EXPERIMENTAL NEUROLOGY **147,** 238–247 (1997)
ARTICLE NO. EN976602

In Vitro Phosphorylation of Cytoskeletal Proteins in the Rat Cerebral Cortex Is Decreased by Propionic Acid

Ângela de Mattos-Dutra, Marta Sampaio de Freitas, Nadja Schröder, Carla Simone Fogaça Lisboa, Regina Pessoa-Pureur, and Moacir Wajner

Departamento de Bioquímica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, 90046-900 Porto Alegre, RS, Brazil

In the present study we demonstrate that propionic acid (PA), a metabolite that accumulates in large amounts in propionic acidemia, is able to decrease *in vitro* incorporation of [^{32}P]ATP into neurofilament subunits (NF-M and NF-L) and α- and β-tubulin. Considering that the endogenous phosphorylating system associated with the cytoskeletal fraction contains cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin protein kinase II (CaMKII), and protein phosphatase 1 (PP1), we first assayed the effect of the acid on the kinase activities by using the specific activators cAMP and Ca^{2+}/calmodulin or the inhibitors PKAI or KN-93 for PKA and CaMKII, respectively. Results demonstrated that the acid totally inhibited the stimulatory effect of cAMP and interfered with the inhibitory effect of PKAI. In addition, PA partially prevented the stimulatory effect of Ca^{2+}/calmodulin and interfered with the effect of KN-93. In addition, we demonstrated that PA totally inhibited *in vitro* dephosphorylation of neurofilament subunits and tubulins mediated by PP1 in brain slices pretreated with the acid. Taken together, these results demonstrate that PA inhibits the *in vitro* activities of PKA, CaMKII, and PP1 associated with the cytoskeletal fraction of the cerebral cortex of rats. This study suggests that PA at the same concentrations found in tissues from propionic acidemic children may alter phosphorylation of cytoskeletal proteins, which may contribute to the neurological dysfunction characteristic of propionic acidemia. © 1997 Academic Press

INTRODUCTION

Propionic acid (PA) is found in large amounts in tissues of patients with propionic acidemia, an inherited disorder of amino acid and lipid metabolism caused by severe deficiency of propionyl-CoA carboxylase activity (14). The levels of the acid in blood and cerebrospinal fluid (CSF) usually are as high as 2.5–5 m*M* during crises and can be much higher in the neuronal cells (24). Encephalopathy is the clinical hallmark of the disease. Among the neurologic symptoms often present, psychomotor delay/mental retardation, focal and generalized convulsions, cerebral atrophy, and EEG abnormalities are the most frequent. The mechanisms underlying the pathophysiology of the neurologic dysfunction of propionic acidemia are unknown.

The cytoskeleton of all cells is formed by microtubules, intermediate filaments, and microfilaments, which are distinct elements made of fibrous polymer structures.

Neurofilaments (NF) are a class of intermediate filaments specially expressed in neuronal cells. Mammalian neurofilaments are composed primarily of three subunits having apparent molecular masses of 200 kDa (NF-H), 160 kDa (NF-M), and 70 kDa (NF-L) (22) as determined by SDS–PAGE. Neurofilaments are believed to play an important role in the maintenance of neuronal shape and determination of the axonal caliber (23, 36). Tubulins and neurofilaments undergo posttranslational modifications that modulate their physiological role in the neuronal cells. The most extensively studied posttranslational modification of these cytoskeletal proteins is phosphorylation. The neurofilament subunits are phosphorylated by different second-messenger-dependent and -independent protein kinases in their head and tail domains, respectively. Phosphorylation in the head domain is probably related to neurofilament assembly, while phosphorylation in the tail domain is considered to be one means by which neurofilaments cross-link and stabilize the axonal cytoskeleton (37, 45).

Microtubules are mainly composed of α- and β-tubulin subunits. Tubulins can be phosphorylated in their carboxyl-terminal regions by multiple protein kinases (49). Phosphorylation is known to stabilize neurofilaments as well as microtubules in the axoplasm by distinct mechanisms ensuring metabolically stable structures for axonal transport (39). Furthermore, phosphorylation plays a central role in the dynamic remodeling of cytoskeleton architecture (37). In the brain, neurofilaments and microtubules are involved in a

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variety of cellular functions, including the axonal transport in the mature neuron (25) and plasticity (1).

The various cytoskeletal components of neurons are thought to have different vulnerability to drugs (18, 44). In addition, several neurotoxicants such as aliphatic hexacarbons (30), β, β′-iminodipropionitrile (IDPN) (21), carbon disulfide (28), and acrylamide (17) produce a central-peripheral neuropathy resulting in the accumulation of neurofilaments proximal to the nodes of Ranvier (31). Although the mechanisms of these neuropathies are still unknown, changes in the phosphorylation state of cytoskeletal proteins are thought to be involved (30).

Alterations in the levels of phosphorylation of cytoskeletal proteins have been demonstrated in various neurodegenerative disorders, including Alzheimer's disease (26). Therefore, in the present investigation we studied the influence of PA on this *in vitro* phosphorylation of cytoskeletal proteins and on the enzymes involved in the phosphorylating system in order to identify a possible underlying mechanism associated with the damage to the brain of propionic acidemic patients. We describe inhibitory effects of PA on the *in vitro* phosphorylation of NF-M, NF-L, and α- and β-tubulins obtained from the Triton-insoluble cytoskeletal fraction of the cerebral cortex of rats. Our results suggest that the inhibitory effect of PA is mediated by protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and protein phosphatase 1 (PP1).

MATERIALS AND METHODS

Animals

Wistar rats were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22°C) colony room. Free water and a 20% (w/w) protein commercial chow were provided. On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments.

