Oligomeric structure of the neutral amino acid transporters KAAT1 and CAATCH1

Elena Bossi, Andrea Soragna, Andreea Miszner, Stefano Giovannardi, Valeria Frangione, and Antonio Peres

Laboratory of Cellular and Molecular Physiology, Department of Structural and Functional Biology, and Center for Neurosciences, University of Insubria, Varese, Italy

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Bassi E, Soragna A, Miszner A, Giovannardi S, Frangione V, Peres A. Oligomeric structure of the neutral amino acid transporters KAAT1 and CAATCH1. Am J Physiol Cell Physiol 292: C1379–C1387, 2007. First published November 29, 2006; doi:10.1152/ajpcell.00473.2006—The highly homologous neutral amino acid transporters KAAT1 and CAATCH1, cloned from the midgut epithelium of the Manduca sexta larva, are members of the Na+/Cl−-dependent transporter family. Recent evidence indicates that transporters of this family form constitutive oligomers. CAATCH1 and KAAT1 give rise to specific kinds of transport-associated currents in the two proteins that represent current depending on the transported amino acid, cotransported ion, pH, and membrane voltage. Different substrates induce notably distinct transport-associated currents in the two proteins that represent useful tools in structural-functional studies. To determine whether KAAT1 and CAATCH1 form functional oligomers, we have constructed four concatameric proteins for electrophysiological analysis, consisting of one KAAT1 protein covalently linked to another KAAT1 (K-K concatamer) or to CAATCH1 (C-K concatamer) and vice versa (C-C concatamer and C-K concatamer), and eight constructs where the two transporters were linked to yellow or cyan fluorescent protein in the NH2 or COOH terminus, to determine the oligomer formation and the relative distance between the different subunits by fluorescence resonance energy transfer (FRET) analysis. Heterologous expression of the concatenated constructs and coinjection of the original proteins in different proportions allowed us to compare the characteristics of the currents to those of the oocytes expressing only the wild-type proteins. All the constructs were fully active, and their electrophysiological behavior was consistent with the activity as monomeric proteins. However, the FRET studies indicate that these transporters form oligomers in agreement with the LeuTα, atomic structure and confirm that the COOH termini of the adjacent subunits are closer than NH2 termini.

transport; oligomerization; Slc6; fret

Cloned from the midgut epithelium of the tobacco hornworm (Manduca sexta) larva, KAAT1 (AF006063) and CAATCH1 (AF013963) are two proteins of 634 and 633 amino acids, respectively, very similar to each other (90% amino acidic identity), and with 35–45% identity to the other members of the NSS, neurotransmitter sodium symporter family (35). They are able to transport different amino acid substrates depending on the cotransported ion (Na+ or K+), the pH, and the membrane voltage.

NSS members have been demonstrated with different approaches to form constitutive oligomers at the plasma membrane of living cells (6, 36, 37, 39, 40). Recent evidence (48) from the high-resolution crystal structure of a bacterial member of the family, the leucine transporter LeuTα (accession no. NP_214423) from Aquifex aeolicus, confirmed that transporters belonging to this family form oligomers. These data indicate that the dimer interface is formed by EL2, TM9, and TM12. The relevance of the last transmembrane domains is further supported by experiments on SERT and GAT1, which indicate that TM12, together with TM11, participates in multimerization (15, 22). Starting from these observations, it is now important to understand the role of this quaternary arrangement and to find the possible determinants involved in the contact sites that stabilize the multimeric structure in the different members of the family.

Although it is reasonably established that NSS members form oligomeric complexes, no conclusive information are available about their functional role. Oligomerization appears to be important for the correct targeting of the transporters to the membrane (6), but only limited experimental evidence for human (h)SERT (17), hNET, and hDAT (11, 19, 44, 45) indicates a functional role for this three-dimensional organization. Furthermore, a countertransport model for monoamine transporters in which the conformational state of one monomer affects the functionality of the other has been recently proposed (38). However, recent results in our laboratory on GAT1 (41), obtained by combining electrophysiology with fluorescent resonance energy transfer (FRET) microscopy, indicated that although structural oligomerization of rat GAT1 occurs, the single subunits operate individually.

