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# Sorbitol Uptake in Plasma Membrane Vesicles Isolated from Immortalized Rabbit TALH Cells: Activation by a $Ca^{2+}$/Calmodulin-dependent Protein Kinase

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**Abstract.** Apical plasma membrane vesicles were isolated from cultures of immortalized thick ascending limb of Henle's loop (TALH) cells and sorbitol uptake was investigated using a rapid filtration technique. In the presence of $Mg^{2+}$, $Ca^{2+}$, ATP, and GTP sorbitol equilibrated within three minutes with the intravesicular space; this uptake was reduced by 75% when the incubation temperature was decreased from 37°C to 4°C. A lower level of uptake was also observed in the presence of 100 μM quinidine and when $Ca^{2+}$ or ATP were omitted from the medium. Membranes preincubated with $Mg^{2+}$, $Ca^{2+}$, ATP, and GTP showed, however, a high sorbitol uptake in ATP-free medium. Staurosporine, but only at high concentrations of 200 nM, inhibited sorbitol uptake when present during the transport experiments or during the preincubation with ATP. Similar results were obtained with 1 μM trifluoperazine. Protein kinase C inhibitory peptide was ineffective whereas 20 nM KT 5926, at low concentrations a specific inhibitor of $Ca^{2+}$/calmodulin-dependent kinase, attenuated the activation. On the basis of these data we suggest that a $Ca^{2+}$/calmodulin-dependent kinase is a mediator of regulation of sorbitol plasma membrane permeability in renal medullary cells.

**Key words:** Thick ascending limb of Henle's loop — Sorbitol transport — Organic osmolytes — Cell volume regulation — $Ca^{2+}$/calmodulin-dependent kinase

## Introduction

During recent years the sugar alcohol sorbitol has been recognized to play an important role in the volume homeostasis of renal medullary cells (Garcia-Pérez & Burg, 1991; Burg, 1994; Kinne et al., 1996). The nature of membrane permeation of sorbitol and its mode of activation are hitherto only poorly understood. In a series of pivotal papers the group of K.R. Spring, using papillary epithelium cells kept in culture, could demonstrate that exposure of the cells to hypotonicity increased both sorbitol efflux and influx, that influx and efflux were stereoselective, but that even at concentrations up to 315 mM no saturation of uptake could be found nor could a competition between substrates be observed (Siebens & Spring, 1989; Garty et al., 1991). They coined the term 'sorbitol permease' for this mechanism which appears to combine properties of a classical carrier with those of a channel. This group also observed that the general inhibitor of arachidonic acid metabolism ETYA (5,8,11,14-eicosa-tetraynoic acid) inhibited the activation of the permease (Napathorn & Spring, 1994). In freshly isolated inner medullary collecting duct cells of rat, the activation of sorbitol efflux was found to be closely related to the level of intracellular calcium, and membrane recycling was invoked in the regulation of permeability (Czekay, Kinne-Saffran & Kinne, 1994; Tinel, Wehner & Sauer, 1994; Mooren & Kinne, 1994; Kinne et al., 1996).

Studies on tonicity-regulated channels are complicated by the variety of organic osmolyte transporters and the diversity of signal transduction pathways used in their regulation (Kinne et al., 1996). We, therefore, opted to approach this question in a simplified system, isolated plasma membranes derived from cells which as described recently (Kinne-Saffran, Pfaff & Kinne, 1995; Eckstein & Grunewald, 1996) predominantly use sorbitol for the maintenance of their cellular volume. The studies presented here demonstrate that a $Ca^{2+}$/calmodulin-dependent protein kinase controls sorbitol permeability in renal medullary cell plasma membranes in vitro. The correlation between cell volume regulation and protein kinase activation remains to be investigated in the future.

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## Materials and Methods

### Chemicals and Reagents

$^3$H-sorbitol (15–20 Ci/mM) was obtained from DuPont de Nemours, NEN Product Division (Brussels, Belgium) and later from American Radiolabeled Chemicals (St. Louis, MO). KT 5926 was purchased from Calbiochem-Novabiochem Int. (La Jolla, CA). The ATP monitoring assay kit was provided by Bio-Orbit (Turku, Finland). All other chemicals were from Sigma (Deisenhofen, Germany) and were of highest purity commercially available. For low speed centrifugations a Sorvall RC 5B and a SS 34 rotor were employed, for high speed centrifugation a Sorvall OTD 65B and an AH 627 swingout rotor were used.

