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Cyclic AMP-dependent protein kinase and Epac mediate cyclic AMP responses in pancreatic acini

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Abstract

The pancreatic acinar cell has several phenotypic responses to cAMP agonists. At physiological concentrations of the muscarinic agonist carbachol (1 μ M) or the CCK analog caerulein (100 pM), ligands that increase cytosolic Ca^{2+} , cAMP acts synergistically to enhance secretion. Supraphysiological concentrations of carbachol (1 mM) or caerulein (100 nM) suppress secretion and cause intracellular zymogen activation; cAMP enhances both zymogen activation and reverses the suppression of secretion. In addition to stimulating cAMP-dependent protein kinase (PKA), recent studies using cAMP analogs that lack a PKA response have shown that cAMP can also act through the cAMP-binding protein, Epac (exchange protein directly activated by cyclic AMP). The roles of PKA and Epac in cAMP responses were examined in isolated pancreatic acini. The activation of both cAMP-dependent pathways or the selective activation of Epac was found to enhance amylase secretion induced by physiological and supraphysiological concentrations of the muscarinic agonist carbachol. Similarly, activation of both PKA or the specific activation of Epac enhanced carbachol-induced activation of trypsinogen and chymotrypsinogen. Disorganization of the apical actin cytoskeleton has been linked to the decreased secretion observed with supraphysiological concentrations of carbachol and caerulein. Although stimulation of PKA and Epac or Epac alone could largely overcome the decreased secretion observed with either supraphysiological carbachol or caerulein, stimulation of cAMP pathways did not reduce the disorganization of the apical cytoskeleton. These studies demonstrate that PKA and Epac pathways are coupled to both secretion and zymogen activation in the pancreatic acinar cell.

Keywords

actin; zymogen; secretion; trypsin; chymotrypsin

Like many other epithelial cells, the pancreatic acinar cell has two major categories of G protein coupled receptors that signal through different second messengers. In the first category, cellular cAMP is increased when ligands such as secretin, vasoactive intestinal polypeptide, and pituitary adenylate cyclase activating peptide bind to their receptors. In the second category, cytosolic Ca^{2+} elevations occur after stimulation of CCK and muscarinic receptors (24). The interplay between these two classes of receptors is complex and the type of downstream response varies.

Several key acinar cell responses are linked to pancreatic physiology and disease. Physiological stimulation of acinar cell enzyme secretion is requisite for the pancreatic secretory response

to a meal and mediated by both classes of G protein-coupled receptors. An increase in cytosolic Ca^{2+} is the principal stimulus for acinar cell secretion; cAMP is known to synergize with Ca^{2+} and potentiate enzyme secretion. Pathological acinar cell stimulation with supraphysiological concentrations of CCK or its analog caerulein or the muscarinic agonist carbachol leads to acinar cell responses that are central to the pathogenesis of acute pancreatitis. These include aberrant Ca^{2+} signaling, activation of zymogens, particularly proteases within the acinar cell, and suppressed secretion (18). The latter leads to retention of the activated zymogens within the acinar cell. Both the pathological activation of zymogens and reduced secretion appear to contribute to acute pancreatitis. Studies by our laboratory and others have shown that cAMP agonists can both enhance caerulein or carbachol stimulated zymogen activation and stimulate enzyme secretion. The net effect is the discharge of activated zymogens from the acinar cell and reduced cell injury. Although the cellular targets of the Ca^{2+} response remain unclear, past work has assigned cAMP-dependent protein kinase (PKA) a role as the mediator of most cAMP responses. Recent studies have identified another major mechanism for cAMP signaling, the Epac (exchange protein directly activated by cAMP) pathway (5).

The Epac signaling mechanism is comprised of cAMP-binding proteins that regulate a GTPase. Epac1 and Epac2 are cAMP-binding proteins with a guanine nucleotide exchange factor domain that regulates the activation of the small G protein RAP1 by promoting its exchange of bound GDP for GTP as well as other cellular targets (20). Epac1 is expressed ubiquitously and has one cAMP-binding domain, whereas Epac2 is found in the brain, liver, and adrenals and has two cAMP-binding domains (12). Proposed functions of Epac/RAP1 include the regulation of insulin secretion from the pancreatic β -cell, modulation of the ryanodine receptor, control of cell morphology and adhesion through interactions with integrins, and regulation of the cell cycle (1,2,10,25). The effects of the Epac pathway in the pancreatic acinar cell are not known.

