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REGULATION OF Na^+/GLUCOSE COTRANSPORTERS

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Summary

Na^+/glucose cotransporters (SGLTs) are expressed in the small intestine and the proximal renal tubule, where they play a central role in the absorption of glucose and galactose from food and the reabsorption of glucose from the glomerular filtrate. The regulation of intestinal sugar absorption occurs over two distinct time scales, one over days and the other over minutes. This review focuses on the mechanisms involved in the shorter-term regulation. Recent studies of the mouse intestine *in vitro* demonstrated that Na^+/glucose cotransport is increased two- to eightfold within minutes by the application of forskolin, an agent that increases intracellular cyclic AMP levels. Here we explore how cyclic AMP may upregulate Na^+/glucose cotransport. Our strategy was to express cloned SGLT1s in *Xenopus laevis* oocytes and then use electrophysiological methods to measure (i) the kinetics of Na^+/glucose cotransport, (ii) the number of cotransporters in the plasma membrane, and (iii) the net rate of exo- and endocytosis before and after activation of protein kinases. To evaluate the role of cotransporter phosphorylation, we have examined the effect of protein kinase activation on various SGLT1 isoforms and other cotransporters. In oocytes expressing rabbit SGLT1, the activation of protein kinase A (PKA) increased the maximum rate of Na^+/glucose cotransport by 30 %, and the activation of protein kinase C (PKC) decreased the maximum rate of transport by 60 %. Changes in maximum transport rates were accompanied by proportional changes in the number of cotransporters in the plasma membrane and by changes in the area of the membrane. We conclude that PKA and PKC regulate rabbit SGLT1 activity by modulating the number of cotransporters in the plasma membrane and that this occurs through regulation of exocytosis and endocytosis. Given the size of intracellular transport vesicles containing SGLT1, 100–120 nm in diameter, and the density of cotransporters in these vesicles, 10–20 per vesicle, we estimate that the net rate of SGLT1 vesicle exocytosis is about 10 000 s^{-1} and that this rate increases 100-fold after activation of PKA. The effect of PKA is independent of the presence or absence of consensus sites for phosphorylation on SGLT1. Surprisingly, the effects of PKA or PKC depend critically on the sequence of the cotransporter being expressed in the oocyte, e.g. activation of PKC inhibited rabbit and rat SGLT1, but stimulated human SGLT1. This dependency suggests that the regulation of vesicle trafficking by protein kinases depends upon the structure of the cotransporter expressed in the oocyte. Similar considerations apply to other classes of cotransporters, such as the neurotransmitter and dipeptide cotransporters. Our working hypothesis is that the regulation of cotransporter expression by protein kinases occurs largely by regulated exo- and endocytosis, and that the effect of the protein kinases is indirect and determined by critical domains in the cotransporter.

Key words: Na^+/glucose symporter, protein kinase A, protein kinase C, cotransporters, *Xenopus laevis* oocytes, vesicle trafficking.

Introduction

Two different types of processes are involved in the short-term regulation of membrane transport proteins. One involves the regulation of the number of transporters in the plasma membrane; examples include the regulation of glucose transporters (GLUT4) in fat and muscle cells by insulin and the regulation of water channels (CHIP28) in renal collecting tubules (see Bradbury and Bridges, 1994). The other involves the activity of the transporters already in the plasma membrane; one example is the activation of the brain glutamate cotransporter (GLT-1) by phosphorylation (Casado *et al.* 1993). Both processes are thought to involve activation of protein kinases.

Cotransporters are an important class of membrane transport proteins that are responsible for the accumulation of many ions and molecules in cells. Although cotransporters are known to contain consensus sites for phosphorylation by protein kinases (Wright *et al.* 1992) and to be regulated by protein kinases, there is little information about the mechanisms of regulation. To remedy this situation, we have studied the effects of protein kinases A and C on the activity of cloned cotransporters

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expressed in *Xenopus laevis* oocytes (Hirsch *et al.* 1996; Loo *et al.* 1996).

