Cloning and characterization of a potassium-coupled amino acid transporter

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Edited by H. Ronald Kaback, University of California, Los Angeles, CA, and approved February 19, 1998 (received for review November 13, 1997)

ABSTRACT  Active solute uptake in bacteria, fungi, plants, and animals is known to be mediated by cotransporters that are driven by Na⁺ or H⁺ gradients. The present work extends the Na⁺ and H⁺ dogma by including the H⁺ and K⁺ paradigm. Lepidopteran insect larvae have a high K⁺ and a low Na⁺ content, and their midgut cells lack Na⁺/K⁺ ATPase. Instead, an H⁺ translocating, vacuolar-type ATPase generates a voltage of approximately −240 mV across the apical plasma membrane of so-called goblet cells, which drives H⁺ back into the cells in exchange for K⁺, resulting in net K⁺ secretion into the lumen. The resulting inwardly directed K⁺ electrochemical gradient serves as a driving force for active amino acid uptake into adjacent columnar cells. By using expression cloning with Xenopus laevis oocytes, we have isolated a cDNA that encodes a K⁺-coupled amino acid transporter (KAAT1). We have cloned this protein from a larval lepidopteran midgut (Manduca sexta) cDNA library, KAAT1 is expressed in absorptive columnar cells of the midgut and in labial glands. When expressed in Xenopus oocytes, KAAT1 induced electrogenic transport of neutral amino acids but excludes α-(methy lamino)isobutyric acid and charged amino acids resembling the mammalian system B. K⁺, Na⁺, and to a lesser extent Li⁺ were accepted as cotransported ions, but K⁺ is the principal cation, by far, in living caterpillars. Moreover, uptake was Cl⁻-dependent, and the K⁺/Na⁺ selectivity increased with hyperpolarization of oocytes, reflecting the increased K⁺/Na⁺ selectivity with hyperpolarization observed in midgut tissue. KAAT1 has 634 amino acid residues with 12 putative membrane domains and shows a low level of identity with members of the Na⁺ and Cl⁻-coupled neurotransmitter transporter family.

To remain alive all cells actively accumulate nutrients across their cell membrane. Since Skou (1) isolated the Na⁺/K⁺ ATPase and Crane (2) showed that sugar transport into mammalian intestinal epithelial cells is driven by Na⁺ glucose cotransport, the dogma has arisen that solute transport into cells occurs via cotransporters that are driven by Na⁺ gradients in animals and Na⁺ or H⁺ gradients in bacteria, fungi, and plants. This dogma was strengthened when the first cDNA encoding a Na⁺-coupled glucose cotransporter, SGLT1 from rabbit small intestine, was cloned (3). Now the Na⁺ dogma is being challenged by validation of the K⁺ and H⁺ paradigm. In the much-studied tobacco hornworm, Manduca sexta, Na⁺ concentrations are very low and Na⁺/K⁺ ATPase is not detectable in midgut cells. Instead, a voltage of −240 mV, generated by an H⁺ vacuolar type ATPase (V-ATPase) (4, 5), secondarily drives exchange (antiport) of K⁺ for 2H⁺, resulting in an inwardly directed electrochemical K⁺-gradient (Fig. 1a), which serves as a driving force for active amino acid uptake into columnar cells (6). The well-characterized K⁺-coupled amino acid uptake across an H⁺ V-ATPase energized membrane, in cells that lack an Na⁺/K⁺ ATPase (6), constitutes an important alternative to the Na⁺ dogma. Mammalian and bacterial cells commonly use Na⁺ or H⁺ gradients to drive uptake of polar solutes across the apolar, lipid barrier via transporter proteins. In far more widely distributed insects and freshwater animals Na⁺ is scarce and, in many cases, H⁺ translocating V-ATPases energize membranes by imposing a transmembrane voltage (6).

Previous in vitro studies had revealed that Na⁺ can replace K⁺ to drive leucine uptake at pH 8.0 in brush border membrane vesicles of M. sexta midgut (7). Taking advantage of the Na⁺ acceptability, expression cloning of the K⁺-coupled amino acid transporter was attempted by measuring labeled leucine uptake in the presence of a Na⁺ gradient in Xenopus oocytes where an inwardly directed K⁺ gradient is not present. This paper reports the cloning and characterization of a unique K⁺-coupled amino acid transporter cDNA.

