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cGMP-phosphodiesterase antagonists inhibit Ca^{2+}-influx in *Dictyostelium discoideum* and bovine cyclic-nucleotide-gated-channel

Daniel F. Lusche[a,*], Hiroshi Kaneko[b], Dieter Malchow[a]

[a]*Department of Biology, University of Konstanz, Universitaetstr.10, D-78457 Konstanz, Germany*
[b]*Department of Molecular Physiology University of Heidelberg, Germany*

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Abstract

We used antagonists of cGMP-phosphodiesterases to examine the role of cGMP for light-scattering oscillations and cAMP-induced Ca^{2+}-influx in *Dictyostelium discoideum*, however, SCH 51866 (cis-5,6a,7,8,9,9a-hexahydro-2-[4-(trifluoromethyl)phenylmethyl]-5-methyl-cyclopent[4,5]imidazo[2,1-b]purin-4(3H)-one) and sildenafil citrate (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1-H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine citrate) were poor inhibitors of cGMP-hydrolysis. Instead, SCH 51866 (IC_{50}=16 μM) and sildenafil, blocked chemoattractant (cAMP)-induced Ca^{2+}-influx as determined with a Ca^{2+}-specific electrode. SCH 51866 (150 μM) affected neither spontaneous cGMP transients during light-scattering-oscillations nor cAMP-mediated K^+-efflux. SCH 51866 and sildenafil are competitive inhibitors of cGMP phosphodiesterases. However, the activity of cGMP-dependent protein kinase Iα (PKGIα) was not altered by SCH 51866 (150 μM). By contrast, patch-clamp measurements of bovine cone cGMP-gated-channels (cyclic-nucleotide-gated-channel, CNGA3), stably expressed in human embryonic kidney cells, HEK 293 cells, revealed reversible, competitive and dose-dependent inhibition of sodium currents by SCH 51866 (IC_{50}=25 μM) and sildenafil, but not by another inhibitor of cGMP-phosphodiesterases, UK 114,542. The possibility that *D. discoideum* cells also express a cGMP-regulated channel is supported by our finding that LY 83583 (6-(phenylamino)-5,8-quinolinedione) (35 μM), known to inhibit cyclic-nucleotide-gated-channels as well as guanylyl-cyclases, reduced cAMP-induced Ca^{2+}-influx in *D. discoideum*, but did not affect cAMP-induced cGMP accumulation. Utilizing a PDED null strain that exhibits a prolonged and elevated cGMP transient following receptor activation, we found that the inhibition of Ca^{2+}-influx by SCH 51866 in the wildtype was absent in the mutant. Our results show that SCH 51866 and sildenafil are antagonists of a Ca^{2+}-permeable channel (CNGA3) and that both compete with cGMP for a regulatory site of Ca^{2+}-influx in *D. discoideum*.
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Keywords: SCH 51866; Sildenafil citrate; Cyclic-nucleotide-gated-channel; cGMP-dependent protein kinase Iα; K^+-efflux

1. Introduction

In *Dictyostelium discoideum*, cAMP functions as a secretable first messenger and chemotactic agent to induce aggregation and to activate distinct signalling pathways within the individual amoebae. In addition, cAMP acts as a second messenger for signal relay, gene expression, terminal stalk cell differentiation, and spore germination (Brown and Firtel, 1999; Brzostowski and Kimmel, 2001; Dormann et al., 2001; Meili and Firtel, 2000). By binding to a serpentine receptor, cAMP-receptor 1, cAMP not only evokes an increase of intracellular cAMP but also a transient ten-fold increase in intracellular cGMP concentration. In contrast to cAMP, cGMP is mainly detected within the cells and only a small amount was found to be secreted (Van Haastert and Kuwayama, 1997; Van Haastert et al., 1983). Recently, progress has been made in understanding the metabolism of cGMP in *D. discoideum* (Bosgraaf and Van Haastert, 2002). Synthesis is mediated by two guanylyl cyclases, a membrane bound and a soluble form, *D. discoideum* GCA and *D. discoideum* sGC, respectively (Roelofs et al., 2001; Roelofs and Van

* Corresponding author. Tel.: +49 7531882478; fax: +49 7531882966.
E-mail address: Daniel.Lusche@web.de (D.F. Lusche).

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Haastert, 2002). Two cGMP-specific phosphodiesterases (PDE) responsible for degradation of cGMP have been cloned (Bosgraaf et al., 2002). *D. discoideum* PDE3 accounts for 20% of the total cGMP-hydrolysing activity of the amoebae. The remaining 78% of cGMP-hydrolysing activity in lysates originates from an enzyme encoded by pdeD that is activated by low cGMP concentrations and to a small extent from another protein PDEE (Kuwayama et al., 2001; Saran et al., 2002). In addition, genes encoding two phosphodiesterases with different substrate preferences have been sequenced. PDE1/PdsA is an enzyme with a higher specificity for cAMP than for cGMP. It exists as a soluble, secreted form and as a membrane-bound form reviewed in Franke and Kessin (1992). In contrast, *D. discoideum* PDE2/Reg A is an intracellular enzyme that specifically degrades intracellular cAMP reviewed in Saran et al. (2002).

cAMP and cGMP are implicated in the regulation of Ca^{2+}-homeostasis. Upon binding of cAMP to its receptor, Ca^{2+} is released from both acidic and inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores leading to a capacitative Ca^{2+}-influx across the plasma membrane and an increase in cytosolic Ca^{2+} concentration (Newell et al., 1995; Schaloske and Malchow, 1997; Schaloske et al., 2000; Schlatterer et al., 2004; Sonnemann et al., 1998). The Ca^{2+}-influx is triggered by cAMP partly via a G-protein dependent and partly via a G-protein independent pathway (Schaloske et al., 1995). Long chain fatty acids contribute to the regulation of Ca^{2+}-entry by releasing Ca^{2+} from the stores and/or possibly by a regulation of calcineurin A ultimately leading to capacitive Ca^{2+}-entry (Kessen et al., 1999). Two types of Ca^{2+}-stores, the acidosomes and inositol 1,4,5-trisphosphate-sensitive stores, are involved in the regulation of Ca^{2+}-influx. Evidence for an involvement of cGMP in the regulation of Ca^{2+}-influx comes from experiments with chemically mutagenized streamer F mutants that lack 80% of cGMP-hydrolyzing activity and display a prolonged and elevated cGMP response after cAMP-stimulation as well as a prolonged and elevated Ca^{2+}-influx (Kuwayama et al., 2001; Menz et al., 1991). By contrast, a pdeD/pde E double knock out (KO) mutant did not show an elevated Ca^{2+}-influx but rather a slight inhibition indicating that streamer F mutants carry a different mutation (Veltman et al., 2003). Using a pdeD KO mutant and physiological concentrations of cAMP for stimulation we even found a strongly reduced Ca^{2+}-influx (Lusche and Malchow, 2005). In a parallel investigation we had planned to apply known membrane permeant cGMP-specific phosphodiesterase and cGMP-regulated phosphodiesterase inhibitors to manipulate the cellular cGMP concentration of *Dictyostelium*. We observed only slight inhibition of cGMP-hydrolysis by these drugs which seems to be due to the different class II catalytic domain and CAP cGMP-binding domain of the *Dictyostelium* PDED. However, although the type 5 phosphodiesterase inhibitors employed in this study act competitively with cGMP, we found that these compounds altered cAMP-induced Ca^{2+}-influx. Therefore, we analysed whether they regulate other cGMP-binding proteins. Bovine protein kinase G (PKG)Iα was not affected. However, the known PDE5/PDE1 inhibitors, SCH 51866 (cis-5,6a,7,8,9,9a-hexahydro-2-[4-(trifluoromethyl)phenylmethyl]-5-methyl-cyclopent[4,5]imidazo[2,1-b]purin-4(3H)-one), and sildenafil citrate (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1-H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine citrate), reversibly inhibited the cGMP-induced current of a bovine cyclic-nucleotide-gated-channel (CNGA3). This finding indicates that the Ca^{2+}-channel present in *D. discoideum* might be directly or indirectly regulated by cGMP.

