The purpose of this study was to identify and characterize functional G proteins that couple regulatory peptides with lacrimal secretory functions. Membranes were prepared from isolated rat exorbital lacrimal gland acini, and guanosine 5'-triphosphate (GTP)-dependence of adenylyl cyclase activity, known to be coupled with regulation of secretion, was measured. The guanine nucleotide GTP produced a biphasic response in the activity of membrane-bound adenylyl cyclase during a 10 min incubation with a maximum stimulation at 10⁻³ mol/l GTP. Significant inhibition occurred at a dose of 10⁻³ mol/l GTP, with cyclic adenosine monophosphate (cAMP) production reduced to less than basal levels. The effect of ADP-ribosylation of membrane proteins by the toxins produced by *Vibrio cholerae* or *Bordetella pertussis* on lacrimal adenylyl cyclase was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, autoradiography, and laser densitometry. Cholera toxin treatment of membranes resulted in dose- (0.5–100 µg/ml) and time-dependent (0–45 min) adenosine diphosphate (ADP)-ribosylation of two membrane proteins with M_r values of 42,000 and 45,000. Pertussis toxin treatment resulted in the specific ADP-ribosylation of a single protein that migrates with an M_r value of 41,000. This was also dose (0.5–25 µg/ml) and time-dependent (0–30 min). Incorporation of ³²P into the 45,000 M_r and 42,000 M_r proteins in the presence of 50 µg/ml cholera toxin was guanine nucleotide dependent, with a two- to threefold increase in labeling when the membranes were incubated with 1 or 0.5 mmol/l GTP. This effect was enhanced in the presence of the nonhydrolyzable GTP analog GTPγS. In contrast, ribosylation of the 41,000 M_r protein by 2.5 µg/ml pertussis toxin was increased 20–25 fold by the addition of GTP. GTPγS resulted in less than 30% of the maximum GTP-dependent incorporation of ³²P in the presence of pertussis toxin. Toxin treatment also resulted in significant activation of adenylyl cyclase in lacrimal membranes. Cholera toxin treatment resulted in a maximum effect at a dose of 10 µg/ml, with an increase in cAMP production from a basal value of 6.6 ± 1.4 pmol/mg protein to 37.6 ± 5.5 pmol/mg protein. Similarly, basal activity of 12.0 ± 4.1 pmol/mg protein was maximally stimulated to 56.2 ± 14.8 pmol/mg protein by adding 5 µg/ml pertussis toxin. In the absence of either toxin, 100 µmol/l GTPγS increased adenylyl cyclase activity five- to sixfold when compared with basal cAMP production. Although the effects of submaximal doses of cholera or pertussis toxin were enhanced by the presence of 100 µmol/l GTPγS, cAMP production was not significantly increased by GTPγS at the maximally effective doses of either toxin. We conclude that the toxin substrates identified by ADP-ribosylation and ³²P incorporation represent the α subunits of G_s and G_i in lacrimal acinar cells that couple peptidergic receptor activation with adenylyl cyclase and the dual regulation of lacrimal secretion.

In the lacrimal gland, control of regulated protein secretion via the cyclic adenosine 3'-5' monophosphate (cAMP) pathway involves stimulation and inhibition of exocytosis. Protein secretion is enhanced by norepinephrine,¹ the β-adrenergic agonist isoproterenol,² the POMC derivatives adrenocorticotropic hormone and α-melanocyte-stimulating hormone,³,⁴ and vasoactive intestinal peptide (VIP).⁵ Associated with cell-surface receptor activation and enhanced protein secretion by these ligands is an alteration in the rate of adenylyl cyclase activity in lacrimal membrane preparations⁶ and resulting changes in the level of intracellular cAMP. The generation of second messengers is linked to exocytosis through cAMP-dependent protein kinases that alter the phosphorylation state of a specific 91,000 M_r, peptide in the basolateral membranes of secretory acinar cells and 26,000 and 20,000 M_r, peptides associated with exocytosis and ribosomal protein S6.⁷,⁸ Inhibition of cholinergic and VIPergic stimulation of lacrimal secretion by d-ala²-met-enkephalinamide⁹ and the inhibition of adenylyl...
cyclase by the synthetic opioid and by derivatives of proenkephalin A\(^{10}\) indicate a dual regulation of the cAMP signal transduction pathway in lacrimal gland. Within this dually regulated cAMP pathway, cell surface receptor activation is known to be coupled to adenylyl cyclase through the heterotrimeric guanine nucleotide-binding proteins \(G_s\) and \(G_i\).\(^{11}\) Coupling of the stimulatory receptors in lacrimal gland through \(G_s\) is implicated by the stimulation of lacrimal adenyl cyclase by NaF, an activator of \(G_{s,12}\) by guanine 5’-0-3’-thiotriphosphate (GTP\(_7\)S), a nonhydrolyzable analog of GTP;\(^2\) and by the GTP-dependence of VIP stimulation of adenylyl cyclase.\(^6\) The GTP-dependence of peptidergic inhibition of adenylyl cyclase in lacrimal gland also is likely, but has not been demonstrated in lacrimal secretory tissues.

