The Commonly Used cGMP-dependent Protein Kinase Type I (cGKI) Inhibitor Rp-8-Br-PET-cGMPS Can Activate cGKI in Vitro and in Intact Cells*

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Small-molecule modulators of cGMP signaling are of interest to basic and clinical research. The cGMP-dependent protein kinase type I (cGKI) is presumably a major mediator of cGMP effects, and the cGMP analogue Rp-8-Br-PET-cGMPS (Rp-PET) (chemical name: β-phenyl-1,N2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer) is currently considered one of the most permeable, selective, and potent cGKI inhibitors available for intact cell studies. Here, we have evaluated the properties of Rp-PET using cGKI-expressing and cGKI-deficient primary vascular smooth muscle cells (VSMCs), purified cGKI isoforms, and an engineered cGMP sensor protein. cGKI activity in intact VSMCs was monitored by cGMP/cGKI-stimulated cell growth and phosphorylation of vasodilator-stimulated phosphoprotein. Unexpectedly, Rp-PET (100 μM) did not efficiently antagonize activation of cGKI by the agonist 8-Br-cGMP (100 μM) in intact VSMCs. Moreover, in the absence of 8-Br-cGMP, Rp-PET (100 μM) stimulated cell growth in a cGKI-dependent manner. Kinase assays with purified cGKI isoforms confirmed the previously reported inhibition of the cGMP-stimulated enzyme by Rp-PET in vitro. However, in the absence of the agonist cGMP, Rp-PET partially activated the cGKIα isoform. Experiments with a fluorescence resonance energy transfer-based construct harboring the cGMP binding sites of cGKI suggested that binding of Rp-PET induces a conformational change similar to the agonist cGMP. Together, these findings indicate that Rp-PET is a partial cGKIα agonist that under certain conditions stimulates rather than inhibits cGKI activity in vitro and in intact cells. Data obtained with Rp-PET as cGKI inhibitor should be interpreted with caution and not be used as sole evidence to dissect the role of cGKI in signaling processes.

cGMP is a cyclic-nucleotide second messenger with multiple targets and functions. Small-molecule modulators of cGMP generators and effectors are important biochemical tools as well as established and prospective drugs for the treatment of human diseases, such as erectile dysfunction, pulmonary hypertension, and various cardiovascular disorders (1–3). cGMP is generated by nitric oxide- or natriuretic peptide-stimulated guanylyl cyclases and can bind to and modulate the activity of at least three classes of cGMP effector proteins: cyclic nucleotide-hydrolyzing phosphodiesterases (PDEs),2 cyclic nucleotide-gated cation channels, and cGMP-dependent protein kinases (cGKs, also known as protein kinase G or PKG) (4).

Based on pharmacological and genetic studies, the cGK type I (cGKI) is considered the major mediator of cGMP signaling in many tissues including the cardiovascular system (5–8). The mammalian cGKI is a cytosolic Ser/Thr protein kinase comprising an N-terminal regulatory domain with two cGMP-binding sites and a C-terminal catalytic domain. It exists in two isoforms termed cGKIα and cGKIβ. The isoforms have identical cGMP-binding sites and catalytic domains but differ in their N-terminal ~100 amino acids, which contribute to homodimerization, sensitivity to cGMP activation, and interaction with anchoring and substrate proteins. Recent in vivo studies with transgenic mice demonstrated that both isoforms can induce smooth muscle relaxation and vasodilation (9), but the respective molecular mechanisms behind these effects are controversial (6, 10). Similarly, opposing effects of cGMP/cGKI signaling have been reported on the growth and phenotype of vascular smooth muscle cells (VSMCs) (11, 12). The inconsistency of the results concerning the function of cGKI might in part be related to unexpected effects of the pharmacological cGKI activators and inhibitors that are commonly used to distinguish between cGKI-dependent and cGKI-independent signaling. For instance, cGMP analogues can bind to multiple targets of cGKI.

