RESEARCH COMMUNICATION

Limited accumulation of cyclic AMP underlies a modest vasoactive-intestinal-peptide-mediated increase in cytosolic $[Ca^{2+}]_i$ transients in GH3 pituitary cells

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The 4-chlorophenylthio analogue of cyclic AMP evoked profound and long-lasting changes in cytosolic $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) in pituitary-derived GH3 cells. However, vasoactive intestinal peptide (VIP), a hormone considered to act via cyclic AMP, was ineffective in modulating $[Ca^{2+}]_i$. The ability of VIP to modulate $[Ca^{2+}]_i$ was enhanced by treatments that increased intracellular cyclic AMP. Much greater concentrations of intracellular cyclic nucleotides were achieved by the analogue than with VIP, under any condition. Thus cyclic AMP may play a prominent role in regulating $[Ca^{2+}]_i$ in these cells, but the ability of hormones to stimulate its synthesis is limited, leading to a weak action on $[Ca^{2+}]_i$.

INTRODUCTION

The regulation of $[Ca^{2+}]_i$ by signals that emanate from inositol phosphate-mediated mechanisms in GH3 pituitary cells is relatively well understood [1,2]. Hormones that increase $InsP_3$ induce a substantial rise in $[Ca^{2+}]_i$, both as a result of direct effects of $InsP_3$ on internal stores [3–5] and as a consequence of the production of diacylglycerol and subsequent modification of the activity of various ion channels [6,7]. There also appears to be a mechanism by which cyclic AMP may increase $[Ca^{2+}]_i$ in GH3 cells, although the details are far from clearly resolved. Single-channel recordings have shown that cyclic-AMP-dependent protein kinase can maintain the activity of L-type Ca^{2+} channels [8] and inhibit the activity of K^+ channels [9]. At the whole-cell level, there are conflicting reports indicating, on the one hand, that dibutyryl cyclic AMP and agents that elevate cyclic AMP decrease the sensitivity of Ca^{2+} channels to inhibition by $[Ca^{2+}]_i$ [10], and on the other that cyclic AMP does not modify Ca^{2+} currents [11]. Therefore it remains to be determined whether in fact there is a direct consequence of the elevation of cyclic AMP on $[Ca^{2+}]_i$. Studies on populations of GH3 cells have indicated that agents that elevate cyclic AMP, such as VIP, also elevate $[Ca^{2+}]_i$ [12]. Of course, these studies do not prove that cyclic AMP is essentially involved in the elevation of $[Ca^{2+}]_i$ by VIP. In addition, experiments conducted in cell populations do not lend themselves to determining the mechanism of action of the agents involved. Single-cell $[Ca^{2+}]_i$ measurements do provide an insightful means for evaluating the link between cyclic AMP and $[Ca^{2+}]_i$. In the present studies, a cell-permeant analogue of cyclic AMP, 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (CPT-cAMP), was compared with VIP to explore the action of cyclic AMP on $[Ca^{2+}]_i$ in individual GH3 cells. Surprisingly, CPT-cAMP elicited a profound and sustained stimulation of $[Ca^{2+}]_i$ -transient firing, contrasting with a modest action that could be induced by VIP. This modest action of VIP on $[Ca^{2+}]_i$ was well correlated with its limited effect on elevating cyclic AMP levels in GH3 cells. Indeed, if the ability of VIP to increase cyclic AMP levels was enhanced, its ability to increase Ca^{2+} transient activity was incrementally increased. These

findings suggest a convincing link between cyclic AMP levels and $[Ca^{2+}]_i$ in GH3 cells.

MATERIALS AND METHODS

Cell culture

GH3/B6 cells were grown in Ham's F-10 medium supplemented with 15% (v/v) donor horse serum, containing 2.5% (v/v) foetal-bovine serum, as described previously [4], on 2.4 cm-diam. dishes, flasks or coverslips, as required.

