Cyclic nucleotides are known to be effluxed from cultured cells or isolated tissues. Two recently described members of the multidrug resistance protein family, MRP4 and MRP5, might be involved in this process, because they transport the 3',5'-cyclic nucleotides, cAMP and cGMP, into inside-out membrane vesicles. We have investigated cGMP and cAMP efflux from intact HEK293 cells overexpressing MRP4 or MRP5. The intracellular production of cGMP and cAMP was stimulated with the nitric oxide releasing compound sodium nitroprusside and the adenylyl cyclase stimulator forskolin, respectively. MRP4- and MRP5-overexpressing cells effluxed more cGMP and cAMP than parental cells in an ATP-dependent manner. In contrast to a previous report we found no glutathione requirement for cyclic nucleotide transport. Transport increased proportionally with intracellular cyclic nucleotide concentrations over a calculated range of 20–600 μM, indicating low affinity transport. In addition to several classic inhibitors of organic anion transport, prostaglandins A1 and E1, the steroid progesterone and the anti-cancer drug estramustine all inhibited cyclic nucleotide efflux. The efflux mediated by MRP4 and MRP5 did not lead to a proportional decrease in the intracellular cGMP or cAMP levels but reduced cGMP by maximally 2-fold over the first hour. This was also the case when phosphodiesterase-mediated cyclic nucleotide hydrolysis was inhibited by 3-isobutyl-1-methylxanthine, conditions in which efflux was maximal. These data indicate that MRP4 and MRP5 are low affinity cyclic nucleotide transporters that may at best function as overflow pumps, decreasing steep increases in cGMP levels under conditions where cGMP synthesis is strongly induced and phosphodiesterase activity is limiting.

The cyclic nucleotides cAMP and cGMP are major second messengers in cells. cGMP is formed from GTP by the action of guanylate cyclase (GC) and cAMP from ATP by adenylyl cyclase (AC). Both GC and AC can be activated by a variety of intracellular and extracellular stimuli. GC is stimulated by nitric oxide (NO), which leads to a build up of cGMP in the cell, whereas AC is activated by the binding of ligands to membrane receptors (1). Phosphodiesterases (PDEs) hydrolyze cGMP and cAMP to 5'-GMP and 5'-AMP, respectively, returning cyclic nucleotide concentrations to basal levels and making the cell susceptible to a new stimulus.

The efflux of cyclic nucleotides, which has been observed in tissues, might also influence intracellular concentrations (2–10). Efflux is most prominent when cells or tissues are incubated with a GC stimulator, such as the nitric oxide (NO) releasing compound sodium nitroprusside (SNP), in combination with a PDE inhibitor, such as 3-isobutyl-1-methylxanthine (IBMX). These results suggest that efflux may serve as an alternative mechanism of cyclic nucleotide reduction under conditions where PDE activity is insufficient, for instance under conditions of PDE saturation or inhibition. Tight regulation of intracellular cyclic nucleotide concentrations is important, because excessive cGMP production in cells is toxic and may cause neurotoxicity (5, 6) or retinal degeneration (11, 12). In addition to a possible role in the regulation of intracellular cyclic nucleotide level, efflux of cyclic nucleotides may also have a signaling function, as suggested by reports showing biological effects of extracellular cGMP (13, 14).

