Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9257–9261, October 1991
Biochemistry

Inhibition of cAMP accumulation by intracellular calcium mobilization in C6-2B cells stably transfected with substance K receptor cDNA

(single-cell calcium imaging/fura-2 fluorescence microscopy/adenylate cyclase/bradykinin/cellular heterogeneity)

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Communicated by Erminio Costa, July 16, 1991

ABSTRACT **C6-2B rat glioma cells were stably transfected with substance K receptor cDNA and used to study interactions between cAMP and Ca^{2+} signaling pathways. Activation of the newly expressed receptors by substance K increased the intracellular free Ca^{2+} concentration, as monitored by single-cell fura-2 imaging, and markedly inhibited agonist-stimulated cAMP accumulation. Blockade of intracellular Ca^{2+} mobilization abolished the substance K receptor-mediated inhibition of isoproterenol-induced cAMP production. Phosphodiesterase inhibitors, down-regulation or inhibition of protein kinase C, and pertussis toxin failed to prevent substance K-induced inhibition of agonist-stimulated cAMP accumulation. An increased intracellular Ca^{2+} concentration caused by either calcium ionophores or activation of endogenous bradykinin receptors was found to markedly reduce cAMP production in wild-type cells. These results demonstrate that elevated intracellular Ca^{2+} concentration can negatively modulate agonist-stimulated adenylate cyclase activity in C6-2B glioma cells.**

Cross talk between second messenger systems certainly plays a crucial role in the complex regulation of multiple signal transduction mechanisms within the cell. cAMP and phosphatidylinositol (PI) pathways appear to share positive (1–5) as well as negative (6–8) modulatory interactions, leading to a finely orchestrated cell response. In particular, a growing amount of experimental evidence indicates the existence of complex interactions between cAMP and Ca^{2+} intracellular pathways (9–17).

In the present study, C6-2B rat glioma cells were stably transfected with the bovine stomach substance K receptor (SKR) cDNA (18) and used as an *in vitro* model system to investigate the interactions between receptor-mediated cAMP and Ca^{2+} signaling pathways. SKRs (or neurokinin 2 receptors) are binding sites for mammalian tackykinins, which preferentially interact with substance K (or neurokinin A). Upon substance K (SK) application, *de novo* expressed SKRs have been shown to lead to PI hydrolysis in murine fibroblasts and to increase the intracellular free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) by promoting Ca^{2+} release from intracellular stores in oocytes (19, 20). In C6-2B cells, stimulation of β-adrenergic receptors (BARs) coupled to adenylate cyclase led to cAMP accumulation, while activation of the newly expressed SKRs, via PI hydrolysis, resulted in intracellular Ca^{2+} mobilization, thus providing a homogeneous cell system where the interactions between second messenger pathways can be investigated in detail upon selective receptor activation (21).

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MATERIALS AND METHODS

Plasmid Construction and Transfection. The 2.5-kilobase *Eco*RI fragment containing the bovine SKR cDNA sequence, the poly(dT-dA) tail, and multiple restriction sites was isolated from pGEM1 (18) and inserted at the *Eco*RI site of the pcDNA1 vector (Invitrogen, San Diego). Alternatively, after digestion of the pcDNA1-SKR cDNA construct with *Not* I and *Hin*dIII, the SKR cDNA-bearing fragment was directionally inserted downstream from the T7 promoter of the pRc/CMV vector (Invitrogen), which carries the neomycin-resistance gene. C6-2B cells, grown as monolayer cultures in Ham's F-10 medium/10% (vol/vol) calf serum (GIBCO), were transfected by both standard $CaPO_4$ (22) and liposome-mediated (23) transfection methods, and stable transformants were selected in G418 (GIBCO) at 300 μg/ml.

Northern Blot Analysis and Receptor Binding. Ten to 20 μg of poly(A)$^+$ RNA was electrophoretically separated on a 1% agarose/6% formaldehyde gel and transferred to a Zeta-Probe nylon membrane (Bio-Rad). Hybridization was performed according to the protocol provided by Bio-Rad. Arbitrary units were defined as a function of the ratio of the peak densitometry area of the SKR mRNA and the p1B15 mRNA (which codes for a structural protein) hybridization bands. For the binding assay, intact cells were incubated in Locke's solution containing 3 mM $MnCl_2$, 0.02% bovine serum albumin, and ^{125}I-labeled SK for 90 min at room temperature.

