Substrate-induced changes in the density of peptide transporter PEPT1 expressed in Xenopus oocytes

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Mertl M, Daniel H, Kottra G. Substrate-induced changes in the density of peptide transporter PEPT1 expressed in Xenopus oocytes. Am J Physiol Cell Physiol 295: C1332–C1343, 2008. First published September 17, 2008; doi:10.1152/ajpcell.00241.2008.—The adaptation of the capacity of the intestinal peptide transporter PEPT1 to varying substrate concentrations may be important with respect to its role in providing bulk quantities of amino acids for growth, development, and other nutritional needs. In the present study, we describe a novel phenomenon of the regulation of PEPT1 in the Xenopus oocyte system. Using electrophysiological and immunofluorescence methods, we demonstrate that a prolonged substrate exposure of rabbit PEPT1 (rPEPT1) caused a retrieval of transporters from the membrane. Capacitance as a measure of membrane surface area was increased in parallel with the increase in rPEPT1-mediated transport currents with a slope of ~5% of basal surface per 100 nA. Exposure of oocytes to the model peptide Gly-L-Gln for 2 h resulted in a decrease in maximal transport currents with no change of membrane capacitance. However, exposure to substrate for 5 h decreased transport currents but also, in parallel, surface area by endocytotic removal of transporter proteins from the surface. The reduction of the surface expression of rPEPT1 was confirmed by presteady-state current measurements and immunofluorescent labeling of rPEPT1. A similar simultaneous decrease of current and surface area was also observed when endocytosis was stimulated by the activation of PKC. Cytochalasin D inhibited all changes evoked by either dipeptide or PKC stimulation, whereas the PKC-selective inhibitor bisindolylmaleimide only affected PKC-stimulated endocytotic processes but not substrate-dependent retrieval of rPEPT1. Coexpression experiments with human Na\(^+\)-glucose transporter 1 (hSGLT1) revealed that substrate exposure selectively affected PEPT1 but not the activity of hSGLT1.

electrophysiology; dipeptide; presteady-state currents; surface expression; endocytosis

THE CAPACITY OF A TRANSPORTER PROTEIN can be regulated either by variation of its membrane abundance or variation of its kinetic properties (e.g., turnover rate, substrate affinity, and ion dependency). Transporter density in the membrane usually involves exocytosis or endocytosis of the proteins delivered to or retrieved from the plasma membrane (26, 29, 33, 53), whereas the kinetic properties can be regulated either by direct phosphorylation and dephosphorylation (8, 28) or by the association of cytosolic regulatory proteins (46). A number of observations have suggested that substrates can, via nonkinetic mechanisms, also regulate the activity of their transporters, but the underlying signaling pathways that cause such substrate-dependent protein regulation are mostly unknown. Such transport regulation processes have even been observed in heterologous expression systems. Xenopus laevis oocytes exposed to a mixture of 20 amino acids at concentrations approximating those in Xenopus plasma (43) caused a downregulation of Na\(^+\)-dependent amino acid transporters (systems B\(^0\), X\(_{AG}\)), whereas the Na\(^+\)-independent transporters for glutamate, glutamine, and glycine displayed a modulation of activity in the opposite direction. The authors suggested that this regulation could prevent amino acid-induced oxidative stress in oocytes by specifically increasing the uptake of amino acids that serve as precursors for glutathione biosynthesis. This regulation of amino acid transport processes has been shown to be independent of membrane potential effects and did not involve changes in mRNA levels or changes in PKC activity status (43).

Another example of substrate-mediated transporter regulation is the neuronal dopamine transporter (DAT). When DAT is expressed in HEK-293 cells, a prolonged exposure of cells to the substrate amphetamine caused a retrieval of human DAT protein from the plasma membrane into the cytosol, thereby reducing total transport capacity (22, 36). Similarly, in Xenopus oocytes expressing DAT, even brief repeated exposures to micromolar concentrations of dopamine or amphetamine reduced DAT-mediated currents markedly. This functional downregulation was attenuated by the PKC inhibitor bisindolylmaleimide I (Bis), suggesting that the substrate effect was mediated via PKC and included endocytotic mechanisms (17, 55).

The intestinal peptide transporter PEPT1 represents the prototype mammalian di-/tripeptide transporter and mediates electrogenic proton-coupled uphill transport of peptides and peptidomimetics in the small intestine and renal tubule. Whereas the transport properties of PEPT1 have been studied in detail (for a review, see Ref. 11), little is known about the cellular mechanisms involved in the regulation of its activity (1, 6, 42). Although the intracellular domains of the rabbit PEPT1 (rPEPT1) protein possess canonical PKA and PKC consensus sites, evidence that these sites undergo phosphorylation and dephosphorylation is lacking.

The aim of the present study was to characterize a phenomenon observed in preliminary experiments that suggested that a prolonged exposure of cells that express PEPT1 to dipeptide substrates affects the transport capacity of the protein. In Xenopus oocytes expressing rPEPT1, transporter velocity, kinetics, and membrane protein density as well as membrane capacitance (\(C_m\)) were measured after exposure of cells for prolonged periods to substrates and inhibitors.

MATERIALS AND METHODS

Expression of rPEPT1 in Oocytes

X. laevis maintenance and oocyte harvest procedures were approved by the local authority for animal care in research (Regierung von Oberbayern, approval no. 211-2531.3-9/99). Oocytes were col-

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lected under anesthesia (immersion in a solution of 0.7 g/l 3-amino-benzoic acid ethyl ester, Sigma) from frogs that had been killed with an anesthetic overdose after the final collection. Oocytes were treated with 1.75 mg/ml collagenase for 90 min and were separated manually thereafter. They were incubated in Barth solution containing (in mM) 88 NaCl, 1 KCl, 0.8 MgSO4, 0.4 CaCl2, 0.3 Ca(NO3)2, 2.4 NaHCO3, and 10 HEPES (pH 7.5) at 17°C overnight. Thereafter, stage V/VI oocytes were injected with 14 ng rPEPT1 cRNA or 23 ng human Na+-glucose transporter 1 (hSGLT1) cRNA and incubated for 3–4 days at 17°C. For coexpression experiments, a mixture containing 13.5 ng of each cRNA was injected in a 27-nl volume.

Electrophysiology

For two-electrode voltage-clamp (TEVC) experiments, oocytes were placed in an open chamber and continuously superfused with Barth solution or with solutions containing the substrates to be studied. In some experiments, the oocyte diameter was measured before the electrical measurements were started by means of an ocular micrometer (accuracy about ±0.03 mm). Oocytes were voltage-clamped, and transport currents were measured at −60 mV using a TEC-05 amplifier and CellWorks software (npi electronic, Tamm, Germany). Current-voltage (I-V) relations were measured in the potential range of −160 to +80 mV, and the current generated by the peptide transport at a given membrane potential was calculated as the difference of the currents measured in the presence and absence of substrate. The kinetic parameters of transport at −60 mV membrane potential and at an extracellular pH of 7.5, Km (in mM) and maximal current (Imax; in nA), were calculated from least-square fits of at least three data points to the Michaelis-Menten equation.