In this study, we examined the mechanism by which cAMP sensitizes acinar cells to zymogen activation and enzyme secretion. To detect contributions by cAMP-dependent protein kinase (PKA), a cell-permeable form of cAMP (8-Br-cAMP) was used in combination with selective PKA inhibitors. Epac was specifically stimulated using 8-pCPT-2'-O-Me-cAMP and other cAMP analogs. We found that both PKA and Epac pathways mediate the effects of cAMP on carbachol-induced zymogen activation and enzyme secretion. Disruption of the apical actin cytoskeleton has previously been linked to the suppressed secretion observed with supraphysiological concentrations of carbachol and caerulein (7,14). Although cAMP agonists enhanced secretion induced by supraphysiological carbachol, we found that they did not prevent disruption of the actin cytoskeleton.

MATERIALS AND METHODS

Preparation of isolated pancreatic acini

Pancreatic acini were isolated as described (13). Briefly, fasted male Sprague-Dawley rats 50–100 g (Charles River Laboratories, Wilmington, MA) were killed by CO_2 via a protocol approved by the Veterans Affairs Connecticut Healthcare Systems Animal Care and Use Committee. The pancreas was minced in buffer containing 40 mM Tris (pH 7.4), 95 mM NaCl, 4.7 mM KCl, 0.6 mM MgCl_2 , 1.3 mM CaCl_2 , 1 mM NaH_2PO_4 , 10 mM glucose, 2 mM glutamine, plus 0.1% BSA, 1 $\times$ MEM-nones-essential amino acids (GIBCO-BRL, San Jose, CA), and 50 U/ml of type-4 collagenase (Worthington, Freehold, NJ) and then incubated for 1 h at 37°C. The digest was filtered through a 300–400 μm mesh (Sefar American, Depew, NY) and distributed in a 24-well Falcon tissue culture plate (Becton Dickinson, Franklin Lakes, NJ). All reagents were purchased from Sigma Biochemical, St. Louis, MO unless otherwise noted.

Acinar cell stimulation

Tissue culture plates containing acini were incubated for 1 h at 37°C under constant O₂ with shaking (80 rpm). After a media exchange and an additional 1 h incubation, acini were stimulated for varying time periods with combinations of carbachol (1–1,000 µM) or caerulein (100 pM), a cAMP analog 8-Br-cAMP (100 µM), the Epac agonists 8-pCPT-2'-O-Me-cAMP and 8-pHPT-2'-O-Me-cAMP (10–1,000 µM; Axxora, San Diego, CA), or PKA inhibitors KT-5720 (1 µM) and myristoylated PKI (1 µM; both from Calbiochem, San Diego, CA).

Enzymatic activity assays

After samples were frozen at –80°C overnight, thawed in ice, and homogenized, protease activity assays were performed using fluorogenic substrates as described (4). Briefly, enzyme substrate (40 µM) (chymotrypsin, Calbiochem, San Diego, CA; trypsin, Peptides International, Louisville, KY) was added to each sample in assay buffer [50 mM Tris (pH 8.1), 150 mM NaCl, 1 mM CaCl₂, 0.01% BSA] and read with a fluorometric plate reader (HTS 7000; Perkin-Elmer Analytical Instruments, Shelton, CT) at excitation wavelength 380 nm and emission 440 nm for 20 measurements over 10 min. The slope of the line, which represents enzyme activity of the homogenate, was normalized to total amylase activity. Amylase activity was determined by use of a commercial kit (Phaebedas kit, Pharmacia Diagnostic, Rochester, NY). Amylase secretion was calculated as percent release into media.

