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Cyclic AMP Inhibits Inositol Polyphosphate Production and Calcium Mobilization in Neuroblastoma × Glioma NG108-15 Cells

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SUMMARY

In the neuroblastoma × glioma hybrid cell line NG108-15, bradykinin (BK) receptor stimulation induced a rapid and concentration-dependent rise in cytosolic free Ca^{2+} levels, as measured with the Ca^{2+}-sensitive fluorescent dye fura-2. The Ca^{2+} transient was present in the absence of extracellular Ca^{2+} and was associated with a concentration-dependent production of inositol phosphates, particularly inositol trisphosphate ($InsP_3$). Pretreatment of intact NG108-15 cells with forskolin or dibutyryl-cAMP plus isobutylmethylxanthine reduced BK-stimulated $InsP_3$ production and the increase in cytosolic free Ca^{2+}. Membranes prepared from forskolin- and [^{3}H]inositol-pretreated NG108-15 cells also showed a diminished production of $InsP_3$ elicited by guanosine 5′-[γ-thio]triphosphate, NaF, or BK plus GTP. On the other hand, the Ca^{2+} sensitivity of membrane-associated phosphoinositide-specific phospholipase C (PI-PLC) was unaffected by forskolin pretreatment of intact NG108-15 cells. Collectively, these results suggest that A-kinase may inhibit receptor-mediated and postreceptor stimulation of PI-PLC in neuron-like cells, perhaps by impairing the coupling between a guanine nucleotide-binding protein and PI-PLC.

In the neuroblastoma × glioma hybrid NG108-15 cell line, the nonapeptide BK has been reported to stimulate PI-PLC, thereby promoting the formation of $InsP_3$ and DAG (1–3). As in most cells, the rise in $InsP_3$ levels serves to mobilize intracellular Ca^{2+} (4, 5), while the DAG stimulates PKC (6, 7). Receptor-mediated activation of PI-PLC in NG108-15 cells is thought to be mediated by a pertussis toxin-insensitive G protein (8). It is also well established that both the BK-induced production of $InsP_3$ and mobilization of Ca^{2+} are attenuated by activation of PKC with the phorbol ester PMA (9). This effect appears to occur distal to the BK receptor, in that PMA does not alter the affinity of BK binding sites and significantly inhibits postreceptor stimulation of PI-PLC in NG108-15 membranes (9). On the basis of these results, it has been suggested that activation of PKC by DAG results in a negative feedback regulation of polyphosphoinositide hydrolysis and Ca^{2+} mobilization, as has been reported in many other cell types (4, 6).

Activation of A-kinase by elevated intracellular cAMP levels has also been reported to reduce agonist-induced production of $InsP_3$ and other responses in a variety of cells, including platelets, neutrophils, and lymphocytes (10, 11). However, the possibility that cAMP may attenuate agonist-induced $InsP_3$ production in a neuronal cell has only recently been addressed (12), and the site(s) of such modulation remains to be determined. Accordingly, the present work was undertaken to explore the effects of cAMP-elevating agents on BK-stimulated $InsP_3$ production and Ca^{2+} mobilization in cultured NG108-15 cells as well as the regulation of membrane-associated PI-PLC activity by Ca^{2+} and guanine nucleotides. The results indicate that forskolin or dibutyryl-cAMP decreases BK-induced $InsP_3$ formation and Ca^{2+} mobilization. These same treatments also attenuated postreceptor G protein-mediated activation of PI-

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ABBREVIATIONS: BK, bradykinin; DAG, diacylglycerol; G_i, guanine nucleotide binding protein that couples inhibitory receptors to adenylate cyclase; G_p, guanine nucleotide-binding protein that couples receptors to phospholipase C; GTPγS, guanosine 5′-[γ-thio]triphosphate (and specified isomers); IBMX, 3-isobutyl-1-methylxanthine; InsP, inositol phosphate; $InsP_3$, inositol trisphosphate; MES, 2-[*N*-morpholino]ethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PI-PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C; HBSS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMA, 12-*O*-tetradecanoylphorbol-13-acetate; PCA, perchloric acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; DMEM, Dulbecco's modified Eagle's medium; $InsP_1$, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_4$, inositol tetrakisphosphate; G protein, guanine nucleotide-binding protein.

PLC, without altering the Ca^{2+} sensitivity of this enzyme. Collectively, the results suggest that elevated cAMP levels may inhibit the responsiveness of PI-PLC to agonists in neuronal cells by impairing its coupling to a G protein. A preliminary report of this work has been published (13).

Experimental Procedures

Materials

Materials used in this study were obtained from the following sources: *myo*-[^{3}H]inositol (10–20 Ci/mmol), [^{3}H]phosphatidylinositol-4,5-bisphosphate, [^{3}H]Ins(4)P_1, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3, and [^{3}H]Ins(1,3,4,5)P_4 from Dupont/New England Nuclear (Boston, MA); Liquiscint from National Diagnostics (Manville, NJ); Dowex from Bio-Rad (Rockville Center, NY); fura-2/AM from Molecular Probes (Eugene, OR); DMEM from GIBCO (Grand Island, NY); penicillin, streptomycin, hypoxanthine, aminopterin, and thymidine from Flow Laboratories (McLean, VA); and L15, ionomycin, saponin, GTPγS, NaF, AMP, ADP, ATP, GMP, GDP, GTP, and bovine albumin from Sigma Chemical Co. (St. Louis, MO). All other reagent grade chemicals were obtained from Fisher Scientific (Pittsburgh, PA).

