Biochem. J. (1991) 279, 521–527 (Printed in Great Britain)

Characterization of Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate (Sp-5,6-DCl-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells

Mårten SANDBERG,*†‡ Elke BUTT,*§‖ Christine NOLTE,* Lilo FISCHER,* Maria HALBRÜGGE,* Jerlyn BELTMAN,‖, Tore JAHNSEN,†‡ Hans-Gottfried GENIESER,§ Bernd JASTORFF§ and Ulrich WALTER*¶

*Medizinische Universitätsklinik, Klinische Forschergruppe, Josef-Schneider-Strasse 2, W-8700 Würzburg, Federal Republic of Germany, †Laboratory for Gene Technology, Institute of Pathology, Rikshospitalet, University of Oslo, N-0027 Oslo, Norway, ‡Institute of Medical Biochemistry, University of Oslo, P.O. Box 1112, N-0317 Oslo, Norway, §Fachbereich Biologie/Chemie, Institut für Organische Chemie, Abteilung Bioorganische Chemie, Universität Bremen, W-2800 Bremen 33, Federal Republic of Germany, and ‖Department of Pharmacology, University of Washington, Seattle, WA 98195, U.S.A.

A newly designed cyclic AMP (cAMP) analogue, Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate (Sp-5,6-DCl-cBiMPS), and 8-(*p*-chlorophenylthio)-cAMP (8-pCPT-cAMP) were compared with respect to their chemical and biological properties in order to assess their potential as activators of the cAMP-dependent protein kinases (cAMP-PK) in intact cells. Sp-5,6-DCl-cBiMPS was shown to be both a potent and specific activator of purified cAMP-PK and of cAMP-PK in platelet membranes, whereas 8-pCPT-cAMP proved to be a potent activator of cAMP-PK and cyclic-GMP-dependent protein kinase (cGMP-PK) both as purified enzymes and in platelet membranes. Sp-5,6-DCl-cBiMPS was not significantly hydrolysed by three types of cyclic nucleotide phosphodiesterases, whereas 8-pCPT-cAMP (and 8-bromo-cAMP) was hydrolysed to a significant extent by the Ca^{2+}/calmodulin-dependent phosphodiesterase and by the cGMP-inhibited phosphodiesterase. The apparent lipophilicity, a measure of potential cell-membrane permeability, of Sp-5,6-DCl-cBiMPS was higher than that of 8-pCPT-cAMP. Extracellular application of Sp-5,6-DCl-cBiMPS to intact human platelets reproduced the pattern of protein phosphorylation induced by prostaglandin E_1, a cAMP-increasing inhibitor of platelet activation. In intact platelets, Sp-5,6-DCl-cBiMPS was also more effective than 8-pCPT-cAMP in inducing quantitative phosphorylation of the 46/50 kDa vasodilator-stimulated phosphoprotein (VASP), a major substrate of cAMP-PK in platelets. As observed with prostaglandin E_1, pretreatment of human platelets with Sp-5,6-DCl-cBiMPS prevented the aggregation induced by thrombin. The results suggest that Sp-5,6-DCl-cBiMPS is a very potent and specific activator of cAMP-PK in cell extracts and intact cells and, in this respect, is superior to any other cAMP analogue used for intact-cell studies. In contrast with 8-pCPT-cAMP, Sp-5,6-DCl-cBiMPS can be used to distinguish the signal-transduction pathways mediated by cAMP-PK and cGMP-PK.

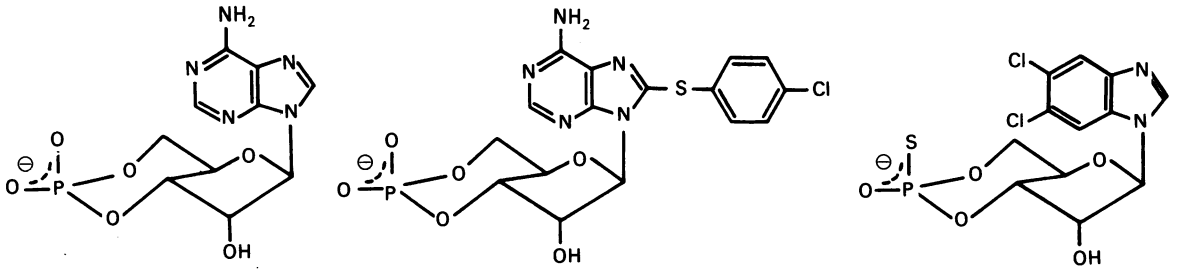
INTRODUCTION

Many cellular events, including intermediary metabolism, motility, ion-channel opening and gene expression, are regulated by the intracellular messengers cyclic AMP (cAMP) and cAMP-dependent protein kinases (cAMP-PK) in eukaryotic cells [1–6]. Cell-membrane-permeant analogues of cAMP are widely used to evaluate whether a given biological response of certain hormones, drugs and factors is mediated by cAMP and its major, if not only, intracellular target protein, the cAMP-PK [1,5]. It is generally assumed that cAMP and cAMP-PK are involved in mediating an investigated biological response if the response can be reproduced by extracellular application of cell-membrane-permeant cAMP analogues to intact cells or tissues. If this assumption is correct, a useful cAMP analogue has to fulfil several criteria [5], such as: (*a*) high lipophilicity, in order to penetrate the cell membrane and to reach an intracellular concentration sufficient to activate the cAMP-PK; (*b*) high specificity for cAMP-PK, to avoid effects on other protein kinases and regulatory proteins [i.e. ion channels and phosphodiesterases (PDEs)]; (*c*) resistance to hydrolysis by PDEs to avoid the accumulation, and possible side effects, of analogue metabolites; (*d*) non-inhibition of PDE catalytic sites to avoid an increase in endogenous cAMP or cyclic GMP (cGMP), with subsequent stimulation of cAMP-PK or cGMP-dependent protein kinase (cGMP-PK); and (*e*) specific and quantitative activation of cAMP-PK-mediated protein phosphorylation in intact cells.