In the present study, cholera or pertussis toxin treatment of membranes prepared from isolated lacrimal acini resulted in the ADP ribosylation of distinct peptides that most likely represent the \(\alpha\) subunits of the guanine nucleotide binding proteins \(G_s\) and \(G_i\). Alterations in adenylyl cyclase activity in the presence of toxins provide evidence for the coupling of \(G_s\) and \(G_i\) to adenylyl cyclase. Therefore, the action of these toxins on the effector enzyme activity also was measured as an indication of the presence of functional lacrimal guanine nucleotide binding proteins that may couple receptor activation with adenylyl cyclase activity and lacrimal secretion.

Materials and Methods

Materials

\[2,8,13\text{H}]\text{-adenosine 3',5' cyclic phosphate (44.5 Ci/ mmol) was obtained from New England Nuclear (Boston, MA), [14C] methylated proteins (34–59 \text{Ci}/\text{mg) was obtained from Amersham Corp. (Arlington Heights, IL), and [adenylate-32P] NAD (250 Ci/ mmol) was from ICN Radiochemicals (Irvine, CA). Forskolin was purchased from Calbiochem (La Jolla, CA). Cholera toxin and pertussis toxin were obtained from List Biological Laboratories (Campbell, CA). Liquid 2% bis-acrylamide (N,N’methylene-bis-acrylamide), liquid 30% acrylamide, N, N, N, N’ tetramethylethylenediamine, sodium dodecyl sulfate (SDS), and ammonium persulfate were obtained from Boehringer-Mannheim (Indianapolis, IN). Fast Stain was from Zioon Research (Alliston, MA), and prestained molecular weight standards were obtained from Bio-Rad (Richmond, CA). Bovine serum albumin (BSA; fraction V) was from ICN (Lisle, IL). All other reagents were obtained from Sigma Chemical (St. Louis, MO).\]

Animals

Male Sprague-Dawley rats (250–300 g) were obtained from Charles River, Wilmington, MA and were used according to the ARVO Resolution on the Use of Animals in Research. The animals were housed in a controlled environment, exposed to a 12 hr light-dark cycle, and provided with standard laboratory rodent chow (No. 5001; Purina, St. Louis, MO) and water ad libitum for at least 1 wk before they were used. Animals were killed by intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight).

Lacrimal Gland Acinar Membrane Isolation

The method for isolating acini is a modification of the procedure of Dartt et al.\(^5\) To obtain the acini, lacrimal glands (100–150 mg) from two animals were removed immediately after the rats were killed. Animals were killed by an intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight). The general procedures were according to the ARVO Resolution on the Use of Animals in Research. The animals were housed in a controlled environment, exposed to a 12 hr light-dark cycle, and provided with standard laboratory rodent chow (No. 5001; Purina, St. Louis, MO) and water ad libitum for at least 1 wk before they were used. Animals were killed by intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight).

