

Conserved tyrosine-147 plays a critical role in the ligand-gated current of the epithelial cation/amino acid transporter/channel CAATCH1

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Summary

CAATCH1 functions both as an amino-acid-gated cation channel and as a cation-dependent, proline-preferring, nutrient amino acid transporter in which the two functions are thermodynamically uncoupled. This study focuses on the ionic channel aspect, in which a Tyr¹⁴⁷ (wild type) to Phe¹⁴⁷ (Y147F) site-directed mutation was investigated by steady-state electrophysiological measurements in the *Xenopus laevis* oocyte expression system. This tyrosine residue is conserved within the third transmembrane domain in members of the Na⁺:neurotransmitter transporter family (SNF), where it plays a role in binding pharmacological ligands such as cocaine to the serotonin (SERT), dopamine (DAT) and norepinephrine (NET) transporters. Epithelial CAATCH1 is a member of the SNF family. The results show that amino acid ligand-gating selectivity and current magnitudes in Na⁺- and K⁺-containing media are differentially altered in CAATCH1 Y147F compared with the wild type. In the absence of amino acid ligands, the channel conductance of Na⁺, K⁺ and Li⁺ that is observed in the wild type was reduced to virtually zero in Y147F. In the wild type, proline binding increased conductance strongly in Na⁺-containing medium and moderately in K⁺-containing medium, whereas in Y147F proline failed to elicit any cation currents beyond those of *N*-methyl-D-

glucamine- or water-injected oocytes. In the wild type, methionine binding strongly inhibited inward Na⁺ currents, whereas in Y147F it strongly stimulated inward currents in both Na⁺- and K⁺-containing media. Indeed, in Na⁺-containing medium, the relative potency ranking for inward current inhibition in the wild type (Met>Leu>Gly>Phe>Thr) was similar to the ranking of ligand-permissive gating of large inward currents in Y147F. In Na⁺-containing medium, current/voltage relationships elicited by ligands in the wild type were complex and reversing, whereas in Y147F they were linear and inwardly rectifying. In K⁺-containing medium, current/voltage relationships remained non-linear in Y147F. Both wild-type and Y147F currents were Cl⁻-independent. Together, these data demonstrate a critical role for Tyr¹⁴⁷ in ligand-binding selectivity and modulation of the ionic channel conductance in CAATCH1. The results support the argument that inhibition of the CAATCH1 conductance by free methionine shares some properties in common with ligand inhibition of DAT, SERT, NET and the γ -aminobutyric acid transporter (GAT1).

Key words: ion channel, potassium, sodium, amino acid, transporter, ionic current, *Xenopus laevis*, *Manduca sexta*.

Introduction

Although CAATCH1 (Feldman et al., 2000; Quick and Stevens, 2001) was cloned from a digestive epithelium cDNA library, it is assigned to the Na⁺:neurotransmitter transporter family (SNF), which consists primarily of neural membrane proteins (Saier, 2001; <http://www-biology.ucsd.edu/~msaier/transport>). Site-directed mutagenesis studies with several SNF proteins from neural tissue collectively suggest that the third transmembrane domain forms a helical pocket that is responsible for binding substrates or psychoactive ligands. Within this pocket, a single tyrosine residue is conserved

(Fig. 1) in all members of the family, and this residue plays a critical role in the behavior of every channel/transporter dual-function protein studied so far. For example, conserved Tyr¹⁷⁶ is associated with cocaine binding in the serotonin transporter (SERT) (Chen et al., 1997). The equivalent position at Tyr¹⁴⁰ in the γ -aminobutyric acid (GABA) transporter (GAT1) is associated with SKF 100330A (a non-transportable GABA analog) blockade of transient currents in Na⁺-containing medium (Bismuth et al., 1997), and the equivalent position at Tyr¹²⁷ in the related glutamate transporter (GLAST) is

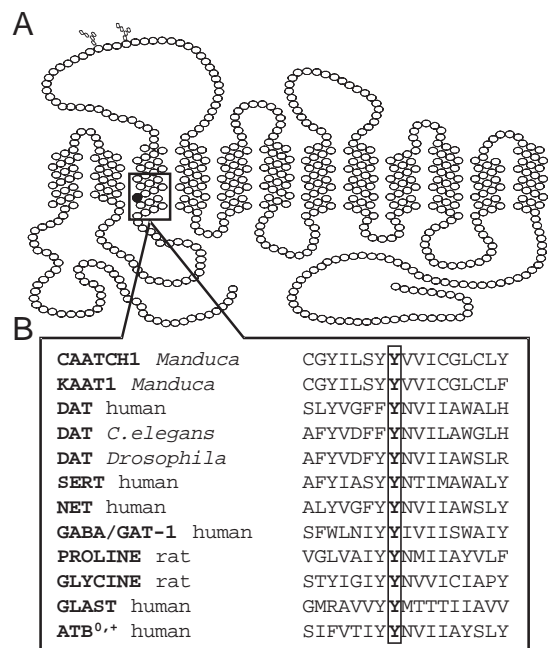


Fig. 1. CAATCH1 (TC 2.A.22.2.4 transporter superfamily classification), Na⁺neurotransmitter transporter family members (SNF; TC 2.A.22) all possess a conserved tyrosine residue (filled circles). (A) Location of Tyr¹⁴⁷ in the third transmembrane domain of CAATCH1. The predicted secondary structure is adapted from Feldman et al. (2000). (B) Alignment of third transmembrane domain residues of CAATCH1 with some representative members of the SNF family showing a universally conserved tyrosine residue representing Tyr¹⁴⁷ in CAATCH1 (<http://ca.expasy.org/cgi-bin/blast.pl?sequence=Q9U5A9>).

responsible for glutamate-elicited ionic currents (Choi and Chiu, 1997).

