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Leukotriene C_4 (LTC_4) does not share a cellular efflux mechanism with cGMP: characterisation of cGMP transport by uptake to inside-out vesicles from human erythrocytes

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Abstract

The transport of cGMP out of cells is energy requiring and has characteristics compatible with an ATP-energised anion pump. In the present study a model with inside-out vesicles from human erythrocytes was employed for further characterisation of the cGMP transporter. The uptake of leukotriene C_4 (LTC_4), a substrate for multidrug resistance protein (MRP), was concentration-dependently inhibited by the leukotriene antagonist MK571 ($IC_{50} = 110 \pm 20$ nM), but cGMP was unable to inhibit LTC_4 uptake. Oxidised glutathione (GSSG) and glutathione *S*-conjugates caused a concentration-dependent inhibition of [^{3}H]cGMP uptake with IC_{50} of 2200 ± 700 μM for GSSG, 410 ± 210 μM for *S*-(*p*-nitrobenzyl)glutathione and 37 ± 16 μM for *S*-decylglutathione, respectively. Antioxidants such as reduced glutathione and dithiothreitol did not influence transport for concentrations up to 100 μM, but both inhibited cGMP uptake with approx. 25% at 1 mM. The cGMP pump was sensitive to temperature without activity below 20°C. The transport of cGMP was dependent on pH with maximal activity between pH 8.0 and 8.5. Calcium caused a concentration-dependent inhibition with IC_{50} of 43 ± 12 μM. Magnesium gave a marked activation in the range between 1 and 20 mM with maximum effect at 10 mM. The other divalent cations, Mn^{2+} and Co^{2+}, were unable to substitute Mg^{2+}, but caused some activation at 1 mM. EDTA and EGTA stimulated cGMP transport concentration-dependently with 50% and 100% above control at 100 μM, respectively. The present study shows that the cGMP pump has properties compatible with an organic anion transport ATPase, without affinity for the MRP substrate LTC_4. However, the blockade of the cGMP transporter by glutathione *S*-conjugates suggests it is one of several GS-X pumps.

Keywords: Cyclic guanosine monophosphate; Leukotriene C_4; Glutathione; GS-X pump; Membrane transport

1. Introduction

Cellular efflux of cGMP is effected by a specialised transport system. Secretion of cGMP has been shown to be unidirectional, to occur against a gradient of high levels of extracellular cGMP [1–4] and to be temperature-sensitive [1,5,6]. These results are compatible with an energy-requiring transport system. Furthermore, it has been shown that cGMP transport was dependent on ATP [7], ATP hydrolysis [8] and inhibited by well-known ATPase inhibitors [9]. Several studies which have shown that probene-

Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidised glutathione; LTC_4, leukotriene C_4; MRP, multidrug resistance protein; SDG, *S*-decylglutathione; SPNBG, *S*-(*p*-nitrobenzyl)glutathione

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cid inhibits cGMP efflux [1,5,6,8,10–14] suggest that the cGMP pump is an anion transporter. This is a property in common with MRP (multidrug resistance protein) [15]. The 190 kDa MRP has been associated with the transport of cysteinyl leukotrienes, such as LTC_4 (leukotriene C_4), and several glutathione *S*-conjugates [16]. MRP has been detected in human erythrocyte membranes by the use of monoclonal antibodies [17] and identified in these cells as the glutathione *S*-conjugate export pump [18]. However, the expansion of the MRP family makes the comprehension of GS-X pumps more complex [19].

The present study was undertaken for further characterisation of the active cGMP transport: first, to determine whether LTC_4, GSSG (oxidised glutathione), SDG (*S*-decylglutathione), SPNBG (*S*-(*p*-nitrobenzyl)glutathione) and cGMP have a common ATP-dependent transport system; second, to examine the effect of the antioxidants GSH (reduced glutathione) and DTT (dithiothreitol) on cGMP transport; third, to study how temperature, pH and divalent ions (Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+}) and chelators (EDTA and EGTA) affect the uptake of cGMP to inside-out vesicles from human erythrocytes.

2. Materials and methods

2.1. Chemicals

[^{3}H]cGMP (spec. act. 16 Ci/mmol) and [^{3}H]LTC_4 (spec. act. 115 Ci/mmol) were purchased from Amersham International (Buckinghamshire, UK) and Du Pont NEN (Boston, MA), respectively. EGTA, EDTA, DTT, GSH, GSSG, SPNBG, SDG and ATP were all obtained from Sigma (St. Louis, MO). MK571 was a kind gift from Merck Sharp and Dome (Drammen, Norway). Other chemicals were of analytical grade.

2.2. Buffers

The following buffers were employed for preparation and incubation of inside-out vesicles: buffer A: 3 mM KCl, 110 mM NaCl and 5 mM Tris-HCl (pH 8.0–8.2); buffer B: 3 mM KCl and 5 mM Tris-HCl (pH 8.0–8.2); buffer C: 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 (pH 7.3–7.4).

