Cyclic GMP potentiates phenylephrine but not cyclic ADP-ribose-evoked calcium release from rat lacrimal acinar cells

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Abstract In the present study, we describe a role for cyclic GMP (cGMP) in the signalling pathway that leads from α-adrenergic receptor activation to intracellular Ca^{2+} mobilization in rat lacrimal acinar cells. The α-adrenergic agonist, phenylephrine, stimulates intracellular Ca^{2+} release which is blocked by inhibitors of guanylate cyclase and cGMP-dependent protein kinase Ia. The membrane-permeable cGMP analogues, dibutyryl-cGMP and 8-bromo-cGMP, potentiate (≈5-fold) the Ca^{2+} response to submaximal phenylephrine stimulation. In contrast, the same cGMP analogues have no effect on cyclic ADP-ribose-evoked Ca^{2+} release from permeabilized lacrimal acinar cells. Collectively, these findings suggest that cGMP, via cGMP-dependent protein kinase Iα, is required for intracellular Ca^{2+} release following α-adrenergic receptor activation in lacrimal acinar cells.

Key words: Cyclic GMP; Lacrimal; Phenylephrine; Cyclic ADP-ribose; Inositol trisphosphate

1. Introduction

Cyclic ADP-ribose (cADPR) is a naturally occurring metabolite of the pyridine nucleotide β-NAD^+ that mobilizes Ca^{2+} via ryanodine-sensitive intracellular Ca^{2+}-induced Ca^{2+} release channels in a number of cell types [1,2], including exocrine acinar cells [3,5]. Although cADPR has Ca^{2+} mobilizing activity, it still remains largely unknown whether it functions as a second messenger for extracellular stimuli. There are indications that cADPR may function as a second messenger in lacrimal acinar cells in response to α-adrenergic receptor activation, since phenylephrine stimulates Ca^{2+} release from intracellular stores via a ryanodine-sensitive mechanism, which is not associated with increased production of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) [6]. However, no unequivocal information about the cellular components constituting this new signalling pathway is yet available.

One clue as to how cADPR synthesis may be regulated has come from a study demonstrating that cGMP mobilizes Ca^{2+} in sea urchin eggs by stimulating cADPR synthesis and it was suggested that cGMP activates, via a cGMP-dependent protein kinase, the ADP-ribosylcyclase that converts β-NAD^+ to cADPR [7]. These observations imply that agonists interacting with receptors which are themselves intrinsic guanylate cyclases, or nitric oxide (NO) which activates soluble guanylate cyclases, may stimulate cADPR production. In the present study we therefore investigated the possibility that cGMP is involved in Ca^{2+} mobilization from intracellular stores in lacrimal acinar cells following α-adrenergic receptor activation.

2. Materials and methods

2.1. Chemicals

Fura-2 acetoxymethyl ester (fura-2/AM) and fura-2 pentapotassium salt were supplied from Molecular Probes (Eugene, OR). cADP-ribose was from Amersham (Buckinghamshire, UK). $Ins(1,4,5)P_3$ was from Alomone Labs (Jerusalem, Israel). 8-(4-Chlorophenylthio)-guanosine-3′,5′-cyclic monophosphorothioate (Rp-8-pCPT-cGMPS) was obtained from Biolog Life Science Institute (Bremen, Germany). LY83583 and N^G-nitro-L-arginine were from Research Biochemicals Inc. (Natick, MA). All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

2.2. Cell preparation and Ca^{2+} measurements

Rat lacrimal glands were extirpated from male Wistar rats (130 g) and dispersed into acini by collagenase and hyaluronidase digestion, as described previously [6]. After cell preparation, the acini were ready for use and all experiments in the present study were performed within 1 h.

For measurements of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in intact cells in suspension, the acini were incubated with 2 μM fura-2/AM (cytocrit ≈ 1%) for 30 min in a shaking water bath at 37°C. The fura-2 loaded cells were washed and resuspended in a HEPES-buffered Krebs-Ringer medium containing (in mM): 142 Na^+, 5 K^+, 1 Ca^{2+}, 1 Mg^{2+}, 125 Cl^-, 20 HCO_3^-, 4 $HPO_4^{2-}/H_2PO_4^-$, 5 HEPES (pH 7.3), before being transferred to a thermostatically controlled cuvette (37°C) with a stirring device mounted on a Perkin-Elmer LS5B fluorescence spectrophotometer. Changes in $[Ca^{2+}]_i$ were measured as described previously [6].

