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Effects of long-term treatment with desipramine on microtubule proteins in rat cerebral cortex

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Abstract

The molecular mechanism of the action of antidepressants beyond the receptor level has not yet been elucidated. We have investigated the effects of long-term treatment with desipramine on the phosphorylation state of microtubule-associated protein 2 (MAP2) and microtubule assembly in the rat cerebral cortex. Phosphorylation of MAP2 was detected by immunoblotting after immunoprecipitation of MAP2 in the soluble fraction. The degree of phosphorylation of serine residues of MAP2 was significantly increased after chronic administration of desipramine without changes in the total concentration of MAP2. Microtubule assembly in crude brain extracts was monitored in terms of changes in turbidity measured at 350 nm using a spectrophotometer. Chronic but not acute treatment with desipramine inhibited microtubule assembly, assayed in the presence of a phosphatase inhibitor, calyculin A, whereas the inhibition was completely nullified in the absence of calyculin A. Desipramine had no direct effect on microtubule assembly in vitro. These results raise the possibility that the changes in the degree of phosphorylation of MAP2 and microtubule assembly represent intracellular modifications involved in functional changes elicited by long-term treatment with desipramine. © 1997 Elsevier Science B.V.

Keywords: Antidepressant; Desipramine; Microtubule-associated protein 2; Microtubule assembly; Phosphorylation

1. Introduction

The molecular mechanism of the action of antidepressants beyond the receptor level has not yet been elucidated. Previously, long-term treatment with desipramine, a tricyclic antidepressant drug, was reported to modify the endogenous phosphorylation of microtubule-associated protein 2 (MAP2) in both soluble and crude microtubule fractions of the rat cerebral cortex (Perez et al., 1989). MAP2 is known to be a substrate for several protein kinases such as type II cyclic AMP (cAMP)-dependent protein kinase (Vallee, 1980), Ca^{2+}/calmodulin-dependent protein kinase II (Goldenring et al., 1983; Schulman, 1984) and Ca^{2+}/phospholipid-dependent protein kinase (Akiyama et al., 1986). Several lines of evidence demonstrate that phosphorylation of MAP2 catalyzed by each of these protein kinases results in the inhibition of microtubule assembly in vitro (Jameson et al., 1980; Yamamoto et al., 1985; Hoshi et al., 1988). Thus, it is of interest to determine the effect of administration of antidepressants on microtubule assembly as a potential target for the action of antidepressants. In this study, we investigated the effects of long-term treatment with desipramine on the phosphorylation state of MAP2 as well as microtubule assembly in the rat cerebral cortex.

2. Materials and methods

2.1. Antibodies

Monoclonal anti-MAP2 (2a-2b) (mouse IgG1) and anti-MAP1 (mouse IgG1) were obtained from Sigma Chemical (USA). Monoclonal anti-tau (mouse IgG1) was purchased from Chemicon International (USA). Monoclonal anti-α-tubulin (mouse IgG1) and anti-β-tubulin (mouse IgG1) were obtained from Oncogene Science (USA). Monoclonal anti-phosphoserine (mouse IgG1), anti-phosphothreonine (mouse IgG2b) and anti-phosphotyrosine (mouse IgG1) were obtained from Bio-Makor (Israel).

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2.2. Animals and drug treatments

Adult male Wistar rats (150–200 g) housed under a 12 h light–dark cycle with food and water available ad libitum were used. In chronic studies, groups of 4–5 rats were subcutaneously administered saline or desipramine (20 mg/kg) once daily for 14 days. The rats were decapitated 24 h after administration of the last dose. In acute studies, separate groups were given saline or desipramine (20 mg/kg) subcutaneously and killed 2 h after injection. The brains were immediately removed and the cerebral cortices were dissected out.

