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Human, rat and chicken small intestinal Na^+–Cl^-–creatine transporter: functional, molecular characterization and localization

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In spite of all the fascinating properties of oral creatine supplementation, the mechanism(s) mediating its intestinal absorption has(have) not been investigated. The purpose of this study was to characterize intestinal creatine transport. [^{14}C]Creatine uptake was measured in chicken enterocytes and rat ileum, and expression of the creatine transporter CRT was examined in human, rat and chicken small intestine by reverse transcription-polymerase chain reaction, Northern blot, *in situ* hybridization, immunoblotting and immunohistochemistry. Results show that enterocytes accumulate creatine against its concentration gradient. This accumulation was electrogenic, Na^+- and Cl^--dependent, with a probable stoichiometry of 2 Na^+: 1 Cl^-: 1 creatine, and inhibited by ouabain and iodoacetic acid. The kinetic study revealed a K_m for creatine of 29 μM. [^{14}C]Creatine uptake was efficiently antagonized by non-labelled creatine, guanidinopropionic acid and cyclocreatine. More distant structural analogues of creatine, such as GABA, choline, glycine, β-alanine, taurine and betaine, had no effect on intestinal creatine uptake, indicating a high substrate specificity of the creatine transporter. Consistent with these functional data, messenger RNA for CRT was detected only in the cells lining the intestinal villus. The sequences of partial clones, and of the full-length cDNA clone, isolated from human and rat small intestine were identical to previously cloned CRT cDNAs. Immunological analysis revealed that CRT protein was mainly associated with the apical membrane of the enterocytes. This study reports for the first time that mammalian and avian enterocytes express CRT along the villus, where it mediates high-affinity, Na^+- and Cl^--dependent, apical creatine uptake.

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Phosphocreatine (PCr) is an important energy buffer, as well as energy transducer, in heart, brain and skeletal muscle (for review see Bessman & Carpenter, 1985; Wallimann *et al.* 1992). However, these tissues, which contain the highest concentrations of PCr, do not synthesize their own creatine or do so to a limited extent (Braissant *et al.* 2001). Most of it is absorbed via a specific creatine transporter (CRT) from the plasma into the respective tissues (for review see Guerrero-Ontiveros & Wallimann, 1998).

Human and other mammals obtain part of their daily creatine requirement by dietary intake. Some species on a creatine-free diet, e.g. ruminants, obtain their creatine by endogenous biosynthesis (see Wyss & Kaddurah-Daouk, 2000). Nevertheless, the absence of creatine in the diet of vegetarians results in low rates of urinary creatine and creatinine appearance (Delanghe *et al.* 1989), and supra-normal creatine retention occurs, at least initially, if oral creatine supplements are added to the diet of those individuals (Green *et al.* 1997). This suggests that endogenous creatine synthesis may not match the creatine requirements of vegetarians and in this respect creatine, which is present also in colostrum and human milk (Hulsemann *et al.* 1987), could be viewed as an essential constituent of a normal diet (Wallimann *et al.* 1999).

Oral creatine supplementation is now used widely by athletes to improve performance. Studies carried out in healthy subjects have demonstrated that oral creatine supplementation improves muscle power output during high-intensity exercise and enhances the increments of muscle mass and muscle strength that result from heavy resistance training (see review by Terjung *et al.* 2000). Not all studies have reported ergogenic benefit of creatine supplementation. In this regard, a number of equally well controlled studies indicated that creatine supplementation does not enhance: single or repetitive sprint performance; work performed during sets of maximal effort muscle contractions; maximal strength; or submaximal endurance exercise (Dawson *et al.* 1995; Cooke *et al.* 1995; Febbraio *et*

al. 1995; Barnett *et al.* 1996; Burke *et al.* 1996; Cooke & Barnes, 1997; Mujika *et al.* 1996; Redondo *et al.* 1996; Thompson *et al.* 1996; Odland *et al.* 1997; Terrilion *et al.* 1997).

The majority of studies on oral creatine supplementation have been on exercise performance in healthy subjects. Recent evidence indicates that creatine may be also useful in the treatment of Gyrate atrophy, cardiovascular and muscular diseases and in rehabilitation of muscle atrophy that results from muscle disuse. This is also the case for diseases such as Parkinson's, Huntington's and mitochondrial cytopathies, and in hypoxia and energy-related brain pathologies (Persky & Brazeau, 2001; Tarnopolsky & Beal, 2001; Hespel *et al.* 2001). Creatine and creatine analogues, such as cyclocreatine, have also been proven to be potent anticancer agents (Teicher *et al.* 1995; Wyss & Kaddurah-Daouk, 2000) and they are currently being investigated as antitumoural, antiviral and neuro-protective agents (see Wyss & Kaddurah-Daouk, 2000, for recent review). They also protect tissues from ischaemic damage, and may therefore have an impact on organ transplantation (Wyss & Kaddurah-Daouk, 2000). However, as recently pointed out by Wyss & Schulze (2002), these results require further confirmation in clinical studies in humans, together with a thorough evaluation of the safety of oral creatine supplementation.

Therefore, a detailed knowledge of the structure, function, localization and regulation at different levels of the CRTs turns out to be highly important for understanding the mechanisms of action of creatine as a cell- and neuro-protective agent, as well as for designing adequate pharmacological and nutritional interventions. The physiological relevance of a detailed knowledge of the CRTs is strengthened by the recent discovery of the first patient, with an X-linked genetic disease, showing defects in the human CRT gene (SLC6A8) (Salomons *et al.* 2001; Hahn *et al.* 2002).

However, in spite of the physiological importance of CRT and of the well established significance of oral creatine supplementation to human health and disease, there is a lack of information regarding intestinal CRT, which represents the first barrier for oral creatine to reach its target tissues. The aim of the current study was to investigate creatine intestinal transport. For the complete functional characterization of the intestinal creatine transport system avian enterocytes were preferred to those from mammals, because: (i) at least in our hands, the rat enterocyte preparations give very low yields, (ii) they do not remain alive for more than 15 min, and (iii) preliminary experiments revealed NaCl-dependent creatine transport in both rat and chicken small intestine. Intact intestine from mammalian and avian sources have been used for immunolocalization and for *in situ* hybridization studies.

A preliminary account of the data described here was given at the Physiological Society meeting in Oxford, UK, March 2001 (Ilundáin *et al.* 2001) and at the 17th EITG meeting in Gerona, Spain, May 2001.

