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Regulation of the mouse retinal taurine transporter (TAUT) by protein kinases in *Xenopus* oocytes

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Abstract The goal was to investigate the role of protein kinases in modulating taurine transporter activity in *Xenopus laevis* oocytes expressing the mouse retinal Na^+/C^-/taurine transporter. The currents generated by the taurine transporter were studied with a two-electrode voltage clamp and we recorded the maximal current (I_{max}), presteady-state charge transfer Q, and membrane capacitance C_m. 8-Br-cAMP, a membrane-permeable activator of the cAMP-dependent protein kinase (PKA), decreased I_{max} (41%), Q (41%) and C_m (10%). Similarly, 1 μM *sn*-1,2-dioctanoylglycerol (DOG), an activator of the Ca^{2+}/diacylglycerol-dependent protein kinase (PKC), decreased I_{max} (56%), Q (37%), and C_m (9%). Calyculin A, a specific inhibitor of protein phosphatases 1 and 2A, also produced effects similar to those of 8-Br-cAMP and DOG, and decreased I_{max} (64%), Q (38%), and C_m (10%). We conclude that the taurine transporter is regulated by activators of PKA and PKC, and regulation occurs largely by changes in the number of transporters in the plasma membrane.

Key words: Na^+/Cl^-/taurine cotransporter regulation; Protein kinase activation; Membrane trafficking

1. Introduction

Taurine (2-aminoethanesulfonic acid) is found in millimolar concentrations in the retina, liver, kidney, heart and brain [1]. While many functions have been attributed to taurine, such as being an essential nutrient for development of the brain and retina, its best understood role is as an osmolyte in the kidney and brain [2]. Isoforms of the taurine transporter have been sequenced and cloned from dog kidney [3], mouse brain [4], mouse retina [5], human placenta [6], and human thyroid [7]. They belong to the family of Na^+- and Cl^--dependent co-transporters, which include neurotransmitter, osmolyte and amino acid transporters [8].

These taurine transporters have one or more putative phosphorylation sites for Ca^{2+}/diacylglycerol-dependent protein kinase and cAMP-dependent protein kinase [3,4], and there is evidence that the kinases are involved in regulation of transport [9,10]. We have used electrophysiological techniques to investigate the role of protein kinases in modulating taurine transport in *Xenopus laevis* oocytes expressing the mouse retinal taurine transporter. We monitored: (1) the maximal rate of Na^+/Cl^-/taurine cotransport; (2) the number of taurine transporters in the plasma membrane [11,12]; and (3) the net rate of exo- and endocytosis [13].

2. Materials and methods

The Na^+/Cl^-/taurine transporter (TAUT) cloned from mouse retina [5] was expressed in *X. laevis* oocytes as described previously [11]. The retinal clone was 100% identical to mouse brain taurine transporter [4]. In a series of experiments in 15 μM taurine, the uptake of [^{3}H]taurine in cRNA-injected oocytes (using methods described in [14]) was 11 ± 2 nM/h (n = 8), and was 20-fold higher than in non-injected oocytes (0.5 ± 0.1 nM/h, n = 10), thus confirming that taurine is transported into cRNA-injected oocytes. Electrophysiological measurements were undertaken 5–7 days post-cRNA injection, using a two-electrode voltage clamp [11]. During experiments, the oocytes were bathed in a standard NaCl buffer (in mM): 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (pH 7.4). Membrane voltage V_m was held at −50 mV. To obtain the steady-state current-voltage relations, V_m was stepped for 100 ms from the holding (V_h −50 mV) to various test values (V_t) between +50 and −150 mV in 20 mV increments, and the steady-state currents at 100 ms were measured. The currents were digitized at 100 μs/point, and the average current of three sweeps was recorded. The steady-state currents generated by the taurine transporter (e.g. Fig. 1C) were obtained as the difference between the steady-state currents in the presence and absence of taurine (Fig. 1A,B). Our preliminary kinetic studies indicate the apparent affinity constant $K_{0.5}$ for taurine in 100 mM external NaCl was 15 μM [15]. To determine the maximal taurine-induced steady-state current I_{max}, a saturating concentration of taurine (500 μM) was added to the bath NaCl buffer.

