

RESEARCH PAPER

The oligopeptide DT-2 is a specific PKG I inhibitor only *in vitro*, not in living cells

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BACKGROUND AND PURPOSE

cGMP is involved in the regulation of many cellular processes including cardiac and smooth muscle contractility, aldosterone synthesis and inhibition of platelet activation. Intracellular effects cGMP are mediated by cGMP-dependent PKs, cGMP-regulated PDEs and cGMP-gated ion channels. PKG inhibitors are widely used to discriminate PKG-specific effects. They can be divided into cyclic nucleotide-binding site inhibitors such as Rp-phosphorothioate analogues (Rp-cGMPS), ATP-binding site inhibitors such as KT5823, and substrate binding site inhibitors represented by the recently described DT-oligopeptides. As it has been shown that Rp-cGMPS and KT5823 have numerous non-specific effects, we analysed the pharmacological properties of the oligopeptide (D)-DT-2 described as a highly specific, membrane-permeable, PKG inhibitor.

EXPERIMENTAL APPROACH

Specificity and potency of (D)-DT-2 to inhibit PKG activity was evaluated using biochemical assays *in vitro* and by substrate phosphorylation analysis in various cell types including human platelets, rat mesangial cells and rat neonatal cardiomyocytes.

KEY RESULTS

Despite potent inhibition of PKGI *in vitro*, (D)-DT-2 lost specificity for PKG in cell homogenates and particularly in living cells, as demonstrated by phosphorylation of different substrates. Instead, (D)-DT-2 modulated activity of other kinases including ERK, p38, PKB and PKC, thereby inducing unpredicted and often opposing functional effects.

CONCLUSIONS AND IMPLICATIONS

We conclude that DT-oligopeptides, as other inhibitors, cannot be used to specifically inhibit PKG in intact cells. Therefore, no specific pharmacological PKG inhibitors are available, and reliable studies of PKG signalling can only be made by using RNA knockdown or genetic deletion methods.

Abbreviations

ANP, atrial natriuretic peptide; GC-A receptor, guanylyl cyclase-A receptor; NRVM, neonatal rat ventricular myocytes; RMC, rat mesangial cells; SNP, sodium nitroprusside; VASP, vasodilator-stimulated phosphoprotein.

Introduction

cGMP is synthesized from GTP by two classes of GCs which include transmembrane particulate GCs, which serve as receptors for natriuretic peptides and the soluble form (sGC), which serves as the cytosolic receptor for NO (Lohmann and

Walter, 2005). cGMP is involved in the regulation of many diverse cellular processes such as cardiac and smooth muscle cell contractility, aldosterone synthesis, inhibition of platelet activation, immune cell response, neuronal excitability and synaptic plasticity (Lohmann and Walter, 2005; Hofmann *et al.*, 2006; 2009; Walter and Gambaryan, 2009).

Intracellular effects of cGMP are mediated by a number of effectors, including cGMP-gated channels, cGMP-regulated PDEs (PDE2 and 3) and cGMP-dependent PKs (PKGs; nomenclature follows Alexander *et al.*, 2011). Most cells contain at least one of the three known PKG subtypes (PKG I α , PKG I β , PKG II) (Hofmann *et al.*, 2009). However, very often, the same cells express cGMP-regulated PDEs, whose activation/inhibition by cGMP can promote or inhibit PKA activity as well. For example, in aldosterone producing cells of the adrenal zona glomerulosa, PKG II activity is involved in the regulation of basal aldosterone production (Gambaryan *et al.*, 2003), whereas activation of PDE2 by cGMP inhibits cAMP-induced aldosterone production (MacFarland *et al.*, 1991; Nikolaev *et al.*, 2005). In cardiac myocytes, PKG I, PDE2 and PDE3 are localized in different compartments and can play even opposing roles in regulation of cardiac contractility (Castro *et al.*, 2010; Mika *et al.*, 2012). In renin-secreting juxtaglomerular cells, cGMP signalling is even more complicated. In these cells, PKG I, PKG II, PDE3 and PDE2 are expressed (Gambaryan *et al.*, 1996; 1998; Castrop *et al.*, 2010). Depending on experimental conditions (*in vivo* experiments, isolated glomeruli or isolated juxtaglomerular cells) and the site of cGMP synthesis (activation of particulate versus soluble GC), cGMP can stimulate or inhibit renin release by activation of PKG or PDEs (Kurtz, 2011). Several approaches (knockout animal models, overexpression of active and inactive PKGs, PKG-specific activators/inhibitors) have been used to characterize PKG-specific effects and distinguish them from other cGMP mediators (Smolenski *et al.*, 1998b; Hofmann *et al.*, 2006; 2009).

Currently used PKG inhibitors can be divided into three classes: cyclic nucleotide-binding site inhibitors such as Rp-phosphorothioate analogues (Rp-cGMPS), ATP-binding site inhibitors such as KT5823 and substrate-binding site inhibitors represented by the recently described DT-oligopeptides. Although in our previous experiments, Rp-cGMPS compounds (Rp-8-pCPT-cGMPS, Rp-8-Br-PET-cGMPS) were potent inhibitors of PKG activity in mesangial cells and platelets (Burkhardt *et al.*, 2000), we later recognized that they also display PKG-independent unspecific effects on platelets (Gambaryan *et al.*, 2004). In smooth muscle cells, Rp-8-Br-PET-cGMPS stimulates PKG I instead of inhibiting its activity (Valtcheva *et al.*, 2009). KT5823, a widely used cell-permeable PKG inhibitor, does not inhibit basal or stimulated PKG activity in platelets, mesangial and smooth muscle cells, but may potentially inhibit other PKs (Wyatt *et al.*, 1991; Komalavilas and Lincoln, 1996; Burkhardt *et al.*, 2000; Bain *et al.*, 2003). DT-oligopeptides, which bind to the substrate-binding site of PKG catalytic domain and inhibit PKG activity by competing with the substrates, were introduced as 'highly specific, membrane permeable peptide blockers of PKG' (Nickl *et al.*, 2010). Here, we show that the oligopeptide (D)-DT-2 was a specific PKG I inhibitor only for purified PKG I α /I β enzymes but did not inhibit PKG activity in intact cells.

Methods

In vitro PK assay

PKA c-subunit (PKAc) and PKG type I α were purified from bovine heart and bovine lung, respectively, as described

earlier (Kaczmarek *et al.*, 1980; Walter *et al.*, 1980). Human PKG I β and PKG II were expressed in Sf9 cells and purified by affinity chromatography (Pohler *et al.*, 1995). The activity of the purified kinases was measured by the phosphocellulose method (Roskoski, 1983). Briefly, PK activity was assayed at 30°C in a total volume of 100 μ L containing 20 mM Tris/HCl buffer pH 7.4, 10 mM MgCl₂, 5 mM β -mercaptoethanol, 0.01% (w/v) bovine serum albumin, 2 nM PK, 20 μ M Vasptide and 5 μ M cGMP (for PKG) or 20 μ M Kemptide (for PKA) as described (Butt *et al.*, 1994), and indicated concentrations of (D)-DT-2. The reaction was started by the addition of 50 μ M [³²P]-ATP (1000 cpm pmol⁻¹) and terminated after 5 min by the addition of 0.1 M EDTA. Phosphorylated peptide was bound to P81-paper (Whatman, Rothenburg, Germany), washed with 75 mM phosphoric acid and quantified by liquid scintillation counting. The half maximal inhibitory concentration (IC₅₀) for PKG I α and PKG I β was determined graphically by plotting kinase activity versus varying (D)-DT-2 concentrations at a constant substrate level and represented the average of three independent experiments.

Preparation of human washed platelets

Our studies with human platelets were approved and recently (24 September 2008) reconfirmed by the local ethics committee of the University of Würzburg (Studies no. 67/92 and 114/04). Blood was obtained from healthy volunteers according to our institutional guidelines and the Declaration of Helsinki.