Pretreatment of Tissue Slices

Slices from cerebral cortex of 17-day-old rats were incubated in the presence or in the absence of 2.5 m*M* buffered PA (Sigma, St. Louis, MO), pH 7.2–7.4. Incubation was carried out in a Dubnoff metabolic shaker for 1 h at 30°C in an atmosphere of 95% O_2 and 5% CO_2. Each flask contained approximately 600 mg of cerebral cortex slices in 2 ml Krebs–Ringer bicarbonate buffer (KRB), pH 7.4, and 5 m*M* glucose, containing all the protease inhibitors described below with 1 μ*M* calpain inhibitor added (Sigma). Incubations were stopped by the addition of 20 vol ice-cold cytoskeletal extraction buffer and performed as described below.

Triton-Insoluble Cytoskeletal Preparation from Cerebral Cortex

The cytoskeletal fraction was prepared as described by de Mattos *et al.* (10). Cerebral cortex (600 mg) was homogenized in 40 ml of ice-cold buffer containing 50 m*M* Tris–HCl, pH 6.8, 5 m*M* EGTA, 1% Triton X-100, and the following protease inhibitors: 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 m*M* benzamidine, 10×10^{-1} μ*M* leupeptin, 7×10^{-1} μ*M* antipain, 7×10^{-1} μ*M* pepstatin, and 7×10^{-7} μ*M* chymostatin (Sigma). The homogenate was centrifuged at 13000*g* for 15 min, at 4°C, in a Sorvall RC-2B centrifuge, SS-34 rotor (Du Pont Instruments, Newtown, CT). The insoluble pellet was resuspended in 40 ml of the same buffer containing 0.85 *M* sucrose and centrifuged under the same conditions. The pellet was dissolved in 50 m*M* Mes (2[*N*-morpholino] ethanesulfonic acid), pH 6.5, and 10 m*M* $MgCl_2$ and the protein concentration was determined by the method of Bradford (3).

In Vitro ^{32}P Incorporation Assays

An enriched cytoskeletal fraction prepared as described previously served as a protein substrate and a source of endogenous protein kinases and phosphatases. Each assay mixture contained 10 μg of protein. Phosphorylation was carried out in 60-μl assay mixtures at pH 6.5 in buffer containing 50 m*M* Mes, 10 m*M* $MgCl_2$, and 1 μ*M* calpastatin (10). The reaction was started by adding 5 p*M* [γ-^{32}P]ATP (5.5×10^{-10} Bq/mmol) (ICN Radiochemicals, Irvine, CA). After 5 min of incubation at 30°C, the reaction was stopped by adding Laemmli sample buffer and the samples were boiled for 3 min. Proteins were analyzed by SDS–PAGE and autoradiograms were obtained from the gels. ^{32}P incorporation into cytoskeletal proteins was measured by liquid scintillation counting in 5.06 *M* toluene, 2.05 *M* ethanol, 12.4 m*M* PPO (2,5-diphenyloxazole), and 0.6 m*M* POPOP [1,4-bis(5-phenyl-2-oxazol)benzene;2,2-*p*-phenylene-bis (5-phenyloxazole)] (Sigma) and 34.5% Triton X-100. The endogenous kinase activities were also assayed using the synthetic peptide substrates syntide 2 and kemptide (Sigma), respectively. The assay mixture was essentially as described previously, except that it contained 40 μ*M* syntide 2 or 30 μ*M* kemptide in the absence or in the presence of 2.5 m*M* PA. After 5 min of incubation at 30°C, reactions were stopped by spotting 20 μl of the assay mixture on P-81 phosphocellulose filters (GIBCO BRL). Filters were washed with 75 m*M* phosphoric acid and the radioactivity was measured in a liquid scintillation counter.

CaMKII and PKA activity assays. The standard assay mixture was the same as described previously. Assays for Ca^{2+}/CaMKII activity contained 1 μ*M* calmodulin (Sigma) and 1 m*M* $CaCl_2$ in the absence or

in the presence of buffered 2.5 m*M* PA (pH 7.2–7.4). Cyclic AMP-dependent PKA activity was determined in reaction mixtures containing 20 µ*M* cAMP (Sigma), 2 m *M* EGTA, and 1 m*M* $MgCl_2$ in the absence or in the presence of PA. The endogenous CaMKII and PKA activities were also assayed in the presence of the exogenous substrates syntide 2 (40 µ*M*) and kemptide (30 µ*M*) using the same conditions as described previously. The effect of inhibitors on the kinase activities was determined by incubation of the assay mixtures with KN93 (Calbiochem Corporation, San Diego, CA) or protein kinase A inhibitor fragment 6-22 amide (PKAI) (Sigma), which are the specific inhibitors of CaMKII and PKA activity, respectively. The CaMKII inhibitory assay contained 10 µ*M* KN93 in the absence or presence of buffered PA and the PKA inhibitory assay contained 80 µ*M* PKAI, also in the absence or in the presence of propionic acid. After 5 min of incubation at 30°C the reactions were stopped as described above and submitted to SDS–PAGE.

Phosphatase activity assay. The standard assay system was essentially as described previously. The effect of PA on the phosphatase activity was determined by adding buffered 2.5 m*M* PA (pH 7.2–7.4) to the reaction mixtures at 0 or 5 min of incubation. The assays that received the acid at 5 min were incubated for 10, 20, or 30 min. Okadaic acid (Sigma) previously prepared as a 50 µ*M* stock solution in 10% dimethylsulfoxide (DMSO) was added to some reaction mixtures to give a final concentration of 0.5 µ*M*, the concentration known to inhibit PP1 activity (7). The reactions were stopped after 5, 10, 20, or 30 min and submitted to SDS–PAGE.

SDS–PAGE

SDS–PAGE was performed on 10% acrylamide according to the discontinuous system of Laemmli (30). Gels were stained with 0.25% (w/v) Coomassie blue R-250 (Sigma), 50% (v/v) methanol, and 10% (v/v) acetic acid and destained overnight in 50% methanol and 10% acetic acid.

