Role of PP2B in cAMP-induced dephosphorylation and translocation of NTCP

CYNTHIA R. L. WEBSTER, CHRISTOPHER BLANCH, AND M. SAWKAT ANWER

Departments of Biomedical Sciences and Clinical Sciences, Tufts University
School of Veterinary Medicine, North Grafton, Massachusetts 01536

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The aim of the present study was to determine whether cAMP activates PP2B in hepatocytes and whether PP2B is involved in cAMP-mediated increases in Na\(^+\)-TC cotransport and NTCP translocation. The role of PP2B was studied using cypemethrin, an inhibitor of PP2A (7). Another known inhibitor of PP2B (16), such as cyclosporin A, was not tested because liver PP2B has been reported to be resistant to cyclosporin A (30). Moreover, a 1-min ex-
posure to cyclosporin A results in a competitive inhibition of TC uptake in hepatocytes (28). Results show that cAMP stimulates PP2B in hepatocytes and cypermethrin inhibits the ability of cAMP to stimulate PP2B and TC uptake, and to dephosphorylate and translocate NTCP. In addition, PP2B directly dephosphorylates NTCP.

MATERIALS AND METHODS

Materials. TC (salt), PP2B (human recombinant), FK-506, and cypermethrin were purchased from Calbiochem (San Diego, CA). 8-(4-Chlorophenylthio)cAMP (CPT-cAMP), apro tinin, leupeptin, okadaic acid, and collagenase were obtained from Sigma (St. Louis, MO). PP2B substrate, a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (DLDVPPIGPR- DRRVSVAAE), and calmodulin were obtained from BioMol (Plymouth Meeting, PA). [24–3H]taurocholic acid (56 mCi/ mmol), [methoxy-3H]inulin (80 Ci/mmol), (γ-32P]ATP (~3,000 Ci/mmol) and carrier-free [32P]orthophosphate (5 mCi/ml) were purchased from New England Nuclear (Boston, MA). Anti-fusion protein antibodies to the cloned NTCP were generated by the laboratories of Drs. F. J. Suchy and P. J. Meier and were prepared as previously described (1, 29). Male Wistar rats (200–300 g) obtained from Charles River Laboratories served as liver donors.

Hepatocyte preparation. Hepatocytes were isolated from rat livers using a previously described collagenase perfusion method (4). Freshly prepared hepatocytes suspended (100 mg wet weight/ml) in a HEPES assay buffer (pH 7.4) containing the following (in mM): 20 HEPES, 140 NaCl, 5 KCl, 1 MgSO4, 1.0 CaCl2, 0.8 K2HPO4 and 5 glucose were incubated for 30 min at 37°C under air before initiating studies. The following experiments were conducted with hepatocytes: 1) effects of the PP2B inhibitor cypermethrin on basal and cAMP-stimulated TC uptake, cAMP-induced dephosphorylation and translocation of NTCP; and 2) basal and cAMP-induced increases in cytosolic [Ca2+] and decreases in mitogen-activated protein kinase (MAPK) activity; and 3) the effect of cypermethrin on NTCP phosphorylation. In addition, the effect of FK-506, another inhibitor of PP2B, on basal and cAMP-stimulated TC uptake was determined. Details of these experiments are given in the legend of each figure. All studies were repeated in at least three different cell preparations.

Isolation of plasma membranes. Plasma membranes were isolated from hepatocytes pretreated with 5 nM cypermethrin in the presence and absence of CPT-cAMP using a Percoll gradient centrifugation method (9), as previously described for hepatocytes (15, 23). Briefly, hepatocytes were homogenized in a buffer (pH 7.4) containing the following (in mM): 5 HEPES, 0.5 EGTA, 250 sucrose, 1 phenylmethylsulfonyl fluoride, 2 KF, and the PP inhibitors leupeptin and apro tinin (10 μg/ml each) followed by centrifugation. The resuspended pellet was subjected to Percoll gradient centrifugation for the isolation of plasma membranes. Final membrane fractions were washed twice in the homogenization buffer and stored at −70°C. Plasma membranes so derived were enriched in ATPase and Na+/K+ -ATPase, and endosomes were not enriched with plasma membrane marker enzymes, as previously reported (23). The enrichment was not significantly affected by pretreatment of hepatocytes with cAMP and/or cypermethrin.

TC uptake in hepatocytes. The initial uptake rate of TC in hepatocytes was determined as previously described (3). Briefly, at various times after incubation of hepatocytes with cyclosporin (or FK-506) and/or CPT-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 [methoxy-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.

