cAMP increases liver Na\textsuperscript{+}-taurocholate cotransport by translocating transporter to plasma membranes

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Mukhopadhyay, Sunil, M. Ananthanarayanan, Bruno Stieger, Peter J. Meier, Frederick J. Suchy, and M. Sawkat Anwer. cAMP increases liver Na\textsuperscript{+}-taurocholate cotransport by translocating transporter to plasma membranes. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G842–G848, 1997.—Adenosine 3',5'-cyclic monophosphate (cAMP), acting via protein kinase A, increases transport maximum of Na\textsuperscript{+}-taurocholate cotransport within 15 min in hepatocytes (S. Grüne, L. R. Engelking, and M. S. Anwer. J. Biol. Chem. 268: 17734–17741, 1993); the mechanism of this short-term stimulation was investigated. Cycloheximide inhibited neither basal nor cAMP-induced increases in taurocholate uptake in rat hepatocytes, indicating that cAMP does not stimulate transporter synthesis. Studies in plasma membrane vesicles showed that taurocholate uptake was not stimulated by the catalytic subunit of protein kinase A but was higher when hepatocytes were pretreated with cAMP. Immunoblot studies with anti-fusion protein antibodies to the cloned transporter recognize a 50-kDa protein in the plasma membrane of hepatocytes (1, 29). In addition, the microsomal epoxy hydrolase (a 49-kDa protein different from Ntcp) has also been proposed to stimulate transporter synthesis. Studies in plasma membrane vesicles showed that taurocholate uptake was not stimulated by the catalytic subunit of protein kinase A but was higher when hepatocytes were pretreated with cAMP. Immunosblot studies with anti-fusion protein antibodies to the cloned Na\textsuperscript{+}-taurocholate cotransport polypeptide (Ntcp) showed that pretreatment of hepatocytes with cAMP increased Ntcp content in plasma membranes but not in homogenates. Ntcp was detected in microsomes, endosomes, and Golgi fractions, and cAMP pretreatment resulted in a decrease only in endosomal Ntcp content. It is proposed that cAMP increases transport maximum of Na\textsuperscript{+}-taurocholate cotransport, at least in part, by translocating Ntcp from endosomes to plasma membranes.

protein kinase A; sodium ion-taurocholate cotransport polypeptide; cycloheximide; rat hepatocytes; plasma membrane vesicles
lar uptake of TC. 3) To determine whether cAMP affects Ntcp content, hepatocytes were treated with 10 µM CPT-cAMP followed by subcellular fractionation and immunoblot analysis. All studies were repeated in at least three different cell preparations.

Subcellular fractionation. Plasma membranes, microsomes, endosomes, and Golgi fractions were isolated from hepatocytes pretreated with cAMP or buffer. All isolation steps were carried out at 40°C, and cAMP pretreatment did not significantly affect marker enzyme enrichment.

Plasma membranes were isolated using a Percoll gradient centrifugation method (10) as described for hepatocytes (17). Briefly, hepatocytes were homogenized in a buffer (pH 7.4) containing (in mM) 5 HEPES, 0.5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 250 sucrose, 1 phenylmethylsulfonyl fluoride (PMSF), and 2 KF and 10 µg/ml leupeptin and aprotinin followed by centrifugation (20 min at 4,400 × g). The resuspended pellet was subjected to Percoll gradient centrifugation (10). The membrane fraction collected at the buffer-10% Percoll interface was recovered, washed twice in the homogenization buffer, and stored at −70°C. The resulting pellet was washed twice with the isolation buffer, and the pooled supernatant fraction (S1) was centrifuged at 33,000 × g for 40 min. The resuspended pellet was subjected to Percoll gradient centrifugation (10). The membrane fraction collected at the buffer-10% Percoll interface was recovered, washed twice in the homogenization buffer, and stored at −70°C. Further subfractionation of crude endosomes was carried out by loading S2 fraction onto sucrose gradient consisting of 1 ml 70% sucrose, 5 ml of 43% sucrose, and a continuous gradient by mixing 7 ml 40% and 7.5 ml 15% sucrose and centrifuging at 97,000 g for 4 h. Three fractions designated as fractions 1, 2, and 3 (corresponding to fractions D, E, and P in Ref. 11) were obtained: fractions 1 and 2 represent low-density endosomes, and fraction 3 is of unknown origin (11). Fraction 3, but not fractions 1 and 2, was enriched in 5′-nucleotidase (2-fold) and Na+·K+-ATPase (3-fold), indicating presence of plasma membranes (Table 1). Pretreatment with cAMP did not result in a significant difference in the enrichment of these marker enzymes.