Recently, two members of the multidrug resistance protein (MRP) family, MRP4 and MRP5, have been shown to transport cAMP and cGMP (15–17). Characterized members of the MRP family are localized in the plasma membrane and function as transporters that couple ATP hydrolysis to the efflux of organic anions (18, 19). In contrast to other MRPs, MRP4 and MRP5 were shown to transport the monophosphorylated form of nucleoside analog drugs (20–22). Subsequently, Jedlitschky et al. (17) showed ATP-dependent transport of cGMP and cAMP in membrane vesicles derived from hamster lung fibroblasts overexpressing human MRP5 cDNA. Other groups obtained similar results with membrane vesicles from Sf9 cells overexpressing human MRP4 cDNA (15, 16), whereas MRP4-overexpressing Madin-Darby canine kidney II cells were demonstrated to efflux cAMP (23). Moreover, because membrane vesicles derived from human erythrocytes display ATP-dependent cGMP transport (24) and contain MRP5 (17), it has been suggested that MRP5 mediates this transport. There are unexplained discrep-
ancies in these studies, however. Although cGMP is reported to be a high affinity substrate for MRP4 and MRP5, it is a poor inhibitor of the transport of other substrates by these transporters (16, 25). Furthermore, GSH was required for transport of cAMP from MRP4-transfected cells (23) but not into inside-out membrane vesicles derived from SH cells (15, 16). To address these discrepancies, we have investigated the efflux of cGMP and cAMP from HEK293 cells overexpressing either MRP4 or MRP5. We find transport properties consistent with a low affinity process that is not influenced by GSH.

EXPERIMENTAL PROCEDURES

Chemicals—Poly-n-lysine, cGMP, cAMP, glutamate, buthionine sulfoximine (BSO), trequinsin (Tq), zarinap, probenecid (PR), sulfipyrazone (SP), sodium nitroprusside (SNP), sodium azide, 2-deoxyglucose, butyrate, 3-isobutyl-1-methylxanthine (IBMX), bromosulfophthalein, bromphenol blue, dexamethasone, indomycin green (ICG), progesterone, protaglandin A (PGA), prostaglandin E (PGE), rose Bengal, and forskolin were obtained from Sigma (Zwijndrecht, The Netherlands). Estramustine phosphate was from Pharmacia & Upjohn BV (Woerden, The Netherlands). Sildenafil was from Pfizer. Bis(pivaloamidomethyl)-9-(2-phosphonomethoxethyl)adenine (bis[POM/P MEA]), 3'-H-labeled at position 8, was obtained from Moravek Chemicals, and 3'-(3H)-labeled bis[POM/PMEA] was a gift from J. Balzarini (Bega Institute, Leuven, Belgium). Acetoxymethyl-cGMP was from Biog (Germany), and interleukin 1B was from Sanver Tech (Heerhugowaard, The Netherlands).

Cell Lines—HEK293 parental cells and HEK293/51 and HEK293/5E cells transfected with MRP5 cDNA and HEK293/4.3 and HEK293/4.63 overexpressing MRP4 cDNA have been described before (21, 22). Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified CO2 incubator. Cells were routinely checked for the absence of mycoplasma and for MRP4 and MRP5 expression levels. Fig. 1B illustrates the degree of overexpression of MRP4/MRP5 in the four transfected cell lines used. The relative overexpression of MRP4 is about 50-fold in the 5I and somewhat lower in 5E; overexpression of MRP5 is about 75-fold in the 4.63 cell line and a little lower in 4.3. It should be stressed, however, that levels on Western blots cannot be directly translated into ability to pump substrate from intact cells, because not all transporter molecules in the secretants are in the plasma membrane (21, 22).

Western Blot Analysis—Western blot analysis was performed essentially as described before (21). Protein from a total cell lysate was quantified using the Bio-Rad protein assay (Bio-Rad, Richmond, CA), and 10 or 50 μg of total protein was electroblotted onto nitrocellulose membrane by electroblotting. After blocking for 1 h in PBS containing 1% nonfat milk powder, 1% bovine serum albumin, and 0.05% Tween 20, rat monoclonal antibodies NKI-12C5 and NKI-12C4 (21) were used to detect MRP5 and MRP4, respectively, with subsequent use of a horseradish peroxidase-labeled goat anti-rat antibody (Santa Cruz Technology) and enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) for detection.