NTCP translocation and phosphorylation studies. To determine whether the PP2B inhibitor cypermethrin affects basal and cAMP-induced changes in NTCP phosphorylation and translocation, hepatocytes were pretreated with 5 nM cypermethrin for 30 min before incubating with 10 μM CPT-cAMP for an additional 15 min followed by isolation of plasma membranes. For studies to determine the effect on NTCP phosphorylation, hepatocytes were incubated with carrier-free [32P]orthophosphate (0.2–0.3 mCi/ml) for 2 h before treatment with cypermethrin and/or CPT-cAMP. Plasma membranes were subjected to immunoblot analysis to determine the effect of cypermethrin and/or CPT-cAMP on NTCP content as well as to immunoprecipitation followed by SDS/PAGE and autoradiography to determine the effect on NTCP phosphorylation. For immunoprecipitation of NTCP, plasma membranes (100 μg protein) were solubilized in 1 ml buffer (10 mM HEPES, 0.5 M NaCl, 0.5% NP-40, 0.3% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 10 μg/ml apro tinin, 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, as previously described (24, 25). Affinity-purified IgG fraction of polyclonal rabbit anti-NTCP antibody (5 μl) was added to the solubilized proteins and incubated at 4°C on a shaker for 2 h. This was followed by the addition of 50 μl of 50% (vol/vol) protein A Sepharose CL4B (Sigma) to each tube, incubation at 4°C for 30 min with occasional gentle mixing, washing three times with membrane solubilization 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 (17). To detect the presence of radiolabeled NTCP, gels were dried and autoradiographed using Kodak X-OMAT AR films at −70°C.

Effect of PP2B on NTCP phosphorylation. To determine whether phosphorylated NTCP is a substrate for PP2B and whether CPT-cAMP-induced NTCP dephosphorylation is mediated via PP2B, [32P]NTCP (phospho-NTCP) was immunoprecipitated from lysate prepared from hepatocytes metabolically labeled with carrier-free [32P]orthophosphate and treated with or without 10 μM CPT-cAMP, as described in NTCP translocation and phosphorylation studies. The immunoprecipitated NTCP attached to protein A Sepharose was incubated for 20 min at 30°C in 25 μl phosphatase reaction buffer containing the following (in mM): 20 Tris, 100 NaCl, 6 MgCl2, 0.5 dithiothreitol, 0.1 CaCl2 and 0.1 mg/ml bovine serum albumin, 100 nM calmodulin, and 10 units of PP2B. The reaction was terminated by 25 μl 2× SDS sample buffer.
followed by boiling and centrifugation. The supernatant (45 μl) was subjected to 10% SDS-PAGE, and the gel was dried and autoradiographed using Kodak XO-MAT AR films at -70°C.

**PP2B assay.** The activity of PP2B was determined in cell lysates using a previously described method (11). Briefly, serine residue of PP2B substrate was phosphorylated by incubating the substrate (150 μM) with the catalytic subunit of cAMP-dependent protein kinase (100 units/ml) and [γ-32P]ATP (1 mM; 30 μCi) in a kinase buffer of the following (in mM): 20 MOPS, 75 MgCl₂, 25 β-glycerophosphate, 5 EGTA, 1 Na₃VO₄, and 1 dithiothreitol for 60 min at 30°C. The 32P-labeled peptide substrate was purified by reversed phase chromatography using a C18 disposable cartridge. The specific activity of fresh preparations was 200–300 μCi/μmol of peptide. The reaction mixture contained 20 μl cell lysates, 5 μl [32P]PP2B substrate (5 μM) and 40 μl assay buffer containing 100 nM calmodulin ± 0.1 mM calcium; 500 nM okadaic acid was added to all reaction mixtures to inhibit PP1 and -2A. The reaction was terminated after 20 min incubation at 30°C by adding 0.5 ml ice-cold stop solution (100 mM phosphate buffer, pH 7.0, in 5% trichloroacetic acid), free inorganic phosphate was isolated by Dowex cation-exchange chromatography and quantitated by scintillation counting. Calcium-dependent release of [32P]phosphate was considered to represent PP2B activity calculated using the specific activity of [32P]phosphopeptide on the day of assay. The activity of PP2B was expressed as picomole of phosphate released per minute per milligram protein.