Capacitance measurements. Cm values were measured by a method adapted from Ref. 37. In brief, the capacitance of voltage-clamped oocytes was calculated from current jumps elicited by membrane potential ramps. The potential function was composed of two asymptotically depolarizing and hyperpolarizing ramp pairs: starting at a holding value of −60 mV, the potential was changed to −40 mV (slope: 2 mV/ms) and then returned to the holding value (slope: −1 mV/ms). After a short delay, a hyperpolarizing ramp pair with a peak of −80 mV and otherwise identical parameters was initiated. As discussed in detail by Schmitt and Koeppel (37), the use of paired ramps has the advantage to yield more accurate capacitance estimates even when the I-V relation is nonlinear in the potential range used. The current signal evoked by the above potential ramp has a resistive component (proportional to the voltage signal) and a capacitive component (square wave jump proportional to the voltage slope). Cm was calculated from current jumps at the beginning and end of each potential ramp as follows: Cm = Imax × dt/dV, where Imax is the current jump and dt/dV is the inverse slope of the membrane potential. Due to the nonideal voltage-clamp conditions and the low-pass characteristics of the recording setup (filter setting: 1 kHz), the recorded signal was delayed with respect to the idealized signal (estimated time constant of the monoeponential signal: 1 ms). To calculate Cm, the theoretical height of the current jumps was calculated by fitting linear functions to the portions of the current signal sufficiently far away from the transition region and extrapolating these functions to the end point of the ramp. In accordance with the time course of potential, six current jumps were fitted by macoroutines written in Excel, and the mean value of the six calculated capacitances (usually not differing more than ±3%) yielded Cm. To avoid any interference from the presteady-state current component that is present in rPEPT1-expressing oocytes (27) (and see below), Cm was measured in the presence of nearly saturating substrate concentrations (also see results).

Prolonged Incubation of Oocytes

To investigate the effects of a prolonged exposure to substrate or by a PKC stimulation on transporter regulation, oocytes were placed in the perfusion chamber and carefully impaled with fine-tipped microelectrodes, and the Cm and potential-dependent transport currents were measured as described above. After −5 min of impalement, the microelectrodes were removed again, and oocytes were transferred into individual wells of 96-well dishes, where they were incubated either in Barth solution (control experiments) or in Barth solution containing either the dipeptide GQ (5 mM) or phorbol-12,13-dibutyrate (PDBu; 1 µM) with or without the selective PKC inhibitor Bis (5 µM) or the microfilm-disrupting drug cytochalasin D (CD; 10 µM) as well as other compounds as noted in the text. After selected incubation times, oocytes were placed back in the perfusion chamber, and the same parameters as before incubation were measured again.

Epitope Tagging

To allow optical measurements of the surface expression of the transporter proteins, a Flag epitope was inserted into the large extracellular loop between transmembrane domains 9 and 10 of rPEPT1. To this end, a Bg/II restriction site was introduced at position 543 by changing glutamate to arginine. The oligonucleotide encoding for the transporter proteins, a Flag epitope was inserted into the large extracellular loop between transmembrane domains 9 and 10 of rPEPT1. To this end, a Bg/II restriction site was introduced at position 543 by changing glutamate to arginine. The oligonucleotide encoding for the Flag epitope was then ligated into the Bg/II site. Surface expression of the tagged transporter after the experimental maneuver was detected...
using the monoclonal primary antibody anti-Flag M2 (Sigma F3165, 10 μg/ml, incubation for 60 min at room temperature) and goat anti-mouse IgG secondary antibody labeled with Alexa fluor 488 (Molecular Probes A-11001, 10 μg/ml, incubation for 45 min). Fluorescence was measured with a Leica TCS SP2 confocal microscope (excitation wavelength: 488 nm and detection wavelength: 500–535 nm). Due to the uneven distribution of transporters over the oocytes surface, the fluorescence of both halves had to be measured. Total fluorescence was calculated from 10 optical sections recorded on each half of the oocyte. Oocytes from the same batches were used to obtain control values for each experimental series.

Amino Acid Analysis

The amino acid and dipeptide content of oocytes was determined with a Biotronik LC3000 analyzer (Eppendorf, Hamburg, Germany). Samples were applied to the ion exchange column, and separation was achieved with a multistep pH and ionic strength gradient.

Chemicals

All chemicals were obtained from Sigma and were added to the solutions in concentrations indicated in the text. After the addition of dipeptides, the pH was readjusted if necessary. Stock solutions of PDBu, Bis, and CD were prepared in DMSO at concentrations of 1 and 5 mM, respectively. The final concentration of DMSO in any solution was not higher than 0.2% (vol/vol), and DMSO at this concentration did not affect any of the measured parameters.

Statistics

Data are given as means ± SE of n = 5–71 oocytes from 2–5 donor animals. Statistically significant differences were determined by ANOVA followed by post hoc multiple-comparison tests. P values of <0.05 were considered as significant.

RESULTS

Cin Values of Oocytes Expressing rPEPT1

Figure 1 shows typical time courses of currents used to calculate capacitance. The mean Cin value of water-injected (WI) oocytes was 224 ± 5 nF (n = 24) and thereby was not significantly different from the Cin value of noninjected (NI) oocytes (217 ± 9 nF, n = 10). Expression of rPEPT1 resulted in a significant increase in capacitance to a mean of 302 ± 6 nF (n = 34, measured in the presence of 20 mM GQ), which related to a peptide-induced maximal current (Imax) in these oocytes of −430 ± 30 nA (n = 34). Figure 2 shows the relationship of Cin of individual WI or rPEPT1-expressing oocytes as a function of peptide-induced Imax. The linear regression line fitted to the data points had a slope of −0.109 nF/nA and an intercept at 238 nA (r² = 0.69, n = 71), equivalent to an increase of Cin by 10.9 nF per −100 nA of inward current. Since the expression of rPEPT1 did not increase the macroscopic diameter of oocytes (1.04 ± 0.03 mm for WI, n = 24, vs. 1.07 ± 0.03 mm for rPEPT1, n = 34), the rise in Cin must have resulted from an increase of the microscopic surface complexity. The calculated surface amplification factor (compared with a smooth sphere) was 6.78 ± 0.31 (n = 24) for WI oocytes and 9.01 ± 0.33 (n = 34) for oocytes expressing rPEPT1. Assuming that rPEPT1 proteins were transported to the plasma membrane in vesicles with a mean diameter of 120 nm, as suggested for other transporters (19), all membrane material of the vesicles remained in the plasma membrane and, taking a PEPT1 turnover rate of 6.6/s (32) into account, the mean number of PEPT1 proteins in a single vesicle was estimated to be ~1,200. In the case of partial reendocytosis of empty vesicles, this number may, of course, be considerably lower.

