospholipid fraction was taken for total radioactivity determination, while the remainder was used for individual phospholipid analysis by two-dimensional chromatography on thin-layer plates developed with chloroform-methanol-NH_4OH (65:25:4, by vol.) followed by chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, by vol.) (23). The positions of the included standards on the plates were visualized with iodine vapor, and the individual areas were scraped and extracted with chloroform-methanol (1:4, v/v) and methanol (24). Following removal of the solvents, the individual phospholipids were analyzed for radioactivity by scintillation spectrometry.

Data analysis

All experiments were carried out in duplicate, and the results are expressed as means ± SD. Student's t-test was used to determine significance, and p values of 0.05 or less were considered significant.

RESULTS

Sublingual salivary gland acinar cells incubated in the presence of [^{3}H]choline incorporated the label into phosphatidylcholine, lysophosphatidylcholine and sphingomyelin. The incorporation of the label into the three phospholipids increased steadily with incubation time with over 93% of the [^{3}H]choline at any time being incorporated into phosphatidylcholine (Table 1). During the course of incubation, the incorporation values for lysophosphatidylcholine did not exceed 2.3% of the [^{3}H]choline labled phospholipids, and that for sphingomyelin reached the value of 3%. Trypan blue uptake indicated that the viability of the acinar cells in culture remained over 98% up to 4h, and the lactate dehydrogenase assays revealed little, if any (0.7—0.9%), cellular damage within this period of incubation. Hence, the labeling time of 2h was chosen for further experiments.

Table 1. Effect of incubation time on the incorporation of [^{3}H]choline into the cellular phospholipid in sublingual salivary gland cells.

Incubation period	[^{3}H]choline incorporation (% total)			
	dpm/assay	PC	LPC	Sph
0.5	15,350 ± 1,320	96.3 ± 7.4	1.0 ± 0.3	0.8 ± 0.2
1	28,100 ± 2,400	94.8 ± 7.7	1.9 ± 0.2	1.5 ± 0.3
2	42,800 ± 3,700	93.6 ± 8.1	2.3 ± 0.4	2.7 ± 0.3
3	51,130 ± 4,860	93.8 ± 7.9	2.2 ± 0.3	3.0 ± 0.4

Values represent means ± SD of six experiments performed in duplicate and expressed as percent of total cell lipid radiolabel. PC-phosphatidylcholine, LPC-lysophosphatidylcholine, Sph-sphingomyelin.

Analysis of the medium and cell fractions for [^{3}H]choline-containing phospholipids indicated that in the absence of any mediators, the secretion of phospholipids increased steadily with time of incubation for at least 1h, and was easily discernible already at 15 min. Therefore, 30 min period was used as standard conditions for the measurements of the secretion of phospholipids in response to various effectors. The secreted phospholipid baseline values under these conditions averaged 1.93% of the total cellular [^{3}H]choline-containing phospholipids (Table 2).

Table 2. Effect of β-adrenergic mediator on the [^{3}H]choline-containing phospholipid secretion and cAMP concentration in sublingual salivary gland cells.

Effector	Phospholipid secretion (% total cellular [^{3}H]phospholipid)	cAMP (pmol/assay
None	1.93 ± 0.21	1.24 ± 0.14
Isoproterenol (5μM)	5.59 ± 0.48*	3.79 ± 0.41*
Alprenolol	1.88 ± 0.17	1.27 ± 0.15
Isoproterenol (5μM) + alprenolol	2.17 ± 0.23	1.32 ± 0.16
Isoproterenol (5μM) + atropine (40μM)	5.34 ± 0.49*	3.67 ± 0.39*
Dibutyryl-cAMP (100μM)	3.22 ± 0.34*	
IBMX (10μM)	2.97 ± 0.28*	2.27 ± 0.31*
Isoproterenol (5μM) + IBMX (10μM)	6.64 ± 0.59*	5.41 ± 0.56*

Cells were labeled for 2h [^{3}H]choline, washed and exposed for 30 min to various mediators. Following centrifugation, the medium was analyzed for the secreted radiolabeled phospholipids, while the pelleted cells were used to measure the incorporation of [^{3}H]choline into the cellular phospholipids and cAMP assay. The cellular incorporation of [^{3}H]choline into the phospholipids following 2h incubation was 42,800 ± 3,700 dpm/assay. Values represent means ± SD of five experiments performed in duplicate.

