Role of Protein Phosphatases in Cyclic AMP-mediated Stimulation of Hepatic Na\(^+\)/Taurocholate Cotransport\(^*\)

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Cyclic AMP has been proposed to stimulate Na\(^+\)/taurocholate (TC) cotransport in hepatocytes by translocating Na\(^+\)/TC cotransport polypeptide (Ntcp) to the plasma membrane and to induce Ntcp dephosphorylation. Whether protein phosphatases 1 and 2A (PP1/2A) are involved in the regulation of Na\(^+\)/TC cotransport by cAMP was investigated in the present study. Okadaic acid and tautomycin, inhibitors of PP1/2A, inhibited cAMP-mediated increases in TC uptake and cytosolic [Ca\(^{2+}\)], and only tautomycin inhibited basal TC uptake. Removal of cAMP reversed cAMP-mediated increases in TC uptake and plasma membrane Ntcp mass. Okadaic acid alone increased Ntcp phosphorylation without affecting Ntcp mass in plasma membranes and homogenates. In the presence of okadaic acid, cAMP failed to increase plasma membrane Ntcp mass, induce Ntcp dephosphorylation, and decrease endosomal Ntcp mass. Phosphorylated Ntcp was detectable in endosomes isolated from okadaic acid-treated hepatocytes but not in endosomes from control and cAMP-treated hepatocytes. PP1 was found to be enriched in plasma membranes, whereas PP2A was mostly in the cytosol. Cyclic AMP did not activate either PP1 or PP2A, whereas okadaic acid inhibited primarily PP2A. These results suggest that 1) the effect of cAMP on Na\(^+\)/TC cotransport is not mediated via either PP1 or PP2A; rather, cAMP-mediated signaling pathway is maintained by PP2A and inhibition of PP2A overrides cAMP-mediated effects, and 2) okadaic acid, by inhibiting PP2A, inhibits cAMP-mediated increases in Na\(^+\)/TC cotransport by decreasing the ability of cAMP to increase cytosolic [Ca\(^{2+}\)]. It is proposed that cAMP-mediated dephosphorylation of Ntcp leads to an increased retention of Ntcp in the plasma membrane, and okadaic acid, by inhibiting PP2A, inhibits cAMP-mediated stimulation of Na\(^+\)/TC cotransport by reversing the ability of cAMP to increase cytosolic [Ca\(^{2+}\)] and to induce Ntcp dephosphorylation.

Efficient hepatic uptake of conjugated bile acids, like taurocholate (TC), is mediated predominantly via Na\(^+\)−coupled cotransport mechanism (1, 2). Two different proteins, namely Na\(^+\)/TC cotransport polypeptide (Ntcp) and epoxy hydrolase, have been shown to mediate Na\(^+\)/TC cotransport into hepatocytes (3, 4). The rat liver Ntcp is a ~51-kDa glycoprotein with seven transmembrane domains (3, 5, 6). Recent studies suggest that Na\(^+\)/TC cotransport and Ntcp are up-regulated by hormones and down-regulated by cholestatic agents. Prolactin increases Na\(^+\)/TC cotransport and Ntcp mRNA in hepatocytes (7). Endotoxin and estradiol decrease TC uptake, Ntcp content of plasma membranes, and Ntcp mRNA (8, 9). These results indicate that Ntcp undergoes transcriptional/translational regulation.

Recent studies also suggest that Ntcp undergoes short term post-translational regulation, but the molecular mechanism underlying this regulation is unclear. In a previous study (10), we reported that cAMP, acting via protein kinase A, stimulates Na\(^+\)/TC cotransport by increasing its maximal transport rate; the effect of cAMP is potentiated by Ca\(^{2+}\)/calmodulin-dependents processes and is down-regulated by protein kinase C. These results would indicate that the stimulation of Na\(^+\)/TC cotransport by cAMP may involve phosphorylation and/or translocation of the transporter as suggested for the glucose transporter (11, 12) and the Na\(^+\)/H\(^+\) exchanger (13). In a recent study (14), we showed that cAMP does not increase transporter synthesis, indicating post-translational regulation of Ntcp. Since treatment of hepatocytes with cAMP results in an increase in plasma membrane and a decrease in endosomal Ntcp mass, we proposed that cAMP increases transport maximum of Na\(^+\)/TC cotransport by translocating Ntcp from an endosomal pool to the plasma membrane (14). In addition, our recent study showed that Ntcp is a serine/threonine phosphoprotein, phosphorylated Ntcp is detectable in the plasma membrane, but not in microsomes and Golgi complex, and treatment of hepatocytes with cAMP results in Ntcp phosphorylation (15). These results raise the possibility that the regulation of Na\(^+\)/TC cotransport by cAMP may also involve phosphorylation/dephosphorylation of Ntcp. However, whether the transport activity and/or translocation of Ntcp is regulated by phosphorylation has not been established.