Cyclic Nucleotide Transport Experiments—Cells were seeded at a density of 2 × 106 per well in poly-n-lysine-coated 12-well plates and grown overnight. On the following day, the cells were washed with PBS and then incubated at 37 °C in 1 ml of pre-warmed Hanks’ buffered salt solution (HBSS, Life Technologies) under the conditions indicated in the text. At the indicated time points the incubation medium was removed, acidified with 1% HCl (end concentration, 0.1 M), and centrifuged, and the supernatant was used for analysis. Intracellular cGMP was extracted by adding 0.1 M HCl (0.5 ml), scraping, and resuspending the cells. Insoluble material was removed by centrifugation. A colorimetric cGMP or cAMP low pH immunoassay (R&D Systems, Abingdon, Oxon, UK) was used for analysis of the intracellular and extracellular cyclic nucleotides. To determine the requirement for ATP in the efflux of cGMP, cells were first stimulated with SNP and IBMX, washed twice with ice-cold PBS, and incubated in 1 ml of pre-warmed HBSS without glucose and supplemented with 10 mM sodium azide and 10 mM 2-deoxy-glucose to deplete intracellular ATP. Samples were taken and analyzed as described above. To deplete glutathione (GSH), cells were incubated overnight with 40 μM BSO in standard growth medium, which led to a reduction of ~90% in GSH levels, and the GSH amount was determined as described previously (26). Extracellularly added GMP was stable under the assay conditions used, because there was a decrease of less than 1% over 4 h, as measured with a validated high-performance liquid chromatographic method (21).

RESULTS

Efflux of cGMP by HEK293 Cells—Because cGMP was reported to be a substrate of MRP4 and MRP5 in vesicle uptake studies, we determined cGMP efflux from HEK293 cells overexpressing MRP4 (4.63 cells) or MRP5 (51 cells) (21). Fig. 1A shows that the 4.63 and 51 cells excreted 3- to 4-fold more cGMP than the parental cells. Fig. 1B shows the relative overexpression of MRP4 and MRP5 for the secretants. Parental HEK293 (293) cells effluxed ~10 pmol of cGMP/106 cells, which was just above the detection limit of the assay. The intracellular levels under these conditions, between 1 and 20 pmol/106 cells could not be determined reliably, which was probably due to the relatively high concentrations of interfering intracellular metabolites. The cell-permeable acetoxymethyl ester of cGMP led to increased intracellular cGMP levels and increased cGMP efflux, but this was still near the detection limit (data not shown).
FIG. 2. Effect of SNP and IBMX on cGMP levels in parental HEK293 cells and extrusion of cGMP from these cells. Parental HEK293 cells were treated with combinations of SNP (1 mM, light gray bar), IBMX (1 mM, dark gray bar), SNP with IBMX (black bar), and vehicle (white bar) for 1 h after which the (A) extracellular and (B) intracellular cGMP levels were determined. Data are the means of five experiments ± S.E., except for the 5E and 4.63 cells which represent the means of three experiments ± S.E. Note the difference in scale between A and B.

To raise both the intracellular and effluxed cGMP to levels that could be reliably determined, we tested the effects of the broad-specificity PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the nitric oxide (NO) releasing agent sodium nitroprusside (SNP). Fig. 2 shows the results for the parental cells. A 1-h incubation with 1 mM SNP or 1 mM IBMX alone readily increased the cGMP efflux of the HEK293 cells by 5- to 6-fold. Incubation with IBMX alone also led to a substantial increase in intracellular cGMP levels, whereas SNP marginally increased the intracellular levels, from ~10 to ~15 pmol/10^6 cells (Fig. 2B). The combination of SNP and IBMX led to the strongest increase of cGMP efflux and intracellular cGMP levels, which increased ~17- and ~135-fold, respectively. Glutamate, butyrate, or interleukin 1β (6, 27, 28), reported for other cell types (e.g. neuronal and colon cells) to induce cGMP synthesis, did not stimulate cGMP production in our HEK293 cells (data not shown). Added extracellularly, cGMP was stable for at least 4 h in our incubation medium (with cells), showing that the amounts of cGMP reported here reflect the actual amounts exported from the cells.

