Cross-talk between calcium and cAMP-dependent intracellular signaling pathways. Implications for synergistic secretion in T84 colonic epithelial cells and rat pancreatic acinar cells.

M Vajanaphanich, … , S J Pandol, K E Barrett

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Research Article

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Cross-Talk between Calcium and cAMP-dependent Intracellular Signaling Pathways

Implications for Synergistic Secretion in T_{84} Colonic Epithelial Cells and Rat Pancreatic Acinar Cells

Mana Vajanaphanich, Carsten Schultz,* Roger Y. Tsien,*‡ Alexis E. Traynor-Kaplan, Stephen J. Pandol,§ and Kim E. Barrett

*Departments of Medicine and *Pharmacology, University of California, San Diego, School of Medicine, San Diego, California 92103; the ‡Howard Hughes Medical Institute, La Jolla, California, 92093 and the §Veteran's Administration Medical Center, San Diego, California, 92161*

Abstract

Treatment of various cells with combinations of agents that increase either cAMP or cytosolic calcium can lead to synergistic responses. This study examined interactions, or cross-talk, between these two intracellular messengers and its implication for signaling in two secretory cell types, T_{84} human colonic epithelial cells and rat pancreatic acinar cells. T_{84} cell chloride secretion was measured in Ussing chambers. Acinar cell activation was monitored as amylase secretion. Cytosolic calcium was assessed via fura-2 microfluorimetry. A cell-permeant analogue of cAMP synergistically enhanced secretory responses to calcium-mobilizing hormones in both cell types, but paradoxically reduced overall calcium mobilization. The reduction in calcium mobilization could be attributed to an inhibition of calcium influx in T_{84} cells, although a different mechanism likely operates in acinar cells. The effects of the cAMP analogue were reproduced by other agents that increase cAMP. Furthermore, econazole, an inhibitor of calcium influx, potentiated secretory responses to calcium-dependent stimulation in T_{84} cells without itself inducing secretion. We conclude that there is cross-talk between calcium and cAMP-dependent signaling pathways at the level of second messenger generation in two secretory cell types. This cross-talk appears to regulate the extent of secretory responses. (*J. Clin. Invest.* 1995. 96:386–393.) Key words: signal transduction • chloride secretion • amylase secretion • exocytosis

Introduction

Changes in the concentration of free intracellular calcium ($[Ca^{2+}]_i$)[1] are widely recognized to control a variety of functions in almost all cell types that have been examined (1). One example of a cellular process that is controlled by $[Ca^{2+}]_i$ is the chloride secretory response of intestinal epithelial cells, as modeled using the human colonic cell line T_{84} (2). In these cells, calcium appears to act in concert with other second messengers that further amplify or inhibit the secretory response (2, 3). Synergism has also been reported in T_{84} and other intestinal epithelial cell lines between the actions of agonists that act through $[Ca^{2+}]_i$, and those that cause increases in cAMP (4, 5). This may therefore represent an example of "cross-talk" between these two signaling pathways. Simultaneous stimulation of such cells with combinations of such agonists induces a greater degree of chloride secretion than would be predicted by simple addition of their independent effects. While this synergism may have significant clinical relevance, in that it provides a means for the amplification of secretory responses and may thus be important in the pathogenesis of secretory diarrhea, its underlying mechanism has not been fully elucidated. One possible mechanism is that cAMP and Ca^{2+} act co-operatively on an apical Cl^- conductance (6). Another possibility is that synergism results from the stimulated opening of a calcium-regulated basolateral potassium channel and the cAMP-regulated apical chloride channel, thereby effectively removing the rate-limiting step for either type of chloride secretory response. This latter mechanism for synergism between calcium-mediated and cAMP-mediated secretagogues was advanced based on previous studies in T_{84} cells (4). A third possible mechanism for the observed synergism would be that these second messenger pathways interact at the level of messenger generation. However, previous studies, based on examining responses of populations of T_{84} cells, did not provide evidence for such interactions (4). However, because they examined mean responses of $\sim 10^6$ cells, these studies may have obscured the details of the calcium response, particularly if an oscillatory response was involved. Moreover, the experiments employed a calcium ionophore to activate calcium-dependent processes, which induces supraphysiologic and non-oscillatory increases in calcium that may not be subject to normal regulatory mechanisms.

More recent studies in T_{84} and other cell types have examined intracellular calcium dynamics in more detail, using single cell imaging approaches and various calcium-binding fluorescent dyes. These experiments have revealed that in many cell types, including T_{84} cells, agonist-stimulated changes in $[Ca^{2+}]_i$ are not exclusively monophasic, but rather may contain oscillatory components (7–11). Such oscillatory changes in $[Ca^{2+}]_i$ have been described to encode signaling information within their frequency (12). Studies have likewise indicated that cAMP is capable of regulating cytosolic calcium via several mechanisms. In guinea pig hepatocytes, cAMP is able to increase the sensitivity of intracellular calcium stores to the calcium-mobilizing messenger *myo*-inositol-1,4,5-trisphosphate

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Address correspondence to UCSD Medical Center, 8414, 200 West Arbor Drive, San Diego, CA 92103-8414. Phone: 619-543-3726; FAX: 619-543-6969; E-mail: kbarrett@ucsd.edu. C. Schultz's present address is University of Bremen, Institute for Organic Chemistry NWII, Leobener Strasse, 28359 Bremen, Germany.

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular calcium concentration; $Ins(1,4,5)P_3$, *myo*-inositol-1,4,5-trisphosphate; Bt_2cAMP, dibutyryl cAMP; Bt_2cAMP/AM, acetoxymethyl ester of dibutyryl cAMP; 8-Br cAMP, 8-bromo cAMP; VIP, vasoactive intestinal polypeptide; CCK, cholecystokinin; CCK-OP, cholecystokinin octapeptide.

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($Ins(1,4,5)P_3$) (13). In lymphocytes, cAMP counteracts agonist-stimulated increases in $Ins(1,4,5)P_3$ (14). In other cells, cAMP may regulate Ca^{2+} influx (15). However, the precise intracellular mechanisms involved in regulating Ca^{2+} influx are still the subject of investigation (16–18).

