Role of cyclic GMP in the control of capacitative Ca^{2+} entry in rat pancreatic acinar cells

Patrick GILON, Johnny F. OBIE, Xiaopeng BIAN, Gary St. J. BIRD and James W. PUTNEY, Jr.*

Calcium Regulation Section, Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, U.S.A.

We have investigated the possible roles of cyclic GMP (cGMP) in initiating or regulating capacitative Ca^{2+} entry in rat pancreatic acinar cells. In medium containing 1.8 mM external Ca^{2+}, thapsigargin activated Ca^{2+} entry and slightly but significantly increased intracellular cGMP concentration. This rise in cGMP levels was prevented by pretreating the cells with the guanylate cyclase inhibitor, LY-83583, or by omitting Ca^{2+} during stimulation by thapsigargin or methacholine. LY-83583 and N^G-nitro-L-arginine (L-NA, an inhibitor of NO synthase) both had a small inhibitory effect on Ca^{2+} entry when they were added after thapsigargin in Ca^{2+}-containing medium, and they reduced by 32 and 48% respectively the thapsigargin-induced capacitative Ca^{2+} entry when added to the cells during a 20 min preincubation period. However, neither dibutyryl cGMP (Bt_2cGMP) nor sodium nitroprusside, an NO mimic, affected either basal intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ or thapsigargin-induced capacitative Ca^{2+} entry. Further, the inhibitory effects observed after preincubation with LY-83583 or L-NA could not be prevented by preincubation with Bt_2cGMP, nor could they be reversed by adding Bt_2cGMP, 8-bromo-cGMP or sodium nitroprusside acutely after activation of capacitative Ca^{2+} entry by thapsigargin. Finally, pretreatment of cells with LY-83583 or L-NA did not affect Ca^{2+} signalling in response to 1 μM methacholine, including the pattern of $[Ca^{2+}]_i$ oscillations. In conclusion, in pancreatic acinar cells, the rise in cellular cGMP levels appears to depend on, rather than cause, the increase in $[Ca^{2+}]_i$ with agonist stimulation.

INTRODUCTION

Ca^{2+} plays a pivotal role in stimulation–response coupling of many cell types. In a wide variety of non-excitable cells, activation of receptors coupled to phosphoinositide hydrolysis induces a biphasic increase in the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). This is due to an initial inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from intracellular stores followed by Ca^{2+} entry across the plasma membrane [1]. A considerable body of evidence suggests that depletion of the intracellular pool is necessary and sufficient to trigger Ca^{2+} entry by a process termed capacitative Ca^{2+} entry [2,3]. The influx of Ca^{2+} presumably involves specific Ca^{2+} channels, and the current evoked by depletion of intracellular stores has been termed I_{CRAC} by Hoth and Penner [4], CRAC designating Ca^{2+}-release-activated Ca^{2+}. The molecular events triggered by pool depletion are unknown, but two types of mechanism have been considered. Influx of Ca^{2+} could result from direct interactions between intracellular stores and plasma membrane via either the cytoskeleton [5] or protein–protein interactions [6]. Alternatively, signalling between intracellular stores and the plasma membrane could occur through the action of a soluble messenger [7,8]. For a review of suggested modes of signalling between stores and plasma membrane, see ref. [3].

cGMP has been proposed as a candidate for either a signal or a modulator of capacitative Ca^{2+} entry [9–13]. One suggested mechanism for capacitative Ca^{2+} entry in pancreatic acinar cells involves activation by pool depletion of an NO synthase which would produce NO. This would in turn activate soluble guanylate cyclase to produce cGMP. Importantly, the production of cGMP should itself be Ca^{2+}-independent, as the data of Xu et al. [13] would indicate. Xu et al. [13] also reported that the effects of cGMP were biphasic because increasing its level initially activated Ca^{2+} entry whereas further increasing its level blocked Ca^{2+} entry.

Following on from the studies cited above, we have used rat pancreatic acinar cells to investigate further the role of cGMP in capacitative Ca^{2+} entry in this cell type. We measured changes in cGMP concentration in cells activated by either methacholine (MeCh) or thapsigargin, a potent inhibitor of the endoplasmic-reticulum Ca^{2+}-ATPase [14,15]. We also examined changes in $[Ca^{2+}]_i$ in fura 2-loaded pancreatic acinar cells activated by these same agonists, and when cGMP levels were altered by various pharmacological means. Our data suggest that cGMP formation in pancreatic acinar cells is a Ca^{2+}-dependent response, and that an increase in cGMP is not able to activate capacitative Ca^{2+} entry on its own and does not appear to be necessary for entry to be activated by agents that deplete intracellular Ca^{2+} pools.

MATERIALS AND METHODS

Preparation of pancreatic acinar cells

Pancreatic acinar cells were prepared by a method similar to that described previously [16]. Pancreata from 80–150 g Sprague–

Abbreviations used: cGMP, cyclic GMP; 8-Br-cGMP, 8-bromo cyclic GMP; Bt_2cGMP, $N^2,2'$-O-dibutyryl cyclic GMP; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; IBMX, isobutylmethylxanthine; IP_3, inositol 1,4,5-trisphosphate; L-NA, N^G-nitro-L-arginine; LY-83583, 6-anilino-5,8-quinolinedione; MeCh, methacholine.

* To whom correspondence should be addressed.

Dawley rats were removed immediately after decapitation. They were injected with a Hepes-buffered solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 10 mm glucose and 0.1% BSA, pH 7.4) supplemented with 10 mM pyruvate and 0.02% soya bean trypsin inhibitor. The tissue was then minced in the same solution and acinar cells were obtained after limited collagenase digestion (2.5 mg of collagenase in 15 ml of oxygenated solution/pancreas) for 10 min at 37 °C in a water bath shaking at 300 rev./min. Digestion was stopped by adding 35 ml of cold solution and the tissue was centrifuged for 3 min at 100 *g*. The supernatant was then discarded and the operation was repeated twice to wash the tissue. The pellet was then resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin and filtered through a nylon mesh to remove undigested tissue. After a last centrifugation, the acinar cells were allowed to attach to glass coverslips covered with Matrigel for $[Ca^{2+}]_i$ measurements or were plated immediately in 60 mm × 15 mm Petri dishes for cGMP experiments.