Methods

Cell culture. Cultures of the neuroblastoma-glioma hybrid cell line NG108-15 were grown to confluence in 75-cm^2 culture dishes for fura-2 calcium and membrane studies or in six-well plates for [^{3}H]inositol labeling studies. Cell cultures were maintained in DMEM with 10% fetal calf serum, supplemented with 25 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM hypoxanthine, 1 μM aminopterin, and 12 μM thymidine, in a humidified atmosphere of 95% O_2/5% CO_2 at 37°. The original cells provided to this laboratory were the generous gift of Dr. M. Nirenberg, National Institutes of Health (Bethesda, MD).

Measurement of intracellular calcium. Confluent monolayers of NG108-15 cells cultures in 75-cm^2 plates were harvested by mild trituration, washed with L15 buffer (supplemented with 15 mM HEPES, 5 mM glucose, and 1 mg/ml bovine albumin), and centrifuged at 25° at 60 × *g* for 10 min. This wash procedure was repeated two more times, after which the cells were resuspended in L15 medium and returned to the incubator (37°, 95% O_2/5% CO_2) for 60 min. The cells were then washed again in L15 medium, resuspended at a concentration of 2–3 × 10^6 cells/ml, and incubated with fura-2/AM (4 μM) for 45 min at 37°. In those experiments involving forskolin pretreatment, forskolin was added for the last 30 min of fura-2 loading. Following the loading period, cells were harvested and centrifuged as before. The supernatant was aspirated, and the cells were washed several times with HBSS (115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.0 mM $CaCl_2$, 15 mM HEPES, 10 mM glucose, 1 mg/ml bovine albumin, pH 7.35), suspended in HBSS, and stored on ice until used.

Fluorescence was continuously monitored using an air turbine fluorimeter constructed by the Biomedical Instrumentation Group, University of Pennsylvania. Fluorescence of the fura-2-loaded NG108-15 cells was measured at excitation wavelengths of 339 and 380 nm and an emission wavelength of 510 nm. The background fluorescence of unloaded cell suspensions was less than 10% of the total fluorescence of fura-loaded cells, and the value for each wavelength was subtracted before calculation of the fluorescence ratio at each excitation wavelength. Calcium calibration was performed by measuring the maximum fluorescence (R_{max}) after addition of ionomycin (10 μM) and calcium (10 mM). This maximum was similar to that observed in saponin (0.03%)-treated suspensions in the presence of 10 mM calcium. Minimum fluorescence, R_{min}, was obtained following saponin addition after addition of excess EGTA. Determination of intracellular calcium concentration was calculated from the recorded fluorescence, *R*, (340/380), using the equation described by Grynkiewicz *et al.* (14). The mean values R_{min} and R_{max} were 0.463 ± 0.2 and 7.7 ± 0.16, respectively, whereas the ratio of free to bound fura-2 at 380 nm was 3.56 ± 0.27 (six determinations for all observations).

***myo*-[^{3}H]Inositol labeling studies.** NG108-15 cells grown to confluency on six-well plates were incubated in DMEM with 5 μCi/ml *myo*-[^{3}H]inositol for 22–24 hr for labeling of membrane phosphoinositides. Each well was washed with 3 ml of HBSS and the cells were then incubated in the same medium for an additional 30 min before addition of BK. The reaction was terminated by rapid aspiration of the medium followed by the addition of 1 ml of ice-cold PCA solution (12%, PCA, 3 mM EDTA, 1 mM DTPA). The cells were harvested by scraping, the wells were washed with 0.5-ml of PCA solution, and the extracts were combined and centrifuged at 215 × *g* for 10 min. The precipitate was analyzed for protein content by the method of Bradford (15), using bovine serum albumin as a standard. The supernatant was neutralized with KOH (3 M), MES (0.25 M), and MOPS (0.25 M) and centrifuged to remove the salt pellet.

InsPs were separated either by ion exchange chromatography on a 0.5-ml Dowex minicolumn (AG1X8 formate from) (16) or by high pressure liquid chromatography (17). Radioactivity in each inositol phosphate fraction was quantified by liquid scintillation counting after addition of 16 ml of Liquiscint. The elution profile of InsPs was characterized by comparison with authentic standards [[^{3}H]Ins(4)P_1, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3, and [^{3}H]Ins(1,3,4,5)P_4].

