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RESEARCH COMMUNICATION

Capacitative Ca^{2+} entry regulates Ca^{2+}-sensitive adenylyl cyclases

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A number of the currently described adenylyl cyclase species can be regulated by Ca^{2+} in the submicromolar concentration range in *in vitro* assays. The regulatory significance of these observations hinges on whether a physiological elevation in intracellular Ca^{2+} can regulate these cyclase activities in intact cells. However, achieving a physiological elevation in cytosolic Ca^{2+} is complicated by the fact that hormonal increases in cytosolic Ca^{2+} can be accompanied by additional effects, such as liberation of $\beta\gamma$-subunits of G-proteins and activation of protein kinase C, which can have disparate type-specific effects on cyclase activities. Therefore we have devised a strategy based on capacitative Ca^{2+} entry to show that, when types I and VI adenylyl cyclase are expressed in human embryonic kidney 293 cells, they are stimulated and inhibited respectively by Ca^{2+} entry. Blockade of Ca^{2+} entry by La^{3+} ions blocks the effects of Ca^{2+} entry on cyclic AMP synthesis. These studies establish that adenylyl cyclases deemed to be sensitive to Ca^{2+} in *in vitro* assays can be regulated by physiological Ca^{2+} entry, and therefore, such cyclases are poised to respond to changes in intracellular Ca^{2+} in tissues in which they are expressed.

INTRODUCTION

Ca^{2+}-sensitive adenylyl cyclases may provide an acute focus for interaction between the Ca^{2+} and cyclic AMP signalling systems. A Ca^{2+}-stimulated adenylyl cyclase has been implicated in learning and memory [1,2], whereas a Ca^{2+}-inhibited adenylyl cyclase has been proposed to yield a valuable negative feedback in cardiac function [3,4]. Two recently cloned adenylyl cyclases, types I and VI, could mediate these physiological effects, since (i) they can be stimulated and inhibited respectively by submicromolar Ca^{2+} in *in vitro* assays [5–7] and (ii) their mRNAs occur at high levels in hippocampus and cardiac tissue respectively [7–11]. In order to entertain the possibility that the Ca^{2+}-sensitivity of these adenylyl cyclases is the feature that confers their physiological effectiveness, it is essential to know whether a physiological elevation of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) would evoke the anticipated effects on cyclic AMP synthesis by these enzymes *in vivo*. Unfortunately, hormones, the intuitive choice to elevate $[Ca^{2+}]_i$, are likely to elicit additional effects, such as liberation of $\beta\gamma$-subunits of G-proteins and activation of protein kinase C, both of which exert disparate type-specific effects on adenylyl cyclases [12–16] that would render results obtained with hormones difficult to interpret. Ionophores, in yielding an unregulated elevation of $[Ca^{2+}]_i$ from indeterminate sources, do not mimic a physiological elevation of $[Ca^{2+}]_i$. In order to resolve this problem, we have developed an approach that is physiological and yet avoids the ambiguities associated with hormone action; we have isolated the Ca^{2+} entry that is stimulated by the emptying of intracellular Ca^{2+} pools ('capacitative Ca^{2+} entry'; [17]) to show that the entry of Ca^{2+} alone is sufficient to stimulate and inhibit respectively type I and VI adenylyl cyclases, when they are expressed in human embryonic kidney (HEK) 293 cells. This strategy not only establishes that Ca^{2+}-sensitive adenylyl cyclases are poised to respond to physiological changes in $[Ca^{2+}]_i$; it also provides a generally applicable method for evaluating putative Ca^{2+}-sensitivities *in vivo*.

METHODS

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was measured in an H&L series 300 spectrofluorimeter, essentially as described in [18], using the change in the 349/380 nm fluorescence ratio of fura-2. Briefly, cells were detached with PBS containing 0.02% EDTA and loaded with 2 μM fura-2 acetoxymethyl ester (AM) and 0.02% Pluronic F-127 for 20 min at room temperature. After washing, the cells were kept at room temperature until use. An aliquot ($\sim 4 \times 10^6$ cells) was diluted with 3 ml of Krebs buffer (composition in mM: NaCl, 120; KCl, 4.75; KH_2PO_4, 1; $NaHCO_3$, 5; $MgSO_4$, 1.44; $CaCl_2$, 1.1; EGTA, 0.1; glucose, 11; Hepes, 25; adjusted to pH 7.4 with Tris) containing 0.1% BSA, centrifuged, resuspended in 3 ml of Krebs buffer and transferred to a stirred cuvette, maintained at 30 °C. After equilibration for 1 min, a baseline was recorded, and additions were made by means of a Hamilton syringe that reached into the cuvette. Ratios were converted into $[Ca^{2+}]_i$ values as described in [18].

