Gi2 and Gi3 Couple Met-Enkephalin to Inhibition of Lacrimal Secretion

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PURPOSE. The intent of this study was to identify the pertussis toxin-sensitive G proteins that couple met-enkephalin to the inhibition of cholinergically stimulated secretion in rabbit lacrimal gland acini.

METHODS. The authors detected G proteins in membranes from freshly isolated glands, freshly isolated acini, and cultured lacrimal acini from rabbits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Antibodies against the α subunits of Gi1, Gi1, and Gi2, or Gi3, were used in cultured acini permeabilized by streptolysin-O to determine the role of the G proteins in met-enkephalin inhibition of cholinergic stimulation of lacrimal acinar protein release.

RESULTS. Western blot analysis showed the presence of the α subunits of Gi2 and Gi3, but not Gi1, in all three membrane preparations. The met-enkephalin analog ω-Ala2-methionine enkephalinamide (DALA) inhibited cholinergic stimulation of secretion by cultured rabbit acinar cells to near basal levels. Inhibition of secretion by DALA was blocked by insertion of antibody to a peptide sequence common to Gia1 and Gia2, but was not blocked by antibody against a specific Gia1 sequence. The inhibitory effect of DALA also was blocked by antibody to a Gia3 sequence. At low doses of anti-Gia1/2 and anti-Gia3 in combination, the effect on reversal of inhibition was additive. However, at higher doses, the effect of the combination was no greater than the effect of either antibody alone.

CONCLUSIONS. These results demonstrate that met-enkephalin inhibition of cholinergic secretion is mediated by way of the pertussis toxin-sensitive G proteins Gi2 and Gi3 in cultured rabbit lacrimal acini. Because the effects of the G proteins are not additive, the intracellular events distal to G protein activation most likely converge at some point before exocytosis. (Invest Ophthalmol Vis Sci. 1998;39:1339-1345)
couple met-enkephalin to inhibition of cholinergic stimulation of secretion.

**METHODS**

**Materials**

Dulbecco’s modified Eagle medium (DMEM)/Ham’s Nutrient Mixture F-12 (1:1), insulin-transferrin-sodium selenite, gentamicin, epidermal growth factor, soybean trypsin inhibitor (STI), aprotinin, dexamethasone, carbamylcholine chloride (carbachol), 1-Ala2-methionine enkephalinamide (DALA) and Hepes were from Sigma Chemical (St. Louis, MO). Certified fetal bovine serum (FBS), collagenase, and streptolysin-O (SLO) were from Life Technologies (Gaithersburg, MD). Hyalurondase was from Worthington Biomedicals (Freehold, NJ) and DNase I was from Boehringer Mannheim (Indianapolis, IN).

Basement membrane matrix was obtained from Collaborative Biomedical (Madrigel; Bedford, MA). G_{	ext{null}2} and G_{	ext{null}3} synthetic C-terminal peptides; G_{	ext{null}1}, G_{	ext{null}2}, and G_{	ext{null}3} antibodies; G_{	ext{null}1}, G_{	ext{null}2}, and G_{	ext{null}3} recombinant subunits; and nonimmune serum were from Calbiochem-Novobiochem (La Jolla, CA).

Precast sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, prestained SDS-PAGE molecular weight standards, polyvinylidene difluoride membranes, alkaline phosphatase–conjugated anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium were from Bio-Rad (Hercules, CA). All other reagents and supplies were from standard sources.