Presteady-state currents due to the expression of the taurine transporter were isolated from the capacitive and leakage currents by fitting the total current $I(t)$ to: $I(t) = I_1 e^{-t/\tau_1} + I_2 e^{-t/\tau_2} + I_{ss}$, where I_1 is the capacitive current with time constant τ_1, t is time, I_2 is the transient current due to the taurine transporter with time constant τ_2, and I_{ss} is the steady-state current [11]. Charge movement Q due to the taurine transporter was calculated by integrating the presteady-state currents at each test voltage V_t. At each test voltage, the ON and OFF charges were equal and opposite. Charge-voltage relations were obtained by fitting the charge movement Q (mean of the ON and OFF charges) at various test voltages with the Boltzmann equation: $(Q-Q_{hyp})/Q_{max} = 1/[1+\exp(z(V_t-V_{0.5})F/RT)]$, in which $Q_{max} = Q_{dep}-Q_{hyp}$, Q_{dep} and Q_{hyp} being Q at depolarizing and hyperpolarizing limits; T, absolute temperature; F, Faraday's constant; R, gas constant; $V_{0.5}$, voltage for 50% charge transfer; and z, the apparent valence of the movable charge. In kinetic experiments, z was 1, $V_{0.5}$ was approx. +50 mV, and Q_{max} varied from 9 to 20 nC. The maximal charge Q_{max} provides a measure of the number of transporters N in the plasma membrane ($Q_{max} = N \times e \times z$, where e is the elementary charge and z the apparent valence of the movable charge [11,12]). Since saturation of the charge vs. voltage relations was not generally reached at +50 mV, we used the charge transfer at +50 mV as an index of the number of transporters in the plasma membrane. Plasma membrane capacitance C_m was determined as the slope of the linear charge vs. voltage relations in the presence of 500 μM external taurine [11].

8-Br-cAMP (8-bromoadenosine 3′:5′-cyclic monophosphate) and DOG (*sn*-1,2-dioctanoylglycerol) were used to activate PKA and PKC [16,17]. Calyculin A, a specific inhibitor of the protein phosphatases 1 and 2A [18] was also used. Presteady-state and steady-state currents were measured in response to external taurine before and after incubation (30 min) of the oocytes in NaCl buffer containing these compounds. All components were purchased from Calbiochem (San Diego, CA) or Sigma (St. Louis, MO).

Summarized data are presented as arithmetic mean values ± S.E.M.