InsPs production in NG108-15 membranes. The production of $InsP_3$ was measured in membranes prepared from NG108-15 cells labeled with *myo*-[^{3}H]inositol as previously described (9, 18). Cells were suspended in a medium containing 10 mM LiCl, 0.5 mM EDTA, 10 mM Tris·HCl (pH 8.0), and 0.1 mM ATP and, after homogenization and centrifugation at 48,000 × *g* at 4° for 20 min, the resulting pellet was resuspended in an assay buffer consisting of 10 mM LiCl, 0.1 mM ATP, 0.25 mM EDTA, 10 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, and a final Ca^{2+} concentration of 1 μM, at a protein concentration of 1 mg/ml. In order to measure $InsP_3$, an aliquot of the membrane suspension (200 μg of protein/assay) was incubated at 37° in a total volume of 300 μl and the reaction was terminated by addition of 300 μl of ice-cold 12% (w/v) PCA, containing 3 mM EDTA and 1 mM DTPA, and 100 μl of 2% bovine serum albumin. InsPs were separated and analyzed by Dowex chromatography.

Measurement of PI-PLC activity. NG108-15 cells were rinsed three times in 20 mM Tris·HCl, pH 7.4, 150 mM NaCl, lysed for 10–15 min at 4° in 20 mM Tris·HCl, pH 7.4, harvested by scraping, and homogenized with a Dounce (glass-glass) homogenizer. The homogenate was then centrifuged at 48,000 × *g* for 30 min. The resultant pellet was resuspended in hypotonic Tris and recentrifuged for 30 min at 48,000 × *g*, and the final pellet was resuspended in 50 mM bis-Tris, pH 7.0, for measurements of PI-PLC activity. PI-PLC activity was measured by the formation of water-soluble products from 1-α[*inositol*-2-^{3}H]phosphatidylinositol-4,5-bisphosphate, as described previously (19, 20). Phosphoinositide substrate was dried under a stream of N_2 and sonicated in 5 mg/ml deoxycholate. Enzymatic activity was measured over 10 min at 37° in 50 mM bis-Tris, pH 7.0, 50 mM KCl, 1 mM $CaCl_2$, 2.4 mM deoxycholate, 20 μM substrate (15,000–25,000 cpm), and 30–50 μg of membrane protein. In those assays using lower Ca^{2+} concentrations, Ca^{2+}-EGTA buffers were prepared as described by Schatzmann (21). Reactions were terminated by the addition of 250 μl of chloroform/methanol/1 M HCl (50:50:0.3) and 75 μl of 1 M HCl containing 5 mM EGTA. Samples were then centrifuged, and 150 μl of the upper aqueous phase were counted in liquid scintillant (Liquiscint; National Diagnostics).

Adenylate cyclase activity. Membranes were prepared from confluent NG108-15 cells as described above. An aliquot of the membrane suspension (approximately 1 mg/ml) was incubated in a total volume of 100 μl of 25 mM Tris-acetate (pH 7.6) containing 0.1 mM [α-^{32}P] ATP (2–6 × 10^6 dpm), 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, 5 mM Mg-acetate, 0.5 mM ATP, 0.05 mM cAMP, 1.0 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.01 mM GTP, and 1.0 mM IBMX. The [^{32}P]cAMP formed was isolated by the column