TC uptake in hepatocytes and plasma membrane vesicles. The initial uptake rate of TC in hepatocytes was determined as previously described (2). Briefly, at various times after incubation of hepatocytes with cycloheximide and/or DB-cAMP, an aliquot of cell suspension (5–8 mg protein/ml) was withdrawn and used to determine the initial uptake rate of TC (20 µM). Transport was initiated by adding cells to the incubation medium containing [14C]TC and [3H]inulin, with uptake determined at different time points. Initial uptake rates were calculated from the slope of the linear portion of time-dependent uptake curves and were expressed in nanomoles per minute per milligram of protein.

A rapid filtration technique was used to determine TC uptake in plasma membrane vesicles (6). Briefly, frozen plasma membrane suspensions were rapidly thawed by immersion in a 37°C water bath, diluted to the desired protein concentration (2–4 mg/ml), and vesiculated by passing membranes 20 times through a 27-gauge needle. To study the effect of PKA on TC uptake, membranes were suspended in a buffer containing the catalytic subunit of PKA (50 U/ml) and ATP (1.0 mM) before vesiculation. An aliquot (20 µl) of membrane vesicles was incubated for 15 min at 37°C, and the uptake was initiated by adding 80 µl of incubation buffer (in mM: 100 sucrose, 0.2 CaCl2, 5 MgSO4, and 20 HEPES-Tris, pH 7.5) containing [3H]TC and [3H]inulin, with uptake determined at different time points. Initial uptake rates were calculated from the slope of the linear portion of time-dependent uptake curves and were expressed in nanomoles per minute per milligram of protein.

Table 1. Effect of cAMP on relative enrichment of marker enzymes in plasma membranes and endosomal fractions

<table>
<thead>
<tr>
<th>Marker Enzymes</th>
<th>Plasma Membranes</th>
<th>Endosomal Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>cAMP</td>
</tr>
<tr>
<td>5′-Nucleotidase</td>
<td>10 ± 1.4</td>
<td>11 ± 1.3</td>
</tr>
<tr>
<td>Na+·K+-ATPase</td>
<td>15 ± 2.3</td>
<td>14 ± 2.2</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>0.3 ± 0.07</td>
<td>0.3 ± 0.09</td>
</tr>
</tbody>
</table>

Values represent enrichment relative to respective whole homogenate and are expressed as means ± SD; n = 3–7 membrane preparations. ND, not determined. Activities of marker enzymes were determined in plasma membranes and endosomal fractions isolated from untreated (control) and cAMP-pretreated (cAMP) hepatocytes.
activity after subtracting nonspecific binding and was expressed in picomoles per milligram of protein.

Immunoblot analysis. Proteins from different subcellular fractions were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (19). Proteins were transferred electrophoretically from SDS gels to nitrocellulose membranes (Transblot, transfer membrane 0.45 µm; Bio-Rad) and probed with the appropriate antibody (anti-Ntcp at 1:2,000 dilution or anti-α1 subunit of Na+-K+-ATPase at 1:1,000 dilution). Peroxidase-conjugated anti-immunoglobulin G was used as the secondary antibody. The immunoblots were developed with the Amersham enhanced chemiluminescence kit according to the manufacturer’s instructions, and a laser densitometer (UltroScan XL, Pharmacia) was used to obtain relative quantitation of the signals.