2. Methods

2.1. Material

Guanosine, dimethylsulfoxid (DMSO) and dithiotreitol (DTT) were purchased from Sigma, St. Louis, (USA); 3′.5′,-cGMP, 3′,5′-cAMP, 5′AMP, 5′GMP, and adenosine were obtained from Boehringer, Mannheim (Germany). [8,5-^{3}H] cGMP was obtained from Amersham, Braunschweig and [2,8-^{3}H]cAMP from ICN, Irvine, Ca (USA). Dipyridamol (6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d] pyrimidine), MY 5445 (1-(3-chlorophenylamino)-4-phenylphthalazine), Vinpocetine (14-ethoxycabonyl-[3α,16α-ethyl)-14,15-eburnamine) and Zaprinast (1,4-dihydro-5-(2-propoxyphenyl)-7H-1,2,3,-triazolo[4,5-d]pyrimidin-7-one) were from Biomol, Hamburg (Germany) and MEQ (4-[[3,4-(methylenedioxy)benzyl]amino]-6,7,8,-trimethoxyquinazoline), Trequinsin (HL 724, 9,10-dimethoxy-2-mesitylimino-3-methyl-2,3,6,7-tetrahydro-4H-pyrimido-(6,1-a)-isoquinolin-4-one) was obtained from Calbiochem/Novabiochem, DMPPO (1,3 dimethyl-6-(2-propoxy-5-methane sulphonylamidophenyl)-pyrazolo[3,4-d]pyrimidin-4-(5H)-one) was kindly provided by H. Coste, Glaxo Wellcome (France), E 4021 (1-[6-chloro-4-(3,4-methylenedioxybenzyl)aminoquinazolin-2-yl]piperidine-4-carboxylate) by M. Takeuchi and T. Saeki, Eisai, Tsukuba-shi, Ibaraki (Japan). SCH 51866 by R. Watkins, Schering-Plough Research Inst. Kenilworth, NJ, (USA), SKF 96231 (2-(2-propoxyphenyl)-6-purinone) by S. Trowbridge, Smith Kline Beecham(UK), sildenafil citrate by E. Bischoff, Bayer AG, Wuppertal (Germany) and UK 114,542 (unpublished) by Mrs. S.D. Srodzinski, Pfizer, Sandwich, Kent (UK); PKGIα and VASPtide were kindly given by Dr. E. Butt and Prof. Dr. U. Walter, Medizinische Universitaetsklinik, Wuerzburg (Germany).

2.2. Cultivation of D. discoideum cells

Cells were grown at 23 °C in axenic medium as described (Schaloske and Malchow, 1997). Growing pdeD KO cells were supplemented with 10 μg/ml blasticidin. Differentiation was induced by washing the cells twice with

ice-cold 17 mM K^+/Na^+ phosphate buffer. Cells were incubated at a cell density of 2×10^7 cells/ml.

2.3. Light-scattering recordings of cells in suspension

Light-scattering measurements of cells were done as described by Gerisch and Hess (1974). The extinction of a cell suspension (2×10^7 cells/ml) aerated in a cuvette was monitored at 500 nm in a Zeiss PM6 spectrophotometer.

cAMP-pulses (1 μM) were applied to a cell suspension (2×10^7 cells/ml) every 6 min while monitoring light-scattering changes. Inhibitor was added after at least three control pulses 1–2 h before the onset of spike-shaped oscillations. For every pulse, the magnitude of the first light scattering change was determined.

2.4. Measurement of cAMP-induced Ca^{2+}-influx and K^+-efflux

Net Ca^{2+}- and K^+-fluxes were measured after cAMP stimulation by means of an electrode sensitive for either K^+ or Ca^{2+} in a cell suspension at 23 °C as described previously (Aeckerle et al., 1985; Bumann et al., 1984). In brief, 5×10^7 cells (Ca^{2+}-electrode) or 2.5×10^8 cells/ml (K^+-electrode) were incubated in nominally Ca^{2+} free buffer (Tricine pH 7.0, supplemented with 5 mM KCl for Ca^{2+}-flux studies). Electrode potentials were recorded with a voltmeter (Metrohm, Herisau, Switzerland). Ca^{2+}-flux was analysed 4–6 h and K^+-efflux 8–9 h after induction of starvation. cAMP was applied 5 min after the addition of SCH 51866 and subsequently cAMP pulses were given every 5 min. Inhibitors were applied in DMSO (0.1%), a concentration that neither effect basal Ca^{2+} concentration nor induced Ca^{2+}-fluxes.

2.5. Extracellular cAMP-hydrolysis assay

[2,8-^{3}H]cAMP hydrolysis was measured during spike-shaped light-scattering oscillations in the presence or absence of the inhibitor. At the maximum of a light-scattering spike 3 μCi/ml of [2,8-^{3}H]cAMP were applied to the cells and samples were taken every 30 s. Hydrolysis was stopped by the addition of a solution containing 0.1 mM EGTA, 5 mM cAMP, 5 mM 5′AMP and 5 mM adenosine and samples were kept on ice. After centrifugation at $12{,}100 \times g$ at 4 °C for 2 min the supernatant was applied to polyethyleneimine (PEI)-cellulose F (Merck, Darmstadt, Germany) sheets and chromatographed in 3.5 M LiCl. Spots were visualized under ultraviolet-illumination, excised and counted in a Beckman scintillation counter.

2.6. Preparation of cell extracts

Cells differentiated for 2 h were washed three times in ice-cold Hepes buffer (20 mM, pH 7.2) and resuspended at a density of 1.5×10^8 cells/ml. Cells were lyzed by passage through a nuclepore filter (5 μm) and collected in buffer containing 3% (w/v) sucrose, 50 mM KCl, 1 mM $MgCl_2$, 20 μg/ml leupeptin, 1 μg/ml aprotinin and 2.5 mM DTT. Extracts were handled as described previously (Schaloske et al., 1998).

2.7. Phosphodiesterase assay

The assay was modified after Thompson et al. and Gulyassy et al. (Gulyassy and Farrand, 1976; Thompson et al., 1974). The cGMP-hydrolysing activity in extracts of *D. discoideum* is due to cGMP-specific and unspecific phosphodiesterase activities. Unspecific phosphodiesterases utilize both cAMP and cGMP as substrate. Unspecific activity is reduced to 5% by preincubation of extracts with 10 mM DTT (Bulgakov and Van Haastert, 1983). The remaining cAMP-hydrolysing activity presumably corresponds to the cAMP-specific enzyme Reg A. Cytosolic extracts were preincubated for 10 min either with or without 10 mM DTT. The reaction-buffer contained 20 mM HEPES-buffer pH 7.2, 50 mM KCl, 1 mM $MgCl_2$, 500 nM cGMP and 3.3 μCi/ml [8,5-^{3}H]cGMP in a total volume of 150 μl. Reactions were started by the addition of the extract and inhibitors or DMSO as a vehicle, respectively. Extracts were diluted until cGMP-hydrolysis of the control was linear. Samples were taken in duplicate after 5 min of incubation at 25 °C. The reaction was terminated by the addition of a stop solution containing 0.1 M EDTA, 5 mM cGMP, 5 mM 5′GMP and 5 mM guanosine. Samples were subsequently treated as described under extracellular cAMP-hydrolysis assay. The running buffer was 75 mM KCl.

2.8. Measurement of cGMP-dependent protein kinase (PKG)Iα activity

Activity of bovine PKGIα was measured according to Butt et al. (1994). Mixtures contained 20 mM Tris/HCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.01% (w/v) bovine serum albumin, 50 ng PKGIα, 5 μM cGMP. Incorporation of phosphate into the specific substrate VASPtide was started by the addition of 50 μM [γ-^{32}P]ATP (100 cpm/pMol). Samples were incubated for 5 min at 30 °C. Addition of 0.3 M EDTA (pH 7.0) and application of the sample onto P-81 cellulose (Whatman) terminated the reaction. After washing with 75 mM phosphoric acid the samples were air-dried and counted. Measurements were performed either in the presence of the inhibitor or the vehicle DMSO. The enzyme activity amounted to 0.5–0.9 μmol/mg protein min.

2.9. Determination of total cGMP

Total cGMP concentrations of developing cells were determined using an enzyme immuno assay (EIA, Amersham Pharmacia, Freiburg, Germany). Roughly 4 h after the onset of starvation spike-shaped oscillations can be recorded by measuring light-scattering of cells in suspension. These

oscillations are accompanied by spike-shaped changes in intracellular cGMP concentrations. Samples were collected during light-scattering oscillations (2×10^7 cells/ml) before and after the addition of the inhibitor and quenched with $HClO_4$ (1 M final concentration). This was followed by neutralization with 3 M $KHCO_3$ (Wurster et al., 1977). Subsequently, the amount of cGMP was determined according to the manufacturer's instructions.

2.10. Patch-clamp recordings

Currents in excised patches of human embryonic kidney (HEK) 293 cells stably expressing bovine cone CNGA3 channels were recorded as described (Weyand et al., 1994). In brief, patches were exposed to a solution containing 140 mM NaCl, 10 mM Hepes, 10 mM EGTA. The same solution was used in the pipette. For channel opening and inhibitor studies the appropriate cGMP concentration was added with or without inhibitor. Leak currents in cGMP free buffer solution were subtracted.