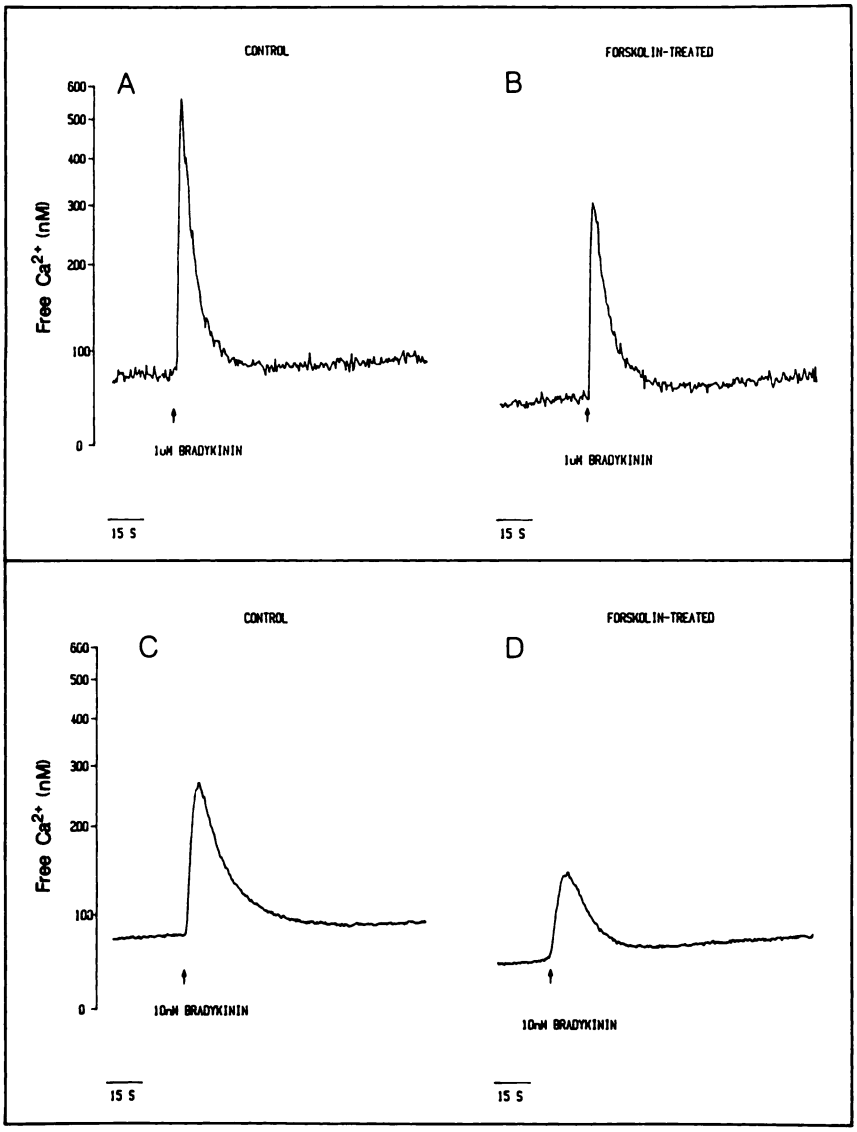

Fig. 1. BK-induced intracellular calcium release in control and forskolin-pretreated cells. NG108-15 cells were loaded with fura-2/AM (4 μM) for 45 min at 37°. Three milliliters of cell suspension (4×10^6 cells total) were pipetted into a 1-cm^2 thermostatically controlled (37°) quartz cuvette and a magnetic stirrer maintained continual suspension of cells. Concentrated stock solutions of BK were added to the cell suspension to achieve the final concentration reported. *Traces A* and *C*, representative agonist responses in controls cells; *traces B* and *D*, representative agonist responses in forskolin-pretreated (100 μM, 30 min) cells.

TABLE 1

Dose-response characteristics of BK-induced peak intracellular calcium and attenuation by forskolin in NG108-15 cells

NG108-15 cells were grown to confluence on 75-cm^2 plates and, after loading with fura-2/AM, were removed from the plates and used at a concentration of 2×10^6 cells/ml for Ca^{2+} measurements. Resting cytosolic free Ca^{2+} concentrations were, on average, 94 ± 5 nM in controls and 71 ± 6 nM in forskolin-treated cells. Results are expressed as the maximum change of cytosolic free Ca^{2+} after addition of BK to control and forskolin-treated (100 μM, 30 min) cells. Each value represents the mean of three to six observations from separate populations of cells. Each value represents the mean ± standard error.

	Ca^{2+}			
	1 nM[a]	10 nM[a]	100 nM[a]	1 μM[a]
	nM			
Control	30 ± 7	227 ± 23	305 ± 14	501 ± 22
Forskolin	32 ± 6	104 ± 18	189 ± 20	285 ± 20
ΔCa^{2+}	+2	−123[b]	−116[b]	−216[b]
Difference (%)	+7	−54	−38	−43

[a] BK concentration.
[b] Statistically significant difference ($p < 0.05$) of forskolin-treated cells from control.

method of Salomon (22). In order to monitor the performance of these chromatographic procedures, [^{3}H]cAMP was added to each tube and counts were subsequently corrected for the efficiency of its recovery.

Statistical analysis. Data were analyzed using either an unpaired Student's *t* test (significance was accepted at $p < 0.05$) or two-way analysis of variance in combination with Dunnett's test (significance was accepted at $\alpha = 0.05$).

Results

BK-induced intracellular calcium release. Fura-2-loaded suspensions of NG108-15 cells displayed stable resting cytosolic free Ca^{2+} levels of 94 ± 7 nM (six determinations). Typical responses of NG108-15 cells to BK are shown in Fig. 1 (A and C). The maximally effective concentration of BK, 1 μM, characteristically produced a rapid transient increase in cytosolic free Ca^{2+} (501 ± 22 nM; four determinations) that reached a peak within 2 sec and declined thereafter to the initial value. Lower concentrations of BK also elicited a sharp rise in Ca^{2+} levels, but the peak height of the response was lower (Fig. 1C). The dose-response characteristics of BK-induced increases in cytosolic free Ca^{2+} are summarized in Table