2.3. Preparation of MAP2 and tau

The cortical tissues were homogenized in three volumes of 50 mM Tris–HCl buffer (pH 7.4 at 4°C) containing 0.8 M NaCl, 25 mM NaF, 5 mM EGTA, 5 mM EDTA, 2.5 mM 2-mercaptoethanol, 50 mM sodium pyrophosphate, 4 mM *p*-nitrophenyl phosphate, 1 μM calyculin A, 10 μg/ml leupeptin, 1 μg/ml pepstatin A and 10 U/ml aprotinin. The homogenates were centrifuged at 100 000 × *g* for 1 h at 4°C. The supernatants were boiled for 5 min and centrifuged at 15 000 × *g* for 30 min to obtain heat-stable proteins (Díez-Guerra and Avila, 1993). It was previously reported that the endogenous protein kinases and protein phosphatases associated with microtubule-associated proteins (MAPs) in cytosolic fractions were inactivated by heat treatment (Murthy and Flavin, 1983; Schulman, 1984; Walaas and Nairn, 1989). MAP2 and tau are heat-stable MAPs (Fellous et al., 1977; Pedrotti et al., 1996). MAP2 or tau was separated from the supernatant fraction subsequent to immunoprecipitation of each protein by the method of Fukunaga et al. (1995) with the following modifications. Briefly, the supernatants (3 mg protein/ml) were incubated for 3 h at 4°C with immunoprecipitation buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 25 mM NaF and 50 mM sodium pyrophosphate, pH 7.4) containing 25% protein A-Sepharose CL-4B (Pharmacia) and anti-MAP2 (2a-2b) monoclonal antibody (1:200 dilution; Sigma Chemical) or anti-tau monoclonal antibody (1:100 dilution; Chemicon International). The samples were then centrifuged at 15 000 × *g* for 30 min. The pellets were washed and repelleted at 15 000 × *g* for 5 min three times.

2.4. Electrophoresis and immunoblot analysis

Immunoprecipitated MAP2 or tau was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Proteins were separated on 7.5% or 3–10% polyacrylamide gels at 20 μg of protein per lane, then electrically transferred to a polyvinylidene difluoride membrane (Millipore) in a semidry blotting apparatus at 0.2 A for 5 h, using a buffer containing 25 mM Tris–HCl (pH 8.3), 192 mM glycine, 0.05% SDS and 20% methanol according to the method of Towbin et al. (1979). The blots were blocked with 5% ovalbumin and incubated with the primary antibody (mouse monoclonal anti-phosphoserine, anti-phosphothreonine or anti-phosphotyrosine, Bio-Makor) diluted 1:50 with 0.1% sodium azide and 0.2% Tween-20 in Tris-buffered saline (20 mM Tris–HCl, 150 mM NaCl, pH 7.5) at room temperature for 3 h. The blots were then washed and incubated for 30 min with a biotinylated anti-mouse secondary antibody (Vector, USA) diluted 1:200. After washing, the blots were stained with antisera by an avidin–biotin–peroxidase complex method (Vector) as described by Hsu et al. (1981). The protein bands were visualized after color development using 4-chloro-1-naphthol. Immunoreactivity of the protein bands was quantified by densitometry. Under our immunoblotting conditions, the immunoreactivity of the protein bands was proportional to the concentration of protein applied to the gel in the range of 5–30 μg of protein.

2.5. Brain crude extract preparation and microtubule assembly

For the investigation of microtubule assembly, we prepared crude extracts from rat cerebral cortex by the method of Qian et al. (1993) with slight modification. The cortical tissues were homogenized using a motor-driven Teflon/glass homogenizer at 4°C in an equal volume of 100 mM PIPES-NaOH buffer (pH 6.9) containing 2 mM EGTA, 1 mM $MgSO_4$, 1 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethyl–sulfonyl fluoride and 10 U/ml aprotinin in the presence or absence of a protein phosphatase inhibitor, calyculin A (1 μM), for comparison of dephosphorylation properties. For the disassembly of microtubules, the homogenates were kept on ice for 1 h and then centrifuged at 100 000 × *g* for 1 h at 4°C. The resulting supernatants were carefully removed and used as the crude extracts. The brain crude extracts were then subjected to gel filtration on a Sephadex G-25 Superfine column (1.6 cm × 2.5 cm) to remove small molecules. The eluted extracts were mixed with 1 μM calyculin A, 1 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethyl-sulfonyl fluoride and 10 U/ml aprotinin. The total protein concentration of the extracts used in all of the microtubule assembly studies was adjusted to 10 mg/ml. The assembly of tubulin into microtubules in crude brain extract in the presence of 1 mM GTP was monitored in terms of the increase in turbidity with time measured at 350 nm and 37°C using a JASCO V-550 spectrophotometer as described by Qian et al. (1993). The initial rate and the final extent of microtubule assembly were determined as previously described (Murthy and Flavin, 1983). To examine the direct effects of desipramine on microtubule assembly in vitro, various concentrations of the drug (final concentration 0.1–100 μM) were included in the assay mixture with 1 mM GTP.