Detection of PKA-phosphorylated proteins by immunoblot

After acini were stimulated for 10 min, they were heated to 95°C for 5 min in Laemmli sample loading buffer and then frozen at –80°C. Immunoblot blot analysis was subsequently performed using a phospho-specific antibody that detects the PKA phosphorylation site on cAMP-responsive element binding protein (CREB; Chemicon International, Temecula, CA). Proteins were separated on 12% SDS-PAGE (Bio-Rad, Hercules, CA) and transferred to Immobilon-P membranes (Millipore, Billerica, MA), blocked for 1 h at room temperature with blocking buffer (TBS, 5% BSA, 0.05% Tween-20), washed in blocking buffer, and probed with the pCREB primary antibody (diluted 1:1,000 from a commercial stock in blocking buffer) overnight at 4°C. After washing with BLOTTO (TBS, 5% nonfat dry milk, 0.05% Tween-20), horseradish peroxidase-labeled goat anti-rabbit IgG secondary in BLOTTO was added for 1 h at room temperature. Signals were detected on membranes by autoradiography using a SuperSignal West Pico Chemiluminescence Kit (Pierce, Rockford, IL).

F-actin staining

Isolated pancreatic acinar cells were stimulated with either carbachol (1 µM or 1 mM) for 1.5 h or caerulein (100 pM or 100 nM) alone for 1 h or costimulated with 8-Br-cAMP (100 nM) or 8-pCPT-2'-O-Me-cAMP (100 µM). Cells were subsequently fixed with 3.7% formaldehyde (in PBS, pH 7.0) with 0.2 mM PMSF and 0.5 mM benzamidine for 15 min, washed with PBS, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 5% goat serum and 50 mM NH₄Cl for 1 h. Rhodamine-phalloidin (1:40 dilution of commercial stock; 0.17 µM final) was used to stain filamentous actin (F-actin) and TOPRO-3 (1:200 dilution of 1 mM stock in DMSO; 5 µM final) was used to stain nuclei (both from Molecular Probes, Eugene, OR). Images were obtained by use of a Zeiss LSM510 laser scanning confocal microscope. Basolateral-to-apical line scans were performed on at least seven cells from each experiment. Line orientation along a polarized axis was standardized by alignment with the middle of the nucleus. The ratio of basolateral to apical intensity was determined by the Profile tool in the LSM510 software and used to quantify the extent of F-actin redistribution in stimulated cells.

Statistical analysis

Data represent means $\pm$ SE of at least three individual experiments unless otherwise noted, with each performed in at least duplicate. Statistical significance was determined by Student's *t*-test analysis, where *P* values < 0.05 were assigned significance.

RESULTS

The pathways that mediate the effects of cAMP in pancreatic acinar cells were examined by using the muscarinic agonist carbachol. Stimulation with physiological concentrations (1 μ M) is associated with maximal enzyme secretion and minimal zymogen activation (4). By contrast, supraphysiological stimulation (1 mM) reduced secretion and induced zymogen activation (19). Most studies were performed with carbachol because, unlike caerulein, it has no effect on cAMP levels.

Effects of PKA on zymogen activation and enzyme secretion

We have previously shown that costimulating with 8-Br-cAMP enhances both carbachol-induced zymogen activation and secretion (4), but 8-Br-cAMP has minimal effects alone (13). To examine the role of PKA in cAMP-mediated zymogen activation and enzyme secretion, isolated pancreatic acini were pretreated for 15 min with two PKA inhibitors, PKI (1 μ M) or KT-5720 (1 μ M), before supraphysiological carbachol and 8-Br-cAMP-enhanced stimulation (Fig. 1). PKI functions as a PKA pseudosubstrate, blocking the interaction of substrates with its catalytic subunit, whereas KT-5720 is a competitive inhibitor of ATP binding to PKA. Neither inhibitor affected activation or secretion in cells stimulated with carbachol alone (not shown), but the inhibitors reduced activation of trypsinogen and chymotrypsinogen induced by carbachol plus 8-Br-cAMP (Fig. 1A). They also reduced the enhanced amylase secretion observed with cAMP addition (Fig. 1B). Notably, the reduction in zymogen activation and amylase secretion by PKI or KT-5720 was not complete; one possible explanation is that cAMP was stimulating PKA-independent pathways.

