

Cyclic AMP raises cytosolic Ca^{2+} and promotes Ca^{2+} influx in a clonal pancreatic β -cell line (HIT T-15)

Marc Prentki, Major C. Glennon, Jean-François Geschwind, Franz M. Matschinsky and Barbara E. Corkey

Department of Biochemistry and Biophysics and Diabetes Research Center, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

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The effect on cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of cAMP analogues and the adenylate cyclase-stimulating agents forskolin, isoproterenol and glucagon has been examined in an insulin-secreting β -cell line (HIT T-15) using fura 2. All these manipulations of the cAMP messenger system promoted a rise in $[\text{Ca}^{2+}]_i$ which was blocked by the Ca^{2+} channel antagonists verapamil and nifedipine or by removal of extracellular Ca^{2+} . The action of the adenylate cyclase activator forskolin was glucose-dependent. The results suggest that cAMP elevates $[\text{Ca}^{2+}]_i$ in HIT cells by promoting Ca^{2+} entry through voltage-sensitive Ca^{2+} channels, not through mobilization of stored Ca^{2+} . Activation of Ca^{2+} influx may be an important component of the mechanisms by which cAMP potentiates fuel-induced insulin release.

cyclic AMP; Forskolin; D-Glucose; cytosolic free Ca^{2+} ; Ca^{2+} channel blocker; Insulin release

1. INTRODUCTION

cAMP is an important modulator of insulin secretion as demonstrated by the ability of agents that elevate cAMP to potentiate fuel-induced insulin release [1-3]. Studies with pure β -cells [4,5] and dealing with the interaction of the nucleotide with the Ca^{2+} -messenger system [6-9] indicate that cAMP may modulate the effectiveness of the Ca^{2+} system and determine the β -cell sensitivity and responsiveness to fuel secretagogues [3]. It also appears that normal β -cell stimulation by fuels requires the presence of A-cells that release some glucagon to maintain β -cell cAMP content at a level necessary for fuels to be effective [4,5]. Thus, cAMP may not only be a potentiator of the secretory process but in addition may play a permissive role in nutrient-induced insulin release [3].

The precise mechanisms by which cAMP exerts

its effects on the β -cell remain largely unsettled. A number of reports using indirect methods suggested that the action of cAMP might be exerted through elevations in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). ^{45}Ca flux measurements in pancreatic islets have led to the proposal that a rise in β -cell cAMP may result in the mobilization of stored Ca^{2+} [10,11], or acceleration of Ca^{2+} influx [12]. Electrical activity recordings of β -cells have shown that the adenylate cyclase activator forskolin potentiates Ca^{2+} -dependent electrical activity induced by glucose, suggesting indirectly that cAMP enhances Ca^{2+} influx [12,13]. In other studies however, cAMP-raising agents failed to affect net ^{45}Ca uptake by pancreatic islets [11,14]. The action of cAMP on cytosolic Ca^{2+} has been tested with the fluorescent indicator quin 2. Using this method, stimulation of adenylate cyclase by forskolin and inhibition of cAMP phosphodiesterase by methylxanthines were not associated with detectable elevations in $[\text{Ca}^{2+}]_i$ in islets of ob/ob mice [15], in RINm5F insulinoma cells [7] or in an HIT β -cell line [16].

Here, using the more sensitive dye fura 2, we

Correspondence address: M. Prentki, Department of Biochemistry and Biophysics, University of Pennsylvania, 36th and Hamilton Walk/G3, Philadelphia, PA 19104, USA

show that a variety of agents which are known to enhance cAMP production promote rapid elevations in $[Ca^{2+}]_i$ by accelerating Ca^{2+} influx in a fuel-sensitive [17-20] hamster β -cell line (HIT T-15). Enhanced Ca^{2+} influx is likely to be a significant process involved in the modulation of nutrient-induced insulin secretion by cAMP.