Other methods. The Lowry method was used to determine cell protein (21). Marker enzymes 5'-nucleotidase (4), Na+-K+-ATPase (27), NADH dehydrogenase (31), and glucose-6-phosphatase (4) were assayed using established methods. All values are expressed as means ± SE. Student’s t-test (or paired t-test) was used to statistically analyze data, with P < 0.05 considered significant.

RESULTS

Effect of cAMP on transporter synthesis. The effect of cycloheximide on TC uptake was studied in isolated hepatocytes to determine whether the stimulatory effect of cAMP involves new protein synthesis. Neither basal nor cAMP-stimulated TC uptake was affected by cycloheximide (50 µg/ml) for up to 120 min (Fig. 1). Thus it is unlikely that cAMP stimulates Na+-TC cotransport by increasing transporter synthesis.

Effect of PKA and cAMP pretreatment on TC uptake in plasma membrane vesicles. PKA may directly affect the transporter and thereby stimulate Na+-TC cotransport by increasing cAMP activity. To test this, the effect of catalytic subunit of PKA on TC uptake in membrane vesicles obtained from untreated hepatocytes was determined. A similar approach was employed by Bae and Verkman (5) to study the regulation of Cl− conductance in endocytic vesicles by cAMP. TC uptake in membrane vesicles containing PKA and ATP was not significantly different from control values (Fig. 2). Thus PKA may not directly alter the transporter activity. To ascertain that PKA was active under the experimental conditions used, plasma membranes were similarly treated with PKA and [32P]ATP, and membrane proteins were subjected to SDS-gel electrophoresis followed by autoradiography. Presence of multiple phosphorylated proteins on the gel (data not shown) indicated that PKA was active.

cAMP may stimulate Na+-TC cotransport by increasing driving forces and/or by inducing stable changes in the transporter at the plasma membrane level. In the former case, plasma membranes isolated from cAMP-treated hepatocytes should not show increased TC uptake. This is because the driving force (Na+ gradient) for TC uptake in plasma membrane vesicles is controlled by the incubation medium. However, plasma membrane vesicles should retain the stimulatory effect of cAMP, if cAMP stably changes the transporter in the plasma membrane. Thus TC uptake was determined in plasma membrane vesicles isolated from hepatocytes pretreated with cAMP (Fig. 2). The initial rate of TC uptake...
uptake was 40% higher \((8.6 \pm 1.3 \text{ vs. } 6.1 \pm 0.95 \text{ pmol} \cdot 10^3 \text{s}^{-1} \cdot \text{mg protein}^{-1}; \text{means} \pm \text{SE}; n = 4)\) in vesicles obtained from cAMP-treated hepatocytes. Thus the stimulation of \(\text{Na}^+\)-TC cotransport by cAMP may involve stable changes in the transporter.

Effect of cAMP on plasma membrane Ntcp content. cAMP may increase the maximum uptake rate of TC by increasing plasma membrane content of Ntcp. Because Ntcp has been shown to mediate \(\text{Na}^+\)-TC cotransport (27), anti-fusion protein antibodies to the cloned Ntcp were used to determine whether cAMP increases plasma membrane content of Ntcp. Two different antibodies from two different laboratories (1, 29) were used. Because results obtained with these antibodies were similar, they are not reported separately. For these studies, hepatocytes were treated with cAMP followed by isolation of plasma membranes. Immunoblot analysis showed that cAMP treatment resulted in a 50% increase in the amount of Ntcp in the plasma membrane (Fig. 3). cAMP did not alter the amount of Ntcp in the whole homogenate (Fig. 3), indicating that the increase in plasma membrane content is not caused by an overall increase in Ntcp. We have also determined the effect of cAMP on plasma membrane content of organic anion transporting protein (Oatp) and the \(\alpha_1\)-subunit of \(\text{Na}^+\cdot\text{K}^+\cdot\text{ATPase}\) (Oatp). Treatment of hepatocytes with cAMP did not result in an increase in plasma membrane content of either Oatp or the \(\alpha_1\)-subunit of \(\text{Na}^+\cdot\text{K}^+\cdot\text{ATPase}\) (Fig. 4). Immunoblot analysis of Oatp using our plasma membrane preparations was conducted in Dr. A. Wolkoff's laboratory. Thus it is unlikely that the increase in Ntcp is caused by a general effect of cAMP on distribution of proteins in plasma membranes. This observed increase in plasma membrane content of Ntcp raises the possibility that cAMP-induced stimulation of \(\text{Na}^+\)-TC cotransport may involve translocation of the transporter from intracellular stores to the plasma membrane.

