

KT5823 Inhibits cGMP-dependent Protein Kinase Activity *in Vitro* but Not in Intact Human Platelets and Rat Mesangial Cells*

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Many signal transduction pathways are mediated by the second messengers cGMP and cAMP, cGMP- and cAMP-dependent protein kinases (cGK and PKA), phosphodiesterases, and ion channels. To distinguish among the different cGMP effectors, inhibitors of cGK and PKA have been developed including the K-252 compound KT5823 and the isoquinolinesulfonamide H89. KT5823, an *in vitro* inhibitor of cGK, has also been used in numerous studies with intact cells to implicate or rule out the involvement of this protein kinase in a given cellular response. However, the efficacy and specificity of KT5823 as cGK inhibitor in intact cells or tissues have never been demonstrated. Here, we analyzed the effects of both KT5823 and H89 on cyclic-nucleotide-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in intact human platelets and rat mesangial cells. These two cell types both express high levels of cGK. KT5823 inhibited purified cGK. However, with both intact human platelets and rat mesangial cells, KT5823 failed to inhibit cGK-mediated serine 157 and serine 239 phosphorylation of VASP induced by nitric oxide, atrial natriuretic peptide, or the membrane-permeant cGMP analog, 8-pCPT-cGMP. KT5823 enhanced 8-pCPT-cGMP-stimulated VASP phosphorylation in platelets and did not inhibit forskolin-stimulated VASP phosphorylation in either platelets or mesangial cells. In contrast H89, an inhibitor of both PKA and cGK, clearly inhibited 8-pCPT-cGMP and forskolin-stimulated VASP phosphorylation in the two cell types. The data indicate that KT5823 inhibits purified cGK but does not affect a cGK-mediated response in the two different cell types expressing cGK I. These observations indicate that data that interpret the effects of KT5823 in intact cells as the major or only criteria supporting the involvement of cGK clearly need to be reconsidered.

Nitric oxide and cGMP have emerged as important signal transduction mediators of the effects of certain hormones, in-

ter- and intracellular signals, toxins, and drugs (1–3). Intracellular cGMP levels may be increased upon binding of natriuretic peptides to their cognate transmembrane receptors (particulate guanylate cyclases) or indirectly via generation and release of nitric oxide that activates soluble guanylate cyclase (4, 5). Increased cGMP may modulate cGMP-dependent protein kinases, cGMP-stimulated or -inhibited phosphodiesterases, cGMP-gated ion channels, and under certain conditions cAMP-dependent protein kinases (2, 3, 6). cGK¹ type I (cGK I, including α and β splice variants) and type II cGK (cGK II) have been identified in mammalian cells (3).

To distinguish among these effectors of cGMP in a given cell type, especially to define which of the physiological effects of cGMP-elevating agents are dependent on cGK, several criteria must be fulfilled. These include agonist-induced up-regulation of cGMP, positive expression of at least one of the cGK-isoforms (endogenously or upon microinjection or transfection), specific activation or inhibition by cGK-specific agonists/antagonists, and lack of this activation or inhibition in corresponding cGK-deficient systems (7). Unfortunately, there are only a few examples that have met all these criteria. Knockout mice lacking endogenous cGK I displayed defective cGMP-mediated inhibition of platelet aggregation and of vascular smooth muscle cell contraction (6, 8). In platelets, cGK-activation has been shown to induce the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and to inhibit agonist-evoked calcium transients and activation of platelets (3, 9–11). In knockout mice lacking endogenous VASP, cGMP-mediated inhibition of platelet aggregation was reduced and agonist-induced platelet activation was enhanced (12, 13). The analysis of VASP phosphorylation by polyclonal antibodies and newly developed monoclonal antibodies, each of which specifically recognize different phosphorylation sites, allows the quantitative measurement of the strength of cGK activation both *in vitro* and in intact cells (9, 14–17). VASP is phosphorylated preferentially at Ser²³⁹ by cGK whereas upon PKA activation Ser¹⁵⁷-VASP is preferentially phosphorylated. However, at very high levels of cyclic nucleotides or their analogs, both phosphorylation sites may be phosphorylated by either protein kinase (15, 17). cGMP-dependent phosphorylation (but not cAMP-dependent phosphorylation) of VASP disappeared in cGK-deficient cells such as