Measurements of $[Ca^{2+}]_i$ in individual pituitary cells

GH3/B6 pituitary cells were grown on glass coverslips in the nutrient medium described above. $[Ca^{2+}]_i$ was determined in individual cells by dual-emission microspectrofluorimetry using indo-1 as the fluorescent intracellular- Ca^{2+} probe [13]. Before loading the cells with indo-1, the culture medium was replaced by modified Hanks' solution containing 142.6 mM-NaCl, 5.6 mM-KCl, 2 mM- $CaCl_2$, 0.8 mM- $MgCl_2$, 5 mM-glucose and 10 mM-Hepes, buffered to pH 7.3 with NaOH. The cells were loaded with indo-1 by exposure to 5 μ M indo-1 acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) and 0.02% Pluronic F-127 (Molecular Probes) in Hanks' solution for 30 min at 37 °C. $[Ca^{2+}]_i$ was estimated from indo-1 fluorescence by the ratio method, by using single-wavelength excitation (340 nm) and dual-wavelength emission (405 and 480 nm) recorded by two separate photometers (model 614; Photon Technology International, South Brunswick, NJ, U.S.A.). Voltage signals corresponding to photon currents were fed into separate channels of an analog-to-digital converter (MacLab 8; Analog Digital Instruments, Castle Hill, N.S.W., Australia), and divided on-line so that the ratio $R (= F_{405}/F_{480})$ was viewed, stored and analysed (Scope V 3.1.2, A-D Instruments) by using a Macintosh IIfx computer. The ratio R was converted into $[Ca^{2+}]_i$ as described previously [13,14].

Solutions containing effectors were administered by pressure ejection from an external micropipette (2–5 μ m tip diameter), with the tip positioned within 10–20 μ m of the recorded cell. The concentrations reported are those in the micropipette. Vasoactive

Abbreviations used: VIP, vasoactive intestinal peptide; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration.

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intestinal peptide (VIP), CPT-cAMP, 3-isobutyl-1-methyl-xanthine (IBMX) and forskolin were purchased from Sigma.

Determination of cyclic AMP accumulation

Cyclic AMP formation in intact cells was generally measured by the method of Evans *et al.* [15]. Cells were incubated in F-10 medium (60 min, 36 °C) with approx. 2 μ Ci of [3 H]adenine per 10^6 cells to label the ATP pool, washed and equilibrated for 30 min at 36 °C. The cells were detached with phosphate-buffered saline (12.1 mM- Na_2HPO_4 , 4 mM- KH_2PO_4 , 130 mM-NaCl, pH 7.4) containing 0.04% EDTA, centrifuged for 4 min at 1000 rev./min in an IEC-HN-SII table-top centrifuge and resuspended in incubation buffer (110 mM-NaCl, 5.4 mM-KCl, 0.8 mM- MgSO_4 , 1.38 mM- CaCl_2 , 0.9 mM- NaH_2PO_4 , 25 mM-D-glucose, BSA 1 mg/ml). The suspension was divided into 900 μ l portions, each containing approx. 9×10^5 cells, and equilibrated for 10 min at 36 °C, followed by another preincubation for 10 min at 36 °C with 2 units of adenosine deaminase/ml and phosphodiesterase inhibitors (500 μ M-IBMX and 100 μ M Ro 20-1724). Cyclic AMP accumulation was initiated by addition of the test compounds (100 μ l) to the cell suspension for the periods indicated, and terminated by addition of 100% (w/v) trichloroacetic acid (50 μ l). Unlabelled cyclic AMP (100 μ l, 10 mM) was added, the mixture was centrifuged and the [3 H]ATP and [3 H]cyclic AMP contents of the supernatant were quantified [16]. The conversion of ATP into cyclic AMP was defined as: conversion = [3 H]cyclic AMP (c.p.m.)/[3 H]ATP (c.p.m.) + [3 H]cyclic AMP (c.p.m.).

In experiments that compared the absolute intracellular levels of cyclic AMP with those of CPT-cAMP, levels were measured by the cyclic AMP binding assay, as previously described [17]. Individual standard curves were carried out by using both cyclic AMP and CPT-cAMP to estimate the levels of the respective nucleotides.

