0026-895X/93/030451-08$03.00/0

MOLECULAR PHARMACOLOGY, 43:451–458

Ca^{2+} Inhibition of β-Adrenergic Receptor- and Forskolin-Stimulated cAMP Accumulation in C6–2B Rat Glioma Cells Is Independent of Protein Kinase C

MARIA A. DEBERNARDI, RAVI MUNSHI, and GARY BROOKER

Department of Biochemistry and Molecular Biology and FIDIA-Georgetown Institute for the Neurosciences, Georgetown University, School of Medicine, Washington, D. C. 20007

Received October 8, 1992; Accepted December 9, 1992

SUMMARY

In C6–2B rat glioma cells, agonist-stimulated cAMP accumulation is potently inhibited after the stimulation of endogenous bradykinin receptors or stably transfected substance K receptors, coupled to phosphatidylinositol hydrolysis. In the present report, pharmacological tools were used to selectively stimulate either protein kinase C or Ca^{2+}, the two final effectors activated upon phosphatidylinositol hydrolysis, and their role in the inhibition of the C6–2B cell cAMP signaling pathway was investigated. Activation of protein kinase C by an acute treatment with phorbol 12-myristate 13-acetate or L-α-1-oleoyl-2-acetoyl-*sn*-3-glycerol did not reduce, but rather enhanced, the cAMP accumulation elicited by forskolin, a direct activator of adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. This effect was antagonized by the protein kinase inhibitor H-7 and mimicked by the protein phosphatase inhibitor okadaic acid. Thapsigargin, a selective microsomal Ca^{2+}-ATPase inhibitor, evoked a sustained increase in the intracellular free Ca^{2+} concentration, with an EC_{50} of 24.8 ± 4.3 nM, and inhibited the cAMP accumulation induced by the β-adrenergic receptor agonist isoproterenol with comparable potency (IC_{50} = 19.3 ± 0.2 nM), strongly suggesting a causal relationship between the two phenomena. The inhibition by thapsigargin of isoproterenol- or forskolin-stimulated cAMP accumulation was not affected by pertussis toxin or down-regulation or inhibition of protein kinase C. Dantrolene, a blocker of Ca^{2+} release from intracellular stores, antagonized 1) the Ca^{2+} transient in response to thapsigargin and substance K and 2) the inhibitory effect of these compounds on isoproterenol- or forskolin-induced cAMP accumulation. Moreover, sequestration of intracellular Ca^{2+} with the cell-permeable Ca^{2+} chelator ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester abolished the cAMP inhibition mediated by thapsigargin. Finally, isoproterenol- or forskolin-stimulated adenylyl cyclase activity in digitonin-permeabilized cells was not affected by either thapsigargin or substance K. These data provide compelling evidence that increases in intracellular free Ca^{2+} concentration without activation of protein kinase C suffice and are responsible for the inhibition of cAMP accumulation in C6–2B cells.

An amazingly complex and cell-specific network of interactions is responsible for integrating multiple transduction signals and translating them into a final biological response within the cell. In particular, evidence has been accumulating that reveals the dualistic potential of Ca^{2+}, as a second messenger *per se* and as a modulator of the cAMP signaling pathway. Beside the well established synergism exerted by Ca^{2+}, via calmodulin, on the adenylyl cyclase activity in brain and some peripheral tissues (1), an antagonistic and apparently direct effect of Ca^{2+} on cAMP formation is now becoming evident in different cell types (2–9). Adenylyl cyclase activity in plasma membranes from a variety of sources (2–6, 8, 9) is inhibited by Ca^{2+} at submicromolar concentrations but is not affected by exogenously added calmodulin (2, 4–6). The potential physiological significance of this is demonstrable in intact cells, wherein stimulation of receptors coupled to PI hydrolysis and intracellular Ca^{2+} mobilization inhibits agonist-induced cAMP accumulation (3, 4).

We have recently reported that in C6–2B rat glioma cells BK and SK, acting via endogenous and stably transfected receptors, respectively, evoke an IP_3-mediated release of intracellular Ca^{2+} and potently inhibit cAMP accumulation elicited by the β-AR agonist ISO or postreceptor agonists (3). This inhibitory effect is not mediated by a PTX-sensitive G protein or by an enhanced phosphodiesterase activity, whereas it is abolished by sequestration of intracellular Ca^{2+} with the cell-

This work was supported by National Institutes of Health Grant HL 28940.

ABBREVIATIONS: PI, phosphatidylinositol 4,5-bisphosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration; SK, substance K; DANT, dantrolene; TG, thapsigargin; ISO, (−)-isoproterenol; FO, forskolin; IP_3, 1,4,5-inositol trisphosphate; PMA, phorbol 12-myristate 13-acetate; PDD, 4-α-phorbol didecanoate; OAG, L-α-1-oleoyl-2-acetoyl-*sn*-3-glycerol; β-AR, β-adrenergic receptor; PTX, pertussis toxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AM, acetoxymethyl ester; IBMX, 3-isobutyl-1-methylxanthine; BK, bradykinin; PKC, protein kinase C; G protein, guanine nucleotide-binding protein; ION, ionomycin.

permeable EGTA/AM, suggesting that the IP_3-mediated increase in $[Ca^{2+}]_i$ is causally related to the cAMP inhibition (3). However, PKC, the other final effector activated by diacylglycerol upon PI hydrolysis, has been shown to either enhance or reduce agonist-stimulated cAMP formation, depending on the cell type (reviewed in Ref. 10). Although phorbol ester and H-7, a protein kinase inhibitor, failed to block the inhibition by SK of cAMP accumulation in C6–2B cells transfected with the SK receptor cDNA (3), we now directly address the question of whether PKC could play a role in the inhibition of the cAMP signaling pathway in wild-type C6–2B cells. To this end, experiments were designed to independently stimulate either $[Ca^{2+}]_i$ increase, with TG, or PKC activity, with PMA, and to test for their effects on agonist-induced cAMP formation. We now provide compelling evidence to support our belief that an increase in $[Ca^{2+}]_i$ without activation of PKC suffices and is responsible for the inhibition of cAMP formation previously described in C6–2B cells (3). A plausible explanation for these results is that in C6–2B cells such inhibition is mediated by the newly described type VI adenylyl cyclase (11), which is negatively regulated by Ca^{2+}.

Materials and Methods

Cell culture. C6–2B cells were grown as monolayers in Ham's F-10 medium/10% calf serum (GIBCO) in an atmosphere of 95% air/5% CO_2 at 37°. C6–2B cells stably transfected with the SK receptor cDNA (A2–3 clone) (3) were grown in the presence of 150 μg/ml G418 (GIBCO) to maintain selective pressure. Cells (passages 12–30) were used at confluency.