METHODS

Tissue preparation

Small intestinal tissues were obtained from 4- to 6-week-old Hubbard chickens, 1-month-old male Wistar rats and adult human. The chickens were killed by decapitation. The rats were anaesthetized with 5–10 min ether inhalation and then decapitated. Rabbits used for the preparation of antibodies were humanely killed at the end of the experiments. The animal experiments were done under the strict supervision of the Veterinary Department of the County of Zürich, Switzerland. Human specimens were obtained from humans undergoing colon resection, who gave written informed consent for the tissue to be used in the experiments. Anatomopathological assays revealed that the human specimens used were normal. The experiments were performed in accordance with the Hospital Virgen del Rocío of Sevilla ethical committee and with the requirements of the European convention for the care and use of laboratory animals.

Chicken enterocyte isolation and creatine uptake

Chicken enterocytes were isolated by hyaluronidase incubation (Calonge *et al.* 1989). Creatine uptake was calculated by measuring the [^{14}C]creatine present in the cell extracts and taking into account the trapped extracellular water volume as previously estimated (Calonge *et al.* 1989). The protein concentrations of the cell pellets were measured by the method of Bradford (1976). Unless otherwise stated, the incubation buffer contained (mM): 100 NaCl, 1 $CaCl_2$, 60 mannitol, 3 K_2HPO_4, 1 $MgCl_2$, 20 Hepes-Tris, pH 7.4, 0.001 [^{14}C]creatine and 1 mg ml^{-1} bovine serum albumin.

Creatine uptake into rat small intestine

Pieces of rat ileum were incubated, at 37 °C with continuous shaking, in Ringer solution (mM): 140 NaCl, 10 $KHCO_3$, 3 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, pH 7.4, and 0.002 [^{14}C]creatine that was continuously bubbled with 95 % O_2–5 % CO_2. Following the incubation, the tissues were washed in ice-cold Na^+-free Ringer solution (mannitol substitution), blotted carefully, weighed wet and extracted by shaking for 15 h in 1 ml of 0.1 M HNO_3. Samples were taken from the bathing solution and from the extracts of the tissues for radioactivity counting.

Brush-border (BBM), basolateral (BLM) and rat heart extract membrane preparations

BBM and BLM were isolated from either human, chicken or rat small intestine as described previously (Dyer *et al.* 1990; Peral *et al.* 1995; De La Horra *et al.* 2001). The purity of the BLM preparation is indicated by the 8-fold enrichment of Na^+–K^+-ATPase and the lack of enrichment (0.6-fold) of sucrase. Rat heart extracts were prepared as described by Neubauer *et al.* (1999). Protein was measured by the method of Bradford (1976).

cDNA cloning and sequencing

Synthesis of *in situ* hybridization riboprobes: two CRT cDNA fragments of 543 bp and 696 bp were generated from rat and human small intestine, respectively, by RT-PCR. Two micrograms of poly(A)$^+$RNA isolated from the rat and human small intestine were primed with a random primer using a SuperScript pre-amplification system kit (Life Technologies, Spain), as described by the manufacturer. The primers designed, based on the rat

nervous system and human kidney CRT cDNA sequences (Mayser *et al.* 1992; Nash *et al.* 1994), were: sense, 5′-CAGCAGCCG-CCTTGCTGTGC-3′ and 5′-CCTCAGGTGTGGATAGATGC-3′, respectively and antisense, 5′-CCTGTGGAGAGCCTCAAGAC-3′ and 5′-GATGCCCATGCAGACCAGC-3′, respectively.

The full length of rat small intestine CRT cDNA was obtained by RT-PCR using the sense 5′-GCCATGGCGAAGAAGAGC-3′ and antisense 5′-CTGTCACATGACACTCTC-3′ primers designed from rat CRT cDNA sequence (Mayser *et al.* 1992).

The PCR profile was 35 cycles of the following: 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, followed by an extension of 10 min 72 °C. The PCR products were gel-purified, subcloned into pGEM-T Easy Vector System (Promega) and sequenced on both strands by using 3100 Genetic Analizer automated sequencer (Applied Biosystems, USA).

RNA preparation and Northern blotting

Total RNA was extracted as described by Chomczynski & Sacchi (1987). Northern hybridization was carried out using human, chicken and rat ileum poly(A)$^+$RNA, as reported previously (Murillo-Carretero *et al.* 1999).

In situ hybridization

Digoxigenin-labelled antisense and sense riboprobes were generated from the human and rat cDNA fragments, described above, by *in vitro* transcription with T7 and SP6 RNA polymerase (Amersham). *In situ* hybridization was performed as described by Durán *et al.* (2002).

Generation of anti-CRT antibodies

Polyclonal antibody against the N-terminal (NH_2-MAKKSA-ENGIYSVSG-) of the rat and human CRT sequence (Mayser *et al.* 1992; Nash *et al.* 1994) was prepared in rabbits as described by Guerrero-Ontiveros & Wallimann (1998). This sequence did not match that of any protein in the sequence data banks.

Western blotting

SDS-PAGE was performed according to Laemmli (1970) on a 7.5 % polyacrylamide gel. Protein samples of heart extracts, intestinal mucosa homogenate and enterocyte apical and basolateral membranes were dissolved in Laemmli sample buffer and boiled 5 min before being loaded, electrophoresed and electrotransferred onto a nitrocellulose membrane (Micron Separations Inc., USA). The membrane was blocked with 3 % non-fat dry milk in TBST buffer (165 mM NaCl, 100 mM Tris/HCl, pH 7.5, and 0.1 % Tween 20) overnight at 4 °C and incubated with the polyclonal antibody anti-N-terminal peptide of rat CRT (1:500 dilution) for 16 h at 4 °C. After washing with TBST buffer, the blot was incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (ICN Cappel, USA) (1 : 1000 dilution) for 1 h at room temperature. The immunoreactive bands were viewed using the enhanced chemi-luminescence procedure (Pierce, USA). The relative abundance of the bands was quantified using PCBAS version 2.0 (Fuji, USA).

Immunohistochemical analysis

Pieces of human, rat and chicken ileum were fixed in PBS containing 4 % paraformaldehyde overnight at 4 °C. Tissue sections (10 μm) were permeabilized with 1 % Triton X-100 for 15 min and washed in PBS for 30 min. The sections were blocked with 5 % bovine serum albumin (BSA) in PBS for 6 h and incubated overnight at 4 °C with the primary antibody anti-N-terminal peptide-CRT or with preimmune serum, both of them 1:50 dilution in PBS containing 5 % BSA. Next, the slides were washed extensively and incubated with the secondary antibody (FITC-conjugated anti-rabbit IgG (Calbiochem), 1: 500 dilution in PBS containing 5 % BSA) for 2 h at room temperature in the dark. Thereafter, the slides were washed extensively in PBS, embedded in 90 % glycerol, 10 % *N*-propyl-gallate in PBS and analysed with a fluorescence microscope (Olympus AH3).

