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ARTICLE

Time-course changes in rat cerebral cortex subcellular distribution of the cyclic-AMP binding after treatment with selective serotonin reuptake inhibitors

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Abstract

Pharmacological investigations have suggested the involvement of the cAMP transduction pathway in the action of antidepressant drugs and in the pathophysiology of mood disorders. We have extended these studies to determine the time-related effects of two selective serotonin reuptake inhibitors, fluvoxamine (15 mg/kg) and paroxetine (5 mg/kg), on the cAMP-binding in rat cerebral cortex, after short and long-term treatments. Photoaffinity labelling experiments with 8-N_3-[^{32}P]cAMP were carried out in cerebrocortical soluble (S1 or S2) and microtubule fractions. In our conditions, both SSRIs administered for 5 days were unable to affect the cAMP-binding in S1, S2, and in microtubule fractions. After 12 days of treatment, paroxetine and fluvoxamine significantly enhanced the cAMP-binding to the 54 kDa protein, corresponding to the type II regulatory subunit of PKA (RII), in the S1 and microtubule fractions. Any modification in respect to controls was observed in S2, the soluble fraction devoid of microtubules. After 21 days of treatment no changes were observed in the soluble S1 fraction and in microtubules, but the cAMP-binding to the RII subunit was found to be significantly higher in the S2 fraction. The high concentration of RII, demonstrated first in microtubules (12 days) and then in the cytosol (21 days), could be the result of a time-related effect of SSRIs on PKA and its translocation from microtubule compartment to the cytosol. The present findings seem to demonstrate the capacity of SSRIs to modulate the subcellular distribution of PKA and support the involvement of the cAMP pathway in the mechanism of action of these drugs.

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Key words: SSRIs, cAMP dependent protein kinase, transduction mechanisms, antidepressants, microtubules.

Introduction

cAMP-dependent protein kinase (PKA) represents the key enzyme of a signalling cascade that links membrane receptor-mediated responses to different intracellular functions (Erlichman et al., 1980; Scott, 1991). The type II isoform of PKA is a major mediator of the actions of cAMP in the central nervous system (CNS) (Rubin et al., 1981). PKA II is maximally expressed in forebrain neurons, where most of the enzyme is anchored to specific proteins termed AKAPs (A Kinase Anchor Proteins), distributed along dendritic microtubules in the proximity of the postsynaptic plasma membrane (Theurkauf and Vallee, 1982; Bregman, 1989; Glantz et al., 1992).

In the absence of cAMP, PKA is organized as a tetrameric complex composed of a regulatory dimer (R) and two catalytic subunits (C). When cAMP rises within the cell and binds the regulatory dimer, the catalytic subunits dissociate and become able to phosphorylate specific proteins.

Pharmacological investigations in different laboratories have pointed out the involvement of the cAMP-dependent signal transduction pathway in the mechanism of

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action of antidepressant drugs and in the pathophysiology of mood disorders. Moyer and colleagues (1986) demonstrated a significant decrease of the soluble, but not particulate, PKA activity in pineal gland extracts from rats subjected to acute and repeated treatment with desipramine (DMI). Also reported was a decreased PKA activity in the soluble, and an increase in the particulate, fractions of rat frontal cortex, determined after chronic administration of imipramine, tranylcypromine or electroconvulsive seizures (Nestler et al., 1989). In addition, our studies clearly indicated that the cAMP-dependent endogenous phosphorylation, as well as the cAMP-binding to the RII subunit in rat cerebral cortex, were altered by long-term treatment with antidepressants (DMI, fluoxetine, oxaprotiline, fluvoxamine, venlafaxine, moclobemide and lithium) (Perez et al., 1989, 1991, 1995; Racagni et al., 1992; Mori et al. (In Press), 1996).

From a clinical point of view, relevant data have been accumulated during recent years indicating the possible involvement of the cAMP-dependent pathway in the pathophysiology of mood disorders.

From our laboratory, data have emerged on cAMP-dependent endogenous phosphorylation, which is augmented in bipolar euthymic patients (Perez et al., 1995) and modulated by lithium treatment (Zanardi et al., 1997). In 1996 Shelton et al. showed a decreased PKA activity measured in the soluble, but not in the particulate, fractions of fibroblasts from patients with major depression. A significant decrease, in respect to control subjects, of the [^{3}H]cAMP-binding has been found in the post-mortem brain of patients affected by bipolar depression (Rahman et al., 1997). Lowther and coworkers (1997) demonstrated that the cAMP-binding in the CNS is unaltered in depressed suicide victims, but is decreased by antidepressant treatments.

