Muscarinic Cholinergic Inhibition of Adenylate Cyclase in the Rabbit Iris–Ciliary Body and Ciliary Epithelium

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The effects of cholinergic agents on hormone-stimulated cyclic AMP (cAMP) accumulation were investigated in iris–ciliary body segments, excised ciliary processes, and isolated ciliary epithelium from albino rabbit eyes. In all three tissue preparations, the cholinergic agonist carbamylcholine markedly inhibited the stimulation of cAMP biosynthesis by vasoactive intestinal peptide VIP—a potent activator of nonpigmented ciliary epithelial adenylate cyclase. Carbamylcholine also attenuated cAMP increases mediated by isoproterenol, prostaglandin E2, and forskolin. The effects of carbamylcholine on VIP-induced cAMP synthesis were concentration dependent (EC50 = 23 nM), mimicked by selective muscarinic cholinergic agonists (oxotremorine, pilocarpine), and antagonized by atropine. Carbamylcholine- and clonidine-mediated inhibition of VIP-stimulated cAMP accumulation in ciliary processes were nonadditive, indicating that inhibitory muscarinic and alpha2-adrenergic receptors coexist on VIP-responsive target cells. These findings suggest that the cholinergic system may have a direct role in modulation of ciliary epithelial adenylate cyclase and aqueous humor secretion. Invest Ophthalmol Vis Sci 31:1103–1108, 1990

The ciliary epithelium of the eye, which carries out the active secretion of aqueous humor, is a key target for regulation by endogenous mediators and antiglaucoma drugs. Histologic evidence indicates that the ciliary processes receive dual innervation by sympathetic and parasympathetic nerves,1–3 and ciliary epithelial cells have been demonstrated to contain both adrenergic and cholinergic receptors.4–8 Although adrenergic mechanisms are believed to play a direct role in regulation of aqueous humor formation, the physiologic function of cholinergic input to the ciliary processes is unclear.10 Pharmacologic studies of the effects of cholinomimetic drugs on aqueous humor production have yielded conflicting results depending on the animal species and methodologies employed.10 Recently, we have provided evidence that cholinergic agonists act prejunctionally to depress sympathetic neurosecretion in the rabbit iris–ciliary body in vitro, suggesting that acetylcholine functions as a neuromodulator as well as an excitatory neurotransmitter in these structures.

Acetylcholine receptors in anterior uveal tissues are predominantly muscarinic. Stimulation of muscarinic receptors in the iris, ciliary processes, or ciliary epithelium7–8,12 has been shown to elicit the rapid turnover of membrane phosphoinositides (PI response), generating the second messengers diacylglycerol and inositol trisphosphate, which have been implicated in the activation of protein kinase C and release of calcium from intracellular stores, respectively.13 Muscarinic receptor activation has also been found to inhibit adenylate cyclase (AC) in a variety of cell types, including cardiac myocytes and neurons.14–16 In view of the proposed involvement of the adenosing 3',5'-cyclic monophosphate (cAMP) pathway in regulation of aqueous humor dynamics, we examined the influence of muscarinic cholinergic agents on cAMP accumulation in the iris–ciliary body, in excised ciliary processes, and in isolated ciliary epithelium from albino rabbits. To investigate adenylate cyclase regulation in situ, we used a radiochemical assay which measures the intracellular conversion of [3H]-ATP to [3H]-cAMP in tissues prelabeled with [3H]-adenine.18,19 A preliminary account of this work has been communicated in abstract form.20

Materials and Methods

Materials

The following drugs were purchased from Sigma (St. Louis, MO): carbamylcholine chloride, isoproterenol, indomethacin, isobutylmethylxanthine, atropine sulfate, oxotremorine sesquifumarate, clonidine hydrochloride, pilocarpine hydrochloride, mec-
amylamine hydrochloride, vasoactive intestinal peptide, and prostaglandin E2. Forskolin was purchased from Calbiochem (San Diego, CA). Other chemicals were purchased from Sigma Aldrich Chemical (Milwaukee, WI), or Fisher Scientific (St. Louis, MO), and were of reagent grade or better.