However, despite the widespread use of cAMP analogues, very few studies have rigorously analysed the specificity and quantitative effects of cAMP analogues in intact cells. In fact, we demonstrate that most cAMP analogues currently used with intact cells do not fulfil the criteria (lipophilicity, specificity, hydrolysis-resistance, quantitative effects on cAMP-PK-

Abbreviations used: cAMP, cyclic AMP; cGMP, cyclic GMP; cAMP-PK, cAMP-dependent protein kinase; cGMP-PK, cGMP-dependent protein kinase; PG-E_1, prostaglandin E_1; PG-I_2, prostaglandin I_2 (prostacyclin); VASP, vasodilator-stimulated protein; 8-Br-cAMP, 8-bromo-cAMP; 8-pCPT-cAMP, 8-(*p*-chlorophenylthio)-cAMP; Sp-5,6-DCl-cBiMPS, Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate; Sp-cAMPS, phosphorothioate stereoisomer of cAMP; Sp-cGMPS, phosphorothioate stereoisomer of cGMP; Bt_2-cAMP, $N^6O^{2'}$-dibutyryl cAMP; PDE, phosphodiesterase; cGI-PDE, cGMP-inhibited PDE; cGS-PDE, cGMP-stimulated PDE; CaM-PDE, Ca^{2+}/calmodulin-dependent PDE.

¶ To whom correspondence should be addressed.



cAMP 8-pCPT-cAMP Sp-5,6-DCl-cBiMPS

Fig. 1. Structures of cAMP and analogues used

mediated protein phosphorylation in intact cells) mentioned above.

We now report that a new cAMP analogue, Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3',5'-monophosphorothioate (Sp-5,6-DCl-cBiMPS), is a useful and specific activator of cAMP-PK in cell extracts and intact-cell preparations. The riboside of Sp-5,6-DCl-cBiMPS, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole ('DRB'), had been used as an inhibitor of RNA synthesis [7]. Our phosphorylation studies were made possible by recent progress in our laboratory with respect to the quantitative analysis of cyclic-nucleotide-dependent protein phosphorylation in intact human platelets [8–11].

Inhibition of the activation and aggregation of human platelets by cAMP-elevating prostaglandins such as prostacyclin (PG-I_2) and prostaglandin E_1 (PG-E_1) is believed to be mediated by cAMP-PK [12–14]. One of the major cAMP-PK substrates in intact platelets, the 46/50 kDa protein termed vasodilator-stimulated phosphoprotein (VASP), was recently characterized and purified [9,10]. Subsequently, an immunological method was developed for the quantitative analysis of the stoichiometric and reversible cAMP-PK-mediated phosphorylation of VASP in intact human platelets [11]. In the present study, 8-(*p*-chlorophenylthio)-cAMP (8-pCPT-cAMP), a widely used cAMP analogue in studies with intact cells, and Sp-5,6-DCl-cBiMPS were compared with respect to their effects on purified cyclic-nucleotide-dependent protein kinases, endogenous protein phosphorylation of platelet membranes, protein phosphorylation in intact platelets, and platelet aggregation.

MATERIALS AND METHODS

Materials

PG-E_1, cAMP, cGMP, 8-bromo-cAMP (8-Br-cAMP) and thrombin were purchased from Sigma (Munich, Germany), and 8-pCPT-cAMP was obtained from Boehringer (Mannheim, Germany). Sp-5,6-DCl-cBiMPS was synthesized as described previously [15], by using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (obtained from Fluka, Buchs, Switzerland) as starting material. [γ-^{32}P]ATP (3000 Ci/mmol), [^{32}P]P_i and ^{125}I-Protein A (30 mCi/mg) were obtained from Amersham Buchler (Braunschweig, Germany), and Kemptide was purchased from Peninsula (Merseyside, U.K.). All other chemicals were from commercial sources as described previously [10,11].

Protein kinase assay

The type II cAMP-PK and the soluble type I cGMP-PK were purified from bovine heart and bovine lung respectively, as described previously [16,17]. The activity of the purified kinases was measured by the phosphocellulose method [18], by using Kemptide as a substrate, with minor modifications [19].

Phosphodiesterase (PDE) assays

Calmodulin-dependent PDE (CaM-PDE), cGMP-stimulated PDE (cGS-PDE) and cGMP-inhibited PDE (cGI-PDE) [20] were purified by using specific monoclonal antibodies [21]. The velocity of hydrolysis (v) was measured by the phosphate-release assay [22] with a final concentration of 500 μM cyclic nucleotide. The hydrolysis rates for cAMP were 0.75 nmol/min for cGS-PDE, 0.25 nmol/min for cGI-PDE and 0.3 nmol/min for CaM-PDE. The phosphodiesterase experiments were carried out in the laboratory of Dr. J. A. Beavo (University of Washington, Seattle, WA, U.S.A.). In addition to the cyclic nucleotide analogues reported here, many others were analysed in these PDE experiments (E. Butt & J. A. Beavo, unpublished work).

Analysis of lipophilicity

Log K_w data of cAMP analogues were determined by reversed-phase h.p.l.c. as described previously [23], by using a 4 mm × 250 mm column containing LiChrosorb RP-18 10 μm material (obtained from Merck, Darmstadt, Germany). The lipophilicity is measured as the partition behaviour between the non-polar bonded stationary phase and the polar eluent [23].