The ability to examine calcium dynamics on a single cell basis prompted us to re-evaluate whether the synergistic chloride secretion induced by combinations of calcium- and cAMP-dependent hormones in T_{84} cells was in any way related to interactions at the level of second messenger generation. The experiments were additionally facilitated by the availability of a novel cell-permeant analogue of cAMP, the acetoxymethyl ester of dibutyryl cAMP (Bt_2cAMP/AM, 19). The improved ability of this analogue to enter cells means that it can be used at much lower concentrations than more conventional analogues (e.g., Bt_2cAMP, 8-Br cAMP). At least for Bt_2cAMP, the use of the novel analogue therefore avoids exposing cells to high concentrations of butyrate that may themselves have biological activity (20, 21). Based on these studies, we now report that cAMP does indeed alter the pattern of agonist-stimulated calcium mobilization in T_{84} cells, and that this ability affects the extent of the chloride secretory response. Further, because T_{84} cells are transformed, and, accordingly, one should extrapolate from findings obtained with this cell type with care, we also sought to extend our findings to an unrelated secretory cell type, the rat pancreatic acinar cell. We therefore also present data that suggest that the ability of cAMP to modify calcium dynamics in secretory cell types is likely a general phenomenon, with similar consequences for the extent of secretion. However, the precise mechanism whereby cAMP regulates calcium dynamics appears to differ between the intestinal and the pancreatic cells.

Methods

Materials. Carbachol, forskolin, and vasoactive intestinal polypeptide (VIP) were purchased from Sigma Chemical Company (St. Louis, MO). Fura-2/AM was obtained from Calbiochem (La Jolla, CA). CCK-JMV180 was purchased from Research Plus (Bayonne, NJ). Bt_2cAMP/AM was synthesized as described previously (19). Pluronic F-127 was a gift from BASF (Wyandotte, MI).

Cells. Methods for the growth and maintenance of T_{84} cells for use in transepithelial electrolyte transport studies have been described previously (22). In brief, monolayers of T_{84} cells were grown on collagen-coated polycarbonate filters (Nuclepore, Pleasanton, CA) for 7 d before experiments. For other studies, T_{84} cells were cultured on collagen-coated coverslips, or in standard 6-well cell culture plates. Rat pancreatic acini were obtained using techniques that have been described previously (23, 24).

Chloride secretion. Studies of transepithelial chloride secretion were performed using monolayers of T_{84} cells, grown on collagen-coated filters, and mounted in Ussing chambers modified for use with cultured cells, as previously described (22). Short-circuit current (I_{SC}) was used to quantitate transepithelial Cl^- secretion. Previous studies using the T_{84} cell line have revealed that this parameter is wholly reflective of the extent of net chloride secretion.

Amylase release. Isolated rat pancreatic acini were suspended in solution Q (consisting of, in mM: NaCl, 120; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; Hepes, 20; pyruvate, 10; ascorbate, 10; and glucose, 10, and supplemented with albumin [1 mg/ml] and soybean trypsin inhibitor [10 μg/ml]) and were incubated at 37°C. The cells were then stimulated with various agents according to the experimental design. Amylase activity in supernatants was determined using a commercially available colorimetric assay (Phadebas amylase test, Pharmacia Diagnostics-AB, Uppsala, Sweden) as described previously (24). Results were expressed as percent amylase release, which was the percentage of total cellular amylase released into the supernatant at a given time of incubation. Total cellular amylase, upon which the calculation of percent amylase release was based, was assessed in an aliquot of cells lysed using a solution containing 4 mM sodium dodecyl sulfate and 1 mM sodium phosphate (pH 7.8).

Measurement of free cytosolic calcium. Levels of cytosolic calcium were measured in single T_{84} or rat pancreatic acinar cells using a digital fluorescence imaging system. In brief, subconfluent monolayers of T_{84} cells on glass cover slips were incubated for 45 min at room temperature with 1 μM fura-2/AM in DME medium containing 10 mM Hepes and 0.02% (vol/vol) pluronic F-127. Rat pancreatic acinar cells were incubated for 45 min at 37°C with 2 μM fura-2/AM in solution Q. Pancreatic acini were immobilized on a glass coverslip previously coated with polylysine (Sigma Chemical Company). For both T_{84} and acinar cells, $[Ca^{2+}]_i$ was monitored continuously on a temperature-controlled microscope stage. Agonists were added in a minimal volume directly to the solution overlying the cells. Fura-2 loaded cells were alternately illuminated with excitation wavelengths of 340 and 380 nm (5 s/cycle), respectively, using a computer-driven system. The relative fluorescence emission at 510 nm was measured for each wavelength, and the ratio between the relative fluorescence at 340 vs. 380 nm was determined. Cells were chosen randomly for analysis, although those exhibiting high levels of resting $[Ca^{2+}]_i$ (likely reflective of damage) were excluded. $[Ca^{2+}]_i$ was calculated using previously published procedures after calibration at the wavelengths used (25).

Determination of $^{45}Ca^{2+}$ uptake in T_{84} cells. To measure agonist-stimulated $^{45}Ca^{2+}$ entry, T_{84} cells were grown to confluence in six-well plates (24 cm^2/well). After rinsing with HBSS containing 10 mM Hepes and 10 mM glucose, cell monolayers were incubated at 37°C in 2 ml of the same buffer containing 1 μCi/ml of $^{45}Ca^{2+}$ and stimulated with various agonists according to the experimental design. $^{45}Ca^{2+}$ uptake was terminated at different times by rapidly washing the cell monolayer three times with 3 ml of ice-cold buffer. Cells were then disrupted by scraping them into 0.5 ml of 0.1 M HCl with a plastic cell scraper and an aliquot was taken to determine $^{45}Ca^{2+}$ by liquid scintillation counting.