Biochemical measurements. Cell cAMP content was measured by the Atto-Flo automated radioimmunoassay (Atto Instruments Inc., Rockville, MD). Briefly, after incubation with drugs at 37° in 10–20 mM HEPES-buffered, serum-free, Ham's F-10 medium (pH 7.4, with 0.3 mM $CaCl_2$) containing IBMX (100–500 μM) and Ro20–1724 (100–200 μM), cells were treated with 0.1 M HCl/0.1 mM $CaCl_2$ for 15 min at room temperature and the acid extract was assayed for cAMP content. cAMP extruded into the medium was extracted with 0.1 M HCl. PI hydrolysis was estimated by measuring the accumulation of labeled inositol phosphates in the presence of 10 mM LiCl in cells that had been preloaded with *myo*-[2-^{3}H]inositol, as described (3). PKC activity was measured using a PKC enzyme assay system from Amersham, following the manufacturer's instructions. In this system, the transfer of the ^{32}P-labeled γ-phosphate group of ATP to a peptide substrate that is highly specific for PKC is measured as an index of the enzyme activity. Adenylyl cyclase activity in 0.01% digitonin-permeabilized cells (12) was measured in the presence of 0.1 mM ATP, 10 μM GTP, 0.1 mM IBMX, and 3 mM $MgCl_2$; the reaction was carried out for 30 min at room temperature. (−)-[^{3}H]CGP-12177 binding was performed with intact cells in Ham's F-10 medium containing 10 mM HEPES, 30 μg/ml bovine serum albumin, and 2 nM radiolabeled ligand, for 90 min at room temperature. Protein was measured by the method of Bradford (13).

Ca^{2+} imaging. Single-cell Ca^{2+} imaging was performed as described previously (14), using an Attofluor digital fluorescence microscopy system (Atto Instruments). Briefly, cells grown on glass coverslips were loaded with fura-2/AM (5 μM; Molecular Probes) for 30 min at 37°, washed, and imaged at 30–32° in Ham's F-10 medium buffered with 20 mM HEPES (pH 7.4).

Reagents. SK and BK were purchased from Peninsula Laboratories, PTX from List Biological Laboratories, TG from Research Biochemical Corp., H-7 from Seikagaku America, FO from Calbiochem, Ro20–1724 from Biomol, OAG from Avanti Polar Lipids, Inc., okadaic acid from GIBCO, fura-2/AM and EGTA/AM from Molecular Probes, and (−)-[^{3}H]CGP-12177 (38 Ci/mmol) and *myo*-[2-^{3}H]inositol (10–20 Ci/mmol) from Amersham. Other chemicals were from Sigma.

Results and Discussion

Role of Ca^{2+} in the regulation of agonist-stimulated cAMP accumulation. To evaluate the contribution of Ca^{2+} in the regulation of the cAMP pathway, we used the non-phorbol ester tumor promoter TG. TG increases $[Ca^{2+}]_i$ by specifically inhibiting microsomal Ca^{2+}-ATPase, thus preventing the reuptake of Ca^{2+} into the sensitive stores (15). Because TG neither stimulates PI hydrolysis (16, 17) nor activates PKC (18, 19), it is an ideal probe to study the specific role played by Ca^{2+} in cell responses. In C6–2B cells, various concentrations of TG transiently elevated $[Ca^{2+}]_i$ (Fig. 1). In the presence of 0.3 mM extracellular Ca^{2+}, both the rate of onset and the peak response were concentration dependent, although the decay kinetics for $[Ca^{2+}]_i$ seemed to be independent of the concentration of TG (Fig. 1). The concentration of TG for half-maximal effect was 24.8 ± 4.3 nM, and the maximal effect was observed at 100 nM (Fig. 1, *inset*). C6–2B$_{A2-3}$ cells, stably transfected with the SK receptor cDNA (3), respond to SK with a transient rise in $[Ca^{2+}]_i$ that is mediated by the activation of phospholipase C and consequent formation of inositol phosphates (3). Unlike TG, SK (1 μM) produced a sharp rise in $[Ca^{2+}]_i$ with much faster decay kinetics (Fig. 2A). When the intracellular stores had been depleted of their Ca^{2+} with the use of TG (100 nM), cells failed to respond to SK, although the peak response to the ionophore ION was not affected (Fig. 2A). The blockade by TG of the SK-evoked $[Ca^{2+}]_i$ increase was not due to an impairment of IP_3 formation, because the stimulation by SK of PI hydrolysis was not inhibited in TG-pretreated cells (Fig. 2B). These data support the notion that the Ca^{2+} stores discharged and inhibited by TG include the IP_3-sensitive pool (16), and they indicate that the transient Ca^{2+} response to SK in C6–2B cells is primarily due to release from internal stores.

In light of our previous observations that, in C6–2B cells, agonist-stimulated cAMP accumulation is inhibited after stimulation of PI hydrolysis and intracellular Ca^{2+} mobilization (3), we investigated the effect of the TG-induced $[Ca^{2+}]_i$ rise, which is associated with neither stimulated PI hydrolysis (Fig. 2B) (16, 17) nor PKC activation (18, 19), on the cAMP pathway. For this purpose, C6–2B cells were exposed to increasing concentrations of TG (1–1000 nM) for 2–5 min and then challenged with different concentrations of ISO (0.1–10 μM) for 5–15 min, and cAMP content was measured. As shown in Fig. 3, TG inhibited the cAMP accumulation elicited by ISO (10 μM) in a concentration-dependent manner, with an IC_{50} of 19.3 ± 0.2 nM, which is in excellent agreement with the EC_{50} (24.8 ± 4.3 nM) of TG for Ca^{2+} release. The possibility that TG could interfere with the binding of ISO to the β-AR was ruled out by the failure of TG to displace (−)-[^{3}H]CGP-12177 binding, as opposed to (−)-propranolol, a potent β-AR antagonist (Fig. 3, *inset*). Moreover, cAMP accumulation elicited by FO, which is known to directly activate adenylyl cyclase, was also significantly inhibited by TG (Fig. 4). The hypothesis that the decreased cell cAMP accumulation in response to ISO and FO resulted from an enhanced activity of the cAMP efflux pump (20) upon exposure of the cells to TG and SK (3) was also considered. However, measurement of agonist-stimulated cAMP levels in the incubation medium from cells pretreated with either TG or SK failed to display an increased efflux,

