SA1

Nucleoside transporters: from physiology to pharmacology and back

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Nucleosides need to be transported into cells either to provide precursors for purine nucleotide biosynthesis by salvage pathways or to regulate the extracellular concentration of adenosine, which in turn modulates purinergic receptors implicated in a variety of physiological processes. High-affinity Na^+-dependent concentrative nucleoside transport was initially thought to be restricted to (re)absorptive epithelia, thus mediating vectorial transport of nucleosides. We kinetically characterized and later cloned a hepatic pyrimidine-preferring nucleoside transporter, CNT1, that appeared to be up-regulated during liver regeneration. CNT1 is targeted to the apical membrane through the hepatic transcytotic pathway, whereas the purine-preferring high affinity nucleoside transporter CNT2 is mostly located at the basolateral membrane of the hepatocyte (Duflot *et al.* 2002), in which it seems to modulate extracellular adenosine availability to A1 type receptors. CNT expression is up-regulated by hepatomitogens and multifunctional cytokines such as IL-6 and TNF-α, both in cultured cells and *in vivo*. Although CNT (particularly CNT1) protein amounts change when progressing into the cell cycle, this may be the result of adaptive responses to alterations in nucleotide metabolism, as recently shown in hepatoma cells synchronized by inhibiting ribonucleotide reductase with hydroxyurea (Valdés *et al.* 2002). Differentiation is indeed the major determinant of nucleoside transporter expression in hepatocytes, as deduced from the analysis of CNT expression in cultured rat fetal hepatocytes induced to differentiate (del Santo *et al.* 2001) and in hepatocarcinogenesis (del Santo *et al.* 1998; Dragan *et al.* 2000). Non-epithelial cells, such as macrophages, also express CNT1, CNT2 and the equilibrative nucleoside transporters ENT1 and ENT2. We recently took advantage of this feature to demonstrate that ENT1 appears to be responsible for nucleoside channelling into DNA and thus required for proliferation, whereas CNT1 and CNT2 are up-regulated after macrophage activation (Soler *et al.* 2001*a*,*b*). Thus, CNT and ENT proteins are differentially regulated and may play different roles in cell physiology. High-affinity uptake of nucleoside-derived anticancer drugs is also mediated by CNT-type proteins, as recently described using the two electrode voltage clamp technique on *Xenopus laevis* oocytes expressing selected CNT isoforms (Lostao *et al.* 2000; Mata *et al.* 2001). Heterologous expression of CNT1 confers sensitivity to CNT1 substrates even in cells overexpressing the ENT1 protein (Mata *et al.* 2001). Although ENT1 activity has recently been correlated with ex vivo sensitivity of chronic lymphocytic leukaemia (CLL) cells to fludarabine (Molina-Arcas *et al.* 2002), retention of high affinity CNT1-mediated transport may confer increased sensitivity to nucleoside-derived drugs. We are currently addressing this issue by analyzing CNT1 expression in human tumors using a tissue array approach.

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SA2

Drug transport and the OATP superfamily

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Numerous transport proteins have been identified and cloned from different tissues during the past years. In the process of their functional characterization it became clear that various drugs are substrates or inhibitors of the so-called drug transporters that include the P-glycoproteins, the multidrug related proteins (Mrps/MRPs) and the organic anion transporting polypeptides (rodents: Oatps; human: OATPs).

Oatps/OATPs form a growing gene superfamily of currently 28 members in man, rat and mouse and mediate Na^+-independent transport of a wide spectrum of mainly amphipathic organic solutes. Based on their amino acid homology they can be grouped into five different families. The proteins with the broadest spectrum of amphipathic transport substrates are clustered in family 1. These OATP1-family members are thought to be part of the overall body detoxification system and help to remove potentially toxic endo- and xenobiotics from the systemic circulation. In the liver, an important organ for the elimination of a wide variety of mainly amphipathic endo- and xenobiotics, several of these family members are expressed at the sinusoidal membrane (rat Oatp1, Oatp2 and Oatp4, human OATP-C and OATP8) and mediate uptake into hepatocytes. Functional characterization revealed that these Oatps/OATPs transport in a sodium-independent way a wide variety of mainly bulky organic anions, neutral compounds, organic cations and several drugs like e.g. statins, cardiac glycosides, ACE-inhibitors, endothelin-antagonists and antihistamines. Besides liver-specific Oatps/OATPs (Oatp4, OATP-C and OATP8) there are others that are expressed in various tissues including intestine (Oatp3), kidney (Oatp1, OAT-K1, OATP-A), brain (Oatp2, Oatp3, OATP-A, and OATP-F) and testis (OATP-F). Their expression in these multiple tissues puts them into a strategic position for absorption, distribution and excretion of drugs and toxins.