2.3. Blood sampling and preparation of inside-out vesicles

Inside-out vesicles were prepared from fresh human blood and all procedures were performed at 4°C. Venous blood (10 ml) was drawn into EDTA vacuum tubes (Vacutainer, Becton Dikinson, Meyland Cedex, France). The cells were separated from plasma by centrifugation ($1000\times g$ for 15 min), washed four times with buffer A, the last one with 4 ml packed cells in 20 ml buffer. The cells were lysed in buffer B (4 ml packed cells in 20 ml buffer). The ghosts were sedimented ($20\,000\times g$ for 10 min) before resuspension in buffer B (1 ml in 20 ml). This procedure was repeated until the membranes appeared milky white with a clear supernatant. The membrane vesiculation was initiated when the ghost pellet (1 ml) was resuspended in 0.5 mM Tris-HCl (1 ml), pH 8.0–8.2, and left for 2 h at 0–4°C. The membranes were sedimented ($100\,000\times g$ for 30 min), then mixed with 0.5 mM Tris-HCl (1 ml), pH 8.0–8.2, and passed five times through a 20 mm long 27-gauge needle. The mixture of vesicles and ghosts were diluted with another 1 ml of 0.5 mM Tris-HCl, pH 8.0–8.2 and layered above a linear gradient with densities from 1.05 to 1.15 g/ml with Nycodenz (Nycomed Pharma, Oslo, Norway). The vesicles and ghosts were the separated during centrifugation for 16–17 h at $100\,000\times g$ at 0–4°C. The layer with the inside-out vesicles was washed with 40 vols. of buffer C, sedimented at $28\,000\times g$ for 30 min and finally resuspended in 0.7–0.8 ml buffer C.

2.4. Uptake studies

The experiments were performed at 37°C (if not otherwise stated) with 50–300 μg protein in 400 μl buffer C, pH 7.4 (if not otherwise stated), including 10 mM $MgCl_2$ (if not otherwise stated), 1 μM [^{3}H]cGMP, with or without 1 mM ATP. The uptake of radioligand was terminated by addition of 10 ml ice-cold 30 mM NaF/0.5 mM Tris-HCl. After centrifugation at $16\,300\times g$ the vesicles were washed once with another 10 ml of this mixture, and after a final centrifugation mixed with 1 ml ice-cold water and

frozen. After 18 h at −20°C, the mixture was thawed, transferred to Eppendorf tubes and centrifuged at 15 000×*g* for 30 min. The radioactivity was determined in 800 μl supernatant transferred to 10 ml Ultima Gold XR (Packard, Groningen, The Netherlands) in a 1900 TR liquid scintillation analyser (Packard, Meridian, MS).

2.5. Radiochemical purity

The radioactivity taken up in the inside-out vesicles was examined with thin-layer chromatography as previously described [7]. It showed a single band with identical R_f value as the native radioisotope.

2.6. Sidedness

The orientation of the vesicles was ensured using acetylcholinesterase activity as a plasma membrane outside marker as reported recently [8].

2.7. Protein concentrations

Protein concentrations were determined by the Coomassie blue method [20] with reagents from Bio-Rad laboratories (Richmond, CA) and BSA from Sigma as standards.

2.8. Statistics

Paired *t*-tests were performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effect of MK571 and cGMP on [^{3}H]LTC$_4$ transport

LTC_4 transport was inhibited by the LTD_4 receptor antagonist MK571 in a concentration-dependent mode (Fig. 1). Under these experimental conditions the concentration (IC_{50}) that reduced LTC_4 uptake to 50% was determined according to Chou [21]. The uptake was defined as the difference in the amount LTC_4 associated with the vesicles in the presence and absence of 1 mM ATP. The analysis gave an IC_{50} value of 110 ± 20 nM (mean ± S.E.M., $n = 3$). For concentrations up to 10 μM cGMP was unable to inhibit LTC_4 uptake (Fig. 1). At 100 μM the transport was reduced to 91 ± 2% of control (mean ± S.E.M., $n = 3$, $P < 0.05$ paired two-tailed *t*-test).

