Differential Inhibition and Posttranslational Modification of Protein Phosphatase 1 and 2A in MCF7 Cells Treated with Calyculin-A, Okadaic Acid, and Tautomycin*

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Calyculin-A (CA), okadaic acid ( OA), and tautomycin (TAU) are potent inhibitors of protein phosphatases 1 (PP1) and 2A (PP2A) and are widely used on cells in culture. Despite their well characterized selectivity in vitro, their exact intracellular effects on PP1 and PP2A cannot be directly deduced from their extracellular concentration because their cell permeation properties are not known. Here we demonstrate that, due to the tight binding of the inhibitors to PP1 and/or PP2A, their cell penetration could be monitored by measuring PP1 and PP2A activities in cell-free extracts. Treatment of MCF7 cells with 10 nM CA for 2 h simultaneously inhibited PP1 and PP2A activities by more than 50%. A concentration of 1 μM OA was required to obtain a similar time course of PP2A inhibition in MCF7 cells to that observed with 10 nM CA, whereas PP1 activity was unaffected. PP1 was predominantly inhibited in MCF7 cells treated with TAU but even at 10 μM TAU PP1 inhibition was much slower than that observed with 10 nM CA. Furthermore, binding of inhibitors to PP2Ac and/or PP1c in MCF7 cells led to differential posttranslational modifications of the carboxyl termini of the proteins as demonstrated by Western blotting. OA and CA, in contrast to TAU, induced demethylation of the carboxyl-terminal Leu309 residue of PP2Ac. On the other hand, CA and TAU, in contrast to OA, elicited a marked decrease in immunoreactivity of the carboxyl terminus of the α-isoform of PP1c, probably reflecting proteolysis of the protein. These results suggest that in MCF7 cells OA selectively inhibits PP2A and TAU predominantly affects PP1, a conclusion supported by their differential effects on cytoskeletal proteins in this cell line.

The serine/threonine protein phosphatase family was initially restricted to four biochemically distinct entities, protein phosphatases-1 (PP1), 2A-2B (PP2A), and 2B (also called calcineurin) (PP2B), a Ca2+-dependent enzyme, and -2C (PP2C), a Mg2+-dependent enzyme (1). This class of enzymes has now been considerably extended by molecular approaches (1, 2). Nevertheless, among its numerous members, PP1 and PP2A can still be considered as the two principal enzymes because of their ubiquitous abundance and broad specificity (1).

PP1 consists of a catalytic subunit (PP1c) associated with various regulatory subunits, forming numerous oligomeric enzymes (1, 3). For instance, in muscles PP1c interacts with the G and M subunits that target it to glyogen and myosin, respectively (reviewed in Refs. 1 and 4). PP1c is a monomeric protein of about 37 kDa. It has a compact, ellipsoidal structure containing in the amino-terminal domain a β-α-β-α-β metal-coordinating unit typical for metal-dependent hydrolases (5).

Four isoforms, α, β (or δ), γ1, and γ2, encoded by three genes (γ1 and γ2 resulting from alternative splicing) (6) are differently expressed in mammalian tissues (7). Protein sequence variations among these isoforms are mainly confined to their carboxyl termini (6), which play a regulatory role in the catalytic activity, as demonstrated by proteolysis (8) and phosphorylation studies (9, 10). Cyclin-dependent protein kinases (cdk) phosphorylate a TPPR consensus sequence present in the carboxyl terminus of all PP1c isoforms and inhibit their activity (9, 10). PP1c is also specifically inhibited by small acidic thermostable proteins, such as inhibitor-1, inhibitor-2, NIPPs (1), and IP (11).

