Calcium store depletion potentiates a phosphodiesterase inhibitor- and dibutyryl cGMP-evoked calcium influx in rat pituitary GH_3 cells

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Received 25 March 1996

Abstract **A role for cGMP in the control of capacitative Ca^{2+} influx was identified in rat pituitary GH_3 cells. Application of 50 μM–1 mM of the non-specific phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), or the specific cGMP-phosphodiesterase inhibitor, zaprinast, induced a dose-dependent increase in the intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ of the pituitary cell line, as assessed by video ratio imaging using fura-2. Response onset times were identical and response profiles were similar in all cells analysed. Application of 50 μM dibutyryl cGMP to GH_3 cells resulted in heterogeneous Ca^{2+} responses, consisting of single or multiple transients with varying onset times. In all cases, increases in $[Ca^{2+}]_i$ were predominantly due to Ca^{2+} influx, since no responses were detected in low Ca^{2+} medium, or following pre-incubation of cells with 1 μM verapamil, or nicardipine. Depleting intracellular Ca^{2+} stores by prior treatment of cells with 1 μM thapsigargin resulted in a dramatic potentiation in the Ca^{2+} influx mediated by both phosphodiesterase inhibitors and dibutyryl cGMP, suggesting that cGMP modulates a dihydropyridine-sensitive Ca^{2+} entry mechanism in GH_3 cells which is possibly regulated by the state of filling of Ca^{2+} stores.**

Key words: Capacitative calcium influx; cGMP; GH_3 cell

1. Introduction

The cyclic nucleotides, cGMP and cAMP, have long been established as important second messengers in a variety of cell signalling pathways [1,2]. Roles for these species in intracellular Ca^{2+} signalling and homeostasis are also apparent. Most notably, cAMP stimulates Ca^{2+} influx through L-type Ca^{2+} channels of rat pancreatic β-cells [3], and cardiac cells [4], whereas cGMP promotes Ca^{2+} influx in vertebrate photoreceptors by interacting with cyclic nucleotide gated (CNG) channels [1], inhibits Ca^{2+} influx in other excitable cells by directly affecting voltage-gated Ca^{2+} channels [4], and modulates Ca^{2+} influx in non-excitable cells [4]. In addition, cGMP has also been shown to stimulate the synthesis of the potent Ca^{2+} mobilizing agent, cylic-ADP-ribose, in sea urchin eggs [5]. Although the above indicate specific roles for cyclic nucleotides in Ca^{2+} mobilization and influx, it is controversial whether cyclic nucleotides are implicated in capacitative Ca^{2+} influx [4,6], the mechanism whereby Ca^{2+} entry is promoted as soon as stored Ca^{2+} is discharged [6]. It is noteworthy that CNG channels are structurally similar to voltage-gated Ca^{2+} channels [7,8], the S5-S6 linker being well conserved in these two [9]. Also, it has been suggested that the calcium-release-activated calcium (CRAC) channel, involved in capacitative Ca^{2+} influx, might be analogous to the transient receptor potential (*trp*) gene product in *Drosophila* photoreceptors [6], which has been shown to possess significant amino acid sequence homology to voltage-gated Ca^{2+} channels [9]. These observations indicate a potential action of cyclic nucleotides on CRAC channels and Ca^{2+} entry channels in excitable cells. Considering the above, we decided to assess whether cyclic nucleotides could evoke Ca^{2+} influx in an excitable cell-line, and whether any influx was regulated by the state of filling of Ca^{2+} stores, employing single cell dynamic Ca^{2+} imaging techniques. Results from this study are consistent with a cGMP-evoked Ca^{2+} influx through a dihydropyridine-sensitive channel in rat pituitary GH_3 cells. This cGMP-mediated increase in $[Ca^{2+}]_i$ was dramatically stimulated by the prior depletion of intracellular Ca^{2+} stores, possibly indicating a role for cGMP and dihydropyridene-sensitive Ca^{2+} channels in capacitative Ca^{2+} entry in excitable cells.

2. Materials and methods

2.1. Preparation of cells

Rat pituitary GH_3 cells were seeded onto polylysine-coated borosilicate glass cover-slips in 6-well plates, and were grown in Hams F10 medium supplemented with 16% foetal calf serum for 3–4 days. For Ca^{2+} imaging experiments, cells were loaded with 2 μM fura-2 AM in 2 ml Hank's medium (pH 7.2) in the presence of 0.1% Pluronic for 45 min at room temperature. Cover slips were rinsed in Hank's medium and were then transferred to a teflon cover-slip holder and 900 μl Hank's medium added. The cover-slip dish was placed on the stage of an inverted Zeiss Axiovert 35 microscope for Ca^{2+} imaging.