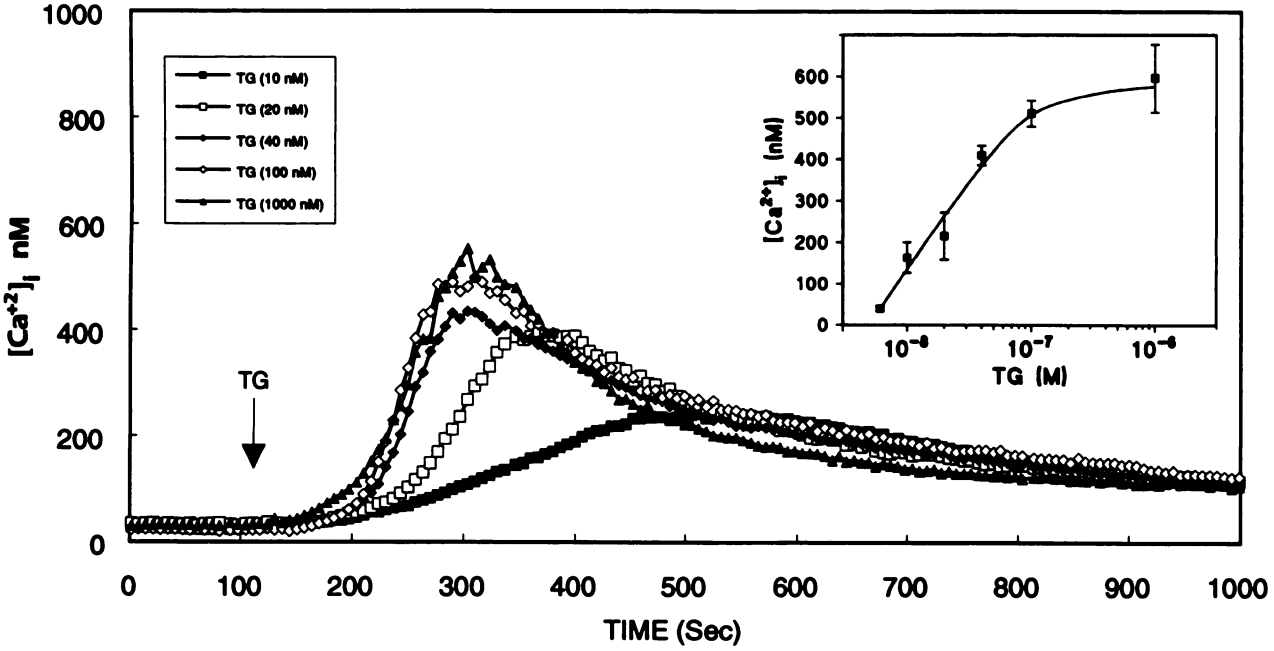

Fig. 1. Concentration-dependent effect of TG on basal $[Ca^{2+}]_i$ in C6–2B cells. Cells were prepared for fluorescence imaging as described in Materials and Methods and were then exposed to various concentrations of TG as shown. The population means of calcium levels in 30 cells from a representative experiment are shown. *Inset*, semilogarithmic concentration-response curve for TG. The EC_{50} for TG was 24.8 ± 4.3 nM. *Points* are the mean ± standard error of four to six different experiments.

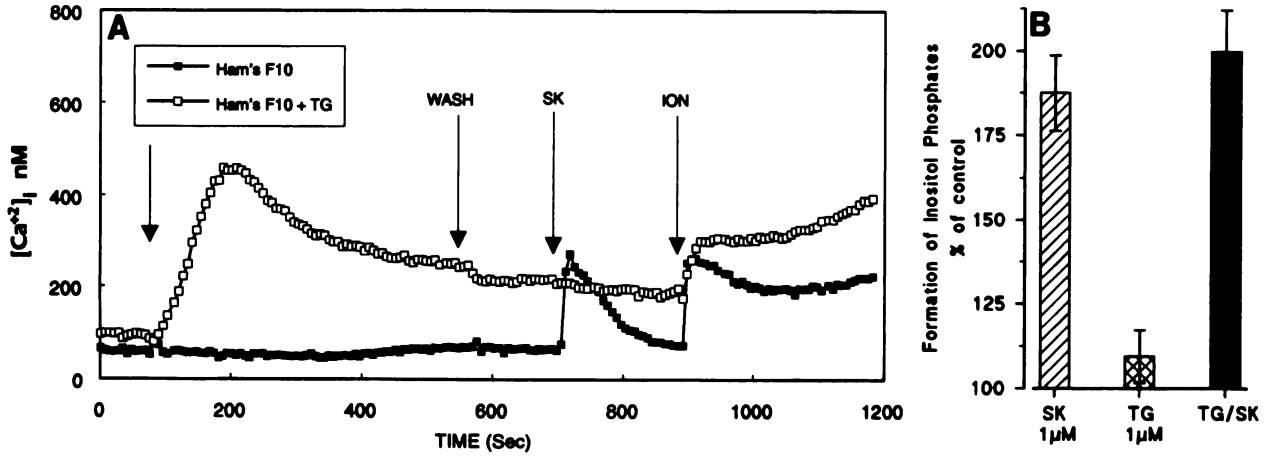

Fig. 2. Effect of TG on SK-induced calcium transient and PI hyrolysis in C6–$2B_{A2-3}$ cells. A, Cells were exposed for 8 min to Ham's F-10 medium in the absence or presence of 100 nM TG, washed for 2 min with Ham's F-10, and then challenged with SK (1 μM) followed by 10 μM ION. The population means of calcium levels in 25 cells from a representative experiment are shown. These data were reproduced in five additional experiments. B, *myo*-[^{3}H]inositol-loaded C6–$2B_{A2-3}$ cells were exposed for 5 min to SK (1 μM) (▨), to TG (1 μM) (■), or first to TG and then to SK (■), in the presence of 10 mM LiCl, and the formation of labeled inositol phosphates was measured as described in Materials and Methods.

compared with the control (data not shown). Moreover, similar to SK (3) and BK (3, 4), TG appears to impair agonist-induced cAMP formation by reducing the synthesis rather than enhancing the degradation of cAMP (21), because the inhibitory effect persists in the presence of two potent phosphodiesterase inhibitors, IBMX (100–500 μM) and Ro20–1724 (100–200 μM). These results, taken together, strongly suggest that the catalytic unit of adenylyl cyclase is the site for TG-induced inhibition of cAMP.

A direct negative effect of TG or SK (3) on adenylyl cyclase, which could account for their inhibition of cAMP formation, was ruled out by the failure of either agent to inhibit ISO-stimulated (data not shown) or FO-stimulated adenylyl cyclase activity in digitonin-permeabilized cells (Fig. 4, *right*). Furthermore, the possibility that TG exerts its effect through coupling with a G protein inhibitory to adenylyl cyclase was investigated. As we have shown previously (3), an overnight treatment with PTX (1 μg/ml) completely prevents subsequent *in vitro* ADP-ribosylation of the toxin-sensitive $G_{i/o}$ protein α subunits in C6–2B cell membranes. In cells pretreated with PTX, cAMP accumulation in response to ISO and FO was enhanced, compared with control (Table 1), strongly suggesting inactivation of the G_i inhibitory input on adenylyl cyclase. However, under these experimental conditions the ability of TG to increase $[Ca^{2+}]_i$ (data not shown) and inhibit ISO- or FO-stimulated cAMP accumulation was not affected (Table 1). These results indicate that, in intact cells, TG and SK (3) impair cAMP formation neither directly nor by a PTX-sensi-

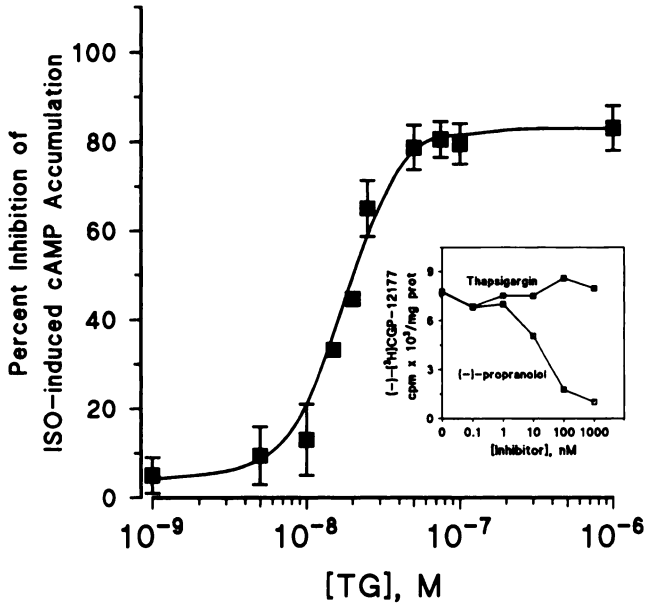

Fig. 3. Concentration-dependent inhibition by TG of ISO-stimulated cAMP accumulation. C6–2B cells were exposed to increasing concentrations of TG for 5 min and challenged with 10 μM ISO, and the cAMP content was measured after 10 min. Data are the mean ± standard error of six determinations from two separate experiments done in triplicate. *Inset*, (−)-[³H]CGP-12177 binding was carried out with intact C6–2B cells in the presence of increasing concentrations of TG or (−)-propranolol.