**Animals and Tissues**

Adult New Zealand white rabbits (2–2.5 kg) of either sex were euthanized by injection of an overdosage of sodium pentobarbital into the marginal ear vein. Anterior eye segments were removed and placed in bicarbonate buffered Krebs solution (composition in mM: NaCl, 118; KCl, 4.8; CaCl2, 1.3; KH2PO4, 1.2; MgSO4, 2.0; NaHCO3, 25; dextrose, 10; ascorbic acid, 0.1; indomethacin, 0.003; pH 7.4) which was gassed continuously with 95% O2/5% CO2 and maintained at 37°C. Iris-ciliary bodies (ICBs) were dissected and cut radially into six segments of approximately equal size. For some experiments, major and minor ciliary processes were excised under a dissection microscope and maintained in oxygenated Krebs solution until subsequent use.

To isolate ciliary epithelium, the anterior segments were placed posterior side-up in a dish of Krebs solution. With forceps, the iris and lens were pulled gently from the adjacent sclera and transferred to a second dish containing buffer. After removal of adherent vitreous from the posterior surface, the tissue was inverted, and with a blunt-pointed pair of corneal scissors, the iris–ciliary body was transected from the pupillary margin to its outer edge. The iris was then gently peeled away from the lens, leaving a pleated sheet of ciliary epithelium attached at the zonules. The epithelial sheet was dissected free of the lens, cut into four sections, and maintained in oxygenated Krebs solution as described above. Histologic examination of the ciliary epithelial preparation by both light and transmission electron microscopy revealed a cellular bilayer (corresponding to the pigmented and nonpigmented epithelial layers) with no appreciable contamination by vascular or stromal cells. A more detailed morphologic and biochemical characterization of this preparation will be given separately (Jumblatt M, Jumblatt J, and Raphael B, submitted for publication).

Rabbits were used in accordance with National Institutes of Health guidelines and the ARVO Resolution on the Use of Animals in Research.

**Assay of 3H-cAMP Accumulation**

Accumulation of 3H-cAMP in tissues preincubated with 3H adenine was assayed by a modification of previously described methods. Isolated ICB segments, ciliary processes (CPs), or ciliary epithelium (CE) were incubated for 60 min at 37°C in Krebs solution containing 2.5 μCi/ml [2,8-3H]-adenine (Amersham, Arlington Heights, IL). After labeling, tissues were rinsed for 15 min with 10 ml Krebs solution, and then placed in individual wells of a multiwell tissue culture plate (Falcon Plastics) maintained at 37°C on a warming table. Tissue segments were pretreated for 10 min with 1 ml Krebs solution supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (Krebs/IBMX). The medium was then replaced with 1 ml fresh Krebs/IBMX solution, with or without test agents, and the incubation continued for an additional 15 min. The incubation was terminated by rapid transfer of tissue segments to vials containing 1 ml 5% w/v trichloracetic (4°C) with 0.3 mM cAMP added as carrier. Samples were sonicated briefly, extracted at 4°C overnight, and then centrifuged for 15 min at 10,000 g. Aliquots (0.5 ml each) of the supernatants were analyzed by sequential chromatography on Dowex 50AG WX4 and neutral alumina columns to isolate 3H-ATP and 3H-cAMP fractions, which then were quantitated by liquid scintillation spectrometry. Radioactivity in 3H-cAMP fractions was corrected based on recoveries of the unlabeled carrier cAMP as determined from the absorbance at 260 nm. Accumulation of 3H-cAMP was expressed as the percentage conversion of 3H-labeled ATP to cAMP during the 15-min drug incubation:

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\% \text{ conversion} = 100 \times \frac{\text{dpm cAMP}}{\text{dpm ATP} + \text{dpm cAMP}}
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In a typical experiment, tissue segments obtained from a single rabbit were pooled and divided randomly into four groups. One group (untreated) was used to determine basal 3H-cAMP accumulation and the other three to measure the effects of activators or inhibitors of adenylate cyclase. Drug-stimulated 3H-cAMP accumulation was estimated by subtraction of basal activity from the total activity measured in the corresponding drug-treated group. Results are expressed as the mean ± SEM, n = number of determinations. Statistical significance of drug responses was evaluated by the student t-test for unpaired observations.