Determination of $^{45}Ca^{2+}$ efflux in rat pancreatic acinar cells. Agonist-stimulated $^{45}Ca^{2+}$ efflux from preloaded rat pancreatic acini was examined using a protocol that has been previously described (26, 27). Briefly, acini were loaded with $^{45}Ca^{2+}$ by incubating them at 37°C in solution Q for 5 min with carbachol (100 μM). Then atropine (10 μM) and $^{45}Ca^{2+}$ (4 μCi/ml) were added and the incubation continued for another 10 min. This procedure completely loads the intracellular stores with $^{45}Ca^{2+}$. The acini were transferred to fresh solution Q without $^{45}Ca^{2+}$, carbachol or atropine, then stimulated with various agonists as indicated in Results. Cellular $^{45}Ca^{2+}$ was measured in aliquots of acini at various time points as previously described (26). Results were expressed as a percent of cell-associated $^{45}Ca^{2+}$ measured in acini immediately prior to stimulus addition.

Data and statistical analyses. Results are expressed as mean values±SEM for a given number of independent experiments. Continuous data were analyzed by Student's t test or by ANOVA, as appropriate. Comparisons between the proportions of T_{84} cells displaying various patterns of calcium responses were made using Fisher's exact test. Differences with an associated probability of < 0.05 were considered to be statistically significant.

Results

Synergism between the effects of carbachol and Bt_2cAMP/AM on chloride secretion. In initial studies, we demonstrated that the novel cell permeant analogue of cAMP, Bt_2cAMP/AM, displayed the same synergistic interaction with a calcium mobilizing stimulus for chloride secretion as has been described previously for VIP in T_{84} cells (4). Fig. 1 depicts the chloride secretion induced by either a maximal dose of carbachol (100 μM), 3 μM Bt_2cAMP/AM, or the combination of these stimuli added simultaneously. Also depicted is the predicted chloride secretory response to the combined agonists calculated on the

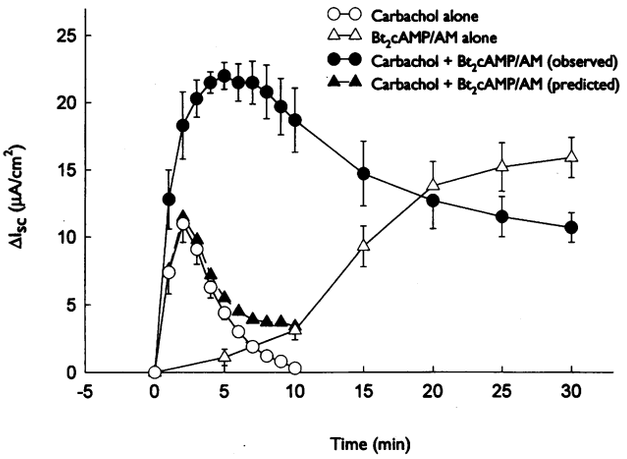

Figure 1. Time course of the effect of carbachol (100 μM) alone (○—○), Bt_2cAMP/AM (3 μM) alone (△—△), or the combination of these stimuli (●—●) on chloride secretion across monolayers of T_{84} cells mounted in Ussing chambers. Chloride secretion was assessed as changes in short circuit current (ΔI_{SC}). Stimuli were added at time zero, which was ~ 20 min after mounting the cells. The predicted response to the combined stimuli calculated by summation of their individual effects is also shown (▲—▲). Values are means ±SEM for nine experiments.

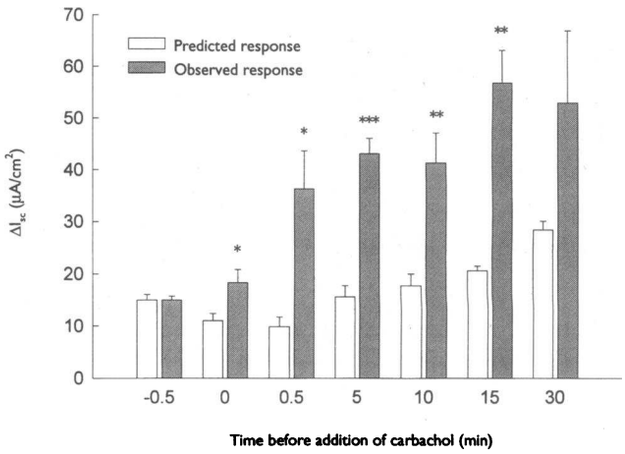

Figure 2. Effect of time of addition of Bt_2cAMP/AM on its ability to synergistically enhance chloride secretory responses to carbachol (100 μM) in T_{84} cells. Bt_2cAMP/AM (3 μM) was added to the cells either 30 s after (*−0.5*), simultaneously with (*0*), or at various times before the addition of carbachol as shown on the *x*-axis. Data are presented as the maximal increase in I_{SC} (ΔI_{SC}). The open bars depict the responses predicted by summation of the independent effects of the agonists; these predicted responses are compared with the corresponding responses that were actually observed, shown in the hatched bars. The data are means±SEM for at least three observations at each time point. Asterisks denote observed responses that significantly exceeded the predicted response ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$ by Student's *t*-test).

basis of summation of the individual responses. It is clear from the figure that the combination of carbachol and Bt_2cAMP/AM induces a markedly synergistic response in T_{84} cells, in that the extent of chloride secretion observed is significantly greater than additive.

The dependence of this synergism on the relative timing of addition of the two stimuli is shown in Fig. 2. There were three experimental paradigms: carbachol addition preceding that of Bt_2cAMP/AM, simultaneous addition of the two agonists, or Bt_2cAMP/AM addition preceding that of carbachol. For the last condition, various time periods of pretreatment with Bt_2cAMP/AM were examined. For each case, results were compared to the maximal responses obtained with each agent added singly. Thus, the observed maximal chloride secretory responses in each case are compared with the predicted additive response. As noted above, significant synergism could be observed if the agonists were added to the cells simultaneously. The extent of this synergism was slightly increased if Bt_2cAMP/AM was added 30 s before carbachol, but longer periods of pretreatment with Bt_2cAMP/AM did not further increase the extent of the synergistic enhancement of chloride secretion. In contrast, no synergism occurred if the addition of carbachol preceded that of Bt_2cAMP/AM.