Plasmids

A mammalian expression vector pCMV-5 [19] was used to express adenylyl cyclases in transfected cells. The construction of pCMV-ACI, which contains the coding sequence of bovine type I adenylyl cyclase in pCMV-5, was described previously [6]. pCMV-ACVI, originally called pCMV-ACV, which contains the coding sequence of mouse type VI adenylyl cyclase in pCMV-5, was described previously [7].

Transfection of HEK cells

HEK 293 cells were maintained in 13 ml of minimum essential medium with 10% (v/v) fetal-bovine serum, penicillin (50 μg/ml), streptomycin (50 μg/ml) and neomycin (100 μg/ml) in plastic flasks (75 cm^2) at 37 °C in a humidified atmosphere of

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+}; Me_2SO, dimethyl sulphoxide; IBMX, 3-isobutyl-1-methylxanthine; HEK, human embryonic kidney; AM, acetoxymethyl ester.

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air/CO_2 (19:1). Transfection was performed by introduction of 26 μg of plasmid DNA into the flasks containing HEK 293 cells at about 50% confluency, as described by Chen and Okayama [20]. After transfection, the cells in each flask (75 cm^2) were harvested with PBS containing 0.02% EDTA and plated in three 35 mm-diameter wells of six-well culture plates using 3 ml of culture medium described above. This gave a confluent monolayer of cells without further growth. The cells were incubated for 2 days before cyclic AMP measurement.

Determination of intracellular cyclic AMP

Cyclic AMP formation in intact cells was measured as described previously, after labelling the intracellular ATP pool with [^{3}H]adenine [21]. Before incubation with test compounds, the cells were preincubated for 10 min at 37 °C with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 500 μM), unless specified otherwise. Test agents, or their solvent, dimethyl sulphoxide (Me_2SO), were added as indicated in the Figure legends. The reaction was terminated by addition of 100% (w/v) trichloroacetic acid (50 μl). For experiments in which $LaCl_3$ was to be used, a Ca^{2+}-, bicarbonate- and phosphate-free buffer was used (composition: 120 mM NaCl, 4.75 mM KCl, 1.44 mM $MgSO_4$, 11 mM glucose and 25 mM Hepes, adjusted to pH 7.4 with 2M Tris base, containing 0.1% BSA). Unlabelled cyclic AMP (100 μl, 10 mM), ATP (10 μl, 65 mM) and [α-^{32}]ATP (~ 5000 c.p.m.) were added to monitor recovery of cyclic AMP and ATP. After pelleting, the [^{3}H]ATP and [^{3}H]cyclic AMP contents of the supernatant were quantified according to the standard Dowex/alumina methodology [22]. The conversion of [^{3}H]ATP into [^{3}H]cyclic AMP was expressed as [^{3}H]cyclic AMP (c.p.m.)/{[^{3}H]ATP (c.p.m.) + [^{3}H]cyclic AMP (c.p.m.)}. The data were expressed as means ± S.D. for three separate determinations. Results presented are representative of two or three similar experiments.