Materials

[^{14}C]Creatine was purchased from American Radiolabeled Chemicals, Inc., and α-^{32}P-UTP from Amersham Pharmacia Biotech. Unless otherwise indicated, the other reagents used in the current study were obtained from Sigma Chemical Co. (Madrid, Spain).

Statistical analysis

Data are presented as means ± S.E.M. for *N* separate animals. In the figures vertical bars, which represent the S.E.M., are absent when smaller than the symbol. Comparison between different experimental groups was evaluated by Student's two-tailed *t* test.

RESULTS

Creatine uptake *versus* time

Rat small intestine takes up [^{14}C]creatine in a NaCl-dependent manner (Fig. 1) and at 60 min the NaCl-dependent : NaCl-independent creatine uptake ratio was approximately 5.

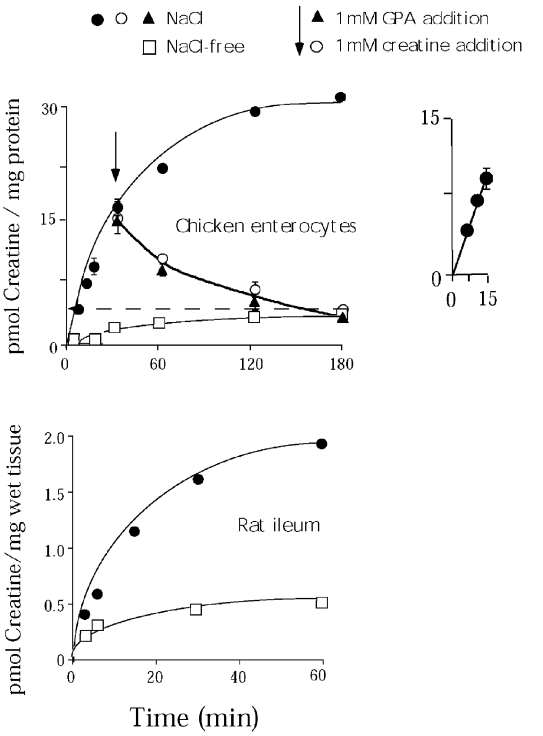

Figure 1. Time course of creatine uptake into either chicken enterocytes or rat ileum

[^{14}C]Creatine uptake was measured in the presence (●, ▲, ○) and absence (□) of extracellular NaCl as a function of time. When required, NaCl was isosmotically substituted by mannitol. At the time indicated by the arrow either 1 mM guanidinopropionic acid (GPA) (▲) or 1 mM unlabelled creatine (○) was added. The dashed line represents the uptake value expected at equilibrium. Inset shows the linearity of uptake below 15 min in the presence of NaCl. Values are means ± S.E.M. of five independent experiments.

Table 1. Specificity of intestinal creatine transport

	% Creatine uptake		
	Chicken		Rat
Modifiers	1 mM	0.02 mM	1 mM
None	100 ± 10	100 ± 10	100 ± 10
Creatine	17 ± 4*	55 ± 1*	18 ± 2*
GPA	16 ± 3*	58 ± 1*	17 ± 3*
Cyclocreatine	50 ± 2*	—	54 ± 2*
Nipecotic acid	72 ± 1.5**	91 ± 1	84 ± 5
GABA	80 ± 10	95 ± 2	85 ± 10
L-Glycine	96 ± 1	—	97 ± 2
Taurine	96 ± 1	—	80 ± 1
β-alanine	87 ± 1	—	88 ± 2
Betaine	97 ± 1	—	100 ± 0.5
Choline	80 ± 9	—	79 ± 2

Uptake of [^{14}C]creatine into either chicken enterocytes (1 μM) or rat ileum (2 μM) was measured during 5 min in the absence and presence of various unlabelled modifiers. Creatine uptake obtained in the absence of modifiers was set at 100%. GPA, β-guanidino-propionic acid. GABA, γ-aminobutyric acid. Values are means ± S.E.M. of three independent experiments. * $P < 0.001$; ** $P < 0.02$, as compared with the first row.

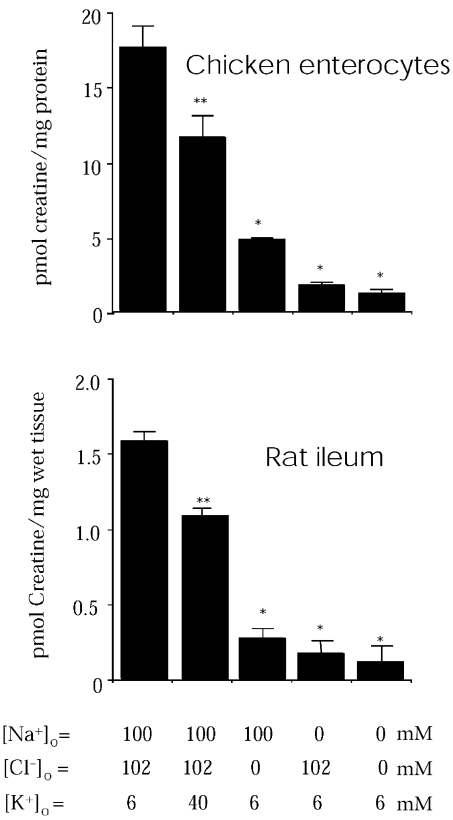

Figure 2. Effect of either electrical, Na^+ or Cl^- gradient on 30 min of intestinal creatine uptake

When required, Na^+ was isosmotically substituted by *N*-methyl-glucamine, Cl^- by gluconate and NaCl by mannitol. Valinomycin (0.02 mM) was added when the external potassium concentration was 40 mM. Other details as in Fig. 1. Values are means ± S.E.M., $N = 3$. *$P < 0.001$; **$P < 0.05$, as compared with control conditions, first column.

