BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 106–111 (1996)
ARTICLE NO. 1137

Expression of Rabbit Ileal N3 Na^+/Nucleoside Cotransport Activity in *Xenopus laevis* Oocytes

Maria J. Redlak,* Zendra E. Zehner,† and Sylvia L. Betcher*,[1]

*Departments of Internal Medicine and †Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Received June 25, 1996

To determine if the Na^+/nucleoside cotransport activity in the distal rabbit intestine has either purine-selective (N1) or broad (N3) substrate specificity, Na^+-dependent inosine uptake was expressed in *Xenopus laevis* oocytes. The rate of expressed Na^+-stimulated inosine uptake saturated with increasing inosine concentration (apparent K_m = 58.2 ± 13.0 μM), was insensitive to inhibition by 6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine, and was partially inhibited by phloridzin. Na^+-dependent uptake was inhibited by guanosine (IC_{50} = 7.1 μM) and thymidine (IC_{50} = 5.5 μM). The Na^+/nucleoside cotransport activity expressed by rabbit ileal mRNA in the *Xenopus* oocyte expression system is most characteristic of the N3 subclass of this family of transport proteins.

Na^+-dependent nucleoside transporters are a functionally heterogeneous group of proteins that transport nucleosides in normal and neoplastic cells (1). Based on differences in specificity of the transported nucleoside and the inhibitory response to NBMPR, five different subclasses, designated N1-N5, have been described (2). To determine the molecular basis for this functional heterogeneity, knowledge of the amino acid sequences for the proteins in each subclass is required.

Expression cloning has resulted in the isolation and sequencing of two cDNAs encoding Na^+/nucleoside cotransporters: one, from rat jejunum, has pyrimidine-selective (N2) substrate specificity (3), and the other, from rat liver, has purine-selective (N1) substrate specificity (4). However, the amino acid sequences of transporters in subclasses N3, N4, and N5 have not yet been reported.

Previous studies of Na^+-dependent adenosine uptake in brush-border membrane vesicles from the distal rabbit ileum have shown that this tissue has a Na^+/nucleoside cotransporter that is either an N1 or an N3 type (5). Thus, the rabbit ileum is a potential source of mRNA enriched in one or both of these subclasses. To determine which one is expressed by the rabbit ileum, we selectively assayed for the N1 and the N3 Na^+/nucleoside cotransporters by expressing Na^+-dependent inosine uptake in *Xenopus laevis* oocytes microinjected with rabbit ileal mRNA. Further characterization showed that expression of the N3 subclass predominates.

MATERIALS AND METHODS

Preparation of mRNA. Total RNA was prepared from the epithelial lining of the distal ileum of New Zealand white rabbits using a one step acid guanidinium thiocyanate-phenol-chloroform extraction method (TRI Reagent) followed by selective precipitation of RNA with LiCl (6). Poly $(A)^+$ RNA (mRNA) was prepared using oligo (dT) cellulose chromatography (6). The absorbance ratio (260/280 nm) of injected mRNA was 1.90 ± 0.06. The quality of the mRNA was verified by gel electrophoresis on denaturing formaldehyde-agarose gels (6). For size fractionation, mRNA

[1] To whom correspondence should be addressed at Department of Medicine, Veterans Administration Medical Center and SUNY Health Science Center, 800 Irving Avenue, Syracuse, NY 13210. Fax: (315) 476-5348.

Abbreviation: NBMPR, 6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine.

