0026-895X/94/020283-08$03.00/0

MOLECULAR PHARMACOLOGY, 46:283-290, 1994.

Human Cyclic GMP-dependent Protein Kinase Iβ Overexpression Increases Phosphorylation of an Endogenous Focal Contact-associated Vasodilator-stimulated Phosphoprotein without Altering the Thrombin-evoked Calcium Response

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Received December 14, 1993; Accepted May 23, 1994

SUMMARY

The role of the cGMP-dependent protein kinase (cGK) and one of its major substrates, the vasodilator-stimulated phosphoprotein (VASP), in the regulation of a receptor-evoked calcium response was investigated. The human type Iβ cGK was stably transfected in human embryonic kidney 293 cells and Swiss mouse 3T6 fibroblasts, which contained significant or no detectable levels of the focal adhesion protein VASP, respectively. Western blot analysis and protein kinase activity measurements demonstrated an 8-fold overexpression of cGK-Iβ in 293 cells (7-fold in 3T6 cells), representing an intracellular cGK concentration of 0.33 μM. In experiments with intact 293 cells expressing cGK-Iβ, β-phenyl-1,N^2-etheno-cGMP and 8-(*p*-chlorophenylthio)-cGMP were capable of converting up to 30–40% of the 46-kDa VASP to its 50-kDa phospho- form, equivalent to results observed with cGMP analogs that cause a marked inhibition of the stimulated Ca^{2+} transient in intact human platelets. In contrast to platelets, preincubation of fura-2-loaded 293 and 3T6 cells with 8-(*p*-chlorophenylthio)-cGMP did not significantly inhibit thrombin-evoked calcium transients, although sufficient cGK-mediated VASP phosphorylation was clearly detectable under these conditions in cGK-Iβ-expressing 293 cells. These results demonstrate that cGK inhibition of agonist-evoked calcium mobilization is not a mechanism common to all cell types and that VASP phosphorylation may not be an essential or sufficient component of the cGK effect on calcium levels. In contrast, the observed VASP phosphorylation mediated by recombinant human cGK-Iβ in intact 293 cells does support the hypothesis that focal adhesions and their associated proteins are important cellular sites of cGK action.

cGMP regulates several signaling pathways, including those involving cGMP-gated channels, certain phosphodiesterases, cGKs, and in certain instances perhaps cAKs (reviewed in Ref. 1). Thus, the soluble form of cGK, consisting of isoforms Iα and Iβ, is clearly only one of several mediators of cGMP action, and more information is required concerning the cellular proteins phosphorylated by cGK and the physiological functions they regulate. The soluble cGK has been proposed to regulate $[Ca^{2+}]_i$ in different cell types by a variety of mechanisms (reviewed in Refs. 1–3). cGK could inhibit Ca^{2+} entry through plasma membrane Ca^{2+} channels (4, 5) or stimulate its efflux across this membrane by activation of a Na^+/Ca^{2+} exchanger (6) or phosphorylation of a 240-kDa protein regulator of Ca^{2+}-ATPase (7). cGK could also inhibit intracellular Ca^{2+} release from the endoplasmic reticulum by inhibition of IP_3 formation (8–11) or stimulate pumping of Ca^{2+} back into its intracellular store by phosphorylation of phospholamban, a regulator of the endoplasmic reticulum Ca^{2+}-ATPase (12). In some of those investigations, functions were examined in response to cGMP or agents that elevate it; thus, no direct effect of cGK was demonstrated. Also, in some cases, results were obtained with *in vitro* experiments and may not represent *in vivo* mechanisms.

This work was supported by the Deutsche Forschungsgemeinschaft (Ko 210/11-3, SFB 176/A11, and the Wilhelm Sander Stiftung).

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ABBREVIATIONS: cGK, cGMP-dependent protein kinase (EC 2.7.1.37); cAK, cAMP-dependent protein kinase (EC 2.7.1.37); 8-Br-cGMP, 8-bromo-cGMP; PET-cGMP, β-phenyl-1,N^2-etheno-cGMP; 8-pCPT-cGMP, 8-(*p*-chlorophenylthio)-cGMP; Sp-5,6-DCl-cBIMPS, Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate; fura-2/AM, fura-2 tetrakis(acetoxymethyl)ester; VASP, vasodilator-stimulated phosphoprotein; $[Ca^{2+}]_i$, intracellular calcium concentration; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; DMEM, Dulbecco's modified Eagle's medium; BHK, baby hamster kidney; CMV, cytomegalovirus; bp, base pair(s); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Mt, metallothionein; CHO, Chinese hamster ovary; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

In certain cases, the effect on $[Ca^{2+}]_i$ of direct introduction of cGK-Iα into cells has been examined. Introduction of cGK into subcultured rat aortic smooth muscle cells deficient in cGK (13) or bovine tracheal smooth muscle cells (14) via osmotic lysis of pinocytotic vesicles lowered $[Ca^{2+}]_i$ in these cells. In the former case it was necessary to activate the cGK with agents that increase cGMP (13); in the latter case a truncated, constitutively active cGK was introduced into cells (14). Micropipette perfusion of cardiac myocytes with truncated, constitutively active cGK was shown by patch-clamp analysis to inhibit cAMP-mediated increases in L-type Ca^{2+} channel current (4). More recently, transfection of cGK into CHO cells was reported to inhibit IP_3 production and calcium transients (10). In contrast to these cases of Ca^{2+} inhibition by cGK, cGK microinjection into snail neurons increased an inward Ca^{2+} current (15).

