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Long Term Blockade of Serotonin Reuptake Affects Synaptotagmin Phosphorylation in the Hippocampus

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SUMMARY

Synaptic vesicle trafficking and transmitter release from presynaptic terminals are precisely regulated by a complex array of protein/protein interactions. Several of these proteins are substrates of endogenous protein kinases present in presynaptic terminals. The activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), one of the kinases involved in the modulation of transmitter release, was previously shown to increase in the hippocampus after long term blockade of 5-hydroxytryptamine (5-HT) reuptake (a treatment known to elicit an increase in 5-HT release in this area). To investigate the changes induced in presynaptic protein phosphorylation by 5-HT reuptake blockade and concomitant CaMKII up-regulation, we analyzed two major CaMKII presynaptic substrates (synapsin I and synaptotagmin). All 5-HT reuptake blockers that we used, which induce an increase in CaMKII activity and autophosphorylation, also caused a large (2–3-fold) increase in the Ca^{2+}/calmodulin-dependent *post hoc* phosphorylation of synaptotagmin. Conversely, the phosphorylation of synapsin I is much less affected. The change in synaptotagmin phosphorylation, as determined through immunoprecipitation and quantitative immunoblot analysis after fluvoxamine treatment, is due exclusively to increased phosphate incorporation (presumably caused by the increased kinase activity) and not to a change in the level of substrate protein after the treatment. Thus, drugs known to induce an increase in 5-HT release simultaneously induce an increase in the activity of presynaptic CaMKII and in the phosphate incorporation (*post hoc*) by a major CaMKII substrate in synaptic vesicles (synaptotagmin). This finding establishes a link between the facilitation of transmitter release induced by antidepressant drugs and the phosphorylation of synaptotagmin by CaMKII.

Protein phosphorylation probably is the most diffused intracellular regulatory mechanism. A great variety of cellular processes are controlled through this post-translational covalent modification (1). In neurons, protein phosphorylation regulates the efficacy of synaptic transmission either by changing the sensitivity of neurotransmitter receptors or by modulating presynaptic release of neurotransmitters (2–4). In this context, there are several ways in which protein kinases may affect presynaptic function. A list of possible regulatory sites includes ion channels, presynaptic transmitter receptors, and proteins of the presynaptic release apparatus controlling vesicle trafficking.

Among the few protein kinases that have been directly implicated in the control of transmitter release and in presynaptic plasticity, CaMKII was shown to increase release when introduced into permeabilized rat brain synaptosomes or injected into squid synaptic terminals (4, 5). Furthermore, long term potentiation in hippocampus, a cellular model of learning and memory that involves a presynaptic modification, is associated with increased activity of CaMKII and increased phosphorylation of a presynaptic substrate (e.g., SYN) (6). Furthermore, mice carrying a mutation for the major isoform of the kinase in forebrain (α-CaMKII) display deficient hippocampal long term potentiation and decreased paired pulse facilitation (a form of presynaptic plasticity), and this is associated with impaired spatial learning (7).

Several presynaptic proteins were found to be substrates for CaMKII *in vitro* (SYN, SYT, synaptobrevin, synaptophysin, rabphilin) (3, 8–11). All of them are SV proteins, involved in the control of vesicle trafficking and in the various regulatory steps regulating fusion/exocytosis. With the exception of SYN, the functional meaning of their phosphorylation by CaMKII has not been investigated. Because modifications in presynaptic CaMKII deeply affect transmitter release and

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ABBREVIATIONS: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; SV, synaptic vesicle; SYN, synapsin I; SYT, synaptotagmin; MAb, monoclonal antibody; 5-HT, 5-hydroxytryptamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

synaptic plasticity, changes in the phosphorylation state of CaMKII substrates are likely to be important in the various steps of the SV cycle.

It was previously found that long term (but not short term) treatment of animals with 5-HT reuptake blockers elicits a large increase in the activity and autophosphorylation of presynaptic CaMKII in the hippocampus (12). These drugs, which are widely used in the treatment of affective disorders, have the capability (after long term treatment) to enhance serotonergic neurotransmission in various brain areas. In particular, it was shown that terminal 5-HT release was increased in several brain areas of serotonergic innervation, including the hippocampus (13, 14). This effect was shown to be linked to the concomitant desensitization of presynaptic terminal 5-HT_{1B} autoreceptors. Because these receptors normally exert an inhibitory constraint on 5-HT release, their desensitization during treatment with 5-HT reuptake inhibitors may elicit a facilitation in 5-HT release, although the underlying intracellular mechanisms are still in large part unknown.