2.2. Analysis of free intracellular Ca^{2+} in single GH_3 cells by video ratio imaging

Video ratio image analysis of GH_3 cell $[Ca^{2+}]_i$ was performed using an ion imaging system supplied by 'Improvision', Coventry, UK, in a similar way as previously described [5]. Cells were loaded with the acetoxymethyl (AM) ester of Fura-2 (Molecular Probes Inc.). Free cytosolic Ca^{2+} concentration was determined by taking the ratio of fluorescence intensities at excitation wavelengths 340 and 380 nm, using an emission wavelength of 510 nm. Pairs of 340 and 380 nm images were captured every 3 s and ratio images were calculated using 'Ionvision' software ('Improvision', Coventry, UK). Experiments were performed at 22°C, with cells maintained in modified Hank's medium. A low temperature was employed for Ca^{2+} measurements with fura-2, since this dye readily compartmentalises into cellular organelles at 32–34°C. Standard $CaCl_2$ solutions were used to calibrate the system, and viscosity corrections were made [10].

2.3. Drugs and solutions

Experiments were performed in Hank's medium (pH 7.2), containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.83 mM $MgSO_4$, 0.42 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 5 mM glucose. For experiments carried out in low Ca^{2+} medium, $CaCl_2$ was omitted from the above, and 3 mM EGTA was added, yielding a free Ca^{2+} concentration of ~1 nM. Fura-2 AM was from Molecular

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PII S0014-5793(96)00413-9

Probes Inc. Phosphodiesterase inhibitors and all other drugs were from Sigma.

3. Results

Under resting conditions the $[Ca^{2+}]_i$ was found to oscillate in a small proportion of GH_3 cells (approx. 15%). In all the following experiments, cells displaying oscillation in their basal $[Ca^{2+}]_i$ were excluded from the analysis, since inclusion may have resulted in ambiguity. Hence, bolus addition of 50 μM–1 mM of the non-specific phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), or the specific cGMP phosphodiesterase inhibitor zaprinast [11] to the incubation bath, resulted in an increase in the $[Ca^{2+}]_i$ of 45–85% of quiescent GH_3 cells, as assessed by video ratio imaging using fura-2 (Fig. 1a,b). The proportion of cells eliciting a response to these inhibitors and response magnitude was dose dependent (Fig. 2a,b). Response onset was the same in all cells, and response profiles were similar, consisting of slow transients and elevated Ca^{2+} plateaus (Fig. 1a,b). Bolus addition of 50 μM of the cell permeant cGMP analogue, dibutyryl cGMP, to the incubation bath resulted in heterogeneous Ca^{2+} responses in 50% of quiescent GH_3 cells, consisting of single or multiple transients with varying onset times (Fig. 1c). In all cases, increases in $[Ca^{2+}]_i$ were due to influx, no responses being detected in low Ca^{2+} medium (0 Ca^{2+}, 3 mM EGTA), or following a 5 min pre-incubation with 1 μM verapamil, or nicardipine. The lack of a zaprinast-induced response following these treatments is shown in Fig. 2b, with similar results being obtained for IBMX and dibutyryl cGMP (data not shown). These combined results suggest that cGMP stimulates Ca^{2+} influx through a dihydropyridine-sensitive Ca^{2+} channel in GH_3 cells.

To determine whether the phosphodiesterase inhibitor- or dibutyryl cGMP-evoked Ca^{2+} influx in GH_3 cells was influenced by Ca^{2+} store depletion, cells were preincubated with 1 μM of the endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, thapsigargin, for 15 min. Following this treatment, Ca^{2+} mobilization induced by 10 μM thyrotropin releasing hormone (TRH) was completely abolished in these cells (data not shown). In these Ca^{2+} store-depleted cells there was a