Although the exact physiological functions of most Oatps/OATPs remain to be elucidated, nearly all Oatps/OATPs of families 2–4 have narrower substrate specificities (e.g. OATP-B, OATP-E) and may serve more specific functions in selected organs such as for example thyroid hormone transport in various peripheral organs, or steroid and steroid metabolite transport in adrenal gland and placenta. Thus, the functional diversity of the OATP superfamily could be very similar to the CYP450 superfamily where only families 1–4 are involved in metabolism and detoxification of drugs and environmental chemicals, whereas the members of the other families catalyse specific pathways in the cholesterol, prostacyclin and/or steroid metabolism.

Based on their selective tissue distribution and substrate specificities, Oatps/OATPs could eventually be used to target drugs to certain organs. However, an accurate determination of their expression as well as an exact delineation of their substrate

specificities is a prerequisite to use them for a more rational drug design.

SA3

Amino acid transport: you say IMINO, I say IMINO

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Although the transport of imino acids across the brush-border membrane of the mammalian small intestinal epithelium has been demonstrated in a large number of studies the identity and nature of the transport mechanism has remained controversial. From the 1960s onwards this transport system has been given a variety of names including the imino acid carrier, the sarcosine carrier, the methionine-insensitive 'sarcosine-glycine-proline' system, and more recently the IMINO system. Most studies demonstrate that this IMINO system is a Na^+-dependent transporter of imino acids and small dipolar amino acids. However, an unusual feature of the IMINO system is that there are marked differences in the ion dependency and substrate specificity between studies using small intestinal tissues from rat, rabbit, hamster or guinea-pig intestine (Munck *et al.* 1994; Stevens & Wright, 1985). The IMINO system is perhaps the only 'classical' amino acid transport system yet to be identified at the molecular level. The IMINO transport system could be linked to the hereditary malabsorption syndrome iminoglycinuria, which is associated with a defect in proline, hydroxyproline and glycine transport (where defects can be observed in both renal transport and intestinal absorption).

To date there is no convincing evidence for the existence of a Na^+-dependent IMINO system in human small intestine. However, a transport system with similar substrate specificity to the rat IMINO carrier has been identified functionally at the apical membrane of the human intestinal epithelial cell line Caco-2. This transport mechanism was named 'system PAT' (for Proton-coupled Amino-acid Transporter) as it functions in an H^+-coupled, pH-dependent, Na^+-independent manner (Thwaites *et al.* 1993; 1995). Substrates for this transporter include a range of small, unbranched, dipolar amino acids (including methylated analogues such as betaine and MeAIB), imino acids, beta amino acids, and potential neuromodulatory amino acids including D-amino acids (e.g. D-cycloserine and D-serine) and GABA (and analogues). The presence of a transport system with such a broad range of transported substrates provides a potential route for nutrient, osmolyte and drug transport across the luminal brush-border membrane.

Recently a cDNA has been isolated (using a probe specific for the LYAAT1 transporter originally identified in rat brain (Sagne *et al.* 2001)) from Caco-2 cells that (when expressed in HRPE cells) is able to induce H^+-coupled amino acid transport (hPAT1, for human Proton-coupled Amino-acid Transporter 1) (Chen *et al.* 2003). hPAT1 has an identical substrate specificity to that measured for system PAT in Caco-2 cell monolayers and the IMINO system in rat small intestine. Immunocytochemistry demonstrates PAT1-like immunoreactivity (IR) localised solely to the apical membrane of Caco-2 cells and human and rat small intestinal enterocytes.

Is PAT1 the IMINO carrier? Despite the similarity in substrate specificity and substrate affinity, there is a clear difference in the apparent ion dependency between studies using the hPAT1 clone or measurements of IMINO transport in intact intestinal tissues. However, our recent studies using intact monolayers of the human intestinal cell line Caco-2 can account for this apparent anomaly (Anderson & Thwaites, 2003). In intact epithelia optimal H^+-coupled PAT1 transport is dependent upon the maintenance of the driving force (H^+-electrochemical gradient) during transport and this is achieved by functional activity of the apically-localised Na^+/H^+ exchanger NHE3. Inhibition of NHE3 (e.g. following Na^+ removal) will thus lead to a reduction in PAT1 capacity and produce the partial Na^+ dependency observed in studies using intact epithelia (e.g. Caco-2 cell monolayers and rat small intestine). In conclusion, the PAT1 clone is responsible for IMINO system transport in human and rat small intestine. The absolute requirement for Na^+ and Cl^- and distinct substrate specificity of the IMINO system in rabbit small intestine suggest that the rabbit IMINO carrier is a non-PAT1 type transporter.