Measurement of $[Ca^{2+}]_i$

After isolation, the cells attached to the coverslips were maintained in the above culture medium for at least 2 h and were always used on the day of isolation. The coverslips were mounted in a Teflon microscope chamber (Bionique Laboratories, Lake Saranac, NY, U.S.A.) and incubated for 30–40 min at room temperature in culture medium supplemented with 1 μM fura 2 acetoxymethyl ester. In some experiments, 10 μM LY-83583 and 2 mM N^G-nitro-L-arginine (L-NA) were added for 20 min at the end of the loading period. Then, the coverslips were washed several times with Hepes-buffered solution, sometimes supplemented with test agents. Single-cell fluorescence was monitored with a Nikon Diaphot inverted microscope equipped with a 40 × Neofluor objective and a photomultiplier-based system (Photon Technologies International Ltd, Princeton, NJ, U.S.A.). $[Ca^{2+}]_i$ was calculated as described previously [17].

Measurement of cGMP levels

After isolation, the acinar cells were maintained for 4–6 h in the above culture medium. In some dishes, 10 μM LY-83583 was added to the culture medium 20 min before the beginning of the experiments. The dishes were then equilibrated for 8 min in the Hepes-buffered medium described above without added Ca^{2+} or in medium containing 1.8 mM Ca^{2+}, with or without 10 μM LY-83583. After 2 min of pretreatment with 1 mM isobutylmethylxanthine (IBMX) in the same medium, the acinar cells were incubated for different periods of time in the presence of 1 mM IBMX with or without agents. At the end of the incubation, the medium was discarded, the dishes were placed on boiling water and 2.5 ml of boiling water was added to each dish. After 5 min, the dishes were placed on ice. cGMP standards treated in the same manner as the pancreatic samples showed no loss of immunoreactivity after this procedure. After attached cells had been scraped off the dishes with a disposable cell scraper, the cellular suspension from each dish was transferred to Eppendorf tubes which were centrifuged at 16000 *g*. Protein content of the pellets was measured by a Bio-Rad assay. The supernatant was then dried in a SpeedVac Concentrator and resuspended in 300 μl of cGMP assay buffer (as specified in the RIA kit from Amersham). The samples were then acetylated and assayed for cGMP content with a ^{125}I-cGMP RIA kit (Amersham).

Materials

LY-83583 and L-NA were purchased from Research Biochemicals International (Natick, MA, U.S.A.). Dibutyryl cGMP (Bt_2cGMP) was obtained from Calbiochem-Novabiochem Co. (San Diego, CA, U.S.A.), and collagenase P was from Boehringer-Mannheim (Mannheim, Germany). Soyabean trypsin inhibitor, 8-bromo-cGMP, IBMX and methacholine (MeCh) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA, U.S.A.), fura 2 acetoxymethyl ester from Molecular Probes (Eugene, OR, U.S.A.), and thapsigargin from LC Laboratories (Woburn, MA, U.S.A.).

Presentation of results

Most of the $[Ca^{2+}]_i$ measurements are illustrated by traces that are representative of results obtained with the indicated number of pancreatic acinar cells. cGMP data were analysed by two-way analysis of variance. For multiple comparisons, the statistical significance between means was assessed by Newman–Keuls test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Levels of cGMP in pancreatic acinar cells

In the presence of the phosphodiesterase inhibitor, IBMX (1 mM), the levels of cGMP in rat pancreatic acinar cells increased with time (Table 1). However, in contrast with findings reported by Xu et al. [13], we observed only slight effects of the Ca^{2+}-store-depleting agent, thapsigargin, on cellular levels of cGMP (Table 2). After 3 and 8 min incubation in the presence of 2 μM thapsigargin plus 1 mM IBMX, the acinar cells contained slightly but significantly higher levels of cGMP than controls incubated for a similar period of time in the presence of 1 mM IBMX alone (Table 2). Experiments were also carried out with the Ca^{2+}-mobilizing agent, MeCh, as a stimulant. A significant increase in cGMP levels was observed for cells treated with 100 μM MeCh for 3 min in the presence of 1 mM IBMX. It should be noted that the phosphodiesterase inhibitor, IBMX, at 1 mM concentration caused greater effects on its own on cellular cGMP levels than did thapsigargin or MeCh; on average in a medium containing

Table 1 Time-dependent effect of IBMX on cGMP levels in pancreatic acinar cells

After 2 min of pretreatment with 1 mM IBMX, the acini were incubated for the indicated period of time in the presence of 1 mM IBMX in medium, without Ca^{2+} or in medium containing 1.8 mM Ca^{2+} and supplemented or not with the guanylate cyclase inhibitor, LY-83583. Levels of cGMP are expressed as fmol/Petri dish of cells (means ± S.E.M. for the number of determinations given in parentheses). Average protein content was 1.55 ± 0.02 mg of protein/Petri dish ($n = 913$). *$P < 0.05$, †$P < 0.01$ versus incubation during the same time without Ca^{2+}; ‡ $P < 0.01$ versus incubation during the same time with 1.8 mM Ca^{2+}.