Experiments aimed to determine the subunit stoichiometry of transporter complexes usually include cross-linking analysis (11, 29, 44) or coprecipitation assays (14, 17, 20) and, more recently, FRET analysis (32, 33, 36, 37, 41, 43). The use of biochemical approaches to study proteins overexpressed in heterologous systems may give ambiguous results because of unnatural aggregation of intracellular proteins. A concatamer strategy also may be useful in investigating oligomerization. This method was first applied to voltage-gated K+ channels (13). Coinjections or linked constructs of cDNA coding for different subunits have been successfully used together with electrophysiology to identify the stoichiometry and the quaternary structure of different electrogenic membrane proteins. Several aspects have been studied with this method, from structure to more subtle features related to the molecular mechanisms of gating and activation (8, 12, 16, 18, 23–26, 30, 46, 49). Many other membrane proteins, such as aquaporins (27), the sodium pump (5), ligand-gated ion channels (28), the...
lactose permease (34), and the renal type IIa Na\(^+\)/P\(_i\) transporter (21), also have been studied using the concatamer approach.

In the present study we used the analysis of electrophysiological properties of concatenated and coinjected constructs together with FRET experiments to investigate the possible relevance of oligomerization in the functioning of two neutral amino acid transporters belonging to the NSS family. To this purpose, the two transporters cloned from *M. sexta* represent valid investigation tools. CAATCH1 and KAAT1, heterologously expressed in *Xenopus laevis* oocytes, give rise to transport-associated currents, which are clearly distinguishable between the two proteins. Differences in amplitude, kinetics, and voltage-dependence have been observed, as well as differences in substrate selectivity, which were confirmed by amino acid uptake assays (1, 3, 4, 7, 31, 42).

To also determine whether KAAT1 and CAATCH1 form functional oligomers, we constructed four concatameric protein constructs consisting of one KAAT1 protein covalently linked to another KAAT1 (K-K concatamer) or to CAATCH1 (K-C concatamer), and vice versa (C-C concatamer and C-K concatamer). Eight constructs in which the two transporters were linked to yellow (YFP) or cyan fluorescent protein (CFP), in either the NH\(_2\) or COOH terminus, were also prepared to investigate the oligomer formation and estimate the relative distance between the different subunits by FRET analysis.

**MATERIALS AND METHODS**

**Construct preparation.** For FRET analysis, KAAT1 and CAATCH1 were subcloned in pECFP-N1 and pEYFP-N1 (Clontech, BD Bioscience, Milan, Italy) between *Hind*III and *Hin*II; these restriction sites were inserted by point mutation (QuickChange site-directed mutagenesis kit; Stratagene) before the ATG site of transporters (NH\(_2\)-) (5'-CC TAA ACA CAT TGC TAG AGT GAC GTG GAC AAA ATG AAT GAC GC C-3' for KAAT1; 5'-A TAC ACC TAA ACA CAT TGC TAG CGT GAT ATT GTG GAC AAA ATG AAT GAC GC C-3' for CAATCH1) and on the stop codon (HindIII) (5'-CGG TCT T GCC TAC AGG CTT AAT ACT ATT CTA TTA ATT AAT AC-3' for KAAT1; 5'-C GGG TGC TTA CAG GCG TAA TAA TTA GAT ATT AAA ATA TG-3' for CAATCH1), in frame with the ATG site of enhanced CFP (ECFP) and EYFP. Fluorescent proteins are in this way fused at their amino termini. A second *Hind*III site was inserted by point mutation at the COOH terminus of KAAT1 and CAATCH1. To link the fluorescent protein at the NH\(_2\) terminus of KAAT1 and CAATCH1, we subcloned the two transporters in pEFP-N3 and pEYFP-N3 (Clontech) between the *Hind*III restriction site, inserted by point mutation on the ATG site of transporters (5'-TC TTT TTT ATT ATT ATT TTT TTT CTT CTA AAG CTT TTC ACC ACC AGT AAT GAC GCC C-3', and the BamHI site.