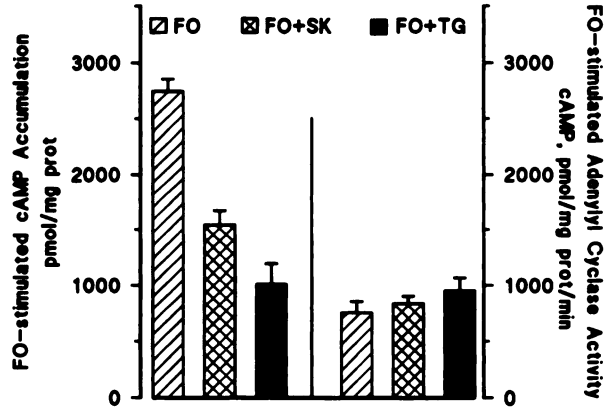

Fig. 4. Effects of SK and TG on FO-stimulated cAMP accumulation and adenylyl cyclase activity. Intact (*left*) or digitonin-permeabilized (*right*) C6–2B_{A2-3} cells were challenged with 10 μM FO alone (▨) or in the presence of 1 μM SK (■) or 1 μM TG (■). cAMP content in intact cells was measured after 5 min and adenylyl cyclase activity was determined as described in Materials and Methods. Data (mean ± standard error) are from one representative experiment done in triplicate; two other experiments gave similar results.

tive $G_{i/o}$ protein-mediated effect on adenylyl cyclase but, rather, by acting via a second messenger (absent or not functional in permeabilized cells), which, in turn, is inhibitory to the adenylyl cyclase. Given that both TG and SK, although through different mechanisms, evoke an increase in $[Ca^{2+}]_i$, it is reasonable to hypothesize that Ca^{2+} plays a key role in the inhibition of cAMP accumulation observed in intact cells after exposure to TG or SK. Furthermore, the inhibitory effects of TG and SK on agonist-induced cAMP formation in intact cells were not additive (data not shown). This evidence, together with the

TABLE 1

Effect of overnight treatment with 1 μg/ml PTX on TG-induced inhibition of cAMP accumulation in C6-2B cells

Treatment	cAMP[a]	
	Control	PTX
	pmol/mg of protein	
None (basal)	13.2 ± 1.5	17.6 ± 1.7
TG (1 μM)	14.0 ± 1.9	12.0 ± 1.7
ISO (10 μM)	5536 ± 701	9099 ± 248
TG/ISO	1250 ± 57 (23 ± 1%)[b]	1936 ± 136 (21 ± 1%)
FO (10 μM)	1804 ± 175	2240 ± 99
TG/FO	1114 ± 151 (61 ± 8%)	1146 ± 56 (51 ± 5%)

[a] cAMP content was measured after a 10-min challenge.
[b] Values in parentheses are the percentage of ISO- or FO-stimulated cAMP accumulation in cells exposed for 5 min to TG, relative to vehicle (0.01% dimethylsulfoxide)-treated cells, in either control or PTX-pretreated cultures.

Ca^{2+} imaging data (see Fig. 2A) showing that TG precludes a subsequent increase in $[Ca^{2+}]_i$ stimulated by SK, further suggests that the two agents act via a common intermediate and mobilize Ca^{2+} from a substantially overlapping intracellular pool.

To address the question of whether the increase in $[Ca^{2+}]_i$ evoked by TG is directly responsible for adenylyl cyclase inhibition, we tested the ability of TG to inhibit cAMP formation under conditions where either the intracellular Ca^{2+} was sequestered or the release of Ca^{2+} from internal stores was blocked. Exposure of C6–2B_{A2-3} cells to EGTA/AM precluded both the increase in $[Ca^{2+}]_i$ and the inhibition of cAMP formation induced by SK (3). In mouse NIH 3T3 fibroblasts, the TG-induced expression of the c-*fos* protooncogene was completely inhibited by chelation of intracellular Ca^{2+} (19). Likewise, sequestration of intracellular Ca^{2+} with EGTA/AM (100 μM, 1 hr) abolished the inhibitory effect of TG on agonist-induced cAMP accumulation in wild-type C6–2B cells (Fig. 5), strongly indicating that the TG-mediated $[Ca^{2+}]_i$ increase is causally associated with adenylyl cyclase inhibition.

The muscle relaxant DANT has been widely used as an inhibitor of intracellular Ca^{2+} mobilization (22). However, the IP_3-releasable pool of Ca^{2+} appears to be sensitive to DANT only in certain cell types (23–25) and not in others (26–28). In C6–2B_{A2-3} cells, DANT (40 μM) virtually abolished both the TG- and SK-induced Ca^{2+} transients measured by fura-2 imaging (Fig. 6), without any appreciable effect on the ability of ION to elicit a response (Fig. 6A). Although DANT itself has been reported to emit fluorescence at 495 nm when excited at 390 nm (29, 30), our results observed with DANT cannot be explained by such fluorescence, because DANT alone (Fig. 6) or DANT and ION (Fig. 6A) increased $[Ca^{2+}]_i$ and the increase in the 334/380 ratio was due to an increase in 334-nm intensity and a decrease in 380-nm intensity. Under the conditions of our experiments, we did not observe any fluorescence of DANT. The possibility that DANT blocks SK-induced Ca^{2+} release by precluding receptor-mediated PI hydrolysis was ruled out by the failure of DANT to inhibit SK-stimulated inositol phosphate formation (data not shown). Consistent with our observations of negative modulation of cAMP levels by Ca^{2+}, we found that DANT antagonized the inhibitory effects of TG and SK on agonist-stimulated cAMP accumulation in our *in vitro* model. A 10-min pretreatment with 50 μM DANT did not appreciably affect the cAMP response to ISO or FO, whereas it reduced the inhibitory effect of TG by 35 ± 10% (six experiments) and that of SK by 65 ± 14% (six experiments). A

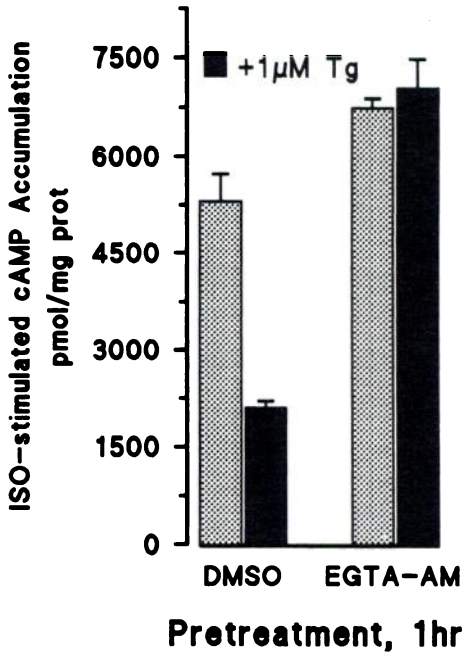

Fig. 5. Effect of EGTA/AM on TG-induced inhibition of ISO-stimulated cAMP. C6–2B cells were treated for 1 hr at 37° with 100 μM EGTA/AM or 0.1% dimethylsulfoxide, washed thoroughly, exposed for 5 min to either vehicle (control) (□) or 1 μM TG (■), and challenged for 10 min with 10 μM ISO. Data are the mean ± standard error from one experiment, done in triplicate, which was repeated two other times with virtually identical results.