The present study concerns the conserved equivalent position at Tyr¹⁴⁷ in CAATCH1 (Fig. 1) and focuses on the role that a site-directed mutation, Tyr¹⁴⁷ (wild type, WT) to Phe¹⁴⁷ (Y147F), plays in CAATCH1 ionic channel gating by a variety of free amino acid ligands. This mutation removes the OH group from tyrosine, leaving the non-polar phenyl ring. The ligand-modulating channel role of CAATCH1 is distinct from its other functional role as a cation-catalyzed, but non-symporting, amino acid transporter that is thermodynamically uncoupled from alkali cation fluxes (Quick and Stevens, 2001).

We previously demonstrated that, in Na⁺-containing medium, free methionine binds to CAATCH1 as an inhibitory ligand, potently blocking its ionic current; conversely, in K⁺-containing medium, methionine acts as an agonist, increasing cationic current (Feldman et al., 2000; Quick and Stevens, 2001). Given that the conserved tyrosine residue of the SNF family affects neural membrane protein functions, we hypothesized that a Y147F mutation would alter the ligand-gating specificity of the epithelial CAATCH1 ionic channel. The results implicate a critical role for Tyr¹⁴⁷ in the channel conductance of CAATCH1 and its gating by amino acids.

Materials and methods

Mutagenesis

Full-length wild-type CAATCH1 (GenBank accession No. AF013963) was originally cloned from a fifth-instar *Manduca sexta* midgut epithelium cDNA library using a polymerase chain reaction (PCR) screening strategy. CAATCH1 WT contains an open reading frame of 1899 nucleotides encoding a membrane polypeptide of 633 amino acid residues. A pBluescript vector containing full-length WT CAATCH1 cDNA (Feldman et al., 2000), including the 5'- and 3'- untranslated regions and poly(A) tail, was used as a template for site-directed mutagenesis. Replacement of Tyr¹⁴⁷ with Phe¹⁴⁷ was conducted by a two-stage PCR protocol using *Pfu* DNA polymerase in the QuikChange system (Stratagene, La Jolla, CA, USA). The mutagenic primers were synthesized by the University of Florida Core Facility, with the A of the sense primer WT sequence being modified to a T in the Y147F mutant: 5'-TACATCCTGTCTTACT7CGTGGTGATCTGTGGT-3'. The mutation was confirmed by sequencing. Plasmids containing full-length CAATCH1 WT, CAATCH1 Y147F or KAAT1 WT (Castagna et al., 1998) cDNA were linearized, and capped-complementary RNA (cRNA) was synthesized *in vitro* using the T3 RNA polymerase promoter with the mMessage mMachine kit (Ambion, Austin, TX, USA). *Xenopus laevis* oocytes at stage V–VI were injected with 50 nl of water with or without 50 ng of cRNA, then incubated at 17 °C in Barth's saline for 3–10 days.

Electrophysiology

Injected oocytes were superfused (22 °C) using a peristaltic pump. The Na⁺-containing medium was 100 mmol l⁻¹ NaCl, 1 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ CaCl₂ and 10 mmol l⁻¹ Taps/*N*-methyl-D-glucamine (NMG⁺), pH 8.0. To determine cation specificity, Na⁺ was completely replaced by K⁺, Li⁺ or NMG⁺; to determine anion specificity, Cl⁻ was completely replaced by gluconate. Transmembrane currents were measured in intact oocytes using a two-electrode voltage-clamp with agar-bridged bath electrodes. Current/voltage (*I/V*) relationships were generated using voltage steps or ramps (36 mV s⁻¹, 1.8 mV per point) between -150 and +30 mV from a holding potential of -60 mV. CAATCH1-associated net ionic currents (ΔI) were obtained by subtracting control current values measured in the absence of amino acids from currents measured in their presence. It should be noted that, for ligands that inhibit constitutive currents, this procedure produces the inverse of the inhibited current, resulting in *I/V* relationships that have negative slopes and have current values with inverted signs compared with conventional *I/V* curves. This phenomenon has been described by for cocaine inhibition of dopamine transporter (DAT) currents (Sonders et al., 1997).

Immunolocalization

CAATCH1 WT and Y147F expressed in *Xenopus laevis* oocytes was immunolocalized using a polyclonal primary antibody (a gift from Dr P. Linser) prepared in rabbits against the N terminus of CAATCH1, which is common to both the

wild type and the mutant. Oocytes in which transport activity had been confirmed electrically were fixed at 22 °C for 2 h in PBSAT buffer (phosphate-buffered saline, 1.5 % bovine serum albumin, 0.1 % Triton X-100) containing 4 % paraformaldehyde. Oocytes were washed three times with PBSAT (22 °C), then incubated at 4 °C for 2 days in PBSAT containing 0.1 % sodium azide buffer, which was changed periodically. The affinity-purified primary antibody (0.2 mg ml⁻¹, diluted 1:100 in PBSAT) was incubated with fixed oocytes for 2 h at 22 °C. Oocytes were washed with PBSAT three times at 22 °C, then twice for 1 h each at 4 °C. The oocytes were then incubated for 2 h at 22 °C with Texas-Red-conjugated, affinity-purified, secondary antibody (goat anti-rabbit, 1.4 mg ml⁻¹, diluted 1:100 in PBSAT; Jackson ImmunoResearch, West Grove, PA, USA). Finally, stained oocytes were washed three times with PBSAT at 22 °C, then incubated with periodic buffer changes at 4 °C for 2 days. Texas Red images were obtained with a BioRad confocal microscope (model 1024 ES) equipped with Olympus IX70 inverted optics. Images were visualized in 2 µm steps with 4X Kalman filtering using the same gain and laser power settings for all oocytes.

Results

Immunohistochemical staining using antibody to KAAT1 WT confirmed that cRNA for both CAATCH1 WT and the Y147F mutant was expressed and that the transport proteins were both localized in the plasma membrane of *Xenopus laevis* oocytes (Fig. 2).