We recently speculated that a possible connection between transmitter reuptake blockade and the increased release of 5-HT may involve an up-regulation of presynaptic CaMKII activity (12). Furthermore, because of the magnitude of the effect observed, we suggested that the change in kinase activity was not restricted to 5-HT terminals but probably extended to other presynaptic terminals in the hippocampus that possessed 5-HT heteroreceptors. In the current study, to further investigate these hypotheses, we studied the phosphorylation of two major SV substrates of CaMKII, SYN and SYT, and found that the increase in activity of the kinase preferentially affects one of them (e.g., SYT).

Materials and Methods

Animal treatment. Male Sprague-Dawley rats (250–300 g at the end of treatment) received daily intraperitoneal injections. Three uptake blockers were used in this study: fluvoxamine and paroxetine, which are selective 5-HT reuptake blockers, and venlafaxine, which is a 5-HT reuptake blocker that also inhibits, although with less potency, norepinephrine reuptake (ratio 5:1; see Ref. 15). Drug doses and timing of treatment were as described previously (12): 5 mg/kg for 19 days for paroxetine, 15 mg/kg for 19 days for fluvoxamine, and 15 mg/kg for 12 days for venlafaxine.

Preparation of subsynaptosomal fractions. Subsynaptosomal fractions were prepared from whole cerebral cortex and hippocampus. Fractions enriched in SVs (LP2), synaptic cytosol (LS2), and synaptosomal membranes (LP1) were prepared through differential centrifugation and ultracentrifugation as previously described (12).

Endogenous phosphorylation of CaMKII substrates. Endogenous protein phosphorylation in the presence of Ca^{2+}/calmodulin was carried out as previously described (8, 12). For densitometry of phosphoprotein bands, phosphorylation reactions were carried out at 30° for 1 min and stopped by the addition of SDS-PAGE sample buffer. For immunoprecipitation of phosphosynaptotagmin, phosphorylation reactions were carried out for 3 min and stopped by the addition of SDS to 0.5% (final concentration).

When purified SYT (immobilized on agarose beads) was phosphorylated, each individual sample contained 850 ng of purified SYT (bound to 30 μl of beads) and 10 μg (total protein) of LS2 fraction from control or treated animals in a total incubation volume of 60 μl. Phosphorylation reaction was carried out for 30 min at 30° and stopped by the addition of EGTA (2 mM final concentration). SYT beads were pelleted, washed three times with cold buffer A (100 mM NaCl, 50 mM HEPES, pH 7.2), and dried. Phosphosynaptotagmin was solubilized by the addition of SDS-PAGE sample buffer and separated by denaturing electrophoresis. Phosphosynaptotagmin band was excised from the gels, solubilized, and counted for liquid scintillation.

Immunoprecipitation of phosphosynaptotagmin. Samples of LP2 fraction from control and fluvoxamine-treated animals were subjected to endogenous protein phosphorylation as described. At the end of the phosphorylation reaction, the equivalent of 90 μg of protein of LP2 was boiled for 3 min, and four volumes of buffer B (150 mM NaCl, 10 mM Tris·HCl, 1 mM EDTA, 1% Triton X-100, pH 7.4) were added at 4°. Then, 100 μl of hybridoma Sn containing MAb 48, which binds SYT I (16, 17), was added, and the samples were incubated for 12 hr on a rotary shaker. A protein A/Sepharose slurry (Pharmacia, Piscataway, NJ) was then added to the incubation mixture (25 μl of beads pre-equilibrated and diluted 1:5 in buffer B), and the incubation was continued for 2.5 hr. At the end of the incubation, the beads were washed three times with buffer B containing 0.6 M NaCl and twice with buffer B. The immunoprecipitate was separated by denaturing electrophoresis, and the SYT band was identified by Coomassie staining and autoradiography, excised from the dried gel, solubilized in 10 ml of 5% Soluene in Ultima gold (Packard, Meriden, CT), and counted in a liquid scintillation instrument.

Quantitative immunoblot of SYT. SYT level in LP2 fraction was determined by the use of MAb 48, with minor modifications of the method previously described (12). Briefly, proteins separated by denaturing electrophoresis and transferred to nitrocellulose were preincubated with TBST buffer (consisting of 20 mM Tris·HCl, 137 mM NaCl, 0.1% Tween 20, 5% milk) for 1 hr. After being washed three times with TBST, the blots were incubated for 1 hr with Sn containing MAb 48 (diluted 1:200 in TBST). The blots were then incubated with peroxidase-coupled antimouse IgG (1:1000) (Sigma, St. Louis, MO). After being washed again with TBST and with TBST without milk, the blots were incubated with substrates for chemiluminescence detection (ECL kit, Amersham, Arlington Heights, IL). Autoradiography films were analyzed as for phosphoprotein autoradiographs (see below).