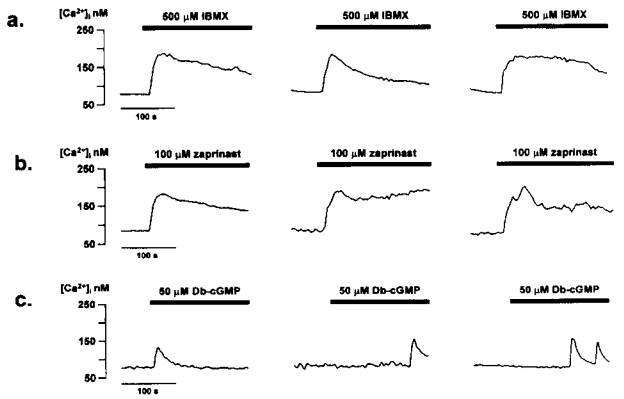

Fig. 1. Intracellular Ca^{2+} responses in single rat pituitary GH_3 cells, mediated by phosphodiesterase inhibitors and dibutyryl cGMP. Traces of $[Ca^{2+}]_i$ vs. time derived from video ratio imaging of fura-2 loaded GH_3 cells treated with 500 μM IBMX (a), 100 μM zaprinast (b), and 50 μM dibutyryl cGMP (c) at the indicated times. For (a–c), traces are representative of 80 responding cells obtained from 4 different experiments.

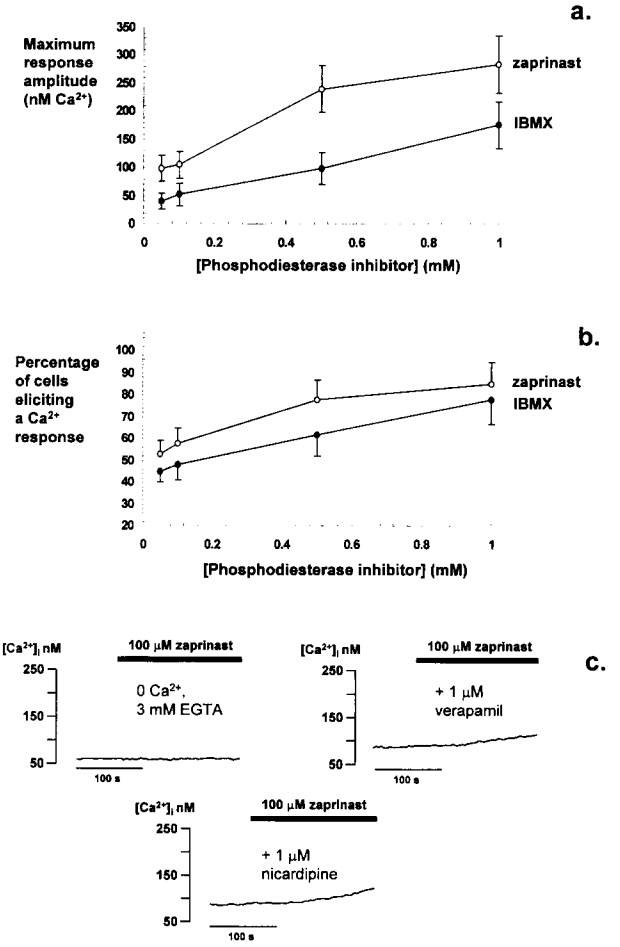

Fig. 2. Dose and extracellular Ca^{2+} dependency of phosphodiesterase inhibitor-evoked Ca^{2+} responses of GH_3 cells. (a) Plot of the mean maximum Ca^{2+} response amplitude ± S.D. vs. [phosphodiesterase inhibitor] for GH_3 cells responding to applications of zaprinast (○) and IBMX (●). Data points were derived from 80 responding GH_3 cells, obtained from 4 separate experiments. (b) Plot of the mean percentage of GH_3 cells eliciting a Ca^{2+} response ± S.D. vs. [phosphodiesterase inhibitor], for cells subjected to bolus applications of zaprinast (○) and IBMX (●). Data points represent the mean of 4 separate experiments, with at least 25 cells in each experiment. (c) Inhibition of the zaprinast-induced Ca^{2+} response by transferring GH_3 cells into low Ca^{2+} medium (0 Ca^{2+}, 3 mM EGTA), or by pre-incubating cells for 5 min with 1 μM verapamil or nicardipine. Out of 80 cells from 4 separate experiments and for each treatment, no responses were detected. IBMX- and dibutyryl cGMP-evoked Ca^{2+} responses were also comprehensively inhibited by these treatments (data not shown).