2.6. Immunoblot analysis of crude brain extract

Since microtubule assembly experiments were performed using a crude extract from the cerebral cortex of rats, we carried out electrophoresis of the crude extract at the end of the polymerization in order to check for the presence of assembled microtubule proteins in the fraction. After 40 min of microtubule assembly in crude brain extracts containing 1 mM GTP at 37°C, the samples were centrifuged at 100 000 × *g* for 90 min at 37°C. The pellets were used as the crude microtubule fraction. The pellets were incubated with Laemmli's sample buffer (Laemmli, 1970) and boiled for 2 min. A 15 μg aliquot of protein was subjected to SDS-PAGE using 3–10% polyacrylamide gels. The gels were either stained with Coomassie Blue or the proteins were transferred to a polyvinylidene difluoride membrane (Millipore) in a semidry blotting apparatus at 0.2 A for 5 h, using a buffer containing 25 mM Tris–HCl (pH 8.3), 192 mM glycine, 0.05% SDS and 20% methanol as described by Towbin et al. (1979). The blots were blocked in Tris-buffered saline containing 10% nonfat dry milk, 0.1% sodium azide and 0.2% Tween-20 for 1 h, then washed with 0.2% Tween-20 in Tris-buffered saline. The blots were incubated with the primary antibody against α-tubulin (1:1000 dilution), β-tubulin (1:1000 dilution), MAP2 (1:250 dilution), MAP1 (1:500 dilution) or tau (1:500 dilution) in Tris-buffered saline containing 0.2% Tween-20 at room temperature for 3 h, and further processed using the avidin–biotin–peroxidase complex kit according to supplier's recommendations (Vector). The protein bands were visualized after color development with 4-chloro-1-naphthol.

2.7. Protein determination

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.8. Statistical analyses

All statistical analyses were determined by analysis of variance with a subsequent Tukey's honestly significant difference test.

3. Results

3.1. Effect of chronic desipramine treatment on MAPs phosphorylation

As shown in Fig. 1A (lanes 1 and 2), the anti-MAP2 monoclonal antibody stained a band of molecular weight 280 kDa of protein after immunoprecipitation of MAP2 from the cerebrocortical boiled supernatant fraction and electrophoresis of the immunoprecipitated MAP2. We observed no significant difference in the immunoreactivity of MAP2 between rats treated chronically with saline or desipramine (Fig. 1A and B, lanes 1 and 2). As shown in Fig. 1A and B (lanes 3 and 4), the immunoreactivity of the phosphoserine residues of immunoprecipitated MAP2 was significantly increased, by approximately 43% ($P < 0.001$), after chronic treatment of rats with desipramine, when compared to that for the controls. In contrast, there was no significant change in the immunoreactivity of phosphothreonine (Fig. 1, lanes 5 and 6) or phosphotyrosine (Fig. 1, lanes 7 and 8) residues of immunoprecipitated MAP2 between control and desipramine-treated rats. A single injection of desipramine failed to alter either the concentration or the degree of phosphorylation of MAP2 (data not shown).

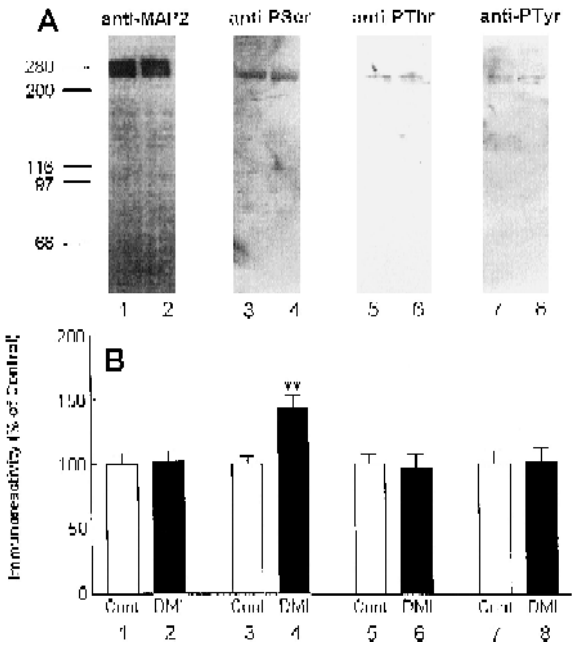

Fig. 1. (A) Western blot analysis of cerebrocortical boiled supernatant fraction with a monoclonal antibody, anti-MAP2 (lanes 1 and 2), anti-phosphoserine (anti-PSer; lanes 3 and 4), anti-phosphothreonine (anti-PThr; lanes 5 and 6) or anti-phosphotyrosine (anti-PTyr; lanes 7 and 8) after immunoprecipitation of MAP2 from the cerebrocortical boiled supernatant fraction from saline (lanes 1, 3, 5 and 7) or desipramine (lanes 2, 4, 6 and 8)-treated rats (20 mg/kg once daily for 14 days). 20 μg of total protein was loaded in each lane and separated by SDS-PAGE on 7.5% polyacrylamide gels. (B) Histograms showing the level of MAP2 (bars 1 and 2), phosphoserine (bars 3 and 4), phosphothreonine (bars 5 and 6) or phosphotyrosine (bars 7 and 8) immunoreactivity quantified by densitometric analysis. Each value represents the mean ± S.E.M. for five animals in the chronic treatment with saline (Cont) or desipramine (DMI) groups. ** $P < 0.001$: significantly different from saline group value.