Assay of CaMKII activity. CaMKII activity in subsynaptosomal fractions was assayed by using the synthetic peptide substrate KKALRRQETVDAL (autocamtide-2; Research Biochemicals, Natick, MA), which reproduces part of the autoinhibitory domain of CaMKII. The procedure was as previously described (12).

Purification of SYT immobilized on Sepharose beads. LP2 fraction prepared from bovine brain was used. All of the procedure, which was carried out at 4°, was a modification (scaled up) of the immunoprecipitation. One milligram of protein of LP2 was solubilized in 2 ml of buffer containing 25 mM HEPES, 10 mM magnesium acetate, 0.1 mM dithiothreitol, and 0.5% SDS, pH 7.4. Four volumes of buffer B (containing 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1 μg/ml chymostatin, 1 μg/ml antipain, 1 mM EGTA) and then 9 ml of hybridoma Sn containing MAb 48 were added, and the sample was incubated for 2.5 hr on a rotary shaker. At the end of the first incubation, 50 μl of protein A/Sepharose (pre-equilibrated and diluted 1:10 with buffer B) was added, and the mixture was incubated for an additional 2.5 hr. The beads were pelleted with a brief centrifugation and washed twice with buffer B containing 0.6 M NaCl and three times with buffer B. SYT beads, which were analyzed by Coomassie blue staining and immunoblot analysis, contained virtually pure SYT plus heavy and light chains of immunoglobulin. SYT bound to beads was quantified in each preparation through staining and densitometry of protein aliquots subjected to electrophoresis, with bovine serum albumin used as a standard. The yield of the procedure was 25 to 50 ng of SYT/μl of beads.

Miscellaneous methods. Denaturing electrophoresis and Western blot analysis were carried out as described previously (12). For quantification of phosphate incorporation in phosphoproteins separated by electrophoresis and of SYT levels in immunoblots, computer images of films were taken with a CCD camera. Individual bands in

the images were quantified by using a computer program for image analysis (Image 1.47, National Institutes of Health, Bethesda, MD). Band densities were within the linear range of the camera sensitivity.

Results

***Post hoc* phosphorylation of SYT and SYN in the hippocampus after long term blockade of 5-HT reuptake.** As shown previously (12), long term treatment of animals with drugs blocking the plasma membrane 5-HT transporter elicits an increase in activity and autophosphorylation of presynaptic CaMKII (located in SVs and synaptic cytosol). The change in presynaptic CaMKII was detected in the hippocampus, an area in which long term 5-HT reuptake blockade has been shown to induce an increase in 5-HT terminal release (13, 14).

In the current study, the Ca^{2+}/calmodulin-dependent phosphorylation of two major substrates of CaMKII in the presynapse was investigated: SYN and SYT. Phosphorylation of CaMKII substrates in total cerebral cortex and hippocampus was screened by densitometric analysis of autoradiographs from electrophoretic gels, as done previously for the kinase (12). Subcellular fractions enriched in SV (LP2) and synaptosomal membranes (LP1) were prepared from animals that had been chronically treated with vehicle or one of three drugs (fluvoxamine, paroxetine, or venlafaxine). The first two compounds are selective inhibitors of 5-HT transporter, and the third (venlafaxine) is an inhibitor of 5-HT and (with less potency) of norepinephrine transporter. LP2 and LP1 samples were subjected to endogenous Ca^{2+}/calmodulin-dependent phosphorylation (*post hoc*). After separation of proteins by SDS-PAGE and autoradiography, phosphoprotein bands were quantified by computer-assisted densitometry.

When phosphate incorporation of SYN and SYT bands was quantified, the following was observed. In the total cerebral

