RESEARCH COMMUNICATION

Predominant expression of type-VI adenylate cyclase in C6-2B rat glioma cells may account for inhibition of cyclic AMP accumulation by calcium

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In C6-2B cells, agonist-stimulated cyclic AMP accumulation is inhibited when the cytosolic Ca^{2+} concentration is increased. We now demonstrate that in C6-2B cells: (i) the early kinetics of the cyclic AMP inhibition by substance K ($t_{1/2} = 35$ s) and thapsigargin ($t_{1/2} = 1.6$ min) closely mimic the kinetics of the cytosolic Ca^{2+} increase evoked by either agent ($t_{1/2} = 25$ s and 1.5 min respectively); (ii) the Ca^{2+} rise and cyclic AMP inhibition by substance K or thapsigargin are similarly affected in EGTA-

containing medium; (iii) PCR detects type-III and type-VI adenylate cyclase cDNAs, and RNAase protection assays show that the mRNA for type-VI adenylate cyclase, an isoform inhibitable by submicromolar Ca^{2+} concentrations, is the predominant species, strongly suggesting that type-VI adenylate cyclase is probably the target molecule for Ca^{2+} -mediated inhibition of cyclic AMP accumulation.

INTRODUCTION

Adenylate cyclase activity in plasma membranes from a variety of sources [1–9] is inhibited by submicromolar Ca^{2+} concentrations and not affected by exogenous calmodulin [4,5,9]. In intact C6-2B (rat glioma) and NCB-20 (mouse neuroblastoma × Chinese hamster brain explant) cells, increased cytosolic free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) following activation of phospholipase C (PLC)-coupled receptors, such as substance K (SK), bradykinin and ATP receptors, cause inhibition of agonist-stimulated cyclic AMP production, thus suggesting a physiological significance to the observation *in vitro* described above [5–8]. The data accumulated from C6-2B and NCB-20 cells, taken together, support the hypothesis that the catalytic unit of adenylate cyclase is the target for the inhibition of cyclic AMP accumulation observed in these cells upon elevation of $[\text{Ca}^{2+}]_i$ [5–8].

Recently, a novel adenylate cyclase (type VI) has been cloned from NCB-20 cells [10]. Among all the adenylate cyclases identified so far [11 and references therein], the type-VI enzyme is the only subtype whose activity is inhibited by submicromolar Ca^{2+} concentrations [10].

Because in C6-2B cells the early kinetics of cyclic AMP inhibition by Ca^{2+} -elevating agents have not been compared with the kinetics of the rise in $[\text{Ca}^{2+}]_i$, and in light of the recent cloning of type-VI adenylate cyclase [10], we asked the following questions: (i) is the inhibition of cyclic AMP accumulation indeed temporally correlated with and dependent upon the initial $[\text{Ca}^{2+}]_i$ rise evoked by Ca^{2+} -mobilizing agents in C6-2B cells?, and (ii) is an adenylate cyclase isoform inhibitable by submicromolar Ca^{2+} concentrations expressed in these cells? We now report that, in C6-2B cells, the inhibition of isoprenaline (ISO, a β -adrenergic-receptor agonist)-stimulated cyclic AMP accumulation by SK and the microsomal Ca^{2+} -ATPase inhibitor thapsigargin (TG) is rapid in onset, with kinetics which closely mimic and strictly depend on the $[\text{Ca}^{2+}]_i$ increase characteristic of either agent.

Evidence is also provided showing a striking abundance of the mRNA coding for type-VI adenylate cyclase, suggesting that this isoform may account for the inhibitory effect of Ca^{2+} on cyclic AMP accumulation observed in C6-2B cells.

MATERIALS AND METHODS

Materials

SK was obtained from Peninsula Laboratories, Belmont, CA, U.S.A., TG from Research Biochemical Incorporated, Natick, MA, U.S.A., Ro20-1724 from Biomol, Plymouth Meeting, PA, U.S.A., and fura-2AM from Molecular Probes, Eugene, OR, U.S.A.; other chemicals were from Sigma.

