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Transfected cGMP-dependent protein kinase suppresses calcium transients by inhibition of inositol 1,4,5-trisphosphate production

(intracellular calcium signaling/phospholipase C/receptor cross-talk/protein phosphorylation/CHO cell transfection)

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ABSTRACT **cGMP is a key regulatory molecule in visual transduction, integration of neuronal response to excitatory neurotransmitters, relaxation of smooth muscle, intestinal secretion of water and salt, and reabsorption of sodium and water in the distal tubules of the nephron. Some of these cellular functions are associated with the activation of cGMP kinase and a decrease in cytosolic calcium levels ($[Ca^{2+}]_i$). The mechanism by which cGMP kinase lowers $[Ca^{2+}]_i$ is controversial. We have used CHO cells stably transfected with cGMP kinase to test several of the proposed $[Ca^{2+}]_i$-lowering mechanisms. Thrombin induces a calcium transient in wild-type and cGMP kinase-expressing CHO cells by releasing calcium from intracellular stores. Preincubation of wild-type cells with 8-bromo-cGMP had no effect on the calcium transient, whereas 8-bromo-cGMP prevented the thrombin-stimulated calcium transient in cGMP kinase-expressing CHO cells. In both cell types 8-bromo-cGMP had no effect on $[Ca^{2+}]_i$ transients induced by replacing extracellular sodium by tetramethylammonium, ruling out an effect of cGMP kinase on Ca^{2+}-ATPases. However, cGMP kinase activation effectively suppressed thrombin-induced stimulation of inositol 1,4,5-trisphosphate production. These results show that cGMP kinase lowers $[Ca^{2+}]_i$ by interfering with the inositol 1,4,5-trisphosphate synthesis.**

Calcium is a primary regulator of the excitability of excitatory neurons (1) and muscle tone (2). In vertebrate smooth muscle a number of compounds such as nitric oxide (NO), atrial natriuretic peptides, and a variety of nitric oxide-containing or -producing drugs elevate cGMP, lower cytosolic calcium concentration ($[Ca^{2+}]_i$), and decrease muscle tone (3, 4). These or similar compounds elevate also cGMP and decrease $[Ca^{2+}]_i$ in platelets (5) and neurons (6). Elevation of cGMP may protect against neuroexcitotoxicity and other harmful conditions (7, 8), presumably by lowering stimulated calcium levels. It is now likely that activation of cGMP kinase is necessary to reduce $[Ca^{2+}]_i$ in smooth muscle cells and platelets (5, 9–13). However, the mechanism of action of cGMP kinase is still unresolved.

Basically three mechanisms have been proposed: (*i*) stimulation of calcium extrusion by activation of the calmodulin-stimulated Ca^{2+}-ATPase (14, 15); (*ii*) stimulation of calcium uptake into the sarcoplasmic reticulum by phosphorylation of phospholamban (16–19); and (*iii*) inhibition of the hormone-triggered generation of second messengers—in particular, phosphatidylinositol breakdown (20–22). The physiological importance of these mechanisms is unclear and may differ between different cell types because none of these mechanisms has been demonstrated to work in a cell-free system. In addition, the analysis of experiments with intact tissues or cells was hampered by the complexities of the cellular signaling systems. The availability of the cDNA for cGMP kinase (23, 24) and modern cell-transfection techniques made it possible to examine in detail the $[Ca^{2+}]_i$-lowering mechanism of cGMP kinase by transfection of Chinese hamster ovary (CHO) cells that do not express cGMP kinase.

MATERIALS AND METHODS

Materials. The CHO dihydrofolate reductase-deficient mutant DG44 cell was provided by L. Chasin, Columbia University, New York. Dialyzed fetal calf serum, HAM F-12 medium, and nonessential amino acids were from GIBCO. Dulbecco's modified Eagle's medium was from Biochrom, Berlin. All other reagents were commercially available.