*$p < 0.05$.

Addition of the β-adrenergic agonist, isoproterenol, led to a dose-dependent stimulation in phospholipid secretion which attained maximum effect at 5μM concentration of the agonist (Fig. 1). At this dose, isoproterenol evoked a 2.9-fold increase in phospholipid secretion, and the effect was accompanied by 3-fold increase in cAMP (Table 2). The increase in phospholipid secretion and the levels of cAMP were also observed with dibutyryl-cAMP and phosphodiestrease inhibitor, IBMX. Furthermore, introduction of IBMX along with isoproterenol produced synergistic effect on both the phospholipid secretion (3.4-fold) and cAMP level (4.4-fold). The stimulatory effect of isoproterenol was inhibited by β-adrenergic antagonist, alprenolol, but not by atropine, the cholinergic antagonist (Table 2).

The effect of cholinergic mediator carbachol on the [^{3}H]choline-containing phospholipid secretion and the levels of cAMP is shown in Table 3. In contrast to isoproterenol, carbachol caused much slower response which reached its maximum at 100 μM (Fig. 2) without causing any effect on cAMP levels (Table 3). This concentration of carbachol resulted only in a 1.3-fold increase in phospholipid secretion and higher concentrations of the agonist had no further

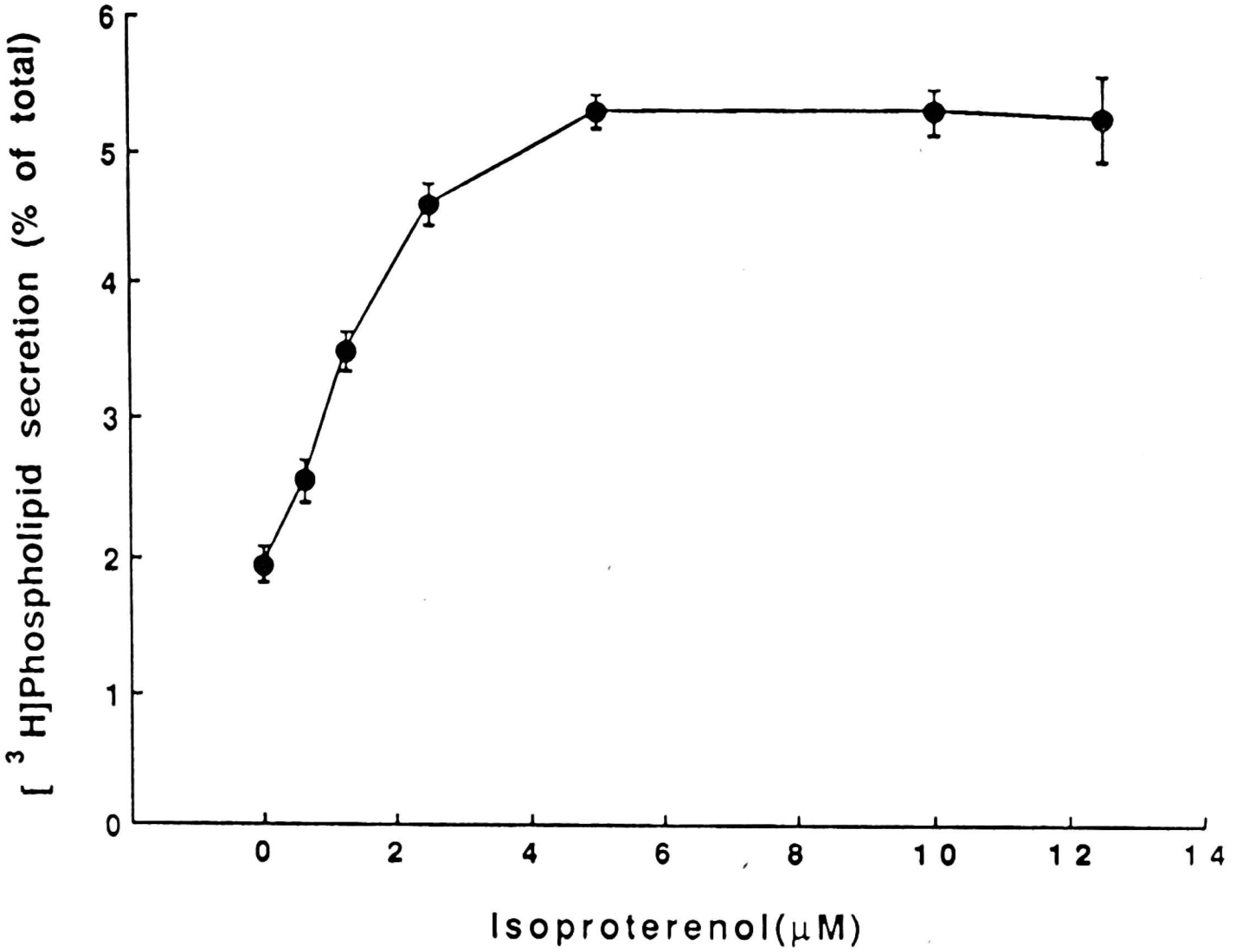

Fig. 1. Effect of isoproterenol concentration on the secretion of [^{3}H]choline-containing phospholipids by sublingual salivary gland cells in culture. Labeled cells were treated with isoproterenol (0—15μM), incubated for 30 min and the medium was anlayzed for radiolabeled phospholipids. The secretion of [^{3}H]choline-labeled phospholipids is expressed as the percentage of cellular phospholipid label. Each point represents the mean ± SD of five experiments performed in duplicate.

stimulatory effect. The inhibition of carbachol effect was achieved with cholinergic antagonist, atropine, while alprenolol had no effect. A significant stimulatory effect on phospholipid secretion was also observed with PMA, a known protein kinase C activator (19), but not with its structural analog, 4α-PDD which does not activate protein kinase C (25).

The effects of cholinergic and β-adrenergic pathway mediators alone and in combination on sublingual salivary gland cell phospholipid secretory responses are depicted in Fig. 3. The results revealed that the stimulatory effect of dibutyryl-cAMP on [^{3}H]choline-containing phospholipid secretion was not affected by 4α-PDD, a structural analog of PMA which does not activate protein kinase C. However, an additive effect on phospholipid secretion was