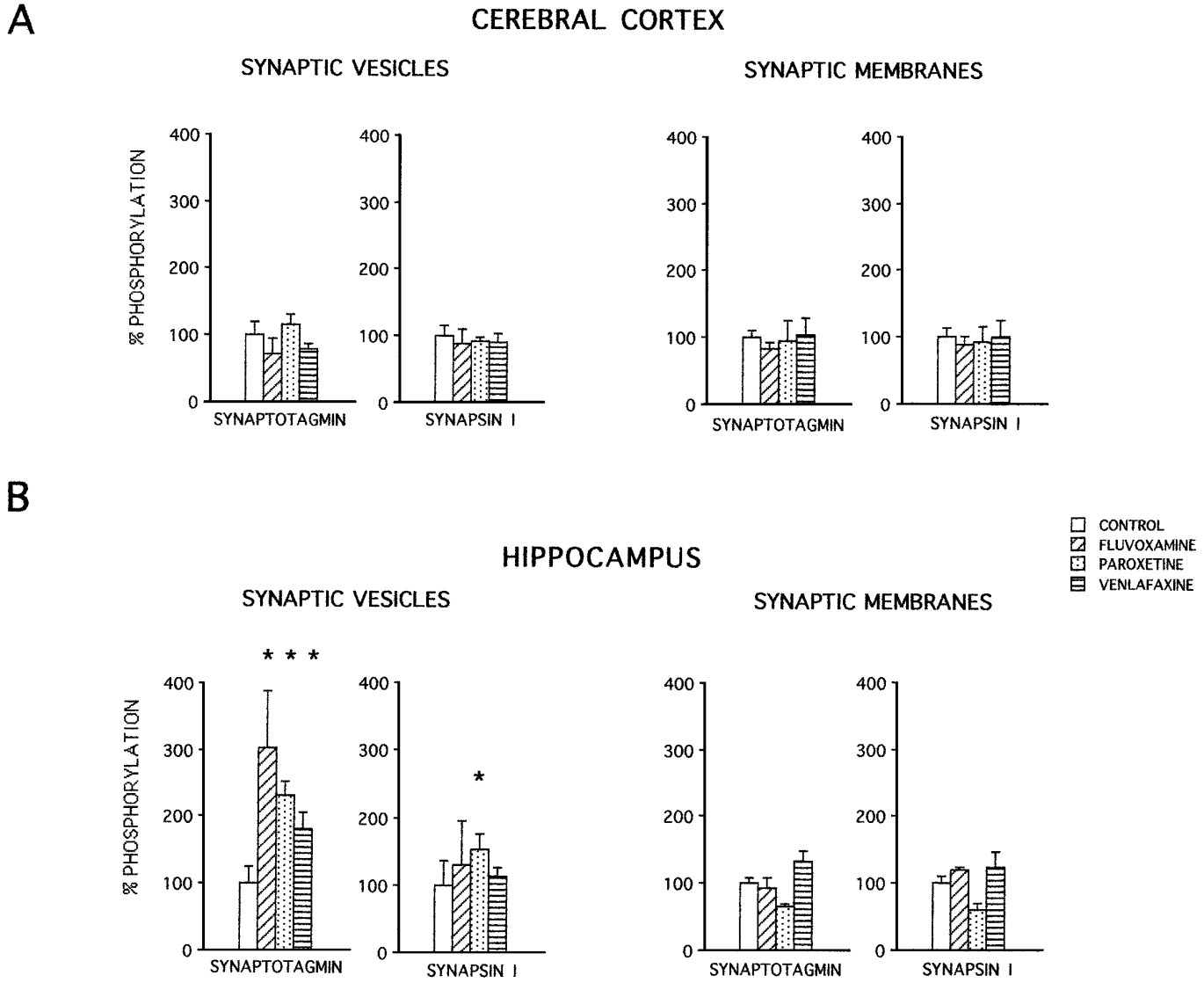

Fig. 1. Quantification of radioactive phosphate incorporation into individual CaMKII presynaptic substrates after separation of phosphoproteins by denaturing electrophoresis, autoradiography, and densitometry. SV- and synaptosomal membrane-enriched fractions were prepared from animals chronically treated with vehicle or a 5-HT reuptake blocker (fluvoxamine, paroxetine, or venlafaxine) and subjected to endogenous phosphorylation (10 μg of total protein) in the presence of Ca^{2+}/calmodulin. Values represent mean $\pm$ standard error percentage of phosphorylation from four to eight experiments. $*$, Significantly different from control ($p < 0.05$ by Student's t test for paired samples).

cortex (Fig. 1A), no significant difference was observed between control and treated animals, as was found previously for CaMKII. In the hippocampus (Fig. 1B), significant increases in the phosphorylation of CaMKII substrates were observed in SVs but not in synaptosomal membranes (again similar to what was previously found for CaMKII autophosphorylation). Interestingly, in SVs, the situation was different for the two substrate proteins. SYT showed a large increase in phosphorylation with all three compounds, ranging from 190 ± 26% (venlafaxine) to 303 ± 86% (fluvoxamine). Conversely, the increase in SYN phosphorylation was more modest and significant only after treatment with paroxetine (152 ± 23%).

SYN is an easily recognizable doublet with an apparent molecular mass of 80–84 kDa. The identity of SYT was assessed by immunostaining with MAb, as done previously (8). The phosphoprotein band has an apparent molecular mass of 60–62 kDa, which easily allows separation of the band from that of α-CaMKII, the major kinase isoform (8). The results of densitometric analysis were later confirmed by phosphosynaptotagmin immunoprecipitation (see below). The β-CaMKII isoform (58 kDa), which could potentially interfere with the SYT band, was only a minor phosphoprotein after SV endogenous phosphorylation and became visible with longer exposures. Indeed, SYT is one of the most abundant proteins of SVs (8% in purified SVs; ref. 18), whereas CaMKII (α and β isoforms) represents ~2% of total SV proteins, with β-CaMKII accounting for only one fourth of this amount (19). However, migration of samples enriched in SYT and CaMKII showed that the two proteins have a different electrophoretic migration (Fig. 2, *right*).