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¹ The abbreviations used are: cGK, cGMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; ANP, atrial natriuretic peptide; MC, mesangial cells; PKA, cAMP-dependent protein kinase; SNP, sodium nitroprusside; P-VASP, phosphorylated VASP; P-Ser¹⁵⁷-VASP, VASP phosphorylated at serine 157; P-Ser²³⁹-VASP, VASP phosphorylated at serine 239; MAPK, mitogen-activated protein kinase.

platelets of cGK I knockout mice (18) and platelets from chronic myelocytic leukemia patients (19), demonstrating that cGMP-dependent phosphorylation of VASP is mediated by cGK and not by activation of PKA. In contrast, cAMP-dependent phosphorylation of VASP is mediated by PKA. In vascular smooth muscle cells, cGK I is thought to counteract the increase in contraction provoked by Ca^{2+} -mobilizing agonists and to inhibit growth factor-induced cell proliferation (20–23).

Specific inhibitors of receptors and enzymes are important tools for evaluating physiological and pharmacological functions including inhibitor peptides for PKA (24, 25). To inhibit cGK activation, two major groups of drugs have been developed; Rp-stereoisomers of cGMP-phosphorothioates and isoquinolinesulfonamide derivatives, protein kinase inhibitors of the so-called H-series. The cGMP analogs prevent activation of cGK at the cGMP binding site of the regulatory domain (7, 26, 27) whereas the second group (*i.e.* the K-252 compound KT5823, H8, H89) interferes at the level of the ATP binding site of the catalytic domain of cGK (28, 29). *In vitro*, inhibitory constants (K_i) of 234 nM (cGK I) and $>10 \mu\text{M}$ (PKA) were determined for KT5823; and for H89, these values are 480 nM (cGK) and 48 nM (PKA) (28, 29).

Based solely on the established cGK-inhibitory activity of KT5823 in cell-free systems without demonstrating the efficacy and specificity of this compound in intact cells, the inhibitor has been extensively used to demonstrate or rule out an involvement of cGK in signaling processes including activation and proliferation of smooth muscle cells (30, 31) and neuronal cells (32), migration of neutrophils and monocytes (33, 34), and regulation of ion channels in epithelial cells and other cell types (35–41). Several inexplicable and partially contradicting conclusions have been reported with KT5823 suggesting that this compound may lack specificity for cGK or may even completely fail to inhibit cGK in several intact cell systems. Previously, both activation of and failure to inhibit cGK in neutrophils were demonstrated as well as lack of cGK-specificity in inhibiting inositol triphosphate receptor phosphorylation (42, 43).

To investigate whether KT5823 inhibits cGK activation not only in broken cell systems but also in intact cells, we determined cGK agonist-induced phosphorylation of VASP in both human platelets and rat mesangial cells, both expressing high levels of cGK I (9, 44). Upon preincubation with KT5823, cGK-mediated phosphorylation of VASP (upon stimulation with SNP, ANP, and 8-pCPT-cGMP) was not inhibited in these cell types but was even further enhanced. Further, activation of PKA by forskolin was also not inhibited in the presence of KT5823 even at high concentrations of inhibitor.

EXPERIMENTAL PROCEDURES

Materials—The cGMP analog 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP) was from BioLog (Bremen, Germany), (*N*-[2-(*p*-bromocinnamyl)aminoethyl]-5-isoquinolinesulfonamide, 2HCl) (H89), atrial natriuretic peptide (ANP) from Calbiochem and KT5823 was either from Calbiochem, Alexis (Läufelfingen, Switzerland), or was kindly provided by Kamiya Biomedical (Seattle, WA). Sodium nitroprusside (SNP) was obtained from Sigma. Polyclonal anti-cGK I antiserum and monoclonal anti-P-Ser²³⁹-VASP 16C2 antibody have been described (17, 45). The monoclonal anti-P-Ser¹⁵⁷-VASP 5C6 antibody was generated as described for the 16C2 antibody.²