dramatic potentiation in the Ca^{2+} influx mediated by IBMX, zaprinast and dibutyryl cGMP. At the inhibitor and dibutyryl cGMP doses displayed in Fig. 3, an approx. 4-fold stimulation in Ca^{2+} influx was witnessed in thapsigargin-treated cells compared to untreated controls (Fig. 3). Although the mean basal $[Ca^{2+}]_i$ was increased from approx. 80 to 120–150 nM in thapsigargin-treated cells (Fig. 3), similar Ca^{2+} influx stimulation was witnessed in single thapsigargin-treated cells whose basal $[Ca^{2+}]_i$ was 80 nM or less. Furthermore, at these IBMX (500 μM) and zaprinast (100 μM) concentrations, all thapsigargin-treated cells elicited a response, compared with approx. 60% for untreated controls. With dibutyryl cGMP (50 μM) application, the proportion of responding cells also increased to 75% for thapsigargin-treated, compared

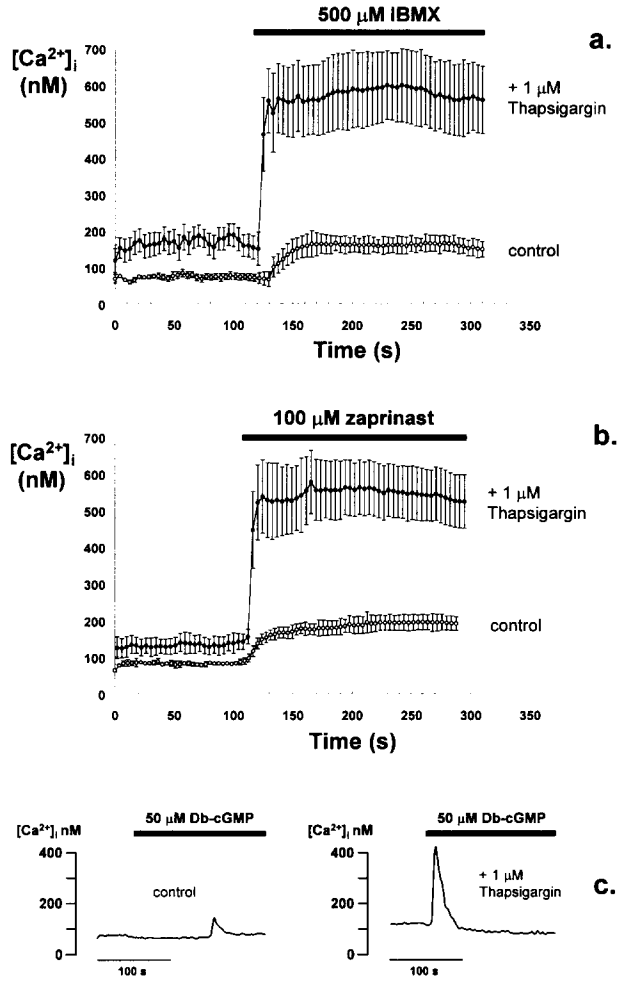

Fig. 3. Stimulation of the phosphodiesterase inhibitor- and dibutyryl cGMP-evoked Ca^{2+} influx of GH_3 cells, following a 10 min pre-incubation with 1 μM thapsigargin. (a) Plot of the mean $[Ca^{2+}]_i \pm$ S.D. vs. time for untreated (○), and thapsigargin-treated (●) GH_3 cells subjected to bolus application of 500 μM IBMX. Data points were derived from 80 responding untreated and 80 responding thapsigargin-treated cells, each from 4 separate experiments. (b) As for (a), except 100 μM zaprinast was applied to the cells instead of IBMX. (c) Single traces of $[Ca^{2+}]_i$ vs. time for an untreated control and a thapsigargin-treated GH_3 cell subjected to bolus application of 50 μM dibutyryl cGMP. Traces are representative of responses from 80 untreated control and 56 thapsigargin-treated cells.

to 50% for untreated cells, and response onset times were reduced (Fig. 3c). All phosphodiesterase inhibitor- and dibutyryl cGMP-evoked Ca^{2+} responses in thapsigargin-treated cells were due to influx, with no responses being detected following the transfer of thapsigargin-treated cells to low Ca^{2+} medium (0 Ca^{2+}, 3mM EGTA), or following pre-incubation with 1 μM nicardipine, or 1 μM verapamil (data not shown). A possible explanation for the observed potentiation in the cGMP-evoked increase in $[Ca^{2+}]_i$ following Ca^{2+} store depletion is a decrease in cytoplasmic Ca^{2+} buffering due to inhibition of Ca^{2+} uptake into stores by thapsigargin. This may seem unlikely. however, since zaprinast-evoked Ca^{2+} influx was increased following Ca^{2+} store depletion by thapsigargin, as assessed by manganese quench of cytosolic fura-2 (data not shown). These combined data strongly suggest that cGMP stimulates a capacitative Ca^{2+} entry mechanism in GH_3 cells.