**MAPK assay.** Cell lysates obtained after treatment of hepatocytes with cypermethrin and/or CPT-cAMP were assayed for MAPK using commercially available assay kits from New England BioLabs (Beverly, MA), as previously described (34). Briefly, whole cell lysates (50–150 μg total protein) were subjected to 10% SDS-PAGE. Separated proteins were transferred electrophoretically from SDS gels to nitrocellulose membranes and probed with the phospho-p44/42 MAPKThr202/Tyr204 antibody (1:1,000 dilution) to detect activated form of MAPK. The blot was stripped and reprobed with p44/42 MAPK antibody (1:1,000 dilution) to detect total MAPK. The immunoblots were developed with an Amersham enhanced chemiluminescence kit according to the manufacturer’s instructions.

**Determination of cytosolic [Ca²⁺].** The effect of cAMP in the presence and absence of cypermethrin on cytosolic [Ca²⁺] was monitored continuously using a Ca²⁺-selective fluorescence indicator, quin 2, as previously described (2). 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 spectrometer fluorocounter (model F2000). Cytosolic [Ca²⁺] was calculated using the standard formula: cytosolic [Ca²⁺] = K_d (F - F_min)/[F_max - F]. The dissociation constant (K_d) was assumed to be 115 nmol/l. F is cell fluorescence, F_max is maximum fluorescence after the addition of Triton X-100 (10 mg/ml), and F_min is minimum fluorescence after the 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 (20). Marker enzymes, 5’nucleotidase (5), Na⁺-K⁺-ATPase (26), and NADH dehydrogenase (32) were assayed using established methods. Blots and autoradiograms were scanned in gray scale using Adobe Photoshop (Adobe, San Jose, CA), and relative band densities were quantitated using SigmaGel (Jandel Scientific Software, San Rafael, CA).

All values are expressed as means ± SE. Paired t-test was used to statistically analyze data with P < 0.05 considered significant.

**RESULTS**

**cAMP activates PP2B.** Basal PP2B activity in freshly isolated hepatocytes was 5.3 ± 0.94 pmol·min⁻¹·mg⁻¹ protein, a value comparable to 3.6 ± 0.6 pmol·min⁻¹·mg⁻¹ reported for rat livers (30). Treatment of hepatocytes with cAMP resulted in a 60% increase in PP2B activity, and this effect was completely inhibited by 5 nM cypermethrin (Fig. 1). Interestingly, cypermethrin did not inhibit basal PP2B activity in hepatocytes (Fig. 1). We were unable to demonstrate inhibition of basal PP2B activity in hepatocytes by ≤1 μM cypermethrin (data not shown). Thus it would appear that cypermethrin does not directly inhibit PP2B in hepatocytes. It has recently been reported that class II pyrethroids, including cypermethrin, do not inhibit bovine brain PP2B in vitro (8).

Cypermethrin and FK-506 inhibit cAMP-stimulated TC uptake. We studied the effect of cypermethrin and FK-506 to determine whether PP2B is involved in cAMP-mediated stimulation of TC uptake. Concentration-dependent studies showed that cypermethrin (0.5 to 5 nM) does not affect basal bile acid uptake (Fig. 2). However, 1 and 5 nM cypermethrin inhibited cAMP-stimulated TC uptake (uptake in the presence of cAMP minus basal uptake) by 50 and 80%, respectively. Similarly, another known inhibitor of PP2B, FK-506, inhibited cAMP-stimulated TC uptake by 40, 75, and 75% at 1, 5, and 10 μM, respectively, without affecting basal TC uptake (Fig. 3). FK-506 has been reported to inhibit liver PP2B activity by 36% at 1 μM (30). Thus inhibition of PP2B is associated with a decrease in TC uptake, indicating a possible role for PP2B. Because 5 nM cypermethrin completely inhibited cAMP-mediated activation of PP2B, further studies were conducted using this concentration of cypermethrin to define the role of PP2B.

**Fig. 1.** Effect of cAMP on protein phosphatase (PP)2B. Hepatocytes were treated with different concentrations of cypermethrin (CM) for 30 min before the addition of 10 μM 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP) or buffer. PP2B activity was determined 15 min after the addition of buffer (basal) or CPT-cAMP. Values are means ± SE (n = 4–6 different cell preparations). *P < 0.05 vs. respective controls.
Cypermethrin reverses cAMP-mediated NTCP translocation and dephosphorylation. We next determined whether cAMP-mediated NTCP translocation is also affected by cypermethrin. For these studies, plasma membranes were prepared from hepatocytes treated with cypermethrin and/or cAMP. Total NTCP content of hepatocytes was not affected by either cypermethrin or cAMP (Fig. 4). Plasma membrane NTCP content was increased by cAMP as previously reported (23), and cypermethrin decreased the ability of cAMP to increase plasma membrane NTCP by 85%, without affecting basal level (Fig. 4). These results suggest that cAMP-mediated increases in TC uptake and NTCP translocation may be regulated by PP2B.

