Binding Characterization of a Putative cGMP Transporter in the Cell Membrane of Human Erythrocytes†

Emmanuel Boadu and Georg Sager*

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

Received November 19, 1996; Revised Manuscript Received July 8, 1997⊗

ABSTRACT: Cyclic GMP is an intracellular signal molecule whose biological and pharmacological role is not well understood. Recent studies with human erythrocytes and other cell types (normal and transformed) have shown that the extrusion of cGMP is an ATP-dependent and saturable process. In this paper, we present our studies on binding of [^{3}H]-cGMP to human erythrocyte ghost and its solubilized extracts. At 4 °C, an apparent dissociation constant of 0.15 μM was found in the samples. Maximum specific binding values in ghost and solubilized extracts were 9.0 pmol/mg of protein and 1.0 pmol/mg of protein, respectively. The low dissociation constant was confirmed by kinetic studies with a value of 0.16 μM. Specific [^{3}H]-cGMP binding was inhibited by cAMP, cGMP, and cIMP with K_D values of 0.22 μM, 0.09 μM, and 0.17 μM, respectively. Unlabeled cGMP and cIMP inhibited [^{3}H]-cGMP binding completely whereas cAMP inhibited only 70%. The membrane-localized cGMP-binding protein discriminates between cyclic and noncyclic nucleotides, since GMP, IMP, and AMP were unable to displace [^{3}H]-cGMP. A zwitterionic detergent, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate), was able to solubilize a protein with identical binding affinity. The results of this study show that erythrocyte ghosts possess a cGMP-binding protein which is not a kinase (due to a similar affinity for cAMP, cGMP, and cIMP) or phosphodiesterase (due to the inability of IBMX, 3-isobutyl-1-methylxanthine, to inhibit specific [^{3}H]-cGMP binding). We hypothesize that this protein is the cell membrane cGMP transporter.

During the last decade, much attention has been focused on cGMP because the endothelium-derived relaxing factor (EDRF), which has been identified as nitric oxide (NO), activates soluble guanylate cyclase while atrial natriuretic peptide (ANP) stimulates particulate guanylate cyclase (Wong & Garbers, 1992; Francis et al., 1994). In addition to their vasodilatory effects on vascular smooth muscle, EDRF and exogenous NO have been found to inhibit platelet functions (Alheid et al., 1987; Radomski et al., 1987a,b; Hogan et al., 1988). Therapeutic nitrates like triglyceryl nitrate (TGN) and isosorbide dinitrate exert their effects by releasing NO from their molecules and activating soluble guanylate cyclase. Elevated levels of extracellular guanosine 3′,5′-cyclic monophosphate (cGMP)[1] have been suggested as a diagnostic tool in cardiac diseases (Vorderwinkler et al., 1991; Abraham et al., 1992; Szekeres et al., 1993; Tsutamoto et al., 1994). Analysis of extracellular cGMP concentrations has been documented as a useful indicator of nitrate tolerance in patients with congestive heart failure (Tsutamoto et al., 1994). Increased levels of cGMP in plasma and urine of patients with various types of cancer have been reported. Extracellular cGMP has been proposed as a biochemical marker for cancer (Guthrie et al., 1979; Chawla et al., 1980; Hunt et al., 1980). Egression of cGMP is inhibited by probenecid, verapamil, and theophylline (Flo et al., 1995; Ørbo et al., 1995b) but stimulated by progesterone (Flo et al., 1995; Ørbo et al., 1994, 1995a). Studies with inside-out vesicles of human erythrocytes have shown the cGMP transporter activity to be ATP-dependent (Sager et al., 1996). The aim of this study was to characterize the binding of cGMP to human erythrocyte ghost and its solubilized extracts with respect to equilibrium and kinetic parameters.

EXPERIMENTAL PROCEDURES

Chemicals. [^{3}H]Guanosine 3′,5′-cyclic monophosphate (specific activity 10 Ci/mM) was obtained from Amersham International (Amersham, Buckinghamshire, U.K.). Cyclic AMP, cGMP, cIMP, GMP, AMP, IMP, and CHAPS were purchased from Sigma Chemicals (St Louis, MO). All other chemicals were of analytical grade.