Human platelets were prepared and used as previously reported (Gambaryan *et al.*, 2010) with small modifications. Blood was collected into 1/7 volume of ACD solution (12 mM citric acid, 15 mM sodium citrate, 25 mM D-glucose and 2 μ M EGTA, final concentrations). PRP was obtained by 5 min centrifugation at 330 \times g. To reduce leukocyte contamination, PRP was diluted 1:1 with PBS and centrifuged at 240 g for 10 min. Subsequently, the supernatant was centrifuged for 10 min at 430 \times g, then the pelleted platelets were washed once in CGS buffer (composition: 120 mM sodium chloride, 12.9 mM trisodium citrate, 30 mM D-glucose, pH 6.5), and resuspended in HEPES buffer (150 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 10 mM D-glucose, 10 mM HEPES, pH 7.4). After 15 min rest in a 37°C water bath, washed platelets were used for experiments.

Animals

All animal care and experimental procedures complied with the guidelines of the Federal German Government and were approved by the Ethical Committee of the University of Würzburg. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 40 animals were used in the experiments described here.

Experiments on rat mesangial cells

Rat mesangial cells (RMC) were isolated from whole kidneys of 1 day old rats (Sprague Dawley; University of Göttingen animal facility) and cultured using a sieving technique as previously described (Burkhardt *et al.*, 2000). Cells were cul-

tured in RPMI1640 containing 20% fetal calf serum, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 5 mM HEPES, pH 7.2, 100 U·mL⁻¹ penicillin, 100 µg·mL⁻¹ streptomycin, 0.1% non-essential amino acids and 0.1% growth supplements (5 µg·mL⁻¹ insulin, 5 µg·mL⁻¹ transferrin, 5 ng·mL⁻¹ sodium selenite). For experiments, cells at passage 5 to 12 at a density of approximately 5×10^5 cells per well (6 well plate) were incubated in RPMI without FCS at 37°C for 3 h. (D)-DT-2 was added 30 min prior to stimulating with ANP, the NO-donor SNP or thrombin for 5 min at indicated concentrations. After washing with PBS, cells were lysed by adding SDS gel loading buffer. For experiments in permeabilized cells, saponin (0.001%) was added to the cells 5 min prior to (D)-DT-2. After 10 min, PKG or PKA was stimulated by 5 µM (final concentration) of cGMP or cAMP, respectively.

Experiments on neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVM) were isolated from 0-1 day old Sprague Dawley rats (University of Göttingen) and cultured as previously described (Grebe *et al.*, 2011). Twenty-four hours after isolation, cells were washed with serum-free media and pre-incubated with 20 µM (D)-DT-2 for 30 min. Next, cells were stimulated with indicated chemicals and processed for Western blot analysis.

FRET experiments on human embryonic kidney (HEK) 293 cells

HEK293a cells were cultured in DMEM medium containing 10% FCS, seeded on 24 mm glass coverslips and transfected with the plasmids for A-kinase activity reporter (AKAR3, kindly provided by Jin Zhang, Baltimore, USA), rat GC-A receptor for ANP and wild-type or dominant-negative (K405A) PKGIβ (Smolenski *et al.*, 2000). Twenty-four hours after transfection, the cells were used for FRET measurements performed exactly as described by Borner *et al.*, (2011). The cells were stimulated with 100 nM ANP, and the amplitudes of FRET responses were analysed.

Confocal microscopy

Confocal microscopy was performed using Zeiss LSM710 microscope (Carl Zeiss MicroImaging, Jena, Germany) equipped with the Plan-Apochromat ×63/1.40 oil-immersion objective. Images were acquired and analysed using ZEN 2010 software (Zeiss). To determine approximate cell volume, 3D stacks of RMC and NRCM transfected with a GFP plasmid were acquired and analysed using the ZEN 2010 software.

Experiments with human washed platelets and RMC lysate

Human washed platelets or RMC were lysed in the buffer (10 mM HEPES, 150 mM NaCl, 2 mM EGTA, 1% Triton ×100, pH 7.4) with protease inhibitors (1 mM PMSE, 10 µg·mL⁻¹ pepstatin A, 20 mg·mL⁻¹ leupeptin) and frozen in liquid nitrogen. In addition, 10 µL of 10× concentrated reaction buffer (100 mM HEPES, 50 mM MgCl₂, 5 mM EGTA, 10 mM dithiothreitol 1 mM ATP, pH 7.4) was added to the 90 µL of cell lysate. The samples were pre-incubated with (D)-DT-2 (20 µM) for 10 min then stimulated with 2 µM of cGMP or

cAMP for 2 min and stopped with 100 µL of 2× SDS gel loading buffer.

Western blot analysis

Washed platelets were pre-incubated for the indicated time/concentration with (D)-DT-2, stimulated with the NO donor DEA-NO, thrombin or collagen and stopped by adding 2× SDS gel loading buffer. RMC were pre-incubated with (D)-DT-2 for 30 min and stimulated with ANP, DEA-NO or thrombin for 10 min. NRVM were pre-incubated with (D)-DT-2 or ERK inhibitor U0126 for 30 min and stimulated with ANP or SNP. An equal volume of 2× SDS gel loading buffer was directly added to platelet suspensions, cells were washed with PBS and lysed in 2× SDS gel loading buffer (500 µL per confluent well of 6 well plate). For Western blotting, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and the membranes were incubated with appropriate primary antibodies [PKG I antibody described by Gambaryan *et al.*, (1996), phospho PDE5-Ser⁹² and total PDE5 (generous gift from Dr S. Rybalkin, University of Washington, Seattle, USA), phospho-VASP-Ser²³⁹, PKG Iα, PKG Iβ, phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²) MAPK antibody, PKC substrate MARCKS phospho-Ser^{159/163} antibody, phospho-ERK (Thr²⁰²/Tyr²⁰), total p38, total ERK phospho-PKB-Ser⁴⁷³ and total PKB] overnight at 4°C. For calculation of PKG Iα/β concentrations in cells, defined amounts of purified kinase and cells were loaded on the same gel. Cell volumes were calculated from confocal microscopic images. For visualization of the signal, goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase was used as secondary antibodies, followed by ECL detection. Blots were scanned using SilverFast software and analysed densitometrically by NIH Image J software for uncalibrated optical density.

Platelet aggregation and calcium measurements

Platelet aggregation was measured using an Apat (LabiTec, Ahrensburg, Germany) aggregometer. Washed human platelets (3×10^8 ·mL⁻¹) were pre-incubated with 10 µM of (D)-DT-2 for 10 min. Platelet aggregation was induced by addition of 0.01 U·mL⁻¹ thrombin or 5 µg·mL⁻¹ collagen. Aggregation was measured with continuous stirring at 1000 rpm and 37°C.

Calcium transients were determined with the fluorescence indicator Fura-2. Platelets were loaded with Fura-2-AM in PRP for 45 min at 37°C. Excess dye and plasma were removed by centrifugation. The pelleted platelets were then resuspended in HEPES buffer and diluted to a density of 2×10^8 platelets·mL⁻¹. The measurement was carried out with a Perkin-Elmer (Waltham, MA, USA) LS50 luminometer. Fura-2 fluorescence was measured at 340 nm excitation. 1 mM Ca⁺⁺ was added immediately before the experiment.