Effect of cAMP on subcellular distribution of Ntcp. To determine intracellular source(s), hepatocytes were treated with cAMP followed by isolation of plasma membrane, microsome, endosome, and Golgi fractions. Immunoblot analysis showed that Ntcp antibody recognized more than one protein band in the general area of 50 kDa (Fig. 5), possibly representing different glycosylated forms (23). Densitometric analysis showed that cAMP-induced increase in plasma membrane content of Ntcp was not associated with a significant decrease in microsomes (data not shown), crude endosomes, and Golgi fractions (Fig. 5). To determine whether a subpopulation of endosomes may be the intracellular source, the crude endosomal fraction was further fractionated on sucrose gradient and the resulting three fractions (fractions 1–3) were subjected to immunoblot analysis (Fig. 6). Results showed that cAMP decreased Ntcp content in fraction 2 and increased Ntcp content in fraction 3 without significantly affecting Ntcp content in fraction 1. Thus cAMP may stimulate translocation of Ntcp from endosomes to plasma membranes.

**DISCUSSION**

CAMP is known to stimulate \(\text{Na}^+\)-TC cotransport in hepatocytes by increasing transport maximum within 15 min. The present study was designed to determine whether this rapid effect of cAMP is caused by increased synthesis of the transporter(s), changes in driving forces, and/or translocation of Ntcp. Results showed that the stimulatory effect of cAMP was not affected by cycloheximide and was still present in...
plasma membranes isolated from hepatocytes pre-treated with cAMP. In addition, cAMP treatment of hepatocytes resulted in an increase in plasma membrane and a decrease in low-density endosomal Ntcp content. These results are discussed in relation to our conclusion that cAMP increases the maximum transport rate of Na\(^{+}\)-TC cotransport, in part, by translocating Ntcp from endosomes to the plasma membrane.

The stimulatory effect of cAMP is unlikely to be caused by increased synthesis of the transporter protein, because cycloheximide (50 µg/ml) failed to inhibit the effect of cAMP (Fig. 1). Moreover, the stimulatory effect of cAMP is maximal within 30 min (Fig. 1) and new protein synthesis usually requires a longer time interval. The same concentration of cycloheximide has been shown to inhibit long-term (>30 min) but not short-term stimulation of L-alanine uptake by glucagon in hepatocytes (8), an effect mediated via cAMP. The long-term stimulation of L-alanine transport by cAMP is believed to be caused by stimulation of transporter synthesis. Because cAMP-stimulated TC uptake was not affected by cycloheximide for 120 min, it is likely that cAMP regulates Na\(^{+}\)-TC cotransport differently than L-alanine transport in hepatocytes. The stimulatory effect of cAMP was retained in plasma membrane vesicles isolated from cAMP-pretreated hepatocytes (Fig. 2). This result would indicate that the effect of cAMP may be independent of cAMP-induced changes in driving forces. However, this result does not rule out the possibility that the effect of cAMP in hepatocytes may, in part, be mediated via changes in the driving force. Studies in hepatocytes indicate that Na\(^{+}\)-TC cotransport is electrogenic (20, 32), and cAMP has been shown to hyperpolarize hepatocytes (9). Thus cAMP is likely to stimulate Na\(^{+}\)-TC cotransport by increasing the electrical gradient, as suggested by our previous preliminary studies (14).