Experimental conditions	cGMP concentration		
	0 min	3 min	8 min
0 mM Ca^{2+}	163 ± 7 (30)	313 ± 33 (22)	512 ± 36 (18)
1.8 mM Ca^{2+}	218 ± 6† (40)	406 ± 25* (32)	597 ± 67 (9)
1.8 mM Ca^{2+} + 10 μM LY-83583	157 ± 5‡ (24)	250 ± 17‡ (21)	338 ± 22†‡ (9)

Table 2 Effects of thapsigargin (2 μM) and methacholine (100 μM) on cGMP levels in pancreatic acinar cells

After 2 min of pretreatment with 1 mM IBMX, the acini were incubated for the indicated period of time with thapsigargin or MeCh in the presence of 1 mM IBMX. Levels of cGMP are expressed as fmol/Petri dish of cells (means ± S.E.M. for the number of determinations given in parentheses). *$P < 0.05$, †$P < 0.01$ versus control within the same experimental condition and for the same time.

	cGMP concentration					
	3 min incubation		8 min incubation		3 min incubation	
Experimental conditions	Control	Thapsigargin	Control	Thapsigargin	Control	MeCh
0 mM Ca^{2+}	346 ± 49 (13)	344 ± 35 (13)	573 ± 59 (9)	549 ± 68 (9)	266 ± 38 (9)	264 ± 21 (9)
1.8 mM Ca^{2+}	403 ± 38 (15)	473 ± 26* (15)	597 ± 67 (9)	728 ± 62* (8)	398 ± 28 (23)	541 ± 47† (23)
1.8 mM Ca^{2+} + 10 μM LY-83583	261 ± 25 (9)	247 ± 20 (9)	338 ± 22 (9)	314 ± 33 (9)	241 ± 24 (12)	235 ± 23 (12)

1.8 mM Ca^{2+}, IBMX increased the cGMP levels by 188 fmol/dish after 3 min of incubation whereas thapsigargin and MeCh induced a further increase of only 70 and 143 fmol/dish respectively (compare Tables 1 and 2). The difference in the magnitude of changes seen in our study and those published by Xu et al. [13] is probably not due to the cGMP assay, since, as expected, the NO-mimicking agent, sodium nitroprusside (1 μM; 8 min), caused substantial increases in cellular cGMP levels (1229 ± 91 fmol/dish; $n = 15$). Also we used the same cGMP extraction and assay procedures in another cellular system, the Jurkat T-cell line, and observed greater than 10-fold increases in cGMP levels after thapsigargin stimulation (P. Gilon, J. Obie, X. Bian, G. St. J. Bird and J. W. Putney, Jr., unpublished work). Finally, it appears that our values and the relative changes seen with IBMX and on cholinergic stimulation are similar to those originally published by Heisler and Lambert [18] using the same preparation.

The small increases in cGMP levels seen with thapsigargin and MeCh appeared to depend on Ca^{2+} entry rather than be the cause of it because, in the absence of external Ca^{2+}, no increase in cGMP was seen with either agonist (Table 2). The increase due to these agonists was also blocked by the guanylate cyclase inhibitor, LY-83583. The inhibitor also attenuated the rise in cGMP due to sodium nitroprusside [$t = 8$ min; 1 μM sodium nitroprusside alone, 1229 ± 91 fmol/dish ($n = 15$); 1 μM sodium nitroprusside plus 10 μM LY-83583, 521 ± 49 fmol/dish ($n = 14$)]. Note that cGMP levels in cells preincubated with 10 μM LY-83583 were significantly lower than those in control cells (Table 1).

$[Ca^{2+}]_i$ responses in pancreatic acinar cells

On addition of 1 μM MeCh to pancreatic acinar cells, $[Ca^{2+}]_i$ increased abruptly and in some cells began to oscillate (Figure 1). This effect was rapidly reversible, as $[Ca^{2+}]_i$ returned to basal levels within a few seconds when MeCh was removed from the medium. Changing from a medium containing Ca^{2+} to one without Ca^{2+} did not affect basal $[Ca^{2+}]_i$ in control cells. Addition of 2 μM thapsigargin to a Ca^{2+}-free medium induced intracellular Ca^{2+} release. Thereafter $[Ca^{2+}]_i$ returned to values similar to or lower than those observed in a Ca^{2+}-free medium (Figures 1, 2, 4). This slight fall in apparent $[Ca^{2+}]_i$ seen in some cells may be due to dye compartmentalization, resulting in a decrease in the fura 2 ratio coming from the intracellular Ca^{2+} stores after the addition of thapsigargin [19]. This mobilization of Ca^{2+} was followed by a sustained elevation of $[Ca^{2+}]_i$ on restoring extracellular Ca^{2+} (Figure 1a). If this capacitative Ca^{2+} entry were mediated by cGMP, it should be mimicked by cGMP analogues or compounds that increase its intracellular level. However, the cell-permeant cGMP analogue, Bt_2cGMP, did not affect basal $[Ca^{2+}]_i$ at concentrations of 5, 50, 500 μM ($n = 3$; results not shown) or 2 mM (Figure 1b). Increasing intracellular cGMP levels with 1 μM (Figure 1c) or 30 μM ($n = 3$ each; not shown) sodium nitroprusside was also without effect on basal $[Ca^{2+}]_i$. We also investigated the effect of decreasing cGMP levels. LY-83583 (10 μM) lowered the basal concentration of cGMP (Table 1) and blocked the effect of agonists on cGMP (Table 2). Note that in the presence of LY-83583 and an agonist, the cellular level of cGMP was actually lower than in control cells treated with neither drug. Addition of 10 μM LY-83583 to medium containing Ca^{2+} did not affect basal $[Ca^{2+}]_i$ (Figure 1d). Likewise, the inhibitor of NO synthase, L-NA (2 mM), was without effect on basal $[Ca^{2+}]_i$ (Figure 1e).

As increasing cGMP levels did not induce capacitative Ca^{2+} entry, we next tested the possibility that it could modulate Ca^{2+} entry when activated by depletion of intracellular Ca^{2+} stores. Thapsigargin was added to the cells, and reagents expected to modify cGMP levels were added after Ca^{2+} entry was fully activated. The addition of 5 μM–2 mM Bt_2cGMP or 0.5 or 30 μM sodium nitroprusside were without effect on steady-state $[Ca^{2+}]_i$ due to Ca^{2+} entry (Figures 2a–2c). However, the addition of 10 μM LY-83583 produced a small slow decrease in $[Ca^{2+}]_i$ in three of eight cells (Figure 2d). In the five other cells, the inhibitor was without any effect. Similarly, 2 mM L-NA had a small inhibitory effect in two of four cells (Figure 2e), being ineffective in the two other cells.

