Muscarinic Receptors and Their Regulation of Cyclic GMP in Corneal Endothelial Cells

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Particulate fractions from fresh bovine corneal endothelium exhibited high affinity, specific binding by a potent muscarinic cholinergic radioligand, [3H]QNB. Particulate fraction binding sites exhibited half maximal binding at approximately 0.3 nM [3H]QNB and reached a maximal binding capacity of 820 fmoles/mg of protein at 3 nM [3H]QNB. Muscarinic cholinergic antagonists and agonists competed with [3H]QNB when incubated concurrently with the tissue, showing relative potencies expected of these agents when binding to muscarinic cholinergic receptors. Particulate fractions prepared from cultured bovine corneal endothelium exhibited qualitatively similar [3H]QNB binding characteristics, but maximal binding capacity was only about one-fifth of its fresh-tissue counterpart. Intact cultured cells showed 3-fold more specific [3H]QNB binding than did their particulate fractions. Incubation of intact corneal endothelial cells with muscarinic cholinergic agonists such as carbachol stimulated cyclic [3H]GMP 3-fold from endogenous [3H]GTP within 1 min of incubation. The effect diminished rapidly and returned to control levels within 8 min. Carbachol stimulation of cyclic [3H]GMP was concentration-dependent, reaching half maximal stimulation at 1 μM. Atropine was a potent, competitive inhibitor of carbachol stimulation of cyclic [3H]GMP in endothelial cultures, requiring only 1 nM to completely block the carbachol response. These experiments demonstrate the existence of muscarinic cholinergic receptors in bovine corneal endothelium and their control of cyclic GMP levels in this tissue. Invest Ophthalmol Vis Sci 31:702-707, 1990

Muscarinic cholinergic (MC) receptors have traditionally been associated with the autonomic nervous system and its target organs.1 However, these receptors have more recently been demonstrated in a variety of other cell types, including erythrocytes,2 lymphocytes3 and fibroblasts.4 In the cornea, the epithelium is known to contain large quantities of acetylcholine,5,6 but the demonstration of MC receptors in this tissue has been inconsistent. Several laboratories have failed to detect epithelial receptors in broken cell preparations,7,8 while muscarinic receptors and their stimulation of cyclic GMP have been reported in epithelial cell cultures.9-10 These agents also stimulated phosphatidylinositol hydrolysis in intact rat corneal epithelium.11

Guanylate cyclase is associated with muscarinic receptors in many cell types.1 In preliminary studies, our laboratory has observed guanylate cyclase activity in particulate fractions of bovine corneal endothelium, but the enzyme was not responsive to muscarinic agonists or other hormones tested.12 Since a loss of receptor-mediated guanylate cyclase stimulation is common in broken cell systems,13 we have searched for the existence of corneal endothelial receptors which could be associated with guanylate cyclase in the intact cell. This paper describes specific, high affinity receptor sites for MC agents in particulate fractions of fresh and cultured bovine corneal endothelium, as well as MC receptors and their control of cyclic GMP levels in cultured, intact cells of this tissue.

Materials and Methods

Endothelial Cell Culture

Bovine eyes were removed at a nearby abattoir within 3 hr postmortem and transported at 4°C in a solution containing 4 mM KH2PO4, 6 mM Na2HPO4 and 0.14 M NaCl, pH = 7.6 (PBS).

The culture of corneal endothelium and the preparation of homogenates of fresh-tissue and cultured cells were performed as described previously.14 Homogenates from each source were then centrifuged at 49,000 g for 30 min. The pellet was rehomenegated in an equal volume of fresh 20 mM Tris-HCl buffer (pH = 7.6) and centrifuged as before. The pellet was then
resuspended in one-third of its original volume of fresh Tris buffer, divided into small samples and stored at −20°C until analyzed.