Effects of Epac on zymogen activation and enzyme secretion

Since there are no available Epac antagonists, the effects of this pathway were examined by using selective agonists and PKA inhibitors. The effects of Epac on zymogen activation and enzyme secretion were assayed using the Epac agonist 8-pCPT-2'-*O*-Me-cAMP. No effect was seen with the addition of the Epac agonist alone (not shown). However, costimulation with supraphysiological carbachol and Epac agonist caused a concentration-dependent increase in trypsinogen and chymotrypsinogen activation (Fig. 2A) and amylase secretion (Fig. 2B) above carbachol alone. There was a tendency for Epac-enhanced activation and secretion to be less than that observed with 8-Br-cAMP. To confirm that Epac mediated the effects of 8-pCPT-2'-*O*-Me-cAMP, two additional studies were performed. First, sensitization to carbachol-induced zymogen activation by 8-pCPT-2'-*O*-Me-cAMP was shown to be insensitive to the PKA inhibitor, PKI (Fig. 3A). Second, another Epac agonist, 8-pHPT-2'-Me-cAMP, was shown to also sensitize acinar cells to carbachol-induced zymogen activation in PKA-independent manner (Fig. 3A). Both putative Epac agonists tended to enhance carbachol-induced amylase secretion. Interestingly, these effects on secretion were not inhibited but might be slightly increased by PKI (Fig. 3B). To further confirm the selectivity of the Epac agonist, 8-pCPT-2'-*O*-Me-cAMP, its effects on the PKA-dependent phosphorylation of CREB were assayed. As shown in Fig. 4, when 8-Br-cAMP was combined with carbachol, there was a prominent increase in CREB phosphorylation. However, the addition of the EPAC agonist to carbachol had only a slight effect on CREB phosphorylation. Together with the PKA inhibitor studies, the findings suggest that the effects of the EPAC agonists are likely mediated by Epac and not PKA.

To determine whether Epac affects responses under physiological conditions, acini were costimulated with 1 μ M carbachol, a concentration that causes maximal enzyme secretion and little to no zymogen activation (Fig. 5, *A* and *B*). Costimulation with 8-pCPT-2'-*O*-Me-cAMP enhanced both zymogen activation and amylase secretion. To confirm the effects of Epac with another Ca^{2+} -mediated agonist, a physiological concentration (100 pM) of caerulein was combined with 8-pCPT-2'-Me-cAMP (Fig. 5, *C* and *D*). This costimulation enhanced both zymogen activation and amylase secretion.

Subapical F-actin disruption is not affected by cAMP

Inhibition of secretion by supraphysiological concentrations of carbachol and caerulein has been linked to disruption of the subapical actin cytoskeleton. Thus the effect of PKA and Epac activation on actin disassembly and redistribution was examined. Under unstimulated conditions (Fig. 6*A*) or after physiological carbachol or caerulein stimulation (not shown), F-actin structures appeared as uninterrupted linear densities that were distributed along the apical pole. However, after supraphysiological carbachol or caerulein stimulation, subapical F-actin decreased to 21 and 50% from maximum, respectively (Fig. 6, *B* and *E*). In addition, the subapical F-actin pattern became irregular and disrupted into short fragments. Furthermore, redistribution of actin to the basolateral pole was observed (Fig. 6, *A* and *D*). When quantified, the basolateral-to-apical ratio increased from 0.21 to 0.54 with carbachol (1 mM; Fig. 6*C*) and 0.13 to 0.45 with caerulein (100 nM; Fig. 6*F*). Coadministration of either 8-Br-cAMP (100 μ M) or the Epac agonist 8-pCPT-2'-*O*-Me-cAMP (100 μ M) did not affect the reduced F-actin staining nor the basolateral-to-apical actin ratio.

DISCUSSION

The present study examines the downstream effects of increasing cellular cAMP in the pancreatic acinar cell. It demonstrates that both PKA and Epac pathways can stimulate cAMP-mediated effects on enzyme secretion and pathological zymogen activation. Inhibition of PKA partially reduced the enhancement of zymogen activation and enzyme secretion observed by costimulation with supraphysiological carbachol and the cAMP analog 8-Br-cAMP. Furthermore, stimulation of the Epac pathway using an Epac-specific cAMP analog also sensitized the acinar cell to carbachol- and caerulein-induced activation and secretion. Taken together, the findings suggest that both the traditional PKA and the newer Epac pathway contribute to the sensitizing effects of cAMP.