Endogenous phosphorylation of platelet membranes

Membranes of human platelets were isolated and used in phosphorylation experiments as described previously [8,10], with minor modifications. The incubation mixture (final volume 140 μl) contained 10 mM-Hepes buffer (pH 7.4), 10 mM-$MgCl_2$ 1 mM-dithiothreitol, 0.2 mM-EDTA, 1 mM-isobutylmethylxanthine, cyclic nucleotides as indicated, 42 μg of membrane protein and 4 μM-[γ-^{32}P]ATP (15800 c.p.m./pmol). The phosphorylation reaction was initiated by addition of ATP, carried out for 2.5 min at 30 °C, terminated by addition of an SDS-containing stop-solution and boiling, and subsequently analysed by SDS/PAGE and autoradiography [8,10].

Phosphorylation experiments with intact human platelets

Washed human platelets were prepared as described previously [9,11] and resuspended in an iso-osmotic buffer containing 10 mM-Hepes (pH 7.4), 137 mM-NaCl, 2.7 mM-KCl, 5.5 mM-glucose and 1 mM-EDTA at a density of 1×10^9 cells/ml. For experiments using ^{32}P-labelled platelets, cells were washed once with the iso-osmotic Hepes buffer and subsequently incubated at a density of 5×10^9 platelets/ml for 1 h at 37 °C in 100 μl of the iso-osmotic Hepes buffer containing 1 mCi of carrier-free [^{32}P]P_i. Excess [^{32}P]P_i was removed by washing, and samples of ^{32}P-labelled platelet were then incubated with reagents for the times indicated at 37 °C, as described previously [9]. Incubations were stopped

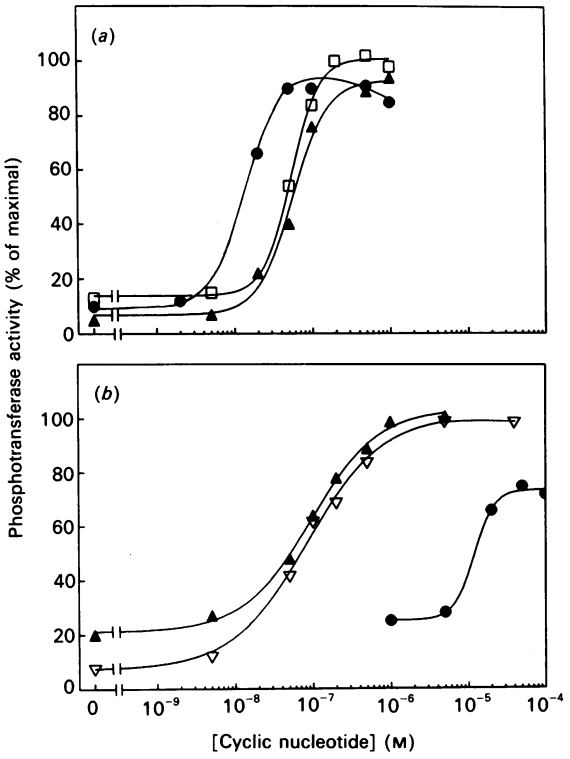


Fig. 2. Effects of cyclic nucleotide analogues on the activity of purified cyclic-nucleotide-dependent protein kinases

The concentration-dependent activation of cAMP-PK (*a*) or cGMP-PK (*b*) by cAMP (□), cGMP (▽), 8-pCPT-cAMP (▲) and Sp-5,6-DCl-cBiMPS (●) is shown. All data are expressed as percentages of the maximal stimulation of cAMP-PK by cAMP (in *a*) or the maximal stimulation of cGMP-PK by cGMP (in *b*). The data represent means of closely agreeing triplicates.

by the addition of an SDS-containing stop-solution and boiling, and were then analysed by SDS/PAGE and autoradiography [9]. For the quantitative analysis of cAMP-PK-mediated VASP phosphorylation [11], platelet suspensions (final density 1×10^9 cells/ml of iso-osmotic Hepes buffer) were incubated at 37 °C with the reagents indicated. At various incubation times, platelet samples were removed, mixed with an SDS-containing stop-solution and boiled. VASP phosphorylation was then measured by SDS/PAGE and Western-blot analysis as described previously [11].

Platelet-aggregation analysis

Human platelets were isolated as described above, except that EDTA was omitted and 1 mM-$CaCl_2$ and 2 mM-$MgCl_2$ were added to the final resuspension buffer. Platelets (final density 1×10^9 cells/ml) were incubated with or without cyclic nucleotides at 37 °C for 10 min before aggregation was induced by the addition of 0.2 unit of thrombin/ml. Aggregation was monitored with a platelet-aggregation profiler PAP 4 (Bio Data Corporation) measuring light transmission.

RESULTS

The structures of cAMP, 8-pCPT-cAMP and Sp-5,6-DCl-cBiMPS are shown in Fig. 1. Compared with the natural cyclic nucleotides cAMP and cGMP, 8-pCPT-cAMP was a potent activator of both cAMP-PK and cGMP-PK (Fig. 2; Table 1). In contrast, Sp-5,6-DCl-cBiMPS stimulated the activity of cAMP-PK even better than cAMP and much better than the phosphorothioate stereoisomers of cAMP and cGMP (Sp-cAMPS and Sp-cGMPS), whereas it was 100-fold less potent than cGMP and 8-pCPT-cAMP as an activator of the cGMP-PK (Fig. 2; Table 1). The analogues had the same effects on endogenous cAMP-PK and cGMP-PK as observed in phosphorylation experiments with membranes from human platelets, which contain, in addition to the kinases, their specific substrates [8–10]. In agreement with previous work [8–10], cAMP stimulated cAMP-PK-mediated phosphorylation of several proteins with relative molecular masses on SDS/PAGE of 240 kDa, 130 kDa, 68 kDa, 50 kDa, 42 kDa and 22 kDa, and this could be reproduced by Sp-5,6-DCl-cBiMPS and 8-pCPT-cAMP (Fig. 3). In contrast, cGMP caused cGMP-PK-mediated phosphorylation of three proteins with molecular masses of 130 kDa, 50 kDa and 46 kDa ([8]; see Fig. 3). Although the 130 kDa and 50 kDa proteins are phosphorylated by both cAMP-PK and cGMP-PK, phosphorylation of the 46 kDa protein in platelet membranes is due only to the activity of cGMP-PK [8,10]. Phosphorylation of the 46 kDa protein in platelet membranes was consistently observed with 8-pCPT-cAMP, but never with Sp-5,6-DCl-cBiMPS (Fig. 3).