Because the effects of LY-83583 and L-NA were very slow and inconsistent when they were added after capacitative Ca^{2+} entry had been activated by thapsigargin, we examined the effect of preincubating the cells for 20 min in the presence of the inhibitors. The inhibitors were also present throughout the whole experiment. Preincubation with 10 μM LY-83583 or 2 mM L-NA attenuated capacitative Ca^{2+} entry induced by 2 μM thapsigargin by 32 and 48% respectively (Figure 3). The extent of this inhibition was again variable from one cell to another (Figure 4). However, the simultaneous addition of 0.5 mM Bt_2cGMP did not prevent the inhibitory effects of either agent (Figure 3). As only a very narrow range of cGMP concentrations has been reported to have a stimulatory effect on capacitative Ca^{2+} entry [13], we could have missed the reversibility of the inhibitory

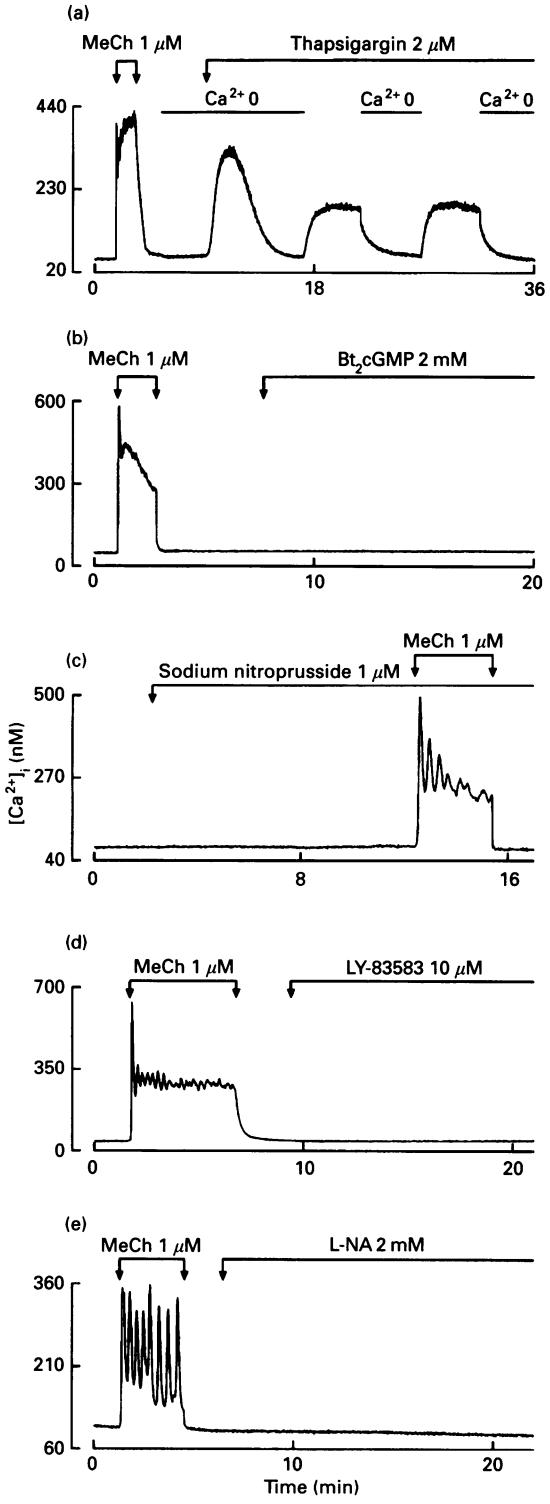

Figure 1 Effects of MeCh, thapsigargin and agents that modify cGMP levels on basal $[Ca^{2+}]_i$ in single rat pancreatic acinar cells

In most experiments, cells were first tested for their responsiveness to 1 μM MeCh. The cells were incubated in medium containing 1.8 mM Ca^{2+} (control condition) or in Ca^{2+}-free solution (Ca^{2+} 0) where indicated. The agents were added during the intervals shown at the top of each panel. (**a**) Thapsigargin was added in the absence of external Ca^{2+}. Thereafter, Ca^{2+} was increased to 1.8 mM, removed from the medium, increased to 1.8 mM and removed again. (**b**)–(**e**) Bt_2cGMP (2 mM), sodium nitroprusside (1 μM), LY-83583 (10 μM) and L-NA (2 mM) were added respectively to medium containing 1.8 mM Ca^{2+}; no effects on basal $[Ca^{2+}]_i$ were observed. These experiments are representative traces obtained with 21 (**a**), three (**b**), four (**c**), eight (**d**) and seven (**e**) cells.