In Vitro Protein Kinase Assay—cGK I was purified from bovine lung as described elsewhere (46, 47). Kinase activity of purified enzyme was determined by using the phosphocellulose method (48) with kemptide as substrate and minor modifications (26). Briefly, KT5823, dissolved in Me_2SO (2 mM) was added to a solution (total volume of 50 μl) containing 10 μg of vasptide (2A3: RRRKVSQKE), 0.3 nM (final) purified bovine cGK I α , and ATP with a specific activity of 6000 Bq/pmol ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$) at varying concentrations in 20 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 prior

to stimulating cGK by adding cGMP at a final 5 μM and incubating at 30 °C. Reactions were stopped by adding EDTA to 100 mM final concentration. Subsequently, incorporated radioactivity was bound to P81 filter paper (Whatman), washed with 75 mM phosphoric acid, and quantified by liquid scintillation counting. The inhibitory constant for KT5823 on cGK I was obtained according to previously described procedures (49, 50).

Preparation of Washed Human Platelets—Washed human platelets were prepared as described (9). Briefly, citrated human blood from healthy donors was centrifuged at $330 \times g$ for 15 min to obtain platelet rich plasma, and platelets were subsequently collected by centrifugation at $400 \times g$ for 10 min and resuspended at a density of 1×10^9 cells/ml in phosphate-buffered saline, pH 7.4 containing 5.5 mM glucose, 1 mM EDTA. Occasionally, platelet aliquots were preincubated at 37 °C for 30 min with inhibitors prior to stimulating with cGK or PKA agonists.

Culture of Rat Mesangial Cells (MC)—MC were isolated and cultured using a sieving technique as described previously (51). Glomeruli were obtained from whole kidneys of newborn rats and were minced finely with a razor blade followed by sequential sieving through 125- and 75- μm pore-size metal sieves and then collected on a 22.5- μm pore size sieve. Cells were cultured in RPMI 1640 containing 20% fetal calf serum (both from Life Technologies, Inc.), 2 mM L-glutamine, 0.1 mM sodium pyruvate, 5 mM HEPES buffer, pH 7.2, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1% non-essential amino acids, and 0.1% growth supplements (5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin, 5 ng/ml sodium selenite, Sigma) on plastic at 37 °C in a moist atmosphere containing 5% CO_2 . The purity of MC was confirmed by immunostaining for desmin, vimentin, smooth muscle actin, Thy1.1, and negative staining for factor VIII (52, 53). For experiments, cells at passage 5–12 at a density of approximately 5×10^5 cells/well (6-well plate) were incubated in RPMI, 5% fetal calf serum at 37 °C for 3 h. Inhibitors were added 30 min prior to stimulating with the membrane-permeant cGMP analog 8-pCPT-cGMP, the adenylate cyclase activator forskolin, atrial natriuretic peptide (ANP, Calbiochem), or sodium nitroprusside (Sigma) for 20 min. After washing with phosphate-buffered saline, cells were lysed by adding SDS-stop solution containing 10% β -mercaptoethanol.

Western Blot Analysis of cGK I and VASP Phosphorylation—After stimulation with agonists of PKA or cGK, SDS gel loading buffer was added to intact cells (10^8 platelets or 5×10^5 MC) and was heated at 95 °C for 10 min, and then protein lysates of 2×10^7 platelets or 10^5 MC were analyzed by SDS-polyacrylamide gel electrophoresis (8% gels) and Western blotting. Western blot nitrocellulose membranes were blocked with 1% hemoglobin or 3% nonfat dry milk (Bio-Rad) in phosphate-buffered saline and divided horizontally to stain the high molecular weight range of proteins with polyclonal anti-cGK I antiserum (diluted 1:3000) and the lower range with monoclonal anti-P-Ser²³⁹-VASP 16C2 or monoclonal anti-P-Ser¹⁵⁷-VASP 5C6 antibodies (1 $\mu\text{g}/\text{ml}$ and 1:100 of hybridoma supernatant, respectively), followed by either horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse secondary antibodies (1:5000, Bio-Rad). Signals were visualized using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). Equal protein loading was confirmed by the Ponceau staining of membrane-bound proteins and by staining the upper part of the horizontally cut membranes for cGK I. For blots with extracts of MC, lysates of (200 μM) 8-pCPT-cGMP-stimulated platelets were used as positive controls.