Digital Fluorescence Microscopy. Cells grown on glass coverslips were imaged at room temperature in Locke's solution (154 mM NaCl/5.6 mM KCl/3.6 mM $NaHCO_3$/2.3 mM $CaCl_2$/1.2 mM $MgCl_2$/5.6 mM glucose/5 mM Hepes, pH 7.4), using an Attofluor digital fluorescence microscopy system (Atto Instruments, Rockville, MD) as described (24). Cells were loaded with the acetoxymethyl ester of fura-2 (fura-2 AM, Molecular Probes; 5 μM) (25) for 30 min at 37°C, washed, and imaged.

Biochemical Measurements and Reagents. Cell cAMP content was measured by the Atto-Flo automated radioimmunoassay as described (26), after incubation with the drugs at 37°C in serum-free medium containing 3-isobutyl-1-methylxanthine (IBMX) (100–500 μM) and/or Ro20-1724 (100 μM) to prevent cAMP breakdown. PI hydrolysis was estimated by measuring [^{3}H]inositol monophosphate accumulation, as described (27). Protein was measured by the method of Bradford (28). After cell permeabilization with 0.01% digitonin (29), adenylate cyclase activity was mea-

Abbreviations: SKR, substance K receptor; PI, phosphatidylinositol; SK, substance K; BAR, β-adrenergic receptor; IBMX, 3-isobutyl-1-methylxanthine; PT, pertussis toxin; Iso, (−)-isoproterenol; G protein, guanine nucleotide-binding regulatory protein; $[Ca^{2+}]_i$, intracellular free $[Ca^{2+}]$; fura-2 AM and EGTA-AM, acetoxymethyl esters of fura-2 and EGTA, respectively.

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sured in the presence of 0.1 mM ATP, 10 μM GTP, 0.1 mM IBMX, and 3 mM $MgCl_2$. Ca-EGTA buffers were used to prepare solutions containing the desired free calcium ion concentration. The *in vitro* ADP-ribosylation assay was performed exactly as described (30).

SK, [D-Pro^2,D-Trp^{7,9}]substance P, and bradykinin were purchased from Peninsula Laboratories; pertussis toxin (PT) and cholera toxin, List Biological Laboratories (Campbell, CA); forskolin, Calbiochem; the acetoxymethyl ester of EGTA (EGTA-AM) and fura-2 AM, Molecular Probes; H-7, Seikagaku America (St. Petersburg, FL); Ro20-1724, Biomol (Plymouth Meeting, PA); ^{125}I-labeled SK (2000 Ci/mmol; 1 Ci = 37 GBq), Amersham.

RESULTS

Characterization of SKR cDNA-Transfected C6-2B Cells. After transfection, G418-resistant clones were screened for SK responsiveness by single-cell fura-2 Ca^{2+} imaging. Upon application of SK, while no response was detected in mock-transfected cells (Fig. 1*A*), two out of three SKR cDNA-transfected clones tested in the experiment shown exhibited a novel increase in $[Ca^{2+}]_i$. However, within each clone, heterogeneity in the number of responsive cells was observed (Fig. 1 *B–D*). The amount of newly expressed SKR mRNA was highest in the fully responding A2-3 group (C6-2B$_{A2-3}$) (Fig. 2*A*). Specific ^{125}I-labeled SK binding, undetectable in control cells, was saturable in SKR-expressing cells, and from Scatchard analysis a K_d of 1.8–3 nM was calculated (Fig. 2*B*). B_{max} values ranged between 15 and 100 fmol/mg of protein among the different clones, proportional to the SKR mRNA content. C6-2B$_{A2-3}$ cells were used for biochemical studies by virtue of their homogeneous $[Ca^{2+}]_i$ imaging response to SK. In these cells, SK increased [^{3}H]inositol monophosphate accumulation and $[Ca^{2+}]_i$, with an EC_{50} of 5–10 nM (Fig. 2 *C* and *D*). The peak of $[Ca^{2+}]_i$ was observed within 1–2 min after the addition of SK, followed by a constant decrease in $[Ca^{2+}]_i$ over a period of several minutes (Fig. 2*D Inset*). The $[Ca^{2+}]_i$ increase also occurred in the absence of extracellular Ca^{2+}, suggesting mobilization from intracellular stores (data not shown).

