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Presynaptic Ca^{2+}/Calmodulin-Dependent Protein Kinase II: Autophosphorylation and Activity Increase in the Hippocampus after Long-term Blockade of Serotonin Reuptake

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SUMMARY

It is known that long-term treatment with antidepressants induces an enhancement of neurotransmission in the pathway projecting from raphe nuclei to the hippocampus. In the case of selective serotonin (5-HT) reuptake inhibitors, this enhancement is due to a desensitization of presynaptic 5-HT autoreceptors and a concomitant increase in 5-HT release in terminal areas. To investigate whether this effect is accompanied by adaptive changes in the molecular machinery regulating transmitter release at serotonergic terminals, autophosphorylation and activity of Ca^{2+}/calmodulin-dependent protein kinase II were measured in subsynaptosomal fractions from hippocampus and total cortex. Long-term treatment with two selective serotonin reuptake inhibitors (paroxetine and fluvoxamine) and with a nonselective reuptake inhibitor (venlafaxine) induces a large increase of kinase autophosphorylation in synaptic vesicles and synaptic cytosol in the hippocampus but not in synaptosomal membranes. No significant change was detected in total cortex. The change is not reproduced by the direct addition of the drugs to the phosphorylation system and is not elicited by acute treatment of the animals. The increase in autophosphorylation is not accounted for by neosynthesis or translocation of the kinase to synaptic terminals. The change is restricted to the kinase located inside the terminals and is not detected in synaptosomal membranes, containing predominantly postsynaptic kinase, suggesting that only presynaptic kinase is affected. In the same fractions, the kinase activity is increased. These results are in agreement with reports suggesting a presynaptic effect for the SSRIs and disclose a new putative site of action for psychotropic drugs.

Many studies in recent years have provided much information about cellular and molecular mechanisms of neuronal function (1–3). As a result, new hypotheses on the neurochemical bases of psychiatric disorders and on the putative sites of action of psychotropic drugs have been proposed (4). However, despite these advancements, little is known about the action of drugs beyond the level of receptors, ion channels, and transmitter carriers, i.e., the plasma membrane level, where the drug action begins.

A good example is the problem of the site of action of antidepressant drugs. In clinical practice, these drugs require long-term treatment to exert their therapeutic effect, although in most cases their known biochemical effects are established within the few minutes or hours after treatment begins (5). Many antidepressant drugs (i.e., TCA and specific transmitter uptake inhibitors) work by blocking plasma membrane transmitter carriers, which are of primary importance in the termination of action of norepinephrine, 5-HT, and dopamine at synapses. Inhibition of transmitter reuptake by these substances is a rapid process, yet none of them show therapeutic efficacy before a few weeks of treatment (6). It is now widely believed that reuptake inhibition and consequent increase of extracellular transmitter concentration represent only the first in a cascade of events, leading to neuronal adaptive changes and modification of neural transmission in a number of affected pathways. These changes include modifications in the sensitivity of presynaptic and postsynaptic receptors (7, 8), in the function of receptor-coupled G proteins (9), in the action of protein kinases controlling several intracellular processes (10–12), and, finally, in the regulation of gene expression at the nuclear level (13).

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ABBREVIATIONS: 5-HT, serotonin; SSRI, selective serotonin reuptake inhibitors; CaMK II, Ca^{2+}/calmodulin-dependent protein kinase II; PAR, paroxetine; FLU, fluvoxamine; VEN, venlafaxine; TCA, tricyclic antidepressants; MAO, monoamine oxidase; SDS, sodium dodecyl sulfate; α-CaMK II-MAb, monoclonal antibody specific to the α isoform of Ca^{2+}/calmodulin-dependent protein kinase II; CNS, central nervous system; SV, synaptic vesicles.

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In recent years, the attention of several investigators involved with these problems was attracted in particular to central serotonergic pathways, which project from the raphe nuclei to most areas of the rostral brain. A novel class of antidepressant agents was developed that preferentially inhibit the reuptake of 5-HT. The effects of SSRIs, TCA, and MAO A inhibitors have been investigated with *in vivo* electrophysiological approaches in the pathway originating in the raphe nuclei and projecting to the dorsal hippocampus (7, 8).

A common result of a long-term treatment with all these drugs is an enhancement of 5-HT neurotransmission in this pathway. TCA sensitize 5-HT_{1A} postsynaptic receptors in the hippocampus (as they do in other areas; Ref. 14). Conversely, MAO A inhibitors and SSRIs desensitize presynaptic 5-HT receptors, somatodendritic 5-HT_{1A} receptors with the former, and both somatodendritic and terminal receptors with the latter (7, 8). Desensitization of presynaptic receptors effects an increase in 5-HT release, but the molecular mechanism by which this is achieved is largely unknown. An adaptive change in serotonergic terminals leading to an increase in 5-HT release would be likely to affect the molecular machinery of release and its regulatory processes.

