Role of cAMP inhibition of p44/p42 mitogen activated protein kinase in potentiation of protein secretion in rat lacrimal gland.

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Supported by NIH grant EY06177
Abstract.

We previously found that addition of cAMP and a Ca\(^{2+}\)/PKC-dependent agonist causes synergism or potentiation of protein secretion from rat lacrimal gland acini. In the present study we determined if cAMP decreases p44/p42 mitogen activated protein kinase (MAPK) activity in the lacrimal gland. As we know that activation of MAPK attenuates protein secretion stimulated by Ca\(^{2+}\)- and PKC- dependent lacrimal gland secretion, we also determined if this activation cause potentiation of secretion. Freshly prepared rat lacrimal gland acinar cells were incubated with dibutyryl cAMP (dbcAMP), carbachol (a cholinergic agonist), phenylephrine (an \(\alpha_1\)-adrenergic agonist) or, epidermal growth factor (EGF). The latter three agonists are known to activate p44/p42 MAPK. p44/p42 MAPK activity and protein secretion were measured. As measured by western blot analysis, dbcAMP inhibited both basal and agonist-stimulated p44/p42 MAPK activity. Cellular cAMP levels were increased by: 1) using two different cell permeant cAMP analogs, 2) activating adenylyl cyclase (L85 8051), or 3) activation of Gs-coupled receptors (VIP). Cell permeant cAMP analogs, L85 8051, and VIP inhibited basal p44/p42 MAPK activity by 50%, 40%, and 40%, respectively. dcAMP and VIP inhibited carbachol- and EGF-stimulated MAPK activity. cAMP, but not VIP, inhibited phenylephrine-stimulated MAPK activity. Potentiation of secretion was detected when carbachol, phenylephrine, or EGF were simultaneously added with dbcAMP. We conclude that increasing cellular cAMP levels inhibits p44/p42 MAPK activity and that this could account for potentiation of secretion obtained when cAMP was elevated and Ca\(^{2+}\) and PKC were increased by agonists.
Keywords.

Lacrimal gland, potentiation protein secretion, cAMP, MAP kinase.
INTRODUCTION.

Cyclic AMP is involved in a variety of cellular processes including the activation of protein kinase A (PKA). One target of cAMP/PKA is the p44/p42 mitogen activated protein kinase (MAPK) pathway. Both stimulatory and inhibitory effects of cAMP on p44/p42 MAPK activity have been described. The effect on the activity of p44/p42 MAPK is cell specific with several distinct pathways and signaling components used (23). (1, 6, 26). (2, 3, 5, 25).

The lacrimal gland is a polarized secretory tissue that secretes proteins, water and electrolytes. This secretion is necessary to maintain the health of the ocular surface and is regulated by parasympathetic and sympathetic nerves. Binding of the parasympathetic neurotransmitter vasoactive intestinal peptide (VIP) to its receptor induces activation of a cAMP dependent pathway (14) through the Gsα G-protein subunit which in turn stimulates adenylyl cyclase (AC) (7). Activating AC with VIP or the AC activator forskolin or preventing cAMP breakdown by inhibiting cyclic nucleotide phosphodiesterase causes an increase in intracellular cAMP levels, which in turn activates PKA (10, 17). That activation of PKA mediates VIP-induced protein secretion was demonstrated using two PKA inhibitors, protein kinase inhibitor (PKI), and H89. VIP also increases the intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in the lacrimal gland (12).

Another parasympathetic neurotransmitter, acetylcholine (Ach), activates M3 muscarinic receptors located on the basolateral membranes of acinar cells (15, 20). The activated M3 receptor then interacts with a Gq/11α G-protein subtype (18), to activate phospholipase Cβ (PLCβ) to break down phosphatidyl inositol bisphosphate (PIP_{2}) into 1,4,5-inositol trisphosphate (1,4,5-IP_{3}) and diacylglycerol (DAG). The 1,4,5-IP_{3} binds to
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IP₃ receptors located on Ca²⁺ stores in the endoplasmic reticulum and increases the [Ca²⁺]i. DAG activates a family of enzymes known as protein kinase C (PKC). The lacrimal gland contains PKC-α, -δ, -ε, -λ and protein kinase D (PKD). Both increasing the [Ca²⁺]i and activation of PKC-α, -δ, and -ε play integral roles in protein secretion stimulated by cholinergic agonists. Activation of M3 receptors also stimulates the non-receptor tyrosine kinases Pyk2 and Src that subsequently stimulate the Ras/Raf, mitogen activated protein kinase kinase (MEK), and p44/p42 MAP kinase (MAPK) pathway. Interestingly our laboratory found that activation of the p44/p42 MAPK pathway attenuates cholinergic agonist-stimulated protein secretion in the lacrimal gland (24).