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min at 40,000 × g. The resulting pellet was washed 3× at 40,000 × g for 20 min. The sedimented membranes were resuspended in the isolation medium, aliquoted, frozen in liquid nitrogen, and stored at −70°C. Membrane protein concentration was determined by the Lowry method.13

Adenylyl Cyclase Activity

Enzyme activity was determined in polypropylene tubes in a total volume of 100 μl of 40 mmol/l Tris (pH 7.5), 4 mmol/l MgCl2, 0.5 mmol/l adenosine triphosphate (ATP), 1 mmol/l DTT, 0.1 mg/ml BSA, 0.1 mmol/l GTP (except where noted), 0.1 mmol/l EDTA, and 0.1 mmol/l IBMX. Additions to the reaction mixture included forskolin or the appropriate vehicle. Forskolin was prepared as a 20 mmol/l stock in 95% ethanol. Enzyme activity was determined in triplicate tubes at 37°C for 10 min, and the reaction was terminated in a boiling bath for 2 min. cAMP was measured by the protein binding assay method of Brown et al.14 Assay tubes contained 100 μl sample or standard, 10 μl 3H-cAMP (50,000 cpm/tube), and 25 μl protein kinase prepared as described previously,6 diluted in 50 mmol/l Tris (pH 6.0), 1 mmol/l DTT, and 9 mmol/l theophylline to give approximately 30% binding. The tubes were incubated at 4°C for 60 min. After this, 500 μl of ice cold 20 mmol/l KH2PO4/K2HPO4 (pH 6.0) 0.4% BSA (pH 5.2), and 0.4% activated charcoal were added to each tube. The tubes were centrifuged at 4°C for 15 min at 3000 rpm and the supernatant containing the bound fraction was counted by liquid scintillation (Beckman LS7500, Irvine, CA). Adenylyl cyclase-specific activities were calculated as picomoles/milligram membrane protein.

[32P] ADP-Ribosylation

Cholera or pertussis toxin was preactivated for 30 min at 30°C with 50 mmol/l DTT in a 50 mmol/l potassium phosphate buffer (pH 7.4). For [32P]ADP-ribosylation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), acinar membranes (100 μg protein) were centrifuged at 4°C for 15 min in a microfuge. The preactivated toxin and ribosylation medium were added to the membrane pellet, resulting in a 100 μl membrane suspension containing 8 μmol/l NAD, 2 μCi [32P]NAD, 10 mmol/l thymidine, 1 mmol/l ATP, 0.4 mmol/l GTP (except where noted), 0.6 mmol/l EDTA, 3 mmol/l MgCl2, 5 mmol/l DTT, and 50 mmol/l potassium phosphate buffer (pH 7.4). The membranes were incubated for 30 min at 30°C. Incubation was terminated by adding 1 ml ice-cold 40 mmol/l Tris (pH 7.4) and centrifuging in a microfuge for 15 min at 4°C. When the effects of toxin-catalyzed ribosylation on adenylyl cyclase activity were determined, [32P]NAD was omitted from the medium, and the NAD final concentration was 1 mmol/l. The membrane was resuspended in 40 mmol/l Tris buffer and assayed for adenylyl cyclase activity.

SDS-PAGE

After the ribosylation reaction, 30 μg pelleted membrane protein was dissolved in gel sample buffer containing 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mmol/l Tris (pH 6.8), and 0.02% bromphenol blue and heated in a boiling bath for 3 min. Membranes were analyzed by SDS-PAGE15 on 0.75 mm slab mini-gels consisting of a 4.5% stacking gel and a 12.5% resolving gel. After electrophoresis, the gels were washed 3× in 45% methanol:10% acetic acid for 10 min and stained with 0.4% amido black to give approximately 30% binding. The gels were incubated at 4°C for 60 min. After this, 500 μl of ice cold 20 mmol/l KH2PO4/ K2HPO4 (pH 6.0) 0.4% BSA (pH 5.2), and 0.4% activated charcoal were added to each tube. The tubes were centrifuged at 4°C for 15 min at 3000 rpm and the supernatant containing the bound fraction was counted by liquid scintillation (Beckman LS7500, Irvine, CA). Adenylyl cyclase-specific activities were calculated as picomoles/milligram membrane protein.

