Autonomic control of lacrimal protein secretion

B. Britt Bromberg

The ability of cholinergic and adrenergic agonists to initiate protein secretion by in vitro slices of rabbit lacrimal gland was examined. The adrenergic response was not inhibited by atropine but was partially inhibited by propranolol and phentolamine. This indicates the presence of both alpha- and beta-adrenergic receptors and their association with the protein secretory response. The cholinergic response was inhibited completely by the muscarinic antagonist atropine. Additionally, the adrenergic antagonists, propranolol and phentolamine, inhibited approximately 70% and 40%, respectively, of the cholinergic response. Dose-response curves obtained for carbachol and isoproterenol indicated that the maximum response to carbachol is greater than that to isoproterenol but that the threshold for response to isoproterenol is much lower than that to carbachol. Additionally, carbachol and isoproterenol acted synergistically in promoting protein secretion. A hypothesis for the regulation of lacrimal gland function is proposed which takes into account the autonomic effects on electrolyte transport and tear flow rates reported by others as well as autonomic control of protein secretion reported in this paper.

Key words: lacrimal gland, cholinergic agonist, adrenergic agonist, protein, secretion, muscarinic response, alpha-adrenergic response, beta-adrenergic response

Corneal clarity is dependent in part on the formation of tears of appropriate volume and composition. The lacrimal portion of tears constitutes the largest fraction of the tear film and consists mainly of water, electrolytes, and proteins. Although the autonomic regulation of lacrimal flow and electrolyte composition has been studied extensively in vivo and in vitro, few studies have focused on the control of protein secretion by the lacrimal gland. These studies have shown that cholinergic agents increase the rate of lacrimal protein secretion.

The major protein found in tears is specific tear prealbumin. Several other proteins, e.g., lysozyme, beta-lysin, lactoferrin, peroxidase, secretory IgA, and IgG are also present and may serve important anti-infective functions. Additionally, Franklin and Bang have identified a nondialyzable, heat-stable factor in tears which may stimulate secretion of mucus by conjunctival goblet cells.

The synthesis and secretion of lacrimal proteins present some interesting problems in ocular physiology which have not yet been addressed. If the regulation of lacrimal secretory protein is under autonomic control, we may investigate the feasibility of manipulat-
cing lacrimal gland function in diseased states such as dry eyes and eye infections. Additionally, because of the success in treating glaucoma with autonomic agonists and antagonists, it may be important to assess the effects of these drugs on lacrimal function. One beta-adrenergic blocking agent, practolol, has been withdrawn because of its side effects, including a dry eye condition.\(^\text{11}\) The adverse effects of practolol may not have been related entirely to its pharmacologic effects; nevertheless, timolol, another beta-adrenergic blocking agent, has also been associated with transient dry eyes in some patients.\(^\text{12}\)

This report demonstrates the effectiveness of cholinergic and adrenergic agents in stimulating the in vitro secretion of protein by rabbit lacrimal gland. Based on these results, a hypothesis concerning the regulation of lacrimal secretions by the autonomic nervous system is proposed.

**Materials and methods**

Carbamylcholine chloride (carbachol), \(\beta\)-isoproterenol HC1, \(\beta\)-norepinephrine bitartrate, atropine sulfate, and \(d,\beta\)-propranolol HC1 were purchased from Sigma Chemical Co., St. Louis, Mo. Phen tolamine HC1 was kindly provided by Mr. Charles A. Brownley, Jr., of CIBA Pharmaceutical Co. (Summit, N.J.). All other compounds were reagent grade.

New Zealand white rabbits (2 to 4 kg) of either sex were heparinized prior to being killed by an overdose of sodium pentobarbital injected intravenously. The head of the rabbit was perfused to reduce the amount of blood in the orbit and within the lacrimal gland. The perfusion solution contained 116 mM NaCl and 5.4 mM KCl and was usually delivered via bilateral common carotid cannulas. Occasionally perfusion was via the left ventricle, aortic arch, or abdominal aorta. After enucleation of the eye, both lacrimal glands were dissected free from the orbits and placed in incubation medium composed of 116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.81 mM MgCl\(_2\), 1.01 mM Na\(_2\)HPO\(_4\), 26.2 mM NaHCO\(_3\), and 5.6 mM dextrose. The medium with the tissue was placed in a 25 ml Erlenmeyer flask on a shaking water bath (Dubnoff Incu-Shaker; Lab-Line Instruments, Inc., Melrose Park, Ill.), maintained at 37\(^\circ\) C, and gassed continuously with 95\% O\(_2\) and 5\% CO\(_2\).

