Expression of the rabbit intestinal N2 Na⁺/nucleoside transporter in *Xenopus laevis* oocytes

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Polyadenylated [poly(A)⁺] mRNA isolated from rabbit small-intestinal mucosa was injected into *Xenopus laevis* oocytes, and expression of the N2 Na⁺/nucleoside co-transporter was assayed by measuring Na⁺-dependent thymidine uptake. Expression of Na⁺-dependent thymidine uptake steadily increased after mRNA injection and was on average increased 11-fold by day 6 over background. Na⁺-dependent thymidine uptake was saturable (apparent $K_m \sim 30 \mu\text{M}$ at 22 °C) and inhibited by uridine and cytidine, but not by guanosine and inosine. These properties of the expressed thymidine transport strongly suggest that the epithelial N2 Na⁺/nucleoside co-transporter can be expressed in *X. laevis* oocytes.

INTRODUCTION

The uptake of nucleosides by mammalian cells is mediated by several distinct systems [1,2]. Two of these systems are broad-specificity facilitated-diffusion mechanisms that differ in their sensitivity to inhibition by nitrobenzylthioinosine (NBMPR). In addition, during the past few years a number of studies have described Na⁺-dependent nucleoside transport in both epithelial and non-epithelial cells [3–13]. On the basis of kinetic and specificity studies, two principal Na⁺-dependent nucleoside transporters in mouse intestinal epithelial cells and rat and bovine renal brush-border membrane vesicles have been proposed [4,5,7,14]. One system, designated N1, accepts purine nucleosides and uridine, whereas the other system, N2, has a substrate specificity for pyrimidine nucleosides, adenosine and analogues of adenosine. Neither system is inhibited by NBMPR [3–7]. As is the case for the NBMPR-insensitive facilitated-diffusion carrier, little is currently known about the structure and molecular properties of these Na⁺/nucleoside co-transporters.

This paper describes studies designed to express the intestinal Na⁺/nucleoside co-transporters functionally in *Xenopus laevis* oocytes. *X. laevis* oocytes faithfully translate heterologous mRNA and have been used in an expression cloning strategy to clone several membrane proteins in the absence of probes such as antibodies and oligonucleotides (for review see [15]). The present investigation demonstrates that injection of rabbit small-intestine polyadenylated [poly(A)⁺] RNA into *X. laevis* oocytes resulted in functional expression of Na⁺-dependent thymidine transport.

METHODS

Isolation of rabbit intestine mRNA

Total RNA was prepared from male New Zealand White rabbit small-intestinal mucosa scrapings by using a guanidium thiocyanate/caesium trifluoroacetate extraction in accordance with the protocol given by Pharmacia/LKB. Poly(A)⁺ RNA (mRNA) was isolated by oligo(dT)–cellulose chromatography [16] and precipitated with ethanol (70 % final concn.). The RNA was dissolved in water (1 $\mu\text{g}/\text{ml}$) and stored at –70 °C until use.

Microinjection of mRNA in oocytes

Fully grown oocytes (stages 5 and 6) were isolated from *X. laevis* by a collagenase digestion procedure [17] with the following

modifications. After digestion, the oocytes were kept overnight in the recovery solution supplemented with 1 % (w/v) BSA, and after 16–24 h the medium was changed with no BSA present. Healthy oocytes were injected with 50 nl of mRNA or water by using a semi-automatic injector (Inject + Matic-system; Gabay, Geneva, Switzerland) and maintained at 18 °C in Ca²⁺-containing medium (96 mM-NaCl, 2 mM-KCl, 1 mM-MgCl₂, 1.8 mM-CaCl₂, 2.5 mM-sodium pyruvate, 5 mM-Hepes, pH 7.6, and 0.1 mg of gentamycin/ml). The oocytes were cultured for up to 6 days with a daily change of medium.

Nucleoside uptake by oocytes

The injected oocytes (up to eight) were incubated for 30 min in Na⁺-free buffer (100 mM-choline chloride, 2 mM-KCl, 1 mM-MgCl₂, 10 mM-Hepes/Tris, pH 7.5) and then placed in 0.2 ml of a Na⁺ buffer (choline chloride replaced by 100 mM-NaCl) or the Na⁺-free buffer containing radioactively labelled thymidine or guanosine (50 $\mu\text{Ci}/\text{ml}$). After incubation for 15–60 min at 22 °C, uptake was terminated by removing the incubation medium and washing the oocytes with 6 $\times$ 1 ml of ice-cold Na⁺-free medium. Each oocyte was dissolved in 0.5 ml of 10 % SDS, and ³H was assayed by liquid-scintillation counting. Uptake at zero time was determined by exposing oocytes to radioactively labelled nucleoside at 1 °C, immediately followed by washing. These blank values were subtracted from measurements associated with the oocytes at 22 °C to determine the uptake rates. The Na⁺-dependent transport activity is defined as the difference between the observed uptake in the Na⁺-containing medium and that in the Na⁺-free medium.

