Cellular Efflux of cAMP and cGMP - A Question about Selectivity

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Abstract: The present paper reviews and discusses selectivity of ABCC4 (MRP4), ABCC5 (MRP5) and ABCC11 (MRP8) as cellular efflux pumps for cAMP and cGMP. These transporters are potential drug targets for selective modulation of cyclic nucleotide action.

Key Words: cGMP, cAMP, ABCC4, ABCC5, ABCC11, transport selectivity.

CYCLIC NUCLEOTIDES AS SECOND MESSENGERS

The discovery of cAMP as a second messenger to hormones and neurotransmitters by Sutherland and co-workers [1] and cGMP few years later [2], led to development of a number of drugs which became a part of modern pharmacotherapy. The impact of cyclic nucleotides in biology and pharmacology was summed up in a more recent review [3]. Knowledge about selectivity in the biokinetic processes (synthesis, enzymatic conversion and cellular efflux) may lead to the development of new and more specific drugs.

CELLULAR EFFLUX OF cAMP

Sutherland and co-workers showed that the pigeon erythrocytes extruded cAMP against a concentration gradient and that the transport was inhibited by probenecid, a non-specific organic anion transport inhibitor [4]. A variety of cells and tissue types, including erythrocytes, fibroblasts, cells from liver and heart, adrenocortical cells, astrocytoma cells, glioma cells, lymphoma cells, adipose tissue, islets of Langerhans, renal tubules and thyroid slices, extruded cAMP [5]. The transport was unidirectional and inhibited by agents such as metabolic inhibitors (iodoacetate, oligomycin and cyanide), inhibitors of membrane transport (probenecid and verapamil) and other compounds (vinblastine, papaverine and prostaglandins) [6-12]. The time course of intra- and extracellular cAMP levels showed different patterns after adenylate cyclase stimulation. Intracellular levels reached a peak after relative short period, whereas, extracellular levels increased linearly for a much longer time, before reaching a plateau [6, 8, 13]. A considerable difference between cell types in their ability to extrude cAMP has also been reported. The export from chromaffin cells limits the rise in cellular cAMP levels [14], whereas, efflux plays a minor role in the control of intracellular cAMP levels in osteoblast-like cells [15]. Values of the first order elimination constant vary between tissues from 0.14 to 0.014 min^{-1}, corresponding to a half-life of intracellular cAMP from a few minutes to almost one hour [16].

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CELLULAR EFFLUX OF cGMP

Cellular cGMP efflux from pancreatic cells, liver and cerebellar slices, glioma and pheochromocytoma cells, vascular smooth muscle cells and endothelial cells showed unidirectional energy-dependent transport against a concentration-gradient and the transport was inhibited by probenecid [13, 17-20]. In airway epithelium, a vectorial cGMP transport was observed, and as much as one-half of the total cGMP was exported from the cells [21]. The apparent increase in total human platelet cGMP content observed after thrombin stimulation, was the result of an increase of cGMP in the extracellular compartment, due to cellular extrusion [22]. When soluble guanylate cyclase was stimulated and cyclic nucleotide phosphodiesterase inhibited in human platelets, intracellular cGMP levels increased almost 5-fold but nearly 20-fold on the extracellular side [23].

An experimental model with inside-out vesicles from human erythrocytes showed that cGMP transport was ATP- and temperature dependent, with K_m-values from 1 to 5 µM [24-26] with an ATPase [27]of m-type [28]. The transport of cGMP was directly linked to hydrolysis of ATP [29]. The low K_m cGMP transport was inhibited by well-known membrane transport modulating agents, like probenecid, sulfinpyrazone, verapamil and progestins [25, 26, 30, 31].

DIFFERENT CHARACTERISTICS OF CELLULAR cAMP AND cGMP EFFLUX

There are many evidences for selective cAMP and cGMP export. Even if intracellular cGMP levels were several times below those of cAMP, its urinary levels were within the same order of magnitude [32]. In a comparative study of endothelial and smooth muscle cells, the extracellular cGMP levels continued to rise after stimulation of guanylate cyclase for several hours, an observation in sharp contrast to the characteristics of cAMP accumulation after adenylate cyclase stimulation, with shorter duration and a peak nearly coincident with that of intracellular levels [13]. In kidney epithelial cells the polarization of cAMP and cGMP transporters and inhibitor (probenecid and nocodazole) potency was markedly different [33]. The first-order kinetic rate constants were 0.139 min^{-1} and 0.022 min^{-1} for cGMP and cAMP, respectively [34]. IBMX reduced the first order cGMP efflux rate six fold whereas that of cAMP was unchanged.