Fig. 2A shows the immunoreactivity of the phosphoserine residues of immunoprecipitated tau from the supernatant fraction of the cerebral cortex containing heat-stable proteins. A slight increase in the level of immunoreactivity of phosphoserine residues of immunoprecipitated tau was observed after chronic treatment of rats with desipramine, however, the increase was not statistically significant (Fig. 2A and B).

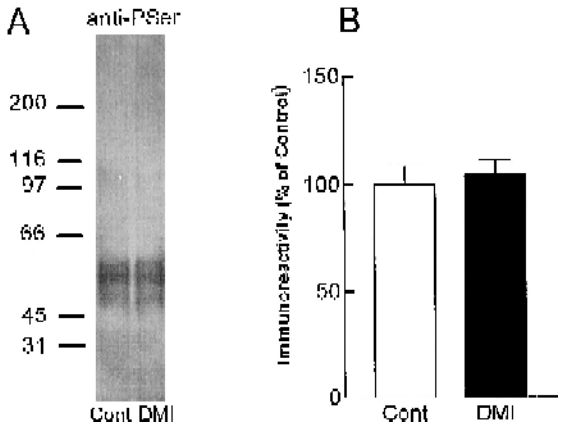

Fig. 2. Effects of long-term treatment with saline (Cont) or desipramine (DMI) for 14 days on the degree of serine phosphorylation of tau in the rat cerebral cortex. The rats were killed 24 h after the last injection. (A) Representative immunoblotting of tau immunoprecipitated from the cerebrocortical boiled supernatant fraction with a monoclonal antibody, anti-phosphoserine (anti-PSer). 20 μg of total protein was loaded in each lane and separated by SDS-PAGE on 3–10% polyacrylamide gels. (B) Histograms showing the level of phosphoserine immunoreactivity quantified by densitometric analysis. Each value represents the mean ± S.E.M. for five animals in each treatment group. There are no significant differences between the two groups.

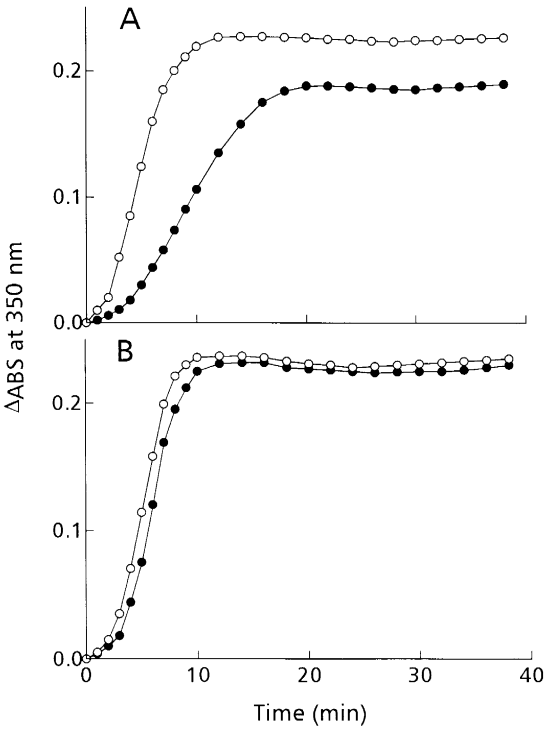

Fig. 3. Effects of long-term treatment with saline (open circles) or desipramine (solid circles) for 14 days on microtubule assembly in the rat cerebral cortex. The crude brain extracts eluted from a Sephadex G-25 column were prepared in the presence (A) or absence (B) of a protein phosphatase inhibitor, calyculin A, as described in the text. The time course of microtubule assembly was monitored by the change in absorbance at 350 nm. Data shown are representative results from one of five separate experiments.

3.2. Effect of desipramine treatment on microtubule assembly

As shown in Fig. 3A and Table 1, long-term treatment with desipramine resulted in significant decreases in the initial rate and the final extent of microtubule assembly, by approximately 56% ($P < 0.001$) and 84% ($P < 0.001$), respectively, compared to that of controls when the crude brain extracts were prepared in the presence of calyculin A. However, when the crude brain extracts were prepared in the absence of calyculin A, inhibition of the initial rate and final extent of microtubule assembly after long-term desipramine treatment was completely nullified (Fig. 3B). Acute treatment with desipramine had no significant effects on microtubule assembly compared with that of the saline-treated controls in either the presence or the absence of calyculin A (Table 1). Desipramine had no direct effect on microtubule assembly at final concentrations ranging from 0.1 to 100 μM (data not shown).