Under a dissecting microscope, the tissue was freed of adhering fat and sliced with a hand-held razor blade into slices of approximately 1 mm\(^3\). The tissue slices (50 to 200 mg wet weight) were then transferred by wide-mouth Pasteur pipette to separate 25 ml siliconized Erlenmeyer flasks containing approximately 4 ml of incubation medium. This solution was replaced with fresh incubation medium to give an incubation volume of 9.9 or 4.9 ml. Each flask was individually and continuously gassed with 95\% O\(_2\) and 5\% CO\(_2\).

After a 10 min preincubation period, the rate of protein secretion into the medium was determined. Agonist (0.5 ml) was then added, and the rate of secretion was determined for five 10 min periods (stimulated rate). The agonists, carbachol, isoproterenol, and norepinephrine, were dissolved in incubation medium containing 10 or 20 mM L-ascorbate (Na\(^+\) salt). Control tissue received 0.5 ml of incubation medium with 10 or 20 mM L-ascorbate (Na\(^+\) salt).

In all these experiments, the agonist was present throughout the stimulatory period.

Even with L-ascorbate present, isoproterenol is
Table I. Protein secretory response to autonomic agonists

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Response*</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol (5)</td>
<td>8.5 ± 1.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Norepinephrine (4)</td>
<td>7.2 ± 1.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Isoproterenol (4)</td>
<td>7.4 ± 0.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control ((12)</td>
<td>2.5 ± 0.31</td>
<td></td>
</tr>
</tbody>
</table>

*Total protein secreted 100 min after stimulation (micrograms of protein per milligram of tissue). Values are mean ± S.E.M.; number of experiments is shown in parentheses.

Results

Response to autonomic agonists. Carbachol, isoproterenol, and norepinephrine stimulated secretion of protein from slices of lacrimal gland (Fig. 1). For each agonist, the secretory rate was highest during the first 20 min period after addition of the agonist (period 2) and thereafter slowed toward baseline levels. During period 2, carbachol stimulated the secretory rate 5.6 times the basal level; norepinephrine- and isoproterenol-stimulated rates were 4.3 and 3.3 times basal, respectively.

The total amount of protein secreted in response to each agonist was significantly greater than that secreted by the control tissue (Table I). No significant differences were seen in the total amount of protein secreted in response to each of the three agonists (Table I), even though the time course may have been different for each agonist (Fig. 1).

Specificity of response. Addition of a specific antagonist 5 min after stimulation showed the reversibility of the secretory response and simulated a pulse of agonist rather than continuous stimulation. In two representative experiments, atropine and propranolol reduced the secretory response to carbachol and isoproterenol, respectively, to basal levels by period 3, the second poststimulation period (Fig. 2).

The specificity of response was tested fur-
ther by preincubation of the tissue with atropine, propranolol, and phentolamine (Table II). Preincubation with 0.1 mM atropine completely inhibited the response to 0.1 mM carbachol but had no significant effect on the response to isoproterenol or norepinephrine. Propranolol (1 mM) inhibited the response to isoproterenol and also reduced the effects of carbachol and norepinephrine by about 66% and 70%, respectively. Phentolamine (1 mM) reduced the response to norepinephrine about 50% and reduced isoproterenol stimulation only slightly. Phentolamine also inhibited about 40% of the response to carbachol.

**Dose-response curves.** The dose-response curves obtained for carbachol showed that, at the highest concentration used (0.1 mM), the secretory rate was about 0.28 µg of protein per milligram of tissue per minute (Fig. 3), more than 2.5 times the maximum secretory rate induced by isoproterenol. However, the threshold of response for isoproterenol was significantly lower than that for carbachol. The half-maximal response to isoproterenol was achieved at about 0.03 µM, whereas that for carbachol occurred at about 3.0 µM. Also, the maximum rate of secretion for isoproterenol, about 0.1 µg of protein per milligram of tissue per minute, was reached at about 10^-6 M. From Fig. 3 it appears that the maximum rate for carbachol may not have been achieved within the limits of these experiments.

To determine whether the actions of carbachol and isoproterenol are additive or synergistic, they were added together at near maximal, half-maximal, and less than half-maximal concentrations. In all cases, the secretory response was greater than that expected from simple additivity (Table III).