In contrast to PP1c, the catalytic subunit of PP2A (PP2Ac) is always associated with a constant regulatory subunit of 65 kDa (PR65 or A subunit). To this dimeric core, various third or variable regulatory subunits can bind and modulate the enzymatic activity of PP2Ac (1, 12–16). PP2A has long been considered as a predominantly cytosolic enzyme, in contrast to PP1 which is distributed among most cellular compartments (1). However, recent studies have questioned this dogma by demonstrating the presence of PP2Ac in fibroblast nuclei (17) and its association with microtubules (18) and neurofilaments (19).

Serine/threonine protein kinases on Tyr307, two residues upstream of its carboxyl terminus (20), is a 35.6-kDa protein. Two highly homologous isoforms, α and β, encoded by two genes, are expressed in mammals (1). Except for their extremities PP2Ac and PP1c are 67% homologous and most of the amino acids crucial for the 3-dimensional structure of PP1c are conserved in PP2Ac (5). The carboxyl terminus of PP2Ac also plays a regulatory role in the enzymatic activity. PP2Ac is phosphorylated by receptor and Src family tyrosine kinases on Tyr307, two residues upstream of its carboxyl terminus (Leu308), which suppresses its activity (20). In vitro PP2Ac can also be inhibited by phosphorylation on Thr residue(s) catalyzed by the autophosphorylation-activated protein kinase (21). Moreover, PP2Ac is methylated on Leu309 by a novel type of carboxyl protein methyltransferase (22–25).
ethylation is catalyzed by a specific protein carboxy-methyltransferase (26). Interestingly, PP2Ac methylation is regulated during the cell cycle (17). Potent protein inhibitors of PP2A, different from those of PP1c, have been recently identified (27–29). The identification of serine/threonine protein phosphatases as the target of okadaic acid (OA), a polyketol fatty acid, provided novel insights into their essential functions (30). OA is a potent tumor promoter (31) that binds to and inhibits PP1c and PP2Ac with dissociation constants (K_i) of 147 and 0.032 nM, respectively (32). Other poly carbonate inhibitors of serine/threo nine protein phosphatases include calyculin-A (CA) and tautomycin (TAU) (31). CA has a higher affinity for PP2A than PP1, K_i ~0.12 and 1 nM, respectively, whereas TAU has the opposite preference, K_i ~30 and 0.5 nM, respectively (32). OA, CA, and TAU appear to bind to the same site on PP1c or PP2Ac (32). The two major protein Ser/Thr phosphatases, PP2B and PP2C, are either weakly sensitive (in the μM range) or completely insensitive, respectively, to these inhibitors. Other classes of Ser/Thr phosphatase inhibitors have been identified (31, 33–37).

CA and OA are often used to demonstrate the involvement of PP1 or PP2A in a biological process. These conclusions are usually based on the in vitro selectivity of CA and OA and extracellular concentration of the inhibitors required to observe an effect on cells. However, such conclusions can be biased by differential cell penetration rates of CA and OA. Here we show that CA, OA, and TAU have very distinct cell permeation properties and induce specific posttranslational modifications of PP1c and/or PP2Ac in accordance to their in vitro selectivity, suggesting that the systematic use of these three inhibitors should facilitate the assignment of the cellular functions of PP1 and PP2A.

EXPERIMENTAL PROCEDURES

Cell Culture, Incubation with Inhibitors, and Labeling with 32P—MCF7 cells were cultured as described previously (24). Where stated, the medium from a 6-cm dish containing approximately 80% confluent medium from a 6-cm dish containing approximately 80% confluent cells was directly lysed in SDS sample buffer containing 4% (w/v) SDS (150 mN per 6-cm dish). Immunoprecipitations were performed exactly as described (24) with either anti-cytokeratin 8 (50 μg/ml) or anti-cytokeratin 18 (10 μg/ml) monoclonal antibodies purchased from Boehringer Mannheim.

Northern Analysis—Northern blot analysis was performed according to Ref. 41 with total RNA isolated from untreated MCF7 cells (at time 0 or 24 h) or cells treated with 100 nM OA or 10 nM CA, every 6 h until 48 h. The probes used were human PP2Ac and α-PP1c cDNA and, as controls, oligonucleotides complementary to the human β-actin gene (nucleotides 4–30 of the coding region, accession number X00351) and a-tubulin gene (nucleotides 1049–1076 of the coding region, accession number R00558) (41).