3.3. Changes in the amount of MAPs in the crude microtubule fraction after chronic desipramine treatment

In order to eliminate the possibility of an effect of chronic desipramine treatment on the amount of MAPs in

Table 1
Effects of acute or chronic treatment with desipramine on microtubule assembly in the rat cerebral cortex

Treatment	*n*	Initial rate (ΔABS/min $\times 10^{-2}$)		Final extent (ΔABS $\times 10^{-2}$)	
		with CaA	without CaA	with CaA	without CaA
Acute control	5	3.3 ± 0.2	4.7 ± 0.2	22.3 ± 0.2	23.3 ± 0.2
Acute DMI	5	3.5 ± 0.3	4.8 ± 0.2	23.8 ± 0.2	24.2 ± 0.1
Chronic control	5	3.4 ± 0.2	4.8 ± 0.3	22.3 ± 0.2	23.3 ± 0.2
Chronic DMI	5	1.7 ± 0.1 [a]	4.3 ± 0.4	18.8 ± 0.3 [a]	23.0 ± 0.2

Rats were killed 2 h (acute) or 24 h (chronic) after the last injection. The crude extracts of brain from saline (Control) or desipramine (DMI)-treated rats, eluted from a Sephadex G-25 column, were prepared in the presence or absence of a protein phosphatase inhibitor, calyculin A (CaA), as described in the text. The initial rate of microtubule assembly was determined from tangents at the steepest points of turbidity curves. The final extent of assembly was determined, after 40 min at 37°C, from the change in absorbance at 350 nm from the zero-time baseline. Values are means ± S.E.M. of individual determinations for five animals.

[a] $P < 0.001$: significantly different from saline group value.

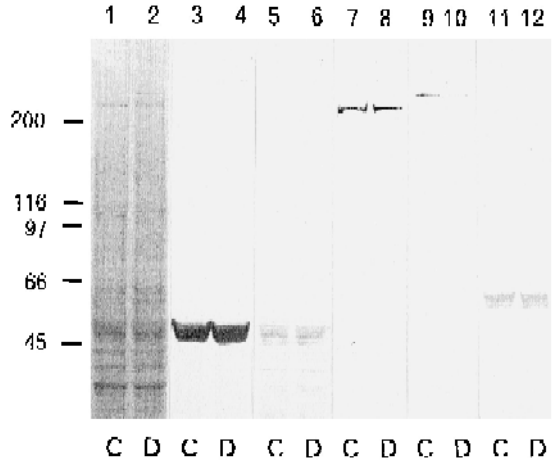

Fig. 4. Western blot analysis of the cerebrocortical crude microtubule fraction isolated from saline (C) or desipramine (D)-treated rats (20 mg/kg once daily for 14 days). Coomassie blue staining of the proteins in the crude microtubule fraction after 3–10% SDS-PAGE (lanes 1 and 2). Immunoblotting with monoclonal antibodies, anti-α-tubulin (lanes 3 and 4), anti-β-tubulin (lanes 5 and 6), anti-MAP2 (lanes 7 and 8), anti-MAP1 (lanes 9 and 10) and anti-tau (lanes 11 and 12). 15 μg of total protein was loaded in each lane.

the crude microtubule fraction, we subjected the crude microtubule fraction to analysis by SDS-PAGE followed by Coomassie Blue staining and immunoblotting using monoclonal antibodies raised against specific microtubule proteins. As shown in Fig. 4 (lanes 1 and 2), the pattern of cerebrocortical crude microtubule proteins separated on 3–10% SDS-PAGE shows no difference in protein composition between rats subjected to chronic treatment with saline or desipramine. Using immunoblot analysis, we identified α-tubulin, β-tubulin, MAP2, MAP1 and tau in the crude microtubule fraction. Fig. 4 shows that there was no significant difference in the immunoreactivity of α-tubulin (lanes 3 and 4), β-tubulin (lanes 5 and 6), MAP2 (lanes 7 and 8), MAP1 (lanes 9 and 10) or tau (lanes 11 and 12) between chronic saline and desipramine-treated rats.