Activity of vesicular and soluble presynaptic CaMKII after long term treatment with fluvoxamine. The experiments summarized in Fig. 1 suggested that a selective increase in *post hoc* phosphorylation occurs for one of the CaMKII substrates investigated (SYT). Therefore, we sought to determine directly the extent of this phosphorylation change. Furthermore, it was relevant to investigate whether the increase in SYT phosphorylation was due to a change in the kinase activity, to a change in the substrate protein level in treated animals, or to both. Therefore, long term treatment of animals was repeated with one of the compounds used previously (i.e., fluvoxamine).

The activity of CaMKII was assayed in subsynaptosomal fractions (SVs, cytosol, synaptosomal membranes) of control and treated animals by using autocamtide-2, a specific peptide substrate of this kinase. No significant change was found in CaMKII activity in total cerebral cortex (not shown), whereas the activity was changed in the hippocampus (Table 1). In this last area, no significant change was detected in the synaptosomal membrane fraction (LP1), which contains mostly postsynaptic CaMKII (highly enriched in postsynaptic densities) (20). Conversely, a large increase (173 ± 23%) was found in SVs, and a more modest but still significant increase (126 ± 8%) was found in the synaptic cytosol. When phosphoproteins in SV fractions were separated by SDS-PAGE and autoradiographed, an increase in phosphorylation of CaMKII and SYT was clearly observed (Fig. 2, *left*).

We then assessed whether the increase in presynaptic cytosolic CaMKII activity, as detected with the peptide substrate, could significantly affect the phosphorylation state of a native substrate (i.e., SYT). To this aim, purified bovine SYT, immobilized on protein A/Sepharose using MAb 48, was phosphorylated *in vitro* using synaptic cytosol from control or treated animals as a source of kinase (Fig. 2, *right*). Consistently more phosphate was incorporated into SYT by cytosolic CaMKII prepared from treated animals (average phosphorylation, 271 ± 12%). This figure, which is quite close to the result obtained in SVs by densitometry, clearly suggests that 1) SYT phosphorylation may be affected by a change in activity of both vesicular and cytosolic kinase, and 2) the increase in cytosolic CaMKII activity seems to be larger when