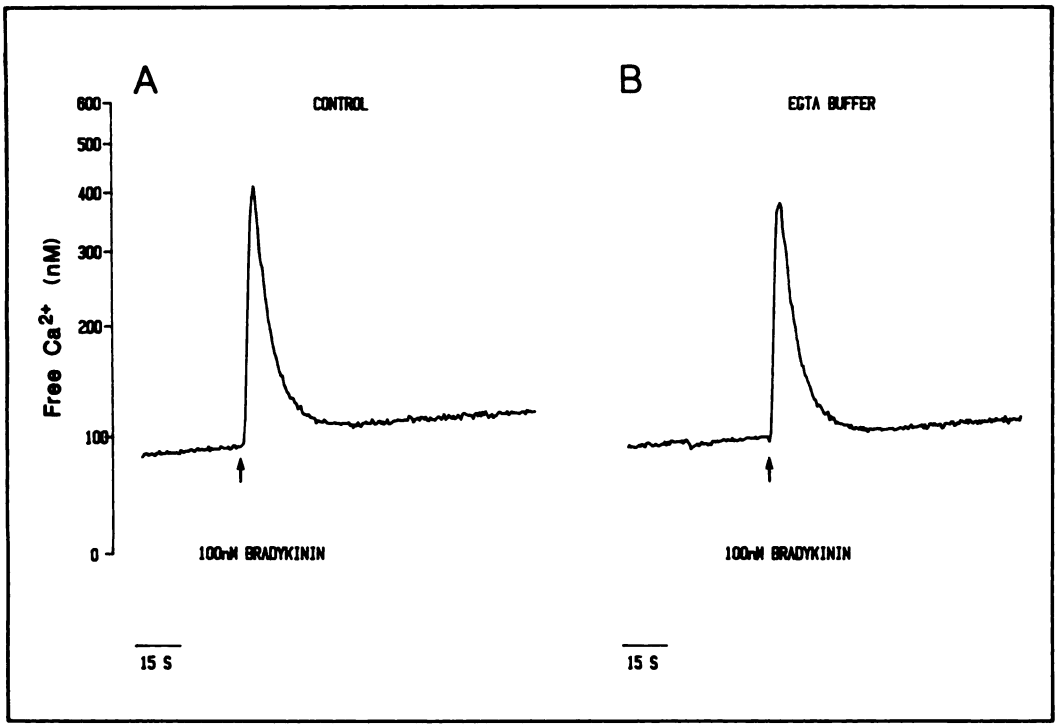

Fig. 2. BK-induced intracellular calcium release in control and EGTA buffer. NG108-15 cells were loaded with fura-2/AM (4 μM) for 45 min at 37°. The experimental conditions were the same as those for Fig. 1. *Trace A*, agonist response in control buffer; *trace B*, agonist responses in EGTA (1 mM) buffer.

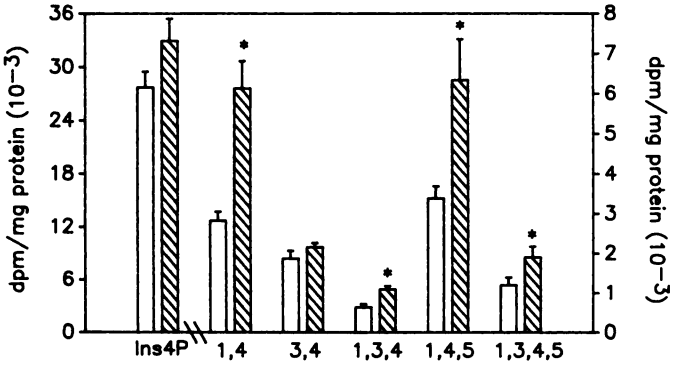

Fig. 3. High pressure liquid chromatographic separation of BK-stimulated (10 μM, 15 sec) InsP isomers in cultured neuronal cells. Neuroblastoma-glioma cells (NG108-15) were incubated in *myo*-[^{3}H]inositol-containing DMEM for 24 hr. Identification of InsP isomers was made by comparison with authentic standards as well as with the absorbance pattern of nucleotide standards. Data are presented as production of tritium-labeled InsP isomers expressed as dpm, normalized to mg of protein. □, Control cells; ▩, agonist-treated cells. *Vertical bars*, standard error of each value; *, statistical significant difference ($p < 0.05$) from control value; five or six determinations for each point from three separate experiments were performed.

1. Ca^{2+} transient responses were also obtained with BK in the presence of 1 mM EGTA (Fig. 2) that were qualitatively and quantitatively similar to those observed in the absence of EGTA, indicating that BK stimulates the mobilization of intracellular Ca^{2+} in NG108-15 cells with a negligible contribution of Ca^{2+} entry to the transient.

Pretreatment of NG108-15 cells with 100 μM forskolin for 30 min increased adenylate cyclase activity from 25 ± 1.3 to 240 ± 16 pmol/min/mg of protein (six determinations). The resultant rise in cAMP levels reduced resting free Ca^{2+} by 23 ± 6 nM and caused a 38–54% decrease in the Ca^{2+} transients produced by all concentrations of BK except the lowest (Table 1). More specifically, forskolin decreased the peak Ca^{2+} without altering

TABLE 2

Forskolin and dibutyryl-cAMP inhibition of BK-stimulated $InsP_3$ production in NG108-15 cells

Confluent monolayers of NG108-15 cells were incubated in DMEM containing *myo*-[^{3}H]inositol (5 μCi/ml), for 24 hr. Forskolin (100 μM, 30 min) was added to monolayers of cells before BK (10 μM, 15 sec) exposure. Likewise, dibutyryl-cAMP (100 μM, 30 min), together with IBMX (500 μM), was added to monolayers of cells preceding BK exposure. Data are presented as [^{3}H]$InsP_3$ production, expressed as dpm/mg of protein. Each value represents the mean ± standard error of six separate observations.

	$InsP_3$		
	Control	Forskolin	Dibutyryl-cAMP
	dpm/mg of protein		
Basal	2571 ± 164	2813 ± 250	2455 ± 250
BK	5331 ± 252[a]	3651 ± 350[a]	4111 ± 405[a]
$\Delta InsP_3$	2760	838[b]	1656[b]

[a] Statistically significant difference ($p < 0.05$) from treatment-matched control cells.