Efflux of cGMP at High Intracellular cGMP Concentrations—Fig. 3 shows the effect of MRP5 and MRP4 on cGMP efflux from 293 cells, if the intracellular cGMP levels were raised by adding IBMX (Fig. 3A) or IBMX plus SNP (Fig. 3B). Under all conditions used and whether cells were incubated for 1 h (white bars) or 4 h (gray bars), overexpression of MRP5 and MRP4 increased cGMP efflux. As the intracellular cGMP concentration increased in the presence of IBMX (Fig. 3A) and more so in the presence of IBMX plus SNP (Fig. 3B, note 7-fold difference in scale) the cGMP efflux increased proportionally. As this increase occurred both in the parental cells and in the transfectants, the relative increase in efflux due to the transfected MRP4 or MRP5 remained relatively modest under all conditions. This is illustrated in Fig. 4. In the MRP5 transfectants cGMP efflux relative to parental cells increased about 2-fold in the presence of IBMX; in the MRP4 transfectants, the relative cGMP efflux was decreased somewhat by IBMX, but this can be attributed to a partial inhibition of MRP4 by IBMX (see below). Note, however, that even though the relative rate of efflux by MRP4 is decreased by IBMX (Fig. 4), the absolute rate is increased, as can be seen by comparing Fig. 1 with Fig. 3. Most of the cGMP was effluxed during the first hour of incubation (Fig. 3A, white bars). The variation of the intracellular cGMP levels over time was similar in all lines: high levels after 1 h decreasing to low levels after 4 h (Fig. 3A, right panel). The decrease might be the result of feedback inactivation of GC, because it was less pronounced when synthesis of cGMP was stimulated with SNP (Fig. 3B, right-hand panel) (29). Activation of a PDE not sensitive to IBMX might also play a role (29, 30).

Two remarkable results presented in Fig. 3 deserve emphasis: First, the presence of high concentrations of MRP4 and MRP5 in the HEK293 transfectants had little effect on the intracellular cGMP concentrations found. At most, there was a 2-fold decrease in cGMP in the MRP4 and MRP5 transfectants relative to parental 293 cells, both in Fig. 3A and Fig. 3B, but this decrease was only visible after 1-h incubation and not after 4 h and showed a relatively large day-to-day variation. When we compared experiments done on the same day the decrease after 1 h was consistently found; in the presence of IBMX alone the decrease was statistically significant for all the transfectants (paired Student’s t test, p < 0.05), whereas with IBMX plus SNP this was only the case for 51 cells. Clearly, MRP4 and MRP5 are unable to lower intracellular cGMP substantially under the conditions tested.

The second remarkable result in Fig. 3 is that the ability of MRP4 and MRP5 to transport cGMP is not saturated even at
very high apparent intracellular cGMP levels. This is unexpected, because the reported \( K_m \) values for MRP4- and MRP5-mediated cGMP transport in vesicle uptake experiments are quite low, 9.7 and 2.1 \( \mu \)M (16, 17), respectively. These values are far below the intracellular cGMP concentrations that can be calculated from the data in Fig. 3. Assuming that 10^6 cells have a volume of about 1 \( \mu \)l and that all cGMP found is free in the cytosol, we calculate that the intracellular cGMP concentrations after 1 h were 40–80 \( \mu \)M in the presence of IBMX alone (Fig. 3A). When the intracellular cGMP concentration was further raised by stimulating the cells with IBMX plus SNP (Fig. 3B), the calculated levels were between 350–600 \( \mu \)M, an approximately increase of 8-to 10-fold over the levels obtained by treatment with IBMX alone (Fig. 3A). The absolute rate of cGMP efflux increased proportionally by 7-to 8-fold in the HERK293 cells and 9-to 10-fold in the 4.63 and 5I cells, a linear increase in transport as a function of cGMP concentration without any indication that the transport capacity was near saturation. We infer from these results that MRP4 and MRP5, as well as the endogenous transporter(s) present in HERK293 cells, have a low affinity for cGMP.