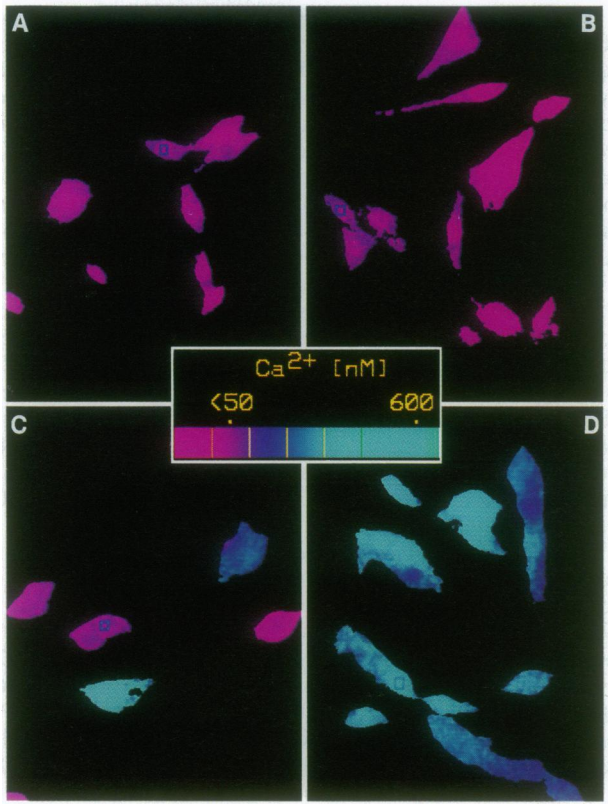

FIG. 1. $[Ca^{2+}]_i$ imaging responses to SK in SKR-transfected C6-2B cells. (*A*) A1-1 control, mock-transfected cells. (*B*) A2-11 clone with no cells responding to SK. (*C*) B2-2 clone where 40% of the cells respond to SK. (*D*) A2-3 clone where 100% of the cells respond to SK. The color scale depicts pixel intensity of the fura-2 ratio (334/380 nm) image: 0–50 nM Ca^{2+} (purple) to 600 nM Ca^{2+} (light blue). Images were obtained 30–50 sec after application of 1 μM SK.

cAMP Accumulation Studies. Basal cAMP levels were similar in wild-type, mock-transfected, and SKR cDNA-transfected cells (0.03–0.04 nmol/mg of protein), whereas accumulation of cAMP stimulated by the BAR agonist (−)-isoproterenol (Iso) was markedly depressed in two out of three SKR-expressing clones compared to the control (Fig. 3*A*). Iso dose–response (1 nM–10 μM) experiments and pindolol binding did not show significant differences from control cells in either the affinity or the number of BARs in SKR-expressing clones (data not shown). In all SKR-expressing clones, when cells were preincubated with SK (1 μM) for 2–5 min and then challenged with Iso (10 μM) for 20 min, a further inhibition, by up to 80%, of the cAMP accumulation was observed (Fig. 3*A*). Inhibition of Iso-induced cAMP accumulation by SK was concentration dependent (EC_{50} = 5 nM) and specific since it was partially antagonized by [D-Pro^2,D-Trp^{7,9}]substance P, a neurokinin antagonist (Fig. 3*B*). Pindolol binding studies in SKR-expressing cells showed no changes in the K_d or in the B_{max} of BARs following exposure to SK (data not shown). In C6-2B$_{A2-3}$ cells, stimulation of intracellular cAMP accumulation by postreceptor agonists, such as cholera toxin (6 nM for 2 hr) and forskolin (100 μM for 20 min), was also greatly impaired by a 5-min pretreatment with 1 μM SK (Table 1).

To better evaluate the role played by intracellular Ca^{2+} mobilization on SKR-mediated inhibition of cAMP accumulation, C6-2B$_{A2-3}$ cells were preincubated for 1 hr at 37°C with the cell-permeant Ca^{2+} chelator EGTA-AM (100 μM), washed, exposed to 1 μM SK for 5 min, and then challenged with Iso (10 μM) for 20 min. As shown in Fig. 4, in cells pretreated with EGTA-AM, the increase in $[Ca^{2+}]_i$ induced by SK was markedly reduced, and the inhibitory effect of SK on cAMP accumulation was totally abolished.

The possibility that Ca^{2+}-enhanced phosphodiesterase activity could account for the decrease in cAMP accumulation after SK exposure was explored. C6-2B$_{A2-3}$ cells were exposed, in the presence or in the absence of IBMX (500 μM) and Ro20-1724 (100 μM), to 1 μM SK for 5 min and then challenged with Iso (10 μM) for 20 min. As shown in Table 1, the extent of SK-induced inhibition of Iso-stimulated cAMP was not significantly affected by the presence of phosphodiesterase inhibitors.