Effect of Bt_2cAMP/AM on calcium mobilization responses in T_{84} cells. Previous studies examining the mechanism of synergistic chloride secretion in T_{84} cells failed to reveal any effect of VIP on calcium mobilization responses (K. E. Barrett, G. Beuerlein, and K. Dharmsathaphorn, unpublished observations). However, these prior experiments only examined average calcium responses of populations of cells, and also used a calcium ionophore to stimulate increases in $[Ca^{2+}]_i$. In order to examine whether an increase in cAMP might in fact modulate calcium mobilization in response to a receptor-mediated stimulus, particularly if examined at a single cell level, we monitored $[Ca^{2+}]_i$ in cells treated with carbachol in the presence or absence of Bt_2cAMP/AM by digital fluorescence microscopy. As shown in Fig. 3 (*upper panel*), when examined on a single cell basis, carbachol had a biphasic effect on $[Ca^{2+}]_i$ in T_{84} cells. There was an initial calcium transient (phase I) that corresponded temporally to the period of chloride secretion induced by this agonist, followed by a more sustained response (phase II) that frequently had an oscillatory character. Others have shown previously that the phase II response in both T_{84} and HT29 cells is associated with influx of calcium from the extracellular milieu (10, 28, 29). This was confirmed in the present studies by monitoring $[Ca^{2+}]_i$ in T_{84} cells treated with carbachol in the absence of extracellular calcium. Under these conditions, the phase II response was abolished (data not shown). The phase I response was unaltered in the absence of extracellular calcium, implying that it is derived from an intracellular pool (data not shown).

We then examined the effect of Bt_2cAMP/AM on carbachol-induced calcium mobilization. As shown in Fig. 3 (*lower panel*), Bt_2cAMP/AM had no effect on $[Ca^{2+}]_i$ by itself, but when added prior to carbachol, it modified the response to the muscarinic agonist to a single, phase I transient. Results from a number of such experiments are summarized in Fig. 4. The majority of cells treated with carbachol alone displayed a biphasic calcium response. In cells pretreated with Bt_2cAMP/AM, the majority of cells displayed only the phase I transient, and this difference was statistically significant ($P < 0.01$ by Fisher's exact test). In data not shown, the effect of Bt_2cAMP/AM could also be reproduced by the cAMP-dependent chloride secretagogues VIP and forskolin.

Effect of Bt_2cAMP/AM on $^{45}Ca^{2+}$ uptake into T_{84} cells. Because the phase II calcium mobilization response in T_{84} cells is derived from the extracellular pool, the ability of Bt_2cAMP/AM to modify agonist-stimulated $^{45}Ca^{2+}$ uptake was next examined. Cells were preincubated with either buffer alone or with

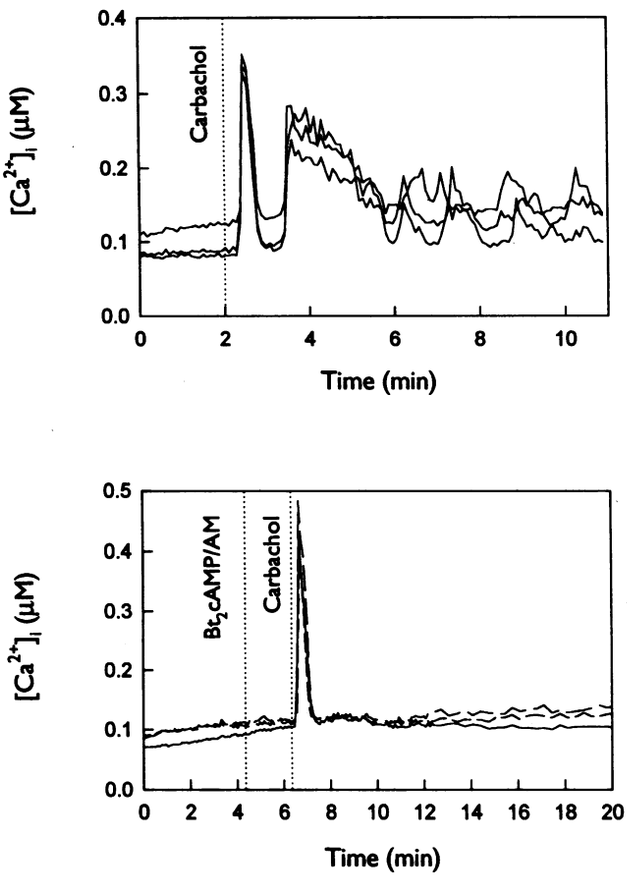

Figure 3. Time course of the effect of carbachol (300 μM) on $[Ca^{2+}]_i$ in individual T_{84} cells either in the absence (*upper panel*) or presence (*lower panel*) of Bt_2cAMP/AM (3 μM). Each trace represents the value for $[Ca^{2+}]_i$ in a single cell, as calculated from fura-2 fluorescence ratios. Agonists were added at the times indicated by the vertical dashed lines. Data are from one experiment representative of at least seven similar experiments.

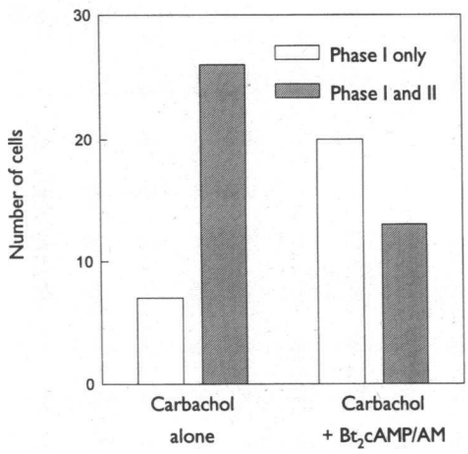

Figure 4. Expression of calcium mobilization responses in individual T_{84} cells in response to carbachol (300 μM) in the absence or presence of Bt_2cAMP/AM (3 μM). The pattern of responses of individual cells monitored in seven separate experiments for each condition were defined as either phase I only (a single, transient increase in $[Ca^{2+}]_i$, analogous to the lower panel in Fig. 3, shown with the open bars) or phase I and II (a single transient followed by a sustained increase in $[Ca^{2+}]_i$ that lasted for several minutes, analogous to the upper panel in Fig. 3, shown with the hatched bars). No cells were observed that displayed only phase II of the response to carbachol. In the presence of Bt_2cAMP/AM, the proportion of cells displaying both phase I and II of the response to carbachol was significantly reduced, with a corresponding increase in the proportion of cells displaying only the phase I transient ($P < 0.01$ by Fisher's exact test).