3. Results

3.1. Effects of SCH 51866 on total cGMP concentration and on cGMP-hydrolysis

In *D. discoideum*, light-scattering oscillations in cell suspension can be measured after about 4 h of differentiation. Light-scattering oscillations mirror transient morphological changes of the cells accompanied by cAMP and cGMP oscillations (Wurster and Kurzenberger, 1989). cGMP concentrations oscillate slightly in advance of cAMP oscillations (Wurster et al., 1977). To address the question whether inhibition of cGMP-phosphodiesterases in wildtype Ax2 cells would lead to a prolonged and elevated increase of cGMP similar to the streamer F mutants, we applied a series of known PDE inhibitors. One compound, SCH 51866, a potent PDE1/PDE5 inhibitor (reference see Table 1), caused strong alterations in spike-shaped light-scattering oscillations (data not shown). Therefore, we were interested whether the cGMP concentration of the Ax2 cells was also affected. In three experiments, the change in total cGMP concentration in the presence of 300 μM SCH 51866 was $105 \pm 39\%$ of control. In cell extracts cGMP-hydrolysis was inhibited by only 17 ± 9 % using 200 μM SCH 51866 and could not be further increased by 600 μM SCH 51866. SCH 51866 specifically inhibited cGMP-hydrolysis as indicated by a specificity ratio of 2.5 (Table 1). We conclude that the inhibition of cGMP-hydrolysis was not sufficient to further raise the transient increase of cGMP concentration.

3.2. Effect of different phosphodiesterase inhibitors on cGMP-hydrolysis

Since SCH 51866 did not inhibit cGMP-hydrolysis in *D. discoideum* to a similar extent as in mammals, we attempted to identify a more potent compound. Currently there are 11 families of phosphodiesterases classified by their pharmacological and kinetic profiles, substrate specificity and cellular as well as subcellular distribution (Rascon et al., 2002 and references therein).

Table 1 summarizes the results obtained for 12 different compounds tested for their potential to inhibit cGMP-

Table 1
Effect of phosphodiesterase inhibitors on cGMP-hydrolysis in *D. discoideum*

Inhibitor	Type of mammalian PDE	IC_{50} for mammalian PDE	Concentration of inhibitor applied for *Dictyostelium* (μM)	Inhibition of *Dictyostelium* cGMP-hydrolysis (%)	Specificity ratio
E 4021	5	0.004 μM (Saeki et al., 1995)	600	35 ± 2	1.9
UK 114,542	5	0.002 μM (unpublished)	300	34 ± 3	1.4
DMPPO	5	0.003 μM (Coste and Grondin, 1995)	300	19 ± 6	0.9
SKF 96231	5	1 μM (Murray et al., 1991)	100	19 ± 3	4.8
MY 5445	5	0.6 μM (Hagiwara et al., 1984b)	100	5 ± 2	0.2
Sildenafil	5	0.0039 μM (Rascon et al., 2002)	75	28 ± 1	2.0
Zaprinast	1, 5	10–30 μM (PDE1); 0.3–1 μM (PDE5) (Silver, 1996)	600	65 ± 6	1.3
MEQ	1, 5	5.5 μM (PDE1); 0.36 μM (PDE5) (Takase et al., 1994)	300	26 ± 13	1.9
SCH 51866	1, 5, 9, 10	0.07 μM (PDE1); 0.063 μM (PDE5); 1.5 μM ; (PDE9); 1 μM (PDE10) (Hetman et al., 2000; Vemulapalli et al., 1996)	200	17 ± 9	2.5
Dipyridamol	5, 6, 8, 10	0.9 μM (PDE5) 0.38 μM (PDE6); 4.5 μM (PDE8); 1.1 μM (PDE10) (Rascon et al., 2002)	300	23 ± 5	1.6
Vinpocetine	1	21 μM (Hagiwara et al., 1984a)	300	32 ± 6	2.6
HL 724	3	250 pM (Ruppert and Weithmann, 1982)	300	54 ± 4	1.1

Inhibitors are listed according to their major target in mammalian cells. PDE5, PDE6 and PDE9 specifically hydrolyse cGMP. PDE1A, PDE1B and PDE10 also degrade cGMP, however, these enzymes accept also cAMP as their substrate. PDE2 is activated by cGMP and PDE3 is inhibited by cGMP. Both enzymes preferably hydrolyse cAMP. IC_{50} values were taken from the literature and references are indicated by parenthesis. The specificity ratio is the percentage of inhibition of cGMP-hydrolysis in Dictyostelium cell lysates in the presence and absence of DTT (see methods) at the indicated concentration. The concentration of cGMP in the assay was 500 nM. Data are means of at least three experiments ± S.D.

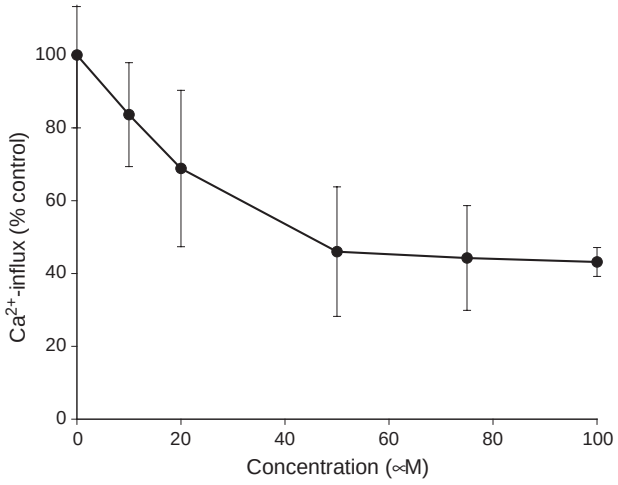

Fig. 1. Dose–response curve for SCH 51866-induced inhibition of cAMP-activated Ca^{2+}-influx Measurements were done as described in the legend of Table 2 ($n \geq 3$). Cells were stimulated with 0.1 μM cAMP after preincubation with SCH 51866 for 5 min. Half maximal inhibition occurred at 16 μM SCH 51866.

hydrolysis in *D. discoideum* extracts. Even at rather high concentrations, the inhibitors only modestly affected cGMP-hydrolysing activity.

The most potent drugs were Zaprinast, and HL 724, yet their specificity was low. Zaprinast inhibited cAMP-hydrolysis with equal potency (data not shown). Except for DMPPO, HL 724 and MY 5445, the majority of the drugs including sildenafil citrate, the most frequently used drug for PDE5 inhibition, were specific for cGMP-hydrolysis. From these experiments we conclude that the commercially available inhibitors of cGMP-specific phosphodiesterases and cGMP-regulated phosphodiesterases are poor inhibitors of *D. discoideum* cGMP-phosphodiesterases.

3.3. SCH 51866 inhibited cAMP-induced Ca^{2+}-influx

We previously reported that *D. discoideum* pdeD KO cells exhibit an elevated and prolonged cGMP transient, a delayed and a reduced Ca^{2+}-influx after cAMP stimulation (Lusche and Malchow, 2005). Therefore, we performed recordings of cAMP-induced Ca^{2+}-influx from cells in suspension in the presence of SCH 51866. SCH 51866 inhibited cAMP-induced Ca^{2+}-influx to more than 50% without altering the kinetics. Fig. 1 shows the dose–response curve for the inhibition of cAMP-induced Ca^{2+}-influx by SCH 51866. The IC_{50} for SCH 51866 was 16 μM and maximum inhibition occurred at 50 μM SCH 51866. Since SCH 51866 is a competitive PDE5 inhibitor, we hypothesized that the competition with cGMP was the reason for the reduction of cAMP-induced Ca^{2+}-influx. The possible existence of a cGMP-regulated Ca^{2+} channel was further supported by experiments done with the soluble guanylyl cyclases inhibitor LY 83583 (6-(phenylamino)-5,8-quinolinedione). This drug is also known to block cyclic-nucleotide-gated-channels in vertebrate cells (Leinders-Zufall et al., 1997). The presence of 35 μM LY 83583 reduced cAMP-induced Ca^{2+}-influx by $34 \pm 6\%$ in three experiments without altering cGMP concentration as shown in Fig. 2, demonstrating that, in *D.*