RESULTS

CPT-cAMP triggers prolonged firing of [Ca^{2+}]_i transients in GH3/B6 cells

A cell-permeant analogue of cyclic AMP, CPT-cAMP, which is a potent activator of cyclic AMP-dependent protein kinase [18], was used to explore the involvement of cyclic AMP in [Ca^{2+}]_i homeostasis. CPT-cAMP was extremely active in inducing Ca^{2+} responses. In spontaneously active cells (9 out of 11 cells) the cyclic AMP analogue evoked an enhancement of [Ca^{2+}]_i spike frequency from 0.1 Hz to about 0.5 Hz within 30 s (Fig. 1a). The firing rate returned to pre-stimulated levels within 9–10 min of CPT-cAMP application. The [Ca^{2+}]_i transients upon CPT-cAMP stimulation were due to Ca^{2+} entry, since no [Ca^{2+}]_i change could be seen when the Ca^{2+} -channel blocker Cd^{2+} (500 μ M) was added to the bathing medium (results not shown). Furthermore, in silent cells, CPT-cAMP triggered a series of [Ca^{2+}]_i transients (12 out of 16 cells). In some of these silent cells the analogue evoked, after a short lag (10–40 s), a series of [Ca^{2+}]_i transients firing at a high rate (0.25–0.4 Hz) that lasted for 8–12 min (Fig. 1b). In the remainder, [Ca^{2+}]_i transients spiking at a lower rate (about 0.05 Hz) were triggered 2–3 min after CPT-cAMP application (Figs. 2a and 2b). However, a subsequent application of CPT-cAMP caused a prominent enhancement of [Ca^{2+}]_i spike frequency (0.5 Hz) after a short lag (20–30 s), so that the response appeared as a 'noisy' plateau rise in [Ca^{2+}]_i (Fig. 2b). The effectiveness of this analogue to evoke [Ca^{2+}]_i transients in GH3 cells prompted an exploration of the action of VIP on Ca^{2+} homeostasis, given that VIP was believed to act via the production of cyclic AMP.

Fig. 1. Effects of CPT-cAMP on [Ca^{2+}]_i transients in spontaneously active and silent GH3/B6 cells

At the time indicated by the horizontal bar, CPT-cAMP (0.5 mM) was applied from a micropipette positioned in the vicinity of (a) a cell showing spontaneous transient increases in [Ca^{2+}]_i and (b) a silent cell. Note that [Ca^{2+}]_i did not return to pre-stimulated levels during [Ca^{2+}]_i firing.

VIP stimulates bursts of [Ca^{2+}]_i transients in spontaneously active GH3/B6 cells pretreated with IBMX and forskolin

VIP applied at a high concentration (100 nM) evoked no significant alteration in [Ca^{2+}]_i in 47 cells tested, even in those showing spontaneous [Ca^{2+}]_i transients (Fig. 3a). In order to determine whether the generation of cyclic AMP in response to VIP was insufficient to mimic the action of CPT-cAMP on [Ca^{2+}]_i, measures were taken to enhance this effect.

In cells in which phosphodiesterase activity was inhibited by IBMX (500 μ M) pretreatment (5–40 min), VIP could modulate [Ca^{2+}]_i. In 11 out of 35 cells, a significant rise in [Ca^{2+}]_i was observed after stimulation with the peptide. Fig. 3(b) illustrates typical recordings from separate cells in which VIP evoked a prolonged enhancement of [Ca^{2+}]_i spiking activity. The response consisted of groups of [Ca^{2+}]_i transients firing at a high rate (referred to as [Ca^{2+}]_i bursts) separated by single [Ca^{2+}]_i transients firing at a lower frequency. It should be noted that all cells showing a VIP response had exhibited spontaneous [Ca^{2+}]_i firing before hormone stimulation.

Cells pretreated not only with IBMX (500 μ M), but also with forskolin at a sub-threshold concentration (1 μ M) to stimulate adenylate cyclase, showed increased spontaneous activity. In these cells, VIP evoked more profound alterations of [Ca^{2+}]_i spiking than in cells pretreated with IBMX alone. Indeed, VIP triggered long-lasting bursts of [Ca^{2+}]_i transients in spontaneously active cells (12 out of 23 cells) (Fig. 3c). [Ca^{2+}]_i bursts lasted for 2–4 min after VIP stimulation. Some bursts due to [Ca^{2+}]_i transients firing at high frequency appeared as a plateau-like increase in [Ca^{2+}]_i (results not shown). However, as noted above, VIP action in increasing [Ca^{2+}]_i, even after exposure to both

Fig. 2. Effects of sequential stimulations with CPT-cAMP on $[Ca^{2+}]_i$

(a) and (b) Two separate cells displaying a delayed and low-frequency $[Ca^{2+}]_i$ transient response on stimulation with CPT-cAMP (0.5 mM). In the cell shown in (b), a second application of CPT-cAMP was made at the second bar.