The role of phosphorylation can be investigated by altering the phosphorylation status of the protein of interest, which is determined by the activity of protein kinases and phosphatases. However, kinases and phosphatases involved in the phosphorylation and dephosphorylation of Ntcp is not known. Protein phosphatases 1 and 2A (PP1/2A) have been shown to affect phosphorylation of a number of cellular proteins (16) and okadaic acid, an inhibitor of PP1/2A (16), has been shown to reverse cAMP-induced dephosphorylation of retinoblastoma protein (17). Thus, in an attempt to define the role of phosphorylation in Ntcp activity and translocation, we studied the effect of okadaic acid on basal and cAMP-induced changes in TC uptake, Ntcp mass, and phosphorylation. Results show that okadaic acid, acting primarily via inhibition of PP2A,
TABLE I

| Treatments | Na⁺/K⁺-ATPase | 5'-Nucleotidase | NADH-dehydrogenase |
|------------|----------------|----------------|-------------------|
| Plasma membranes |                  |                |                   |
| Control    | 17 ± 2.1        | 9 ± 2.8        | 0.2 ± 0.06       |
| cAMP       | 19 ± 2.8        | 10 ± 1.3       | 0.3 ± 0.09       |
| Okadaic acid | 16 ± 1.8        | 11 ± 1.5       | 0.2 ± 0.08       |
| CAMP + okadaic acid | 16 ± 2.2        | 11 ± 1.5       | 0.4 ± 0.05       |
| Low density endosomes |          |                |                   |
| Control    | 0.8 ± 0.17      | 1.0 ± 0.11     | 0.1 ± 0.03       |
| cAMP       | 0.9 ± 0.08      | 1.1 ± 0.15     | 0.2 ± 0.04       |
| Okadaic acid | 0.9 ± 0.13      | 0.9 ± 0.11     | 0.2 ± 0.05       |
| CAMP + okadaic acid | 0.8 ± 0.12      | 1.0 ± 0.13     | 0.1 ± 0.02       |

Ntcp Translocation and Phosphorylation Studies—To determine whether protein phosphatase inhibitor, okadaic acid, affects basal, and CAMP-induced changes in Ntcp phosphorylation and translocation, hepatocytes were pretreated with 100 nM okadaic acid for 15 min before incubating with 10 μM CPT-cAMP for an additional 15 min followed by isolation of plasma membranes and endosomes. For studies to determine the effect on Ntcp phosphorylation, hepatocytes were preincubated with carrier-free [32P]orthophosphate (0.2–0.3 mCi/ml) for 2 h before treatment with okadaic acid and/or CPT-cAMP. Plasma membranes and endosomes were subjected to immunoblot analysis to determine the effect on Ntcp content as well as to immunoprecipitation followed by SDS-PAGE and autoradiography to determine the effect on Ntcp phosphorylation.

For immunoblot analysis, proteins (5–20 μg) from plasma membranes, homogenates, and endosomes were subjected to 12% SDS-PAGE by the method of Laemmli (22) as described previously (14). Proteins were transferred electrophoretically from SDS gels to nitrocellulose membranes (Trans-Blot, transfer membrane, 0.45 μm, from Bio-Rad) and probed with the Ntcp antibody (1:2000 dilution). Peroxidase-conjugated anti-IgG was used as the secondary antibody. The immunoblots were developed with the Amersham Pharmacia Biotech ECL kit according to the manufacturer’s instructions.