The cAMP analogues investigated differ also in their apparent lipophilicity and in their capacity to serve as substrates for three different cyclic nucleotide PDEs (Table 1). Both 8-Br-cAMP and

Table 1. Some biological and chemical properties of cyclic nucleotides

The cyclic nucleotides shown were analysed with respect to their half-maximal activation (K_a) of the type II cAMP-PK or type I cGMP-PK, hydrolysis by the cGS-PDE, cGI-PDE or CaM-PDE and with respect to their lipophilicity ($\log K_w$) as measured by reversed-phase h.p.l.c. The apparent activation constant K_a is the cyclic nucleotide concentration required for half-maximal activation (* partial antagonist). The K_a values represent means of at least three separate experiments. The hydrolysis data are expressed as the velocity of hydrolysis of the analogues relative to cAMP hydrolysis [$v' = v$(analogue)/v(cAMP)]. Hydrolysis data represent the means of triplicates of two separate experiments. Some of the data concerning Sp-cAMPS and Sp-cGMPS have been reported previously [19].

Derivative	K_a (μM)		Hydrolysis (v')			Lipophilicity ($\log K_w$)
	cAMP-PK	cGMP-PK	cGS-PDE	cGI-PDE	CaM-PDE	
cAMP	0.08	39.0	1.0	1.0	1.0	1.05
cGMP	60.00	0.11	1.0	0.3	0.7	0.68
8-Br-cAMP	0.05	5.8	0.11	0.68	0.23	1.42
8-pCPT-cAMP	0.05	0.11	0.07	0.38	0.17	2.76
Sp-5,6-DCl-cBiMPS	0.03	10.0	0.008	None	None	> 3.1
Sp-cAMPS	1.8	*	None	None	None	1.35
Sp-cGMPS	27.00	27.0	0.006	None	0.017	1.04

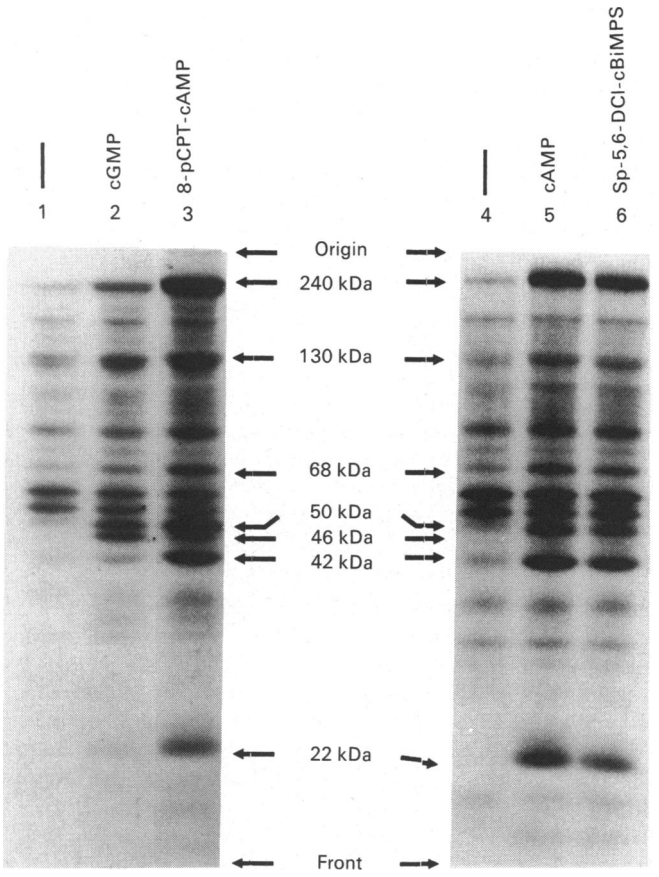


Fig. 3. Autoradiograph showing the effects of cyclic nucleotides on the endogenous phosphorylation in platelet membranes

Phosphorylation of platelet membrane proteins (42 μg) was performed under standard conditions in the absence of cyclic nucleotides (lanes 1 and 4) or in the presence of 1 μM-cGMP (lane 2), 1 μM-8-pCPT-cAMP (lane 3), 1 μM-cAMP (lane 5) or 1 μM-Sp-5,6-DCl-cBiMPS (lane 6). ^{32}P-labelled proteins were separated by SDS/PAGE and detected by autoradiography. Arrows indicate the positions and apparent molecular masses (kDa) of proteins whose phosphorylation was consistently stimulated by these cyclic nucleotides.

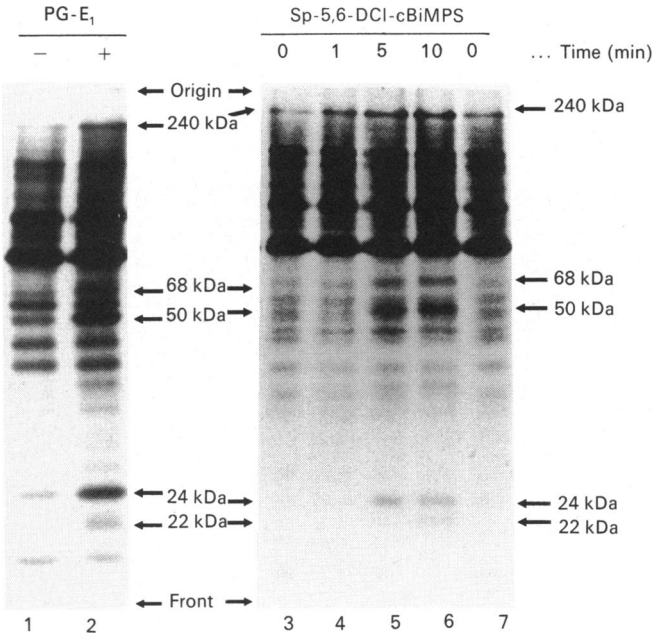


Fig. 4. Autoradiograph showing the regulation of protein phosphorylation in intact platelets by PG-E_1 and Sp-5,6-DCl-cBiMPS