Fluvoxamine and paroxetine both explain a selective inhibition of the serotonin reuptake (SSRIs) and are effectively employed in the pharmacotherapy of different psychiatric disorders, such as major depression, obsessive-compulsive disorders (OCD) and panic attacks. A precise time-course for the clinical response to these drugs has been identified (Greist et al., 1995; Mundo et al., 1997; Ware, 1997). It has, in fact, been suggested that uptake inhibition, which occurs from the first day of administration, could not explain by itself the clinical effects of these drugs, which become evident after at least 3 wk of therapy (Quitkin et al., 1984) and intracellular events, which are slower to develop, could account for the pharmalogical efficiency.

This study reports the time-related effects of 5-HT reuptake blockers (fluvoxamine and paroxetine) on the cAMP-binding in rat cerebral cortex, evaluated after short and long-term treatment.

Materials and Methods

Materials

Fluvoxamine was a gift from Solvay Duphar and paroxetine was purchased from Smith Kline Beecham. Male Sprague–Dawley rats were from Charles River Laboratories (Calco, Italy). EGTA, EDTA, morpholin ethansulphonic acid (MES), piperazine-*N*,*N*′-(2-ethansulphonic) acid (Pipes), 3-isobutyl-1-methylxantine (IBMX), aprotinin, pepstatin A, phenylmethylsulphonyl fluoride (PMS-F), GTP, cAMP, bovine serum albumin (BSA), glycerol and methylcellulose were from Sigma Chemical Corporation.

8-azido-[^{32}P]cAMP (8-N_3-[^{32}P]cAMP; 66.7 Ci/mmol) was from ICN (Irvine, CA, USA). Hyperfilm b-max were from Amersham (UK). Molecular weight standards and all materials for gel electrophoresis were from Bio-Rad. The bicinchoninic acid protein assay (BCA^{TM}) was from Pierce.

Animals and the treatment schedule

Male Sprague–Dawley rats (initial weight 150 g; Charles River, Calco, Italy) were used for all experiments. Animals were housed 4 per cage with free access to water and food and exposed to a 14-h light/dark cycle. They received injections of saline, vehicle or drug for 5, 12 or 21 days during the same calendar period. Paroxetine was prepared in saline and 1% methylcellulose, and was given at the dose of 5 mg/kg i.p. once daily; the dose used for fluvoxamine was 15 mg/kg diluted in saline. Each group of rats were sacrificed by decapitation after the treatment period. Brains were quickly removed, dissected on ice and stored at −80 °C until used. Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (U.S. Institute, Bethesda).

Preparation of subcellular fractions

Cerebrocortical soluble and microtubule fractions were prepared for analysis as previously described by Perez et al. (1991). Frontal cortex obtained from several controls and treated animals were weighed and homogenized in 1 volume of ice-cold buffer Pipes pH 6.8 (100 mM Pipes, 2 mM EDTA, 1 mM DTT, 100 μg/ml leupeptine, 100 μg/ml aprotinin, 0.1 mM PMS-F). The homogenate was centrifuged at 40 000 rpm at 4 °C for 60 min. Part of the supernatant obtained by centrifugation (S1) was used as the soluble fraction, the rest was incubated at 37 °C for 30 min in the presence of 1 mM GTP and 4 M glycerol. At the end of the incubation period the assembled proteins were collected by centrifugation (40 000 rpm for