### Cell Line and Culture Procedure

TALH cells were isolated from kidneys of New Zealand white rabbits as described in detail by Eveloff et al. (1980). Immortalized clones were obtained by cotransfecting these cells with DNA from plasmid pSV2neo plus that from PSVE and PSVE2 which contain the early regions of SV40 virus (Scott et al., 1987; Scott et al., 1989; MacDonald et al., 1991). The immortalized TALH cell line was maintained as a monolayer culture in Minimum Essential Medium with Dulbecco's salts (DMEM; Gibco BRL-Life Technologies, Eggenstein, Germany) with 4.5 g/l D-glucose and supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1% L-glutamine, 1% nonessential amino acids, 1% pyruvate, 0.1% β-mercaptoethanol, $10^{-7}$ M/l vasopressin and $10^{-7}$ M/l calcitonin. Cells were routinely cultured in 75 cm$^2$ Falcon 3111 flasks (Becton Dickinson, Heidelberg, Germany) at 37°C and 7.5% $CO_2$. Confluent cultures were trypsinized with phosphate buffered saline (PBS) containing 0.25% trypsin and 0.2% EDTA. For experiments, cells were seeded in 75 cm$^2$ Falcon 3111 flasks at a density of approximately $2 \times 10^6$ cells/flask. The medium was changed every other day. The cells were allowed to grow for 5 days (300 mOsm/kg) by which time they had reached confluency. The cells were then used for preparation of a microsomal plasma membrane fraction or uptake measurements as described below.

For culture on filters $2 \times 10^5$ cells were seeded on cell culture inserts (ø 25 mm, pore size 0.4 μ; Falcon, #3090) and placed in a 6-well plate designed for these inserts (Falcon, #3502). As above, 2 ml of culture medium were added to both sides of the inserts. Osmolality of the medium was elevated to 600 mOsm/kg by addition of 150 mM NaCl. Culture medium was changed after 3 days and then every second day until confluency was reached (8–10 days of culture).

### Organic Osmolyte Efflux Experiments

To determine the location of the sorbitol transporter efflux experiments under hypotonic shock were performed with confluent cell cultures. Medium was removed and the cell layers were rinsed twice with 600 mOsm/kg PBS and then exposed to 1 ml of 300 mOsm/kg PBS for 10 min added to both sides of the culture inserts. After 10 min PBS was removed from the upper and lower compartment and examined for its sorbitol content (*see below*). To determine the remaining intracellular sorbitol the cells were extracted with 6% $HClO_4$.

### Determination of Sorbitol with HPLC

Sample preparation for HPLC on a Sugar Pak 1-column was carried out according to Wolff et al. (1989). Supernatants of the samples were passed through Alumina A light cartridges (Waters, Eschborn, Germany) to remove salts and acids and were then filtered through a 0.45 μM acrodisc (Gelman Sciences, Rossdorf, Germany). The cell extracts in 6% $HClO_4$ were neutralized with 5 M KOH. For delipidation these samples were measured with a Waters HPLC equipment consisting of pump (510), autosampler (717 plus), differential refractometer (410) and the Milennium 2010 software. The column oven was from LKB (Freiburg i.Br., Germany). A sugar Pak 1-column (6.5 × 300 mm; Waters) was used as stationary phase and HPLC grade water with 50 mg/ml calcium. 50 μl of each sample were injected. Appropriate standards in the range of 0.01-1 mM were used for calibration.

