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PERSISTENT ALTERATIONS OF CALMODULIN KINASE II ACTIVITY IN CHICKENS AFTER AN ORAL DOSE OF TRI-*o*-CRESYL PHOSPHATE

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Abstract—Calmodulin kinase II has been found to be involved in the increased phosphorylation of brain microtubule and spinal cord neurofilament triplet proteins following treatment of animals with organophosphorus compounds that are capable of producing organophosphorus compound-induced delayed neurotoxicity (OPIDN). In this report, chickens were given a single oral neurotoxic dose of 750 mg/kg tri-*o*-cresyl phosphate (TOCP), and killed after 1 or 21 days of treatment. Crude calmodulin kinase II from brain cytosol as well as phosphocellulose-purified microtubules were prepared from control and treated animals. Phosphorylation reactions were started by adding protein into the phosphorylation buffer in the presence of Mg^{2+}, Ca^{2+}, calmodulin or trifluoperazine, and [γ-^{32}P]ATP. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to autoradiography. The extent of the calmodulin kinase II autophosphorylation as well as the Ca^{2+}/calmodulin-dependent phosphorylation of the purified microtubules was investigated. The enzyme activities isolated from control and treated animals were compared. Autophosphorylation of calmodulin kinase II was found to be higher in both 1-day and 21-day TOCP-treated animals than in control animals. The activity of the kinase to phosphorylate exogenous substrates such as tubulin and microtubule-associated protein-2 (MAP-2) was also higher in the treated hens than in the controls. The increased activity of the kinase was noted at day 1 following treatment when no clinical signs were observed and persisted until day 21 when the animals were paralyzed completely. This finding supports the significance of altered calmodulin kinase II in the pathogenesis of OPIDN.

Protein phosphorylation has been implicated in modulating diverse physiologic processes such as membrane transport, cell division, metabolic pathways, gene transcription, and synaptic transmission [1–3]. A multifunctional kinase that is particularly abundant in brain is calmodulin kinase II, which may constitute up to 2% of protein in the hippocampus [4, 5]. This kinase has been shown to phosphorylate various cytoskeletal proteins, such as tubulin, microtubule-associated protein-2 (MAP-2), and neurofilament triplet proteins [6–8].

Organophosphorus compounds, such as trio-*o*-cresyl phosphate (TOCP), may interfere with protein kinases by competing with ATP as phosphoryl group donor and phosphorylating their serine or threonine hydroxyl residues. TOCP is one of the many organophosphorus compounds capable of producing a delayed neuropathy, termed organophosphorus compound-induced delayed neurotoxicity (OPIDN), in sensitive species, such as human, cat, and chicken [9]. Rodents have been found to be less sensitive to OPIDN [9]. A Wallerian-type degeneration of the axon with subsequent demyelination is observed in OPIDN. Clinical signs include flaccid paralysis which develops distally in the legs 1–2 weeks following exposure and which progresses to spasticity and ataxia. Similar signs have been observed in chickens, the test animal of choice for OPIDN. Results from this laboratory have shown a dose- and time-dependent increase of protein phosphorylation in chickens treated with TOCP [10, 11]. An increase in a calcium/calmodulin-dependent phosphorylation of brain microtubules as well as spinal cord neurofilament proteins was found in TOCP-treated animals [12, 13]. This increase in phosphorylation was calmodulin kinase II mediated [13]. Since no alteration was found in the phosphorylation sites of the substrates [13], we attempted to study the significance of the kinase activity in the development of OPIDN. Two time points were chosen: 1 day after TOCP oral dosing, when no neurological deficit was observed, and 21 days, when animals were paralyzed completely. The present work suggests that a persistent alteration in calmodulin kinase II activity plays a role in the development of OPIDN.

MATERIALS AND METHODS

Treatment of animals

Eight adult leghorn hens (*Gallus gallus* domesticus, Featherdown Farms, Raleigh, NC) were given a single oral dose of 750 mg/kg TOCP (99% pure, Eastman Kodak, Rochester, NY) in gelatin capsules. Another group of eight served as control animals. Animals were observed for neurological deficit. One day after treatment, a group of four TOCP-treated and four control hens were anesthetized with CO_2 and killed by decapitation. The rest of the animals were killed 21 days after treatment.

Preparation of protein

Brain cytosol. Immediately following sacrifice,

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brains were rapidly removed and homogenized in 100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), pH 6.9, 10 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA), 10 mM EDTA in a ratio of 1 g/mL buffer [13]. The homogenate was centrifuged at 100,000 *g* for 60 min. Some of the supernatant was used for protein phosphorylation.

Phosphocellulose purified tubulin. Brain cytosol from individual animals was loaded onto a phosphocellulose column (3 mL bed volume, P-11, Whatman). Protein was eluted with 30 mM PIPES, pH 6.9, and determined by absorbance at 280 nm [14]. The first fractions of eluate were rich in tubulin and were used for phosphorylation.

Crude calmodulin kinase II. After tubulin had been eluted, the P-11 column was subsequently washed with 150 and 500 mM NaCl in 30 mM PIPES, pH 6.9 [15]. Again protein was detected by measuring absorbance at 280 nm. The 350 mM NaCl fraction contained calmodulin (CaM) kinase II as well as MAP-2 and some other proteins.