RESULTS

Selectivity of CA, OA, and TAU toward PP1 and PP2A Activities in the Soluble Fraction of MCF7 Cell Extracts—Since we were interested in the selectivity of CA, OA, and TAU in MCF7 cells, we first analyzed their apparent affinity for PP1...
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and PP2A in cell extracts. To assay PP2A activity we used a synthetic phosphorylated heptapeptide whose sequence, LRRAASVA, corresponds to the protein kinase A phosphorylation site in pyruvate kinase. About 85% of the total soluble phosphorylase phosphatase activity from MCF7 cell extracts (specific activity without inhibitor: 1.77 ± 0.08 units/mg protein, ± S.E., n = 36) was inhibited by 5 nM OA (Fig. 1A). The 15% phosphorylase phosphatase activity insensitive to 5 nM OA was not suppressed by higher concentrations of OA (up to 1 µM), indicating that this activity could not be attributed to PP1. CA exhibited inhibitory properties similar to OA. In contrast, TAU was approximately 10-fold less potent than OA and CA (Fig. 1A). Henceforth PP2A activity is defined as the phosphorylase phosphatase activity inhibited by 10 nM OA.

Both PP1 and PP2A are active toward phosphorylase a and account for 100% of the cellular activity (42). Total phosphorylase a phosphatase activity in the soluble fraction of MCF7 cells (1.57 ± 0.05 units/mg protein, ± S.E., n = 5) was very sensitive to CA, as expected from the similar high affinity of CA for both PP1 and PP2A (32) (Fig. 1B). With OA an intermediate plateau of inhibition at about 1 nM was observed, PP2A activity corresponding to the phosphatase activity most sensitive to OA (≤1 nM), 0.54 ± 0.06 units/mg protein (± S.E., n = 21) (32), and PP1 activity to the least OA-sensitive (≥1 nM OA), 1.03 ± 0.12 units/mg protein (± S.E., n = 21). When an increasing amount of inhibitor-2 was added, which specifically affects PP1 (1, 39), the inhibition reached a plateau, corresponding to a suppressed PP1 activity of 0.85 ± 0.07 units/mg protein (± S.E., n = 21) (not shown). The exact reason for the difference consistently observed between OA and inhibitor-2 (around 0.2 units/mg protein, see above) is unclear (longer preincubation times with inhibitor-2 did not alter the results) but might be related to the presence of PP1-like activities relatively insensitive to inhibitor-2, as described previously in other systems (43–45). PP1 activity is defined in all further experiments as the phosphorylase a phosphatase activity that was inhibited by a saturating concentration of inhibitor-2. TAU inhibited phosphorylase a phosphatase activity with a potency slightly weaker than that of CA. Addition of 1 nM OA to 1 nM TAU almost completely suppressed the activity (92% in comparison with 66% inhibition observed in the presence of TAU alone). In contrast, inhibitor-2 negligibly increased caused (4%) the inhibition promoted by 1 nM TAU, demonstrating that the activity more sensitive to TAU was catalyzed by PP1 (32).

Treatment of MCF7 Cells with OA Induces a Concentration- and Time-dependent Inhibition of PP2A Activity in Cell-free Extracts—In our experimental conditions to assay PP2A activity from MCF7 cells a concentration of 3 nM OA was required to completely inhibit PP2A activity (Fig. 1A). This value is much higher than the dissociation constant of OA for PP2Ac (Kd 40 pM) (32). Consequently, upon MCF7 cell treatment with OA, the inhibitor bound to PP2Ac should not dissociate during the preparation of MCF7 cell extracts, since the concentration of PP2Ac is always higher than its Ki for OA. Incubation of cells with OA indeed induced a concentration- and time-dependent inhibition of PP2A (Fig. 2A).