2 The abbreviations used are: PDE, phosphodiesterase; cGKI, cGMP-dependent protein kinase type I; PET, 8-Br-PET-cGMPS; Rp-PET, Rp-8-Br-PET-cGMPS; 8-Br-PET-cGMP, β-phenyl-1,N2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphate; Rp-8-Br-PET-cGMPS, 8-(4-chlorophenylthio)-guanosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer; Rp-8-Br-PET-cGMPS, β-phenyl-1,N2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; VASP, vasodilator-stimulated phosphoprotein; VSMC, vascular smooth muscle cell; MTs, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; ESI-MS, electrospray ionization-mass spectrometry; FRET, fluorescence resonance energy transfer; Mes, 2-morpholineethanesulfonic acid.

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cGMP receptors and can elevate the cAMP level by inhibiting the cAMP-degrading PDE3 (8, 13, 14). Moreover, there is evidence that one of the most frequently used cGKI inhibitors, KT5823, does not at all inhibit cGKI activity both in vitro and in intact cells and may in fact inhibit other protein kinases (15–17). Consequently, other classes of cGKI inhibitors are increasingly used for intact cell studies. These inhibitors are based on cell-permeable modified peptide substrates (18) or on the allosteric activator cGMP (see Fig. 1A) (19). Agonistic cGMP analogues have been converted to antagonists by exchanging one oxygen atom of the cyclic phosphate moiety with sulfur in the equatorial position with respect to the sugar ring (20). The resulting antagonistic Rp-phosphorothioate cGMP analogues are supposed to bind to the cGMP-binding sites of cGKI without inducing the conformational change crucial for allosteric activation of the enzyme (21, 22). For instance, the cGMP analogue 8-Br-PET-cGMP (PET) is a cGKI agonist (23), whereas Rp-8-Br-PET-cGMPS (Rp-PET) (see Fig. 1B) competitively inhibits activation of purified cGKI by cGMP (24). Rp-PET is currently considered one of the most permeable, selective, and potent cGKI inhibitors available for intact cell studies (14, 19).

In the present study, we intended to validate the selectivity and efficacy of Rp-PET as a cGKI inhibitor in intact cells by comparing its effects on cGKI-expressing and cGKI-deficient VSMCs. Surprisingly, Rp-PET did not efficiently inhibit but rather stimulated cGKI-mediated processes in VSMCs. In vitro experiments with purified cGKI isozymes and an engineered cGKI-based cGMP sensor protein supported these findings, suggesting that Rp-PET is a partial agonist rather than an antagonist of cGKIA.

**EXPERIMENTAL PROCEDURES**

**Materials**—cGMP (catalogue number G 001), 8-Br-cGMP (catalogue number B 004), 8-Br-PET-cGMP (PET; catalogue number P 003), Rp-8-Br-PET-cGMPS (Rp-PET; catalogue number P 007), and Rp-8-PCPT-cGMPS (catalogue number C 013) were purchased from Biolog Life Science Institute. Compounds were dissolved in water, and stock solutions (100 mM for cGMP and 8-Br-cGMP and 10 mM for the other compounds) were stored at −20 °C.

**Intact Cell Studies (Cell Culture, Growth Assays, Western Blotting, and Antibodies)**—Primary VSMCs were obtained from aortae of 3–6-week-old cGKI-deficient (cGKI-/-) or litter-matched control (cGKI+/-) mice or from aortae of 8-week-old SM-1α or SM-1β smooth muscle rescue mice (9). Cells were isolated by enzymatic digestion and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin as described previously (26).

To determine cell growth, primary VSMCs were plated on 96-well culture plates (20,000 cells/well) in the absence or presence of 8-Br-cGMP and/or Rp-PET. After 72 h, the cell number was determined by the MTS assay (CellTiter 96® AQueous Pro-mega) or by staining with toluidine blue O. The MTS assay was performed according to the manufacturer’s protocol. Briefly, cells were washed once with serum-free medium to remove non-adherent cells. Subsequently, 20 μl of the MTS reagent were added to 100 μl of serum-free medium in each well. The absorbance at 492 nm was measured after a 30- and 60-min incubation at 37 °C in a humidified, 6% CO₂ atmosphere. For toluidine blue O staining, cells were washed once with phosphate-buffered saline and then fixed and stained for 10 min in 100 μl of ice-cold toluidine blue O solution (0.5% (w/v) toluidine blue O in phosphate-buffered saline containing 0.2% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde). Excess dye was removed by five washes with phosphate-buffered saline. Stained cells were incubated in 100 μl of 1% (w/v) SDS to release the dye and the A₆₂₀ was determined.

Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) was detected by Western blotting via the band shift to a higher apparent molecular weight when VASP is phosphorylated at Ser-157 (8). Primary VSMCs were plated on 6-well culture plates (100,000 cells/well) and grown for 7 days to a confluence of 80–90%. Subsequently, cells were maintained in serum-free medium for a further 48 h. Then cells were preincubated for 30 min with Rp-PET or vehicle followed by a 30-min incubation in the presence or absence of 8-Br-cGMP. Cells were washed once with phosphate-buffered saline and lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 0.7% (w/v) SDS, 1.7% (v/v) β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated for 5 min at 95 °C and used for SDS-PAGE and Western blot analysis with polyclonal rabbit antibodies against VASP (Alexis Biochemicals, catalogue number Alx-210-725, 1:2000), Akt (Cell Signaling Technology, catalogue number 9272, 1:1000), and cGKI (1:5000). The rabbit polyclonal cGKI antiserum, termed “cGKI common (DH),” detects both cGKIα and cGKIβ. It was raised against recombinant bovine cGKIα and affinity-purified using cGKIα-coupled...
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FIGURE 2. Effects of Rp-PET on cGKI-mediated processes in intact primary VSMCs. Cell growth (upper panels) and VASP phosphorylation (lower panels) were monitored in control (ctr, left panels) and cGKI-deficient (ko, right panels) VSMCs. Cells were incubated with or without 8-Br-cGMP (100 μM) and Rp-PET (100 μM) as indicated. The cell number was determined after 72 h by the toluidine blue assay and normalized to control growth in the absence of drugs. Data of two to five separate cell preparations were pooled and presented as means ± S.E. The number of wells measured under each condition (n) is indicated in the respective columns. *** indicates p < 0.001 versus control in the absence of drugs (Student’s t test). The cell number in the presence of 8-Br-cGMP and Rp-PET was not significantly different from 8-Br-cGMP alone (Student’s t test). Similar results were obtained by the MTS assay (data not shown). Phosphorylation of VASP was measured after a 30-min preincubation with or without Rp-PET followed by 30 min with or without 8-Br-cGMP. Phospho-VASP (p-VASP) was monitored by immunodetection of the band shift to a higher apparent molecular weight when VASP is phosphorylated at Ser-157 (8). Staining of cGKI confirmed the presence and absence of the kinase in control and cGKI-deficient cells, respectively. Akt was used as a loading control. rel. cell number, relative cell number.

to BrCN-Sepharose. The cGKIα protein was expressed in Sf9 insect cells and purified as described (27).

In Vitro Kinase Assay—A radioactive assay was used to determine the kinase activity of purified recombinant bovine cGKIα and cGKIβ. Both isozymes were expressed in Sf9 insect cells and purified by affinity chromatography (27, 28). The phosphorylation reaction was carried out at 30 °C in a total volume of 100 μl. The reaction mix contained 50 mM Mes, 0.4 mM EGTA, 1 mM magnesium acetate, 10 mM NaCl, 10 mM dithiothreitol, 0.1% (w/v) bovine serum albumin, 0.1 mM ATP (~100 cpm/pmol [γ-32P]ATP), 40 μM substrate peptide GRTGRRNSI-amide, and various concentrations of cGMP and/or Rp-PET. Reactions were started by adding 10 ng of purified enzyme. After 5 min, 80 μl of the reaction mix were spotted onto Whatman P81 phosphocellulose paper (2.5 × 3.0 cm). Then the filter papers were washed three times for 10 min in 85 mM phosphoric acid, dried, and put into scintillation vials to measure 32P incorporation. Activity was calculated as μmol of phosphate transferred per minute and mg of kinase. Kᵦ values for reaching half-maximal activity were determined from the inflection points of the activation curves. Kᵦ values for inhibition of the enzyme to half-maximal activity were determined by Dixon plots (29).