For immunoprecipitation of Ntcp, plasma membranes, endosomes, or homogenates (50–400 μg of protein) were solubilized in 1 ml of solubilizer buffer (10 mM HEPES, 0.5 mM NaCl, 0.5% Nonidet P-40, 0.3% Triton x-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mM okadaic acid, and 0.6 mM orthovanadate (pH 7.4)) by incubating at 4 °C for 16–18 h on a rocker platform. Affinity-purified IgG fraction of polyclonal rabbit anti-Ntcp antibody (5 μl) was added to the solubilized proteins and incubated at 4 °C for 2 h. The mixture was subjected to 50% (v/v) protein A-Sepharose CL-4B (Sigma) to each tube, incubation at 4 °C for 30 min with occasional gentle mixing, washing three times with membrane solubilizer buffer, and centrifugation. The pellet was mixed with 60 μl of SDS-sample buffer, boiled for 5 min, and centrifuged. The supernatant was collected and subjected to 10% SDS-PAGE using the method of Laemmli (22). To detect the presence of radiolabeled Ntcp, gels were dried and autoradiographed using Kodak X-Omat AR film at −70 °C.

PP1/2A Assay—The activity of phosphatases was determined in cell homogenate and plasma membranes using glycogen phosphorylase a as a substrate (Protein Phosphatase Assay System from Life Technologies, Inc.). Hepatocytes were incubated in HEPES assay buffer with or without 10 μM CPT-cAMP (15 min). Cells were then washed followed by isolation of plasma membranes as described above. The enzyme activity was determined by measuring the release of inorganic [32P]phosphate from [32P]phosphorylase a at 30 °C (23). The phosphatase activity measured by this method is essentially due to PP1/2A. [32P]Phosphorylase a was prepared by incubating phosphorylase b with phosphorylase kinase and [γ-32P]ATP using the protocol provided by Life Technologies Inc. Briefly, the enzyme reaction was started by adding [32P]labeled phosphorylase a to appropriately diluted homogenate or plasma membranes in protein phosphorylase assay buffer (20 mM imidazole-HCl, 0.1% β-mercaptoethanol, 0.1 mM EDTA, and 1 mg/ml albumin (pH 7.4)). Following 10-min incubation at 30 °C, the reaction was stopped by adding ice-cold 20% trichloroacetic acid. The mixture was centrifuged at 4 °C, and the supernatant was counted for radioactivity. Protein phospho-
phatase activities were calculated after subtracting appropriate blank counts and expressed as nmol of P$_i$ released per min/mg of protein.

To distinguish between the effect on PP1 and PP2A, the phosphatase activity was also determined in the presence of 2 nM okadaic acid, which completely inhibits PP2A activity (23, 24). The remaining phosphatase activity represents PP1 activity.

**Determination of Cytosolic [Ca$^{2+}$]**—The effect of cAMP in the presence and absence of phosphatase inhibitors on cytosolic [Ca$^{2+}$] was monitored continuously using a Ca$^{2+}$-selective fluorescence indicator, Quin-2, as described previously (25). Briefly, hepatocytes were loaded with Quin-2 by incubating with 100 µM Quin-2 AM for 15 min at 37 °C. The fluorescence intensity (excitation 340 nm; emission 500 nm) was continuously monitored using a Hitachi fluorescence spectrofluorometer (F2000). Cytosolic [Ca$^{2+}$] was calculated using a standard formula:

$$\text{Cytosolic [Ca}^{2+}\text{]} = K_\text{d}/(F - F_{\text{min}})/(F_{\text{max}} - F).$$

The dissociation constant ($K_d$) was assumed to be 115 nmol/liter. $F$ is cell fluorescence, $F_{\text{max}}$ is maximum fluorescence after addition of Triton X-100 (10 mg/ml), and $F_{\text{min}}$ is minimum fluorescence after addition of EGTA/Tris (pH > 8.0). All fluorescence values were corrected for autofluorescence.

**Other Methods**—The Lowry method was used to determine cell protein (26). Marker enzymes, 5'-nucleotidase (27), Na$^+$, K$^+$-ATPase (28), and NADH dehydrogenase (29) were assayed using established methods. The blots and autoradiograms were scanned in gray scale using Adobe Photoshop® (Adobe System Incorporated, San Jose, CA), and the relative band densities were quantitated using Signal Gen® (Jandel Scientific Software, San Rafael, CA). All values are expressed as mean ± S.E. Paired t test was used to statistically analyze data with $p < 0.05$ considered significant.