Muscarinic Receptor Studies

Receptor binding activity was assessed by incubating particulate fractions or intact cells with the indicated concentration of [3H]quinuclidinyl benzilate ([3H]QNB), a potent MC antagonist. Unless otherwise indicated, [3H]QNB binding to particulate fractions (80–150 µg protein/assay) took place in 40 mM Tris-HCl (pH = 8.0) at 23°C for 35 min in a 1 ml final volume. Some assays contained other agents (as specified) to observe competition for receptors and assess nonspecific binding. Reactions were terminated by rapid filtration through Whatman GF/B filters and three successive 5 ml rinses with ice-cold buffer, using a Brandel (Gaithersburg, MD) Cell Harvester, Model 24. Total filtration time was approximately 6 sec. Filters were then placed in a plastic vial with 10 ml of Budget Solve (Fisher Scientific, Pittsburgh, PA), shaken for 1 hr and kept in the dark for an additional hour before counting in a liquid scintillation spectrometer.

Binding to intact cells was performed analogously, except for the following changes: Incubation took place at 37°C in PBS. Reactions were terminated by quickly aspirating the incubation buffer and rinsing with three 5 ml portions of ice-cold PBS. After the final aspiration, the cellular contents were dissolved in 2 N NaOH and the neutralized samples were measured for protein and radioactivity.

Specific binding of [3H]QNB was calculated by subtracting the radioactivity associated with fractions incubated with radioligand in the presence of 1 nM atropine (nonspecific binding), from the radioactivity associated with parallel assays incubated with radioligand alone (total binding).

Measurement of Cyclic GMP

Changes in cyclic GMP content of intact endothelial cell cultures were investigated by essentially using the method described by Richelson and co-workers. Cells in plates of 24 miniwells were preincubated with 1 µCi/well of [3H]guanosine for 60 min under otherwise normal culture conditions in order to generate an intracellular pool of [3H]GTP. Preliminary experiments showed that 69.8 ± 0.3 pmol [3H]GTP/mg of cellular protein were produced under these conditions. After removing the preincubation media and washing cells briefly, cells were incubated in fresh media containing 50 mM HEPES (pH = 7.6) at 37°C in a water bath for an additional 30 min before adding the specified MC receptor agents for the time indicated. Reactions were terminated by the addition of TCA to 10% final concentration. Plates were frozen, thawed and sonicated for 2 sec to release intracellular cyclic [3H]GMP. An aliquot of each well was then applied to 0.8 × 8 cm columns of Dowex 50-H+ (AG50W-X2, 200–400 mesh) to isolate the cyclic [3H]GMP. The appropriate fraction was then counted for radioactivity using standard liquid scintillation techniques. Preliminary experiments showed the efficiency of the columns for isolating cyclic [3H]-GMP to be 82 ± 2%. Protein determinations were made using the method of Lowry et al.

[3H]QNB and [3H]guanosine were purchased from New England Nuclear (Boston, MA) and ICN (Irvine, CA), respectively. Other reagents were products of Sigma Chemical Company (St. Louis, MO), except cyclopentolate (Cyclogyl) was a gift from Leo R. Landhuis, MD.

Results

Characterization of Receptors From Fresh Tissue

The binding of [3H]QNB to the corneal endothelial particulate fraction is shown in Figure 1 as a function of incubation time. Specific binding activity reaches a steady state level of approximately 600 fmol/mg protein within 20 min at the conditions employed. At least 85% of total binding appears to be specific in nature.

The specific binding response of endothelial particulate fractions to increasing concentrations of [3H]-
QNB is shown in Figure 2. These preparations showed concentration-dependent binding between 0.03 and 3.0 nM. Receptors appeared to saturate at 3 nM. Rosenthal analysis of the data (inset) indicates that fresh tissue exhibits a maximal binding capacity (Bmax) of 820 fmoles [3H]QNB/mg protein and half-maximal binding (Kd) at 290 pM.