Electrospray Ionization Mass Spectrometry (ESI-MS)—Aqueous Rp-PET and PET stock solutions (10 mM, sodium salt) were stored at −20 °C. Before MS analysis, they were allowed to thermally equilibrate at room temperature for 30 min. For ESI-MS, stock solutions were diluted with water/acetonitrile (50:50, v/v) to a final concentration of 0.5 mM. For spiking, PET was added to the Rp-PET aliquot in a 30:70 (v/v) ratio. Solutions were infused (4 μl/min) into the ESI source using a Parmer Infusion 74900 series syringe pump (Cole-Parmer Instrument Co.). Mass spectra were acquired in the negative ion mode using a HCT Plus ion trap mass spectrometer equipped with a standard ESI source (Bruker Daltonics). Spectra (50–1000 m/z) were acquired in the standard enhanced mode (scan rate 8100 m/z per second). Dry gas (5 liters/min) temperature was set to 300 °C, the nebulizer was set to 10.0 p.s.i., and the electrospray voltage was set to 4000 V. Maximal accumulation time was set to 200 ms. Loading of the trap was controlled by the instrument (ICC 70000).

Fluorescence Resonance Energy Transfer (FRET) Measurements—A fusion protein (cGi-500, Ref. 30) consisting of the tandem cGMP-binding domains of bovine cGKI sandwiched between the cyan and yellow fluorescent proteins CFP and YFP, respectively, was transiently expressed in HEK-293 cells using the FuGENE 6 transfection reagent according to the instructions of the manufacturer (Roche Applied Science). 1 × 10⁷ cells were lysed in homogenization buffer (25 mM triethanolamine/HCl, pH 7.4, containing 2 mM dithiothreitol and a 100-fold dilution of protease inhibitor mixture, Sigma-Aldrich) by sonication (1 pulse, 5 s), and a cytosolic fraction was monitored by immunodetection of the band shift to a higher apparent molecular weight when VASP is phosphorylated at Ser-157 (8). Staining of cGKI confirmed the presence and absence of the kinase in control and cGKI-deficient cells, respectively. Akt was used as a loading control. rel. cell number, relative cell number.

RESULTS AND DISCUSSION

The effects of Rp-PET (Fig. 1B) on intact cells were studied in murine primary aortic VSMCs, which express both cGKIα and cGKIβ (31). VSMCs obtained from control or cGKI-deficient mice (25) were compared. This cell culture system has been proven useful to identify cGKI-dependent functions. Previous studies have shown that the stimulation of cell growth and VASP phosphorylation by the membrane-permeable cGMP analogue 8-Br-cGMP is indeed mediated via activation of cGKI
The concentration (31–33). Therefore, it was tested whether Rp-PET could inhibit cGMP/cGKI-stimulated VSMC growth and phosphorylation of VASP. As expected, the cGKI agonist 8-Br-cGMP (100 μM) induced cell growth and VASP phosphorylation in cGKI-expressing cells (Fig. 2, left panels) but was ineffective in cGKI-knock-out cells (Fig. 2, right panels). Surprisingly, Rp-PET (100 μM) had no significant effect on 8-Br-cGMP (100 μM)-induced growth and phospho-VASP. When applied under basal conditions, i.e. in the absence of 8-Br-cGMP, Rp-PET even stimulated cell growth and VASP phosphorylation in control VSMCs (Fig. 2, left panels). Growth stimulation by Rp-PET alone was reproducible and highly significant, but clearly weaker than with 8-Br-cGMP. Importantly, Rp-PET had no effects on cGKI-deficient cells (Fig. 2, right panels), demonstrating that its apparent agonistic activity in control VSMCs was indeed mediated by cGKI.