After SDS–PAGE, cytoskeletal proteins were quantified as described by Rubin *et al.* (42). Briefly, destained, dried SDS–PAGE gels were scanned with a densitometer (Hoefer Scientific Instruments GS 300 Transmittance/Reflectance Scanning Densitometer, San Francisco, CA) equipped with a chart recorder. The relative distribution of cytoskeletal proteins was calculated by cutting out and weighing the area under each peak of the densitometric scan and calculating its percentage contribution to the total area. Therefore, cytoskeletal protein concentrations were calculated from percentage values considering the total protein concentration of cytoskeletal fraction measured by the method of Lowry *et al.* (35) to correspond to 100% (total area of densitometric scan).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the *F* test was significant ($P < 0.05$). All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

RESULTS

The autoradiograph shown in Fig. 1 demonstrates that under our experimental conditions, cytoskeletal proteins from rat cerebral cortex are phosphorylated when incubated with [^{32}P]ATP. We observe that the 150-kDa neurofilament subunit (NF-M), as well as α- and β-tubulins, is a good substrate for the endogenous phosphorylation system. Furthermore, as seen in Table 1, the exogenous substrates syntide 2 and kemptide are also good substrates for the endogenous phosphorylation system. When assays contained the protein kinase activators cAMP and Ca^{2+}/calmodulin, we observed that syntide 2 acted as a good substrate for both PKA and CaMKII, whereas calmodulin did not stimulate ^{32}P incorporation into kemptide.

Table 2 shows the effect of 2.5 m*M* PA on *in vitro* ^{32}P incorporation into the cytoskeletal fraction. The acid inhibited this incorporation by approximately 27%. PA was also observed to have the same effect when the exogenous substrates syntide 2 (39%) and kemptide (42%) were added to the cytoskeletal fraction.

Next we preincubated brain slices in the presence of 2.5 m*M* PA and studied the *in vitro* ^{32}P incorporation

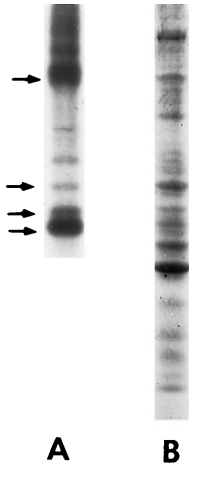

FIG. 1. Polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography of the cytoskeletal fraction. (A) Autoradiography obtained after *in vitro* incorporation of ^{32}P into the cytoskeletal fraction. The positions of 150- and 68-kDa neurofilament subunits, and α- and β-tubulin (top to bottom) are marked by arrows at left in lane A. (B) SDS–PAGE of the cytoskeletal fraction stained with Coomassie blue.

TABLE 1

Effect of cAMP and Ca^{2+}/CaM on the *in Vitro* Incorporation of ^{32}P into the Exogenous Substrates Syntide 2 and Kemptide

Exogenous substrates	^{32}P incorporation (cpm)	% of incorporation
Syntide 2 + EGTA	13,466	100
Syntide 2 + EGTA + cAMP	26,555	197
Syntide 2	19,991	100
Syntide 2 + Ca^{2+} + CaM	35,349	177
Kemptide + EGTA	8,532	100
Kemptide + EGTA + cAMP	74,854	877
Kemptide	19,225	100
Kemptide + Ca^{2+} + CaM	19,713	102

Note. Values correspond to a representative experiment. Cytoskeletal fractions were incubated in the presence of syntide 2 and kemptide.

into various proteins of the cytoskeletal fraction in order to identify which proteins were affected by the metabolite. We detected a significant diminution of phosphorylation in the neurofilament subunits M (NF-M) and L (NF-L) and α-tubulin and β-tubulin (Fig. 2).

The effect of PA on cAMP-dependent PKA and Ca^{2+}/CaMKII was also assayed by adding the specific protein kinase activators, cAMP and Ca^{2+}/calmodulin, respectively, to the incubation system in the presence or in the absence of the acid (Fig. 3). We first verified that cAMP enhances *in vitro* ^{32}P incorporation into NF-M, NF-L, and tubulins. It is also clear in Fig. 3 that 2.5 m*M* PA inhibits *in vitro* ^{32}P incorporation into these proteins and also prevents the activating effect of cAMP. These results suggest that the inhibition of ^{32}P incorporation

TABLE 2

Effect of Propionate (PA) on ^{32}P *in Vitro* Incorporation into the Cytoskeletal Fraction and Exogenous Substrates

Treatment	Substrates		
Control	Cytoskeletal fraction	Cytoskeletal fraction + syntide 2	Cytoskeletal fraction + kemptide
	48,988 ± 403	65,843 ± 2285[a]	62,983 ± 530[a]
MMA	Cytoskeletal fraction + PA	Cytoskeletal fraction + syntide 2 + PA	Cytoskeletal fraction + kemptide + PA
	35,788 ± 528[a]	39,939 ± 1544[a,b]	36,807 ± 3025[b,c]

Note. Data represent ^{32}P incorporation (cpm) into substrates and are expressed as means ± SEM values from three to six experiments. The cytoskeletal fraction was prepared as indicated under Materials and Methods and 2.5 m*M* propionate was added at the beginning of the experiments. Comparison between means was calculated by the Student *t* test.

[a] Statistically significant differences from control for $P < 0.01$.

[b,c] Statistically significant differences from cytoskeletal fraction + syntide 2 or kemptide, respectively, for $P < 0.01$.