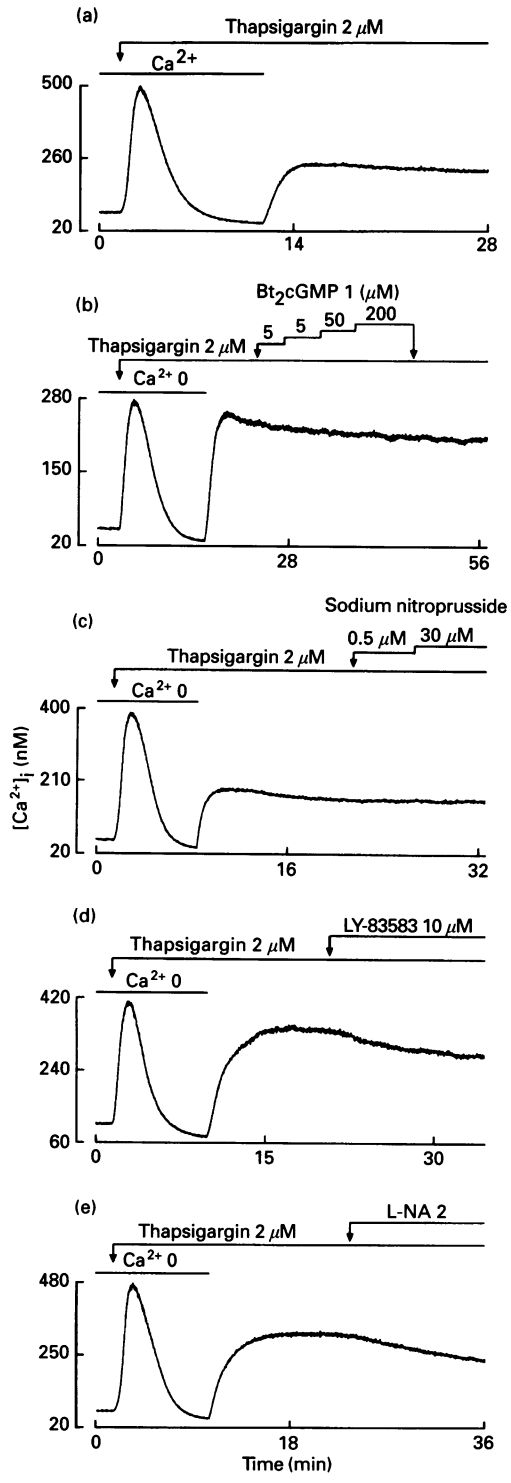

Figure 2 Effects of a cGMP analogue and agents that alter cGMP levels on thapsigargin-induced Ca^{2+} entry in single rat pancreatic acinar cells

The cells were incubated in medium containing 1.8 mM Ca^{2+} or in Ca^{2+}-free solution (Ca^{2+} 0) as indicated. The agents were added during the periods indicated at the top of the panels. Thapsigargin was always added to a Ca^{2+}-free medium, and the Ca^{2+} concentration of the medium was increased to 1.8 mM thereafter. (**a**) Control cells showed a sustained increase in $[Ca^{2+}]_i$. (**b**)–(**e**) Bt_2cGMP (5, 50, 500, 2000 μM), sodium nitroprusside (0.5, 30 μM), LY-83583 (10 μM) and L-NA (2 mM) were added respectively to medium containing 1.8 mM Ca^{2+} after thapsigargin addition. Bt_2cGMP and sodium nitroprusside did not affect $[Ca^{2+}]_i$, but LY-83583 and L-NA caused a slow decrease in three of eight and two of four cells respectively. These experiments are representative traces obtained with 21 (**a**), three (**b**), six (**c**), three (**d**) and two (**e**) cells.

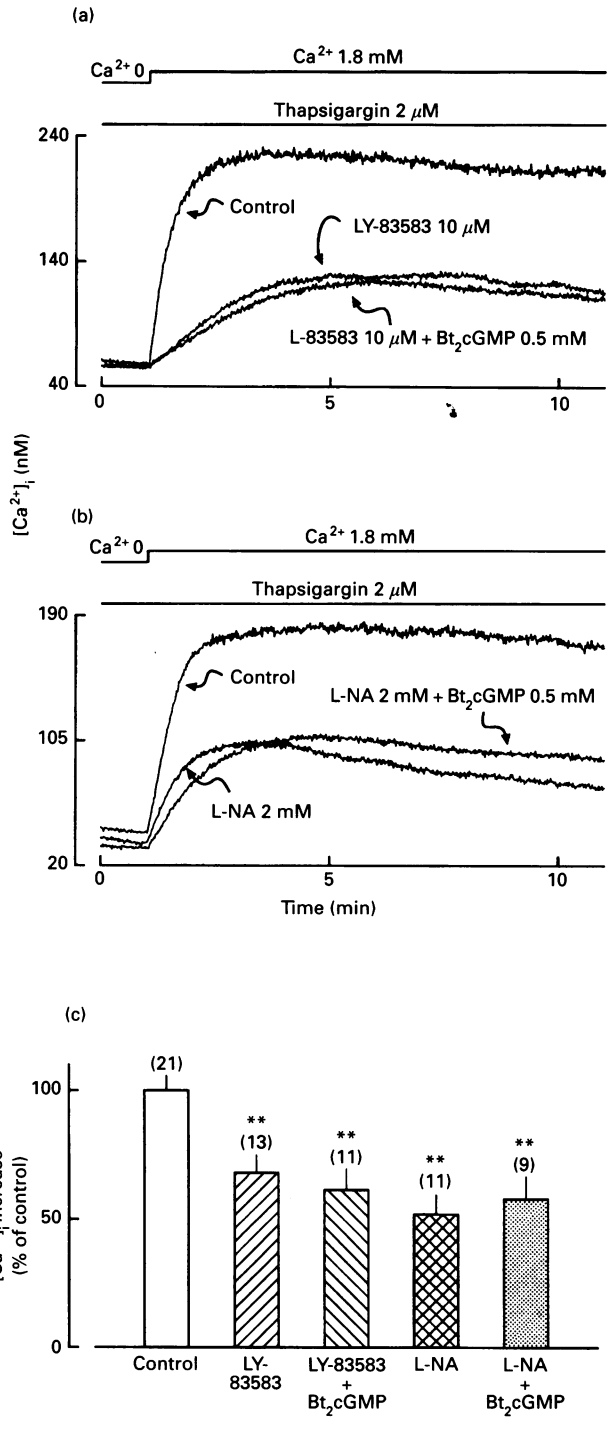

Figure 3 Inhibitory effects of preincubation with LY-83583 and L-NA on thapsigargin-induced $[Ca^{2+}]_i$ increase in single rat pancreatic acinar cells and lack of reversal of this effect by preincubation with Bt_2cGMP

The cells were preincubated for 20 min in control medium or medium containing 10 μM LY-83583 or 2 mM L-NA (supplemented or not with 0.5 mM Bt_2cGMP). All agents were present throughout the period shown as well as during the preincubation periods. For all cells, thapsigargin was added to a Ca^{2+}-free medium (before $t = 0$) (Ca^{2+} 0), and the Ca^{2+} concentration in the medium increased to 1.8 mM thereafter as indicated at the top of the panels. (**a**), (**b**) $[Ca^{2+}]_i$ changes just after mobilization of Ca^{2+} induced by thapsigargin and 1 min before Ca^{2+} readmission. (**c**) Summary of results from several such experiments, presented as percentage of control $[Ca^{2+}]_i$. The percentages were calculated by measuring the maximum $[Ca^{2+}]_i$ reached after Ca^{2+} readmission to thapsigargin-treated cells, which, in control cells, averaged 205 ± 18 ($n = 21$). The number of experiments in each group is indicated in parentheses above the bars in (**c**). $^{**}P < 0.01$ compared with the control.