One mechanism by which cAMP can induce stable changes that will result in increased maximum transport activity is increasing the number of transporter in the plasma membrane. This possibility is supported by our result that cAMP increases the Ntcp content in plasma membranes (Fig. 3). Because Ntcp has been shown to mediate Na\(^{+}\)-TC cotransport (23), it is most likely that cAMP-induced change in Ntcp is involved in the regulation of this cotransporter in hepatocytes. cAMP did not affect the Ntcp content of the whole homogenate, indicating that the increase in plasma membrane content is not caused by an increase in Ntcp synthesis. This result is consistent with our finding that cycloheximide did not affect the stimulatory effect of cAMP. This result also suggests that the increase in plasma membrane content is likely to be caused by...
translocation of the transporter from intracellular stores.

The present study also showed that the increase in plasma membrane Ntcp by cAMP was associated with a decrease in endosomal fraction 2 (Fig. 6). This result may indicate that cAMP stimulates translocation of Ntcp from endosomes to the plasma membrane. However, cAMP also increased Ntcp content of fraction 3 (Fig. 6), raising the possibility that these changes may result from redistribution of Ntcp in different endosomal compartments by cAMP. Although this possibility cannot be ruled out, it seems unlikely for the result from redistribution of Ntcp in different endosomal fractions 1–3 when compared with homogenate (Table 1). Determination of marker enzyme activity showed that fraction 3 is enriched in 5’-nucleotidase and Na$^{+}$-K$^{+}$-ATPase (Table 1), indicating the presence of canalicular as well as sinusoidal plasma membranes in this fraction. On the other hand, the activities of these enzymes were not higher in fractions 1 and 2 compared with homogenate (Table 1). Thus, it is possible that the observed increase in the Ntcp content of fraction 3 is caused by the presence of basolateral membranes. On the basis of these results, we propose that cAMP stimulates Na$^{+}$-TC cotransport in part by translocating Ntcp, most likely from endosomes, to plasma membranes. Regulation of transport activity by translocation has been proposed for glucose transporter. For example, the ability of insulin to activate glucose transport in adipocytes and myocytes is mainly caused by translocation of GLUT-4 to plasma membranes (28), and cAMP has also been shown to acutely stimulate translocation of glucose transporter (GLUT-4) from low-density microsomal membranes to the plasma membranes in rat adipocytes (18).

The activity of a transporter can also be regulated by phosphorylation (12). Whether cAMP also regulates Na$^{+}$-TC cotransport by altering phosphorylation status is not known. Our preliminary study indicates that Ntcp is a phosphoprotein (25). Because PKA failed to stimulate TC uptake in plasma membrane vesicles (Fig. 2), it is unlikely that the stimulation of Na$^{+}$-TC cotransport by cAMP is caused by PKA-mediated direct phosphorylation of the transporter. This, however, does not rule out regulation by phosphorylation/dephosphorylation, because the phosphorylation status of the transporter may be affected by kinases and phosphatases that are, in turn, regulated by PKA. The potential role of phosphorylation is currently under investigation. In the present study, antibodies against Ntcp were used to determine the effect of cAMP on Na$^{+}$-TC cotransporter protein. However, another protein (microsomal epihydrolase) unrelated to Ntcp has been proposed to mediate plasma membrane Na$^{+}$-TC cotransport (30). Whether cAMP also affects this protein remains to be determined. It may, however, be mentioned that Ntcp appears to represent the major Na$^{+}$-TC cotransport system in the rat liver (23), and Ntcp has been shown to be downregulated in cholestasis associated with decreased Na$^{+}$-TC cotransport (13, 24).

In summary, the present study showed that the stimulation of Na$^{+}$-TC cotransport in hepatocytes by cAMP is unlikely to be caused by synthesis or PKA-mediated direct phosphorylation of the transporter. It is proposed that cAMP increases the transport maximum by translocating Ntcp from intracellular stores to the plasma membrane.

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