Buffers. The composition of Krebs−Ringer (KR) bicarbonate buffer was 121 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25.3 mM $NaHCO_3$, and 1.3 mM $CaCl_2$, pH 8.0 at 20−22 °C, and that of Krebs−Ringer (KR) phosphate buffer was 122 mM NaCl, 4.9 mM KCl, 1.3 mM $CaCl_2$, and 15.9 mM NaH_2PO_4, pH 7.4 at 20−22 °C.

Preparation of Erythrocyte Ghosts. Blood from healthy human donors was collected directly into EDTA-vacutainer tubes and centrifuged at 600*g* for 15 min at 4 °C. Plasma and buffy coat were removed before the erythrocytes were resuspended in the KR bicarbonate buffer (pH 8.0) to restore the original hematocrit. The cells were washed and resuspended in the same buffer at 4 °C. Unsealed membranes were prepared from washed erythrocytes by slight modifications of the method of Dodge (Dodge et al., 1963). The packed erythrocytes were lysed by resuspension in ice-cold deionized water and centrifuged at 3 × 26000*g* for 10 min.

† This work was supported by The Norwegian Cancer Society.

⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1997.

[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cIMP, inosine 3′,5′-cyclic monophosphate; AMP, adenosine 5′-monophosphate; GMP, guanosine 5′-monophosphate; IMP, inosine 5′-monophosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate; IBMX, 3-isobutyl-1-methylxanthine.

S0006-2960(96)02853-X CCC: $14.00

Membranes (milky white) were resuspended in the KR bicarbonate buffer (pH 8.0) or in solubilization buffer (100 mM potassium phosphate, 150 mM NaCl, pH 7.8 at 20−22 °C).

Membrane Solubilization. Preparation of solubilized membrane proteins by 1% CHAPS (w/v) was performed using the method of Doige (Doige et al., 1992) with slight modifications. After shaking the membrane−detergent suspension at 4 °C for 30 min, the mixture was centrifuged at 4 °C for 1 h at 105000*g*. The resulting supernatant (S_1) containing the cGMP-binding protein as well as other proteins was removed carefully without pellet contamination. This extract was stored at −20 °C and used for subsequent ligand-binding studies. The pellet was resuspended in solubilization buffer (100 mM potassium phosphate, 150 mM NaCl, pH 7.8 at 20−22 °C) to give a final protein concentration of 0.4−0.5 mg/mL and 1% CHAPS (w/v) added. The sample was incubated for 30 min at 4 °C and then pelleted at 15000*g* for 15 min at 4 °C. The resulting supernatant (S_2), was stored at −20 °C and used for ligand-binding assays. Binding activities of both extracts were maintained even after 6 months of storage at −20 °C.

Determination of Protein Concentrations. Protein concentrations in the unsealed membranes and soluble extracts were determined by a dye binding assay (Bradford, 1976) using reagents from Bio-Rad Laboratories (Richmond, CA) and BSA from Sigma Chemicals as standards.

[^{3}H]-cGMP Binding to Unsealed Ghosts. Unsealed ghosts in KR bicarbonate buffer were incubated in duplicates in four separate experiments with various concentrations (0.04−1.28 μM) of [^{3}H]-cGMP at 4 °C in a total volume of 500 μL with a protein concentration of 1.0−1.2 mg/mL. Nonspecific binding was determined in parallel incubations with an excess of unlabeled cGMP (32 μM). After 2 h, the incubation mixture was diluted with 2 mL of ice-cold KR phosphate buffer and immediately filtered through a 25 mm single Whatman GF/C glass fiber filter which had previously been soaked in KRP buffer. The filtration rate was 12 mL/min. The filters were washed twice with 10 mL of KR phosphate buffer and placed in scintillation vials containing 0.5 mL of 1.0 M HCl and 9.5 mL of scintillation liquid (Ultima Gold XR, Packard Instrument B. V., The Netherlands). Radioactivity was washed out of filters by gentle shaking for 20 h, and measured in a Packard Tri-Carb scintillation spectrometer, Model 1900TR.

Specific binding was defined as the difference between total and nonspecific binding and analyzed according to the method of Scatchard (1949).

To further ascertain the binding parameters (K_D and B_{max}) of [^{3}H]-cGMP binding to unsealed ghosts, other assay systems like centrifugation and equilibrium dialysis were employed.