RESULTS AND DISCUSSION

The Ca^{2+} entry that is promoted either by the microsomal Ca^{2+}-ATPase inhibitor thapsigargin [23] or the muscarinic-receptor agonist carbachol was compared in order to validate the use of thapsigargin in further experiments, as outlined below (Figure 1). Carbachol evokes a biphasic $[Ca^{2+}]_i$ rise; an initial rapid rise, presumably reflecting release of intracellular stores, is followed by a prolonged entry phase (Figure 1a). The release and entry phase can be observed separately by initially including EGTA in the extracellular medium and subsequently introducing an excess of $CaCl_2$ (Figure 1b). Under such conditions, a sharp spike in $[Ca^{2+}]_i$ is elicited by carbachol; thereafter, a low basal level of $[Ca^{2+}]_i$ is maintained until the addition of excess $CaCl_2$, which results in a rapid burst of Ca^{2+} entry. Thapsigargin elicits a slow initial elevation in $[Ca^{2+}]_i$, resulting from emptying of the Ca^{2+} stores [24,25], which is accompanied by a sustained elevation in $[Ca^{2+}]_i$ (Figure 1c). Thapsigargin depletes the stores accessible to carbachol, since addition of carbachol subsequent to thapsigargin cannot elicit a $[Ca^{2+}]_i$ rise (Figure 1c). These data show that carbachol and thapsigargin access a common intracellular store of Ca^{2+}. When EGTA is added during the sustained $[Ca^{2+}]_i$ elevation elicited by thapsigargin, $[Ca^{2+}]_i$, levels immediately decrease and remain low until an excess of $CaCl_2$ is added to the external medium, upon which there is a rapid, capacitative Ca^{2+} influx (Figure 1d; [17,24,25]). This response is very similar to that elicited by carbachol (cf. Figure 1b). The apparent rate of Ca^{2+} entry can be accelerated by increasing the extracellular concentration of $CaCl_2$ in this latter experiment (Figure 2).

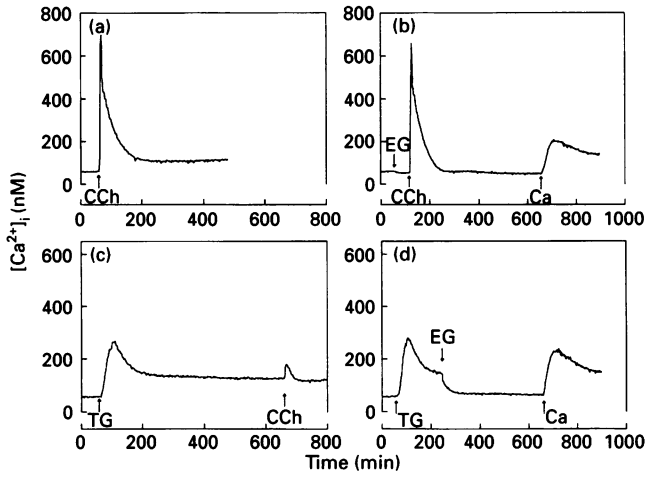

Figure 1 Comparison of the effects of carbachol (CCh) and thapsigargin (TG) on $[Ca^{2+}]_i$ in HEK 293 cells

$[Ca^{2+}]_i$ levels were measured in a suspension of ~ 4×10^6 HEK 293 cells, utilizing the fluorescent properties of fura-2 in a Krebs medium containing 1.1 mM $CaCl_2$, as described in the Methods section. (**a**) Carbachol (0.1 mM) was added at the arrow; (**b**) EGTA (EG) (2.5 mM) was added at the first arrow, followed by carbachol (0.1 mM; second arrow) and 3 mM $CaCl_2$ (Ca) (third arrow); (**c**) thapsigargin (1 μM) was added at the first arrow, followed by carbachol (0.1 mM; second arrow); (**d**) thapsigargin (1 μM) was added (first arrow) followed by EGTA (2.5 mM; second arrow) followed by 3 mM $CaCl_2$. (Note: the regulation of Ca^{2+} homoeostasis by carbachol and thapsigargin was identical in untransfected and transfected cells; results not shown.)