If cAMP-mediated dephosphorylation of NTCP is mediated via PP2B, then this should be inhibited by cypermethrin. This hypothesis was tested by studying the effect of cypermethrin on NTCP phosphorylation. Cyclic AMP decreased plasma membrane NTCP phosphorylation by 50%, and this effect was reversed to 80% of the basal level when hepatocytes were pretreated with 5 nM cypermethrin (Fig. 5), indicating a role for PP2B in cAMP-mediated NTCP dephosphorylation.

Cypermethrin alone did not affect NTCP phosphorylation (Fig. 5), consistent with the result that cypermethrin does not inhibit basal PP2B activity (Fig. 1).

**PP2B directly dephosphorylates NTCP.** If cAMP dephosphorylates NTCP by activating PP2B, then the ability of PP2B to dephosphorylate should be less for NTCP obtained from cAMP-treated hepatocytes compared with control hepatocytes. This hypothesis was tested by incubating PP2B with phospho-NTCP, immunoprecipitated from control and cAMP-treated hepatocytes. PP2B dephosphorylated phospho-NTCP.

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**Fig. 2.** Effect of CM on basal and cAMP-stimulated taurocholate (TC) uptake. Freshly prepared hepatocytes were treated with different concentrations of CM for 30 min before the addition of 10 μM CPT-cAMP or buffer. TC (20 μM) uptake was determined 15 min after the addition of buffer (basal) or CPT-cAMP. Values are means ± SE (n = 5 different cell preparations). *P < 0.05 vs. respective controls.

**Fig. 3.** Effect of FK-506 on basal and cAMP-stimulated TC uptake. Freshly prepared hepatocytes were treated with different concentrations of FK-506 for 30 min before the addition of 10 μM CPT-cAMP or buffer. TC (20 μM) uptake was determined 15 min after the addition of buffer (basal) or CPT-cAMP. Values are means ± SE (n = 3 different cell preparations). *P < 0.05 vs. respective controls.

**Fig. 4.** Effect of CM on cAMP-mediated Na"'-TC-cotransporting polypeptide (NTCP) translocation. Hepatocytes were treated with DMSO or 5 nM CM for 30 min, followed by the addition of buffer (control or CM) or 10 μM CPT-cAMP (cAMP or CM + cAMP). After an additional 15-min incubation, homogenates (total) and plasma membranes (PM) were prepared and subjected to NTCP immunoblot analysis using 10–50 μg protein. **Top**, typical NTCP immunoblots; **bottom**, results of densitometric analysis (means ± SE; n = 7). *P < 0.05 vs. respective controls (Con).

**Fig. 5.** Effect of CM on cAMP-mediated dephosphorylation of NTCP. Hepatocytes were incubated with carrier-free [32P]orthophosphate (0.2–0.3 mCi/ml) for 2 h before treatment with 5 nM CM and/or 10 μM CPT-cAMP as in Fig. 2. NTCP was immunoprecipitated followed by SDS-PAGE and autoradiography as described in NTCP translocation and phosphorylation studies. **Top**, typical autoradiogram of phospho-NTCP (pNTCP); **bottom**, results of densitometric analysis (means ± SE; n = 3). *P < 0.05 vs. control.
from control hepatocytes by 60% (Fig. 6). In contrast, PP2B failed to dephosphorylate phospho-NTCP immunoprecipitated from cAMP-treated hepatocytes (Fig. 6). These results strongly suggest that phospho-NTCP is a direct substrate for PP2B and cAMP dephosphorylates NTCP via PP2B.

Cypermethrin does not affect cytosolic \([Ca^{2+}]\). Results showing that cypermethrin inhibits cAMP-stimulated but not basal PP2B activity raise the possibility that cypermethrin may act by inhibiting the ability of cAMP to increase cytosolic \([Ca^{2+}]\). We thus determined the effect of cypermethrin on basal and cAMP-stimulated increases in cytosolic \([Ca^{2+}]\) (Fig. 7). Thus it is unlikely that the effect of cypermethrin on cAMP-stimulated PP2B activity is due to an inhibition of the ability of cAMP to increase cytosolic \([Ca^{2+}]\).