Equilibrium [^{3}H]-cGMP binding to unsealed ghosts was measured in three separate experiments using the method of Gorga and Lienhard (1981) with slight modifications. Unsealed ghosts in KR bicarbonate buffer were incubated in duplicate with varying concentrations (0.04−1.28 μM) of [^{3}H]-cGMP at 4 °C in a total volume of 500 μL with a protein concentration of 1.5 mg/mL. Nonspecific binding was determined in parallel incubations with an excess of unlabeled cGMP (32 μM). After 2 h, the incubation mixture in the Eppendorf tube was pelleted by centrifugation at 13000*g* for 10 min at 4 °C. Aliquots of 125 μL were taken from each sample before and after centrifugation to determine the total and free [^{3}H]-cGMP concentrations. The concentration of bound [^{3}H]-cGMP was defined as the difference between the total and free [^{3}H]-cGMP concentrations.

Three separate equilibrium dialysis experiments were performed in duplicate using a Dianorm equilibrium dialyzer with the cell type Macro 1s and the method of Zoccoli (Zoccoli et al., 1978) with slight modifications. The two compartments in each cell were separated by a M_r 12 000−14 000 cutoff Spectra/Por dialysis membrane from Spectrum Medical Industries, Inc. (Houston, TX). One compartment was loaded with 1000 μL of unsealed ghosts in KR bicarbonate buffer with a protein concentration of 2.6 mg/mL. The other compartment was loaded with 1000 μL of varying concentrations (0.04−1.28 μM) of [^{3}H]-cGMP in the same buffer with or without an excess of unlabeled cGMP (32 μM). In the control cell, one compartment was loaded with 1000 μL of 0.04 μM [^{3}H]-cGMP in KR bicarbonate buffer, and the other compartment was loaded with only buffer. The cells were rotated at 12 rpm for 4 h at 4 °C, and then 500 μL aliquots of each sample removed from the chambers were added to 4.5 mL of scintillation liquid (Ultima Gold XR, Packard Instrument B. V., The Netherlands) for the measurement of radioactivity.

Binding of [^{3}H]-cGMP to Solubilized Extracts. Solubilized extracts in solubilization buffer (100 mM potassium phosphate, 150 mM NaCl, pH 7.8 at 20−22 °C) were incubated in duplicate in two separate experiments with various concentrations (0.04−1.28 μM) of [^{3}H]-cGMP in the presence or absence of excess (32 μM) unlabeled cGMP at 4 °C. The final volume of the reaction mixture was 500 μL with a protein concentration of 0.2−0.4 mg/mL. After 2 h, the incubation mixture was diluted with 2 mL of ice-cold solubilization buffer (100 mM potassium phosphate,150 mM NaCl, pH 7.8 at 20−22 °C) and filtered through a 25 mm cellulose ester (Millipore) filter with a 0.45 μm pore size. The filtration rate was 24 mL/min. Filters were washed twice with 10 mL of solubilization buffer and placed in scintillation vials, and extract-bound [^{3}H]-cGMP in the filters was measured as described for unsealed membranes.

RESULTS

Binding of [^{3}H]-cGMP to Erythrocyte Ghosts. Incubation of unsealed human erythrocyte ghosts with increasing concentrations of [^{3}H]-cGMP for 2 h at 4 °C showed that specific membrane binding was a saturable process. The results obtained from the ultrafiltration experiments are shown in Figure 1. When analyzed according to Scatchard (1949), a dissociation constant (K_D) of 0.15 ± 0.10 μM was found with maximum binding (B_{max}) of 8.3 ± 2.2 pmol/mg of protein (mean ± SD, $n = 4$). Parallel experiments with 0.2 mM of the nonspecific phosphodiesterase inhibitor IBMX gave a similar hyperbolic curve using the ultrafiltration method. Scatchard analysis of the saturation experiment gave a linear relationship with similar K_D (0.15 ± 0.16 μM) and B_{max} (8.8 ± 2.6 pmol/mg of protein, mean ± SD, $n = 4$).

Binding was also determined by centrifugation and equilibrium dialysis. The centrifugation experiments gave a K_D of 0.25 ± 0.04 μM with a B_{max} of 8.7 ± 3.8 pmol/mg of protein. Using the equilibrium dialysis technique, the K_D and B_{max} values obtained were 0.23 ± 0.09 μM and 13.3 ± 4.2 pmol/mg of protein, respectively.