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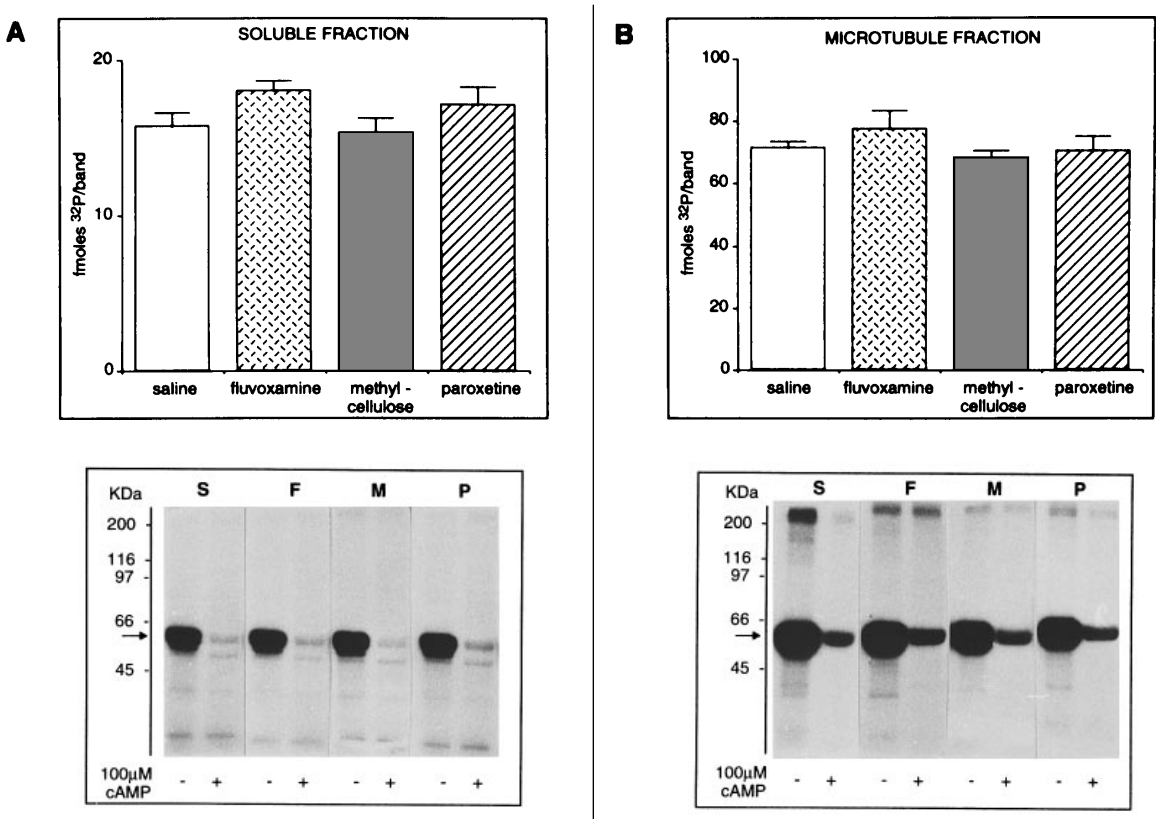

Figure 1. Photoaffinity labelling in the S1 soluble fraction (A) and in microtubules (B) from rat cerebral cortex after 5 days of treatment with fluvoxamine and paroxetine. Four experiments for each fraction were performed in duplicate. *Lower panel*: Autoradiographies (12 h of exposure), after SDS–PAGE, show the total (−cAMP) and non-specific (+cAMP) [^{32}P] incorporation into the 54 kDa protein band (indicated by arrow) from rats treated with saline (S), 15 mg/kg fluvoxamine (F), methylcellulose (M) and 5 mg/kg paroxetine (P). *Upper panel*: Histograms represent the specific [^{32}P]cAMP covalent binding to the 54 kDa protein band, expressed as fmol of [^{32}P]/band. The values are the means ± standard deviation.

60 min at 4 °C). Microtubule pellets were resuspended in the appropriate buffer and the supernatant (S2) was dialysed overnight. All fractions were kept at −80 °C until assay. Total protein concentration was determined using BCA™ protein assay; bovine serum albumin was used as the standard.

Photoaffinity labelling

Photoaffinity labelling was performed as described elsewhere (Perez et al., 1991). The reaction mixture, containing 40 μg of proteins in MES buffer pH 6.2 and 2 μM 8-N_3-[^{32}P]cAMP was incubated for 60 min in the dark at 4 °C, whether or not 100 μM unlabelled cAMP was present. The samples were then irradiated with UV lamp (Spectroline, ENF/24F) at 254 nm for 10 min. The reaction was blocked with an equal volume of Laemmli buffer (Laemmli, 1970). An equivalent amount of proteins were fractionated by one-dimensional SDS–PAGE on 10% acrylamide. The dried gels were subjected to autoradiography and the photoactivated [^{32}P]cAMP-binding to the protein bands was quantified by liquid scintillation. The covalent binding was linear in a range of 5–75 μg of soluble or microtubule proteins.

Statistical analysis

For each fraction and for each time of drug administration, four experiments in duplicate or triplicate have been performed. Differences in drug effects between groups were determined by Student's t test. The criterion for significance was taken as $p \leqslant 0.05$.

Results

This study was designed to evaluate the time-related influence of fluvoxamine and paroxetine on the cAMP-binding in rat cerebral cortex.