Thymidine metabolism

Oocytes were incubated with 50 μM -[³H]thymidine for 30 min at 22 °C, and the reaction was terminated as described above. The oocytes were lysed and deproteinized by addition of 0.1 ml of ice-cold 4 % (w/v) HClO₄. The precipitate was removed by centrifugation (2 min, 13000 g) and the supernatant neutralized (5 M-KOH). The centrifugation step was repeated to remove the resulting KClO₄. Possible [³H]thymidine metabolites associated with the supernatant were assessed by t.l.c. as described previously [5].

Chemicals

[6-³H]Thymidine (25 Ci/mmol) and [8-³H]guanosine (11 Ci/mmol) were purchased from Amersham International,

Amersham, Bucks., U.K., and ICN Radiochemicals, Irvine, CA, U.S.A., respectively. Guanidinium thiocyanate, caesium trifluoroacetate, oligo(dT)-cellulose, collagenase (type II) and gentamycin were obtained from Fluka, Glossop, Derbyshire, U.K., Pharmacia LKB, Milton Keynes, U.K., Boehringer, Mannheim, Germany, and Sigma Chemical Co., Poole, Dorset, U.K., respectively. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

Preliminary experiments during June to September with oocytes injected with water established that 50 μM -thymidine uptake was low and similar in the presence (0.58 ± 0.16 pmol/oocyte per h; mean $\pm$ s.d. of a total of 56 determinations derived from 3 oocyte preparations) and absence (0.55 ± 0.24 pmol/oocyte per h) of an inwardly directed NaCl gradient. These results demonstrate that *X. laevis* oocytes do not express measurable levels of endogenous Na⁺-dependent thymidine transport and are thus suitable to monitor the expression of nucleoside co-transporters.

The time course of expression of thymidine uptake by oocytes following the injection of 50 ng of total mRNA from the rabbit small intestine is shown in Fig. 1. In the presence of Na⁺, thymidine uptake increased at a steady rate between 1 and 6 days after injection, a profile similar to that observed for other membrane transporters [18]. After 6 days of incubation, Na⁺-dependent thymidine uptake had increased 11-fold, from 0.28 (at day 1) to 3.0 pmol/oocyte per h. In contrast, there was only a slight increase in thymidine influx in the presence of choline chloride, from 0.72 to 1.14 pmol/oocyte per h, and this increase was not significantly different from that observed with water-injected oocytes. The time course of 50 μM -Na⁺-dependent thymidine uptake by mRNA-injected oocytes was linear for at least 1 h (results not shown). Thus, in subsequent studies 30 min incubation times were chosen to measure initial rates of thymidine uptake using mRNA-injected oocytes cultured for 4–5 days.

The results of Fig. 1 are consistent with the suggestion of functional expression of a rabbit small-intestinal Na⁺/nucleoside co-transport system by the *X. laevis* oocyte. It is conceivable that expression of thymidine uptake resulted from the translation of a regulatory subunit acting on an endogenous carrier. Such an explanation is unlikely, because we were unable to demonstrate any endogenous thymidine transport in oocytes (see above). An alternative possibility, that the increased thymidine uptake was due to increased metabolism of thymidine, was tested and rejected by determining whether 50 μM -[³H]thymidine was metabolized by injected oocytes. Over 90% of the radioactivity associated with oocytes after 30 min incubation with [³H]thymidine co-migrated with thymidine on t.l.c. plates. Thus no significant metabolism of [³H]thymidine was observed.