The hypothesis that SK-induced inhibition of cAMP accumulation was mediated through a guanine nucleotide-binding regulatory protein (G protein), such as $G_{i/o}$, was also investigated. *In vitro* ADP-ribosylation results showed that PT-sensitive $G_{i/o}$ units are present in C6-2B cells and that they could be labeled by [^{32}P]NAD in control membranes (Fig. 5*A*, lane 3; M_r, 41,000) but not in membranes from cultures pretreated overnight with PT (Fig. 5*A*, lane 4). Overnight treatment of C6-2B$_{A2-3}$ cells with PT at 1 μg/ml resulted in an enhanced cAMP response upon a 20-min challenge with 10 μM Iso, as shown in Fig. 5*C*, suggesting that $G_{i/o}$ units had been inactivated. However, PT failed to prevent both SK-induced intracellular Ca^{2+} mobilization and inhibition of Iso-elicited cAMP accumulation (Fig. 5 *B* and *C*). Also, cholera toxin, at the dose (6 nM) and for the time (2 hr) maximally effective in stimulating cAMP production (Table 1), did not affect the ability of C6-2B$_{A2-3}$ cells to increase $[Ca^{2+}]_i$ in response to SK (data not shown).

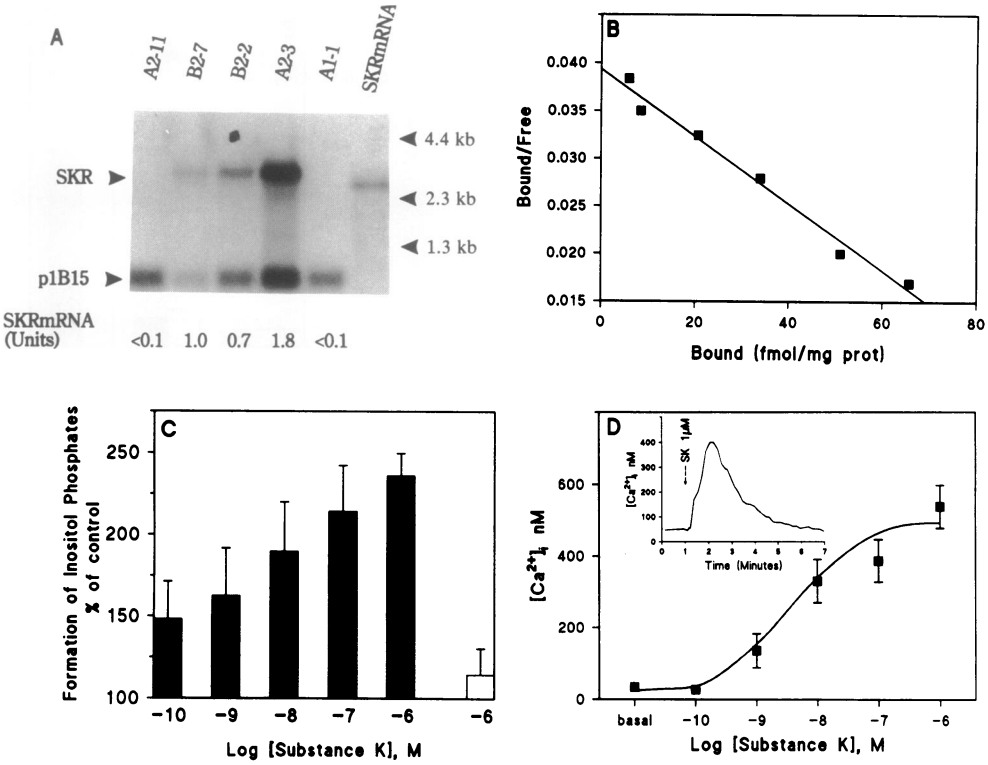

FIG. 2. (*A*) Northern blot analysis of poly(A)$^+$ RNA from control and SKR cDNA-transfected C6-2B cells. A1-1, control cells; A2-3 (100%), B2-2 (40%), B2-7 (90%), and A2-11 (0%), SKR cDNA-transfected clones with respective percentage of cells responding to SK by Ca^{2+} imaging. SKRmRNA, 1 ng of SKR mRNA transcribed *in vitro* from the plasmid used for transfection. (*B*) Scatchard analysis of ^{125}I-labeled SK binding in intact C6-2B$_{A2-3}$ cells. Values for K_d and B_{max} calculated in this experiment were 1.8 nM and 69 fmol/mg of protein, respectively. (*C*) SK-induced inositol phosphate formation in C6-2B$_{A2-3}$ cells. [^{3}H]Inositol monophosphate accumulation elicited by a 5-min treatment with SK was concentration dependent in the A2-3 clone (solid bars), while it was not stimulated by 1 μM SK in mock-transfected cells (open bar). (*D*) Dose–response curve of SK for the $[Ca^{2+}]_i$ increase in C6-2B$_{A2-3}$ cells. $[Ca^{2+}]_i$ was calculated by fura-2 Ca^{2+} imaging. (*Inset*) Time course of the response to 1 μM SK.

