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Cholinergic Stimulation of Phosphatidic Acid Formation by Rat Cornea In Vitro

Alan D. Proia,* Sophia M. Chung,* Gordon K. Klintworth,* and Eduardo G. Lapetina†

The cornea has one of the highest acetylcholine (ACh) concentrations of any tissue but the function of the ACh has remained enigmatic. During studies on corneal arachidonic acid metabolism, we observed that ACh stimulates formation of labeled phosphatidic acid in rat corneas whose phospholipids were pre-labeled with [¹⁴C]arachidonate. ACh did not affect the metabolism of free [¹⁴C]arachidonate. [¹⁴C]Arachidonyl-phosphatidic acid formation was doubled after 10 min of incubation in the presence of ACh concentrations of 10⁻⁴ M or greater. The stimulation by ACh could be completely blocked by atropine and scopolamine and partially blocked by d-tubocurarine. These studies suggest that intact rat cornea has muscarinic cholinergic receptors and that the enzymes of the inositol phospholipids pathway are present since phosphatidic acid is an obligatory intermediate in that cycle of reactions. Invest Ophthalmol Vis Sci 27:905-908, 1986

Bovine and rabbit corneas have one of the highest acetylcholine concentrations of any body tissue.^{1,2} Choline acetyltransferase, the enzyme responsible for acetylcholine synthesis, has been demonstrated in corneal epithelium of rabbits, cows, and humans, but little or no enzyme activity could be detected in feline corneal epithelium.³ The function of the acetylcholine is uncertain, but proposed roles have included sensory mediation and regulation of epithelial sodium ion transport.¹ Recently, Cavanagh, and Colley showed that cultured rabbit corneal epithelial cells have muscarinic cholinergic receptors⁴ and that addition of acetylcholine to cultures increases intracellular guanosine-3',5'-cyclic monophosphate concentrations and enhances [³H]thymidine and [¹⁴C]leucine incorporation.⁵

In the pancreas, parotid gland, brain, and other tissues, cholinergic stimulation results in inositol phospholipid hydrolysis^{6,7}; this hydrolysis has been implicated in the generation of calcium signals that trigger cellular processes such as secretion, contraction, and metabolism.⁶⁻⁸ Phosphatidylinositol hydrolysis forms diacylglycerol which is subsequently phosphorylated

to produce phosphatidic acid.⁶⁻⁸ Diacylglycerol activates protein kinase C, a potential regulator of cell proliferation and differentiation.⁹ Phosphatidic acid is a putative calcium ionophore.^{8,10-16} In the experiments reported here, we have examined the effect of cholinergic stimulation on phosphatidic acid formation by rat cornea in vitro.

Materials and Methods

Materials

[¹⁴C]Arachidonic acid (specific activity 60.1 mCi/mmol) was purchased from Amersham; Arlington Heights, IL, and used without further purification. Bovine albumin (essentially fatty acid free; Cohn fraction V), acetylcholine bromide, eserine (free base), atropine (free base), scopolamine hydrochloride, and d-tubocurarine chloride were from Sigma Chemical Co.; St. Louis, MO.

Preparation of Corneas

Corneas were excised from enucleated eyes of 200-250-gm Sprague Dawley male rats (Harlan Sprague Dawley Inc.; Indianapolis, IN) and were weighed after gentle blotting to remove adherent fluid. Rats were cared for and treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Radiolabeling of Corneal Phospholipids

Isolated corneas were incubated for 3 hr at 37°C in 0.5 ml of modified Tyrode's solution,¹⁷ pH 7.4, containing 1.8 mM CaCl₂, 1.0% albumin, and 1.0 μCi [¹⁴C]arachidonic acid. After labeling the phospho-