Cation-dependent, *I/V* relationships were drastically altered in Y147F compared with WT (Figs 3–5). In the absence of amino acids (Fig. 3), the slopes of the *I/V* relationships for WT for total current in Na⁺-, K⁺- and Li⁺-containing media were similar to each other and steeper than in NMG⁺-containing medium (Fig. 3A). The WT reversal potentials were approximately -5 mV for Li⁺-, -15 mV for Na⁺-, -45 mV for K⁺- and -80 mV for NMG⁺-containing media. The net cation-induced CAATCH1-specific inward currents in WT (Fig. 3B) were subsequently obtained by subtracting the outward K⁺ current component of the total current in NMG⁺-containing medium; the resulting Na⁺-, K⁺- and Li⁺-specific currents were strictly inwardly rectifying at positive potentials. The NMG⁺ curve in Y147F (Fig. 3C) was the same as that in water-injected controls (data not shown) and similar to that in WT (Fig. 3A), with a reversal potential

of approximately -60 mV. Notably, in Y147F, the Na⁺, K⁺ and Li⁺ curves were all very similar to that of NMG⁺, each yielding reversal potentials of approximately -50 mV. The positive net current values observed at depolarizing potentials are due primarily to outward K⁺ current (Quick and Stevens 2001), and subtracting the currents obtained in NMG⁺-containing medium effectively reduced net cation-specific Y147F currents in the absence of ligands to zero at all potentials (Fig. 3D). These data collectively indicate that, in the absence of any amino acid ligand, the Na⁺, K⁺ and Li⁺ conductances observed in WT are reduced to nearly zero in Y147F.

The effects of various free amino acids on *I/V* relationships were also investigated in both Na⁺- and K⁺-containing media by subtracting the small control values from values obtained with each ligand (Figs 4, 5). As we have shown previously (Feldman et al., 2000; Quick and Stevens, 2001) for WT in Na⁺-containing medium (Fig. 4A), the proline-evoked inward currents were the largest elicited by any test ligand at all potentials. In contrast, methionine elicited a potent blockade of inward Na⁺ current (Fig. 4A), as shown by inverted *I/V* curves with negative slopes (due to the subtraction protocol), representing the algebraic inverse of the inhibited current (Feldman et al., 2000; Quick and Stevens, 2001). The methionine block of WT inward current appears only in Na⁺-containing medium and is not observed in K⁺- (Fig. 5A) or Li⁺-containing medium, as shown previously (Feldman et al., 2000).

In WT in Na⁺-containing medium, leucine produced

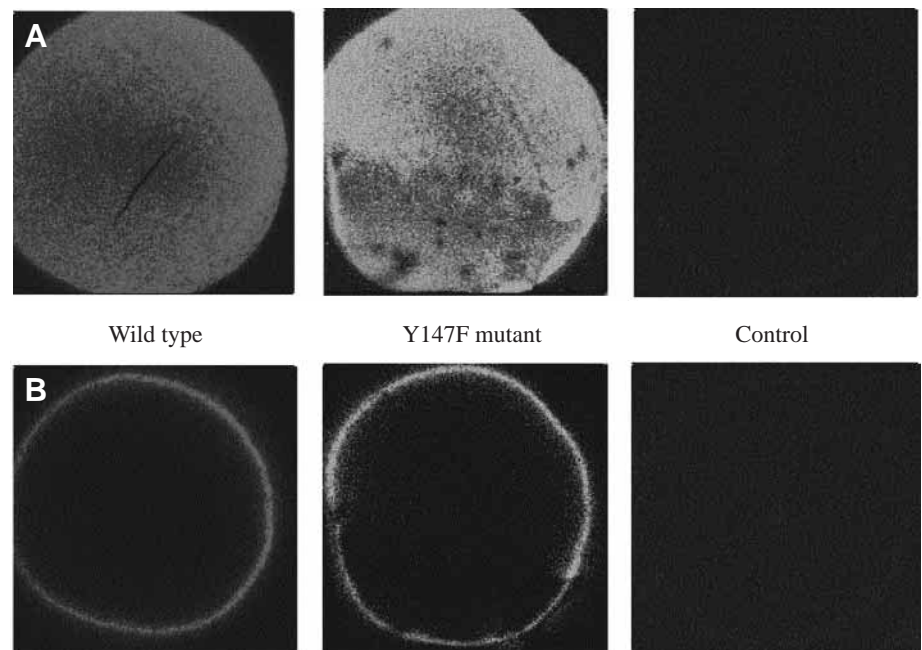


Fig. 2. Immunohistochemistry of CAATCH1 wild type (WT) and mutant Y147F expressed in *Xenopus laevis* oocytes. (A) Confocal scanning micrographs of the outer membrane surface of oocytes expressing CAATCH1 (WT or Y147F) and a water-injected control. (B) Cross sections of intact oocytes from A were obtained by confocal microscopy scans focused using depths of field of 2 µm.

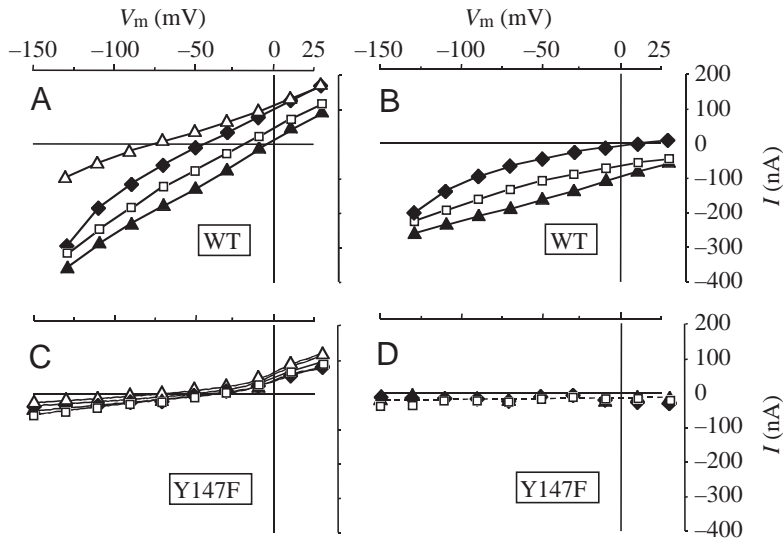


Fig. 3. Voltage-dependence of CAATCH1 steady-state currents in the absence of free amino acid ligands. Net total currents measured in representative oocytes expressing WT or Y147F were obtained without amino acids in the presence of $100 \text{ mmol l}^{-1} \text{ Na}^+$ (open squares), K^+ (filled diamonds), Li^+ (filled triangles) or NMG^+ (open triangles). (A) Total WT currents (adapted from Feldman et al., 2000). (B) Net cation-specific WT-dependent currents obtained by subtracting the NMG^+ currents in A at each voltage. (C) Total Y147F currents. (D) Net cation-specific Y147F-dependent currents obtained by subtracting the NMG^+ currents in C at each voltage. V_m , membrane potential; I , current. The experiment was repeated 3 times.