Finally, the possible contribution of protein kinase C in the SK-mediated inhibition of cAMP production was investigated. C6-2B$_{A2-3}$ cells were treated with phorbol 12-myristate 13-acetate (0.1–1 μM) overnight or preincubated with H-7 (10 μM) or staurosporine (1 μM) and then exposed to increasing concentrations of SK (1–1000 nM). None of the above agents blocked the increase in $[Ca^{2+}]_i$ and the inhibition of Iso-induced cAMP accumulation caused by SK (data not shown).

Effect of Elevated $[Ca^{2+}]_i$ on the cAMP Pathway in C6-2B Wild-Type Cells. Ca^{2+} influx promoted by either A23187 (10 μM) or ionomycin (1 μM) severely impaired cAMP accumulation elicited by a 20-min challenge with Iso (10 μM), forskolin (100 μM), or prostaglandin E_1 (10 μM) (Fig. 6*A*). Moreover, stimulation of endogenous bradykinin receptors, coupled to PI hydrolysis (31), resulted in mobilization of intracellular Ca^{2+} in both wild-type and A2-3 clonal C6-2B cells (Fig. 6*B*) and a concentration-dependent inhibition of Iso-induced cAMP accumulation (Fig. 6*A*) in wild-type cells. Finally, increasing the Ca^{2+} concentration potently inhibited Iso-stimulated adenylate cyclase activity in digitonin-permeabilized C6-2B cells, as shown in Fig. 7.

DISCUSSION

The interactions between the Ca^{2+} and cAMP second messenger pathways were investigated by examining the effect of receptor-mediated $[Ca^{2+}]_i$ increase on the agonist-induced cAMP accumulation in intact C6-2B cells transfected with SKR cDNA. Single-cell Ca^{2+} imaging revealed heterogeneity in the number of cells responding to SK in stably transfected clones, which by virtue of the G418-resistance phenotype,

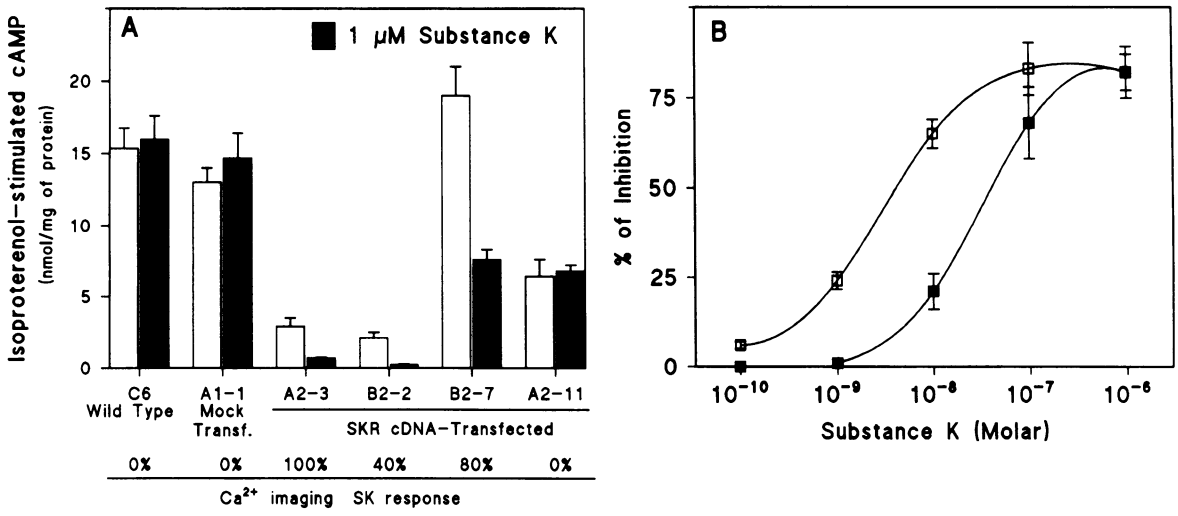

FIG. 3. (*A*) SK effect on cAMP accumulation. Cells were incubated, in serum-free medium containing 100 μM IBMX, in the presence (solid bars) or in the absence (open bars) of 1 μM SK for 5 min. Iso (10 μM) was then added, and the cAMP content was measured after 20 min. Data are the mean ± SEM of at least three separate experiments done in triplicate. (*B*) Concentration-dependent inhibition by SK of Iso-induced cAMP in C6-2B$_{A2-3}$ cells. After a 5-min treatment with increasing concentrations of SK in the presence (■) or in the absence (□) of 10 μM [D-Pro^2,D-Trp^{7,9}]substance P, C6-2B$_{A2-3}$ cells were challenged for 20 min with 10 μM Iso.