Chicken enterocytes also take up [^{14}C]creatine in a NaCl-dependent manner, reaching a cell concentration value of 30.80 ± 0.06 pmol (mg protein)$^{-1}$ (8.00 ± 0.02 μmol l^{-1}) at 120 min. This corresponds to an inside : outside creatine ratio of 8 : 1 (Fig. 1). In nominally NaCl-free conditions creatine uptake was undetectable before 15 min and cell creatine concentration reached equilibrium with the incubation medium at 120 min. Since in the presence of NaCl, creatine uptake increased linearly up to 15 min (Fig. 1, inset), a 5 min incubation time was adopted to determine the initial rate of creatine uptake into isolated enterocytes.

Creatine was neither significantly metabolized nor bound to intracellular components, since the addition of either guanidinopropionic acid (GPA), a high-affinity substrate of CRT, or cold creatine to the outside medium released the previously accumulated creatine (Fig. 1).

Energy dependence and effect of either ouabain, Na^+, Cl^- or electrical membrane potential on creatine uptake

Rat and chicken intestinal creatine transport was inhibited by 40 mM external K^+ plus 20 μM valinomycin, a condition expected to depolarize the cells (Fig. 2). Isosmotic replacement of either Na^+, Cl^- or NaCl with *N*-methyl-glucamine (NMG^+), gluconate or mannitol, respectively, significantly reduced rat and chicken intestinal creatine uptake (Fig. 2). Inhibition induced by the absence of either Na^+ or Cl^- was similar to that seen under NaCl-free conditions.

The Na^+–K^+-ATPase inhibitor ouabain (1 mM) decreased [^{14}C]creatine uptake into chicken enterocytes from 1.02 ± 0.01 to 0.260 ± 0.001 pmol min^{-1} (mg protein)$^{-1}$.

The metabolic inhibitor iodoacetic acid (15 mM) reduced creatine uptake into chicken enterocytes from 1.00 ± 0.01 to 0.270 ± 0.001 pmol min^{-1} (mg protein)$^{-1}$. Ice-bath incubation of the cells prevented creatine uptake completely.

These findings indicate that the intestinal transport of creatine is active and electrogenic, and that the transporter requires the presence of both Na^+ and Cl^- in the extracellular buffer.

Specificity of intestinal creatine uptake

Creatine uptake in either chicken enterocytes or rat ileum was measured in the absence and presence of the compounds listed in Table 1. In any of the concentrations tested, cold creatine and β-guanidinopropionic acid (GPA) were the more potent inhibitors of creatine uptake. The creatine analogue cyclocreatine also significantly inhibited creatine uptake. GABA, betaine, β-alanine, glycine, choline, nipecotic acid and taurine had, if any, only a small inhibitory effect on creatine uptake.

Na^+ : Cl^- : creatine stoichiometry

The observation that changes in the electrical membrane potential affected intestinal creatine uptake suggests that

net transfer of charge across the membrane occurs during the uptake process. The Na^+:Cl^-:creatine stoichiometry was therefore evaluated. The dependence of the initial rate of creatine uptake into chicken enterocytes on the extracellular concentration of either Na^+ (keeping the external concentration of Cl^- constant at 80 mM) or Cl^- (keeping the external concentration of Na^+ constant at 80 mM) was measured. To minimize the effects of membrane potential on the uptake rate, the electrical membrane potential was clamped by increasing external K^+ to 40 mM and adding 20 μM valinomycin.

Figure 3 reveals that the initial rate of creatine uptake increased with increasing extracellular Na^+ concentration. The number of Na^+ ions interacting with the carrier was determined by fitting the data to a Hill-type equation:

$$V = \frac{V_{max}[Na^+]^n}{K^n_{0.5} + [Na^+]^n},$$

where V is the initial rate of uptake, V_{max} is the maximal initial uptake rate, $K_{0.5}$ is the concentration of Na^+ necessary for half-maximal activation and n is the Hill coefficient. Plots of V *vs.* $V/[Na^+]^n$ were constructed for a Na^+ concentration range of 5–80 mM. When n was equal to 1.9, the plot was a straight line, suggesting that at least two Na^+ ions are involved per transport of one creatine molecule. The approximate value of $K_{0.5}$ for Na^+ was 32 mM.

Increasing the extracellular concentration of Cl^- increased creatine uptake (Fig. 4). A plot of V *vs.* $V/[Cl^-]^n$, for a Cl^- concentration range of 5–80 mM, was linear ($r = 0.99$) when $n = 1.06$, suggesting the involvement of one Cl^- per transport of one creatine molecule. The approximate value of $K_{0.5}$ for Cl^- was 23 mM.

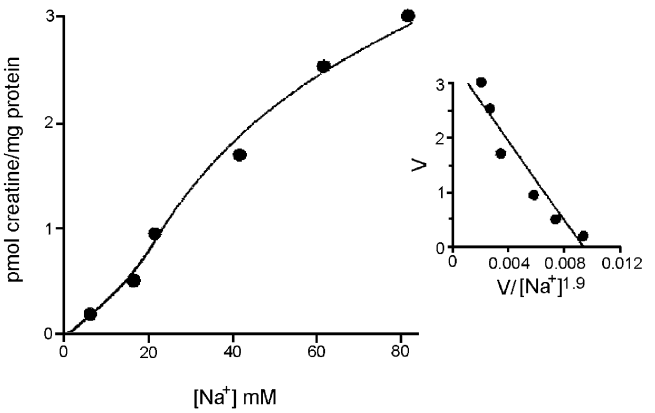

Figure 3. Na^+-dependent creatine uptake into chicken enterocytes *vs.* Na^+ concentration

Uptake of 1 μM [^{14}C]creatine was measured for 5 min. The incubation buffer contained (mM): 34 KCl, 1 $CaCl_2$, 3 K_2HPO_4, 1 $MgCl_2$, 20 Hepes–Tris, pH 7.4, 20 mannitol, 0.02 valinomycin, 1 mg ml^{-1} bovine serum albumin and different concentrations of Na^+, which were made by substitution of NaCl with NMGCl. The Na^+-dependent component for creatine uptake was calculated by subtracting the uptake measured in the absence of Na^+ from that measured in its presence. Inset: Hill-type plot of the data, in which the initial velocity (V) was plotted against $V/[Na^+]^{1.9}$, $r = 0.96$. Values are means ± S.E.M., $N = 5$.