Stable Transfection of CHO Cells. The CHO cells were transfected by electroporation with plasmid p91023Iα (24), which contains the coding sequence of the cGMP kinase Iα isozyme and that of the dihydrofolate reductase in a second open reading frame. cGMP kinase was amplified in two stably transfected cell clones, as described (25). Cell clone I-8 [CHO cells stably transfected with the cGMP kinase plasmid (CHO-cGK cells)] was chosen for further experiments. Control cells [wild-type CHO (CHO-WT)] were grown in HAM F-12 medium supplemented with the heat-inactivated fetal calf serum.

Assays. Cytosols were obtained by harvesting confluent CHO-WT and CHO-cGK cells with a cell scraper. The cells were washed twice with ice-cold phosphate-buffered saline and processed as described (24, 25). cGMP binding, cGMP kinase-activity measurement, and immunoblotting were done as described (24–26).

Measurement of Calcium Transients in CHO Cells. *Method A.* Cells were grown for 2 days on coverslips and incubated for 0.5–1.5 hr with 5 μM fura-2-AM. Coverslips were then washed with buffer A (25 mM $NaHCO_3$, pH 7.4/115 mM NaCl/4.7 mM KCl/1.2 mM KH_2PO_4/1.18 mM $MgSO_4$/2 mM $CaCl_2$/10 mM glucose) and placed in the light path of an Axiovert microscope equipped with a xenon light source. Fura-2 fluorescence of a single cell was monitored on a Deltascan system and stored on a personal computer. Changes in 340/380 nm ratio were smoothed by Savitzky–Golay smoothing with 9-point buffer. The coverslips were continuously superfused at 36°C with buffer A at a flow rate of 3–4 ml/min. Apparent $[Ca^{2+}]_i$ values were calculated according to ref. 27.

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Abbreviations: $[Ca^{2+}]_i$, cytosolic calcium concentration; CHO-WT cells, Chinese hamster ovary wild-type cells; CHO-cGK cells, CHO cells stably transfected with the cGMP kinase plasmid; InsP_3, inositol 1,4,5-trisphosphate; TMA, tetramethylammonium; BAPTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate.

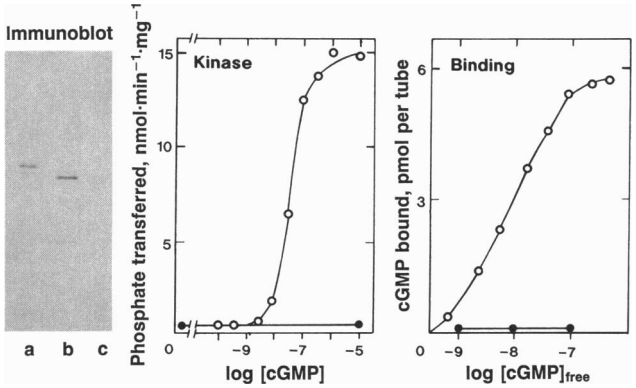

FIG. 1. Characteristics of cGMP kinase-expressing CHO cells. (*Left*) Immunoblot of cGMP kinase-expressing cells. The samples were loaded onto a 7.5% SDS/PAGE as follows: soluble protein (100 μg) from CHO-cGK cells (lane a), purified bovine lung cGMP kinase (50 ng) (lane b), and soluble protein (100 μg) from CHO-WT cells (lane c). The poly(vinylidene difluoride) membrane was probed with an affinity-purified antiserum against the carboxyl terminus of cGMP kinase (25). (*Middle*) cGMP kinase activity. The soluble-protein (5 μg) fraction of the CHO-cGK (○) or CHO-WT (●) cells was incubated for 20 min with and without the indicated cGMP concentration. (*Right*) Binding of ^{3}H-labeled cGMP by the supernatant of CHO-cGK (○) or CHO-WT (●) cells. Each tube contained 75 μg of soluble protein.