### Preparation of the Microsomal Membrane Fraction

Cells from passages #32–50 were rinsed once with PBS. Ice-cold ST-buffer (250 mM sucrose, 10 mM triethanolamine, pH 7.4 adjusted with $H_2SO_4$) was added and the cells were harvested with the help of a rubber policeman. Cells from 5 culture flasks were then pelleted at 1,500 × *g* for 10 min in a cooled cell centrifuge (Beckman, model GS 6KR). The supernatant was replaced by 5 ml fresh ST-buffer and the cells were frozen overnight at −70°C. For preparation of the microsomal plasma membrane fraction the frozen cell pellets were rapidly thawed at 37°C, put back on ice and immediately homogenized 20 times in a tight fitting Dounce homogenizer. After an interval of 2 min the homogenization was repeated once. Membranes were prepared by differential centrifugation at 4°C. After a centrifugation for 10 min at 700 × *g*, the supernatant was centrifuged at 16,000 × *g* for 20 min. The resulting supernatant was saved and the sediment was resuspended in 10 ml ST-buffer by homogenization in a lose fitting Dounce homogenizer and spun for 20 min at 16,000 × *g*. This procedure was repeated and all resulting supernatants were combined and centrifuged for 1 hr at 100,000 × *g*. The pellet was suspended in vesicle buffer containing 100 mM sucrose and 20 mM HEPES (N-2-hydroxethylpiperazine-N′-2-ethanesulfonic acid) (pH 7.4 with Tris [hydroxyaminomethane]) by repeated passage through a 26-gauge needle and used immediately for transport studies.

### Determination of Enzyme Activities and Protein Content

The microsomal plasma membrane fractions were assayed for the marker enzymes ADPase, Na,K-ATPase, acid phosphatase, and succinate dehydrogenase by previously described methods (König, Ricapito & Kinne, 1983). Protein content was determined by the method of Lowry et al. (1951) after precipitation of the membranes with 10% trichloroacetic acid, using bovine serum albumin as standard.

### Transport Studies

Sorbitol uptake into plasma membrane vesicles was determined by a rapid filtration technique as described previously (Hopfer et al., 1973). The incubation medium contained generally (in mM): 100 sucrose, 100 KCl, 0.1 sorbitol, and 20 HEPES, adjusted with Tris to pH 7.4. Differences in the condition of incubation media and/or preincubation steps are indicated in the figure legends and the description of tables. The uptake was initiated by adding 20 μl of membranes kept on ice to 130 μl of incubation medium containing 15 μCi of $^3$H-sorbitol. Incubation took place either at 37°C or at 4°C. The transport reaction was terminated at timed intervals by removal and rapid dilution of 20 μl of the reaction mixture into 1 ml of cold stop solution (in mM): 100 sucrose, 120 KCl, 0.1 pCMB (p-chloromercuribenzoic acid),

**Table 1.** Sorbitol release from immortalized rabbit TALH cells grown on filter supports

| Experimental maneuver | % Total cellular sorbitol | |
|---|---|---|
| | Apical compartment | Basolateral compartment |
| 600 ⇨ 600 | 5.3 ± 1.1% ($n$ = 3) | 3.1 ± 0.6% ($n$ = 3) |
| 600 ⇨ 300 (high calcium) | 17.9 ± 3.5%[a] ($n$ = 3) | 5.7 ± 0.2% ($n$ = 3) |
| 600 ⇨ 300 (calcium omitted) | 10.5 ± 2.1%[a,b] ($n$ = 3) | 3.7 ± 0.7% ($n$ = 3) |

TALH cells grown at 600 mOsm/kg to confluency on filter inserts were exposed for 10 min at 37°C to media varying in osmolarity (change in NaCl content). Sorbitol content in the compartments is given as mean values ± SD in % of total sorbitol detected in the cells and both fluid compartments. [a] $P < 0.05$ compared to control. [b] $P < 0.05$ compared to the high calcium medium. The cells contained 986.9 ± 91.9 μM sorbitol/mg protein ($n$ = 10).

0.1 quinidine, 0.1 niflumic acid, and 20 HEPES, adjusted to pH 7.4 with Tris. The diluted samples were immediately placed onto a cellulose nitrate filter, pore size of 0.45 μM (Schleicher + Schüll, Dassel, Germany), and the filter was washed rapidly with 3 ml of cold stop solution. The filters were placed into scintillation fluid and counted by standard liquid scintillation techniques.

In preliminary experiments, it was found that sorbitol could be reproducibly determined first after 15 sec of incubation. Therefore, an incubation period of 15 sec was used in all subsequent experiments where 20 μl of membranes were added to 50 μl of incubation medium. After 15 sec 1 ml of cold stop solution was added to the reaction mixture which was subsequently placed onto the filter and washed with 3 ml of stop solution.