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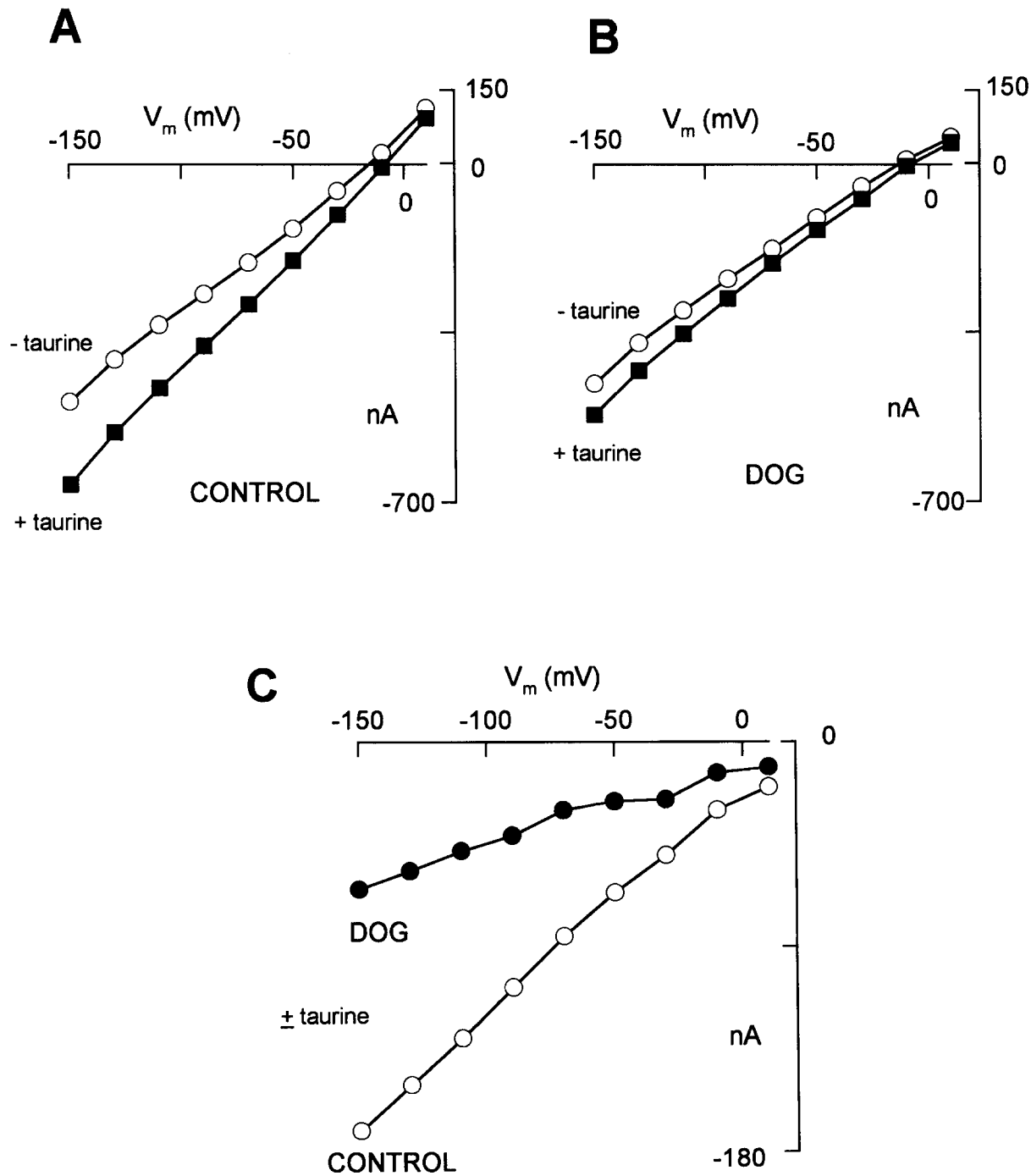

Fig. 1. Effect of DOG on steady-state currents. The experiment was performed on an oocyte injected with 50 ng of the mouse retinal taurine transporter cRNA. The oocyte was bathed in a NaCl solution at 20°C. The holding potential (V_h) was −50 mV and test voltages (V_t) were applied for 100 ms. Shown are the steady-state currents (measured at 100 ms after the onset of the voltage pulse) corresponding to V_t between +10 and −150 mV. The taurine-induced steady-state current is the difference in steady-state current in the presence (+) and absence (−) of 500 μM taurine. (A) Was obtained before, and (B) after incubating the oocyte in 1 μM DOG for 30 min. (C) Steady-state current-voltage relation of the taurine-induced current before and after DOG treatment.

with n referring to the number of observations. A paired t-test was used to test for statistically significant differences, $p \leq 0.05$ was set as significance level.

3. Results

3.1. PKC activation

The steady-state currents induced by taurine (500 μM) in a cRNA-injected oocyte before and after exposure to DOG are shown in Fig. 1. The taurine-induced current was obtained as the difference in current in the presence and absence of taurine (Fig. 1A). This current (Fig. 1C, open symbols) was inward, increased with hyperpolarizing voltages, and was largest at the most negative voltage applied (−150 mV). After DOG incubation, the baseline or background current was unaffected (open symbols, Fig. 1B), but there was a 60% reduction in