**RESULTS**

**Effect of Protein Phosphatase Inhibitors on TC Uptake**—When hepatocytes were pretreated with okadaic acid or tautomycin (inhibitors of PP1/2A), basal TC uptake remained unaltered except for 100 nM tautomycin, which decreased basal uptake by 30%. However, both okadaic acid and tautomycin decreased the ability of cAMP to stimulate TC uptake in a dose-dependent manner (Fig. 1). Norokadaic acid (100 nM), an inactive analogue of okadaic acid, did not affect either the basal or cAMP-induced TC uptake. Note that 100 nM okadaic acid completely inhibited the stimulatory effect of cAMP, whereas 100 nM tautomycin decreased cAMP effect by only 70%. In contrast, dephostatin, an inhibitor of protein tyrosine phosphatase, did not affect either the basal or cAMP-stimulated TC uptake. Thus, the effect of cAMP may not involve protein tyrosine phosphatase.

To determine whether the inhibitory effect of okadaic acid persists once TC uptake is stimulated by cAMP, TC uptake was determined in hepatocytes first treated with CPT-cAMP for 20 min and then with 100 nM okadaic acid for an additional 20 min (Fig. 2). When Me$_2$SO instead of okadaic acid was added, TC uptake remained elevated. However, when okadaic acid was added, TC uptake returned to the pretreatment value. Thus, okadaic acid prevents as well as inhibits the ability of cAMP to stimulate and maintain stimulated TC uptake.

When hepatocytes were washed to remove added CPT-cAMP, TC uptake also returned to the pre-cAMP value within 20 min (Fig. 2), and this was associated with a decline in plasma membrane Ntcp mass to the basal level (Fig. 3). Thus, the reversibility of cAMP effect on TC uptake following washing (Fig. 2) is likely to be due to decreased plasma membrane Ntcp mass.

**Effect of Okadaic Acid on cAMP-induced Ntcp Dephosphorylation and Translocation**—When hepatocytes were treated with cAMP, Ntcp mass increased by 52% in plasma membrane, decreased by 50% in endosomes, and did not change in homogenates (Fig. 4), as observed previously (14). Okadaic acid alone did not affect either plasma membrane or endosomal Ntcp mass. However, in the presence of okadaic acid, cAMP failed to increase plasma membrane and to decrease endosomal Ntcp mass (Fig. 4). Treatment of hepatocytes with cAMP resulted in 38 and 42% decrease in Ntcp phosphorylation in plasma membranes and homogenates, respectively (Fig. 5). Ntcp phosphorylation increased by 65–70% in both plasma membranes and homogenates, when hepatocytes were treated with 100 nM okadaic acid (Fig. 5). Treatment of hepatocytes with okadaic acid followed by cAMP also resulted in 55–60% increase in plasma membrane and homogenate Ntcp phospho-
rylation. The effect of okadaic acid on plasma membrane and homogenate Ntcp phosphorylation in the presence and absence of cAMP was not significantly different. Similar results were obtained when hepatocytes were first treated with cAMP followed by okadaic acid, i.e. okadaic acid inhibited the ability of cAMP to increase plasma membrane Ntcp mass and to induce Ntcp dephosphorylation (data not shown). Phosphorylated Ntcp was barely detectable in endosomes isolated from control or cAMP-treated hepatocytes, but was detectable in endosomes isolated from okadaic acid-treated hepatocytes (Fig. 5). Thus, phosphorylated Ntcp is located primarily in the plasma membrane and is detectable in endosomes only when PP1/2A are inhibited.