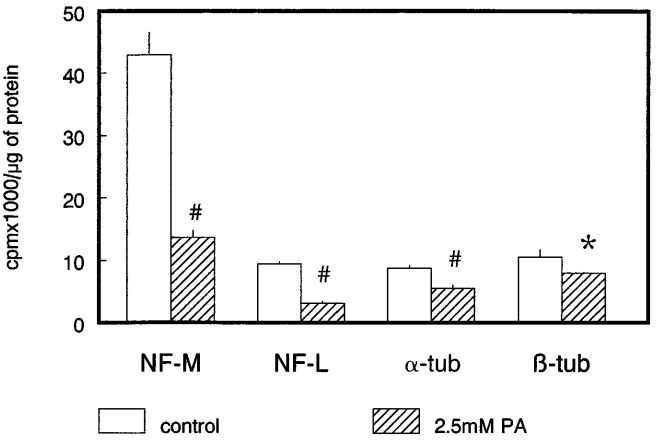

FIG. 2. Effect of pretreatment of brain slices with PA on *in vitro* ^{32}P incorporation into the Triton-insoluble cytoskeletal fraction proteins. 150-kDa neurofilament subunit, NF-M; 68-kDa neurofilament subunit, NF-L; α- and β-tubulins, α- and β-tub. Tissue slices were incubated for 1 h at 30°C with 2.5 m*M* PA. The cytoskeletal fraction was incubated in the presence of the acid and *in vitro* ^{32}P incorporation measured as described under Materials and Methods. Data are means ± SEM values from five or six experiments. Statistically significant differences from controls, as determined by the Student *t* test, are indicated: #, $P < 0.01$, *$P < 0.05$.

induced by PA might be mediated by cAMP-dependent protein kinase.

In order to better clarify the inhibitory effect of the metabolite on the phosphorylation system associated with the cytoskeletal fraction, we tested the effect of PA on CaMKII activity (Fig. 4). We first verified that Ca^{2+}/calmodulin activates incorporation of ^{32}P into the cytoskeletal proteins. When PA was added to the incubation system in the presence of Ca^{2+}/calmodulin, we observed a differential effect on the various proteins. The *in vitro* ^{32}P incorporation into NF-M and α-tubulin was partially blocked by the metabolite in the presence of Ca^{2+}/calmodulin (Figs. 4A and 4C). However, *in vitro* ^{32}P incorporation into NF-L and β-tubulin was not affected by PA in the presence of the activators (Figs. 4B and 4D).

We also investigated the influence of PKAI (Fig. 5A) and KN93 (Fig. 5B), inhibitors of PKA and CaMKII, respectively, on the effect of PA on ^{32}P incorporation into cytoskeletal proteins. For these experiments, brain slices were first preincubated with PA, the cytoskeletal fraction was obtained as usual, and the incorporation assays were carried out in the presence of PA and PKAI or KN93. Controls were identical but with no acid added. Figure 5A shows that both PKAI and PA added separately to the incubation system decrease *in vitro* incorporation of ^{32}P into the cytoskeletal proteins. However, when PKAI was added to the incubation assay after preincubation of brain slices with PA, the effects were not additive, suggesting that the mechanisms of action of PA and PKAI are somehow related.