Bt_2cAMP/AM (3 μM), then $^{45}Ca^{2+}$ was added with or without carbachol (100 μM). As shown in Fig. 5, carbachol alone stimulated a significant increase in $^{45}Ca^{2+}$ uptake at 1 and 2 minutes after addition compared with cells treated with buffer alone. When cells were preincubated with Bt_2cAMP/AM, the carbachol-stimulated increase in $^{45}Ca^{2+}$ uptake was completely abolished. An essentially similar pattern was observed at 5 and 10 minutes after carbachol addition.

Effect of econazole on responses of T_{84} cells to carbachol. Because cAMP appeared to modify agonist-stimulated calcium uptake into T_{84} cells, we hypothesized that other blockers of this process should have similar effects on the chloride secretory response. We therefore tested whether econazole, an inhibitor of calcium uptake in a number of other systems (16, 30, 31), had any effect on carbachol-stimulated chloride secretion. As shown in Fig. 6 (*left*), econazole had no effect on chloride secretion when added alone, but significantly enhanced and prolonged the secretory response evoked by carbachol. This effect was dose-dependent, as shown in the right panel of Fig. 6. To confirm that econazole does in fact reduce calcium influx in T_{84} cells, $^{45}Ca^{2+}$ uptake experiments were performed. As shown in Fig. 7, carbachol-stimulated $^{45}Ca^{2+}$ uptake was significantly inhibited by pretreatment with econazole.

Effect of Bt_2cAMP/AM on secretory responses of pancreatic acinar cells. Because T_{84} cells are transformed, and may therefore display signaling mechanisms that are not wholly reflective of those in normal cells, we also sought to extend our

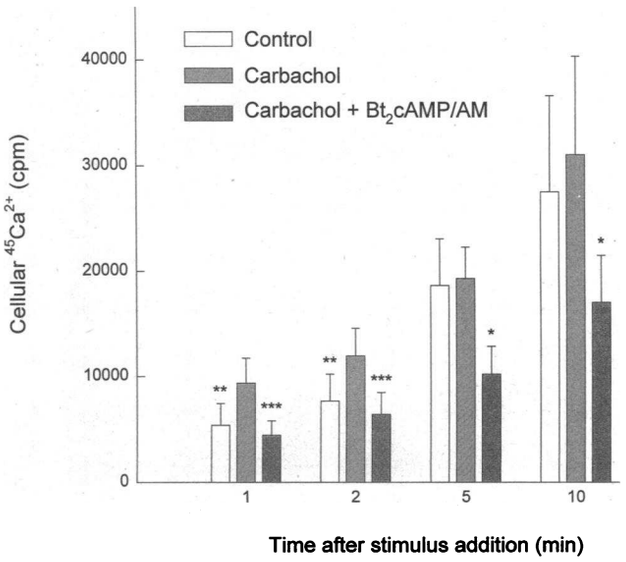

Figure 5. Time course of $^{45}Ca^{2+}$ uptake into T_{84} cells treated with buffer alone (control), carbachol (100 μM) alone, or pretreated for 5 min with Bt_2cAMP/AM (3 μM) then stimulated with carbachol. The *x*-axis depicts the time after carbachol addition. The data are means±SEM for seven experiments. Asterisks denote responses that differ significantly from those observed with carbachol alone (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by ANOVA).

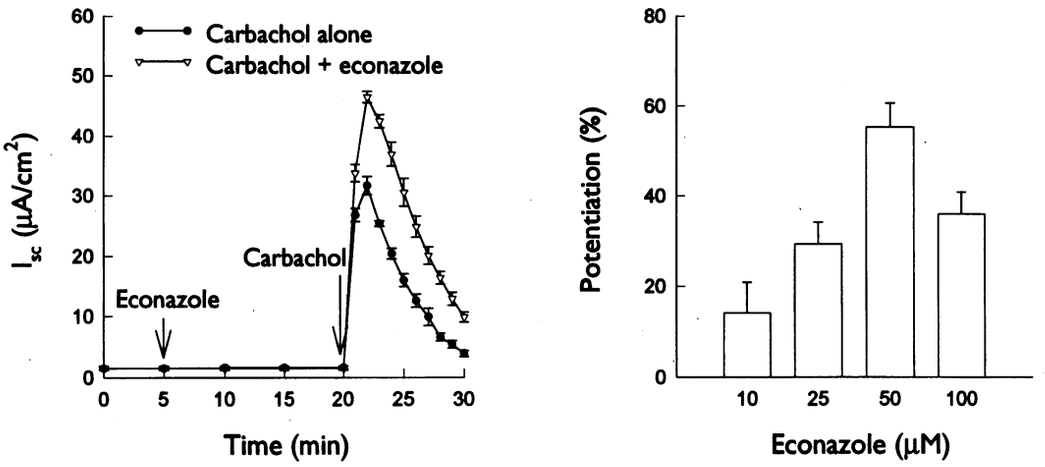

Figure 6. (*Left*) Time course of effect of econazole (50 μM) on chloride secretory responses of T_{84} cells to carbachol (100 μM). Stimuli were added at the times indicated by the arrows. The data are means±SEM for four experiments. The response in the presence of econazole is significantly greater than that induced by carbachol alone at all time points after carbachol addition, $P < 0.05$ by Student's t test). (*Right*) Dose dependency of the effect of econazole on chloride secretory responses to carbachol in T_{84} cells. The data are presented as the percent potentiation of the response to carbachol alone observed in the presence of the stated dose of econazole. Values are means±SEM for four experiments.

studies to an examination of responses in a normal cell type. The effect of Bt_2cAMP/AM on calcium-dependent secretory responses of rat pancreatic acinar cells was therefore examined. As shown in Fig. 8, pancreatic acini treated with the cholecystokinin (CCK) analogue JMV-180 show a progressive release of amylase. Bt_2cAMP/AM had little effect on amylase release when added alone at a concentration of 3 μM, but synergistically enhanced the response to JMV-180 such that greater than additive secretory responses were observed.