RESULTS

Inhibition of cGK by KT5823 in Vitro—To confirm the previously published cGK-inhibitory effects of KT5823 *in vitro*, purified cGK I α was incubated in the presence of increasing concentrations of inhibitor, and kinase activity was determined by measuring the kinetics of kemptide phosphorylation. Previously, the inhibitory constant for KT5823 of approximately 250 nM was reported (28). However we observed that the potency of cGK inhibition varied with the batch used. Some were essentially inactive. For the most active batches of KT5823, a K_i of 50 to 100 nM was found. This is similar to the published report and well below the KT5823 concentrations used with intact cells. To address the discrepancy for our *in vitro* data with those obtained with intact cells (see below), we used only those batches of KT5823 that were highly active *in vitro*, for experiments with intact cells.

VASP Phosphorylation in Intact Human Platelets—Site-spe-

² U. Walter and H. Hoschuetzky, manuscript in preparation.

cific phosphorylation of VASP reflects the activation of cGK and PKA *in vitro* and in intact cells including platelets. Using phosphorylation-specific monoclonal anti-P-Ser¹⁵⁷-VASP 5C6 and anti-P-Ser²³⁹-VASP I6C2 antibodies revealed cGK- and PKA-specific phosphorylation patterns as described previously, *i.e.* preferential phosphorylation of Ser²³⁹-VASP upon activation of cGK and of Ser¹⁵⁷-VASP upon activation of PKA (Figs. 1 and 2). Phosphorylation of Ser¹⁵⁷-VASP caused a shift in the apparent molecular weight on SDS-gels from 46 to 50 kDa (15). Using the newly developed anti-P-Ser¹⁵⁷-specific 5C6 antibody, a signal was revealed at 50 kDa upon stimulation with either forskolin or 8-pCPT-cGMP as expected.

Effects of KT5823 and H89 on cGMP- and Forskolin-induced VASP Phosphorylation in Intact Platelets—In contrast to cell-free systems, the actual intracellular concentrations of inhibitors may vary among different cell types, which may be caused by differences in membrane permeabilities, physiological turnover of the inhibitor, compartmentalization, and intracellular accumulation. To determine whether H89 and KT5823 actually inhibited PKA- and cGK-mediated VASP phosphorylation in intact cells, washed human platelets were incubated in the presence of either KT5823 (0.2 to 200 μ M) or H89 (5 to 100 μ M) for 30 min prior to adding the membrane-permeant cGMP analog 8-pCPT-cGMP or forskolin (activating adenylate cyclase) for 20 min and determining VASP phosphorylation as above. Whereas H89 dose-dependently inhibited both PKA- and cGK-activation with similar efficacy as seen in the reduced Ser¹⁵⁷- and Ser²³⁹-VASP phosphorylation, KT5823 at concentrations from 0.5 to 200 μ M not only did not inhibit but further enhanced 8-pCPT-cGMP-induced VASP phosphorylation (Fig. 1B, upper and middle panels). Similarly, activation of PKA by forskolin was not inhibited by KT5823 (Fig. 1B, lower panel). In contrast, H89 inhibited both forskolin and 8-pCPT-cGMP-induced phosphorylation. Preincubation of platelets with KT5823 also accelerated 8-pCPT-cGMP-induced VASP phosphorylation (data not shown). Adding the same amount of the solvent Me₂SO had no effect (data not shown).

VASP Phosphorylation in Rat Mesangial Cells—Mesangial cells of the kidney represent a specialized type of contractile smooth muscle similar to cells that show high expression levels of cGK I. In contrast to vascular smooth muscle cells as well as several other cell types, cGK expression was not down-regulated upon isolation and *in vitro* cultivation for more than 10 passages (Fig. 2A and data not shown). Comparing cGK-specific signals of MC extracts with a standard curve of purified recombinant cGK protein revealed cGK expression levels of approximately 0.3 ng of cGK I protein per μ g of total protein, which is approximately 25% of the concentration in platelets (1.36 ng of cGK per μ g of protein, data not shown).