**Isolation and Culture of Lacrimal Acini**

All experimental procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult male New Zealand white rabbits were sedated with an intramuscular injection of 30 mg/kg ketamine and 3.0 mg/kg xylazine, and killed with an intracardiac injection of 70 mg/kg sodium pentobarbital. Isolation of acini was accomplished by a modification of the procedures for single cell isolations with the omission of EDTA chelation in a calcium- and magnesium-free medium as previously described. Intraorbital glands from a single rabbit were removed and placed in DMEM/F12 supplemented with gentamicin (0.01 mg/ml), dexamethasone (1 µM), STI (0.1 mg/ml), and aprotinin (1.0 µg/ml). The glands were minced, and the fragments were transferred to 95% O2/5% CO2-gassed DMEM/F12 digestion medium (3 ml) containing collagenase (150 U/ml), hyaluronidase (402 U/ml), STI (0.4 mg/ml), and DNase (2 U/ml). The tissue was incubated in a shaking water bath at 37°C for 60 minutes in 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride for preparation of membranes from cultured acini, the medium was removed and washed twice with 2 ml of DMEM/F12, and the pelleted proteins were solubilized by incubation on ice for 60 minutes in 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and 0.9% sodium cholate, pH 8.0. The suspensions were then centrifuged at 13,800g for 3 minutes. Protein concentration of the solubilized samples was determined with the Bio-Rad protein reagent. The proteins were resolved by SDS-PAGE on 12.5% acrylamide or 9% acrylamide, 6 M urea mini-gels at 200 V and transblotted on polyvinylidene difluoride membranes at 100 V for 2 hours in a Bio-Rad transblot apparatus. Membranes were blocked for 1 hour with biotin and incubated for 18 hours at room temperature with the primary antiserum diluted 1:1000. Membranes were washed three times and incubated with alkaline phosphatase–conjugated goat anti-rabbit IgG (1:15000) for 2 hours. Immunoreactive proteins were visualized with 0.15 mg/ml alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate, and 0.33 mg/ml nitroblue tetrazolium in 100 mM Tris and 0.5 mM MgCl2, pH 9.5.

**Preparation of Membranes**

Membranes were prepared from freshly isolated lacrimal gland fragments, freshly isolated acini, and cultured acini as previously described. Lacrimal glands from several rabbits were pooled, minced, and portioned into three aliquots. Membranes were prepared directly from gland fragments or from acini isolated as described above. Remaining acini were cultured for 3 days and membranes were prepared from the cultured acini. Lacrimal gland fragments or freshly isolated acini were placed in ice-cold isolation medium containing 5% sorbitol, 0.5 mM EDTA, 5 mM histidine-imidazole buffer (pH 7.5), 9 µg/ml aprotinin, 3 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. For preparation of membranes from cultured acini, the medium was removed and washed six times, and acini were incubated with 1.5 ml dispase at 37°C for 2 hours. The cells were collected, washed three times, and resuspended in the membrane isolation medium. Tissues (gland fragments, freshly isolated acini, or cultured acini) were homogenized (using a Tissumizer at a setting of 45, Model TK-10 power control; Tekmar, Cincinnati, OH) for 15 minutes on ice. All subsequent isolation procedures were conducted at 4°C. The supernatants from two centrifugations at 1,000g and 10 minutes were combined and centrifuged for 20 minutes at 40,000g. The resulting pellet was washed three times at 40,000g for 20 minutes. The pelleted membranes were resuspended in the isolation medium, aliquoted, frozen in liquid nitrogen, and stored at —70°C. Membrane protein concentration was determined by the method of Lowry.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting**

G protein subunits were identified by SDS-PAGE and western blot analysis using commercially available antisera developed against peptide sequences of the α subunits of G_{	ext{null}1}, G_{	ext{null}2}, and G_{	ext{null}3}. Membranes were centrifuged at 13,800g for 15 minutes, and the pelleted proteins were solubilized by incubation on ice for 60 minutes in 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and 0.9% sodium cholate, pH 8.0. The suspensions were then centrifuged at 13,800g for 3 minutes. Protein concentration of the solubilized samples was determined with the Bio-Rad protein reagent. The proteins were resolved by SDS-PAGE on 12.5% acrylamide or 9% acrylamide, 6 M urea mini-gels at 200 V and transblotted on polyvinylidene difluoride membranes at 100 V for 2 hours in a Bio-Rad transblot apparatus. Membranes were blocked for 1 hour with biotin and incubated for 18 hours at room temperature with the primary antiserum diluted 1:1000. Membranes were washed three times and incubated with alkaline phosphatase–conjugated goat anti-rabbit IgG (1:15000) for 2 hours. Immunoreactive proteins were visualized with 0.15 mg/ml alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate, and 0.33 mg/ml nitroblue tetrazolium in 100 mM Tris and 0.5 mM MgCl2, pH 9.5.