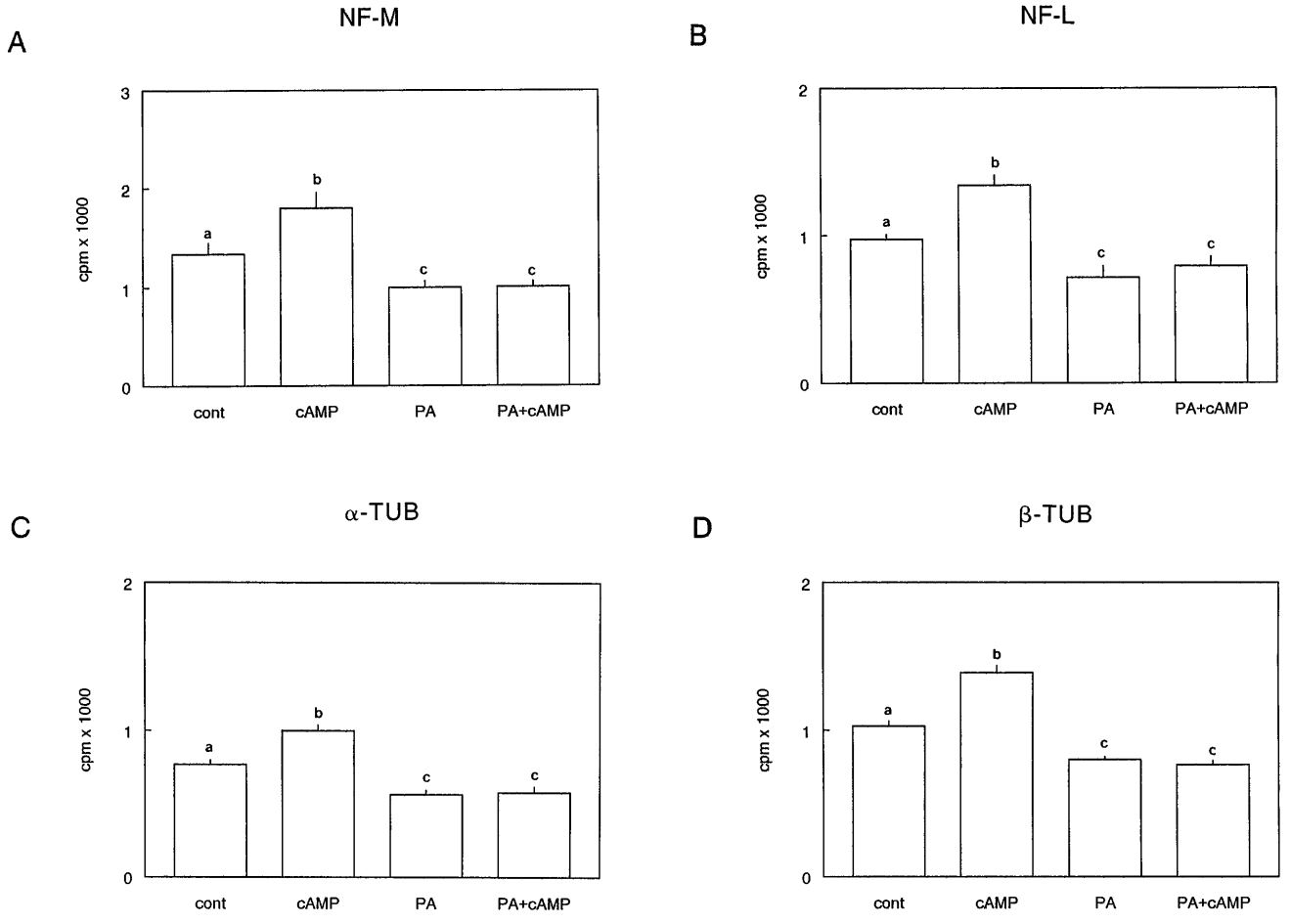

FIG. 3. Effect of pretreatment of brain slices with PA on cAMP-dependent *in vitro* incorporation of ^{32}P into cerebral cortex cytoskeletal proteins. 150-kDa neurofilament subunit, NF-M; 68-kDa neurofilament subunit, NF-L; α-tubulin, α-tub; β-tubulin, β-tub. Tissue slices were incubated for 1 h at 30°C with 2.5 m*M* PA. Controls did not contain the acid. The cytoskeletal fraction obtained was incubated in 60-μl assay mixtures containing 50 m*M* Mes, pH 6.5, 1 m*M* $MgCl_2$, and 2 m*M* EGTA in the presence of 20 μ*M* cyclic AMP or 2.5 m*M* PA or both (PA + cAMP). The *in vitro* ^{32}P incorporation was measured as described under Materials and Methods. Data are means ± SEM values for four experiments. Statistically significant differences were determined by ANOVA, followed by the Duncan multiple range test. Statistically significant differences between means of various groups: $P < 0.05$; $a \neq b \neq c$.

Similar results were obtained when the incorporation assays were undertaken in the presence of KN93, a specific CaMKII inhibitor, following PA pretreatment. Under these conditions, inhibition was similar to that obtained only with KN93 suggesting that the inhibitions caused by KN93 and PA are also related (Fig. 5B).

Finally we studied the effect of PA on the phosphatase activity associated with the cytoskeletal fraction (Fig. 6). The figure exhibits the *in vitro* dephosphorylation of NF-M, NF-L, and α- and β-tubulin proteins obtained from brain slices preincubated in the absence or in the presence of PA. The *in vitro* phosphorylation level of all studied cytoskeletal proteins extracted from control tissue slices decreased during incubation (30 min) in the absence of okadaic acid (OA). We also observed a constant rate of phosphorylation over a period of 30 min when the phosphatase inhibitor okadaic acid was added to the system. Next, we tested the action of PA added at time zero (PA 0) or 5 min (PA 5) after ^{32}P incubation. When tissue slices were preincubated with the drug at the beginning of the incubation (PA 0), the *in vitro* phosphorylation level was decreased compared to controls and remained so throughout the entire experiment. These results are in agreement with our previous experiments demonstrating that the drug inhibits *in vitro* ^{32}P incorporation. When PA was added after 5 min of ^{32}P incubation (PA 5), the phosphorylation level was higher and remained the same throughout incubation, indicating an inhibited phosphatase activity, as we can observe by comparison with the dephosphorylation pattern obtained in the presence of 0.5 μ*M* okadaic acid, a drug known to inhibit protein phosphatase. These findings indicate an additional effect of PA inhibiting PP1.