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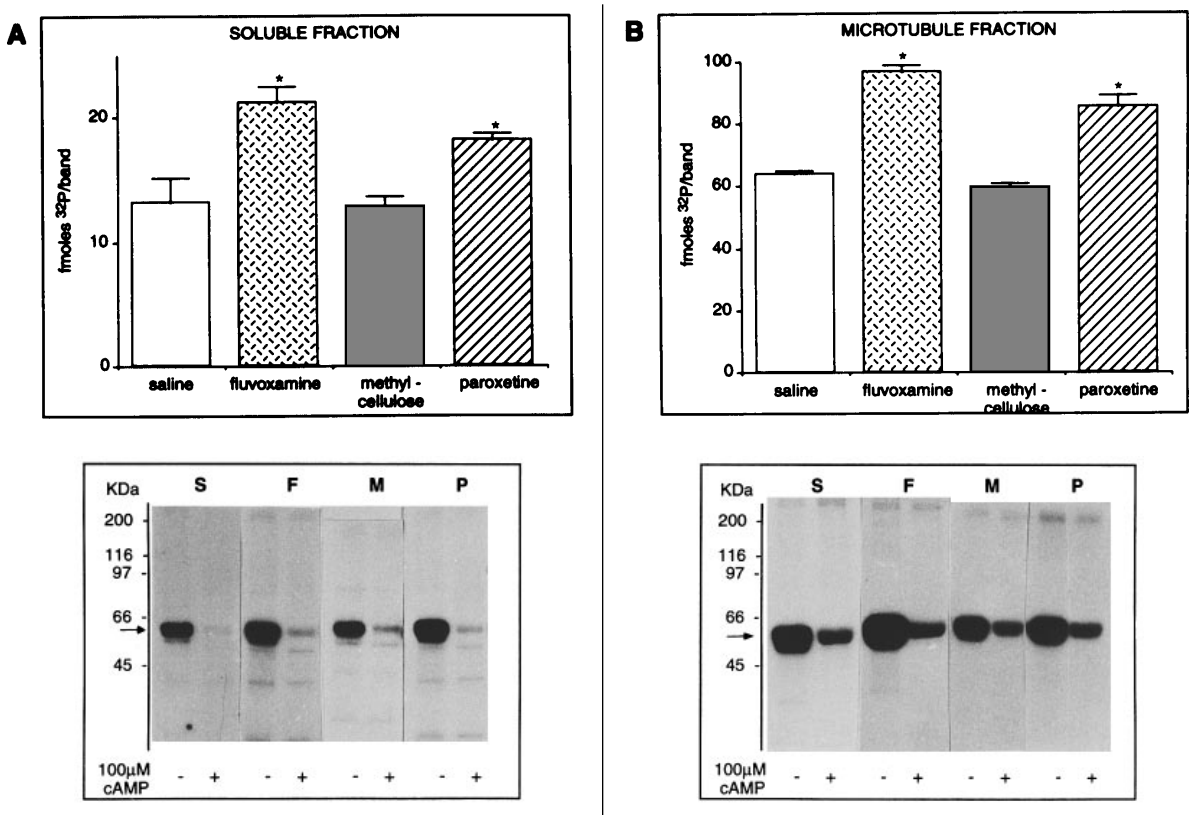

Figure 2. Photoaffinity labelling in the S1 soluble fraction (A) and in microtubules (B) from rat cerebral cortex after 12 days of treatment with fluvoxamine and paroxetine. Four experiments for each fraction were performed in triplicate. *Upper panel*: Autoradiographies (13 h of exposure), after SDS–PAGE, show the total (−cAMP) and non-specific (+cAMP) [^{32}P] incorporation into the 54 kDa protein band (indicated by arrow) from rats treated with saline (S), 15 mg/kg fluvoxamine (F), methylcellulose (M) and 5 mg/kg paroxetine (P). *Lower panel*: Histograms represent the specific [^{32}P]cAMP covalent binding to the 54 kDa protein band, expressed as fmol of [^{32}P]/band. The values are the means ± standard deviation. $^{*}p \leqslant 0.01$.

The first approach was to analyse the pattern of cAMP-binding proteins in rat cerebrocortical soluble and microtubule fractions after short-term treatment of 5 days with fluvoxamine (15 mg/kg) and paroxetine (5 mg/kg), adopting the photoaffinity labelling technique.

By rapid centrifugation of the homogenates (1 g/ml), a soluble fraction (S1) was obtained, from which microtubules were pelletted after one cycle of temperature-dependent polymerization. The remaining soluble proteins (S2), deprived of microtubules, were collected for assay.