The Na^+/glucose cotransporter

The Na^+/glucose cotransporter (SGLT1) is arguably the premier member of the cotransporter family (Wright *et al.* 1994). The cotransporter is mainly expressed in the brush-border membrane of the small intestine where it is responsible for the absorption of glucose and galactose from food. The uphill transport of sugar is coupled to Na^+ transport down its electrochemical potential gradient across the plasma membrane. SGLT1 is a member of a growing gene family of Na^+ cotransporters expressed in both bacteria and animal cells; family members are responsible for the transport of sugars, amino acids and anions into cells. The genes code for proteins containing 482–718 amino acid residues, and secondary structure analysis suggests that SGLT1 consists of 14 transmembrane α-helices (Turk *et al.* 1996). The SGLT1 secondary structure model is shown in Fig. 1 with both the N- and C-terminal residues facing the extracellular side of the plasma membrane. On the basis of consensus sequences (Kennelly and Krebs, 1991), SGLT1 contains a number of potential protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites: there are five consensus PKC sites in rat and human and four in rabbit; and one PKA site in rabbit and human, and none in rat (Fig. 1).

The transport properties of rabbit, rat and human SGLT1s are very similar (Hirayama *et al.* 1996): at membrane potentials between −70 and −150 mV, the K_m for glucose (α-methyl-D-glucopyranoside) is between 0.2 and 0.5 mmol l^{-1}, and the K_m for Na^+ is between 2 and 7 mmol l^{-1}. Phlorizin is a very specific, competitive inhibitor of Na^+/glucose cotransport by all three isoforms, and the K_i ranges from 10 nmol l^{-1} for rat to 1 μmol l^{-1} for rabbit SGLT1.

Expression of cotransporters in oocytes

The *Xenopus* oocyte expression system has proved to be very powerful in the study of cloned membrane proteins such as the cotransporters. It is relatively simple to inject cRNA into these large cells and to measure transporter activity by radioactive tracer and electrophysiological techniques. In the case of SGLT1, the rate of Na^+/glucose transport into oocytes injected with SGLT1 cRNA is usually 500–1000 times greater than that into non-injected oocytes (Hediger *et al.* 1987). Furthermore, electrophysiological methods have been developed to obtain a complete kinetic profile of a cloned transporter in a single oocyte (e.g. Umbach *et al.* 1990; Parent *et al.* 1992). For example, the K_m for sugar and the maximum rate of transport, I_{max} or J_{max}, are obtained by measuring the sugar-induced inward Na^+ currents as a function of sugar concentration. In the same oocyte, the number of cotransporters expressed in the plasma membrane is obtained from SGLT1 charge measurements in the absence of sugar (Loo *et al.* 1993; Zampighi *et al.* 1995). The maximum amount of charge movement, Q_{max}, is proportional to the number of cotransporters, n, by the relationship $n=Q_{max}/(ze)$, where z is the valence of the protein (3.5) and e is the elementary charge. The ratio J_{max}/Q_{max} is then simply the turnover number of the transporter.