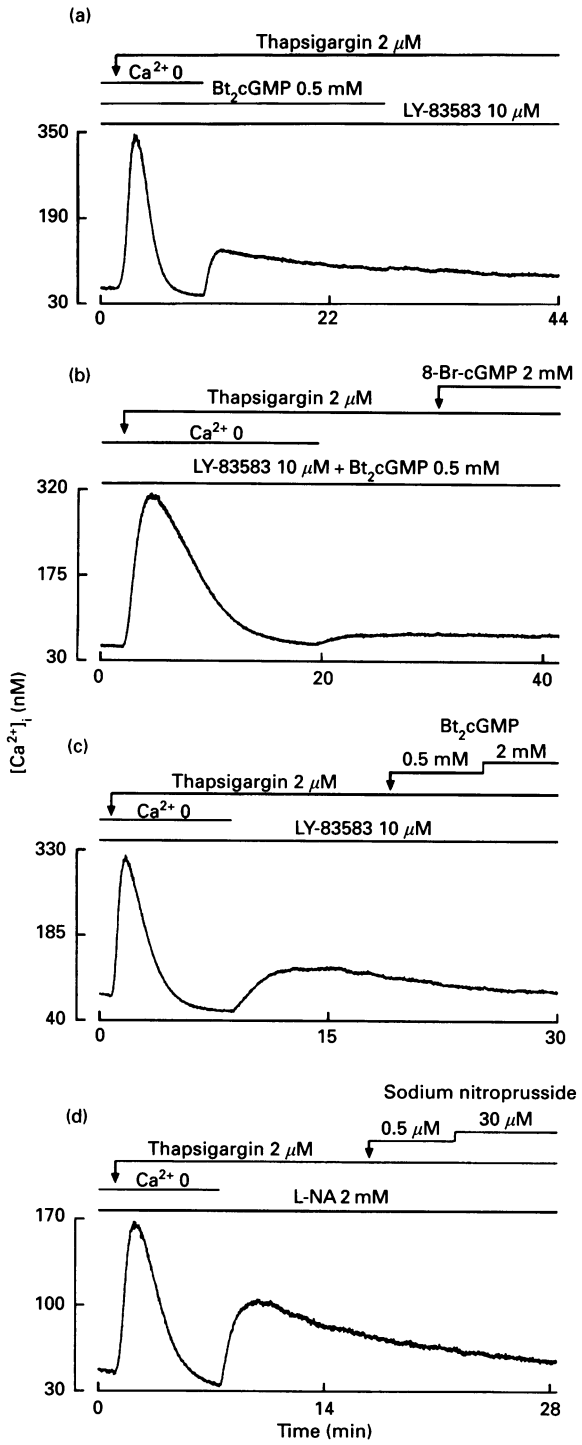

Figure 4 Failure of cGMP analogues or sodium nitroprusside to reverse LY-83583- or L-NA-induced inhibition of Ca^{2+} entry in single rat pancreatic acinar cells

The cells were incubated in medium containing 1.8 mM Ca^{2+} or in Ca^{2+}-free solution (Ca^{2+} 0) as indicated. The agents were added during the intervals shown at the top of the panels. In each case, thapsigargin was added to Ca^{2+}-free medium, and the Ca^{2+} concentration in the medium was then increased to 1.8 mM. (**a**) The cell was preincubated for 20 min with 0.5 mM Bt_2cGMP and 10 μM LY-83583. Both agents were present when thapsigargin was added, but Bt_2cGMP was washed out after Ca^{2+} addition. (**b**) The cell was pretreated as in (**a**), and 8-Br-cGMP was added after Ca^{2+} was readmitted to the medium. (**c**), (**d**) After the loading period, the cells were preincubated for 20 min with 10 μM LY-83583 (**c**) or 2 mM L-NA (**d**). Bt_2cGMP (**c**) or sodium nitroprusside (**d**) was added where indicated. These experiments are representative traces obtained with three (**a**), three (**b**), six (**c**) and four (**d**) cells.