In the present study, we tested the function of a protein kinase directly involved in the regulation of vesicle exocytosis in synaptic terminals: CaMK II. It is well known that stimulation of synaptic terminals is accompanied by autophosphorylation of the kinase, a step that makes it in part independent on Ca^{2+}/calmodulin (16). Injection of this autonomous kinase into the terminals stimulates transmitter release (17, 18). Several specific proteins (e.g., synapsin I and synaptotagmin) (19, 20), components of the release molecular machinery, are substrates of CaMK II, which is present in the presynapse. Synapsin I, which links the vesicles to the actin-based cytoskeleton, regulates the number of vesicles available for exocytosis. Its phosphorylation by vesicle-associated CaMK II increases the number of vesicles available (19). Synaptotagmin is a putative calcium sensor in the presynaptic release machinery, and it has multiple interactions with other presynaptic proteins (including calcium channels) (21). Phosphorylation by CaMK II could be a key step in the regulation of its function (22).

To investigate whether long-term (and short-term) treatment with SSRIs modifies the function of presynaptic CaMK II in the hippocampus (and in total cortex), rats were treated with PAR and FLU, two 5-HT reuptake blockers, and VEN, a mixed 5-HT and norepinephrine reuptake inhibitor. All of them are drugs that are widely used for the treatment of affective illnesses. Fractions enriched in synaptosomal membranes, SV, and cytosol were prepared from the presynaptic compartment, and the kinase autophosphorylation and activity were investigated in each fraction.

Materials and Methods

Animal treatment. For the long-term treatment group, male Sprague-Dawley rats (250–300 g at the end of the treatment) were randomly assigned to groups of 10 and received intraperitoneal injections of drug every 24 hr. Two courses of treatments were performed. The drug doses were 5 mg/kg for 19 days (PAR), 15 mg/kg for 19 days (FLU), and 15 mg/kg for 12 days (VEN). These doses (PAR and FLU) have been shown to inhibit efficiently and selectively 5-HT uptake from treated brain tissue *ex vivo* (23, 24). The VEN dose used was within the dose range used in previous animal studies (25). For each treatment group, an equal number of control rats were injected with vehicle (1% carboxymethylcellulose in saline for PAR and saline for FLU and VEN). For the short-term treatment groups, the animals were injected once with the same drug dose as for long-term treatment and killed 3 hr later.

Preparation of synaptosomes and subsynaptosomal fractions. Forebrains were collected, and brain areas were quickly dissected and placed onto ice. Pooled areas (hippocampus or cortex) were homogenized 1:10 at 4° in a Potter homogenizer with a loose-fitting pestle (10 strokes) in 0.32 M sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, containing the following protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1 μg/ml chymostatin, and 1 μg/ml antipain. A crude synaptosomal fraction (P2) and fractions enriched in synaptic membranes (LP1), SV (LP2), and synaptic cytosol (LS2) were prepared by differential centrifugation according to Huttner *et al.* (26), with minor modifications. LP1 and LP2 fractions were resuspended in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, containing the cocktail of protease inhibitors. All fractions were aliquoted and frozen at −80°. Protein content was measured with the BCA assay kit (Pierce).

CaMK II endogenous phosphorylation. Endogenous phosphorylation in the presence or absence of Ca^{2+}/calmodulin was carried out as previously described (20) in a total volume of 50 μl, containing 10–20 μg protein, 0.2 mM $CaCl_2$, and 50 μg/ml calmodulin. The reaction was started by the addition of [γ-^{32}P]ATP (final concentration, 5 μM, 40–80 μCi/ml), continued for 1 min at 30°, and was terminated by the addition of half of the volume of SDS-electrophoresis buffer 3× concentrated, followed by incubation in boiling water for 2 min. Phosphoproteins were separated by denaturing electrophoresis on 8% or 10% polyacrylamide minigels (Bio-Rad); 25 μl/sample were loaded. Gels were stained with Coomassie blue, dried, and autoradiographed. Phosphoprotein bands were analyzed and quantified with the use of CCD camera images of films and a computer program for image analysis (NIH Image 1.47). Band densities were within the linear range of the camera sensitivity (not shown).