4. Discussion

From previous studies it is generally accepted that capacitative Ca^{2+} entry is switched on by a variety of stimuli which share a common property of releasing stored Ca^{2+}. Ca^{2+} entry therefore appears to be regulated by the state of filling of Ca^{2+} stores [6]. An inhibitor of ER Ca^{2+}-ATPase pumps, thapsigargin, is well established as an agent that effectively depletes intracellular Ca^{2+} stores [12-14], and consequently promotes capacitative Ca^{2+} influx. Results from this study suggest that thapsigargin-induced depletion of Ca^{2+} stores leads to stimulation of a cGMP-evoked Ca^{2+} influx in GH_3 cells. This influx appears to be dihydropyridine-sensitive, suggesting that cGMP might be modulating a channel with structural and possibly functional similarity to dihydropyridine-sensitive voltage-gated channels. Whether cGMP is acting either directly or indirectly on CRAC channels in the GH_3 cells of this study is uncertain, since these channels are classically dihydropyridene-insensitive.

Various models have been put forward to explain capacitative Ca^{2+} influx [6]. These fall into two categories; those that propose the existence of a diffusible factor (cytoplasmic influx factor, or CIF) that transmits information across the gap separating the ER from the plasma membrane, and those that propose direct information transfer between the ER and the plasma membrane through a protein-protein interaction [6]. Considering cGMP-evoked Ca^{2+} influx was dramatically stimulated following Ca^{2+} store depletion in this study, it appears that emptying of Ca^{2+} stores somehow enhances cGMPs efficacy. If cGMP stimulates CRAC channels directly, then it is possible that Ca^{2+} store emptying may promote cGMP binding by inducing a conformational change in these channels, either through a direct store-channel interaction, or indirect CIF-channel interaction. Alternatively, cGMP may potentiate the action of CIF or messengers released from Ca^{2+} stores following the emptying process. Similar observations to this study have been noted with low concentrations of the nitric oxide (NO) promoter, sodium nitroprusside (SNP) and cGMP in colon epithelial cells [16]. In cells with intact stores, SNP and cGMP increased $[Ca^{2+}]_i$ by only a small amount, but in cells with depleted stores, these species had a more profound effect. Additionally Xu et al. [17] have provided evidence that depletion of Ca^{2+} stores activates nitric oxide synthase (NOS) leading to the generation of cGMP, which then stimulates Ca^{2+} influx in pancreatic acini. These data further reinforce a potential role for cGMP in the control of capacitative Ca^{2+} influx. It is also noteworthy that capacitative Ca^{2+} influx can be modulated following injection of a non-metabolizable analogue of GTP, guanosine 5′-3-*O*-(thio)-triphosphate (GTPγS) [15,18-20]. Since cGMP is formed in mammalian cells by the guanylate cyclase catalysed hydrolysis of GTP [21], the above procedure may have resulted in the inhibition of guanylate cyclase, and a corresponding decrease in the free cytoplasmic level of cGMP in the above studies. If cGMP acts as a positive modulator of capacitative Ca^{2+} influx, in line with the findings of this study, then inhibition of capacitative Ca^{2+} influx might be expected with injection of GTPγS, as long as this procedure does not result in a G-protein coupled activation of phospholipase C. Previous re-

ports suggest both activation and inhibition of capacitative Ca^{2+} influx with GTPγS injection. Stimulatory effects are thought to be ascribed to activation of heterotrimeric G-proteins responsible for activating phospholipase C to generate inositol 1,4,5-trisphosphate (IP_3), leading to Ca^{2+} store depletion and the onset of Ca^{2+} entry [6]; inhibitory effects are thought to result from the parallel formation of diacylglycerol (DAG) and subsequent activation of protein kinase C (PKC), which then inhibits capacitative Ca^{2+} influx [6]. Although the effect of GTPγS on capacitative Ca^{2+} influx appears complex and is probably concentration-dependent and/or dependent on cell type, it is still possible that observed inhibitory effects might also be due to guanylate cyclase inhibition and a corresponding decrease in cytoplasmic cGMP levels.

Acknowledgements: A.G. is supported by the Wellcome Trust.

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