To further investigate the potential partial agonistic activity of Rp-PET, its effects on the activity of purified cGKIα and cGKIβ were examined. Both enzymes displayed cGMP-dependent kinase activity with characteristic $K_i$ values for stimulation with cGMP ($K_i \sim 0.1$ and $\sim 1 \mu M$ for cGKIα and cGKIβ, respectively; Fig. 3, no inhibitor, and Fig. 4). Increasing concentrations of Rp-PET caused a right shift of the cGMP activation curves for both the cGKIα isoform (Fig. 3A, upper panel) and the cGKIβ isoform (Fig. 3B, upper panel), as expected for an inhibitor. By using Dixon plots (29), $K_i$ values of 0.03 and 0.05 μM were determined for cGKIα (Fig. 3A, lower panel) and cGKIβ (Fig. 3B, lower panel), respectively. These $K_i$ values were similar to $K_i$ values reported previously for the inhibition of the cGKI isoforms by Rp-PET in vitro (24). Interestingly, inhibition of cGMP-activated cGKIα by Rp-PET was less complete than inhibition of cGKIβ (Fig. 3, upper panels, black arrows). Moreover, when added in the absence of cGMP, Rp-PET appeared to increase the activity of cGKIα but not cGKIβ above basal levels (Fig. 3, upper panels, broken arrows). These findings were consistent with the partial agonistic effect of Rp-PET observed in intact cells and suggested that Rp-PET might preferentially activate the cGKIα isozyme. Indeed, Rp-PET alone increased the kinase activity of cGKIα in a concentration-dependent manner with a $K_a$ value of 1 μM (Fig. 4A). In line with a partial agonistic activity, the maximal kinase activity that could be induced with Rp-PET was lower than...
with cGMP, reaching ~38% of the enzyme activity in the presence of saturating cGMP concentrations. In contrast, Rp-PET did not significantly alter the kinase activity of the cGK1β isoform (Fig. 4B).

It seemed possible that the apparent activation of purified cGK1α by Rp-PET was caused by spontaneous exchange of the sulfur atom of the cyclic phosphorothioate moiety of Rp-PET with oxygen, resulting in conversion of the Rp-analogue to the corresponding agonistic compound, PET (Fig. 1B; see Technical Information about Rp-8-Br-PET-cGMPs, update October 15, 2007, Biolog Life Science Institute). To exclude this possibility and to analyze the homogeneity of the Rp-PET compound, the aliquot used for the kinase assays was examined by ESI-MS. Only peaks at m/z ratios corresponding to the molecular mass of Rp-PET could be detected (Fig. 5, left panel), indicating that the compound was pure and that exchange of sulfur with oxygen or other chemical alterations had not occurred. Spiking of Rp-PET with the potential conversion product PET, which is 16 Da lighter, confirmed that these compounds could clearly be resolved by the experimental setup (Fig. 5, right panel). Thus, the mass spectrum confirmed that Rp-PET itself exerted the partial agonistic effect on cGK1α that was observed in the kinase assays.

To further verify its partial agonistic activity, the effects of Rp-PET on the isolated cGMP-binding domains of cGKI were studied by FRET measurements. For these measurements, the two cGMP-binding sites of bovine cGKI were sandwiched between CFP and YFP, respectively (30). This construct contains only the cGMP-binding sites, which are identical in cGK1α and cGK1β but lacks the N-terminal region that differs between the isozymes. Binding of the agonist cGMP to the FRET construct induces a conformational change that can be monitored as altered FRET signal (30), whereas binding of an antagonist does not produce a FRET change. In line with a partial agonistic activity of Rp-PET, increasing concentrations of Rp-PET caused a similar, albeit weaker, FRET change as the known agonists cGMP and PET (Fig. 6).

The combined results of the in vitro kinase assays with purified cGK1 isoforms (Figs. 3 and 4) and of the FRET measurements (Fig. 6) suggest the following model. Rp-PET binds to the cGMP-binding sites of both cGK1 isozymes. Although it acts as an antagonist of cGK1β, its binding to cGK1α induces a conformational change that partially activates the enzyme. To confirm that Rp-PET activates cGK1α but not cGK1β in intact cells, growth assays were performed with so-called SM-1α or SM-1β smooth muscle rescue VSMCs, which express only the cGK1α or the cGK1β isoform, respectively (9). Indeed, Rp-PET (100 μM) stimulated the growth of SM-1α but not SM-1β cells (Fig. 7). 8-Br-cGMP (100 μM) was effective in both cell preparations,
FIGURE 8. Inhibition of 8-Br-cGMP-stimulated VSMC growth (upper panel) and VASP phosphorylation (lower panel) by Rp-PET. The experiments were performed with cGKI-expressing primary VSMCs as described in the legend for Fig. 2, with the exception that the concentration of 8-Br-cGMP was reduced to 50 μM and that of Rp-PET was increased to 200 μM. Cell growth was determined by the MTS assay, and p-VASP (Ser-157) was determined by the upshift of the VASP band. Relative cell numbers (rel. cell number) are given as means ± S.E. The number of wells measured under each condition (n) is indicated in the respective columns. *** indicates p < 0.001 versus 8-Br-cGMP (Student’s t-test).