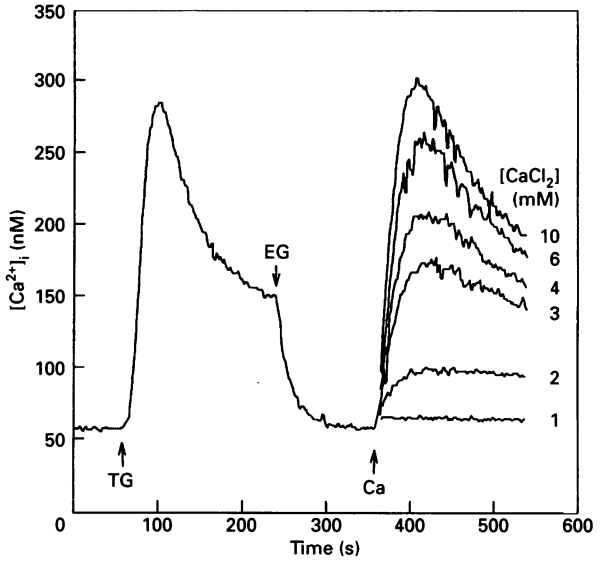

Figure 2 Effects of increasing concentrations of extracellular $CaCl_2$ on Ca^{2+} entry

Thapsigargin (TG; 1 μM) was added (first arrow) followed by EGTA (EG). (2.5 mM; second arrow) followed by the $CaCl_2$ (Ca) concentrations indicated (third arrow), and $[Ca^{2+}]_i$ was measured as described in Figure 1. The data from six separate runs were compiled for presentation purposes beyond the 360 s time point. The data presented over the first 360 s are from one of the runs, all of which were virtually identical. [Note: the regulation of Ca^{2+} homoeostasis by thapsigargin was identical in control cells, control-transfected cells and cells transfected with adenylyl cyclase cDNAs (R. Mahey, K. Fagan and D. M. F. Cooper, unpublished work).]

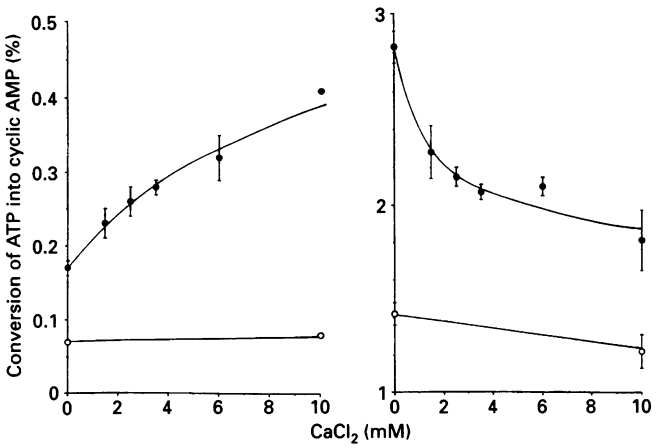

Figure 3 Effects of increasing $CaCl_2$ concentrations on cyclic AMP accumulation in cells transfected with type I and VI adenylyl cyclase cDNAs

Cells transfected with either the mammalian expression vector pCMV-5 (○) or this vector encoding either type I adenylyl cyclase, pCMV-ACI (●, **a**), or type VI adenylyl cyclase, pCMV-ACVI (●; **b**), respectively were pretreated with 1 μM thapsigargin and 500 μM IBMX for 3 min, followed by 2.5 mM EGTA for 12 min. Cyclic AMP accumulation, stimulated by either 10 μM forskolin (**a**) or 10 μM forskolin plus 10 μM prostaglandin E_1 (**b**) in the presence of the indicated added concentrations of $CaCl_2$, was measured for 1 min. (Note: the medium bathing the cells contained 1.1 mM $CaCl_2$ prior to the addition of EGTA; see the Methods section.) The data are triplicate determinations from one of three similar experiments. All of the values determined in the presence of added $CaCl_2$ are significantly different ($P < 0.01$) from values without added $CaCl_2$.

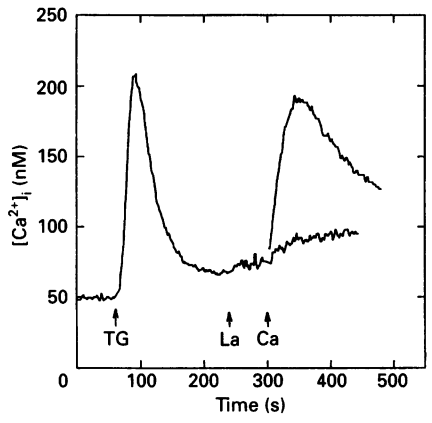

Figure 4 Effect of $LaCl_3$ (La) on thapsigargin (TG)-mediated Ca^{2+} entry

$[Ca^{2+}]_i$ was measured in HEK 293 cells, as described in Figure 1, except that a Ca^{2+}-free Krebs buffer was used as described in the Methods section. Thapsigargin (TG; 1 μM) was added at the first arrow, followed by 3 mM $CaCl_2$ (Ca) with (lower trace) or without (upper trace) 100 μM $LaCl_3$, as indicated (second arrow). The data from two separate runs are combined beyond 300 s for clarity of presentation. The data for the first 300 s were virtually identical in both runs.