During the analysis of Cin of oocytes expressing rPEPT1 but not in WI oocytes, we observed that Cin values were ~17% larger when they were measured in the absence of substrate compared with values obtained in the presence of saturating dipeptide concentrations (see, e.g., Fig. 1). Since rPEPT1 has been shown to generate presteady-state currents in the absence of substrate (27, 32) and because of our own results (see below), we expected that the calculated surplus capacitance under substrate-free conditions resulted from the presteady-state current component, which has a component with a similar time course as the Cin current. To test this, we measured transport currents and Cin values in the presence of increasing concentrations of substrate and fitted the data to the Michaelis-Menten equation. At a pH of 7.5, the calculated Km for the current rise was 0.41 mM and the EC50 for the capacitance decrease was 0.49 mM (mean of 5 oocytes), whereas at a pH of 8.5, the respective values were 3.31 mM (current) and 2.83 mM (capacitance). These Km values are very close to those reported previously (25), and the match of the Km values for current and EC50 values for capacitance strongly suggest that the surplus capacitance results from the presteady-state current component.

A subsequent series of experiments with the high-affinity competitive inhibitor Lys[Z(NO2)]-Pro further strengthened this observation. Cin values were measured in the presence of substrate (GQ, 1 mM) and in the presence of 50 μM of the high-affinity competitive inhibitor Lys[Z(NO2)]-Pro, which has an apparent Ki for rPEPT1 of 10 μM (24). Both GQ and the inhibitor reduced the initial Cin value of 311 ± 7 nF (measured in the absence of substrate, n = 17) by 31 ± 3 and 36 ± 3 nF, respectively, whereas only GQ induced a transport current of 216 ± 16 nA (n = 17). These data suggest that the presteady-state current component and, in consequence, the virtual component of the capacitance is inhibited by both a transported substrate and a nontransported inhibitor.
Residence Time of rPEPT1 Transporters in the Oocyte Membrane

It has been shown for renal outer medullary K⁺ channels (ROMK channels) expressed in Xenopus oocytes that if the plasma membrane insertion of newly synthesized channel proteins was blocked by brefeldin A (BFA), ROMK currents decreased with a half-time of 6 h (54). To test whether a similar turnover rate exists also for rPEPT1, we incubated rPEPT1-expressing oocytes starting 3 or 4 days after cRNA injection for 3, 6, 9, or 24 h in Barth solution containing 10 μM BFA. Oocytes incubated in Barth solution without BFA served as controls. *I*$_{\text{max}}$ values were measured at the beginning and end of the incubation period. In control Barth solution, transport current slightly increased (23 ± 3%, 24 ± 7%, 19 ± 5%, and 9 ± 3% above the initial current value after 3, 6, 9, and 24 h, respectively). The addition of BFA did not decrease transport currents but resulted even in a modestly increased current after 3 h (147 ± 5%), whereas exposure times of 6, 9, and 24 h yielded 111 ± 5%, 105 ± 5%, and 94 ± 7% of initial currents. These data show that there is no further significant insertion of newly synthesized rPEPT1 proteins into plasma membranes 3–4 days after cRNA injection. The BFA data, however, do not exclude that there is a constitutive exchange of transporters between the plasma membrane and a putative intracellular pool. To test this, we used monensin, a hydrophobic ionophore known to dissipate the pH gradient across the membrane of early sorting endosomal vesicles and thus preventing trafficking to the recycling compartment. Since monensin could also have an effect on the plasma membrane Na⁺ conductance resulting in an increased cytosolic Na⁺ concentration, in accompanying experiments using Na⁺ concentration jumps we first tested if this was the case. Neither the small hyperpolarization (2.50 ± 0.11 mV, *n* = 11) of the open circuit membrane potential observed after replacement of 88 mM extracellular Na⁺ by choline nor the small outward current (17 ± 4 nA, *n* = 9) measured in oocytes clamped at −60 mV was significantly altered by monensin, suggesting no change in the composition of cytosolic cations. However, incubation of oocytes for 5 h in Barth solution containing 50 μM monensin reduced transport current to 34 ± 5% (*n* = 5, *P* < 0.01) and after 18 h of incubation to 38 ± 4% (*n* = 11, *P* < 0.01), suggesting constitutive endocytosis via clathrin-coated vesicles and recycling.

Changes of Transport Capacity in Response to Prolonged Exposure to Substrate

Prolonged (1–3 days) exposure of confluent layers of Caco-2 cells to dipeptide substrate resulted in an increase of membrane abundance of human PEPT1 (hPEPT1) (44, 49). To test whether a similar effect exists in Xenopus oocytes expressing rPEPT1, oocytes were incubated after injection with rPEPT1 cRNA for 3 days in Barth solution containing varying low concentrations of GQ (20–50 μM). Thereafter, transport currents were measured in response to 5 mM GQ. Instead of the expected increase, we observed a concentration-dependent decrease of transport currents (20 μM: −28%, 30 μM: −48%, 40 μM: −55%, and 50 μM: −64% compared with oocytes incubated in substrate-free solution; *n* = 6–8). To investigate the mechanism causing this decline in current in more detail, we exposed the oocytes to 5 mM GQ and reduced the incubation time to 5 h (at 18°C). As shown in Fig. 2, this treatment resulted in a drastic reduction of the GQ-generated current from −505 ± 49 nA before incubation to −149 ± 16 nA after incubation. The mean capacitance also decreased from 342 ± 6 to 223 ± 13 nF (*n* = 11). Although the large decrease of transport current suggested a decrease in *I*$_{\text{max}}$, we determined *K*$_{\text{m}}$ values before and after the exposure to substrate to exclude the possibility that the effect was caused by a reduction of the substrate binding affinity. Prolonged incubation in GQ resulted in a small decrease of the apparent *K*$_{\text{m}}$ at −60 mV (from 1.10 ± 0.07 to 0.87 ± 0.09 mM, *n* = 6), which might be the result of the slight acidification of the cytosol (see below); thus, these data clearly suggest that the observed decrease of transport current most likely reflects a reduction in the number of transporter units in the membrane.

To test whether the effects of GQ incubation and monensin were additive, we incubated oocytes for 5 h in Barth solutions containing either 5 mM GQ or 5 mM GQ together with 50 μM monensin. The reduction of transport current after GQ exposure (to 44 ± 6%, *n* = 7) was not different from the reduction when monensin was also present (48 ± 14%, *n* = 7). This suggests that prolonged exposure to GQ resulted in the inhibition of the same recycling pathway that was also blocked by monensin.