Cyclic AMP measurement

C6-2B cells, wild type or stably transfected with the SK-receptor cDNA (A2-3 clone), were cultured as previously described [7,8]. After incubations with drugs at 37 °C in 20 mM Hepes-buffered, serum-free Ham's F-10 medium (GIBCO BRL, Gaithersburg, MD, U.S.A.) (pH 7.4; 0.3 mM CaCl_2) containing 100–500 μM 3-isobutyl-1-methylxanthine and 100 μM Ro20-1724 to prevent cyclic AMP breakdown, cyclic AMP content was measured using the Atto-Flo automated radioimmunoassay system (Atto Instruments Inc., Rockville, MD, U.S.A.). Protein was measured by the method of Bradford [12].

Digital fluorescence microscopy

Single-cell Ca^{2+} imaging was performed as described previously [13] using an Attofluor digital fluorescence microscopy system (Atto Instruments Inc.). Cells were loaded with the acetoxymethyl ester of fura-2 (fura-2AM, 5 μM) for 30 min at 37 °C, washed and imaged at 30–32 °C in Ham's F-10 medium buffered with 20 mM Hepes (pH 7.4).

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; SK, substance K; TG, thapsigargin; ISO, isoprenaline; PLC, phospholipase C.

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PCR and RNAase protection assay

Poly(A)⁺ RNA was prepared from C6-2B cells by using the FastTrak mRNA isolation kit (Invitrogen). PCR was performed using degenerate primers *adc4* and *adc5*, as described previously [10]. The identity of isolated clones was confirmed by DNA sequencing [14]. ³²P-labelled antisense RNA probes were prepared using isolated cDNA clones corresponding to type III and type VI in pBluescriptII SK (–) (Stratagene) and T3 RNA polymerase. The specific radioactivity of the probes was about 3×10^8 c.p.m./ μ g. Hybridization and RNAase digestion were carried out using a commercial kit (Ambion) according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Early kinetics of SK- and TG-mediated inhibition of cyclic AMP accumulation

In previous studies, C6-2B cells, wild type or stably transfected with the SK-receptor cDNA (A2-3 clone), were exposed to SK or TG for 2–5 min and then challenged with ISO or forskolin; the cyclic AMP accumulation measured after a standard incubation of 10–20 min was found to be markedly inhibited [7,8]. Single-cell Ca^{2+} imaging has shown that C6-2B_{A2-3} cells respond to SK with a sharp rise in $[\text{Ca}^{2+}]_i$ ($\text{EC}_{50} = 5\text{--}10$ nM), characterized by fast kinetics, such that $[\text{Ca}^{2+}]_i$ peaks at between 30 s and 1 min after the application of SK and returns to resting levels within 5 min [7,8]. On the other hand, the $[\text{Ca}^{2+}]_i$ increase evoked by TG ($\text{EC}_{50} = 24.8 \pm 4.3$ nM) is slower in both onset and decay: $[\text{Ca}^{2+}]_i$ peaks at 2–3 min and rarely returns to the resting level, but rather stabilizes slightly above the baseline level [8]. To characterize clearly the temporal relationship between the increase in $[\text{Ca}^{2+}]_i$ and inhibition of cyclic AMP accumulation, the effects of SK and TG on the early kinetics of ISO-stimulated cyclic AMP formation were now investigated. In the presence of 1 μ M SK (IC_{50} for SK-mediated cyclic AMP inhibition is 5–10 nM [7]), inhibition of cyclic AMP accumulation was detected at as early as 30 s, peaked at 5 min and remained stable up to 30 min (Figure 1a). A detailed analysis of the early kinetics (30 s to 5 min, Figure 1c) of the cyclic AMP inhibition caused by SK, from three separate experiments, showed an apparent $t_{1/2}$ of 35 s, consistent with the fast kinetics of the $[\text{Ca}^{2+}]_i$ rise produced by SK ($t_{1/2}$ for SK-induced peak of $[\text{Ca}^{2+}]_i$ increase is approx. 25 s; see Figure 2a, open diamonds). On the other hand, the inhibition of ISO-induced cyclic AMP accumulation by 1 μ M TG (IC_{50} for TG-mediated cyclic AMP inhibition is 19.3 ± 0.2 nM [8]) required 2 min to develop and, similar to the inhibitory effect elicited by SK, reached the maximum at 5 min and persisted up to 30 min (Figure 1b). From early kinetic analysis (Figure 1c), TG was found to inhibit cyclic AMP formation with an apparent $t_{1/2}$ of 1.6 min, which very likely reflects the slower kinetics of the $[\text{Ca}^{2+}]_i$ response evoked by this agent ($t_{1/2}$ for TG-induced peak of the $[\text{Ca}^{2+}]_i$ increase is approx. 1.5 min; see Figure 2b, open diamonds).