Although these are non-physiological extracellular levels of $CaCl_2$, they can be exploited to accelerate the rate and extent of initial Ca^{2+} entry (note that the $[Ca^{2+}]_i$ achieved by these $CaCl_2$ concentrations is still well within the intracellular physiological range).

Since the Ca^{2+} entry induced by thapsigargin followed by EGTA (as in Figures 1d and 2) was so similar to the Ca^{2+}-entry process mediated by carbachol (e.g. Figure 1b), these conditions were used to determine the effects of $[Ca^{2+}]_i$ elevation on adenylyl cyclase activity, without any of the repercussions associated with

Table 1 Ability of La^{3+} to attenuate effects of Ca^{2+} entry on cyclic AMP accumulation in cells transfected with types I and VI adenylyl cyclase cDNAs

Cells transfected (as in Figure 3) with plasmids encoding either type I or type VI adenylyl cyclase were pretreated with 1 μM thapsigargin and 500 μM IBMX for 15 min, in a Krebs buffer that did not contain added $CaCl_2$, bicarbonate or phosphate salts (see the Methods section). Cyclic AMP accumulation, stimulated by 10 μM forskolin (type I) or 10 μM forskolin plus 10 μM prostaglandin E_1 (type VI) in the presence or absence of 3 mM $CaCl_2$ and 0.1 mM $LaCl_3$, as indicated, was measured subsequently over 1 min. [There was no effect of either $CaCl_2$ or $LaCl_3$ in control transfected cells, in which cyclic AMP synthesis was 0.12% (10 μM forskolin) and 2.3% (10 μM forskolin plus 10 μM prostaglandin E1 respectively).] The data are triplicate determinations in a representative of four similar experiments.

		Conversion of ATP into cyclic AMP (%)	
Adenylyl cyclase	$[LaCl_3]$ (mM)	No addition	Plus 3 mM $CaCl_2$
Type I	0	0.28 ± 0.03	0.90 ± 0.04
	0.1	0.29 ± 0.01	0.41 ± 0.02
Type VI	0	5.47 ± 0.28	4.18 ± 0.18
	0.1	5.89 ± 0.31	5.97 ± 0.26

hormone-receptor occupancy. cDNAs encoding type I and type VI adenylyl cyclase were transfected into HEK 293 cells, and the effect of the thapsigargin-promoted Ca^{2+} entry on cyclic AMP accumulation was determined (Figure 3). Increasing extracellular $CaCl_2$, subsequent to the addition of thapsigargin and EGTA, which results in increased levels of $[Ca^{2+}]_i$ (see Figure 2), exerts prominent effects on cyclic AMP synthesis in transfected cells. The synthesis of cyclic AMP in cells expressing type I adenylyl cyclase activity is stimulated by at least 2-fold, whereas synthesis is inhibited by > 30% in cells expressing type VI activity (Figure 3). These effects of elevating $[Ca^{2+}]_i$ are similar to the effects of Ca^{2+} on these adenylyl cyclases in plasma-membrane preparations in *in vitro* assays [6,7]. {Acute treatment of transfected cells with carbachol (1 mM) for 1 min in Ca^{2+}-containing medium also significantly stimulated cyclic AMP accumulation in cells expressing type I adenylyl cyclase and elicited a modest, but significant, inhibition of cyclic AMP accumulation in cells expressing type VI adenylyl cyclase (results not shown). Such results are as would be anticipated if the effects of carbachol were mediated by a rise in $[Ca^{2+}]_i$; however, for the reasons discussed above, they do not exclude the participation of other processes.}