### Statistical Analysis

Data from paired experiments were compared using Student's $t$-test, $P$ values < 0.05 were considered as statistically significant.

## Results

### Sorbitol Fluxes in Intact TALH Cells

We first investigated sorbitol release from TALH cells grown on permeable filters (*see* Table 1). Under control conditions only a slow sorbitol release was found. When cells adapted to 600 mOsm/kg were exposed for 10 min to a medium of lower osmolality (300 mOsm/kg) a clear stimulation of total release was observed. Moreover, a directionality was found in that flux across the apical cell surface was about three times higher than across the basal-lateral surface. As shown in Table 1 the apical sorbitol efflux was also affected by changes in the calcium concentration of the efflux medium. Efflux into a medium containing 5 mM $Ca^{2+}$ was 1.7-fold higher than into a medium from which calcium had been omitted.

Thus, in TALH cells sorbitol release occurs predominantly across the apical membrane and shows a distinct $Ca^{2+}$ dependence. In view of these results we attempted to obtain a plasma membrane fraction for transport studies enriched in luminal membranes.

**Table 2.** Enzyme activities in a microsomal plasma membrane fraction isolated from TALH cells grown at 300 mOsm/kg and in the starting homogenate

| Enzyme | Homogenate | Microsomal plasma membrane fraction |
|---|---|---|
| ADPase (EC 3.6.1.6) | 1.04 ± 0.09 | 6.45 ± 0.61 |
| Enrichment | | 6.2 |
| Recovery | | 86.8 ± 9.0 |
| Na,K-ATPase (EC 3.6.1.3) | 1.71 ± 0.11 | 2.40 ± 0.34 |
| Enrichment | | 1.4 |
| Recovery | | 92.0 ± 4.9 |
| Succinate dehydrogenase (EC 1.3.99.1) | 0.71 ± 0.09 | 0.69 ± 0.07 |
| Enrichment | | 0.97 |
| Recovery | | 79.1 ± 10.7 |
| Acid phosphatase (EC 3.1.3.2.) | 1.38 ± 0.10 | 2.69 ± 0.24 |
| Enrichment | | 1.9 |
| Recovery | | 82.9 ± 2.4 |

Enzyme activities are expressed as μmoles substrate hydrolyzed/h · mg protein and represent mean values ± SD derived from at least 4 different membrane preparations. The recoveries (sum of the activities found in all fractions) are expressed as percent of the total activity found in the homogenate.

### Sorbital Fluxes in Isolated Microsomal Plasma Membranes

#### *Enzymatic Characterization of the Membrane Fraction*

As depicted in Table 2 the plasma membrane fraction employed in the transport studies was highly enriched in ADPase, a marker for apical membranes (Novikoff et al., 1962), only low enrichments or lack of enrichment were found for marker enzymes of lysosomes (acid phosphatase), mitochondria (succinate dehydrogenase), and basal-lateral plasma membranes (Na,K-ATPase).

#### *Basic Requirements of Sorbitol Uptake*

In a series of preliminary experiments the conditions to observe reproducibly sorbitol transport into the plasma membrane vesicles were investigated. As shown in Fig. 1 at 37°C in the presence of $Mg^{2+}$, $Ca^{2+}$, GTP, and ATP sorbitol uptake was significantly higher than the uptake observed at 4°C. Under the former conditions, sorbitol