Phosphorylation assays

Phosphorylation assays were carried out as described previously [13]. Briefly, phosphorylation reactions were initiated by addition of a 65-μL sample containing 100 μg of brain cytosolic protein, P-11 purified tubulin or crude calmodulin kinase II to the reaction mixture. In some assays, proteins from control and treated animals were mixed in a ratio of 1:1 for the cytosol, P-11 tubulin and crude kinase or 1:3 for crude kinase: P-11 purified tubulin. The reaction mixture contained, in a final volume of 200 μL, 10 mM PIPES, pH 7.4, 1 mM EDTA, 2 mM EGTA, 5 μM [γ-^{32}P]ATP (2900 Ci/mmol, New England Nuclear) and, where indicated, 10 mM $MgCl_2$, 5 mM $CaCl_2$ and 2 μg of calmodulin (Sigma) or 50 μM trifluoperazine (TFP, Sigma). Reactions were incubated at 37° and terminated after 1 min by the addition of 100 μL of sodium dodecyl sulfate (SDS) stop solution [0.125 M Tris–HCl, pH 6.8, 4.5% SDS, 20% (v/v) glycerol and 10% (v/v) β-mercaptoethanol].

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed as described earlier [12] by the method of Laemmli [16] on 8% resolving gel. Gels were stained with Coomassie blue, dried under vacuum, and subjected to autoradiography for the same period of time for all gels. The amount of proteins as well as the amount of phosphoproteins in each autoradiographic band was quantified by integration of the area under the corresponding peak in the densitometric scan, obtained by using an LKB Ultrascan Laser Densitometer interfaced with a Recording Integrator (LKB Instruments, Gaithersburg, MD).

RESULTS

Clinical assessment

In the 21-day TOCP-treated hens, ataxia was observed by day 7 and complete paralysis by day 14. On the contrary, the 1-day treated animals did not show any clinical signs of neurotoxicity.

Phosphorylation pattern

Twenty-one-day TOCP-treated hens. Figure 1 shows a comparison of phosphorylation patterns of proteins isolated from cytosol, phosphocellulose-purified tubulin, and crude kinase extracts. The bands identified at 55, 65 and 300 K were identified tentatively as the α and β subunits of calmodulin kinase II and MAP-2 in the crude kinase preparation. However, due to the overlap of the α and β subunits of tubulin with those of the calmodulin kinase II subunits, a definitive identification in the cytosolic and phosphocellulose-purified tubulin preparation was not possible in this figure. The phosphorylation patterns of brain cytosol isolated from control, treated, and control plus treated hens (1:1 ratio) are presented in Fig. 1. Proteins from either control or treated hens were mixed at a 1:1 ratio to determine the relative contribution of kinase activity to phosphorylate substrates. Treated animals showed more phosphorylation than controls; the percentage increase was 233% for the 55 K, 164% for the 65 K and 102% for the 300 K (Table 1). When cytosol from control was mixed with treated, the percentage increase was only significant for the 55 K (174%); the extent of phosphorylation of the 65 K and 300 K proteins in this mixture was between control and treated values. The phosphocellulose-purified microtubule fraction isolated from control animals showed more phosphorylation than that from treated animals (Fig. 1). There was a 300 K protein that copurified with this preparation. The changes in phosphorylation in the treated animals were −50, −47 and −69% of control for the 55, 65, and 300 K, respectively (Table 1). Partially purified calmodulin kinase II isolated from treated animals showed a significant increase in autophosphorylation as compared to that from controls. The percentage increase was quantified in Table 1 (140 and 78% for the 55 and 65 K). Phosphorylation of microtubule-associated protein-2 (MAP-2) that copurified in this preparation was also increased by 19% (Fig. 1, Table 1).

Interesting results were seen upon incubating the enzyme with phosphocellulose-purified microtubule protein. When kinase isolated from control was added to the purified microtubule from the same control animal, it showed an increase in phosphorylation when compared with its activity when added to microtubule isolated from a treated animal. This change in phosphorylation, however, was not statistically significant for the 55 and 65 K proteins (Fig. 2, Table 2). On the contrary, a decrease in phosphorylation of the 300 K protein was found in the treated animal; the decrease was 36% of the control value (Table 3). The same results were found when kinase isolated from treated animals was incubated with the purified microtubule isolated from control or treated animals. The stimulation of phosphorylation did not change significantly for proteins of M_r 55 and 65 K. There was, however, a decrease in the phosphorylation of the 300 K protein isolated from the treated animal. The decrease was 44% of the control value.