Effect of IBMX on Transport by MRP4 and MRP5—The results in Fig. 4 suggest that IBMX inhibits MRP4- but not MRP5-mediated cGMP transport in intact cells. However, because IBMX also increases intracellular cGMP levels and does not completely inhibit MRP4, the degree of inhibition was difficult to assess based on the cGMP efflux alone. We therefore tested the effect of 1 mM IBMX on efflux of another substrate of MRP4 and MRP5, PMEA (20, 22). Fig. 5A shows that the drug decreased PMEA efflux from parental cells and from the MRP4 and MRP5 transfectants. However, if the results for the transfectants are corrected for the substantial inhibition of the efflux from parental cells (Fig. 5B), only the MRP4 transfectant was inhibited by about 50%, whereas the MRP5 transfectant was unaffected. Addition of 1 mM SNP together with IBMX did not affect PMEA efflux (not shown), showing that PMEA transport was inhibited by IBMX itself and not by the increased cGMP levels. Similar results were obtained with an independent MRP4 (4.3) and an independent MRP5 (Fig. 5E) transfectant, respectively (not shown). The high inhibition of endogenous PMEA transport by IBMX also appears to apply to the cGMP transport, as suggested by the results in Fig. 2 (compare light and dark gray bars).

**Active Transport by MRP4 and MRP5**—To show that transport by MRP4 and MRP5 was ATP-dependent and to determine whether this was also the case for the endogenous cGMP transport, we determined transport under normal and under ATP-depleting conditions. Fig. 6 shows the time-dependent and ATP-dependent efflux of cGMP for 293, 5I (MRP5), and 4.63 (MRP4) cells, after inducing high intracellular cGMP levels with IBMX and SNP. Inhibition of ATP production strongly decreased the cGMP efflux by MRP5 and MRP4 as well as by the endogenous transporter(s).

**Inhibition of MRP4- and MRP5-mediated cGMP Transport**—Many studies have investigated the effects of a wide variety of compounds on the transport of cyclic nucleotides (2–10, 17, 31). Fig. 7A shows the inhibition of endogenous, MRP4-, or MRP5-mediated cGMP efflux by a range of compounds. Probenecid (0.5 mM), sulfipyrazone (0.5 mM), bromosulphthalein (50 \( \mu \)M), bromphenol blue (150 \( \mu \)M), rose bengal (100 \( \mu \)M), and indocyanine green (100 \( \mu \)M) inhibited both the endogenous and MRP4-mediated cGMP efflux but MRP5 most effectively. PGA_1 (10 \( \mu \)g/ml) and PGE_1 (10 \( \mu \)g/ml) strongly inhibited the endogenous and MRP4-mediated cGMP efflux, whereas only PGA_1 strongly inhibited MRP5-mediated efflux. The steroids progesterone (100 \( \mu \)M) and estramustine (100 \( \mu \)M) inhibited the endogenous and the MRP5-mediated cGMP efflux strongly, but only progesterone inhibited MRP4. In contrast, dexamethasone (150 \( \mu \)M) was only a moderate inhibitor of cGMP efflux in all
cell lines. The PDE5 inhibitors zaprinast (10 μM), sildenafil (10 μM), and trequinsin (10 μM) were poor inhibitors of cGMP efflux. Fig. 7B shows that none of the compounds tested strongly affected the intracellular cGMP levels, excluding the possibility that a decrease in cGMP production led to the observed decreases in cGMP efflux. Because sildenafil, zaprinast, and trequinsin were reported to be high affinity inhibitors of MRP5-mediated cGMP transport in vesicular uptake (17), the lack of inhibition in our cells was unexpected. This was not because these compounds did not enter the cells, because they inhibited PDE activity in the absence of IBMX. Incubation of HEK293 cells for 1 h with 10 μM each of sildenafil, zaprinast, and trequinsin led to 30-, 20-, and 6-fold increases in the intracellular cGMP levels, respectively. In contrast, neither probenecid (0.5 mM) nor sulfipyrazone (0.5 mM) increased the intracellular cGMP levels in the absence of IBMX (data not shown).