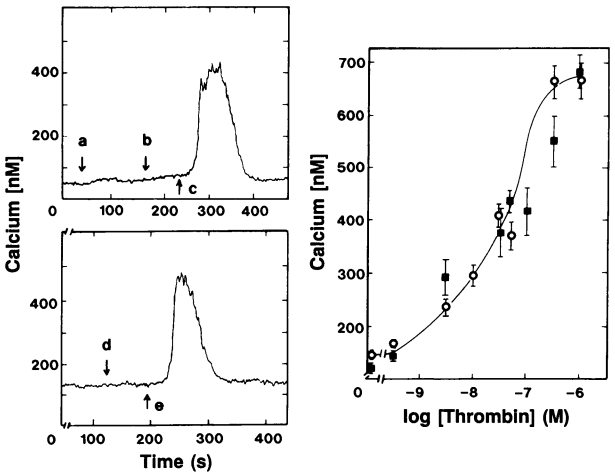

FIG. 2. Thrombin stimulates intracellular release of calcium. (*Upper left*) Extracellular potassium concentration was changed for 2 min from 6 to 66 mM KCl with a concomitant change in sodium concentration from 140 to 80 mM (arrow a). After return (washout) to 6 mM KCl (arrow b), 30 nM thrombin was added for 30 s (arrow c). A CHO-cGK cell was used. (*Lower left*) The extracellular calcium (2 mM) was chelated by 4 mM EGTA (arrow d). The CHO-cGK cell was then stimulated in the presence of 4 mM EGTA by 30 nM thrombin for 30 s (arrow e). (*Right*) A thrombin dose–response curve for CHO-WT (■) and CHO-cGK (○) cells. Values are means ± SEMs for 6–23 cells.

Method B. Experiments were done at 23–26°C, as described (28).

Inositol 1,4,5-Trisphosphate ($Ins P_3$) Determination. The change in $Ins P_3$ concentrations was determined in a stop-flow system containing three syringes (29). The $Ins P_3$ concentrations were determined by the procedure of Palmer *et al.* (30).

RESULTS AND DISCUSSION

Characteristics of cGMP Kinase-Expressing CHO Cells. CHO cells were transfected stably with the coding sequence of bovine tracheal cGMP kinase Iα (24). Immunoblots of cell extracts showed that CHO-cGK cells contained three closely spaced cGMP kinase bands with an approximate upper molecular mass of 83 kDa, whereas CHO-WT cells did not contain cGMP kinase-specific bands (Fig. 1 *Left*). The extracts from CHO-cGK cells contained a protein kinase activity that was stimulated 10-fold by cGMP with apparent K_a and V_{max} values of 50 nM and 12.5 nmol/min per mg of extract protein, respectively (Fig. 1 *Middle*). The activity of cAMP kinase was blocked by adding the inhibitor peptide from the heat-stable inhibitor protein. The CHO-cGK cell extract bound cGMP specifically with apparent K_d and B_{max} values of 19 nM and 72.7 pmol/mg of protein, respectively (Fig. 1 *Right*). The expressed enzyme had the characteristics of the cGMP kinase Iα isozyme. Dissociation kinetics of bound cGMP revealed a slowly and rapidly cGMP-exchanging binding site in the presence of 1 mM cGMP. The expressed enzyme was not stained by an Iβ isozyme-specific antibody (24, 25) in immunoblots. cGMP kinase was not detected by immunoblotting, kinase activity, or cGMP-binding assay in the extract of CHO-WT cells (Fig. 1) or in CHO cells that were transfected with an unrelated cDNA. The concentration of the expressed cGMP kinase holoenzyme (150 kDa) was calculated by using either the specific catalytic activity of the purified cGMP kinase (5.1 μmol of phosphate transferred per min per mg of enzyme) or four