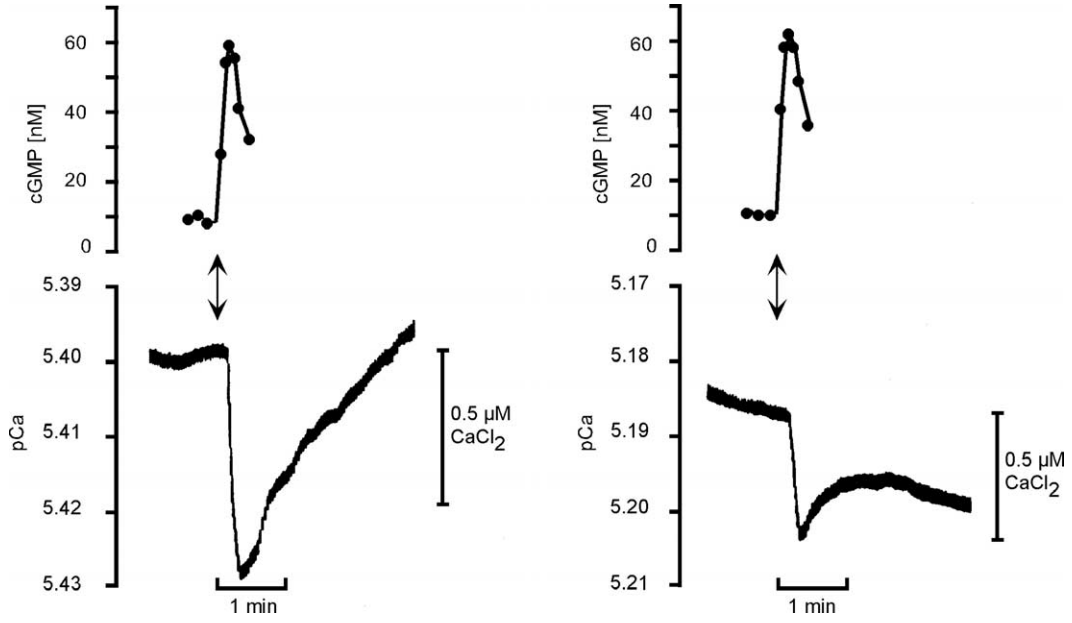

Fig. 2. LY83583 inhibited cAMP-induced Ca^{2+}-influx but did not effect transient cGMP elevations after cAMP-stimulation. One out of three experiments is shown. The addition of cAMP to a cell suspension as indicated by an arrow induced a Ca^{2+}-influx peaking after 30 s. LY83583 (right) was applied for 5 min before the cAMP-pulse was given. Samples for determination of total cGMP were taken before and after application of cAMP. The cGMP concentration increased only for $5 \pm 9\%$ compared to untreated cells. Experiments were performed and samples processed as described in the methods section ($n = 3$).

discoideum, the soluble guanylyl-cyclase is not blocked by LY 83583. The inhibitory effect of SCH 51866 was specific for the cAMP-induced Ca^{2+}-influx. We measured K^+ efflux elicited by 1 μM cAMP in the presence and absence of the inhibitor. At 100 μM SCH 51866, a concentration that maximally inhibited cAMP-induced Ca^{2+}-influx, inhibition amounted to $-2 \pm 28\%$ ($n=3, \pm$ S.D.) and at 150 μM SCH 51866 to $17 \pm 6\%$ ($n=3, \pm$ S.D.).

3.4. Effect of SCH 51866 on cAMP-induced light-scattering changes, extracellular cAMP-hydrolysis and PKGIα

Given the fact that SCH 51866 competes with cGMP for its binding site and the lack of an SCH 51866-induced cGMP increase, we wondered whether the cause for the inhibition of cAMP-induced Ca^{2+}-influx by SCH 51866 is mediated by a cGMP-binding protein.

Non-specific binding of SCH 51866 to the cAMP receptor cAR1 via its guanine base moiety was excluded as the height of the amplitude of the light-scattering responses upon addition of cAMP remained unchanged in the presence of the inhibitor. The height of the amplitude in the presence of 300 μM SCH 51866 was $99.5 \pm 2.5\%$ compared to the control amplitude in four experiments ($\pm$ S.D.). SCH 51866 therefore acts either downstream of the receptor or possibly on the extracellular phosphodiesterase.

To test for inhibition of extracellular PdsA phosphodiesterase activity by SCH 51866 we measured [2,8-^{3}H]cAMP-hydrolysis during light scattering oscillations in cell suspension. 300 μM SCH 51866 only weakly affected cAMP-hydrolysis with $18 \pm 15\%$ inhibition after 30 s and only $6 \pm 10\%$ after 60 s. It is unlikely that this inhibition accounted for the observed changes in the oscillatory

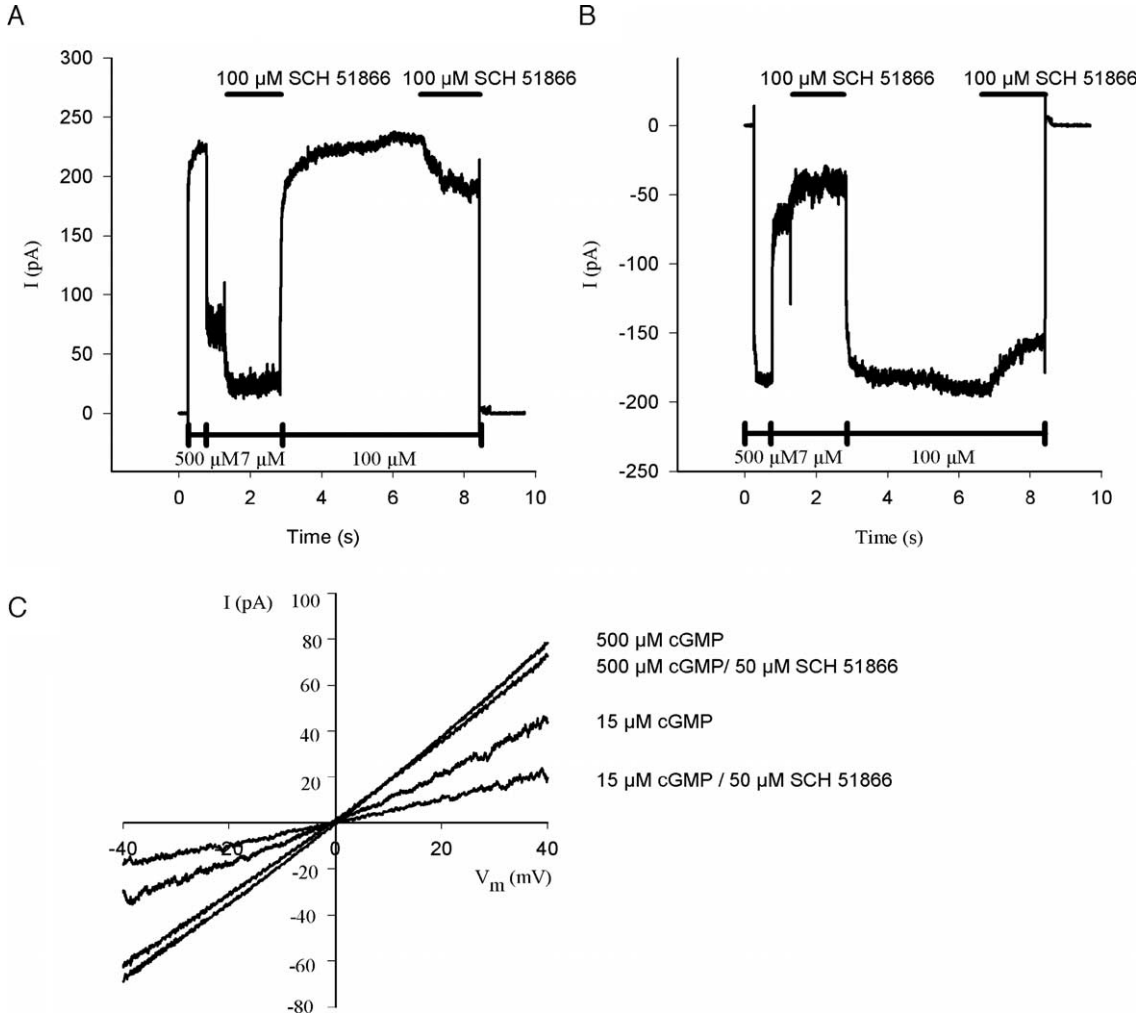

Fig. 3. Current recordings of CNGA3 stably expressed in HEK 293 cells. Sodium-currents through CNG-channels in inside-out patches were induced by the addition of cGMP. SCH 51866 (100 μM) was applied in the presence of 7 μM and 100 μM cGMP as indicated by the beams. Reversibility was tested by washing the patch with buffer solution. One out of five experiments over time is shown for a patch clamped at (A) positive and (B) negative holding potential (−40 mV), respectively. A I–V_m relation of a single cell out of three independent patches is shown in (C). Recordings for the SCH 51866 treated and untreated patch are shown.