demonstrating that, in principle, both cGKI isoforms can promote VSMC growth.

The differential sensitivity of cGKIα versus cGKIβ to the partial agonistic effect of Rp-PET must be related to their different N termini, which apparently differ in their ability to couple ligand binding to activation of the catalytic region. Indeed, the N termini determine the differential sensitivity of the isoforms to cGMP activation, the cGKια isoform being ~10 times more sensitive to cGMP than cGKιβ (34). Target-specific effects have also been reported for the Rp-phosphorothioate cGMP analogue Rp-8-pCPT-cGMPS, which is an antagonist of the olfactory cyclic nucleotide-gated channel but an agonist of the photoreceptor cyclic nucleotide-gated channel (35). Because Rp-8-pCPT-cGMPS is also used as CGK inhibitor (36), we tested its effect on cGKI activity in the absence of cGMP. Similar to Rp-PET, Rp-8-pCPT-cGMPS did not alter the basal kinase activity of cGKιβ but partially activated the cGKια isozyme to ~35% of the maximal cGMP-stimulated activity with a Kₜ value of 1 μM (data not shown). In the original reports on the use of Rp-8-pCPT-cGMPS (36) and Rp-PET (24) as cGK inhibitors, the effects of these compounds on cGKI activity in the absence of cGMP were not determined. However, in line with the present results, other investigators have noticed partial agonistic effects of both Rp-PET and Rp-8-pCPT-cGMPS on cGKI (37, 38). Partial agonistic activity has also been reported for Rp-phosphorothioate cAMP analogues that are used as inhibitors of the cAMP-dependent protein kinase (39).

As a partial agonist, Rp-PET should, in principle, be able to inhibit cGMP-activated cGKI in intact cells. The failure of our initial attempts to inhibit cGKI-stimulated VSMC growth and VASP phosphorylation in the presence of 100 μM 8-Br-cGMP by an equimolar concentration of Rp-PET (Fig. 2) might be related to an inappropriate intracellular ratio of 8-Br-cGMP to Rp-PET. Indeed, when the nominal 1:1 ratio was decreased to 1:4, i.e. 50 μM 8-Br-cGMP and 200 μM Rp-PET, cGMP/cGKI-mediated cell growth and VASP phosphorylation were significantly inhibited by Rp-PET (Fig. 8). These results are consistent with a previous study showing that Rp-PET can inhibit cGMP-activated cGKI in intact cells under certain conditions (24). Considering the 46-fold higher lipophilicity (14) and the 4-fold higher concentration of Rp-PET versus 8-Br-cGMP used in our experiment, it appears that the inhibitory potential of Rp-PET in intact cells is quite moderate and that it must be present in large excess to inhibit the effect of a strong agonist such as 8-Br-cGMP.

Taken together, the present study indicates that the cGMP analogue Rp-PET, which is frequently used as cGKI inhibitor, is a cGKιβ antagonist and partial cGKια agonist and, therefore, affects cGKI activity in a complex isoform-dependent manner. In intact cells, Rp-PET may either inhibit or activate cGKI-mediated pathways depending on the intracellular cGMP level and the prevalence of the cGKια or cGKιβ isoform. Other studies have shown that Rp-PET can also exert cGKI-independent effects (40, 41), perhaps via modulation of PDEs (3, 14). The unpredictable behavior of Rp-PET complicates the interpretation of intact cell studies. Rp-PET should be used with caution as cGKI inhibitor and not as sole proof for the involvement of cGKI in signaling pathways.

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