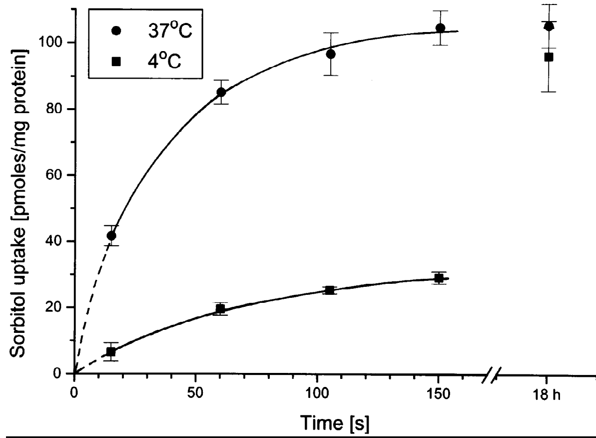


**Fig. 1.** Time course of $^3$H-sorbitol uptake by the microsomal plasma membrane fractions isolated from immortalized TALH cells from rabbit kidney at 4°C (■) and 37°C (●), respectively. The membranes were prepared (in mM): 100 sucrose and 20 HEPES-Tris (pH 7.4) and incubated in a medium containing (in mM): 100 sucrose, 100 KCl, 1.5 $MgSO_4$, 0.5 $CaCl_2$, 0.1 ATP, 0.1 GTP and 20 HEPES-Tris (pH 7.4). Data collected at 18 hr are considered equilibrium values. Values are means ± SD of 3 individual preparations, with experiments performed in duplicate.

equilibrated within 3 min across the membrane without an overshoot. The uptake at 4°C was slow and equilibration was achieved only after 18 hr of incubation. At 37°C uptake after 15 sec was also inhibited by 100 μM quinidine (35.01 ± 4.0 pmoles/mg protein versus 18.4 ± 1.7 pmoles/mg protein, $n = 2$). No change in equilibrium uptake was observed (74.2 ± 7.1 pmoles/mg protein vs. 72.6 ± 9.1 pmoles/mg protein, $n = 2$).

In the following the role of the individual components added to the transport medium was evaluated. The results of these experiments are compiled in Table 3.

*Role of Divalent Cations*

As depicted in Table 3 the removal of $Ca^{2+}$ from the transport medium led to a reduction of sorbitol uptake to the level observed at 4°C, thus the temperature-sensitive flux was completely inhibited. When $Mg^{2+}$ was omitted from the incubation medium a decrease of the temperature-sensitive flux by 43.0 ± 5.7% ($n = 3$) was observed.

*Role of Nucleotides*

With regard to the nucleotide requirement of the temperature-sensitive sorbitol flux, first of all the concentration dependence with regard to ATP was determined. Increasing the ATP concentration from 0.1 mM to 1 mM or 3 mM did not change the sorbitol uptake significantly. In a representative experiment values were 44.1, 40.7, and 41.0 pmoles/mg protein · 15 sec. Thus 0.1 mM ATP appeared to be sufficient for the maximum effect. We

**Table 3.** Dependence of sorbitol influx on divalent cations and ATP analogues

| | 37°C | 4°C |
|---|---|---|
| Experimental series 1 | | |
| 0.5 mM $Ca^{2+}$ | 38.9 ± 3.0 | 10.8 ± 2.5* |
| | ($n = 6$) | ($n = 6$) |
| no $Ca^{2+}$ + 0.1 mM EGTA | 9.5 ± 1.5* | N.D. |
| | ($n = 6$) | |
| Experimental series 2 | | |
| 1.5 mM $Mg^{2+}$ | 48.6 ± 4.5 | 9.6 ± 0.3* |
| | ($n = 3$) | ($n = 3$) |
| no addition of $Mg^{2+}$ | 31.6 ± 0.8* | N.D. |
| | ($n = 3$) | |
| Experimental series 3 | | |
| ATP | 38.7 ± 4.8 | 6.1 ± 1.4* |
| | ($n = 3$) | ($n = 3$) |
| ATPγS | 9.7 ± 0.8* | 4.05 ± 0.1* |
| | ($n = 3$) | ($n = 3$) |
| AMP-PCP | 12.0 ± 2.8* | N.D. |
| | ($n = 3$) | |

Sorbitol uptake is given in pmoles/mg protein · 15 sec as mean values ± SD derived from $n$ experiments. ND = not determined. In experimental series 1 and 2 GTP and ATP were present during the uptake measurements, in series 3 ATP was replaced in the presence of $Ca^{2+}$, $Mg^{2+}$ and GTP by the respective analogues. * $P < 0.05$ compared to control. AMP-PCP = adenosine 5′-β,γ-methylene-triphosphate.

then investigated whether ATP could be replaced by nonhydrolyzable analogues such as ATP γS or AMP-PCP. As shown in Table 3 in the presence of these analogues, temperature-sensitive sorbitol flux was strongly reduced, suggesting that ATP hydrolysis was required for the increase in sorbitol flux.