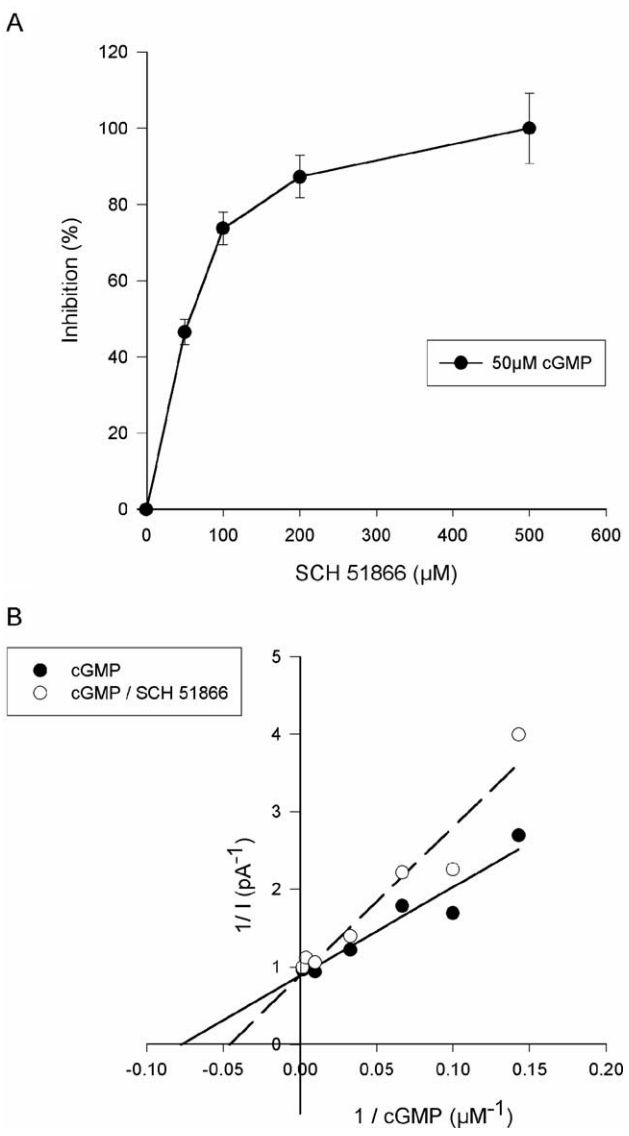

Fig. 4. SCH 51866 competitively and dose-dependently inhibited CNGA3. (A) Dose–response curve for SCH 51866-mediated inhibition of CNGA3. Ion currents were induced by application of 50 μM cGMP ($n \geq 5$). (B) Lineweaver-Burke diagram generated for currents induced by variable amounts of cGMP in the presence of 100 μM SCH 51866 at positive holding potential ($n \geq 5$). Data in the linear range of the dose–response curve were calculated using the Hill-equation (Weyand et al., 1994).

pattern, since cAMP was readily degraded within 90 s long before the next spike arose at 420 s.

We then analysed whether SCH 51866 targeted a protein kinase G (PKG). Since the activity of the partially purified *D. discoideum* PKG is very unstable (Wanner and Wurster, 1990), the activity of bovine PKGIα was measured (Butt et al., 1994). We found that there was no significant alteration of the cGMP-activated enzyme at concentrations of 75–300 μM SCH 51866. The enzyme activities in the absence and presence of SCH 51866 were 0.8 ± 0.2 vs. 0.9 ± 0.2 (μmol/mg min, control and 75 μM SCH 51866 ± S.D., respectively); 0.5 ± 0.2 vs. 0.5 ± 0.2 (μmol/mg min, control and 150 μM SCH 51866 ± S.D., respectively) and 0.5 ± 0.2, 0.6 ± 0.1 (μmol/mg min, control and 300 μM SCH 51866 ± S.D., respectively).

3.5. SCH 51866 is an inhibitor of bovine CNGA3

Another target for SCH 51866 could be a cyclic-nucleotide-gated-cation-channel. These proteins contain a cyclic nucleotide-binding site at their C-terminus. Moreover, although CNG-channels are nonselective cation channels in vitro, the currents recorded from these channels are mainly carried by Ca^{2+} in vivo (Ohyama et al., 2000). Since no CNG-channels were identified in *D. discoideum* to date, we used bovine CNGA3 stably expressed in HEK 293 cells to record cGMP-induced currents. Indeed, inhibition of the current occurred when SCH 51866 was applied to the intracellular side of excised patches in the presence of cGMP as shown in Fig. 3A,B. SCH 51866 itself was inactive in the absence of cGMP (data not shown). Without SCH 51866, channels were completely opened by excess cGMP (500 μM). 30% of the maximal current was recorded at 7 μM cGMP which is close to the k_m of 8.3 μM measured by Weyand et al. (1994). The current at 7 μM was reduced by 100 μM SCH 51866 applied to the patch. The inhibition in the presence of 100 μM SCH 51866 was stronger at positive compared to negative potentials (Fig. 3A,B). Inhibition by SCH 51866 could be reversed by the addition of cGMP in the absence of the inhibitor. Inhibition was generally stronger at a positive than at a negative potential as shown by the I–V_m relation in Fig. 3C, indicating that the charge of the SCH 51866 molecule partly contributes to the inhibitory effect on the channel. Fig. 4A shows a dose response curve for the inhibition by SCH 51866 at +40 mV. Half maximal inhibition of CNGA3 occurred at 50 μM SCH 51866. Inhibition was complete at 500 μM SCH 51866. Since increasing amounts of cGMP reduced the inhibitory effect of SCH 51866, we conclude that both compounds compete for the same binding site. We found that the activity of CNGA3 in the presence of 100 μM SCH 51866 could be completely restored by the addition of excess cGMP (data not shown). A Lineweaver-Burke plot (Fig. 4B) confirmed competition. Maximal currents remained

Table 2
Inhibition of cAMP-induced Ca^{2+}-influx by phosphodiesterase inhibitors

Inhibitor	Concentration(μM)	Inhibition of Ca^{2+}-influx(%)
SCH 51866	50	54 ± 18 (n=4)
Sildenafil	100	41 ± 6 (n=5)
UK 114,542	150	44 ± 6 (n=4)

The inhibition by different phosphodiesterase inhibitors was assessed 4–6 h after induction of development. cAMP-induced Ca^{2+}-influx was measured as described in the methods section. Pulses of 0.1 μM cAMP were applied in the presence or absence of inhibitor at the same extracellular Ca^{2+}-concentration. After preincubation with inhibitor for 5 min the first pulse of cAMP was added. Extracellular Ca^{2+} concentration ranged from 1–3 μM. The numbers of experiments ± S.D. are given in parenthesis.

unchanged whereas the k_m shifted to higher concentrations of cGMP.

3.6. Effects of sildenafil citrate and its derivative on bovine CNGA3 and cAMP-induced Ca^{2+}-influx in D. discoideum

Two of the most potent inhibitors of PDE5, sildenafil citrate and another inhibitor of cGMP-phosphodiesterase, UK 114,542, affected light-scattering oscillations similar to SCH 51866 in *D. discoideum* (data not shown). Both drugs like SCH 51866 inhibited cAMP-induced Ca^{2+}-influx in *D. discoideum* for more than 50% as shown in Table 2. This finding prompted us to analyse the effects of these compounds on CNGA3. Sildenafil also proved to inhibit CNGA3, as shown in Fig. 5. Channel activity was reduced by 58 ± 14% in three independent experiments with 30 μM sildenafil at +40 mV and by 20 ± 1% at 20 μM in two experiments. Inhibition could be reversed by replacing the bathing solution containing the inhibitor with a mock solution. At −40 mV sildenafil was less potent (Fig. 5). The derivative of sildenafil, UK 114,542 at 100 μM, did not inhibit cGMP-induced currents (data not shown), indicating structural requirements for the inhibition of CNGA3 by SCH 51866 and sildenafil. These data reveal that the competitive phosphodiesterase inhibitors SCH 51866 and sildenafil are novel inhibitors of CNGA3.

3.7. The antagonism of SCH 51866 on cAMP-induced Ca^{2+}-influx is reversed by cGMP

In a pdeD KO strain cGMP concentrations are higher than in the parental strain and the cGMP transients are prolonged (Meima et al., 2002). Receptor-mediated Ca^{2+}-influx, however, was delayed and smaller than in the wildtype (Lusche and Malchow, 2005). We used this mutant strain to investigate whether the elevated cGMP concentration acts either in concert or competes with SCH 51866 for a regulatory site of Ca^{2+}-influx. Table 3 shows that 25 μM SCH 51866, that blocks about 40% of Ca^{2+}-influx in the wildtype and is a concentration within the linear range of the dose response curve, did not inhibit Ca^{2+}-influx in the mutant in contrast to the wildtype. In the presence of the drugs the mean influx was 116 ± 35% (n=4, ± S.D.). We infer from this experiment that SCH 51866 and cGMP do not act independently but rather seem to compete for the same site.

Table 3
Lack of inhibition of cAMP-induced Ca^{2+}-influx by SCH 51866 in PDED-KO cells

Experiment	Ca^{2+}-influx(pmol/10^7 cells)		Inhibition (%)
	Control	SCH 51866	
I	25 ± 3	41 ± 7	−64
II	45 ± 4	61 ± 3	−35
III	22 ± 1	16 ± 2	27
IV	32 ± 1	30 ± 4	7

Inhibition of receptor-mediated Ca^{2+}-influx by SCH 51866 was absent in PDED-KO cells. Ca^{2+}-influx was measured as described for wt cells (see legend of Table 2 and methods for details). Four independent experiments are shown. Compared to the control the mean influx amounted to 116 ± 35% (n=4).