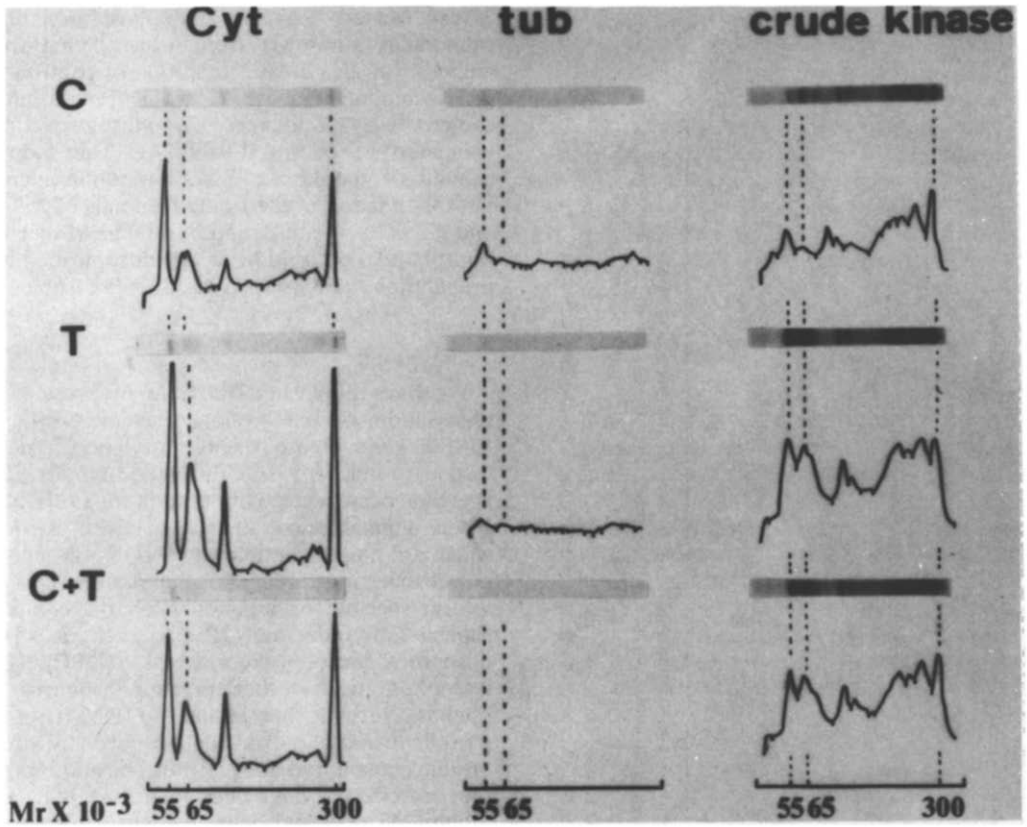

Fig. 1. Autoradiographs and densitometric tracings of proteins phosphorylated under the standard conditions in the presence of Ca^{2+} and calmodulin. Key: C, control; T, TOCP-treated; C + T, control samples mixed with treated samples in a 1:1 ratio. Different preparations were studied: Cyt = cytosolic proteins; tub = phosphocellulose-purified tubulin; and crude kinase = partially purified calmodulin kinase II. The 300 K protein was MAP-2. The sensitivity of the densitometer was set at 100× more sensitive to scan the autoradiographs of the cytosol and purified microtubule than to scan the crude kinase. Gels from the cytosolic and purified microtubule protein preparations were also exposed to the X-ray films 24× longer than the gel from crude kinase.

Comparing the activity of kinase isolated from control to that from treated animals, a significant change was found. Kinase isolated from treated animals showed more activity than that of control animals regardless of the source of substrate protein. Since we used the same amount of kinase as well as the same amount of substrate proteins, comparison of stimulation in phosphorylation of the substrates may be used as an indicator of kinase activity (Table 2). The percentage increase of kinase activity isolated from treated animals to that from control animals was 88, 160 and 99% for the 55, 65 and 300 K proteins, respectively, with control microtubule proteins as the substrates. When microtubule protein isolated from TOCP-treated animals was used as substrates, the percent increase of treated kinase activity over the control kinase was 95% for the 55 K, 112% for the 65 K, and 74% for the 300 K proteins.

In one-dimensional gel electrophoresis it is difficult to ascertain the relative contributions to a phosphorylation pattern of α and β tubulin as well as the α and β subunits of calmodulin kinase II since their molecular weights have some overlap. Two-dimensional gel electrophoresis was carried out to at least qualitatively relate their respective contributions to the degree of phosphorylation. Figures 3 and 4 show that there was some kinase activity which copurified with the 150 mM NaCl fraction of phosphocellulose-purified microtubules. The kinase seemed to be associated more in the control than in treated animals (Fig. 3). Figure 4 shows that, by using a phosphocellulose column, calmodulin kinase II was enriched in the 350 mM NaCl fraction, with tubulin completely separated from the kinase.

The two-dimensional gel electrophoresis of the kinase isolated from control and treated animals using the same substrate protein is shown in Fig. 5. The 55 and 65 K proteins were identified as α and β tubulin. The autophosphorylation of calmodulin kinase II was enhanced in the treated animals. The phosphorylation of tubulin was also stimulated in the preparation using the kinase isolated from treated animals.

One-day TOCP-treated hens. As was found in the

Table 1. ^{32}P Incorporation into brain proteins isolated from control and 21-day tri-*o*-cresyl phosphate-treated chickens

Fraction	Protein mass (kD)	Phosphorylation			% Change of treated from control	% Change of C + T
		C	T	C + T (1:1)		
Brain cytosol	55	0.91 ± 0.37	3.04 ± 0.62	2.49 ± 0.60	233*	174†
	65	1.12 ± 0.46	2.97 ± 0.63	1.54 ± 0.38	164†	NS§
	300 (MAP-2)	1.27 ± 0.23	2.56 ± 0.34	1.56 ± 0.17	102‡	NS
Purified microtubule	55	1.74 ± 0.27	0.87 ± 0.10		−50‡	
	65	0.48 ± 0.09	0.21 ± 0.07		−47†	
	300 (MAP-2)	0.45 ± 0.11	0.14 ± 0.04		−69†	
Crude kinase	55	4.45 ± 1.47	10.6 ± 1.52	7.83 ± 1.34	140*	NS
	65	11.30 ± 2.71	20.08 ± 2.76	14.63 ± 1.60	78†	NS
	300 (MAP-2)	8.89 ± 0.50	10.55 ± 0.43	9.22 ± 0.72	19†	NS