This assumed ATP hydrolysis could either serve directly as an energy source for the uptake or lead to an activation of a sorbitol uptake pathway. To distinguish between these possibilities we investigated whether the presence of ATP was required at the time sorbitol uptake was determined or whether ATP could exert its action prior to the flux measurements. For this purpose membranes were preincubated with ATP (in the presence of $Ca^{2+}$, $Mg^{2+}$ and GTP) and subsequently sorbitol uptake was investigated in the virtual absence of ATP. The latter condition was achieved by omitting ATP from the uptake medium and including hexokinase and D-glucose as scavenger for any residual ATP transferred from the preincubation medium. ATP content in the final uptake medium, as determined enzymatically, was at least 10,000 times lower than in the preincubation medium.

As shown in Table 4 sorbitol flux was highest when the membranes were preincubated with ATP and uptake was measured in the absence of ATP. Uptake after 15 sec into membranes which were exposed to ATP only during the uptake phase was significantly lower (by 34.5%), but was in the range observed previously. Omission of the nucleotides during the preincubation pe-

**Table 4.** Stimulation of sorbitol flux by preincubation with ATP

| Preincubation conditions | Uptake conditions | Sorbitol flux [pmoles/mg protein] | | |
|---|---|---|---|---|
| | | 15 sec | 1 min | Equilibrium |
| +ATP (0.1 mM)<br>+GTP (0.1 mM)<br>$+Ca^{2+}$ (0.5 mM)<br>$+Mg^{2+}$ (1.5 mM) | No ATP<br>+GTP<br>$+Ca^{2+}$<br>$+Mg^{2+}$ | 47.8 ± 7.3 ($n$ = 4) | 54.5 ± 4.2 | 61.5 ± 7.8 |
| No ATP<br>No GTP<br>$Ca^{2+}$<br>$Mg^{2+}$ | No ATP<br>+GTP<br>$+Ca^{2+}$<br>$+Mg^{2+}$ | 11.0 ± 0.8* ($n$ = 2) | 12.0 ± 3.4* | 60.6 ± 15.8 |
| No ATP<br>No GTP<br>No $Ca^{2+}$<br>No $Mg^{2+}$ | +ATP (0.1 mM)<br>+GTP (0.1 mM)<br>$+Ca^{2+}$ (0.5 mM)<br>$+Mg^{2+}$ (1.5 mM) | 31.1 ± 4.7* ($n$ =2) | 34.6 ± 4.3* | 61.4 ± 9.2 |
| +ATP<br>+GTP<br>No $Ca^{2+}$<br>$+Mg^{2+}$ | No ATP<br>+GTP<br>$+Ca^{2+}$<br>$+Mg^{2+}$ | 9.5* | 9.3* | 64.1 |

Preincubation was performed for 5 min at 37°C in vesicle buffer and the additions indicated above, membrane protein amounted to 6 mg/ml. At time zero of the uptake period 50 µl of the membrane suspension were transferred into 325 µl of transport medium containing 100 mM sucrose, 100 mM KCl, 20 mM HEPES-Tris, pH 7.4, and the additions indicated above. For ATP scavenging the transport medium contained in addition 1.5 mM D-glucose and 20 mU hexokinase. 50 µl of the uptake suspension were removed at the indicated time points and filtered as described above. * $P < 0.05$ compared to control.

riod and the uptake period reduced the uptake even further to 23.1%, a value similar to the basal uptake found before. A similar low uptake was observed when $Ca^{2+}$ was already removed during the preincubation period. None of these maneuvers changed the equilibrium value, suggesting that the membrane vesicles maintained their integrity. These results clearly demonstrate that ATP hydrolysis is not the driving force for sorbitol uptake but probably leads to an activation of the transporter.

It should also be noted that the uptake of mannitol was not influenced significantly in vesicles subjected to preincubation in the presence of ATP, GTP, $Ca^{2+}$, and $Mg^{2+}$ (uptake after 15 sec, 11.4 *vs.* 9.1 pmoles/mg protein; uptake after 1 min 11.9 *vs.* 11.8 pmoles/mg protein). Thus the activated transport system seems to be rather specific for sorbitol.