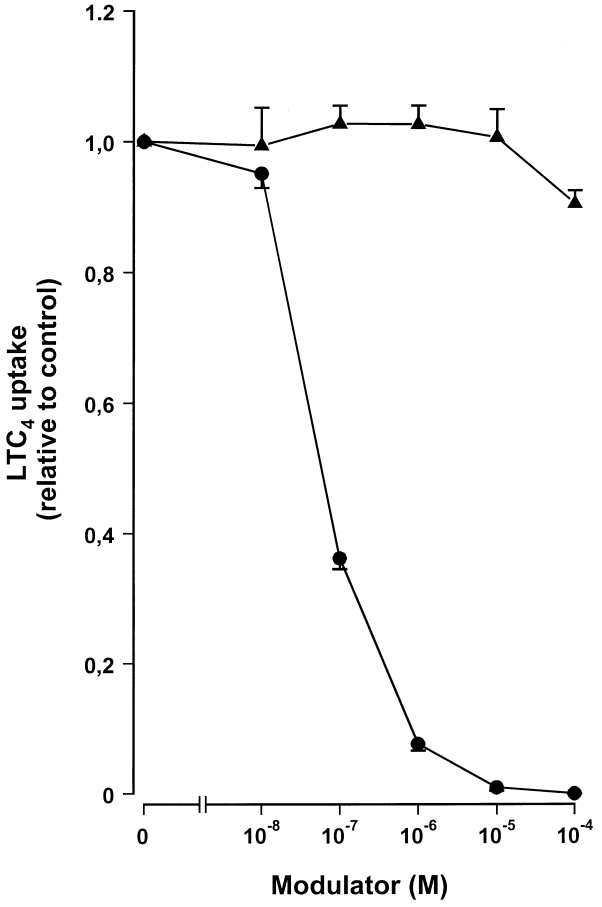

Fig. 1. The ability of cGMP and MK571 to inhibit [^{3}H]LTC_4 uptake to inside-out vesicles after 120 min co-incubation at 37°C. Concentrations of MK571 (●) and cGMP (▲) from 0.01 to 100 μM were added together with 1 μM [^{3}H]cGMP with or without 1 mM ATP. The results are presented as mean ± S.E.M. ($n = 3$).

3.2. Effect of GSSG, SPNBG and SDG on [^{3}H]cGMP transport

The ability of GSSG and glutathione *S*-conjugates to inhibit the uptake of cGMP was investigated. The uptake was defined as the difference in the amount of cGMP associated with the vesicles in the presence and absence of 1 mM ATP. Fig. 2 shows that

GSSG had no marked effect below 100 μM, but from 100 μM to 1 mM, the uptake was reduced from 87 ± 3% to 53 ± 9% of control (mean ± S.E.M., $n = 8$). Two glutathione *S*-conjugates were also tested for their ability to reduce cGMP uptake. SPNBG and SDG were more potent inhibitors than GSSG (Fig. 2). Under these experimental conditions the concentration (IC_{50}) that reduced uptake to 50% was determined according to Chou [21]. The IC_{50} values (mean ± S.E.M.) were 2200 ± 700 μM ($n = 8$) for GSSG, 410 ± 210 μM ($n = 4$) for SPNBG and 37 ± 16 μM for SDG ($n = 4$).

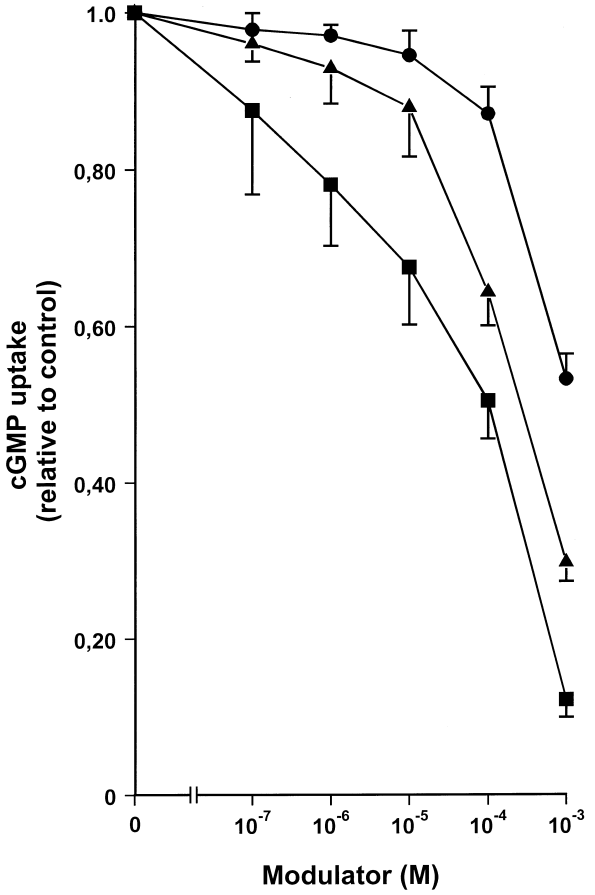

Fig. 2. The effect of GSSG, SPNBG and SDG on [^{3}H]cGMP on the uptake of cGMP to inside-out vesicles after 120 min co-incubation at 37°C. Concentrations of GSSG (●), SPNB-glutathione (▲) and SD-glutathione (■) from 0.1 μM to 1 mM were added together with 1 μM [^{3}H]cGMP with or without 1 mM ATP. The results are presented as mean ± S.E.M. ($n = 4$).