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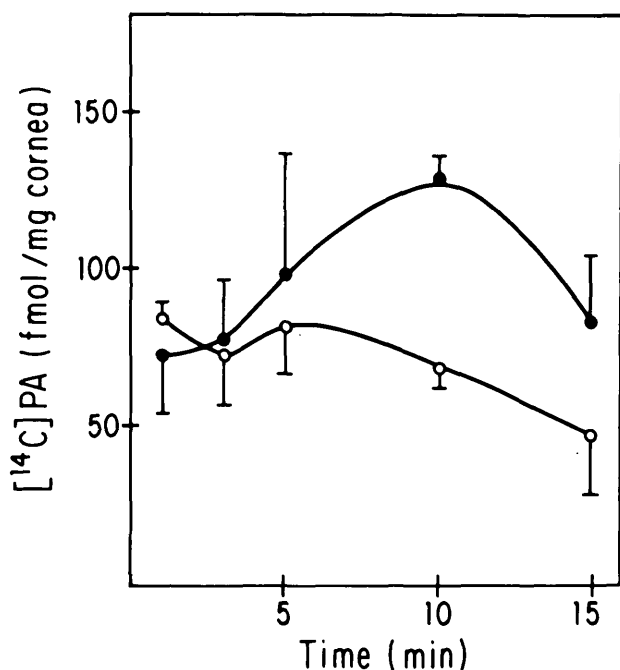


Fig. 1. Effect of incubation time on acetylcholine stimulation of phosphatidic acid (PA) formation. Rat corneas whose phospholipids were prelabeled with [¹⁴C]AA were incubated with (●—●) or without (○—○) 1.0 mM acetylcholine (ACh) for varying time periods. Data are expressed as femtomoles of [¹⁴C]PA formed per milligram of cornea (wet weight). Each point represents the mean (\pm SEM) of three (1 and 3 min), four (5 and 15 min), or nine (10 min) determinations.

lipids, the majority of the free arachidonate was removed by washing four times with 1.0 ml of ice-cold modified Tyrode's solution. Using this procedure, thin layer chromatography showed that 65–80% of the [¹⁴C]arachidonate was usually in the phospholipid fraction, 15–30% in the free arachidonate fraction, and approximately 5% in triacylglycerols.

Phospholipase Activity Assay

Labeled corneas (washed) were incubated at 37°C in 0.5 ml of modified Tyrode's solution, pH 7.4, containing 0.1% albumin to "trap" potential arachidonate metabolites.¹⁸ Our routine assay contained 1.0 mM acetylcholine and 50 μ M eserine (to inhibit acetylcholinesterase). In control experiments, there was no difference in [¹⁴C]arachidonyl-phosphatidic acid formation when corneas were incubated with or without 50 μ M eserine, in the absence of acetylcholine. In addition, incubation of radiolabeled corneas with 10 μ M atropine (in the absence of acetylcholine) did not alter phosphatidic acid formation. Incubations were terminated by adding 1.8 ml of chloroform:methanol (1:2) and five drops of concentrated formic acid (final pH about 3.0). After standing at room temperature for 30 min

with occasional mixing, the lipids were extracted using an adaptation of the Bligh and Dyer procedure.¹⁹

Thin Layer Chromatography (TLC)

Chloroform extracts were dried under N₂ gas and applied to silica gel G TLC plates (LK5D Linear-K plates, Whatman Chemical Separation Inc.; Clifton, NJ) using chloroform. TLC plates were used without prior heat activation. TLC plates were developed using the organic phase of ethyl acetate:isooctane:acetic acid:water (90:50:20:100) acidified by adding 1.0 ml glacial acetic acid to each 100 ml of organic phase.^{20,21} R_F values were phospholipids = 0–0.04, phosphatidic acid = 0.09, 12-HETE (12S-hydroxy-5,8,10,14-eicosatetraenoic acid) = 0.67, arachidonic acid = 0.75, and triacylglycerol = 0.86. TLC bands were visualized using autoradiography with Kodak (Rochester, NY) XAR-5 film, and fractions were scraped and quantitated using a liquid scintillation counter. For each set of assays, controls consisting of corneas boiled for 5 mins were assayed; background radioactivity in the control phosphatidic acid fraction was subtracted from the other assays.

Results

Using rat corneas whose phospholipids were prelabeled with [¹⁴C]arachidonic acid (AA), [¹⁴C]arachidonyl-phosphatidic acid (PA) formation was increased 86% after 10 min of incubation in the presence of 1.0 mM ACh (Fig. 1). There were no significant differences in the [¹⁴C]AA incorporation into the phospholipid, 12-HETE, or triacylglycerol fractions when controls were compared with samples incubated in the presence of ACh.