[b] Significant difference from BK-treated control cells.

the shape of the transient (Fig. 1, B and D). Further, pretreatment of NG108-15 cells with dibutyryl-cAMP (100 μM; 30 min) plus IBMX (500 μM) resulted in a diminution of the BK-induced (1 μM) Ca^{2+} transient that was comparable to that obtained with forskolin (data not shown). Taken together, these data suggest that an increase in cAMP attenuates intracellular Ca^{2+} release in NG108-15 cells. Therefore, further studies were aimed at elucidating possible mechanisms for this effect.

BK-induced $InsP_3$ production. In a series of preliminary studies, we confirmed the work of previous investigators (1–3, 8, 9) by demonstrating that BK produced a dose- and time-dependent rise in $InsP_3$ levels, with the maximal increase occurring between 1 and 10 μM after approximately 15 sec of agonist exposure (data not shown). For instance, Fig. 3 shows that the levels of [^{3}H]Ins(1,4,5)P_3 were significantly increased by 10 μM BK after 15 sec, as were those of another IP_3 isomer, Ins(1,3,4)P_3. The levels of Ins(1,4,5)P_3 were 7-fold greater than those of Ins(1,3,4)P_3, indicating that Ins(1,4,5)P_3 is the predom-

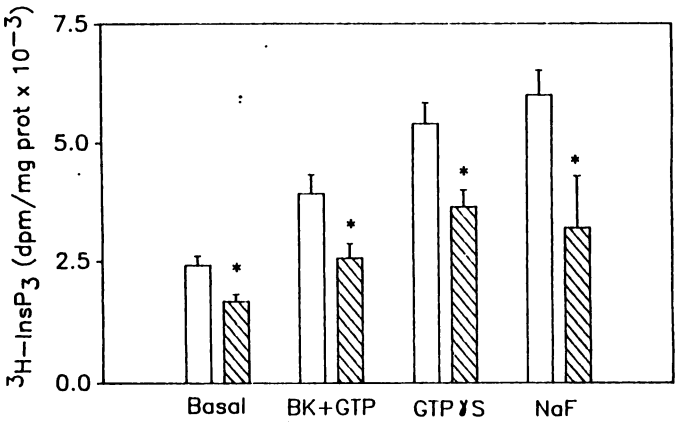

Fig. 4. Effect of forskolin on basal and agonist-, GTPγS-, and NaF-stimulated $InsP_3$ formation in NG108-15 membranes. Intact cells were incubated in *myo*-[^{3}H]inositol-containing DMEM, membranes were prepared, and InsPs were separated by Dowex chromatography. Membrane aliquots (200 μg of protein) were exposed to BK (10 μM) and GTP (100 μM, 5 min), GTPγS (100 μM, 2 min), or NaF (10 mM, 5 min). $InsP_3$ levels are expressed as dpm (× 10^{-3}), normalized to mg of protein. □, Control responses; ▩, forskolin-treated cells (100 μM1, 30 min). *, Statistically significant difference ($p < 0.05$) versus control. Each point represents the mean of four to eight observations from four separate experiments.

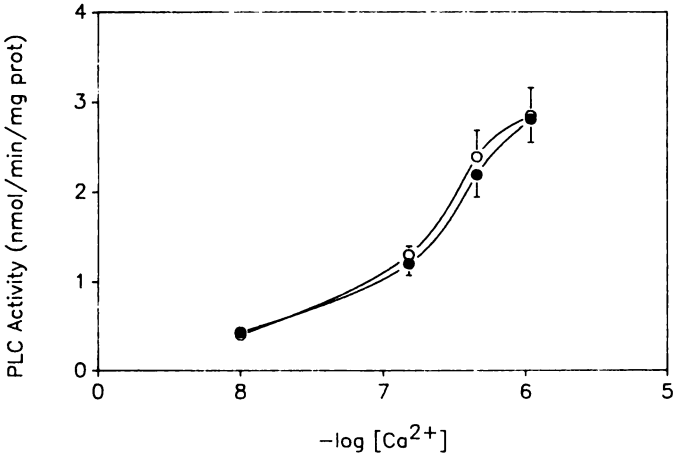

Fig. 5. Effect of calcium on PI-PLC activity in membranes obtained from control and forskolin-treated NG108-15 cells. PI-PLC activity was assayed using 20 μM phosphatidyl bisphosphate at various calcium concentrations. ○, control responses; ●, forskolin-treated cells. Each point represents the mean and standard error of six to nine observations from three separate experiments.

inant $InsP_3$ isomer produced initially in agonist-stimulated cells. BK also elevated the levels of Ins(1,3,4,5)P_4, consistent with the formation of this isomer from Ins(1,4,5)P_3 by a 3-kinase activity with subsequent hydrolysis by a 5-phosphatase to Ins(1,3,4)P_3 (23). Ins(1,4,)P_2 levels increased upon BK stimulation in these cells, whereas Ins(3,4)P_2, the product of Ins(1,3,4)P_3 hydrolysis, as well as Ins(4)P_1 remain unchanged at this early time point.