0006-291X/96 $18.00

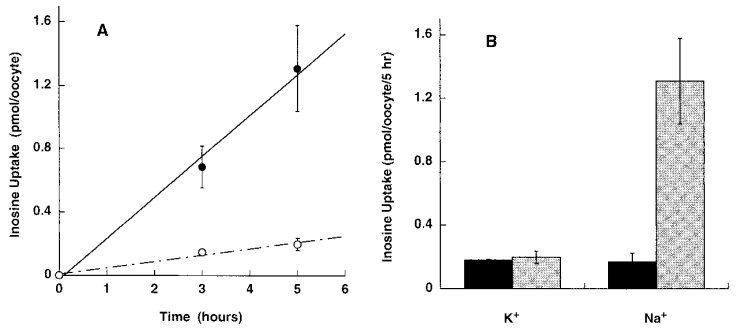

FIG. 1. Na^+-dependent inosine uptake in *Xenopus* oocytes injected with rabbit ileal poly $(A)^+$ RNA. (A) Individual oocytes were injected with mRNA (30 ng / 50 nl) or water (50 nl, data shown in B). On day 5 the uptake of radiolabeled inosine (0.5 μM) was measured at 3 and 5 hours in the presence of either 100 mM K^+ (0) or 100 mM Na^+ (●). (B) Individual oocytes were injected with water (50 nl; solid bars) or mRNA (30 ng / 50 nl; shaded bars). On day 5 the uptake of radiolabeled inosine (0.5 μM) was measured after 5 hours in the presence of either 100 mM K^+ or 100 mM Na^+. Data are shown as means ± S.E. for 2 separate experiments (5 oocytes/data point/experiment).

and total RNA were electrophoresed on a 0.7% denaturing agarose gel. The mRNA region corresponding to the ribosomal RNA bands of total RNA was cut into slices. The mRNA was electroeluted from individual gel slices for injection into oocytes.

Isolation and injection of oocytes. Xenopus laevis oocytes were obtained by surgical removal of egg sacs from adult female toads (7). The sacs were exposed to 1% collagenase (Type V, EC 3.4.24.3) in oocyte Ringer's solution (82.5 mM NaCL, 1.0 mM $MgCl_2$, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 5 mM HEPES), pH 7.31, plus penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). After separation, individual oocytes were injected with sterile water, or mRNA (50 nl, 30-50 ng), using a pressure-driven microinjection apparatus (PLI-100, Medical Systems Corp.). Injected oocytes were maintained at 4°C for 1-6 days prior to assay.

Nucleoside uptake assay. 5-7 oocytes were placed in 0.5 ml Na^+-Ringer's solution (100 mM NaCl, 2.0 mM KCl, 1.0 mM $CaCl_2$, 10 mM Hepes, pH 7.2 at 22°C) containing [2,8-^{3}H]-inosine (37 Ci/mmol). For uptakes in the absence of sodium, the oocytes were transferred to K^+-Ringer's solution (102 mM KCl, 1.0 mM $CaCl_2$, 10 mM Hepes pH 7.2 at 22°C). For inhibitor studies, the indicated concentration of the inhibitor was included in the assay solution. At the desired time, media was quickly removed by pipeting. Oocytes were washed five times with ice-cold aliquots (3 ml) of either Na^+- or K^+-Ringer's solution, dissolved in 10% sodium dodecyl sulfate (0.5 ml) at 60°C, and counted. Background counts, obtained by removing a group of oocytes immediately from the uptake medium, were subtracted from each measurement.

Data analysis. The data are presented as means with standard errors for the numbers of oocytes indicated in the respective figure legend. Statistics were performed using Student's t-test.

Materials. Adult *Xenopus leavis* female toads were purchased from Nasco. TRI Reagent was obtained from Molecular Research Center, Inc. Type V collagenase, sodium dodecyl sulfate, oligo dT cellulose, nucleosides, and inhibitors were purchased from Sigma Chemical Co. [2,8-^{3}H]-Inosine was from Moravek Biochemicals, Inc.

RESULTS

Expression of Na^+-Stimulated Inosine Uptake in Xenopus Oocytes

After injection with rabbit ileal mRNA, *Xenopus* oocytes accumulate radiolabeled inosine linearly at a rate 6.6-fold greater in Na^+-containing medium than in K^+-containing medium: 1.308 ± 0.271 pmole/oocyte/5 hr *vs.* 0.198 ± 0.038 pmole/oocyte/5 hr (Fig. 1A). The rate of uptake in water-injected control oocytes was similar to that of mRNA-injected oocytes assayed in K^+-containing medium: 0.167 ± 0.057 or 0.180 ± 0.004 pmole/oocyte/5 hr in the presence of Na^+ or K^+, respectively (Fig. 1B). Expression of Na^+-dependent inosine uptake increased daily for at least six days after injection (Fig. 2). These results show that Na^+-stimulated inosine uptake is expressed in *Xenopus* oocytes following injection with rabbit ileal mRNA.