METHODS

Expression Cloning. Total RNA was extracted from fifth instar M. sexta larval midgut by the guanidinium isothiocyanate method using cesium trifluoroacetate. Poly(A)⁺ RNA was isolated and injected into collagenase-treated Xenopus oocytes. Rapid filtration tracer uptake measurements were made in pools of 6–8 collagenase-treated oocytes 4 days after injection of 50 ng of poly(A)⁺ RNA or K⁺-coupled amino acid transporter (KAAT1) cRNA. Oocytes were incubated for 1 h in 750 μl of uptake solution (100 mM NaCl/2 mM KCl/1.8 mM CaCl₂/1 mM MgCl₂/5 mM Tris-HCl, pH 8) containing 200 μM 14C-leucine. Uptake of 14C-leucine was enhanced in cRNA-injected oocytes as compared with water-injected controls (Fig. 1b). The poly(A)⁺ RNA was size-fractionated by using preparative gel electrophoresis and assayed in oocytes. A directional cDNA library was constructed from the active fraction of poly(A)⁺ RNA, using the SuperScript Plasmid System (Life Technologies). cDNAs were ligated into the NotI and SalI restriction enzyme sites of the expression vector pSPORT1 and electroporated into ElectroMax DH10B cells (Life Technologies). Plasmid DNA was transcribed in vitro from pools of 500 clones, and the resulting cRNA was injected into Xenopus oocytes.

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This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: KAAT1, K⁺-coupled amino acid transporter; GABA, γ-aminobutyric acid; GAT1, rat GABA transporter.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF006063).

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Electrophysiological Studies. Stage V–VI oocytes were obtained from mature *Xenopus* females that had been anesthetized with tricaine (1 g/liter). Oocytes were defolliculated by collagenase treatment (1 mg/ml, Boehringer Mannheim) in Ca$^{2+}$-free Ringer’s solution. Capped mRNA was synthesized from linearized plasmids containing the coding region of KAAT1 and approximately 50 ng of RNA in 50 ml of water was injected into the oocytes. The expression was assayed 3–6 days later by two-microelectrode voltage-clamp recording. Data acquisition and analysis used the PCLAMP program suite (Axon Instruments, Foster City, CA). During the two-electrode voltage-clamp recordings, oocytes were first clamped at a holding voltage of $-250$ mV in the uptake solution containing 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM Tris-HCl, pH 7.4 and then perfused with the same solution containing 100 mM KCl instead of NaCl at pH 8.0 in which the experiments were performed (except where otherwise indicated).

Northern Blot Analysis. Total RNA (10 µg), prepared from fifth instar *M. sexta* larval midgut tissue, was separated on a formaldehyde/agarose gel, blotted onto a nylon filter, and hybridized with full-length KAAT1 cDNA labeled with $^{32}$P-dCTP. Hybridization was performed at 42°C in 2× standard saline citrate (SSC)/50% formamide hybridization solution and washed at high stringency (65°C in 0.1× SSC/0.1% SDS).