For electrophysiological investigations, the two transporters were concatenated in homo- and heteroconcatameric constructs by a linker peptide (LST). Briefly the second cDNA coding for KAAT1 or CAATCH1, mutated to insert an *Hind*III site before the ATG (5'-CGG TCT TGCC TAC AGG CTT AAT ACT ATT CTA TTA ATT AAT AC-3' for KAAT1; 5'-CGG TCT TGCC TAC AGG CTT AAT ACT ATT CTA TTA ATT AAT AC-3' for CAATCH1), was inserted in KAAT1-pAMV-PA or CAATCH1-pAMV-PA between two *Hind*III sites, one created by point mutation, to eliminate the stop codon of the transporters (5'-CGG TCT TTT TTT ATT ATT ATT TTT TTT CTT CTA AAG CTT TTC ACC ACC AGT AAT GAC GCC C-3', and the BamHI site.

All constructs were verified by sequencing (MWG Biotech, Sequencing Service, Ebersberg, Germany). All enzymes were supplied by Promega Italia (Milan, Italy).

**cRNA preparation and *Xenopus laevis* oocyte expression.** The experimental procedure has been described in detail elsewhere (2). The cDNAs encoding the original cotransporters and the concatenated construct were linearized with *Nco*I. cRNAs were in vitro synthesized in the presence of Cap Analog and 200 units of T7 RNA polymerase.

*X. laevis* frogs were anesthetized in 0.10% (wt/vol) MS222 (tricaine methanesulfonate) solution in tap water; portions of ovary were removed through an incision on the abdomen, and the frogs were humanely killed after the collection. The oocytes were treated with 1 mg/ml collagenase (type IA; Sigma) in Ca\(^2+\)/free ND-96 for at least 1 h at 18°C. Healthy looking stage V and VI oocytes were selected and injected with 12.5 ng of the appropriate cRNA in 50 nl of water, using a manual microinjection system (Drummond). In coinjection experiments, oocytes were injected with 6.25 ng of each cRNA for a 1:1 ratio and with 3.125 and 9.375 ng for a 3:1 ratio. The oocytes were incubated at 18°C for 3–4 days in ND solution (ND-96 solution: 96 mM Na\(_2\)SO\(_4\), 1.8 mM KCl, 1.8 mM CaCl\(_2\), 1.1 mM MgCl\(_2\)) and single HEPES supplemented with 50 μg/ml gentamicin and 2.5 mM Na-pyruvate at pH 7.6 before electrophysiological studies were performed.

**Physiology and data analysis.** A two-microelectrode voltage clamp was used to perform electrophysiological experiments (Geneclamp 500B; Axon Instruments, Union City, CA). The holding potential was kept at −60 mV, and the typical protocol consisted of 200-ms voltage pulses spanning the range from −160 to +20 mV in 20-mV steps. Two pulses were averaged at each potential; signals were filtered at 1 kHz and sampled at 2 kHz. Experimental protocols, data acquisition, and analysis were done using the pCLAMP 8 software (Axon Instruments) (9).

**Cell culture and transfection.** tsA201 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μM penicillin, and 100 μg/ml streptomycin and were kept in a 5% CO\(_2\) humidified atmosphere at 37°C. Cells were plated in 35-mm dishes (1.4 × 10\(^5\) cells/dish) and transfected with 1 μg of total DNA per dish using Lipofectamine-2000 (Invitrogen, Milan, Italy). In single transfection experiments, 400 ng of plasmid were used. In cotransfection experiments, the different constructs were always used in a ratio of 1:1, at 400 ng each. An empty vector (pcDNA3.1; Invitrogen) was used to normalize the total amount of transfected plasmid. Cells were replated on round glass coverslips 24 h after the transfection and used 24 h after the replating procedure.