Table 1
Effect of antioxidants on [^{3}H]cGMP uptake to inside-out vesicles

Conc. (μM)	Uptake of [^{3}H]cGMP (% of control)	
	GSH	DTT
1	98 ± 3 ($n = 3$)^{ns}	95 ± 3 ($n = 3$)^{ns}
10	95 ± 2 ($n = 3$)^{ns}	92 ± 4 ($n = 3$)^{ns}
100	86 ± 2 ($n = 7$)^{ns}	90 ± 4 ($n = 4$)^{ns}
1000	75 ± 2 ($n = 4$)**	76 ± 5 ($n = 5$)*

The inside-out vesicles were co-incubated with [^{3}H]cGMP and GSH or DTT for 120 min at 37°C. The results are presented as mean ± S.E.M.
*Paired *t*-test, two-tailed *P*-value: $^{ns}P > 0.05$, $*P < 0.025$, $**P < 0.001$.

3.3. Effect of antioxidants on [^{3}H]cGMP transport

GSH and DTT showed a concentration-dependent reduction in the uptake (Table 1). However, a significant reduction in the transport of cGMP was seen only for the highest tested concentration (1 mM).

3.4. Effect of temperature on [^{3}H]cGMP transport and binding

We studied the temperature sensitivity of cGMP uptake for temperatures ranging from 0–4°C to 37°C. The uptake increased linearly with temperature above 20°C and no uptake occurred below this temperature (Fig. 3). At 15°C there was no difference in the association in the presence and absence of ATP (Table 2).

The binding of cGMP to the inside-out vesicles (association in the absence of ATP) reached an equilibrium within 15 min for temperatures between 15 and 37°C. At 0–4°C a slower association was observed (results not shown). The binding of cGMP (association to inside-out vesicles in the absence of ATP) in the absence of ATP was not dependent on temperature (Table 2). After 120 min incubation at 0–4°C, ATP apparently inhibited the binding of cGMP (Table 2).

3.5. Effect of pH on [^{3}H]cGMP transport

The present data demonstrate that the cGMP uptake was pH-sensitive with a maximal accumulation

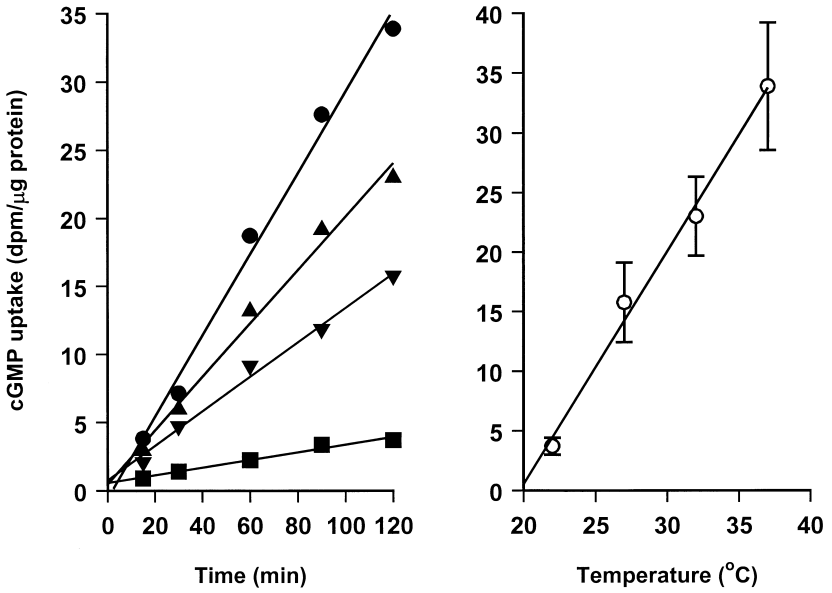

Fig. 3. The effect of temperature on the uptake of cGMP to inside-out vesicles from 15 to 120 min. The vesicles were incubated with 1 μM [^{3}H]cGMP with and without 1 mM ATP. Left panel: accumulation of cGMP at 22°C (■, $n=4$), 27°C (▼, $n=6$), 32°C (▲, $n=4$) and 37°C (●, $n=6$) is presented as mean values. Right panel: correlation between uptake and temperature after 120 min incubation, $y=a*x+b$ where $a=1.95$ and $b=-38.6$, $r=0.996$, $P<0.005$. The regression line intercepts with the abscissa at 19.7°C.

at pH 8.0–8.5 (Fig. 4). The amount of cGMP taken up after 120 min at pH 7.5 was 43.3 ± 9.8 dpm/μg protein (mean ± S.E.M., $n=6$). The uptake at pH 7.1 and 8.0 was 27 ± 8% ($P<0.05$) lower and 24 ± 5% higher ($P<0.05$) than that observed at pH 7.5 (mean ± S.E.M., $n=6$). The difference between the uptake at pH 8.0 and 8.5 was not statistically significant ($P>0.05$). The cGMP binding was unaffected by pH ($P>0.05$ compared to the value at pH 7.5) with the exception of a marked reduction at pH 6 (approx. 85% below the value at pH 7.5, data not shown).

3.6. *Effect of divalent ions on [^{3}H]cGMP transport and binding*

Calcium inhibited the transport of cGMP in a concentration-dependent manner at 37°C (Fig. 5).