Results

Guanine Nucleotide-Dependent Stimulation and Inhibition of Adenylyl Cyclase

The activity of adenylyl cyclase in lacrimal acinar membranes (Fig. 1) was determined by measuring cAMP production during a 10 min incubation in the presence of increasing concentrations of GTP (0–1 mmol/l). Basal cAMP production in the absence of the guanine nucleotide was 6.1 ± 2.8 pmol/mg protein. Adding increasing concentrations of GTP resulted in a biphasic response made up of a stimulatory and an inhibitory phase. In the stimulatory phase, production of cAMP increased with the addition of increasing GTP. The maximum production of cAMP was 14.8 ± 1.4 pmol/mg protein. Adding increasing concentrations of GTP resulted in a biphasic response made up of a stimulatory and an inhibitory phase. In the stimulatory phase, production of cAMP increased with the addition of increasing GTP. The maximum production of cAMP was 14.8 ± 1.4 pmol/mg protein.
determined by Student's t-test.

*Significantly different from control, P < 0.05, or **P < 0.01. The means ± standard errors of four experiments were calculated.

Fig. 1. Biphasic effect of GTP on adenyl cyclase activity in lacrimal acinar membranes. Membranes were incubated for 10 min at 37°C. Basal (open circles) and 30 μmol/l forskolin-stimulated (filled circles) cyclic adenosine monophosphate production was measured in the presence of increasing concentrations (0-10⁻³ mol/l) of GTP. Statistically significant differences indicated by densitometry.

The initial incorporation of [³²P] by the cholera toxin substrates was linear with increasing incubation time. A maximum ribosylation occurred at 30 min at a dose of 75 μg/ml cholera toxin (Fig. 2B). Pertussis toxin-dependent ADP-ribosylation resulted in [³²P] incorporation into a single protein that migrated with an M, of 41,000 (Fig. 3A, Top). Significant ribosylation occurred in the presence of as little as 0.5 μg/ml pertussis toxin. Maximum ribosylation in the presence of pertussis toxin required a dose of 5 μg/ml; the degree of ribosylation was linear with time up to 30 min at a dose of 2.5 μg/ml pertussis toxin (Fig. 3B, Bottom). The intensity of radioactivity incorporated into the 41,000 dalton protein in the presence of pertussis toxin was much greater than the intensity of the 42,000 and 45,000 dalton proteins ribosylated in the presence of cholera toxin at all doses and equivalent incubation periods. Incubation of lacrimal membranes with 10 μg/ml cholera toxin plus 1 μg/ml pertussis toxin (Fig. 4) resulted in the ribosylation of three bands corresponding to the proteins ribosylated in the presence of cholera toxin or pertussis toxin. The differential ribosylation by cholera and pertussis toxins and the associated M, values indicated these peptides are the stimulatory and inhibitory guanine nucleotide regulatory proteins Gs and Gi.

Further differentiation of the G proteins was achieved by incubating membranes with [³²P]NAD and cholera or pertussis toxin in the absence of guanine nucleotides or with the addition of GTP or GTPγS. ADP-ribosylation by 50 μg/ml cholera toxin (Fig. 5, Top) was minimal in the absence of any guanine nucleotide. Incorporation of [³²P] into the 45,000 and 42,000 M, proteins by cholera toxin was guanine nucleotide dependent with a two- to threefold increase in labeling when the membranes were incubated with either concentration (1 or 0.5 mmol/l) of GTP. This effect was enhanced twofold in the presence of the nonhydrolyzable GTP analog GTPγS (Fig. 5, Bottom). In contrast, ribosylation of the 41,000 M, protein by 2.5 μg/ml pertussis toxin was increased 20- to 25-fold by the addition of GTP (Fig. 6). GTPγS was much less effective, resulting in less than 30% of the maximum GTP-dependent incorporation of [³²P].
Fig. 2. Adenosine diphosphate (ADP)-ribosylation of lacrimal membrane proteins by cholera toxin. Membranes were incubated with [32P]NAD in the presence of cholera toxin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, autoradiography, and laser densitometry. (A) Membranes were incubated with increasing (0–100 μg/ml) concentrations of cholera toxin for 30 min. Data shown represent n = 4. (B) Membranes were incubated with 75 μg/ml cholera toxin for increasing (0–45 min) incubation times. Curves in the bottom figures were derived by measurement of the 42 kD and 45 kD bands as a single band. Data shown represent n = 2.