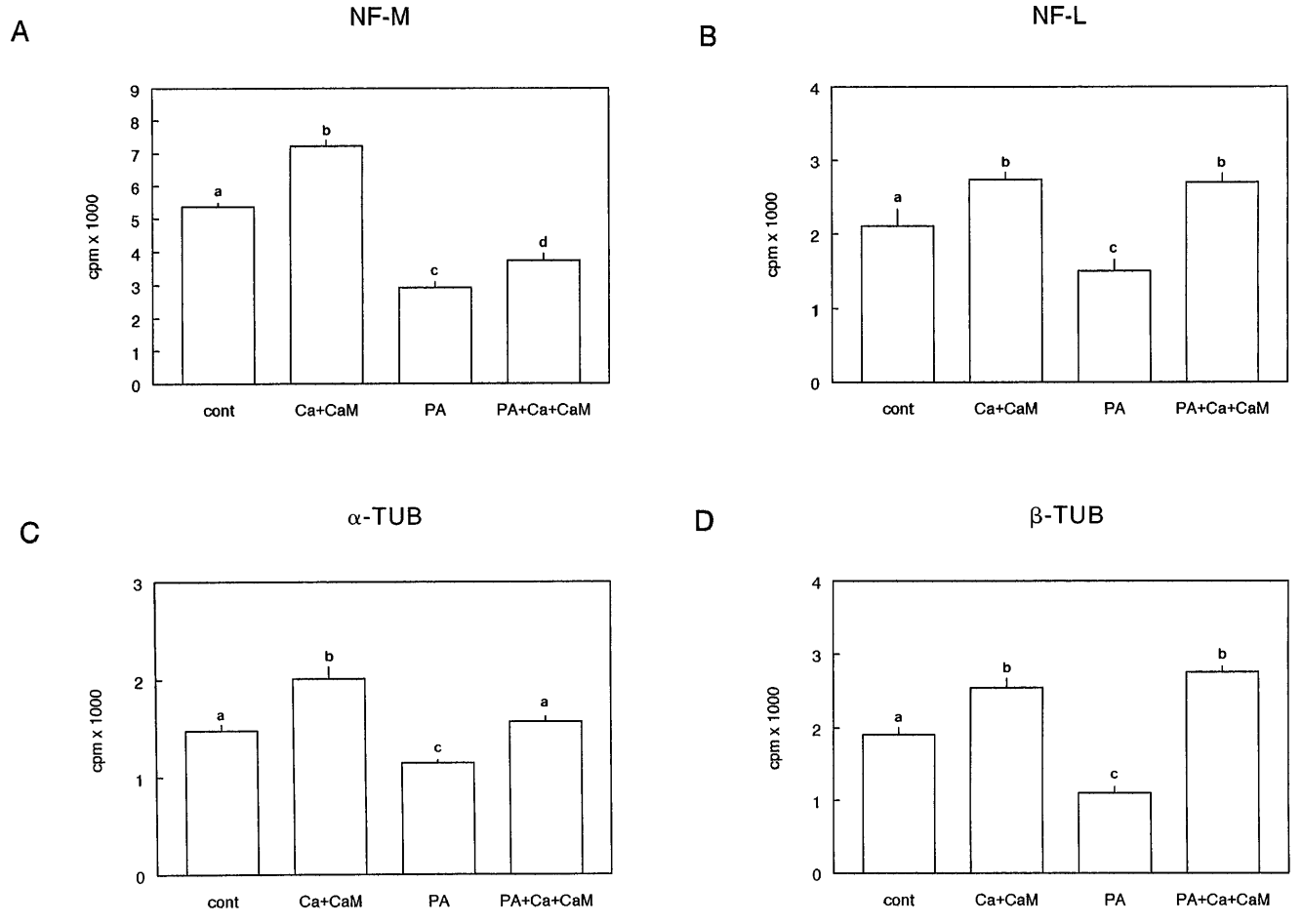

FIG. 4. Effect of pretreatment of tissue slices with PA on Ca^{2+}/calmodulin-dependent *in vitro* incorporation of ^{32}P into cerebral cortex cytoskeletal proteins. 150-kDa neurofilament subunit NF-M; 68-kDa neurofilament subunit, NF-L; α-tubulin, α-tub; β-tubulin, β-tub. Tissue slices were incubated for 1 h at 30°C with 2.5 m*M* PA. The cytoskeletal fraction obtained was incubated in 60-μl assay mixtures containing 50 m*M* Mes, pH 6.5, and 10 m*M* $MgCl_2$, in the presence of 1 μ*M* calmodulin, 1 m*M* $CaCl_2$ (Ca + CaM) or 2.5 m*M* PA, or both (PA + Ca + CaM). Data are means ± SEM values for four experiments. Statistically significant differences were determined by ANOVA, followed by the Duncan multiple range test. Statistically significant differences between means of various groups: $P < 0.05$; $a \neq b \neq c \neq d$.

DISCUSSION

Propionic acidemia is biochemically characterized by high levels of propionate in blood (2.5–5 m*M*) and other tissues of affected individuals and clinically characterized by severe encephalopathy. The disorder has been recently called a "cerebral" organic acidemia, because propionic acid can be produced and accumulated in the neuronal cells (24). Therefore it is likely that the metabolite concentrations are even higher in brain, and this may explain the delayed myelination and cerebral atrophy (2, 14) present in propionic acidemic patients.

To our knowledge, no study has specifically been done to investigate the pathophysiology of the neurological dysfunction of propionic acidemia, although some reports describe the influence of PA on carbohydrate and lipid metabolism in tissues other than brain (4, 6, 12, 13, 15, 19, 32, 33, 47).

The present study demonstrated that propionate inhibits the endogenous phosphorylation system associated with the Triton-insoluble cytoskeletal fraction obtained from the cerebral cortex of young rats *in vitro*. Our results are probably due to the metabolite itself, since conversion of propionate to propionyl-CoA in large amounts in the brain is unlikely. We showed that the drug is effective in inhibiting ^{32}P incorporation into endogenous NF subunits and tubulins and also into the exogenous substrates syntide 2 and kemptide. It should be noted that kemptide is more selective for PKA, whereas syntide 2 is a substrate for CaMKII, CaMKIV, PKA, and PKC (27, 51). One possible explanation for the inhibitory effect of PA on *in vitro* ^{32}P incorporation into endogenous and exogenous substrates could be the inhibition of protein kinase activities associated with the cytoskeletal fraction. We therefore carried out a more detailed study on the effect of the drug on the