Several potential competitors for the corneal endothelial MC receptor sites were tested against 1 nM [3H]QNB. Figure 3 summarizes the results of this series of experiments. Cyclopentolate appeared to be equipotent with [3H]QNB toward the receptors, requiring only 1 nM to reduce [3H]QNB binding to 50% of its original level (IC50). Atropine also showed high affinity for the receptor, exhibiting an IC50 of 5 nM. The receptor agonists, pilocarpine and carbachol, expressed IC50 values of 1 nM and 10 μM, respectively. Nicotine had no effect on [3H]QNB binding until 1 mM levels were employed, which is consistent with its known properties as a specific nicotinic cholinergic agonist.

Receptors in Cultured Endothelial Particulate Fractions

A partial characterization of muscarinic receptors in primary cultured bovine corneal endothelium was performed using the same assay conditions as in the fresh tissue studies. Figure 4 shows the specific binding response of the cultured cells' particulate fraction to increasing concentrations of [3H]QNB. Binding increased with radioligand concentration in generally the same range as seen with fresh tissue preparations. The primary difference between fresh and cultured tissue is that cultured particulate fractions showed substantially less maximal binding capacity (Bmax = 150 fmoles/mg), but expressed a somewhat higher affinity (Kd = 130 pM) for [3H]QNB. Receptor specificity and other characteristics of the cultured cell preparation were virtually identical to those seen in fresh tissue (not shown).

A direct comparison of [3H]QNB binding in cultured endothelial broken cell preparations versus intact cells is made in Table 1. Intact cells exhibited specific binding of [3H]QNB. In fact, the level of binding was 3-fold higher than observed in particulate fractions prepared from identical cultures.

Muscarinic Cholinergic Control of Cyclic GMP

The effect of MC agents on cyclic GMP levels of intact, cultured corneal endothelium was investigated in experiments depicted by Figures 5–7. Carbachol stimulated cyclic GMP in these cells from approximately 5 to 15 pmoles/mg protein between 20 and 60 sec of incubation (Fig. 5). Maximal stimulation oc-
Fig. 4. Specific [3H]QNB binding to particulate fractions of cultured bovine corneal endothelium as a function of radioligand concentration. Data presentation and Rosenthal analysis are as described in Figure 2.

curred at 1 min and cyclic GMP returned to essentially basal values within 8 min. Surprisingly, the potent cyclic nucleotide phosphodiesterase inhibitor, isobutyl-1-methylxanthine (IBMX), had no appreciable effect on the magnitude or the duration of the cyclic GMP response.

The potency of carbachol in stimulating cyclic GMP in corneal endothelial cell cultures was established (Fig. 6). Cyclic GMP levels increased in a dose-dependent manner between 0.1 and 100 μM carbachol, with half-maximal stimulation occurring at approximately 1 μM.

Carbachol stimulation of cyclic GMP was extremely sensitive to inhibition by MC antagonists such as atropine. Figure 7 shows that atropine inhibited the carbachol stimulation of cyclic GMP content in a concentration-dependent manner. The inhibition could be observed with as little as 0.1 nM atropine and carbachol-stimulated activity was completely inhibited using 1 μM of the antagonist.

**Discussion**

These data demonstrate the presence of MC receptors in bovine corneal endothelium which regulate
levels of cyclic GMP in this tissue. This finding was somewhat surprising, since searches by our laboratory of rabbit, feline and human corneal endothelial particulate fractions failed to show consistent, specific \( ^{3}H \)QNB binding (not shown). Furthermore, MC agonists had no effect on guanylate cyclase in preliminary studies using particulate fractions of fresh tissue bovine corneal endothelium. It is common for broken cell preparations to be unresponsive to guanylate cyclase receptor agonists. The lack of specific \( ^{3}H \)QNB binding in particulate fractions from the other species tested suggests either that not all species contain muscarinic cholinergic receptors or that technical problems preclude observation of binding activity in at least some of the nonresponding species. We believe that the second possibility is more likely. Our reasons are that: first, intact cultured rabbit endothelium show specific \( ^{3}H \)QNB binding in preliminary studies. Second, specific \( ^{3}H \)QNB binding is substantially reduced in broken cell preparations of cultured bovine endothelial cells. The latter may be compared to intact cell binding levels in Table 1.