Table 1. Activation of cGMP kinase inhibits thrombin-induced calcium transients

Cell	Method	Preincubation	Cells, no.		$[Ca^{2+}]_i$, nM		*n*
			Total	Responder	Control	Thrombin	
CHO-WT	A	Buffer	11	11	173 ± 32	582 ± 61	11
	A	8-Br-cGMP	21	21	149 ± 10	477 ± 40	21
	A	8-Br-cAMP	10	9	68 ± 8	421 ± 26	9
	B	Buffer	10	7	63 ± 6	319 ± 54	7
	B	8-Br-cGMP	11	8	138 ± 11	462 ± 125	8
CHO-cGK	A	Buffer	33	32	132 ± 11	401 ± 31	32
	A	8-Br-cGMP	35	9*	135 ± 10	159 ± 12	26
	A	8-Br-cAMP	12	11	101 ± 10	406 ± 58	11
	B	Buffer†	8	6	175 ± 34	995 ± 185	6
	B	8-Br-cGMP†	7	1*	198 ± 18	198 ± 18	6

Individual cells were preincubated for 10 min with buffer, 1 mM 8-bromo-cGMP (8-Br-cGMP), or 1 mM 8-bromo-cAMP (8-Br-cAMP) and then stimulated with 30 nM or 800 nM (†) thrombin. Control values were taken directly before thrombin addition. Thrombin values are the peak value reached after thrombin addition. Values are the means ± SEMs; *n* is number of cells used for calculation. Total number and number of responder cells refer to total number of cells used and number of cells showing a thrombin-stimulated calcium transient, respectively. Methods A and B were used independently in München and Göttingen, respectively.

*Cells in which preincubation with 8-bromo-cGMP did not completely suppress the thrombin-stimulated calcium transient.

cGMP-binding sites per holoenzyme. Both calculations yielded values of 16.1 and 18.2 pmol of cGMP kinase per mg of extract protein. The average cGMP kinase concentration of 17.2 pmol per mg of protein corresponds to a cytosolic concentration of 0.8 μM, assuming that the extract protein equals 5% of the wet weight of these cells. This is a physiologically relevant enzyme concentration that has been estimated at 0.1, 0.35, and 3.5 μM, respectively, in pig coronary arteries (9), adult bovine tracheal smooth muscle cells (10), and human platelets (31).

cGMP Kinase Prevents Thrombin-Stimulated Calcium Transients. The basal $[Ca^{2+}]_i$ was similar between CHO-cGK and CHO-WT cells and ranged between 0.06 and 0.19 μM (Fig. 2, Table 1). Spontaneous opening of voltage-dependent calcium channels did not contribute significantly to basal or stimulated $[Ca^{2+}]_i$ levels. Depolarization of individual cells by increasing the extracellular potassium concentration from 6 to 66 mM did not significantly increase calcium-dependent fura-2 fluorescence, whereas 30 nM thrombin increased the $[Ca^{2+}]_i$ to 0.48 μM (Fig. 2 *Upper left*). In the presence of 1 μM thrombin, peak $[Ca^{2+}]_i$ values reached 0.68 μM (Fig. 2 *Right*). Thrombin activates phospholipase C and releases intracellular calcium through an $InsP_3$-mediated pathway in CHO cells (32). As expected, the stimulatory effect of thrombin was reversible and was not prevented by removal of extracellular calcium (Fig. 2 *Lower left*). Thrombin stimulated the $[Ca^{2+}]_i$ 6- to 8-fold with an EC_{50} of 30 nM in CHO-WT and CHO-cGK cells (Fig. 2 *Right*). Preincubation of a CHO-WT cell for 10 min with 1 mM 8-bromo-cGMP did not affect the response to 30 nM thrombin (Fig. 3 *Left*). However, preincubation of CHO-cGK cells with 1 mM 8-bromo-cGMP completely prevented the thrombin-stimulated calcium transient (Fig. 3 *Right* and Table 1). Thrombin stimulated $[Ca^{2+}]_i$ in >90% of all tested cells. Preincubation with 8-bromo-cGMP suppressed the thrombin-induced calcium transient in 72–86% of all tested CHO-cGK cells (Table 1). In the remaining cells, the transients were only partially suppressed. Suppression of the calcium transients required activation of cGMP kinase, as demonstrated by the ability of thrombin to increase $[Ca^{2+}]_i$ in CHO-cGK cells without 8-bromo-cGMP (Table 1). The effect of 8-bromo-cGMP was specific for cGMP kinase because 8-bromo-cAMP did not influence the calcium transients (Table 1), although 8-bromo-cAMP should have activated cAMP-dependent protein kinase at the used concentration. These results also exclude the possibility that 8-bromo-cGMP worked indirectly through modulation of a cGMP-regulated phosphodiesterase.