La^{3+} ions were used to confirm that the entry of Ca^{2+}, as such, was required for the observed regulation of transfected adenylyl cyclases. These experiments were performed in nominally Ca^{2+}-free medium, since EGTA cannot be used in combination with La^{3+}. Ca^{2+} entry is promoted in this medium simply by treating with thapsigargin to deplete stores. Thus, in nominally Ca^{2+}-free medium, thapsigargin causes an elevation of $[Ca^{2+}]_i$ that returns to baseline within 3 min, reflecting the net release from intracellular stores and extrusion into the medium (Figure 4). Reintroduction of $CaCl_2$ to the medium causes a rapid entry of Ca^{2+}. This entry can be blocked by inclusion of 100 μM $LaCl_3$ (Figure 4, lower trace). These conditions were then used to determine whether the effects of Ca^{2+} entry on cyclic AMP accumulation could also be blocked by $LaCl_3$.

Cyclic AMP synthesis was stimulated (by ~ 3-fold) and inhibited (by ~ 25%) in HEK 293 cells transfected with types I or VI adenylyl cyclase respectively in response to Ca^{2+} entry under conditions that were directly analogous to those used in Figure 4 (Table 1). Inclusion of $LaCl_3$ markedly attenuated both the stimulation of type I and the inhibition of type VI. This result

strongly supports the contention that Ca^{2+} entry as such regulates transfected adenylyl cyclases.

The results presented demonstrate that an elevation of $[Ca^{2+}]_i$ alone is sufficient to regulate Ca^{2+}-sensitive adenylyl cyclases, without the involvement of any process linked to Ca^{2+}-mobilizing hormones. However, it might be argued that even the present, non-hormonal, means of elevating $[Ca^{2+}]_i$ might activate protein kinase C indirectly, by stimulating phospholipid hydrolysis, leading to diacylglycerol production [25]. However, in separate studies, we and others have demonstrated that protein kinase C, while it can stimulate type II adenylyl cyclase, is without effect on types I and VI [14–16].

The simplest means of elevating $[Ca^{2+}]_i$ is by the use of ionophores. However, the elevation of $[Ca^{2+}]_i$ achieved by ionophores is unlikely to duplicate the kinetics, intracellular distribution, concentration or action of physiologically elevated $[Ca^{2+}]_i$ [26]. In contrast, the Ca^{2+} entry provoked by thapsigargin-mediated store depletion reflects both the normal cellular Ca^{2+}-storage capacity and the routes of Ca^{2+} entry [17,23,24,27]. Thus the thapsigargin-based approach is an instructive strategy for evaluating the intracellular consequences of a discrete facet of hormonal elevation of $[Ca^{2+}]_i$. This approach could profitably be applied to other situations in which intracellular actions of Ca^{2+} are investigated, given that both the ambiguity associated with the use of hormones and the artefacts involved with the use of ionophores are avoided, while a critical aspect of cellular handling of Ca^{2+} is preserved. (Using the same strategy, we find that type V adenylyl cyclase, which is closely related to type VI [9–11], can also be inhibited by Ca^{2+} entry [28].)

Overall, these results show that capacitative Ca^{2+} entry, by itself, is *sufficient* to stimulate or inhibit type I and type VI adenylyl cyclase respectively. In the future it may be of interest to determine whether Ca^{2+} entry (rather than internal release) is *required* to regulate these adenylyl cyclases in cells that naturally express these activities. This issue is raised to resolve two alternative explanations for the sensitivity of Ca^{2+}-regulated adenylyl cyclases to Ca^{2+} entry: (i) it is possible that the high local concentrations of Ca^{2+} achieved around Ca^{2+}-entry channels, so-called 'I_{CRAC}s' [29], are necessary to regulate plasma-membrane-localized adenylyl cyclases; (ii) it is also conceivable that adenylyl cyclases themselves, given their transport-like structure [5], may possess the weak Ca^{2+}-conducting activities of I_{CRAC}s [28] and by such means be intimately sensitive to Ca^{2+} entry. For the present it may be asserted unambiguously that physiological changes in $[Ca^{2+}]_i$ will mediate changes in cyclic AMP synthesis in tissues that express Ca^{2+}-sensitive species of adenylyl cyclase.

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