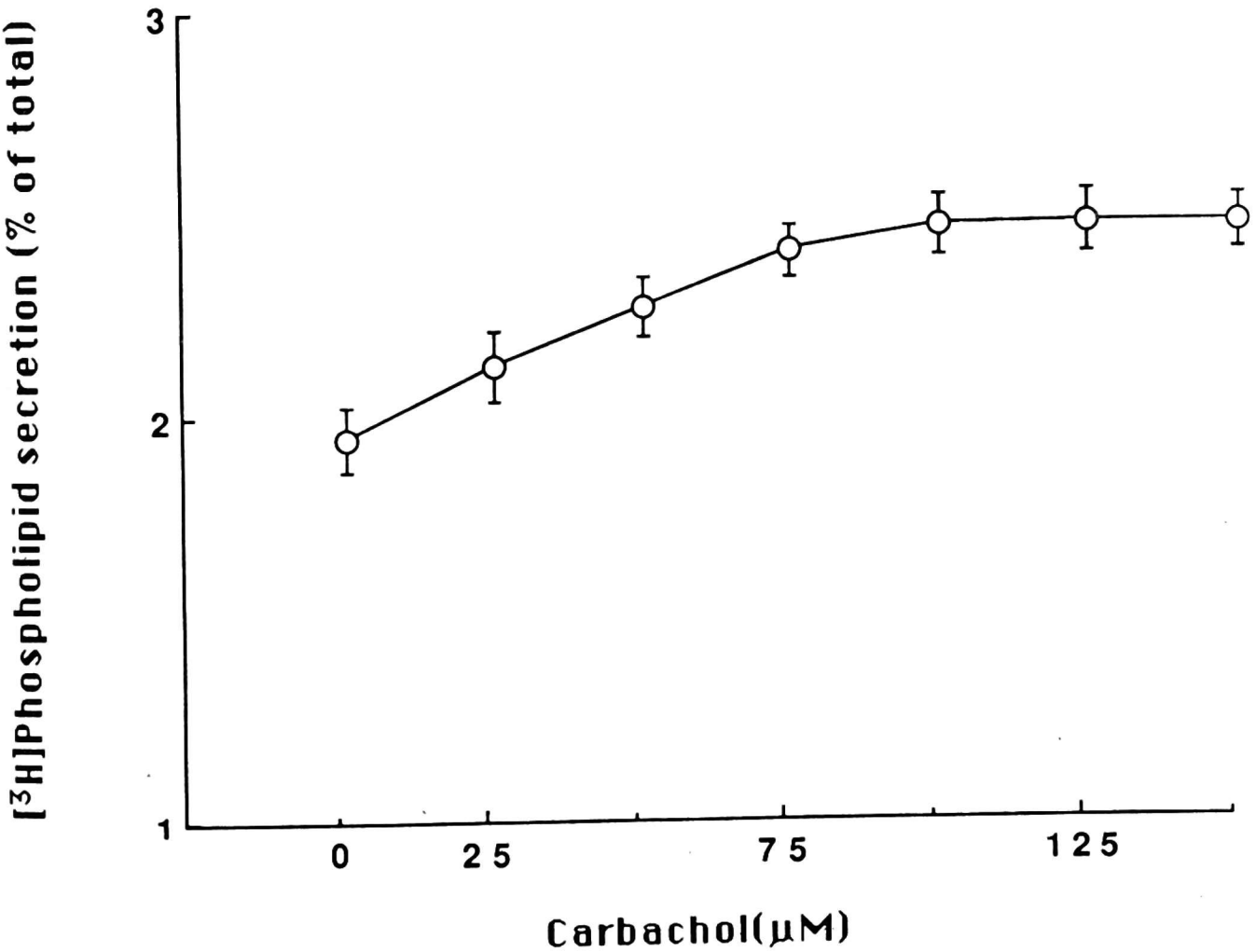

Fig. 2. Effect of carbachol concentration on the secretion of [^{3}H]choline-containing phospholipids by sublingual salivary gland cells in culture. Labeled cells were treated with carbachol (0—150 μM), incubated for 30 min and the medium was analyzed for radiolabeled phospholipids. The secretion of [^{3}H]choline-labeled phospholipids is expressed as the percentage of cellular phospholipid label. Each point represents the mean $\pm$SD of five experiments performed in duplicate.

observed when dibutyryl-cAMP was combined with PMA, thus indicating the involvement of protein kinase A and protein kinase C in the phospholipid secretory responses of salivary gland.

The composition of [^{3}H]choline-containing phospholipids secreted by sublingual salivary gland acinar cells in response to cholinergic and β-adrenergic mediators is presented in Table 4. The results indicated that in the absence of mediators, 87.7% of the secreted [^{3}H]choline-containing lipids consisted of phosphatidylcholine, 4.4% by lysophosphatidylcholine, and 11.5% by sphingomyelin. Isoproterenol, cAMP analog, dibutyryl-cAMP, and phosphodiesterase inhibitor, IMBX all caused a significant decrease in lysophosphatidylcholine. The phospholipids secreted in response to carbachol showed significant increase in lysophosphatidylcholine (2.8-fold) and sphingomyelin (1.4-fold), and a 23% decrease in phosphatidylcholine (Table 4). A marked increase in lysophosphatidylcholine and sphingomyelin, and a decrease in

Table 3. Effect of cholinergic mediator on the [^{3}H]choline containing phospholipid secretion and cAMP concentration in sublingual salivary gland cells in culture

Effector	Phospholipid secretion (% total cellular [^{3}H]phospholipid)	cAMP (pmol/assay)
None	1.93±0.21	1.24±0.14
Carbachol (100 μM)	2.45±0.21*	1.21±0.12
Atropine (40 μM)	1.89±0.17	1.20±0.12
Carbachol (100 μM)$^+$ atropine (40 μM)	2.01±0.20	1.19±0.12
Carbachol (100 μM)$^+$ alprenolol (40 μM)	2.46±0.20*	1.21±0.15
PMA (0.1 μM)	2.27±0.18*	1.25±0.13
4α-PDD (0.1 μM)	1.95±0.17	1.23±0.11