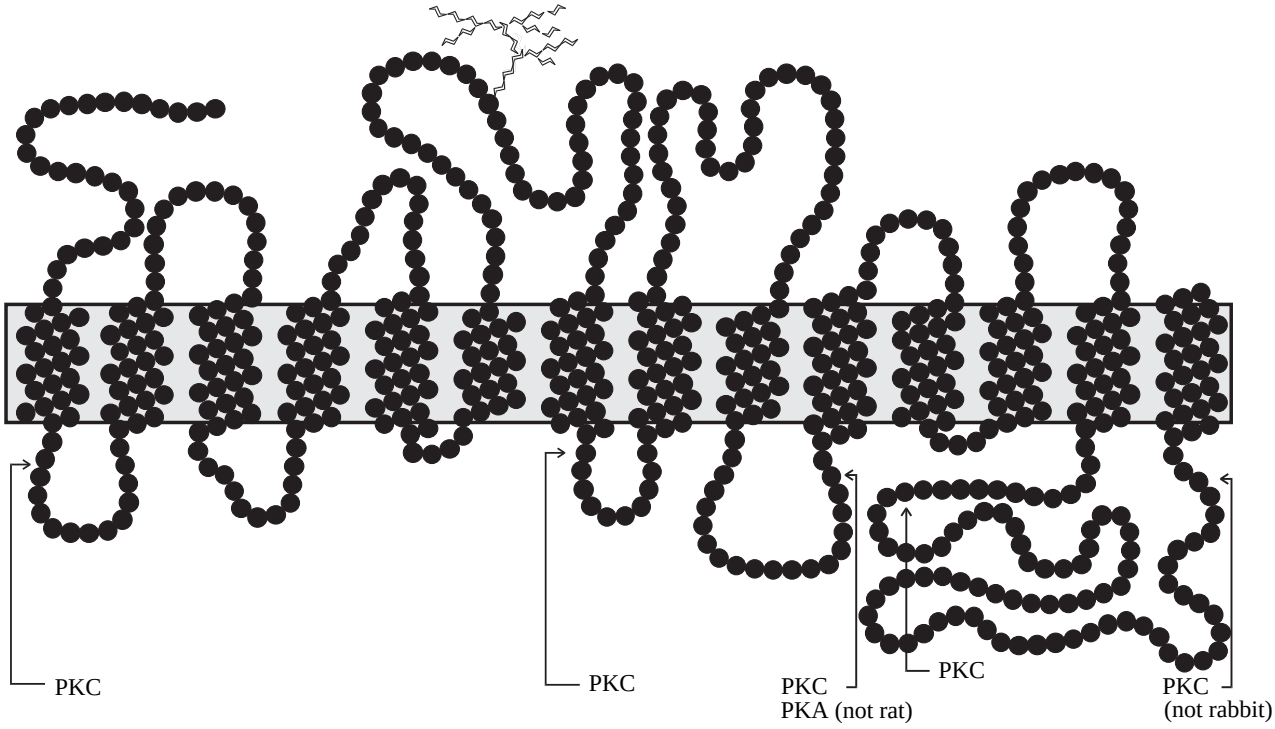

Fig. 1. A secondary structure model of SGLT1 (Turk *et al.* 1996) showing the location of the putative protein kinase C (PKC) and protein kinase A (PKA) phosphorylation sites.

In a recent study, we have shown that the expression of SGLT1 in *Xenopus* oocytes increased linearly from 1 to 14 days after injection of rabbit SGLT1 cRNA (Hirsch *et al.* 1996): I_{max} increased linearly to 2500 nA per oocyte and Q_{max} to 150 nC. The turnover number, I_{max}/Q_{max}, was 20 s^{-1}. At 6 days, the number of cotransporters in the plasma membrane was 1×10^{11}. This number corresponds to an insertion rate of 250 000 transporters s^{-1} into the membrane, and this rate can be observed directly using freeze–fracture electron microscopy (Zampighi *et al.* 1995). Fig. 2 shows the increase in density of the 7.5 nm diameter SGLT1 intramembrane particles in the P-face of the oocyte plasma membrane with the increase in SGLT1 expression.

Associated with the increase in expression of SGLT1 in the oocyte plasma membrane, there is an increase in the electrical capacitance of the oocyte (Hirsch *et al.* 1996). Over 14 days, the oocyte capacitance increased from 300 to 1250 nF. Given that the specific capacitance of biological membranes is 1 μF cm^{-2}, this value is equivalent to an increase in plasma membrane area of 60 $\mu m^2 s^{-1}$. Morphological studies have confirmed that oocyte capacitance accurately reflects the plasma membrane area (Zampighi *et al.* 1995; Isom *et al.* 1995; Vasilets *et al.* 1990) There was no increase in the capacitance of control oocytes with time.

A correlation between the change in number of plasma membrane cotransporters and the change in plasma membrane area is expected for a process where transporters are inserted and retrieved from the plasma membrane by exo- and endocytosis. Thin-section electron micrographs of oocytes show a high density of 120 nm diameter vesicles close to, and attached to, the plasma membrane (Fig. 3A,B). Fusion of 1500 of these vesicles per second with the plasma membrane would account for the increase in plasma membrane area (60 $\mu m^{-2} s^{-1}$). However, to account for the rate of cotransporter insertion (250 000 s^{-1}), each vesicle would need to contain 200 cotransporters. In fact, there are only about 20 SGLT1 particles per vesicle (Fig. 3C,D), a number close to the maximum expected for 75 kDa membrane proteins in a 120 nm vesicle. This number suggests that the rate of delivery of transport vesicles to the plasma membrane, by exocytosis, is an order of magnitude higher, 10 000 s^{-1}, than the net rate of vesicle delivery. Although this rate appears high, it should be recalled that the area of the oocyte is greater than 30×10^6 μm^2.