Under these experimental conditions the concentration (IC_{50}) that reduced uptake to 50% was determined according to Chou [21] and gave a value of 43 ± 12 μM (mean ± S.E.M., $n=9$). Calcium had no concentration-dependent effects on the binding of cGMP at 37°C or at 0–4°C (results not shown).

Fig. 6 (left panel) shows that the divalent cations

Table 2
Association of [^{3}H]cGMP (1 μM) to inside-out vesicles in the presence and absence of ATP (1 mM) at 15 and 4°C after 15 and 120 min co-incubation

Temperature	Binding/uptake of cGMP (dpm/μg protein)			
	After 15 min		After 120 min	
	+ATP	−ATP	+ATP	−ATP
37°C[a]	11.5 ± 0.7	7.9 ± 1.6**	41.8 ± 4.5	7.9 ± 1.6**
15°C[b]	8.2 ± 0.8	8.1 ± 1.2[ns]	8.4 ± 1.1	8.1 ± 1.3[ns]
0–4°C[b]	6.3 ± 0.9	7.4 ± 0.4[ns]	7.5 ± 0.9	8.4 ± 0.7*

The results are presented as mean ± S.E.M.
Difference between association with and without ATP. Paired t-test with two-tailed P-value: [ns]$P>0.5$, *$P<0.02$, **$P<0.005$.
[a]Six separate experiments.
[b]Five separate experiments.

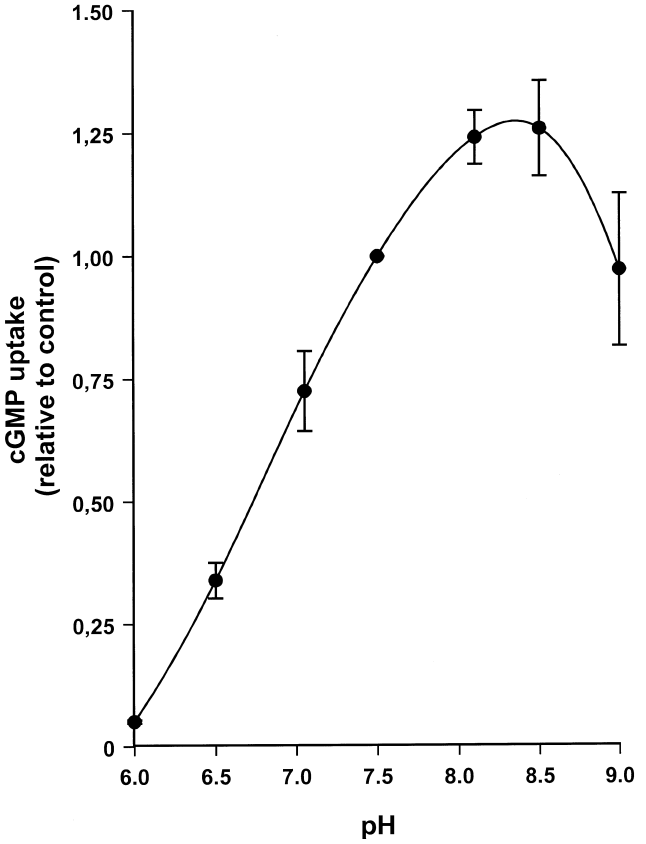

Fig. 4. The effect of pH on the uptake of cGMP to inside-out vesicles after 120 min incubation at 37°C. The vesicles were incubated with 1 μM [^{3}H]cGMP with and without 1 mM ATP. Accumulation of cGMP is given relative to the values obtained at pH 7.4 (mean ± S.E.M., $n = 6$).

Mg^{2+}, Mn^{2+} and Co^{2+} were activators of the cGMP uptake, but with a different profile. Magnesium was markedly more potent and showed an activation for a much broader concentration range as compared with Mn^{2+} and Co^{2+}. The maximum activation (1180 ± 400% above control, mean ± S.E.M., $n = 5$) was seen at Mg^{2+} concentrations of 8–10 mM ($P < 0.05$, paired t-test, two-tailed P-value). The maximum activation for Mn^{2+} and Co^{2+}, observed at 1 mM, was 600 ± 60% ($P < 0.001$) and 530 ± 70% ($P < 0.01$) above control (mean ± S.E.M., $n = 5$, paired t-test, two-tailed P-value), respectively. With increasing concentrations of the divalent cations, the activation diminished (Fig. 6, left panel). At the highest Mg^{2+} concentration tested (20 mM), some activation (590 ± 240% above control) appeared to exist, but the elevation was not statistically significant ($P > 0.05$, paired t-test, two-tailed P-value). The two other divalent cations (20 mM) seemed to reduce the transport below the control situation, but the reductions were not statistically significant ($P > 0.05$, paired t-test, two-tailed P-value) with values of 69 ± 40% and 53 ± 18% (mean ± S.E.M., $n = 5$) of control for Mn^{2+} and Co^{2+}, respectively.