Evidence for Carrier-Mediated Transport

When the rate of Na^+-stimulated inosine uptake was measured over a range of substrate concentrations (2-250 μM), Na^+-dependent uptake saturated at inosine concentrations greater

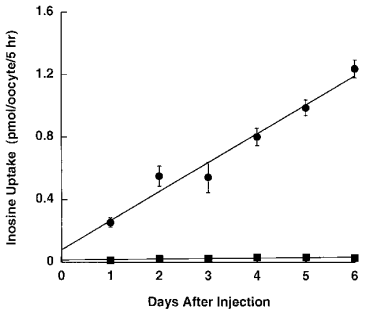

FIG. 2. Effect of time after injection on the rate of Na^+-dependent inosine uptake. The rate of radiolabeled inosine uptake (0.5 μM) was measured daily in the presence of 100 mM Na^+ in individual oocytes injected with either rabbit ileal mRNA (30 ng / 50 nl; ●) or water (50 nl; ■). Data are shown as means ± S.E. for 15 oocytes.

than 100 μM (Fig. 3). Linear analysis using Hanes-Woolf plots predicted a single saturable transport process with a K_m of 58.2 ± 13.0 μM (not shown). The kinetics of the expressed Na^+-stimulated inosine uptake are characteristic of a carrier-mediated transporter with an affinity for inosine about 3-fold less than that measured for adenosine uptake in brush-border membrane vesicles (5).

Specificity of the Expressed Na^+-Dependent Inosine Uptake

The observed Na^+-dependent inosine uptake could be due to transport on a protein of either the N1 or the N3 subclass. To differentiate between the two types, the effects of increasing concentrations of the pyrimidine ribonucleoside, thymidine, and the purine ribonucleoside, guanosine, were determined. Both nucleosides inhibited Na^+-stimulated inosine uptake in a dose-dependent manner (Fig. 4A and 4B). The IC_{50} for inhibition by each nucleoside is shown in Table 1. High affinity inhibition by both nucleosides defines the expressed activity as that of the N3 Na^+/nucleoside cotransporter (2).

Effect of Inhibitors

The effect of NBMPR and dipyridamole on the expressed Na^+-dependent inosine uptake was determined. Na^+-stimulated inosine uptake was not inhibited by either NBMPR (20 μM),

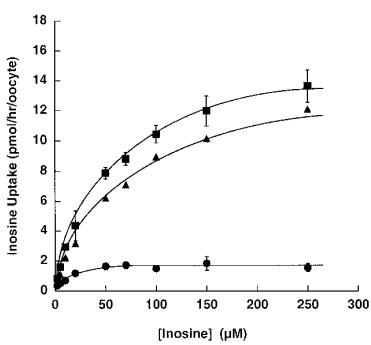

FIG. 3. Dependence of Na^+-stimulated inosine uptake on inosine concentration. Rates of radiolabeled inosine uptake (2, 5, 10, 20, 50, 70, 100, 150, or 250 μM) were measured in the presence of 100 mM Na^+ (■) or 100 mM K^+ (●) on day 5 after injection with rabbit ileal mRNA (50 ng / 50 nl / oocyte). The difference represents the Na^+-dependent uptake (▲). Data are shown as means ± S.E. for 17-19 oocytes (2-100 μM inosine) or 3-5 oocytes (150-250 μM inosine).

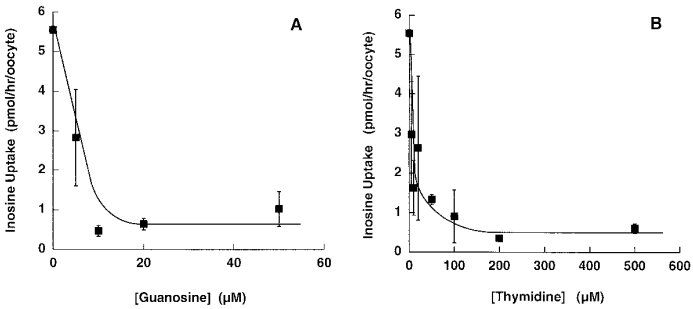

FIG. 4. Effect of guanosine and thymidine on expressed Na^+-dependent inosine uptake. Individual oocytes were injected with rabbit ileal mRNA (50 ng / 50 nl). On day 5 the uptake of radiolabeled inosine (20 μM) was measured in the presence of 100 mM Na^+ and 5, 10, 20, or 50 μM guanosine (A), or 100 mM Na^+ and 5, 10, 20, 50, 100, 200, or 500 μM thymidine (B). The data are shown as means ± S.E. for 2 separate experiments (6-7 oocytes/data point/experiment).

during a 5 hour uptake period (Fig. 5), or dipyridamole (250 μM), during a 3 hour uptake period (not shown). Lack of inhibition by NBMPR and dipyridamole is a characteristic of Na^+-dependent nucleoside transporters in subclasses N1 through N4 (1, 2). Although a significant reduction in radiolabeled inosine accumulation occurred after a five hour exposure to dipyridamole ($p<0.001$), this effect may be nonspecific: a similar decrease in Na^+-dependent adenosine uptake was observed in ileal brush-border membrane vesicles after preincubation with dipyridamole (5).