In studies with intact human platelets, cGMP-dependent (11) and endothelial-dependent (16) inhibition of $[Ca^{2+}]_i$ has been shown to involve inhibition of both Ca^{2+} mobilization from internal stores and the secondary store-related Ca^{2+} influx in response to either thrombin or ADP, but not inhibition of the ADP receptor-operated cation channel. The effect of cGMP is believed to be mediated by cGK, because a specific activator of cGK, 8-pCPT-cGMP, inhibited the rise in $[Ca^{2+}]_i$ caused by activation of normal platelets but not that caused by activation of cGK-deficient platelets from patients with chronic myelogenous leukemia (17). Protein phosphorylation in intact platelets has identified a protein designated VASP, which is one of the very few well characterized proteins phosphorylated by cGK in intact cells. VASP is found in highest concentrations in platelets, and VASP phosphorylation in intact human platelets correlates well with inhibition of both Ca^{2+} mobilization and platelet activation (11, 17). VASP has been observed in a wide variety of other cells and tissues and has been purified and found to be associated with microfilaments and focal adhesions (18).

To further investigate the activation properties of cGK-Iβ and the role of cGK-Iβ and VASP in $[Ca^{2+}]_i$ regulation, we examined the effect of overexpression of cGK-Iβ in cultured cells, an approach made possible by the recent cloning of cGK isoforms (19, 20). After *in vitro* characterization of expressed recombinant human cGK-Iβ activity, an important goal was to achieve activation and regulation of cGK-Iβ in intact cells. A second important goal was to investigate the regulation of calcium transients by cGK and its major substrate VASP. For the stable transfection studies two different cell lines were therefore used, which were found in preliminary studies to contain significant or no detectable levels of VASP. The results indicate that cGK inhibition of calcium transients is not common to all cell types and that VASP phosphorylation may not be an essential or sufficient component of the cGK effect on calcium, but they do support the hypothesis that focal adhesions are important sites of cGK action.

Experimental Procedures

Materials. Human embryonic kidney 293 cells (CRL 1573; American Type Culture Collection) and BHK-21 (C-13) cells (CCL 10; American Type Culture Collection) were obtained from H. Hauser (GBF—Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). Swiss mouse 3T6 fibroblasts were from I. Horak (University of Würzburg, Germany). Parent expression vectors were obtained from Invitrogen (pRc/CMV) and from F. Melchers (Basel, Institute of Immunology, Switzerland) (pBMGNeo) (21). Lipofectin was purchased from GIBCO/BRL, and cAMP, cGMP, 8-Br-cGMP, and fura-2/AM from Sigma. PET-cGMP, 8-pCPT-cGMP, and Sp-5,6-DCl-cBIMPS were from Biolog. The peptide RRKVSKQE, designated 2A3, was synthesized by D. Palm (University of Würzburg, Germany).

Cell culture. Cells were cultured in DMEM containing either 5% (BHK and 3T6 cells) or 10% (293 cells) fetal calf serum, at 37° in a humidified atmosphere with 5% CO_2.

Eukaryotic expression vector constructs. The expression vectors pMM9 and pMM11 were constructed by inserting the complete coding sequence of soluble cGK-Iβ into the parent vectors pRc/CMV and pBMGNeo, respectively. To obtain a construct containing the complete cGK-Iβ coding sequence, a *Bam*HI-*Nco*I fragment from clone 3 (20) was inserted into clone 2 (20), which had been cut with the same enzymes. Due to the discovery of a nucleotide mutation in clone 2, a *Bsm*I-*Pac*I restriction fragment from clone 1 (20) was used to repair the defect in clone 2. In the pMM9 vector containing a constitutive human CMV promoter, the final cGK-Iβ insert (*Apa*I-*Not*I fragment) contained 58 bp of the 5′ untranslated region of cGK-Iβ, the entire coding region (2061 bp), and 55 bp of the 3′ untranslated region. The pMM11 vector contained the same cGK-Iβ insert as an *Eco*RI fragment and contained the regulatable mouse Mt1 promoter, which was induced by Zn^{2+} in our experiments. Both expression vectors contained the *neo*^{r} gene.

Transient and stable transfection of cells. All cell lines were transfected using the lipofection procedure (22). After an additional 24 hr in culture in medium containing fetal calf serum, transiently transfected cells were either harvested (those transfected with the constitutive pMM9 vector) or stimulated with Zn^{2+}, at about 70% confluency, for an additional 24 hr (those transfected with the regulated pMM11 vector) before harvesting. For BHK and 293 cells 90 μM Zn^{2+} was used, whereas only 80 μM Zn^{2+} was well tolerated by 3T6 cells. Stable transfectants were selected using 1 mg/ml (or 0.7 mg/ml for 293 cells) G418 beginning 3 days after transfection. Induction of pMM11 expression with Zn^{2+} before harvesting of stable transfectants was performed as described for transiently transfected cells. Both transient and stable transfectants were analyzed for expression of cGK-Iβ by Western blot analysis. Stable transfectants were used in all other experiments.

Western blot analysis of cGK-Iβ. Cells on 10-cm plates were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7) and then harvested in PBS by scraping. Aliquots of the cell suspension were added to 90° stop solution for SDS-PAGE processing or to 0.2 N NaOH for Lowry protein determination (23). Samples of extracts from transfected cells and standards of purified cGK-Iβ were subjected to SDS-PAGE using a 3.5% stacking gel and 8% separating gel, transferred to nitrocellulose, and radioimmunolabeled using well characterized antibodies made against purified bovine lung cGK, as described previously (24).