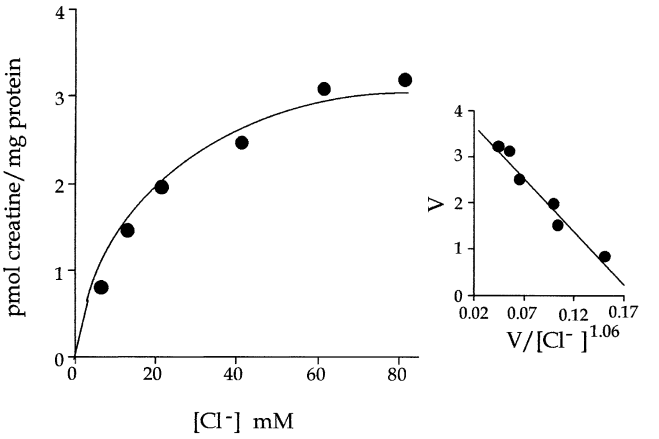

Figure 4. Cl^--dependent creatine uptake into chicken enterocytes *vs.* Cl^- concentration

The different concentrations of Cl^- were made by substitution of NaCl with sodium gluconate. Other details as in Fig. 3. Inset: Hill-type plot of the data, in which the initial velocity (V) was plotted against $V/[Cl^-]^{1.06}$, $r = 0.991$. Values are means ± S.E.M., $N = 5$.

Kinetic study of creatine uptake

Initial rates of enterocyte creatine uptake were measured at varying concentrations of creatine, both in the presence and absence of NaCl (Fig. 5). The results obtained in the presence of NaCl were analysed using a non-linear regression data analysis programme (Enzfitter, Elsevier Biosoft,

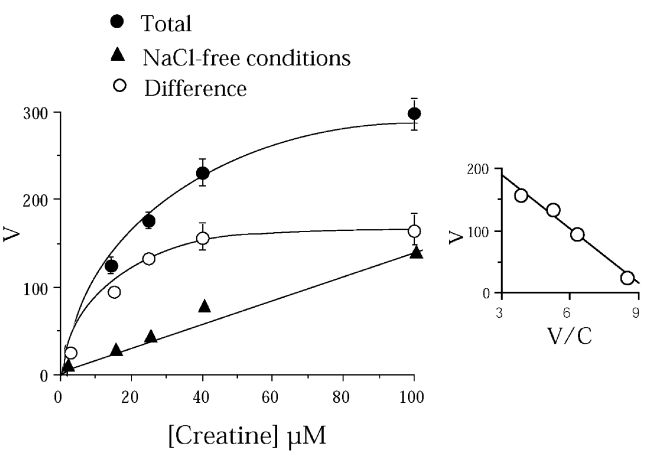

Figure 5. Kinetics of creatine uptake into chicken enterocytes

Initial rate (5 min) of chicken enterocyte creatine uptake *vs.* increasing concentrations of external creatine measured in the presence (●) and absence (▲) of extracellular NaCl. Difference: total uptake minus that measured in NaCl-free conditions. Inset: Eadie–Hofstee plot of the difference data. Values are means ± S.E.M., $N = 5$.

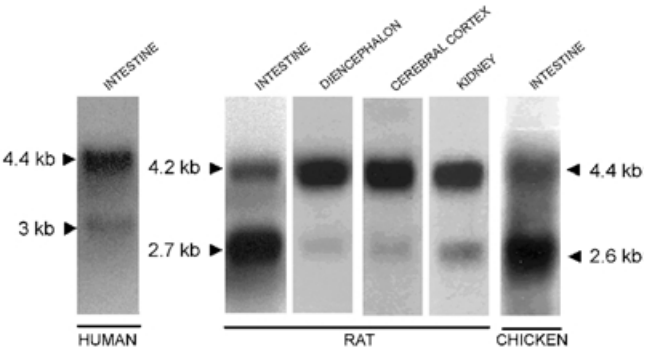

Figure 6. Northern blot analysis of CRT in rat, human and chicken ileum

Equal amounts (10 μg) of poly(A$^+$)RNA were loaded onto the gel per lane. The size of the transcripts was determined by ribosomal RNA. The CRT transcripts displayed a motility corresponding to a size of *ca* 4.4–3 kb for human, 4.2–2.7 kb for rat and 4.4–2.6 kb for chicken ileum.

Cambridge, UK). The best fit ($r = 0.997$) was obtained with a transport model comprising a single saturable transport system according to:

$$V = [V_{max} S/(K_m + S)] + K_d S,$$

where V is the initial rate of uptake, S the external creatine concentration, V_{max} the maximal initial uptake rate, K_m the Michaelis–Menten constant and K_d is the apparent diffusion constant. The calculated apparent K_m and V_{max} for creatine were 29 ± 2.0 μM and 55 ± 3.5 pmol min^{-1}(mg protein)$^{-1}$, respectively.

Creatine uptake measured in the absence of NaCl showed a linear relationship with its extracellular concentration (Fig. 5). The difference between total creatine uptake and that observed in the absence of NaCl follows first-order kinetics. The Eadie–Hofstee plot of the saturable component (Fig. 5, inset) yielded a linear relationship, consistent with the existence of a single saturable creatine transporter.

Cloning and sequencing of the complete CRT cDNA from rat small intestine

The CRT cDNA fragments generated from rat and human small intestine by RT-PCR were identical to the CRT cDNAs cloned from rat nervous system and human kidney, respectively (Mayser *et al.* 1992; Nash *et al.* 1994).

To verify whether CRT, and not a partial identical gene, is transcribed in the small intestine, the full length of CRT was cloned from rat small intestine as described in Methods. A cDNA with an insert of 1.9 kb that contained the complete open reading frame of CRT was obtained. Nucleotide sequencing and a search in the GenBank database revealed that this cDNA was identical to the cDNA coding for rat nervous system CRT (Mayser *et al.* 1992).