FACS analysis of platelet integrin αIIbβ3 activation

FACS analysis was performed on a Becton Dickinson FACS-calibur using CELLQuest software, version 3.1f (Becton Dickinson, Heidelberg, Germany). Washed platelets were pre-incubated with (D)-DT-2 for 10 min and stimulated with thrombin (0.01 U·mL⁻¹) or collagen for 1 min; incubations were then simultaneously terminated for Western blot and

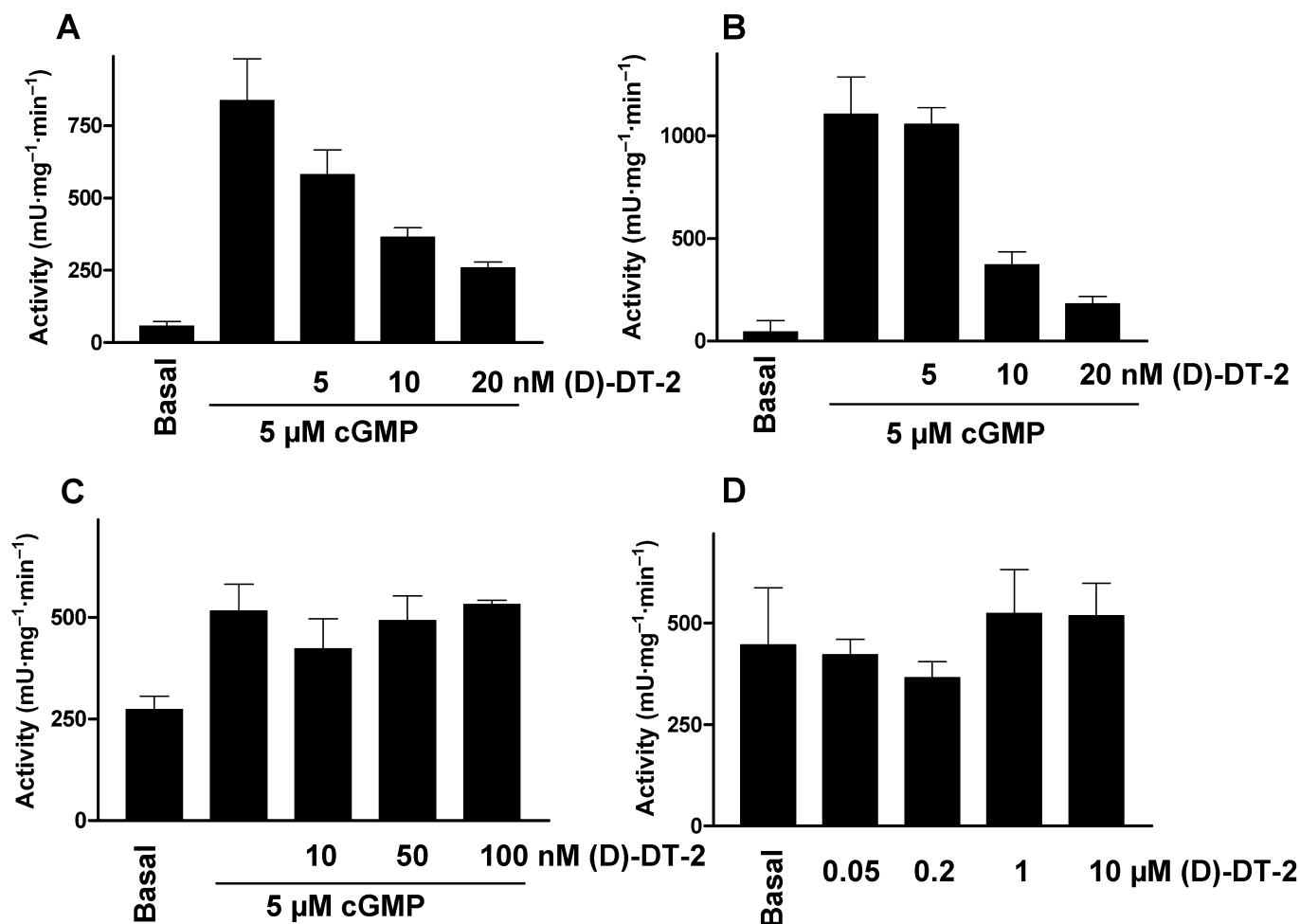


Figure 1

In vitro, (D)-DT-2 selectively inhibits PKG I α and PKG I β but not PKG II or PKA. Indicated concentrations of (D)-DT-2 were added to purified (A) PKG I α , (B) PKG I β , (C) PKG II and (D) PKAc (2 nM each). PKGs were stimulated by addition of 5 μ M cGMP (PKAc is catalytically active). Kinase activity was determined by phosphorylation of Vasptide (for PKGs) or Kemptide (for PKAc), both used at 20 μ M.

FACS analysis. For FACS analysis, after agonist stimulation, washed platelets were labelled for 5 min at room temperature with FITC-conjugated antibody against activated α IIb β 3 integrins, PAC-1-FITC (BD-Bioscience Heidelberg, Germany). Stimulation was stopped by diluting platelets (1:10) in PBS/5.5 mM glucose/0.5%, and platelets were analysed by flow cytometry.

Data analysis

All experiments were performed at least three times and summary data are expressed as means \pm SEM. Differences between groups were analysed by ANOVA followed by Bonferroni's test, and Student's *t*-test was used when appropriate. *P* < 0.05 was considered statistically significant.

Materials

(D)-DT-2 was from Biolog (Bremen, Germany); atrial natriuretic peptide (ANP), sodium nitroprusside (SNP), saponin, phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²) MAPK antibody were from Sigma

(Deisenhofen, Germany); thrombin was from Roche (Mannheim, Germany); DEA-NO was from Alexis Biochemicals (Lörrach, Germany); collagen was from Nycomed (Linz, Austria); tat peptide was from Antibodies-online (Aachen, Germany); U0126 was from Calbiochem (Schwalbach, Germany); phospho-VASP-Ser²³⁹, PKG I α , PKG I β antibodies were from Nanotools (Teningen, Germany); PKC substrate MARCKS phospho-Ser^{159/163} antibody was from Epitomics (Hamburg, Germany); phospho-ERK (Thr²⁰²/Tyr²⁰), total p38, total ERK, phospho-PKB-Ser⁴⁷³, total PKB antibodies were from Cell Signaling (Frankfurt am Main, Germany)

Results

In vitro characterization of (D)-DT-2 effects on PKG (I α , I β , II) and PKA activity

The oligopeptides DT-2 (Dostmann *et al.*, 2000) and (D)-DT-2 (Nickl *et al.*, 2010) have been characterized *in vitro* as highly

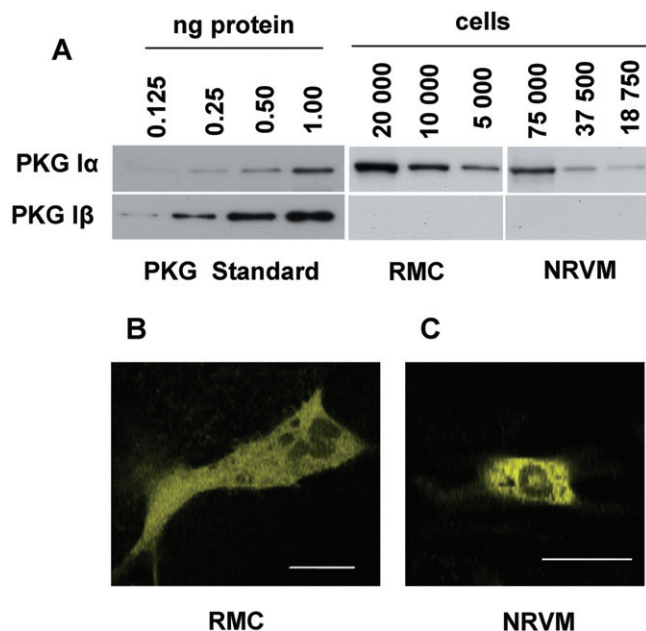


Figure 2

Quantitative characterization of PKG expression in RMC and NRVM. Indicated concentrations of purified PKG I α /I β and cells were loaded on the gel and processed for Western blotting. Blots were scanned using SilverFast software and analysed densitometrically by NIH Image J software for uncalibrated optical density. Cell volumes calculated from confocal 3D stacks (representative microscopy images are shown in B and C. Scale bars, 10 μ M) were 6.3 pL for RMC and 3.5 pL for NRVM. In both cell types, only PKG I α is expressed in concentrations of $0.36 \pm 0.03 \mu\text{M}$ for RMC and $0.30 \pm 0.02 \mu\text{M}$ for NRVM.