*Effect of Kinase Inhibitors on Sorbitol Flux*

To further characterize the nature of the activation of the transporter, the effect of protein kinase inhibitors on sorbitol uptake in the presence of ATP was investigated. The results of these studies are compiled in Fig. 2. Staurosporine, an inhibitor with a broad specificity (Yanagihara et al., 1991), inhibited the action of ATP almost completely at 200 nM, at 40 nM the inhibition was

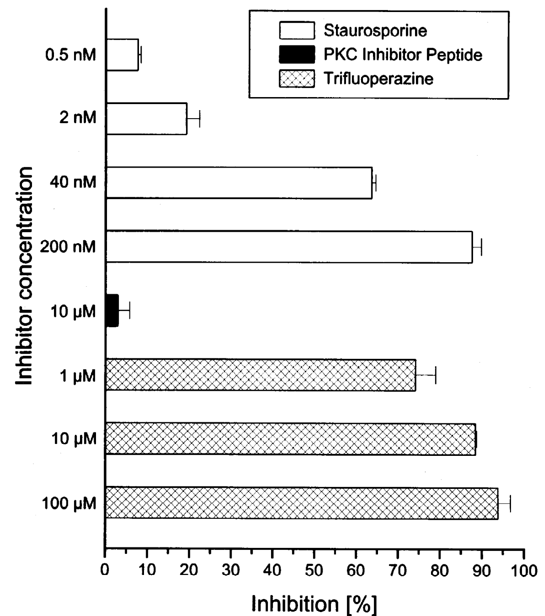


**Fig. 2.** Effect of different inhibitors on temperature-dependent $^3$H-sorbitol uptake (difference between the uptake at 37°C and 4°C) by the microsomal membrane fraction isolated from immortalized TALH cells.

slightly more than 50%. The high concentration of staurosporine required for the inhibition suggests that neither protein kinase A (PKA) nor protein kinase C (PKC) but probably $Ca^{2+}$/calmodulin-dependent kinases are involved in augmenting sorbitol flux (Tamaoki et al., 1986; Schächtele, Seifert & Osswald, 1988; Tischler, Ruzicka & Perlman, 1990; Yanagihara et al., 1991). PKA could already be excluded on the basis of the $Ca^{2+}$ dependence of action. The lack of effect of the PKC inhibitory peptide even at 10 $\mu$M shown in Fig. 2 also supports the notion that PKC does not activate the transporter. Further evidence for the action of $Ca^{2+}$/calmodulin-dependent kinases can be derived from the experiments using trifluoperazine (TFP), which interferes with calmodulin-dependent reactions. As depicted in Fig. 2 TFP turned out to be a potent inhibitor of the ATP-dependent increase of sorbitol influx; at 1 $\mu$M still an inhibition of 75% was observed. It should be noted that none of the inhibitors changed the sorbitol uptake at 4°C, indicating that the inhibitors or solvents did not destroy the vesicular integrity.

Some inhibitors were also investigated in preincubation experiments. TFP (10 $\mu$M) and staurosporine (200 nM) prevented the stimulation of sorbitol uptake by preincubation completely ($n = 2$). The same result was obtained, when KT 5926, a rather specific inhibitor for $Ca^{2+}$/calmodulin-dependent kinases (Nakanishi et al., 1990), was used. In the presence of 50 nM KT 5926 the preincubation with ATP, GTP, $Ca^{2+}$ and $Mg^{2+}$ did not stimulate the subsequently (in the absence of ATP) determined sorbitol flux. Again, the equilibrium values for sorbitol uptake were not altered by the inhibitors. Based on these data we suggest that a phosphorylation reaction mediated by a $Ca^{2+}$/calmodulin-dependent kinase activates sorbitol flux across (luminal) plasma membranes of the thick ascending limb of Henle's loop.