Norepinephrine, a sympathetic neurotransmitter, activates both α- and β-adrenergic receptors. Activation of β-adrenergic receptors by isoproterenol stimulates the cAMP signaling pathway to induce protein secretion (19). Activation of α₁D-adrenergic receptors in the lacrimal gland by phenylephrine stimulates production of nitric oxide (NO). NO activates guanylate cyclase to increase guanosine 3´, 5´-cyclic monophosphate (cGMP) to induce secretion (13). α₁D-Adrenergic receptors also activate the p44/p42 MAPK pathway by increasing EGF ectodomain shedding to transactivate the EGF receptor to stimulate p44/p42 MAPK activity. Similar to its effect on cholinergic agonist-induced secretion, activation of MAPK inhibits protein secretion stimulated by α₁D-adrenergic agonists, similarly to (4, 22).

Another stimulus of protein secretion in the lacrimal gland is epidermal growth factor (EGF) (20). EGF binds to the EGF receptor (EGFR) to recruit adapter molecules that activate the three major, downstream signaling pathways: MAPK, phosphatidylinositol-3 kinase (PI-3K), or PLC-γ. To stimulate the p44/p42 MAPK
pathway, the adapter proteins Shc and Grb2 are recruited to the phosphorylated EGFR leading to activation of Ras, Raf, MEK, and MAPK (24-26). EGF simulates lacrimal gland protein secretion by stimulating PLC-γ to increase the [Ca^{2+}]_{i} and activate PKC, but not PI-3K or p44/p42 MAPK (24).

In 1984, our laboratory found that if a Ca^{2+}- or PKC- dependent agonist, such as carbachol or phenylephrine, was combined with a cAMP-dependent agonist, such as VIP, synergism or potentiation of secretion occurred. In other words, the response to the two agonists was greater than the sum of the individual responses (8). The potentiation also occurred if cAMP was increased independently of receptor activation. Potentiation of protein secretion also occurs when α₁-adrenergic agonists and β-adrenergic agonists were used together (9, 18).

Several possible mechanisms by which potentiation of protein secretion could occur include potentiation of the increase in the [Ca^{2+}]_{i}; potentiation of the activation of PKC; and potentiation of the cellular cAMP concentration. However, all of these possibilities have been disproven (7, 9). We now hypothesize that an inhibition of p44/p42 MAPK activity could cause potentiation of protein secretion by relieving its attenuating effects on agonist-stimulated secretion.

In the present study we used freshly isolated, non-transfected lacrimal gland acinar cells and found that potentiation of secretion and inhibition of p44/p42 MAPK occurred when cAMP analogs and Ca^{2+}/PKC- dependent agonists were added simultaneously. Increasing cellular cAMP levels inhibited both basal and agonist-induced p44/p42 MAPK activity. Agonists known to increase cAMP in lacrimal gland cells also inhibited p44/p42 MAPK activity. We conclude that inhibition of p44/p42 MAPK by
cAMP could be responsible for the potentiation of secretion that occurs when cAMP is elevated in conjunction with Ca^{2+}/PKC-dependent agonists.

MATERIALS AND METHODS

Materials. Dibutyryl cAMP (dbcAMP), Sp-5, 6-Dcl-cBiMPS, IBMX [3-isobutyl-1-methylxanthine] are from Biomol (Plymouth Meeting, PA), 8-Bromo-cAMP and 8-pCPT-2Me-cAMP are from Tocris (Ellisville, MI), L-858051 (7 - Deacetyl - 7 - [ O - ( N - methylpiperazio ) - γ - butyryl ] - , Dihydrochloride) and vasoactive intenstinal peptide are from Calbiochem (San Diego, CA). Carbachol and phenylephrine are from Sigma (St Louis, MO), and epidermal growth factor is from Pepro Tech (Rocky Hill, NJ). For western blot analysis, antibodies against phosphorylated p44/p42 MAPK and total p42 MAPK are from Santa Cruz Biotechnology Inc (Santa Cruz, CA), and monoclonal antibodies against total CREB and phosphorylated CREB are from Affinity Bio Reagents (Golden, CO). For measurement of peroxidase secretion, the fluorescent molecule Amplex Red (Molecular Probes, Eugene, OR) was acquired from Molecular Probes.

Preparation of lacrimal gland acini.

All experiments were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Both exorbital lacrimal glands were removed from male Sprague-Dawley rats that had been anesthetized with CO_{2} for 1 min and then decapitated. Lacrimal glands were trimmed of fatty and connective tissue and fragmented into small pieces 2–3 mm in diameter. The pieces were then washed at 37 °C in Krebs-Ringer bicarbonate (KRB) buffer (in mM: 119 NaCl, 4.8 KCl, 1 CaCl_{2}, 1.2 MgSO_{4}, 1.2 KH_{2}PO_{4},
and 25 NaHCO₃) supplemented with 10 mM HEPES, 5.5 mM glucose, and 0.5 % BSA (KRB-HEPES), pH 7.4. Lacrimal gland acini were prepared by incubating tissue pieces with collagenase (CLSIII; 150 U/ml) in 10 ml of KRB-HEPES buffer for 40 min at 37 °C under a stream of 95 % O₂-5 % CO₂. Lacrimal gland lobules were subjected to gentle pipetting ten times at regular time intervals through tips of decreasing diameter. The preparation was then filtered through nylon mesh, and the acini were pelleted with a 3-min centrifugation at 5000 rpm. The pellet was washed twice by centrifugation (5,000 rpm, 3 min) through a 4% BSA solution made in KRB-HEPES buffer. The dispersed acini were allowed to recover for 60 min in 5 ml of fresh KRB-HEPES buffer containing 0.5 % BSA.