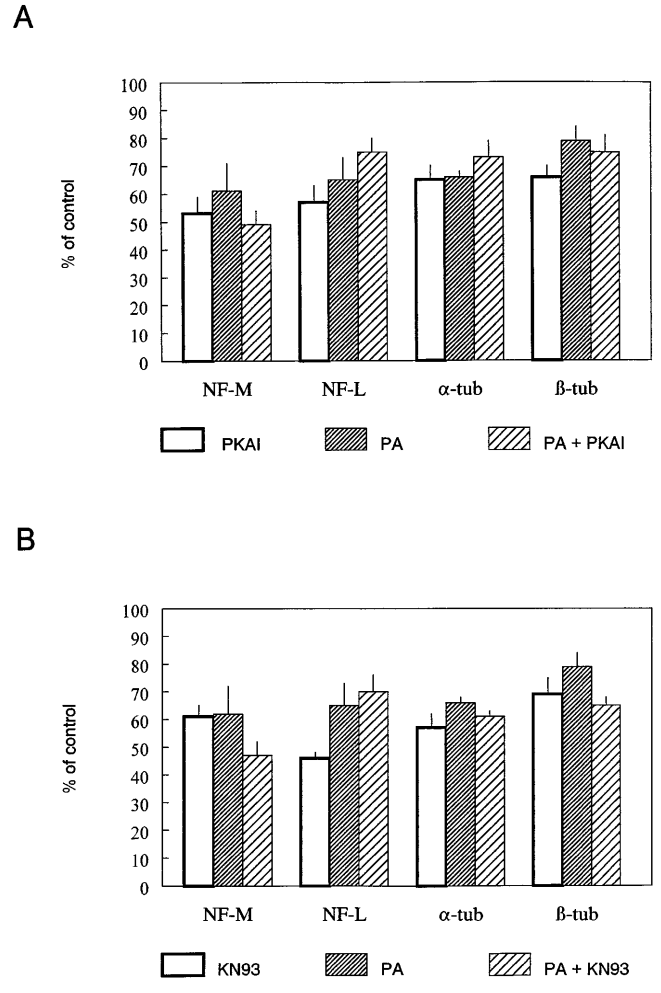

FIG. 5. Comparison between the effects of PA and the inhibitors of PKA (PKAI) and CaMKII (KN93) on the ^{32}P *in vitro* incorporation into the cytoskeletal proteins. 150-kDa neurofilament subunit, FN-M; 68-kDa neurofilament subunit, NF-L; α-tubulin, α-tub; and β-tubulin (β-tub). (A) Cytoskeletal fractions were incubated with [^{32}P]ATP (2 μCi/10 μg of protein) in the presence of the protein kinase A inhibitor PKAI, or 2.5 m*M* PA separately, or in the presence of both drugs. (B) Cytoskeletal fractions were incubated with [^{32}P]ATP in the presence of the Ca^{2+}/calmodulin kinase II inhibitor KN93, or 2.5 m*M* PA alone, or with both drugs in the assay. The *in vitro* ^{32}P incorporation was measured as described under Materials and Methods. Data (means ± SEM values for four experiments) were analyzed statistically by ANOVA followed by the Duncan multiple range test. Statistically significant differences between means of various groups: $P < 0.05$; $a \neq b$.

endogenous kinases associated with the cytoskeletal fraction, which are a calcium/calmodulin-dependent protein kinase II and a cyclic AMP-dependent protein kinase as demonstrated in our laboratory (7). We used two distinct approaches: (i) the specific kinase activators cAMP and Ca^{2+}/calmodulin and (ii) the kinase inhibitors PKAI and KN93 in the absence or in the presence of PA. PKAI is a competitive inhibitor of the catalytic subunit of cAMP-dependent protein kinase (16), whereas KN93 is a CaMKII inhibitor (48).

Our experiments showed that pretreatment of tissue slices with 2.5 m*M* PA totally prevented the activation of PKA activity by exogenous cAMP into NF-M, NF-L, and α- and β-tubulins (Fig. 3). In addition, when PKAI was added to the incubation system, its inhibitory activity on ^{32}P uptake was similar to that observed when the acid was tested alone or combined with the inhibitor (Fig. 5A). Since there was no additive inhibitory effect of the two drugs on this kinase, it is possible that PA may act like PKAI on the phosphorylation system.

As shown in Figs. 4A and 4C, the activating effect of Ca^{2+}/calmodulin on CaMKII activity was partially blocked by PA in NF-M and α-tubulin. Therefore, this effect was probably not due to a decreased CaMKII activity since the ^{32}P incorporation into NF-L and β-tubulin was apparently not affected by the acid (Figs. 4B and 4D). The reasons for this apparent substrate dependence of the inhibitory action of PA are unknown. Furthermore, when pretreated brain slices were incubated with the protein kinase inhibitor KN93, we did not observe additive inhibitory effects in the presence of PA as would be expected if the inhibitory mechanisms were independent (Fig. 5B). These findings indicate superposed mechanisms of action as suggested for PKAI. Taken together, these results suggest that PKA and CaMKII activities are somehow involved in the *in vitro* inhibition by PA of ^{32}P incorporation into the cytoskeletal proteins.

Furthermore, in addition to interfering with kinase activities, we clearly demonstrated that PA is able to inhibit the phosphatase activities present in the phosphorylation system. The presence of the acid in the incubation system consistently prevented dephosphorylation of neurofilament subunits and tubulins (Fig. 6).

The precise mechanisms underlying the inhibitory effect of PA on these enzymatic activities are still far from understood. Possible explanations could be as follows. With respect to kinase activities, PA might partially block the active site or alternatively bind to other sites on the enzymes, producing an indirect action on the catalytic site. Inducible conformational changes in the enzyme leading to a decreased second messenger or substrate binding to the enzyme and therefore to a diminished enzyme activity is a possibility. Evidence for this hypothesis appears in the experiments using cAMP as PKA activator (Fig. 3). Nonetheless we cannot exclude the alternative that the drug binds directly to second messengers or to the inhibitor molecule(s), preventing their action. Otherwise, considering the differential effects of PA on the stimulatory actions of Ca^{2+}/calmodulin on CaMKII in distinct cytoskeletal phosphoproteins, it is tempting to speculate