Our routine assays were performed using 1.0 mM ACh which was sufficient to give maximal stimulation of PA formation (Fig. 2). The stimulation by ACh could be completely blocked by the muscarinic antagonists atropine and scopolamine and partially blocked by the nicotinic antagonist d-tubocurarine (Fig. 3). The stimulation by ACh in the presence of d-tubocurarine was significantly less than that observed with ACh alone but was significantly greater than control assays in which ACh was absent.

Acetylcholine did not significantly affect the formation of PA when corneas were incubated with free [¹⁴C]AA (Fig. 4). Metabolism of [¹⁴C]AA into phospholipids, 12-HETE, and triacylglycerol was not affected by the presence of ACh.

Discussion

These studies demonstrate that ACh stimulates formation of labeled phosphatidic acid from phospholip-

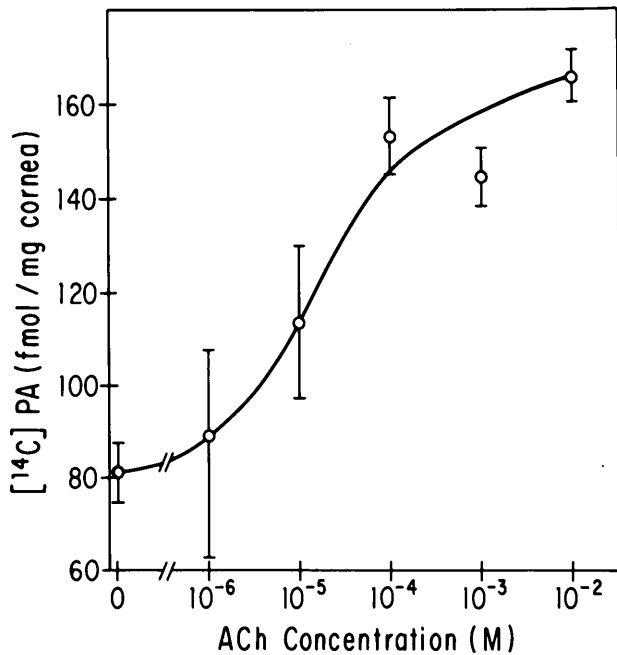


Fig. 2. Concentration curve of ACh stimulation of PA synthesis. Rat corneas whose phospholipids were pre-labeled with [¹⁴C]AA were incubated for 10 minutes in the presence of varying concentrations of ACh. Each point is the mean (\pm SEM) of four assays.

ids pre-labeled with [¹⁴C]AA and suggest that this process is mediated by muscarinic cholinergic receptors. Cholinergic stimulation of PA formation in pancreas,²² avian salt gland,²³ brain synaptosomes,²⁴ and smooth muscle^{25,26} is associated with increased phosphatidylinositol (PI) turnover. PI hydrolysis yields diacylglycerol and some investigators have hypothesized that this is the primary reaction stimulated by ACh and the rate-limiting step for PA production.²⁷ According to this view, increased PA formation is a "marker" for the activity of the inositol phospholipids pathway. However, other authors have proposed that cholinergic stimulation of radioisotopic labeling of PA is due to diacylglycerol kinase activation²⁸ or reflects an increased availability of diacylglycerol resulting from stimulation of phosphatidic acid phosphohydrolase activity.²⁹ Our results indicate that ACh was not stimulating phosphatidic acid phosphohydrolase activity in rat cornea since that would have decreased [¹⁴C]PA levels and not increased them as observed. Studies with purified enzyme preparations from rat cerebral cortex have indicated that cholinergic activation of diacylglycerol kinase is unlikely.³⁰ The presence of a phospholipase C activity catalyzing phosphodiesteratic cleavage of phosphatidylinositol in rat cornea³¹ is in accord with the cholinergic stimulation of PA formation being mediated via the PI cycle, but our data do not exclude that possibility that PA is being formed by phospholipase C cleavage of other phospholipids.