We next determined the time dependence for the substrate-induced changes in *C*$_{\text{m}}$ and *I*$_{\text{max}}$. Since oocytes did not tolerate continuous recordings over prolonged periods, they were impaled with electrodes only for a few minutes at the beginning and end of the incubation periods. The results of these exper-
ments are shown in Fig. 3. Incubation in a dipeptide-free control solution for either 2 or 5 h did not significantly change dipeptide-induced \( I_{\text{max}} \) and induced only a minute reduction in \( C_m \), resulting in no change in surface-related current density over 2 h and only a modest increase after 5 h. In contrast, the \( C_m \) rate was unchanged when 25 unidentified regulatory proteins, since the current reduction equal rates, with no changes of the current densities in this period. The decrease did not depend on the synthesis of some unidentified regulatory proteins, since the current reduction rate was unchanged when 25 \( \mu \)M cycloheximide was added to the GQ-containing incubation solution (decrease of transport current to 52 \pm 6\%, \( n = 7 \), in the absence of cycloheximide and 53 \pm 7\%, \( n = 6 \), in the presence of cycloheximide). These findings suggest that the current reduction in the first 2 h was caused by a mechanism that did not affect membrane surface area, whereas the proportional reduction of the membrane area and the transport capacity between 2 and 5 h resulted presumably from the inhibition of the recycling of transporters removed from the plasma membrane by constitutive endocytosis.

**Transporter Density in the Membrane as Assessed by Presteady-State Charge Movement and Immunofluorescence Measurements**

Presteady-state currents are generated by the potential-dependent reorientation of empty carriers within the membrane and from the binding and dissociation of the cotransported ion to its binding site on the transporter protein and are thus proportional to the amount of carriers in the membrane. Therefore, we determined presteady-state charge movements as described in MATERIALS AND METHODS in oocytes before and after incubation for 5 h in GQ-containing solution to test for the expected reduction of transporter number in the membrane. Oocytes were incubated in Barth solution, and the parameters of the simultaneously measured \( C_m \) current components served as controls.

As expected, the time constant of the \( C_m \) current component was independent of the presence of substrate (1.08 \pm 0.05 ms without and 1.05 \pm 0.05 ms with substrate, \( n = 26 \)), was equal for the on and off responses, and, with the exception of those measured at jumps to +115 mV, was also independent of membrane potential (1.04 \pm 0.05 ms at −135 mV and 1.06 \pm 0.05 ms at +65 mV, \( n = 26 \)). At potential jumps from −60 to +115 mV, the time constant of the presteady-state component was strongly reduced (see below), so that at this potential the capacitive and presteady-state time constants became comparable and could not reliably be separated.

The time constant of the presteady-state current for the on response was highly potential dependent and reached its maximum when the potential was jumped from −60 to −135 mV (18.4 \pm 0.9 ms calculated with method 1 or 15.2 \pm 0.4 ms calculated with method 2, \( n = 26 \)) and declined to 5.42 \pm 0.22 ms at +15 mV and to 2.36 \pm 0.09 ms at potential jumps from −60 to +115 mV (\( n = 26 \), method 2). In addition, time constants differed for the on and off responses (off: 11.3 \pm 0.4 ms at −135 mV with method 2). Since the presteady-state charge movement showed only a slight tendency of saturation in the voltage range investigated (see also Ref. 32), the calculation of the maximal charge movement and of the midpoint voltage (usually denoted as \( Q_{\text{max}} \) and \( V_{0.5} \), respectively (see Ref. 32)) from the individual measurements were generally not possible. Therefore, the charge movement at voltage jumps from −60 to −135 mV determined by method 2 was used for further calculations.

The suitability of presteady-state currents to quantify the amount of transporters in the membrane was tested by calculating the regression coefficient between transport current and presteady-state charge movement for the on response. The slope of the regression line was 32.2 pC/nA, and the coefficient of determination (Pearson \( R^2 \) coefficient) was 0.736 (\( n = 26 \)). The correlation was highly significant. On and off responses at jumps from −60 to −135 mV were not different (on: 12.8 \pm
DIPEPTIDE SUBSTRATES REDUCE PEPTIDE TRANSPORT BY PEPT1

0.7 nC and off: 13.1 ± 0.8 nC, n = 26) and showed a high correlation (R² = 0.92, n = 26).

In the following experiments, 9 oocytes were incubated in Barth solution and 17 oocytes were incubated in a solution containing 5 mM GQ for 5 h. Maximal transport and presteady-state currents of each sample were measured before and after incubation. The results shown in Table 1 show that incubation without GQ neither altered transport current nor presteady-state charge movement. In contrast, incubation in GQ-containing solution reduced the transport current to 37.7 ± 2.2% and presteady-state charge movement to 49.4 ± 2.6% (n = 17). The slope of the regression line of charge movement versus transport current before incubation was 31.2 pC/nA (R² = 0.67) and after incubation was 41.8 pC/nA (R² = 0.67).

The large reduction of the presteady-state charge movement clearly shows a reduced number of transporters in the membrane after incubation, but the still larger decrease of transport current and the increase of the slope of the regression line suggest, in addition, that after incubation in GQ, the turnover rate of each rPEPT1 was reduced, in agreement with the observations made after 2 h of incubation in GQ.

Although the incomplete saturation of the presteady-state charge movement within the potential range investigated impeded the calculation of Qmax for each individual measurement (see above), a fit of a Boltzmann function to the average values was still possible (Fig. 4). This calculation yielded Qmax = 58.5 nC and V0.5 = −71 mV (before incubation in GQ) and Qmax = 22.2 nC and V0.5 = −114 mV (after incubation in GQ). The turnover rate at –60 mV (defined as transport current/Qmax) was 6.6 1/s, nearly identical before and after exposure to GQ and identical to the value found by Nussberger and coworkers (32).

To complement the electrophysiological measurements on substrate-induced rPEPT1 retrieval from the cell membrane, immunofluorescence detection experiments of surface expression of Flag-tagged rPEPT1 were performed. Preliminary experiments established that insertion of the short Flag tag into the large extracellular loop of rPEPT1 did not alter the expression rate or Km or other transport properties of rPEPT1 (data not shown). Measurements of fluorescence and GQ-induced transport current on the same oocytes showed a correlation of these parameters for oocytes harvested from the same frog but different slopes of the regression lines belonging to different frogs (not shown). Since fluorescence detection does not allow multiple measurements (i.e., paired experiments), oocytes from the same batches were used to determine control values. Exposure of oocytes to GQ for 5 h resulted in a fluorescence intensity of 3,765 ± 355 arbitrary units (n = 25) compared with the control value of 6,065 ± 340 arbitrary units (n = 24), a decrease by an estimated 38%, very similar to the reduction of Cm (as shown in Fig. 3).