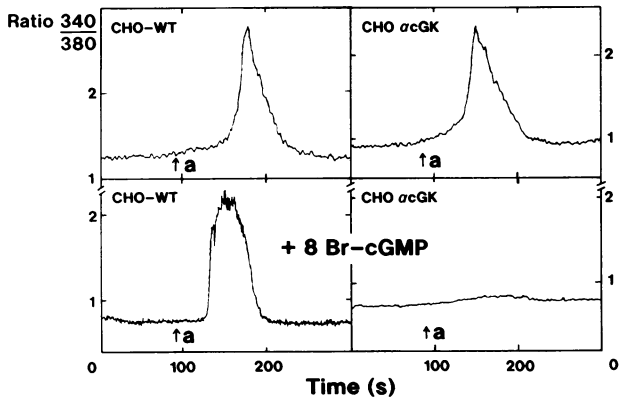

FIG. 3. 8-Bromo-cGMP lowers $[Ca^{2+}]_i$ only in CHO-cGK cells. Individual coverslips were preincubated for 10 min with buffer (*Upper*) or 1 mM 8-bromo-cGMP (*Lower*). An individual cell was then stimulated by 30 nM thrombin (arrow a) for 30 s. Responses of individual CHO-WT (*Left*) and CHO-cGK cells (*Right*) are shown.

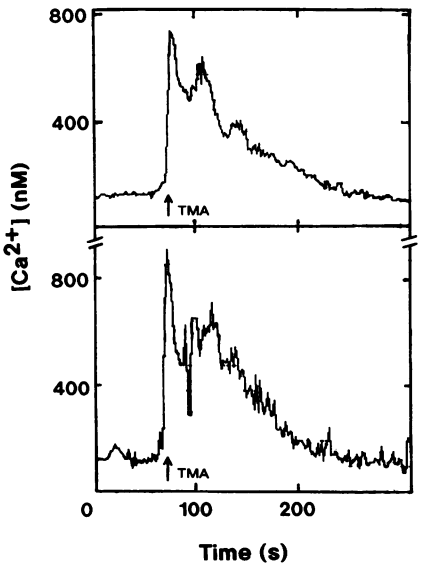

FIG. 4. TMA-induced Ca^{2+} transients are not affected by activation of cGMP kinase. Two CHO-cGK cells were superfused with Hepes buffer (5 mM Hepes, pH 7.4/10 mM glucose/2 mM $CaCl_2$/140 mM NaCl/1.18 mM $MgSO_4$/5.9 mM KCl). At the arrow, cells were superfused with the Hepes buffer in which sodium was replaced by TMA. The cell was pretreated for 10 min with buffer (*Upper*) or 1 mM 8-bromo-cGMP (*Lower*); 8-bromo-cGMP was also present in the TMA-containing buffer (*Lower*).

Ca^{2+}-ATPase Activity Is Not Affected by cGMP Kinase. These experiments suggest that cGMP kinase needs to phosphorylate one or several proteins in CHO-cGK cells to decrease $[Ca^{2+}]_i$. Several potential targets such as L-type calcium (33), potassium, or chloride channels (34) can be neglected because depolarization of the CHO cells by potassium had no significant effect on the basal $[Ca^{2+}]_i$ (see Fig. 2). The plasma membrane (14, 15) and sarcoplasmic reticulum Ca^{2+}-ATPases (16–19) are suggested targets for cGMP kinase. Direct phosphorylation of these ATPases has not been observed (35–37), but cGMP-dependent phosphorylation of a 240-kDa membrane protein (15) and of phospholamban (16–19), a protein that regulates the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase in heart, has been implicated to increase the activity of the respective ATPase and to increase thereby the rate by which calcium is removed from the cytosol. The *in vitro* phosphorylation of smooth muscle phospholamban slightly stimulates calcium uptake into the sarcoplasmic reticulum (16, 17). Immunoblots of crude-membrane fractions and cytosols from CHO-WT and CHO-cGK cells did not identify phospholamban-specific bands when the blots were probed with a phospholamban-specific antiserum. The activation of cGMP kinase should stimulate