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SA4

The glutamine–glutamate cycle

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The high rates of firing at many synapses require mechanisms to replace the released neurotransmitter. At excitatory synapses (and probably many inhibitory synapses as well), this involves recycling through supporting glial cells and the intermediate glutamine. Transport systems N and A exhibit properties that suggest important roles in this cycle. An unrelated family of proteins contributes to the loading of synaptic vesicles with glutamate in the final step of the cycle

SA5

The role of neuroendocrine interactions for signalling in the gut

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Vital functions of the gut are under the control of nerves. These nerves belong to the enteric nervous system (ENS) which is a truly autonomous nervous system able to act independently from inputs of the central nervous system. The ENS is located within the wall of the gut and consists of two ganglionated plexus referred to as the myenteric and the submucous plexus. While the former is primarily responsible for regulation of muscle activity the latter is mainly concerned with the regulation of epithelial functions. Nerves of both plexus interact with the enteric immune system and are therefore important components from ensuring an intact gut barrier system as a first line of defence against noxious stimuli. Although the ENS is structurally

and functionally very similar to the brain and able to conduct complex functions, ENS activity is heavily modulated by signals from non-neuronal cells. Among them are smooth muscle cells, epithelial cells, immune competent cells and glia, all of which influence ENS activity via endocrine and/or paracrine routes.

One of the most important neuroendocrine interactions is the signalling from enterochromaffine cells (EC cells) to enteric nerves. EC cells are strategically located in the epithelial layer to have relatively close contact to nerve endings. Their role is to code chemical and/or mechanical stimuli and transmit them to nerves. That means that if there is distortion of the epithelium or chemicals in the lumen, the EC cells will release serotonin, which in turn activates nerve endings in the ENS. These nerve endings belong to sensory nerves, also referred to as intrinsic primary afferents. Beside this serotonin also excites vagal or spinal afferents which are part of the extrinsic nerve supply to the gut. Serotonergic transmission in the ENS involves complex pharmacology since 5-HT1P, 5-HT3 and 5-HT4 receptor have been demonstrated post as well as presynaptically. At least in the stomach, 5-HT1P-mediated activation occurs in inhibitory neurones whereas 5-HT3-mediated activation is confined to excitatory neurones. This explains in part the differential effects of serotonin which may inhibit or activate smooth muscle activity. 5-HT4 receptors play a role in the presynaptic facilitation of acetylcholine release and activation of this receptor increases motility as well as chloride secretion. In the human submucous plexus 5-HT excites neurones and this effect is almost entirely mediated by 5-HT3 receptors, yet the finding that activation of 5-HT3 receptors do not affect secretion in the human colon was unexpected. Serotonin and its mode of action has gained a lot of attention recently because it is not only crucial for the physiological behaviour of the gut but is involved in the pathophysiology of functional bowel disease, in particular in irritable bowel.

Nutritive signalling molecules like fatty acids or glycine exert direct postsynaptic effects in the enteric nervous system. Application of butyrate, even in doses less than 1 mM, exert excitation of enteric nerves. Many of them have been identified as sensory neurones with multipolar morphology. The excitatory effect of glycine is mediated by strychnine-sensitive receptors. The basis of this unusual excitatory glycine effect is the relatively high intracellular chloride concentration in enteric neurones. Very likely glycine is not a transmitter in the ENS because no glycinergic neurones and no glycine-mediated synaptic transmission could be demonstrated. Functionally, we could identify a glycine-evoked activation of colonic motility, an effect which requires intact nerves.

Other important molecules involved in interaction between non-neuronal cells and nerves are inflammatory mediators like histamine or prostaglandins. Prostaglandins have a powerful postsynaptic excitatory effect in the ENS and they often evoke long term excitation of the nerve cells with oscillations in their excitability. This is paralleled by an activation of mucosal chloride secretion thereby favouring diarrhoea. Prostaglandin-evoked secretion is to about 70 % mediated by nerves and only a small components of their pro-secretory effects is direct activation of epithelial cells. In this way harmful antigen attacks in the gut are antagonised by dilution of the irritant and by rapid expulsion because motility is also enhanced. The ENS is a crucial part of such an alarm system and inflammatory mediators are integral parts of neuro-immune-interactions in the gut. Histamine has a similar excitatory effect though a presynaptic inhibition of acetylcholine release has been observed in addition to its powerful postsynaptic activation of enteric nerves. The prominent effect of inflammatory mediators has been supported by the finding that application of a mediator cocktail contained in the supernatant of stimulated human mast cells excites enteric nerves. In summary, although the ENS is vital for normal gastrointestinal functions one has to consider that changes in the environment of the ENS have powerful influences on enteric nerves. A substantial number of mediators from non-neuronal cells are important to maintain signalling in the ENS. Identification of their mode of action will advance our knowledge on the physiology of gut functions but is also crucial to understand malfunctions in the diseased gut.

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