Several lines of evidence suggest that the MC receptor regulation of cyclic GMP could be physiologically relevant in the corneal endothelium. The relative affinities of the receptor for \( ^{3}H \)QNB and other MC ligands as well as its lack of affinity for a nicotinic cholinergic agonist (Fig. 3) are similar to physiologically relevant receptors in many other tissues. Stimulation of cyclic GMP by carbachol took place at concentrations consistent with its affinity for binding to the receptor (Fig. 3, 6). Furthermore, atropine competitively inhibited carbachol stimulation of cyclic GMP at low concentrations (Fig. 7) as predicted by its affinity for the receptor (Fig. 3). Yet, it could be argued that this system must not be relevant in the corneal endothelium, since the literature shows no consistently demonstrable physiological effects of either MC agonists or antagonists in laboratory experiments or after prolonged clinical use. The experiments herein may provide some insight into this paradox. First, the cyclic GMP response to carbachol under the conditions of study was relatively short: less than 8 min (Fig. 5). Many physiological techniques of the corneal endothelium (eg, maintenance of corneal deturgescence) are not designed to detect a cyclic GMP-mediated physiological effect during this brief period. Furthermore, phosphodiesterase inhibitors can not be assumed to enhance and prolong a cyclic GMP effect in this system (Fig. 5). The lack of effect of IBMX on the cyclic GMP responses was surprising, but it was consistent with its inability to enhance cyclic \( ^{32}P \)GMP synthesis in particulate fractions of bovine corneal endothelium. In fact, no guanylate cyclase activity could be observed in our previous work with broken cells unless a large quantity of unlabelled cyclic GMP was added to assays in order to spare hydrolysis of the radiolabeled product. Although IBMX is usually the most potent of methylxanthine inhibitors of cyclic nucleotide phosphodiesterase, the potencies of these drugs vary considerably from tissue to tissue. While it is possible that higher concentrations of IBMX may have enhanced the carbachol stimulation of cyclic GMP, it is nonetheless noteworthy that the commonly employed concentration of 0.1 mM IBMX does not guarantee significant inhibition of cyclic nucleotide phosphodiesterase in all tissues.

Another factor which could limit or preclude detection of a physiological effect is receptor desensitization, which occurs in response to large pharmacological doses of agonists. This loss of cyclic GMP response to carbachol has been observed by our group in bovine corneal endothelial cultures (unpublished experiments) and in other tissues.

A physiological role for muscarinic receptor on corneal endothelium would require an endogenous source of acetylcholine to interact with the receptors. It may be possible for acetylcholine to reach the endothelium from the ciliary body and/or iris via the aqueous. Another intriguing possibility may be the large stores of acetylcholine in the epithelium which might reach the endothelium during epithelial trauma or other stimulus.

In other cell types, cyclic GMP is thought to play a role in cellular proliferation, cellular responses to UV light, cell death and fluid transport. All of

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**Fig. 7. Effect of atropine on carbachol stimulation of cyclic GMP in cultured corneal endothelium.** Cells were incubated without drugs (-- O --) or with 0.1 mM carbachol and the indicated concentration of atropine (-- • --) under otherwise standard conditions. Each data point indicates the mean of nine assays, generated in three separate experiments.
these effects are of great importance in corneal physiology. It remains to be determined whether cyclic GMP actually regulates any of the above processes in the corneal endothelium, or whether it even exists in other species. However, it appears that traditional pharmacological approaches of study may need to be modified in order to appreciate the physiological role of cyclic GMP in this tissue.

**Key words:** cornea, endothelium, cholinergic, cyclic GMP, receptors

**Acknowledgments**

The authors would like to recognize the expertise of Ms. Frances Wester in the execution of these experiments and Ms. Janet Hussey in the preparation of this manuscript.

**References**