Thus, these results clearly show that the kinetics of the early cyclic AMP inhibition by SK or TG closely mimic those displayed by the initial $[\text{Ca}^{2+}]_i$ rise characteristic of each agent. The rapid onset of the Ca^{2+} -mediated inhibition of cyclic AMP accumulation in C6-2B cells, as well as in NCB-20 cells [5,6], supports the hypothesis that $[\text{Ca}^{2+}]_i$, once elevated to submicromolar concentrations, rapidly and, perhaps directly, acts to inhibit adenylate cyclase. However, an interesting feature of C6-2B cells is the prolonged duration of the Ca^{2+} -mediated inhibition of cyclic AMP formation (Figures 1a and 1b), such that cyclic AMP

synthesis remains depressed after $[\text{Ca}^{2+}]_i$ returns to resting values (Figure 2, open diamonds). A sustained $[\text{Ca}^{2+}]_i$ elevation, localized at the site of adenylate cyclase (yet not detectable with the standard fura 2 AM Ca^{2+} imaging resolution), could account for

Figure 1 Effect of TG on the time course of ISO-stimulated cyclic AMP accumulation

C6-2B cells, A2-3 clone (a) or wild type (b), were challenged with 10 μ M ISO in the presence of vehicle (open symbols), 1 μ M SK (a, closed squares) or 1 μ M TG (b, closed triangles), and cellular cyclic AMP content was measured at the indicated time points (0–30 min) as described in the Materials and methods section. Insets: the cyclic AMP accumulation determined at 0–2 min in the same experiment is shown. Results are the mean $\pm$ S.E.M. from a representative experiment, performed in triplicate. (c) Mean $\pm$ S.E.M. values of the percentage of inhibition by SK (closed squares) and TG (closed triangles) of ISO-stimulated cyclic AMP accumulation at early time points (30 s to 5 min) from three independent experiments performed in triplicate.

Figure 2 Kinetics of SK- and TG-induced $[Ca^{2+}]_i$ transients in the presence and absence of nominal extracellular Ca^{2+}

C6-2B_{A2.3} cells loaded with fura2-AM were exposed to 1 μ M SK (a) or 1 μ M TG (b) in standard incubation medium (Ham's F-10/Hepes) in the absence (open diamonds) or presence of 2 mM EGTA (closed triangles). A separate coverslip was pre-incubated for 15 min in 2 mM EGTA-containing medium and SK or TG were then added in the presence of the Ca^{2+} chelator (open squares). Single-cell Ca^{2+} imaging was performed as described in the Materials and methods section. Results represent the population mean of $[Ca^{2+}]_i$ from a representative experiment which was repeated once (b) or twice (a) with similar results. Nine to 24 cells per coverslip were imaged and 4–6 coverslips per incubation condition were used. The mean $\pm$ S.E.M. of peak $[Ca^{2+}]_i$ (expressed as $\Delta [Ca^{2+}]_i$; net $[Ca^{2+}]_i$ increase above the resting levels) from 2–3 experiments is shown in Table 1.

the sustained cyclic AMP inhibition. Alternatively, such a long-lasting effect could suggest the involvement of an intermediate factor whose activation, triggered by a transient $[Ca^{2+}]_i$ increase, leads to persistent inhibition of adenylate cyclase. These possibilities are currently being explored.