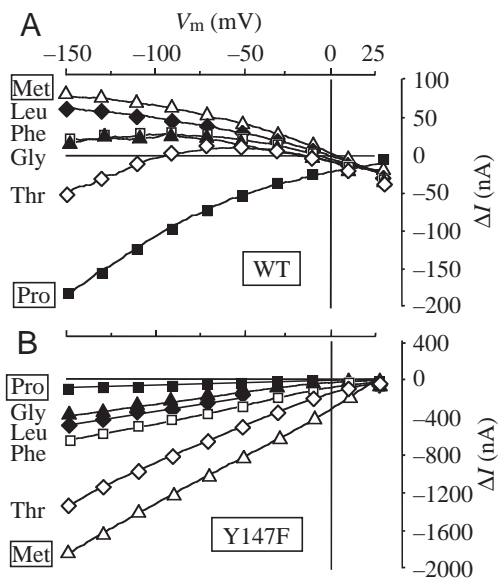


Fig. 4. Representative current/voltage (I/V) relationships elicited by free amino acids in $100 \text{ mmol l}^{-1} \text{ Na}^+ / 0 \text{ mmol l}^{-1} \text{ K}^+$ medium. The ΔI data represent total currents obtained by subtracting control I/V data from the I/V data in the presence of 0.5 mmol l^{-1} amino acids for (A) CAATCH1 WT and (B) CAATCH1 Y147F. For A, in the case of methionine, leucine, phenylalanine and glycine, the blockade of inward current is represented by inverted I/V data as a result of the subtraction protocol representing the algebraic inverse of the inhibited current. V_m , membrane potential.

inhibitory effects similar to methionine, whereas threonine, phenylalanine and glycine all produced complex I/V curves characterized by negatively sloping domains at depolarized voltages and positively sloping domains at hyperpolarized voltages, to produce inverted U-shaped I/V relationships. These complex I/V relationships suggest a voltage-dependent modulation of gating such that, at depolarized potentials, these amino acids inhibit currents, but at hyperpolarized potentials they increase currents. The WT relative inward current inhibition ranking for the ligands that have pronounced negatively sloping I/V domains was: $\text{Met} > \text{Leu} > \text{Phe} > \text{Gly} > \text{Thr}$. These effects were observed for WT in $100 \text{ mmol l}^{-1} \text{ Na}^+$ -containing medium completely lacking K^+ (Fig. 4A); addition of as little as $2 \text{ mmol l}^{-1} \text{ K}^+$ attenuated the net outward current elicited by these ligands (data not shown). For WT in K^+ -containing medium lacking Na^+ (Fig. 5A), all test amino acids, except leucine, yielded inwardly rectifying currents, with threonine eliciting the greatest current. In some oocytes expressing WT, leucine in K^+ -containing medium (Fig. 5A) elicited an apparently inverted, negatively sloping I/V relationship (produced by the subtraction protocol) with reversal potentials near -100 mV , suggesting a possible current inhibition.

In stark contrast to WT, there was no current block in the Y147F mutant by any test amino acid in Na^+ -containing medium, with all ligands instead eliciting solely inwardly rectifying currents (Fig. 4B). Unlike the complex I/V curves observed in WT (Fig. 4A), the curves for Y147F were linear with all test ligands in Na^+ -containing medium (Fig. 4B). Also in contrast to WT, proline failed to elicit currents in either Na^+ - or K^+ -containing media beyond NMG^+ values in Y147F (Figs 3B, 4B, 5B) or water-injected control oocyte currents. For Y147F, the relative potency ranking for permissive gating of inward current in Na^+ -containing medium ($\text{Met} > \text{Thr} > \text{Phe} > \text{Leu} > \text{Gly} > \text{Pro}$) (Fig. 4B) was similar to the ranking order of WT inward current inhibition (Fig. 4A). It is notable that, in Na^+ -containing medium, methionine elicited inward currents in Y147F (Fig. 4B), rather than blocking it as in WT (Fig. 4A). Furthermore, the magnitude of methionine-elicited inward currents in Y147F (Fig. 4B) was the largest measured for any test amino acid at hyperpolarizing potentials in all trial oocytes expressing CAATCH1 WT or Y147F.

In K^+ -containing medium, methionine permitted large inwardly rectifying currents in Y147F (Fig. 5B), much like in WT (Fig. 5A). In contrast to WT in K^+ -containing medium, none of the test amino acids blocked Y147F inward currents (Fig. 5B). For WT in K^+ -containing medium, leucine demonstrated a mild inward blocking effect with a considerable negative reversal potential in some oocytes (Fig. 5A), whereas for Y147F in K^+ -containing medium leucine also yielded both inward and outward currents