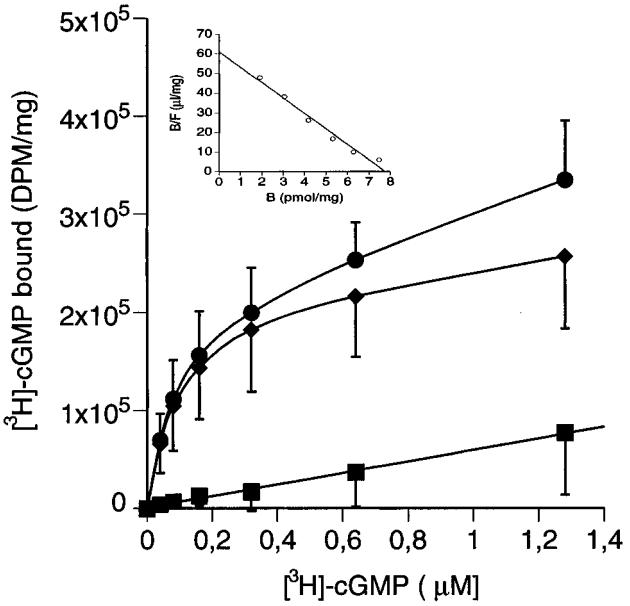

FIGURE 1: Binding of [^{3}H]-cGMP as a function of radioligand concentration at 4 °C to human red cell ghost at constant protein concentration. Total binding (●) and nonspecific binding (■) represent binding of the radioligand in the absence and presence of 32 μM cGMP, respectively. Specific binding of [^{3}H]-cGMP is given as the difference between total and nonspecific binding (◆). The nonspecific binding can be described by the linear correlation $y = 60189x$ ($r = 0.99$). Results are presented as mean ± SD ($n = 4$). Inset: Scatchard plot (Scatchard, 1949) of specifically bound [^{3}H]-cGMP. The least-squares regression line is given ($r = -0.98$).

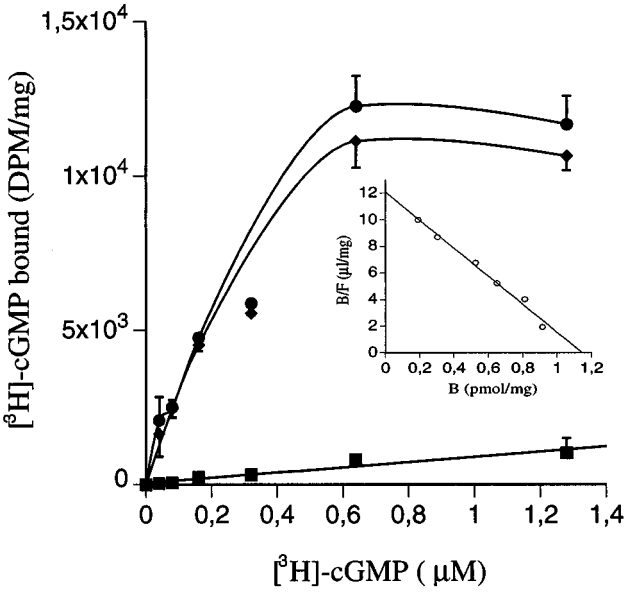

FIGURE 2: Binding of [^{3}H]-cGMP as a function of radioligand concentration at 4 °C to the first CHAPS extract (S_1) at constant protein concentration. Total binding (●) and nonspecific binding (■) represent binding of the radioligand in the absence and presence of 32 μM cGMP, respectively. Specific binding of [^{3}H]-cGMP is given as the difference between total and nonspecific binding (◆). The nonspecific binding can be described by the linear correlation $y = 901x$ ($r = 0.98$). Results are presented as mean ± SD ($n = 2$). Inset: Scatchard plot (Scatchard, 1949) of specifically bound [^{3}H]-cGMP. The least-squares regression line is given ($r = -0.98$).