Reinsertion of Transporters Into the Membrane During Recovery

To explore the fate of the transporters removed from the plasma membrane, we tested whether substrate-exposed oocytes could recover their transport capacity and, if so, whether the recovery depended on the de novo synthesis of transporter proteins and whether it occurred by recycling of endocytosed vesicles. Oocytes exposed for 5 h to a GQ-containing solution (resulting in a decrease of transport current to 43 ± 3% and of Cm to 66 ± 4% of their respective control values) were transferred into a dipeptide-free solution and allowed to recover for 15–16 h at 18°C (group A). In part of the experiments, the recovery solution contained, in addition, 25 μM cycloheximide for the inhibition of protein synthesis (group B). In control experiments, GQ was omitted in the incubation period, and oocytes were kept for 20 h in dipeptide-free solution without (group C) or with added cycloheximide (group D). GQ-induced transport currents, and, in part of the experiments, also Cm values, were measured at the beginning of the experiment and at the end of the recovery period; however, we forwent measuring for a third time after the GQ incubation period, since three impalements were generally not well tolerated by the oocytes. The currents measured at the end of recovery period as a percentage of the initial current for the

Table 1. Transport currents, presteady-state charge movements (on responses), and presteady-state time constants measured before and after incubation in control Barth solution or in solution containing 5 mM GQ

<table>
<thead>
<tr>
<th>Experimental State</th>
<th>Transport Current, nA</th>
<th>Ratio of Transport Current After Versus Before Incubation</th>
<th>Presteady-State Charge Movement After Versus Before Incubation</th>
<th>Ratio of Presteady-State Charge Movement After Versus Before Incubation</th>
<th>Presteady-State Charge Movement After Versus Before Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Barth solution incubation</td>
<td>387 ± 52</td>
<td>13.5 ± 0.4</td>
<td>17.1 ± 0.7</td>
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<tr>
<td>After Barth solution incubation</td>
<td>447 ± 73</td>
<td>1.18 ± 0.13 * (n = 9)</td>
<td>13.4 ± 1.4</td>
<td>1.03 ± 0.09 * (n = 9)</td>
<td>15.0 ± 0.8</td>
</tr>
<tr>
<td>Before GQ solution incubation</td>
<td>383 ± 31</td>
<td>12.4 ± 0.8</td>
<td>14.2 ± 0.3</td>
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<tr>
<td>After GQ solution incubation</td>
<td>142 ± 11</td>
<td>0.38 ± 0.02 * (n = 17)</td>
<td>6.1 ± 0.4</td>
<td>0.49 ± 0.03 * (n = 17)</td>
<td>21.4 ± 0.7</td>
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Transport currents were measured with 20 mM Gly-L-Gln (GQ) at –60-mV membrane potential. Presteady-state parameters (charge movements and time constants) were determined from voltage jumps from –60 to –135 mV (see also Fig. 1, inset). *Not significant; †P < 0.01.
above groups were as follows: group A, 119 ± 8%; group B, 112 ± 4%; group C, 120 ± 8%; and group D, 129 ± 6% (n = 12–14). ANOVA did not reveal a significant difference among these groups. Cm values were measured in six oocytes of group A and showed a recovery to 87 ± 1% of the control value. In another series of experiments, the recovery period was reduced to 5 h, and currents were measured at the end of the GQ incubation and recovery periods. Recovery in Barth solution alone resulted in an increase of current to 241 ± 17% (n = 9, P < 0.01, current after GQ incubation: 100%), whereas in the presence of cycloheximide, BFA, or monensin, the corresponding values were 222 ± 17% (n = 11, P < 0.01), 187 ± 16% (n = 7, P < 0.01), and 66 ± 8% (n = 6, P < 0.01), respectively. These data suggest that transporters retrieved from the cell membrane were not submitted to degradation but recycled to the membrane after substrate removal from the extracellular surface. This recycling was inhibited only by monensin but was independent of protein synthesis or protein transport from the endoplasmic reticulum to the Golgi.

Comparison of the Effects of Exposure to Substrate and of PKC Stimulation

Activation of PKC has been found to increase endocytosis rates and thereby reduce the number of transporters expressed in oocytes and other cell systems (15, 18, 19, 26, 33, 52). To analyze whether the reduction of rPEPT1 transport activity is mediated by PKC, we first tested if stimulation of PKC evokes the withdrawal of rPEPT1 transporters from the oocyte membrane and then used a PKC-specific inhibitor to test the involvement of this kinase in the dipeptide-evoked response. Oocytes were exposed for 1 h to the phorbol ester PDBu in the absence of peptide substrates, and GQ-induced transport currents and Cm values were recorded immediately before and after PDBu treatment. As shown in Fig. 3, right column, PDBu treatment resulted in large and almost equal decreases in Imax and Cm with no change in current density. Cm was reduced to 145 ± 9 nF (n = 9), i.e., to a value significantly lower than the average Cm of NI control oocytes (217 ± 9 nF, n = 10) or oocytes exposed to dipeptide (223 ± 13 nF, n = 11). Activation of PKC therefore induces endocytosis, which affects not only the membrane surface area during exocytotic delivery of rPEPT1 but also the basal membrane surface area present in NI oocytes. Interestingly, exposure of NI oocytes to PDBu resulted in a moderate increase of Cm (from 201 ± 2 to 230 ± 4 nF, n = 8, P < 0.01). The electrophysiological data were also confirmed by immunofluorescence measurements. Oocytes exposed for 1 h to PDBu induced transport currents of 196 ± 20 nA (n = 15) and possessed a fluorescence intensity of 7,830 ± 440 arbitrary units, with corresponding values of control oocytes from the same batches of 360 ± 24 nA (n = 15) and 10,790 ± 560 arbitrary units. This corresponded to a reduction of Imax by 45%, nearly equal to that shown in Fig. 3, and a reduction of surface expression of the protein by 27%.

The data shown in Fig. 3 were calculated from current values measured at a membrane potential of −60 mV. In addition, at each experimental state, I-V relations were recorded in the potential range of −160 to +80 mV. Both exposure to dipeptide and stimulation of PKC resulted in comparable changes of the static I-V relationship. Figure 5 shows the comparison of I-V relations before and after 5 h of exposure of oocytes to 5 mM GQ. Before incubation, the I-V relation was similar to that previously documented (3, 13). The direction of current reversed at a potential of +15.7 mV. By assuming that the intracellular pH is similar to that outside (7.5), the concentration of GQ in the very neighborhood of the inner membrane surface was estimated from the reversal potential and the Nernst equation to be −2.8 mM. The presence of an outward current at membrane potentials above the reversal potential verified our previous observation that rPEPT1 can transport in both directions (25). As shown in Fig. 5, prolonged incubation in GQ reduced the inward current at positive and negative membrane potentials. The decrease in the outward transport direction (e.g., at +80 mV) was even larger than in the inward direction, and the reversal potential of the I-V relationship shifted to +54.9 mV. A similar shift of the reversal potential was observed already after 2 h of incubation in GQ, whereas the incubation in the absence of substrate for up to 5 h had no effect (data not shown). PDBu treatment of oocytes also caused a shift of the reversal potential toward more positive values (from 0.1 to 46.3 mV; not shown). This shift was not observed when PDBu and CD were applied simultaneously (data not shown).