4. Discussion

Although the therapeutic efficacy of antidepressants in the treatment of depression is well established, their precise mechanism of action has not yet been elucidated. A single administration of antidepressants induces an inhibition of monoamine reuptake within minutes to hours, whereas the clinical response to antidepressants usually requires 1 to 3 weeks to become evident (Heninger and Charney, 1987). The dissociation between the acute effect of antidepressants and the delayed onset of clinical action has led to a number of studies on the biochemical mechanisms involved in the chronic action of antidepressants. In the 1980s, particular attention was focused on defining the long-term modifications in the sensitivity of presynaptic and postsynaptic receptors after prolonged antidepressant treatment. A large number of receptor binding studies have indicated that long-term, but not short-term, treatment with clinically effective antidepressants commonly results in a decrease in β-adrenoceptor and 5-HT_2 receptor binding in the rat cerebral cortex (Peroutka and Snyder, 1980; for review, see Charney et al., 1981; Heninger and Charney, 1987). However, these receptor binding changes represent only the first step in the signal transduction pathway cascade. For determining the functional changes in neurons after long-term treatment with antidepressants, studies at the level of receptors have limited value. Therefore, recent studies have focused on intracellular signal transduction processes beyond the level of receptors as potential target sites for the action of antidepressants. It has been demonstrated that long-term treatment with antidepressants leads to changes in and modifications of the function of receptor-coupled GTP-binding proteins (Ozawa and Rasenick, 1989), in the action of protein kinases and phosphoproteins (Nestler et al., 1989; Perez et al., 1989, 1991; Racagni et al., 1992; Mann et al., 1995; Popoli et al., 1995) and in the regulation of gene expression at the nuclear level (Morinobu et al., 1995; Nibuya et al., 1995, 1996). At present, it is a generally accepted belief that the long-term adaptive neuronal mechanisms are of relevance to the therapeutic actions of antidepressants (Duman et al., 1994; Nibuya et al., 1995, 1996), although the precise mechanisms remain unidentified.

Our present study focused on the molecular mechanism of action of antidepressants involving the phosphorylation of an endogenous substrate and its function after phosphorylation. The results demonstrate that long-term administration of desipramine induces an increase in the degree of serine phosphorylation of MAP2 in vivo and has an inhibitory effect on microtubule assembly in the rat cerebral cortex. Furthermore, these changes in the degree of phosphorylation of proteins associated with microtubules were observed following chronic, but not acute or in vitro, treatment with desipramine, and correlated with the time course of the clinical action of antidepressants.

It has become increasingly clear that chronic but not acute treatment with several classes of antidepressants elicits specific changes in the cAMP-dependent phosphorylation system in the rat cerebral cortex (Nestler et al., 1989; Perez et al., 1989, 1991; Racagni et al., 1992). Perez et al. (1989) have shown that prolonged administration of desipramine can affect the degree of cAMP-dependent endogenous phosphorylation of MAP2 without changes in MAP2 concentration in the cerebrocortical soluble fraction. Findings in the present study support those of Perez et al. (1989) and we can now further characterize the phosphorylation state of MAP2 after chronic administration of desipramine. Moreover, the observed inhibition of microtubule assembly may represent a novel post-receptor

action of antidepressants in the intracellular signal transduction pathway caused by chronic treatment with desipramine.

cAMP-dependent protein kinase is reported to phosphorylate MAP2 exclusively on serine residues as is the case with Ca^{2+}/phospholipid-dependent protein kinase (Goldenring et al., 1985; Akiyama et al., 1986; Walaas and Nairn, 1989), whereas Ca^{2+}/calmodulin-dependent protein kinase II phosphorylates MAP2, not only on serine residues but also on threonine residues (Goldenring et al., 1985; Yamamoto et al., 1985; Walaas and Nairn, 1989). It is therefore possible that the increased degree of serine phosphorylation of MAP2 detected after repeated administration of desipramine is attributable to the increased activity of a specific serine–threonine kinase, possibly cAMP-dependent protein kinase, Ca^{2+}/phospholipid-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase II. However, it has been reported that chronic treatment with imipramine (a tricyclic antidepressant) or tranylcypromine (a monoamine oxidase inhibitor) results in a decrease in the level of cAMP-dependent protein kinase activity in the cytosol of the rat frontal cortex (Nestler et al., 1989). In addition, a recent study has shown that chronic administration of desipramine to rats results in a reduction in the level of Ca^{2+}/phospholipid-dependent protein kinase activity in both the soluble and particulate fractions of the cerebral cortex of the rats (Mann et al., 1995). Moreover, long-term treatment with other antidepressants, such as fluvoxamine (a selective serotonin reuptake inhibitor) or venlafaxine (a mixed inhibitor of serotonin and norepinephrine reuptake), fails to alter the level of presynaptic Ca^{2+}/calmodulin-dependent protein kinase II activity in the cortical subsynaptosomal fractions (Popoli et al., 1995). Regardless of these findings, the results of our study suggest that chronic treatment with desipramine brings about activation of certain protein kinases and/or inactivation of certain protein phosphatases in the soluble fraction of the rat cerebral cortex. In order to further understand the precise mechanism underlying the increase in the degree of MAP2 phosphorylation evoked by desipramine, it is necessary to investigate the changes in distribution and activity of cAMP-dependent protein kinase, Ca^{2+}/phospholipid-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase II associated with the phosphorylation of MAP2 after long-term treatment with desipramine.