The binding of cGMP to the membrane endoside was also affected differently by the divalent ions. No effect was seen in the presence of Mg^{2+} (20 mM) with a binding of 95 ± 8% (mean ± S.E.M., $n = 5$) of control ($P > 0.05$, paired t-test, two-tailed P-value). In contrast, Mn^{2+} and Co^{2+} caused a concentration-dependent inhibition of binding (Fig. 6, right panel). At the highest concentration tested (20 mM) these ions reduced the binding to 34 ± 8% and 34 ± 7% (mean ± S.E.M., $n = 5$) of control for Mn^{2+} and Co^{2+}, respectively. This effect was highly significant ($P < 0.005$, paired t-test, two-tailed P-value).

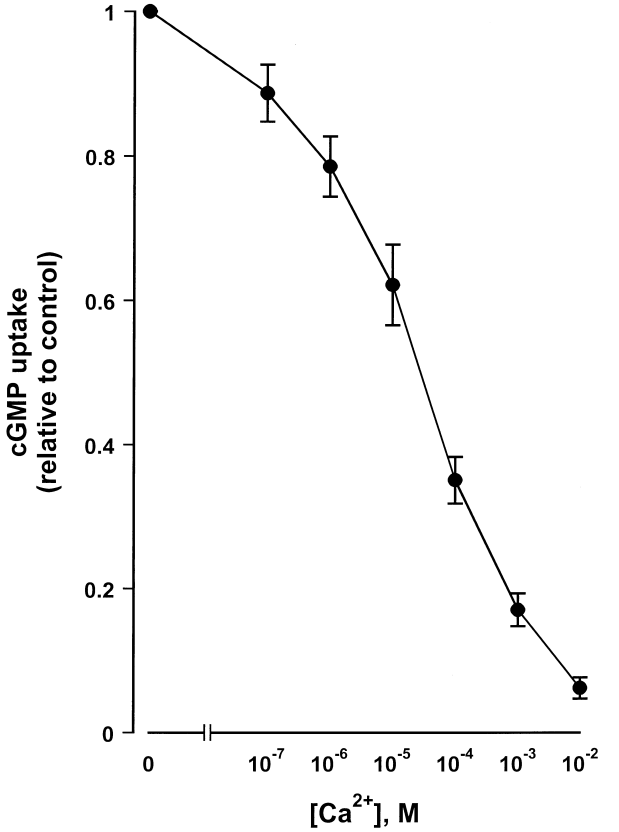

Fig. 5. The effect of Ca^{2+} on the uptake of cGMP to inside-out vesicles after 120 min co-incubation at 37°C. Concentrations of calcium from 0.1 μM to 10 mM were added together with 1 μM [^{3}H]cGMP with or without 1 mM ATP. The results are presented as mean ± S.E.M. of nine separate experiments.

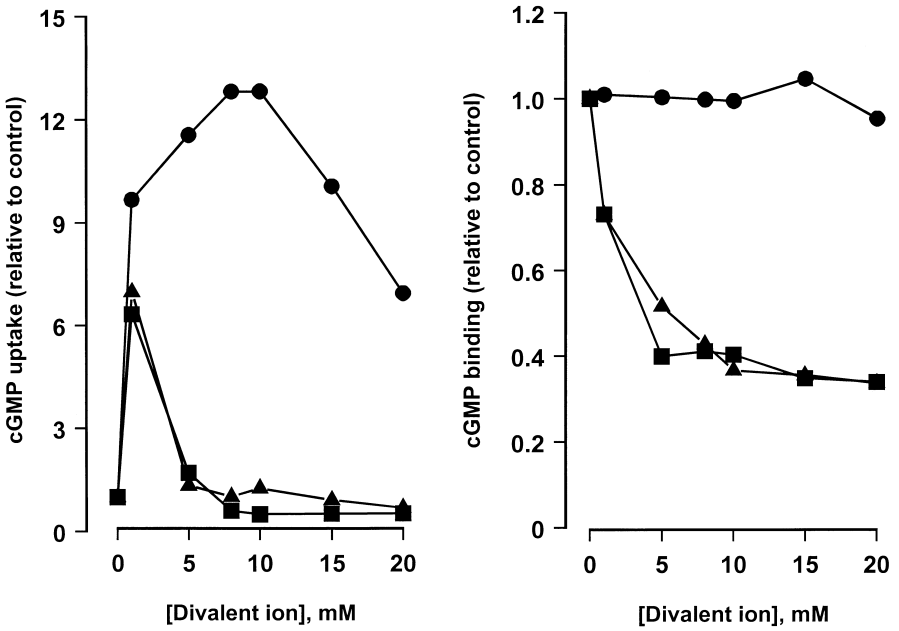

Fig. 6. The effect of Mg^{2+}, Mn^{2+} and Co^{2+} on the uptake (left panel) and binding (right panel) of cGMP to inside-out vesicles after 120 min co-incubation at 37°C. Mg^{2+} (●), Mn^{2+} (▲) and Co^{2+} (■) were added at concentrations from 1 to 20 mM together with 1 μM [^{3}H]cGMP with or without 1 mM ATP. The results are presented as mean values of five separate experiments.

