# Cyclic AMP Stimulates the Cyclic GMP Egression Pump in Human Erythrocytes: Effects of Probenecid, Verapamil, Progesterone, Theophylline, IBMX, Forskolin, and Cyclic AMP on Cyclic GMP Uptake and Association to Inside-Out Vesicles†

Christin Schultz, Svanhild Vaskinn, Hanne Kildalsen, and Georg Sager*

*Department of Pharmacology, Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway*

*Received June 5, 1997; Revised Manuscript Received October 8, 1997*

ABSTRACT: The knowledge about the structure and function of the protein families responsible for cGMP synthesis and metabolic conversion has grown vastly the last years, whereas little is known about proteins that account for the cellular export of cGMP. In the present study, we have employed a model with inside-out vesicles prepared from human erythrocytes to characterize modulation and regulation of cellular cGMP extrusion. The active transport was saturable ($K_m$ of 2.4 ± 0.2 $\mu$M, mean ± SEM, $n = 3$) and coupled to ATP hydrolysis since no accumulation was detected in the presence of ATP-$\gamma$-S and AMP-PNP. The observation that 100 $\mu$M of cAMP caused a minimal inhibition (14.4 ± 0.3%) of active cGMP transport showed that the extrusion system for cGMP was not shared with cAMP, but a competitive interaction occurred for the ATP-independent association to the inside out vesicles. In contrast, the lowest, but physiological relevant cAMP concentrations (0.1−5 $\mu$M) stimulated the active cGMP transport with 30−35%, an observation that suggests cAMP as an allosteric regulator of the cGMP transporter. Several well-known modulators of other energy-requiring membrane transport systems caused a competitive and concentration-dependent inhibition, including verapamil ($K_i = 13.0 \pm 2.4$ $\mu$M), forskolin ($K_i = 13.5 \pm 1.4$ $\mu$M) and probenecid ($K_i = 27.0 \pm 1.3$ $\mu$M). Progesterone, which was the most potent inhibitor ($K_i = 2.2 \pm 0.3$ $\mu$M), interacted with the active cGMP transport in a noncompetitive manner. The highest concentration (100 $\mu$M) of IBMX and theophylline reduced the active cGMP uptake with 29.5 ± 1.9% and 21.6 ± 2.1%, respectively. None of these substances interfered with the association of cGMP to the vesicles in absence of ATP. The present results show that human erythrocytes possess a cell membrane cGMP transporter which is coupled to an ATPase. Its activity is regulated by cAMP in an apparent allosteric manner and inhibited by substances previously known to interact with other membrane transport systems.

The cellular eliminatory pathways of cyclic nucleotides comprise metabolic conversion and cellular secretion. An overwhelming knowledge about the function and structure of the phosphodiesterases has accumulated (Conti et al., 1991, 1995; Thompson, 1991; Sonnenburg & Beavo, 1994; Manganiello et al., 1995). In contrast, much less attention has been paid to the secretory pathway, even if cyclic nucleotides were identified in extracellular fluids, like urine 25 years ago (Butcher & Sutherland, 1962; Ashman et al., 1963).

The fact that cGMP (Kapoor & Krishna, 1977; Tjörnhammar et al., 1983; Hamet et al., 1989) is extruded against a concentration gradient is consistent with active transport. In accordance, an ATP-dependent uptake in inside-out vesicles from human erythrocytes has been reported (Sager et al., 1996). The first question was, therefore, whether ATP hydrolysis was needed for the cGMP accumulation in inside-out vesicles.

In studies of intact human erythrocytes, probenecid and verapamil inhibited the egression of cGMP, whereas progesterone appeared to stimulate the efflux (Flo et al., 1995). Assuming that these substances interact with the cytosolic part of the transporter, the inside-out vesicles represent an ideal model to obtain the characteristics of the interaction and our second questions were if substances such as probenecid, verapamil, progesterone, IBMX,[1] and theophylline interact directly or indirectly with the pump in a competitive or noncompetitive manner?

Some evidence exists that supports the idea of a common transport system responsible for the cellular extrusion of cAMP and cGMP (Hamet et al., 1989; Patel et al., 1995). This assumption was based on the fact that forskolin is a potent activator of adenylate cyclase. However, it is well-known that forskolin is a nonspecific inhibitor of several transport systems, including P-glycoprotein (Morris et al., 1991b), the glucose transporter (Joost & Steinfelder, 1987), and the L-type calcium channel (Boutjdir et al., 1990). The third question was, therefore, may the apparent inhibition by cAMP be explained by a direct interaction between forskolin and the cGMP transporter?

† This work was supported by The Norwegian Cancer Society.
* Corresponding author.

[1] Abbreviations: DMSO, dimethyl sulfoxide; IBMX, 3-isobutyl-1-methylxanthine.

S0006-2960(97)01340-8 CCC: $15.00 
Published on Web 01/13/1998

Finally, we asked whether cAMP directly affects the cGMP uptake in inside-out vesicles from human erythrocytes.