**Fig. 1.** (a) Schematic model of the midgut epithelium of lepidopteran larvae showing the relationship between KAAT1 and other apical membrane components. The apical membrane of goblet cells contains an H$^+$-vacuolar type ATPase in parallel with a K$^+$/2H$^+$ antiporter that secretes K$^+$ into the lumen while maintaining a transapical voltage of $\sim -270$ mV. The voltage, attenuated to $\sim -240$ mV, appears across the apical (brush border) membrane of electrically coupled columnar cells where it drives K$^+$-coupled amino acid transport from lumen to cell via KAAT1. (b) Na$^+$-coupled uptake of 200 µM $^{14}$C-leucine (1 h) in *Xenopus* oocytes injected with mRNA prepared from *M. sexta* larval midgut or with cRNA synthesized by *in vitro* transcription using KAAT1 cDNA. Open bars indicate water injection. The black bar in the control group indicates injection of total RNA. (Inset) Uptake of 200 µM $^{14}$C-leucine (1 h) in oocytes injected with size-fractionated poly(A)+ RNA from *M. sexta* larval midgut. All data points represent the mean ± SEM from 6–8 oocytes. (c) Leucine (1 mM) evoked uptake currents mediated by KAAT1. A representative oocyte expressing KAAT1 was voltage-clamped at $-50$ mV and superfused with uptake solution containing 100 mM Na$^+$ or specified concentrations of K$^+$. When K$^+$ was being tested, Na$^+$ was replaced with choline$^+$. Leucine was applied where indicated by black bars.
In situ Hybridization. In situ hybridization histochemistry was performed on 7-μm cryosections (postfixed with 4% paraformaldehyde) of fifth instar *M. sexta* larval midgut tissue. Digoxigenin-labeled antisense and sense cRNAs were generated from full-length KAAT1 cDNA by using the Genius Kit (Boehringer-Mannheim) and hydrolyzed to about 200 nts. Sections were hybridized at 65°C for 18 h and washed at the same temperature. The probes were visualized by using antidi- goxigenin Fab fragment and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/nitroblue tetrazolium chloride as a substrate. The sections were developed after 24 h and photographed.

**RESULTS**

Expression Cloning of KAAT1 cDNA. Previous *in vitro* studies had established that K⁺, Na⁺, and to a lesser extent Cs⁺ are accepted but Rb⁺ and Li⁺ are rejected as a coupling ion by *M. sexta* brush border membrane vesicles (7). By taking advantage of the Na⁺ selectivity of KAAT1, amino acid transporters were attempted in *Xenopus* oocytes by measuring labeled leucine uptake in the presence of a Na⁺ gradient. Injection into oocytes of poly(A)⁺ RNA from *M. sexta* larval midgut induced a Na⁺-dependent uptake of 14C-L-leucine that was twice as large as that in water-injected controls (Fig. 1b), with maximal stimulation in sizes ranging from 2.5 to 3.5 kbp (Fig. 1b, Inset). This fraction was used to construct a cDNA library from which ~5,000 clones were sequentially screened by expression in oocytes until the cRNA constructs (Fig. 1c), with maximal stimulation in sizes ranging from 2.5 to 3.5 kbp (Fig. 1b, Inset). This fraction was used to construct a cDNA library from which ~5,000 clones were sequentially screened by expression in oocytes until the cRNA constructs (Fig. 1c). Replacement of Na⁺ by choline⁺ in the bathing solution completely abolished the leucine-evoked transport currents (data not shown). More significantly, a large inward current also was induced by leucine perfusion when Na⁺ was replaced by K⁺; moreover, the amplitude of the current increased with increasing concentrations of external K⁺ (Fig. 1c). The leucine-evoked inward currents at ~50 mV must have been carried solely by K⁺, because Na⁺ was absent. No currents were evoked by leucine in water-injected control oocytes (data not shown). The clone isolated therefore was designated KAAT1.

Analysis of KAAT1 Amino Acid Sequence. KAAT1 cDNA contained 2,881 nts with an ORF from nucleotides 103-2004 that encodes a 634-aa residue protein of 70 kDa. A search of available databases revealed weak, but significant, identity of KAAT1 with amino acid transporters belonging to the Na⁺/K⁺/Cl⁻ superfamily, with 38% overall identity to rat GABA trans-  synthase, and 33% identity to human brain specific L-proline transporter HPROT (8–11). Like KAAT1, that couples K⁺ to Na⁺ and H⁺ to one amino acid, KAAT1 seems to be selective for one amino acid. The substrate specificity of KAAT1 was tested by applying various candidate amino acids under voltage clamp condition. KAAT1 is stereospecific, because L-isomers were preferred. It transports all neutral amino acids tested but not α-(methyl-amino)isobutyric acid. It rejects L-arginine and L-lysine, which would be cationic at the pH tested; it rejected L-glutamate and even proline. This spectrum is reminiscent of system B in mammalian intestinal and renal epithelia (12) from which no corresponding cDNA has yet been isolated.