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No competition was observed despite high intracellular cyclic nucleotide levels, after submaximal co-stimulation of guanylate cyclase and adenylate cyclase [34]. Together, these studies provide strong arguments for two separate transport systems.

ABCC4, ABCC5 AND ABCC11

Members of the organic anion ABCC transporters (ATP-Binding-Cassette transporter, subfamily C), previously called multidrug resistance associated proteins (MRP), are responsible for the cyclic nucleotide transport out of cells. The recognition and initial characterization of ABCC4 (MRP4) [35, 36], ABCC5 (MRP5) [35, 37, 38] and ABCC11 (MRP8) [39-41] showed that these efflux pumps have a P-glyco-protein-like core, consisting of two TMDs and two nucleotide-binding domains, distinct from other members of the ABCC subfamily which have an amino-terminal TMD (TMD_0).

ABCC4, ABCC5 AND ABCC11 AS TRANSPORTER PROTEINS FOR cGMP

Low and virtually identical K_m-values were reported for cGMP uptake into human erythrocyte inside-out vesicles [25, 26, 30, 31, 42, 43]. However, since both ABCC4 [42] and ABCC5 [44] coexist in human erythrocytes, the respective roles of these proteins were unclear, but direct evidence of ABCC5 being the low K_m transporter for cGMP was reported with a K_m-value of 2.1 µM in cells overexpressing ABCC5 [44]. Furthermore, cGMP transport was reduced after treatment of membrane extracts with antibodies against ABCC5 [45]. In agreement, ABCC5 operates as a cGMP-selective pump in pial arteriolar smooth muscle [46] and in pituitary cells [47]. Taken together, these reports show that ABCC5 transports cGMP with high affinity. The low affinity cGMP transport effectuated by ABCC5 [48] may be identical with the second transport component (K_m: 170 – 280 µM) reported for cGMP uptake into human erythrocyte inside-out vesicles [24, 30]. This process proved to be non-specific [30].

The features of cGMP transport by ABCC4 are less consistent. A study of human erythrocyte inside-out vesicles concluded that the cGMP transport had properties similar to those for MRP4 [42]. In addition to a minor high affinity transport component (K_m of 0.5-2.5 µM), a dominant low affinity component of cGMP transport with K_m values of 50-80 µM was reported. In a follow up study, both high affinity (3.3 µM) and low affinity (330 µM) cGMP transport were identified [43]. In cells overexpressing ABCC4 cGMP was transported with a K_m value for 180 µM [49]. The possibility exists that ABCC4 is responsible for the low affinity cGMP transport component (K_m-values of 170 – 280 µM) [24, 30] since ABCC4 is present in human erythrocytes [42]. In many studies with cells overexpressing ABCC4 low affinity transport for cGMP has been reported. High cGMP concentrations (>300 µM) were needed to reduce methotrexate transport markedly (53%) [50]. The maximal stimulation of ATPase activity by cGMP was modest (75% above basal) [51]. No sign of saturation of cGMP transport was observed for concentration up to 600 µM [48] and 1 mM cGMP was required to exceed 50 % inhibition of estradiol 17ß-glucuronide transported by ABCC4 [52].

However, some studies have also reported relatively high affinity cGMP transport by ABCC4. A K_m-value of 9.8 µM was calculated, but the affinity might have been overestimated, since the highest cGMP concentration tested was 25 µM [53]. A more recent study on human erythrocytes [54] showed a time- and concentration-dependent inhibition of cGMP transport by the irreversible serine protease inhibitor AEBSF. AEBSF blocked transport by ABCC4 but not by ABCG2. It has not been tested against ABCC5 and the data cannot be used to exclude a role for ABCC5 [54] since these cells contain both ABCC4 and ABCC5 [42, 44]. In smooth muscle cells wherein ABCC4 was silenced by siRNA, a clear increase (230%) in the intra- to extracellular cGMP ratio was observed [55] but this may rather reflect ABCC4 low affinity cGMP transport due to high intracellular cyclic nucleotide levels as a result of PDE inhibition by 100 µM IBMX.