**Results**

**Effects of Carbamylcholine on Basal and Mediator-Stimulated 3H-cAMP Accumulation**

Vasoactive intestinal peptide (VIP) has been reported previously to potently stimulate CAMP biosynthesis in rabbit ciliary processes and nonpigmented ciliary epithelium. Accordingly, VIP
Fig. 1. Effects of carbamylcholine on basal and VIP-stimulated 3H-cAMP accumulation in iris-ciliary body segments, ciliary processes, and ciliary epithelium. Rabbit iris-ciliary body segments (ICB), excised ciliary processes (CP), or isolated ciliary epithelium (CE) were prelabeled with 3H-adenine, treated for 10 min with isobutylmethylxanthine (0.5 mM), and then exposed for 15 min to carbamylcholine (10^{-5} M) and/or VIP (10^{-7} M) as indicated. Bars represent the means ± SEM, n = 4-16 determinations. *, P < 0.01; **, P < 0.001 relative to corresponding values for VIP-stimulated cAMP accumulation.

(10^{-7} M) induced 2-4-fold increases in 3H-cAMP accumulation in ICB sections, CP, and isolated CE (Fig. 1). In each case, the response to VIP was significantly inhibited (P < 0.05) by coadministration of the cholinergic agonist carbamylcholine (10^{-5} M). The magnitude of cholinergic inhibition (ie, a 40–60% reduction of the VIP-induced response) was similar for the three tissue preparations. Carbamylcholine also appeared to decrease basal 3H-cAMP accumulation in ICB and CP preparations, although the effects were not statistically significant.

The inhibitory action of carbamylcholine was not restricted to VIP-stimulated cAMP synthesis. As shown in Figure 2, 10^{-5} M carbamylcholine significantly reduced the stimulation of 3H-cAMP accumulation in ICB segments by prostaglandin E_2 (PGE_2; 10^{-6} M), isoproterenol (10^{-6} M), and forskolin (5 X 10^{-6} M). The percentage inhibition of the induced response varied from 15% (for forskolin response) to > 60% (for the VIP and isoproterenol responses), with no apparent correlation between the degree of AC stimulation and the magnitude of inhibition.

The inhibitory effect of carbamylcholine on VIP-stimulated cAMP synthesis in ICB sections was concentration-dependent, as shown in Figure 3. Significant inhibition (P < 0.05) was observed at 10^{-9} M carbamylcholine, and was maximal at 10^{-6} M or greater, corresponding to a 61% reduction of the VIP-induced response. Half-maximal inhibition (EC50) was estimated to occur at 23 nM carbamylcholine.

Effects of Selective Cholinergic Agonists and Antagonists on VIP-Stimulated 3H-cAMP Accumulation

Carbamylcholine-mediated inhibition of VIP-stimulated cAMP accumulation in ICB segments was mimicked by two selective muscarinic agonists—pilocarpine and oxotremorine (Fig. 4). Oxotremorine was the most potent of the cholinergic agonists tested, consistent with its relatively high binding affinity for muscarinic receptors in rabbit iris membranes.22 As also indicated in Figure 4, carbamylcholine-mediated inhibition was selectively antagonized by the muscarinic antagonist atropine (10^{-6} M), but was unaffected by the nicotinic antagonist mecamylamine (10^{-6} M).
Nonadditivity Between Muscarinic and Alpha₂-adrenergic Inhibition of \( ^3 \)H-cAMP Accumulation

Results from previous investigations indicate that rabbit ciliary processes possess alpha₂-adrenergic receptors negatively linked to adenylate cyclase.\(^{19,24,25}\) To investigate possible additivity between inhibitory muscarinic and alpha₂-adrenergic responses, the individual and combined effects of clonidine (a selective alpha₂-adrenergic agonist) and carbamylcholine on VIP-stimulated cAMP accumulation were compared using ICB segments. Both agonists were applied at relatively high concentrations to insure maximum stimulation of their corresponding receptors. As shown in Figure 5, inhibition of VIP-stimulated \(^3\)H-cAMP accumulation by combined treatment with clonidine (\(10^{-5}\) M) plus carbamylcholine (\(10^{-5}\) M) did not exceed the effect of either agent alone, suggesting that muscarinic and alpha₂-adrenergic receptors coregulate adenylate cyclase in the same VIP-responsive target cells.