3.7. *Effect of EGTA and EDTA on [^{3}H]cGMP transport and binding*

Fig. 7 shows that both EDTA and EGTA increased the cGMP uptake in a concentration-dependent mode. EGTA was more potent than EDTA which appeared to reach a plateau at 10 μM. For a chelator concentration of 100 μM, the uptake of cGMP was $50 \pm 10\%$ (mean ± S.E.M., $n = 3$, one-tailed $P < 0.05$) and $100 \pm 20\%$ (mean ± S.E.M., $n = 4$, one-tailed $P < 0.025$) above control for EDTA and EGTA, respectively. The binding of cGMP to the vesicles was not influenced (data not shown).

4. Discussion

The transport of molecules across lipid membranes is an essential function of all living organisms. The plasma membrane is virtually impermeable to extracellular cyclic nucleotides in physiological concentrations, but the appearance of these intracellular signal molecules in extracellular fluids has been known for decades. Evidence exists that the transport system for cGMP is closely linked to an ATPase since ATP, but not the non-hydrolysable ATP analogues ATP-γ-S and AMP-PNP, promotes cGMP transport [8]. This idea is also supported by the observations that well-known ATPase inhibitors caused a concentration-dependent reduction in the cGMP transport [9]. These observations suggest that the cGMP pump is a member of the ATP-dependent organic ion transporters, such as P-glycoprotein and MRP. Unlike P-glycoprotein, but similar to MRP, the non-selective inhibitor of anion transport systems, probenecid, inhibits the cGMP pump [1,5,6,8,10–14].

In the present study we investigated the possibility that the cGMP was transported by MRP. The finding that cGMP was unable to inhibit LTC_4 transport strongly suggests that two non-identical transport routes exist, since LTC_4 has been identified as an endogenous substrate with high affinity for MRP [15]. However, the ability of the LTD_4 receptor antagonist MK571 to reduce the uptake of LTC_4 to inside-out vesicles in a concentration-dependent mode is in agreement with the observations of MRP in human erythrocytes [17,18]. The inhibitory constant (IC_{50}) for MK571 of approx. 0.1 μM, obtained in the present study, is similar to that reported previously for human erythrocytes [18].

GSSG and *S*-conjugates of glutathione are sub-

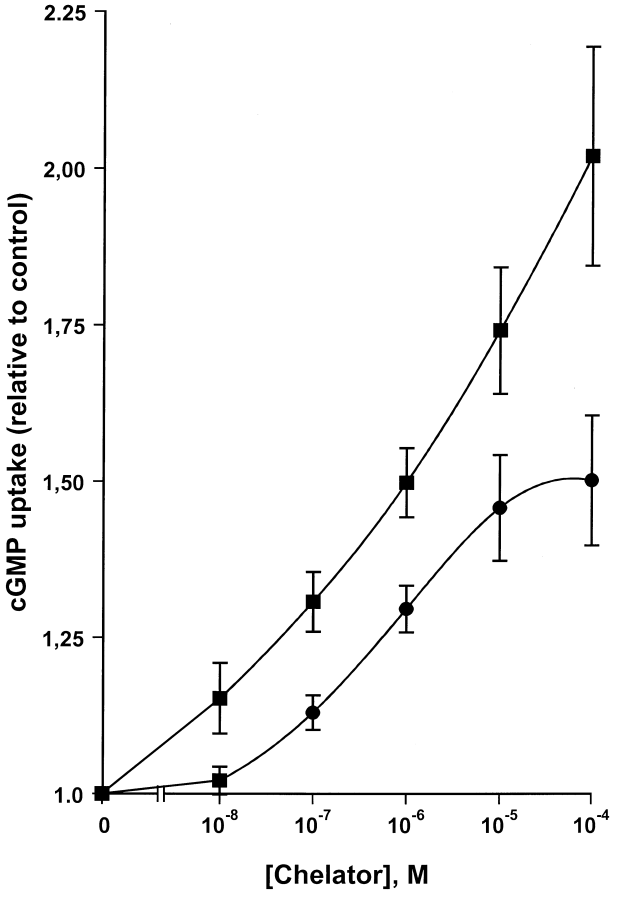

Fig. 7. The effect of EDTA and EGTA on the uptake of cGMP to inside-out vesicles after 120 min co-incubation at 37°C. Concentrations of the divalent ions from 0.01 to 100 mM were added together with 1 μM [^{3}H]cGMP with or without 1 mM ATP. The results are presented as mean values of three and four separate experiments for EDTA (●) and EGTA (■), respectively.

strates for various members of the MRP family [19]. In human erythrocytes MRP has been identified as a glutathione *S*-conjugate transporter [18]. The most potent conjugate in the present study was SDG which caused a half-maximal inhibition at about 40 μM. This compound has been reported to inhibit LTC_4 transport with a K_i of 50–120 nM [16,22]. Furthermore, GSSG inhibited cGMP uptake with a IC_{50} value of about 2 mM, but LTC_4 transport with K_i values of 0.1 mM [16,22]. Taken together these observations support the idea that the cGMP transporter is different from MRP. However, human erythrocytes possess several transport systems for glutathione conjugates [23] and the cGMP transport system appears to be a type of GS-X pump.