Comparing platelets and rat MC, full induction of VASP phosphorylation was observed at lower concentrations of 8-pCPT-cGMP in MC (Fig. 2A) and therefore in order to submaximally induce cGK, lower concentrations of the cGMP analog (20 μ M for MC *versus* 50 μ M for platelets), were used for inhibitor studies (see below). Whereas in non-stimulated MC, no phosphorylation was apparent at Ser²³⁹-VASP, a positive signal was observed with the Ser¹⁵⁷-VASP-specific 5C6 antibody indicating partial constitutive phosphorylation of this site in non-stimulated MC, which was further increased by both cGK and PKA agonists (Fig. 2A).

Similar as in platelets, cGK agonist-induced phosphorylation of VASP (by 20 μ M 8-pCPT-cGMP, 1 μ M SNP, or 100 nM ANP; Fig. 2, B and C) was not inhibited upon preincubating MC for 20 min in the presence of KT5823, and 8-pCPT-cGMP-stimulated VASP phosphorylation was further increased as seen by a relative increase of the 50-kDa *versus* 46-kDa Ser²³⁹-VASP as

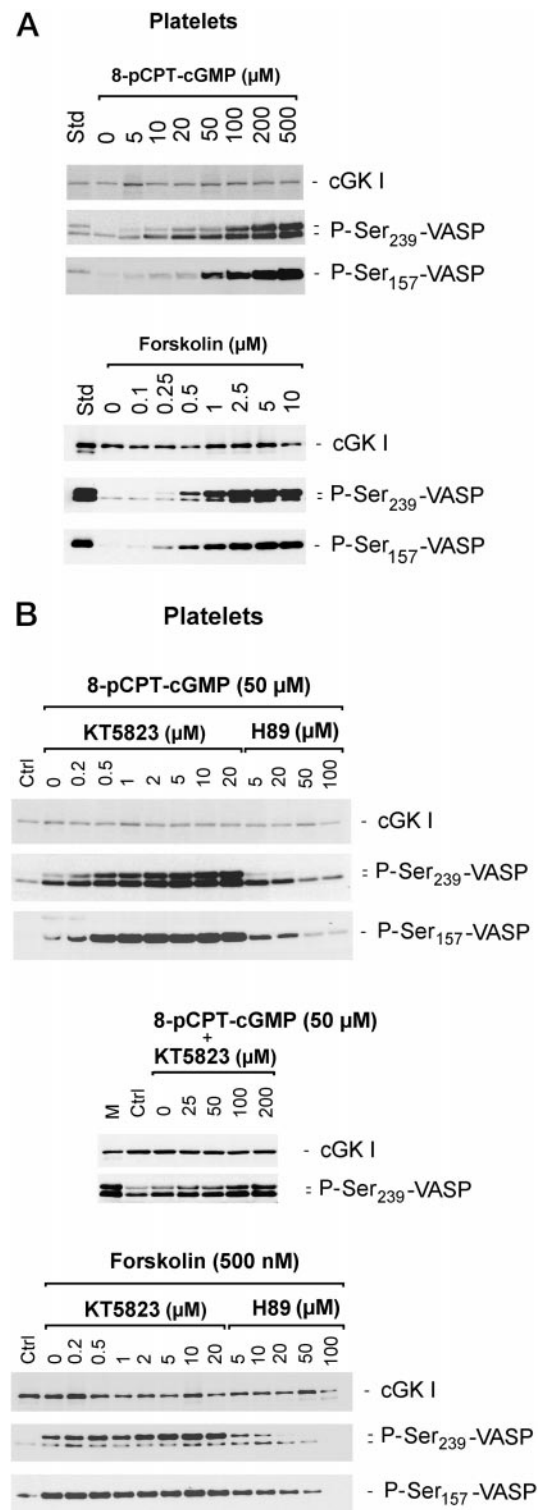


FIG. 1. VASP phosphorylation in human platelets. A, 100 μ l of washed human platelets (10^8 cells/ml) were incubated with the membrane-permeant cGMP analog 8-pCPT-cGMP or forskolin at indicated concentrations for 20 min. Cells (collected by centrifugation and immediately harvested in SDS-gel loading buffer) were subsequently analyzed (10^7 platelets/lane and 10^5 MC/lane) for cGK I expression (upper part of horizontally cut membranes) and VASP phosphorylation (lower part) using the monoclonal phosphorylation-specific anti-P-Ser²³⁹-VASP I6C2 and anti-P-Ser¹⁵⁷-VASP 5C6 antibodies. The apparent shift of the VASP signal from 46 to 50 kDa is caused by phosphorylation at Ser¹⁵⁷ (the only band recognized by the 5C6 antibody)(15). The results are representative of three independent experiments. For inhibitor studies (B), KT5823 or H89 at indicated concentrations were preincubated for 30 min prior to stimulating with cGMP and cAMP agonists and analyzing VASP phosphorylation.