To examine whether the forskolin and dibutyryl-cAMP attenuation of BK-induced mobilization of intracellular Ca^{2+} was associated with a similar decrease of $InsP_3$ production, monolayers of cells were incubated with 100 μM forskolin for 30 min before the addition of 10 μM BK for 15 sec. BK alone increased $InsP_3$ levels by 2-fold and this effect was substantially attenuated by pretreatment of the cells with forskolin (Table 2). BK-stimulated $InsP_3$ levels were also significantly reduced after a 30-min preincubation of the cells with dibutyryl-cAMP (100 μM) and IBMX (500 μM (Table 2), although the magnitude of the decrement was less than that observed in forskolin-treated cells. Forskolin or dibutyryl-cAMP pretreatment also reduced the levels of $InsP_2$ in agonist-stimulated cells (data not shown).

NaF and GTPγS stimulation of $InsP_3$ production in NG108-15 membranes. Preliminary studies with membranes containing endogenously labeled [^{3}H]inositol lipids indicated that NaF (10 mM) produced a rise in $InsP_3$ levels within 2 min and attained a peak after 5 min (98% increase over control). Similarly, GTPγS (100 μM) stimulated a rapid increase in $InsP_3$ levels, reaching a peak after 2 min of exposure, with the magnitude of this increase being comparable to that observed with NaF. The agonist BK (10 μM) also caused a rapid rise in $InsP_3$ levels in membranes (59% increase over control at 5 min) but only in the presence of exogenous GTP (100 μM), which itself was virtually ineffective (data not shown).

In order to continue the analysis of the effects of cAMP-elevating agents on phosphoinositide hydrolysis, we measured basal and agonist- and postreceptor-stimulated $InsP_3$ production of NG108-15 membranes prepared from control and forskolin-pretreated NG108-15 cells. In control membranes, basal production of $InsP_3$ was 2.4 × 10^3 dpm/mg of protein (Fig. 4). The agonist BK (10 μM) together with GTP (100 μM) significantly increased PI-PLC activity by 63%. Moreover, stimulation with GTPγS (100 μM) or NaF (10 mM), which bypass agonist receptors and presumably stimulate the putative G protein involved in PI-PLC activation, increased $InsP_3$ production by 124% and 149%, respectively, in NG108-15 membranes. Finally, forskolin pretreatment significantly decreased the basal production of $InsP_3$ and attenuated the increases induced by the agonist BK as well as GTPγS and NaF.

PI-PLC activity. Preliminary studies indicated that membrane-associated PI-PLC activity was approximately 5% of total PI-PLC activity in NG108-15 cells, as has been reported previously for other cell types (19, 20). Moreover, the specific activity of membrane-associated PI-PLC was 2.85 ± 0.31 nmol/min/mg of protein, whereas that in NG108-15 cytosolic fractions was 6.91 0.72 nmol/min/mg of protein in the presence of optimal Ca^{2+} concentration.

Because forskolin pretreatment of intact cells was associated with a small decline in the basal production of $InsP_3$ in NG108-15 membranes, we examined the effects of forskolin on the Ca^{2+} sensitivity of membrane-associated PI-PLC. As expected, with control NG108-15 cells, the activity of membrane-associated PI-PLC was markedly stimulated by Ca^{2+} within the physiological range. More specifically, PI-PLC activity increased from 0.4 nmol/min/mg of protein at 10 nM Ca^{2+} to a maximum of 2.89 nmol/min/mg of protein at 1 μM Ca^{2+} (Fig. 5). However, pretreatment of intact NG108-15 cells with forskolin had no effect on PI-PLC activity or its sensitivity to Ca^{2+}, subsequently measured in membranous fractions.

Discussion

In a number of different cell types, activation of adenylate cyclase has been shown to modulate cellular functions that are mediated by InsP production and calcium mobilization. For instance, in platelets (11), glomerulosa cells (24), PC12 cells (25), neutrophils (10), dorsal root ganglion hybrid cells (12), and lymphocytes (10), concurrent activation of adenylate cyclase decreases agonist responses that are, in part, the result of

phosphoinositide hydrolysis. In most cases, this attenuation is associated with a diminution of agonist-induced production of inositol polyphosphates, although the site(s) of cAMP-mediated modulation appear to vary from cell to cell. For instance, in rat glomerulosa cells, elevations of cAMP levels induced by cholera toxin reduce vasopressin and angiotensin II-mediated $InsP_3$ formation, apparently by decreasing agonist receptor number (24). Alternatively, in PC12 cells, cAMP may impair responses mediated by phosphoinositide hydrolysis and the subsequent mobilization of Ca^{2+} by decreasing the activity of Ca^{2+}/calmodulin kinases (25). Other reports indicate that G proteins, PI-PLC, and $InsP_3$ receptors may be likely sites of cAMP modulation (26–28).