***In vitro* protein kinase assay.** Stably transfected cells (10-cm plates) were washed twice with PBS, harvested by scraping in a buffer containing 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 15 mM β-mercaptoethanol, and 20 units/ml Trasylol, homogenized, and centrifuged at 100,000 × *g* for 1 hr at 4°. Aliquots of the supernatants (20 μl) were used for protein kinase activity assay and for protein measurements by the method of Lowry *et al.* (23).

The activity of cGK in cell extracts and in preparations of bovine lung cGK-Iα was analyzed using the assay for binding of phosphorylated substrate to P-81 phosphocellulose, with modifications (25). The substrate used for selective measurement of cGK activity was a synthetic peptide designated 2A3 (RRKVSKQE), which is nearly identical to a VASP phosphorylation site and is a much better substrate for cGK than cAK (26). Briefly, phosphorylation of 55 μM (based on the peptide concentration in a 1 mg/ml solution of 64% pure compound) 2A3 peptide was assayed at 30° for 5 min in a total volume of 100 μl containing 20 mM Tris·HCl buffer, pH 7.4, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.01% (w/v) bovine serum albumin, and 50 ng of cGK. The reaction was started by addition of 50 μM ATP containing

100 cpm/pmol [γ-^{32}P]ATP. The activity of cGK in the absence and presence of 5 μM cGMP was measured in triplicate in all experiments. One unit of enzyme activity is the amount capable of phosphorylating 1 μmol of substrate/min. K'_a values, defined as the cyclic nucleotide concentration causing half-maximal cGK activation, were automatically calculated from the activation curves drawn by the GraphPad Inplot program, version 3.0.

Analysis of the phosphorylation of the cGK substrate VASP in intact cells. While still attached to 6-cm plates, stably transfected cells were washed twice with serum-free DMEM and incubated in the same medium at 37° in 5% CO_2, in the presence and absence of cell membrane-permeant, hydrolysis-resistant, cGMP analogs. Before this step, cGK-Iβ expression from the pMM11 vector in 3T6 cells had been induced for 24 hr with 80 μM Zn^{2+}. At the indicated times, medium was removed from the cells so that they could be harvested in the presence of 90° stop solution for SDS-PAGE. Samples were subjected to SDS-PAGE and Western blotting as described previously (24), using an antibody against VASP shown to detect both the dephospho- and phospho- forms of VASP, which migrate as 46- and 50-kDa proteins, respectively, on SDS-PAGE. Parallel plates were harvested with 0.2 N NaOH for protein determination.

Calcium measurements with fura-2. Stably transfected 293 and 3T6 cells still attached to 15-cm plates were washed twice with serum-free DMEM and then incubated for 45 min with 2 μM fura-2/AM dissolved in 1% (v/v) dimethylsulfoxide, at 37° in 5% CO_2. Before this step, cGK-Iβ expression from the pMM11 vector in 3T6 cells had been induced for 24 hr with 80 μM Zn^{2+}. After being loaded with fura-2, the cells were washed twice with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% glucose, and 0.1% bovine serum albumin, shaken loose, and resuspended in the same buffer to 5×10^6 cells/ml. Cells (2 ml) were then preincubated, with shaking, either in the absence or in the presence of cGMP analogs for 15–20 min at 37°. Fluorescence measurements were then performed with this concentration of cells in a Perkin-Elmer LS50 luminescence spectrometer, in which the cells could be stirred in a 37° thermostatted cuvette holder, as described previously (11). In certain experiments, the 1 mM $CaCl_2$ in the external medium was chelated by addition of 4 mM EGTA before stimulation of cellular $[Ca^{2+}]_i$ with 0.1–1.0 unit/ml (1.4–14 nM) thrombin. Reagents added to the medium were dissolved in water except for cGMP analogs, which were dissolved in dimethylsulfoxide (1%, v/v, final concentration in experiments). For each experimental condition, a control was performed with the vehicle alone. The fluorescence excitation wavelength was 340 nm at a bandwidth of 2.5 nm, and emission was collected at 510 nm with a bandwidth of 6 nm. Results were expressed as arbitrary fura-2 fluorescence units obtained at the emission wavelength of 510 nm.

Results

Two different expression vectors for cGK-Iβ were prepared as described in Experimental Procedures. The major differences between these vectors are their promoters and mechanisms of replication in eukaryotic cells. The pMM9 vector contains the human CMV promotor and can integrate into cellular genomes, whereas the pMM11 vector contains the mouse Mt1 promotor, which can be regulated by Zn^{2+}, and can replicate episomally. Initial transient transfection results (not shown) indicated that pMM9 and pMM11 were strongly expressed in BHK and 293 cells but that pMM9 was only weakly expressed in mouse 3T6 cells. For additional experiments, cells stably transfected with cGK-Iβ were selected with G418. Western blot analysis demonstrated strong stable expression of cGK-Iβ by the pMM9 vector in 293 cells and by the pMM11 vector in 3T6 cells (Fig. 1). Only weak stable expression of pMM9 in BHK cells was observed (data not shown). BHK (data not shown) and 3T6 cells contained no detectable endogenous cGK (Fig. 1, pBMGNeo control vector-transfected cells), whereas 293 cells