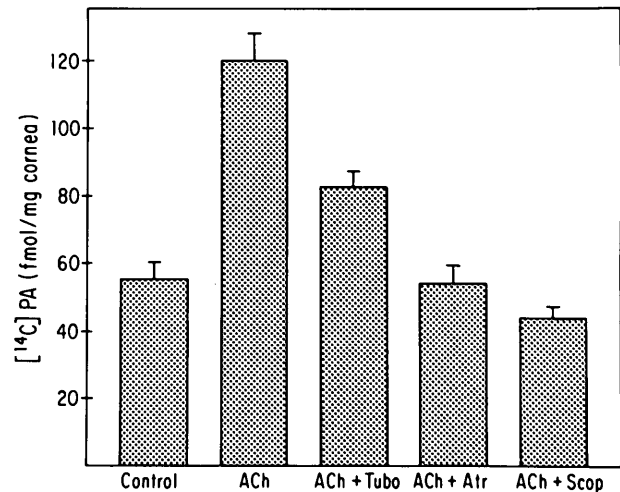


Fig. 3. Effect of nicotinic and muscarinic cholinergic antagonists on ACh stimulation of PA formation. Rat corneal phospholipids were pre-labeled with [¹⁴C]AA and the corneas were then incubated for 10 min in buffer alone (control) or in the presence of either 1.0 mM ACh, 1.0 mM ACh + 10 μ M d-tubocurarine (Tubo), 1.0 mM ACh + 10 μ M atropine (Atr), or 1.0 mM ACh + 10 μ M scopolamine (Scop). Results are the mean (\pm SEM) of four assays except for ACh alone (five assays).

Phosphatidic acid has at least two potential roles in cellular metabolism: as a calcium ionophore^{8,10-16} and as a source of arachidonic acid.³² The importance of phosphatidic acid for the release of arachidonic acid has been inferred from studies in platelets.³² In these cells, production of phosphatidic acid precedes for-

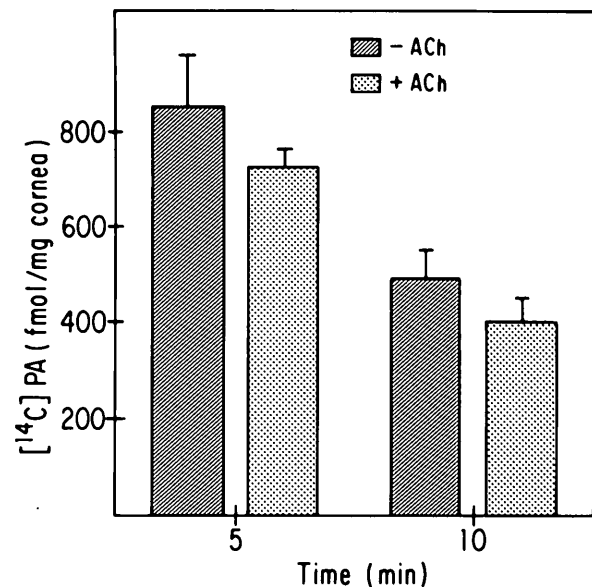


Fig. 4. Incorporation of free [¹⁴C]AA into PA in the presence or absence of 1.0 mM ACh. Rat corneas were incubated for either 5 or 10 min with 1.0 mM ACh and 1.0 μ Ci [¹⁴C]AA. Data are shown as [¹⁴C]PA formation (mean \pm SEM) for triplicate assays.

mation of arachidonate during thrombin activation,²¹ and there is a phospholipase A₂ activity specific for phosphatidic acid.³² The possibility that phosphatidic acid is a source of arachidonic acid for metabolism by rat cornea is currently being investigated in our laboratory.

Attempts to detect corneal cholinergic receptors using radioligand-binding assays have yielded positive results with cultured rabbit corneal epithelial cells,⁴ but negative results using a membrane fraction prepared from rabbit corneas.³³ Our data are consistent with the presence of muscarinic cholinergic receptors on rat cornea and suggest that they are involved in regulation of the phosphatidylinositol cycle. By modulating this cycle, ACh may regulate production of inflammatory mediators from arachidonic acid and ACh may be important in regulating cell proliferation during reepithelialization.

Key words: acetylcholine, cornea, phosphatidic acid, cholinergic receptors, phosphatidylinositol

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