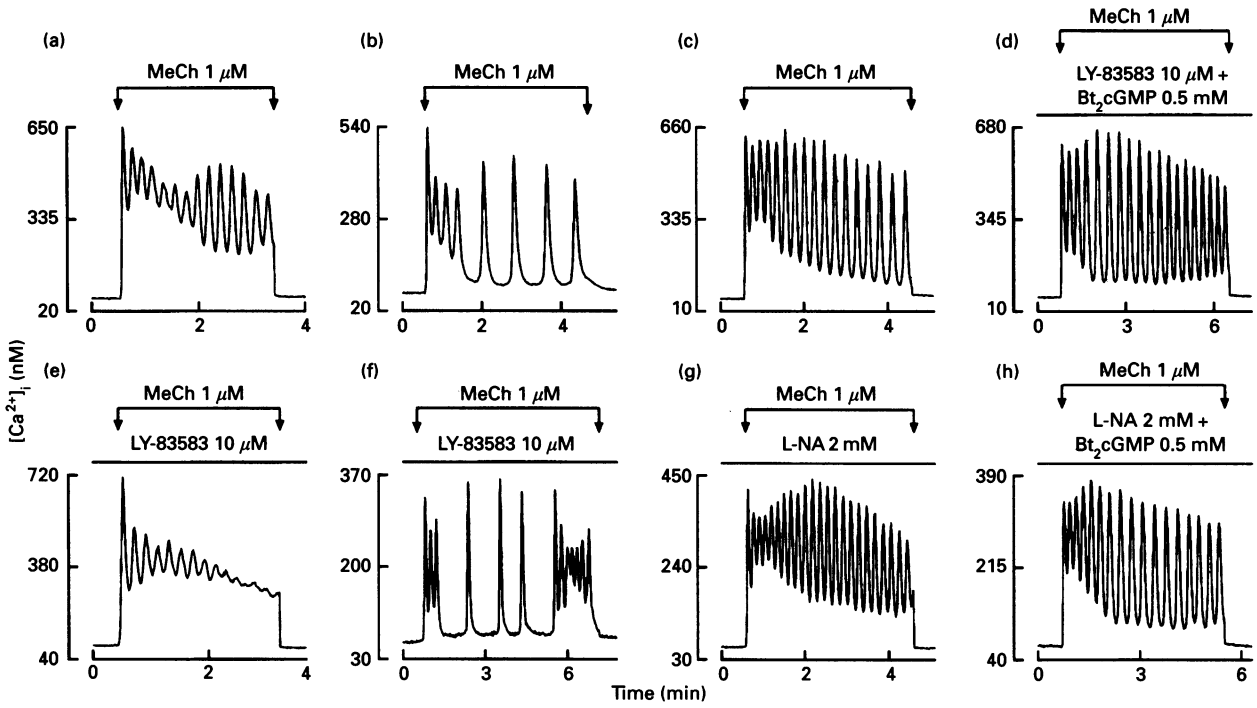

Figure 5. Lack of effect of LY-83583 or L-NA on $[Ca^{2+}]_i$ oscillations induced by 1 μM MeCh in single rat pancreatic acinar cells

The Ca^{2+} concentration of the medium was 1.8 mM throughout all experiments. The cells were preincubated for 20 min in control medium (**a–c**), or with a medium containing 10 μM LY-83583 or 2 mM L-NA supplemented or not with 0.5 mM Bt_2cGMP, as indicated (**d–h**). These experiments are representative traces obtained with ten (**a–c**), seven (**d**), seven (**e, f**), six (**g**) and six (**h**) cells.

effects of LY-83583 and L-NA by using a single concentration of Bt_2cGMP which could have been either too high or too low. We therefore removed Bt_2cGMP during Ca^{2+} entry to determine if this might transiently re-activate Ca^{2+} entry; however, this was without effect (Figure 4a). Various other manipulations designed to increase cellular cGMP failed to augment Ca^{2+} entry in cells previously treated with inhibitors of cGMP formation. These included further increasing the concentration of Bt_2cGMP to 2 or 5 mM ($n = 2$; not shown), addition of another cell-permeant cGMP analogue, 8-Br-cGMP (2 mM), to cells pretreated with LY-85383 and 0.5 mM Bt_2cGMP (Figure 4b), or addition of either 0.5 or 30 μM sodium nitroprusside to cells pretreated with L-NA and 0.5 mM Bt_2cGMP ($n = 5$; not shown). We also tested the effect of increasing cGMP levels while capacitative Ca^{2+} entry was underway in cells preincubated with LY-83583 or L-NA. Again the addition of 0.5–2 mM Bt_2cGMP did not reverse the inhibition of capacitative Ca^{2+} entry produced by LY-83583 (Figure 4c). Similar results were obtained with 0.5 and 2 mM 8-Br-cGMP ($n = 2$; not shown). Sodium nitroprusside, at 0.5 and 30 μM, was also unable to overcome the inhibition produced by L-NA (Figure 4d).

We also tested the effects of manipulations of cellular cGMP concentration on $[Ca^{2+}]_i$ responses to MeCh, an agonist acting on the surface-membrane phospholipase C-coupled muscarinic receptor. Xu et al. [13] suggested that, because of the biphasic effect of cGMP on capacitative Ca^{2+} entry, cGMP could provide cells with a negative feedback on Ca^{2+} influx which could contribute to the frequently observed $[Ca^{2+}]_i$ oscillations. We found that 1 μM MeCh induced a sustained increase in $[Ca^{2+}]_i$ in some cells (four of 14 cells; Figure 1b), or, as previously described [20,21], initiated oscillations of $[Ca^{2+}]_i$ (10 of 14 cells; Figure 5). The pattern of $[Ca^{2+}]_i$ oscillations was variable from cell to cell (Figures 5a–5c). Pretreatment of cells with either 10 μM LY-83583 or 2 mM L-NA did not appreciably alter the magnitude of MeCh-induced $[Ca^{2+}]_i$ signals. Further, a similar percentage of cells oscillated when they were pretreated with LY-83583 (seven of 11 with oscillations; Figures 5e–5f), L-NA (six of nine with oscillations; Figure 5g), LY-83583 plus 0.5 mM Bt_2cGMP (seven of ten with oscillations; Figure 5d) or L-NA plus 0.5 mM Bt_2cGMP (six of eight with oscillations; Figure 5h). The frequency and oscillatory patterns of these cells, although variable, were comparable with those of control cells. Finally, sodium nitroprusside did not noticeably affect the $[Ca^{2+}]_i$ signals, nor did it prevent $[Ca^{2+}]_i$ oscillations (Figure 1c).

DISCUSSION

The present study was prompted by recent reports suggesting that capacitative Ca^{2+} entry is initiated by activation of NO synthase which would sense luminal Ca^{2+} in intracellular stores and would thus be stimulated by intracellular pool depletion [12,13]. The resulting production of NO would activate soluble guanylate cyclase which in turn would increase cellular levels of cGMP. Pandol and co-workers [9,11,12] suggested that cGMP could then act as a signal to open plasma-membrane Ca^{2+} channels. On the other hand, Xu et al. [13] proposed that cGMP may act as a modulator of capacitative Ca^{2+} entry, perhaps by activation of cGMP-dependent protein kinase.

NO is synthesized from L-arginine by NO synthase [22]. Three isoforms of this enzyme have been described so far [23]. The brain-type (NOS-I) and the endothelium-type (NOS-III) are

constitutively expressed and are stimulated after interaction with Ca^{2+} and calmodulin. The macrophage-type (NOS-II) is inducible after immunological activation and does not seem to be sensitive to Ca^{2+}. The constitutive forms are present in most tissues. Their Ca^{2+}-dependence is consistent with myriad observations from the early literature that suggested that the production of cGMP occurs as a consequence of Ca^{2+} mobilization [18,24,25]. Our observation that MeCh and thapsigargin increased cGMP levels only in the presence of external Ca^{2+} are consistent with this idea, but the data of Xu et al. [13] contradict this rather general finding for reasons that are not clear. This is an important issue, because the Ca^{2+}-dependence that we and the vast majority of other investigators have observed argues against a role for cGMP as a primary signal for the activation of Ca^{2+} entry.