Binding of [^{3}H]-cGMP to Solubilized Extracts. Equilibrium binding of [^{3}H]-cGMP to the first solubilized extract (S_1) is presented in Figure 2. Scatchard analysis revealed a K_D of 0.11 ± 0.01 μM (mean ± SD, $n = 2$), which is similar to that in membranes, and a maximum binding capacity of

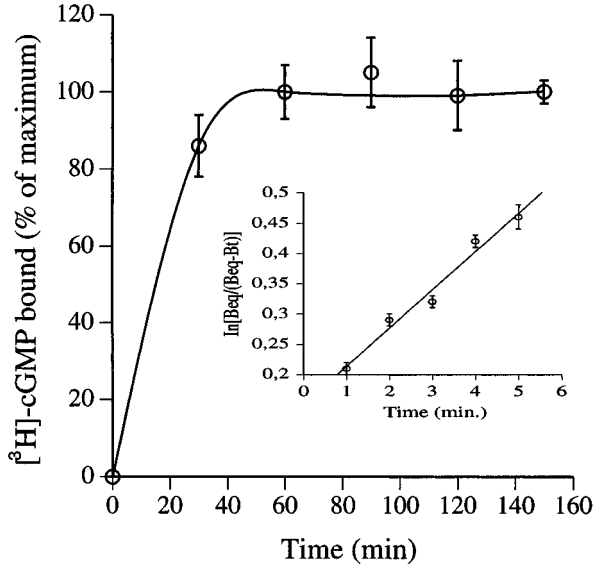

FIGURE 3: Time course for association of specific [^{3}H]-cGMP binding to human erythrocyte ghosts at 4 °C was determined in incubations containing 1.28 μM radioligand in the absence or presence of 32 μM cGMP. The reaction was terminated at various time intervals up to 240 min. Specific [^{3}H]-cGMP binding at 180 min is given as 100%. Equilibrium was obtained at approximately 90 min and was maintained for 150 min. *B*eq = concentration of specifically bound [^{3}H]-cGMP at 150 min. *Bt* = concentrations of bound [^{3}H]-cGMP at the stated time. The results are given as mean ± SD ($n = 4$). Inset: Pseudo-first-order plot of specific binding and the least-squares regression line is shown ($r = 0.98$).

1.03 ± 0.1 pmol/mg of protein. The binding of [^{3}H]-cGMP to the second solubilized extract (S_2) is also saturable and characterized by a dissociation constant of 0.15 ± 0.05 μM (mean ± SD, $n = 2$), which is of the same order of magnitude as that in membranes (data not shown). The B_{max} of [^{3}H]-cGMP (1.3 ± 0.4 pmol/mg of protein) is similar to that in the first solubilized extract (S_1) but lower in both extracts when compared to that in membranes. However, the similarity in dissociation constants observed in extracts and membranes indicates the ability of CHAPS in solubilizing the cGMP membrane-localized binding protein.

Kinetics of [^{3}H]-cGMP Binding to Unsealed Ghosts. Kinetic studies were carried out to confirm the dissociation constant (K_D) obtained from equilibrium studies. Specific binding of [^{3}H]-cGMP reached an equilibrium at approximately 90 min at 4 °C (Figure 3) and remained unchanged for the next 150 min. Kinetic data were used to calculate the rate constant, k_1, for the association process: R + L → RL where L represents unbound [^{3}H]-cGMP, R free binding sites, and RL occupied binding sites.

The slope, k_{obs} (Figure 3, inset), is an estimate of the observed forward rate constant for a pseudo-first-order reaction (Williams et al., 1976) since the concentration of binding sites determined from the binding studies was much lower than the concentration of [^{3}H]-cGMP. The least-squares regression method gave a k_{obs} value of 0.06 ± 0.01 min^{-1} (mean ± SD, $n = 3$), and dissociation rates (k_{21} and k_{22}) of [^{3}H]-cGMP from the binding sites (Figure 4) were measured by diluting the ligand in the equilibrium mixture with an excess of KR bicarbonate buffer. The resulting dissociation curve appeared to be biexponential (Figure 4) and resolved into rapid and slow dissociation components by plotting the fraction of [^{3}H]-cGMP bound versus time. Based on the late time points (4, 5, 15 min), the slow phase