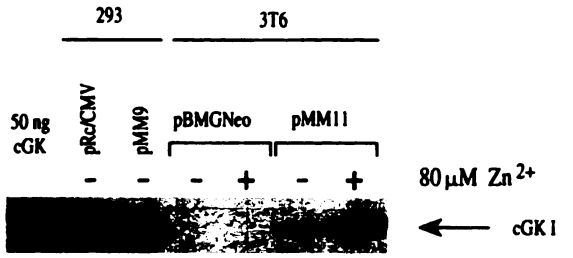

Fig. 1. Western blot analysis of stable cGK-I expression in transfected cells. The stable cGK-Iβ expression in 293 cells transfected with the constitutive pMM9 vector or in 3T6 cells transfected with the Zn^{2+}-inducible (Mt1 promoter) pMM11 vector is shown. In comparison, basal endogenous cGK-I expression is observed in cells transfected with the corresponding control vectors, pRc/CMV for 293 cells and pBMGNeo for 3T6 cells. All lanes contain 300 μg of protein. *Left lane*, a standard of cGK-Iα purified from bovine lung.

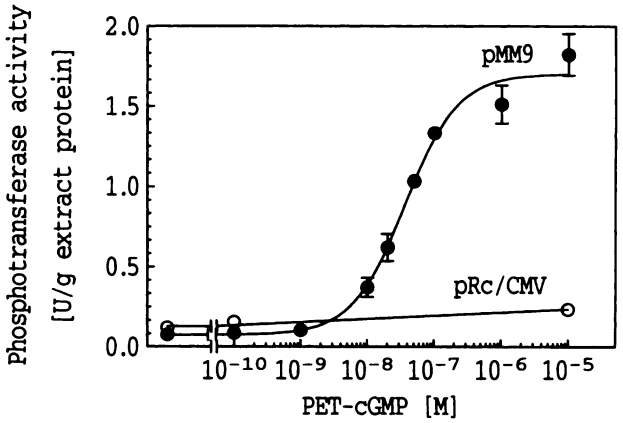

Fig. 2. Concentration-dependent activation by PET-cGMP of stably transfected cGK-Iβ in 293 cell extracts *in vitro*. Soluble extracts from 293 cells stably transfected with the pMM9 vector for cGK-Iβ (●) or with the control vector pRc/CMV (○) were assayed for cGK activity *in vitro*, using the synthetic peptide 2A3, in the presence of increasing concentrations of PET-cGMP. Specific activity (units/g of cell extract protein) is reported. The data shown (mean ± standard deviation) are derived from two separate kinase assays, each containing triplicate determinations. In some cases the standard deviation is not visible because it was smaller than the data point plotted by the computer program.

did (Fig. 1, pRc/CMV control vector-transfected cells). Laser densitometry analysis showed that the ratio of cGK present in 293 cells transfected with the pMM9 vector, compared with the pRc/CMV control vector, was 6.2 ± 1.5 (mean ± standard deviation) for three experiments. The Zn^{2+} stimulation of cGK expression from the pMM11 vector was an average of 5.9 ± 0.7 for three experiments.

The cGK-Iβ stably expressed in 293 cells (Fig. 2; Table 1) and 3T6 cells (data not shown) was demonstrated to contain catalytic activity by *in vitro* protein kinase assays performed on cell extracts. The 2A3 peptide substrate used in these assays had a specificity index for cGK, in comparison with cAK, of 21 (26), resulting in very little background kinase activity, e.g., in extracts from cells transfected with cGK-Iβ assayed in the absence of cGMP or in extracts from cells transfected with control vectors assayed in the presence of cGMP. The K_m and V_{max} of the 2A3 peptide for purified cGK-Iα were 27 μM and 6.8 μmol/min/mg, respectively, in contrast to the values for cAK of 264 μM and 3.2 μmol/min/mg, respectively. The specificity index of the peptide was calculated by dividing its V_{max}/K_m for cGK by that for cAK. Concentration-dependent activation of cGK-Iβ in cell extracts by cGMP, cAMP, and

TABLE 1

Comparison of the K'_a values for activation of cGK isoforms Iα and Iβ by cyclic nucleotides

K'_a values, defined as the concentration of cyclic nucleotide that produced half-maximal cGK activation, were calculated automatically from individual activation curves like that shown in Fig. 2, drawn using the GraphPad Inplot program. K'_a values for cGK-Iβ were measured in cell extracts prepared from stably transfected (pMM9) 293 cells as described in Experimental Procedures and represent the means of two or three separate kinase assays, each performed with triplicate determinations. For comparison, the K'_a values obtained with the same cyclic nucleotides and purified bovine lung cGK-Iα are shown. Except for the results with cGMP and PET-cGMP, the cGK-Iα K'_a data were taken from our other recent reports.