**Fluorescence microscopy and image acquisition.** tsA201 cells were observed 48 h after transfection. Fluorescence images were acquired with a Retiga 2000R cooled charge-coupled device (CCD) camera (QImaging, Burnaby, BC, Canada) mounted on a Zeiss Axiosvert IM35 inverted microscope and through a Zeiss fluor × 40 oil-immersion objective. Excitation was obtained by a 150-W xenon arc lamp coupled to a filter wheel (CAIRN Research, Faversham, UK) delivering the light to the microscope through a light guide. Excitation filters were 430/25 nm for ECFP and 500/20 nm for EYFP; a 50% neutral density filter was used on the 500-nm excitation optic corridor. A double dichroic filter (68002 BS) was placed on the excitation light path to reflect both excitation wavelengths and pass both emissions. Emitted fluorescence reached an optospilt image splitter (CAIRN Research) fitted with a 495DCLP beam splitter and two emission filters at 470/30 and 535/30 nm for ECFP and EYFP, respectively. With this configuration, donor, acceptor, and FRET images could be obtained by changing only the excitation filters. The image splitter allowed us, for each excitation wavelength, to capture the two images (through the 470- and 535-nm emission filters) in the same frame on the CCD chip. To minimize photobleaching effects, we interposed an electronically driven shutter between the excitation filters and the dichroic mirror so that the cells were only excited for the time
necessary to acquire the images. Under these conditions, significant photobleaching of YFP occurred only after long-lasting exposures (>30 min). Images of the same set were acquired under identical conditions. All filters were obtained from Chroma Technology (Rockingham, VT).

Images analysis and FRET calculation. Images were analyzed with Image Pro Plus software (Media Cybernetics, Silver Spring, MD). NFRET images were constructed using the three-filter set method, according to the following analytical procedure introduced by Xia and Liu (47). Relative background images were subtracted (obtained by acquiring images in an area of the sample without cells, with the respective excitation configuration and CCD integration time). Independent thresholding on the images was necessary to avoid image-processing artifacts. Donor and acceptor fluorescence bleed-through were calculated from cells transfected with KAAT1-ECFP and KAAT1-EYFP alone, according to Gordon et al. (10), and were subtracted from the FRET image to obtain the net FRET (nF) image; nF normalization was then performed by dividing the nF image by the square root of the donor/acceptor product (47);

$$NFRET = \frac{I_{FRET} - I_{YFP}^a - I_{CFP}^b}{\sqrt{I_{YFP} I_{CFP}}}$$

where NFRET is the normalized FRET value and I_{FRET}, I_{YFP}, and I_{CFP} are the intensities in each area of interest detected using FRET, EYFP, and ECFP filter sets, respectively. The constants a (I_{FRET}/I_{YFP}) and b (I_{FRET}/I_{CFP}) represent correction coefficients to account for KAAT1-ECFP and KAAT1-EYFP bleed-through, respectively, under the FRET filter combination, calculated when only the donor (b) or the acceptor (a) were present. NFRET calculated by cotransflecting KAAT1-ECFP and 5HT1A-EYFP (generous gift of Dr. E Ponimaskin, Göttingen University, Germany) was used as negative control. Data are presented as means ± SE. Experimental setup and analysis procedures have been optimized using a CFP-YFP tandem (generous gift of Dr. H.H. Sitte, Medical University of Vienna, Vienna, Austria), which was constructed as a positive control for FRET imaging (36), and consequently, ECFP and EYFP bleed-through coefficients were calculated in cells transfected only with ECFP and EYFP, respectively.

Solutions. In electrophysiological experiments, the external control solution had the following composition (in mM): 98 NaCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES free acid; in the other solutions, NaCl was replaced by KCl or tetramethylammonium (TMA)-Cl. The pH was adjusted to 7.6 by adding the corresponding hydroxide for each alkali ion and TMAOH for TMA solution. Amino acids (leucine, threonine, proline) at 500 μM were added to induce transport-associated currents. Solutions were superfused by gravity onto the oocyte by a pipette tip placed very close (1–2 mm) to the cell. In fluorescence experiments, the extracellular control solution contained (in mM) 135 NaCl, 4 KCl, 1 MgCl2, 2 CaCl2, 6 glucose, and 10 HEPES-NaOH at pH 7.35.