The dipeptide-induced and PKC-mediated changes in currents and capacitance were found to depend on an intact cytoskeleton, as proven by the treatment of oocytes with CD in the absence or presence of dipeptides or PDBu. The duration of the CD application during long-term exposure to substrates had to be restricted to the last 3 h of the 5-h period since a longer application of CD destroyed virtually all oocytes. Figure 6 shows that CD treatment fully inhibited the PDBu-induced changes. In case of dipeptide-induced alterations, CD also antagonized the effects significantly, although not fully (probably due to the shorter exposure time). Thus, the reduction of Imax and Cm changes in both experimental settings required an intact functional cytoskeleton.

Fig. 5. Effects of prolonged substrate exposure on current-voltage (I-V) relations. The potential dependency of the dipeptide-induced current was measured before (-) and after (●) 5 h of exposure to 5 mM GQ. Positive currents denote the outward transport of dipeptides from the oocyte. Mean values are of 13 paired measurements (3 frogs). Arrows show reversal potentials. Please note the decrease of the outward current at positive membrane potentials after substrate incubation.
Despite the similarity in changes in transport current, $C_m$, and $I-V$ relationship evoked by either PKC activation or long-term substrate exposure, different mechanisms appeared to cause these effects. Application of the PKC-selective inhibitor Bis (5 μM) completely reversed the PDBu-evoked endocytosis effects, as shown in Fig. 6, third column, but failed to prevent the decline in $I_{\text{max}}$ and the capacitance reduction when those were induced by GQ exposure, as shown in Fig. 6, sixth column. Furthermore, the effects of GQ and PDBu seemed to be at least partially additive: the reduction of the transport current was larger (59 ± 3%, $n = 4$) when GQ and PDBu were applied simultaneously (PDBu was present in the last hour of the GQ exposure lasting 5 h) compared with the GQ-evoked reduction alone (44 ± 3%, $n = 12$). Whether the similar changes in reversal potential shifts observed under PDBu treatment and substrate exposure indicate a common mechanism downstream of PKC needs further study.

Assessing the Possible Mechanism Underlying the Dipeptide-Evoked Transport Reduction

Does a cytosolic accumulation of dipeptides or amino acids inhibit transporter recycling? The shift of reversal potential toward more positive values after long-term dipeptide exposure (Fig. 5) was unexpected since accumulation of the dipeptide in the oocytes should have changed the reversal potential to more negative values and should have increased the outward current component. To determine the intracellular concentration of dipeptide before and after long-term substrate exposure, oocyte contents were analyzed by HPLC analysis. In WI oocytes, two major peaks representing aspartate and glutamate were identified (Fig. 7). Oocytes expressing rPEPT1 and exposed to GQ for 5 h revealed, in addition, high intracellular concentrations of glycine and glutamine but no detectable intact dipeptide. This suggests a complete hydrolysis of GQ in the oocytes (see the arrow in Fig. 7 marking the expected location of the GQ peak). Although this lack of intact dipeptide in the oocyte may explain why no increased outward current was observed at positive membrane voltages (Fig. 5), the reason for the shift in reversal potential to more positive values still remains unknown.

![Fig. 6](image1.png)

Fig. 6. Effects of exposure of oocytes expressing rPEPT1 to dipeptides in the absence or presence of cytochalasin D (CD) or bisindolylmaleimide I (Bis). Changes of transport current, $C_m$, and current density in response to incubation in PDBu or dipeptide solution were measured in the absence or presence of CD or Bis. Mean values are from 6–16 measurements (2–3 frogs). Significant differences with respect to the control period are shown as *$P < 0.05$ or **$P < 0.01$; significant differences with respect to PDBu or GQ periods, respectively, are shown as +$P < 0.05$ or ++$P < 0.05$.

![Fig. 7](image2.png)

Fig. 7. Analysis of amino acid contents in oocytes after exposure to a dipeptide (absorbance vs. elution time). Oocytes were incubated in 5 mM GQ solution for 5 h. Top: WI oocytes. The peaks show the high concentrations of aspartate and glutamate. Bottom: rPEPT1-expressing oocytes. In addition to aspartate and glutamate, these oocytes contained comparable amounts of glycine and glutamine but not the unhydrolyzed dipeptide GQ (see the arrow marking the expected location of GQ).
The complete intracellular hydrolysis of the dipeptide substrate raises the question of whether the amino acids resulting from the hydrolysis were involved in the endocytosis of rPEPT1. To test this, we exposed oocytes for 5 h to cefadroxil (20 mM), a substrate of PEPT1 that is not hydrolyzed by any of the peptidases. This maneuver resulted in a marked decrease of the GQ-induced transport current (from 452 ± 46 to 258 ± 34 nA, n = 16, P < 0.01) and of the Cm (from 302 ± 7 to 180 ± 7 nF, n = 16, P < 0.01), very similar to what was observed in response to incubation in GQ. Thus, the endocytosis of the transporters is independent of the cytosolic accumulation of amino acids. This result was confirmed by another series of experiments, where 9.2 nmol GQ (37 nl of 250 mM solution) was directly injected into the oocyte and the transport current was measured before and 5 h after the injection. Injection did not result in a decrease but rather in a moderate increase of transport current (321 ± 36 before vs. 431 ± 59 nA after, +34 ± 8%, n = 10). This result shows that not the presence of substrate in the oocyte but the translocation of it via the transporter triggers the observed endocytosis.

The availability of the high-affinity competitive inhibitor Lys[Z(NO$_2$)$_2$]-Pro allowed to test whether long-lasting occupation of the substrate-binding domain without transport suffices to activate the endocytosis process. Exposure for 5 h to 5 mM Lys[Z(NO$_2$)$_2$]-Pro did not significantly reduce GQ-induced current (426 ± 59 nA before and 383 ± 35 nA after incubation, n = 14, not significant) and reduced Cm only slightly (from 345 ± 7 to 280 ± 10 nF, n = 14, P < 0.01). Therefore, transport and not only substrate binding to PEPT1 is driving the retrieval of the protein from the membrane.