Cyclic nucleotides	K'_a	
	cGK-Iα	cGK-Iβ
	μM	
cGMP	0.110	0.300
8-Br-cGMP	0.010[a]	0.960
PET-cGMP	0.038	0.035
8-pCPT-cGMP	0.040[a]	0.550
cAMP	39.000[b]	56.000
Sp-5,6-DCl-cBIMPS	10.000[b]	23.000

[a] From Ref. 25.
[b] From Ref. 27.

certain of their analogs was observed. The average maximal cGMP-stimulated activity of cGK-Iβ in 293 cell extracts (four experiments with triplicate determinations each) was 8 times that of the endogenous cGK in cells transfected with the control vector (data not shown). An example of the results obtained for PET-cGMP are shown in Fig. 2. The kinase activities in the absence and presence of 10^{-5} M PET-cGMP were 76 and 233 milliunits/g of extract protein for the pRc/CMV transfectants and 70 and 1822 milliunits/g of extract protein for the pMM9 transfectants, respectively. Calculated with respect to the activity of purified bovine lung cGK-Iα (1.33 μmol/min/mg of 150-kDa cGK holoenzyme added to extracts of pRc/CMV control vector-transfected cells), and assuming that protein is 5% of cellular wet weight, the 293 cell extracts contained an average concentration of 6.6 pmol of cGK/mg of protein, equivalent to 0.33 μM cGK. In comparison, the maximal activity of cGK-Iβ found in transfected 3T6 cells treated with Zn^{2+} was about 70% (data not shown) of that in the 293 cells.

The K'_a values for activation, by various cyclic nucleotides, of cGK-Iβ in transfected cells and cGK-Iα purified from bovine lung were clearly different (Table 1). The K'_a for activation of cGK-Iβ by 8-substituted cGMP derivatives was much higher (10-fold for 8-pCPT-cGMP and 100-fold for 8-Br-cGMP) than that for cGK-Iα. The decreasing order of potency for stimulation of cGK-Iβ was PET-cGMP > cGMP > 8-pCPT-cGMP > 8-Br-cGMP. In contrast, all of these cGMP analogs were better activators of cGK-Iα than was cGMP itself. Of these and other cGMP derivatives tested, none were found that were specific activators of cGK-Iβ. As shown, although PET-cGMP was the best activator for cGK-Iβ (K'_a = 0.035 μM), it had an almost identical K'_a (0.038 μM) for cGK-Iα. Until now these values for PET-cGMP had been determined only with purified cGK-Iα and -Iβ (K'_a = 0.026 and 0.020 μM, respectively) (28), which are difficult to separate from one another except by high performance liquid chromatography. Our data, however, show that indeed PET-cGMP is a very potent activator of both cGK isoforms. In contrast to cGMP and its analogs, the K'_a values for cAMP and one of its derivatives, Sp-5,6-DCl-cBIMPS, were very high. It should be noted that such high concentrations of cAMP and its analogs interfere with the kinase ATP binding site, maximal catalytic activity, and thus the K'_a determination.

The focal adhesion protein VASP, a well characterized substrate of cGK in intact platelets (11, 17, 18), was examined in 293 and 3T6 cells. Whereas endogenous VASP was observed in 293 cells, no significant amount of VASP was detected in 3T6 cells (Fig. 3). Incubation of cGK-Iβ-transfected (pMM9) 293 cells with 0.5 mM PET-cGMP for 10 min caused VASP phosphorylation, which was detected as a molecular mass shift of VASP from 46 kDa to 50 kDa (dephospho- and phospho-VASP, respectively) on SDS-PAGE analyzed by Western blotting. A small amount of VASP was also phosphorylated by the endogenous cGK found in 293 cells transfected with the control vector (pRc/CMV). VASP phosphorylation increased somewhat by 30 min but was essentially constant at all later time points checked (60 and 120 min; data not shown). Similar results were also obtained when 0.5 mM 8-pCPT-cGMP was used instead of PET-cGMP (Fig. 4). VASP phosphorylation measured by laser densitometry in pMM9-transfected cells in

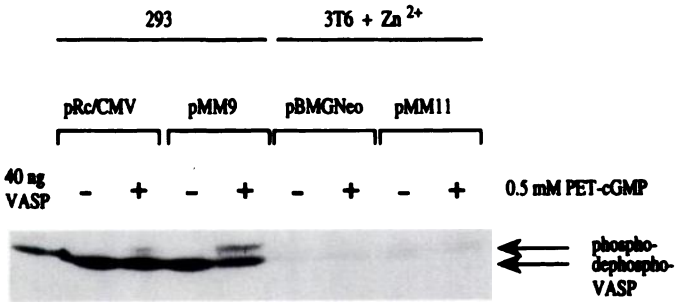

Fig. 3. Western blot analysis of the phosphorylation of VASP in cells stably transfected with cGK-Iβ. The VASP phosphorylation observed in 293 cells stably transfected with cGK-Iβ (pMM9 vector) or the control vector (pRc/CMV) and incubated in the presence (+) or absence (−) of 0.5 mM PET-cGMP for 10 min is shown. Identical incubations were performed with 3T6 cells stably transfected with cGK-Iβ (pMM11 vector) or the control vector (pBMGNeo), which had been induced for 24 hr with 80 μM Zn^{2+}. All lanes contain 300 μg of protein. VASP antibody detected both the phospho- and dephospho- forms of VASP (50 kDa and 46 kDa, respectively). *Left lane*, a standard of dephospho-VASP purified from human platelets.