The incorporation of ^{32}P into brain proteins (100 μg total) was determined using the standard phosphorylation assay in the presence of Mg^{2+}, Ca^{2+} and calmodulin. Autoradiographs were scanned using a densitometer. The sensitivity of the densitometer was set at 100× more sensitive to scan the autoradiographs of the cytosol and purified microtubule than to scan the crude kinase. Values are in arbitrary units ± SEM of at least eight independent observations.
Abbreviations: C, control; T, treated; C + T (1:1) a mixture of control (50 μg) and treated proteins (50 μg).
*–‡ Statistics were done using Student's *t*-test: * $P < 0.02$, † $P < 0.05$ and ‡ $P < 0.01$. There were no significant changes in protein staining.
§ NS = no significant change.

21-day treated hens, the autophosphorylation of calmodulin kinase II from 1-day TOCP-treated animals was also greater than that of controls (Fig. 6). Calmodulin kinase II from treated animals showed a 241% increase in phosphorylation as compared to control (Table 4). The individual subunit of the kinase also showed an increased phosphorylation in the treated animals of 259, 245, and 225% for the α, β, and β′ subunit, respectively. Phosphorylation of MAP-2, which copurified in this preparation, was also increased (96%, Table 4).

DISCUSSION

We have implicated the role of protein phosphorylation in the pathogenesis of OPIDN [9–13, 17]. The increase in phosphorylation of nervous system proteins of TOCP-treated animals fits the age and species selectivity criteria for OPIDN [11]. Young animals such as chicks, as well as rodents, which are not susceptible to OPIDN, did not show any changes in protein phosphorylation. This effect is also specific for organophosphorus compounds capable of inducing OPIDN such as TOCP, diisopropylphosphorofluoridate (DFP), and leptophos, while other organophosphorus compounds which do not induce OPIDN (tri-*p*-cresyl phosphate and paraoxon) do not have any effect on protein phosphorylation [11, 17]. The major proteins that are affected have been identified as α- and β-tubulin, MAP-2 and neurofilament triplet proteins [12, 13]. Because all these cytoskeletal proteins are phosphorylated by Ca^{2+}/calmodulin kinase II, this enzyme may be the primary target for OPIDN [12, 13]. Furthermore, studies have ruled out ATPase inhibition, phosphatase activation or a change in substrate phosphorylation sites as an explanation for TOCP-induced endogenous protein phosphorylation [11, 13].

In the present work, we reported an increase in Ca^{2+}/calmodulin-mediated protein phosphorylation in brain microtubules, which was consistent with the previous findings [12, 13]. Upon mixing the brain cytosolic proteins isolated from control and treated animals in a ratio of 1:1, we found that the stimulation of tubulin and MAP-2 was somewhere between that of the control and the treated animals. Since the same amount of proteins was loaded and there was no change in protein staining, this may be an early indication of the activity of the kinase being altered. To further confirm this hypothesis, we ran a phosphorylation assay of phosphocellulose-purified microtubules incubated with crude calmodulin kinase II.

Interestingly, phosphocellulose-purified microtubules isolated from control animals showed more phosphorylation than those of treated animals. This may be explained by the finding that the kinase was associated more tightly with tubulin in control animals, as shown in Fig. 3. Also, calmodulin kinase II has been reported to exist in two forms, one of which is associated tightly with cytoskeletal proteins such as microtubules and another which is not [18]. Similarly, MAP-2 that copurified with this preparation was also less phosphorylated in the treated animals than in the controls (Table 1).