Having established that SK- and TG-induced $[Ca^{2+}]_i$ responses are temporally related to the onset of inhibition of cyclic AMP accumulation, we then addressed the question as to whether the initial $[Ca^{2+}]_i$ rise evoked by either agent was indeed the event triggering cyclic AMP inhibition. To this end, Ca^{2+} imaging was performed on fura 2-AM-loaded C6-2B_{A2.3} cells exposed to 1 μ M SK or 1 μ M TG in (a) standard incubation medium (Ham's F-10/Hepes, pH 7.4, containing 0.3 mM $CaCl_2$), (b) medium supplemented with 2 mM EGTA, or (c) medium as in (b) after a 15 min pre-incubation in 2 mM EGTA-containing medium (Figure 2). As one would expect from the activation of PLC-coupled

Table 1 Effect of depletion of extracellular Ca^{2+} on SK- and TG-evoked $[Ca^{2+}]_i$ increase and inhibition of cyclic AMP accumulation

C6-2B_{A2.3} cells, loaded with fura 2-AM, were challenged with 1 μ M SK or 1 μ M TG and used for Ca^{2+} imaging. Parallel cultures were challenged with 10 μ M ISO for 5 min in the absence (control) or presence of 1 μ M SK or 1 μ M TG and cellular cyclic AMP was measured.

Incubation conditions*	$\Delta [Ca^{2+}]_i$ † (nM)	ISO-stimulated cyclic AMP formation‡ (pmol/mg of protein)
A Control	—	700 $\pm$ 28
SK	383 $\pm$ 49	504 $\pm$ 14
TG	268 $\pm$ 45	461 $\pm$ 29
B Control	—	686 $\pm$ 13
SK	311 $\pm$ 49	523 $\pm$ 28
TG	67 $\pm$ 15	770 $\pm$ 87
C Control	—	676 $\pm$ 23
SK	94 $\pm$ 12	749 $\pm$ 47
TG	58 $\pm$ 4	805 $\pm$ 63

* A: standard medium; B and C: 2 mM EGTA-containing medium; C: cells were pre-incubated for 15 min in 2 mM EGTA-supplemented medium before drug exposure.

† Net $[Ca^{2+}]_i$ increase above the resting levels (31–48 nM); data are the mean $\pm$ S.E.M. from 2–3 experiments each with two coverslips.

‡ Basal cell cyclic AMP levels were 21–47 pmol/mg of protein. Data are the mean $\pm$ S.E.M. from one representative experiment done in triplicate; three other experiments gave similar results.

receptors, which causes the release of Ca^{2+} from internal stores, the application of SK in Ca^{2+} -depleted medium resulted in a transient rise in $[Ca^{2+}]_i$ with a magnitude comparable with that recorded in Ca^{2+} -containing medium (Figure 2a and Table 1). However, the decay kinetics were much faster in Ca^{2+} -depleted medium than in Ca^{2+} -containing medium, consistent with the notion that a large component of the decay phase of the $[Ca^{2+}]_i$ rise which follows an initial emptying of the stores is due to capacitative Ca^{2+} entry [15]. When the cells were pre-incubated for 15 min in Ca^{2+} -depleted medium, the peak $[Ca^{2+}]_i$ increase elicited by SK was remarkably reduced both in magnitude and duration (Figure 2a and Table 1), probably reflecting a progressive emptying of the internal stores following chelation of extracellular Ca^{2+} . Application of 1 μ M TG in Ca^{2+} -containing medium caused an increase in $[Ca^{2+}]_i$ (Figure 2b) with kinetics similar to those previously described [8]. On the other hand, when TG was applied to the cells in the presence of EGTA, either simultaneously or after a 15 min pre-incubation in Ca^{2+} -depleted medium, the rise in $[Ca^{2+}]_i$ was dramatically reduced (Figure 2b and Table 1). Using a similar paradigm in the biochemical studies, the inhibition by SK of ISO-stimulated cyclic AMP accumulation was found to be comparable in Ca^{2+} -containing and Ca^{2+} -depleted medium, consistent with the ability of SK to evoke a similar peak $[Ca^{2+}]_i$ increase under either incubation condition (Table 1, incubation conditions A and B). Pre-incubation for 15 min in 2 mM EGTA-containing medium, which significantly reduced the SK-evoked $[Ca^{2+}]_i$ increase, abolished the inhibitory effect of SK on ISO-induced cyclic AMP accumulation (Table 1, incubation condition C). TG markedly inhibited the cyclic AMP synthesis stimulated by ISO in standard medium, but it was ineffective in Ca^{2+} -depleted medium, consistent with its impaired ability to increase $[Ca^{2+}]_i$ under these conditions (Table 1).