Aliquots of proteins from the soluble (S1 and S2) and the microtubule fractions were subjected to photoaffinity labelling with 8-N_3-[^{32}P]cAMP, in the absence (−cAMP) or presence (+cAMP) of 100 μM cAMP, fractionation by SDS–PAGE and autoradiography (lower panels).

The autoradiograms of the gels in Figures 1 and 3 reveal that both SSRIs given for 5 days were unable, compared to controls, to modify the binding of cAMP to the 54 kDa protein, the type II regulatory subunit of PKA (RII), corresponding to the major cAMP-binding protein in cerebral cortex. No changes were detected either in the soluble S1 (fluvoxamine 14.3 ± 3.57%, paroxetine 10.9 ± 2.1%: Figure 1A) and S2 (fluvoxamine −21 ± 1.98%, paroxetine 5.3 ± 0.76%: Figure 3A) fractions or in microtubules (fluvoxamine 7.8 ± 1.96%, paroxetine 3.0 ± 0.2%: Figure 1B). The mean values of the specific binding, measured as [^{32}P] incorporated in the RII subunit, is shown in the histograms (Figures 1A, 1B and 3A, upper panels).

In the second set of experiments, the effect of fluvoxamine and paroxetine on the cAMP-binding in rat cerebral cortex after 12 days of treatment was analysed. As indicated by the histograms (Figure 2A, upper panel), a significant enhancement of the [^{32}P]cAMP-specific binding to the 54 kDa-RII subunit was detected either in the cerebrocortical cytosolic fraction (S1) (fluvoxamine 60.5 ± 4.9%, paroxetine 40.5 ± 1.9%) or microtubules (fluvoxamine 50.0 ± 2.17%, paroxetine 43.5 ± 3.4%) (Figure 2B). No differences between control and treated animals were observed by photoaffinity labelling in the S2 fraction (fluvoxamine −1.1 ± 0.26%, paroxetine 14.77 ± 0.25%) after 12 days of treatment (Figure 3B).

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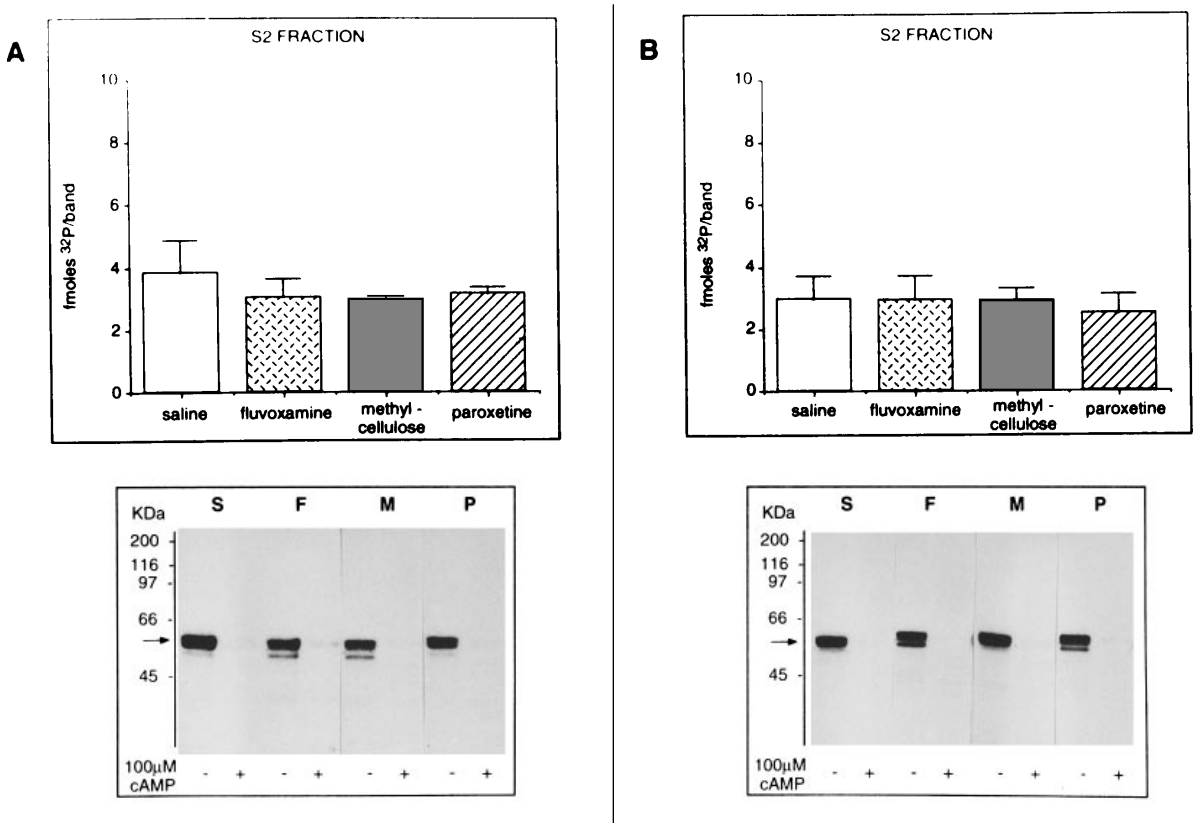