^{32}P-labelled platelets were incubated without (lane 1) or with (lane 2) 5 μM-PG-E_1 for 5 min or with 0.5 mM-Sp-5,6-DCl-cBiMPS for the times indicated (lanes 3–7). ^{32}P-labelled proteins were separated by SDS/PAGE and observed by autoradiography. The positions and apparent molecular masses of proteins whose phosphorylation was consistently stimulated by PG-E_1 and Sp-5,6-DCl-cBiMPS are shown.

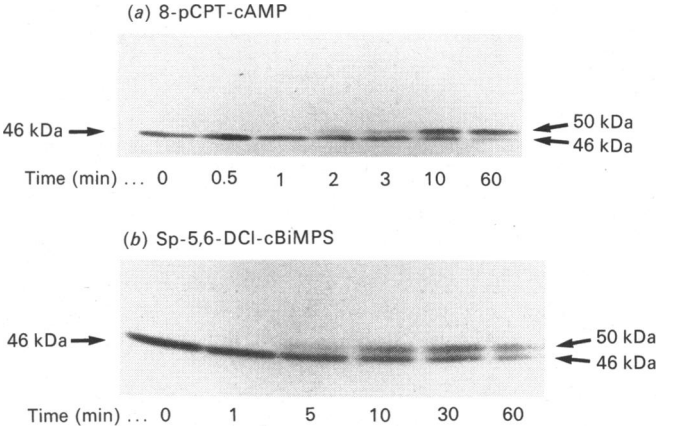


Fig. 5. Autoradiograph showing the time course of VASP phosphorylation in intact platelets incubated with 8-pCPT-cAMP or Sp-5,6-DCl-cBiMPS

Washed human platelets were incubated with 1 mM-8-pCPT-cAMP (*a*) or 0.5 mM-Sp-5,6-DCl-cBiMPS (*b*) for the times indicated. Platelet samples were analysed for VASP phosphorylation by the Western-blot method. VASP phosphorylation was detected by the shift of VASP from the dephospho-form (46 kDa protein) to the phospho-form (50 kDa protein).

8-pCPT-cAMP were hydrolysed to significant extents by the CaM-PDE, and even more so by the cGI-PDE, whereas Sp-5,6-DCl-cBiMPS was either not used, or not significantly used, as a substrate by any of the three types of PDEs investigated (Table 1). The apparent lipophilicity (relative distribution between a non-polar and a polar phase) of Sp-5,6-DCl-cBiMPS was even higher than that of 8-pCPT-cAMP (Table 1).

To investigate whether extracellularly applied Sp-5,6-DCl-cBiMPS is capable of activating the intracellular cAMP-PK, phosphorylation experiments were carried out with intact human platelets, an established model system for the analysis of cAMP-PK-mediated protein phosphorylation [9,11,12,14]. In intact human platelets, the cAMP-elevating PG-E_1 consistently stimulated the phosphorylation of several proteins with molecular masses of 240 kDa, 68 kDa, 50 kDa, 24 kDa and 22 kDa, and this could be reproduced by 0.5 mM extracellular Sp-5,6-DCl-cBiMPS (Fig. 4). These phosphorylation data also indicated that the maximal Sp-5,6-DCl-cBiMPS-induced protein phosphorylation obtained with intact ^{32}P-labelled platelets was observed within 5–10 min after addition of 0.5 mM analogue (Fig. 4; other results not shown).

One of the major cAMP-PK substrates in intact human platelets, the 46/50 kDa protein termed VASP (the 50 kDa phosphoprotein in Fig. 4), was recently purified and characterized by immunological and other methods [10,11]. Phosphorylation of VASP at one site alters the apparent mobility of this protein in SDS/PAGE, resulting in the appearance of a 50 kDa phosphoprotein and the concomitant disappearance of the 46 kDa dephosphoprotein [10,11]. This property (shift of apparent molecular mass in SDS/PAGE after phosphorylation) and the

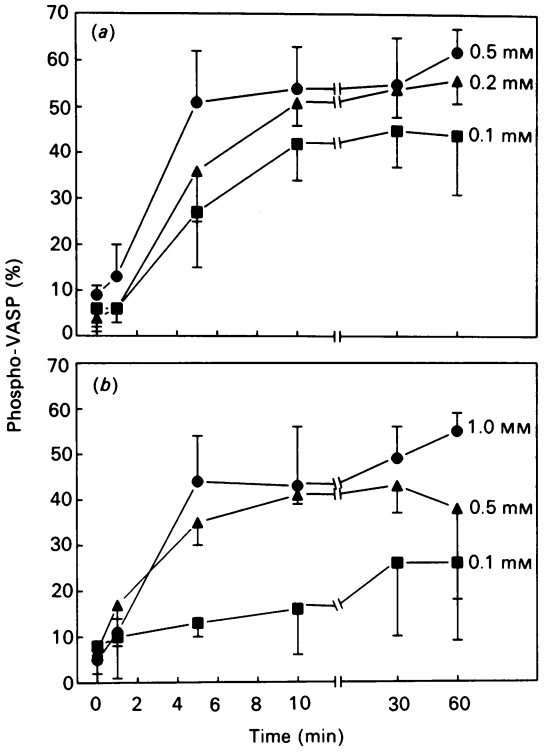


Fig. 6. Quantitative analysis of VASP phosphorylation in platelets incubated with Sp-5,6-DCl-cBiMPS or 8-pCPT-cAMP

Intact washed human platelets were incubated with various concentrations of Sp-5,6-DCl-cBiMPS (*a*) or 8-pCPT-cAMP (*b*) for the times indicated. Platelet samples were analysed for VASP phosphorylation by the Western-blot method. VASP phosphorylation is expressed as phospho-VASP (50 kDa protein) percentage of total VASP (46 kDa + 50 kDa protein). The data represent means ± S.D. of three separate experiments.