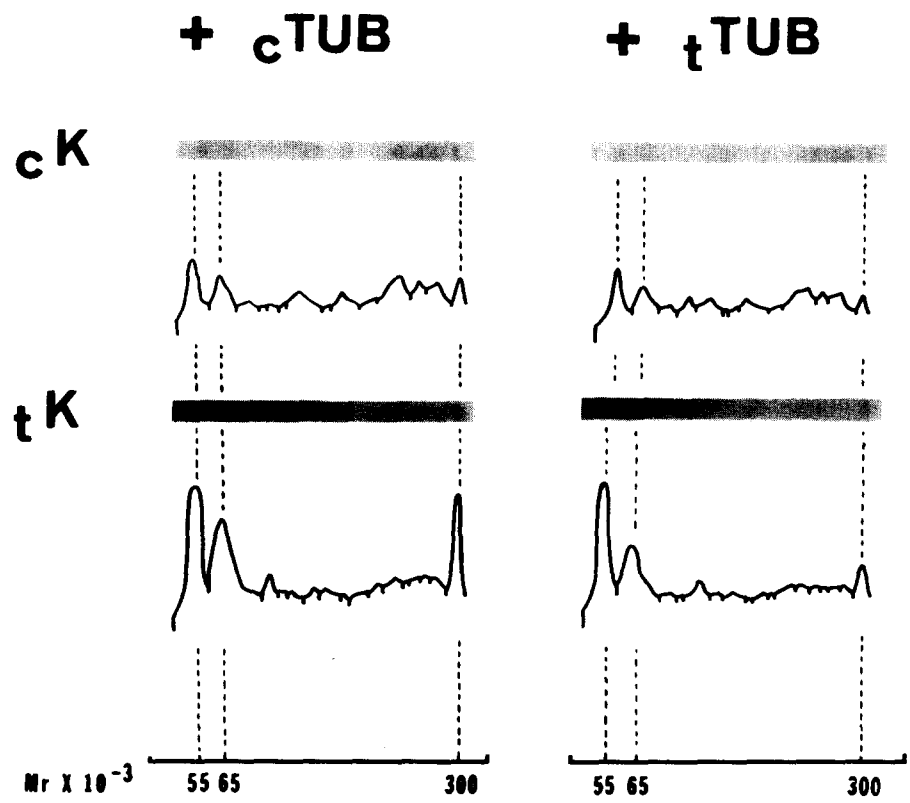

Fig. 2. Comparison between the control/treated tubulin phosphorylated with either control or treated kinase. $_c$Tub = control tubulin; $_t$Tub = treated tubulin; $_c$K = control kinase; $_t$K = treated kinase. Note that tubulin, regardless of the source, was phosphorylated more upon addition of treated kinase. The molecular mobility of 55–65 K is the range for the kinase as well as the tubulin. The 300 K protein is MAP-2. Gels were exposed to X-ray films for the same period of time.

Table 2. ^{32}P Incorporation into microtubule protein: Comparison of kinase activity between control and tri-*o*-cresylphosphate-treated animals

	Protein mass (kD)	Phosphorylation		Kinase activity (% increase)
		+ Control kinase	+ Treated kinase	
Control tubulin	55	3.56 ± 0.68	6.70 ± 1.18	88*
	65	2.06 ± 0.55	5.35 ± 1.22	160*
Treated tubulin	55	3.04 ± 0.52	5.93 ± 0.92	95†
	65	1.82 ± 0.46	3.85 ± 0.63	112*

Microtubule proteins were incubated under the standard phosphorylation assay in the presence of Mg^{2+}, Ca^{2+} and calmodulin. Phosphocellulose-purified microtubules (75 μg) were incubated in the presence of partially purified calmodulin kinase II (25 μg) isolated from control and TOCP-treated animals. The activities of kinase isolated from control and treated animals, using the same substrates, were compared. Also, the stimulation of protein phosphorylation of microtubules isolated from treated animals was compared to that of control animals. Numbers are in arbitrary units ± SEM of at least eight independent observations.

*† Statistics were done using Student's *t*-test: * $P < 0.05$, and † $P < 0.02$. There were no significant changes in protein staining.

Autophosphorylation of calmodulin kinase II isolated from 1-day as well as 21-day TOCP-treated animals was found to be significantly higher than that from control. The significance of autophosphorylation on its activity to stimulate phosphorylation of exogenous substrates depends on the stability of the autophosphorylated enzyme [1, 2]. Some investigators have found autophosphorylation to cause an inhibition of activity [19–22], an activation [23], or no effect [24]. In this

Table 3. ^{32}P Incorporation into microtubule-associated protein-2: Comparison of kinase activity and substrate phosphorylation between control and tri-*o*-cresyl phosphate-treated animals

	Protein mass (kD)	Phosphorylation		Kinase activity (% increase)
		+ Control kinase	+ Treated kinase	
Control MAP-2	300	1.36 ± 0.14	2.72 ± 0.47	99*
Treated MAP-2	300	0.86 ± 0.09	1.51 ± 0.30	74*
Substrate phosphorylation (% decrease)	300	36†	44*	

Microtubule proteins were incubated under the standard phosphorylation assay in the presence of Mg^{2+}, Ca^{2+} and calmodulin. Phosphocellulose-purified microtubules (75 μg) were incubated in the presence of partially purified calmodulin kinase II (25 μg) isolated from control and TOCP-treated animals. The activities of kinase isolated from control and treated animals, using the same substrates, were compared. Also, the stimulation of protein phosphorylation of microtubules isolated from treated animals was compared to that of control animals. Numbers are in arbitrary units ± SEM of at least eight independent observations.

*† Statistics were done using Student's *t*-test: * $P < 0.05$, and † $P < 0.01$. There were no significant changes in protein staining.