Table 1. Effect of 1 μM SK on C6-2B$_{A2-3}$ cell cAMP content

Treatment	cAMP, pmol/mg of protein	
	Control cells	SK-pretreated cells
None (basal)	35 ± 5.5	40 ± 3.8
Forskolin (100 μM)	5,783 ± 611	2250 ± 323
Cholera toxin (6 nM)	4,659 ± 171	1768 ± 167
IBMX (500 μM) + Ro20-1724 (100 μM)	64 ± 5.2	71 ± 6.7
Isoproterenol (10 μM)	4,172 ± 833	828 ± 40
Iso + IBMX + Ro20-1724	12,650 ± 1340	3800 ± 332

Northern analysis, and binding studies, were expected to homogeneously express SKR. A fully responding clone (A2-3) was then identified, wherein 100% of the cells exhibited a novel concentration-dependent increase in $[Ca^{2+}]_i$ upon stimulation with SK. Inositol 1,4,5-trisphosphate receptor-mediated Ca^{2+} release seems to be the mechanism underlying the $[Ca^{2+}]_i$ increase induced by SK, since PI hydrolysis was also stimulated upon SK application.

Agonist-induced intracellular cAMP accumulation was greatly reduced in two out of three SKR-expressing clones compared to both wild-type and mock-transfected cells. The biochemical event(s) responsible for the depressed cAMP response observed even in the absence of SK, the ligand of the newly introduced receptor, is not yet clear to us. However, neither a loss of BARs nor a shift in K_a for the agonist was observed in SKR-expressing clones. Moreover, basal cAMP levels in SKR-transfected clones were not appreciably different from control cells, making the possibility of a direct negative effect of the newly expressed receptor *per se* on adenylate cyclase activity unlikely. Pretreatment with SK further inhibited, in a concentration-dependent manner by up to 80%, cAMP accumulation elicited by Iso in all SKR-expressing clones. No changes in BAR affinity or number were detected in SKR-expressing cells following SK incubation. Moreover, cAMP accumulation induced by forskolin and cholera toxin was also depressed by SK, strongly suggesting that activation of the newly expressed SKR leads to events resulting in reduced adenylate cyclase activity.

Chelation of intracellular Ca^{2+} by EGTA-AM greatly or totally inhibited the $[Ca^{2+}]_i$ rise evoked by SK and prevented the inhibition of cAMP accumulation in SK-treated cells, indicating that, in C6-2B$_{A2-3}$ cells, the SKR-mediated increase in $[Ca^{2+}]_i$ is causally associated with the inhibition of

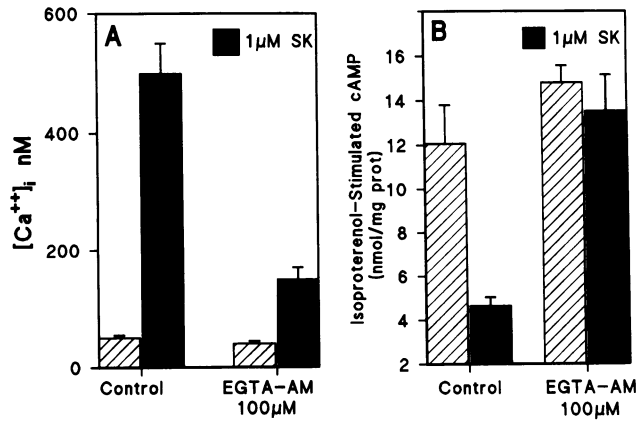

FIG. 4. Effect of EGTA-AM on the SK-evoked $[Ca^{2+}]_i$ rise and the SK-induced inhibition of cAMP accumulation in C6-2B$_{A2-3}$ cells. Cells were incubated, in serum-free medium containing 500 μM IBMX and 100 μM Ro20-1724, for 1 hr at 37°C in the absence (control) or in the presence of 100 μM EGTA-AM, extensively washed, and either subjected to fura-2 Ca^{2+} imaging (*A*) or exposed for 5 min to 1 μM SK and then challenged with 10 μM Iso for 20 min (*B*).