Phloridzin inhibited Na^+-dependent inosine influx in a biphasic manner (Fig. 6). This inhibition pattern suggests heterogeneity in the binding of phloridzin to the expressed nucleoside transporter and is similar to the pattern reported for phloridzin inhibition of uridine fluxes in oocytes injected with rat jejunal mRNA (8).

Size Selection of mRNA Associated with Na^+-Dependent Inosine Accumulation

After size-fractionation of rabbit ileal mRNA, individual mRNA fractions were injected into *Xenopus* oocytes and assayed for Na^+-dependent inosine uptake (Fig. 7). The expressed Na^+-dependent inosine uptake was greatest in fractions corresponding to mRNA of 2-3 kb.

DISCUSSION

We have demonstrated Na^+-stimulated inosine uptake in *Xenopus* oocytes after microinjection of rabbit ileal mRNA. The initial rates of Na^+-dependent inosine uptake fit the Michaelis-Menten equation, a finding consistent with the expression of a carrier-mediated transport mechanism. Inhibition by both guanosine (IC_{50} = 5.5 μM), and thymidine (IC_{50} = 7.1 μM)

TABLE 1
Half-Maximal Inhibitory Concentrations for Inhibitors of Expressed Na^+-Dependent Inosine Uptake

Inhibitor	IC_{50} (μM)
Guanosine	5.5
Thymidine	7.1
Phloridzin	<16

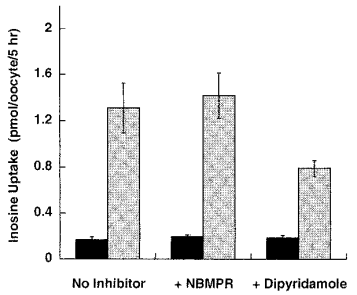

FIG. 5. Effect of nucleoside transport inhibitors on expressed Na^+-dependent inosine uptake. Individual oocytes were injected with water (50 nl; solid bars) or rabbit ileal mRNA (50 ng / 50 nl; shaded bars). On day 5 the uptake of radiolabeled inosine (0.5 μM) was measured in the presence of 100 mM Na^+, Na^+ plus NBMPR (20 μM), or Na^+ plus dipyridamole (250 μM). Data are shown as means ± S.E. for 10 oocytes from 2 separate experiments. Inhibitors did not affect uptake in water-injected oocytes. Dipyridamole significantly reduced inosine uptake at 5 hours ($p<0.001$).

defines the rabbit ileal Na^+/nucleoside cotransporter as belonging to the N3 subclass (2). The size of the mRNA associated with this activity is similar to that encoding clones for the rat jejunal N2 Na^+-dependent nucleoside transporter (3) and the rat liver N1 Na^+-dependent nucleoside transporter (4)

The demonstration of Na^+/nucleoside cotransport activity of the N3 subclass in the rabbit intestine contrasts with two other studies that used the *Xenopus* oocyte expression system to measure Na^+-dependent nucleoside transport (9-11). Jarvis selectively assayed the N2 Na^+/nucleoside cotransporter by using radiolabeled thymidine as the transported substrate (9,10). He concluded that N1 transporters were not expressed by the rabbit intestine, since Na^+ did not stimulate guanosine uptake (9). Because inosine failed to inhibit Na^+-dependent uridine uptake in mRNA-injected oocytes, another study concluded that N2 is the predominant Na^+-dependent nucleoside transporter in the rabbit intestine (11). The differences could be explained if transcripts for N3 Na^+/nucleoside cotransport activity are more abundant in the distal ileum and if the mRNA used in these two studies were derived mainly from the proximal intestine. This hypothesis suggests that subtypes of the Na^+/nucleoside cotransporter are localized to specific segments along the rabbit intestine.