4. Discussion

We demonstrated that two potent cGMP-phosphodiesterases antagonists are also inhibitors of bovine cone CNGA3. SCH 51866 as well as sildenafil competed with the physiological ligand cGMP for regulation of CNGA3 in excised patches of HEK 293 cells, albeit the concentration was approximately 3000 times higher for sildenafil than required for inhibition of mammalian PDE5 (Rascon et al., 2002).

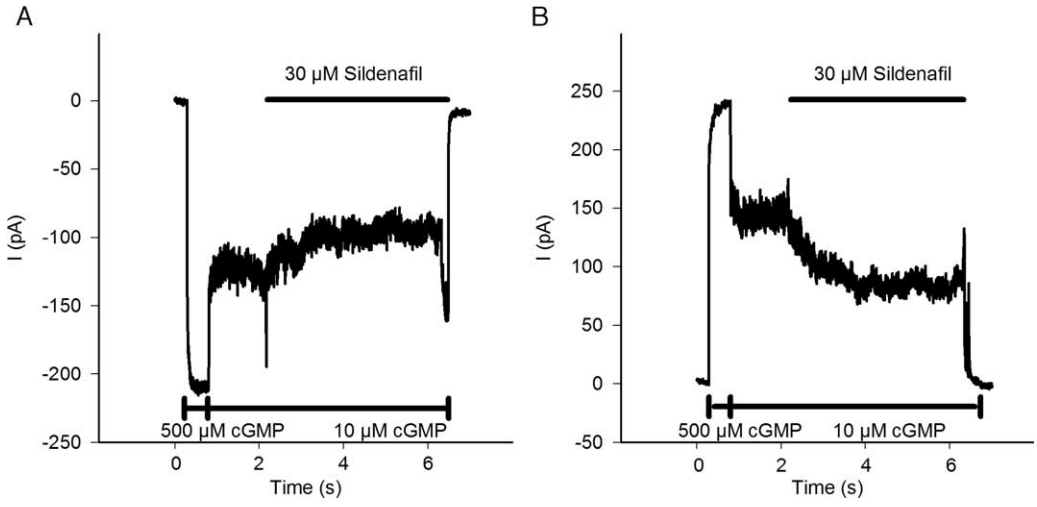

Fig. 5. Sildenafil-induced inhibition of CNGA3. Sildenafil (30 μM) was applied to the bathing solution in the presence of 10 μM cGMP. One out of three experiments at negative (A) as well as at positive (B) holding potential is shown.

For scrutinizing signalling pathways involving cyclic-nucleotide-gated-channels (CNG-channels) there is a need for specific drugs that alter channel activity. Inhibitors have been described for CNG-channels (Kleene, 1994; Leinders-Zufall et al., 1997), yet none of them was found to act competitively. The most widely used inhibitor, L-*cis*-diltiazem, is a pH, voltage and subtype specific CNG-channel blocker acting at the cytoplasmic site of the channel (Lee et al., 2001) and references therein]. Polyamines were also reported to block CNG-channels but competition with the ligand was not tested for (Lu and Ding, 1999). SCH 51866 and sildenafil inhibited CNGA3 at micromolar concentrations. However, the drugs might also block other CNG-channel subtypes more potently.

Although the phosphodiesterase inhibitors share some structural similarities, the drugs do not generally inhibit CNG-channels. Rather, the inhibition required structural prerequisites in addition to the common guanine base moiety. Thus UK 114,542 did not inhibit CNGA3 at all. The specificity of CNG-channel inhibition by sildenafil and SCH 51866 with respect to other phosphodiesterase inhibitors is supported by Womack et al. (2000) who did not find inhibition of inositol 4,5-bisphosphate and ATP regulated currents through rod CNG-channels by Zaprinast and IBMX in oocyte patches.

Inhibition of phosphodiesterases has been subject to intensive investigations. The design of specific compounds for potential clinical treatments continues to be necessary because of the variety of phosphodiesterases present in various tissues. Albeit the tested inhibitors specifically and effectively inhibit mammalian PDEs, they failed to potently reduce cGMP-hydrolysis in *D. discoideum*. Either much higher concentrations were required and/or the specificity was low. Taken together, we conclude that the cGMP-activated cGMP-phosphodiesterase activity in *D. discoideum* extracts represent unusual enzymes that are pharmacologically different to their mammalian counterparts. Recently genes corresponding to the majority of the cGMP-hydrolysis activity have been cloned (Bosgraaf et al., 2002; Meima et al., 2002). cGMP-hydrolysis and cGMP-binding has been shown. Their detailed biochemical characterisation still remains to be investigated. However, PDED and PDEE are phosphodiesterases with a Zn^{2+}-binding domain. Both of them might be targeted by Zaprinast, a putative Zn^{2+} chelator, and the most potent inhibitor in this study. The lack of inhibition by the available mammalian PDE inhibitors could be due to structural differences in the cGMP-binding sites. In fact, mammalian enzymes exhibit a class I catalytic domain, whereas the catalytic domains of the main Dictyostelium enzymes belong to class II. Only *D. discoideum* PDE3 shows considerable sequence homology to mammalian PDEs within this domain.

Although SCH 51866 caused slight but specific inhibition of cGMP-hydrolysis, there was no additional increase of the cGMP concentration evident during spike-shaped oscillations. An increase in the cGMP concentration was therefore not the cause for the observed SCH 51866-induced changes in light-scattering oscillations. This lack of increase in total cGMP is in contrast to mammalian cells, where small amounts of the PDE5 inhibitors elevate intracellular cGMP-concentrations in a variety of tissues. For instance, SCH 51866 competitively inhibited PDE1 and PDE5 leading to an increase in intracellular cGMP-concentration in blood platelets. Thus, SCH 51866 prevented collagen-induced platelet aggregation and proliferation of vascular smooth muscle cells in vitro (Vemulapalli et al., 1996). Sildenafil inhibited PDE5 in smooth muscle of the corpus cavernosum leading to an NO-dependent increase in cGMP, muscle relaxation and subsequent increase in blood flow resulting in penile erection (Maggi et al., 2000). Nevertheless, the competitive nature of these drugs with respect to cGMP led us to presume that in *D. discoideum* the putative target (s) responsible for the SCH 51866-induced alterations of the light-scattering spikes might contain a cGMP-binding site.

We then found that PDE inhibitors can alter Ca^{2+}-homeostasis in *D. discoideum*. SCH 51866 and also sildenafil strongly inhibited cAMP-induced Ca^{2+}-influx. This implies the presence of a cGMP-regulated step in the signalling cascade leading to Ca^{2+}-influx. In excised patches of *D. discoideum* ion channels could not be observed reproducibly due to the difficulties in patch formation (Müller et al., 1986). Experimental access to a possible cGMP-binding site of plasma membrane channels is therefore restricted.

As known from other organisms it is possible that cGMP acts either directly or indirectly at a plasma membrane channel or at Ca^{2+}-stores. Indirect effects might be mediated by PKG. PKG mediated regulation of L-type calcium channels and modulation of inositol 1,4,5-trisphosphate-induced Ca^{2+}-release were reported (Biancani, 1998; Jiang et al., 2000). Given the very small activity changes of bovine PKGIα, we conclude that SCH 51866 less likely affects *D. discoideum* PKG. However, the participation of *D. discoideum* PKG cannot be excluded as the specificity of this enzyme may differ from the mammalian PKG.

In vivo, Ca^{2+} carries considerable fractions of currents through CNG channels in rods and cones (Ohyama et al., 2000). A similar type of Ca^{2+}-permeable channel regulated by cGMP and targeted by SCH 51866 and sildenafil might exist in *D. discoideum*. In favour of this hypothesis we have shown that LY 83583 also inhibited cAMP-induced Ca^{2+}-influx in *D. discoideum* in the absence of inhibition of guanylyl-cyclase activity. LY 83583 was shown to inhibit CNG-channels directly in salamander olfactory receptor neurons with a K_d of 1.4 μM, presumably by acting inside the channel pore (Leinders-Zufall et al., 1997). A regulation of Ca^{2+}-influx by cGMP is in agreement with the time course of both responses to cAMP. The cGMP rise is maximal after 10 s while Ca^{2+}-influx peaks 30 s after cAMP

stimulation. In the pdeD KO strain the cGMP rise was maximal after 50–90 s and Ca^{2+}-influx was delayed for 30–90 s and reduced. Here we found that SCH 51866 and sildenafil inhibited receptor-mediated Ca^{2+}-influx in the parent strain, but had no effect on the kinetics of the influx. Since these drugs act competitively with cGMP but, on the other hand, are not identical with cGMP, they might not interfere with all targets of cGMP.