**Discussion**

Several investigators have reported the effects of autonomic stimulation on electrolyte transport, acinar cell membrane resistance and potential, and fluid flux from the lacrimal gland. Cholinergic stimulation invariably alters cell membrane properties and increases electrolyte and water flux from the gland. In contrast, the effects of sympathetic stimulation are usually small and dependent on species and route of stimulation.

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**Table II. Specificity of secretory response**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Carbachol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Isoproterenol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Norepinephrine&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.078 ± 0.020</td>
<td>0.136 ± 0.033</td>
</tr>
<tr>
<td>Propranolol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.105 ± 0.005&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.006 ± 0.002&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.043 ± 0.019&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phentolamine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.194 ± 0.009&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.075 ± 0.015&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.079 ± 0.012&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.312 ± 0.026</td>
<td>0.068 ± 0.018</td>
<td>0.148 ± 0.018</td>
</tr>
</tbody>
</table>

Values are micrograms of protein per milligram of tissue per minute above background rate; mean ± S.E.M. of 4 experiments.

<sup>a</sup>Concentration = 0.1 mM.

<sup>b</sup>Concentration = 1 mM, except for atropine = 0.1 mM.

Tissue was preincubated for 5 to 10 min with the antagonist prior to exposure to agonist as described in Materials and methods.

<sup>c</sup>p value vs. controls: <0.001; <0.01; <0.02; <0.05.

**Table III. Synergism of carbachol and isoproterenol**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Secretory rate (µg protein/mg tissue/min)</th>
<th>n*</th>
</tr>
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<tbody>
<tr>
<td>0.1 mM Carbachol</td>
<td>0.287 ± 0.061</td>
<td>3</td>
</tr>
<tr>
<td>0.01 mM Isoproterenol</td>
<td>0.113 ± 0.013</td>
<td>3</td>
</tr>
<tr>
<td>0.1 mM Carbachol + 0.01 mM isoproterenol</td>
<td>0.750 ± 0.260</td>
<td>3</td>
</tr>
<tr>
<td>10 µM Carbachol</td>
<td>0.218 ± 0.040</td>
<td>3</td>
</tr>
<tr>
<td>0.1 µM Isoproterenol</td>
<td>0.094 ± 0.017</td>
<td>3</td>
</tr>
<tr>
<td>10 µM Carbachol + 0.1 µM isoproterenol</td>
<td>0.504 ± 0.060</td>
<td>3</td>
</tr>
<tr>
<td>1 µM Carbachol</td>
<td>0.067</td>
<td>2</td>
</tr>
<tr>
<td>0.01 µM Isoproterenol</td>
<td>0.037</td>
<td>2</td>
</tr>
<tr>
<td>1 µM Carbachol + 0.01 µM isoproterenol</td>
<td>0.232</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>1</sup>Number of experiments.

<sup>1</sup>Mean ± S.E.M.

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Fig. 3. Dose-response curve for isoproterenol and carbachol. Values are mean ± S.E.M., and numbers in parentheses are numbers of replicates for each point.

Inhibition of the response to isoproterenol and norepinephrine by propranolol and phentolamine suggests that isoproterenol acts almost exclusively as a beta-adrenergic agonist whereas norepinephrine has both alpha- and beta-adrenergic activity. Indeed, the norepinephrine response is about 1.5 to 2 times that of isoproterenol (Table II). The action of carbachol is more complex, as indicated by its significant inhibition not only by atropine but also by propranolol and phentolamine.

In contrast to the observations reported here (Table II), Keryer and Rossignol observed no antagonism by propranolol or phentolamine of the carbachol-induced secretion of protein by slices of rat lacrimal gland. Three possibilities exist for this discrepancy. First, there is the difference in species. The major rat lacrimal gland (the exorbital gland) lies outside the orbit proper, and the functional anatomy and physiology may be different from those of the rabbit. Second, Keryer and Rossignol measured protein secretion by release into the medium of newly incorporated [14C]-leucine. Possibly the newly synthesized protein belonged to a separate pool which was responsive only to carbachol. Third, the dose used by Keryer

acetylcholine and are consistent with the observations reported here.