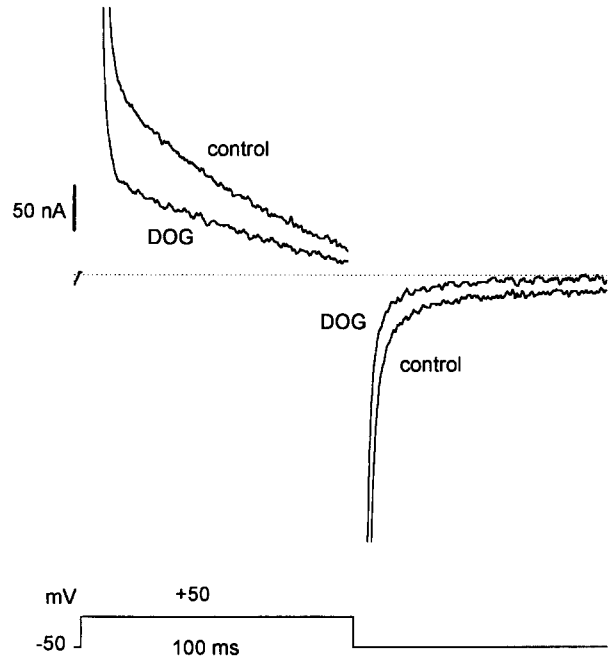

Fig. 2. Effect of DOG on presteady-state currents. Shown are the current relaxations after membrane voltage was stepped from −50 mV (V_h) to +50 mV (V_t) for 100 ms before (control) and after incubating the oocyte in 1 μM DOG. The current consisted of an initial capacitive transient with time constant $\tau = 1.2$ ms followed by a slower decay ($\tau = 49$ ms) to the steady state. The current relaxations were obtained from the total oocyte current by subtraction of the background steady-state currents and have been filtered at 300 Hz for display. The dashed line indicates the zero current level. The presteady-state currrent of the taurine transporter is isolated from the current relaxations by subtraction of the capacitive current as described in Section 2, and the charge transfer Q is obtained as the integral of the presteady-state transients. In this experiment, DOG treatment reduced maximum transport rate I_{max} by 54% and Q by 45%, but there were no differences in the relaxation time constants τ_{ON} and τ_{OFF} for the presteady-state current: they were 49 ± 1 (error of the fit) and 19 ± 1 ms before, and 49 ± 2 and 19 ± 2 ms, respectively, after DOG.

the steady-state current induced by taurine at every membrane potential (Fig. 1C, filled symbols). In 5 experiments, the maximal current (at −150 mV) induced by taurine I_{max} before and after incubation in DOG was 88 ± 17 and 35 ± 4 nA, a reduction of 56 ± 5% (Fig. 3).

Fig. 2 shows the current records in response to a step change in membrane voltage from the holding potential −50 to +50 mV in the absence of taurine. The voltage jump elicited a capacitive transient which decayed to the steady-state with a time constant $\tau \approx 1$ ms, and was followed by a slower decay ($\tau \cong 50$–70 ms) to the steady-state. This slower component was not observed in non-injected oocytes, and was abolished by addition of 500 μM taurine (not shown). The presteady-state current for both the ON and the OFF pulses were dramatically reduced after DOG treatment (Fig. 2). The charge transfer Q, the integral of the presteady-state current, was reduced 36 ± 4% ($n = 4$). The decrease in taurine transport due to activation of PKC was accompanied by a significant reduction in the oocyte membrane capacitance of 9 ± 1% from 414 ± 16 to 377 ± 19 nF ($n = 5$). DOG had no effect on the relaxation kinetics of the presteady-state currents. In 5 experiments, the relaxation time constant τ_{ON} (for a step jump in membrane voltage from −50 to +50 mV) was 60 ± 3 ms before, and 55 ± 3 ms after incubation with DOG; the time constant for the off-response τ_{OFF} (the return from +50 to −50 mV) was 26 ± 3 ms before, and 24 ± 2 ms after DOG.