Effect of Bt_2cAMP/AM on calcium responses in pancreatic acinar cells. CCK JMV-180 is known to elicit oscillatory calcium responses in pancreatic acinar cells (32). To determine whether cAMP is capable of modulating such responses, as seen for the T_{84} cells, we examined $[Ca^{2+}]_i$ in pancreatic acinar cells

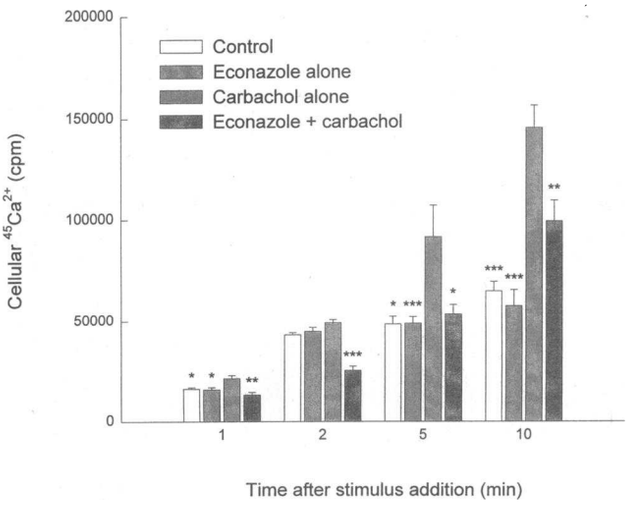

Figure 7. Effect of econazole on carbachol-induced influx of $^{45}Ca^{2+}$ in T_{84} cells. Cells were treated with either buffer or econazole (50 μM) for 15 min, then $^{45}Ca^{2+}$ was added with or without carbachol (100 μM) as indicated and the incubation continued for the times noted on the x-axis. Data are means±SEM for four experiments. Asterisks denote values that differ from those in the presence of carbachol alone ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$ by ANOVA).

by fluorescence imaging techniques. In fact, pretreatment with Bt_2cAMP/AM (3 μM) modified the response to JMV-180, resulting in a single transient, as seen for the effect of the nucleotide analogue on carbachol-induced responses in T_{84} cells (Fig. 9, *upper panel*). Further, Bt_2cAMP/AM could promptly terminate oscillatory responses to JMV-180 in pancreatic acinar cells after they had been elicited. These effects of Bt_2cAMP/AM were also reproduced by 10 nM VIP (data not shown).

Based on previous studies, and in contrast to the effect of carbachol in T_{84} cells described above, JMV-180 is thought to induce oscillations in $[Ca^{2+}]_i$ in pancreatic acinar cells via repetitive release and uptake of calcium from intracellular stores, rather than inducing influx of calcium from the extracellular compartment (33). Therefore, it was unlikely that the effect of Bt_2cAMP/AM on calcium mobilization in the pancreatic acinar cells reflected an action on calcium influx. We therefore sought an alternative mechanism. Acinar cells were preloaded with $^{45}Ca^{2+}$, and cellular $^{45}Ca^{2+}$ monitored over time after transfer to $^{45}Ca^{2+}$-free medium in control cells and those stimulated with either the cAMP-dependent agonist VIP (10 nM), JMV-180 (1 μM), or the combination of JMV-180 and VIP (Fig. 10). Some cells were also treated with a maximal dose of CCK-octapeptide (CCK-OP, 10 nM), as a control to fully release intracellular calcium stores (CCK-OP at this dose does not cause oscillations in $[Ca^{2+}]_i$ in acinar cells) (34). VIP had no effect on calcium efflux, compared with controls. As expected, CCK-OP caused a rapid and large efflux of calcium. JMV-180 alone had a rapid but smaller effect on calcium efflux lasting one minute, with no further enhancement of the rate of calcium efflux occurring with continued stimulation with JMV-180. These results are consistent with recurrent release and re-uptake of $^{45}Ca^{2+}$ into intracellular stores during sustained secretion with JMV-180. Less $^{45}Ca^{2+}$ would therefore be available to leave the cells than in the case of treatment with CCK-OP. When cells were treated with the combination of VIP and JMV-180, the rate of $^{45}Ca^{2+}$ efflux did not differ significantly from that caused by JMV-180 alone.

Discussion

The goal of this study was to re-examine possible mechanisms of synergistic chloride secretion in the T_{84} cell model, by de-