In addition to member 4 and 5 of the ABCC family, overexpression of ABCC11 causes an increased cGMP extrusion [56]. Based on inhibition studies [57] the estimated IC_{50} values for cGMP are 25-50 µM. This shows that ABCC11 is transporting cGMP with medium to low affinity.

ABCC4, ABCC5 AND ABCC11 AS TRANSPORTER PROTEINS FOR cAMP

Assuming that low cGMP concentrations (1 µM) are mainly transported by ABCC5 in human erythrocyte inside-out vesicles, uptake was inhibited 15% by 100 µM and 50% by 300 µM cAMP [25, 42]. In cells with ABCC5 overexpression, cAMP caused low affinity inhibition of cGMP transport (K_i = 379 µM) [44] and of alaninyl-d4TMP transport (31% inhibition with 850 µM cAMP) [52]. No saturation of cAMP export from intact cells overexpressing ABCC4 was seen for intracellular concentrations up to 600 µM [48]. These studies show that ABCC5 transports cAMP with low affinity.

On the other hand, several studies support the idea that ABCC4 is a main cAMP transporter. Studies on cells overexpressing ABCC4 gave a) K_m-value of 45 µM [53], b) fourfold increase in ratio between extra- and intracellular cAMP levels after forskolin stimulation [58] and c) transported the cAMP analog (fluo-cAMP) with high affinity (K_m-value of 5.3 µM) [59]. An order of potency like PGA_1 > PGE1 > $PGF_{1\alpha}$ was reported for inhibition of an ABCC4 substrate (17-ß estradiol glucuronide) [60]. This is consistent with studies two decades ago wherein PGA_1 was an effective inhibitor of cAMP efflux [10, 61] with an order of potency like PGA_1 > PGB_1 > PGE_1 > $PGF_{1\alpha}$ [11, 62, 63]. When ABCC4 was silenced by siRNA a clear increase between intra- and extracellular cAMP levels (289%) appeared [55].

ABCC11 transports cAMP with moderate to low affinity [56, 57] with estimated IC_{50}-values (25 - 50 µM) similar to those of cGMP.

MOLECULAR MODELS OF cAMP ANDS cGMP

The planar structures of cGMP and cAMP are similar (Fig. (**1**)), but there is considerable degree of selectivity among protein kinase, some phosphodiesterases and cation

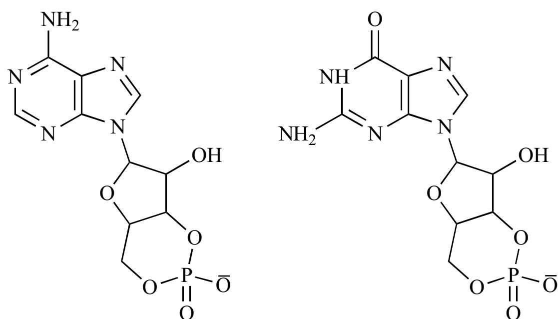

Fig. (1). Chemical structures of cAMP (panel A) and cGMP (panel B).

channels [64]. Differences in stereological conformations may account for this. In order to support this idea, molecular models were constructed based upon the crystal structures of cAMP bound to a cyclic nucleotide regulated potassium channel [65] and cGMP bound to a hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channel [66]. The energy minimized cAMP and cGMP crystal structures differed distinctly in spatial appearances and in the electrostatic potential surface (EPS) (Fig. (**2**)). When the cyclic nucleotides were flipped 180° along the y-axis, cAMP showed an oval, but linear shape (anti-conformation), whereas cGMP had a distinct hinge in the mid molecule (syn-conformation). Compared to the cAMP surface area which was neutral, cGMP had a prominent negative surface in the ribose-phosphate area. This shows that stereological requirements for selectivity exist.