Fig. 1. A: representative traces of membrane currents recorded at a holding potential (Vh) of –60 mV in 6 representative oocytes exposed to leucine (Leu), threonine (Thr), and proline (Pro) at 500 μM, applied in a solution in which the main cation was Na+. B: schematic representation of the transport protein wild type (wt) and concatamers (solid, KAAT1; shaded, CAATCH). From top to bottom: KAAT1 wt, KAAT1_KAAT1 (K-K concatamer), KAAT1_CAATCH1 (K-C concatamer), CAATCH1_KAAT1 (C-K concatamer), CAATCH1_CAATCH1 (C-C concatamer), and CAATCH1 wt.

AJP-Cell Physiol • VOL 292 • APRIL 2007 • www.ajpcell.org
RESULTS

Electrophysiology. The activity of the constructs was tested by measuring the response to threonine, a good substrate for both KAAT1 and CAATCH1. All constructs were able to transport this amino acid and to elicit transport-associated currents in Na\(^+\) and in K\(^+\), although variability in the potency of the response among batches and concatamers was observed. The substrate selectivity order, or the reversal in leucine-induced current direction, allowed us to identify an unequivocal profile for the two native transporters. To distinguish between the cooperative and the independent functionality of these proteins, we investigated the activity of forced dimers, comparing it with that of the wild-type proteins and the coinjections. A strict relationship, dependent on the number of the oligomer-forming units, between the current induced by leucine and the proportion of the two cRNA injected or concatenated, is expected. That is, if an oligomer containing one or more CAATCH1 subunits shows a full block of the leucine transport-associated current, this is assumed to indicate a functional interaction among subunits. Although strict quantitatively reliable results may not be expected from the independent coinjection of cRNA because of the uncertainties in the expression efficiencies of the two transporters, the use of the concatameric constructs circumvents this problem by forcing equal expression levels of the two proteins, allowing the possible function and structural oligomerization to be tested by electrophysiological and FRET experiments.

Transport-associated currents in the presence of Na\(^+\). The behavior of KAAT1, CAATCH1, the homoconcatamers K-K (KAAT1_KAAT1) and C-C (CAATCH1_CAATCH1) and the

![Fig. 2. Current-voltage relationships from wild-type and concatenated transporters, as indicated, in the presence of Na\(^+\). A–H: data were obtained by subtracting the traces in the absence from those in the presence of the indicated substrate (500 \(\mu\)M). The data were normalized to the value in proline at -160 mV for each oocyte before averaging and are presented as means ± SE from 6–10 oocytes in each group. Coinjection curves were obtained from oocytes injected with equal amounts of cRNA of KAAT1 and CAATCH1 (E) and the mean of the sum of 4 oocytes injected with KAAT1 and 4 oocytes injected with CAATCH1 was normalized as described.]

AJP-Cell Physiol • VOL 292 • APRIL 2007 • www.ajpcell.org
heteroconcatamers C-K (CAATCH1_KAAT1) and K-C (KAAT_CAATCH1) was initially investigated by measuring the transport-associated currents at a holding potential of \(-60\) mV when a Na\(^+\) solution containing 500 \(\mu\)M of one of the three amino acids (leucine, threonine, or proline) was applied. The results are shown in Fig. 1, where it is possible to compare the electrophysiological profiles of the wild-type proteins with those of the forced constructs. On the right side of the traces, a schematic representation of the corresponding constructs is illustrated.

At the top of Fig. 1, in KAAT1 wild-type and in K-K concatamer, the selectivity profiles of the currents induced by the indicated amino acid may be superimposed, i.e., leucine elicited an inward current, threonine induced the maximal current, and proline at this potential gave rise to a response that is \(\sim 50\%\) of the threonine-induced current. Conversely, in the bottom two traces of Fig. 1A, in CAATCH1 and in its homoconcatamer C-C, leucine reduced the holding current, interpreted as a block of the sodium leakage current (7), whereas the threonine-induced current was \(\sim 80\%\) of the maximal current elicited by proline. In the middle of Fig. 1A, the behavior of the heteroconcatamers K-C and C-K is illustrated. Their selectivity profiles are similar but do not correspond to any one of the original proteins: whereas leucine induced very small inward currents, the currents associated with the transport of the other two amino acids at this voltage were quite similar. Figure 1 also shows that the current amplitudes among the recordings are different: in addition to the individual variability among oocytes, the currents generated by the concatenated constructs are smaller than those from the wild-type proteins in oocytes from the same batch. This effect was already observed in electrophysiological experiments with concatenated transport proteins (21) and may be due to impaired translation of constructs of larger dimensions.