Immunoblot of α-CaMK II. Levels of α-CaMK II in LP2 and LS2 fractions were determined with α-CaMK II-MAb (Boehringer), essentially as described previously (27). Proteins separated by denaturing electrophoresis (5–10 μg) were electrically transferred to nitrocellulose (0.45 μm). The blots were preincubated in TBST buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 0.1% Tween 20, 5% milk) for 1 hr and then incubated with α-CaMK II-MAb in TBST (5 μg/ml) for 1 hr. After being washed three times with TBST, the blots were incubated for 1 hr with anti-mouse IgG-peroxidase conjugated 1:1000 (Sigma). After being washed three additional times with TBST and twice with TBST without milk, the α-CaMK II band was detected with an enhanced chemiluminescence method (ECL, Amersham). The films were analyzed as for autoradiographs (see above).

Immunoprecipitation of α-CaMK II. LP2 samples (10 μg) were phosphorylated with [γ-^{32}P]ATP as described. The reaction was stopped by the addition of SDS to a final concentration of 0.5%. The samples were heated in boiling water for 3 min, and then 4 volumes of buffer A (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) at 4° were added. To each sample, 1.5 μg of α-CaMK II-MAb were added in 6 μl, and the samples were incubated for 3 hr at 4° with mixing. Then, 15 μl of pelleted protein A-Sepharose beads (Pharmacia) were added, and incubation continued for 2.5 hr. The beads were washed three times with 1 ml of buffer A containing 0.6 M NaCl and twice with buffer A. Bound kinase was solubilized by SDS-electrophoresis buffer and separated by denaturing electrophoresis. The radioactive band was excised from the gel and quantified with liquid scintillation counting.

Assay of CaMK II activity. CaMK II activity in subsynaptosomal fractions was assayed with the synthetic peptide substrate KKALRRQETVDAL (autocamtide-2, Research Biochemicals Int.)

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based on the sequence of the autoinhibitory domain of CaMK II. Phosphorylation reactions were carried out in a volume of 50 μl as described, containing 0.2 mM $CaCl_2$, 20 μM autocamtide-2, 20 μg/ml calmodulin, 20 μM [γ-^{32}P]ATP (0.3–0.6 μCi), and 2–5 μg of sample. The reaction was carried out for 30 sec at 30° (linear up to 2 min; not shown) and stopped by the addition of cold TCA to a final concentration of 5%. Samples were centrifuged for 2 min in a microcentrifuge, and 10 μl of the supernatant was spotted onto phosphocellulose P 81 paper (Whatman). The paper was washed four times in 75 mM phosphoric acid and dried. Then, scintillation fluid was added, and the samples were counted for liquid scintillation. For each experiment, blanks were prepared omitting the peptide from the incubation mixture.

Results

Ca^{2+}/calmodulin-dependent protein phosphorylation of synaptosomes and subsynaptosomal fractions in control and long-term treated rats. CaMK II in the CNS is highly enriched at synapses, in both presynaptic terminals and dendritic spines. In one of the most purified synaptosome preparations, it was estimated that 44% of the total CaMK II present is outside the nerve terminal, associated with the postsynaptic densities that remain attached to the synaptosomes (28). Inside the terminals, the kinase is associated with SV and membranes and present in the cytosol. In SV, CaMK II was shown to be tightly bound to the membrane, composing ~2% of total protein (29). We prepared crude synaptosomal fractions (P2) and fractions enriched in SV (LP2), synaptosomal membranes (LP1), and synaptic cytosol (LS2) from cortex and hippocampus of rat brain. These fractions were endogenously phosphorylated as previously reported (20), showing a labeling pattern similar to that reported previously (29) (Fig. 1). Autophosphorylation of CaMK II and phosphorylation of major substrates were dependent on the presence of Ca^{2+}/calmodulin and inhibited by the specific peptide CaMK II (281–302) (Fig. 1A). LP2 shows the typical Ca^{2+}/calmodulin-dependent phosphorylation pattern of SV, with three major phosphorylated bands: α-CaMK II (50 kDa), synapsin I (80–84 kDa), and a third (intermediate) band containing synaptotagmin (20) and β-β′CaMK II (58–60 kDa; Fig. 1A). As expected, synaptic cytosol (LS2) contains no vesicle or membrane proteins, and predominantly α and β isoforms of CaMK II are labeled (Fig. 1A).

When phosphorylation patterns of fractions from control and long-term treated rats were compared, the following was observed. In the cortex, there was no major difference (not shown). In the hippocampus (Fig. 1B), phosphate incorporation into both CaMK II and its substrates was greatly increased in SV and cytosol, whereas there was no difference in synaptosomal membranes and in synaptosomes (not shown).

Using computer-assisted densitometry, we quantified phosphate incorporation into the α isoform of CaMK II, the most abundant isoform of the kinase in forebrain (16). As shown in Fig. 2, in the hippocampus, autophosphorylation increase was similar in both vesicles and cytosol for the three drugs, ranging from 108% to 146% in the vesicles and from 87% to 133% in the cytosol. No significant variation was found in synaptosomal membranes from hippocampus (Fig. 2) and in all subsynaptosomal fractions from cortex (not shown).