**Effect of Okadaic Acid on Cytosolic [Ca²⁺]**—In a previous study (10) we observed that cAMP-stimulated TC uptake is potentiated by cAMP-induced increases in cytosolic [Ca²⁺]. Others have reported that okadaic acid inhibits agonist (epinephrine, angiotensin II, and vasopressin)-mediated inositol phosphate formation and Ca²⁺ transients in isolated hepatocytes (30, 31). Thus, the effect of okadaic acid on cAMP-stimulated TC uptake may be due to its effect on cAMP-mediated increases in cytosolic [Ca²⁺]. However, the effect of okadaic acid on cAMP-induced increases in cytosolic [Ca²⁺] in hepatocytes has not been previously reported. Pretreatment of hepatocytes with 100 nM okadaic acid did not affect the basal cytosolic [Ca²⁺], but decreased the ability of cAMP to increase cytosolic [Ca²⁺] with 70% inhibition of initial spike and complete inhibition of the following plateau (Fig. 6). These effects are similar to those described for the inhibition of agonist-induced increases in cytosolic [Ca²⁺] by okadaic acid (31). The
The effect of okadaic acid on initial spike was concentration-dependent (Fig. 6). Tautomycin at 10 and 100 nM also decreased cAMP-induced initial spike by 31 and 73%, respectively (data not shown). These results indicate that the inhibition of cAMP-stimulated TC uptake by inhibitors of PP1/2A may, in part, be due to the inhibition of cAMP-induced increases in cytosolic [Ca\(^{2+}\)].

**Effect of cAMP and Okadaic Acid on Protein Phosphatases 1 and 2A**—To determine whether okadaic acid inhibits PP1/2A in the presence of cAMP, the total activity of PP1 and PP2A was determined in homogenate and plasma membranes prepared from hepatocytes pretreated with 10 μM CPT-cAMP for 15 min in the presence and absence of 100 nM okadaic acid. Okadaic acid decreased the total activity (PP1 + PP2A) in homogenate and plasma membranes by 50 ± 10 and 15 ± 2%, respectively, in the presence as well as in the absence of CAMP. However, the total activity in neither homogenate (1.24 ± 0.072 versus 1.19 ± 0.053) nor plasma membranes (1.31 ± 0.093 versus 1.39 ± 0.076) was significantly affected by cAMP. Since cAMP is known to inhibit PP1 by activating inhibitor 1 (32), the effect of cAMP on total activity may be due to activation of PP2A. To determine whether cAMP affected the activity of either PP1 or PP2A, the individual activity of PP1 and PP2A was determined in homogenate and plasma membranes prepared from hepatocytes treated with 10 μM CPT-cAMP for 15 min. The activity of neither PP1 nor PP2A was significantly affected by cAMP (Fig. 7). Thus, cAMP does not affect the activity of PP1 or PP2A in either homogenate or plasma membranes of hepatocytes under our experimental conditions. Note that the distribution of PP1 and PP2A in the homogenate was significantly different from that in the plasma membrane. PP2A represented 60% of total PP1 + PP2A activity in the homogenate and only 13% of the total PP1 + PP2A activity in the plasma membrane. The relative enrichment of PP1 and PP2A in the plasma membrane was 2.5- and 0.2-fold, respectively. Thus, PP2A is mostly in the cytosol, and PP1 is mostly in the plasma membrane of hepatocytes as reported for adipocytes (33).

**DISCUSSION**

The present study showed that inhibitors of PP1/2A decreased the ability of cAMP to stimulate TC uptake in hepatocytes. Norokadaic acid, an inactive analogue of okadaic acid, did not affect either the basal or cAMP-stimulated TC uptake, indicating that the effect of okadaic acid is specific. Okadaic acid also inhibited the ability of cAMP to increase cytosolic [Ca\(^{2+}\)] and plasma membrane Ntcp mass and to induce Ntcp dephosphorylation. These results suggest that PP1/2A are involved in the regulation of this cotransporter by cAMP.

The effect of okadaic acid and tautomycin on cAMP-stimulated TC uptake is most likely mediated via inhibition of PP2A in hepatocytes, although both agents are known inhibitors of PP1 and 2A (16, 34). Okadaic acid has been shown to inhibit only PP2A in hepatocytes (24), and this is further supported by our present findings. For example, PP2A represents 60 and 13% of total PP1/2A activity in homogenate and plasma membranes, respectively (Fig. 7), and okadaic acid inhibits 50 and 15% of the total PP1/2A activity in homogenate and plasma membranes, respectively. Moreover, okadaic acid was more effective in inhibiting cAMP-stimulated TC uptake than tautomycin (Fig. 1), and okadaic acid is a more effective inhibitor of PP2A than tautomycin. The reported IC\(_{50}\) (Calbiochem catalog, 1998, p. 286) for okadaic acid is lower for PP2A (0.1 nM) than PP1 (10–15 nM), and IC\(_{50}\) for tautomycin is higher for PP2A (10 nm) than PP1 (1 nM). Based on these data we propose that PP2A rather than PP1 is involved in cAMP-mediated stimulation of Na\(^+\)/TC cotransport.