Fig. 3. Adenosine diphosphate (ADP) ribosylation of lacrimal membrane proteins by pertussis toxin. Membranes were incubated with [32P]NAD in the presence of pertussis toxin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, autoradiography, and laser densitometry. (A) Membranes were incubated with increasing (0–25 μg/ml) concentrations of pertussis toxin for 30 min. Data shown represent n = 3. (B) Membranes were incubated with 2.5 μg/ml pertussis toxin for increasing (0–30 min) incubation times. Data shown represent n = 2.
Fig. 4. Adenosine diphosphate ribosylation of distinct proteins in lacrimal membranes by cholera or pertussis toxin. Membranes were incubated with [32P]NAD in the presence of 1 μg/ml pertussis toxin plus 10 μg/ml cholera toxin, 10 μg/ml cholera toxin, 1 μg/ml pertussis toxin, or no toxin for 30 min. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The gel shown represents n = 2.

Effect of Cholera and Pertussis Toxins on Lacrimal Adenylyl Cyclase

In additional experiments, lacrimal membranes were incubated in the presence of increasing concentrations of cholera toxin or pertussis toxin. Because toxin-induced ribosylation results in characteristic modifications of the physiologic activity of the G proteins, the membranes were assayed for adenylyl cyclase activity after the ribosylation reactions. Cholera toxin treatment of membranes resulted in a dose-dependent (0–25 μg/ml) activation of adenylyl cyclase activity (Fig. 7). Maximum adenylyl cyclase activity occurred at a dose of 10 μg/ml cholera toxin. At this dose, cAMP production was increased from a basal value of 6.6 ± 1.4 pmol/mg protein to 37.6 ± 5.5 pmol/mg protein (P < 0.001). To determine if activation by cholera toxin was the result of a Gα-mediated mechanism, adenylyl cyclase was measured with 100 μmol/l GTPγS in addition to increasing concentrations of the toxin.

In the absence of cholera toxin, GTPγS increased cAMP production from the basal value to 37.1 ± 4.2 pmol/mg protein (P < 0.001). Significant increases resulted from the addition of GTPγS at doses of 0.5, 1, 2, and 5 μg/ml cholera toxin (P < 0.001, in all cases). Maximum stimulation by cholera toxin at 10 μg/ml was not significantly increased by adding 100 μmol/l GTPγS to the adenylyl cyclase reaction mixture. The response of cAMP production through activation of adenylyl cyclase in the presence of increasing concentrations of pertussis toxin (0–10 μg/ml)
with or without 100 μmol/l GTPγS (Fig. 8) was similar to that obtained with cholera toxin. Basal activity, (12.0 ± 4.1 pmol/mg protein) was maximally stimulated to 56.2 ± 14.8 pmol/mg protein (P < 0.01) by a dose of 5 μg/ml pertussis toxin. The presence of 100 μmol/l GTPγS resulted in an increase from the basal value to 63.5 ± 16.4 pmol/mg protein (P < 0.01). Although the submaximal effect of pertussis toxin at 0.5 and 1 μg/ml was enhanced by the addition of GTPγS, activity was not significantly increased by GTPγS at the maximally effective dose of pertussis toxin.

Discussion

In lacrimal gland acinar membrane preparations, the guanine nucleotide GTP produced a dose-dependent biphasic response in the activity of membrane-bound adenylyl cyclase. The biphasic response to GTP is typical of dually regulated systems coupled through distinct guanine nucleotide binding proteins to the regulation of adenylyl cyclase and intracellular levels of cAMP.15 In addition, the presence of separate and distinct GTP binding sites that are localized to the α subunits of the stimulatory and inhibitory G proteins is suggested by the persistence of the GTP-dependent inhibitory phase in the presence of forskolin.