For measurements of Ca^{2+} release from permeabilized lacrimal acinar cells, aliquots of cells were permeabilized (3 min at 37°C) in 2.5 ml of intracellular-like (IC) solution containing (in mM): 20 Na^+, 100 K^+, 1 Mg^{2+}, 120 Cl^-, 1 SO_4^{2-}, 20 HEPES (pH 7.20) and 22.5 μg/ml saponin. Following permeabilization, the cells were washed and resuspended in 100 μl of IC solution before being transferred to a cuvette containing 2.4 ml of IC solution with 1 mM ATP, 10 U/ml creatine phosphokinase, 10 mM phosphocreatine and 1 mM fura-2 pentapotassium salt added and Ca^{2+} release was measured as described previously [4].

2.3. Data analysis

Results are presented as mean values ± S.D. for the indicated number of experiments. Statistical significance's were evaluated using Student's *t*-test for paired observations.

3. Results and discussion

Fig. 1A shows the effect of sequential stimulation with maximal concentrations of phenylephrine (20 μM) and acetylcholine (10 μM) on $[Ca^{2+}]_i$ in a suspension of rat lacrimal acinar cells. Phenylephrine stimulated a rapid transient rise in $[Ca^{2+}]_i$ from a resting level of 133–352 nM, which declined towards a plateau level slightly higher than the pre-stimulatory $[Ca^{2+}]_i$. Subsequent stimulation with acetylcholine induced a new transient rise in $[Ca^{2+}]_i$, which reached a peak of 756 nM

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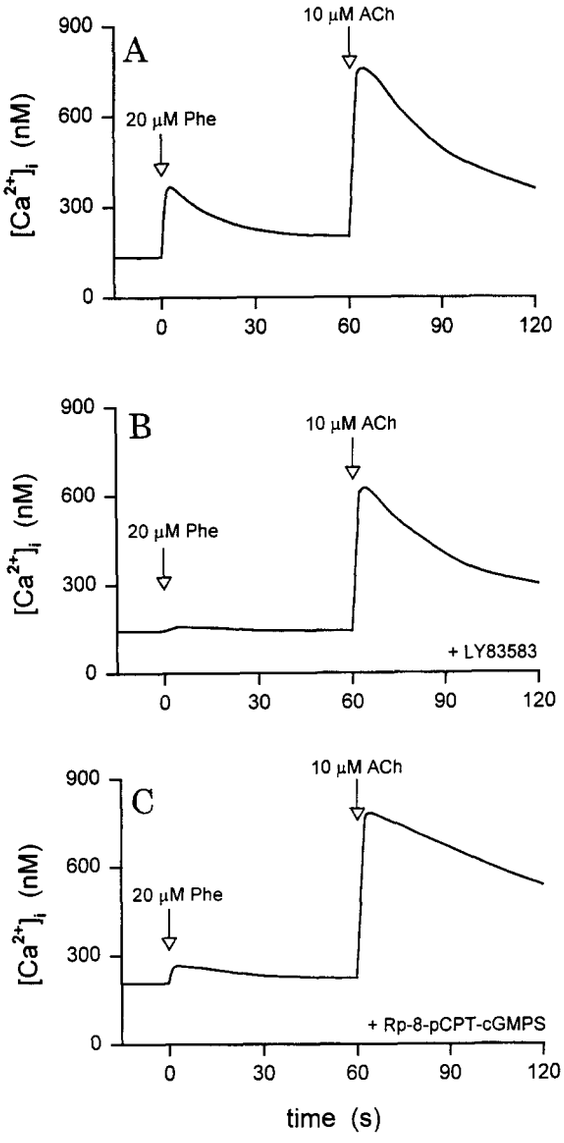

Fig. 1. Effect of LY83583 and Rp-8-pCPT-cGMPS on the changes in $[Ca^{2+}]_i$ evoked by sequential phenylephrine and acetylcholine stimulations. Changes in $[Ca^{2+}]_i$ in suspensions of lacrimal acinar cells in (A) control cells or in cells pre-treated with (B) 20 μM LY83583 or with (C) 50 μM Rp-8-pCPT-cGMPS for 3 min before stimulation with 20 μM phenylephrine (Phe) followed by 10 μM acetylcholine (ACh). The traces are representatives of 5–7 experiments from three different cell preparations.