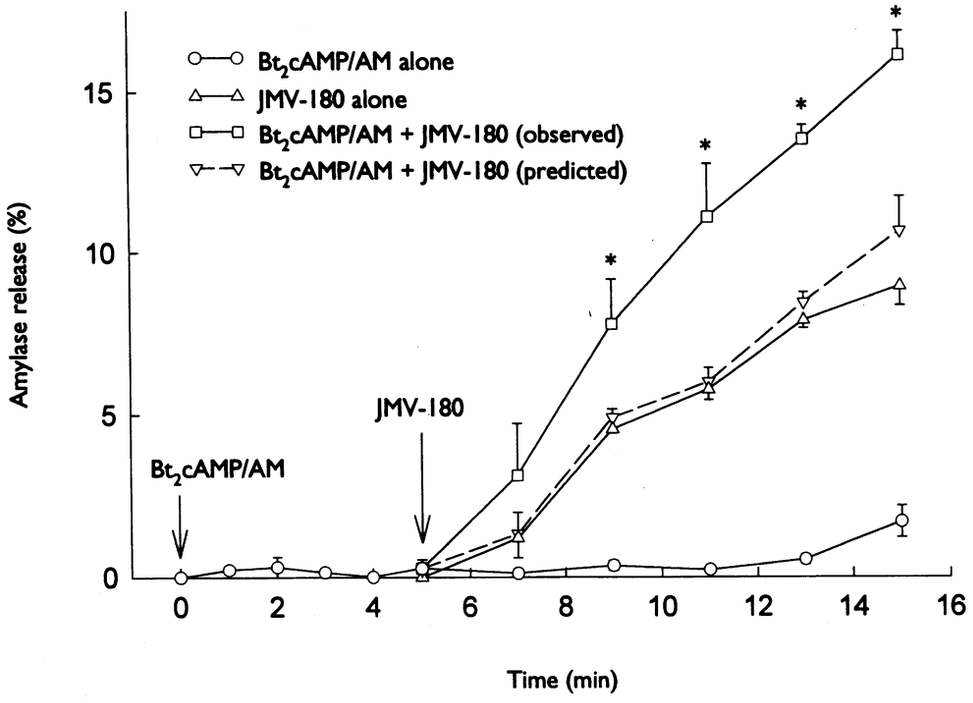

Figure 8. Time course of amylase release from rat pancreatic acini induced by either Bt_2cAMP/AM (3 μM) alone (○—○), JMV-180 (1 μM) alone (△—△), or the combination of these stimuli (□—□). The response to the combined stimuli predicted by summation of their individual effects is also shown (▽–▽). Agonists were added at the times indicated by the arrows. Data are presented as the percent of total cellular amylase released at a given time point, and are corrected for the release of amylase occurring in the absence of stimulation. Data are means±SEM for four experiments. Asterisks denote observed responses to the combined agonists that significantly exceed the predicted response ($P < 0.05$ by Student's t test).

termining whether there were any effects at the level of second messenger generation that could account for the observed synergistic response to combinations of calcium- and cAMP-elevating agonists. We have accordingly uncovered an ability of cAMP to modify calcium mobilization responses to the muscarinic agonist, carbachol. Carbachol is a prototypic calcium-dependent agonist in this system, acting via an increase in $Ins(1,4,5)P_3$ and a consequent elevation in $[Ca^{2+}]_i$. The impact of cAMP is somewhat paradoxical in light of the end result, i.e. synergism; in fact, overall calcium mobilization induced by carbachol was reduced in cells pretreated with Bt_2cAMP/AM or cAMP-mobilizing agonists such as VIP or forskolin, apparently as a result of decreased calcium influx. Moreover, the characteristics of the calcium response were changed such that the initial calcium transient, mobilized by carbachol from intracellular pools, remained intact, and a later phase of calcium oscillations was abolished by the nucleotide. However, there is precedent for our findings in that others have reported that cells respond differently to calcium derived from the intracellular *vs.* the extracellular pool (11).

It would appear that the effect of cAMP in the T_{84} cell system is to reduce calcium influx from the extracellular pool. This conclusion is supported by the fact that the second phase of calcium mobilization induced by carbachol in T_{84} cells is known to be dependent on extracellular calcium (28, as also shown here), and by the observation that Bt_2cAMP/AM abolished carbachol-stimulated ^{45}Ca uptake. One possible explanation for the ability of cAMP to reduce calcium influx in this system is that the nucleotide stimulates chloride efflux from the cells, which in turn would reduce the electrical driving force for calcium entry. While this explanation might indeed account for part of the effect of cAMP on calcium influx into T_{84} cells, it is unlikely to be the only mechanism of action. Thus econazole, a known inhibitor of receptor-mediated calcium influx pathways (16, 30, 31), did not by itself activate chloride secretion, but enhanced chloride secretion in association with a reduction in carbachol-stimulated calcium influx.

In fact, the second phase of calcium mobilization activated by carbachol in T_{84} cells (i.e., the influx phase) is temporally dissociated from the effect of carbachol on chloride secretion. Chloride secretory responses to carbachol are transient, and correspond to the first phase of calcium mobilization from intracellular pools. The second phase of calcium mobilization corresponds to a time when chloride secretion has returned to basal values. Thus this second phase either coincides with, results in, or itself constitutes a negative signal for chloride secretion. We have previously shown that *myo*-inositol-3,4,5,6-tetrakisphosphate is a candidate negative signal for calcium-dependent chloride secretion in T_{84} cells (35). Calcium derived from the extracellular pool might be necessary for the production of this negative signal, perhaps by participating in the activation of phospholipase C (36) or $Ins(1,4,5)P_3$-kinase (37). Thus, the ability of cAMP to reduce calcium influx would limit the negative arm of the calcium-dependent signaling pathway and thus result in a synergistic enhancement of secretion, at least during the period when calcium-dependent chloride secretion is occurring. This last point also relates to the time course for the synergistic effect of cAMP. If Bt_2cAMP/AM was added as rapidly as 30 s after carbachol, no synergism of chloride secretion was observed (Fig. 2). At this time point after carbachol addition, activation of chloride secretion as a result of the carbachol-stimulated release of calcium from intracellular stores is already resolving.

The present studies have also shown that cross-talk between cAMP and calcium mobilization, with a consequence of an enhanced secretory response, may be a generalizable phenomenon in that a similar interaction could also be demonstrated in nontransformed, freshly isolated rat pancreatic acinar cells. The combination of Bt_2cAMP/AM with an agonist that causes calcium oscillations in acinar cells, JMV-180, led to a synergistic enhancement of amylase release. This synergism was again correlated with a prompt cessation of calcium oscillations. However, the mechanism whereby cAMP alters calcium mobilization in acinar cells is likely to be somewhat dissimilar from that