Regulation of cotransport

To study the role of protein kinase A (PKA) and protein kinase C (PKC) in the regulation of cotransporters in oocytes, we measured (i) transporter kinetics, (ii) the number of cotransporters in the membrane, and (iii) the area of the plasma membrane before and after exposure of single oocytes to membrane-permeable activators of PKA and PKC (Hirsch *et al.* 1996). We used 8-bromo-cyclic-AMP (8-Br-cAMP) to activate PKA, *sn*-1,2-dioctanoylglycerol (DOG) to activate PKC, and calyculin A to block the dephosphorylation of serine and threonine residues targeted by PKA and PKC. All of these agents produced their effects on oocytes expressing SGLT1 within 30 min. The effects were reversible and the agents showed no effects on the membrane potential, input impedance or capacitance of control oocytes.

8-Br-cAMP increased the maximum rate of Na^+/glucose cotransport by 27±9 % (mean ± S.E.M., N=3) and DOG decreased the maximum rate of transport by 62±5 % within minutes. These effects were sustained for at least 2 h (Fig. 4). The effect of 8-Br-cAMP is consistent with the effect of forskolin on mouse small intestine (Grubb, 1995). The activation of PKA and PKC did not produce any measurable change in any other kinetic property of SGLT1, including the

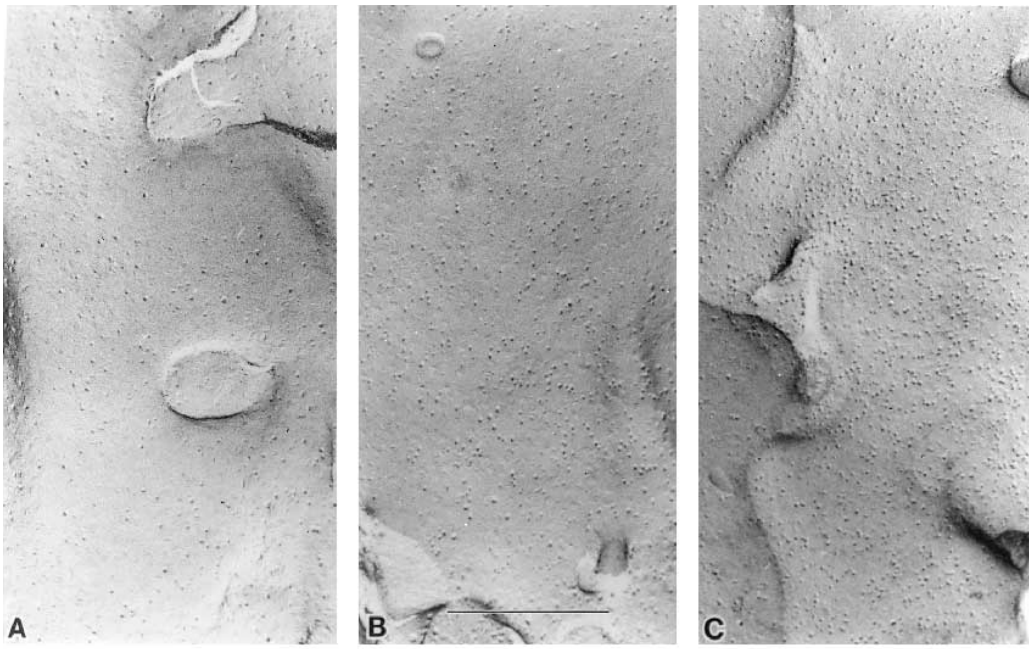

Fig. 2. The density of rabbit SGLT1 intramembrane particles in the P-face of the plasma membrane from *Xenopus laevis* oocytes injected with SGLT1 cRNA. The density of intramembrane particles increased with the level of expression. In A, where Q_{max} was 4 nC, the density of SGLT1 particles was 210±55 μm^{-2}; in B, where Q_{max} was 11 nC, the density was 556±108 μm^{-2} (N=12); and in C, where Q_{max} was 16 nC, the density was 655±67 μm^{-2} (N=8). In all cases, the density refers to the increase in density above the control value of 212±48 μm^2 (N=14). Magnification, 10 000×; scale bar, 0.3 μm. Taken with permission from Zampighi *et al.* (1995).