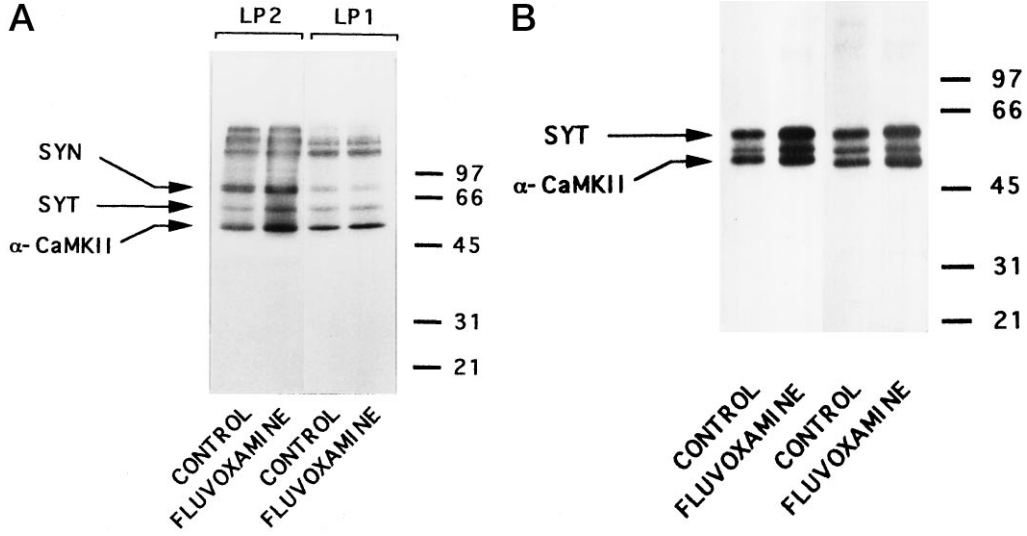

Fig. 2. *Left*, Ca^{2+}/calmodulin-dependent endogenous protein phosphorylation of SV- (LP2) and synaptosomal membrane- (LP1) enriched fractions. The samples (10 μg of total protein) were from control and fluvoxamine-treated animals. Molecular mass given in kDa. *Right*, Ca^{2+}/calmodulin-dependent phosphorylation *in vitro* of purified SYT bound to protein A/Sepharose beads. Each sample contained 850 ng of SYT bound to 30 μl of beads (dry weight) and was incubated with synaptic cytosol (10 μg of total protein) from control or fluvoxamine-treated animals. The additional phosphoprotein bands are autophosphorylated CaMKII (α and β isoforms). After binding to and phosphorylation of SYT, the kinase was not removed by washing with buffer containing 0.1 M NaCl; during immunoprecipitation of SYT from the phosphorylated vesicle fraction (LP2), the kinase was removed from the immunoprecipitate by washing with buffer containing 0.6 M NaCl (see Fig. 3).

TABLE 1

Assay of CaMKII activity after long term blockade of 5-HT (hippocampus)

Values are mean ± standard error.

SVs		Synaptic cytosol		Synaptic membranes	
Control	Treated	Control	Treated	Control	Treated
		%			
100.0 ± 9.7	173.5±22.7[a]	100.0±4.6	125.9±8.0[a]	100.0±1.9	114.6±7.2

[a] Significantly different from control ($p < 0.05$ by paired student's *t* test for four experiments).

measured by using a native substrate. This last result implies that, whenever possible, it may be advisable to measure protein kinase activities with endogenous rather than peptide substrates.

Immunoprecipitation of SYT from phosphorylated SVs after long term blockade of 5-HT reuptake. A screening of CaMKII substrates phosphorylation by densitometry showed that long term blockade of 5-HT reuptake affected SYT and SYN to different degrees. Because SYT phosphorylation seemed to be preferentially changed, the treatment was repeated (see above) to assess the extent of the change in SYT phosphorylation with a more straightforward approach. The protein was immunoprecipitated by using MAb 48, the immunoprecipitate was separated by denaturing electrophoresis, and the amount of phosphate incorporated into the SYT band was determined by counting of radioactivity. As shown in Fig. 3A, electrophoretic separation of SYT immunoprecipitate resolved a clearly identifiable phosphosynaptotagmin band. No other Coomassie-stained bands were present on gels. Phosphate incorporation was greatly increased in treated animals (Fig. 3B), showing a value (291 ± 70%) quite similar to that found with densitometric measurements.

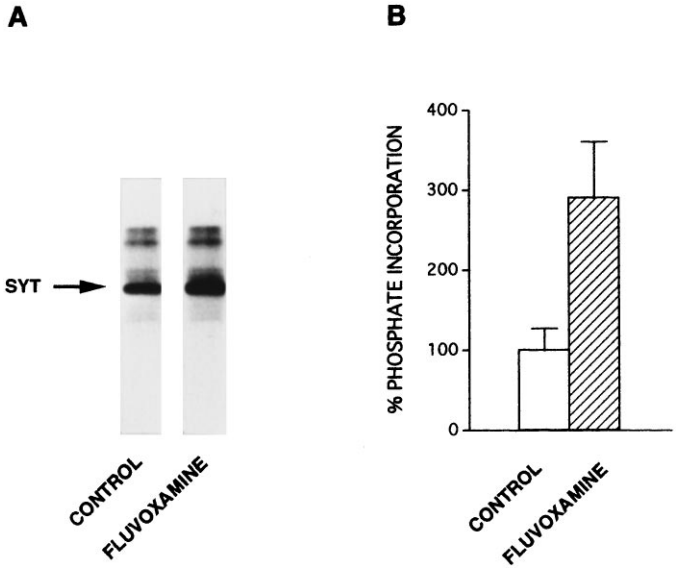

Fig. 3. A, Immunoprecipitation of phosphosynaptotagmin from SV fractions (90 μg of total protein) prepared from control and fluvoxamine-treated animals and phosphorylated *in vitro* in the presence of Ca^{2+}/calmodulin. A representative experiment is shown (autoradiography). B, Quantification of radioactive phosphate incorporated into SYT bands. Individual bands were excised from gels, solubilized, and counted for liquid scintillation. Data are expressed as mean ± standard error percentage of phosphate incorporation. Treated samples were significantly different from controls ($p < 0.05$, Student's *t* test for paired samples for four experiments).

Synaptotagmin protein level after long term blockade of 5-HT reuptake. In principle, the phosphorylation change detected in SYT could be accounted for by a modification in phosphate incorporation (due to the increase in CaMKII activity), by a change in SYT expression during the treatment, or by both. Because both events could be relevant to the modifications induced by reuptake blockers in the presynaptic release apparatus (and in neurotransmission), a quantitative immunoblot study was performed (12). The protein level of SYT, as measured by immunoblot analysis with MAb 48, did not show any significant difference between control and treated animals (Fig. 4). Therefore, it can be concluded that no change in SYT expression was induced by the treatment and that the change effected by long term 5-HT reuptake blockade was entirely due to increased phosphate incorporation during *post hoc* phosphorylation.