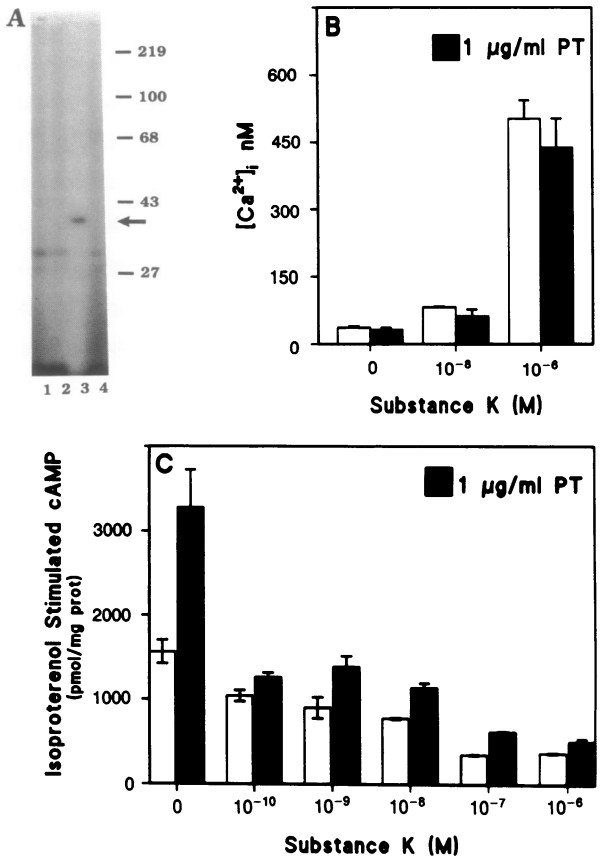

FIG. 5. Effect of PT on SK-induced $[Ca^{2+}]_i$ and SK-mediated inhibition of cAMP production in C6-2B$_{A2-3}$ cells. (*A*) Autoradiogram of *in vitro* [^{32}P]NAD-labeled proteins from membranes of control (lanes 1 and 3) and PT-pretreated (1 μg/ml, 17 hr; lanes 2 and 4) cells. Activated PT was added to the samples in lanes 3 and 4. The arrow indicates the M_r 41,000 $G_{i/o}$ unit. Molecular weight standards ($M_r \times 10^{-3}$) are indicated at right. (*B*) PT-pretreated cells were washed, loaded with fura-2 AM, and analyzed by Ca^{2+} imaging. (*C*) Control and PT-pretreated cells were exposed to increasing concentrations of SK for 5 min and challenged with 10 μM Iso.

cAMP production. The transient rise in total cell Ca^{2+} induced by SK occurs within 5 min, and yet the inhibition of cAMP accumulation can be detected after a 20-min challenge with Iso (or even after 60 min; data not shown). This discrepancy in time between $[Ca^{2+}]_i$ increase and inhibition of adenylate cyclase suggests that a transient rise in Ca^{2+} could set in motion a series of events, which leads to a sustained inhibition of adenylate cyclase activity.

Moreover, phosphodiesterase inhibitors failed to prevent SK-induced inhibition of cAMP accumulation in C6-2B$_{A2-3}$ cells, which strongly suggests an impairment in cAMP formation rather than an enhancement in cAMP degradation.

Activation of protein kinase C by phorbol esters has been shown to cause a reduction in receptor-mediated intracellular cAMP increase in NCB-20 cells (32). In our study, the possible contribution by protein kinase C as the alternative final effector of SKR-mediated PI hydrolysis, was ruled out due to the failure of protein kinase C inhibitors and phorbol 12-myristate 13-acetate to block the SK effect.

The coupling of the newly expressed SKR to its effector seems to be mediated by neither a classical PT-sensitive $G_{i/o}$ protein nor a cholera toxin-sensitive G_s protein.

In wild-type C6-2B cells, the $[Ca^{2+}]_i$ increase by bradykinin receptor activation led to inhibition of Iso-induced cAMP accumulation, thus indicating that Ca^{2+} mobilization by endogenous agonists might negatively modulate the cAMP pathway. Interestingly, bradykinin was not as efficacious as