OA Treatment of MCF7 Cells Affects the Immunoreactivity of PP2Ac—The possibility that OA altered the level of PP2Ac in MCF7 cells was investigated by Western blot analysis of the soluble and particulate fractions with Ab169/182. With these antibodies (as well as with Ab60/182), no signal could be detected in the particulate fraction from untreated or OA-treated cells (see Ref. 17 for factors affecting the distribution of PP2A.
in cell-free extracts), whereas in the soluble fraction from cells treated with 100 nM OA for 24 h the immunoreactivity of PP2Ac was 1.7-fold ± 0.15 (± S.E., n = 7) higher than that from untreated cells (Fig. 2B). This OA-induced increase was due to an up-regulation of the protein since it could be almost prevented by cotreatment with the protein synthesis inhibitor cycloheximide (Fig. 2C, upper panel) or puromycin (data not shown). The precise mechanism underlying this modest increased expression of PP2Ac upon treatment of MCF7 cells with OA is unknown. Northern blot analysis did not reveal any significant effect of OA treatment on the level of mRNA transcripts encoding PP2Ac (see “Experimental Procedures,” data not shown).

PP2Ac is reversibly methylated on its carboxyl terminus Leu309 residue (22–26) and phosphorylated on Tyr507 (20). We have demonstrated that carboxyl methylation of PP2Ac negatively affects the affinity of Ab299/310 for the protein (17, 24). It is probable that tyrosine phosphorylation has similar consequences. Upon cell treatment with 100 nM OA, the immunoreactivity of the carboxyl terminus of PP2Ac progressively increased with prolonged incubation time to reach 420% ± 115 (± S.E., n = 14) in comparison with that of untreated cells (Fig. 2B). In contrast to what was previously observed with Ab509/182, cycloheximide (or puromycin) did not affect the large OA-induced increase of the carboxyl terminus immunoreactivity (Fig. 2C, lower panel). Since PP2Ac is predominantly carboxyl-methylated and dephosphorylated in asynchronously growing MCF7 cells (24), results obtained with Ab299/310 indicate that the degree of methylation of PP2Ac was lower in OA-treated cells than in untreated cells. Similar demethylation of PP2Ac in response to OA treatment was observed in COS-1 and BHK-21 cells (data not shown).

OA-mediated Inhibition of PP2Ac—Is Due to the Tight Binding of the Inhibitor to PP2Ac—Association of OA with PP2Ac is reversible but very slow (32). Therefore, we tested whether precipitation of soluble extracts with 80% ethanol, which releases the regulatory subunits from PP2Ac (38), could also dissociate OA from PP2Ac. PP2A activity in the soluble fraction from OA-treated cells increased after one ethanol precipitation but recovery was incomplete. It reached a plateau only after three to four successive ethanol precipitations (Fig. 3A, lower panel). After these repetitive ethanol precipitations PP2A activity from OA-treated cells was slightly higher (1.6-fold ± 0.1, ± S.E., n = 3) than that from untreated cells, whereas the OA-insensitive phosphopeptide phosphatase activity was similar in both extracts (Fig. 3A, upper panel). Addition of 100 nM OA to extracts from untreated cells, before starting the ethanol precipitations, did not induce a comparable elevation in PP2A activity ruling out a stabilization effect of OA during the precipitation-solubilization cycles. Ethanol precipitation of PP2A in MCF7 cell-free extracts also elicits demethylation of PP2Ac, which should negatively influence PP2Ac activity (24, 25). Western blot analyses with Ab299/310 of the effect of three successive ethanol precipitations indicated that demethylation, monitored by an increase in immunoreactivity of the carboxyl terminus of PP2Ac, mainly occurred during the first ethanol precipitation/solubilization (Fig. 3B). Therefore, the progressive recovery of PP2A activity in OA-treated samples by repetitive ethanol precipitation/solubilization cycles was essentially due to the dissociation of OA from PP2Ac and could not be related to the degree of methylation of PP2Ac.