Discussion

The presence of muscarinic acetylcholine receptors in ciliary epithelium was first indicated by radioligand binding studies performed with bovine tissue.\(^{6}\) More recently, muscarinic acetylcholine receptors coupled to increased phosphoinositide hydrolysis have been described in rabbit CPs and epithelial cells,\(^{8,12}\) and in a virus-transformed, human ciliary epithelial cell line.\(^{7}\) The present results demonstrate that rabbit ciliary processes and isolated ciliary epithelium contain muscarinic acetylcholine receptors linked to inhibition of AC. Carbamylcholine and selective muscarinic agonists were observed to inhibit the stimulation of cAMP biosynthesis by several mediators, including VIP, isoproterenol, PGE\(_2\), and forskolin. Muscarinic and alpha₂-adrenergic effects on cAMP accumulation were nonadditive, suggesting that the underlying receptors share a common site or mechanism for inhibition of AC. Because both VIP and beta adrenergic agonists have been shown to interact preferentially with nonpigmented ciliary epithelial cells,\(^{4,6,21}\) it is likely that the inhibitory muscarinic receptors also are located on these cells, although their exact cellular distribution remains to be established.

While this report was in preparation, a paper by Tobin and Osborne\(^{26}\) appeared, describing muscarinic cholinoceptors linked to inhibition of AC in rabbit ICB segments. Their findings are generally in agreement with ours with respect to cholinergic inhibition of VIP-, forskolin-, and isoproterenol-induced cAMP biosynthesis, but suggest a much higher EC\(_{50}\) for carbamylcholine-mediated inhibition (>\(10^{-6}\) M) than was observed in the current study (\(2.3 \times 10^{-8}\) M; Fig. 3). This discrepancy might reflect the inclusion, in our experiments, of indomethacin (\(3 \times 10^{-6}\) M) to block tissue cyclooxygenase. Muscarinic agonists have been reported to stimulate arachidonic acid release and synthesis of prostanoids (mostly PGE\(_2\)) in rabbit irides in vitro.\(^{27}\) Because PGE\(_2\) can stimulate cAMP biosynthesis in this tissue (eg, Fig. 2), its formation in response to muscarinic activation might mask the inhibitory action of carbamylcholine on AC and thereby shift the concentration dependence for inhibition to the right. A similar explanation has been proposed to account for the paradoxical stimulation of AC by muscarinic agonists in other systems.\(^{28}\) The EC\(_{50}\) indicated by our data for carbamylcholine-me-
diated inhibition of cAMP formation is in excellent agreement with values reported for cardiac myocytes and various other cell types,14-16,29 and >100-fold lower than the EC_{50} for stimulation of phosphoinositide hydrolysis in rabbit irides or ciliary processes.8,12

Muscarinic acetylcholine receptors comprise a family of distinct receptor subtypes, three of which can be distinguished pharmacologically30,31 and five genetically.28,32,33 Although several systems for pharmacologic classification of muscarinic receptors have been proposed,30 we prefer that of Doods et al.31 which is based primarily on radioligand binding data rather than on functional studies, and which corresponds to the nomenclature (M_1, M_2, M_3, etc) used by Bonner's laboratory for genetic classification of rat muscarinic receptor subtypes.3 It should be noted that this classification differs from that of Peralta et al.3 which is based primarily on radioligand binding data and corresponds to the nomenclature (M_1, M_2, M_3, etc) used by Bonner's laboratory for genetic classification of rat muscarinic receptor subtypes.3

In conclusion, we have presented evidence that rabbit CPs and CE contain muscarinic acetylcholine receptors linked to inhibition of AC. Additional studies are needed to evaluate the significance of these receptors in terms of 1) their biologic function in regulation of ciliary epithelial transport; 2) their contributions to the intraocular responses to cholinergic drugs; and 3) their relevance to the human eye. Such studies will be aided by the growing repertoire of new, subtype-selective muscarinic agents now becoming available.

Key words: muscarinic, cholinergic, adenylate cyclase, iris, ciliary epithelium

References

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