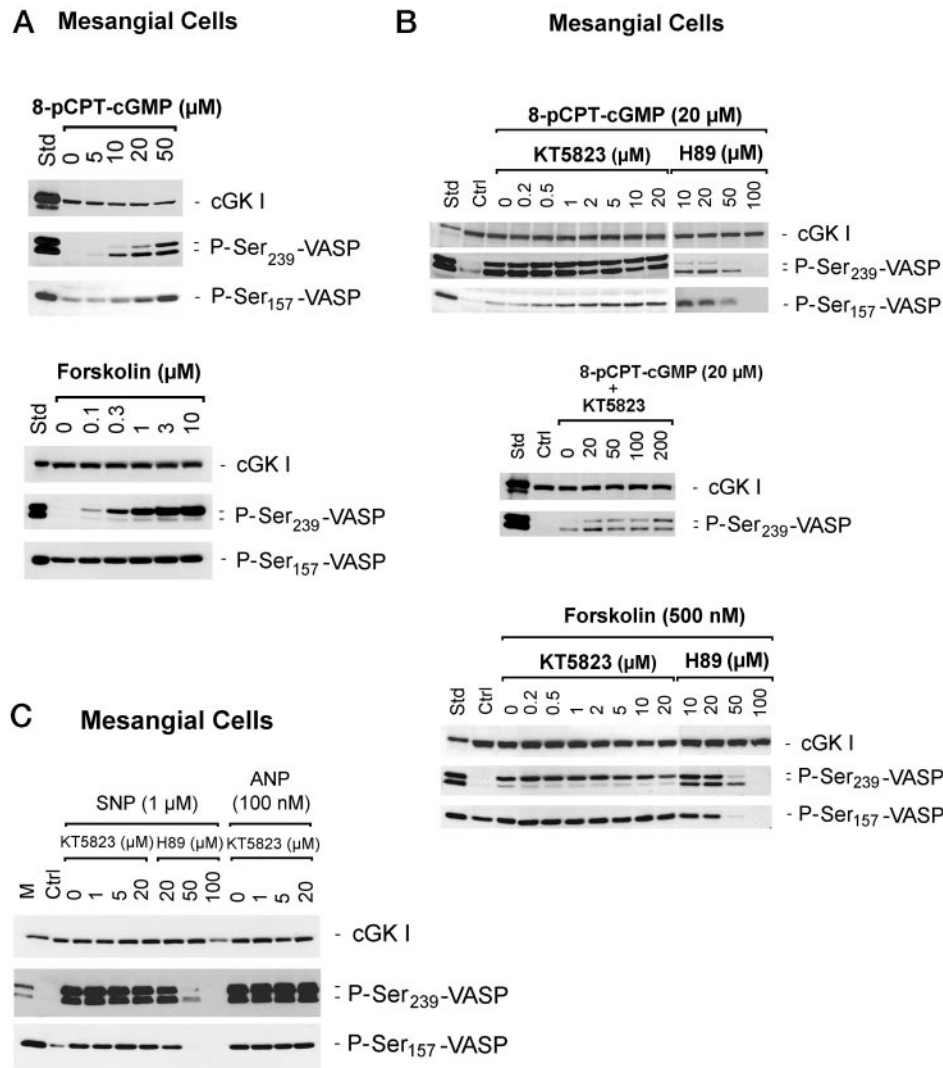


FIG. 2. VASP phosphorylation in MC. *In vitro* cultured rat MC (passage 10) were incubated with indicated concentrations of 8-pCPT-cGMP or forskolin for 20 min, and VASP phosphorylation was determined as in Fig. 1. For inhibitor studies (*B* and *C*) cells were preincubated for 30 min in the presence of KT5823 or H89 at indicated concentrations prior to adding 8-pCPT-cGMP at final 20 μM , forskolin at 500 nM, SNP at 1 μM or atrial natriuretic peptide at 100 nM and incubating for a further 20 min. Cellular lysates were subjected to Western blot analysis of cGK I and phosphorylated VASP was analyzed as in Fig. 1. Lysates of 200 μM 8-pCPT-cGMP-stimulated platelets were used as positive control for MC blots (*Ctrl*). The data shown are representative of four (*B*) and three (*C*) independent experiments using different cell preparations. Data shown for the low and high concentrations of KT5823 (*upper* and *middle*) are from different experiments.

well as the Ser¹⁵⁷-VASP signals. PKA-mediated VASP phosphorylation (stimulated by 500 nM forskolin) was not affected by KT5823 (Fig. 2*B*, lower panel) and both cGMP- (8-pCPT-cGMP, SNP, ANP) and cAMP-induced signals were inhibited in the presence of H89 (Fig. 2, *B* and *C*).

DISCUSSION

In this study, we have demonstrated cGK- and PKA-mediated VASP phosphorylation at Ser²³⁹ and Ser¹⁵⁷ in human platelets and rat mesangial cells. KT5823 did not inhibit and even enhanced cGMP/cGK-mediated VASP phosphorylation. We conclude that KT5823 does not inhibit cGK in two intact cell systems, human platelets and rat mesangial cells, which both express high levels of cGK I.