PP1 Activity and Immunoreactivity—Is Unaffected in OA-treated MCF7 Cells—Treatment of MCF7 cells with 100 nM OA for 24 h did not affect PP1 activity (Fig. 4A). Western blot analyses with Abα317/330 did not reveal any significant changes due to cell treatment with 100 nM OA when compared with untreated cells (Fig. 4B).

CA Has Comparable Effects to Those Induced by OA on PP2A Activity and Immunoreactivity—If the observed effects of OA on PP2A were a direct consequence of its binding to the protein, similar results should be obtained with other PP2A inhibitors. Treatment of MCF7 cells with 10 nM CA also led to the inhibition of PP2A activity. CA apparently entered the cells much faster than OA, but a plateau was reached when about 60–70% of PP2A activity was inhibited (Fig. 4A). The time course of penetration was dependent on CA concentration as with OA, but even at 100 nM CA inhibition was incomplete. To obtain a

![FIG. 3. Recovery of PP2A activity from the soluble fraction of OA-treated MCF7 cells by repetitive ethanol precipitations. A, PP1 activity, defined as the phosphorylase α phosphatase activity inhibited by inhibitor-2, was assayed in the soluble fraction from untreated cells (white bars), untreated cells to which 100 nM OA was added after their homogenization (shaded bars), or cells treated with 100 nM OA (black bars) for 24 h, before and after successive ethanol precipitations as described under “Experimental Procedures.” B, Western blot analysis with Ab299/310 of the soluble fraction from untreated cells (−) or cells treated with 100 nM OA (+) for 24 h before (0) and after successive ethanol precipitations (1–3) as indicated on the top of the panel. The position of size markers is indicated on the left.](http://www.jbc.org/)

![FIG. 4. Treatment of MCF7 cells with 100 nM OA does not affect PP1 activity and α-PP1c immunoreactivity. A, PP1 activity, defined as the phosphorylase α phosphatase activity inhibited by inhibitor-2, was assayed in the soluble fraction from cells incubated for increasing periods without inhibitor (W, dotted line) or with 100 nM OA (A). 100% activity corresponded to 1.68 ± 0.07 units/mg protein (± S.D., n = 4). B, Western blot analysis with Abα317/330 of the soluble (S) and particulate (P) fractions (5% of each fraction from a 6-cm dish cell culture) from untreated cells or cells treated with 100 nM OA for the indicated period. The position of size markers is indicated on the left.](http://www.jbc.org/)
CA-induced inhibition of PP1 and PP2A activities and changes in immunoreactivity of PP1c and PP2Ac in MCF7 cells.

A, PP2A activity was assayed in the soluble fraction from cells incubated for increasing periods with 10 nM CA (■), 100 nM CA (○), or 1 μM OA (▲), 100% activity corresponded to 2.28 ± 0.19 units/mg protein (± S.E., n = 3). B, Western blot analysis of the soluble fraction (50 μg of protein) from cells treated with or without 10 nM CA for the indicated period. The same membrane was consecutively incubated with Ab169/182 and Ab33k/309, as described under “Experimental Procedures.” PP2A activity after 24 h treatment with 10 nM CA was 20% of controls. C, PP1 activity, defined as the phosphatase α phosphatase activity inhibited by inhibitor-2, was assayed in the soluble fraction from cells incubated for increasing periods with 10 nM CA (■), 100 nM CA (○), or 1 μM OA (▲). 100% activity corresponded to 1.69 ± 0.01 units/mg protein (± S.E., n = 3). D, Western blot analysis with Ab33k/309 of the soluble (S) and particulate (P) fractions (5% of each fraction from a 6 cm dish cell culture) from cells treated with 10 or 100 nM CA for the indicated period. The position of size markers is indicated on the left.