Cypermethrin does not affect MAPK activity. cAMP inhibits MAPK activity in hepatocytes (34), and cypermethrin may have reversed the effect of cAMP by inhibiting its effect on MAPK. The effect of cypermethrin on MAPK activity was determined from the phosphorylation of p42 and p44 MAPK (also known as extracellular regulated kinases). Cypermethrin did not affect either basal or cAMP-mediated decreases in MAPK activity (Fig. 8). Thus the effect of cypermethrin is not mediated via alterations in MAPK activity.

DISCUSSION

The present study showed for the first time that cAMP stimulates PP2B in hepatocytes and PP2B directly dephosphorylates NTCP. In addition, our study showed that the inhibition of cAMP-mediated stimula-

![Fig. 6. Effect of PP2B on NTCP phosphorylation. Phospho-NTCP immunoprecipitated from either control and CPT-AMP (cAMP)-treated hepatocytes prelabeled with \(^{32}P\) was incubated with PP2B (+PP2B). The reaction product was subjected to SDS-PAGE followed by autoradiography. Top, typical autoradiogram of phospho-NTCP (pNTCP); bottom, results of densitometric analysis (means ± SE; \(n = 3\)). *\(P < 0.05\) vs. control.](http://ajpgi.physiology.org/)

![Fig. 7. Effect of CM on cytosolic \([Ca^{2+}]\) concentration (\([Ca^{2+}])\). Hepatocytes pretreated with DMSO (control) or various concentrations of CM for 30 min were transferred to a cuvette. After a 100-s recording to determine basal cytosolic \([Ca^{2+}]\), 10 \(\mu\)M CPT-cAMP was added, and recording continued for another 5 min. Data represent basal cytosolic \([Ca^{2+}]\) and peak \([Ca^{2+}]\) after the addition of CPT-cAMP (cAMP). Values are means ± SE; \(n = 4\).](http://ajpgi.physiology.org/)

![Fig. 8. Effect of CM on cAMP-mediated inhibition of phosphorylated mitogen-activated protein kinase (pMAPK) kinase. Cell lysates were prepared from hepatocytes treated with 5 nM CM and/or 10 \(\mu\)M CPT-cAMP as in Fig. 2 and then assayed for MAPK. Top, representative blot of phosphorylated p42 and p44 MAPK; bottom, results of densitometric analysis expressed as relative values (means ± SE; \(n = 4\)). *\(P < 0.05\) respective controls.](http://ajpgi.physiology.org/)
Moreover, we have previously reported that cAMP to dephosphorylate NTCP (24) and calmodulin antagonists inhibit cAMP-induced increases in TC uptake (14). Taken together, these results suggest that cAMP-mediated dephosphorylation of NTCP involves cAMP-mediated increases in cytosolic [Ca\(^{2+}\)] followed by Ca\(^{2+}\)/calmodulin-dependent activation of PP2B.

These results, however, do not rule out the possibility of other mechanisms of NTCP dephosphorylation. This is because although 5 nM cypermethrin completely inhibited the ability of cAMP to activate PP2B (Fig. 1), cypermethrin failed to reverse cAMP-mediated NTCP dephosphorylation completely (Fig. 5). Because cAMP decreases MAPK activity in hepatocytes (Fig. 8), it is possible that MAPK phosphorylates NTCP and that cAMP-mediated dephosphorylation, at least in part, involves inhibition of MAPK activity. In a previous study (25) we reported that okadaic acid, an inhibitor of PP2A in hepatocytes, increases NTCP phosphorylation. Preliminary in vitro studies showed that PP2A can dephosphorylate NTCP (unpublished observations). Thus PP2A may also be involved in NTCP dephosphorylation. However, cAMP-mediated dephosphorylation of NTCP is unlikely to be mediated via PP2A, because cAMP does not activate PP2A in hepatocytes (25).