Effect of cAMP on Cyclic Nucleotide Transport by MRP4 and MRP5—Effect of cAMP by HEK293 cells under control conditions was detected, but the levels were at the limits of detection (extracellular 0.19 ± 0.14 pmol/10⁶ cells/h and intracellular 14 ± 4 pmol/10⁶ cells). Equivalent intracellular and extracellular cAMP levels were found in all cells, and these levels were not altered in the presence of SNP and IBMX (data not shown). Incubation with forskolin (100 μM) strongly induced cAMP production in the HEK293 cells (extracellular 365 ± 130 pmol/10⁶ cells/h and intracellular 1730 ± 185 pmol/10⁶ cells). Relative to the parental cells, efflux of cAMP was strongly increased in the 5I and 4.63 cells, but MRP4 and MRP5 overexpression did not result in a decrease in intracellular cAMP levels (Fig. 8).

Effect of GSH Depletion on Cyclic Nucleotide Transport by MRP4 and MRP5—Recently it was reported that depletion of intracellular GSH led to decreased cAMP efflux from MRP4-overexpressing cells (23). In contrast, we find no effect of GSH depletion on the resistance to PMEA mediated by either transporter. To determine whether cGMP or cAMP efflux from HEK293 cells was dependent on intracellular GSH, we depleted GSH by overnight incubation with BSO. The HEK293, 4.63, and 5I cells showed a similar ~90% drop in intracellular GSH/GSSG levels from 6.6 pmol of GSH/mg of protein without BSO, to 0.4 pmol of GSH/mg of protein with BSO. Under these conditions, there was no change in cyclic nucleotide transport by the three cell lines (Fig. 9A and B), indicating that intracellular glutathione did not influence the transport of cyclic nucleotides by MRP4, MRP5, or endogenous transporters.

DISCUSSION

The cyclic nucleotides cGMP and cAMP are second messengers involved in mediating the response to numerous stimuli, and large families of enzymes regulate their intracellular concentrations (reviewed in Ref. 1). Although the efflux of cyclic nucleotides from cells has been recognized for decades, transporters capable of mediating this process have only recently been identified. Several reports have shown that both MRP4 and MRP5 transport cGMP and cAMP in vesicular uptake experiments (15–17). We have investigated the efflux of cyclic nucleotides from intact HEK293 cells and lines overexpressing either MRP4 or MRP5 and found that both MRP4 transport cGMP and cAMP from intact cells in an ATP-dependent manner. Under the experimental conditions used MRP5 exports cGMP somewhat faster from 293 cells than cAMP, whereas the reverse is true for MRP4 (see Fig. 8). Neither cAMP nor cGMP transport was affected by GSH depletion, strongly suggesting that GSH is not essential for cyclic nucleotide transport. This contrasts with the MRP4-mediated cAMP efflux found by Lai and Tan (23) but is consistent with the lack of GSH requirement in MRP4- and MRP5-mediated cGMP and cAMP transport in vesicular uptake studies (16).

Published kinetic analyses of MRP4- and MRP5-mediated cyclic nucleotide transport in vesicular uptake have suggested that MRP4 has a high affinity for both cGMP (Km = 9.7 μM) and cAMP (Km = 44.5 μM) (16), whereas MRP5 has a very high affinity for cGMP (Km = 2.1 μM) and lower affinity for cAMP (Km = 379 μM) (17). In contrast, our results show that, in intact cells, both MRP4 and MRP5 mediate low affinity extrusion of cyclic nucleotides. In fact, MRP4 and MRP5 transport rates increased nearly proportionally up to calculated intracellular concentrations of ~0.6 mM. The notion that cGMP and cAMP are low affinity substrates in intact cells is supported by the observation that an extra 10-fold increase in intracellular cGMP levels brought about by addition of SNP to cells incubated with IBMX did not lead to any further inhibition of PMEA efflux (Fig. 5). In addition, in vesicular uptake studies we need almost millimolar concentrations of cGMP and cAMP to inhibit MRP4 and MRP5 (32), supporting the finding that cGMP and cAMP are relatively low affinity substrates for MRP4 and MRP5. Possible reasons for this apparent difference in affinities between our cGMP transport and that found by others are discussed elsewhere (32). We consider our results with intact cells robust, and, because the intact cell is the natural environment of these transporters, we conclude that the apparent low affinities we find for MRP4 and MRP5 transport of cGMP is the relevant affinity in the in vivo situation.