**KAAT1 mRNA Expression and Tissue Distribution.** Northern analysis using full-length KAAT1 cDNA as a probe revealed a strong hybridization signal of 2.9 kb in size in both anterior and posterior regions of the midgut where amino acid occurs, and a weak signal in the labial glands where amino acid uptake may be involved in the secretion of a proteinaceous semiliquid fluid (Fig. 4a) (27). In situ hybridization was used to determine the cell type in the midgut that expresses KAAT1. Fig. 4 b and c illustrate that KAAT1 is expressed only in the columnar cells where K⁺-coupled amino transport takes place, and no signal was detected in the goblet cells.

**DISCUSSION**

Amino acid uptake in cells has been investigated for many years. Until now a variety of amino acid transport proteins and corresponding cDNAs have been identified and categorized into many gene families (13). In general, amino acids are transported by coupling to the downhill movement of specific cations, mostly Na⁺ and H⁺. By using the expression cloning strategy, we report here the cloning of a unique transporter, KAAT1, that couples K⁺ rather than Na⁺ or H⁺ to amino acid transport in the midgut of lepidopteran *M. sexta* larvae.

K⁺-coupled amino acid transport systems have been found in several species of phytophagous lepidopteran larvae (14) whose hemolymph is characterized by a high [K⁺] to [Na⁺] ratio, in contrast to blood of mammals. Absorption of amino acid in these insects therefore is driven by a K⁺ electrochemical gradient maintained by a luminal directed active potassium transporter located at the apical border of goblet cells (6) (see Fig. 1a). Characterization of amino acid uptake induced by KAAT1 in *Xenopus* oocytes is in good agreement with the previous physiologic studies in brush border membrane vesicles showing that the expression in amphibian oocytes does not change remarkably the features of this protein.

The kinetic analysis of leucine uptake in the oocytes expressing KAAT1 revealed values that are all in good agreement with those measured in brush border membrane vesicles (7, 15, 16). The *Kₘ* for sodium was five times lower than that for potassium. The high affinity of KAAT1 for Na⁺ possibly allows the midgut cells to take up this ion even from the low concentrations (<5 mM) found in the lumen of *M. sexta* (17).
Although it is not yet known how sodium can reach the hemolymph in the absence of a conventional Na\(^+\)/K\(^+\) ATPase, Na\(^+\) uptake is essential, because a relatively high Na\(^+\) concentration is present in a special compartment surrounding nerve and muscle cells that operate by a classical Hodgkin–Huxley mechanism (18, 31). Nevertheless, the affinity of the transporter for K\(^+\) and the high concentration of this cation in the gut lumen ensure that the transporter is coupled mainly to K\(^+\) in vivo.

The pattern of cation and anion selectivity and the membrane potential dependence of KAAT1 expressed in oocytes are in good agreement with the properties of K\(^+\)-coupled amino acid transport systems described for midgut brush border membrane vesicles (7). K\(^+\), Na\(^+\), and to a lesser extent Li\(^+\), but not Rb\(^+\), were accepted as cotransported ions (Fig. 3c). K\(^+\) and Na\(^+\) were accepted equally well at \(-250\) mV whereas K\(^+\) was preferred over Na\(^+\) at \(-150\) mV, which approaches the physiological value for columnar cells of \(-240\) mV. Studies addressing the anion dependence of amino acid transport in midgut brush border membrane vesicles did not reveal chloride dependence of transport (7). However, voltage clamp analysis showed that KAAT1 is Cl\(^-\) dependent, because replacement of Cl\(^-\) by gluconate suppressed the amino acid-evoked currents. This finding suggests a K\(^+\)/Cl\(^-\) amino acid stoichiometry of at least 2:1:1. The Cl\(^-\) dependence of KAAT1 indicates that there is not only a structural but also a functional similarity between KAAT1 and Na\(^+\)/Cl\(^-\)-dependent transporters of the GABA family. However, K\(^+\) coupling appears to be unique to KAAT1. Furthermore, among the tested anions, KAAT1 specificity was: I\(^-\) > SCN\(^-\) > NO\(_3\) > SO\(_4\)\(^2-\) (Fig. 3d). In particular at hyperpolarized membrane potentials the selectivity for I\(^-\) and SCN\(^-\) was significantly increased whereas the pattern for other anions was not changed (Fig. 3d). Overall these observations suggest that the membrane potential is not only the driving force of the K\(^+\)-dependent amino acid transport but it also influences the properties of KAAT1.