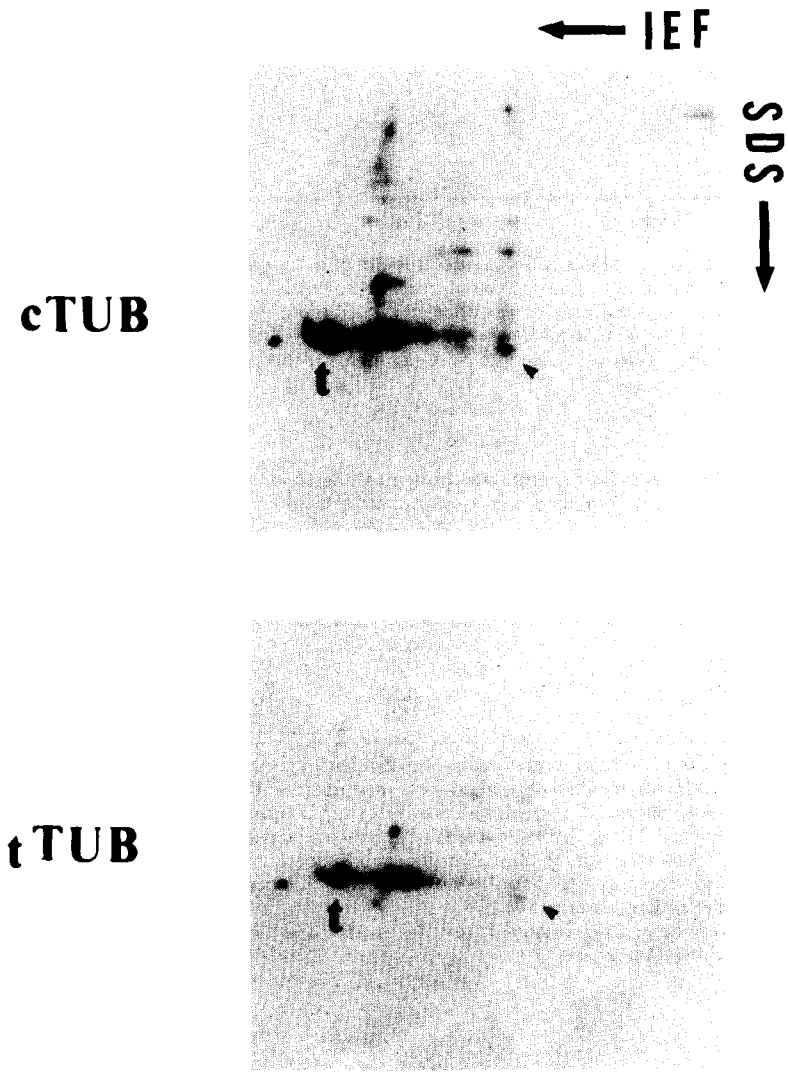

Fig. 3. Two-dimensional gel electrophoresis of phosphorylated phosphocellulose-purified tubulin (t) M_r 55 K. Arrows indicate kinase which copurified in the preparation. Note that control tubulin ($_c$Tub) was phosphorylated more than treated tubulin ($_t$Tub). Also, the copurified kinase seemed to be more tightly associated in the control tubulin preparation than in the treated preparation.

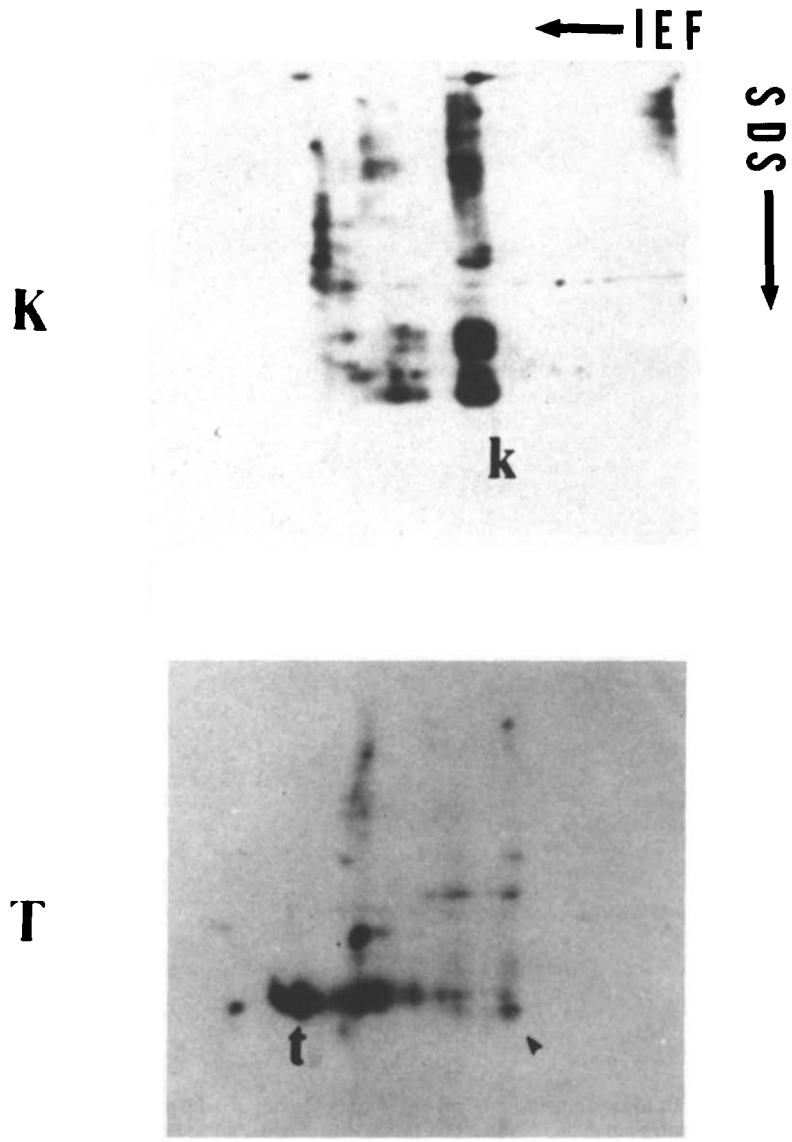

Fig. 4. Autoradiographs of two-dimensional gel electrophoresis of partially purified calmodulin kinase II (K) and phosphocellulose-purified tubulin (T) phosphorylated under the standard conditions. Note that column chromatography separated the tubulin from the kinase preparation, but only a trace amount of kinase copurified with the tubulin. Both tubulin and kinase had a mobility range of 55–65 K. Gels from purified tubulin were exposed to X-ray films 20× longer than those from crude kinase.