Figure 3. Photoaffinity labelling in the S2 soluble fraction from rat cerebral cortex after 5 (A) and 12 (B) days of treatment with fluvoxamine and paroxetine. Four experiments were performed in triplicate. *Upper panel*: Autoradiographies (24 h of exposure), after SDS–PAGE, show the total (−cAMP) and non-specific (+cAMP) [^{32}P] incorporation into the 54 kDa protein bands (indicated by arrow) from rats treated with saline (S), 15 mg/kg fluvoxamine (F), methylcellulose (M) and 5 mg/kg paroxetine (P). *Lower panel*: Histograms represent the specific [^{32}P]cAMP covalent binding to the 54 kDa protein band, expressed as fmol of [^{32}P]/band. The values are the means ± standard deviation.

Figures 4 and 5 show the photoaffinity labelling experiments after 21 days treatment with fluvoxamine (15 mg/kg) and paroxetine (5 mg/kg). Both drugs (Figure 4) were able to induce a significant enhancement of the cAMP-binding to the regulatory subunit of PKA in the S2 soluble fraction devoid of microtubules (fluvoxamine 211 ± 8.9%, paroxetine 115.8 ± 5.9%).

The analysis of the S1 fraction (fluvoxamine −17.09 ± 0.88%, paroxetine −8.18 ± 1.08%: Figure 5A) and microtubule components (fluvoxamine −1.11 ± 0.03%, paroxetine 2.5 ± 0.02%) (Figure 5B) revealed that the levels of [^{32}P]cAMP specifically bound (upper panels) were unchanged by the treatment.

Discussion

During the last few years new concepts have emerged about the mechanism of action of antidepressants. They seem to regulate not only the extracellular levels of neurotransmitters by blocking monoamine oxidase activity and monoamine reuptake, but also modulate their receptors and the intracellular mechanisms responsible for neuronal function, such as G proteins, adenylate cyclase and protein phosphorylation systems (Lesch et al., 1991; Ozawa and Rasenick, 1989; Guitart and Nestler, 1992).

Among signal transduction, the cAMP-dependent system proved extremely sensitive to antidepressant medications. Our previous studies had demonstrated that subchronic, but not acute, treatment with DMI or fluoxetine modulates cAMP, augmenting the [^{32}P]cAMP-binding to the 54 kDa type II regulatory subunit of PKA in rat cerebrocortical soluble fraction (S1) and microtubules (Perez et al., 1989, 1991). Keeping in mind the ability of antidepressants to influence the cAMP-dependent protein kinase, the present study has been undertaken to determine the time-related changes in the subcellular distribution of the cAMP-binding in rat cerebral cortex after treatment with SSRIs.

Fluvoxamine and paroxetine administered for 5 days were ineffective to alter the cAMP-binding to the 54 kDa

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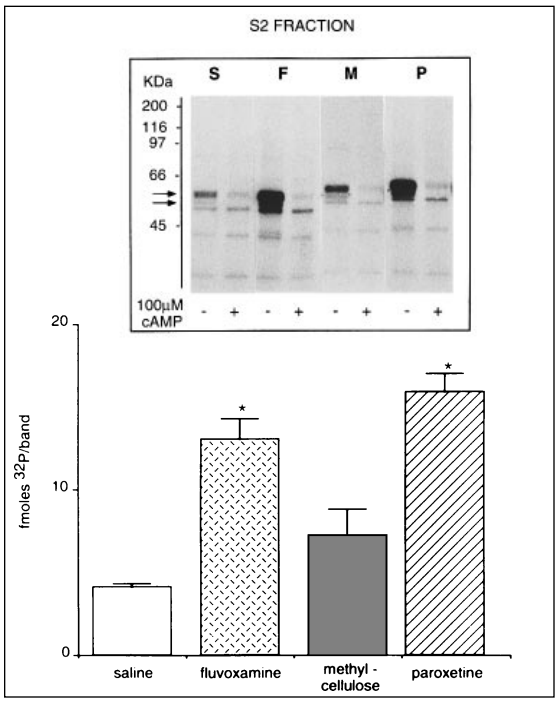