Detection of MAPK and CREB activation by Western blotting.

Lacrimal gland acini were incubated for the indicated time period with agonists at the indicated concentrations. To terminate the reaction, ice cold KRB-HEPES buffer without BSA was added. The acini were centrifuged, and 100 µl of ice-cold RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % sodium deoxycholate, 1 % Triton X-100, 0.1 % SDS, 1 mM EDTA, 100 µg/ml PMSF, 30 µl/ml aprotinin, and 1 mM Na₃VO₃) was added. The pellet was then sonicated. After a 10,000 rpm centrifugation for 30 min at 4 °C, proteins in the supernatant were removed and separated by SDS-PAGE (10 % acrylamide gels) and transferred to nitrocellulose membranes. Activated p44/p42 MAPK and CREB were detected with antibodies that specifically recognize the phosphorylated (activated) pools of enzymes. A secondary antibody conjugated to HRP conjugated secondary was using and detected by the enhanced chemiluminescence method. Films were scanned and
analyzed using NIH Image software. Values for phosphorylated enzymes (amounts for p42 and p44 MAPK were added together) were normalized to the amount of total enzyme by using antibodies to total enzyme (phosphorylated and nonphosphorylated) and were compared to the control value that was set at 1.

*Measurement of peroxidase secretion.*

Lacrimal gland acini were incubated in 0.5 % BSA-KRB-HEPES buffer in duplicate for 20 minutes with agonists or no additions, or simultaneous addition of two compounds. After a brief centrifugation, supernatant was collected, and peroxidase activity was measured in duplicate in both the supernatant and the pellet fraction using Amplex Red. Briefly 100 µl of sample was incubated with 4 mM hydrogen peroxide and 0.4 Amplex Red in 50 mM Tris-HCl, pH. 8.0. Oxidation of Amplex Red by peroxidase in the presence of hydrogen peroxide produces a highly fluorescent molecule, resorufin. The amount produced was read using a fluorescence microplate reader (model FL600; BioTek, Winooski, VT) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

*Data presentation and statistic analysis.*

Results are expressed as means ± SEM. Data were analyzed by Student’s t-test. P<0.05 was considered statistically significant different.

RESULTS
Time- and concentration-dependency of cell-permeable cAMP analogs on p44/p42 MAPK and CREB activity in freshly isolated lacrimal gland acinar cells.

We characterized the effect of cAMP on p44/p42 MAPK activity. We increased cellular cAMP levels by incubating lacrimal gland acini with the cell membrane permeant cAMP analog dbcAMP at $10^{-3}$ M for 0, 1, 5, 10 and 30 min. With no additions basal p44/p42 MAPK levels did not significantly change over the 30 min incubation (Fig. 1A, B). Addition of dbcAMP significantly decreased basal p44/p42 MAPK activity to 0.7 ± 0.2, 0.6 ± 0.2, and 0.5 ± 0.1 fold compared to zero time at 5, 10 and 30 min respectively. In contrast to dbcAMP, the positive control carbachol ($10^{-4}$ M) increased p44/p42 MAPK activity to 1.6 ± 0.9-fold above basal after a 5 min incubation (Fig 1B). When lacrimal gland acini were incubated for 30 min with increasing concentrations of dbcAMP (Fig. 1C, D), basal p44/p42 MAPK activity was significantly inhibited by dbcAMP in a concentration-dependent manner to 0.5 ± 0.1, and 0.5 ± 0.1 fold at $10^{-3}$ M and $3 \times 10^{-3}$ M dbcAMP respectively compared to no additions. The positive control EGF ($10^{-7}$ M) increased p44/p42 MAPK activity 2.3 ± 0.7-fold above basal (Fig 1D).

Two other cell permeant cAMP analogs, Sp-5, 6-DCI-cBiMPS and 8Br-cAMP also decreased basal p44/p42 MAPK activity. Sp-5, 6-DCI-cBiMPS at $10^{-3}$ M significantly decreased basal p44/p42 MAPK activity to 0.4 ± 0.7 fold compared to basal at $10^{-3}$ (Fig. 2A, B). 8Br-cAMP significantly decreased basal p44/p42 MAPK activity to 0.6 ± 0.1, and 0.5 ± 0.08 fold compared to basal at $10^{-3}$, and $10^{-2}$ M respectively (Fig. 2C). In these experiments carbachol ($10^{-4}$ M) stimulation, the positive control, caused a 1.5 ± 0.1-fold increase over basal (Fig. 2D).
We also examined activation and phosphorylation of the common PKA substrate cAMP-responsive element binding protein (CREB) (11). dbcAMP increased basal phosphorylated CREB in a time dependent manner by 1.7 ± 0.2, 2.5 ± 0.2 and 2.6 ± 0.3 fold compared to zero time at 5, 10 and 30 min incubation respectively (n=3, Fig. 3A, B), and in a concentration dependent manner to 1.4, 1.6, and 1.7 fold above basal at 3x10^{-4} M, 10^{-3} M and 3x10^{-3} M respectively (n=2, Fig. 3C, D).