The densitometric measurement of the phosphorylated

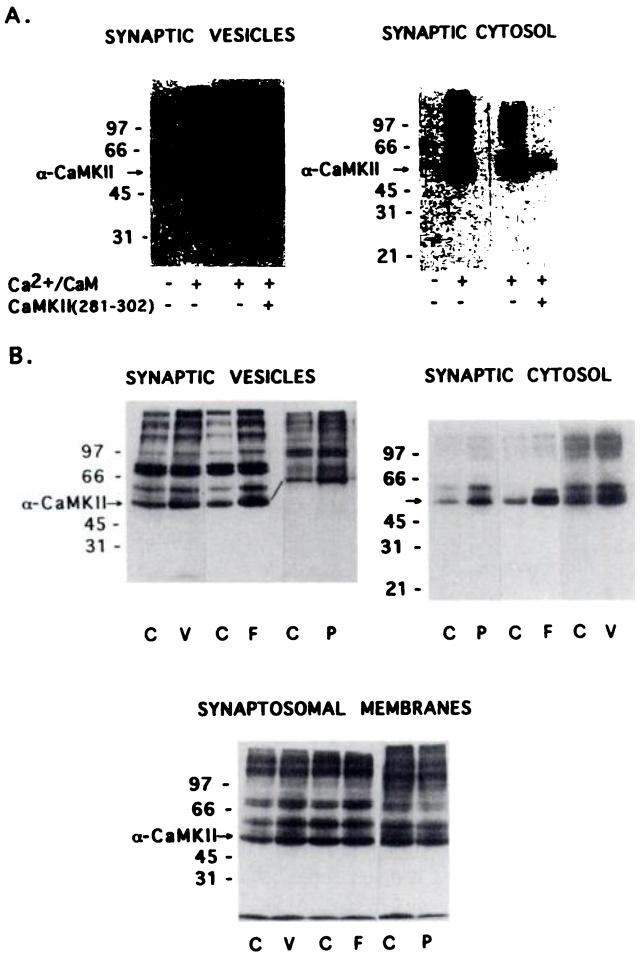

Fig. 1. A, Ca^{2+}/calmodulin (CaM)-dependent protein phosphorylation of synaptic vesicle fraction (LP2) and synaptic cytosol fraction (LS2). Synaptosomal membranes (LP1) have a Ca^{2+}/calmodulin phosphoprotein pattern similar to LP2. Three major phosphoprotein bands are seen in LP2: α-CaMK II (50 kDa), synapsin I (80–84 kDa), and a third band containing synaptotagmin and β-β′-CaMK II (58–60 kDa); some high-molecular-weight bands in LP2 are probably contaminating proteins, as they are usually absent from more purified SV (20, 26). Major phosphoprotein bands in LS2 are predominantly α- and β-β′-CaMK II. Protein (10 μg) was endogenously phosphorylated in the presence or absence of Ca^{2+}/calmodulin or CaMK II (281–302) peptide (100 μM for LP2, 60 μM for LS2). Exposure time was 2 hr for LP2 and 15 hr for LS2. B, Ca^{2+}/calmodulin-dependent protein phosphorylation of subsynaptosomal fractions in control and long-term treated animals. Protein (20 μg for SV and synaptosomal membranes or 10 μg for synaptic cytosol) was phosphorylated. *C*, control; *V*, VEN; *F*, FLU; *P*, PAR. Exposure time was 1.5 hr for LP2, 8 hr for LS2, and 5 hr for LP1.

α-CaMK II band could not exclude that other minor phosphoproteins, comigrating with the kinase, interfered with the measure. This is especially true for SV, where a higher number of phosphoproteins are present. As a control, α-CaMK II was immunoprecipitated with a monoclonal antibody from phosphorylated vesicles (FLU- and VEN-treated animals), the immunoprecipitate was separated by SDS-gel electrophoresis, and the amount of radioactive phosphate incorporated into the kinase band was determined (see Materials and Methods). The result of this procedure confirmed that a large increase in α-CaMK II autophosphorylation occurs in SV from the hippocampus of animals treated with 5-HT reuptake blockers (+108% for FLU- and +84% for VEN-treated animals). This result indicates that in SV samples,