## MATERIALS AND METHODS

*Chemicals.* The following substances were employed: [$^3$H]cGMP from Amersham International plc. (Buckinghamshire, U.K.) and acetylthiocholine chloride, ATP, ATP-$\gamma$-S, AMP-PNP, cAMP, DL-cysteine, DMSO, 5,5′-dithiobios-(2-nitrobenzoic acid), forskolin, glyceraldehyde-3-phosphate, IBMX, $\beta$-NAD, probenecid, progesterone, sodium arsenate, theophylline, Triton X-100, and (±)-verapamil from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of analytical grade.

*Buffers.* Medium A included KCl, 3 mM; NaCl, 110 mM; and Tris-HCl, 5 mM, pH 8.0−8.2. Medium B included KCl, 3 mM and Tris-HCl, 5 mM; pH 8.0−8.2. Phosphate buffered saline (PBS) included NaCl, 140 mM; KCl, 3 mM; $Na_2HPO_4$, 10 mM; and $KH_2PO_4$, 1.8 mM; pH 7.3−7.4.

*Blood Sampling and Preparation of Inside-Out Vesicles.* The preparation of inside-out vesicles was virtually performed as previously described (Steck et al., 1970). Venous blood (10 mL) was sampled in EDTA vacuum tubes (Vacutainer, Becton Dickinson, Meylan Cedex, France). The cells were separated from plasma by centrifugation (1000*g* for 15 min), washed three times (4 mL packed cells in 20 mL medium A), and lysed (4 mL packed cells in 20 mL medium B). The ghosts were sedimented (20000*g* for 10 min) and resuspended (1 mL in 20 mL medium B) until the membranes appeared milky white and the supernatant was clear. The invagination of the cell membrane started when a 1 mL ghost pellet was resuspended in 39 mL of 0.5 mM Tris-HCl, pH 8.0−8.2, and left for 2 h at 0−4 °C. The membranes were sedimented (100000*g* for 30 min), then mixed with 1 mL of 0.5 mM Tris-HCl (pH 8.0−8.2) and vesiculated by passing them five times through a 20 mm-long 27-gauge needle. The mixture obtained from a 1 mL ghost pellet was diluted with 1 mL of 0.5 mM Tris-HCl (pH 8.0−8.2). The vesicles and ghosts were separated on linear gradients, with densities from 1.05 to 1.15 g/mL (Nycodenz, Nycomed Pharma, Oslo, Norway) during centrifugation for 16−17 h at 100000*g* and 0−4 °C. The vesicles were aspirated and washed with 40 vol of PBS and sedimented at 28000*g* for 30 min and finally resuspended in PBS (0.7−0.8 mL).

*Uptake Studies.* The experiments were performed at 37 °C with 100−200 $\mu$g of protein in 400 $\mu$L PBS including 10 mM $MgCl_2$ and 1 $\mu$M [$^3$H]cGMP, with or without 2 mM ATP. The substances tested for their ability to modulate transport were dissolved in DMSO (0.1−0.5% v/v), and identical concentrations of the vehicle were used in the control samples. The uptake of radioligand was terminated by addition of 10 mL of ice-cold 30 mM NaF/0.5 mM Tris-HCl, one washing with 10 mL of this mixture. The samples were mixed with 1 mL of ice-cold water and freezed. After 18 h at −20 °C, the mixture was thawed, centrifuged at 15000*g* for 30 min and radioactivity determined in the supernatant (800 $\mu$L to 10 mL Ultima GoldTM, XR, Packard, Groningen, The Netherlands) in a 1900 TR liquid scintillation analyzer (Packard, Meridian, MS). The radioactivity taken up in the inside-out vesicles was examined with thin-layer chromatography as described previously (Sager et al., 1996) and comprised a single band with a $R_f$ value identical with the native radioisotope.

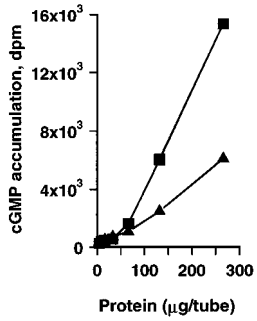


FIGURE 1: The effect of inside-out vesicle protein concentrations on cGMP uptake. Inside-out vesicles were incubated for 120 min with 1 $\mu$M [$^3$H]cGMP in the presence (■) and absence of 2 mM ATP (▲) at 37 °C with varying vesicle protein concentrations (5−270 $\mu$g/tube). The results are given as mean value of two separate experiments in duplicate.

*Sidedness.* The orientation of the vesicles was determined by using acetylcholinesterase activity for an outside plasma membrane marker and glyceraldehyde-3-phosphate dehydrogenase for an inside plasma membrane marker. The previously described assays (Steck & Kant, 1974) were slightly modified with exchange of all phosphate solutions with PBS. The acetylcholinesterase assay comprised 0.63 mM 5,5′-dithiobios-(2-nitrobenzoic acid) and 0.78 mM acetylthiocholine chloride dissolved in PBS. The glyceraldehyde-3-phosphate dehydrogenase assay comprised of 2.5 mM cysteine, 10 mM sodium arsenate, 0.8 mM $\beta$-NAD, and 1.25 mM glyceraldehyde-3-phosphate dissolved in PBS. After a 1 min preincubation in the absence or presence of Triton X-100 (0.2% v/v), the reaction (20−22 °C) was followed spectrophotometrically (Ultraspec III, Pharmacia LBK, Biochrom Ltd., Cambridge, U.K.) at 412 and 340 nm for the acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase assay, respectively. The pH optimum of glyceraldehyde-3-phosphate dehydrogenase activity is about 8.4 (Schrier et al., 1975), but the activity was determined at pH 7.4−7.6 to obtain comparable conditions in the assays of sidedness and transport.