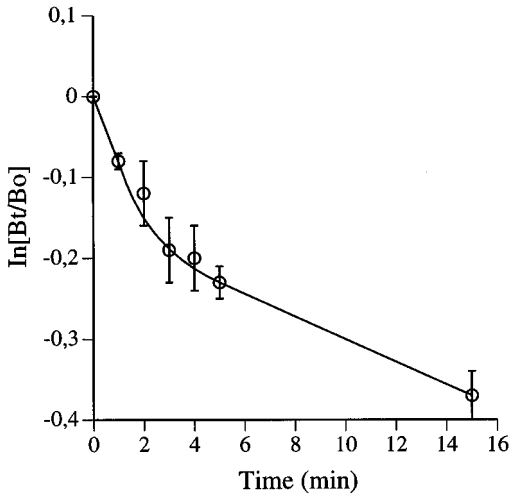

FIGURE 4: Time course for dissociation of specifically bound [^{3}H]-cGMP from human erythrocyte ghosts at 4 °C. Maximum binding (*Bo*) refers to the amount of [^{3}H]-cGMP specifically bound at equilibrium prior to dilution with 4.5 mL of KRB buffer. The erythrocyte ghosts were incubated with 0.4 μM radioligand for 120 min. The dissociation process was initiated by diluting samples with 4.5 mL of KRB buffer. Nonspecific binding was determined with 32 μM cGMP. *Bt* = concentration of specifically bound [^{3}H]-cGMP at stated times. The results are given as mean ± SD (n = 3).

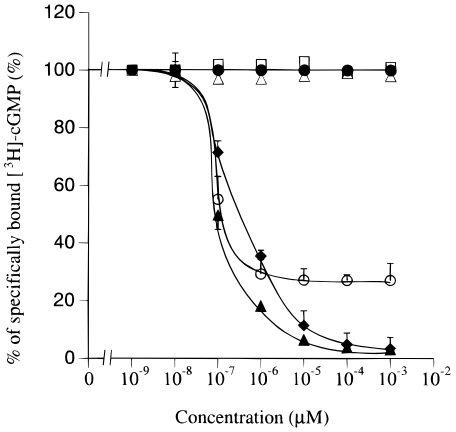

FIGURE 5: Inhibition of specifically bound [^{3}H]-cGMP binding to human erythrocyte ghosts at 4 °C by increasing concentrations of nucleotides. The ligands were added just prior to the addition of 0.4 μM radioligand. The reaction was terminated after 2 h of incubation. Inhibition (mean ± SD, n = 3) is given as percent of that observed without unlabeled ligand. (○) cAMP, (◆) cGMP, (▲) cIMP, (□) GMP, (△) AMP, (●) IMP.

was extrapolated back to zero. A line representing the rapid dissociation phase was then derived by subtracting the extrapolated slowly dissociation component from the observed dissociation data at the early time points (0, 1, 2, 3 min). The dissociation rate constants obtained for the rapid and slow phases were $k_{21} = 0.057 \pm 0.007$ min^{-1} and $k_{22} = 0.018 \pm 0.001$ min^{-1} (mean ± SD, n = 3), respectively. The second-order association rate constant could be calculated (Jencks, 1970) from the values of k_{obs} and the dissociation rate constants using the equation:

$$k_1 = \frac{k_{obs} - k_2}{[L]}$$

The values obtained were $k_{11} = (0.008 \pm 0.001) \times 10^6$ M^{-1} min^{-1} and $k_{12} = (0.112 \pm 0.002) \times 10^6$ M^{-1} min^{-1} (mean ± SD, n = 3), respectively. Using the individual values of k_1 and k_2 from three separate experiments, the dissociation constants (K_D) for the low- and high-affinity sites were calculated to be 7 ± 0.13 μM and 0.161 ± 0.01 μM (mean ± SD, n = 3), respectively.

Inhibition of [^{3}H]-cGMP Binding by Nucleotides. The effect of some nucleotides on the specific binding of [^{3}H]-cGMP to unsealed ghosts is presented in Figure 5. Among the nucleotides tested, cAMP, cIMP, and cGMP effectively displaced [^{3}H]-cGMP with similar potency. In contrast, AMP, IMP, and GMP did not have any effect on [^{3}H]-cGMP binding. When the data with cGMP as inhibitor were analyzed according to Hofstee (1952), the displacement curve could be dissected into two components, one with high affinity (K_D = 0.085 ± 0.002 μM) and a second one with lower affinity (K_D = 9.2 ± 0.4 μM). The other cyclic nucleotides fitted best with the one-site model with apparent K_D values of 0.22 ± 0.12 μM and 0.17 ± 0.05 for cAMP and cIMP, respectively.