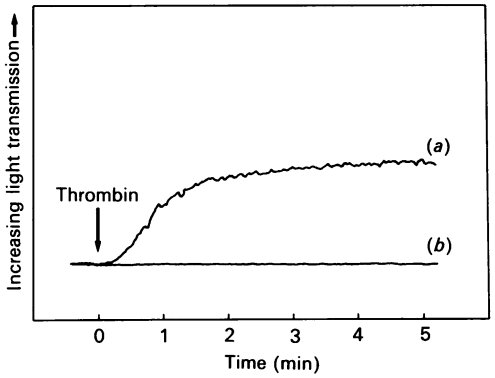


Fig. 7. Effect of Sp-5,6-DCl-cBiMPS on platelet aggregation

Washed human platelets were incubated for 10 min in the absence (*a*) or presence (*b*) of 0.2 mM-Sp-5,6-DCl-cBiMPS before 0.2 unit of thrombin/ml was added (indicated by the arrow). Platelet aggregation was monitored by the increase in light transmission.

availability of a specific antibody which recognizes both the phospho- and dephospho-forms can be used to measure quantitatively cAMP-PK-mediated VASP phosphorylation in intact human platelets [11]. Both 8-pCPT-cAMP and Sp-5,6-DCl-cBiMPS caused the time-dependent appearance of the 50 kDa phospho-VASP and the concomitant decrease of the 46 kDa dephospho-VASP (Fig. 5). The quantitative analysis of VASP phosphorylation in intact platelets in response to different concentrations of 8-pCPT-cAMP and Sp-5,6-DCl-cBiMPS is shown in Fig. 6. The time course and maximal extent of VASP phosphorylation were similar when 0.2–0.5 mM-Sp-5,6-DCl-cBiMPS and 0.5–1.0 mM-8-pCPT-cAMP were used (Fig. 6), and the extent of phosphorylation was similar to the maximal extent of cAMP-PK-mediated VASP phosphorylation in response to high concentrations of PG-E_1 [11]. It is noteworthy that analogue concentrations (i.e. 0.1 mM) that were relatively ineffective with 8-pCPT-cAMP produced significant VASP phosphorylation when Sp-5,6-DCl-cBiMPS was used (Fig. 6). The effect of Sp-5,6-DCl-cBiMPS on platelet aggregation was also investigated, since cAMP-elevating prostaglandins such as PG-E_1 inhibit the platelet aggregation induced by agonists such as thrombin, collagen and others [12,14]. Pretreatment of washed human platelets with 0.2 mM-Sp-5,6-DCl-cBiMPS for 10 min prevented the subsequent aggregation in response to thrombin (Fig. 7). Under similar conditions, lower concentrations, i.e. 0.05 mM- and 0.1 mM-Sp-5,6-DCl-cBiMPS, caused partial and complete inhibition, respectively, of thrombin-induced platelet aggregation (results not shown). With 8-pCPT-cAMP, concentrations of 0.25–0.50 mM were required to observe complete inhibition of thrombin-induced platelet aggregation [11].

DISCUSSION

Although the use of cAMP analogues has been very helpful in defining and characterizing the cyclic-nucleotide-binding sites of the cAMP-PK (reviewed in [5]), quantitative data with respect to specific cAMP-PK-mediated protein phosphorylation caused by cAMP analogues with intact cells are very rare [5,24]. Most often, it is assumed that the effects of cAMP analogues with intact cells are mediated by the activation of cAMP-PK. The cAMP analogues most commonly used for intact cell studies are $N^6,O^{2'}$-dibutyryl cAMP (Bt_2-cAMP), 8-Br-cAMP, and more recently 8-pCPT-cAMP. None of these cAMP analogues fulfils the criteria ([1,5]; see the Introduction) for an ideal activator of the cAMP-PK in intact cells. Bt_2-cAMP, although relatively lipophilic, is a very poor activator of the cAMP-PK and has to be metabolized intracellularly to the more potent cAMP-PK activator N^6-monobutyryl cAMP by releasing free butyrate [25]. Since this required metabolism may differ from cell type to cell type, and the released free butyrate itself may have effects on the cell function studied [26,27], Bt_2-cAMP cannot be considered as an ideal activator of cAMP-PK in intact cells. 8-Br-cAMP, although a specific and potent activator of cAMP-PK with purified kinases (Table 1) and cell extracts (results not shown), is not very lipophilic (Table 1), and, compared with 8-pCPT-cAMP and Sp-5,6-DCl-cBiMPS, less effective with respect to cAMP-PK-mediated protein phosphorylation in intact platelets (results not shown). In contrast with earlier studies, 8-Br-cAMP is efficiently hydrolysed by at least three types of PDEs, the cGI-PDE, the CaM-PDE and a cAMP-specific PDE from yeast (Table 1; [28,29]). Both Sp-cAMPS and Sp-cGMPS are not very useful for the activation of cyclic-nucleotide-dependent protein kinases in intact cells, owing to their low lipophilicity and relatively low potency in activating these kinases (Table 1).