**Does a decrease in intracellular pH reduce transport current and transporter retrieval?** The uptake of dipeptide substrate by PEPT1 is accompanied by proton influx, which might exceed the buffer capacity of the cell and thus acidify the cytosol. Since the pH gradient across the membrane is one of the driving forces of the transport, we determined the extent of intracellular acidification in response to the prolonged exposure to GQ and tested if an acute acidification by the same extent has, per se, any effect on the transport current. The cytosolic pH was measured in oocytes expressing rPEPT1 after 5 h of exposure to 5 mM GQ and was compared with the pH of control oocytes exposed to Barth solution without substrate. The average cytosolic pH of control oocytes was 7.71 ± 0.05 (n = 5) and was thus within the range reported by others (9, 35, 41), whereas substrate-exposed oocytes had an average pH value of 7.15 ± 0.10 (n = 6), significantly (P < 0.01) lower than the control value. The direct effects of the cytosolic pH reduction were tested by incubating oocytes in a solution containing 30 mM butyrate, which has been reported to induce a fast intracellular acidification of similar magnitude as reported above (14). This maneuver caused, within 7–8 min, a shift of cytosolic pH from 7.31 ± 0.03 to 6.72 ± 0.06 (difference of 0.59 ± 0.05, n = 7, P < 0.01), but the current induced by 5 mM GQ remained unchanged (360 ± 44 nA before and 379 ± 62 nA after butyrate application, n = 12, not significant). We repeated these experiments with a lower butyrate concentration (10 mM) but with a prolonged incubation time (2–3 h). Under these conditions, pH was reduced from 7.33 ± 0.02 to 6.91 ± 0.03 (n = 9) but transport currents were not reduced; they even slightly increased (292 ± 23 nA before and 378 ± 15 nA after incubation, n = 9, P < 0.05). Thus, at nearly saturating substrate concentrations, cytosolic acidification by ~0.6 pH units did not significantly reduce the overall driving force for transport. The missing direct effect of acidification on the transport rate does not exclude, however, the possibility that the low cytosolic pH affected some processes that finally led to the endocytosis of PEPT1.

**Does membrane depolarization trigger transporter endocytosis?** The exposure of oocytes expressing rPEPT1 to high concentrations of substrate leads, due to electrogenic proton symport, to a depolarization of the cell membrane. Our next experiments aimed, therefore, to determine whether this plays a role in the substrate-induced retrieval of rPEPT1. To investigate the role of membrane depolarization, we compared the changes evoked by long-lasting exposure to substrate in oocytes expressing rPEPT1 or hSGLT1. Since the cotransport of Na$^+$ via hSGLT1 leads to a similar membrane depolarization but to no intracellular acidification, we could distinguish between the effects caused by potential or pH change.

To estimate the extent of depolarization, we first impaled oocytes to record the response of membrane potential to substrate exposure. Thereafter, transport current was measured in the same oocytes in the TEVC configuration. The results showed that the average depolarization in rPEPT1-expressing oocytes was 61 ± 4 mV (from −35 ± 2 mV in the absence to +26 ± 3 mV in the presence of substrate, n = 13). The mean transport current generated by these oocytes was 409 ± 44 nA (at 5 mM GQ), in the same range as in our previous experiments, and there was no correlation between the extent of depolarization and the transport current (slope: −0.053 mV/nA).

Oocytes expressing hSGLT1 exposed to 5 mM α-methyl-D-glucopyranoside (α-MDG) responded with a substrate-induced depolarization of 59 ± 2 mV (n = 11), which was nearly identical to the corresponding value in rPEPT1-expressing oocytes. α-MDG evoked in these oocytes a steady current of 965 ± 73 nA. Thus, we could expect similar depolarization in rPEPT1-expressing and hSGLT1-expressing oocytes. If the observed endocytosis was triggered by the long-lasting membrane depolarization, a similar decrease of transport activity in oocytes expressing hSGLT1 or rPEPT1 should be expected. This was, however, not the case: 5 h of exposure of oocytes expressing hSGLT1 to α-MDG did not result in a reduction but even in a slight elevation of transport current from 510 ± 35 nA before exposure to 629 ± 39 nA after exposure (n = 20, P < 0.01). A similar increase in current was also seen in hSGLT1-expressing control oocytes exposed to solutions containing no substrate (506 ± 66 nA before and 579 ± 53 nA after exposure, n = 7, P < 0.01).

**Does prolonged exposure to the dipeptide also affect other transporters?** The objective of the next series of experiments was to clarify whether the observed endocytotic retrieval that was assumed to result from a cytosolic pH reduction uniformly affects all transporters expressed in the plasma membrane. Coexpression of rPEPT1 and hSGLT1 resulted in comparable currents for both transporters (294 ± 16 nA in response to 5 mM GQ and 237 ± 19 nA in response to 5 mM α-MDG, n = 26). The current in the simultaneous presence of both substrates was 536 ± 20 nA, i.e., the currents of both transporters were additive. Exposure of these oocytes to 5 mM GQ for 5 h markedly inhibited the GQ-induced transport current (to 119 ± 9 nA, corresponding to a decrease by 60 ±
2%, \( P < 0.01 \) but did not significantly reduce the \( \alpha \)-MDG-induced current (to 191 ± 9 nA or by 9 ± %, not significant).

Another set of oocytes coexpressing rPEPT1 and hSGLT1 was also exposed to \( \alpha \)-MDG. Before incubation, transport currents were 284 ± 10 nA (rPEPT1) and 353 ± 15 nA (hSGLT1, \( n = 12 \)), respectively. After exposure of these oocytes to 5 mM \( \alpha \)-MDG for 5 h, the transport current via hSGLT1 did not change (373 ± 17 nA after exposure, \( n = 12 \), not significant), whereas the transport current of rPEPT1 even increased to 426 ± 19 nA (\( n = 12 \), \( P < 0.01 \)). The reason for this increase is unknown but could possibly be caused by a rise of cytosolic Na\(^+\) concentration, which has been reported to stimulate the expression of transporters in Xenopus oocytes (39). In summary, the coexpression experiments provided strong evidence that long-time exposure to GQ does not effect SGLT1 but is specific for rPEPT1.

**DISCUSSION**

Whereas the transport properties of PEPT1 have been analyzed in detail, little is known about the cellular mechanisms involved in the regulation of this carrier protein (1, 16, 20, 23, 31, 40). In the present study, we show, for the first time, that the transporter density and, in turn, the activity of rPEPT1 expressed in *Xenopus* oocytes is downregulated by both prolonged exposure to substrates and by activation of PKC. Expression of rPEPT1 in oocytes was accompanied by a marked increase of the oocyte surface area (compared with WI oocytes), and the reduction of transport activity was paralleled by a marked reduction of this surplus of surface enlargement. Disturbing the actin cytoskeleton inhibited the substrate-dependent changes as well as PKC-evoked changes, suggesting the involvement of endocytic processes. However, the PKC-mediated and substrate-evoked changes appeared to act via different signaling processes on transporter retrieval since only the PKC pathway could be inhibited by the specific inhibitor Bis. The specificity of the effects on PEPT1 was established by demonstrating that 1) only PEPT1 but not the Na\(^+\)-coupled electrogenic transporter hSGLT1 showed this type of substrate-evoked regulation, 2) the effects are independent of the nature of the substrate (hydrolyzable or nonhydrolyzable), and 3) an occupation of the substrate-binding domain without transport is not sufficient to elicit the transporter retrieval.