The mechanism of inhibition of cAMP-mediated activation of PP2B by cypermethrin is unclear. Because cypermethrin does not inhibit basal PP2B activity, a direct effect on PP2B is unlikely. Cypermethrin may inhibit a step involved in the activation of PP2B by cAMP. According to our current knowledge (16), the activation of PP2B is dependent on Ca\(^{2+}\) and calmodulin. It is believed that the activation of PP2B involves the Ca\(^{2+}\)-dependent binding of calmodulin to PP2B resulting in a displacement of an autoinhibitory domain. However, because cypermethrin does not affect cAMP-mediated increases in cytosolic [Ca\(^{2+}\)] (Fig. 7), its site of action is likely to be subsequent to the increase in cytosolic [Ca\(^{2+}\)]. It is possible that cypermethrin inhibits Ca\(^{2+}\)-dependent binding of calmodulin to PP2B and/or displacement of the autoinhibitory domain. It can, however, be ruled out that cypermethrin affected cAMP-mediated increases in PP2B activity by altering cAMP-mediated decreases in MAPK activity (Fig. 8). Irrespective of the mechanism of action of cypermethrin, results of the present study suggest that PP2B is involved in cAMP-mediated dephosphorylation of NTCP.

Whether cypermethrin inhibits other phosphatases is not known. While developing the assay for PP2B, we observed that cypermethrin did not inhibit dephosphorylation of PP2B substrate (induced by cell lysate) in the absence of calcium and okadaic acid, an inhibitor of PP1 and -2A. Because cAMP does not activate either PP1 or PP2A, and okadaic acid inhibits cell lysate-induced dephosphorylation of PP2B substrate, it is unlikely that the effect of cypermethrin is mediated via inhibition of either PP1 or PP2A. In addition, cypermethrin did not inhibit cAMP-induced activation of protein kinase B (data not shown) or inhibition of MAPK (Fig. 8). It would thus appear that the effect of cypermethrin on cAMP-stimulated PP2B activity is relatively specific.

Results of the present and previous studies (14, 23, 25) raise the possibility that cAMP-mediated dephosphorylation may facilitate translocation of NTCP to the plasma membrane and this may explain the finding that cAMP stimulates TC uptake by increasing maximal velocity. In a previous study (14), we observed that cAMP-mediated stimulation of TC uptake is dependent on the ability of cAMP to increase cytosolic [Ca\(^{2+}\)]. However, cytosolic [Ca\(^{2+}\)] mobilizing agents like thapsigargin do not stimulate TC uptake, although thapsigargin potentiates the effect of cAMP on TC uptake. Thus the stimulation of TC uptake by cAMP is potentiated, but not mediated, by cytosolic [Ca\(^{2+}\)] (14). Another study (24) showed that the inhibition of cAMP-mediated increases in cytosolic [Ca\(^{2+}\)] inhibited the ability of cAMP to dephosphorylate and translocate NTCP. Results of the present study showed that the ability of cAMP to stimulate TC uptake and translocate NTCP (Figs. 2–4) is diminished with the inhibition of cAMP-mediated NTCP dephosphorylation (Fig. 5). These results raise the possibility that translocation may be related to dephosphorylation. Other studies (33) have suggested that the PI3-K signaling pathway is involved in cAMP-mediated NTCP translocation in hepatocytes. Thus cAMP may facilitate NTCP translocation by simultaneously activating two pathways. On the one hand, cAMP dephosphorylates NTCP by activating Ca\(^{2+}\)/calmodulin-dependent PP2B, and on the other hand, cAMP stimulates NTCP translocation by activating the PI3-K signaling pathway. These two steps can be linked if translocation is dependent on Ca\(^{2+}\)/calmodulin-dependent activation of PP2B. However, because cypermethrin does not inhibit Ca\(^{2+}\)/calmodulin-dependent activation of PP2B (14). It is assumed that dephosphorylation favors retention of NTCP in the plasma membrane, as discussed previously (25). CAM, calmodulin. POK, phosphoinositide-dependent kinase.
dephosphorylation. It can then be speculated that cAMP-mediated NTCP dephosphorylation favors the insertion of NTCP in the plasma membrane and the insertion process is enhanced by the PI3-K signaling pathway (Fig. 9). Such a mechanism can explain why decreases in cytosolic [Ca\(^{2+}\)] alone are not sufficient to stimulate TC uptake and why cytosolic [Ca\(^{2+}\)] can potentiate the effect of cAMP. It is, however, also possible that dephosphorylation and translocation are two independent events and that dephosphorylation by itself increases intrinsic transport activity of NTCP.

In summary, results of the present study suggest that cAMP dephosphorylates NTCP by activating PP2B, a Ca\(^{2+}\)/calmodulin-dependent PP, and that PP2B-mediated NTCP dephosphorylation may be involved in cAMP-mediated NTCP translocation.

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