This conclusion is further supported by our $[Ca^{2+}]_i$ measurements in pancreatic acinar cells. The cell-permeant analogue of cGMP, Bt_2cGMP, and the NO donor agent, sodium nitroprusside, tested at a variety of concentrations, were unable to activate capacitative Ca^{2+} entry. Finally, the abilities of MeCh and thapsigargin to induce Ca^{2+} entry were not abolished by pretreating the cells with the NO synthase inhibitor, L-NA [26], nor were they abolished by the guanylate cyclase inhibitor, LY-83583 [27], which prevented the rise in cGMP levels induced by MeCh and thapsigargin.

We also considered the possibility that cGMP might exert a modulatory effect on Ca^{2+} entry. We used thapsigargin which maximally depletes intracellular Ca^{2+} stores and which avoids complications of interpretation that might arise with phosphoinositide hydrolysis-linked agonists if changes in cGMP levels affect transduction mechanisms brought about after receptor activation. Unlike Xu et al. [13], we did not use Mn^{2+} quench to assess Ca^{2+} entry. In our hands, Mn^{2+}-quench experiments were difficult to interpret because there was considerable variability in the rate of quench from cell to cell, and the ability of thapsigargin and agonists to increase the rate of quench was slight and not statistically reliable. We thus used the increase in steady-state $[Ca^{2+}]_i$ as a measure of Ca^{2+} entry, and found that application of different concentrations of Bt_2cGMP or induction of an increase in cGMP levels by sodium nitroprusside did not affect thapsigargin-induced Ca^{2+} entry. These results are inconsistent with the suggestion of Xu et al. [13] that a high concentration of cGMP inhibits Ca^{2+} influx.

When we tested the effects of a decrease in cGMP levels on capacitative Ca^{2+} entry, we observed that L-NA and LY-83583 induced a small inhibitory effect on $[Ca^{2+}]_i$ when they were added after thapsigargin. The very small effect observed when L-NA and LY-83583 were added acutely after thapsigargin could be due to slow penetration of the inhibitors into the cells and/or to the slow inhibition of NO synthase and guanylate cyclase. More substantial inhibition was seen after pretreatment with these inhibitors. However, in contrast with Xu et al. [13], we still did not observe complete inhibition of Ca^2 entry with L-NA and LY-83583 despite the fact that the inhibitors would have lowered cGMP levels in this experiment to an even lower value than that in resting unstimulated cells. More importantly, we could not reverse this inhibition by pretreating or acutely treating the cells with any of a variety of concentrations of Bt_2cGMP, 8-Br-cGMP or sodium nitroprusside. This lack of reversibility strongly suggests that the effects of L-NA and LY-83583 are not related to their effects on cGMP levels. We note that LY-83583 was first isolated as an inhibitor of leukotriene release [28], and this might underlie its inhibitory actions in the pancreas. It is not clear why other studies have shown that a component of the Ca^{2+} entry inhibited by LY-83583 could be reversed by Bt_2cGMP [9], whereas we saw no such effect. However, we would point out that equally contradictory results for the action of LY-83583 have been reported from within a single laboratory [9,10]; this agent was found to augment the increase in $[Ca^{2+}]_i$ in pancreatic acinar cells induced by the Ca^{2+} ionophore, Br-A23187, whereas it inhibited the $[Ca^{2+}]_i$ signal due to agonists, while in both cases it inhibited the rise in cGMP levels. As the $[Ca^{2+}]_i$ signal induced by Ca^{2+} ionophores is believed to be due at least in part to activation of capacitative Ca^{2+} entry [29], it is not clear how one can arrive at an internally consistent interpretation of these observations.

Another suggestion of Xu et al. [13] was that the high concentrations of cGMP reached during high $[Ca^{2+}]_i$ may provide the cell with a negative feedback mechanism for Ca^{2+} entry and this could contribute to the appearance of $[Ca^{2+}]_i$ oscillations. Again, our results do not support this hypothesis. We tested the effect of pretreatment with L-NA or LY-83583, alone or together with Bt_2cGMP, or of sodium nitroprusside on the oscillations of $[Ca^{2+}]_i$ induced by 1 μM MeCh. If fluctuations in cGMP levels were required to induce oscillations, we should have blocked them by clamping intracellular cGMP concentrations at low levels with the inhibitors or at high levels with Bt_2cGMP or sodium nitroprusside. However, we observed no effects of these treatments on the oscillatory behaviour of the cells.

The conclusion that cGMP does not play a major role in signalling in the exocrine pancreas is not without precedent. In previous studies on exocrine pancreas [18,24,25,30], augmentation of cellular cGMP levels by various agents was found to neither potentiate nor inhibit the Ca^{2+}-mediated secretory responses to cholecystokinin or cholinergic agonists. In pancreatic acinar cells, 10 μM sodium nitroprusside did not alter the increase in $^{45}Ca^{2+}$ uptake induced by carbachol [10]. In *Xenopus* oocytes, 8-Br-cGMP did not activate Ca^{2+} current [7], and in a mast cell line, inclusion of cGMP in the patch pipette did not modify I_{CRAC} induced by ionomycin [31]. Finally, in platelets, increases in GMP caused by sodium nitroprusside did not augment, but rather inhibited thapsigargin-activated Ca^{2+} entry [32].

In conclusion, the present study argues against a role for cGMP as a signal for or a modulator of capacitative Ca^{2+} entry in pancreatic acinar cells. The increase in cGMP levels induced by intracellular-pool-depleting agents appears to be a consequence rather than a cause of the influx of Ca^{2+}. Many effects of cGMP have now been identified in a wide variety of tissues (for reviews, see refs. [23,33]). They not only include modulation of ion channels, but also indirect effects through regulation of cAMP phosphodiesterase activity or protein kinase G. As stated by Williams [34], it appears that in the exocrine pancreas 'cyclic GMP is still a messenger in search of a function.'

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