study, the phosphorylation of MAP-2 which copurified with calmodulin kinase II was found to be increased in both 1-day and 21-day TOCP-treated hens. Thus, the increased autophosphorylation of calmodulin kinase II in TOCP-treated animals also caused an activation of the phosphorylation of its substrates, such as MAP-2. The consequence of the alteration in calmodulin kinase II activity to the physiological function is still unknown. MAP-2 has been reported to form cross-bridges between microtubules and neurofilaments [25, 26], and this interaction has been suggested to be mediated by phosphorylation [6, 27]. The altered phosphorylation of MAP-2 can thus be speculated to alter the delicate interaction between cytoskeletal proteins.

To further support the hypothesis of calmodulin kinase II activity being altered in TOCP-treated animals, we incubated kinase isolated from control or treated animals with phosphocellulose-purified tubulin from control or treated animals. All four possible combinations were investigated (control kinase + control tubulin, control kinase + treated tubulin, treated kinase + control tubulin and treated kinase + treated tubulin). When control kinase plus control tubulin was compared with control kinase plus treated tubulin, there was a non-significant decrease in the phosphorylation of tubulin from treated animals. However, there was a decrease in the phosphorylation of the 300 K protein from the treated animal. The decrease in phosphorylation was

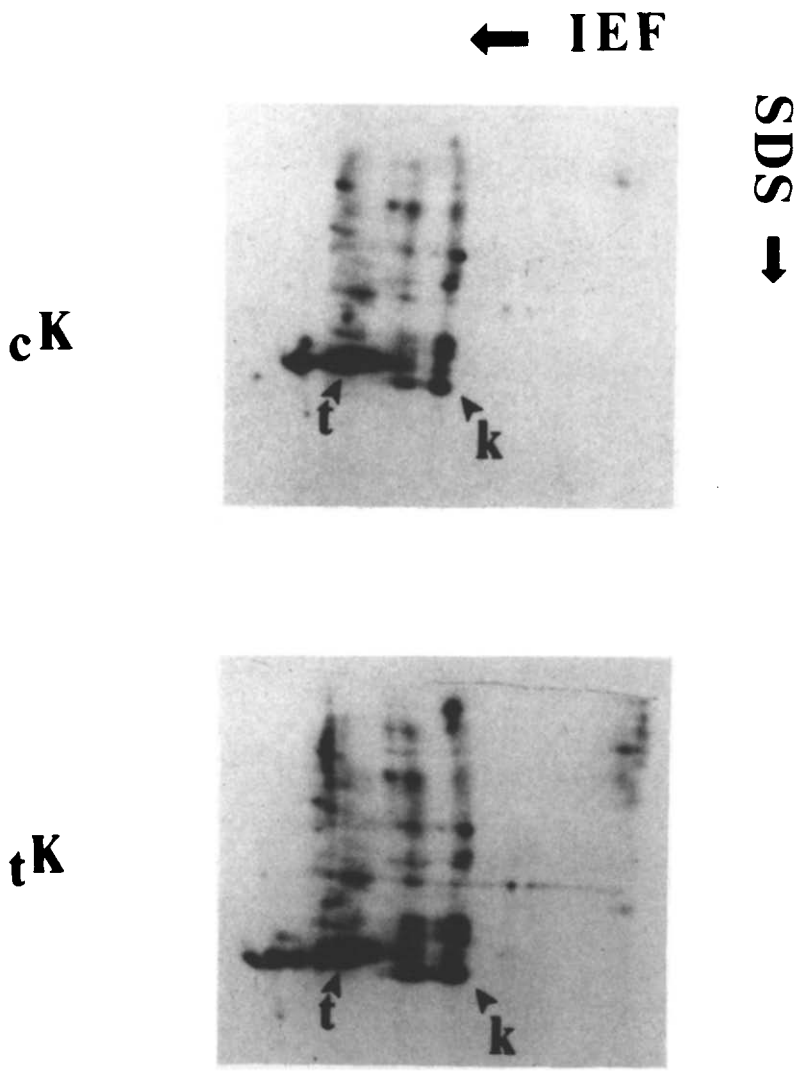

Fig. 5. Autoradiographs of two-dimensional gel electrophoresis of partially purified calmodulin kinase II (k) isolated from control animals ($_cK$) and TOCP-treated animals ($_tK$) when incubated with the same source of tubulin (t). Arrows indicate kinase (k) and tubulin (t); both have mobility of 55–65 K. Note that kinase seemed to have more autophosphorylation, and also that tubulin was more phosphorylated in the presence of treated kinase.