specific inhibitors ($IC_{50} = 12.0 \pm 0.8 \text{ nM}$) for 2 nM of PKG I α (selectivity of more than 15 000-fold compared to PKA). Using *in vitro* PK assays, we tested the commercially available oligopeptide (D)-DT-2 for its ability to inhibit all known PKG isoforms (I α , I β and II) and the catalytic subunit of PKA. (D)-DT-2 concentration-dependently and equally inhibited PKG I α and PKG I β , (Figure 1A, B), but did not inhibit PKG II (Figure 1C) or PKA (Figure 1D), even at high concentrations. Calculated IC_{50} values for 2 nM of PKG I α and PKG I β were $9 \pm 2 \text{ nM}$ and $7.5 \pm 1.8 \text{ nM}$, respectively, indicating that (D)-DT-2 concentrations should be at least three times higher than the kinase concentration to reach half maximal inhibition.

In human platelets, only PKG I β is expressed at a concentration of $7.3 \pm 0.8 \mu\text{M}$ (Eigenthaler *et al.*, 1992). In RMC and in NRVM, no PKG I β was detected, while the concentrations of PKG I α were $0.36 \pm 0.03 \mu\text{M}$ for RMC and $0.30 \pm 0.02 \mu\text{M}$ for NRVM, as calculated by Western blotting according to the standard concentrations of the purified kinase (Figure 2A) and by measuring cell volume by confocal microscopy (Figure 2B, C). Therefore, we used micromolar concentrations of (D)-DT-2 in all other experiments. Next, we tested whether the selectivity of (D)-DT-2 for PKG I α /I β was still present in cell homogenates. We used whole cell homogenates from human platelets, which express PKG I β , and RMC, which express

PKG I α , to assess PKG and PKA activity by phosphorylation of the well-known PKG and PKA substrate vasodilator-stimulated phosphoprotein (VASP). Phosphorylation of VASP at Ser¹⁵⁷, the preferred PKA site, alters the apparent molecular mass of VASP on SDS/PAGE from 46 kDa to 50 kDa, whereas phosphorylation on Ser²³⁹, the preferred site for PKG, can be analysed by phospho-Ser²³⁹-specific monoclonal VASP antibodies, which can be used as a marker of PKG activity *in vitro* and in intact cells (Smolenski *et al.*, 1998a). However, both kinases (PKA and PKG) can phosphorylate VASP at Ser²³⁹ and Ser¹⁵⁷. Unexpectedly, in contrast to purified kinases (Figure 1), in both cases (homogenates from platelets and RMC), (D)-DT-2 lost its specificity and potently and concentration-dependently inhibited both PKG and PKA activities (Figure 3).

Experiments on intact washed human platelets

In intact human platelets, PKG I β concentration has been calculated as $7.3 \mu\text{M}$ (Eigenthaler *et al.*, 1992); therefore, we used very high (up to $100 \mu\text{M}$) concentrations of (D)-DT-2 to evaluate the inhibition of PKG in intact platelets (Figure 4A). As fluorescein-labelled DT-2 was translocated into smooth muscle cells within 30 min (Dostmann *et al.*, 2000), we also incubated our cells with (D)-DT-2 for up to 30 min (Figure 4B). In both cases, (D)-DT-2 failed to inhibit DEA-NO-stimulated PKG activity assessed by phosphorylation of the established PKG substrates VASP and PDE5. Next, we tested whether (D)-DT-2 could inhibit basal (unstimulated) PKG activity and its effect on several other PKs including MAP kinases (p38 and ERK), PKC and PKB, which are important markers of platelet activation. Platelets were stimulated by thrombin or collagen, which do not activate PKG, and platelet activation was assessed with the PAC-1 antibody which binds only to activated integrin $\alpha\text{IIb}\beta_3$. Kinase activities were evaluated by phospho-specific antibodies which recognize activated forms of kinases (p38, ERK, PKB), or by phosphorylation of the established kinase substrates (VASP for PKG and MARKS for PKC). (D)-DT-2 had no effect on the basal PKG activity, but concentration-dependently inhibited thrombin-stimulated activation of p38, ERK, PKB and PKC (Figure 5A). Inhibition of these kinases correlates with the inhibition of integrin activation, aggregation (Figure 5B) and calcium mobilization (Figure 5C). In collagen-stimulated platelets, (D)-DT-2 had an opposite effect on platelet activation. Starting from $5 \mu\text{M}$, (D)-DT-2 enhanced PKB and PKC activity and PAC-1 binding (Figure 5A), which corresponds to an increased platelet aggregation (Figure 5D). However, (D)-DT-2 had no effect on collagen-induced calcium mobilization (Figure 5E).

In additional experiments performed with the tat peptide, which was added to the (D)-DT-2 sequence to make it cell permeable (Nickl *et al.*, 2010), we could show that (D)-DT-2 effects on platelets were not mediated by the tat-peptide, but were directly connected with (D)-DT-2 (Figure 5F). Our data clearly indicated that (D)-DT-2 did not inhibit PKG activity in intact platelets but, unexpectedly, did inhibit (in the case of thrombin stimulation) or enhance (after collagen stimulation) the activation of several other PKs (p38, ERK, PKC, PKB).