Fig. 3. Current-voltage relationships from wild-type and concatenated transporters and coinjection at 1:1, as indicated, in the presence of K\(^+\). A–H: data were obtained by subtracting the traces in the absence from those in the presence of the indicated substrate (500 \(\mu\)M). The data were normalized to the value in threonine at \(-160\) mV for each oocyte before averaging and are presented as means \(\pm SE\) from 4–6 oocytes in each group. Coinjection curves were obtained from oocytes injected with equal amounts of cRNA of KAAT1 and CAATCH1 (E), and the mean of the sum of 4 oocytes injected with KAAT1 and 4 oocytes injected with CAATCH1 was normalized as described.
Current-voltage relationships. The behavior at a single membrane potential, however, may be misleading, because the transport-associated currents elicited by different amino acids have characteristic voltage dependencies. Following these initial observations, the investigation was extended to other voltages, to the currents in the presence of K/H11001, and also including oocytes coinjected with cRNA coding for the wild types in different proportions. Figures 2 and 3 show the current-voltage relationships derived from experiments in the presence of Na/H11001 and K/H11001, respectively, with the indicated amino acids for the wild-type KAAT1 and K-CATCH1, for the concatenated construct KK, KC, CC, and CK, and with 1:1 coinjection; furthermore, the calculated sum of the currents recorded from the wild-type proteins is also shown. The data for the leucine current at $-160$ mV, from the other coinjection proportions, are shown in Fig. 4.

The current-voltage graphs were obtained by subtracting the current in Na$^+$ or K$^+$ solution from the current recorded in the presence of the amino acid in the same ionic solution. For each condition, the data were collected from at least four batches of oocytes and normalized to the value at $-160$ mV in proline for Na$^+$ and in threonine for K$^+$ recordings.

Confirming the results at $-60$ mV, it is possible to observe that the homoconcatenated constructs K-K and C-C show the same behavior as the wild-type proteins, in both Na$^+$ (Fig. 2, A, B, G, and H) and in K$^+$ (Fig. 3, A, B, G, and H), maintaining the most important characteristics at all membrane potentials. In the presence of Na$^+$, leucine generates inward currents $-20$ to $-25\%$ of the maximal current recorded in threonine for KAAT1 and K-K (Fig. 2, A and B), whereas for CATCH1 as well as the concatamer C-C, leucine blocks the leakage current producing an apparent outward current, and the maximal trans-
port current is associated with the transport of proline (Fig. 2, G and H).

In the presence of K⁺, in KAAT1 and the concatamer K-K (Fig. 3, A and B), the maximal current is associated with the leucine transport, whereas the proline-induced current is ten times less at −160 mV. In CAATCH1 and its homoconcatamer C-C, leucine blocks the potassium leakage current, and the current associated with the proline transport is about one-third of the maximal current obtained in the presence of threonine at −160 mV (Fig. 3, G and H).

In the presence of K⁺/H11001, in KAAT1 and the concatamer K-K (Fig. 3, A and B), the maximal current is associated with the leucine transport, whereas the proline-induced current is ten times less at −160 mV. In CAATCH1 and its homoconcatamer C-C, leucine blocks the potassium leakage current, and the current associated with the proline transport is about one-third of the maximal current obtained in the presence of threonine at −160 mV (Fig. 3, G and H).

In the middle of Figs. 2 and 3, where the heteroconcatamers (C and D), the coinjection data (E), and the calculated sum of KAAT1 and CAATCH1 independently injected (F) are shown, it is possible to notice that in every case the current-voltage curves are very similar at all potentials tested, but again they do not correspond to those obtained from the wild-type and homoconcatenated proteins.