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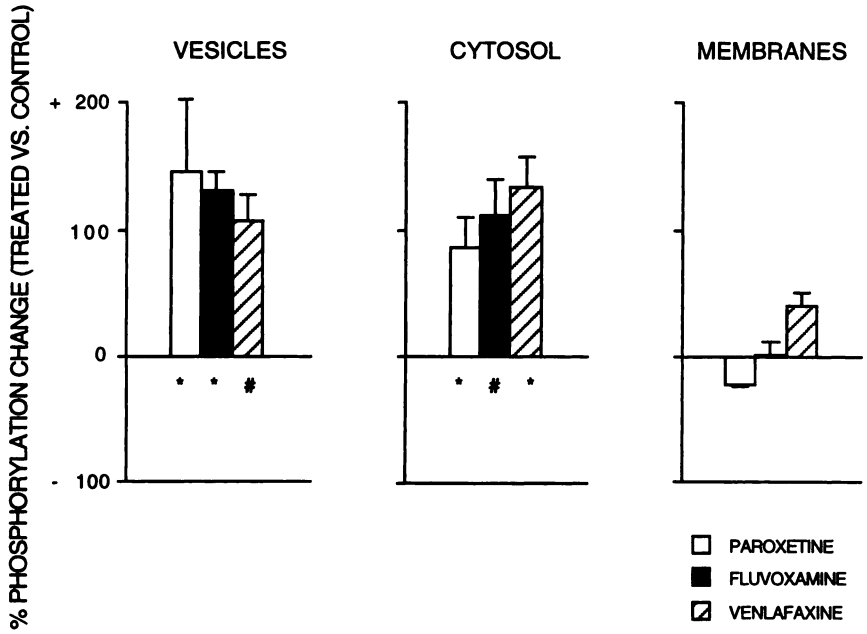

Fig. 2. Quantification of CaMK II autophosphorylation in the presynapse after long-term blockade of 5-HT reuptake. Values represent mean ± standard error percentage change (treated versus control) of three to five experiments. *, $p < 0.05$; #, $p < 0.01$ by paired Student's t test.

the densitometric measurement is substantially correct, slightly overestimating the autophosphorylation increase in α-CaMK II.

Effect of reuptake inhibitors *in vitro* and of short-term treatment of animals on presynaptic CaMK II autophosphorylation. The finding that long-term treatment with transmitter reuptake inhibitors elicited in the hippocampus a large increase in the autophosphorylation of presynaptic α-CaMK II raised several questions. First, it was important to assess whether these drugs have any direct effect on CaMK II. It could be that they stimulate phosphorylation per se and that this effect is not related to a cascade of events developing in the pathways affected during long-term treatment. Second, it was of interest to determine the effect of short-term treatment, i.e., to investigate whether the kinase autophosphorylation increases immediately (partially or totally) or the effect develops with time.

To answer the first question, SV fractions prepared from hippocampus of control rats were phosphorylated as usual but in the presence of either one of two concentrations of PAR, FLU, or VEN (see Materials and Methods) (Fig. 3A). With all three drugs, there was no significant variation of phosphate incorporation into α-CaMK II, with the exception of 100 μM PAR, which strongly inhibited autophosphorylation of the kinase. We believe that it is unlikely that such a high concentration is reached in the cerebral tissue during