Table 2. Summary of TMA-treated cells

$[Ca^{2+}]_e$	Preincubation	$[Ca^{2+}]_i$, nM Control	$[Ca^{2+}]_i$, nM TMA	Interval, s	*n*
0	Buffer	126 ± 17	676 ± 63	196 ± 20	9
0	8-Br-cGMP	129 ± 8	704 ± 49	250 ± 34	8
2	Buffer	109 ± 8	800 ± 90	323 ± 18	20
2	8-Br-cGMP	142 ± 8	1073 ± 92	288 ± 18	18

CHO-cGK cells were superfused and pretreated as described in the legend to Fig. 4. In some experiments, calcium was replaced by 2 mM EGTA in the Hepes buffer. Control values were taken directly before replacing extracellular sodium with TMA. TMA values are the peak values reached after replacement. The interval is the time until $[Ca^{2+}]_i$ reached control level. Values are the means ± SEMs; *n* is the number of cells. $[Ca^{2+}]_e$, extracellular $[Ca^{2+}]$; 8-Br-cGMP, 8-bromo-cGMP.

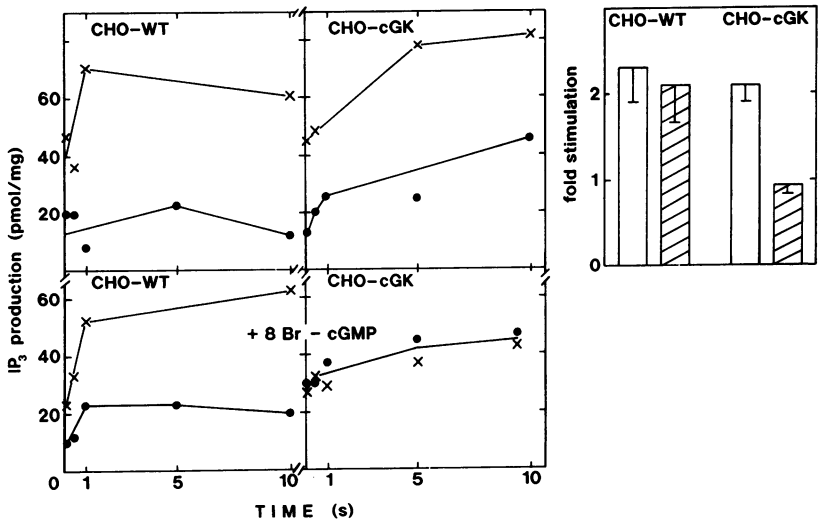

FIG. 5. Activation of cGMP kinase inhibits thrombin-stimulated InsP_3 (IP_3) production. (*Left*) CHO-WT (*Left*) and CHO-cGK (*Right*) cell suspensions were preincubated for 10 min with buffer (*Upper*) or 1 mM 8-bromo-cGMP (*Lower*) before incubation without (●) or with (×) 800 nM thrombin. The reaction was stopped at the indicated time points. (*Right*) Summary of InsP_3 experiments. The stimulation factor was calculated for the 5- or 10-s time points by dividing the thrombin value by the control value. The open and hatched columns represent the CHO cells pretreated with buffer and 8-bromo-cGMP, respectively. Values are the means ± SEMs for experiments done with three different cell passages. In most experiments, the values for individual time points were determined in duplicate.