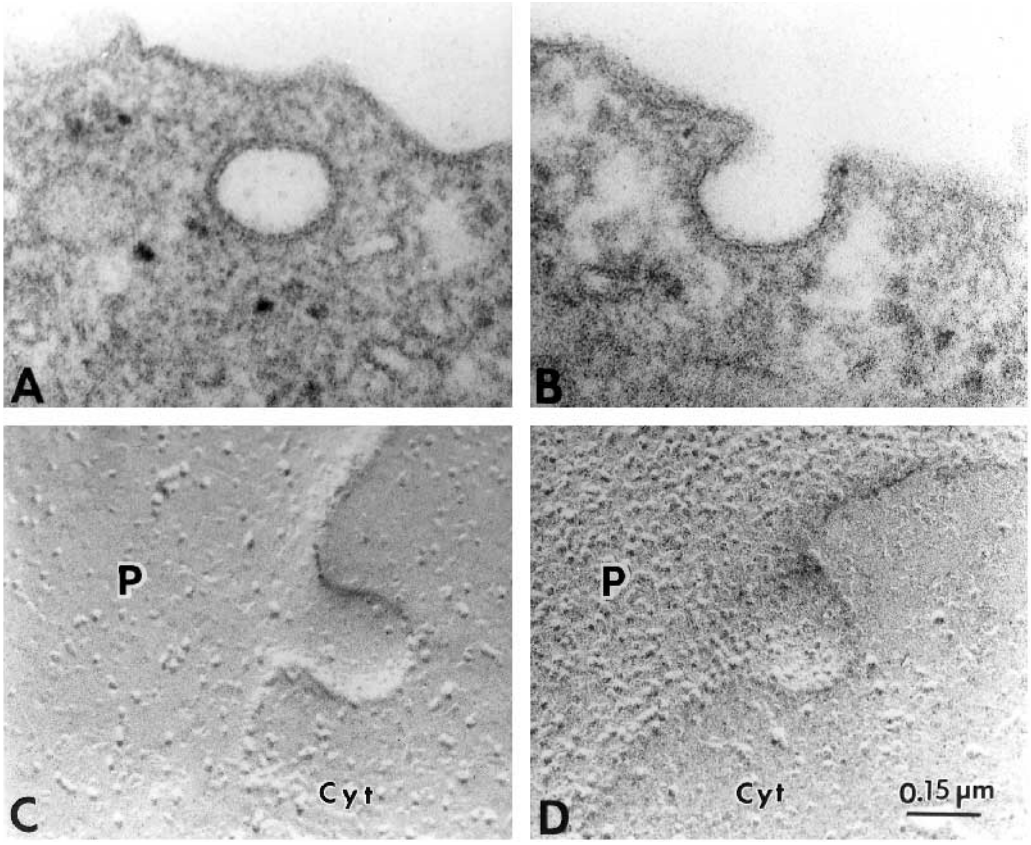

Fig. 3. Thin-section electron micrographs (A,B) and freeze–fracture electron micrographs (C,D) showing membrane vesicles in *Xenopus laevis* oocytes expressing rabbit SGLT1. A and B show 120 nm diameter vesicles approaching and fusing with the plasma membrane, and C and D show the P-face (P) of similar vesicles containing 7.5 nm diameter SGLT1 intramembrane particles (see also Fig. 2). Cyt, cytoplasm. Magnification, 150 000×; scale bar, 0.15 µm.

K_m for glucose, the K_i for phlorizin or the turnover number. This result suggested that the changes in maximum rate were due to changes in the number of transporters. This conclusion was confirmed by the Q_{max} measurements: 8-Br-cAMP increased Q_{max} by 19±7 % and DOG decreased Q_{max} by 41±4 %. A plot of the change in maximum rate against the change in number of transporters in this series of experiments was linear with a slope of 0.9±0.1. These effects of 8-Br-cAMP and DOG on the rate of transport and the number of transporters in the plasma membrane were accompanied by proportional changes in plasma membrane area. We conclude that PKA and PKC regulate Na^+/glucose transport by regulating the number of cotransporters in the plasma membrane, and that the mechanism of this regulation is exocytosis and/or endocytosis.