Northern blots and *in situ* hybridization

The presence of CRT mRNA in mammal and avian small intestine was examined by Northern blot hybridization using poly(A$^+$)RNA and high (mammal) and low (avian)

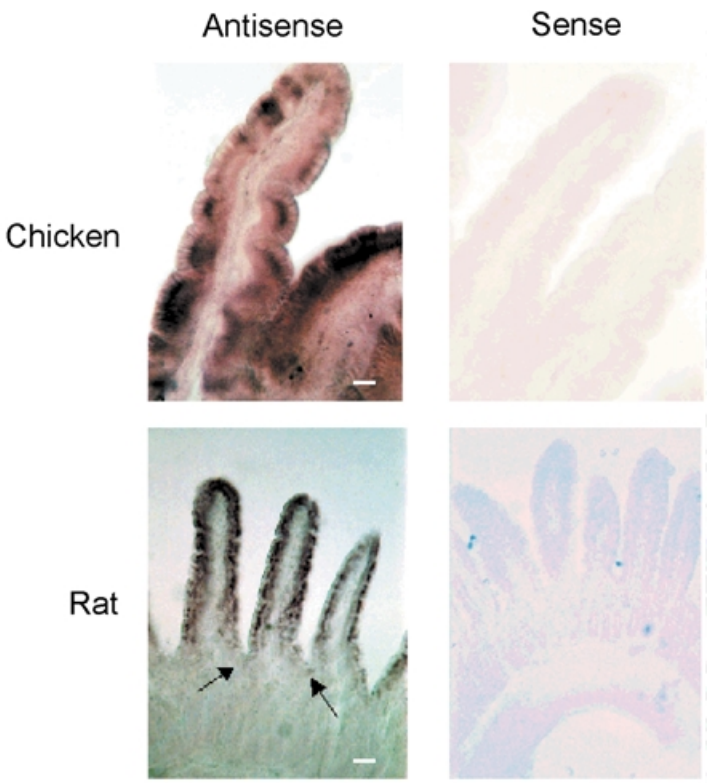

Figure 7. Expression of CRT mRNA in chicken and rat ileum

Panels are bright-field photomicrographs of rat and chicken small intestine which have been *in situ* hybridized with either antisense or sense digoxigenin-UTP-labelled riboprobes. The arrows indicate the intestinal crypts. Scale bars: 100 μm for chicken; 50 μm for rat; bars also apply to right panels.

stringency conditions. Figure 6 reveals that the antisense riboprobes hybridized to two transcripts of 4.4 and 3 kb in human ileum, of 4.2 and 2.7 kb in several rat tissues, including rat ileum, and of 4.4 and 2.6 kb in chicken ileum.

The cell location of CRT mRNA in the small intestine was determined by *in situ* hybridization. Chicken intestinal sections were hybridized with CRT riboprobes generated from human cDNA because they produced stronger hybridization signals than those obtained from rat CRT cDNA. The results revealed that CRT mRNA expression was restricted to the cells lining the villus and was absent from the crypt cells (Fig. 7, antisense panels). There was only low background labelling with the sense riboprobe used on adjacent sections of the tissue (Fig. 7, sense panels).

Immunohistochemistry and Western blot analysis

The antibody used here detects polypeptides of 52, 57 and 72 kDa in skeletal and cardiac muscle, brain, kidney and liver (Guerrero-Ontiveros & Wallimann, 1998; Walzel *et al.* 2000, 2002). Western blotting (Fig. 8) revealed that the previously reported 52, 57 and 72 kDa bands were all present in mammal heart extracts and small intestine homogenate (H) and brush-border membranes (BBM), whereas in chicken the immunoreactive proteins were of 56, 64 and 74 kDa. Figure 8 also shows that the band most highly enriched in the BBM as compared with the homogenate was the 57 kDa band in human and rat (approx. 6-fold enrichment) and the 64 kDa band in chicken (approx. 4-fold enrichment). Rat small intestine

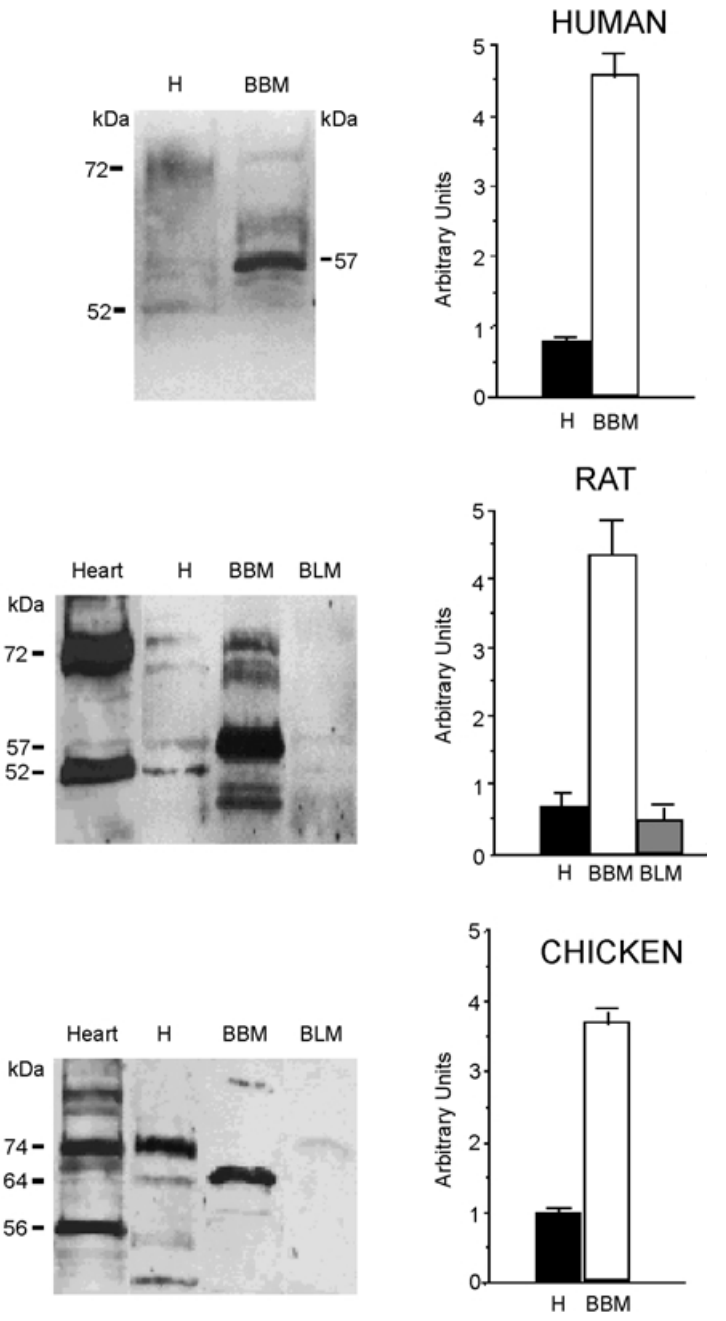

Figure 8. Western blot analysis of heart extracts, intestinal homogenate (H) and apical (BBM) and basolateral (BLM) membranes

A total of 70 μg protein was loaded to each lane. The blots were probed with the polyclonal anti-N-terminal anti-CRT antibody, as described in the Methods section. Histograms represent the relative abundance of CRT protein in homogenate (H), apical (BBM) and basolateral (BLM) membrane. Values are means ± S.E.M., $N = 3$.

basolateral membranes (BLM) present a very faint band of 57 kDa that may represent apical membrane contamination.