(Fig. 1A). Similar transient rises in $[Ca^{2+}]_i$ were observed in a series of seven experiments with the amplitude of the phenylephrine-induced rise in $[Ca^{2+}]_i$ amounting in average to 39% of that observed following acetylcholine stimulation (Table 1). We have previously demonstrated that the observed rise in $[Ca^{2+}]_i$ in suspensions of rat lacrimal acinar cells following stimulation with phenylephrine and acetylcholine is due to mobilization from intracellular stores and not from influx of Ca^{2+} since both agonists induced changes in $[Ca^{2+}]_i$ of similar magnitudes when measured in either Ca^{2+}-free or Ca^{2+}-containing media [6]. The acetylcholine-evoked rise in $[Ca^{2+}]_i$ in lacrimal acinar cells involves activation of phospholipase C and Ins(1,4,5)P_3 production [6,8]. In contrast, the phenylephrine-induced Ca^{2+} mobilization is mediated without any inositol phosphate production and therefore a different signalling pathway seems to be activated following α-adrenergic receptor activation [6,9]. In this context, it has been suggested that cADPR could act as the Ca^{2+} mobilizing second messenger activated by α-adrenergic receptor stimulation [4,6].

It was recently demonstrated that cGMP is an endogenous regulator of cADPR synthesis in sea urchin eggs [7] and we therefore tested the effect of the specific guanylate cyclase inhibitor, LY83583, on the phenylephrine- and acetylcholine-induced rise in $[Ca^{2+}]_i$. Pre-treatment of lacrimal acinar cells with 20 mM LY83583 for 3 min had no effect on the resting $[Ca^{2+}]_i$ but strongly and selectively inhibited the phenylephrine-evoked $[Ca^{2+}]_i$ response by 93 ± 2% ($P < 0.001$, $n = 6$) (Fig. 1B). In accordance with previous observations on pancreatic acinar cells [10,11], LY83583 had no significant effect on the initial Ca^{2+} response following acetylcholine stimulation (Table 1).

These results suggest a crucial role for cGMP in the phenylephrine-induced Ca^{2+} response and we investigated the possibility that cGMP mediates its action through activation of a cGMP-dependent protein kinase. Inhibition of cGMP-dependent protein kinase Iα by pre-treatment for 3 min of lacrimal acinar cells with 50 μM of the membrane-permeant inhibitor Rp-8-pCPT-cGMPS [2,12] strongly inhibited the phenylephrine-evoked rise in $[Ca^{2+}]_i$ (Fig. 1C). In a series of five experiments, we found that Rp-8-pCPT-cGMPS inhibited the phenylephrine-induced rise in $[Ca^{2+}]_i$ by 75 ± 1% ($P < 0.001$) without affecting the amplitude of the acetylcholine-induced $[Ca^{2+}]_i$ response (Table 1).

These data suggest that phenylephrine promotes Ca^{2+} release from intracellular stores through a pathway involving activation of guanylate cyclase and cGMP-dependent protein kinase Ia. In order to further explore the role that cGMP plays in the cascade of events initiated by α-adrenergic receptor activation, we monitored the effects of membrane-permeant forms of cGMP on the phenylephrine- and acetylcholine-induced rise in $[Ca^{2+}]_i$. Exposure of lacrimal acinar cells to 1 mM dibutyryl-cGMP did not affect the resting $[Ca^{2+}]_i$ but strongly potentiated the increase in $[Ca^{2+}]_i$ following stimulation with a submaximal phenylephrine concentration (5 μM) (Fig. 2A,B). A submaximal agonist concentration was applied in order to fully reveal the potentiating effect of cGMP on $[Ca^{2+}]_i$. In accordance with the results depicted in Fig. 1, the potentiating effect of cGMP on $[Ca^{2+}]_i$ was specific for

Table 1
Effect of inhibitors of guanylate cyclase and cGMP-dependent protein kinase Iα on phenylephrine- and acetylcholine-induced changes in $[Ca^{2+}]_i$

Condition	Parameter $\Delta[Ca^{2+}]_i$ (nM)
Phe (20 μM)	202 ± 37 (7)
LY83583 (20 μM)+Phe (20 μM)	14 ± 6 (6)*
Rp-8-pCPT-cGMPS (50 μM)+Phe (20 μM)	55 ± 11 (5)*
ACh (10 μM)	498 ± 75 (7)
LY83583 (20 μM)+ACh (10 μM)	432 ± 29 (6) n.s.
Rp-8-pCPT-cGMPS (50 μM)+ACh (10 μM)	538 ± 48 (5) n.s.

