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Modulation of high affinity ATP-dependent cyclic nucleotide transporters by specific and non-specific cyclic nucleotide phosphodiesterase inhibitors

Lena Aronsen [a,b], Elin Orvoll [a], Roy Lysaa [a], Aina W. Ravna [a], Georg Sager [a,b,*]

[a] Medical Pharmacology and Toxicology, Department of Medical Biology, Faculty of Health sciences, University of Tromsø, The Arctic University of Norway, Norway

[b] Clinical pharmacology, Department of Laboratory Medicine, Division of Diagnostic services, University Hospital of North Norway, Tromsø, Norway

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ABSTRACT

Intracellular cyclic nucleotides are eliminated by phosphodiesterases (PDEs) and by ATP Binding cassette transporters such as ABCC4 and ABCC5. PDE5 and ABCC5 have similar affinity for cGMP whereas ABCC5 has much higher affinity for cGMP compared with cAMP. Since the substrate (cGMP) is identical for these two eliminatory processes it is conceivable that various PDE inhibitors also modulate ABCC5-transport. Cyclic GMP is also transported by ABBC4 but the affinity is much lower with a K_m 50–100 times higher than for that of ABBCC5. The present study aimed to determine K_i-values for specific or relative specific PDE5 inhibitors (vardenafil, tadalafil, zaprinast and dipyridamole) and the non-specific PDE inhibitors (IBMX, caffeine and theophylline) for ABCC5 andABCC4 transport. The transport of [^{3}H]-cGMP (2 μM) was concentration-dependently inhibited with the following K_i-values: vardenafil (0.62 μM), tadalafil (14.1 μM), zaprinast (0.68 μM) and dipyridamole (1.2 μM), IBMX (10 μM), caffeine (48 μM) and theophylline (69 μM). The K_i-values for the inhibition of the [^{3}H]-cAMP (2 μM) transport were: vardenafil (3.4 μM), tadalafil (194 μM), zaprinast (2.8 μM), dipyridamole (5.5 μM), IBMX (16 μM), caffeine (41 μM) and theophylline (85 μM). The specificity for ABCC5 we defined as ratio between K_i-values for inhibition of [^{3}H]-cGMP and [^{3}H]-cAMP transport. Tadalafil showed the highest specificity (K_i-ratio: 0.073) and caffeine the lowest (K_i-ratio: 1.2).

1. Introduction

The major pharmacotherapeutic progress during the last 50 years includes drugs which modulate signalling systems. One example is the drugs that influence cyclic nucleotide biokinetics with effects on the balance between synthesis and elimination (Beavo and Brunton, 2002). The cellular elimination of cyclic nucleotides comprises PDEs responsible for biotransformation and ABC-transporters which account for the cellular efflux of unmodified molecule. In humans 11 PDE families with different tissue distribution and substrate specificity exist (Francis et al., 2011). Significant progress has been made in developing specific inhibitors of PDEs to obtain more selective effects and minimise adverse effects. The feasibility of these enzymes as drug targets is exemplified by the commercial and clinical successes of the erectile dysfunction drugs, sildenafil, tadalafil and vardenafil. However, still non-specific PDE inhibitors are in clinical use (Savai et al., 2010).

During the last decades the mechanisms that regulate the intracellular levels of cGMP have been clarified. A finely tuned balance between synthesis, degradation and efflux with compartmentalisation of the biokinetics components (cyclases, phosphodiesterases and efflux pumps) exist (Cheepala et al., 2013). The signature substance of specific PDE5 inhibitors, sildenafil, prevents the high affinity cellular cGMP efflux (Sundkvist et al., 2002) by inhibition of ABCC5 (Jedlitschky et al., 2000). Cyclic GMP is transported by ABCC5 with high affinity (K_m-value ≈ 2 μM) (Jedlitschky et al., 2000), and clearly higher than ABCC4-mediated transport. It is possible that an early report of high affinity (K_m ≈ 10 μM) might have overestimated the ABCC4 affinity for cGMP by limiting the concentrations to 25 μM (Chen et al., 2001). Much higher K_m-values have been reported: 170–180 μM (Klokouzas et al., 2003; van Aubel et al., 2005) and a K_i-value of 280 μM (Orvoll et al., 2013). In agreement, Jedlitschky et al. (2000) reported that inhibition of ABCC5-mediated cGMP transport of cAMP was detected only at concentrations above 100 μM. On the other hand ABCC4 has a preference for cAMP in low concentrations with K_m-values 30–45 μM (Chen et al., 2001; Orvoll et al., 2013).

* Correspondance to: Medical Pharmacology and Toxicology, Department of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway.
Tel.: +47 77644708; fax: +47 7764 5310.
E-mail address: georg.sager@uit.no (G. Sager).

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Several reports exist on interactions between PDE inhibitors and ABC-transporters; inhibition of ABCC5 by zaprinast (Jedlitschky et al., 2000), ABCB1 by sildenafil, vardenafil and tadalafil (Shi et al., 2011; Ding et al., 2011) and ABCG2 by sildenafil (Shi et al., 2011). In the present paper the term selective has been used to distinguish between molecular targets (i.e. PDEs versus ABCC transporters) and specificity to distinguish between the individual enzymes or transporters within the same family. Recently, we reported that sildenafil and sildenafil analogues inhibit high affinity [^{3}H]-cGMP transport (Sager et al., 2012) and that, at least some of these, exhibit a specificity in their inhibition of ABCC4 and ABCC5 (Orvoll et al., 2013). To extend these observations the present study aimed to obtain K_i-values of specific and non-specific PDE inhibitors of ABCC4 and ABCC5.