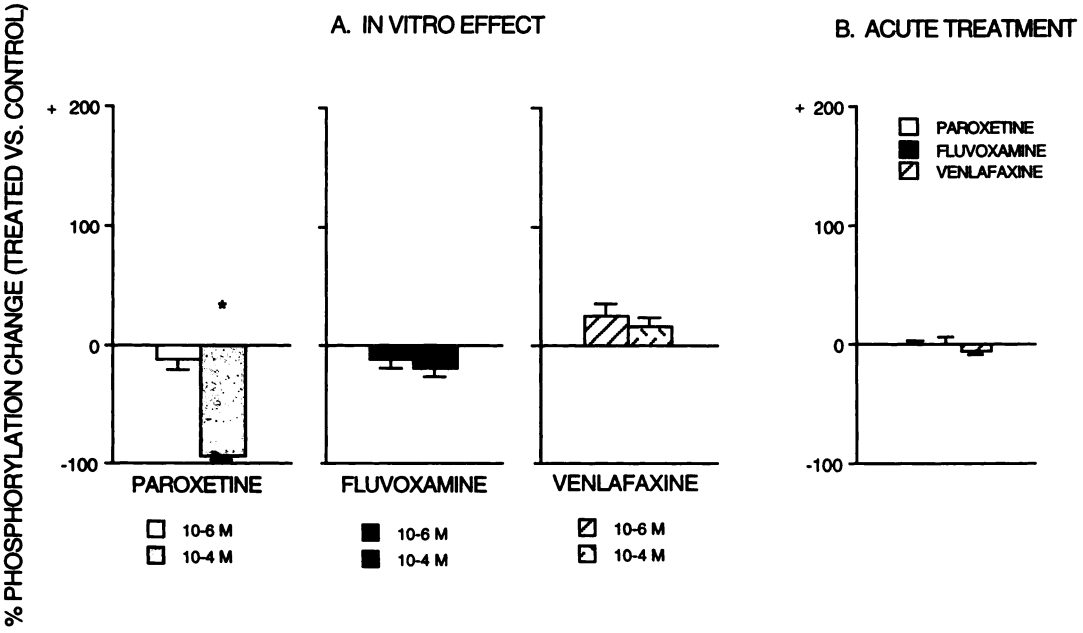

Fig. 3. A, CaMK II autophosphorylation change in the presence of 5-HT reuptake inhibitors (hippocampus SV from control animals). Data (in this and other figures) are expressed on the same scale as in Fig. 2 to allow comparison. *, $p < 0.01$ (four experiments). B, CaMK II autophosphorylation change after short-term blockade of 5-HT reuptake (hippocampus SV from control animals) (four experiments).

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the treatment. However, PAR, having an inhibitory effect on phosphorylation at this concentration, does not appear to stimulate CaMK II autophosphorylation per se (this is true for the other two drugs). This inhibitory effect of PAR at high concentrations may reflect a general effect of the substance on protein kinases because a similar result was obtained in our laboratory with brain cAMP-dependent protein kinase and microtubule-associated protein-2 as a substrate.[1]

To determine the effect of short-term treatment, rats were injected with a single dose of drug or vehicle and killed 3 hr later (Fig. 3B). In SV from hippocampus, there was no significant difference in α-CaMK II autophosphorylation between control and treated rats. Therefore, short-term treatment does not elicit even minimally the effect of long-term treatment. This suggests that the modification in CaMK II phosphorylation system develops with time during the treatment.

Protein levels of CaMK II in hippocampal presynapses of control and long-term treated rats. The large increase of phosphate incorporation in CaMK II α isoform, measured in SV and cytosol from hippocampus after treatment, could reflect one or more of the following events: (a) increased phosphate incorporation into the kinase, (b) increased kinase neosynthesis, and (c) kinase translocation. To investigate whether more CaMK II is neosynthesized and transported (or translocated) to synaptic terminals during the treatment, we measured levels of CaMK II α isoform with quantitative immunoblot in control and treated rats. As shown in Fig. 4, after treatment there was no significant difference in the levels of the kinase in both vesicles and cytosol from hippocampus, with a slight increase in fractions prepared from rats treated with FLU. In this case, the level of α-CaMK II was increased by 20% in the vesicles and by 29% in the cytosol. However, this increase could account for 15% and 26%, respectively, of the total autophosphorylation increase measured in vesicles and cytosol after FLU treatment. Therefore, we concluded that in treatment with the three drugs, the large increase in α-CaMK II autophosphorylation is mostly if not exclusively due to increased phosphate incorporation into the kinase.

Activity of CaMK II in hippocampal presynapses after long-term inhibition of 5-HT reuptake. The finding that CaMK II autophosphorylation increases after long-term inhibition of 5-HT reuptake prompted us to investigate whether and how the kinase activity changes after the treatment. The correlation between autophosphorylation and activity is not simple in CaMK II because the activity can increase or decrease depending on phosphorylation conditions and on the residues phosphorylated (16). The activities in control and FLU- and VEN-treated animals were assayed with the peptide autocamtide-2, which is a selective substrate for the kinase, allowing sensitive measurement in subcellular fractions and even in tissue homogenates (30). As shown in Table 1, CaMK II activity increased in SV and cytosol from hippocampus, whereas no major change was detected in synaptic membranes from hippocampus and in all subsynaptosomal fractions prepared from cortex (data not shown). At variance with what observed in autophosphorylation experiments, where the increase in SV was comparable for the different drugs, the percentage increase in VEN-treated was much higher than in FLU-treated animals. It is a matter of speculation whether this quantitative difference is due to different specificities of the two drugs.

[1] J. Perez, unpublished observations.

Discussion

The main findings of the present study can be summarized as follows. First, long-term treatment with the 5-HT reuptake blockers PAR and FLU elicits a large increase in the autophosphorylation of presynaptic CaMK II and in the phosphorylation of selected substrates (not shown) in the hippocampus. Second, the effect of a third drug, VEN (a mixed inhibitor of 5-HT and norepinephrine reuptake), on presynaptic CaMK II is qualitatively indistinguishable from that of SSRIs. Third, the effect is obtained with long-term treatment, but not with short-term treatment, and is not due to direct stimulation of kinase activity by the drugs. Fourth, the increase in CaMK II phosphorylation is accounted for by increased phosphate incorporation and not by an increase of CaMK II protein level in subsynaptosomal fractions. Fifth, assay of the kinase activity in FLU- and VEN-treated animals with exogenous peptide substrate shows that the activity of presynaptic CaMK II is increased.