In recent years, 8-pCPT-cAMP has been increasingly used as an activator of the cAMP-PK in intact cells. However, not only is 8-pCPT-cAMP hydrolysed by the PDEs studied (Table 1), it is also a potent activator of the purified cGMP-PK (Fig. 1, Table 1), and it stimulates both cGMP-PK- and cAMP-PK-mediated protein phosphorylation in platelet membranes (Fig. 3). The potent activation of cGMP-PK by 8-pCPT-cAMP has been noted previously [30], and in that study it was speculated that perhaps the relaxation of smooth muscle caused by 8-pCPT-cAMP was mediated by cGMP-PK rather than by cAMP-PK

[30]. Clearly, 8-pCPT-cAMP must not be used when the effects of cAMP-PK and cGMP-PK should be distinguished. This is particularly the case in studies with smooth- and cardiac-muscle cells, platelets, and certain neurons and epithelial cells, for which the presence of cGMP-PK has been established and a functional role for the cGMP-PK is strongly suspected [14,31]. In these cell types, cAMP-PK and cGMP-PK may have similar [14] or opposite [31] effects on the cellular response studied.

The data presented here suggest that Sp-5,6-DCl-cBiMPS is a very potent and specific activator of the cAMP-PK in intact cells. Sp-5,6-DCl-cBiMPS binds to cAMP-binding sites of both cAMP-PK types I and II, with a relative selectivity for the *C*-terminal site B [32], and is a powerful and specific activator of purified cAMP-PK and of cAMP-PK-mediated protein phosphorylation in platelet membranes (Fig. 2, Table 1, Fig. 3). In particular, Sp-5,6-DCl-cBiMPS was a very poor activator of the cGMP-PK (Figs. 2 and 3). As expected for cyclic monophosphorothioates [33], Sp-5,6-DCl-cBiMPS was hydrolysed very poorly, or not at all, by three types of PDEs tested (Table 1). However, not all known members of the still growing family of PDEs have been tested with respect to Sp-5,6-DCl-cBiMPS hydrolysis, and it cannot be excluded that this analogue may alter the hydrolysis of cAMP or cGMP by these PDEs in intact cells. These questions require investigation (E. Butt & J. A. Beavo, unpublished work). A first series of experiments indicated that Sp-5,6-DCl-cBiMPS at relatively high concentrations ($> 10\ \mu$M) had inhibitory effects on the catalytic site, but not on the allosteric site of certain phosphodiesterases. The high lipophilicity of Sp-5,6-DCl-cBiMPS observed by h.p.l.c. would predict that this analogue is capable of penetrating cell membranes and activating the intracellular cAMP-PK. Indeed, incubation of intact human platelets with Sp-5,6-DCl-cBiMPS resulted in the phosphorylation of several proteins (Fig. 4) which are known to be phosphorylated by the cAMP-PK in response to cAMP-elevating agents such as PG-E_1 [9,11]. The phosphorylation of one of these cAMP-PK substrates, the 50 kDa vasodilator-stimulated phosphoprotein (VASP), could be quantitatively analysed by recently developed immunological methods [11]. Extracellular Sp-5,6-DCl-cBiMPS (0.1–0.5 mM) caused a 10-fold elevation of the level of phospho-VASP within 5–10 min (Fig. 6), a cAMP-PK-mediated response normally seen only with maximal concentration of cAMP-elevating prostaglandins [11]. Interestingly, 0.1 mM-Sp-5,6-DCl-cBiMPS was more effective than 0.1 mM-8-pCPT-cAMP in stimulating VASP phosphorylation in intact platelets (Fig. 6), consistent with the better lipophilicity of Sp-5,6-DCl-cBiMPS observed experimentally (Table 1). Sp-5,6-DCl-cBiMPS could reproduce the inhibitory effects of the cAMP-elevating PG-E_1 on platelet aggregation (Fig. 7), which are thought to be mediated by the cAMP-PK [12,14].

Although some cAMP analogues bind to purified cAMP-PK with binding constants in the nM range [32], it should be noted that the intracellular concentration of cAMP-binding sites due to cAMP-PK is in the μM range [3,34], and may be as high as 6 μM in human platelets ([35]; M. Eigenthaler & U. Walter, unpublished work). This would predict that for studies with intact cells a good cAMP-analogue lipophilicity is more important than binding constants of these analogues for the cAMP-PK in the nM range, since μM concentrations of cAMP and analogues are presumably required to activate significant amounts of the intracellular cAMP-PK. In this respect, Sp-5,6-DCl-cBiMPS is more lipophilic than other cAMP analogues studied extensively with intact cells. Since Rp-cAMPS is an antagonist of cAMP-PK [36,37], the Rp-diastereoisomer of 5,6-DCl-cBiMPS was also briefly investigated. Surprisingly, Rp-5,6-DCl-cBiMPS was found to be a partial agonist of cAMP-PK of platelet membranes and of purified type II cAMP-PK, an unexpected result which needs further investigation.

In conclusion, several cAMP analogues previously and currently used in many studies have certain drawbacks when used as specific activators of cAMP-PK with intact cells. Sp-5,6-DCl-cBiMPS, owing to its chemical and biological properties described here, appears to be a potent and specific activator of cAMP-PK when used with human platelets (cell extracts and intact cells). Certainly, additional cellular responses and other cell types than those used here have to be analysed in order to evaluate the full potential and specificity of Sp-5,6-DCl-cBiMPS as an activator of cAMP-PK in intact cells.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (Ko 210/11-1 and SFB 176). B.J. is supported by the Fonds der Chemischen Industrie. The work of M.S. in Würzburg was made possible by a NATO travel grant (0826/88). E.B. is a recipient of a DFG postdoctoral fellowship. Some of the work was carried out while E.B. was a visiting scientist in the laboratory of Dr. J. A. Beavo (University of Washington, Seattle, WA, U.S.A.). We thank Dr. Beavo for multiple discussions and for his support of this project (via N.I.H. grants DK 21723 and EY 08197). We also thank Monika Krieger for many protein kinase assays and Silvia Ebert for the preparation of the manuscript.