DISCUSSION

The existence of a cellular cGMP transporter has been shown in different cell types (Tjörnhammer et al., 1986; Wu et al., 1993; Flo et al., 1995; Sager et al., 1996). The cyclic GMP transporter not only may serve the purpose of returning cGMP levels to normal after stimulation of the cell by hormones or neurotransmitters, but also may act as an intercellular signal molecule for the released cyclic nucleotide.

In this paper, we have demonstrated the existence of saturable [^{3}H]-cGMP-binding sites on unsealed ghosts employing three separate assays (centrifugation, equilibrium dialysis, and ultrafiltration). Using separate methods (saturation of binding at equilibrium by increasing radioligand or by increasing unlabeled cGMP and kinetic studies), a high-affinity component with a K_D of 85−200 nM was obtained for [^{3}H]-cGMP binding at 4 °C. Similar dissociation constants have been reported for bovine aorta cGMP-dependent protein kinase isoenzymes and the noncatalytic site of bovine rod PDE (Ruth et al., 1991; Wild et al., 1995). But the noncatalytic site of cGMP-binding PDE (cG-BPDE) is known to be approximately 100-fold more selective for cGMP than for cAMP (Thomas et al., 1992). Moreover, the membrane-bound form of cyclic nucleotide phosphodiesterase is specific for cAMP (Suzuki et al., 1980). The inability of IBMX to influence the binding of [^{3}H]-cGMP to ghosts supports that the ligand did not bind to a phosphodiesterase. Cyclic nucleotide-dependent protein kinases are 200-fold cyclic nucleotide-specific (Øgreid et al., 1983; Corbin et al., 1986). Therefore, the similarities in the affinities of the cyclic nucleotides suggest that the radioligand did not bind to either cG-BPDE or PKG but to a protein which has a binding capacity in a range of physiological relevant concentrations. Nucleotide specificity studies have demonstrated the existence of selective transport mechanisms for cAMP and cGMP, respectively, and the inability of increased cAMP level by glucagon in inhibiting cGMP efflux (Tjörnhammer et al., 1983). The differential distribution pattern of cyclic nucleotides across the membrane also suggests different transport mechanisms (Ørbo et al., 1993). Furthermore, a previous study of cAMP transport showed a passive carrier-mediated transport out of human erythrocytes with an apparent K_D of 475 μM (Holman, 1978). These reports suggest that cGMP and cAMP do not share a common efflux pump. In contrast, reduction of cGMP

egression by forskolin was assumed to be the result of displacement of cGMP from the transport binding site by cAMP after forskolin had activated adenylate cyclase (Hamet et al., 1989). Preliminary experiments in our laboratory, however, have shown that forskolin inhibits the cGMP transporter activity. A similar effect has been reported for other transporters, such as P-glycoprotein (Morris et al., 1991).

In the present study, cGMP and cIMP give 100% inhibition whereas cAMP is not able to inhibit [^{3}H]-cGMP binding to a full degree (70%). It appears that cAMP displaces cGMP bound to its high-affinity site, but not the low-affinity binding site. This raises the question of whether cAMP and cGMP have a common binding site, and if they do, is it the transport site or an allosteric regulatory site.

In contrast to the inhibition curves for cIMP and cAMP, the curve for cGMP could be dissected into two components. This agrees with the results obtained by the kinetic experiments, but the low-affinity component was not seen in the saturation binding experiments with increasing concentrations of the tracer. We suggest that the concentration used to define nonspecific binding was too low to unhide the low-affinity component. The binding of cGMP to the low- and high-affinity sites may represent the initial step (recognition) in the cellular export of cGMP, recently characterized by a high- and low-K_m process (Sager et al., 1996). Studies to purify and characterize this putative cGMP transporter are in progress.

REFERENCES

Abraham, W. T., Hensen, J., Kim, J. K., Dürr, J., Lesnefsky, E. J., Groves, B. M., & Schrier, R. (1992) *J. Am. Soc. Nephrol. 2*, 1697−1703.

Alheid, U., Frölich, J. C., & Förstermann, U. (1987) *Thromb. Res. 47*, 561−571.