Cels were labeled for 2h with [^{3}H]choline, washed and exposed for 30 min to various mediators. At the end of incubation, the medium was analyzed for radiolabeled phospholipids and the pelleted cells were used to measure the incorporation of [^{3}H]choline into cellular phospholipids and cAMP assay. The incorporation of [^{3}H]choline into the cellular phospholipids at the end of 2h incubation was 42,800 ± 3,700 dpm/assay. Values represent means ± SD of five experiments performed in duplicate. *$P<0.05$.

Table 4. Effect of β-adrenergic and cholinergic mediators on the [^{3}H]choline-containing phospholipid distribution in the secretion of sublingual salivary gland cells.

Effector	[^{3}H]choline incorporation (% total)		
	PC	LPC	Sph
None (control)	83.1±6.8	4.4±0.5	11.5±1.6
Isoproterenol (5 μM)	87.7±7.1	2.1±0.2*	9.3±1.4
Carbachol (100 μM)	63.7±6.4*	12.4±1.2*	16.0±1.8*
Dibutyryl cAMP (100 μM)	85.6±7.0	2.7±0.3*	10.2±1.4
IBMX (10 μM)	85.4±6.9	3.0±0.4*	10.7±1.3
PMA (0.1 μM)	70.8±6.6*	10.4±1.1*	15.9±1.5*
4 *a*-PDD (0.1 μM)	82.8±7.1	4.6±0.5	11.9±1.4

Cells were labeled for 2h with [^{3}H]choline, washed and exposed for 30 min to various mediators. At the end of incubation, the medium was removed and analyzed for radiolabeled phospholipid distribution. Values represent means ± SD of six experiments performed in duplicate. *$P < 0.05$. PC-phosphatidylcholine, LPC-lysophosphatidylcholine, Sph-sphingomyelin.