IBMX and forskolin, was limited to cells that showed spontaneous $[Ca^{2+}]_i$ transients before agonist stimulation.

Taken together, these findings seem to imply that the relative low efficiency of VIP to stimulate $[Ca^{2+}]_i$ rises in GH3/B6 cells is related to a limited production of cyclic AMP, rather than to an insignificant action of cyclic AMP on $[Ca^{2+}]_i$.

Higher levels of cyclic AMP are achieved with CPT-cAMP than with VIP

The above results, showing an increased efficacy of treatments that might be expected to elevate cyclic AMP to elicit progressively more efficient actions on $[Ca^{2+}]_i$, could suggest a tight linkage between the absolute levels of cyclic AMP achieved by various stimuli and their effects on $[Ca^{2+}]_i$. In order to evaluate this inference directly, measurements were made of the levels of cyclic AMP attained in response to various stimuli.

VIP elicits an increase of approx. 2-fold in cyclic AMP over 1 min under control conditions (Table 1). In the presence of the phosphodiesterase inhibitor IBMX, the stimulation is increased to 4-fold. When forskolin is also included, this response is enhanced an additional 4-fold (Table 1). Thus these cyclic AMP levels are progressively greater, with increasing degrees of stimulation. However, the percentage conversion of ATP into cyclic AMP is low. Furthermore, at 5 min, the levels of cyclic AMP are not well sustained (Table 1). These low levels of cyclic AMP, which agree with previous studies in these cells [12,19], may suggest that the endogenous cyclic AMP-generating system is poorly able to maintain levels of cyclic AMP. Detailed time courses were performed to evaluate the accumulation of cyclic AMP. Under the maximally effective stimulation condition, a very muted rate of increase in cyclic AMP is observed, which is maximal at 1 min (Fig. 4). This unusual cyclic AMP time course was explored further by comparing the intracellular levels of cyclic AMP achieved by VIP, IBMX and forskolin in com-

Fig. 3. Effects of VIP on $[Ca^{2+}]_i$ spiking activity in spontaneously active cells

VIP (100 nM) was applied to cells in which cyclic AMP production was either not primed (a) or primed by pretreatment in the bathing medium with 500 μ M-IBMX (b) or 500 μ M-IBMX plus 1 μ M-forskolin (c). Note that only cells showing spontaneous $[Ca^{2+}]_i$ transients would respond to VIP.

Table 1. Effects of forskolin, IBMX and VIP on cyclic AMP accumulation in GH3 cells

Cells were incubated for either 1 or 5 min in the absence or presence of 200 nM-VIP, 500 μ M-IBMX or 1 μ M-forskolin in the combinations indicated. The conversion of $[^3H]$ ATP into cyclic $[^3H]$ AMP was measured as outlined in the Materials and methods section (IBMX was not included in the 10 min preincubation). S.D. values of triplicate determinations are indicated in parentheses.

Condition	Conversion of ATP into cAMP (%)	
	1 min	5 min
Control	0.11 (0.01)	0.09 (0.01)
VIP	0.28 (0.03)	0.18 (0.01)
IBMX	0.19 (0.02)	0.19 (0.02)
IBMX + forskolin	0.49 (0.01)	0.58 (0.03)
IBMX + VIP	0.46 (0.01)	0.32 (0.02)
IBMX-VIP + forskolin	1.84 (0.06)	1.2 (0.02)

bination with exposure to CPT-cAMP over time. The permeant cyclic AMP analogue achieves a far greater (> 20-fold) level of cyclic AMP than can be achieved by stimulation of cyclic AMP synthesis (Fig. 4). This finding explains the high efficacy of this treatment in elevating $[Ca^{2+}]_i$, i.e. since CPT-cAMP achieves a 20-fold higher intracellular level of cyclic AMP than can be elicited by the most extreme stimulation of adenylate cyclase, it suggests that the VIP control of $[Ca^{2+}]_i$ in GH3 cells is limited by cyclic AMP generation, rather than by, for instance, the levels of cyclic AMP-dependent protein kinase.