Cholera toxin treatment resulted in the ADP ribosylation of two proteins with Mr values of 42,000 and 45,000. Because the α-subunit is the site of cholera toxin-catalyzed ADP-ribosylation,17 these results indicate two major forms of the α subunit of Gs in lacrimal acinar membranes. Multiple forms of Gsα within a single cell type is not uncommon and apparently is the result of alternative splicing of a single precursor mRNA.18 Alternate forms can bind and hydrolyze GTP. However, the kinetics of receptor-stimulated rates of GDP dissociation and GTP activation may differ.18 The physiologic significance of the presence of alternate forms in any cell type is not entirely clear. However, regulation of ion fluxes, known to be important in lacrimal secretion, may be important. In contrast to the presence of two distinct cholera toxin ribosylated proteins, pertussis toxin treatment resulted in the ADP ribosylation of a single protein that migrates with a Mr value of 41,000. This protein clearly differs in electrophoretic mobility and in its specificity as a substrate for pertussis toxin-mediated ADP ribosylation from the 42,000 Mr subunit of Gαs that was ADP-ribosylated by cholera toxin. Unlike Gαs, at least three genes encode Gαi subunits, as evidenced by the isolation of multiple cDNAs.19 All three Gαi subunits may be present in a single cell type.18

Although characterization of Gαi in lacrimal gland remains to be determined, at least Gαi2 probably is present. This subunit is known to mediate opioid receptor activation and inhibition of adenylyl cyclase20 and recently was localized to the basolateral membranes of an epithelial cell line.21 The suggested presence of Gsαi in lacrimal membranes is consistent with the localization of adenylyl cyclase and cAMP-dependent protein kinases to the basolateral membranes22 of lacrimal acinar cells as well as the inhibition of lacrimal secretion and adenylyl cyclase activity by enkephalins.5,10

The effect of the guanine nucleotide GTP and its nonhydrolyzable analog, GTPγS, on ADP ribosylation of Gαs and Gαi agrees with the known mechanistic
similarities and differences in the physical and biochemical behavior of subunit associations of the G proteins. One consequence of the binding site occupation of the Gs or G, G subunit by GTP is the dissociation of the α and βγ subunits, which affects the degree of toxin-dependent ADP ribosylation. Our results of enhanced Gs ribosylation in the presence of GTP with an effect of an even greater magnitude by GTPγS are consistent with those reported by others22 and lend additional support to identifying the cholera substrates as the Gα subunits. While the dissociated form of Gs serves as the substrate for cholera toxin, pertussis toxin catalyzes the ribosylation of Gi in its inactive GDP-bound form.23 The GTPγS-bound α subunit dissociates from the βγ subunits,24 and this dissociated form of Gi does not serve as an optimal substrate for pertussis toxin ribosylation in rabbit corpora lutea25 or in platelet membranes.24 The present study indicates that this also is the case in lacrimal acinar membranes.

In the present study, toxin enhancement of adenylyl cyclase paralleled incorporation of 32P by ADP-ribosylation; both actions depended upon the concentration of toxin with which the membranes were pretreated. Stimulation of adenylyl cyclase by the binding of GTPγS to the α subunits was equivalent to the maximum effect of pretreatment with either toxin. Furthermore, the effects of toxin treatment (ADP ribosylation) and persistent activation by the nonhydrolyzable analog GTPγS were not additive. Thus, toxin treatment effects on adenylyl cyclase activity in lacrimal gland acinar membranes were not separate from the mechanisms regulated by the binding of guanine nucleotide.

In summary, we have shown that lacrimal gland acinar cells contain cholera toxin and pertussis toxin substrates that appear to represent the α subunits of Gs and Gi coupled to regulation of adenylyl cyclase. The lacrimal gland contains the peptides VIP10,26 and enkephalin,10,26 and these peptides regulate secretion via alterations in adenylyl cyclase activity.6 Thus, these peptides and possibly others known to affect secretion through alterations in intracellular cAMP probably are coupled through Gs and Gi to this effector. The characterization of coupling of specific cell surface receptors to the effector enzyme adenylyl cyclase through G proteins also may be important in elucidating the mechanism of interactions among the components of transmembrane signalling pathways mediated by G proteins, including cross-talk between the cAMP and IP3/Ca2+ pathways.27

Key words: adenylyl cyclase, exocrine gland, G proteins, Gα, Gi, lacrimal gland

References