3.2. PKA activation

The taurine-induced inward currents were markedly re-

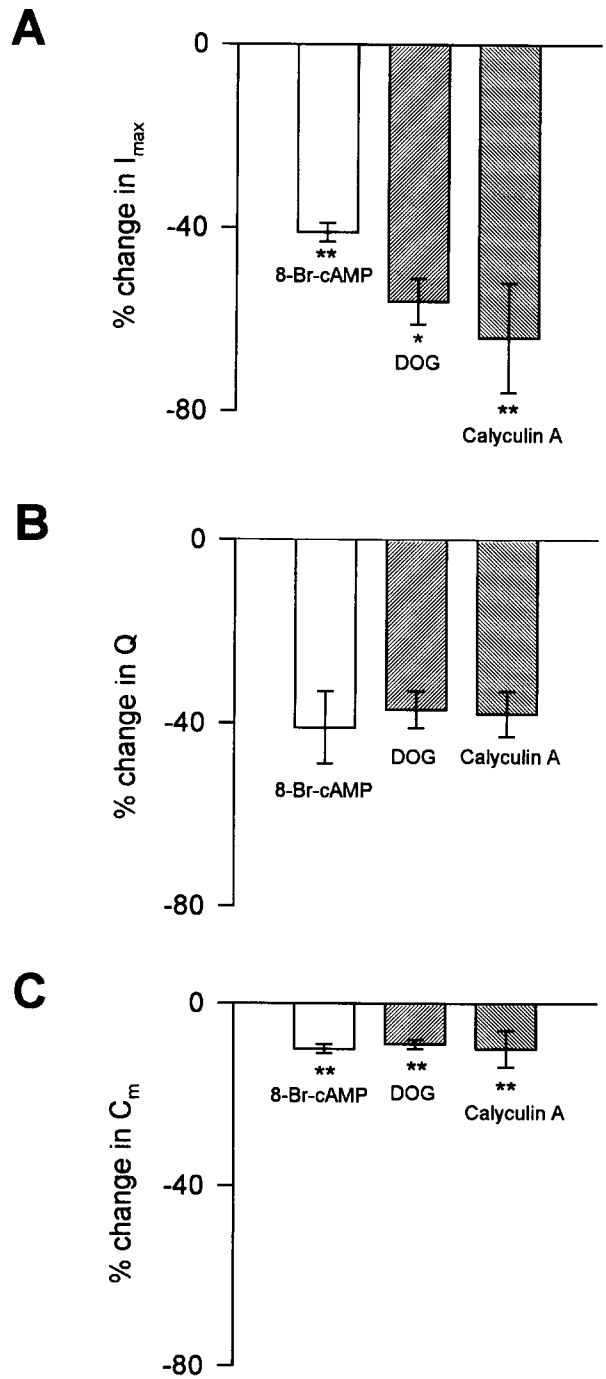

Fig. 3. Summary of the inhibition of the taurine transporter by different effectors of signalling pathways. (A) Inhibitory effect of 0.1 mM 8-Br-cAMP, 1 μM DOG and 1 μM calyculin A on I_{max} of the taurine transporter. (B,C) Similar effects for these three effectors on Q_{max} and C_m, respectively. Data are presented as mean values ± S.E.M., $n = 5$, except in B, where $n = 4$ for the effect of DOG on Q. Paired t-tests were performed on I_{max} and C_m before and after treatment by the different effectors, and (*) and (**) denote p values < 0.02 and 0.004, respectively. There was no significant difference between the percentage changes in Q and I_{max}.

duced after 8-Br-cAMP incubation (Fig. 3). I_{max} was inhibited from 92 ± 6 to 55 ± 4 nA ($n = 5$) with a concurrent reduction of the presteady-state charge transfer Q by 41 ± 8% ($n = 5$). Similar to DOG, the reduction in taurine-induced steady-state current was voltage independent, and there was no change in the presteady-state kinetics: in 5 experiments τ_{ON} and τ_{OFF} were 70 ± 4 and 21 ± 2 ms before, and 70 ± 6 and 24 ± 2 ms, respectively, after incubation in 8-Br-cAMP. The membrane capacitance of the oocyte was also reduced significantly by 10 ± 1% from 398 ± 19 to 362 ± 17 nF ($n = 5$).

3.3. Inhibition of protein phosphatases 1 and 2A by calyculin A

Calyculin A (1 μM) also resulted in 64 ± 12% inhibition of I_{max}, 38 ± 5% ($n = 5$) reduction in Q, and a reduction in the membrane capacitance by 10 ± 4%.

In non-injected control oocytes there was no measurable TAUT currents and no changes in membrane capacitance with short-term incubation of the oocytes in 1 μM DOG ($n = 3$), 0.1 mM 8-Br-cAMP ($n = 3$) or 1 μM calyculin A ($n = 3$).