In summary, we have expressed a Na^+-dependent inosine transport activity from rabbit ileal mRNA that can be characterized as belonging to the N3 subclass of the Na^+/nucleoside cotransporter family. The amount of N3 transport activity expressed suggests that the rabbit

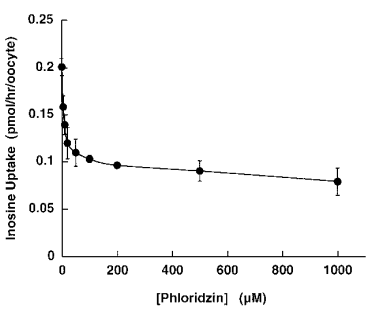

FIG. 6. Effect of phloridzin on expressed Na^+-dependent inosine uptake. Individual oocytes were injected with rabbit ileal mRNA (50 ng / 50 nl). On day 5 the uptake of radiolabeled inosine (0.5 μM) was measured in the presence of 100 mM Na^+, or 100 mM Na^+ plus phloridzin (5-200 μM). Data are shown as means ± S.E. for 2 separate experiments (7 oocytes/data point/experiment).

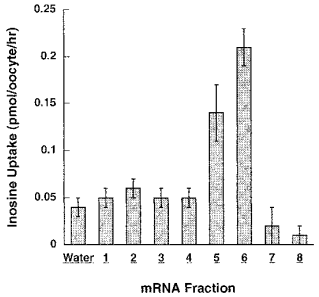

FIG. 7. Effect of messenger size on expression of Na^+-dependent inosine uptake. Individual oocytes were injected with water (50 nl) or rabbit ileal mRNA fractions (50 ng / 50 nl) obtained by electroelution from agarose gel slices. Inosine (0.5 μM) uptake was measured in the presence of 100 mM Na^+ on day 3. The results for each fraction are shown as the mean of Na^+-dependent inosine uptake ± S.E. for 5 oocytes.

ileum should be a good source of mRNA for expression cloning of the N3 Na^+/nucleoside cotransporter. Cloning of the N3, N4 and N5 subtypes will permit sequence comparison to be used to analyze potential structure-function relationships in this family of transport proteins.

ACKNOWLEDGMENTS

This work was supported by research grants J-246 from the Thomas F. and Kate Miller Jeffress Memorial Trust (S.L.B.) and IN-105R from the American Cancer Society (S.L.B.), and has been published in abstract form: Redlak, M. J., Zehner, Z. E., and Betcher, S. L. (1994) *Clin. Res.* **42,** 319A.

REFERENCES

1. Belt, J. A., Marina, N. M., Phelps, D. A., and Crawford, C. R. (1993) *Advan. Enzyme Regul.* **33,** 235–252.
2. Cass, C. E. (1995) *in* Drug Transport in Antimicrobial and Anticancer Chemotherapy (Georgopapadakou, N. H., Ed.), pp. 403–451, Marcel Dekker, Inc., New York.
3. Huang, Q-Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E., and Young, J. D. (1994) *J. Biol. Chem.* **269,** 17757–17760.
4. Che, M., Ortiz, D. F., and Arias, I. M. (1995) *J. Biol. Chem.* **270,** 13596–13599.
5. Betcher, S. L., Forrest, Jr., J. N., Knickelbein, R. G., and Dobbins, J. D. (1990) *Am. J. Physiol.* **259,** G504–G510.
6. Ausubel, F. M., *et al.* (Eds.) (1992) Short Protocols in Molecular Biology, 2nd ed., pp. 4–9 to 4–24, Greene Publishing Associates and John Wiley & Sons, New York.
7. Colman, A. (1984) *in* Transcription and Translation: A Practical Approach (Hames, B. D., and Higgins, S. J., Eds.), pp. 271–302, IRL Press, Oxford.
8. Huang, Q-Q., Harvey, A. M., Paterson, A. R. P., Cass, C. E., and Young, J. D. (1993) *J. Biol. Chem.* **268,** 20613–20619.
9. Jarvis, S. M., and Griffith, D. A. (1991) *Biochem. J.* **278,** 605–607.
10. Griffith, D. A., and Jarvis, S. M. (1991) *in* Purine and Pyrimidine Metabolism in Man (Harkness, R. A., *et al.*, eds.), Vol. 7, Part A, pp. 431–434, Plenum Press, New York.
11. Terasaki, T., Kadowaki, A., Higashida, H., Nakayama, K., Tamai, I., and Tsuji, A. (1993) *Biol. Pharm. Bull.* **16,** 493–496.