Figure 4. Photoaffinity labelling in the S2 soluble fraction from rat cerebral cortex after 21 days of treatment with fluvoxamine and paroxetine. Four experiments were performed in triplicate. *Upper panel*: Autoradiographies (11 h of exposure), after SDS–PAGE, show the total (−cAMP) and non-specific (+cAMP) [^{32}P] incorporation into the 52 kDa and 54 kDa protein bands (indicated by arrows) from rats treated with saline (S), 15 mg/kg fluvoxamine (F), methylcellulose (M) and 5 mg/kg paroxetine (P). *Lower panel*: Histograms represent the specific [^{32}P]cAMP covalent binding to the 54 kDa protein band, expressed as fmol of [^{32}P]/band. The values are the means ± standard deviation. $^{*}p \leqslant 0.001$.

RII subunit of PKA in both soluble fractions (S1 and S2: Figures 1A and 3A) and in microtubules (Figure 1B), whereas a treatment of 12 days significantly enhanced the [^{32}P]cAMP-binding to the RII protein in the soluble S1 and microtubule fractions (Figures 2A and 2B). This increase in the S1 fraction seems to depend on the augmented binding of [^{32}P]cAMP to the RII subunit associated with microtubules, since the labelling was unchanged in the S2 soluble fraction devoid of microtubules (Figure 3B). Even if previous studies have reported that both soluble and membrane fractions contained low amounts of endogenous cAMP (Walter et al., 1978), the possibility that the observed modifications may reflect changes in the affinity of the RII subunit for the ligand cannot be ruled out. Alternatively, this result could be ascribed to an increased expression of the PKA regulatory protein.

No differences between controls and treated animals were obsrved, relative to the RII-cAMP binding in the soluble (S1) and microtubule fractions after 21 days of treatment with paroxetine and fluvoxamine (Figures 5A and 5B). The analysis of the S2 fraction otherwise revealed a significant enhancement of the cAMP-binding in two major proteins of 52 and 54 kDa, possibly corresponding to the a and b or to the dephospho/phospho isoforms of the type II regulatory subunit of PKA.

The lack of effect on microtubules, compared to that observed after 12 days, and the higher presence of the RII subunit in the S2 fraction could be interpreted as a translocation of RII from the cytoskeletal compartment to the cytosol. It is known that the primary function of the regulatory subunit of PKA is to bind and control the activity of the catalytic subunit. In particular, the RII isoform seems to be responsible also for the localization of the kinase holoenzyme, through its binding to specific anchor proteins (Scott, 1991; Mochly-Rosen, 1995), among which microtubule-associated protein 2 (MAP2) is one of the most important in neurons. This translocation of RII from microtubules to the cytosol could represent an intermediate step necessary for the passage of the catalytic subunit from the cytosol to the nucleus, as described by the results of Nestler and coworkers (1989), indicating that in rat cerebral cortex, chronic antidepressants could stimulate the translocation of this kinase activity from the cytosol to the particulate nuclear fraction.

It is known that the major percentage of PKAII is associated with microtubules (Theurkauf and Vallee, 1982; Rubino et al., 1989; Perez et al., 1993). The reason why the binding of cAMP was not increased in the S1 fraction at 21 days seems to be because, in this fraction, the amount of cytosolic RII subunit was masked by the large presence of the RII subunit associated with microtubules, which did not undergo any modification after 21 days of SSRIs treatment. On the contrary, after 12 days, the increase in [^{32}P]cAMP-binding to the RII subunit associated with microtubules paralleled an increase also in the S1 fraction.

Moreover, it has been suggested by Schwarts and Costa (1980) that microtubules could be actively involved in the nuclear transfer of PKA activity induced by isoproterenol and blocked by vinblastine, an inhibitor of microtubule polymerization. The high concentration of RII subunit, found first in microtubules (12 days) and then in the cytosol (21 days), could be the result of a time-related effect of SSRIs on the translocation of PKA regulated by microtubules.