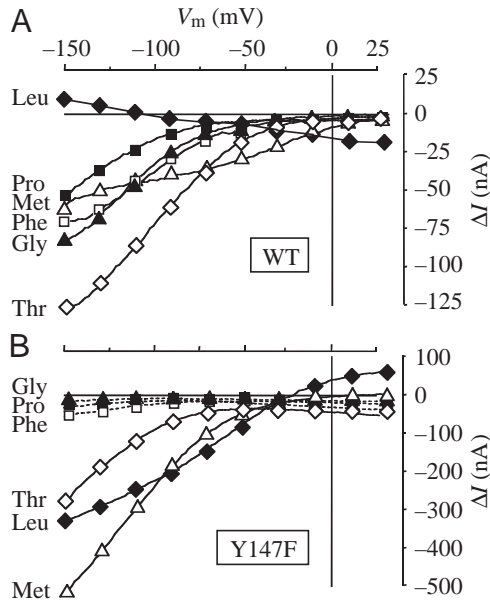


Fig. 5. Representative current/voltage (I/V) relationships elicited by free amino acids in $100\text{ mmol l}^{-1}\text{ K}^+$ / $0\text{ mmol l}^{-1}\text{ Na}^+$ medium. The ΔI data represent total currents obtained by subtracting control I/V data from the I/V data in the presence of 0.5 mmol l^{-1} amino acids for (A) CAATCH1 WT and (B) CAATCH1 Y147F. In B, data were obtained using the same oocyte as Fig. 3C,D, demonstrating that currents elicited by Gly, Pro and Phe were similar to those in the presence of NMG^+ alone. V_m , membrane potential.

(Fig. 5B), but the I/V relationships were complex and reversed at approximately -25 mV .

We have previously demonstrated that amino acid ligands bind CAATCH1 with varying affinities, thereby modifying the degree of charge transfer across the membrane (Feldman et al., 2000; Quick and Stevens, 2001). In Fig. 6, we depict the ligand-binding selectivity of WT and Y147F, as revealed by the evoked inward or blocked inward (i.e. data reported as apparently outward as a result of the subtraction protocol) currents with 0.5 mmol l^{-1} test amino acids. Current was measured at -60 mV , and data were normalized to the value for the preferred ligand in Na^+ -containing medium, proline (Fig. 6A), or to the value for threonine in K^+ -containing medium (Fig. 6B). Three striking results emerge in Na^+ -containing medium. First, all relative inward currents elicited by all test amino acids were much greater in Y47F than in WT. Second, the inward current block phenomenon was eliminated in Y147F for all ligands. Third, the inhibition of inward current by methionine in WT was converted in Y147F to a strictly inward current that was nearly 30 times greater than the inward current elicited by proline. Thus, the largest currents in Na^+ -containing medium were produced by methionine in Y147F, but by proline in WT. In K^+ -containing medium, notable differences also exist between the WT and Y147F variants (Fig. 6B). First, glycine, proline and phenylalanine in K^+ -containing medium were ineffective or poor gating ligands in

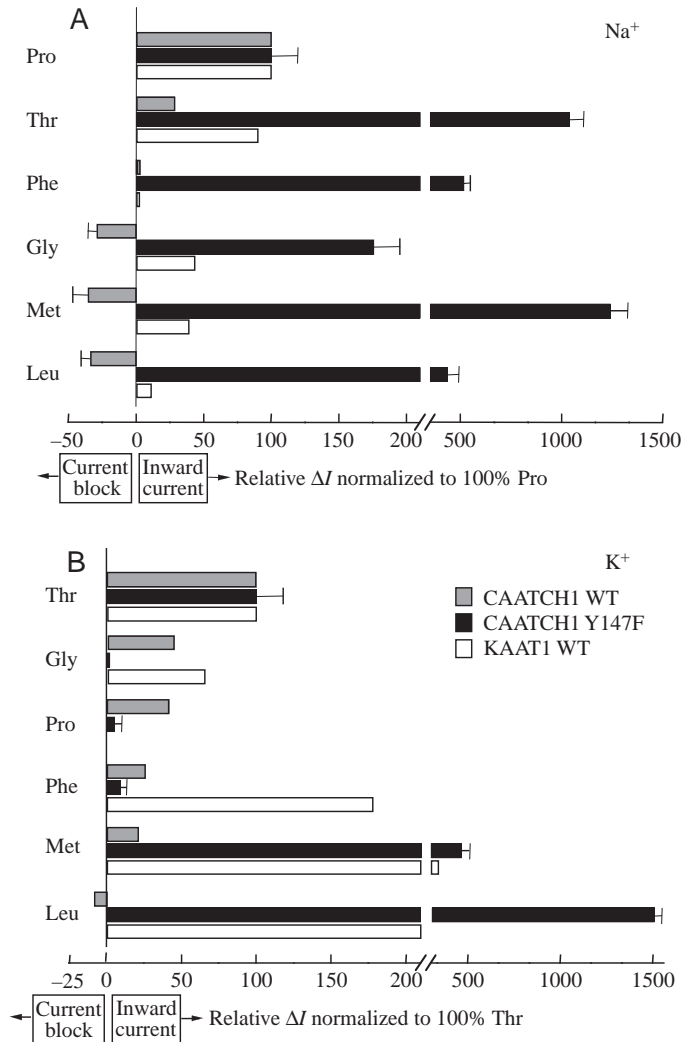


Fig. 6. Net change in currents (ΔI) elicited by 0.5 mmol l^{-1} free amino acids in 100 mmol l^{-1} (A) Na^+ - or (B) K^+ -containing medium obtained at a holding potential of -60 mV . Negative values represent blockade of net inward current, as described in Materials and methods. Values were normalized to the CAATCH1 WT preferences (Feldman et al., 2000; Quick and Stevens, 2001) for gating amino acids in Na^+ -containing (Pro) or K^+ -containing (Thr) medium. KAAT1 WT values were also measured for comparison using the same electrophysiological apparatus. Values are means \pm S.E.M., $N=3$.

Y147F compared with WT. Second, in K^+ -containing medium, methionine and leucine are by far the preferred ligands in Y147F.

Although CAATCH1 WT and Y147F display quite different current-gating behavior, they are alike in at least one respect, the absence of Cl^- does not influence amino-acid-gated currents (Fig. 7). When Cl^- was completely replaced by gluconate in all media, there were no significant effects on (i) the WT proline-elicited current in Na^+ -containing medium; (ii) the Y147F methionine-elicited current in Na^+ -containing medium or (iii) the Y147F leucine-elicited current in K^+ -containing medium.