These data indicate that an increase in the cellular cAMP levels by membrane permeant cAMP analogs inhibited p44/p42 MAPK activity in lacrimal gland acinar cells while increasing the amount of phosphorylated CREB. The increase in CREB activity is consistent with the cell permeant cAMP analog increasing intracellular cAMP levels.

**Effect of activating AC and inhibiting PDE on p44/p42 MAPK activity.**

Another method for increasing intracellular cAMP concentrations is to prevent its degradation by inhibiting PDE or stimulate its synthesis by activating AC. To determine the effects of inhibiting PDE or activating AC on p44/p42 MAPK activity, we increased cAMP levels by PDE with IBMX or activating AC with the forskolin analog, L-85 8051. As shown in Fig. 4, a 30 min incubation with dbcAMP at 10^{-3} M and L-85 8051 at 10^{-4} M significantly inhibited basal p44/p42 MAPK activity by 22 ± 5.0%, and 39 ± 3.0% compared to no addition. The decrease in basal p42/p44 MAPK activity was not significant with IBMX at 10^{-3} M. Simultaneous addition of IBMX (10^{-3} M) and L-85 8051 (10^{-4} M), or all three compounds (dbcAMP (10^{-3} M), IBMX (10^{-3} M), and L-85 8051 (10^{-4} M)) to acini significantly decreased basal p44/p42 MAPK activity by 68 ± 6.0%, and 66 ± 4.0% respectively. As a positive control, carbachol (10^{-4} M) increased p44/p42
MAPK activity 2.0 ± 0.4-fold over basal (Fig 4B). Thus increasing the cAMP concentration by activating AC decreases p44/p42 MAPK activity. Simultaneous inhibition of PDE and activation of AC gives a greater inhibition than the use of permeable cAMP analogs, AC activators, or PDE inhibitors alone.

Effect of VIP on p44/p42 MAPK activity and CREB activity.

To determine if increasing the cAMP concentration by a receptor-mediated process also decreased basal p44/p42 MAPK activity, acini were incubated with VIP for various times. As shown in Fig. 5A and B, VIP (10⁻⁸ M) significantly decreased p44/p42 MAPK activity to 0.7 ± 0.09, 0.6 ± 0.09 fold compared to zero time at 1, 5 min.

VIP also significantly decreased basal p44/p42 MAPK activity in a concentration dependent manner in lacrimal gland acini to 0.6 ± 0.08, 0.7 ± 0.07, 0.7 ± 0.01, and 0.7 ± 0.09 fold compared to no addition at VIP 10⁻⁹ – 10⁻⁶ M, respectively (Fig 5C, D).

Effect of increasing cAMP levels on agonist-stimulated p44/p42 MAPK activity.

We next determined the effect of increasing cAMP levels on agonist-induced p44/p42 MAPK activity. In Fig 6A and B, the membrane permeant cAMP analog dbcAMP (10⁻³ M) significantly inhibited basal p44/p42 MAPK activity by 30 ± 4.0% when incubated for 5 min. To determine if agonist-induced p44/p42 MAPK activity is also inhibited by cAMP, lacrimal gland acini were incubated simultaneously with dbcAMP (10⁻³ M) and either carbachol (10⁻⁴ M), phenylephrine (10⁻⁴ M), or EGF (10⁻⁷ M) for 5 min. We previously showed that these three compounds each activated p44/p42 MAPK (22). Carbachol significantly increased p44/p42 MAPK activity by 2.2 ± 0.5-fold.
Addition of dbcAMP (10^{-3} M) significantly inhibited this increase by 56%. Phenylephrine significantly increased p44/p42 MAPK activity by 1.3 ± 0.1-fold. Addition of dbcAMP completely inhibited this response. EGF significantly increased p44/p42 MAPK activity by 1.8 ± 0.3-fold. Addition of dbcAMP also completely inhibited EGF-stimulated increase in p44/p42 MAPK activity.

These data show that increasing cellular cAMP levels significantly reduced basal and agonist-induced p44/p42 MAPK activation. Thus, inhibition of p44/p42 MAPK could account for the potentiation of protein secretion.

Effect of VIP on agonist-stimulated p44/p42 MAPK activity.

To determine if VIP had an effect on agonist-induced p44/p42 MAPK activity, lacrimal gland acini were incubated simultaneously with VIP (10^{-8} M) and either carbachol (10^{-4} M), phenylephrine (10^{-4} M), or EGF (10^{-7} M) for 5 min. VIP alone significantly inhibited basal p44/p42 MAPK activity (Fig 7A, B, C). Carbachol increased p44/p42 MAPK activity to 2.5 ± 0.3 fold above basal which was significantly decreased by 58 ± 13% by the simultaneous addition of VIP (Fig 7A). Phenylephrine increased p42/p44 MAPK activity to 1.8 ± 0.6 fold above basal. Interestingly, VIP did not have any effect on phenylephrine-induced increase in p44/p42 MAPK activity (n=3, Fig 7B). EGF increased p42/p44 MAPK activity to 2.1 ± 0.3 fold above basal that was completely inhibited by the simultaneous addition of VIP (Fig 7A).