**Protein Secretion by Cultured Acini**

Cultured acini were washed three times with a K+-Krebs-Ringer–Hepes buffer (permeabilization buffer) containing 145

**Insulin, 5.0 µg/ml; transferrin, 5.0 µg/ml; sodium selenite, 5.0 ng/ml and epidermal growth factor (10 ng/ml). Medium was replaced every 1 to 2 days.**
mM KCl, 2.0 mM MgCl₂, 1.2 mM KH₂PO₄, 10 μM CaCl₂, 10 mM glucose, 10 mM Hepes, 0.2% BSA, and 0.1% soybean trypsin inhibitor, pH 7.0. Acini were permeabilized by exposure to 0.4 U/ml SLO in the permeabilization buffer for 30 minutes at 37°C alone or in combination with antibodies or nonimmune rabbit serum as described previously. Control experiments were conducted in which antibodies were coincubated with the carboxyl terminal peptides of Gα₁/₂ or Gα₃ (1 μM) for 30 minutes at 37°C before introduction into the cells. The acini were then washed three times with DMEM/F12 and were equilibrated for a 20-minute period at 37°C with 5% CO₂. After a final 20-minute period of incubation with fresh medium supplemented with the appropriate agonist or vehicle, the medium was removed and assayed for total protein with the Bio-Rad protein reagent (Hercules, CA). In all secretion experiments, at least three experiments were performed with duplicate wells for each condition.

**Statistical Analysis**

Data are expressed as the mean ± SE. Statistical significance of P < 0.05 was determined by Student’s t-tests for unpaired data.

**Results**

We have previously demonstrated that met-enkephalin inhibition of carbachol-induced secretion in rabbit lacrimal gland is suppressed by pertussis toxin-catalyzed ADP-ribosylation of G proteins and that pertussis toxin-sensitive G proteins are present in freshly isolated rat lacrimal gland. To determine whether the pertussis toxin-sensitive G proteins that are present in freshly isolated lacrimal gland also occur in cultured rabbit lacrimal gland acini, membrane proteins from tissue fragments, freshly isolated acini and cultured acini were separated by SDS-PAGE and compared by western blots reacted with antibodies against specific decapeptide sequences of the α subunits of G. Immunoreactivity with the antibody against Gα₁, was not detected in any of the three lacrimal membrane preparations, however, the antibody reacted strongly with recombinant Gα₁ (Fig. 1, top). Antibody against a peptide sequence common to the α subunit of Gα₁ and Gα₂ was immunoreactive with proteins in membranes derived from freshly isolated gland fragments, freshly isolated acini, and cultured acini as well as with recombinant Gα₂ (Fig. 1, middle). Separation of the proteins by urea SDS-PAGE allowed discrimination between Gα₁ and Gα₂ with slower migration of Gα₂ as compared with Gα₁. The mobility of the lacrimal proteins corresponded to the faster migrating Gα₂. With the urea gel, all the bands appeared as doublets. Because mammalian Gα₁ subunits and the recombinant α subunits are myristoylated, it is possible that the doublets represent myristylated and nonmyristoylated forms, which are resolved on the urea gels. Alternatively, the doublets may represent differences in binding of guanine nucleotide to the proteins that would vary the molecular weight by approximately 500. Proteins that were immunoreactive with Gα₃ antibody were also detected in membranes from the three preparations of lacrimal gland with an electrophoretic mobility that was similar to that of Gα₃ (Fig. 1, bottom). For Gα₃ and Gα₃, staining was more intense with membranes derived from cultured acini than with membranes derived from fragments or freshly isolated acini.