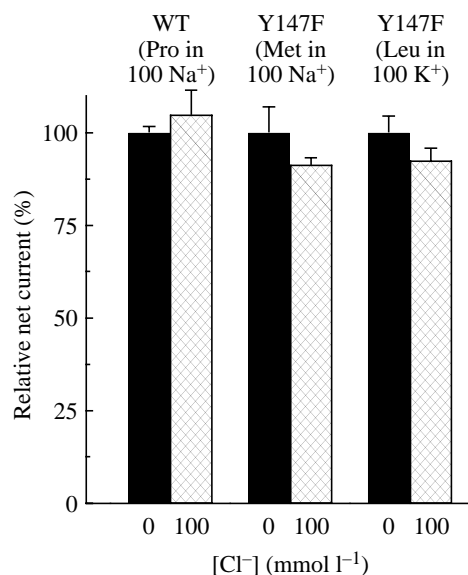


Fig. 7. Cl^- -independence of amino-acid-elicited inward currents of WT or Y147F in 100 mmol l^{-1} Na^+ - or K^+ -containing medium. In all media, Cl^- salts were replaced by gluconate salts. There was no significant differences ($P \leq 0.05$) between currents in Cl^- - and gluconate-containing media. Values are means \pm S.E.M., $N=3$.

Discussion

Neural members of the SNF family of channel/carrier proteins (Saier, 2001) share a conserved tyrosine in the third transmembrane domain (Fig. 1), and this residue plays a critical role in their physiological and pharmacological activities. The results of the present study extend this tyrosine requirement to include CAATCH1, which is a membrane protein from a digestive epithelium and a member of the SNF family. Attention is focused in this study on the ionic channel aspect of dual-function CAATCH1, in which conductance in Na^+ - and K^+ -containing media is shown to be differentially affected by a variety of free amino acid ligands upon mutating Tyr¹⁴⁷ to Phe¹⁴⁷.

SNF proteins expressed *in vitro* clearly exhibit ionic currents, but the interpretation of these currents in the literature varies with the experimental evidence for a particular protein. A range of models (DeFelice et al., 2001; Amara, 1996; Cao et al., 1998; DeFelice and Blakely, 1996; DeFelice and Galli, 1998; Fairman and Amara, 1999; Galli et al., 1996, 1997, 1998; Kanner et al., 2001; Kavanaugh, 1998; Mager et al., 1996; Petersen and DeFelice, 1999; Sonders and Amara, 1996; Sonders et al., 1997; Su et al., 1996; Zhu et al., 1997) has been put forward for various channel/transporters. The current has been interpreted as (i) a transport-associated ionic current that arises from secondary active cotransport of organic solutes that are stoichiometrically coupled with activator ions, (ii) ion leakage through a saturable cotransporter as the result of slippage (uncoupling), (iii) organic ligand-gating of constitutive leak channels exclusively serving inorganic ions, (iv) channel movement of charged organic solutes singly or in

clusters, (v) transient currents from the protein's conformational shifts and/or (vi) multiple conductance states of one or more of the above. CAATCH1 fits the last category. It is a dual-function digestive epithelial membrane protein that operates as both (i) an amino-acid-gated alkali cation channel and (ii) a stoichiometrically uncoupled non-symporting, but Na^+ -dependent, proline-preferring amino acid transporter (Feldman et al., 2000; Quick and Stevens, 2001).

Although the ionic channel and transport functions of CAATCH1 are not thermodynamically coupled (Quick and Stevens, 2001), they are interdependent. Thus, alkali metal cations modulate specific amino acid transport and vice versa. This interdependence is likely to be complex. Our earlier steady-state electrophysiological studies with CAATCH1 (Feldman et al., 2000) initially demonstrated that methionine unmasked constitutive cationic current pathway(s). Our subsequent transient current/radiotracer studies (Quick and Stevens, 2001) confirmed that CAATCH1 WT possesses non-saturating Na^+ and K^+ channel activity which behaves according to the Nernst-Planck relationship and displays voltage-dependent transient currents with symmetrical ON/OFF rates.

It is not known whether CAATCH1 possesses one or more than one channel serving Na^+ and K^+ . A variety of free amino acids bind to CAATCH1 ligand-gating site(s), thereby modulating its channel conductance (Quick and Stevens, 2001); e.g. proline binding increases the conductance of both Na^+ and K^+ , whereas methionine and other amino acids inhibit cation conductance in Na^+ -containing medium but potentiate it in K^+ -containing medium. Ligand-binding affinities and ON/OFF transient rate constants are both dependent on the concentrations of Na^+ and K^+ . However, the maximal transient charge transfer by CAATCH1 is affected neither by organic solute ligands nor by Na^+ (Quick and Stevens, 2001), unlike 'prototypical' cotransporters such as the Na^+ /glucose cotransporter SGLT1 (Parent et al., 1992; Quick et al., 2001) or the γ -aminobutyric acid transporter GAT1 (Hilgemann and Lu, 1999). Thus, the ionic conductance of CAATCH1 is thermodynamically uncoupled from amino acid transport, but the physical relationship between ionic channel and amino acid transport events is unknown.

The results of the present study are summarized in the schematic model of Fig. 8. In the absence of any gating amino acid ligand, Y147F conductance in Na^+ -, K^+ - or Li^+ -containing media is effectively reduced to zero (Fig. 3B), compared with the open-channel *I/V* relationships with these alkali cations of WT (Fig. 3A). The absence of measurable Y147F constitutive currents in the absence of gating ligands (Fig. 3B) represents an inherent property of the mutated protein because protein translation and trafficking to the membrane in the heterologous oocyte expression system appear to be the same for both Y147F and WT (Fig. 2). Moreover, free methionine strongly stimulated inward currents of Y147F in both Na^+ - and K^+ -containing media, whereas it strongly inhibited inward currents of WT in Na^+ -containing medium (Figs 4–6). Furthermore, although proline increased inward currents of WT strongly in