In detail, comparing the current-voltage plots of the hetero-complexes with the KAAT1 and K-K currents in the presence of Na⁺, the currents associated with the transport of threonine and proline can be superimposed at all potentials, whereas the leucine current is reduced to ~15% of the maximal current (Fig. 2, C–F). Greater changes are visible in K⁺, where the reduction of the leucine transport-associated current is more marked. The heteroconcatameric transporters K-C (Fig. 3C) and C-K (Fig. 3D) again showed the same behavior of the coinjection, which is also similar to the sum of the currents measured in oocytes separately expressing the two proteins, as indicated by the fact that in these current-voltage relationships, leucine caused a 50% inhibition of the KAAT1 wild-type current. Threonine becomes the most potent substrate in the oocytes expressing CAATCH1 and KAAT1 either as forced dimer or as independent coexpression as in those expressing CAATCH1 alone. Finally, proline induced an inward current at negative potentials in all subunit compositions.

The relative leucine currents at −160 mV (I_{Leu}/I_{Pro} in the presence of Na⁺ and I_{Leu}/I_{Thr} in the presence of K⁺) for wild-type, homo-, and heteroconcatamers and from different ratios of coinjection are shown in Fig. 4. The leucine-induced currents are generally in good agreement with the percentage of injected CAATCH1, suggesting an independent functioning of each subunit.

**Fluorescence microscopy and FRET analysis.** To validate the independent functionality of the subunits revealed by electrophysiology, we needed to determine the oligomerization of KAAT1 and CAATCH1 and also whether they may form heteroligomers. We therefore tagged both proteins with CFP and YFP at either the NH₂ or COOH termini and tested all the constructs for FRET on an epifluorescence setup equipped with a CCD camera. Independent transfection of tsA201 cells with

<table>
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<th>Cotransfection</th>
<th>N_{sample}</th>
<th>N_{FRET}</th>
<th>Scheme</th>
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<td>Caatch1_CFP Caath1_YFP</td>
<td>12</td>
<td>0.79 ± 0.01*</td>
<td>![C/Y](shaded, KAAT1; solid, CAATCH1).</td>
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<td>0.79 ± 0.02*</td>
<td>![C/Y](shaded, KAAT1; solid, CAATCH1).</td>
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<tr>
<td>CFP_Kaat1 YFP_Kaat1</td>
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<td>0.35 ± 0.01*</td>
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<tr>
<td>CFP_Kaat1 Kaat1_YFP</td>
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<td>0.87 ± 0.02</td>
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<td>0.02 ± 0.01</td>
<td>![C/Y](shaded, KAAT1; solid, CAATCH1).</td>
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Fig. 6. N_{FRET} values were calculated as explained in MATERIALS AND METHODS. All the mean N_{FRET} values are statistically significant (Student’s t-test, *P < 0.05) compared with the control (5HT1A_YFP KAAT_CFP). Schematic representations show the transport linked to yellow (Y) or cyan (C) fluorescent protein (shaded, KAAT1; solid, CAATCH1).
the different constructs gave rise to fluorescence signals mainly localized at the plasma membrane (not shown). The membrane distribution, however, may be visualized by looking at the CFP and YFP filter channels in Fig. 5, an example of the FRET experiment. As a further control, all the constructs also were tested electrophysiologically in patch-clamp experiments (data not shown): both KAAT1 and CAATCH1 with the fluorescent proteins linked at either the NH2 or the COOH termini behaved in a wild-type fashion, indicating that the presence of the CYP/YFP does not influence the ability of the two proteins to transport neutral amino acids. Having demonstrated the full functionality of the tagged transporters, we next assessed whether KAAT1 and CAATCH1 exist as oligomers.