These data show that increasing cellular cAMP levels with VIP significantly reduced basal and carbachol- and EGF-induced p44/p42 MAPK activation.
Effect of cAMP added with carbachol, phenylephrine, or EGF on peroxidase secretion.

We previously found that VIP potentiated peroxidase secretion stimulated by cholinergic or α₁D-adrenergic agonists (8). To test whether cAMP also potentiated peroxidase secretion by affecting the muscarinic pathway, the α₁D-adrenergic pathway, or the EGF pathway, dbcAMP (10⁻³ M) was added alone or with carbachol (Cch, 10⁻⁵ M and 10⁻⁴ M), phenylephrine (Ph, 10⁻⁵ M and 10⁻⁴ M), or EGF (10⁻⁸ M and 10⁻⁷ M) for 20 minutes. Theoretical additivity was calculated as experimental value of dbcAMP-stimulated protein secretion plus experimental value of each agonist-stimulated protein secretion value minus the experimental basal value. As shown in Fig. 8A dbcAMP at 10⁻³ M and carbachol at 10⁻⁴ M and 10⁻⁵ M significantly increased peroxidase secretion compared to the basal value. Simultaneous addition of dbcAMP with carbachol at both concentrations, potentiated secretion by 181% at 10⁻⁴ M carbachol and 208% at 10⁻⁵ M carbachol compared to the theoretical additivity values. In Fig. 8B, phenylephrine at 10⁻⁴ M and 10⁻⁵ M significantly increased secretion compared to the basal value. When dbcAMP 10⁻³ M was added simultaneously with phenylephrine, secretion was significantly potentiated by 240% at 10⁻⁴ M and 287% at 10⁻⁵ M phenylephrine compared to the theoretical additivity values. EGF at 10⁻⁸ M significantly increased peroxidase secretion. Simultaneous addition of dbcAMP 10⁻³ M with EGF, significantly potentiated secretion by 380% increase at 10⁻⁷ M EGF and 323% increase at 10⁻⁸ M EGF compared to the theoretical additivity values (n=7) (Fig. 8C).

These results confirm previous findings that simultaneous addition of a cAMP dependent agonist with a Ca²⁺/PKC- dependent agonist causes potentiation of lacrimal
Experimental data from different laboratories have demonstrated that in the lacrimal gland cAMP- and Ca\(^{2+}\)-/PKC-dependent pathways interact to potentiate protein secretion (7, 8, 18). Previous work showed that potentiation of secretion occurred when cAMP dependent agonists were used with cholinergic and \(\alpha_{1D}\)-adrenergic agonists that are known to increase the \([Ca^{2+}]_i\) and activate PKC\(\alpha\) and activate MAPK, even though the cellular mechanisms of action for these agonists differ. We now show that potentiation of secretion can also occur with a cAMP dependent stimulus and the growth factor EGF, which increases protein secretion by increasing the \([Ca^{2+}]_i\) and activating PKC. The potentiation of protein secretion under all these conditions did not appear to occur at 1) the receptor level, as activating AC or membrane permeable cAMP analogs could replace cAMP dependent agonists increasing potentiation of secretion, 2) at the level of the increase in \([Ca^{2+}]_i\), as the \([Ca^{2+}]_i\) was not potentiated, or 3) at the cAMP level, as the cAMP level was not potentiated (9). In the present study, we identified a possible mechanism that could account for potentiation of secretion that of inhibition of basal and agonist-activated p44/p42 MAPK activity through an increase in cellular cAMP concentration. Since increasing p44/p42 MAPK activity attenuates agonist stimulated protein secretion (24), decreasing p44/p42 MAPK activity would increase protein secretion. This mechanism of potentiation is supported by our previous findings that inhibition of p44/p42 MAPK with the MEK inhibitor U0126 increased cholinergic and
α₁-adrenergic agonist as well as EGF induced-protein secretion (22, 24). In the present study, simultaneous addition of a membrane permeable cAMP analog with a cholinergic agonist, an α₁D-adrenergic agonist, or EGF potentiated protein secretion and inhibited agonist induced p44/p42 MAPK activity. That cAMP can mediate an inhibitory response and alter another signaling pathway has also been shown by Burgering et al in NIH 3T3 cells (2). They demonstrated that elevation of the intracellular cAMP levels results in the inhibition of growth factor-induced mitogenesis. In these cells cAMP regulation of the MAPK cascade provided important crosstalk between hormone and growth factor signaling, as occurs in the lacrimal gland.