Additional insight into the regulatory process is gained by quantitative considerations. For example, the 30 % increase in transport caused by 8-Br-cAMP is produced by the insertion of approximately 2.5×10^{10} new SGLT1 transporters into the plasma membrane within 30 min, or $1\times10^7\ s^{-1}$. This rate is equivalent to a 100-fold increase above the constitutive insertion rate of protein insertion. Furthermore, these results suggest that there is an intracellular pool of vesicles containing SGLT1 that are ready and available for regulated insertion into the plasma membrane. Similar considerations apply to the inhibition of Na^+/glucose transport by DOG.

Similar increases in Na^+/glucose transport were obtained with 8-Br-cAMP on oocytes expressing human and rat SGLT1s (Fig. 5) even though there are no consensus PKA sites in the rat sequence (Fig. 1). This relationship suggests that phosphorylation at PKA consensus sites is not required for regulation. A different result was observed with activation of PKC (Fig. 5): whereas DOG inhibited transport for both rabbit

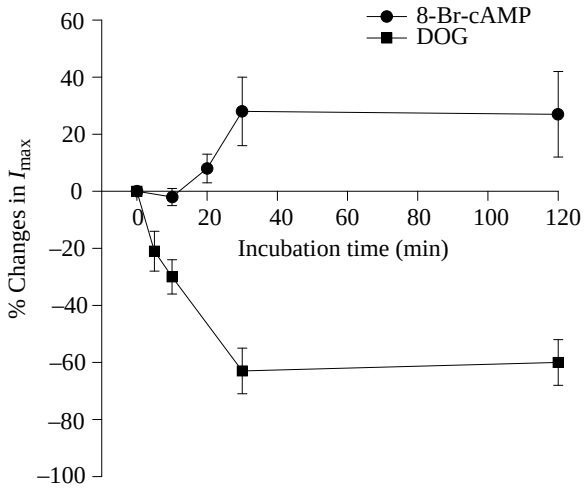

Fig. 4. The effect of 8-Br-cAMP and DOG on the maximum rate of transport by rabbit SGLT1 expressed in *Xenopus laevis* oocytes. The changes in maximum transport rates (I_{max}) after incubation of oocytes with 0.1 mmol l^{-1} 8-Br-cAMP or 1 µmol l^{-1} DOG for 5 –120 min are shown. The control value of the maximum sugar-induced current in this batch of oocytes was 1670±200 nA. Taken with permission from Hirsch *et al.* (1996). Values are means ± S.E.M., N=3.