Similar rate of PP2A inhibition to that induced by 10 nM CA, a concentration of 1 μM OA was necessary (Fig. 5A). Upon treatment of MCF7 cells with CA the level of PP2Ac, monitored with Ab33k/309, slightly increased while the degree of methylation of the protein, estimated with Ab317/330, diminished (Fig. 5B).

CA Negatively Affects PP1 Activity and Immunoreactivity in MCF7 Cells—PP1 activity in the soluble fraction of cells treated with CA was inhibited in a concentration- and time-dependent manner as for PP2A (Fig. 5C). Similar to the inhibition of PP2A activity by CA, the suppression of PP1 activity in response to cell incubation with CA was also incomplete. A plateau was reached when about 75% of the activity was inhibited (Fig. 5C).

Surprisingly, treatment of cells with CA resulted in a time- and concentration-dependent decrease in immunoreactivity of the carboxyl terminus of α-PP1c both in the soluble and particulate fractions (50% decrease after about 60 and 90 min, respectively, with 10 nM CA and 40 and 60 min, respectively, with 100 nM CA) (Fig. 5D). A similar phenomenon was observed in the four other cell lines we tested, namely HeLa, COS-1, BHK-21, and REF-52 (data not shown). Since a concentration of 1 μM OA was required to obtain a similar time course of PP2A inhibition to that of 10 nM CA, we also tested the effects of 1 μM OA on PP1c. Neither PP1 activity (Fig. 5C) nor α-PP1c immunoreactivity was affected (data not shown).

CA Induces the Down-regulation of PP1c—The decrease in immunoreactivity detected by Ab317/330 could reflect changes in either the level of the whole protein or a posttranslational modification occurring at the carboxyl terminus. To discriminate between these two hypotheses we used Ab317/330, which are polyclonal antibodies raised against PP1c whose carboxyl terminus (about 30 residues) was cleaved off due to partial proteolysis during its purification (M, 33,000–35,000) (8). Such analysis revealed that PP1c was down-regulated following 10 nM CA treatment (Fig. 6A). The disappearance rate of PP1c (50% decrease after 5 and 3 h in the soluble and particulate fraction, respectively) was slower than that observed with Ab317/330. Further analysis of the mode of action of CA on PP1c indicated that the level of PP1c transcripts in MCF7 cells was unaltered by 10 nM CA treatment for 24 h (see “Experimental Procedures,” data not shown). Moreover, protein synthesis inhibitors (cycloheximide or puromycin) did not mimic the negative effect of 10 nM CA on the carboxyl immunoreactivity of α-PP1c (Fig. 6B). These results suggest that CA treatment promoted the degradation of PP1c.