Increases in cAMP are known to stimulate exocytic secretion in a number of tissues. PKA mediates the effects of cAMP by directly regulating exocytic machinery and indirectly by affecting factors such as ion channels and replenishment of the secretory granule pool (20). However, a role for Epac in regulating exocytosis has only recently been described. Studies demonstrating PKA-independent secretion of insulin were the first to suggest an alternate cAMP-regulated mechanism for secretion (16). Subsequent studies have also shown that PKA-independent exocytosis mediates a component of cAMP-dependent secretion from neurons (17). Similar to our findings in the exocrine pancreas, cAMP appears to mediate insulin secretion by both PKA- and Epac-dependent mechanisms. Although the small GTPase Rap1 is the best characterized target of Epac, it may not be involved in regulation of secretory function in most systems. Thus the interaction of Epac with the proteins Rim2 and Piccolo and not Rap1 appears to regulate insulin secretion (21). These proteins may form a complex with the small GTPase Rab3 to regulate exocytosis of insulin granules from the β -cell. Epac may also regulate secretion by affecting ion transporters. For example, β -cell insulin secretion is also dependent on Epac regulation of SUR, a protein that activates specific chloride channels on insulin-containing secretory granules (6). Epac also appears to modulate Ca^{2+} release from stores in the endoplasmic reticulum of the pancreatic β -cell through the ryanodine receptor

(11). In this context, our laboratory has reported that Ca^{2+} release through the ryanodine receptor is a major mediator of zymogen activation in the pancreatic acinar cell (8). Finally, we have also found that activation of a vacuolar ATPase mediates acinar cell zymogen activation (23). It is possible that either PKA or Epac might mediate an ion transporter such as the vacuolar ATPase that mediates zymogen activation. In this context, our unpublished studies suggest that cAMP agonist may enhance assembly of the vacuolar ATPase in the acinar cell, a condition required for the activation of this proton transporter. Together, these studies indicate that Epac has the potential to regulate exocytic secretion and zymogen activation by a number of distinct mechanisms.

Notably, both PKA and Epac pathways were able to increase the suppressed secretion observed with supraphysiological concentrations of carbachol or caerulein. Past studies have linked this suppression of secretion to actin cytoskeleton disruption, a phenomenon not observed with physiological stimulation (3,7,9,14). Furthermore, serine protease inhibitors cause F-actin redistribution and also block enzyme secretion (22). Thus there has been speculation that actin disruption caused the observed inhibition of enzyme secretion. In addition, a protective role was ascribed to cAMP in preventing actin disassembly (22). However, cAMP or Epac did not affect the cytoskeletal disruption associated with supraphysiological carbachol or caerulein and suppressed secretion. There are two potential explanations for this observation. First, cAMP may be causing secretion by a pathway that is calcium independent and that does not require an intact apical actin network. Second, as suggested by others, disruption of the actin network may not be causally related to inhibition of enzyme secretion (15).

In summary, these studies demonstrate that cAMP has two major intracellular effector mechanisms in the pancreatic acinar cell: the traditional PKA pathway and the recently described Epac mechanism. Both pathways appeared to mediate the two key acinar cell responses: enzyme secretion and pathological intracellular activation of zymogens. Although the molecular targets of the PKA and Epac pathways have been defined in a few other systems, the effector molecules in the pancreatic acinar cell remain unclear and require further study. On the basis of the present study and other recent publications, an effect on the acinar cell actin cytoskeleton does not appear to be a target of either pathway. One possible conclusion from our studies is that the acinar cell may have functional redundancy with respect to the actions of cAMP. Although the explanation for such an organization is unclear, it is possible that these represent backup mechanisms for critical cellular responses. Alternatively, the PKA and Epac pathways might mediate secretion or activation of different cellular pools that cannot be distinguished using our present assays. Such a function would be consistent with the proposed regulation by PKA and Epac of insulin secretion from distinct pools in the β -cell (20).

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Fig. 1.

PKA inhibitors reduce the maximal effects of the cAMP analog 8-Br-cAMP on carbachol-stimulated zymogen activation and amylase secretion. Pancreatic acini were stimulated with either supraphysiological carbachol (1 mM) alone or costimulated with 8-Br-cAMP (100 μ M). Samples were pretreated with KT-5720 (1 μ M) or PKI (1 μ M) to inhibit PKA activity. Trypsin and chymotrypsin activities (A), as well as amylase secretion (B) were assayed. Data are means $\pm$ SE from 3 independent experiments, performed in duplicate. *, #, ♦ $P < 0.05$ compared with carbachol + 8-Br-cAMP for trypsin and chymotrypsin activity and amylase secretion, respectively.