## Discussion

The current paper adds the thick ascending limb of Henle's loop to the still rather short list of cells in which sorbitol permeases have been found (Stahl, Wiesinger & Hamprecht, 1989; Garty et al., 1991; Wiesinger, Schuricht & Hamprecht, 1991; Kracke, Preston & Stanley, 1994). The basic features of the sorbitol flux in the TALH cell membranes are very similar to those described in the papillary epithelium cell line. The flux across the membranes is strongly reduced when the temperature is decreased and quinidine inhibits the transport. These two observations suggest that the transfer of sorbitol across the membranes is mediated by a transport system with a certain specificity for sugar alcohols, as studied in detail by Napathorn and Spring (1994), which as in other cells, is characterized by a lack of saturability. In addition, the flux system is found in apical membranes of the TALH cells, corresponding to the findings in the papillary epithelium cells. The apical location was observed in the cells grown on filters where a correct sorting of apical transport systems, such as the Na-K-2Cl cotransporter and basal-lateral transport systems, such as Na,K-ATPase, to their appropriate membranes occurs. Futhermore the membrane fraction isolated from the immortalized TALH cell culture is very similar to the one obtained by Eveloff and Kinne (1983) and König et al. (1983) from TALH cells isolated from rabbit kidney medulla. It is also characterized by a high content of ADPase, a marker enzyme for luminal membranes and a low content of Na,K-ATPase, a marker for contraluminal membranes. In the former fraction Na-K-2Cl cotransport has been demonstrated (König et al., 1983; Kinne et al., 1986), supporting its origin from the apical surface of the cells.

In our attempts to find a mechanism that might be responsible for the activation of sorbitol flux we were guided first by the observations reported for TALH cells in this paper and those made recently for IMCD cells, where a $Ca^{2+}$ dependence of sorbitol release has been established (Tinel et al., 1994; Ruhfus, Tinel & Kinne, 1996; Kinne et al., 1996) and second, by the finding that lack of intracellular ATP, induced by the addition of 2-deoxy-D-glucose, attenuated the activation of sorbitol efflux (Ruhfus, 1996). These results taken together prompted us to include $Ca^{2+}$, ATP, and $Mg^{2+}$ in the influx medium, in order to mimic the optimum intracellular conditions for sorbitol flux. We furthermore included GTP since an involvement of G-proteins has been described (Ruhfus et al., 1996).

Out of these additions ATP and $Ca^{2+}$ turned out to be the most important. Their omission lead to a strong reduction of sorbitol influx to the level observed at 4°C. Lack of $Mg^{2+}$ only partially inhibited uptake. This might be due to the fact that $Mg^{2+}$ had not been completely removed from the medium or that $Ca^{2+}$ can replace $Mg^{2+}$ at its active site as for example reported for the Na,K-ATPase (Kinne-Saffran et al., 1993). The role of GTP is still unclear, results of experiments omitting GTP were inconclusive, therefore, its role in the activation process remains to be determined.

Another new finding was that a preincubation of the membranes with $Ca^{2+}$, $Mg^{2+}$, ATP, and GTP was sufficient to activate the transport, subsequent removal of ATP had no effect. These results strongly suggested that a phosphorylation reaction was involved in the activation process. The studies employing the kinase inhibitors support this notion. The high concentration for staurosporine required to inhibit the activation, its sensitivity to low concentrations of KT 5926 and to trifluoperazine indicate that a $Ca^{2+}$/calmodulin-dependent kinase might be the mediator. Since the current studies have been performed in an *in vitro* system, where possible side effects

of inhibitors that occur in intact cells are minimized and the concentration of the inhibitors can be controlled rather well, the results with the inhibitors in conjunction with the prephosphorylation experiments are considered as quite strong evidence for such conclusions. Furthermore, a variety of transport systems such as Na/H exchanger (Cohen et al., 1990) have been recently shown to be regulated by phosphorylation catalyzed by the $Ca^{2+}$/calmodulin-dependent kinase (for review *see* Braun & Schulman, 1995).

It has to be emphasized, however, that further studies are required to determine whether $Ca^{2+}$/calmodulin-dependent kinase is involved in the hypotonicity-induced increase in sorbitol permeability in TALH cells. Since the current investigations were performed on isolated plasma membranes only one potential mechanism of activation could be revealed. Other mechanisms, for example those involving arachidonic acid (Tinel, Wehner & Kinne, 1997) or its metabolites (Furlong, Moriyama & Spring, 1991) could also be part of the chain of events leading to a change of the sorbitol transport properties of the plasma membranes.

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