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

Chawla, R. K., Shlaer, S. M., Lawson, D. H., Murray, T. G., Schmidt, F., Shoji, M., et al. (1980) *Cancer Res. 40*, 3915−3920.

Corbin, J. D., Ogreid, D., Miller, J. P., Suva, R. H., Jastroff, B., & Doskeland, S. O. (1986) *J. Biol. Chem. 261*, 1208−1214.

Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119−130.

Doige, C. A., Yu, X., & Sharom, F. J. (1992) *Biochim. Biophys. Acta 1109*, 149−160.

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

Francis, S. H., & Corbin, J. D. (1994) *Adv. Pharmacol. 26*, 115−170.

Gillespie, P. G., & Beavo, J. A. (1988) *J. Biol. Chem. 263*, 8133−8141.

Gorga, F. R., & Lienhard, G. E. (1981) *Biochemistry 20*, 5108−5113.

Guthrie, D., Isah, H., Latner, A. L., & Turner, G. A (1979) *IRCS Med. Sci. 7*, 209.

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

Hofstee, B. H. J. (1952) *Science 116*, 329−331.

Hogan, J. C., Lewis, M. J., & Henderson, A. H. (1988) *Br. J. Pharmacol. 94*, 1020−1022.

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

Hunt, N. H., Smith, B., & Pembrey, R. (1980) *Clin. Sci. 58*, 463−467.

Jencks, W. P. (1970) *Catalysis in Chemistry*, & *Enzymology*, pp 555−614, McGraw-Hill Book Co., New York.

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

Øgreid, D., Døskeland, S. O., & Miller, J. P. (1983) *J. Biol. Chem. 258*, 1041−1049.

Ørbo, A., Jaeger, R., & Sager, G. (1993) *Int. J. Cancer 55*, 957−962.

Ørbo, A., Jaeger, R., & Sager, G. (1994) *Gynecol. Oncol. 52*, 320−325.

Ørbo, A., Jaeger, R., Kjørstad, K. E., & Sager, G. (1995a) *Anticancer Res. 15*, 1905−1909.

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

Radomski, M. W., Palmer, R. M. J., & Moncada, S. (1987a) *Biochem. Biophys. Res. Commun. 148*, 1482−1489.

Radomski, M. W., Palmer, R. M. J., & Moncada, S. (1987b) *Br. J. Pharmacol. 92*, 639−646.

Ruth, P., Landgraf, W., Keilbach, A., May, B., Egleme, C., & Hofmann, F. (1991) *Eur. J. Biochem. 202*, 1339−1344.

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

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660−672.

Suzuki, K., Terao, T., & Osawa, T. (1980) *Biochim. Biophys. Acta 602*, 78−86.

Szekeres, T., Artner-Dworzak, E., Puschendorf, B., Fritzer, M., Havel, M., Gassner, A., & Fridrich, L. (1993) *Eur. J. Clin. Chem. Clin. Biochem. 31*, 69−74.

Thomas, M. K., Francis, S. H., Beebe, S. J., Gettys, T. W., & Corbin, J. D. (1992) *Advances in Second Messenger and Phosphoprotein Research* (Strada, S. J., & Hidaka, H., Eds.) Vol. 25, pp 45−53, Raven Press, Ltd., New York.

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

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

Tsutamoto, T., Kinoshita, M., Ohbayashi, Y., Wada, A., Maeda, Y., & Adachi, T. (1994) *Circulation 90*, 823−829.

Vorderwinkler, K.-P., Artner-Dworzak, E., Jakob, G., Mair, J., Dienstl, F., Pichler, M., & Puschendorf, B. (1991) *Clin. Chem. 37*, 186−190.

Wild, N., Herberg, F. W., Hofmann, F., & Dostmann, W. R. G. (1995) *FEBS Lett. 374*, 356−362.

Williams, L. T., Jarett, L., & Lefkowitz, R. J. (1976) *J. Biol. Chem. 251*, 3096−3104.

Wong, S. K.-F., & Garbers, D. (1992) *J. Clin. Invest. 90*, 299−305.

Wu, X.-B., Brüne, B., Von Appen, F., & Ullrich, V. (1993) *Mol. Pharmac*ol. *43*, 564−568.

Zoccoli, M. A ., Baldwin, S. A., & Lienhard, G. E. (1978) *J. Biol. Chem. 253*, 6923−6930.

BI9628533