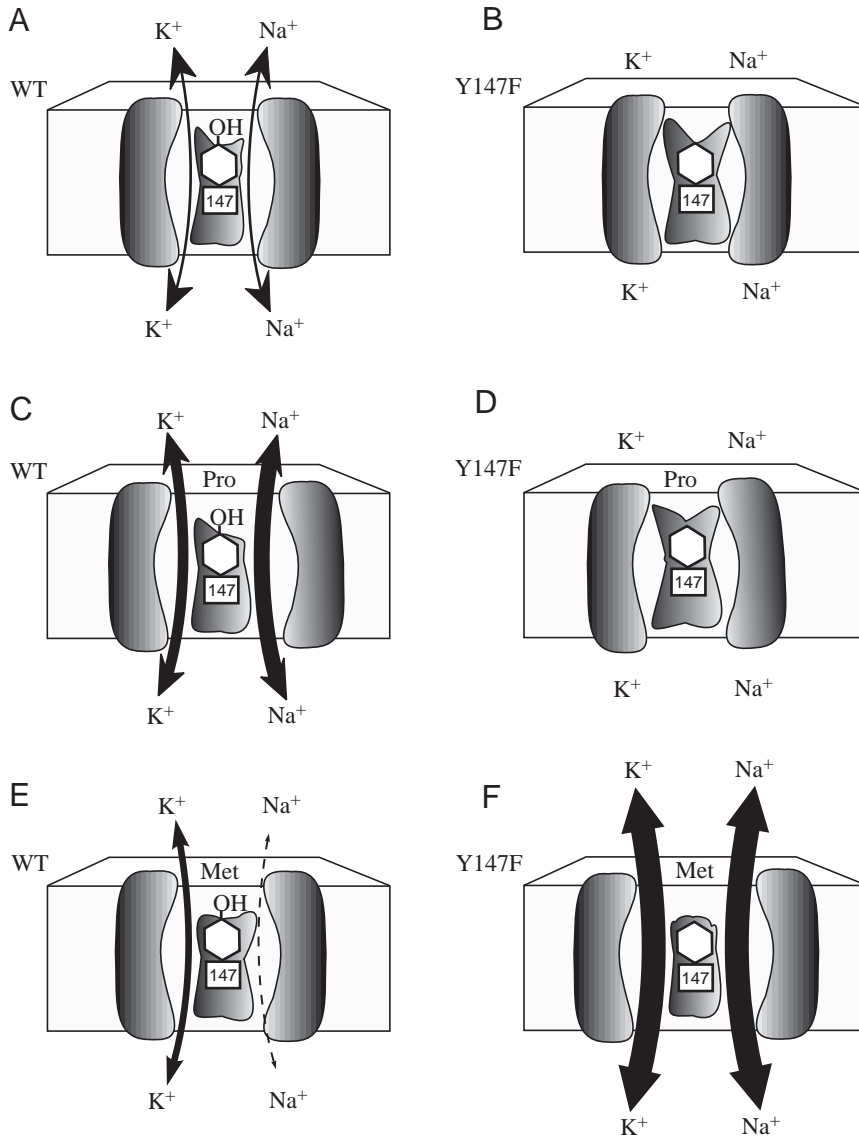


Fig. 8. Schematic representation of amino acid ligand-gating of cationic channel conductance in CAATCH1 (Tyr¹⁴⁷) and the Y147F (Phe¹⁴⁷) mutant. (A) Endogenous WT currents in the absence of ligands. (B) No Y147F current in the absence of ligands. (C) Increased WT currents gated by extracellular L-proline. (D) No Y147F current gated by extracellular L-proline. (E) WT Na⁺ inward current blocked by extracellular L-methionine. (F) Y147F currents enhanced by extracellular L-methionine.

Na⁺-containing medium and moderately in K⁺-containing medium, it failed to elicit currents of Y147F in either Na⁺- or K⁺-containing media beyond values in NMG⁺-containing medium or in water-injected control oocytes (Figs 3–6). Thus, the Y147F mutation alters the modulation of ionic conductance induced by the binding of particular amino acid ligands so that antagonists of conductance in the wild type become agonists and wild-type agonists lose efficacy in the mutant. One property shared by both WT and Y147F is the Cl[−]-independence of CAATCH1 channel activity (Fig. 7), in contrast to KAAT1 and neural SNF family members, which are Cl[−]-dependent.

It is remarkable that, in contrast to WT, there is no current block in Y147F by the test amino acids. This is especially notable since the Y147F mutation consists simply of removing the polar OH group from tyrosine, leaving the non-polar phenyl ring of phenylalanine (Fig. 8). The functional effects of a single mutation of the conserved tyrosine residue could be attributed (i) to the binding of ligand amine nitrogen to the protein's hydroxyl side chain itself, as suggested for other SNF members (Bismuth et al., 1997), (ii) to steric alterations of the site or pore geometry as a result of hydrophobic shifts within the transmembrane pore or, more likely, (iii) to a complex combination of many biophysical/structural perturbations. Considered together with other SNF mutagenesis studies, it is noteworthy that this conserved tyrosine residue is required for appropriate physiological behavior among such a wide spectrum of SNF proteins. Because the residue is highly conserved, it is possible that naturally occurring nucleotide substitutions, even single substitutions, elsewhere in the sequence could give rise to completely different functional phenotypes for a particular SNF protein isoform, either genetically programmed by design as a splice variant or by an unintended mutation.