Discussion

A major aim of the current study was to investigate whether the increase in activity and autophosphorylation of presynaptic CaMKII induced by 5-HT reuptake blockers (12) is mirrored by changes in the phosphorylation state of presynaptic substrates of the kinase. Such an investigation may be relevant for both the physiology and the pharmacology of the presynapse.

5-HT reuptake blockers are widely used in psychiatry for the treatment of affective disorders, although the mechanism of action of these, as well as of other psychotropic drugs, is

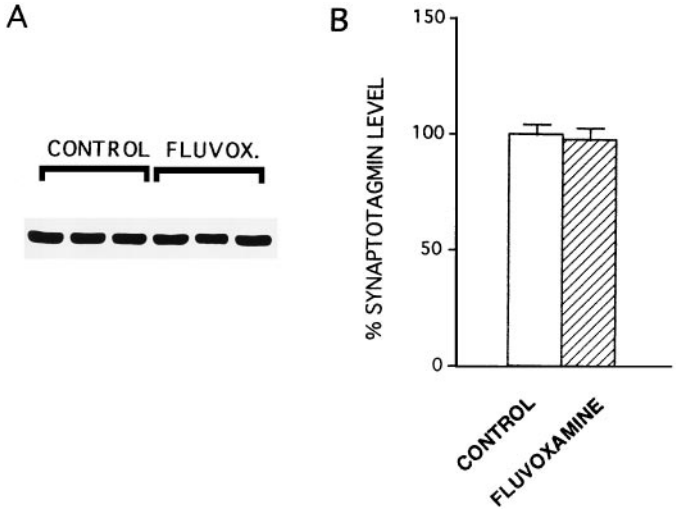

Fig. 4. A, Immunoblot of SYT in SV fractions prepared from control and fluvoxamine-treated animals (10 μg of total protein/sample). A representative experiment is shown. B, Quantification of SYT level (data are expressed as percentage level ± standard error for five experiments).

still poorly understood at the neuronal level (21). What seems clear is that the therapeutic effect is not simply due to the action on the plasma membrane 5-HT transporter because blockade of the transporter is quite fast, whereas several weeks of treatment are usually required to achieve therapeutic efficacy. This discrepancy has been explained by the suggestion that the sustained elevation in extracellular transmitter concentration in turn induces adaptive changes in receptors and, most notably, in signal transduction mechanisms beyond receptors (21, 22). In this context, the therapeutic action of drugs may be considered to be drug-induced neural plasticity. Several lines of evidence have confirmed this hypothesis (23–25).

In hippocampus, a brain area that is crucial for cognitive functions and motivation, it was shown that long term blockade of 5-HT reuptake enhances neurotransmission in serotonergic neurons that originate in medial raphe nuclei (13). This enhancement is at least in part accounted for by an increase in 5-HT terminal release (14). Because presynaptic CaMKII is a major protein kinase that is involved in the control of release (4, 5), it was relevant to investigate whether 5-HT reuptake blockade would effect changes in the phosphorylation of CaMKII substrates that are components of the presynaptic release apparatus. The results of this work are summarized as follows. 1) Long-term blockade of 5-HT reuptake induces in the hippocampus an increase in the phosphorylation (*post hoc*) of CaMKII substrates in SVs (SYT and SYN) concomitant with an increase in the Ca^{2+}/calmodulin-dependent activity of the kinase (both vesicular and cytosolic). The phosphorylation increase is much larger in SYT than in SYN. 2) With one of the compounds used (fluvoxamine, a selective 5-HT reuptake inhibitor), SYT phosphorylation was increased ~3-fold compared with controls. This change was quantified by two independent methods (densitometry and immunoprecipitation), with nearly coincident results. In addition, phosphorylation of purified immobilized SYT with cytosolic CaMKII from control and treated animals resulted again in a ~3-fold incorporation ratio (treated/control). This suggests that the increases in vesicular and cytosolic kinase activity, as measured with endogenous substrate, are equivalent. 3) The increase in SYT phosphorylation is accounted for by increased phosphate incorporation, not by a change in SYT expression during the treatment. Therefore, the hyperphosphorylation of SYT seems to be entirely due to the increase in kinase activity.