$\Delta[Ca^{2+}]_i$ is the difference between peak and basal $[Ca^{2+}]_i$.
Phe, phenylephrine; ACh, acetylcholine. LY83583 and Rp-8-pCPT-cGMPS were added 3 min before agonist stimulation.
*$P < 0.001$; n.s. (not significant).

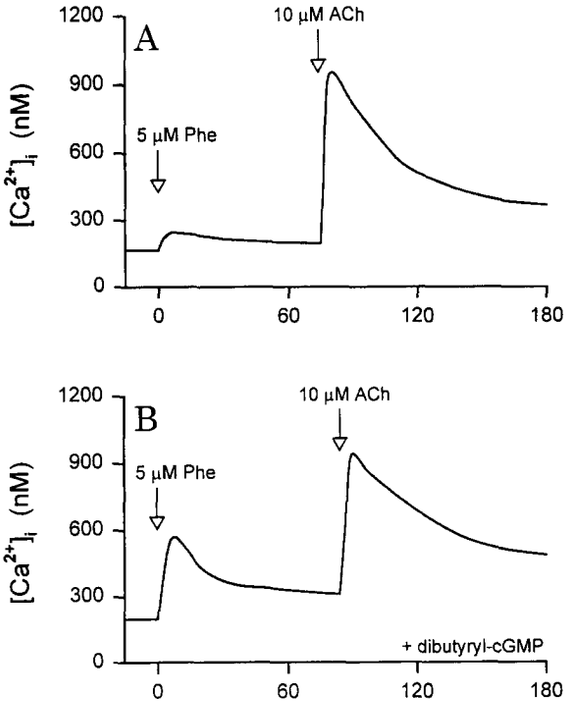

Fig. 2. Cyclic GMP selectively potentiate the phenylephrine-evoked increase in $[Ca^{2+}]_i$. Effect of sequential phenylephrine (Phe; 5 μM) and acetylcholine (ACh; 10 μM) stimulation in either (A) control cells or in (B) cells pre-treated with 1 mM dibutyryl-cGMP for 3 min. The traces are representatives of three and four individual experiments from at least three different cell preparations.

α-adrenergic receptor activation and did not affect the acetylcholine-induced rise in $[Ca^{2+}]_i$ (Fig. 2B and Table 2). Similar potentiating effects on intracellular Ca^{2+} handling were observed in cells pre-treated with 8-bromo-cGMP, but not with 8-bromo-cAMP (Table 2).

These results indicate that a rise in the intracellular cGMP concentration is not per se sufficient to stimulate an increase in $[Ca^{2+}]_i$ and that α-adrenergic receptor activation is essential for revealing the potentiating effect of cGMP. It has been suggested that cADPR might mediate its effects through a path involving NO and with cGMP as an intermediate [2]. Accordingly, NO would activate soluble guanylate cyclases, resulting in elevated cytoplasmic cGMP levels. This would lead to stimulation of cGMP-dependent protein kinase resulting in enhanced cADPR production [2]. However, application of various concentrations (1–500 μM) of the NO-synthase inhibitor, N^G-nitro-L-arginine, to lacrimal acinar cells had

Table 2
Effect of pharmacological interventions on phenylephrine-induced changes in $[Ca^{2+}]_i$

Condition	Parameter $\Delta[Ca^{2+}]_i$ (nM)
Phe (5 μM)	72 ± 20 (5)
db-cGMP (1 mM)+Phe (5 μM)	314 ± 81 (3)*
8-Br-cGMP (1 mM)+Phe (5 μM)	359 ± 64 (4)**
8-Br-cAMP (1 mM)+Phe (5 μM)	96 ± 40 (3) n.s.

$\Delta[Ca^{2+}]_i$ is the difference between peak and basal $[Ca^{2+}]_i$.
Phe, phenylephrine; db-cGMP, dibutyryl-cGMP; 8-Br-cGMP, 8-bromo-cGMP; 8-Br-cAMP, 8-bromo-cAMP. db-cGMP, 8-Br-cGMP and 8-Br-cAMP were added 3 min before phenylephrine stimulation.
*$P < 0.025$; **$P < 0.005$; n.s. (not significant).

no effect on either the resting cytoplasmic Ca^{2+} concentration or the phenylephrine-evoked rise in $[Ca^{2+}]_i$ (data not shown). Thus, it appears that the potentiating effect of cGMP on the phenylephrine-induced $[Ca^{2+}]_i$ response in lacrimal acinar cells does not involve activation of soluble guanylate cyclases and that the effect of LY83583 (see Fig. 1) on this signalling pathway must result from inhibition of another type of guanylate cyclase.