2. MATERIALS AND METHODS

HIT cells (subclone T-15) [17] were a gift from Dr A.E. Boyd, iii (Houston, TX). Cells were grown in monolayer cultures as described [19]. Cells were detached from culture flasks by incubation in the presence of EDTA without trypsin [21] for 5 min at 37°C. They were subsequently washed twice in modified L-15 medium (Gibco) containing 15 mM Hepes and 0.5% bovine serum albumin (BSA fraction V, Sigma). HIT cells harvested from two confluent T-75 flasks were then resuspended in 5 ml L-15 medium and incubated in the presence of 1 μ M fura 2/AM at 37°C. After one wash, cells were incubated for an additional 10 min in the absence of fura 2/AM to allow completion of fura 2/AM hydrolysis by nonspecific esterases. Fura 2-loaded cells were further washed in L-15 medium and resuspended in 0.5 ml modified Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) containing 5 mM $NaHCO_3$, 2 mM $CaCl_2$, 10 mM Hepes and 0.5% BSA, and kept on ice until use. The fluorescence of fura 2-loaded cells was measured in acrylic cuvettes using an MB-2 four filter air turbine fluorescence spectrophotometer (Johnson Foundation, University of Pennsylvania) at the excitation wavelengths of 340 nm (Ca^{2+} -fura 2 complex) and 380 nm (free fura 2), and at an emission wavelength of 510 nm. Cell suspensions (approx. 0.5 mg protein) were incubated at 26°C in 2 ml KRB supplemented with 10 mM glucose. Leakage of fura 2 from the cells at 37°C was considerable since it was about 3-times the leakage at 26°C that is shown in fig.1d. We therefore routinely measured fura 2 fluorescence at 26°C and have verified that all findings reported here were also observed at 37°C. None of the agents tested induced autofluorescence in HIT cells that had not been loaded with fura 2. $[Ca^{2+}]_i$ was calibrated from data obtained at an excitation wavelength of 340 nm using a K_d for Ca^{2+} of 224 nM [22]. Cells were lysed with 0.04% Triton

X-100 to obtain the maximal fluorescence. This was followed by addition of Tris base to bring the medium pH to 7.9 and 5 mM EGTA to determine minimal fluorescence. Corrections for extracellular fura 2 were made from measurements of the immediate decrease in fluorescence after addition of 50 μ M $MnCl_2$ to quench external fura 2 [22]. The traces shown in the figures are representative experiments that have been repeated at least three times. All reagents were of analytical grade. Forskolin and fura 2/AM were obtained from Calbiochem-Behring. Glucagon was purchased from Lilly (Indianapolis, IN) and 8-(4-chlorophenylthio)-cAMP from Boehringer Mannheim (FRG).

3. RESULTS

To examine the action of cAMP on $[Ca^{2+}]_i$ in HIT cells, we tested the effect of a cAMP analogue and several agents that promote rises in cAMP by different mechanisms. Fig.1 illustrates a series of experiments in which we used the cAMP analogue 8-(4-chlorophenylthio)-cAMP (cpt-cAMP) which has been shown to be a very potent activator (nearly 100-times more effective than cAMP) of rat liver cAMP-dependent protein kinase [23]. The analogue cpt-cAMP rapidly increased $[Ca^{2+}]_i$. A peak elevation in Ca^{2+} was reached in about 2 min, and $[Ca^{2+}]_i$ remained elevated for more than 10 min (fig.1a). Dibutyl-cAMP caused similar changes in $[Ca^{2+}]_i$, but was approx. 10-times less effective at the same concentration. Prior addition of the voltage-sensitive Ca^{2+} channel blocker verapamil or chelation of medium Ca^{2+} with EGTA suppressed the Ca^{2+} transient induced by cpt-cAMP or by a depolarizing concentration of KCl (fig.1b,c). In contrast, the action of the muscarinic agonist carbamylcholine was unaffected by verapamil or EGTA. The data demonstrate that cpt-cAMP promoted a net Ca^{2+} influx in HIT cells and did not mobilize stored Ca^{2+} . Increases in cytosolic Ca^{2+} elicited by cpt-cAMP appear to occur as a result of accelerated Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and not by inhibition of Ca^{2+} extruding transport systems that are present in the cell membrane. Thus, the action of cpt-cAMP was blocked by verapamil (fig.1b) and cpt-cAMP did not modify $[Ca^{2+}]_i$ when it was added after a Ca^{2+} transient

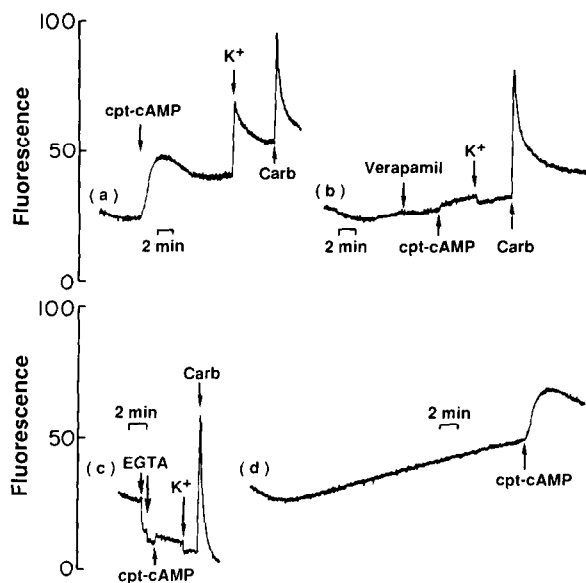


Fig.1. Effect of verapamil and EGTA on cytosolic Ca^{2+} transients induced by a cAMP analogue, high K^+ and carbamylcholine. The traces show fura 2 measurements (expressed in arbitrary units) made at an excitation wavelength of 340 nm and at an emission wavelength of 510 nm. An increase in fluorescence indicates an elevation in cytosolic Ca^{2+} . Arrows denote the times of addition of 200 μM 8-(chlorophenylthio)-cAMP (cpt-cAMP), 35 mM KCl (K^+), 200 μM carbamylcholine (Carb), 25 μM verapamil, and 5 mM EGTA. Isolated HIT cells in suspension were incubated in the presence of 10 mM glucose as described in section 2.