**Membrane Surface Area Changes in Xenopus Oocytes**

The *Xenopus* oocyte model provides a simple electrical system composed of membrane conductance paralleled by \( C_m \). These parameters can be measured and analyzed without extensive modelling as necessary, e.g., in complex epithelial preparations. The range of average \( C_m \) values measured in native oocytes extends from 158 to 236 nF (2, 15, 38, 47, 55). The \( C_m \) values measured in the present study were also in this range. Simultaneous measurements of the diameter and \( C_m \) indicated a surface amplification of about six times over a smooth sphere with 1.1-mm diameter.

Heterologous expression of transporter or channel proteins has been reported to be accompanied by an increase of \( C_m \) and surface area [CFTR Cl\(^-\) channel (51), epithelial Na\(^+\) channel (ENaC) (2, 38), rabbit SGLT1 (19), and native oocyte channels activated by the marine poison maitotoxin (50)]. These observations were also confirmed by electron microscopy displaying increases in the density and size of oocyte microvilli simultaneously with the functional expression of proteins (21, 48).

In contrast with the above data, the expression of some other transporters resulted in no alterations of the membrane area [human DAT (55), hSGLT1 (47), Na\(^+\)-phosphate cotransporter NaPi II (15), and an amino acid transporter cloned from insect larvae (4)].

From the increase of \( C_m \) observed for rabbit SGLT1 and from the estimated diameter of transport vesicles, Hirsch and coworkers (19) estimated some 200 protein molecules per fused vesicle. However, electron microscopic analysis revealed only ~20 SGLT1 units per vesicle, which seems to indicate that the exocytosis rate is even much higher and ~90% of the fused vesicle membrane area was withdrawn from the plasma membrane (19). We calculated a number of ~1,200 rPEPT1 proteins per transport vesicle, but the reuptake rate of empty vesicles is not known and the actual number could be considerably lower.

**Regulation of Heterologously Expressed Transporters by PKC in Xenopus Oocytes**

In *Xenopus* oocytes, PKC has been found to induce endocytosis and thus to downregulate a variety of heterologously expressed membrane transport proteins [Na\(^+\)-K\(^+\)-ATPase (48), SGLT1 (19), the taurine transporter TAUT (26), NaPi II (15), the Na\(^+\)-dicarboxylate cotransporter NaDC-1 (33), human DAT (hDAT) (55), the glutamate transporter EAAC1 (45), and cardiac Ca\(^{2+}\) or K\(^+\) channels and ENaC (2, 5, 30)]. However, other transporters did not change density and/or activity upon PKC stimulation in oocytes, including the endogenous Na\(^+\)/H\(^+\) exchanger, the rat GABA transporter GAT1, the glutamate transporter EAAT3, or hSGLT1 (7, 10, 12, 53). In the case of GAT1, even a redistribution of transport proteins from the intracellular pool to the plasma membrane has been reported (10). This suggests that the regulation of endocytosis and vesicle trafficking by PKC is not identical for all transporter proteins expressed in the oocyte membrane.

**Substrate-Induced Transporter Regulation**

To our knowledge, the only data demonstrating substrate-induced adaptive regulation of transporter activity in *X. laevis* oocytes have been reported by Taylor et al. (43) and Gulley et al. (17). Exposure of oocytes to a mixture of amino acids caused a downregulation of Na\(^+\)-dependent amino acid-transporting systems but upregulated Na\(^+\)-independent systems, suggesting that *Xenopus* oocytes possess endogenous signaling mechanisms for selectively modulating the activity of amino acid transport proteins expressed in the cell membrane (43). In oocytes expressing hDAT, a repeated exposure for short periods to low concentrations of dopamine, amphetamine, or tyramine also led to a marked reduction of transport-mediated currents. The effect was attenuated by the application of the PKC inhibitor Bis. The reduction in transport currents was paralleled by reduced radioligand binding to the oocyte surface indicating a retrieval of DAT from the cell surface (17). Very similar results have been observed in HEK-293 cells stably transfected with yellow fluorescent protein-tagged hDAT (22).

In the present study, we showed by means of electrophysiology as well as immunostaining that rPEPT1 upon prolonged exposure of oocytes to substrates is retrieved from the oocyte...
membrane via endocytosis. This process is independent of the nature of the substrate but needs the transport process and, moreover, is reversible. Analysis of the underlying mechanisms revealed that neither the membrane depolarization nor the transport-mediated intracellular acidification caused by substrate exposure drove this internalization. Despite the fact that PKC activation can mimic the effect on rPEPT1 retrieval, the substrate-induced alterations appeared not to involve the same pathways.

The effects of a prolonged substrate exposure on the transport activity of hPEPT1 has also been investigated in confluent monolayers of human intestinal Caco-2 cells (44, 49). In both studies, long-term (1 or 3 days, respectively) incubation in a dipeptide-containing culture medium (GQ or Gly-Sar) increased the $V_{\text{max}}$ of hPEPT1 with no change in $K_m$. The underlying mechanism was proposed to include increased transcription of the pept1 gene leading to an increased density of the protein. Short-term (2 h) exposures of cells did not cause any changes in PEPT1 activity (44).

In our study, the exposure of oocytes expressing rPEPT1 to low concentrations of GQ for 3 days or to high concentrations for 5 h reduced transport activity, and this was the result of a change in $V_{\text{max}}$ and transporter protein density while $K_m$ was not altered. The findings in the Caco-2 cell model showed opposite changes in transporter density in the cell membrane. Since the oocyte does not express endogenously peptide transporters, and it cannot respond like a mammalian cell with alterations in gene transcription, the type of adaptation observed in Caco-2 cells cannot be expected in oocytes. It therefore could be that the retrieval of peptide transporter proteins from the cell membrane upon prolonged substrate exposure is simultaneously compensated by an increased de novo synthesis of new carrier proteins.

In summary, we report here that the insertion of rPEPT1 transport proteins into the Xenopus oocyte cell membrane results in a significant increase of the membrane surface area. Moreover, membrane surface density of PEPT1 proteins seems to be controlled by the transport function of PEPT1 as prolonged substrate exposure resulting in an increased cytosolic amino acid and proton concentration and in membrane depolarization triggered an as-yet-undefined intracellular signaling pathway that, via endocytosis, leads to a reversible retrieval of the transporters from the plasma membrane and to a reduction of the oocyte surface area.

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