In secretion studies performed with cultured acini, we tested the effect of DALA on release of protein induced by 100 μM carbachol (Fig. 2A). Basal secretion of 0.06 ± 0.006 mg/ml was significantly increased by carbachol to 0.45 ± 0.015 mg/ml. The addition of DALA to the cells immediately before exposure to carbachol resulted in a dose-dependent reversal of stimulation with a statistically significant reduction to 0.30 ± 0.02 mg/ml at 10 nM DALA. Stimulated secretion was reduced to near basal levels (0.07 ± 0.005 mg/ml) by the addition of 10 μM DALA. In contrast to the dose-dependent effect of DALA on
carbachol-stimulated secretion, the enkephalin analog alone at doses of $10^{-10}$ M to $10^{-5}$ M had no effect on secretion by cultured acini at any dose tested (Fig. 2B).

To determine the physiological role of G_{ia} in inhibition of secretion, permeabilization of acini with SLO and insertion of antibodies was performed. Permeabilization of cultured acini has been used successfully in lacrimal cells with no significant differences in stimulated secretion by nonpermeabilized and permeabilized cells. In this study, in nonpermeabilized cells (Fig. 3), basal protein secretion in the presence of nonimmune serum was $0.11 \pm 0.01$ mg/ml. Carbachol induced a significant increase with the release of $0.69 \pm 0.04$ mg/ml. The addition of $10 \mu$M DALA significantly reduced stimulation of protein release to near basal levels of $0.13 \pm 0.005$ mg/ml. Exposure of the acini to antibody to G_{ia1}, G_{ia1/2}, or G_{ia3} in the absence of SLO had no significant effect on inhibition by DALA.

In the permeabilized cells (Figs. 4, 5, 6) basal, carbachol-induced, and DALA-inhibited protein releases were similar to secretion in the nonpermeabilized cells. However, exposure of the acini to anti-G_{ia1/2} or anti-G_{ia3} antisera, but not anti-G_{ia1}, antisera in the presence of SLO had significant effects on met-enkephalin inhibition of secretion. In the case of anti-G_{ia1} antiserum, basal secretion in the permeabilized cells was $0.13 \pm 0.023$ mg/ml, which was significantly increased to $0.57 \pm 0.08$ mg/ml by $100 \mu$M carbachol. DALA inhibited carbachol-induced secretion to near basal levels, and the effect was not reversed by the addition of anti-G_{ia1} antiserum at dilutions from 1:200 to 1:50. In similar experiments (Fig. 5), basal secretion was increased from $0.06 \pm 0.006$ mg/ml to $0.62 \pm 0.065$ mg/ml by carbachol. This increase was inhibited by DALA to $0.08 \pm 0.009$ mg/ml. In contrast to the lack of effect by anti-G_{ia1} antiserum, the addition of anti-G_{ia1/2} antiserum resulted in a dose-dependent reversal of inhibition with the maximum effect ($0.34 \pm 0.033$ mg/ml) at a dilution of the antiserum of 1:100. No further increase in secretion was ob-

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933207/)  
**FIGURE 2.** Effect of D-Ala²-methionine enkephalinamide (DALA) on protein secretion by cultured acini. Protein release was measured in acini that were (A) exposed to vehicle, $100 \mu$M carbachol (Cch), or $100 \mu$M Cch with increasing concentrations of DALA ($10^{-10}$ M to $10^{-5}$ M) or (B) exposed to vehicle or increasing concentrations (10⁻¹ M to 10⁻⁵ M) of DALA alone. Values are the mean ± SE of three experiments.

*Significantly different from Cch.
In this work we have demonstrated that the profile for protein subunits is identical in membranes obtained from freshly isolated glands, freshly dissociated acini, and cultured acini. Separation of membrane proteins and immunoreaction with antibody directed against a peptide sequence common to subunits and allowed the detection of a protein band that was not labeled in blots reacted with antibody directed against only. Thus , but not , is present in rabbit lacrimal gland. was also identified in all three lacrimal membrane preparations. The mobility of the lacrimal proteins identified by immunopositive reactions as and was identical with that of the recombinant subunits whose reported apparent molecular masses are approximately 40 kDa.