Since SCH 51866 did not affect K^+-fluxes, this also argues against an unspecific binding of the drug to cAR1 that should prevent cAMP-induced Ca^{2+}-influx. Inhibition of extracellular cAMP-hydrolysis and therefore possible inhibitory cAMP accumulation did also not occur in the presence of SCH 51866. With respect to the finding that SCH 51866 and sildenafil inhibited CNGA3 and that inhibition could be reversed by excess cGMP we propose that cGMP participates in the regulation of Ca^{2+}-influx at the plasma membrane. In support of this we found that inhibition of Ca^{2+}-influx by SCH 51866 in the wildtype was absent in a mutant with elevated cGMP concentrations, indicating that cGMP competes with SCH 51866 for a regulatory site also in *D. discoideum*. Thus, elevated cGMP concentrations seem to have two effects on the Ca^{2+}-channel activity: it regulates the onset and magnitude of Ca^{2+}-influx. The magnitude of influx may be regulated either directly or indirectly at the channel because the PDE antagonists inhibited the current through bovine Ca^{2+}-permeable channels as well as Ca^{2+}-influx in *D. discoideum*. However, the PDE antagonists did not interfere with the onset of Ca^{2+}-influx as did elevated cGMP concentrations in the mutant. This action of cGMP may be due to a more complete binding at the channel site or by binding to another target, for instance, a PKG.

Although Sildenafil and SCH 51866 are antagonists of cGMP for the CNG-channel they seem to be agonists of cGMP for inhibition of receptor-mediated Ca^{2+}-influx in *D. discoideum*. Clearly, the structural features of both cGMP-binding sites are distinct: (1) SCH 51866 is inactive in the absence of cGMP at the CNG channel. (2) UK 114,542 does not interfere with CNG-channel activity whereas both drugs inhibited receptor-mediated Ca^{2+}-influx.

Although the IC_{50} for inhibition of cGMP-induced currents of the bovine photoreceptor channel by SCH 51866 and sildenafil are in the micromolar range, small amounts of SCH 51866 and sildenafil at the target site might already interfere with CNG-dependent pathways in vivo. Vemulapalli et al. reported an increase of SCH 51866 concentration of up to 5 μM after 2 h in blood platelets after injection of 10 mg SCH 51866/kg in rats (Vemulapalli et al., 1996). Sildenafil is reported to cause headache and facial flushing (Cheitlin et al., 1999). It was also shown to affect vision (Laties and Zrenner, 2002). Sildenafil is readily taken up and distributed in the tissue. Plasma levels account to 40% bioavailability and are enhanced in patients older than 65 years and in patients with hepatic impairment. According to this and another report high levels of sildenafil are bound to plasma proteins (Nichols et al., 2002) and the free plasma concentration calculated to 43 nM is low. However, the bound sildenafil makes up a large reservoir and might also bind to proteins within the membrane. It will also be interesting to elucidate the potency and selectivity of these drugs on different subtypes of CNG-channels as well as on native channels and to perceive the consequences for patients following the application of these drugs. The results of this study should also encourage the search for derivatives of SCH 51866 and sildenafil that might act more potently on CNGA3 and presumably on other CNG-channel subtypes.

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References

Aeckerle, S., Wurster, B., Malchow, D., 1985. Oscillations and cyclic AMP induced changes of the K^+ concentration in *Dictyostelium discoideum*. EMBO J. 4, 39–43.

Biancani, P., 1998. Diversity of Ca^{2+}-mobilizing mechanisms focus on "cGMP-mediated Ca^{2+}-release from IP3-insensitive Ca^{2+} stores in smooth muscle". Am. J. Physiol. 274, C1196–C1198.

Bosgraaf, L., Van Haastert, P.J., 2002. A model for cGMP signal transduction in *Dictyostelium* in perspective of 25 years of cGMP research. J. Muscle Res. Cell Motil. 23, 781–791.

Bosgraaf, L., Russcher, H., Snippe, H., Bader, S., Wind, J., Van Haastert, P.J., 2002. Identification and characterization of two unusual cGMP-stimulated phosphodiesterases in *Dictyostelium*. Mol. Biol. Cell 13, 3878–3889.

Brown, J.M., Firtel, R.A., 1999. Regulation of cell-fate determination in *Dictyostelium*. Dev. Biol. 216, 426–441.

Brzostowski, J.A., Kimmel, A.R., 2001. Signaling at zero G: G-protein-independent functions for 7-TM receptors. Trends Biochem. Sci. 26, 291–297.

Bulgakov, R., Van Haastert, P.J.M., 1983. Isolation and partial characterization of a cyclic GMP-dependent cyclic GMP-specific phosphodiesterase from *Dictyostelium discoideum*. Biochim. Biophys. Acta 756, 56–66.

Bumann, J., Wurster, B., Malchow, D., 1984. Attractant-induced changes and oscillations of the extracellular Ca^{2+} concentration in suspensions of differentiating *Dictyostelium* cells. J. Cell Biol. 98, 173–178.

Butt, E., Abel, K., Krieger, M., Palm, D., Hoppe, V., Hoppe, J., Walter, U., 1994. cAMP-and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator -stimulated phosphoprotein (VASP) in vitro and intact human platelets. J. Biol. Chem. 269, 14509–14517.

Cheitlin, M.D., Hutter Jr., A.M., Brindis, R.G., Ganz, P., Kaul, S., Russell Jr., R.O., Zusman, R.M., 1999. Use of sildenafil (Viagra) in patients with cardiovascular disease. Technology and Practice Executive Committee. Circulation 99, 168–177.

Coste, H., Grondin, P., 1995. Characterization of a novel potent and specific inhibitor of type V phosphodiesterase. Biochem. Pharmacol. 50, 1577–1585.

Dormann, D., Kim, J.Y., Devreotes, P.N., Weijer, C.J., 2001. cAMP receptor affinity controls wave dynamics, geometry and morphogenesis in *Dictyostelium*. J. Cell. Sci. 114, 2513–2523.

Franke, J., Kessin, R.H., 1992. The cyclic nucleotide phosphodiesterases of *Dictyostelium discoideum*: Molecular genetics and biochemistry. Cell. Signal. 4, 471–478.

Gerisch, G., Hess, B., 1974. Cyclic AMP -controlled oscillations in suspended *Dictyostelium* cells: their relation to morphogenetic cell interaction. Proc. Natl. Acad. Sci. U. S. A. 71, 2118–2122.

Gulyassy, P.F., Farrand, J.R., 1976. Comparison of batch and chromatographic assays of cyclic nucleotide phosphodiesterases. J. Chromatogr. 129, 107–113.

Hagiwara, M., Endo, T., Hidaka, H., 1984a. Effects of vinpocetine on cyclic nucleotide metabolism in vascular smooth muscle. Biochem. Pharmacol. 33, 453–457.

Hagiwara, M., Endo, T., Kanayama, T., Hidaka, H., 1984b. Effect of 1-(3-chloroanilino)-4-phenylphthalazine (MY 5445), a specific inhibitor of cyclic GMP phosphodiesterase, on human platelet aggregation. J. Pharmacol. Exp. Ther. 228, 467–471.

Hetman, J.M., Soderling, S.H., Glavas, N.A., Beavo, J.A., 2000. Cloning an characterization of PDE7B, a cAMP-specific phosphodiesterase. Proc. Natl. Acad. Sci. U. S. A. 97, 472–476.

Jiang, L.H., Gawler, D.J., Hodson, N., Milligan, C.J., Pearson, H.A., Porter, V., Wray, D., 2000. Regulation of cloned cardiac L-type calcium channel by cGMP-dependent protein kinase. J. Biochem. 275, 6135–6143.

Kessen, U., Schaloske, R., Aichem, A., Mutzel, R., 1999. Ca^{2+}/Calmodulin-independent activation of calcineurin from *Dictyostelium* by unsaturated long chain fatty acids. J. Biol. Chem. 274, 37821–37826.

Kleene, S.J., 1994. Inhibition of olfactory cyclic nucleotide-activated current by calmodulin antagonists. Br. J. Pharmacol. 111, 469–472.

Kuwayama, H., Snippe, H., Derks, M., Roelofs, J., Van Haastert, P.J.M., 2001. Identification and characterization of DdPDE3, a cGMP-selective phosphodiesterase from *Dictyostelium*. J. Biochem. 353, 635–644.

Laties, A., Zrenner, E., 2002. Viagra (sildenafil citrate) and ophthalmology. Prog. Retin. Eye Res. 21, 485–506.

Lee, H.M., Park, Y.S., Kim, W., Park, C.S., 2001. Elektrophysiological characteristics of rat gustatory cyclic nucleotide-gated channel expressed in *Xenopus* oocytes. J. Neurophysiol. 85, 2335–2349.