The effect of cAMP on inhibition of p44/p42 MAPK was robust. It occurred when intracellular cAMP levels were increased by diverse mechanisms. These included the increasing receptor mediated activity with VIP, using 3 different cell permeable cAMP analogs (dbcAMP, Sp-5, 6-DCI-cBiMPS and 8Br-cAMP) and activating adenylyl cyclase with a forskolin analog. Furthermore, if cAMP levels are increased by the use of a membrane permeant cAMP analog, activation of AC, and inhibition of cAMP phosphodiesterase simultaneously, basal p44/p42 MAPK activity was inhibited to a greater extent than compared the effect of the membrane permeant analog alone. It is not clear why IBMX alone did not have a significant effect on basal p44/p42 MAPK activity. It is possible that the lack of effect could be due to the fact that multiple isoforms of phosphodiesterases exist, some of which are insensitive to IBMX (21). It is not know which types of phosphodiesterases are present in the lacrimal gland. Regardless, these data indicate that increasing cAMP levels by multiple different mechanisms inhibits basal p44/p42 MAPK activity.
The inhibition of basal p44/p42 MAPK activity is functional as prevention of cAMP breakdown by inhibiting cAMP phosphodiesterase on its own stimulates protein secretion as does activation of AC and use of the membrane permeant analog (7). The stimulation of secretion by increasing cAMP levels could be due to an effect of cAMP directly in the secretory process, on MAPK, or both. Unfortunately we cannot use U0126 that inhibits MEK to test this hypothesis as both U0126 and cAMP inhibit p44/p42 MAPK. Therefore inhibition of MAPK by cAMP cannot be reversed by inhibition of MEK with U0126. Thus MEK inhibitors cannot be used to demonstrate the cAMP induced secretion or cAMP induced potentiation of secretion occurs via p44/p42 MAPK.

To ensure that the membrane permeant cAMP analog dbcAMP increased intracellular cAMP levels in rat lacrimal gland acinar cells, we measured CREB (cyclic AMP responsive element binding protein) activity. It is well known that CREB is stimulated by protein kinases such as PKA (36). While CREB can be activated by a number of different kinases and [Ca^{2+}]_i (16), only PKA should be activated in unstimulated acinar cells incubated with cAMP analogs. The increase, then, is consistent with an increase in intracellular cAMP.

In the present study VIP inhibited p44/p42 MAPK. In addition, simultaneous addition of VIP with cholinergic agonists and EGF inhibited the increase in MAPK activity normally seen with these stimuli. This supports the hypothesis that MAPK is responsible for potentiation of secretion under these conditions. However, simultaneous addition of VIP and α1-adrenergic agonists had no effect on α1D-adrenergic agonist-stimulated MAPK activity despite the fact that VIP potetiated α1D-adrenergic agonist-
stimulated protein secretion. It is well established in the lacrimal gland that cholinergic and α₁D-adrenergic agonists stimulate protein secretion by activating different signaling pathways. Cholinergic agonists increase [Ca²⁺]ᵢ and activate PKCα and -δ while α₁D-adrenergic agonists increase both the amount of NO/cGMP and the activity of PKCe. (13, 27) It is also known that VIP increases the amount of intracellular cAMP concentration in the cell and causes a small increase in [Ca²⁺]ᵢ. (14) The increase in [Ca²⁺]ᵢ could activate enzymes in other pathways in addition to the cAMP pathway. It is possible then that the VIP and α₁D-adrenergic agonists pathways interact at another step in the secretory process to cause the potentiation of secretion seen with these two agonists.

Potentiation of secretion occurred when a membrane permeable cAMP analog was used simultaneously with a cholinergic agonist, an α₁D-adrenergic agonist, and EGF, agonists known to activate p44/p42 MAPK. This suggests that potentiation of secretion occurs not only in neurally stimulated secretion, but also when growth factors were used. As the pathways used by these agonists and EGF are different, it is not surprising that the effect of exogenous cAMP analog was slightly different for each agonist. For secretion, greater potentiation occurred with cAMP and either EGF or the α₁D-adrenergic agonist than cAMP with the cholinergic agonist. This is similar to the inhibition of p44/p42 MAPK that occurred with cAMP and either EGF or the α₁D-adrenergic agonist that was greater than cAMP with the cholinergic agonist. EGF and α₁D-adrenergic agonists both activate the EGFR, but EGF causes a greater activation of p44/p42 MAPK than the α₁D-adrenergic agonist. Interestingly cholinergic agonists caused a greater activation of p44/p42 MAPK than EGF or the α₁D-adrenergic agonist, but caused less potentiation of
secretion. This could be explained by cholinergic agonists not activating EGFR, as do the other two agonists indicating that there may be a second mechanism, a non-cAMP pathway, used to cause potentiation of secretion. The existence of a second mechanism for cholinergic agonists is consistent with dbcAMP only partially inhibiting cholinergic agonist activation of MAPK.