In previous studies we have found that met-enkephalin inhibits carbachol-induced secretion of peroxidase from rat lacrimal gland fragments in a dose-dependent manner. Met-enkephalin also inhibits the release of secretory protein from cultured rabbit lacrimal gland acini. Recently, dose-dependent stimulation of lacrimal protein secretion by leu-enkephalin has been reported in porcine lacrimal gland. In this study, we found a dose-dependent inhibition of carbachol stimulation of secretion by the met-enkephalin analog DALA, with no effect on secretion by enkephalin alone. The inhibition of carbachol-stimulated secretion by met-enkephalin is completely blocked by pertussis toxin, and we show in this study that antibodies to pertussis toxin-sensitive and , but not , block met-enkephalin inhibition of carbachol-induced secretion, indicating coupling of the receptor to multiple G proteins. The reversal of inhibition by antibody to subunits in lacrimal acini was not complete at the highest dose of antibody tested. This result indicates that inhibition by met-enkephalin may be only partially mediated by . It is possible that may also be involved because the subunit of this G protein is present in

**DISCUSSION**

The specificity of the antibodies was established by pre-incubation of antisera at the dilution with a 1-μM concentration of the synthetic carboxyl terminal decapptide used to produce the antibodies (Table 2). The effect of anti- and anti- antisera on met-enkephalin inhibition was completely blocked by the corresponding peptides with no detectable cross-reactivity.
rabbit lacrimal gland acinar cell membranes, is pertussis toxin sensitive, and couples opiate receptors to intracellular effectors. The nonadditive effect of $G_{io}$ suggests that the mechanisms by which $G_{io}$ and $G_{ic}$ effect inhibition converge before exocytosis.

In neuronal and non-neuronal cells, $G$-coupled opioid receptor activation to negative regulation of adenyl cyclase and inhibition of Ca$^{2+}$ channels. However, we have not been able to demonstrate met-enkephalin inhibition of VIP or forskolin stimulation of secretion and inhibition of Ca$^{2+}$ channels. This hypothesis remains to be tested.

In conclusion, this work demonstrates that $G_{io}$ and $G_{ic}$, but not $G_{ia}$, are present in cultured rabbit lacrimal acinar cells and in freshly isolated fragments and freshly isolated acini. The met-enkephine enkephalin analog DALA has no effect on rabbit lacrimal protein secretion alone, but significantly inhibits carbachol-stimulated secretion. Inhibition of secretion is linked to met-enkephalin receptor activation by $G_{io}$ and $G_{ic}$ in a nonadditive manner that indicates convergence before exocytosis. The effect of inhibition is unknown, but may involve direct regulation of Ca$^{2+}$ channels, independent of CAP effects.

### References


### Table 1. Effect of Anti-$G_{io1/2}$ and Anti-$G_{io3}$ Antiserum on $\alpha$-Ala$^2$-Methionine Enkephalinamide

<table>
<thead>
<tr>
<th>Nonimmune Serum + Ccb</th>
<th>Nonimmune Serum + Ccb/DALA</th>
<th>Anti-$G_{io1/2}$ Antiserum + Ccb/DALA</th>
<th>Anti-$G_{io3}$ Antiserum + Ccb/DALA</th>
<th>Both Antisera + Ccb/DALA</th>
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<tr>
<td>0.06 ± 0.01</td>
<td>0.48 ± 0.01</td>
<td>0.34 ± 0.01*</td>
<td>0.31 ± 0.01*</td>
<td>0.39 ± 0.01* (1:100)</td>
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<tr>
<td>0.23 ± 0.02*</td>
<td>0.21 ± 0.02*</td>
<td>0.16 ± 0.01*</td>
<td>0.32 ± 0.02* (1:300)</td>
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DALA, $\alpha$-Ala$^2$-methionine enkephalinamide; Ccb, carbachol. Nonimmune serum or antiserum (1:100 dilution) was incubated with or without 1 $\mu$M peptide for 60 minutes at 37°C before introduction into acini. Release of protein (mg/ml) was measured in the presence of vehicle, 100 $\mu$M Ccb, or 100 $\mu$M carbachol plus 10 $\mu$M DALA (Ccb-DALA). *Significantly different from Ccb-DALA plus corresponding antiserum. Values are the mean ± SE of three experiments.