Specific inhibitors of protein kinases are invaluable tools for evaluating physiological and pharmacological cell functions as has been emphasized with respect to cAK inhibitor peptides and mitogen-activated protein kinases (MAPKs/ERKs) or stress-activated protein kinases (SAPKs, Ref. 54). However, no inhibitors of cGK with comparable specificity and activity have been available. To date, an increasing number of reports include KT5823 as a tool to define cGK involvement in intracel-

lular signaling processes. Of a total of more than 160 citations found in the public medical literature databases, more than 95% have used KT5823 in intact cell systems, and frequently involvement of cGK in signaling processes was concluded solely by showing inhibition of these pathways by KT5823. However, although KT5823 inhibited cGK-induced activation in cell-free systems, it did not inhibit and even enhanced cGMP/cGK-mediated phosphorylation of VASP in both intact platelets and rat mesangial cells.

The reasons for the failure of KT5823 to inhibit cGK in intact cells may arise from the following: (i) failure to access cGK in intact cells because of compartmentalization of the drug; (ii) effects of KT5823 on enzymes other than those that were investigated resulting in counteracting effects; (iii) longer incubation times (in comparison to broken cell systems) that are necessary to enable diffusion/transport of KT5823 through the cellular membrane; (iv) accumulation of KT5823 within cells resulting in much higher intracellular concentrations than was added to the medium because of the hydrophobicity of the compound; and (v) generation of metabolites of KT5823 (perhaps by P450 oxidases) with differing substrate specificities

and perhaps cGK-activating functions that might depend on intact membrane structures.