Potentiation of secretion occurred when either maximal or submaximal concentrations of agonists were used with the membrane permeant cAMP analog. The use of a membrane permeant cAMP analog and an activator of AC is in contrast to previous findings in which VIP was used to increase cellular cAMP levels (8). When VIP was used with a maximal concentration of a cholinergic or \( \alpha_{1D} \)-adrenergic agonist, potentiation of secretion did not occur, although it occurred at submaximal concentrations of these latter two agonists. A possible explanation is that receptor mediated interactions are limited by saturation of the agonist-receptor interaction. Another possible explanation is that there is another overlap, in addition to MAPK, between the VIP dependent and cholinergic agonist, \( \alpha_{1D} \)-adrenergic agonist, and EGF signaling pathways. This overlap could be the increase in \([Ca^{2+}]_i\), as stimulation of all four pathways increases \([Ca^{2+}]_i\), whereas use of endogenous cAMP analogs does not. This is consistent with our previously published work showing that increasing cAMP by activating AC with forskolin potentiated secretion when used with both maximal and submaximal concentrations of cholinergic agonists.

We conclude that increasing intracellular cAMP levels by multiple mechanisms inhibits p44/p42 MAPK activity, and this could alleviate the inhibitory effect of p44/p42
MAPK on agonist-stimulated lacrimal gland secretion and account for the potentiation of secretion that occurs when cellular cAMP levels are elevated by a receptor mediated activation, activation of AC, use of permeable cAMP analogs along with the simultaneous stimulation with a Ca\(^{2+}\) / PKC - dependent agonist.
FIGURE LEGENDS

**Figure 1.** Effect of the membrane permeant cAMP analog dbcAMP on basal p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with dbcAMP (10^{-3} M) for various times (A, B) or at increasing concentrations of dbcAMP for 30 minutes (C, D), and the amount of phosphorylated p44/p42 MAPK were measured. B and D are representative blots from a single experiment. A and C are the mean ± SEM from three to four independent experiments. *Statistically significant difference from basal level. B; Basal, D; dbcAMP, Ch; carbachol, E; EGF.

**Figure 2.** Effect of the membrane permeant cAMP analog Sp-5, 6-DCl-cBiMPS and 8Br-cAMP on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with increasing concentrations of Sp-5, 6-DCl-cBiMPS (A and B) or 8Br-cAMP for 30 minutes (C and D), and the amount of phosphorylated p44/p42 MAPK were measured. B and D are representative blots from a single experiment. Data are the mean ± SEM from three to four independent experiments. *Statistically significant difference from basal level. B; Basal, D; dbcAMP, Ch; carbachol, Sp; Sp-5, 6-DCl-cBiMPS.

**Figure 3.** Effect of the membrane permeant cAMP analog dbcAMP on CREB activity. Rat lacrimal gland acini were incubated with dbcAMP (10^{-3} M) for various times (A, B) or at various concentrations of dbcAMP for 30 minutes (C, D), and the amount of phosphorylated CREB were measured. B and D are representative blots from a single experiment. Data in A are the mean ± SEM of results from three independent experiments. Data in C is the mean from two experiments. B; Basal, D; dbcAMP.
Figure 4. Effect of activation of AC with the forskolin analog L-85 8051 and the phosphodiesterase inhibitor IBMX on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with dbcAMP (10^{-3} M), IBMX (10^{-3} M), L-85 8051 (10^{-4} M) or these compounds together for 30 minutes. Data are the mean ± SEM from four independent experiments. B is representative blot from a single experiment. *Statistically significant difference from basal level. B; Basal, D; dbcAMP, IB; IBMX, L; L-85 8051, Ch; carbachol.

Figure 5. Effect of VIP on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with VIP 10^{-8} M for the indicated times (A and B) or indicated concentrations (C and D), and western blot analysis was performed for phospho p44/p42 MAPK. Data are the mean ± SEM from seven independent experiments in A and four experiments in C. B and D are representative blots from single experiments. *Statistically significant difference from basal level. B; Basal, V; VIP.

Figure 6 Effect of simultaneous addition of the membrane permeant cAMP analog dbcAMP and Ca^{2+}/PKC dependent agonists on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated simultaneously with presence or absence of dbcAMP 10^{-3} M alone or with carbachol (Cch, 10^{-4} M), phenylephrine (Ph, 10^{-4} M), or EGF (10^{-7} M) for 5 minutes. A; a representable blot. B; summary of results. Data are the mean ±SEM from four to seven independent experiments. *Statistically significant difference from basal
level. *Statistically significant difference from agonist-stimulated p44/p42 MAPK activity.

**Figure 7** Effect of simultaneous use of VIP and Ca^{2+}/PKC dependent agonists on p44/p42 MAPK activity in rat lacrimal gland. Rat lacrimal gland acini were incubated simultaneously with VIP (V, 10^{-8} M) and carbachol (C, 10^{-4} M) (A), phenylephrine (P, 10^{-4} M) (B), and EGF (E, 10^{-7} M) (C) for 5 min. Western blot analysis was performed for phosphor-p44/p42 MAPK. Data are mean ±SEM of results in 3-5 dependent experiments. *Statistically significant difference from basal level. #Statistically significant difference from agonist-stimulated peroxidase secretion.