The present results demonstrate that the BK-induced accumulation of $InsP_3$ as well as the Ca^{2+} transient were attenuated by forskolin or dibutyryl-cAMP in cultured NG108-15 cells. These data suggest that the attenuation of the Ca^{2+} transient may result from the decreased production of $InsP_3$. Consistent with this view, forskolin also decreased BK-induced $InsP_3$ production in NG108-15 membranes. Moreover, the stimulatory effect of GTPγS and NaF, which mimics the actions of GTP, were similarly attenuated in membranes, suggesting that a likely site of A-kinase action was located distal to the BK receptor and proximal to the Ca^{2+} release mechanisms, possibly at the level of the putative G protein (G_p) that couples agonist receptors to PI-PLC (29, 30).

In addition to the dimunition of stimulated activity, the basal production of $InsP_3$ in NG108-15 membranes was also decreased by forskolin pretreatment, raising the possibility of an additional effect of A-kinase on membrane-associated PI-PLC. However, under assay conditions that measured membrane-associated PI-PLC activity directly in the absence of agonist or G protein regulation of this enzyme, forskolin pretreatment did not alter enzymatic activity or its sensitivity to Ca^{2+}. On the basis of these results, it seems unlikely that cAMP-elevating agents directly affect the activity of membrane-associated PI-PLC isozymes in NG108-15 cells. Rather, it would appear that the A-kinase-mediated modulation of phosphoinositide hydrolysis may require a membranous environment that permits interactions between G proteins and PI-PLC. Recently, a similar mechanism was proposed in HL-60 cells, in which it was demonstrated that activated cAMP-dependent protein kinase inhibited receptor-mediated and GTPγS stimulation of $InsP_3$ production without altering increased $InsP_3$ formation induced by elevated Ca^{2+} in plasma membranes (31).

The inhibitory effects of elevated cAMP levels and presumed activation of A-kinase on BK-induced phosphoinositide hydrolysis and Ca^{2+} mobilization in NG108-15 cells are similar to those produced by activation of PKC. For instance, Osugi *et al.* (9) demonstrated that the phorbol ester PMA rapidly inhibited BK-induced $InsP_3$ formation and the attendant mobilization of Ca^{2+}, without altering the density of BK receptors. PMA also significantly reduced $InsP_3$ formation in membranes even when the receptor was bypassed with GTPγS, suggesting that a postreceptor site was the best unifying hypothesis for the location of the PMA effect. Recent work in other cells has supported the involvement of postreceptor events in the regulatory actions of PKC. For instance, phorbol esters have been reported to attenuate vasopressin-induced $InsP_3$ formation in A-10 cells (32). This effect was associated with an abolition of GTP regulation of agonist binding, indicating that the coupling of the receptor to G_p was altered. Additional observations suggesting that a G_p-PLC complex may be a site of PKC interaction include reports of the inhibition of GTPγS stimulation of phosphoinositide hydrolysis in plasma membrane preparations by phorbol esters or purified PKC (20, 33, 34), whereas a G protein that reacts with antibodies to G_z (35) has been shown to be phosphorylated after PMA treatment in platelets (36). Moreover, it has been demonstrated that a M_r 62,000 PLC from guinea pig uterus can be phosphorylated *in vivo* by incubation with phorbol esters (19), and Rhee *et al.* (37) have recently reported the presence of multiple forms of PLC, which are similarly phosphorylated by PKC.

The evidence in support of A-kinase-directed phosphorylation of G_p or PI-PLC is less complete. With respect to G proteins in general, it has been shown that G_i can be phosphorylated *in vitro* by both PKC (38) and A-kinase (39), which may prevent receptor-mediated inhibition of adenylate cyclase (40, 41). More recently, it has been suggested that A-kinase may phosphorylate the G protein involved in PLC activation in platelets. In the study of Lazarowski and Lapetina (26), NaF stimulated PLC activity as measured by the formation of InsPs and phosphatidic acid, and these responses were inhibited by forskolin, dibutyryl-cAMP, and iloprost. Moreover, the iloprost-induced inhibition of platelet PLC activity was associated with the phosphorylation of a *ras*-related M_r 22,000–24,000 G protein, which may be a putative G_p in platelets (42), raising the possibility that it was inactivated by cAMP-dependent phosphorylation.

On the other hand, recent work in human platelets has suggested that PI-PLC may not be phosphorylated by A-kinase (27). In these studies, dibutyryl-cAMP was shown to inhibit GTPγS stimulation of PLC, and this inhibition was reversed by H-8, an inhibitor of A-kinase. The attenuation of stimulated PLC activity was not associated with A-kinase-mediated phosphorylation of PLC purified from platelet membranes (43), nor was the binding of 8-N^3-[γ-^{32}P]GTP to these and the small G proteins (M_r 21,000–27,000), which may be involved in PLC activation in platelets (42, 44, 45), altered. On the basis of these results, it was suggested that the A-kinase-induced inhibition of PLC activity did not result from the phosphorylation of either G_p or PI-PLC. Initial attempts in our laboratory to determine whether a G protein or PLC is phosphorylated by activation of A-kinase in NG108-15 cells have been similarly unsuccessful. However, with the development of suitable immunoprecipitating antibodies to distinct G proteins and isoforms of PLC, it should be possible to define the mechanisms responsible for the cross-talk between the cAMP and Ca^{2+} signalling pathways in greater detail.

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