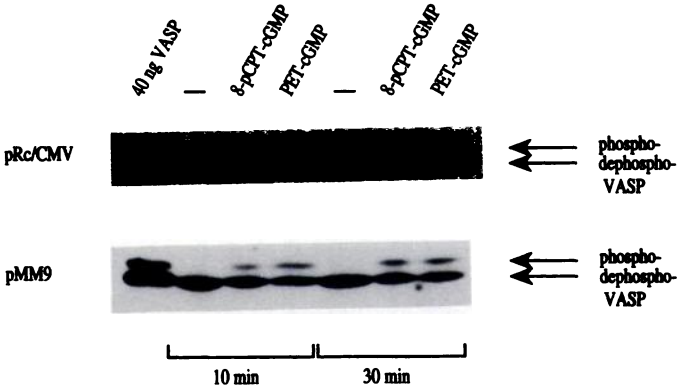

Fig. 4. VASP phosphorylation in cells stably transfected with cGK-Iβ, in response to PET-cGMP and 8-pCPT-cGMP. Shown are the phospho- and dephospho- forms of VASP (50 kDa and 46 kDa, respectively) observed in Western blots of 293 cells stably transfected with cGK-Iβ (pMM9 vector) or the control vector (pRc/CMV) and incubated in the absence (−) or presence of 0.5 mM 8-pCPT-cGMP or 0.5 mM PET-cGMP for 10 and 30 min. All lanes contain 300 μg of protein. *Left lane*, a standard of human platelet VASP containing phospho- and dephospho-forms.

three experiments was 22 ± 0.9% (mean ± standard deviation) at 10 min and 31 ± 1.0% at 30 min for 8-pCPT-cGMP and was 36 ± 1.5% at 10 min and 39 ± 1.4% at 30 min for PET-cGMP.

The effect of stably transfected cGK-Iβ on $[Ca^{2+}]_i$ in 293 cells (pMM9 vector) and 3T6 cells (Zn^{2+}-induced pMM11 vector) was examined with fura-2 fluorescence measurements. Preincubation of cGK-Iβ-transfected cells with 1 mM 8-pCPT-cGMP had no reproducible effect on the ability of 0.1 unit/ml thrombin to increase $[Ca^{2+}]_i$, either in the presence (data not shown) or in the absence (extracellular 4 mM EGTA) (Fig. 5) of extracellular Ca^{2+}. These data are representative of results obtained in five separate experiments with multiple determinations, all demonstrating no significant effect of 8-pCPT-cGMP or 8-Br-cGMP stimulation of cGK-Iβ on thrombin elevation of $[Ca^{2+}]_i$ in these cells. PET-cGMP was not useful for these experiments because its autofluorescence interfered with the fura-2 measurements. Similar experiments (data not shown) also showed no effect of transfection with the respective control vectors (pRc/CMV and pBMGNeo) on thrombin-stimulated cellular $[Ca^{2+}]_i$.

Discussion

Properties of the recombinant human cGK-Iβ isoform stably expressed in 293 and 3T6 cells. Properties of cGK-Iβ, as well as effects of cGMP that can be mediated by cGK-Iβ, were examined by overexpression of cGK-Iβ protein from transfected vectors. The results demonstrated the first evidence of phosphorylation of a major endogenous substrate, VASP, by the cGK-Iβ isoform in intact cells. Previously, assessment of the functions of individual endogenous isoforms of cGK was difficult because the cGK-Iα and -Iβ isoforms have extremely similar protein molecular masses and mRNA sizes, hindering quantitation of the composition of isoforms in a given cell or tissue type. Here, cGK-Iβ was overexpressed in cell lines using vectors with constitutive (CMV in pMM9) or inducible (Mt1 in pMM11) promoters, both of which produced high levels of cGK-Iβ in stably transfected cells. The holoenzyme concentration achieved in 293 cells was 0.33 μM and in 3T6 cells was 0.23 μM, which is in the range observed in tissues for which a physiological role of cGK is postulated [0.03 μM in rat cardiac myocytes (4), 0.1 μM in pig coronary arteries (29), 0.36 μM in adult bovine tracheal smooth muscle cells (14), and 3.6 μM in human platelets (24)]. Furthermore, various cell-permeant cyclic nucleotide analogs were shown to activate cGK-Iβ in extracts made from transfected cells, indicating that such analogs were suitable for studying cGK-Iβ functions in intact cells. PET-cGMP (K'_a = 0.035 μM) was a 15-fold better stimulator of cGK-Iβ than was 8-pCPT-cGMP (K'_a = 0.55 μM) *in vitro* (Table 1).

Activation of human cGK-Iβ and phosphorylation of VASP in intact 293 cells. Phosphorylation of endogenous VASP in cGK-Iβ-transfected 293 cells after treatment of intact cells with cell membrane-permeant cGMP analogs was demonstrated. The level of VASP phosphorylation (approximately 30–40%) by cGK-Iβ in 293 cells, observed as the VASP shift to a 50-kDa protein in SDS-PAGE, was equivalent to that observed in response to cGMP analogs in intact human platelets (11, 25). For still unknown reasons, only a fraction of VASP in intact cells can be converted from the 46-kDa form to the 50-kDa phospho- form by cGK, whereas there is complete conversion of purified VASP to the 50-kDa phospho- form by cGK *in vitro* (30). cGK directly phosphorylates VASP *in vitro* and in intact cells, as shown by comparison of normal and cGK-deficient platelets (17) and, more extensively, by two-dimensional gels (30) and peptide purification and sequencing (26). Both purified VASP and VASP in intact cells are phosphorylated at three sites, of which only one site causes the shift in size from 46 kDa to 50 kDa in SDS-PAGE (26, 30). Interestingly, serine 2 (the shift-causing site) is preferentially phosphorylated by cAK, whereas cGK preferentially phosphorylates serine 1. Peptide analysis indicates that, when 30–40% of VASP is shifted to the 50-kDa form, the total cGK-dependent VASP phosphorylation is much higher, because the cGK phosphorylation rate for serine 1 is greater than that for serine 2, which