In several reports it has been suggested that MRP-mediated cGMP and cAMP efflux from cells might be able to reduce the intracellular concentrations of these second messengers following stimulation (4, 8, 9, 27, 33). In our experiments, however, MRP4- and MRP5-mediated cGMP transport had only a marginal effect on intracellular cGMP. Our results are in line with the conclusion that cyclic nucleotide efflux is not a general mechanism by which cells control intracellular cyclic nucleotide levels. This conclusion was also reached in studies comparing the rate of cGMP hydrolysis by various PDE iso-enzymes with the rate of cGMP efflux by aortic smooth muscle cells (34, 35) and is further substantiated by observations that inhibition of efflux, even combined with PDE inhibition, did not result in increased intracellular cyclic nucleotide concentrations (2, 4, 7, 8, 10). Taken together, these findings suggest that
intracellular cyclic nucleotide levels are controlled by a combination of feedback inactivation of GC and regulation of PDE activity (29, 30, 36, 37) rather than by extrusion of cyclic nucleotides by MRPs. In the light of the apparent low affinity of MRP4 and MRP5 for cyclic nucleotides in intact cells, these transporters would only be expected to make a modest contribution to the lowering of intracellular cyclic nucleotide peak concentrations levels under extreme conditions or in cells that lack sufficient PDE activity and/or GC feedback inhibition.

With the exception of its role in the development of the multicellular form of Dictyostelium discoideum (38) the extracellular function of cyclic nucleotides remains unclear. D. discoideum has at least four cAMP receptors, but vertebrate orthologs have yet to be identified. There are, however, reports showing that cGMP and cAMP influence several processes in cultured cells. In cultured mouse cerebellar neurons extracellular cGMP inhibited a kainate-activated response and in rat astrocytes cGMP inhibited a Na+/H+ exchanger (13, 14). In the kidney, extracellular conversion of cAMP to adenosine could also be involved in receptor-mediated effects on renal function (39, 40). The localization of MRP4 in the brush-border membrane of proximal tubule cells and the high renal expression of MRP5 is compatible with a role for these pumps in renal homeostasis. Because excretion of cGMP by the kidney keeps plasma levels is low (3), this would enable locally and temporally increased cyclic nucleotide levels to act as signaling events. There are also reports suggesting that cGMP has systemic effects. For instance, during pregnancy plasma cGMP levels increase (41), and relapsed cervical cancer patients have somewhat elevated cGMP levels (42).
Several studies have investigated the effects of a wide variety of compounds on the efflux of cyclic nucleotides from different cell types. Similar to previous reports (4, 7, 8, 43) we found that 0.5 mM probenecid inhibited cGMP efflux from HEK293 cells. This was due to inhibition of MRP4 and MRP5 as well as endogenous transporters. In addition, bromphenol blue and bromosulfophthalein were strong inhibitors of MRP5 and endogenous transporters but weaker inhibitors of MRP4. Rose bengal inhibited both MRP4 and MRP5 but had less influence on endogenous efflux, whereas indocyanine green strongly inhibited all efflux.