representative experiment is presented in Fig. 7, which also shows that the inhibitory effect of BK (1 μM) (3) on ISO-stimulated cAMP formation was blocked by DANT. Because DANT precludes the increase in $[Ca^{2+}]_i$ elicited by TG and SK (see Fig. 6), these data further support the involvement of Ca^{2+} in the inhibition of agonist-stimulated cAMP accumulation by these two agents. However, whereas we observed a good reversal by DANT of the SK- and BK receptor-mediated cAMP inhibition, the effect of TG on cAMP accumulation was less efficiently blocked by DANT, the reason for which is presently unclear.

We then addressed the possibility that PKC, which is known to either positively or negatively regulate the cAMP pathway (reviewed in Ref. 10), participates in the inhibition by TG of the C6–2B cell cAMP accumulation. Continuous exposure of C6 glioma cells to PMA results in a time- and dose-dependent loss of PKC activity, such that after 24-hr treatment with 200 nM PMA cells are essentially depleted of PKC activity (31). As shown in Fig. 8A, in C6–2B cells an overnight treatment with PMA (1 μM) resulted in an 80% loss of PKC activity, compared with control cells exposed to the inactive PDD (1 μM). However, under these conditions the ability of TG to mobilize Ca^{2+} (data not shown) and inhibit agonist-induced cAMP formation was unaffected (Fig. 8B). The involvement of PKC in the inhibition by TG of the cAMP signaling pathway was also ruled out by the failure of the protein kinase inhibitor H-7 (10 μM) to block the TG response (data not shown). Finally, the negative modulation by Ca^{2+} of the C6–2B cell cAMP pathway was not affected by calmidazolium, a calmodulin antagonist (data not shown), further suggesting a direct effect of Ca^{2+} on the cAMP formation. However, the possibility that the inhibitory effect of Ca^{2+} is mediated through a Ca^{2+}-binding protein other than calmodulin cannot be excluded at this time.

Taken together, these results 1) provide strong evidence for a direct inhibitory effect of elevated $[Ca^{2+}]_i$ on cAMP formation and 2) suggest that, because the action of FO and receptor-mediated events are similarly affected, the catalytic unit of adenylyl cyclase is the most probable site for Ca^{2+}-mediated inhibition of cAMP accumulation. Our earlier evidence that adenylyl cyclase activity measured in digitonin-permeabilized C6–2B cells is inhibited by submicromolar Ca^{2+} concentrations (3) also supports this interpretation.

Role of PKC activation in the cAMP signaling pathway. Exposure of C6 glioma cells to 100 nM PMA caused PKC translocation to the plasma membrane with a $t_{1/2}$ of 15 min (32). In our experiments, C6–2B cells were exposed to either the inactive phorbol ester PDD (1 μM) or PMA (1 μM) for 30 min and then tested for PKC activity and FO-stimulated cAMP formation. In the experiment shown in Fig. 9A, a 3.2-fold increase in PKC activity was detected in PMA-treated cells, compared with control PDD-treated cells. Under these conditions, the intracellular cAMP content in PMA-treated cells was enhanced, compared with control, both under basal conditions (54 ± 16% increase, four experiments) and upon FO challenge (72.3 ± 30% increase, six experiments; Fig. 9B). Likewise, when cells were exposed to the synthetic diacylglycerol OAG (100 μM) FO-stimulated cAMP accumulation was potentiated (Fig. 10). The enhanced cAMP formation was not due to decreased cAMP efflux from the cells, because the level of cAMP in the medium, measured after agonist exposure, was also found to be increased in PMA- and OAG-treated cultures, compared with control (Figs. 9B and 10). The presence in the incubation medium of two potent phosphodiesterase inhibitors, IBMX (100–500 μM) and Ro20–1724 (100–200 μM), makes unlikely, although does not completely rule out, the possibility that the increased cAMP levels are due to a reduced degradation of cAMP, secondary to inhibition of cAMP phosphodiesterases by PKC (Refs. 10 and 33 and references cited therein). Furthermore, the enhanced cAMP accumulation induced by PMA and OAG was 1) antagonized by 10 μM H-7 (79 ± 10% decrease of the intracellular cAMP enhancement, six experiments; Figs. 9 and 10) and 2) mimicked by 1 μM okadaic acid (Table 2), a specific inhibitor of phosphatases 1 and 2A (34), thus suggesting the involvement of a phosphorylation-mediated event. These results demonstrate that in C6–2B cells an acute activation of PKC does not inhibit, but rather enhances, FO-stimulated cAMP accumulation. Whether the potentiation by PKC of the cAMP response in C6–2B cells is the result of inactivation of G_i (35) or a direct effect on the adenylyl cyclase (36) remains to be elucidated.

Conclusions

In the present study, TG and PMA were used as pharmacological tools to selectively study the role played by Ca^{2+} and PKC, respectively, the two final effectors activated upon PI hydrolysis, in the inhibition of the cAMP signaling pathway in C6–2B cells. An acute activation of PKC by PMA or OAG led to an enhancement, rather than an impairment, of the FO-stimulated cAMP accumulation, and this was antagonized by H-7 and mimicked by okadaic acid. TG transiently mobilized intracellular Ca^{2+} (EC_{50} = 24.8 ± 0.2 nM) in the absence of any significant increase in inositol phosphate formation and inhib-