induced by a depolarizing concentration of KCl (fig.2). These observations suggest that cpt-cAMP and high KCl promoted activation of similar type(s) of voltage-dependent Ca^{2+} channels. There was some leakage of fura 2 from HIT cells, since the progressive increase in fluorescence shown in fig.1d was immediately suppressed upon addition of Mn^{2+} to quench extracellular fura 2 (see also fig.3).

Additional evidence demonstrates that rises in cellular cAMP activated Ca^{2+} influx. Consistent with observations using cpt-cAMP, the adenylate cyclase activator forskolin also induced rises in $[\text{Ca}^{2+}]_i$ (fig.3). The half-maximally effective concentration of forskolin was 5 μM . The divalent cation Mn^{2+} is known to permeate Ca^{2+} channels [24]. In contrast to Ca^{2+} , Mn^{2+} binding to fura 2 causes quenching of the fura 2 signal [22]. Fig.3

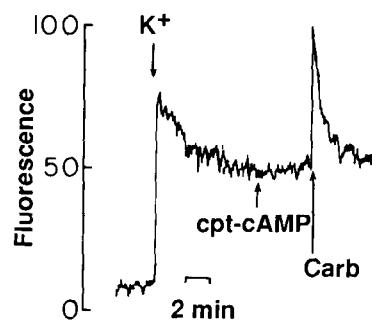


Fig.2. A cAMP analogue does not cause an elevation in cytosolic Ca^{2+} after a Ca^{2+} transient induced by a depolarizing concentration of KCl. Arrows denote the additions of 35 mM KCl (K^+), 200 μM 8-(chlorophenylthio)-cAMP (cpt-cAMP), and 200 μM carbamylcholine (Carb).

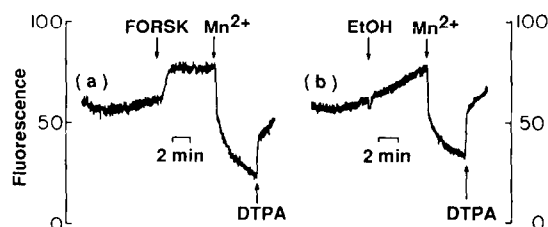


Fig.3. Forskolin-treated cells display an accelerated quenching of intracellular fura 2 fluorescence upon addition of MnCl_2 . Arrows denote the additions of 25 μM forskolin (FORSK) dissolved in ethanol (EtOH), 100 μM MnCl_2 (Mn^{2+}) and 200 μM diethylenetriaminepenta-acetic acid (DTPA).

shows that Mn^{2+} elicited an immediate decrease in fluorescence due to quenching of contaminating extracellular fura 2. This was followed by a slower progressive decrease in fluorescence that was accelerated in cells which had been treated with forskolin (fig.3). Subsequent addition of DTPA, a high-affinity non-permeant heavy metal chelator [25], reversed the action of Mn^{2+} on the Ca^{2+} signal. Thus, an immediate increase in fluorescence occurred due to chelation of extracellular Mn^{2+} and therefore restoration of the signal due to contaminating extracellular fura 2 signal. This was followed by a progressive increase in fluorescence that is presumably a consequence of slow extrusion of cellular Mn^{2+} . The rate of intracellular fura 2 quenching by Mn^{2+} (calculated from the slope of the decrease in fluorescence following immediate quenching of extracellular fura 2) was measured in

the absence and presence of forskolin and high KCl. This slope may reflect the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels since Mn^{2+} has been shown to permeate voltage-sensitive Ca^{2+} channels. Compared to basal conditions, forskolin (25 μM) increased the initial rate of intracellular fura 2 quenching by $54 \pm 2\%$ and KCl (35 mM) by $240 \pm 47\%$ (mean $\pm$ SE of 3 experiments).