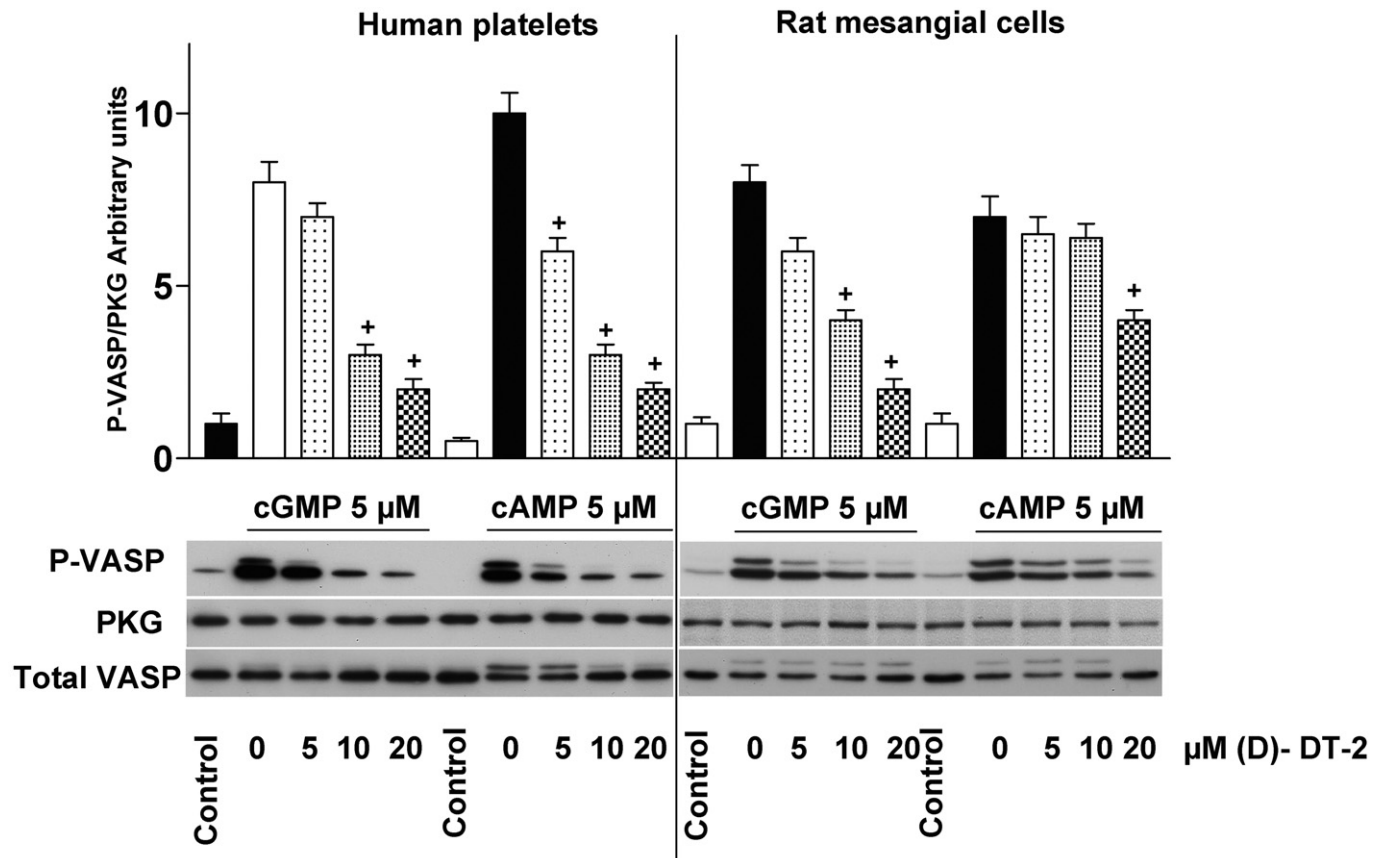


Figure 3

(D)-DT-2 loses its selectivity against PKG and PKA in cell homogenates. Lysates of human platelets or RMC were pre-incubated (10 min) with indicated concentrations of (D)-DT-2. PKG and PKA were stimulated by cGMP or cAMP, respectively (for 5 min, 5 μM each). Samples were processed for Western blotting. PKG activity was evaluated by VASP-Ser²³⁹ phosphorylation (P-VASP). The apparent shift of the VASP signal from 46 kDa to 50 kDa is due to phosphorylation at Ser157. PKG I and total VASP expression are served as loading control. Blots were scanned using SilverFast software and analysed densitometrically by NIH Image J software for uncalibrated optical density. The intensity of P-VASP signal was normalized to the total VASP signal, and then this ratio for each sample was expressed relative to the control which was designated as unity. Results are mean ± SEM, $n = 5$, $+P < 0.05$ (Student's *t*-test). In contrast to *in vitro* assays (Figure 1), (D)-DT-2 inhibits both PKA and PKG activation in the whole cell lysate.

Experiments on RMC and NRVM

To test the ability of (D)-DT-2 to inhibit PKG Iα in intact cells, we used RMC and NRVM which only express PKG Iα at concentrations of 0.36 μM and 0.30 μM, respectively. In these cells, PKG is activated by stimulation of two different receptors: membrane-bound GC-A (by ANP) and soluble GC (by SNP). Activation of both receptors similarly stimulated PKG activity (Figure 6A, B). Pre-incubation with (D)-DT-2 (20 μM, for 30 min) had no effect on PKG activity (as evident from P-VASP blot). However, it slightly potentiated basal and thrombin-induced ERK activation in RMC (Figure 6A), whereas in NRVM, it strongly, and comparable to the specific ERK inhibitor (U0126), inhibited ERK activation (P-ERK blot). Next, we tested whether the lack of PKG inhibition in RMC originates from a limited diffusion of the peptide across the cell membrane. RMC were permeabilized by 0.001% saponin, and PKG or PKA were activated by cGMP or cAMP, respectively. In intact cells, cGMP and cAMP added to the media did not stimulate PKG or PKA,

while in permeabilized cells, both kinases were strongly activated by the cyclic nucleotides (Figure 6C). Pre-incubation with (D)-DT-2 (20 μM, 30 min), in contrast to the cell lysates (compare with Figure 3), did not inhibit PKG or PKA activity in permeabilized cells (Figure 6C).

FRET experiments in HEK 293 cells

To directly identify possible inhibitory effects of (D)-DT-2 in living cells, we used a FRET-based PKA substrate sensor AKAR3 (Zhang *et al.*, 2005; Allen and Zhang, 2006), which in our experiments, was also phosphorylated by PKG Iβ in transfected HEK293 cells. HEK293 cells do not express detectable amounts of PKG I and GC-A. Therefore, in addition to AKAR3, the cells were co-transfected with GC-A and PKG Iβ. The specificity of AKAR3 phosphorylation by PKG was tested in experiments where the dominant-negative PKG (dnPKG Iβ) was transfected instead of active PKG Iβ. Activation of GC-A by ANP strongly enhanced the FRET signal of AKAR3, without any detectable changes in FRET response in the cells

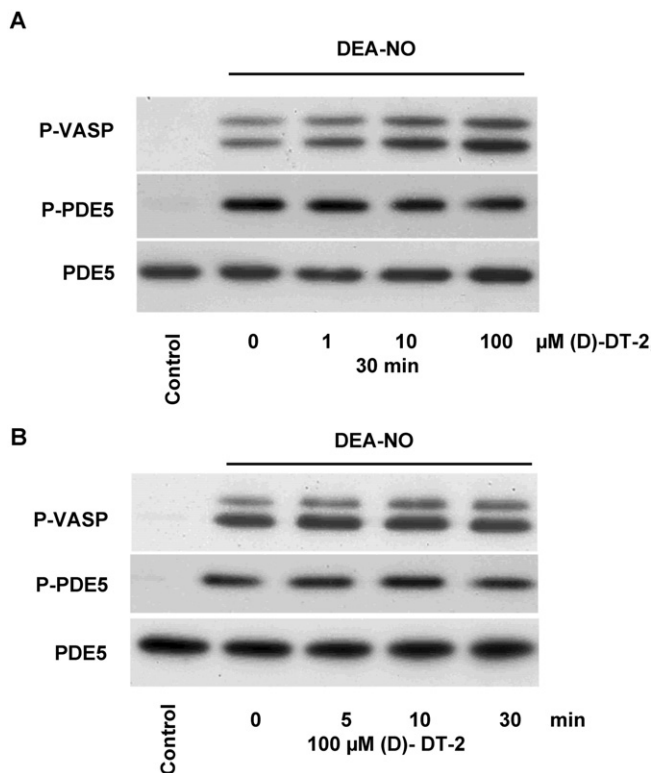


Figure 4

(D)-DT-2 does not inhibit PKG activity in intact human platelets. 100 μL of washed human platelets (2×10^8 cells·mL⁻¹) were pre-incubated with indicated concentrations of (D)-DT-2 for 30 min (A), or with 100 μM of (D)-DT-2 for indicated time points (B). PKG was activated by DEA-NO (1 μM , 1 min). Samples were processed for Western blotting and PKG activity was evaluated by VASP-Ser²³⁹ (P-VASP) and PDE5-Ser⁹² (PDE5) phosphorylation. PDE5 expression served as a loading control. The results are representative of four independent experiments.

transfected with dnPKG I β . Again, pre-incubation with (D)-DT-2 did not inhibit PKG activity measured with the FRET sensors in these cells (Figure 7).