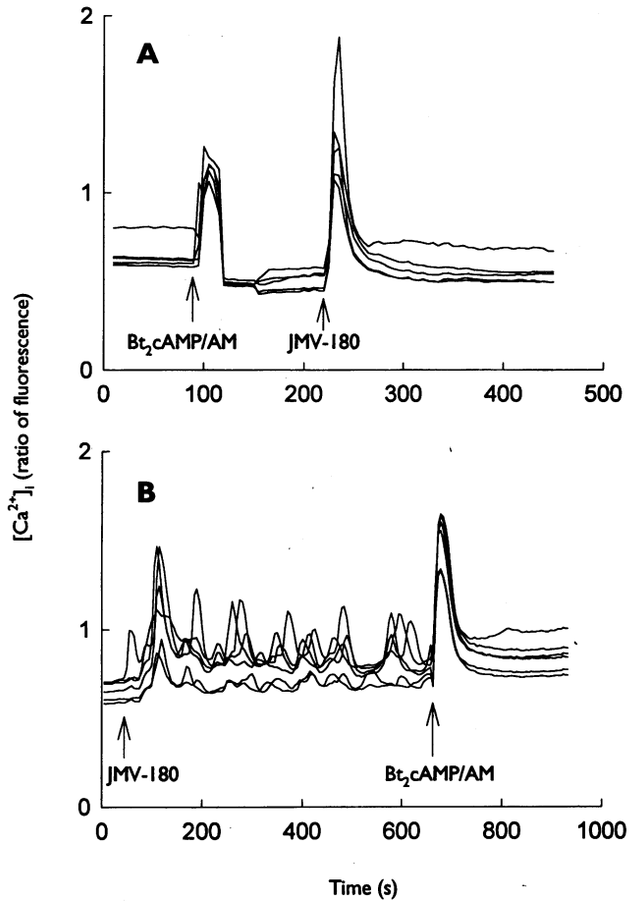

Figure 9. Time course of changes in $[Ca^{2+}]_i$ induced by Bt_2cAMP/AM (3 μM) and JMV-180 (1 μM) in rat pancreatic acini, as monitored by changes in the ratio of fura-2 fluorescence in individual cells. In *A*, Bt_2cAMP/AM was added before JMV-180 addition as indicated by the arrows; in *B*, the order of agonist addition was reversed. Each trace represents data for a single cell. The data are for one experiment each, representative of at least three experiments for each condition.

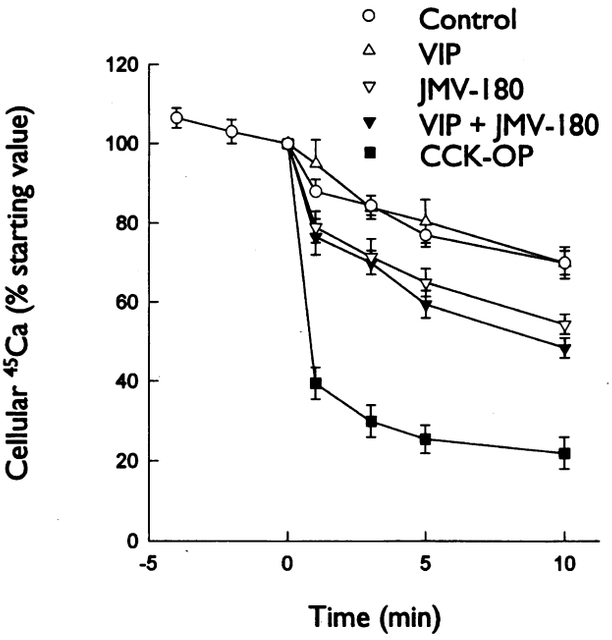

Figure 10. Time course of $^{45}Ca^{2+}$ efflux from preloaded rat pancreatic acini following stimulation with either buffer alone (○), VIP (10 nM, △), JMV-180 (1 μM, ▽), the combination of VIP and JMV-180 (▼), or CCK-OP (10 nM, ■). Data are expressed as a percent of the cellular content of label at the time of stimulus addition (time zero) and are means±SEM for four experiments.

in T_{84} cells, since the pool from which the calcium driving oscillatory responses was derived differed between the two cell types. In T_{84} cells, second phase calcium oscillations were entirely dependent on extracellular calcium, and thus likely represent calcium influx. In contrast, the ability of JMV-180 to evoke oscillations in $[Ca^{2+}]_i$ in acinar cells was entirely independent of extracellular calcium, and therefore must represent release and re-uptake from an intracellular pool. In the latter model, we attempted to determine whether cAMP might block re-uptake into the intracellular pool. Rates of calcium efflux in stimulated acinar cells diluted into calcium-free medium were compared when cells were treated with JMV-180 alone, or with JMV-180 plus the cAMP-dependent agonist, VIP. In fact, VIP did not modify the rate of calcium efflux induced by JMV-180. Thus, it is unlikely that cAMP modifies uptake into the pool. If that had been the case, we would have expected that VIP would have enhanced the rate of JMV-180 stimulated ^{45}Ca efflux, since more ^{45}Ca would have been available in the cytosol to be lost from the cell. Instead, the observed effects of cAMP on calcium oscillations in acinar cells may be due to an action of cAMP on calcium-dependent release of calcium from the intracellular pool, or via an as yet undetermined mechanism.

In summary, we have shown that there is cross-talk between cAMP and calcium-dependent signaling pathways at the level of calcium mobilization in two distinct secretory cell types, and that this cross-talk appears to encode information that controls the extent of secretion. In the case of the intestinal epithelial cell, this cross-talk may act in addition to the previously described cooperative opening of potassium and chloride channels (2, 4), to further enhance the extent of secretory responses. The existence of a mechanism leading to synergistic chloride secretion in the intestine appears to have both physiological and pathophysiological significance. Under normal conditions, synergism provides for a greater response to a given level of a hormone or neurotransmitter. Synergism may also provide for a rapid and extensive coordinated secretory response at times of threat to the intestine, such as colonization by pathogenic microorganisms. The extensive secretion thereby produced might serve as a primitive, non-immune defense mechanism that "flushes" the intestine free of an insult. Conversely, the existence within the epithelium of intrinsic mechanisms that markedly limit the extent of calcium-dependent chloride secretion, unless coordinated with a cAMP-dependent response, may serve to prevent inappropriately excessive secretory responses to luminal, calcium-dependent secretagogues, such as bile acids.

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