Our study provides some insight into the mechanism by which okadaic acid, by inhibiting PP2A, may affect cAMP-stimulated TC uptake. Based on our previous studies (14), we suggested that cAMP increases Na\(^+\)/TC cotransport maximum by translocating Ntcp to the plasma membrane. The role of translocation is further supported by the present finding that...
cAMP-induced increases in plasma membrane Ntcp content and TC uptake return to the basal level following removal of cAMP (Figs. 2 and 3). Since okadaic acid inhibited the ability of cAMP to increase plasma membrane Ntcp content and to dephosphorylate Ntcp, PP2A is involved in cAMP-mediated Ntcp translocation and/or dephosphorylation. The effect of cAMP is, however, not mediated via PP2A, since cAMP failed to activate PP2A. Rather, cAMP-mediated signaling pathway may be maintained by PP2A and is derailed when PP2A is inhibited. Possible mechanisms by which PP2A may affect cAMP-stimulated Na+/TC cotransport are discussed below.

One mechanism may be that PP2A is required for cAMP-mediated translocation of Ntcp to the plasma membrane and hence stimulation of Na+/TC cotransport. This is supported by the present finding that cAMP fails to stimulate Na+/TC cotransport (Figs. 1 and 2) and to increase plasma membrane Ntcp content in the presence of okadaic acid (Fig. 4). PP2A may affect multiples sites in the signaling pathway involved in cAMP-mediated translocation of Ntcp. We have reported previously (10) that the stimulatory effect of cAMP on Na+/TC cotransport is potentiated by cAMP-induced increases in cytosolic [Ca2+]. We also observed that cAMP failed to increase plasma membrane Ntcp when cytosolic Ca2+ was buffered (15). Since okadaic acid inhibited the ability of cAMP to increase cytosolic [Ca2+] (Fig. 6), it may inhibit cAMP-mediated translocation of Ntcp, at least in part, by decreasing cAMP-induced increases in cytosolic [Ca2+]. This effect of okadaic acid also suggests that the activity of PP2A is necessary for cAMP to increase cytosolic [Ca2+]. Okadaic acid has been shown previously to block the ability of cAMP to enhance inositol 1,4,5-trisphosphate binding to its receptor in the presence of Ca2+ (30). PP2A has been proposed to regulate microtubule-dependent vesicle movement in hepatocytes (24). Thus, it is also possible that okadaic acid inhibits cAMP-mediated Ntcp translocation by disrupting vesicle movement. Although likely, a dependence of cAMP-stimulated Na+/TC cotransport and Ntcp translocation on microtubule has not been reported.

Our result that cAMP-mediated Ntcp dephosphorylation and stimulation of TC uptake was reversed by okadaic acid raises the possibility that phosphorylation of Ntcp may also be involved in the regulation of Na+/TC cotransport. Theoretically, phosphorylation may affect the activity and/or translocation of Ntcp. However, results of the present study do not allow a definite conclusion. This is, in part, because protein kinases and phosphatases involved are not known. Moreover, since cAMP does not activate PP2A, it is unclear whether cAMP-mediated Ntcp dephosphorylation is due to inhibition of a kinase or activation of another protein phosphatase. Nevertheless, based on results of the present study (summarized in Table II) it appears likely that phosphorylation regulates Ntcp translocation as discussed below.

The plasma membrane Ntcp mass under basal state should be the result of an equilibrium between the rate of insertion into and the rate of removal from the plasma membrane. Thus, cAMP-mediated increases in plasma membrane Ntcp mass should result from an increase in the rate of insertion or a decrease in the rate of removal. Either of these processes may be influenced by phosphorylation of Ntcp, and our results suggest that dephosphorylation decreases the removal of Ntcp from the plasma membrane. Phosphorylation does not seem to be the signal for insertion, since cAMP induces dephosphorylation of Ntcp. Moreover, it has been proposed that phosphorylation of insulin-regulatable glucose transporter promotes its internalization (35). Thus, dephosphorylation of Ntcp may result in either decreased removal from or increased insertion into the plasma membrane. Our study showed that phosphorylated Ntcp is located primarily in the plasma membrane (Fig. 5). Thus, cAMP-mediated dephosphorylation should occur in the plasma membrane and may lead to decreased removal of Ntcp from the plasma membrane resulting in increased Ntcp mass (see Fig. 8). This explanation is consistent with our finding that cAMP, in the presence of okadaic acid, fails to increase TC uptake and plasma membrane Ntcp mass and to induce Ntcp dephosphorylation.