Amino acid uptake studies using *Manduca sexta* midgut brush-border membrane vesicles (for reviews, see Giordana et al., 1989; Wolfersberger, 2000) indicated the presence of a variety of amino acid transporters with widely overlapping specificities. This pattern is consistent with the possible existence of multiple isoforms of CAATCH1-like transporter/channels representing simple splice variants (Z. Liu and W. R. Harvey, unpublished data). The only other amino acid transporter cloned from *Manduca sexta* midgut is KAAT1, which is approximately 90% identical to

CAATCH1 but exhibits a battery of properties distinct from those of CAATCH1 (Castagna et al., 1998). The effects of several KAAT1 mutations (Z. Liu and W. R. Harvey, unpublished data) were similar to those of the equivalent GAT1 mutations, with one notable exception: mutation of conserved Tyr¹⁴⁰ to Phe¹⁴⁰ (Y140F) in GAT1 led to a complete loss of GABA uptake activity and prevented the non-transportable GABA analog SKF 100330A from blocking transient currents in Na⁺-containing medium (Bismuth et al., 1997). However, the equivalent Y147F mutation in KAAT1 led to a fivefold increase in steady-state currents in Na⁺-containing medium and to changes in the substrate amino acid

specificity (Z. Liu and W. R. Harvey, unpublished data). These differential effects of amino acids on KAAT1 currents in either Na^+ - or K^+ -containing media are therefore quite different from those in CAATCH1 Y147F and WT (Fig. 6). Nonetheless, in some respects, CAATCH1 Y147F resembles KAAT1 WT (Fig. 6). Notably, both differ from CAATCH1 WT in that they demonstrate only inward currents with no current block for the test amino acid ligands. Also, in both cases, in K^+ -containing medium, methionine and leucine are the favored current-gating ligands whereas proline is relatively inert.

CAATCH1 shares several biophysical properties with carrier/channel proteins such as the serotonin transporter (SERT), the dopamine transporter (DAT), the norepinephrine transporter (NET) and the γ -aminobutyric acid transporter (GAT1). However, whereas neural ionic channels are blocked by pharmacological agents such as cocaine, CAATCH1 is a digestive epithelial channel that is blocked by nutrient amino acids, exemplified by methionine. Our previous studies with CAATCH1 (Quick and Stevens, 2001) clearly demonstrated that the transport of amino acids is thermodynamically uncoupled from cation fluxes, although amino acid substrate binding is catalyzed by Na^+ , with the ion channel aspect being differentially gated by amino acids. Thus, both neural tissue SNF members and epithelial CAATCH1 can regulate current movement by organic ligands while also transporting organic substrates. CAATCH1 may be an evolutionary transition protein at a branch point separating neurotransmitter transporters and nutrient transporters, implying the existence of undiscovered endogenous, naturally occurring inhibitors/modulators of SNF proteins in neural tissues.

Several other epithelial membrane, cation-dependent amino acid and cation-independent cotransporters displaying ionic channel activity have been cloned (Avisar et al., 2001; Broer et al., 2000; Kekuda et al., 1996; Wagner et al., 2001; Zerangue and Kavanaugh, 1996). However, $\text{B}^{0,+}$ (Sloan and Mager, 1999), CAATCH1 and KAAT1 appear to be the only epithelial members of the SNF family to be cloned to date. $\text{B}^{0,+}$ transports amino acids in a digestive/absorptive epithelium (Nakanishi et al., 2001; Sloan and Mager, 1999), but there is no evidence that any ligand gates currents in $\text{ATB}^{0,+}$ in the manner of CAATCH1. It is reasonable to hypothesize that, like the neural SNF transporter/channels and CAATCH1, pharmacological or natural antagonistic ligands that block an ionic current in $\text{ATB}^{0,+}$ may exist.

What do the channel properties of CAATCH1 and their drastic alteration by a point mutation contribute to our understanding of the physiology of insect larvae that normally possess alkaline (pH 11) midguts? This information sheds light on two processes, namely amino acid uptake in posterior midgut and lumen alkalization in anterior midgut.

In the posterior midgut of living caterpillars such as *Manduca sexta*, from which CAATCH1 was cloned, the epithelial intracellular compartment is 240 mV negative with respect to the lumen. Luminal $[\text{K}^+]$ is nearly 300 mmol l^{-1} , whereas $[\text{Na}^+]$ is less than 3 mmol l^{-1} (Dow et al., 1984; Harvey et al., 1975). Under these conditions, a flow of K^+ through the

CAATCH1 channel would be inward and would compete electrically with the transport of K^+ through a K^+ -coupled amino acid cotransporter such as KAAT1. Blockade of the channel function of CAATCH1 by certain amino acids (e.g. methionine inhibition of Na^+ conductance in WT) could conceivably prevent this competition while allowing the solute transporter aspect to remain functional in promoting nutrient amino acid uptake. With respect to gating ionic currents, CAATCH1 is unlike KAAT1 in that KAAT1 appears to operate in an uncoupled conductance mode without ligands, but then switches to a thermodynamically coupled ion-cotransport current in the presence of amino acid co-substrates (Bossi et al., 1999).

In larval anterior midgut, the channel aspect of CAATCH1 may play a role in midgut alkalization. Bicarbonate/chloride exchange has been suggested as a mechanism for accumulating bicarbonate in the lumen and accounting for alkalization up to pH 8.5 (Moffett and Cummings, 1994). However, to account for measured luminal pH values approaching 11 (Dow, 1984), a proton would have to be removed from bicarbonate to yield carbonate. H^+ flow from the lumen to the cell through the CAATCH1 channel, driven by the apical transmembrane potential of -240 mV (Dow and Harvey, 1988), would accomplish this end. We have shown that CAATCH1 is at least partially conductive to H^+ (Feldman et al., 2000), and future studies with CAATCH1 should directly address the proton conductance and its gating by amino acid ligands.

To conclude, we have shown that Phe¹⁴⁷ substitution of SNF-family-conserved Tyr¹⁴⁷ differentially alters the amino acid ligand selectivity for gating CAATCH1 channel conductance in Na^+ - and K^+ -containing media, while leaving Cl^- -independence unaffected. The removal of a hydroxyl group at this critical residue alters the association between amino acid binding and ionic conductance modulation, such that amino acids that normally inhibit the conductance become agonists of conductance. In the absence of any amino acid ligands, the Y147F mutant constitutive currents are virtually eliminated, in contrast to the freely conducting ionic channel(s) of WT. Given the tissue milieu in which CAATCH1 resides *in situ*, CAATCH1 probably plays an important physiological role in ion homeostasis *in vivo*. The results support the idea that methionine inhibition of ionic conductance in CAATCH1 parallels the phenomenon of organic solute ligand pharmacological inhibition of ionic conductance in neural SNF family members.

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