*Protein Concentrations.* Protein concentrations were determined by the coomassie brilliant blue method (Bradford, 1976) with reagents from Bio-Rad Laboratories (Richmond, CA) and BSA from Sigma Chemical Co. (St. Louis, MO) as standards.

## RESULTS

*Preliminary Considerations of cGMP Transport: Vesicle Protein Concentration, DMSO Concentrations, and Sidedness.* Active transport, defined as the difference in accumulation when ATP was present and absent, could not be detected below 50 $\mu$g of protein/incubate in the present experimental setup (Figure 1). Above this lower limit, the active transport increased linearly with protein concentration. The protein concentrations in the experiments were in the range between 100 and 250 $\mu$g/incubate.

The substances which were tested for their ability to affect cGMP transport were dissolved in DMSO, and the possibility that the solvent affected cGMP membrane transport was examined in separate experiments. Table 1 shows that 1% v/v DMSO depressed the transport, whereas the concentra-

Table 1: Effect of DMSO on the cGMP Uptake in Inside-Out Vesicles from Human Erythrocytes[a]

| % DMSO (v/v) | +ATP (% of control) | −ATP (% of control) | active uptake (% of control) |
|---|---|---|---|
| 0.05 | 102 ± 1 | 100 ± 2 | 103 ± 2 |
| 0.1 | 100 ± 3 | 100 ± 6 | 101 ± 3 |
| 0.3 | 98 ± 3 | 101 ± 5 | 99 ± 4 |
| 0.5 | 96 ± 1 | 93 ± 4 | 97 ± 1 |
| 1.0 | 81 ± 4 | 89 ± 3 | 76 ± 5 |

[a] The vesicles were incubated for 120 min at 37 °C in the presence or absence of 2 mM ATP with various DMSO concentrations. In the absence of DMSO the cGMP uptake was 45.7 ± 0.7 and 14.7 ± 2.9 dpm/$\mu$g protein in the presence and absence of ATP, respectively. The active uptake was 30.4 ± 2.6 dpm/$\mu$g protein. The results are shown as mean value ± SEM, $n = 4$.

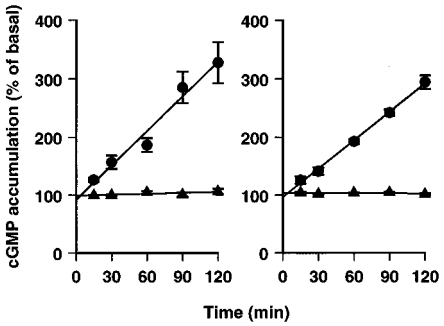


FIGURE 2: Effect of nonhydrolyzable ATP-analogues on cGMP uptake. The time course of [$^3$H]cGMP uptake was determined in the absence and presence of 2 mM ATP, ATP-$\gamma$-S, and AMP-PNP. (Left panel) ATP (●) and ATP-$\gamma$-S (▲). (Right panel) ATP (●) and AMP-PNP (▲). The results are presented as accumulation of cGMP given as percent of the uptake in the absence of ATP. The results are given as mean ± SEM, $n = 5$ for ATP-$\gamma$-S and $n = 3$ for AMP-PNP.

tions used in the present study (0.5% v/v or below) did not influence the cGMP uptake.

The sidedness of the employed vesicles was verified by using markers for the cell membrane ectoside (acetylcholinesterase activity) and the cell membrane endoside (glyceraldehyde-3-phosphate dehydrogenase activity). The respective activities of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase were 9−12% and 67−72% of that in the presence of Triton X-100. These values are in close correspondence with previously reported results for human erythrocyte inside-out vesicles (Steck & Kant, 1974).

*Effect of Nonhydrolyzable ATP-Analogues on cGMP Uptake.* The observation that cGMP accumulated linearly in the presence of ATP with a model of inside-out vesicles (Sager et al., 1996) suggested that the transport system comprised of an ATPase. In the present study, a linear accumulation was observed with ATP, but in contrast, no uptake was evident with the nonhydrolyzable ATP-analogues, neither ATP-$\gamma$-S nor AMP-PNP (Figure 2).

This demonstrates that hydrolysis of ATP is needed for cGMP to be transported into the vesicles.

*Effect of Probenecid on cGMP Uptake.* Probenecid is a well-known inhibitor of anion transport, and previous studies on intact cells have demonstrated a concentration-dependent inhibition of cellular cGMP egression (Tjörnhammar et al., 1983, 1986; Flo et al., 1995; Patel et al., 1995; Millul et al., 1996). However, the effect of probenecid on cGMP uptake to inside-out vesicles has not been determined previously.