Others have reported the in vitro secretion of proteins from rat lacrimal tissue in response to carbachol⁴,⁵; however, the effects of adrenergic agonists were not tested directly as was done here. Both cholinergic and adrenergic agonists initiate protein secretion from rabbit lacrimal slices, and similar amounts of protein are secreted in response to each agonist (Table I). Preliminary analysis by gel electrophoresis indicates that no differences exist among the proteins secreted in response to the different autonomic agonists. Similarly, amylase secretion by parotid gland²² and exocrine pancreas²³ can be stimulated by cholinergic and adrenergic agonists, and even by peptides. Even though the ultimate physiological response, protein secretion, is the same for all three agonists, differences clearly exist in the time course and pharmacological specificity of the response.

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and Rossignol, 1 μM carbachol, may not have been sufficient to cause release of native nor-
epinephrine (see below); hence no inhibition
was seen by propranolol or phentolamine. In
this study, the response to 1 μM carbachol in
rabbit tissue was found to be less than half-
maximal (Fig. 3).

At least two interpretations are possible
concerning the mode of action of carbachol
on rabbit lacrimal gland. One is that car-
bachol acts on two separate muscarinic recep-
tors. That portion of the response which is
not inhibited by propranolol or phentolamine
may be the result of direct muscarinic stimu-
lation of the protein secretory acinar cells.
The remainder of the response, which is in-
hibited by both atropine and the adrenergic
agonists, can be attributed to the car-
bachol-induced release of endogenous nor-
epinephrine from sympathetic nerve termi-
nals. Thus carbachol could bind to muscarinic
receptors on the sympathetic nerves and
cause the release of native norepinephrine.
Atropine would inhibit the muscarinic re-
lease of norepinephrine, whereas the adren-
ergic antagonists would prevent the secretory
response by blocking sympathetic receptors
on the secretory cells.

A second possibility is that the entire re-
response may be mediated through sympa-
thetic receptors activated by norepinephrine
released as described above, with no direct
action of carbachol on protein secretion by
acinar cells. This idea is suggested because
the sum of the remaining activity after pro-
pranolol and phentolamine inhibition of the
carbachol responses (Table II, column 1) is
about equal to the entire uninhibited car-
bachol response. A similar model has been
proposed by Burn and Rand4 to explain the
cholinergically mediated response of the cat
nictitating membrane, spleen, uterus, rat
mesenteric artery, and other systems. This
hypothesis will be tested on denervated or
adrenergically depleted lacrimal glands and
in tissue preparations preloaded with 3H-
norepinephrine.

The significance of the nonsaturating dose-
response curve for carbachol (Fig. 3) is unclear
and requires further experimentation. The
inhibition by atropine (Fig. 2, Table II) sup-
ports the pharmacological specificity of the
response. It is possible that the high secretry
rate due to carbachol is the result of stimula-
tion of electrolyte transport and water flux into
the secretory ducts and hence, in effect, the
washing out of the protein secreted into the
ducts under the influence of carbachol-
induced release of norepinephrine.

Cholinergic augmentation of norepineph-
rine release could be responsible, at least in
part, for the synergism between carbachol and
isoproterenol (Table III). Carbachol, act-
ing directly on the sympathetic nerve termi-
nals, would greatly increase the concentra-
tion of adrenergic agonist in the microen-
vironment of the acinar cells. In the presence
of exogenous isoproterenol, the normal path-
ways for uptake and degradation of native
norepinephrine would already be saturated.
At high concentrations of carbachol plus iso-
proterenol, where isoproterenol is already
saturating, the synergism is less easily un-
derstood. Again, the explanation probably
rests with the ability of carbachol to elicit
both high rates of electrolyte transport with
ensuing water flux and protein secretion via
sympathetic pathways.

The significant in vitro response of the
rabbit lacrimal tissue to adrenergic agonists
suggests a new explanation for the dual in-
ervation (adrenergic and cholinergic) of lac-
rimal gland. As indicated above, the effects of
cholinergic stimulation on flow and elec-
trolyte content of lacrimal effluent are well
established. On the other hand, because the
effect on basal lacrimal flow of adrenergic
stimulation is small and inconsistent, the
main site of action was thought to be vascu-
lar. However, the observation that the thresh-
old for lacrimal protein secretion is lower for
isoproterenol than for carbachol (Fig. 3) and
that part or all of the carbachol response is
mediated through adrenergic mechanisms
appears to contradict this. Additionally, a
significant synergism exists between isopro-
terenol and carbachol. These data suggest
that, in vivo, acetylcholine and norepineph-
rine act together to form the appropriate
lacrimal secretion. It may be that the para-
sympathetic tone regulates the flow rate and electrolyte content of the lacrimal effluent whereas the sympathetic tone regulates the macromolecular content.

REFERENCES