We tested all the combinations, as reported in Fig. 6, i.e., with both fluorescent proteins linked at either the COOH or the NH2 termini and also with one fluorescent protein at the NH2 terminus while the other one was attached at the COOH terminus. The N_fRET values reported in Fig. 6 show that both KAAT1 and CAATCH1 exist as oligomers, since the signal is significantly higher than that of the two negative controls used: the cotransfections of the CFP and YFP and the cotransfection of two proteins believed not to interact (the serotonin receptor 5-HT1A and KAAT1). Furthermore, that higher N_fRET values were obtained when the transporters were tagged at the COOH termini, indicating that these regions of the subunits are closer to each other than the NH2 termini, in agreement with the three-dimensional structure of LeuT_Aa (48).

We next investigated whether KAAT1 and CAATCH1 may assemble in a heteroligomeric complex. For this experiment, we used the COOH-tagged transporters because of the high value of N_fRET achieved in this condition.

Cotransfection of KAAT1-YFP and CAATCH1-CFP (and also CAATCH1-YFP and KAAT1-CFP) revealed high N_fRET values (not statistically different from the COOH-terminus tagged transporters forming homoligomers), therefore indicating the ability of the two proteins to form mixed oligomers in addition to their ability to exist as complexes composed of identical subunits.

DISCUSSION

This study investigates the formation of structural and functional units in the two neutral amino acid transporters KAAT1 and CAATCH1, which, because of their electrophysiological characteristics, appear well suited to this purpose. In fact, leucine is a transported substrate for the first but acts as a blocker of the latter (7, 31, 42). To analyze the behavior of heteroligomers possibly generated by coinjection or by forced constructs, it is necessary to assume that the lack of activity of a single subunit should affect the transport capability of the entire functional complex (effect of negative dominance) and that the subunits of the two proteins aggregate randomly (21, 26).

With these assumptions, and considering that the structure is probably a tetramer (a dimer of dimer) as suggested by the FRET data and by the crystal structure of LeuT_Aa (48), the leucine current is expected to be ~25% of KAAT1 wild type when 50% of KAAT1 and 50% of CAATCH1 are expressed in the oocyte membrane together as coinjection or concatamers. The observed 50% loss of function (Figs. 2–4) for the 1:1 ratio is instead consistent with the existence of functionally independent transport pathways in each subunit.

The superimposability between the current-voltage curves obtained by coinjecting the two wild-type proteins in equal proportions, although subject to the assumption of equal expression efficiency of the two cRNAs, and by injecting the forced heteroligomers, clearly points to an independent behavior of the two components. The FRET analysis results tell us that homo- and heteroligomerization may occur between KAAT1 and CAATCH1. Furthermore, for all the cotransfections, higher N_fRET values were obtained when the fluorescent proteins were positioned at the COOH terminus. N_fRET gradually decreases with the displacement of one or both the fluorescent proteins to the NH2-terminal positions of the transporters. The value of N_fRET, independently of the subunit composition (i.e., in homo- and heteroligomers), suggests the closeness of the COOH termini of the different subunits in the three-dimensional organization, in very good agreement with the recent structural model of LeuT_Aa (48).

The negative control obtained cotransfecting two plasma membrane proteins not believed to interact (KAAT1 and 5HT1A) shows, however, relatively higher N_fRET values compared with the cotransfection of the fluorescent proteins alone (see Fig. 6). This result is in agreement with previous observations (10, 47) and may be explained as a possible protein-protein weak interaction between the transporter and the serotonin receptor or, most likely, to transient fluorophore interaction by diffusion, a side effect of the overexpression of the two proteins, in this case further enhanced by the structural constraint of the membrane localization.

On the whole, the functional and FRET results reported in this study indicate that the M. sexta neutral amino acid transporters KAAT1 and CAATCH1 form structural oligomers in which, however, each subunit maintains its own independent transport activity, similar to the behavior observed in other transporters of the NSS superfamily (41).

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Present addresses: A. Soragna, Laboratory of Membrane Physiology, Institute of General Physiology and Biological Chemistry “G. Esposito,” University of Milan, Via Trentacoste 2, 20134 Milan, Italy; V. Frangione, Dipartimento di Scienze Cliniche e Biologiche, University of Insubria, Via Dunant 5, 21100 Varese, Italy.

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