The phosphorylation and dephosphorylation of MAP2 by several protein kinases and protein phosphatases are known to play an important role in the regulation of the microtubule assembly–disassembly cycle (Yamamoto et al., 1985, 1988). In addition, it has been shown that phosphorylation of MAP2 by cAMP-dependent protein kinase (Nishida et al., 1982), Ca^{2+}/calmodulin-dependent protein kinase II (Yamamoto et al., 1985), Ca^{2+}/phospholipid-dependent protein kinase (Hoshi et al., 1988) or epidermal growth factor receptor kinase (Nishida et al., 1987) results in reduced microtubule nucleation and a decrease in both the initial rate and the final extent of microtubule assembly in vitro (Jameson et al., 1980). Therefore, the inhibition of microtubule assembly observed only in the presence of a protein phosphatase inhibitor after chronic desipramine administration may reflect the inhibition of microtubule assembly caused by increased phosphorylation of MAP2 in vivo.

MAP2 is composed of two major domains termed the microtubule binding domain and the projection domain, both of which can be phosphorylated (Vallee, 1980). It has been demonstrated that approximately one-third of soluble type II cAMP-dependent protein kinase in brain is tightly associated with microtubules through the binding of the regulatory subunit (RII) to the projection domain of MAP2 (Vallee, 1980; Theurkauf and Vallee, 1982). Perez et al. (1989) found that chronic, but not acute, administration of desipramine induced an increase not only in the amount of RII but also in the degree of cAMP-dependent endogenous phosphorylation of MAP2 in the soluble fraction of the rat cerebral cortex, suggesting that RII could be an intracellular target site of desipramine. However, these findings do not convincingly indicate a direct correlation of increased amounts of RII with effects on MAP2 phosphorylation, since the changes in the activity of type II cAMP-dependent protein kinase after long-term treatment of rats with desipramine are unclear. If chronic administration of desipramine induces an increase in the degree of phosphorylation of the microtubule binding domain of MAP2, the polymerization of tubulin would be inhibited as observed in our results. Thus, further studies are needed to define the exact phosphorylation sites of MAP2 which could modulate the binding affinity to microtubules during long-term treatment with desipramine.

In the present study, we used different methods to prepare extracts for use in MAP2 phosphorylation and microtubule assembly experiments. The reason why we boiled the cytosolic fraction to isolate MAP2 from brain tissue was that heat treatment without temperature dependent microtubule assembly–disassembly cycles was the best method of preventing the dephosphorylation of MAP2 during the experimental procedure (Tsuyama et al., 1986; Walaas and Nairn, 1989). Moreover, we could inactivate both the endogenous protein kinases and the protein phosphatases associated with MAP2 in the cytosolic fractions by heat treatment (Murthy and Flavin, 1983; Schulman, 1984; Tsuyama et al., 1986; Walaas and Nairn, 1989). For microtubule assembly experiments, we did not use purified MAP2, but a crude extract from the cerebral cortex of rats. Therefore, we could not exclude the possibility that some phosphoproteins other than MAP2 were present in the crude extract and consequently related to the inhibition of microtubule assembly. Indeed, this crude extract contained some microtubule proteins such as tubulin, MAP2, MAP1 and tau, but we observed no changes in the concentration of each of these proteins following chronic desipramine

administration (Fig. 4). The phosphorylation of tau has also been thought to be a key factor in the regulation of microtubule assembly (Nishida et al., 1982; Yamamoto et al., 1985; Hoshi et al., 1987). We therefore determined the phosphorylation state of tau after chronic desipramine treatment of rats. Our finding that the degree of phosphorylation of tau in the soluble fraction of the cerebral cortex between control and desipramine-treated rats was not significantly different (Fig. 2) suggests that the observed inhibition of microtubule assembly is likely not due to the phosphorylation of tau, but rather appears to be a consequence of the increased degree of MAP2 phosphorylation in response to chronic treatment with desipramine. However, we cannot completely exclude the involvement of changes in the level of phosphorylation of several other endogenous phosphoproteins such as tubulin (Yamamoto et al., 1985; Wandosell et al., 1986; Khan and Ludueña, 1996), neurofilament proteins (Minami and Sakai, 1985), MAP1B (Pedrotti et al., 1996) and MAP4 (Illenberger et al., 1996) in the inhibition of microtubule assembly in crude brain extracts after chronic treatment of rats with desipramine.