4. Discussion

These electrophysiological experiments demonstrate that activators of PKA and PKC inhibit taurine transport by the cloned retinal Na/Cl/taurine transporter expressed in oocytes. The maximum rate of taurine transport, as judged by the current induced by saturating concentrations of taurine (I_{max}), was inhibited 40–60% within 30 min of adding membrane permeable activators of the kinases. The fact that calyculin A, a potent inhibitor of protein phosphatases 1 and 2A, also inhibited taurine transport within the same time scale (Fig. 3) strongly suggests that the effects of 8-Br-cAMP and DOG are indeed activating the protein kinases. Similar inhibitory effects of PKA and PKC on taurine transport have been reported in LLC-PK1, Caco-2 and HT-29 cells, and in astrocytes [10,19–21], but the mechanism is not understood.

Protein kinase C activation have also been recently shown to modulate the activity of other cloned cotransporters expressed in oocytes and HEK 293 cells [22–26]. The Na/P_i (NaP_i-2), Na/Cl/glycine (GLYT1b) cotransporters were inhibited [23,24] and the Na/Cl/GABA (GAT1) cotransporter was stimulated after activation of PKC [22]. The modulation of the GABA and glycine cotransporters resulted in changes in V_{max}, similar to our observations on the taurine transporter. Are protein kinase effects direct or indirect? All the evidence indicates indirect effects: (1) removal of the consensus PKC phosphorylation sites in GAT1, GLYC1b and NaP_i-2 by site-directed mutagenesis did not eliminate the effects of PKC activation [22–24]; (2) cell fractionation experiments indicate that PKC modulation caused a redistribution of GAT1 in oocytes between cytoplasmic compartments and the plasma membrane [22]; and (3) our charge transfer measurements with TAUT1 suggest that the PKA- and PKC-induced reductions in the V_{max} are due to a concomitant decrease in the number of cotransporters in the plasma membrane. The decrease in the number of plasma membrane transporters (Fig. 3) was also accompanied by a decrease in the membrane capacitance of the oocyte, strongly suggesting that the transporters are being internalized by endocytosis. Morphometric measurements have validated capacitance measurements as indications of the surface area of the oocyte, and changes in membrane capacitance as due to exo- and endocytosis [27,28].

Since activation of PKA and PKC had no effect on the presteady-steady kinetics (τ), the steady-state current-voltage curves of the taurine-induced currents, or the turnover number of the taurine transporter (I_{max}/Q_{max} [11]), this indicates that the kinetics of taurine transport have not been altered. Thus, the major effect of the activation of PKA and PKC is on the retrieval of the transporters from the plasma membrane. We cannot preclude a direct effect of protein kinases on the taurine transporter and this may account for some of the variations observed in the changes in maximum transport rate, number of transporters, and the area of the plasma membrane (Fig. 3) with PKA and PKC stimulation.

We conclude that the activity of the cloned retinal taurine cotransporter expressed in oocytes is inhibited by activation of protein kinase A and C, and that this occurs by a reduction of the number of cotransporters in the plasma membrane. While our capacitance measurements indicate that the decrease in number of transporters is due to endocytosis, it is not clear how the protein kinases regulate this process. The effect of a kinase depends on the transporter and the isoform being expressed in the oocyte: activation of PKC reduces the number of mouse taurine and rabbit glucose cotransporters, but increases the number of rat GABA and human glucose cotransporters in the oocyte plasma membrane (Fig. 3 [22,26]); arachidonic acid reduces the maximal rate of transport by the human Na/glutamate transporter (EAAT1) but stimulates transport by the human EAAT2 isoform [29]. The action of the kinases also depends on the cell type: PKC stimulates the rat GABA transporter expressed in oocytes but inhibits it HEK 293 cells [22,25]. The physiological significance of the differential modulation of cotransporter isoforms by kinases, and the dependence of the kinase action on the cell type are puzzling. Nevertheless, our results indicate that the kinases regulate the taurine transporter by regulating the number of transporters in the plasma membrane.

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