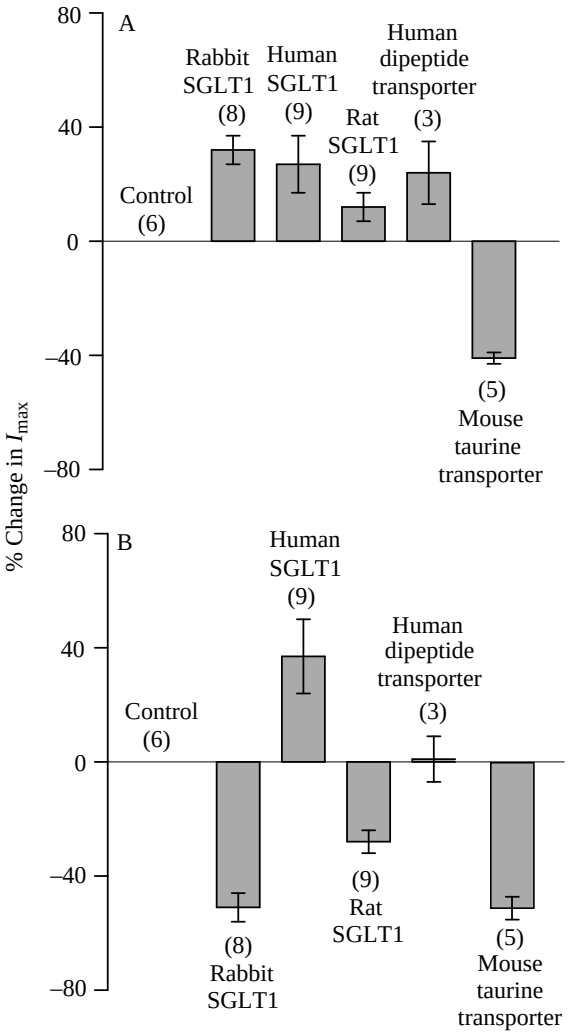

Fig. 5. Effect of 8-Br-cAMP (A) and DOG (B) on the maximum rates of transport (I_{max}) of rabbit SGLT1, human SGLT1, rat SGLT1, human dipeptide transporter (PEPT1) and mouse taurine transporter (TAUT1) expressed in *Xenopus laevis* oocytes. The currents produced by saturating substrate concentrations were recorded at –150 mV before and after treatment of oocytes with 0.1 mmol l^{-1} 8-Br-cAMP or 1 μmol l^{-1} DOG. Data from Hirsch *et al.* (1996), Loo *et al.* (1996) and J. R. Hirsch, D. D. F. Loo and E. M. Wright (unpublished results). Values are means ± S.E.M.; values of *N* are given in parentheses.

and rat SGLT1 isoforms, DOG *stimulated* transport by 40 % by human SGLT1.

Regulation of other cotransporters

We have extended our studies to other types of cotransporters to determine the generality of our observations with SGLT1. The results show that the kinase activators also regulate the activity of other cotransporters by regulating the number of cotransporters in the oocyte plasma membrane. However, the direction of the effect, stimulation or inhibition, was unpredictable. The human H^+/dipeptide transporter (hPEPT1) was activated by 8-Br-cAMP, but there was no effect of DOG (J. R. Hirsch, unpublished observations). In contrast, activation of PKA and PKC both inhibited transport by the mouse Na^+/Cl^-/taurine cotransporter (TAUT1) by 40–50 % (Fig. 5) (Loo *et al.* 1996).

As in the case of rabbit SGLT1, changes in the maximum rates of transport were accompanied by proportional changes in both the number of transporters and the area of the oocyte plasma membrane. However, there was no change in area with 8-Br-cAMP in oocytes expressing rat SGLT1. This difference is perhaps not surprising in view of the small increases in transport rate and the number of cotransporters (12 %) and the fact that the rates of exo- and endocytosis are estimated to be an order of magnitude higher than the net rate of vesicle insertion or retrieval (see above).

Further evidence that 8-Br-cAMP and DOG act through PKA- and PKC-mediated phosphorylation events is provided by the effects of calyculin A on oocytes expressing human SGLT1 or mouse TAUT1 (Hirsch *et al.* 1996; Loo *et al.* 1996). This specific inhibitor of phosphatases 1 and 2A, which dephosphorylates PKA and PKC threonine and serine residues, mimicked the effects of 8-Br-cAMP and DOG on the maximum rate of transport, the number of transporters and the area of the oocyte.

Others groups using different techniques have shown that activation of PKC modulates the activity of cloned cotransporters expressed in heterologous expression systems, and their results are consistent with the conclusions presented here. The Na^+/phosphate cotransporter (NaPi-2) expressed in oocytes (Hayes *et al.* 1995) and the Na^+/Cl^-/glycine cotransporter (GLYT1b) expressed in HEK 293 cells (Sato *et al.* 1995) were both inhibited by activation of PKC, whereas the Na^+/Cl^-/GABA cotransporter (GAT1) expressed in oocytes (Corey *et al.* 1994) was stimulated by PKC. The maximum rates of γ-aminobutyric acid (GABA) and glycine transport were modified by PKC, and the removal of the consensus PKC phosphorylation sites in GAT1, GLYT1b and NaPi-2 by site-directed mutagenesis did not eliminate the effects. In the case of the GABA transporter, cell fractionation experiments provided evidence that stimulation of PKC caused a redistribution of GABA transporters from an intracellular compartment to the plasma membrane (Corey *et al.* 1994). On the basis of these parallel findings, it is reasonable to suggest that the cloned cotransporters are regulated in oocytes and HEK 293 cells by similar mechanisms, and that this occurs by regulated exo- and endocytosis. A question yet to be resolved concerns the site of action of the protein kinases. For example, does PKA activation increase the rate of delivery of SGLT1 transport vesicles to the plasma membrane or decrease the rate of SGLT1 retrieval from the plasma membrane? This question may be answered in the future by recording the expected unitary changes in membrane capacitance produced by vesicle fusion and retrieval.