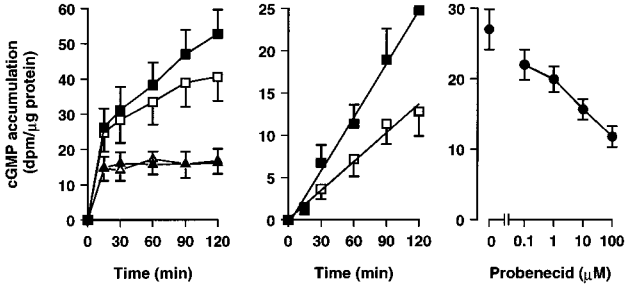


FIGURE 3: Effects of probenecid on cGMP uptake. Inside-out vesicles were incubated with 1 $\mu$M [$^3$H]cGMP at 37 °C. (Left panel) Time course of total cGMP uptake, in the presence of 2 mM ATP without (■) and with 100 $\mu$M probenecid (□), and the uptake in the absence of ATP without (▲) and with 100 $\mu$M probenecid (△), $n = 7$. (Middle panel) Time course of ATP-sensitive cGMP uptake without (■) and with 100 $\mu$M probenecid (□), $n = 7$. (Right panel) Concentration-dependent effect on ATP-sensitive cGMP uptake in the presence of increasing probenecid concentrations (0.1−100 $\mu$M) after 120 min incubation, $n = 7$. The results are given as mean ± SEM.

The present study shows that the active transport is sensitive to probenecid, whereas the ATP-independent uptake is insensitive (Figure 3, left panel). The time course of cGMP uptake in inside-out vesicles shows an instantaneous effect with a constant fractional inhibition up to 120 min (Figure 3, middle panel). Probenecid reduced the activity of the cGMP pump in a concentration-dependent manner (Figure 3, right panel).

*Effect of Verapamil and Progesterone on cGMP Uptake.* In a study of intact human erythrocytes verapamil inhibited, whereas progesterone apparently stimulated, the export of cGMP (Flo et al., 1995). The ATP-independent cGMP uptake, in the present study, was not influenced of these substances (results not shown). In the present study, with inside-out vesicles from human erythrocytes, both verapamil and progesterone reduced the active transport. The inhibition was seen instantly with a constant fractional inhibition throughout the accumulation period (Figure 4, left panels). Both substances inhibited the active uptake in a concentration-dependent manner (Figure 4, right panels) with progesterone the most potent inhibitor (Tables 2 and 3).

*Effect of Forskolin on cGMP Uptake.* The presence of forskolin has been reported to depress cellular cGMP efflux (Hamet et al., 1989; Patel et al., 1995). This effect was explained by an indirect mechanism where the elevated cAMP levels after forskolin-activated adenylate cyclase competitively inhibited the cGMP export. In the present study, a direct inhibitory effect of forskolin was observed. During the accumulation period of 120 min, a constant fractional depression was seen and the forskolin inhibited the active cGMP uptake in a concentration-dependent way (Figure 5) with similar affinity as probenecid and verapamil (Tables 2 and 3). The substance had no effect on cGMP uptake in the absence of ATP (results not shown).

*Competitive and Noncompetitive Inhibition of Active cGMP Uptake.* In an attempt to determine the mode of inhibition, a series of experiments with cGMP concentrations from 0.5 to 5 $\mu$M was performed in the absence and presence of ATP and in the absence and presence of 100 $\mu$M of probenecid, verapamil, progesterone, and forskolin. Lineweaver−Burk plots showed that probenecid, verapamil, and forskolin inhibited the active cGMP uptake by a

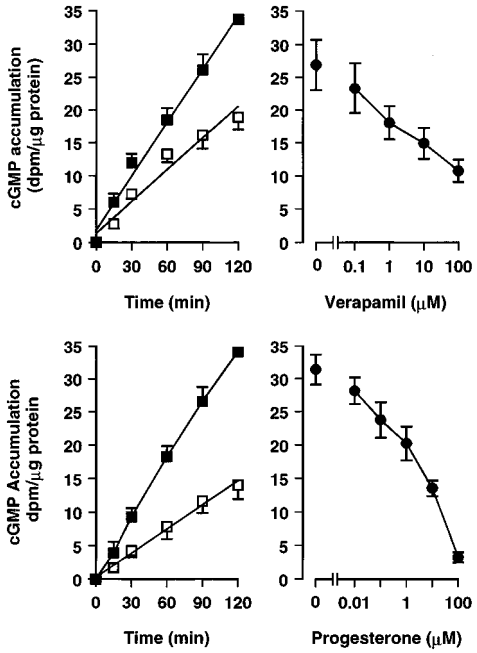


FIGURE 4: The effect of verapamil and progesterone on active cGMP transport. Inside-out vesicles were incubated with 1 $\mu$M [$^3$H]cGMP at 37 °C in the absence or presence of modulator. (Left panels) Time course of ATP-sensitive cGMP uptake. (Upper panel) Control (■) and 100 $\mu$M verapamil (□), $n = 6$. (Lower panel) Control (■) and 10 $\mu$M progesterone (□), $n = 8$. (Right panels) The effect of increasing the concentrations of modulators on ATP-sensitive cGMP uptake after 120 min incubation. (Upper panel) Verapamil (0.1−100 $\mu$M), $n = 8$. (Lower panel) Progesterone (0.01−100 $\mu$M), $n = 8$. The results are given as mean ± SEM.