Ca^{2+}-ATPase activity and thereby lower basal $[Ca^{2+}]_i$ levels. However, the basal $[Ca^{2+}]_i$ level was not changed when a CHO-cGK cell was incubated for 25 min in the presence of 1 mM 8-bromo-cGMP (see Fig. 6*A*). Twenty minutes after the addition of 1 mM 8-bromo-cGMP, the $[Ca^{2+}]_i$ levels were 106 ± 9% (n = 5) of the values measured before adding 8-bromo-cGMP. Despite this apparent lack of phospholamban and cGMP kinase on basal $[Ca^{2+}]_i$, the potential contribution of the Ca^{2+}-ATPases was tested further by a different experimental approach (Fig. 4). The activity of the Na^+/Ca^{2+} exchanger that controls the basal calcium level in many cells was blocked by replacing extracellular sodium with tetramethylammonium (TMA), which is not transported by the Na^+/Ca^{2+} exchanger. The replacement of Na^+ by TMA induced a rapid calcium transient (Fig. 4). The transient was caused by a leak of calcium from intracellular stores because removal of the extracellular calcium did not affect this transient (Table 2). The $[Ca^{2+}]_i$ depends, under this condition, only on the activity of the two Ca^{2+}-ATPases. Preincubation of CHO-cGK cells with 8-bromo-cGMP—this should stimulate the ATPase activities—had no effect on the peak or the time course of the calcium transient (Fig. 4, Table 2) regardless of the presence or absence of extracellular calcium (Table 2). These negative results rule out the possibility that cGMP kinase lowers $[Ca^{2+}]_i$ by increasing activity of a Ca^{2+}-ATPase.

Activation of cGMP Kinase Inhibits Thrombin-Stimulated InsP_3 Production. The remaining major potential mechanism—namely, inhibition of the signal-transduction pathway between the thrombin receptor and InsP_3 synthesis (20–22, 38)—was investigated further using the stop-flow technique (29) to measure rapid changes in InsP_3 synthesis (Fig. 5). Activation of cGMP kinase completely suppressed thrombin-induced stimulation of InsP_3 synthesis in CHO-cGK cells. 8-Bromo-cGMP had no effect on the thrombin-induced InsP_3 synthesis in CHO-WT cells. In agreement with the experiments on the calcium transients (Table 1), the activation of cGMP kinase suppressed InsP_3 synthesis stimulated at a maximal (800 nM) thrombin concentration (Fig. 5) but had no effect on basal InsP_3 levels. This observation rules out the