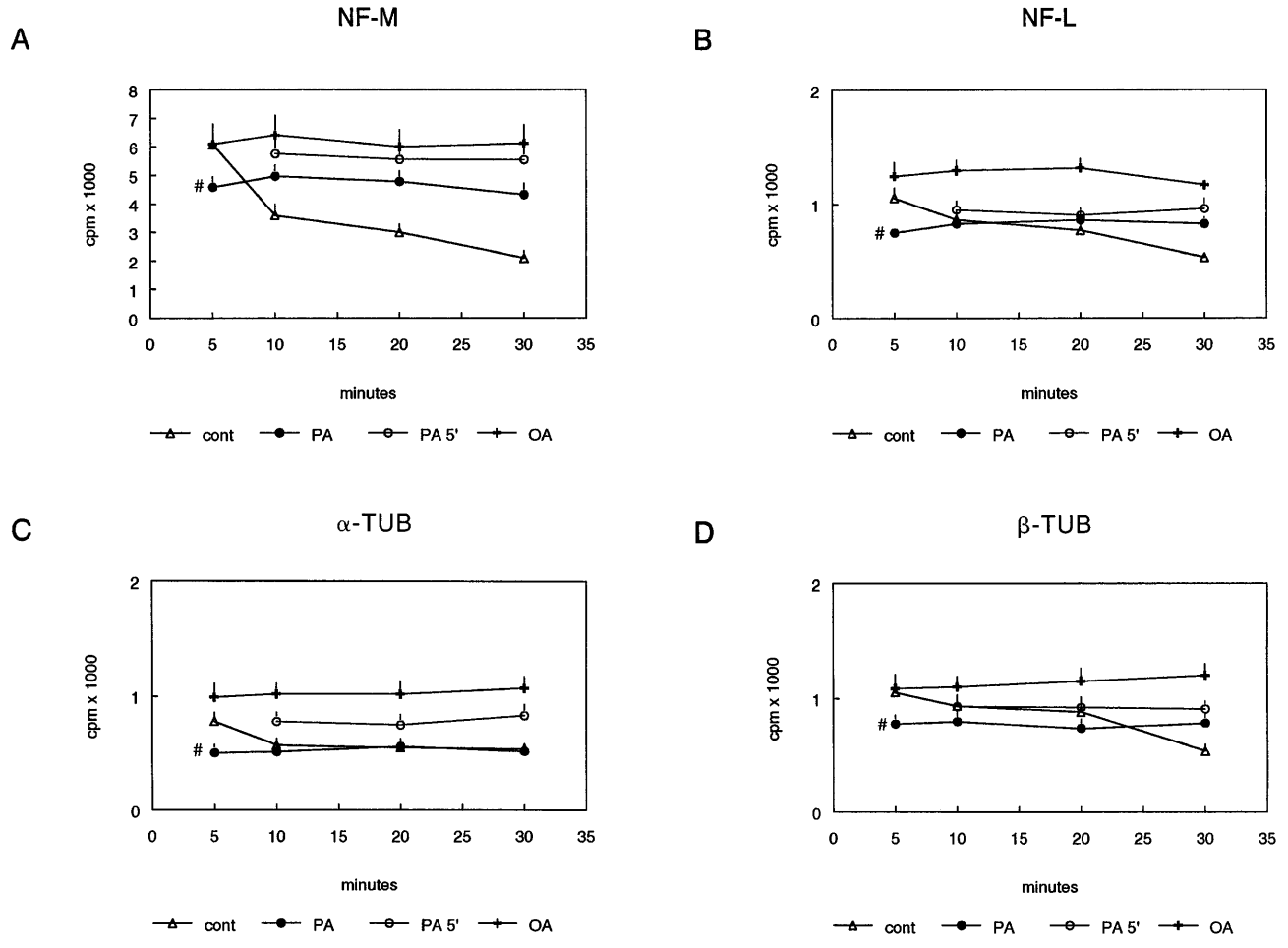

FIG. 6. Effect of pretreatment of tissue slices with PA on the time-course dephosphorylation of cytoskeletal fraction proteins of cerebral cortex. 150-kDa neurofilament subunit, NF-M (A); 68-kDa neurofilament subunit, NF-L (B); α-tubulin, α-tub (C); and β-tubulin β-tub (D). Cytoskeletal fractions obtained from control or PA-pretreated tissue slices were incubated with [^{32}P]ATP (2 μCi/10 μg of protein) and the reaction was stopped at specific intervals (5, 10, 20, and 30 min) by adding Laemmli sample buffer (controls). 2.5 m*M* PA was added to the incubation mixture at 0 min (PA) or after 5 min (PA 5′). In order to inhibit the endogenous phosphatase activity, okadaic acid was added to a final concentration of 0.5 μ*M* in some assays. Data are expressed as means ± SD values for three experiments. Statistically significant differences from controls, as determined by the Student *t* test, are indicated: #$P < 0.01$.

that the metabolite has different affinities for the distinct enzyme complexes.

Although the mechanisms by which PA inhibits the CaMKII and PKA phosphorylation of NF-M, NF-L, and tubulins are obscure, this decreasing phosphorylation may well play a significant role in the effects of PA on cerebral function.

There is evidence in the literature that phosphorylation of cytoskeletal proteins modulates the reciprocal interactions of their filamentous components, i.e., microfilaments, microtubules, and intermediate filaments (40). The cAMP-dependent phosphorylation of the neurofilament proteins NF-L and NF-M inhibits their coassembly into filaments *in vitro* (46). Furthermore, it is widely known that the phosphorylation level of the substrate protein regulates calpain-mediated hydrolysis (34). Pant (39) suggested that phosphorylation protects neurofilaments against the action of calpain in the axoplasm, while dephosphorylation enhances their susceptibility to degradation by calpain in the nerve terminals.

On the other hand, various studies have demonstrated that phosphorylation/dephosphorylation of cytoskeletal proteins from the central and peripheral nervous system can be disturbed by external factors such as drugs, as well as by neuropathologies (8, 9, 26, 41, 50). Other studies have demonstrated a relationship between the phosphorylation of neurofilaments and the axonal caliber (5). Hypomyelination in mutant mice is correlated with reduction in phosphorylation of neurofilaments and inhibition of radial growth (11). In this context, since hypo- or demyelination is characteristi-

cally a pathologic finding in the central nervous system of patients with propionic acidemia (2), a disturbed myelogenesis in affected individuals may contribute to the altered phosphorylation of cytoskeletal proteins demonstrated in our results. We should emphasize that even though alterations in the phosphorylation system are demonstrated under our experimental conditions, the question of whether these findings are relevant to the etiology or the neurologic dysfunction related to propionic acidemia remains unclear.

In conclusion, the present study shows that PA at doses equal to or even lower than those found in blood, brain, and other tissues from patients with propionic acidemia markedly affects the phosphorylation system associated with cytoskeletal proteins in rat cerebral cortex. The presence of poorly phosphorylated neurofilaments and other cytoskeletal phosphoproteins may affect the transport of neurofilaments, their interaction with other cytoskeletal proteins, and their turnover due to resistance to proteolysis (19, 38). All of these factors may contribute to PA neurotoxicity. However, further research is necessary to clarify the mechanisms underlying these effects, although inhibition of protein phosphatases and possibly protein kinases by the metabolite occurs, as shown by our data. Even though it is difficult to extrapolate our results to the human condition, if this is the case, we presume that interference with this system would probably lead to the deleterious action of PA on the brain, a fact that might explain at least in part the pathogenesis of the severe neurological dysfunction of propionic acidemic patients. Therefore, although the mechanisms of brain damage in patients with propionic acidemia are still unknown, we may propose that interference with the phosphorylating system may be related to the pathophysiology of the disorder in the brain of these patients.

ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científco e Tecnológico (CNPq); Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Pró-Reitoria de Pesquisa e Pós-Graduação da Universidade Federal do Rio Grande do Sul (PROPESP-UFRGS).

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