To evaluate further the characteristics of expressed thymidine uptake, we measured the kinetics and substrate-specificity of the transport activity. The concentration-dependence of thymidine uptake in the presence of 100 mM-NaCl or 100 mM-choline chloride is shown in Fig. 2. In the presence of choline chloride, thymidine uptake was linear with increasing thymidine concentration. In contrast, thymidine influx in the presence of NaCl could be resolved into two components: (i) a linear component, and (ii) a saturable component. The Na⁺-dependent component of transport (i.e. uptake values in the presence of Na⁺ minus those in the presence of choline) was saturable and conformed to Michaelis–Menten kinetics (apparent K_m 36 ± 12 μM and V_{max} 15.5 ± 2.2 pmol/oocyte per h). In a separate experiment with a different batch of oocytes and mRNA an apparent K_m of

28 ± 8.5 μM was determined. These K_m values are similar to those previously reported for Na⁺-dependent nucleoside transport activity in enterocytes and isolated membrane vesicles [3–8,19], further supporting the suggestion of the functional expression of the intestinal N2 nucleoside carrier in oocytes.

As discussed in the Introduction, two distinct Na⁺-dependent nucleoside transporters have been characterized [3–7]. Table 1 demonstrates that Na⁺-dependent thymidine uptake was blocked by uridine and cytidine, but guanosine and inosine were without

Fig. 1. Time-dependence of thymidine uptake expression in *X. laevis* oocytes

Oocytes were injected with 50 nl of poly(A)⁺ RNA (1 $\mu\text{g}/\mu\text{l}$) from rabbit small-intestinal mucosa or water and maintained at 18 °C for various periods of time as indicated. After the incubation period, the uptake of 50 μM -[³H]thymidine at 22 °C for 30 min was measured in the presence of 100 mM-NaCl (●, ■) and 100 mM-choline chloride (○, □). Uptake values presented are the means $\pm$ S.E.M. for 8 oocytes. Symbols: ● and ○, mRNA-injected oocytes; ■ and □, water-injected oocytes.

Fig. 2. Concentration-dependence of thymidine uptake in mRNA-injected oocytes

Oocytes were treated as described in the legend to Fig. 1, and after 5 days of incubation [³H]thymidine uptake was measured in the presence of 100 mM-NaCl (●) or 100 mM-choline chloride (▲). The Na⁺-dependent flux was taken as the rate in the presence of NaCl minus the rate in the presence of choline chloride for each thymidine concentration. The kinetic constants of saturable Na⁺-dependent thymidine uptake were determined by non-linear least-squares fitting of the Michaelis–Menten equation by using the computer program Enzfitter (Elsevier Biosoft). The kinetic constants were K_m 36 ± 12 μM and V_{max} 15.5 ± 2.2 pmol/oocyte per h. The uptake values shown represent means $\pm$ S.E.M. for 8 oocytes.

Table 1. Effect of nucleosides on Na⁺-dependent thymidine uptake in mRNA-injected oocytes

Oocytes were injected with 50 ng of rabbit intestinal poly(A)⁺ RNA and incubated for 5 days. Uptake of 50 μ M-[³H]thymidine was determined at 22 °C for 30 min in the presence or absence of unlabelled nucleoside in the presence of 100 mM-NaCl or 100 mM-choline chloride. Na⁺-dependent thymidine-uptake rates (flux in the presence of NaCl minus that in the presence of choline chloride) are presented (means $\pm$ S.E.M. for eight oocytes).

Nucleoside	Na ⁺ -dependent thymidine uptake (pmol/oocyte per h)
Control	2.5 $\pm$ 0.88
Inosine (1 mM)	2.0 $\pm$ 0.26
Guanosine (1 mM)	2.5 $\pm$ 0.28
Uridine (1 mM)	0.3 $\pm$ 0.003
Cytidine (1 mM)	0

effect. These results are consistent with the properties expected for the expression of the N2 Na⁺-dependent nucleoside-transport system. In other experiments, we measured the uptake of 50 μ M-[³H]guanosine by mRNA-injected oocytes. Guanosine is a model permeant for the N1 Na⁺/nucleoside co-transporter [5], but no Na⁺-stimulated guanosine uptake was observed in oocytes injected with rabbit small-intestine mRNA (results not shown).

In conclusion, the present study demonstrates that injection of total rabbit small-intestinal mRNA into *X. laevis* results in functional expression of Na⁺-dependent thymidine uptake. The characteristics of this expressed transport activity, namely Na⁺-dependency, saturability with a high affinity and selective inhibition by pyrimidine nucleosides, are entirely consistent with the properties of the N2 Na⁺/nucleoside co-transporter system in intact enterocytes and membrane vesicles [3–7,14]. These observations suggest that it should be possible to use the cloning strategy

of functional expression in *X. laevis* oocytes to isolate and clone cDNAs encoding the N2 Na⁺-dependent nucleoside transporter.

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