36%. The same result was seen with treated kinase plus control tubulin compared to treated kinase plus treated tubulin. Again, there was a non-significant decrease in the phosphorylation of the 55 and 65 K proteins. However, a significant decrease in the phosphorylation of MAP-2 (300 K) was observed. This indicated that the phosphorylation of MAP-2 was most likely altered *in vivo*. MAP-2 was probably more phosphorylated *in vivo* in the treated animals than in controls; therefore, less *in vitro* phosphorylation of MAP-2 from treated animals was observed. Since MAP-2 is the best substrate for this kinase and has eight distinct phosphorylation sites [27], the effect is more significant in MAP-2 than with tubulin, another, albeit poorer, substrate of the kinase.

When control kinase plus control tubulin was compared with treated kinase plus control tubulin, we found a significant increase in phosphorylation with the treated kinase. Again, the same effect was seen when control kinase plus treated tubulin was compared to treated kinase plus treated tubulin. The activity of kinase isolated from treated animals was consistently higher than in the controls, independent of the substrate tubulin, i.e. either from control or treated.

It is noteworthy that the increased activity of the kinase was very persistent; at day 1 there was an increase in autophosphorylation of 241% and on day 21 a 95% increase was noted. The ability of the kinase to phosphorylate MAP-2 was also comparable in both the 1-day and 21-day TOCP-treated animals.

In summary, we have reported a persistent increase in calmodulin kinase II activity in TOCP-treated

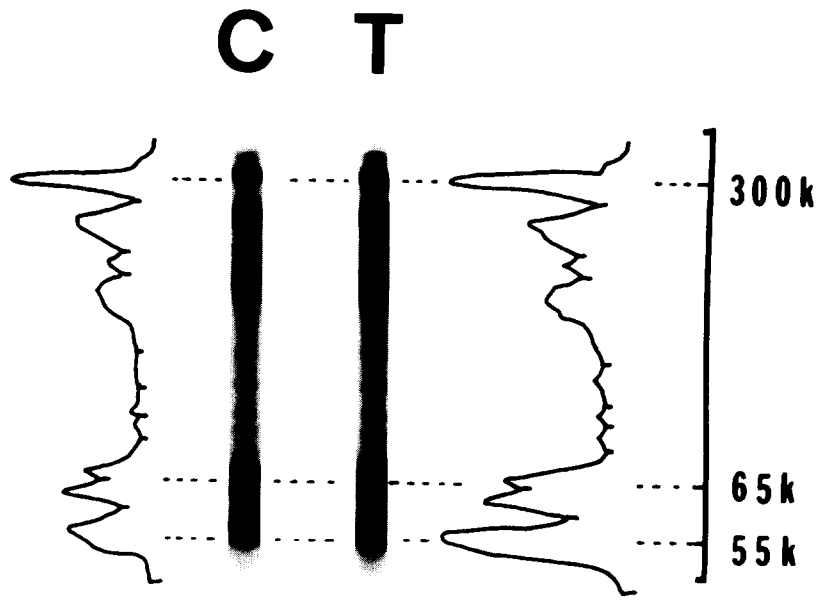

Fig. 6. Autoradiographs and densitometric scans of the autophosphorylation of partially purified calmodulin kinase II. Key: C = control; T = 1-day TOCP-treated. The range for the molecular mobility of the kinase was 55–65 K, while 300 K was the range for MAP-2 which copurified with the enzyme preparation.

Table 4. Activity of partially purified calmodulin kinase II isolated from control and 1-day tri-*o*-cresyl phosphate-treated animals

Protein	Control	Treated	% Increase*
55 K (α)	3.00 ± 1.16	10.78 ± 1.60	259
62 K (β)	1.56 ± 0.57	5.37 ± 0.86	245
67 K (β')	2.32 ± 0.96	7.55 ± 1.15	225
55 K, 62 K, 67 K (Total)	6.94 ± 3.09	23.69 ± 3.77	241
300 K (MAP-2)	2.47 ± 0.29	4.85 ± 0.48	96

The autophosphorylation of partially purified calmodulin kinase II isolated from 1-day TOCP-treated animals was compared to that isolated from control. Phosphorylation reactions were carried out as described in the presence of Mg^{2+}, Ca^{2+}, and calmodulin. Proteins were loaded onto polyacrylamide gels. All gels were exposed on X-ray film for the same period of time. Autoradiographs were scanned using a densitometer. Numbers are in arbitrary units ± SEM of at least eight independent observations. α, β, and β' refer to the subunits of calmodulin kinase II.

* Each value in the column was statistically significant at $P < 0.02$ (Student's *t*-test). There was no change in protein staining.

animals. TOCP may stabilize the autophosphorylated state of the enzyme, rendering it Ca^{2+}/calmodulin independent. The Ca^{2+}/calmodulin independence of autophosphorylated calmodulin kinase II has been studied extensively [1, 21]. Changes produced by autophosphorylation may cause the kinase to function as a switch, remaining active after the decay of an initial calcium signal. The increase in autophosphorylation of calmodulin kinase II isolated from TOCP-treated animals results in increased *in vitro* phosphorylation of its exogenous substrates, such as tubulin, MAP-2, and neurofilaments. An increase in *in vivo* phosphorylation of MAP-2 in TOCP-treated animals occurred as well. These findings further support the significance of altered phosphorylation in the development of OPIDN. The increase in phosphorylation of cytoskeletal proteins produced by calmodulin kinase II may alter interaction between the cytoskeleton components and cause an axonopathy.

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