Table 2: Calculated $K_i$ Values for Inhibition of Low $K_m$ ATP-dependent [$^3$H]cGMP (1 $\mu$M) Uptake in the Presence of Various Concentrations of the Tested Substances for 120 min at 37 °C[a]

| substance | $K_i$ ($\mu$M) |
|---|---|
| probenecid, $n = 7$ | 28.6 ± 1.4 |
| verapamil, $n = 8$ | 13.8 ± 2.5 |
| progesterone, $n = 8$ | 2.3 ± 0.3 |
| forskolin, $n = 8$ | 14.2 ± 1.5 |

[a] The $IC_{50}$ values were obtained according to Chou (1976) and corrected according to Cheng and Prusoff (1973) using the $K_m$ value obtained in the present study (Table 4). The results are given as mean ± SEM.

Table 3: Order of Inhibitory Potency[a]

| substance | uptake (% of control) |
|---|---|
| progesterone, $n = 8$ | 8.0 ± 1.0 |
| verapamil, $n = 13$ | 45.8 ± 2.8 |
| forskolin, $n = 3$ | 46.0 ± 1.0 |
| probenecid, $n = 14$ | 47.6 ± 2.8 |
| IBMX, $n = 4$ | 70.5 ± 1.9 |
| theophylline, $n = 6$ | 78.4 ± 2.1 |
| cAMP, $n = 3$ | 85.6 ± 0.3 |

[a] The effect of 100 $\mu$M progesterone, verapamil, forskolin, probenecid, IBMX, and theophylline on active transport of [$^3$H]cGMP was determined at 37 °C after 120 min. The results are given as mean ± SEM.

competitive interaction, whereas progesterone affected the transport by a noncompetitive interaction (Figure 6 and Table 4).

*Effect of the Methylxanthines on cGMP Uptake.* Theophylline and IBMX were the least potent inhibitors among the tested exogenous substances in the present study (Table 3). Both substances were without effect on the ATP-independent uptake, whereas the active transport was reduced in a concentration-dependent way (data not shown). The active transport of cGMP was 31.8 ± 2.9 to 25.0 ± 2.4 dpm/$\mu$g protein (mean ± SEM, $n = 6$) in the absence and presence of 100 $\mu$M theophylline and 26.4 ± 0.9 to 18.6 ± 0.7 dpm/$\mu$g protein (mean ± SEM, $n = 4$) in the absence and presence of 100 $\mu$M IBMX, respectively.

*Effect of cAMP on cGMP Uptake.* To determine whether cAMP shared the low $K_m$ transport system with cGMP, two sets of inhibition studies were carried out. In the first experiments, we tested a narrow range of cAMP concentrations (1−8 $\mu$M), where inhibition would be expected to occur if the extrusion pump had similar $K_m$ for cAMP and cGMP.

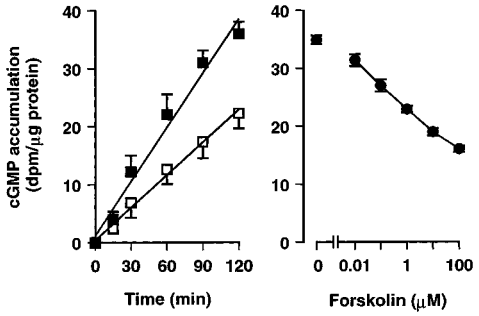


FIGURE 5: The effect of forskolin on active cGMP transport. Inside-out vesicles were incubated with 1 $\mu$M [$^3$H]cGMP at 37 °C in the absence or presence of forskolin. (Left panel) Time course of ATP-sensitive cGMP uptake: control (■) and 10 $\mu$M forskolin (□), $n = 5$. (Right panel) The effect of 0.1−100 $\mu$M forskolin ($n = 8$) on ATP-sensitive cGMP uptake after 120 min incubation. The results are given as mean ± SEM.

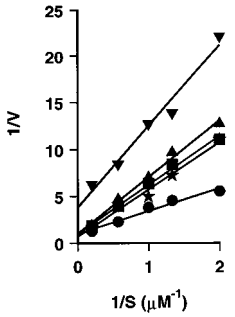


FIGURE 6: Lineweaver−Burk plots of active cGMP uptake in the absence (●) and presence of probenecid (■), verapamil (▲), progesterone (▼), and forskolin (★). Inside-out vesicles were incubated with radioligand and competitor for 120 min at 37 °C. The $V_{max}$ of probenecid, verapamil, progesterone, and forskolin is given relative to the $V_{max}$ of control. The absolute vales are given in Table 4. The results are given as mean value, $n = 3$.