TAU Treatment of MCF7 Cells Strongly Affects PP1 but Not PP2A—TAU significantly inhibited cellular PP1 activity at concentrations ≥3 μM. The inhibition was both concentration- and time-dependent (Fig. 7A). Treatment of cells with 10 μM TAU resulted in complete inhibition of PP1 activity. TAU promoted a decrease in immunoreactivity of the carboxyl terminus of α-PP1c both in the soluble and particulate fractions (50% decrease in 10 and 7 h, respectively) (Fig. 7B). However, the effect of TAU was not as great as that induced by CA, despite the complete inhibition of PP1. The level of PP1c, tested with Ab317/330, was not significantly altered upon treatment with TAU (data not shown). In contrast to OA and CA, TAU had only a weak effect toward PP2A in MCF7 cells. PP2A was inhibited by approximately 20% after a 24 h treatment of cells with 10 μM TAU (Fig. 7A). Neither the methylation state of PP2Ac (Fig. 7C) nor the level of the protein (data not shown) was altered in MCF7 cells incubated with 10 μM TAU for 24 h. Hyperphosphorylation of Intermediate Filaments in MCF7 Cells upon Treatment with CA and OA but Not TAU—To confirm the apparent selectivity of OA for PP2A and TAU for PP1 in MCF7 cells deduced from our previous experiments, we analyzed their effects, as well as those of CA, on potential substrates for PP1 and/or PP2A, the intermediate filaments. Intermediate filaments are abundant proteins that become rapidly hyperphosphorylated and disorganized following the addition of protein phosphatase inhibitors to various cells (46–
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50). This hyperphosphorylation results in migration shifts on SDS-PAGE that can be easily detected. MCF7 cells express three intermediate filaments, cytokeratin 8, 18, and 19, with $M_r$ of 52,500, 45,000, and 40,000, respectively (51). SDS-PAGE analysis of whole extracts from MCF7 cells treated with 10 nM CA, 100 nM OA, or 10 μM TAU for 24 h revealed that the migration of two abundant proteins, $M_r$ 52,000 and 44,000, was altered by treatment with either CA or OA (Fig. 8A). Their identity with cytokeratin 8 and 18 was indicated by Western blotting with various monoclonal anti-cytokeratin antibodies (Fig. 8B). The correlation between SDS-PAGE migration shift and hyperphosphorylation of cytokeratins induced by CA and OA in MCF7 cells was confirmed by labeling the cells with $^{32}$P, and immunoprecipitating the intermediate filament proteins with either anti-cytokeratin 8 or anti-cytokeratin 18 antibodies (see “Experimental Procedures”) (data not shown). The mobility of the third cytokeratin expressed in MCF7 cells, cytokeratin 19, $M_r$ 40,000, which migrated just beneath an abundant protein identified by Western blotting as actin, was apparently not affected by cell treatment with any protein phosphatase inhibitor (Fig. 8, A and B). TAU did not significantly alter the migration of any cytokeratins (Fig. 8A), indicating that hyperphosphorylation of cytokeratins was not a consequence of PP1 inhibition and supporting the previously drawn conclusions that PP1 is selectively inhibited by TAU and PP2A by OA in MCF7 cells.

**DISCUSSION**

Our data show that PP2A activity is inhibited in cell-free extracts upon treatment of MCF7 cells with CA or OA, whereas PP1 inhibition is observed after cell incubation with CA or TAU. A similar decrease in PP2A activity in cell-free extracts upon treatment of GH4 rat pituitary cells and human fibroblasts with OA has also been observed (52, 53). As demonstrated for cell treatment with OA, PP2A inhibition is due to the tight binding of the inhibitor to PP2Ac. Upon cell incubation with CA, binding of the inhibitor to both PP1 and PP2A could also be released by repetitive ethanol precipitations (data not shown). Based on theoretical considerations (32), similar conclusions can be drawn to explain the inhibition of PP1 after cell treatment with TAU. It is, therefore, possible to take advantage of the tight binding of CA and TAU to PP1, as well as CA and OA to PP2A, to estimate their rate of cellular penetration. It cannot, however, be excluded that part of the inhibitors becomes trapped in membranes or compartments separated from protein phosphatases and gains access to the enzymes only after cell homogenization (measurement of the cellular uptake of radioactively labeled inhibitor could not distinguish between bound and unbound inhibitor). Nevertheless, OA, despite its hydrophobicity, does not significantly distribute to artificial membranes (54). Our results indicate that OA must be 100-fold more concentrated than CA and TAU 100-fold more concentrated than OA to cross the membrane at a similar rate.

The structural characteristics underlying the distinct permeation properties of CA, OA, and TAU is unknown. OA permeates through dipalmitoylphosphatidylcholine membranes only when in a liquid crystalline state (54). It has been recently
reported that various factors such as pH and temperature affect the penetration rate of OA through rabbit erythrocyte membranes and that CA passes across these membranes much faster than OA (55). No similar studies with artificial or biological membranes have been performed with TAU. TAU, such a mixture of two tautomers in solution. It is unclear whether both tautomers are inhibitory to PP1c and PP2Ac. Moreover, because of their distinct electrostatic charges, their membrane permeability is probably dissimilar. In contrast to OA and TAU, CA contains a phosphate group in its chemical structure. This peculiarity might be a determinant for the unique properties of CA, very rapid cell penetration but incomplete protein phosphatase inhibition.