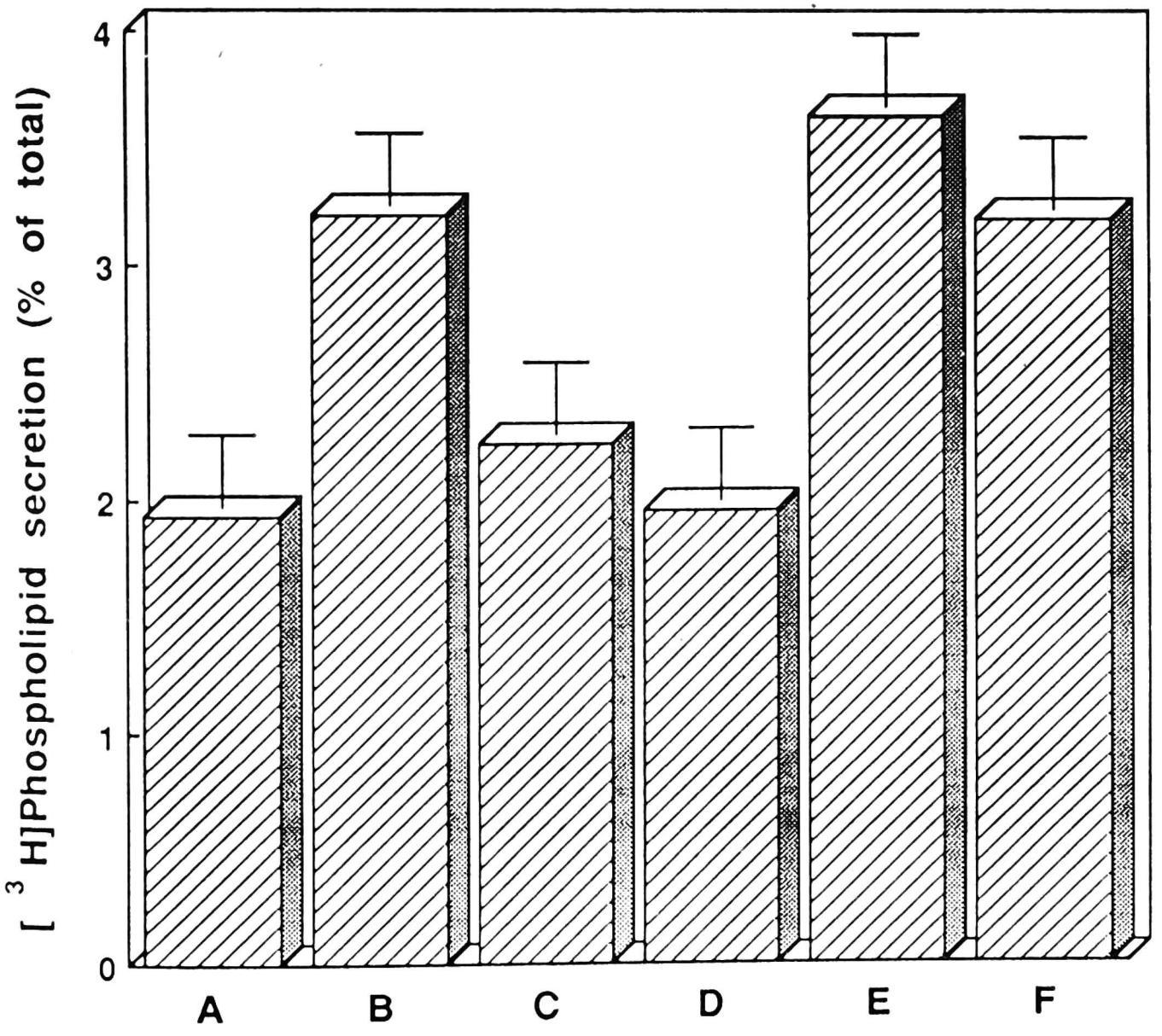

Fig. 3. Combined effects of dibutyry-cAMP, PMA and 4α-PDD on [^{3}H]choline-containing phospholipid secretion in sublingual salivary gland cells in culture. The [^{3}H]choline labeled cells were stimulated for 30 min with various mediators as indicated below. A, control; B, 100 μM dibutyryl cAMP; C, 0.1 μM PMA; D, 0.1 μM 4α-PDD; E, 0.1 μM PMA and 100 μM dibutyryl-cAMP; F, 0.1 μM 4α-PDD and 100 μM dibutyryl-cAMP. The secretion of [^{3}H]choline-labeled phospholipids is expressed as the percentage of cellular phospholipid label.

phosphatidylcholine also occurred in the phospholipids secreted in response to protein kinase C activator, PMA, whereas 4α-PDD which does not activate protein kinase C had no effect.

DISCUSSION

Lipids of major and minor salivary gland secretions play an important role in the functional properties of saliva (1, 5, 7, 26). The elevated levels of salivary lipids are associated with the high incidence of caries, and the development of plaque, calculus and periodontal disease (5, 27). Considerable variation in lipid

content of saliva has been observed in cystic fibrosis (2), and the elevated phospholipid levels characterizes the parotid saliva of patients with Sjogren's syndrome (3). Investigations into the function of lipids in saliva indicate that lipids, and phospholipids in particular, readily enter into heterotypic interaction with other salivary constituents to form a dynamic continuum which determines many physicochemical and biological properties of saliva (1). Included among these are viscosity, buoyant density, permselectivity, hydrophobicity, proteolytic susceptibility, and the tenacity of tooth enamel pellicle and oral mucosal mucus coat (3—6, 28). As the salivary secretions remain under neural control (9—12, 16), the study presented herein was directed towards elucidation of the role of cholinergic and β-adrenergic meditors in the secretion of salivary phospholipids.