Table 4: Apparent $K_m$ and $V_{max}$ Values Obtained from Competition Studies Where [$^3$H]cGMP (0.5−5 $\mu$M) Was Incubated with and without 100 $\mu$M Probenecid, Verapamil, Forskolin, and Progesterone for 120 min at 37 °C[a]

| competitor | apparent $K_m$ ($\mu$M) | apparent $V_{max}$ (fmol/mg of protein/min) |
|---|---|---|
| none | 2.4 ± 0.2 | 170 ± 46 |
| probenecid | 5.1 ± 0.6 | 167 ± 43 |
| verapamil | 6.1 ± 0.7 | 169 ± 43 |
| progesterone | 2.2 ± 0.1 | 46 ± 2 |
| forskolin | 4.7 ± 0.7 | 176 ± 48 |

[a] The results are given as mean value ± SEM, $n = 3$.

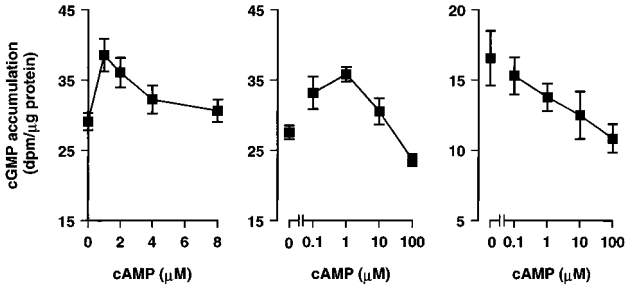


FIGURE 7: The effect of cAMP on cGMP uptake. Inside-out vesicles were incubated with 1 $\mu$M [$^3$H]cGMP at 37 °C in the absence or presence of cAMP for 120 min. (Left panel) The effect of 1−8 $\mu$M cAMP on ATP-sensitive cGMP uptake ($n = 4$). (Middle panel) The effect of 0.1−100 $\mu$M cAMP on ATP-sensitive cGMP uptake ($n = 3$). (Right panel) The effect of 0.1−100 $\mu$M cAMP ($n = 3$) on ATP-independent cGMP uptake/association. The results are given as mean ± SEM.

However, the opposite effect was observed. The active uptake of cGMP was markedly increased (Figure 7, left panel). In the next experiments, a broader concentration range (0.1−100 $\mu$M) was tested. The stimulatory effect on active transport was verified for low cAMP concentrations (0.1−10 $\mu$M). However, only the highest cAMP concentration caused a slight inhibition (Figure 7, middle panel and Table 3). In contrast, cAMP inhibited the ATP-independent cGMP uptake in a concentration-dependent manner (Figure 7, right panel). The estimated concentration ($IC_{50}$) needed to reduce the ATP-independent uptake with 50% was estimated to be about 2−4 mM.

## DISCUSSION

In a recent study, we observed an ATP-sensitive uptake of cGMP in inside-out vesicles from human erythrocytes (Sager et al., 1996). The present results extend this observation. In the presence of nonhydrolyzable ATP-analogues, the time-dependent accumulation of cGMP was absent. This is compatible with a tight coupling between cGMP transport and ATPase activity, and it is possible that the cGMP pump is a member of the superfamily of ABC-transporters [for review, see Ames et al. (1992)].

Sparse information exists about effects of exogenous compounds on the cGMP pump activity. Probenecid has been employed in numerous studies of tissues and intact cells, including nerve tissue (O'Dea et al., 1978; Tjörnhammar et al., 1986), liver tissue (Tjörnhammar et al., 1983), smooth muscle cells (Hamet et al., 1989), transformed renal cells (Millul et al., 1996), fibroblasts and kidney cells (Patel et al., 1995), erythrocytes (Flo et al., 1995), and transformed cells from the uterine cervix (Ørbo et al., 1995). In agreement with the previous studies, probenecid inhibited the cGMP extrusion in a concentration-dependent mode. However, the effect of probenecid has not, to our knowledge, been tested in inside-out vesicles before. The finding of competitive inhibition suggests that the interaction occurs at the transport recognition site at the endoside of the membrane. This may also explain why the inhibitory constants are more than 10-fold higher in intact cells where probenecid was applied to the ectoside (Patel et al., 1995; Millul et al., 1996).

Verapamil has also been shown to inhibit cGMP efflux, whereas progesterone appeared to enhance cGMP extrusion from normal and transformed cells (Flo et al., 1995; Ørbo et al., 1995). In the present experimental model, verapamil was slightly more potent than probenecid. Thus, verapamil appears to be a nonselective inhibitor of various membrane transport systems such as P-glycoprotein (Gottesman & Pastan, 1993) and cAMP transport (Henderson & Strauss, 1991).