The mechanism of the effects seen with KT5823 is not known though similar inexplicable results have been reported before. KT5823 was shown to induce cellular shape changes in human neutrophils without inhibitory effects on cGK-dependent phosphorylation of vimentin (31, 43, 55), whereas dibutyl cGMP-induced activation of p38 MAPK was inhibited by either KT5823 or H89 (56). Others reported inhibition of endothelin- or carbon monoxide-induced neutrophil migration by KT5823 (33, 57). In the proximal tubule of rat kidneys, KT5823 not only failed to inhibit ANP- and 8-Br-cGMP-induced Cl^- channel activation but acted stimulatory by itself (35). Further, KT5823 failed to abolish inhibitory effects of ANP and 8-Br-cGMP on a Ca^{2+} -activated K^+ ion channel in kidney cells (36), whereas the same group reported inhibition of cGMP/ATP-activated K^+ channels in the basolateral membrane of rat collecting duct by KT5823 (37). In cGK-transfected 293 cells (derived from human embryonic kidney) cGMP-stimulated Ca^{2+} -activated K^+ (BK_{Ca}) channels were inhibited by KT5823 (38) and evidence for cGK involvement was provided by showing cGK-specific phosphorylation of BK_{Ca} protein *in vitro*. Interestingly, whereas sodium nitroprusside-induced channel activity was inhibited by KT5823, a potentiating effect of KT5823 was observed upon stimulation with another nitric oxide donor, DETA/NO making it unlikely that cGK was directly affected by KT5823. Similarly, interferon γ - or plasmin-induced monocyte/macrophage motility, which was mediated by increased nitric oxide release that led to increased cGMP concentrations, was inhibited by KT5823 (34, 58), but cGK activation was not directly investigated. Inhibition of cGMP-stimulated cell proliferation by KT5823 as well as by Rp-8-pCPT-cGMP was shown in sensory neuron precursor cells as well as in PC12 cells transfected with adenoviral cGK I encoding vectors (32). Adenovirus-mediated overexpression of cGK rendered rabbit pulmonary artery smooth muscle cells sensitive to inhibition of proliferation and sensitized to NO/cGMP-induced apoptosis, which could be inhibited by KT5823 (30).

In some studies, modulatory effects of cGMP-increasing substances were thought to be mediated via cGK because KT5823 abolished the effects even though cGK expression was not shown and was not observed by other investigators in these cell types. In a recent report for instance, 8-Br-cGMP-induced activation of cardiac L-type Ca^{2+} -channels expressed in *Xenopus* oocytes was shown to be inhibited by KT5823 (59); however, expression of endogenous cGK in this system was not found by other investigators (60).

Clearly, the use of an inhibitor alone is not sufficient to prove the involvement of particular enzymes like cGK in signaling processes, and data obtained from *in vitro* experiments cannot prove specificity and efficacy of inhibitors in intact cells. The fact that in both platelets and mesangial cells, KT5823 not only failed to show any inhibitory effect on cGK activation but even enhanced cGK-induced VASP phosphorylation underlines this assumption. Although several processes that may be associated with cGK-activation were abolished upon pretreating cells with KT5823, other processes were not affected or were even further increased. Clearly, the majority of published studies that used KT5823 to prove cGK-dependent effects should be reevaluated considering these new aspects.

No perfect inhibitor of cGK has been developed that would be generally applicable for intact cells. The usefulness of membrane-permeable stereoisomers of cGMP analogs such as the Rp-8-pCPT-cGMPs may be restricted to some intact cells whereas in others, because of altered membrane and perhaps abundant lipophilic vesicles, they may not work. Because of

uncertainties in appropriately adjusting effective intracellular concentrations that would not affect other enzymes such as PKA, as well as inhibitors that compete either for ATP binding or for cGMP binding, it seems generally unlikely that such classes of inhibitors could fulfill the requirements of the "magic bullet" to selectively inhibit cGK.

In conclusion, although under well defined conditions and complementary to other assays some of the currently available cGK inhibitors might be useful, at present there is no perfect cGK inhibitor available that would be highly specific and generally applicable. The worst among these inhibitors is KT5823 because this compound completely lacks any cGK-inhibitory effect in intact cells. If KT5823 is used in future investigations with intact cells, data demonstrating selective inhibition of cGK in these systems are required. The development of a new class of cGK-specific inhibitors that would overcome the current problems would therefore be highly desirable both to avoid future misinterpretations of signaling pathways as well as to develop new drugs that might be useful to study the action of vasodilators and/or modulators of coagulation.

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