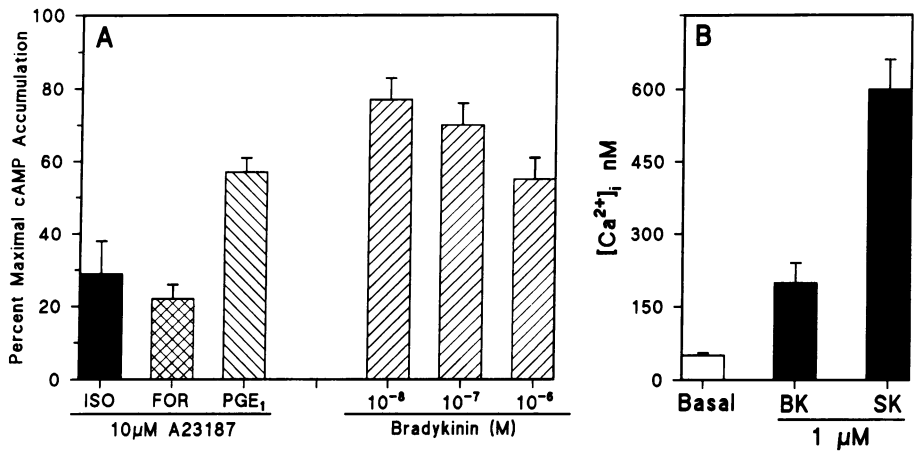

FIG. 6. (*A*) Effect of ionophoretic Ca^{2+} influx and bradykinin on agonist-induced cAMP accumulation in wild-type C6-2B cells. Cells were treated for 5 min with 10 μM A23187 and then challenged with Iso (10 μM), forskolin (FOR; 100 μM), or prostaglandin E_1 (PGE_1; 10 μM) for 20 min (left group of bars). Cells were exposed to increasing concentrations of bradykinin for 5 min, and Iso (10 μM) was then added for 20 min (right group of bars). Data are expressed as the percent of agonist-induced cAMP accumulation in cells either not pretreated with A23187 or not exposed to bradykinin. (*B*) Effect of bradykinin on $[Ca^{2+}]_i$ in the C6-2B_{A2-3} clone. Fura-2 AM-loaded cells were exposed to 1 μM bradykinin or SK and imaged, and the $[Ca^{2+}]_i$ was calculated.

SK in evoking a $[Ca^{2+}]_i$ rise and inhibited Iso-elicited cAMP accumulation to a lesser extent than SK, probably reflecting differences in the number of receptors or in second messenger coupling efficiency. Finally, adenylate cyclase activity measured in permeabilized C6-2B cells demonstrated that physiological concentrations of Ca^{2+} could directly inhibit Iso-stimulated adenylate cyclase.

In conclusion, our study demonstrates that elevated intracellular Ca^{2+} levels, whether they are due to receptor-mediated Ca^{2+} release or ionophoretic Ca^{2+} influx, are inhibitory to stimulated adenylate cyclase activity in C6-2B glioma cells. In biological systems wherein the final biochemical response is the result of multiple transduction signals within the cell, this interaction between the cAMP and Ca^{2+} pathways is very likely to play a crucial regulatory role. Furthermore, based upon a recent report showing cAMP-mediated release of intracellular Ca^{2+} in human T lymphocytes (33), the possibility of a Ca^{2+}-mediated feedback in the cAMP pathway is, although only speculative at the present, very appealing. Upon adenylate cyclase activation, a cAMP-sensitive pool of Ca^{2+} would be released which, in turn, would potentially inhibit further cAMP accumulation, thus giving (*i*) cAMP the ability to regulate Ca^{2+} and (*ii*) Ca^{2+}, as shown in this report, the ability to regulate cAMP.

Note. While this manuscript was in preparation, a paper by Boyajian *et al.* (34) appeared showing that bradykinin stimulates intracellular Ca^{2+} release in NCB-20 cells and leads to inhibition of adenylyl cyclase.

We wish to thank Drs. S. Nakanishi and R. J. Milner for the generous gift of the plasmids as well as Drs. I. Mocchetti, R. Raulli, and W. Wojcik and Ms. N. Buckley for their precious help. This study was supported by National Institutes of Health Grant HL 28940.

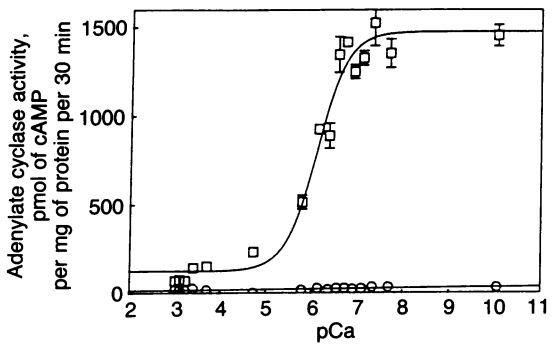

FIG. 7. Effect of increasing free Ca^{2+} concentrations on adenylate cyclase activity in permeabilized wild-type C6-2B cells. After permeabilization with 0.01% digitonin, cell adenylate cyclase activity stimulated by 10 μM Iso was measured in the absence (○) or in the presence (□) of 10 μM GTP. pCa, − log[Ca^{2+}].

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