The two sulfhydryl protecting agents GSH and DTT inhibited cGMP egression similarly at high concentrations. This effect may be explained by a protection of sulfhydryl groups at the membrane endoside since DTT, but not GSH, is membrane permeable. The recently reported effect of NEM [9] suggests a role for sulfhydryl groups in the regulation of the cGMP transport.

In agreement with previous reports [1,6], the present study showed that the transport of cGMP was temperature sensitive and with no detectable transport below 18–20°C. This is unlike the transport of dinitrophenyl-glutathione where a significant activity was observed at 20°C [24,25].

On the other hand, the ATP-independent association of cGMP to the inside-out vesicles was not temperature dependent. We define this component as binding, and not passive diffusion, since (a) concentration gradients between 100 and 1000 were needed to load intact erythrocytes with cGMP [6], (b) a rapid equilibrium occurs (within 15 min at 37°C) and (c) the association rate is markedly slowed down at low temperature (0–4°C). This binding may represent specific (such as binding to active sites of the cGMP pump, phosphodiesterases or protein kinases) or non-specific interactions.

Transport ATPases are dependent on pH and may be characteristic for the individual enzyme [26]. The present study showed that the high affinity transport of cGMP has a pH optimum at pH 8.0–8.5. This is similar to that reported for dinitrophenyl-glutathione transport [24,27,28], but in contrast to the transport system for GSSG with an optimum at acidic pH [29].

Calcium has a regulatory function in a variety of cellular processes, including ATP-dependent transport systems [26] and inhibits the transport of dinitrophenyl-glutathione with an IC_{50} value of about 0.7 μM [28]. A markedly lower sensitivity to calcium (IC_{50} value of about 40 μM) was found for cGMP uptake to inside-out vesicles. However, both these values are above the normal cytosolic free calcium concentrations (about 100 nM). In contrast to the present observation, incubation of liver slices with the Ca^{2+} ionophore A23187 [5] and platelets with thapsigargin, that inhibits ER Ca^{2+} ATPases and thereby increases intracellular Ca^{2+} [30], leads to increased extracellular cGMP levels. The reasons for these apparently conflicting results are not clear.

Magnesium is a well-known activator of various ATPases, but other divalent cations may, at least partly, substitute Mg^{2+} to maintain enzyme activity [26]. Recently, we showed that ATP energised the cGMP transport with a K_m value of 0.3–0.5 mM and that Mg^{2+} caused an activation of transport for concentrations up to 10 mM [9]. The present study verifies this effect of Mg^{2+} and shows clearly that Mn^{2+} and Co^{2+} were unable to substitute Mg^{2+} and the low intracellular concentrations of Mn^{2+} and Co^{2+} make their role as regulators of little relevance. Similar, but not identical results were obtained in a study of P-glycoprotein [31].

Another difference was that Mn^{2+}/Co^{2+} were potent inhibitors of cGMP binding to membrane endoside, whereas Mg^{2+} had no effect. If the binding of cGMP reflects an interaction with the cGMP transport binding site, the effect of Mn^{2+}/Co^{2+} could explain the transport inhibition at the highest concentrations.

EDTA is a chelator with affinity for both Ca^{2+} and Mg^{2+}, whereas EGTA is relatively calcium-specific. The present study revealed that both EGTA and EDTA enhanced cGMP efflux. On the background of the present observed Ca^{2+} and Mg^{2+} effects, it is conceivable that the apparent stimulatory effect of chelators is an indirect effect due to Ca^{2+} binding. The source of Ca^{2+} in the present experiments is unclear, since no such was added, but release from cell membrane stores or impurities within the chemicals or solvents are possible explanations. The final effect of EDTA is a result of reduced stimulation by binding of Mg^{2+} and reduced inhibition by binding of Ca^{2+}. This may explain why EDTA is less potent than EGTA. The ATPase responsible for cGMP egression must be different from Ca^{2+} ATPases since EGTA is referred to as a relatively specific inhibitor of these. The present observation of a stimulatory effect of EGTA on cGMP transport is in close agreement with a recent report [9].

In conclusion, the present study shows that the cGMP transport is dependent on an ATPase with characteristics typical for other transport/traffic ATPases (sensitive to temperature and pH, and regulated by divalent cations). Although the cGMP transporter has properties similar to that of glutathione conjugate transporters (GS-X pumps), it is different from MRP.

Acknowledgements

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