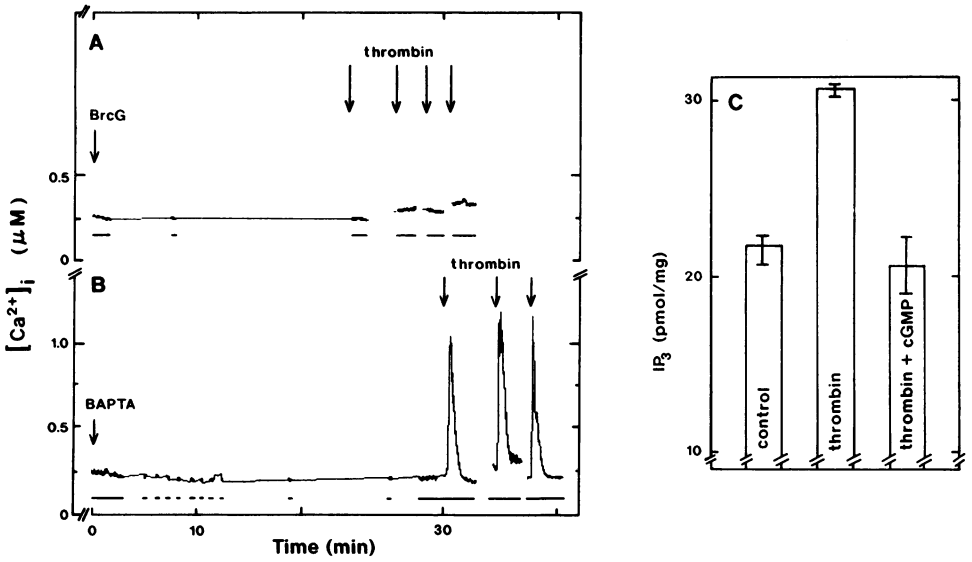

FIG. 6. Effect of 8-bromo-cGMP and BAPTA-AM on thrombin-stimulated $[Ca^{2+}]_i$ and InsP_3 (IP_3) production. Coverslips with CHO-cGK cells were incubated with 1 mM 8-bromo-cGMP (BrcG) (*A*) or 100 nM BAPTA-AM (*B*) for the indicated times. Fluorescence data were collected only at the time points underlined in one cell, which was stimulated at the end with 60 nM (*A*) or 800 nM (*B*) thrombin. Thereafter other individual cells from the same preincubated coverslip were stimulated by thrombin. (*C*) CHO-cGK cell suspensions were preincubated for 30 min with 100 nM BAPTA-AM and for 10 min with buffer or 1 mM 8-bromo-cGMP. The preincubated cells were then incubated in a stop-flow apparatus, as described in *Methods*, with or without 800 nM thrombin. Data shown were collected 5 s after mixing the cell suspension with thrombin or buffer and are the means ± SEMs for three experiments with two different cell passages.

possibility that cGMP kinase affects basal InsP_3 metabolism. Although unlikely, it was possible that the cGMP kinase-dependent inhibition of InsP_3 production was caused by a negative effect of cGMP kinase on the calcium-release mechanism. According to the positive feed-forward regulation suggested by Meyer (39), a decreased calcium release would diminish $[Ca^{2+}]_i$ and thereby decrease phospholipase C activity and InsP_3 production. Whether or not this positive feed-forward regulation is operative in CHO cells is unknown. To test its potential contribution, CHO-cGK cells werepreincubatedwithbis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetate (BAPTA)-AM, which should buffer part of the released calcium and thereby mimic a decreased calcium release. After a preincubation for 30 min, the cells were challenged with thrombin. Preincubation of CHO-cGK cells with 100 nM BAPTA-AM lowered $[Ca^{2+}]_i$ by 26 ± 4% (n = 6) but did not significantly affect the thrombin response (Fig. 6*B*). Even preincubation of a CHO-cGK cell with 5 μM BAPTA-AM, which lowered $[Ca^{2+}]_i$ by 44%, did not prevent thrombin-stimulated increase in $[Ca^{2+}]_i$ (data not shown). However, the thrombin-stimulated InsP_3 production was completely suppressed by activation of cGMP kinase in cells loaded with BAPTA-AM (Fig. 6*C*). These results do not support the hypothesis that cGMP kinase affects the synthesis of InsP_3 by activation of a Ca^{2+}-ATPase or by inhibition of calcium release.

The experiments presented here demonstrate that (*i*) cGMP kinase lowers hormonally stimulated increases in $[Ca^{2+}]_i$, (*ii*) cGMP kinase does so by interfering with the transduction pathway between the thrombin receptor and InsP_3 production, and (*iii*) these effects are specific for cGMP kinase and cannot be mimicked by cAMP kinase. The results could explain the older observation that cGMP analogs relax hormonal-induced contractions more easily than contractions induced by membrane depolarization (9). Thrombin stimulates InsP_3 synthesis in CHO cells through a pertussis toxin-sensitive G_p protein (G protein that activates phospholipase C) (32). It is, therefore, likely that cGMP kinase phosphorylates either a member of the G_p protein family or an isozyme of the phospholipase C family. cGMP kinase inhibits the opening probability of an amiloride-sensitive Na^+ channel in kidney collecting ducts through a pertussis toxin-sensitive G protein (40), suggesting that the mechanism established for CHO cells may also be present in other tissues. The results reported here are intriguing because they suggest that cGMP kinase does interfere not only with the InsP_3 pathway but also with the activation of protein kinase C. Interference with the activation of protein kinase C may not be important for the relaxation of smooth muscle but may be important for the postulated neuroprotective effect of cGMP against excitotoxicity (7, 8).

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