Fig. 2.

The Epac agonist 8-pCPT-2'-O-Me-cAMP enhances supraphysiological (1 mM) carbachol-stimulated zymogen activation and amylase secretion. Acini were either stimulated with supraphysiological carbachol (1 mM) or costimulated with 8-pCPT-2'-O-Me-cAMP (10–1,000 μ M). Trypsin and chymotrypsin activity (A) and amylase secretion (B) were assayed. Data are means $\pm$ SE from 3 independent experiments, performed in duplicate. *,# P < 0.05 compared with carbachol alone for trypsin and chymotrypsin activity and for amylase secretion, respectively. ■ in B, basal amylase secretion.

Fig. 3.

PKA inhibition does not affect Epac-dependent zymogen activation or amylase secretion. Pancreatic acini were stimulated with either supraphysiological carbachol (1 mM) alone or costimulated with 8-pCPT-2'-O-Me-cAMP (100 μM) or 8-pHPT-2'-O-Me-cAMP (100 μM) in the absence or presence of PKI (1 μM). Trypsin and chymotrypsin activities (A), as well as amylase secretion (B) were assayed. Although 8-pCPT-2'-O-Me-cAMP and 8-pHPT-2'-O-Me-cAMP both enhanced carbachol-induced zymogen activation and amylase secretion, PKI did not affect these responses. Data are means ± SE from 3 independent experiments, performed in duplicate. NS, not statistically significant; * $P < 0.05$ compared with carbachol + 8-pHPT-2'-O-Me-cAMP.

Fig. 4.

Carbachol with 8-Br-cAMP, but not an Epac agonist, causes a prominent PKA-dependent increase in cAMP-responsive element binding protein (CREB) phosphorylation. Pancreatic acini were stimulated with either or 8-pCPT-2'-O-Me-cAMP (100 μ M) or supraphysiological carbachol (1 mM) alone or costimulated with 8-Br-cAMP (100 μ M) or 8-pCPT-2'-O-Me-cAMP (100 μ M) in the absence or presence of PKI (1 μ M). Only the combination of carbachol and 8-bromo-cAMP caused a significant increase in CREB phosphorylation, and this increase in phosphorylation was blocked by preincubation with PKI. Data are means $\pm$ SE from 3 independent experiments. * P < 0.05 compared with control; # P < 0.05 compared with carbachol + 8-bromo-cAMP.

Fig. 5.

The Epac agonist 8-pCPT-2'-O-Me-cAMP enhances physiological secretagogue-stimulated zymogen activation and amylase secretion. Acini were stimulated with either physiological carbachol (1 μ M) or costimulated with 8-pCPT-2'-O-Me-cAMP (100 μ M). Trypsin and chymotrypsin activity (A) and amylase secretion (B) were assayed. Similar studies were performed using physiological concentrations of caerulein (100 pM; C and D). Data are means + SE from 3 independent experiments, performed in duplicate. Zymogen activity was normalized to a maximum for each secretagogue [carbachol (1 mM) or caerulein (100 nM)]. *P < 0.05 compared with carbachol (1 μ M) for trypsin and chymotrypsin activity and for amylase secretion.

Fig. 6.

Disruption of subapical F-actin is unaffected by 8-Br-cAMP or Epac. **A** and **D**: pancreatic acini were stimulated with either carbachol or caerulein with or without 8-Br-cAMP (100 μ M) or the Epac agonist 8-pCPT-2'-O-Me-cAMP (100 μ M). Cells were fixed and stained with rhodamine-phalloidin (red) for F-actin and TOPRO-3 (blue) for nuclei. Representative images from 3 independent experiments are shown. **B** and **E**: apical intensities were measured for each sample and expressed relative to unstimulated cells. **C** and **F**: ratio of fluorescence intensities of the apical to basolateral regions were determined for each condition. Unstim., unstimulated; Carb, carbachol. Seven cells were analyzed per condition and expressed as means $\pm$ SE.

*, #, $\blacklozenge$ $P < 0.05$ for either carbachol (1 mM) alone, with 8-Br-cAMP, or with 8-pCPT-2'-O-Me-cAMP compared with unstimulated, respectively.