Fig. 4. Levels of cyclic nucleotides achieved in GH3 cells over time

GH3 cells plated on 35 mm dishes were either stimulated with 200 nM-VIP, 1 μ M-forskolin and 500 μ M-IBMX in combination (○) or exposed to 1 mM-CPT-cAMP (●) for the times indicated. After incubation, the cells were rapidly washed with medium to remove extracellular cyclic nucleotides, before termination of the incubation with trichloroacetic acid. Cyclic nucleotide levels were measured by a cyclic-AMP-binding assay, as outlined in the Materials and methods section.

DISCUSSION

Both CPT-cAMP and hormones linked to cyclic AMP can increase $[Ca^{2+}]_i$ in individual GH3 cells. The nature of the responses explains why in studies of populations of these cells only modest effects of VIP are seen [12]; i.e. an increase in frequency of $[Ca^{2+}]_i$ transients can be induced in previously active cells. The blockade of the responses by Cd^{2+} , as well as the kinetic features of the Ca^{2+} transients, are consistent with these Ca^{2+} responses comprising action-potential-linked Ca^{2+} entry, as had been proposed from electrophysiological studies [20].

The level of cyclic AMP appears to be critical in determining whether a response is evoked; for instance, VIP alone, which elicits only a modest increment in cyclic AMP, could not elevate $[Ca^{2+}]_i$ in any cells tested. However, if the ability of VIP to increase cyclic AMP was augmented by inhibition of phosphodiesterase, or a sub-maximal stimulation of adenylate cyclase, a progressively greater likelihood of increasing $[Ca^{2+}]_i$ was encountered. Consistent with this indication was the finding that CPT-cAMP, which achieved sustained high levels of intracellular cyclic AMP, elicited dramatic and sustained elevation in $[Ca^{2+}]_i$.

The reason for the limited ability of GH3 cells to sustain accumulation of cyclic AMP is puzzling. In many intact cells, a robust stimulation of adenylate cyclase activity in plasma membranes is mirrored by linear increases in cyclic AMP accumulation, particularly upon inhibition of phosphodiesterase activity. In GH3 plasma membranes, VIP causes such a robust stimulation in adenylate cyclase activity. In a similar plasma-membrane preparation derived from another cell line, NCB-20 cells, stimulated adenylate cyclase activity is 70–90 pmol/min per mg, compared to 400–500 in GH3-cell membranes under similar assay conditions [17,21]. However, whereas the accumulation of cyclic AMP in NCB-20 cells is steady and linear with time, in GH3 cells this is severely curtailed. Therefore, it is a paradox of the present studies that VIP cannot elicit a sustained increase in cyclic AMP in intact cells, whereas it evokes a profound stimulation of adenylate cyclase in membranes. This deficiency

appears to underlie the impotence of VIP to elevate $[Ca^{2+}]_i$. The mechanism underlying this paradoxical effect on cyclic AMP remains elusive, but tantalizing. The general inhibitor of phosphodiesterases, IBMX, as used in the present studies, would be expected to combat endogenous phosphodiesterases. However, it is conceivable that rapid and, possibly co-operative, cyclic-AMP-stimulated degradation might be less susceptible to the phosphodiesterase inhibitor [22]. It is also conceivable that the levels of $[Ca^{2+}]_i$ under the plasma membrane are such that adenylate cyclase activity is chronically inhibited in these cells, given that GH3 cells express an adenylate cyclase activity that is inhibited by sub-micromolar Ca^{2+} [21]. Beyond these simple explanations, other mechanisms relating to structural constraints in the intact cell may yet be present. Whatever the mechanism, it seems clear that cyclic AMP is quite capable, when sufficient levels are achieved, of exerting profound effects on $[Ca^{2+}]_i$ in these cells. Therefore, as with the phospholipase C pathway, cyclic-AMP-mediated mechanisms can modulate $[Ca^{2+}]_i$ in GH3 cells. The use of analogues, such as CPT-cAMP, may permit the clarification of the details of the regulation.

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