Table 1 summarizes the effects of different agonists which were tested at a maximally effective concentration. The adenylate cyclase activator forskolin, the β -adrenergic agonist isoproterenol and the peptide hormone glucagon raised $[\text{Ca}^{2+}]_i$ to approximately the same level. The pattern and magnitude of $[\text{Ca}^{2+}]_i$ changes caused by these agents were similar to the effect of cpt-cAMP. All induced peak elevations in $[\text{Ca}^{2+}]_i$ that were about 50% above basal levels. The action of these adenylate cyclase activators was abolished by the Ca^{2+} channel blockers verapamil (25 μM) and nifedipine (3 μM) or chelation of medium Ca^{2+} with EGTA (not shown).

cAMP potentiates fuel-induced insulin secretion [1-3]. We therefore investigated whether the action of forskolin was dependent on the presence of glucose. As observed previously using quin 2 [18], glucose (10 mM) by itself did not cause detectable changes in $[\text{Ca}^{2+}]_i$ in HIT cells when added to glucose-deprived cells. The action of forskolin on $[\text{Ca}^{2+}]_i$ was, however, dependent on glucose since

in the absence of glucose forskolin increased $[\text{Ca}^{2+}]_i$ by only $43 \pm 4\%$ (mean $\pm$ SE of 3 experiments) of the effect observed at 10 mM glucose.

4. DISCUSSION

Agents that elevate cellular cAMP such as forskolin or the cAMP-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) markedly potentiate fuel-induced insulin secretion in insulin-secreting cells [1-5], including HIT cells [16,18,20]. Recent studies using quin 2 suggested that cAMP modulates secretion at a site distal to Ca^{2+} since several agents that promote an elevation in cAMP did not cause detectable changes in $[\text{Ca}^{2+}]_i$ [7,15,16]. Using the more sensitive dye fura 2, the present study shows unambiguously that cytosolic Ca^{2+} rises when HIT cells are stimulated with cAMP agonists. Several lines of evidence indicate that cAMP promotes Ca^{2+} entry through voltage-sensitive Ca^{2+} channels in HIT cells and that it does not mobilize stored Ca^{2+} . First, the effect was suppressed by the voltage-sensitive Ca^{2+} channel blockers verapamil and nifedipine or by removal of external Ca^{2+} with EGTA. Second, cpt-cAMP did not elevate Ca^{2+} when cells were depolarized with high KCl. Third, net Ca^{2+} entry appear to be accelerated in forskolin-treated cells as shown in studies (fig.3) in which the rate of intracellular fura 2 quenching by Mn^{2+} was measured. It appears therefore that accelerated Ca^{2+} entry through voltage-sensitive Ca^{2+} channels might be an important mechanism by which cAMP potentiates the insulin secretory process. Additional mechanism(s) could also be of importance. Studies with permeabilized islets [8,9] or RINm5F insulinoma cells [26] have shown that cAMP stimulates insulin secretion under conditions where medium Ca^{2+} is fixed with Ca^{2+} /EGTA buffers. In addition, it has been shown that forskolin slightly stimulates insulin release from islets incubated in the absence of Ca^{2+} with EGTA present [14,27]. Therefore, cAMP may facilitate secretion both by promoting Ca^{2+} entry and by sensitizing the releasing machinery to the action of Ca^{2+} or other messengers.

The observation that the action of forskolin on $[\text{Ca}^{2+}]_i$ is glucose-dependent is of interest. It is noteworthy in this respect that several reports have

Table 1
 $[\text{Ca}^{2+}]_i$ in HIT cells under various experimental conditions

Condition	$[\text{Ca}^{2+}]_i$ (nM)
Basal	61 ± 4 (24)
cpt-cAMP (200 μM)	95 ± 12 (8)
Forskolin (25 μM)	92 ± 13 (10)
Isoproterenol (1 μM)	86 ± 22 (4)
Glucagon (0.1 μM)	97 (2)
Carbamylcholine (200 μM)	147 ± 15 (9)
KCl (35 mM)	251 ± 29 (8)

HIT cells were incubated with 10 mM glucose. The results of basal and peak $[\text{Ca}^{2+}]_i$ measurements made in the presence of a variety of agents are summarized. Cytosolic $[\text{Ca}^{2+}]_i$ was calibrated as described in section 2. Means $\pm$ SE of $[\text{Ca}^{2+}]_i$ are given for the indicated number (*n*) of separate experiments

documented that insulin secretion cannot be elicited by cAMP-raising agents in the absence of fuel stimuli, in particular glucose [1,2,28]. The reason for this glucose dependency is not known. It is plausible that permissive non-stimulatory levels of nutrients are needed to maintain the energy status of β -cells or basal levels of various messengers.

The exact mechanism by which cAMP promotes Ca^{2+} influx in insulin-secreting cells remains to be determined. By comparison with data obtained in other tissues, the most likely possibility is that cAMP alters Ca^{2+} or K^{+} channel activities. Using the patch-clamp technique it has been demonstrated that cAMP enhances transmembrane Ca^{2+} influx in heart cells [29] and corticotropin-secreting cells [30] by increasing the number and opening probability of voltage-sensitive Ca^{2+} channels [29]. K^{+} channels have also been reported to be modulated by cAMP in lymphocytes [31]. Further studies using the patch-clamp technique should help to define the mechanism by which cAMP accelerates Ca^{2+} influx in insulin-secreting cells.

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