It follows from the above discussion that increased phosphorylation of Ntcp should result in increased presence of phosphorylated Ntcp intracellularly and decreased Ntcp mass in

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**Table II**

Summary of results from the present study

| Parameters | Subcellular fractions | Hepatocytes treated with |
|------------|-----------------------|-------------------------|
|            |                       | cAMP | Okadaic acid | cAMP + okadaic acid |
| Ntcp activity | Hepatocytes | Increase | No change | No change |
| Ntcp mass  | Homogenate | No change | No change | No change |
|            | Plasma membranes  | Increase | No change | No change |
|           | Endosomes | Decrease | No change | No change |
| Pntcp     | Homogenate | Decrease | Increase | Increase |
|           | Plasma membranes  | Decrease | Increase | Increase |
|           | Endosomes | Undetectable | Detectable | Detectable |
the plasma membrane. Phosphorylated Ntcp was detectable in endosomes isolated from okadaic acid-treated hepatocytes, but not in endosomes isolated from control or cAMP-treated hepatocytes. These results may suggest that any internalized phosphorylated Ntcp is rapidly dephosphorylated and is only detectable when PP2A is inhibited. Our result that okadaic acid-induced increases in Ntcp phosphorylation in the absence of cAMP were not associated with a decline in plasma membrane Ntcp may suggest that phosphorylation does not promote removal of Ntcp from the plasma membrane. However, okadaic acid-induced phosphorylation sites may differ in the presence and absence of cAMP. For example, cAMP-mediated dephosphorylation of a critical amino acid(s) may result in an increased retention of Ntcp in the plasma membrane. Inhibition of PP2A-mediated dephosphorylation by okadaic acid under this condition may lead to rephosphorylation of the critical amino acid(s), resulting in reestablishment of basal level of Ntcp mass in the plasma membrane. Such a mechanism will be consistent with our finding that okadaic acid produces the same effect whether added before or after stimulation by cAMP. Under the basal state, this particular amino acid(s) is already phosphorylated and inhibition of PP2A by okadaic acid may lead to phosphorylation to additional sites without affecting either the activity or net translocation of Ntcp. Studies to test such a possibility, however, will require a prior knowledge of specific amino acids that are phosphorylated and dephosphorylated by okadaic acid and cAMP, respectively.

The possibility that dephosphorylation may affect the transport activity of Ntcp is consistent with our results that cAMP-stimulated TC uptake is associated with cAMP-stimulated phosphorylation of Ntcp. Thus, it can be argued that cAMP increases Na\(^+\)/TC cotransport by inducing Ntcp dephosphorylation. In that case, dephosphorylated Ntcp may represent the active form. The dephosphorylated form of Na\(^+\),K\(^-\)-ATPase has been proposed to be the active form of the enzyme in renal tubule cells (36, 37). However, okadaic acid alone increased Ntcp phosphorylation without inhibiting the basal TC uptake, indicating that increased phosphorylation due to inhibition of PP2A does not affect Ntcp activity. Whether Ntcp activity is affected by phosphorylation resulting from inhibition of other phosphatases remains to be established.

In summary, the present study showed that okadaic acid inhibits cAMP-mediated increases in TC uptake, cytosolic [Ca\(^{2+}\)], and plasma membrane Ntcp mass. In addition, cAMP-mediated dephosphorylation of Ntcp is reversed by okadaic acid. It is proposed that CAMP-mediated dephosphorylation of Ntcp leads to an increased retention of Ntcp in the plasma membrane and okadaic acid, by inhibiting PP2A, inhibits cAMP-mediated stimulation of Na\(^+\)/TC cotransport by reversing the ability of cAMP to increase cytosolic [Ca\(^{2+}\)] and to induce Ntcp dephosphorylation.

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