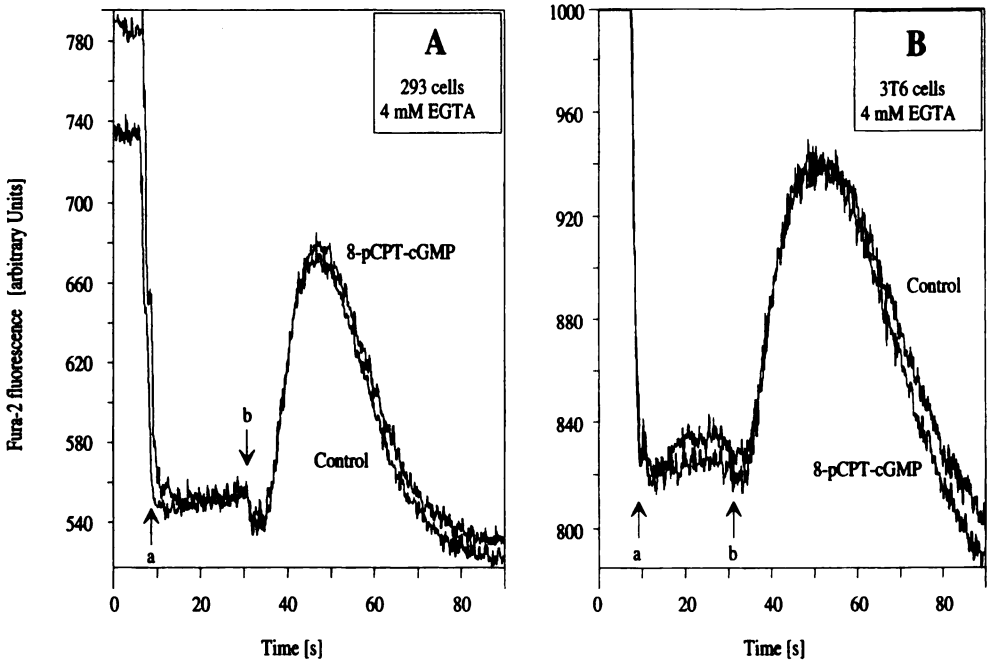

Fig. 5. Fura-2 analysis of $[Ca^{2+}]_i$ in 293 (A) and 3T6 (B) cells stably transfected with cGK-Iβ. Cells (10^7) stably transfected with cGK-Iβ (pMM9 vector for 293 cells or Zn^{2+}-induced pMM11 vector for 3T6 cells) were loaded with fura-2/AM (45 min, 37°) and preincubated for 15 min at 37° with 1 mM 8-pCPT-cGMP or 1% (v/v) dimethylsulfoxide (diluent control) before experiments. At the times shown, 4 mM EGTA (*arrows a*) was added, followed by 0.1 unit/ml thrombin (*arrows b*). Fura-2 fluorescence at the emission wavelength (510 nm) is shown in arbitrary units.

causes the shift (26). The third phosphorylation, on a threonine, could not be further stimulated in intact platelets, perhaps due to prior endogenous phosphorylation (26).

In contrast to their different effects on cGK-Iβ activity in *in vitro* kinase assays (Table 1) (28), 8-pCPT-cGMP and PET-cGMP had roughly equivalent effects on VASP phosphorylation in the intact cell studies shown in Figs. 4 and 5 and in similar experiments analyzed at earlier time points, at which submaximal phosphorylation was observed. The activation kinetics of cyclic nucleotide-dependent protein kinases in intact cells are also dependent on the intracellular concentration of the kinase. Under conditions in which the concentration of a cyclic nucleotide-binding protein (kinase) is relatively high, in comparison with the ligand (cyclic nucleotide or analog) concentration, the binding site concentration is an important determinant of K'_a (24). In our experiments, 0.33 μM cGK holoenzyme corresponds to 1.2 μM cGMP binding sites, which is significantly higher than the K'_a obtained *in vitro* using conditions of ligand excess over kinase. The similar potencies of the two cGMP analogs for activating cGK in the intact cell experiments are a function of this relatively high (compared with that *in vitro*) intracellular cGK concentration and the similar lipophilicities and biological stabilities of the analogs themselves (25, 31).[2]