2. Materials and methods

2.1. Chemicals

Cyclic GMP, cyclic AMP, zaprinast, dipyridamole, theophylline, theobromine, caffeine, IBMX (3-isobutyl-1-methylxanthine), ATP magnesium, acetyl thiocoline chloride and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma-Aldrich (Schnelldorf, Germany), tadalafil from Toronto Research Chemicals Inc. (Ontario, Canada), [^{3}H]-cGMP and [^{3}H]-cAMP from Perkin Elmer Inc. (Boston, MA, USA). Vardenafil was a gift from Bayer Health Care AG (Leverkusen, Germany). Other chemicals were of analytical grade.

2.2. Preparation of inside out vesicles

Fresh human EDTA blood was obtained from healthy donors (Department of laboratory medicine, University hospital of North Norway). The blood cells were separated from plasma by centrifugation and washed with 5 mM Tris–HCl, 113 mM KCl (pH 8.1) three times before lysis. The cells were lysed in 10 volumes of 5 mM Tris–HCl, 0.5 mM EGTA, 4 mM KCl (pH 8.1) and washed in the same buffer (20.000*g* for 20 min) until ghosts were milky white. Inside-out vesicles were prepared using a modification of the procedure originally described by Steck et al. (1970). The membrane vesiculation was initiated by adding 39 volumes of a hypertonic buffer (500 mM Tris–HCl, pH 8.2) to one volume of cell suspension and this process was completed after several hours. All the steps above were performed at 2–4 °C. After centrifugation the suspension was forced through a 27 gauge syringe needle to enhance homogenisation of the membranes. Inside-out vesicles were separated from right side out vesicles and ghosts by ultracentrifugation (100.000*g*) overnight using a density gradient from 1.048 g/ml to 1.146 g/ml (Histodenz, Sigma-Aldrich, Schnelldorf, Germany) in 5 mM Tris, 3 mM KCl and 0.3 mM EGTA. The uppermost band was collected, washed and resuspended in 1.47 mM KH_2PO_4, 81 mM K_2HPO_4 and 140 mM KCl (pH 7.6). Sidedness was verified using acetylcholinesterase accessibility with small modifications of the original method (Ellman et al., 1961).

2.3. Transport assay

Transport assays were performed by incubating the inside-out vesicles at 37°C with or without 2.0 mM ATP at various inhibitor concentrations. All assays were carried out in triplicates. Inhibition assays for transport of 2 μM [^{3}H]-cAMP or 2 μM [^{3}H]-cGMP were performed for dipyridamole (10 nM–1 mM), vardenafil (1 nM–1 mM), tadalafil (1 nM–100 μM), IBMX (1 nM–1 mM), zaprinast (1 nM–100 μM), caffeine (10 nM–1 mM), theophylline (10 nM–1 mM), theobromine (100 μM). After 60 min incubation in the presence of the appropriate inhibitor the transport was stopped by adding ice cold buffer with pH 7.6 (1.47 mM KH_2PO_4, 8.1 mM K_2HPO_4, 140 mM KCl). The inside-out vesicles suspension was filtered and washed with the use of nitrocellulose membranes (0.22 μm GSWP, Millipore, Billerica, MA) and the filters with inside-out vesicles were dissolved in ethylene acetate and the radioactivity was quantified (Packard 1900 TR Liquid Scintillation analyser), after addition of scintillation fluid (Ultima Gold XR, Packard, Groningen, The Netherlands).

2.4. Data analysis

The IC_{50} values were determined according to Chou (1976). These data were transformed to K_i-values according to Cheng and Prusoff (1973). Substrate concentration was 2 μM for both cyclic nucleotides whereas the K_m-values used were 2.6 μM and 30.8 μM for cGMP and cAMP, respectively, based on recently reported values (Orvoll et al., 2013).

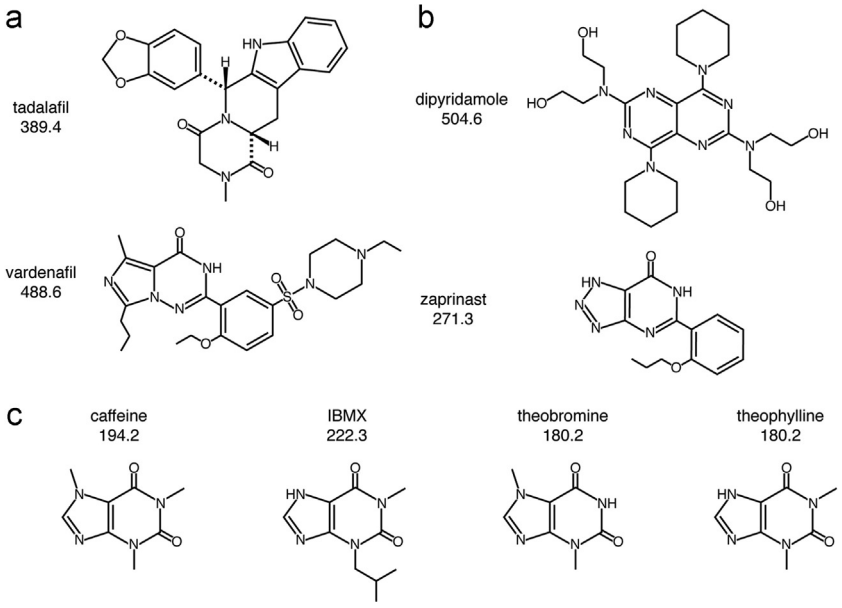

Fig. 1. Structures of specific PDE5 inhibitors (panel A), assumed specific PDE5 inhibitors (panel B) and non-specific PDE inhibitors (panel C).