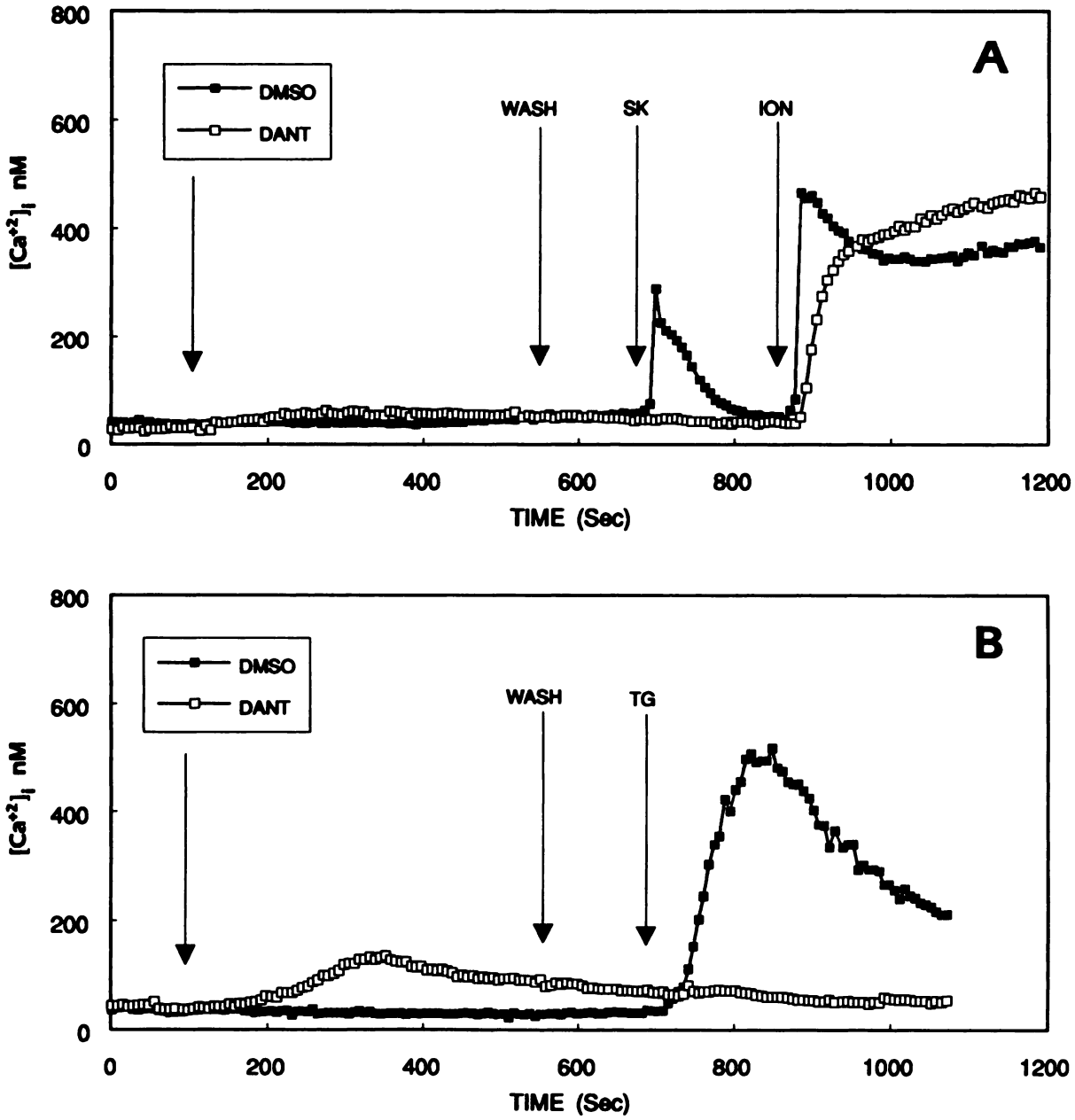

Fig. 6. Effect of DANT on SK- and TG-induced calcium transients in C6–2B$_{A2-3}$ cells. Cells were pretreated for 8 min with either vehicle [0.4% dimethylsulfoxide (*DMSO*)] or DANT (40 μM), washed for 2 min, and then challenged with SK (1 μM) followed by 10 μM ION (A) or 100 nM TG (B). Population means from 20–45 cells are shown. Similar results were obtained in four to six experiments.

ited agonist-stimulated cAMP accumulation (IC_{50} = 19.3 ± 0.2 nM). The excellent agreement between the half-maximal concentration of TG required for increases in $[Ca^{2+}]_i$ and inhibition of cAMP accumulation is strong evidence for a causal relationship between the two phenomena. The inhibition by TG of cAMP formation was neither due to a direct negative effect of TG on adenylyl cyclase nor mediated through a PTX-sensitive $G_{i/o}$ protein or PKC activation. The blockade of Ca^{2+} release from internal stores with DANT and the sequestration of intracellular Ca^{2+} with EGTA/AM antagonized and abolished, respectively, the inhibitory effect of TG on cAMP accumulation.

Therefore, we conclude that, in C6–2B glioma cells, 1) submicromolar increases in $[Ca^{2+}]_i$, achieved intracellularly by either PI-coupled receptor activation (3) or Ca^{2+} discharge from internal stores (results from this study), "directly" inhibit adenylyl cyclase and 2) an acute activation of PKC appears to play no role, either alone or in combination with Ca^{2+}, in the inhibition of adenylyl cyclase. The inhibitory effect of submicromolar concentrations of Ca^{2+} on the adenylyl cyclase activity of digitonin-permeabilized C6–2B cells (3) provides compelling evidence in support of our conclusion. Moreover, consistent with this interpretation are the recent data from our laboratory showing that the $[Ca^{2+}]_i$ increase observed upon stimulation by UTP of P_{2U} purinergic receptors also results in a PTX-insensitive inhibition of ISO- and FO-induced cAMP formation in C6–2B cells.[1]

[1] R. Munshi, M. A. DeBernardi, and G. Brooker. P_{2U} nucleotide receptors in C6-2B glioma cells: Sequentials changes in the levels of inositol phosphates, intracellular Ca^{2+} and cAMP are modulated by protein kinase C. Manuscript in preparation.

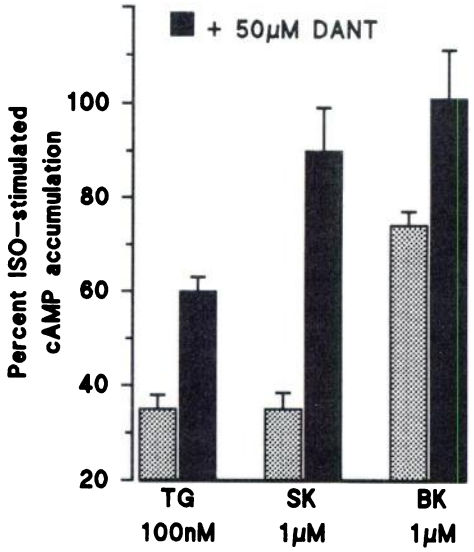

Fig. 7. Effect of DANT on the inhibition by TG, SK, and BK of ISO-stimulated cAMP accumulation. Cells were preincubated for 10 min with 50 μM DANT (■) or 0.5% dimethylsulfoxide (□), treated for 5 min with 100 nM TG, 1 μM SK, or 1 μM BK, and challenged for 10 min with 10 μM ISO. Data (mean ± standard error) are expressed as percentage of the cAMP accumulation elicited by ISO in the absence of TG, SK, or BK. The results from one experiment (representative of six for SK and TG and three for BK) are shown.

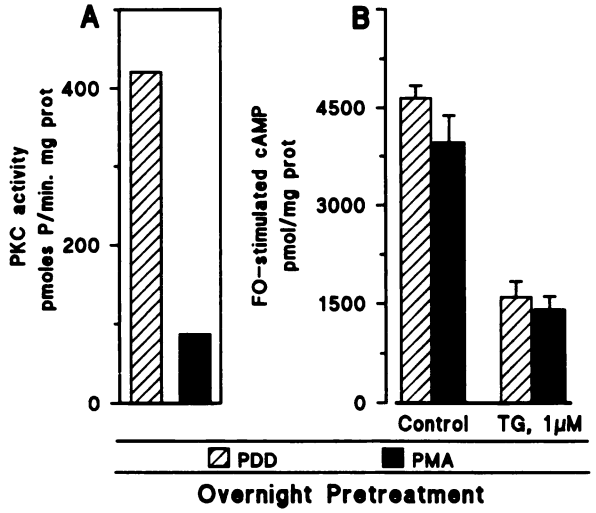

Fig. 8. Effect of overnight treatment with PMA on PKC activity and TG-mediated inhibition of FO-stimulated cAMP formation. C6–2B cells were exposed overnight to 1 μM PDD (▨) or 1 μM PMA (■) and processed for assay of PKC activity (A) or washed thoroughly, treated for 5 min with 1 μM TG or vehicle (control), and challenged for 10 min with 10 μM FO (B). PKC activity was measured in duplicate samples. cAMP results are representative of at least five experiments done in triplicate.