Prostaglandins have been reported to inhibit cyclic nucleotide efflux from several cell types. Both prostaglandin A1 (PGA1) and PGE1 inhibited cAMP efflux from avian erythrocytes, whereas only PGA1 inhibited cAMP efflux from C-6 rat glioma cells (43–45). In our HEK293 cells PGA1 inhibited the cyclic nucleotide efflux mediated by MRP4 and MRP5, as well as endogenous efflux, whereas PGE1 inhibited MRP4 and endogenous transport more potently than MRP5 transport. Intra-cellular PGA1 is readily converted to a glutathione conjugate (PGA1-SG), and this conjugate most likely inhibits the cAMP efflux from erythrocytes (10). Because MRP1, a transporter closely related to MRP4/5, has been shown to transport PGA1-SG (46), it is likely that PGA1-SG partially inhibits the cGMP efflux in our cells. In contrast, PGE1 does not form a glutathione conjugate showing that glutathione conjugation is not a required for inhibition. Prostaglandins are poorly membrane permeable (47) but must be secreted to act as local hormones. Our inhibition data suggest that some prostaglandins are MRP4 and MRP5 substrates and that these transporters might be involved in prostaglandin secretion. In this respect it is noteworthy that indocyanine green, which inhibited the cGMP efflux by MRP4 and MRP5, has been used to inhibit prostaglandin transport (48), further supporting the idea that the MRPs may be involved in prostaglandin secretion.

More recently, Sundkvist et al. (31) reported that several steroid-based compounds potently inhibit the ATP-dependent cGMP transport into vesicles derived from human erythrocytes. They reported an inhibition constant (Ki) of 1.7 μM for progesterone and a Ki of 0.2 μM for the structurally related mifepristone. We found that progesterone inhibited the cGMP efflux from all cells studied to a similar extent. Estramustine, a structurally related drug used in the treatment of prostate cancer, also inhibited transport, especially that mediated by MRP5. These data raise the possibility, that, like prostaglandins, steroid hormones might be physiological substrates of MRP4 and MRP5. Our results show that HEK293 cells contain endogenous transporters for cGMP that differ in their affinities for inhibitors from MRP4 and MRP5. Because MRP4 and MRP5 are detected in parental 293 cells (21), they may be in part responsible for the endogenous cyclic nucleotide efflux. Two recently identified MRPs, MRP8 and MRP9 (49–51), might also contribute, although their substrate specificity remains to be determined.

In their report of MRP5-mediated cGMP transport into vesicles, Jedlitschky et al. (17) found that the PDE inhibitors sildenafil, trequinsin, and zaprinast were particularly potent inhibitors of MRP5. In contrast, we did not observe any appreciable inhibition of the cGMP efflux with either sildenafil or trequinsin. Neither the endogenous nor the MRP4- and MRP5-mediated cGMP efflux was inhibited using relatively high concentrations (10 μM), and only slight inhibition was observed in the presence of zaprinast. Because the concentration of sildenafil that we used inhibited PDE in our cells and is similar to the clinically attained plasma concentration (52), we conclude that inhibition of MRP4 and MRP5 does not significantly contribute to the characteristic increase of intracellular cGMP levels that is brought about by sildenafil. The inhibition of MRP4 and MRP5 by some inhibitors, such as Pr, Sp, and Tq, but not by all (e.g. ICG or PGA1), tended to increase the intracellular cGMP levels, and this was comparable to the 2-fold decrease in intracellular cGMP levels mediated by these pumps in the absence of inhibitors (Fig. 3).

In summary, we have shown that MRP4 and MRP5 transport cAMP and cGMP from intact cells with low affinity in an ATP-dependent, but GSH-independent, manner. Overexpression of MRP4 and MRP5 did not lead to a substantial decrease in intracellular cGMP and cAMP levels, even when these levels were high. Conversely, when transport by MRP4 and MRP5 was inhibited this did not lead to substantial increases in intracellular cGMP levels. Our results suggest that cyclic nucleotide efflux by MRP4 and MRP5 does not play a role in modulating intracellular signals that are propagated through cGMP or cAMP.

Acknowledgments—We thank Hugo de Jonge (Erasmus University, Rotterdam, The Netherlands) and Jan de Vente (University of Maastricht, The Netherlands) for helpful suggestions and discussions and Hein te Riele, Henk de Vries, and Noam Zelcer for reading the manuscript.

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