The biochemical consequences of the inhibition of microtubule assembly by chronic desipramine treatment are unclear at present. However, it has been demonstrated that tubulin dimers, the primary constituent of microtubules, act as regulators of the adenylate cyclase signal transduction system in rat cerebral cortex membranes (Rasenick and Wang, 1988; Hatta et al., 1995). Tubulin dimers are associated with the synaptic membrane and participate in the stimulatory or inhibitory regulation of adenylate cyclase by the transfer of guanine nucleotides from tubulin to cortex membrane GTP-binding proteins (Rasenick and Wang, 1988; Roychowdhury et al., 1993; Hatta et al., 1995). These effects can be observed only for the dimer form, and not for the polymer form of tubulin (Rasenick and Wang, 1988). Previous studies have demonstrated that chronic treatment of rats with desipramine enhanced activation of adenylate cyclase by increasing the degree of interaction between stimulatory GTP-binding proteins and the adenylate cyclase catalytic moiety in rat cortical synaptic membranes (Menkes et al., 1983; Ozawa and Rasenick, 1989). More recently, Kamada et al. (1997) have shown that long-term treatment with antidepressants induces enhanced interaction between tubulin dimers and GTP-binding proteins via transfer of GTP from tubulin to α subunits of stimulatory GTP-binding proteins in the cerebral cortex of rats. Thus, these studies appear to indicate that tubulin dimers may be involved in the mechanism which elicits enhanced coupling between stimulatory GTP-binding proteins and adenylate cyclase following chronic antidepressant treatment. It is suggested that the inhibition of tubulin polymerization after chronic desipramine treatment (Fig. 3A) may represent an increase in either the rate of formation or the amount of the unpolymerized forms of tubulin, such as tubulin dimers or oligomers. Therefore, it is intriguing to speculate that chronic treatment of rats with desipramine may induce an increase in the rate of formation of tubulin dimers or in the amount of tubulin dimers in the cytoplasm of the cerebral cortex and consequently modulate their association with adenylate cyclase via GTP-binding proteins in the synaptic membrane of the cerebral cortex of rats.

Among the neuronal cytoskeletal proteins, MAP2 has unique properties relating to its subcellular localization. The high molecular weight form of MAP2 is present in high concentrations in dendrites where it appears in association with microtubules, actin filaments and neurofilaments (see Johnson and Jope, 1992 for review). Recently, correlation between dendrite sprouting and an increase in the degree of MAP2 phosphorylation has been observed in cultured hippocampal neurons (Díez-Guerra and Avila, 1995). It has been reported that in lamprey neurons microtubule destabilization and changes in protein phosphorylation precede dendritic sprouting (Hall et al., 1991). Nakamura (1990) has shown that repeated administration of desipramine to rats induces sprouting of noradrenergic neurons in the cerebral cortex of the rats. Taken together, we propose that changes in the degree of MAP2 phosphorylation after chronic treatment with desipramine can influence the stability of the microtubular network and hence induce a morphological change in dendrites.

Although we raised the possibility that our results reflect the biochemical effects of desipramine, it might be impossible to determine whether these effects are involved in the clinical action of antidepressants. Thus, it is necessary to check whether other classes of antidepressants have the same effects. Moreover, we must determine the effect of treatment with neuroleptics or anxiolytics on microtubule proteins in order to confirm that the observed effects associated with MAP2 and microtubules are limited to antidepressant drugs.

In conclusion, our results suggest that long-term treatment of rats with desipramine results in the inhibition of microtubule assembly in the cerebral cortex and that this effect appears to be mediated by the increase in the degree of phosphorylation of MAP2. However, it remains to be seen what kinases are implicated in the increase in the degree of serine phosphorylation of MAP2 as well as the activity of such enzymes. Moreover, we must determine whether long-term treatment with desipramine affects the phosphorylation state of the microtubule binding domain of MAP2 in order to establish a relationship between an increased degree of MAP2 phosphorylation and the inhibition of microtubule assembly in vivo. Finally, changes in the degree of phosphorylation of MAP2 and microtubule assembly observed in this study may represent a possible role of cytoskeletal proteins in mediating functional and/or morphological changes following chronic treatment with desipramine. Further studies are required for investigation of the second messenger-phosphorylation system associated with other phosphoproteins which may regulate long-

term adaptive or plastic changes in the molecular mechanism of action of antidepressant drugs.

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