Figure 9 shows the localization by immunofluorescence of CRT protein in rat, chicken and human ileum at the light microscopy level. There was a strong specific signal at the apical membrane of cells lining the villus, corroborating the notion that the 57 and 64 kDa CRT subspecies are located in the plasma membrane of the mammal and avian enterocytes, respectively. Labelling was very weak or absent at the basolateral membrane, in the cytoplasm, in the crypts and at the goblet cells.

DISCUSSION

Oral supplementation of creatine is routinely used by many athletes in conjunction with resistance training to improve exercise performance and muscle mass. Nowadays the use of oral creatine supplementation has been extended to the medical field to prevent and/or treat mitochondrial, neuromuscular, cardiovascular and brain diseases, and as antitumoural and antiviral agent (for references see Introduction).

Creatine transport has been investigated in several cell types (Fitch *et al.* 1968; Daly & Seifter, 1980; Ku & Passow, 1980; Loike *et al.* 1986; Möller & Hamprecht, 1989; Masson & Quistorff, 1994; Odoom *et al.* 1996; Syllm-Rapoport *et al.* 1980). However, in spite of the increasing physiological relevance of oral creatine supplementation, the mechanisms involved in creatine intestinal transport have not been investigated.

We present evidence indicating that the small intestine has an active, saturable and electrogenic Na^+:Cl^-:creatine cotransporter located at the brush-border membrane. Only in the presence of extracellular NaCl do intestinal cells accumulate free-creatine against a concentration gradient. This concentrative creatine uptake was prevented by metabolic inhibitors and reduced by membrane depolarization. Earlier studies carried out in different cells types did not evaluate the role of Cl^- on creatine active transport and only showed the Na^+ dependence of the transport system (Fitch *et al.* 1968; Daly & Seifter, 1980; Ku & Passow, 1980; Syllm-Rapoport *et al.* 1980; Loike *et al.* 1986; Möller & Hamprecht, 1989; Masson & Quistorff, 1994; Odoom *et al.* 1996). However, expression of the CRT transporter in either COS-7 cells (Guimbal & Kilimann, 1993), HeLa cells (Saltarelli *et al.* 1996) or in *Xenopus laevis* oocytes (Dai *et al.* 1999) and measurements of creatine transport in rat renal cortex (García-Delgado *et al.* 2001) revealed that creatine uptake is Na^+ and Cl^- dependent and that, as in the current work, at least two Na^+ and one Cl^- are required to transport one creatine molecule.

The calculated K_m value for intestinal creatine uptake is similar to those found in red blood cells (Ku & Passow, 1980), in monocytes and macrophages (Loike *et al.* 1986), in COS-7 cells expressing brain CRT (Guimbal & Kilimann,

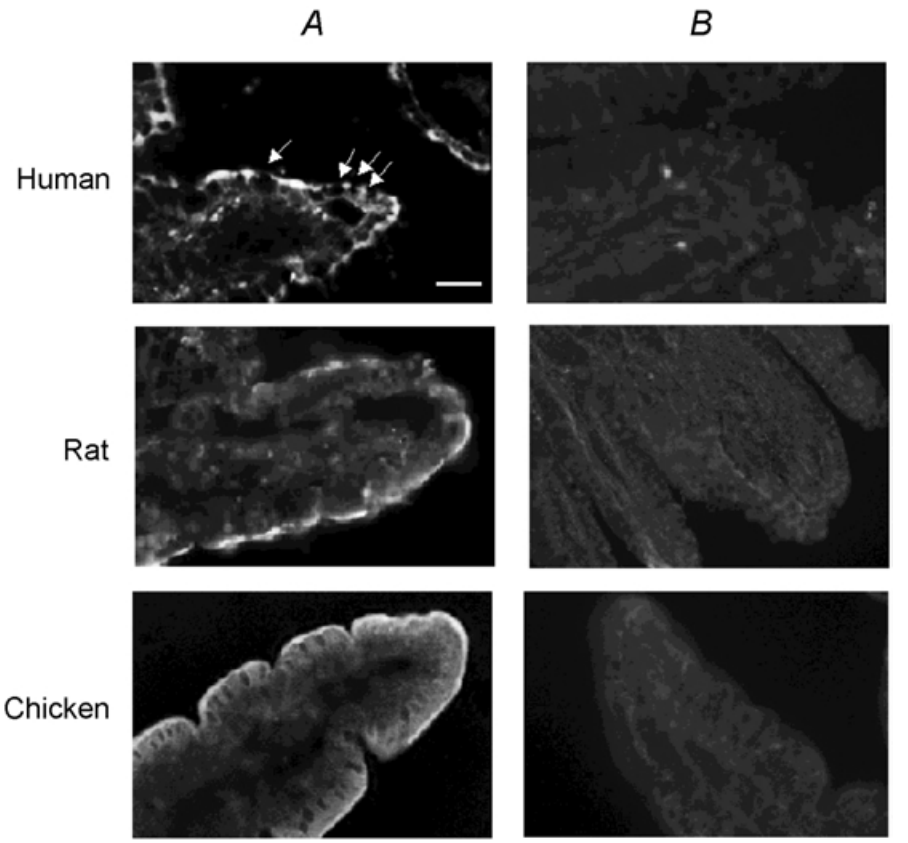

Figure 9. Immunolocalization of CRT in chicken, rat and human small intestine

Sections of ileum were immunostained with the polyclonal CRT antibody (*A*) and with preimmune serum (*B*), as indicated in the Methods. The arrows indicate the goblet cells. Scale bar represents 100 μm and applies to all panels.

1993; Sora *et al.* 1994) or in *Xenopus* oocytes expressing human heart CRT (Dai *et al.* 1999), and is lower than those obtained in astroglia cell culture (Möller & Hamprecht, 1989), red blood cells (Syllm-Rapoport *et al.* 1980), L6 myoblasts (Loike *et al.* 1988) and in HEK-293 cells expressing CRT (Schloss *et al.* 1994), and an order of magnitude lower than that measured in myoblast G8 cell line (Odoom *et al.* 1996) and in HEK-293 cells transfected with cDNA CRT (Dodd *et al.* 1999). The reason for this diversity is not entirely clear, but it may indicate the existence of different CRT subspecies and/or differential regulation of these transporters in different tissues and cells (Guerrero-Ontiveros & Wallimann, 1998).