Considerable discrepancy exists among the reported ability of methylxanthines to depress cGMP egression. In rat mesenteric smooth muscle, IBMX (100 $\mu$) had no effect on basal cGMP excretion, but after stimulation with ANP, the extracellular levels were reduced with about 20% (Hamet et al., 1989). In contrast, two studies with transformed cells reported a reduction with about 60 and 80% in the presence of theophylline (1 mM) and IBMX (1 mM), respectively (Ørbo et al., 1995; Millul et al., 1996). Methylxanthines appear to depress cAMP efflux through adenosine receptors (Fehr et al., 1990). If the same mechanism applies to their effect on the cellular cGMP export, an overexpression of these receptors in transformed cells may account for the more pronounced inhibition.

However, in the present model, application of theophylline (100 $\mu$M) and IBMX (100 $\mu$M) to the cellular endoside, being virtually inaccessible to potential adenosine receptors, caused a reduction in active cGMP uptake of about 20 and 30%, respectively. This shows that methylxanthines at least exert some of their effect by a direct interaction with the transport protein.

In previous studies, we found that progesterone increased extracellular cGMP levels in studies of intact human erythrocytes and human cells derived from carcinoma of the uterine cervix (Flo et al., 1995; Ørbo et al., 1995). Those observations agreed with the finding of elevated levels of cGMP in plasma and urine sampled from women in the luteal phase of the menstrual cycle (Lebeau et al., 1975 Turner et al., 1982). However, in striking contrast, progesterone appeared to be a very potent inhibitor of active cGMP uptake when applied to the endoside of the cell membrane. In agreement with the present finding, progesterone is also a potent inhibitor of P-glycoprotein (Gottesman & Pastan, 1993), but is not itself a substrate for the pump (Ueda et al., 1992). This fits with the present results which showed a noncompetitive inhibition of the cGMP transport. The apparent discrepancy between the progesterone as a stimulator of cGMP-efflux in intact cells and inhibitor in inside-out vesicles is puzzling. However, a similar adversative role of progesterone has been reported for P-glycoprotein (Jancis et al., 1993). In transformed cells that overexpressed P-glycoprotein, progesterone stimulated rhodamine 123 efflux. Certainly, a nongenomic mechanism must account for the effects on cGMP uptake in inside-out vesicles from human erythrocytes.

The issue of specificity of the cGMP pump has to some extent been addressed. Both glucagon and forskolin elevate intracellular cAMP levels due to activation of adenylate cyclase. Whereas glucagon was ineffective (Tjörnhammar et al., 1983), forskolin caused a marked reduction in the cGMP efflux (Hamet et al., 1989; Patel et al., 1995). The effects of forskolin led to the concept that cGMP and cAMP shared a transport system in the cell membrane. However, it has been shown that forskolin inhibits several transport systems (Morris et al., 1991a,b) and the possibility existed

that forskolin directly interacted with the cGMP transporter. The present results confirmed this assumption and weaken the arguments for a shared transport system for cyclic nucleotides based on the effects of forskolin in whole cells (Hamet et al., 1989; Patel et al., 1995).

The absence of inhibition in the presence of a wide range of cAMP concentrations in the present study exclude the possibility of a shared high-affinity transport system for cAMP and cGMP. Unexpectedly, we found that low cAMP concentrations (0.1−5 $\mu$M) stimulated the active cGMP uptake. This observation suggests the presence of an allosteric regulatory binding site and corresponds to the situation for some members of the cyclic nucleotide phosphodiesterase family with cGMP as an allosteric regulator (Conti et al., 1995). Recently, we reported that cAMP showed an incomplete inhibition of cGMP high-affinity binding to the putative cGMP transporter (Boadu & Sager, 1997). Together with the present findings, this is compatible with a shared high-affinity regulatory site, but not a shared transport recognition site. The weak inhibition of active transport that occurred for the highest tested and supraphysiological concentration (100 $\mu$M) may represent a shared transport system with low affinity. A previous study of human erythrocyte ghosts reported an apparent $K_m$ of 0.4−0.5 mM for cellular cAMP export (Holman, 1978). This might be identical with the high $K_m$ transport process observed for cGMP also with a $K_m$ of 170 $\mu$M (Sager et al., 1996).

In contrast to the other tested substances in this study, cAMP caused a concentration-dependent inhibition of ATP-independent uptake of cGMP. The fact that cGMP equilibrates rapidly (within 15 min) with the inside-out vesicles, observed in the present and in a former study (Sager et al., 1996), suggests that this uptake represents binding to the endoside of the membrane and not transport into the vesicles since the membrane permeability of cyclic nucleotides is extremely low. The interaction between cAMP and cGMP in the absence of ATP may reflect one of the steps in the complex cycling of the cGMP transporter, being the low affinity ("release") site recycled from the ecto- to the endoside. Such a hypothesis is only valid if ATP converts the low-affinity binding site into a high-affinity state with high degree of selectivity for cGMP ("recognition site"). However, future and comprehensive studies are obviously needed to unveil details about the structure and function of the cGMP transporter.

## ACKNOWLEDGMENT

The technical assistance by Ragnhild Jaeger and Elisabeth Sundkvist is acknowledged.

## REFERENCES

Ames, G. F., Mimura, C. S., Holbrook, S. R., & Shyamala, V. (1992) *Adv. Enzymol. Relat. Areas Mol. Biol. 65*, 1−47.