**Figure 8** Effect of simultaneous use of the membrane permeant cAMP analog dbcAMP and Ca^{2+}/PKC dependent agonists on peroxidase protein secretion from rat lacrimal gland acinar cells. Rat lacrimal gland acini were incubated simultaneously with dbcAMP (10^{-3} M) and (A) a maximal (10^{-4} M) or submaximal (10^{-5} M) concentration of carbachol (Cch); (B) a maximal (10^{-4} M) or submaximal (10^{-5} M) concentration of phenylephrine (Ph), or (C) a maximal (10^{-7} M) or submaximal (10^{-8} M) concentration of EGF for 20 minutes. Data are the mean ±SEM of results in seven to ten independent experiments *Statistically significant difference from basal level. #Statistically significant difference from theoretical additivity value, indicated by dotted line.
REFERENCES


Effect of the membrane permeant cAMP analog dbcAMP on basal p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with dbcAMP (10^{-3} M) for various times (A, B) or at increasing concentrations of dbcAMP for 30 minutes (C, D), and the amount of phosphorylated p44/p42 MAPK were measured. B and D are representative blots from a single experiment. A and C are the mean ± SEM from three to four independent experiments. *Statistically significant difference from basal level. B; Basal, D; dbcAMP, Ch; carbachol, E; EGF.
Effect of the membrane permeant cAMP analog Sp-5, 6-DCI-cBiMPS and 8Br-cAMP on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with increasing concentrations of Sp-5, 6-DCI-cBiMPS (A and B) or 8Br-cAMP for 30 minutes (C and D), and the amount of phosphorylated p44/p42 MAPK were measured. B and D are representative blots from a single experiment. Data are the mean ± SEM from three to four independent experiments. *Statistically significant difference from basal level. B; Basal, D; dbcAMP, Ch; carbachol, Sp; Sp-5, 6-DCI-cBiMPS.
Effect of the membrane permeant cAMP analog dbcAMP on CREB activity. Rat lacrimal gland acini were incubated with dbcAMP (10^{-3} M) for various times (A, B) or at various concentrations of dbcAMP for 30 minutes (C, D), and the amount of phosphorylated CREB were measured. B and D are representative blots from a single experiment. Data in A are the mean ± SEM of results from three independent experiments. Data in C is the mean from two experiments. B; Basal, D; dbcAMP.
Effect of activation of AC with the forskolin analog L-85 8051 and the phosphodiesterase inhibitor IBMX on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with dbcAMP (10-3 M), IBMX (10-3 M), L-85 8051 (10-4 M) or these compounds together for 30 minutes. Data are the mean ± SEM from four independent experiments. B is representative blot from a single experiment. *Statistically significant difference from basal level. B; Basal, D; dbcAMP, IB; IBMX, L; L-85 8051, Ch; carbachol.
Effect of VIP on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated with VIP 10^{-8} M for the indicated times (A and B) or indicated concentrations (C and D), and western blot analysis was performed for phospho p44/p42 MAPK. Data are the mean ± SEM from seven independent experiments in A and four experiments in C. B and D are representative blots from single experiments. *Statistically significant difference from basal level. B; Basal, V; VIP.
Effect of simultaneous addition of the membrane permeant cAMP analog dbcAMP and Ca2+/PKC dependent agonists on p44/p42 MAPK activity. Rat lacrimal gland acini were incubated simultaneously with presence or absence of dbcAMP 10-3 M alone or with carbachol (Cch, 10-4 M), phenylephrine (Ph, 10-4 M), or EGF (10-7 M) for 5 minutes. A; a representable blot. B; summary of results. Data are the mean ±SEM from four to seven independent experiments. *Statistically significant difference from basal level. #Statistically significant difference from agonist-stimulated p44/p42 MAPK level.
Effect of simultaneous use of VIP and Ca2+/PKC dependent agonists on p44/p42 MAPK activity in rat lacrimal gland. Rat lacrimal gland acini were incubated simultaneously with VIP (V, 10^{-8} M) and carbachol (C, 10^{-4} M) (A), phenylephrine (P, 10^{-4} M) (B), and EGF (E, 10^{-7} M) (C) for 5 min. Western blot analysis was performed for phosphor-p44/p42 MAPK. Data are mean ±SEM of results in 3-5 dependent experiments. *Statistically significant difference from basal level.
Effect of simultaneous use of the membrane permeant cAMP analog dbcAMP and Ca2+/PKC dependent agonists on peroxidase protein secretion from rat lacrimal gland acinar cells. Rat lacrimal gland acini were incubated simultaneously with dbcAMP (10-3 M) and (A) a maximal (10-4 M) or submaximal (10-5 M) concentration of carbachol (Cch); (B) a maximal (10-4 M) or submaximal (10-5 M) concentration of phenylephrine (Ph), or (C) a maximal (10-7 M) or submaximal (10-8 M) concentration of EGF for 20 minutes. Data are the mean ±SEM of results in seven to ten independent experiments *Statistically significant difference from basal level. #Statistically significant difference from theoretical additivity value, indicated by dotted line.