The CRT is a member of the superfamily of proteins that includes the family of Na^+- and Cl^--dependent transporters, responsible for the uptake of certain neurotransmitters (e.g. dopamine, GABA, serotonin and noradrenaline) and amino acids (e.g. glycine) (Nelson & Lill, 1994; Guimbal & Kilimann, 1994). Within this family, the human CRT is strongly related to a subfamily that includes the transporters of taurine, betaine and GABA. The current study reveals that intestinal uptake of radioactive creatine was abolished by a 100-fold excess of either unlabelled creatine or GPA, a well characterized alternative CRT substrate in several tissues (Fitch *et al.* 1968; Daly & Seifter, 1980; Loike *et al.* 1986). However, GABA, betaine, β-alanine, glycine, choline, nipecotic acid and taurine had, if any, only a small effect on creatine transport. These observations indicate that the small intestine has a creatine-preferring transport system, different from the GABA, betaine and taurine transporters. Intestinal creatine transport was also inhibited by cyclo-creatine, a creatine analogue that exhibits antitumoural activity (Teicher *et al.* 1995; Wyss & Kaddurah-Daouk, 2000).

Consistent with these functional data, we isolated CRT cDNA from the small intestine and detected expression of CRT messenger RNA. The nucleotide sequence of the CRT cDNA fragments cloned from rat and human small intestine, and that of the full-length CRT cDNA cloned from rat small intestine, were identical to the CRT cDNAs previously cloned from rat nervous system and human kidney, respectively (Mayser *et al.* 1992; Nash *et al.* 1994; Sora *et al.* 1994). The full-length CRT cDNA predicts a CRT protein of 635 amino acids (approx. 70.5 kDa).

Northern hybridization analysis revealed the presence of two CRT mRNA transcripts in rat (4.2–2.7 kb), human (4.4–3.0 kb) and chicken small intestine (4.4–2.6 kb). The two 4.2–2.7 kb transcripts were also detected in several other rat tissues. Previous studies have found two CRT mRNA transcripts in various tissues of human (4.0–3 kb), rabbit (4.5–3.1 kb) and rat (4.8–3.2 kb) (Guimbal & Kilimann, 1993; Nash *et al.* 1994; González & Uhl, 1994; Schloss *et al.* 1994; Saltarelli *et al.* 1996). Although CRT expression has been detected in human (Nash *et al.* 1994) and rat (González & Uhl, 1994) small intestine by Northern hybridization, other studies failed to show CRT expression in rabbit (Guimbal & Kilimann, 1993) and human (Gonzalez & Uhl, 1994) small intestine. The presence of different transcripts is believed to arise from alternative polyadenylation (Nash *et al.* 1994). On the other hand, the differences between the transcript lengths of the various species have been considered to be due to differences in the lengths of the non-coding region of the gene or to the experimental procedures. Since we have used the same experimental procedures, the differences detected in the lengths of the two transcripts among the different species must be species specific.

Northern blot analysis, however, does not provide information regarding the type of cells expressing CRT within the epithelium. *In situ* hybridization studies corroborate the presence of CRT mRNA in small intestine and reveal that this expression is restricted to the cells lining the intestinal villus. The crypt cells do not express CRT mRNA.

The Western blots revealed 52, 57 and 72 kDa proteins in mammal intestinal membranes and in heart extract preparations. In chicken, however, the immunoreactive proteins identified by the antibody were of 56, 64 and 74 kDa. The immunoreactive bands identified in mammal tissues were previously detected in skeletal and cardiac muscle, brain, kidney and liver (Guerrero-Ontiveros & Wallimann, 1998; Walzel *et al.* 2000, 2002) by the antibody used here. Tran *et al.* (2000), using a different anti-CRT antibody, identified a 55 and a 70 kDa immunoreactive band in cultured muscle cells. The functional significance of the presence of different CRT isoforms within cells has to be resolved. Recent evidence indicates that the 57 kDa CRT and the 55 kDa CRT are located at the cell membrane of mammal skeletal muscle (Walzel *et al.* 2000) and cultured myocytes (Tran *et al.* 2000), respectively, whereas the 52 and 72 kDa are mitochondrial CRTs (Walzel *et al.* 2000). In agreement with these observations, we found that the bands most highly enriched in Western blots of BBM, compared with that from the homogenate, were the 57 kDa band in both human and rat, and the 64 kDa band in chicken. These observations support the view that the 57 and 64 kDa proteins correspond to the mammal and avian apical CRT, respectively. The difference in mass may be due to species differences in the processing of the proteins.

The immunohistochemistry analyses corroborate the immunoblot data, for fluorescence mostly appears along the apical membrane of the epithelial cells lining the villus, with no or very little fluorescence signal observed either in the cytoplasm or in the basolateral border, respectively (Fig. 9). Fluorescence was absent from both goblet and crypt cells.

Since creatine once absorbed by the high-affinity apical CRT has to be transported into the blood, it seems likely that a creatine transport system should also exist at the basolateral side of the enterocytes. Such a transporter cannot be CRT because the combined Na^+ and Cl^- gradients will move creatine in the opposite direction, that is from the blood into the cell. The nature of such transporter is unknown.

Creatine is most likely also needed by intestinal epithelial cells to build up high-energy PCr for proper function. Relatively high concentrations of brain-type cytosolic creatine kinase, as well as mitochondrial creatine kinase, have been identified in these cells (Keller & Gordon, 1991), indicating that a PCr circuit is at work in intestinal epithelial cells, where creatine kinase is coupled to circumferential contractile ring myosin (Gordon & Keller, 1992). Whether the enterocytes have CRT in the basolateral membrane, to import creatine from the plasma to fulfil their creatine requirements independently of the presence of creatine in the diet, remains unknown. Our anti-CRT antibody does not detect to a significant extent basolateral membrane proteins, which may indicate that CRT is not present at the basolateral side or it is at a much lower concentration.

In conclusion, this is the first study reporting that the small intestine expresses an active, Na^+- and Cl^--dependent, electrogenic and high-affinity creatine transport system, localized at the brush-border membrane, and identifies it as CRT. This transporter mediates the first step of intestinal creatine absorption and has driving forces, a pattern of inhibition and kinetics similar to those described for the nervous system, heart and kidney CRT. This represents an important step towards understanding the molecular basis of the normal creatine absorption process and contributes to the knowledge of CRT function, localization and regulation.

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