We have previously demonstrated that the effect of phenylephrine on $[Ca^{2+}]_i$ involves activation of ryanodine-sensitive Ca^{2+} release channels and proposed that cADPR might mediate this process [6]. Therefore, we measured Ca^{2+} release from permeabilized lacrimal acinar cells in order to explore the possibility that the potentiating effect of cGMP results from sensitization of these intracellular Ca^{2+} release channels. As shown in Fig. 3, this is unlikely to be the case since cGMP (in the presence of β-NAD^+, the substrate for the cADPR-synthesizing enzyme, ADP-ribosyl cyclase) did not by itself stimulate Ca^{2+} release, nor did it affect Ca^{2+} release in response to subsequent cADPR and $Ins(1,4,5)P_3$ stimulations. The Ca^{2+} responses following the addition of 5 μM cADPR amounted in control cells to 122 ± 23 nM ($n = 3$) and to 127 ± 27 nM ($n = 4$) in cells pre-treated with 1 mM cGMP. The corresponding Ca^{2+} responses following the addition of 5 mM $Ins(1,4,5)P_3$ amounted to 508 ± 75 nM ($n = 3$) in control cells and to 481 ± 24 nM ($n = 4$) in cells incubated with cGMP (not significant). Furthermore, treatment of intact acinar cells with 1 mM dibutyryl-cGMP for 3 min before permeabilization had no effect on the extent of Ca^{2+} release in response to cADPR stimulation (data not shown).

The data in the present study indicate that cGMP and activation of cGMP-dependent protein kinase Ia are essential for intracellular Ca^{2+} mobilization following α-adrenergic receptor activation in lacrimal acinar cells. These findings are in line with an earlier study demonstrating cGMP-mediated cADPR production in sea urchin eggs [7]. The potentiating effect of cGMP on the phenylephrine-evoked rise in $[Ca^{2+}]_i$ in lacrimal acinar cells does not occur via sensitization of ryanodine-sensitive intracellular Ca^{2+} release channels, since cGMP had no effect on the magnitude of the Ca^{2+} response

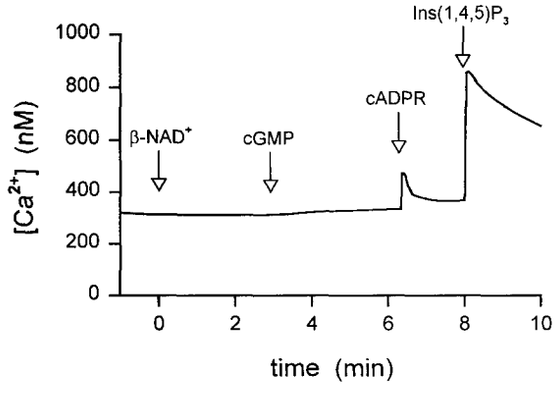

Fig. 3. Ca^{2+} release from permeabilized lacrimal acinar cells following addition of cADPR and $Ins(1,4,5)P_3$. Aliqots of cells were permeabilized with saponin and following Ca^{2+} accumulation and baseline stabilization, β-NAD^+ (1 mM, final concentration) was added. After 3 min cGMP (1 mM) was added followed by 5 μM cADPR and 5 μM $Ins(1,4,5)P_3$. The trace is representative of four individual experiments from four different cell preparations.

to cADPR stimulation. However, it has been demonstrated that cGMP-dependent protein kinases can induce rapid phosphorylation (and sensitization) of ryanodine-sensitive Ca^{2+} channels [13,14]. Therefore, it seems more likely that the potentiating effect of cGMP on the phenylephrine-evoked rise in $[Ca^{2+}]_i$ results from an enhanced cADPR production. At the present time it is not clear how cGMP can potentiate the phenylephrine-evoked Ca^{2+} response without in itself stimulating a rise in $[Ca^{2+}]_i$. However, it seems that α-adrenergic receptor activation is essential for initiating the cascade of reactions leading to Ca^{2+} mobilization from intracellular stores and that cGMP somehow enhances the flow of information through this signalling pathway. Thus, it appears that cGMP has a *permissive* rather than a *decisive* role in the signalling pathway stimulated by α-adrenergic receptor activation in lacrimal acinar cells.

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