Overall, these results are in agreement with the reports pointing toward an effect of 5-HT reuptake blockers on presynaptic cells, in the pathway from raphe nuclei to hippocampus (7). It would be interesting to investigate whether other drugs with a presynaptic effect in the same system (i.e., MAO A inhibitors) and drugs devoid of presynaptic effect (i.e., TCA) accordingly affect presynaptic CaMK II. This will be the subject of future studies. With regard to the action of VEN, it is unknown whether this is due to a prevalence of its serotonergic effect or to a concomitant desensitization of α_2-heteroreceptors located on 5-HT terminals (31).

We detected no significant increase in the kinase level (as measured with immunoblot) in SV and cytosol of treated animals and only a trend toward increase in the presynapses of FLU-treated animals. As shown, this increase in CaMK II level may account for only a minor portion of the large autophosphorylation increase. However, it could be argued that the small amount of neosynthesized or translocated kinase has a higher phosphorylation stoichiometry, significantly affecting the measure. We believe that this is unlikely because the new kinase should possess a stoichiometry 4–6-fold higher to account for the total phosphorylation increase observed in treated animals. However, a contribution of kinase neosynthesis or translocation in FLU-treated animals cannot be absolutely excluded.

The change was detected in a selected area, hippocampus, where it is known that 5-HT reuptake blockers increase the efficacy of 5-HT neurotransmission (7). It was reported that long-term treatment with SSRIs induces an increase of 5-HT release in hippocampus, frontal cortex, and other areas (32, 33). Although we found that CaMK II is in large part modified in the hippocampus, we did not find such a modification in total cortex. A reason for this could be that only selected areas of cortex are involved and in an examination of the entire cortex, any detectable effect is diluted. Future studies should investigate whether CaMK II is modified in selected cortex areas (i.e., frontal cortex).

It is remarkable that CaMK II was altered only in SV and cytosol, whereas it appeared to be unchanged in total synap-

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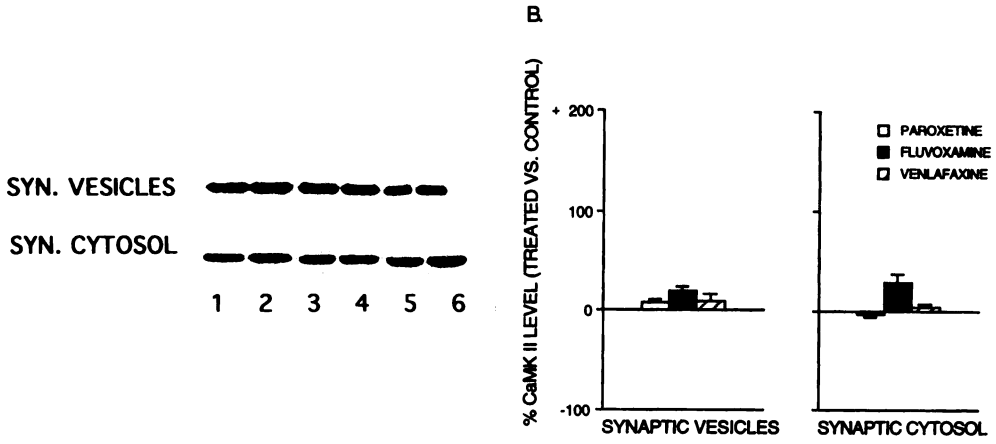

Fig. 4. A, Immunoblot of α-CaMK II (hippocampus) after long-term blockade of 5-HT reuptake. *Odd numbers*, Controls. *Even numbers*, Treated samples. *1* and *2*, VEN. *3* and *4*, PAR. *5* and *6*, FLU. B, Data expressed as percent change in the level of α-CaMK II (treated versus control). Values are mean ± standard error of three to six experiments.

tosomes and synaptic membranes, because this suggests an effect on the presynaptic kinase only. As we reported, the postsynaptic membrane, which copurifies with synaptosomes, contains an estimated 44% of the total CaMK II present in the preparation (28). Of the remaining presynaptic kinase, a minor portion is associated with SV (16%), and some is present in synaptic cytosol (20%). When synaptosomes are lysed and subsynaptosomal fractions are separated, the fraction of synaptosomal membranes contains more postsynaptic than presynaptic CaMK II. The finding that the kinase was not modified in membrane fraction compared with vesicular and soluble kinase suggests that postsynaptic CaMK II was unchanged (Fig. 2).