Role of cGK activation and VASP phosphorylation in the regulation of thrombin-evoked calcium transients. Overexpression of transfected cGK-Iβ did not inhibit thrombin-stimulated $[Ca^{2+}]_i$ in cells in which VASP was present and sufficiently phosphorylated (293 cells) or absent (3T6 fibroblasts). The absence of an effect of cGK on thrombin-stimulated $[Ca^{2+}]_i$ was unexpected, because the 30–40% VASP phosphorylation by cGK-Iβ in 293 cells, observed as the VASP shift to a 50-kDa protein in SDS-PAGE, was equivalent to that observed in response to cGMP analogs or sodium nitroprusside, which caused marked inhibition of the Ca^{2+} transient in intact human platelets (11, 17). Also, direct introduction of purified cGK into smooth muscle cells by osmotic lysis of pinocytotic vesicles (13, 14) or transfection of recombinant cGK-Iα into CHO cells (10) was reported to inhibit agonist-stimulated calcium levels. Patch-clamp analysis of rat cardiac ventriculocytes demonstrated that cGK inhibited Ca^{2+} influx via an L-type Ca^{2+} channel (4); in contrast, microinjection of cGK into non-mammalian (snail *Helix aspersa*) neurons stimulated a Ca^{2+}-dependent action potential (15). All of the investigations using purified cGK used that from bovine lung, which has been characterized by antibody analysis to be primarily the Iα isoform (32). Although our transfection experiments were performed with cGK-Iβ, we nevertheless do not think this could account for the absence of a cGK effect on $[Ca^{2+}]_i$, because cGK-Iα and -Iβ seem to have similar substrate specificities, at least *in vitro* (33). The Iα and Iβ forms differ only in their amino-terminal amino acids (approximately the first 100), in a domain that regulates holoenzyme dimerization and cooperativity between cGMP binding sites and that contains autophosphorylation sites and a region that inhibits catalytic activity in the absence of cGMP (reviewed in Refs. 1, 2, and 34). The different amino-terminal end of cGK-Iβ causes cGK-Iβ to have a lower affinity cGMP binding site 1, compared with that of cGK-Iα (35), although the amino acid sequences in both cGMP binding sites 1 and 2 of cGK-Iβ are identical to those of cGK-Iα (19, 20). Moreover, similar to the results presented here, our other recent observations (36) also suggest that VASP phosphorylation in human neutrophils cannot be correlated with any inhibition of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-evoked Ca^{2+} mobilization from intracellular stores in these cells. These results indicate that only certain cells (human platelets, smooth muscle cells, etc.) contain the intracellular components necessary for the inhibition of calcium mobilization by cGK.

The only transfection experiments comparable to those shown here have reported cGK-Iα inhibition of thrombin-evoked calcium transients and IP_3 production in cGK-Iα-transfected CHO cells (10). In that report, bovine cGK-Iα was used instead of human cGK-Iβ and fura-2 measurements were performed on single cells rather than on cell populations. Although we do not anticipate different results from use of cGK-Iα instead of -Iβ, as discussed above, analyses of different cell population sizes could have certain consequences. For example, individual pheochromocytoma (PC-12) cells (37) studied with fura-2 imaging microscopy have been shown to have very heterogeneous receptor-induced $[Ca^{2+}]_i$ responses. Comparison of agonist-dependent $[Ca^{2+}]_i$ elevation in a relatively small number of single cells with inhibitory effects of cGK in a separate set consisting also of a relatively small number of single cells may give a nonrepresentative view. In our experiments, the thrombin-evoked calcium response of 10^7 cells was being compared with the ability of cGK-Iβ to inhibit that response in 10^7 cells, which may more accurately reflect the physiological responses from a population of cells *in vivo*. We cannot, however, rule out the possibility that there are intrinsic differences among all of the various cell types being studied and that some cells have more functional, cGK-dependent, inhibitory pathways for calcium regulation than do others.

There may be other fundamental problems involved in measuring effects on calcium regulation with transfected proteins that are not yet understood. For example, in 3T3 cells in which PLC-γ was overexpressed, platelet-derived growth factor induced tyrosine phosphorylation of PLC-γ and increased generation of IP_3 but caused no increase in $[Ca^{2+}]_i$ (38). There may be cellular counter-regulatory mechanisms that modify the expected $[Ca^{2+}]_i$ increase in this case or the expected $[Ca^{2+}]_i$ decrease caused by overexpression of cGK in our experiments.

In conclusion, the results show that the transfected cGK-Iβ isoform expressed in intact cells phosphorylates the focal adhesion protein VASP to a similar extent as has been observed for endogenous cGK in platelets. The data further suggest that regulation of $[Ca^{2+}]_i$ by a cGK inhibitory pathway involving VASP is not well developed in all cell types. Although this pathway appears to exist in platelets, the presented results and others emphasize the need to investigate additional potential mechanisms by which cGK and VASP phosphorylation are involved in platelet inhibition. The association of VASP with the cytoskeleton and focal adhesions (18) suggested more than one site at which VASP could participate in platelet inhibition, only one of which would involve inhibition of $[Ca^{2+}]_i$. Thrombin stimulation of platelets causes an increase in the activity of certain enzymes, including PLC, associated with the platelet cytoskeleton (39) and, if VASP phosphorylation were to inhibit this process, it might thus prevent elevation of $[Ca^{2+}]_i$ by PLC. However, thrombin, in addition to stimulating PLC, also acti-

[2] H.-G. Genieser, unpublished observations.

vates the fibrinogen receptor (an integrin, GPαIIbβ3). This initiates stimulation of a focal adhesion kinase, c-Src, and other nonreceptor tyrosine kinases that phosphorylate a number of cytoskeletal proteins before cell adhesion, which precedes platelet aggregation (40). It is possible that VASP phosphorylation in intact platelets is more closely associated with the inhibition of aggregation by regulating the fibrinogen receptor[3] than by inhibiting calcium mobilization. Our present results and the recent identification of VASP as a focal adhesion protein suggest that the cell lines used here may be useful for investigating the regulation of focal adhesion structure and function by the cGMP signal transduction cascade. Because VASP phosphorylation is not accompanied by gross morphological alterations in platelets or fibroblasts (18), more detailed examination of VASP interactions with cytoskeletal and focal adhesion proteins is needed. The recent cloning of VASP cDNA in our laboratory[4] will further expand the approaches that can be used for this examination.

Acknowledgments

We thank S. Ebert for assistance with the manuscript.

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