Thus, the early kinetics of the SK- or TG-induced inhibition of cyclic AMP formation indeed share the same temporal profile

Figure 3 Expression of type-III and type-VI adenylate cyclase mRNAs

Poly(A)⁺ RNA (4 μ g) from C6-2B cells was analysed using 3×10^5 c.p.m. of antisense RNA probes prepared from type-III and type-VI cDNA clones. The control RNAase digestion was carried out with 10 μ g of yeast RNA. After electrophoresis on polyacrylamide gel containing 8 M urea, autoradiograms were obtained by exposure for 3 h at -70°C . Type-III probe was used in lanes 1 to 3: lane 1, probe; lane 2, yeast RNA; lane 3, C6-2B cell RNA. Type-VI probe was used in lanes 4 to 6: lane 4, probe; lane 5, yeast RNA; lane 6, C6-2B cell RNA. (Exposure of the gel for 18 h revealed a small signal in lane 3, indicating the presence of trace amounts of type-III mRNA.)

and Ca^{2+} requirements of the Ca^{2+} response characteristic of each agent, providing strong evidence that the peak $[\text{Ca}^{2+}]_i$ increase is the trigger for the SK- or TG-mediated inhibition of the cyclic AMP signalling pathway in C6-2B cells.

Identification of adenylate cyclase molecular forms in C6-2B cells

For PCR analysis, degenerate oligonucleotides (adc4 and adc5 [10]), designed against a conserved region in the second cytoplasmic domain in three mammalian adenylate cyclases (types I [16], II [17], and III [18]), were used to amplify cDNA from C6-2B poly(A)⁺ RNA. Nucleotide sequence analysis performed on 12 cDNA clones isolated after PCR revealed that nine clones were 95% identical to the mouse type-VI sequence from NCB-20 cells [10] and 100% identical to the recently published rat type-VI adenylate cyclase sequence [19], the other three clones were 100% identical to rat type-III sequence [18] (results not shown). Finally, RNAase protection assay using type-III and type-VI probes revealed that type-VI adenylate cyclase mRNA is by far the major species expressed in C6-2B cells (Figure 3). Type-VI adenylate cyclase has been shown to be most abundant in heart [10,20], wherein sarcolemma adenylate cyclase activity is also sensitive to inhibition by low Ca^{2+} concentration [4,21]. Thus, it is likely that the high degree of expression of type-VI

adenylate cyclase, inhibitable by submicromolar Ca^{2+} concentrations [10], accounts for the profound inhibitory effect exerted by Ca^{2+} on cyclic AMP formation in C6-2B cells [7,8]. By analogy, the apparently modest cyclic AMP inhibition observed in NCB-20 cells upon hormone-induced $[\text{Ca}^{2+}]_i$ elevation [5,6] could represent the net result of the effects of Ca^{2+} on the three different adenylate cyclases (types III, IV, and VI) present in these cells [10].

In conclusion, in this study, we (i) provide strong evidence for a temporal as well as causal relationship between the early rise in $[\text{Ca}^{2+}]_i$ and the onset of inhibition of cyclic AMP formation in C6-2B cells, and (ii) propose C6-2B cells, by virtue of their abundant and predominant expression of type-VI adenylate cyclase, as a useful model to investigate the mechanism(s) whereby Ca^{2+} negatively regulates this particular form of the enzyme.

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