Discussion

The main function of PKs is the phosphorylation of target proteins (substrates) by covalent addition of inorganic phosphate to serine, threonine or tyrosine at a distinct consensus substrate sequence. Therefore, inhibition of specific substrate phosphorylation in intact cells should be regarded as the only reliable criteria for PK inhibitors. Unfortunately, in many, if not most, of the studies involving PKG inhibitors, conclusions about the mediation by PKG of the effects of cGMP are drawn from the blockade or abolition of these effects. Such conclusions solely rely on the observed functional effects of the inhibitors used, without prior demonstration of their specificity for PKG and inhibition of PKG substrate phosphorylation. From the updated list of PKG substrates presented in a recent review (Francis *et al.*, 2010),

it becomes evident that each cell type contains at least some of the identified PKG substrates, and against some of them, including VASP, LASP, PDE5, IRAG, phospho-specific antibodies are available. Among them, VASP, which is expressed in many tissues and cell types (Gambaryan *et al.*, 2001), is one of the best characterized PKG and PKA substrates (Butt *et al.*, 1994). A peptide, corresponding to the PKG phosphorylation site of VASP (LRKVSKQE, also called Vasptide) is used as a substrate for *in vitro* determination of purified PKG activity (Butt *et al.*, 1994). The oligopeptides DT-2 (YGRKKRRQRRPP-**LRKKKKKH**, sequence of DT-2 is in bold) and (D)-DT-2 are stable against proteolysis (Nickl *et al.*, 2010) and were developed from the peptide library as pseudo-substrates for PKG which can bind to the substrate binding site of the PKG catalytic domain and competitively inhibit PKG activity. High selectivity of DT-2 for PKG over PKA was shown by *in vitro* assays. However, the selectivity of DT-2 against other Ser/Thr PKs has not been investigated. In addition, the *in vivo* inhibitory potential of DT compounds was shown only at the functional level (Dostmann *et al.*, 2000; Krieg *et al.*, 2005), without demonstrating inhibition of PKG-specific substrate phosphorylation. We chose (D)-DT-2 as the best characterized PKG inhibitor to analyse PKG-specific effects in both cell lysate and intact cells. We used human platelets, RMC and cardiac myocytes (NRVM) because these cells have been used earlier by us (Burkhardt *et al.*, 2000; Gambaryan *et al.*, 2004) and others (Wyatt *et al.*, 1991; Valtcheva *et al.*, 2009) to demonstrate that the PKG inhibitors KT5823 (competitive binding at ATP-site) and Rp-cGMPs (competitive binding at cGMP binding sites) did not inhibit the kinase but had some PKG-independent functional effects.

(D)-DT-2 is a highly specific inhibitor for purified PKG I α / β enzymes which does not inhibit PKA *in vitro* (Figure 1). The calculated IC₅₀ values for PKG I α / β in these experiments are in agreement with the published data (Dostmann *et al.*, 2000). Binding of (D)-DT-2 to PKG I α was mapped by photoaffinity cross linking to the catalytic core on residues 356–372, also known as the glycine-rich loop which is essential for ATP binding (Pinkse *et al.*, 2009). This domain is highly conserved in all species and homologous between various PKG isoforms (Uhler, 1993). Nevertheless, PKG II activity is not blocked by (D)-DT-2. Differences in binding affinity are also observed in the highly homologous cyclic nucleotide-binding domains for cGMP that vary from 0.07 μM for PKG II to 0.1 μM for PKG I α and 0.9 μM for PKG I β and is most likely due to structural differences between the isoforms (Osborne *et al.*, 2011). Therefore, it is tempting to speculate that there might be also some structural differences in the catalytic site of all three PKGs that would allow differentiation between substrates. An example of such differences in substrate recognition is a novel PKG substrate, LASP-1. While VASP is phosphorylated by all three PKGs to similar extent, LASP-1 is preferred by PKG I β (Butt *et al.*, 2003). As the (D)-DT-2 peptide was designed and screened for PKG I α inhibition (Dostmann *et al.*, 2000), the observed preferential inhibitory potential for PKG I is possible.

However, in intact human platelets, expressing PKG I β , even at very high (up to 100 μM) concentrations and at up to 30 min incubation time, which is sufficient for this peptide to enter the cells (Dostmann *et al.*, 2000), (D)-DT-2 did not