Changes in the subcellular localization of PKA could also determine the phosphorylation of new substrates by the kinase and the consequent regulation of different cellular functions. One of these proteins could be the transcription factor, cAMP-responsive element-binding protein (CREB), which is the mediator of cAMP in the

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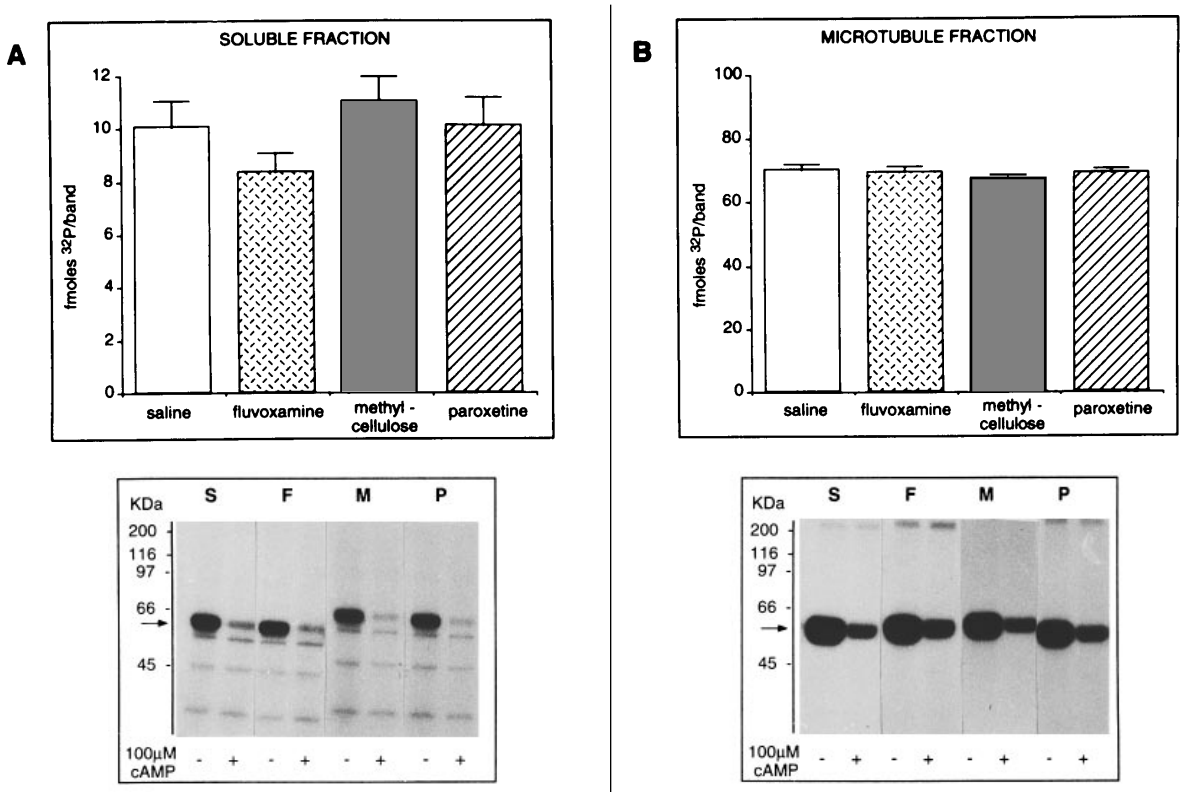

Figure 5. Photoaffinity labelling in the S1 soluble fraction (A) and in microtubules (B) from rat cerebral cortex after 21 days of treatment with fluvoxamine and paroxetine. Four experiments for each fraction were performed in duplicate. *Upper panel*: Autoradiographies (12 h of exposure), after SDS–PAGE, show the total (−cAMP) and non-specific (+cAMP) [^{32}P] incorporation into the 54 kDa protein band (indicated by arrow) from rats treated with saline (S), 15 mg/kg fluvoxamine (F), methylcellulose (M) and 5 mg/kg paroxetine (P). *Lower panel*: Histograms represent the specific [^{32}P]cAMP covalent binding to the 54 kDa protein band, expressed as fmol of [^{32}P]/band. The values are the means ± standard deviation.

regulation of gene expression and which could be itself influenced by antidepressant treatment, as described by Duman et al. (1997).

Overall, the results here presented suggest that SSRIs, besides their ability to affect serotoninergic neurotransmission, seem to influence the subcellular distribution of PKA, an enzyme which could be a target for antidepressant drugs.

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