REFERENCES

1. Sutherland, E. W. (1972) Science **177**, 401–408
2. Krebs, E. G. & Beavo, J. A. (1979) Annu. Rev. Biochem. **48**, 923–959
3. Lohmann, S. M. & Walter, U. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **18**, 63–117
4. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) Annu. Rev. Biochem. **56**, 567–613
5. Beebe, S. J. & Corbin, J. D. (1986) Enzymes 3rd Ed. **17**, 43–111
6. Meinecke, M., Büchler, W., Fischer, L., Lohmann, S. M. & Walter, U. (1990) in Cellular and Molecular Biology of Myelination (Jeserich, G., Althaus, H. H. & Waehneldt, T. V., eds.), pp. 201–215, Springer Verlag, Berlin
7. Chodosh, L. A., Fire, A., Samuels, A. & Sharp, P. (1989) J. Biol. Chem. **264**, 2250–2257
8. Waldmann, R., Bauer, S., Göbel, C., Hofmann, F., Jakobs, K. H. & Walter, U. (1986) Eur. J. Biochem. **158**, 203–210
9. Waldmann, R., Nieberding, M. & Walter, U. (1987) Eur. J. Biochem. **167**, 441–448
10. Halbrügge, M. & Walter, U. (1989) Eur. J. Biochem. **185**, 41–50
11. Halbrügge, M., Friedrich, C., Eigenthaler, M., Schanzenbächer, P. & Walter, U. (1990) J. Biol. Chem. **265**, 3088–3093
12. Haslam, R. J. (1987) in Thrombosis and Haemostasis 1987 (Verstraete, M., Vermylen, J., Lijnen, R. & Arnout, J., eds.), pp. 147–174, Leuven University Press, Leuven
13. Vane, J. R., Gryglewski, R. J. & Botting, R. M. (1987) Trends Pharmacol. Sci. **8**, 491–496
14. Walter, U. (1989) Rev. Physiol. Biochem. Pharmacol. **113**, 41–88
15. Genieser, H.-G., Dostmann, W., Bottin, U., Butt, E. & Jastorff, B. (1988) Tetrahedron Lett. **29**, 2803–2804
16. Kaczmarek, L. K., Jenning, K. R., Strumwasser, F., Nairn, A. C., Walter, U., Wilson, F. D. & Greengard, P. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 7487–7491
17. Walter, U., Miller, P., Wilson, F. D., Menkes, D. & Greengard, P. (1980) J. Biol. Chem. **255**, 3757–3762
18. Roskoski, R. (1983) Methods Enzymol. **99**, 3–6
19. Butt, E., van Bemmelen, M., Fischer, L., Walter, U. & Jastorff, B. (1990) FEBS Lett. **263**, 47–50
20. Beavo, J. A. & Reifsnyder, D. H. (1990) Trends Pharmacol. Sci. **11**, 150–155
21. Harrison, S. A., Reifsnyder, D. H., Gallis, B., Cadd, G. G. & Beavo, J. A. (1986) Mol. Pharmacol. **25**, 506–514
22. Gillespie, P. G. & Beavo, J. A. (1989) Mol. Pharmacol. **36**, 773–781
23. Braumann, T. & Jastorff, B. (1985) J. Chromatogr. **350**, 105–118
24. Beebe, S. J., Blackmore, P. F., Segaloff, D. L., Koch, S. R., Burks, L. E., Limbird, L. E., Granner, D. K. & Corbin, J. D. (1986) Horm. Cell Regul. **139**, 159–180
25. Kaukel, E. & Hilz, H. (1972) Biochem. Biophys. Res. Commun. **46**, 1011–1018
26. Kruh, J. (1982) Mol. Cell. Biochem. **42**, 65–82
27. Yusto, B., Ortiz-Caro, J., Pascual, A. & Aranda, A. (1988) J. Neurochem. **51**, 1808–1815

28. MacFarland, R. T., Zelus, B. D. & Beavo, J. A. (1991) J. Biol. Chem. **266**, 136–142
29. Van Lokkeren-Campagne, M. M., Villalba Diaz, F., Jastorff, B., Winkler, E., Genieser, H.-G. & Kessin, R. H. (1990) J. Biol. Chem. **265**, 5847–5854
30. Francis, S. H., Noblett, B. D., Todd, B. W., Wells, J. N. & Corbin, J. D. (1988) Mol. Pharmacol. **34**, 506–517
31. Mery, P.-F., Lohmann, S. M., Walter, U. & Fischmeister, R. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 1197–1201
32. Dostmann, W. R. G., Taylor, S. S., Genieser, H.-G., Jastorff, B., Doskeland, S. O. & Ogreid, D. (1990) J. Biol. Chem. **265**, 10484–10491
33. Jastorff, B. (1982) in Cell Regulation by Intracellular Signals (Swillens, S. & Dumont, J. E., eds.), pp. 195–207, Plenum Press, New York
34. Hofmann, F., Bechtel, P. J. & Krebs, E. G. (1977) J. Biol. Chem. **252**, 1441–1447
35. Eigenthaler, M., Friedrich, C., Schanzenbächer, P. & Walter, U. (1990) Eur. J. Clin. Invest. **20** (2), A16
36. De Wit, R. J. W., Hoppe, J., Stec, W. J., Baraniak, J. & Jastorff, B. (1982) Eur. J. Biochem. **122**, 95–99
37. De Wit, R. J. W., Hekstra, D., Jastorff, B., Stec, W., Baraniak, J., Van Driel, R. & Van Haastert, P. J. M. (1984) Eur. J. Biochem. **142**, 255–260

Received 19 April 1991/7 June 1991; accepted 14 June 1991