Inhibition and countertransport studies of M. sexta midgut brush border membrane vesicles led to the identification of distinct K\(^+\)-coupled transport systems (19–22). The substrate
specificity of KAAT1 expressed in oocytes corresponds to the B-like neutral amino acid transport system based on the following criteria: KAAT1 is stereospecific, because L-isomers are preferred; it transports all neutral amino acids tested but not α-(methylamino)isobutyric acid; it rejects L-arginine and L-lysine, which would be cationic at the pH tested; it rejected L-glutamate and even proline. This spectrum is also highly reminiscent of system B in mammalian intestinal and renal epithelia (12) from which no corresponding cDNA has yet been isolated.

K⁺-coupled amino acid uptake has been reported only in the endoderm-derived midgut and not in the ectoderm-derived, cuticle-lined foregut and hindgut of lepidopteran larvae. KAAT1 mRNA expression followed this same pattern. The

Fig. 3. (a) and (b) External K⁺ and leucine concentration dependence of KAAT1-evoked uptake currents. Data are steady-state currents obtained at −150 mV by using a voltage jump protocol that steps the membrane potential to −150 mV for 100 ms from a holding potential of −50 mV. Data points (mean ± SEM, n = 3) were normalized against the maximal current from the same oocyte and fitted by least-squares according to the equation: I/I_{max} = [S]^n / (K_{n0}^n + [S]^n). (c and d) Cation and anion dependency of KAAT1-mediated leucine uptake. The figure shows steady-state current-voltage relations of a representative oocyte between ±50 and −150 mV, obtained by subtraction of control currents from the corresponding currents in the presence of 200 μM leucine. (e) Substrate specificity of KAAT1. An oocyte expressing KAAT1 was held at −50 mV in the uptake solution containing 100 mM KCl, and specific substrates were applied at 200 μM concentration. Data represent the mean ± SEM of three separate experiments and were normalized against the uptake current induced by 200 μM L-leucine.
prominent 2.9-kb band was present in both anterior and posterior regions of the midgut but not in foregut or hindgut. In situ hybridization indicated that KAAT1 was expressed only in the columnar cells where K⁺-coupled amino transport takes place. The transcript was not found in the goblet cells, which are responsible for energizing the entire apical membrane and for K⁺ secretion into the lumen.

KAAT1 appears to be a new piece in the largely unsolved puzzle of transport protein evolution that may represent an adaptation to the high K⁺/Na⁺ ratio present in the diet and hemolymph of lepidopteran larvae. The peculiar features of KAAT1 suggest that its inhibitors would represent a new family of caterpillar gut poisons that would spare mammals and could lead to a new class of environmentally safe insecticides. On the other hand, KAAT1 may be closely related to system B of mammalian intestine and kidney. In that case it might be used as a probe to clone this broad scope amino acid transport system from mammals. An intriguing finding is that KAAT1 has sequence homology with members of the GABA transport family; apparently transporters that mediate neuro-transmitter amino acid uptake and those that mediate metabolic amino acid uptake had a common ancestor before their divergence during evolution. The pathway and extent of this divergence can be clarified now that KAAT1 probes are available to hasten the search for new pieces of the evolutionary puzzle. Moreover, structure–function studies of KAAT1 will stimulate the investigation of mammalian K⁺-coupled systems, including those of Na, K, 2Cl cotransporters (23), Na⁺- and K⁺-dependent glutamate transporters (24) and Na, K, ATPases.

We thank Daniel D. Spaeth for excellent technical help of larval tissue preparation. This research was supported by National Institutes of Health Grants RO1 DK43171 (M.A.H.) and RO1 AI30464 (W.R.H.). D.T. was supported by a long-term fellowship from Telethon-Italia.