CONCLUSIONS

The basis of development of new drugs is a selectivity for endogenous compounds between molecular targets. The existing data on cyclic nucleotides and ABCC transporters can be summarized like this: 1) ABCC4 is a selective moderate high affinity transporter for cAMP, 2) ABCC5 is a selective high affinity transporter for cGMP, 3) ABCC4 and ABCC5 have additionally a transport site with high K_m for both nucleotides, i.e. they are non-selective low affinity transporters and 4) ABCC11 is a non-selective low affinity transporter for cAMP and cGMP.

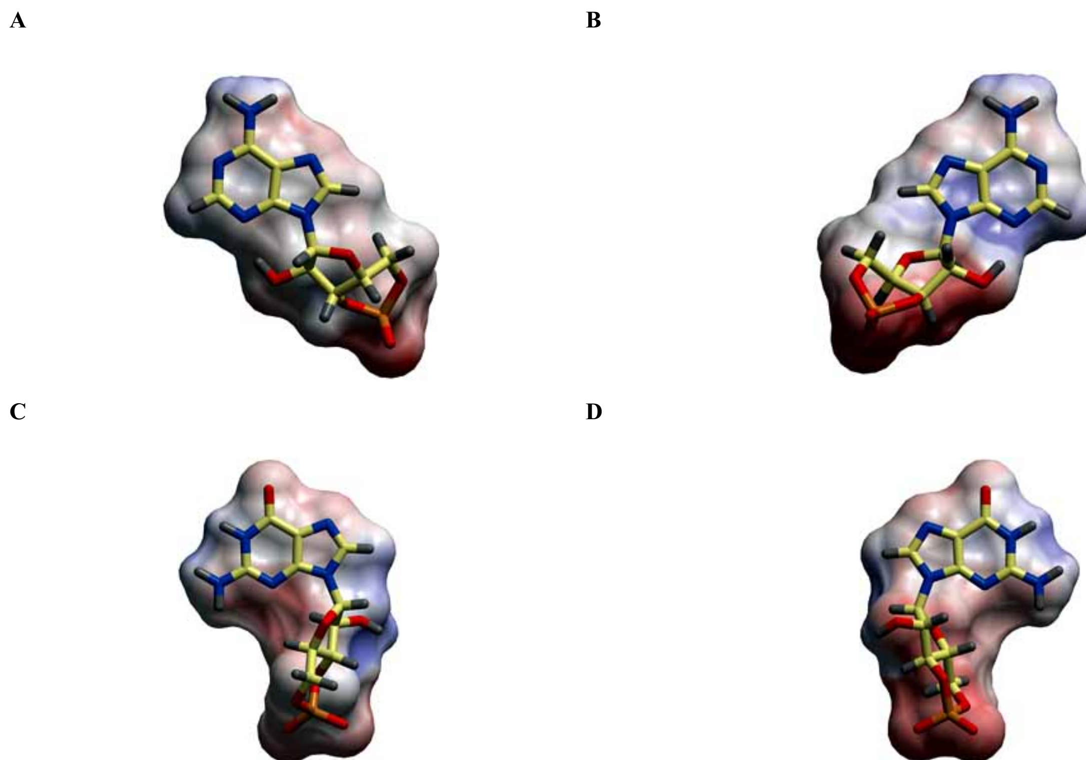

Fig. (2). Chemical structures of cAMP (panel **A** and **B**) and cGMP (panel **C** and **D**) superimposed on their respective EPS models. In panels B and D, the molecules are flipped 180° along the y-axis. The crystal structures of cAMP, bound to a cyclic nucleotide regulated potassium channel [65] and cGMP, bound to a hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channel [65] were used as input pdb structures for the X-windows graphical interface of the xleap shell scripts, and the Antechamber and tleap programs of the AMBER 8.0 program package. Atomic charges and atom types were assigned by the Antechamber program. The Sander program of the AMBER 8.0 program package was used for energy minimization of the ligands. The electrostatic potential surface (EPS) of cAMP and cGMP were calculated and visualized by the ICM program after converting the AMBER energy minimized structures to ICM objects *via* the ICM molecular editor.

In the structure formulas the different atoms are indicated as follows; carbon, oxygen, nitrogen, phosphor and hydrogen with yellow, red, blue, orange and grey, respectively. In the EPS-models blue, white and red areas indicate positive, neutral and negative areas.

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