These experiments do not preclude direct effects of PKA and PKC on cotransporters. In fact, there is strong evidence that one neurotransmitter cotransporter, the glutamate (GLT-1) cotransporter (Casado *et al.* 1993), is phosphorylated by PKC and that phosphorylation increases the rate of transport.

Furthermore, mutation of the serine residue to asparagine in the only PKC consensus phosphorylation site abolished the stimulation of transport by phorbol ester. Similar experiments with SGLT1, and with the other cotransporters studied here, will need to be conducted to address this question. However, the effects we have described here are not correlated with the presence of consensus phosphorylation sites, and the expected changes in kinetic parameters, e.g. turnover numbers, due to phosphorylation were not observed.

Conclusions

Studies on several cloned cotransporters expressed in *Xenopus* oocytes demonstrate that activation of PKA and PKC with membrane-permeable reagents regulates the functional expression of the transporters. The changes in maximum rates of transport are proportional to the changes in number of cotransporters in the plasma membrane, and changes in membrane area suggest that the number of cotransporters expressed is determined by the rates of exocytosis and endocytosis. The combination of biophysical and structural techniques suggests that rabbit SGLT1 is delivered to the plasma membrane by 120 nm diameter transport vesicles containing about 20 SGLT1 cotransporters. The basal rate of insertion, about 10 000 vesicles s^{-1}, is increased by 8-Br-cAMP to about 100 000 s^{-1}. Similar results were obtained with human and rat SGLT1 and with mouse TAUT1. There was no correlation between the effect of 8-Br-cAMP and the presence of consensus PKA phosphorylation sites on the transporter. These studies indicate that, in oocytes at least, the regulation of cotransporters by PKA and PKC occurs by the regulation of cotransporter trafficking between an intracellular compartment and the plasma membrane.

A surprising outcome of these experiments is the finding that the effect of PKA and PKC depends critically on the cotransporter expressed: activation of PKA increased the maximum rate of transport by all three SGLT1 isoforms and the human H^+/dipeptide cotransporter, but decreased the rate of transport by mouse TAUT1; while activation of PKC reduced transport by rabbit SGLT1, rat SGLT1 and mouse TAUT1, had no effect on human PEPT1 and increased transport by human SGLT. There are several important implications of these results. One is that the nature of the cargo (cotransporter) determines the effect of PKA and PKC on exocytosis and endocytosis. This conclusion is reinforced by recent studies on human SGLT1 mutants (J. R. Hirsch, unpublished data), showing that the responses to PKA and PKC can be radically changed by replacement of single residues, e.g. replacing Ala166 in the third extracellular hydrophilic loop completely reverses the effect of PKC. The implication is that flag sequences on the cotransporter determine the interactions with the chaperones involved in regulating exocytosis and endocytosis. Another major implication is that the transport vesicles only contain one type of cargo, i.e. since the trafficking of rabbit and human SGLT1s between intracelluar vesicles and the plasma membrane is regulated in opposite directions by PKC, it follows that each isoform is carried by its own private transport vesicle. This deduction can be tested by the co-expression of two cotransporters, e.g. rabbit SGLT1 and mouse TAUT1, and determining the effect of 8-Br-cAMP on functional expression. PKA activation should increase glucose-induced currents by 30 % and simultaneously reduce taurine-induced currents by 40 %.

What is the machinery responsible for the trafficking of cotransporters in oocytes? We anticipate that the molecular mechanisms are very similar to those for other cells. In both neuronal and non-neuronal cells, it is clear that vesicles for the transport of materials between the *trans*-Golgi apparatus and the plasma membrane are directed by molecules containing unique addresses and zip-codes, SNAPS and SNARES (see Rothman, 1994; Südhof, 1995). In addition, small GTP-binding proteins of the Ras family play essential roles. The recent experiments of Schmalzing *et al.* (1995) provide the most pertinent information that is available on the recycling of Na^+ pumps between an intracellular compartment and the plasma membrane in oocytes. These authors provide compelling evidence that the GTP-binding protein RhoA is an essential component of a PKC-regulated endocytotic pathway. It will certainly be interesting to see whether these observations can be extended to the cloned cotransporters.

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