Leinders-Zufall, T., Rand, M.N., Shepherd, G.M., Greer, C.A., Zufall, F., 1997. Calcium entry through cyclic nucleotide-gated channels in individual cilia of olfactory receptor cells: spatiotemporal dynamics. J. Neurosci. 17, 4136–4148.

Lu, Z., Ding, L., 1999. Blockage of retinal cGMP-gated channel by polyamines. J. Gen. Physiol. 13, 35–43.

Lusche, D.F., Malchow, D., 2005. Developmental control of cAMP-induced Ca(2+)-influx by cGMP: influx is delayed and reduced in a cGMP-phosphodiesterase D deficient mutant of *Dictyostelium discoideum*. Cell Calcium 37, 57–67.

Maggi, M., Filippi, S., Ledda, F., Magini, A., Forti, G., 2000. Erectile dysfunction: from biochemical pharmacology to advances in medical therapy. Eur. J. Endocrinol. 143, 143–154.

Meili, R., Firtel, R.A., 2000. *Dictyostelium*: a model for regulated cell movement during morphogenesis. Curr. Opin. Genet. Dev. 10, 421–427.

Meima, M.E., Biondi, R.M., Schaap, P., 2002. Identification of a novel type of cGMP phosphodiesterase that is defective in the chemotactic stmF mutants. Mol. Biol. Cell 13, 3870–3877.

Menz, S., Bumann, J., Jaworski, E., Malchow, D., 1991. Mutant analysis suggest that cyclic GMP mediates the cyclic AMP-induced Ca^{2+} uptake in *Dictyostelium*. J. Cell. Sci. 99, 187–191.

Müller, U., Malchow, D., Hartung, K., 1986. Single ion channels in the slime mold *Dictyostelium discoideum*. Biochim. Biophys. Acta 857, 287–290.

Murray, K.J., Eden, R.J., England, P.J., Dolan, J., Grimsditch, D.C., Stutchbury, C.A., Patel, B., Reeves, M.L., Worby, A., Torphy, T.J., 1991. Potential use of selective phosphodiesterase inhibitors in the treatment of asthma. Agents Actions Suppl. 34, 27–46.

Newell, P.C., Malchow, D., Gross, J.D., 1995. The role of calcium in aggregation and development of *Dictyostelium*. Experientia 51, 1155–1165.

Nichols, D.J., Muirhead, G.J., Harness, J.A., 2002. Pharmacokinetics of sildenafil after single oral doses in healthy male subjects: absolute bioavailability, food effects and dose proportionality. Br. J. Clin. Pharmacol. 53, 5S–12S.

Ohyama, T., Hackos, H.D., Frings, S., Hagen, V., Kaupp, U.B., Korenbrot, J.I., 2000. Fraction of the dark current carried by Ca^{2+} through cGMP-gated ion channels of intact rod and cone photoreceptors. J. Gen. Physiol. 116, 735–753.

Rascon, A., Soderling, S.H., Schaefer, J.B., Beavo, J.A., 2002. Cloning and characterization of a cAMP-specific phosphodiesterase (TbPDE2B) from *Trypanosoma brucei*. Proc. Natl. Acad. Sci. U. S. A. 99, 4714–4719.

Roelofs, J., Van Haastert, P.J.M., 2002. Characterization of two unusual guanylyl cyclases from *Dictyostelium*. J. Biol. Chem. 277, 9167–9174.

Roelofs, J., Snippe, H., Kleineidam, R.G., Van Haastert, P.J.M., 2001. Guanylate cyclase in *Dictyostelium discoideum* with the topology of mammalian adenylate cyclase. Biochem. J. 354, 697–706.

Ruppert, D., Weithmann, K.U., 1982. HL 725, an extremely potent inhibitor of platelet phosphodiesterase and induced platelet aggregation in vitro. Life Sci. 31, 2037–2043.

Saeki, T., Adachi, H., Takase, Y., Yaoshitake, S., Souda, S., Saito, I., 1995. A selective type V phosphodiesterase inhibitor, E 4021, dilates porcine large coronary artery. J. Pharmacol. Exp. Ther. 272, 825–831.

Saran, S., Meima, M.E., Alvarez-Curto, E., Weening, K.E., Rozen, D.E., Schaap, P., 2002. cAMP signaling in *Dictyostelium*. Complexity of cAMP synthesis, degradation and detection. J. Muscle Res. Cell Motil. 23, 793–802.

Schaloske, R., Malchow, D., 1997. Mechanism of cAMP-induced Ca^{2+}-influx:role of phospholipase A2. J. Biochem. 327, 233–238.

Schaloske, R., Sordano, C., Bozzaro, S., Malchow, D., 1995. Stimulation of calcium influx by platelet activating factor in *Dictyostelium*. J. Cell. Sci. 108, 1597–1603.

Schaloske, R., Sonnemann, J., Malchow, D., Schlatterer, C., 1998. Fatty acids induce release of Ca^{2+} from acidosomal stores and activate capacitative Ca^{2+} entry in *Dictyostelium discoideum*. J. Biochem. 322, 541–548.

Schaloske, R., Schlatterer, C., Malchow, D., 2000. A Xestospongin C-sensitive Ca^{2+} store is required for cAMP-induced Ca^{2+} influx and cAMP oscillations in *Dictyostelium*. J. Biol. Chem. 275, 8404–8408.

Schlatterer, C., Happle, K., Lusche, D.F., Sonnemann, J., 2004. Cytosolic [Ca^{2+}]-transients in *Dictyostelium discoideum* depend on the filling state of internal stores and on an active SERCA Ca^{2+}-pump. J. Biol. Chem. 279, 18407–18414.

Silver, P.J., 1996. Inhibition of phosphodiesterase Isoenzymes and cell function by selective PDE 5 inhibitors. In: Schudt, C., Dent, G., Rabe, K.F. (Eds.), Phosphodiesterase Inhibitors. Academic Press, pp. 127–134.

Sonnemann, J., Aichem, A., Schlatterer, C., 1998. Dissection of the cAMP induced cytosolic calcium response in *Dictyostelium discoideum*: the role of cAMP receptor subtypes and G protein subunits. FEBS Lett. 436, 271–276.

Takase, Y., Saeki, T., Watanabe, N., Adachi, H., Souda, S., Saito, I., 1994. Cyclic GMP phosphodiesterase inhibitors: 2. Requirement of 6-substitution of quinazoline derivatives for potent and selective inhibitory activity. J. Med. Chem. 37, 2106–2111.

Thompson, W.J., Brooker, G., Appelman, M.M., 1974. Assay of cyclic nucleotide phosphodiesterases with radioactive substrates. Methods Enzymol. 38, 205–212.

Van Haastert, P.J.M., Kuwayama, H., 1997. cGMP a second messenger during *Dictyostelium* chemotaxsis. FEBS Lett. 410, 25–28.

Van Haastert, P.J.M., Van Lookeren Campagne, M.M., Kesbeke, F., 1983. Multiple degradation pathways of chemoattractant mediated cyclic

GMP accumulation in *Dictyostelium*. Biochim. Biophys. Acta 756, 67–71.

Veltman, D.M., De Boer, J.S., Van Haastert, P.J., 2003. Chemoattractant-stimulated calcium influx in *Dictyostelium discoideum* does not depend on cGMP. Biochim. Biophys. Acta 1623, 129–134.

Vemulapalli, S., Watkins, R.W., Chintala, M., Davis, H., Ahn, H.S., Fawzi, A., Tulshian, D., Chiu, P., Chatterjee, M., Lin, C.C., Sybertz, E.J., 1996. Antiplatelet and antiproliferative effects of SCH 51866, a novel Type1 and type 5 phosphodiesterase inhibitor. J. Cardiovasc. Pharmacol. 28, 862–869.

Wanner, R., Wurster, B., 1990. Cyclic GMP-activated protein kinase from *Dictyostelium discoideum*. Biochim. Biophys. Acta 1053, 179–184.

Weyand, I., Godde, M., Frings, S., Weiner, J., Müller, F., Altenhofen, W., Hatt, H., Kaupp, U.B., 1994. Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm. Nature 368, 859–863.

Womack, K.B., Gordon, S.E., Feng, H., Wensel, T.G., Lu, C.C., Hilgemann, D.W., 2000. Do phosphatidylinostides modulate vertebrate phototransduction? J. Neurosci. 20, 2792–2799.

Wurster, B., Kurzenberger, W., 1989. Periodic cell aggregation in suspensions of *Dictyostelium discoideum*. Differentiation 41, 1–4.

Wurster, B., Schubiger, K., Wick, U., Gerisch, G., 1977. Cyclic GMP in *Dictyostelium*. Oscillations and pulses in response to folic acid and cyclic AMP. FEBS Lett. 76, 141–144.