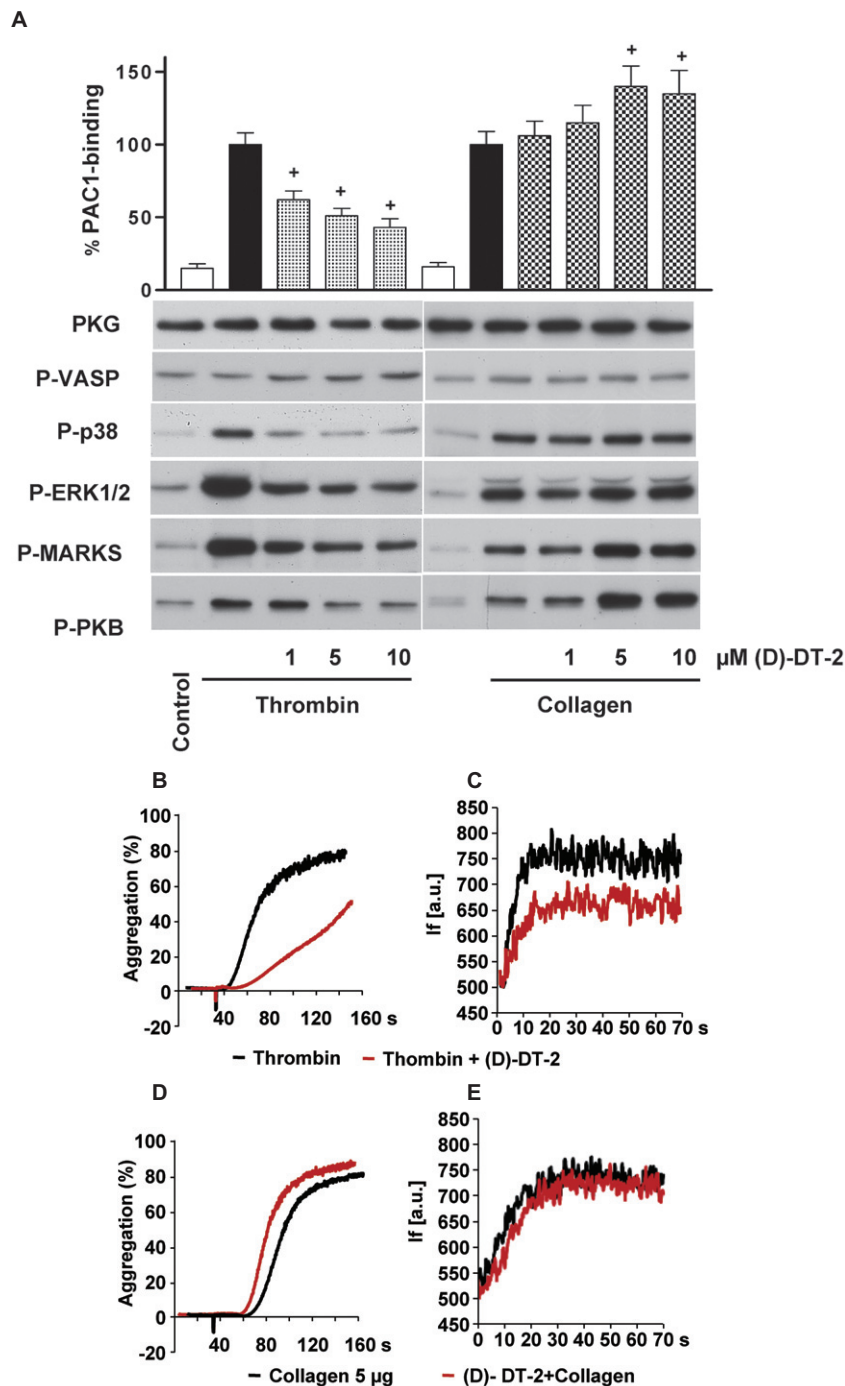


Figure 5

(D)-DT-2 PKG-independently inhibits thrombin-stimulated and enhances collagen-stimulated platelet activation. (A) Human washed platelets were pre-incubated with indicated concentrations of (D)-DT-2 for 10 min and then stimulated with thrombin ($0.01 \text{ U}\cdot\text{mL}^{-1}$, 1 min) or collagen ($5 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$, 1 min). $20 \text{ }\mu\text{L}$ of platelet suspension was used for FACS analysis of integrin $\alpha\text{IIb}\beta_3$ activation (PAC-1 binding). The rest of the samples were processed for Western blotting. (D)-DT-2 does not inhibit basal PKG activity in both cases (P-VASP panel); however, it strongly and concentration-dependently inhibits thrombin-stimulated platelet activation assessed by integrin activation (PAC-1 binding). In addition, (D)-DT-2 inhibits MAP kinases (P-p38 and P-ERK1/2 panels), PKC (inhibition of established PKC substrate, P-MARKS panel) and PKB (P-PKB panel). In collagen-stimulated platelets, (D)-DT-2 starting from $5 \text{ }\mu\text{M}$ significantly enhances integrin activation which corresponds to increased activation of PKC and PKB. These data correspond to (D)-DT-2 effects on platelet aggregation (B) and calcium mobilization (C) induced by thrombin, or collagen (D, E). Pre-incubation of (D)-DT-2 ($10 \text{ }\mu\text{M}$, 10 min) strongly inhibits thrombin-induced platelet aggregation (B) and calcium mobilization (C) and slightly enhances collagen-induced aggregation (D), without affecting calcium mobilization (E). (F) shows the same experiment as in (A) except that (D)-DT-2 was replaced by the tat peptide. All results are representative of at least three independent experiments. Data on PAC-1 binding (A, F) are means \pm SEM, $n = 4$, $+P < 0.05$ (ANOVA followed by Bonferroni's test).

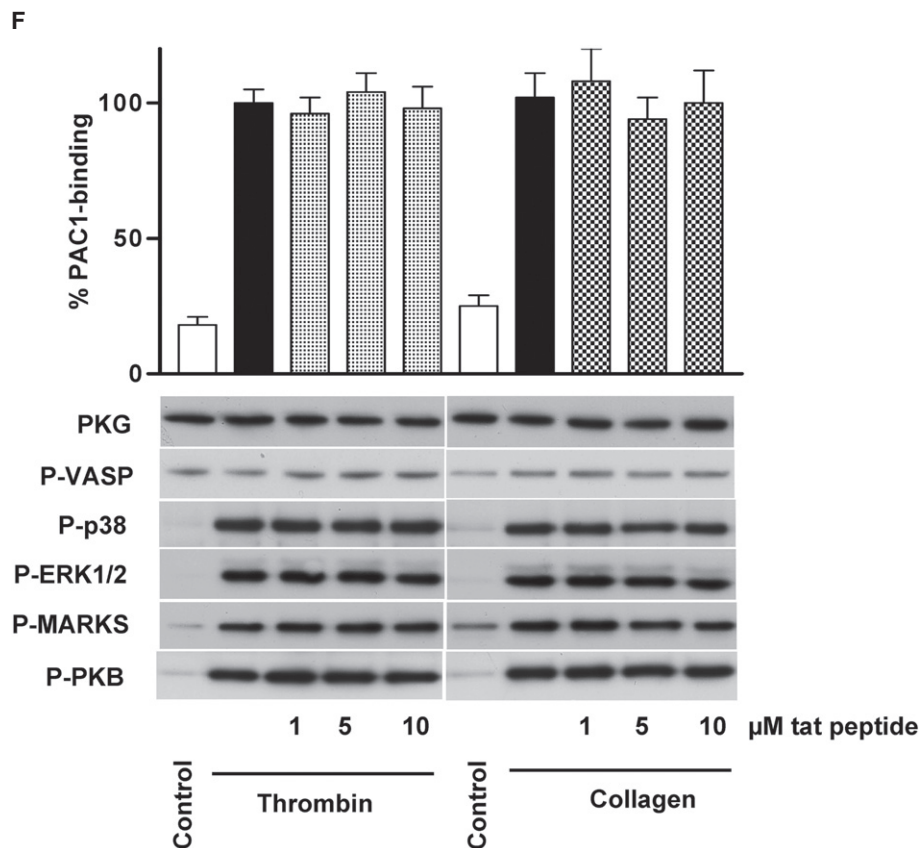


Figure 5

Continued.

inhibit the PKG-mediated phosphorylation of the established PKG substrates VASP and PDE5 (Figure 4). In contrast to intact platelets, in whole cell lysates, (D)-DT-2 dose-dependently inhibited PKG activity. However, even in this case, it cannot be used as PKG-specific inhibitor, because it also inhibits PKA activity. Next, we tested whether (D)-DT-2 inhibited basal PKG activity and/or interacted with other PKs. We used platelet agonists (thrombin, collagen) which do not activate PKG but stimulate the activity of several other PKs. Surprisingly, we found that this 'highly specific' PKG inhibitor modulated other kinases such as PKC, PKB and MAP kinases (Figure 5).

We can only speculate about the exact mechanism of (D)-DT-2 mediated inhibition of thrombin-activated platelets or the potentiation of collagen-activated platelets. However, these effects are obviously not connected with the tat peptide, because the tat peptide itself had no effect on platelets used under the same conditions (Figure 5F). Possibly, in platelets, (D)-DT-2 can interact with thrombin receptors, thereby inhibiting thrombin-induced platelet activation, whereas binding to collagen receptors might further potentiate collagen-induced platelet activation. In contrast, experiments on RMC and NRVM, where (D)-DT-2 also PKG-independently and unexpectedly potentiated (RMC, Figure 6A) or inhibited (NRVM, Figure 6B) basal ERK activity,

suggested that these effects of (D)-DT-2 might be intracellular and not connected to cell membrane receptors. Importantly, in NRVM, the oligopeptide inhibited basal ERK activity (Figure 6B) potentially (comparable to the specific ERK inhibitor U0126) but independently of PKG.

One possible explanation for this cross-inhibition might be the high similarity of the catalytic domain between Ser/Thr kinases. They all contain a common catalytic domain with an ATP-binding site characterized by the Rossmann fold motif GXGXXG and a substrate recognition site. Binding of (D)-DT-2 to PKG I α was mapped by photoaffinity cross linking to the catalytic core on residues 356–372, which is essential for ATP binding (Pinkse *et al.*, 2009). This domain is highly homologous among Ser/Thr kinases and conserved between all three PKGs (Uhler, 1993). Another important aspect, which was not taken into account by the developers of (D)-DT-2, is the substrate specificity of the kinases. Several Ser/Thr kinases including PKA, PKG, PKC, PKB, Pim1, RSK1, SLK1, DMPK and ZIPK have a conspicuous overlapping substrate specificity with a preference for basic amino acids N-terminally at positions –2 and –3 from the phosphorylated serine or threonine residue and varying amino acids at position –1 (Miller and Blom, 2009). A further disadvantage of the (D)-DT-2 is its competition with other PKG substrates in intact cells. To date, more than 50 PKG substrates have