The change in presynaptic CaMK II induced by 5-HT reuptake blockers appears to be a long-lasting modification because it is elicited by long-term but not by short-term treatment and it is detectable after the time necessary to prepare subsynaptosomal fractions (4–5 hr). Therefore, it is different from the autophosphorylation induced by stimulation of synaptic terminals. In the latter case, CaMK II phosphorylates itself on a single threonine residue, becoming independent of the presence of Ca^{2+}/calmodulin, but this autonomous activity only lasts ~30–60 sec after the depolarization (34). Here, a long-lasting modification appears to be involved, as for postsynaptic CaMK II after protocols of long-term potentiation (35, 36) or for total CaMK II after various models of ischemic insult. In the latter case, in one of the various models used it was shown that the change (a large decrease in CaMK II phosphorylation) is due to a post-translational modification, decreasing the kinase affinity for ATP (37). It is a matter of speculation whether a similar modification, albeit of opposite sign, may account for the change in presynaptic CaMK II induced by 5-HT reuptake blockers.

TABLE 1

Assay of presynaptic CaMK II activity after long-term blockade of 5-HT reuptake

Activity of CaMK II was assayed with the peptide substrate autocamtide-2. Values are mean ± standard error percent change in kinase activity (treated versus control). *p* Values were determined by paired Student's *t* test (three or four experiments performed in duplicate).

	% Change in activity	
	FLU	VEN
SV (LP2)	+60.2 ± 5.4[a]	+145.2 ± 5.9[b]
Synaptic cytosol (LS2)	+70.0 ± 11.6[c]	+81.6 ± 16.2[d]

[a] $p < 0.05$
[b] $p < 0.05$
[c] $p < 0.05$
[d] $p < 0.01$

It is difficult to envisage the sequel of molecular events that, starting with desensitization of terminal 5-HT_{1B} or somatodendritic 5-HT_{1A} receptors (7), may lead to an increase in presynaptic CaMK II activity. A clue may be the fact that both types of receptors are negatively coupled to adenylate cyclase (15). A desensitization of these receptors may increase cAMP concentration in selected compartments, leading to drastic metabolic changes. On the other hand, it is somewhat easier to speculate how an increase in presynaptic CaMK II activity may increase transmitter release and potentiate neurotransmission. As we reported, several substrates of CaMK II have been identified in the presynapse, including synapsin I and synaptotagmin (19, 20). Both effectors could change the extent of release, although via different mechanisms. Synapsin I may alter the number of vesicles available for release (19), and synaptotagmin may change the Ca^{2+} sensitivity of the release machinery (22). It will be interesting to investigate the chain of events involved, both upstream and downstream from presynaptic CaMK II. As a first approach, we are investigating whether any substrate of CaMK II is preferentially phosphorylated in treated animals.

A major question arising from the results reported is whether the modification induced in CaMK II phosphorylation system in the hippocampus is restricted to 5-HT terminals or extends to other terminals as well. The magnitude of the change is so great that it is difficult to imagine that only serotonergic terminals are involved. Presynaptic terminal 5-HT receptors (mainly 5-HT_{1B} in rat CNS) are also present as heteroreceptors on terminals releasing acetylcholine and glutamate (38, 39). It has been proposed that these heteroreceptors may predominate, as lesions of serotonergic neurons do not produce significant losses of 5-HT_1 binding in most areas (15). In rat hippocampus, 5-HT_{1B} receptors have been described in the terminals of cholinergic neurons originating in the medial septum (38). As they inhibit acetylcholine release, their desensitization after SSRI treatment would bring about an increase in acetylcholine release. Therefore, in the hippocampus the treatment may produce changes in the release apparatus of serotonergic, cholinergic, and possibly other terminals possessing 5-HT_{1B} heteroreceptors. This may explain the magnitude of the effect observed after the treatment. A consequence of this would be that not

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only 5-HT neurotransmission is enhanced but also the interplay between 5-HT and other transmitters is changed in the hippocampus after the treatment. A way to distinguish between CaMK II changes in serotonergic and other transmitter terminals in the hippocampus could be a selective lesion of afferent pathways (i.e., cholinergic), and this will be the subject of a future study.

In summary, this finding may help elucidate the mechanism of action of antidepressant drugs, implicating protein kinases as key elements in the action of psychotropic drugs (11).

In a recent report (40), a large increase (~80%) in CaMK II activity was described in striatal synaptic membranes of animals chronically treated with haloperidol. This increase appeared to be associated with an increase in glutamate release in the corticostriatal tract. We believe that those findings may have relevance for the present study. In both cases, desensitization or block of presynaptic heteroreceptors, leading to an increase of transmitter release, brings about an increase in the activity of CaMK II. We wonder whether this is a mechanism of action common to different drugs, promoting an increase in transmitter release in different brain areas as well as in different physiopathological situations.

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