While this work was in progress, Yoshimura and Cooper (11) reported the cloning from a NCB-20 cell cDNA library of a novel Ca^{2+}-inhibitable adenylyl cyclase (type VI). In purified plasma membranes from 293 cells transfected with the cDNA coding for type VI adenylyl cyclase, FO-stimulated adenylyl cyclase activity displayed inhibition by submicromolar Ca^{2+} that was unaffected by the presence of added calmodulin (11), similar to what was originally observed in NCB-20 cells (4). The presence of mRNAs of similar size in NCB-20 cells, cardiac tissue, and pituitary-derived GH_3 cells (11), all systems in which Ca^{2+} negatively modulates the cAMP pathway (4–6), strongly indicates that this novel adenylyl cyclase isoform is the com-

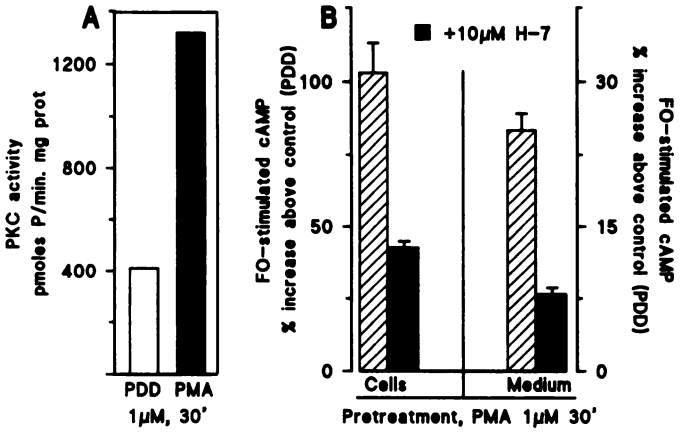

Fig. 9. Effect of an acute exposure to PMA on PKC activity and FO-stimulated cAMP accumulation in C6–2B cells. A, Cells treated for 30 min with 1 μM PDD (□) or 1 μM PMA (■) were processed for assay of PKC activity as described in Materials and Methods. B, Cells were preincubated for 20 min in serum-free medium in the presence (■) or in the absence (▨) of 10 μM H-7, treated for 30 min with 1 μM PDD or 1 μM PMA, washed thoroughly, and challenged for 10 min with 10 μM FO. Where indicated, H-7 was present throughout the experiment; H-7 did not inhibit the FO response in control PDD-treated cells. The results in B are expressed as percentage of increase above FO-stimulated cAMP accumulation, either intracellularly or in the medium, in control (PDD-treated) cells. Data are the mean (± standard error) of duplicate (A) or triplicate (B) determinations from a representative experiment.

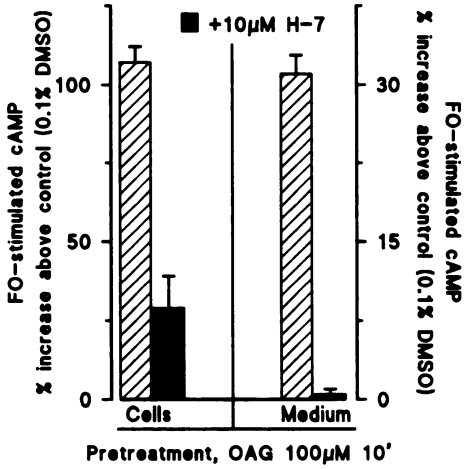

Fig. 10. Effect of OAG on FO-stimulated cAMP accumulation. C6–2B cells were treated for 10 min with 100 μM OAG and challenged for 10 min with 10 μM FO either in the presence (■) or in the absence (▨) of 10 μM H-7. H-7 was applied, where indicated, 20 min before the exposure of the cells to OAG. The results are expressed as percentage of increase above FO-stimulated cAMP accumulation, either intracellularly or in the medium, in control (0.1% dimethylsulfoxide-treated) cells. Data are the mean ± standard error from one experiment. Three other experiments yielded similar results.

TABLE 2

Effect of a 30-min pretreatment with 1 μM okadaic acid on cAMP accumulation in C6-2B cells

Treatment (10-min)	cAMP	
	0.1% DMSO*	Okadaic acid
	pmol/mg of protein	
None (basal)	55.7 ± 4.2	65.3 ± 3.0
FO (10 μM)	3447 ± 467	9198 ± 981
ISO (10 nM)	1524 ± 25	2193 ± 138
PGE_1 (10 μM)	174 ± 7.3	246 ± 19

* DMSO, dimethylsulfoxide; PGE_1, prostaglandin E_1.

mon site of Ca^{2+}-mediated inhibition. This appears to be indeed the case, in that C6–2B cells also express the newly identified type VI cyclase,[2] thus very likely accounting for the Ca^{2+}-mediated inhibition of cAMP formation described in the present report.

Acknowledgments

The authors wish to thank Dr. J. Wroblewsky and Ms. I. Iacaci for their help with the measurement of PI and PKC activity and Dr. D. M. F. Cooper for critical discussion.

[2] M. A. DeBernardi, R. Munshi, M. Yoshimura, D. M. F. Cooper, and G. Brooker. Type VI adenylyl cyclase mediates Ca^{2+} inhibition of the cAMP signaling pathway in rat C6-2B glioma cells. Manuscript in preparation.