The absence of inhibition in vitro of either PP1 or PP2A after cell treatment with OA or TAU, respectively, does not necessarily mean that the enzymes were not inhibited within the cell. Because of the higher cell, respectively, does the 10 nM CA treatment with 10 nM CA could not be reversed by incubating MCF7 cells with fresh medium for more than 24 h (data not shown).

Anomalous immunoreactivity induced by ethanol precipitation of MCF7 cells. The absence of inhibition in vitro of either PP1 or PP2A after cell treatment with OA or TAU, respectively, does not necessarily mean that the enzymes were not inhibited within the cell. Because of the higher cell, respectively, does the 10 nM CA treatment with 10 nM CA could not be reversed by incubating MCF7 cells with fresh medium for more than 24 h (data not shown).

A plausible mechanism for the disappearance of the carboxyl-terminal immunoreactivity of α-PP1c is partial proteolysis. The carboxyl terminus of PP1c is very sensitive to proteolysis in vitro after dissociation from regulatory subunits (8, 38). However, we could not detect the presence of a partially proteolyzed form of PP1c, and incubation of cell extracts (prepared without protease inhibitors) in the presence of CA at 37 °C did not affect the integrity of PP1c. Therefore, it cannot be excluded that the decrease in the immunoreactivity of the carboxyl terminus of PP1c-isoform of PP1c (40). Consequently, the α-isoform of PP1c might be more (or exclusively) susceptible to a posttranslational modification than other isoforms, if expressed in MCF7 cells. For instance, only α-PP1c is phosphorylated by tyrosine protein kinases (59). The use of specific antibodies toward the other PP1c isoforms should answer this question.

The apparent lack of effect of TAU on PP2Ac and OA on PP1c, in MCF7 cells is in agreement with their in vitro selectivity for both protein phosphatases and strongly suggests that they can be used to investigate the physiological roles for each type of protein phosphatase. Studies on smooth muscle contraction have illustrated their selectivity in vivo. OA up to 1 μM inhibits the Ca2+-dependent contraction of the actomyosin fibers in contrast to CA and TAU, which induce a Ca2+-independent contraction of smooth muscles (60). At concentrations higher than 1 μM, OA apparently mimics the action of CA and TAU, suggesting that PP1 is involved in the dephosphorylation of myosin light chains, a conclusion also supported by biochemical studies (61).

The apparent selectivity of OA and TAU for PP2Ac and PP1, respectively, is confirmed by their differential effects on intermediate filaments in MCF7 cells. Our results suggest that inhibition of PP2A, induced by cell treatment with OA or CA, is responsible for the hyperphosphorylation of these cytoskeletal proteins. This conclusion is supported by the demonstration of the association of PP2A with neurofilaments that belong to the family of intermediate filaments (19). Based on comparison between in vitro affinities of OA, dinophysistoxin (35-methyl-OA), and CA for PP1 and PP2A and the extracellular concentrations required to induce the hyperphosphorylation of the intermediate filament protein vimentin in BHK-21 cells, Eriksson et al. (46) came to the reverse conclusion, PP1 being probably responsible for the steady dephosphorylation of vimentin. However, the time course of cell permeation by these inhibitors was not taken into account. In summary, in MCF7 cells, depending on their concentrations, (i) TAU initially inhibits PP1 and weakly promotes the down-regulation of PP1, (ii) OA specifically inhibits PP2A and induces the demethylation of PP2A, and (iii) CA strongly affects both PP1 and PP2A simultaneously. We suggest that the systematic use of these three inhibitors, while taking into account their cell membrane permeation properties by measuring
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