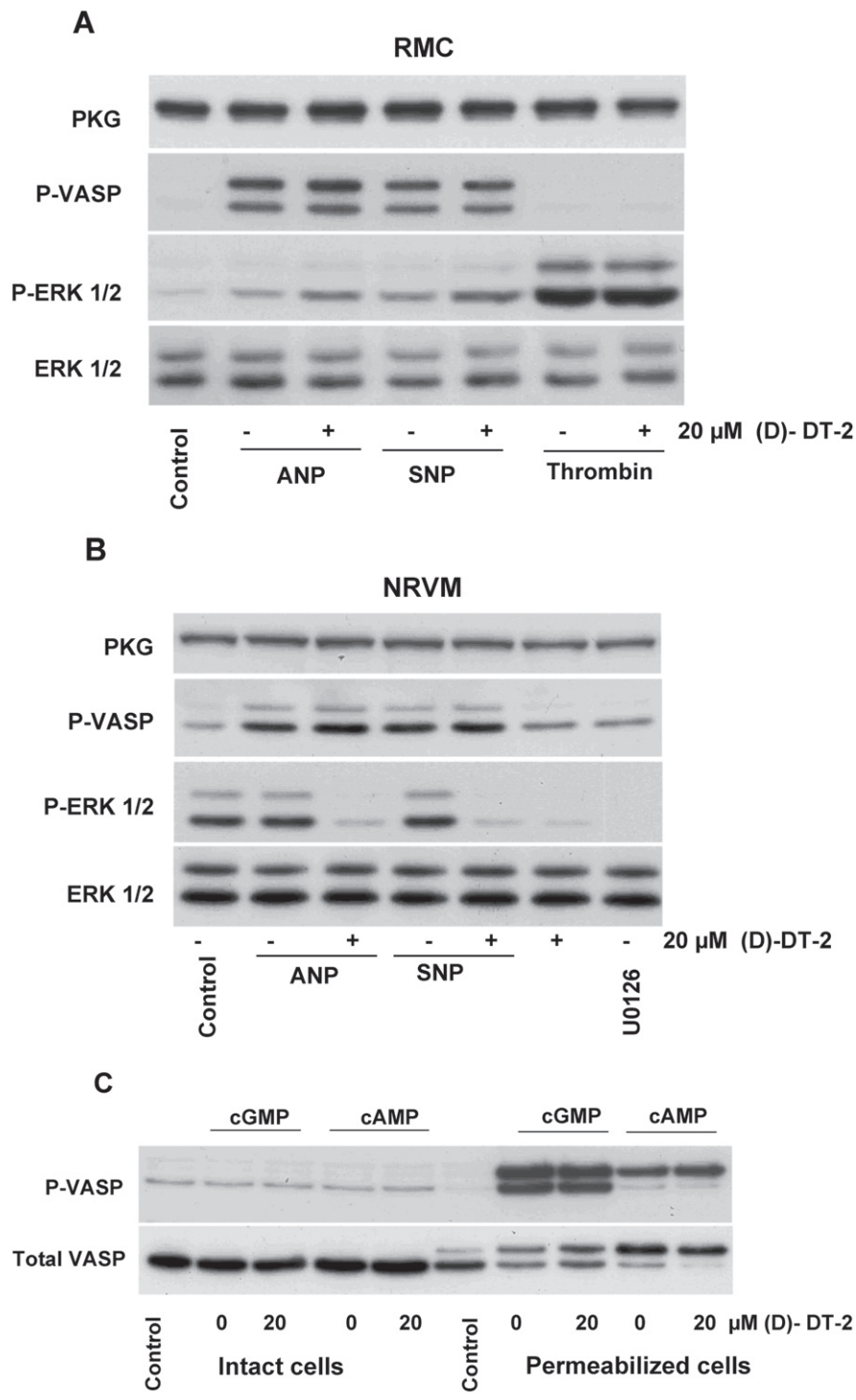


Figure 6

(D)-DT-2 does not inhibit PKG activation in intact and permeabilized RMC and in NRVM. (A) Intact RMC, (B) NRVM and (C) saponin-permeabilized RMC were pre-incubated for 30 min with 20 μ M of (D)-DT-2 or ERK inhibitor U0126 (only for NRVM, 1 μ M, 5 min) before 5 min stimulation with ANP (10 nM), SNP (5 μ M), thrombin (0.1 U·mL⁻¹) (in A), cGMP or cAMP (in C). Cells were washed with PBS and processed for Western blot analysis for VASP and ERK phosphorylation and PKG expression. In intact RMC, (D)-DT-2 does not inhibit PKG activity (P-VASP panel) and slightly enhances basal and thrombin-induced ERK1/2 phosphorylation. In NRVM, (D)-DT-2 also does not inhibit PKG activity (P-VASP panel). However, in contrast to RMC (compared to A), it potently (comparable to the specific ERK inhibitor U0126) inhibits ERK phosphorylation. In permeabilized RMC (C), in contrast to cell lysate (compare with Figure 3), (D)-DT-2 did not inhibit PKG or PKA activated by cGMP or cAMP, respectively. Incubation of non-permeabilized cells with cGMP or cAMP does not activate PKG or PKA. The data shown are representative of four independent experiments using different cell preparations.

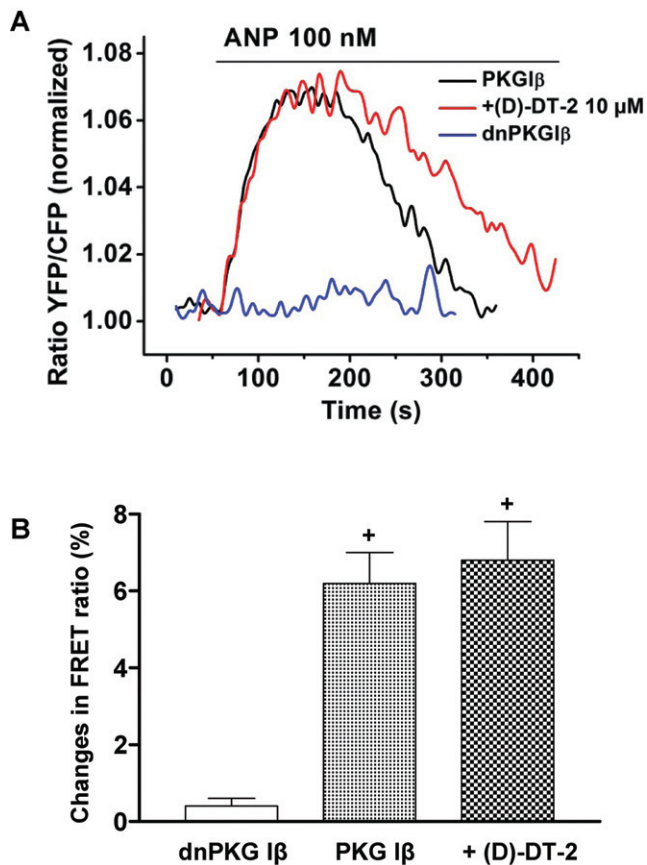


Figure 7

(D)-DT-2 does not inhibit the ANP-induced FRET response of the PKA/PKG activity sensor AKAR3. HEK293 cells transfected with AKAR3, GC-A and active PKG β , or dominant-negative dnPKG β plasmids, were stimulated with 100 nM ANP. FRET responses were recorded from single cells and compared with those measured in cells after 30 min pre-incubation with 10 μ M (D)-DT-2. Representative FRET recordings are shown in (A). Quantification of the FRET data is in (B). Data shown are means \pm SEM, $n = 5$, $+P < 0.05$ (Student's t -test).

been identified (Francis *et al.*, 2010). They are expressed at various intracellular concentrations ranging from nanomolar amounts up to 25 μ M for VASP (Egenthaler *et al.*, 1992) and 18 μ M for PDE5 in human platelets (Wangorsch *et al.*, 2011). Accordingly, (D)-DT-2 as a PKG inhibitor should compete with many endogenous substrates. Therefore, it is not surprising that (D)-DT-2 is not a specific PKG inhibitor in intact cells and can modulate the activity of several other Ser/Thr kinases. All the data reviewed above indicate that the use of peptide-based pseudo-substrates as PK inhibitors for intact cells is probably not the correct approach.

In summary, we showed that the 'highly specific' PKG inhibitor (D)-DT-2 was even less specific than Rp-cGMPS or KT5823 because (i) PKG activity was not inhibited in several types of intact cells; (ii) PKG specificity over PKA was lost in whole cell homogenates; and (iii) PKG-independent inhibition/stimulation of other PKs was rather unpredictable. Therefore, replacing PKA by PKG in the title of the recent

comprehensive review on PKA inhibitors (Murray, 2008), we can clearly affirm that 'Pharmacological PKG inhibition: all may not be what it seems'.

Acknowledgements

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Conflicts of interest

None.

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