Since phosphatidylcholine, lysophosphatidylcholine and sphingomyelin account for over 50% of salivary phospholipids (1, 5), we investigated the secretion of these choline-containing phospholipids in sublingual salivary gland acinar cells in culture. The obtained data revealed that the cells maintained in the presence of [3H]choline incorporate over 93% of the phospholipid label into phosphatidylcholine with the remaining being distributed among lysophosphatidylcholine and sphingomyelin. In the absence of mediators, the secretion of [3H]choline phospholipids into the incubation medium over 30 min period averaged about 2% of the total cellular [3H]choline labeled phospholipids. Both cholinergic and β-adrenergic agonists evoked increase in the secretion of [3H]choline phospholipids. This increase was apparently due to acinar cells response to the mediators rather than the result of any cellular damage, as demonstrated by lactate dehydrogenase assay. The maximum stimulation of phospholipid secretion with β-adrenergic agonist occurred at 5 μM and above, at which concentrations a 2.9- fold increase in [3H]choline phospholipids over the control was attained. Carbachol, a cholinergic agonist, caused much slower response and at its optimal concentration of 100 μM and higher evoked only 1.3-fold increase in phospholipids. The action of each agonist was sensitive to inhibition by the appropriate antagonist and neither agonist caused cross-stimulation. The variations in salivary gland cells response to the two types of mediators appear to be related to the differences in the mechanism of action. The β-adrenergic agonist, such as isoproterenol, exerts its action through cAMP-dependent protein kinase and hence evokes an increase in cAMP levels (9, 10). Our data indicate that the increase in phospholipid secretory response is also associated with increase in cAMP, and that similar effect could be achieved with dibutyryl-cAMP, an analog of cAMP. Furthermore, inhibition of phosphodiesterase with IBMX in the presence of isoproterenol led not only to an increase in cAMP, but also in secretory phospholipids. Similar phospholipid secretory responses to β-adrenergic mediators were reported for lung epithelial cells (18, 19).

Although carbachol evoked much slower phospholipid secretory response, the data obtained suggest that protein kinase C mediated signal transduction system also participates in salivary phospholipid secretion, as stimulatory effect was attained with protein kinase C activator, PMA, but not with its analog 4α-PDD which is not a protein kinase C activator (25). This effect apparently occurs independently of cAMP-mediated pathway, since no changes in cAMP levels were observed in response to carbachol or PMA. Moreover, addition of PMA, but not 4α-PDD in conjunction with dibutyryl-cAMP, produced a synergistic increase in phospholipid secretion. That cAMP-dependent protein kinase and protein kinase C often phosphorylate different sites on the same protein to cause secretory response is well established (29).

The cholinergic and β-adrenergic mediators also evoked different responses with respect to the phospholipid composition. In comparison to the controls, the phospholipids secreted in response to isoproterenol exhibited a significant decrease in lysophosphatidylcholine, while those secreted in response to carbachol were markedly enriched in this phospholipid, contained considerably more sphingomyelin and showed lower content of phosphatidylcholine. The compositional changes evoked by isoproterenol were mimicked by dibutyryl-cAMP and IBMX, and that of carbachol by PMA. The enrichment of the secreted phospholipids in lysophosphatidylcholine in response to carbachol suggests that the cholinergic stimulation of submandibular salivary gland acinar cells leads to protein kinase C dependent activation of phospholipase A_2. Indeed, studies show that protein kinase C is involved in the regulation of the expression of phospholipase A_2 for the breakdown of phosphatidylcholine and hence the generation of lysophosphatidylcholine (30, 31). The mechanism of this regulation apparently involves lipocortin, a known endogeneous phospholipase A_2 inhibitor (32). The phosphorylation of lipocortin by protein kinase C abolishes the inhibitory effect resulting in the activation of phospholipase A_2 (30, 31). On the other hand, the observed decrease in lysophosphatidylcholine content of the phospholipids secreted in response to isoproterenol may be due to β-adrenergic mediated inhibitory effect of cAMP dependent protein kinase on phospholipase A_2 enzyme (29).

Studies on the functional properties of saliva indicate that its physicochemical and protective characteristics are influenced not only by the content, but also by the composition of phospholipids (5, 28). The viscoelastic and hydrophobic properties of the secretions are detrimentally affected by the content of lysophospholipids (1, 5), and the elevated salivary levels of lysophosphatidylcholine have been shown to be a factor in enhancing glucosyltransferase activity associated with the cariogenic potential of bacteria (33). Thus, the cholinergic and adrenergic modulation of the makeup of saliva is of direct relevance to the ability of this fluid to maintain the oral health.

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