References

1. Brostrom, C. O., Y. C. Huang, B. McL. Breckenridge, and D. J. Wolff. Identification of a calcium-binding protein as a calcium dependent regulator of brain adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **72**:64–68 (1975).
2. Caldwell, K. K., C. L. Boyajian, and D. M. F. Cooper. The effects of Ca^{2+} and calmodulin on adenylyl cyclase activity in plasma membranes derived from neuronal and non-neuronal cells. *Cell Calcium* **13**:107–121 (1992).
3. DeBernardi, M. A., T. Seki, and G. Brooker. Inhibition of cAMP accumulation by intracellular calcium mobilization in C6–2B cells stably transfected with substance K receptor cDNA. *Proc. Natl. Acad. Sci. USA* **88**:9257–9261 (1991).
4. Boyajian, C. L., A. Garritsen, and D. M. F. Cooper. Bradykinin stimulates Ca^{2+} mobilization in NCB-20 cells leading to direct inhibition of adenylyl cyclase. *J. Biol. Chem.* **266**:4995–5003 (1991).
5. Colvin, R. A., J. A. Oibo, and R. A. Allen. Calcium inhibition of cardiac adenylyl cyclase: evidence for two distinct sites of inhibition. *Cell Calcium* **12**:19–27 (1991).
6. Boyajian, C. L., and D. M. F. Cooper. Potent and cooperative feedback inhibition of adenylate cyclase activity by calcium in pituitary-derived GH_3 cells. *Cell Calcium* **11**:299–307 (1990).
7. Pepperell, J. R., and H. R. Behrman. The calcium-mobilizing agent, thapsigargin, inhibits progesterone production in rat luteal cells by a calcium-independent mechanism. *Endocrinology* **127**:1818–1824 (1990).
8. Narayanan, N., B. Lussier, M. French, B. Moor, and J. Kraicer. Growth hormone-releasing factor-sensitive adenylate cyclase system of purified somatotrophs: effects of guanine nucleotides, somatostatin, calcium, and magnesium. *Endocrinology* **124**:484–495 (1989).
9. Giannattasio, G., R. Bianchi, A. Spada, and L. Vallar. Effect of calcium on adenylate cyclase of rat anterior pituitary gland. *Endocrinology* **120**:2611–2619 (1987).
10. Houslay, M. D. "Crosstalk": a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. *Eur. J. Biochem.* **195**:9–27 (1991).
11. Yoshimura, M., and D. M. F. Cooper. Cloning and expression of a Ca^{2+}-inhibitable adenylyl cyclase from NCB-20 cells. *Proc. Natl. Acad. Sci. USA* **89**:6716–6720 (1992).
12. DeBernardi, M. A., and G. Brooker. Diphtheria toxin prevents catecholamine desensitization of A431 human epidermoid carcinoma cells. *Proc. Natl. Acad. Sci. USA* **84**:2246–2250 (1987).
13. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
14. De Erausquin, G., H. Manev, A. Guidotti, E. Costa, and G. Brooker. Gangliosides normalize distorted single-cell free Ca^{2+} dynamic after toxic doses of glutamate in cerebellar granule cells. *Proc. Natl. Acad. Sci. USA* **87**:8017–8021 (1990).
15. Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA* **87**:2466–2470 (1990).
16. Jackson, T. R., S. I. Patterson, O. Thastrup, and M. R. Hanley. A novel tumor promoter, thapsigargin, transiently increases cytoplasmatic free Ca^{2+} without generation of inositol phosphates in NG115–401L neuronal cells. *Biochem. J.* **253**:81–86 (1988).
17. Takemura, H., A. R. Hughes, O. Thastrup, and J. W. Putney. Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.* **264**:12266–12271 (1989).
18. Hakii, H., H. Fujiki, M. Suganuma, M. Nakayasu, T. Tahira, T. Sugimura, P. L. Scheuer, and S. B. Christensen. Thapsigargin, a histamine secretagogue, is a non-12-*O*-tetradecanoylphorbol-13-acetate (TPA) type tumor promoter in two-stage mouse skin carcinogenesis. *J. Cancer Res. Clin. Oncol.* **111**:177–181 (1986).
19. Schonthal, A., J. Sugarman, J. H. Brown, M. R. Hanley, and J. R. Feramisco. Regulation of *c-fos* and *c-jun* protooncogene expression by the Ca^{2+}-ATPase inhibitor thapsigargin. *Proc. Natl. Acad. Sci. USA* **88**:7096–7100 (1991).
20. Rindler, M. J., M. M. Bashor, N. Spitzer, and M. H. Saier. Regulation of adenosine 3′:5′-monophosphate efflux from animal cells. *J. Biol. Chem.* **253**:5431–5436 (1978).
21. Harden, T. K., T. Evans, J. R. Hepler, A. R. Hughes, M. W. Martin, R. B. Meeker, M. M. Smith, and L. I. Tanner. Regulation of cyclic cAMP metabolism by muscarinic cholinergic receptors. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:207–220 (1985).
22. Ward, A., M. O. Chaffman, and E. M. Sorkin. Dantrolene: a review of its pharmacological and therapeutic use in malignant hyperthermia, the neuroleptic malignant syndrome and an update of its use in muscle spasticity. *Drugs* **32**:130–168 (1986).
23. Kojima, I., K. Koijma, D. Kreutter, and H. Rasmussen. The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. *J. Biol. Chem.* **259**:14448–14457 (1984).
24. Takeda, K., H. Meyer-Lehnert, J. K. Kim, and R. W. Schrier. Effect of angiotensin II on Ca^{2+} release kinetics and contraction in cultured rat glomerular mesangial cells. *Am. J. Physiol.* **254**:F254-F266 (1988).
25. Bouchelouche, P., B. Belhage, A. Frandsen, J. Drejer, and A. Schousboe. Glutamate receptor activation in cultured cerebellar granule cells increases cytosolic free Ca^{2+} by mobilization of cellular Ca^{2+} and activation of Ca^{2+} influx. *Exp. Brain Res.* **76**:281–291 (1989).
26. Biden, T. J., M. Prentki, R. F. Irvine, M. J. Berridge, and C. B. Wollheim. Inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+} from permeabilized insulin-secreting cells. *Biochem. J.* **223**:467–473 (1984).
27. Joseph, S. K., and J. R. Williamson. Characteristics of inositol trisphophate-mediated Ca^{2+} release from permeabilized hepatocytes. *J. Biol. Chem.* **261**:14658–14664 (1986).
28. Rossier, M. F., K. H. Krause, P. D. Lew, A. Capponi, and M. B. Vallotton. Control of cytosolic free calcium by intracellular organelles in bovine adrenal glomerulosa cells. *J. Biol. Chem.* **262**:4053–4058 (1987).
29. Dehpour, A. R., S. Mofakham, and M. Mahmoudian. *In vitro* binding of dantrolene to sarcoplasmic reticulum of rabbit skeletal muscle. *Biochem. Pharmacol.* **31**:965–968 (1982).
30. Nohmi, M., K. Kuba, and S. Y. Hua. Ultraviolet light activates blocking actions of dantrolene on intracellular Ca^{2+} release in bullfrog sympathetic neurones. *J. Biol. Chem.* **266**:22254–22259 (1991).
31. Fishman, P. H., M. Sullivan, and J. Patel. Down-regulation of protein kinase C in rat glioma C6 cells: effects on the β-adrenergic receptor-coupled adenylate cyclase. *Biochem. Biophys. Res. Commun.* **144**:620–627 (1987).
32. Patel, J., and S. Kassis. Concanavalin A prevents phorbol-mediated redistribution of protein kinase C and β-adrenergic receptors in rat glioma C6. *Biochem. Biophys. Res. Commun.* **144**:1265–1272 (1987).
33. Abou-Samra, A. B., J. P. Harwood, V. C. Manganiello, K. J. Catt, and G. Aguilera. Phorbol 12-myristate 13-acetate and vasopressin potentiate the effect of corticotropin-releasing factor on cyclic AMP production in rat anterior pituitary cells. *J. Biol. Chem.* **262**:1129–1136 (1987).
34. Bialojan, C., and A. Takai. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* **256**:283–290 (1988).
35. Katada, T., A. G. Gilman, Y. Watanabe, S. Bauer, and K. H. Jakobs. Protein kinase C phosphorylates inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur. J. Biochem.* **151**:431–437 (1985).
36. Yoshimasa, T., D. R. Sibley, M. Bouvier, R. J. Lefkowitz, and M. G. Caron. Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. *Nature (Lond.)* **327**:67–70 (1987).

Send reprint requests to: Dr. Gary Brooker, Department of Biochemistry and Molecular Biology, Georgetown University Medical School, Washington DC 20007.