Ashman, D. F., Lipton, R., Melicow, M. M., & Price, T. D. (1963) *Biochem. Biophys. Res. Commun. 11*, 330−334.

Boadu, E., & Sager, G. (1997) *Biochemistry 36*, 10954−10958.

Boutjdir, M., Mery, P. F., Hanf, R., Shrier, A., & Fischmeister, R. (1990) *Mol. Pharmacol. 38*, 758−765.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Butcher, R. W., & Sutherland, E. W. (1962) *J. Biol. Chem. 237*, 1244−1250.

Cheng, Y.-C., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099−3108.

Chou, T.-C. (1976) *J. Theor. Biol. 39*, 253−276.

Conti, M., Jin, S.-L. C., Monaco, L., Repaske, D. R., & Swinnen, J. V. (1991) *Endocrine Rev. 12*, 218−234.

Conti, M., Nemoz, G., Sette, C., & Vicini, E. (1995) *Endocrine Rev. 16*, 370−389.

Fehr, T. F., Dickinson, E. S., Goldman, S. J., & Slakey, L. L. (1990) *J. Biol. Chem. 265*, 10974−10980.

Flo, K., Hansen, M., Ørbo, A., Kjorstad, K. E., Maltau, J. M., & Sager, G. (1995) *Scand. J. Clin. Lab. Invest. 55*, 715−721.

Gottesman, M. M., & Pastan, I. (1993) *Annu. Rev. Biochem. 62*, 385−427.

Hamet, P., Pang, S. C., & Tremblay, J. (1989) *J. Biol. Chem. 264*, 12364−12369.

Henderson, G. B., & Strauss, B. P. (1991) *J. Biol. Chem. 266*, 1641−1645.

Holman, G. D. (1978) *Biochim. Biophys. Acta 508*, 174−183.

Jancis, E. M., Chen, H. X., Carbone, R., Hochberg, R. B., & Dannies, P. S. (1993) *Biochem. Pharmacol. 46*, 1613−1619.

Joost, H. G., & Steinfelder, H. J. (1987) *Mol. Pharmacol. 31*, 279−283.

Kapoor, C. L., & Krishna, G. (1977) *Science 196*, 1003−1005.

Lebeau, M., Dumont, J. E., & Golstein, J. (1975) *Horm. Metab. Res. 7*, 190−194.

Manganiello, V. C., Murata, T., Taira, M., Belfrage, P., & Degerman, E. (1995) *Arch. Biochem. Biophys. 322*, 1−13.

Millul, V., Prie, D., GeniteauLegendre, M., Verpont, M. C., Baudouin, B., & Ronco, P. M. (1996) *Am. J. Physiol.-Cell Physiol. 39*, C1051−C1060.

Morris, D. I., Robbins, J. D., Ruoho, A. E., Sutkowski, E. M., & Seamon, K. B. (1991a) *J. Biol. Chem. 266*, 13377−13384.

Morris, D. I., Speicher, L. A., Ruoho, A. E., Tew, K. D., & Seamon, K. B. (1991b) *Biochemistry 30*, 8371−8379.

O'Dea, R. F., Gagnon, C., & Zatz, M. (1978) *J. Neurochem. 31*, 733−738.

Ørbo, A., Kjørstad, K. E., Jaeger, R., & Sager, G. (1995) *Int. J. Oncol. 6*, 1279−1282.

Patel, M. J., Wypij, D. M., Rose, D. A., Rimele, T. J., & Wiseman, J. S. (1995) *J. Pharmacol. Exp. Ther. 273*, 16−25.

Sager, G., Ørbo, A., Pettersen, R. H., & Kjørstad, K. E. (1996) *Scand. J. Clin. Lab. Invest. 56*, 289−293.

Schrier, S. L., Junga, I., & Johnson, M. (1975) *Life Sci. 17*, 735−738.

Sonnenburg, W. K., & Beavo, J. A. (1994) *Adv. Pharmacol. 26*, 87−114.

Steck, T. L., & Kant, J. A. (1974) in *Biomembranes* (Fleischer, S., & Packer, L., Eds.) Vol. 31, pp 172−180, Academic Press, New York.

Steck, T. L., Weinstein, R. S., Straus, J. H., & Wallach, D. F. H. (1970) *Science 168*, 255−257.

Thompson, W. J. (1991) *Pharmacol. Ther. 51*, 13−33.

Tjörnhammar, M.-L., Lazaridis, G., & Bartfai, T. (1983) *J. Biol. Chem. 258*, 6882−6886.

Tjörnhammar, M.-L., Lazaridis, G., & Bartfai, T. (1986) *Neurosci. Lett. 68*, 95−99.

Turner, G. A., Ellis, R. D., Guthrie, D., Latner, A. L., Monaghan, J. M., Ross, W. M., Skillen, A. W., & Wilson, R. G. (1982) *J. Clin. Pathol. 35*, 800−806.

Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T., & Hori, R. (1992) *J. Biol. Chem. 267*, 24248−24252.

BI9713409