We showed previously (12) that no endogenous phosphorylation of CaMKII substrates occurs when a CaMKII peptide inhibitor is added to the phosphorylation reaction or when the reaction is performed in the absence of Ca^{2+}/calmodulin. Furthermore, the consensus site for SYT phosphorylation by casein kinase II (the only other kinase that was shown to phosphorylate SYT *in vitro*) is completely different, making it very unlikely that what was observed here was phosphorylation induced by casein kinase II. These reasons suggest that under the study conditions, SYT phosphorylation is due entirely to CaMKII and not to other kinases present in the presynaptic terminal. It is also possible that a change in the activity of a specific protein phosphatase simultaneous with the change in kinase activity is responsible for the modifications in protein phosphorylation.

It is surprising that the increase in CaMKII activity seems to affect preferentially one of the two substrates investigated (SYT). We wondered whether this may be due to a dissociation of SYN from the vesicles in treated animals. However, densitometric measurement of SYN in gels of control and treated LP2 (not shown) clearly demonstrated that the amount of SYN associated to the vesicles was not changed. It is known that CaMKII is associated with SYN in SVs, where it works as an anchoring protein for SYN. Also, we have preliminary evidence that CaMKII associates with SYT *in vitro* (Fig. 2, *right*).[1] It can be speculated that the different phosphorylation of the two substrates is due to the existence of different kinase subpopulations associated to respective substrates in SVs.

Like before (12), it must be mentioned that the effect observed after treatment is too great to be restricted to 5-HT terminals in hippocampus. The presence of 5-HT heteroreceptors in terminals releasing other transmitters (acetylcholine, glutamate) has been demonstrated (26, 27). Also, it was shown that 5-HT may modulate the release of norepinephrine in hippocampal slices, in this case by activating 5-HT_3 heteroreceptors (28). Therefore, it seems likely that the changes observed in the Ca^{2+}/calmodulin phosphorylation system connected to proteins of the presynaptic release apparatus are not restricted to a single class of nerve terminals. It would be interesting to assess which pathways are involved because this could give more insight into the specific action of drugs in a major brain area.

All of the several substrates of CaMKII in SVs intervene in the protein/protein interactions that are thought to carry out the various steps in the cycle of SVs (29). Among the two substrates investigated in this study, the function of SYN has been thoroughly studied recently (3). Phosphorylation of this protein by CaMKII is thought to regulate the availability of vesicles for exocytosis, controlling the number of vesicles translocating from the reserve pool to the docked pool, although there is disagreement with this hypothesis (30).

SYT seems to be a multifunctional protein, playing a role in docking, fusion, and endocytosis of SVs (31, 32). The best characterized properties of this protein are the Ca^{2+}/phospholipid binding and the interaction with syntaxin and voltage-gated calcium channels (33, 34). The first property makes SYT the best candidate for the role of Ca^{2+} sensor in the release apparatus. The second property (which is also Ca^{2+} dependent) could fulfill the function of maintaining in register Ca^{2+} sensor and Ca^{2+} channel in the last steps of the SV cycle, just before fusion (35). The finding that treatment with 5-HT reuptake inhibitors (and consequential CaMKII up-regulation) preferentially affects SYT phosphorylation suggests that modifications in docking, priming, and/or fusion of SVs may be responsible for the increased 5-HT release found in the hippocampus (14). However, other possible functions of SYT (e.g., SV endocytosis) cannot be excluded. It was proposed that phosphodephosphorylation may regulate SYT function (36, 37). We are investigating the hypothesis with regard to Ca^{2+}/phospholipid and syntaxin binding activity.[2] It is also noteworthy that for both CaMKII (12) and SYT, phosphorylation but not protein expression seemed to be modulated by reuptake inhibition. This confirms that protein phosphorylation has a major regulatory role at the presyn-

[1] M. Popoli, C. Vocaturo, and L. Buffa, unpublished observations.
[2] M. Popoli, A. Venegoni, L. Buffa, and G. Racagni. Manuscript in preparation.

aptic level in the physiology of the release apparatus as well as in the pharmacology of drugs affecting release.

However, it is difficult at this stage to debate the phosphorylation state of SYT *in vivo* after the treatment. Although the kinase increase in both *post hoc* phosphorylation and activity suggests that the treatment induces an up-regulation of CaMKII, the situation is not so clear cut for SYT. The finding that *post hoc* phosphorylation of the protein is increased might be due to a decreased phosphorylation *in vivo*, although the persistent increase in CaMKII activity after long term treatment and the association of kinase and substrate in the same compartment suggest that SYT phosphorylation is also increased. Only a back-phosphorylation type of procedure could settle this matter (38, 39). Unfortunately, this method is not applicable to an integral membrane protein such as SYT. We are currently investigating whether SYT can be extracted from brain areas in a manner that preserves its *in vivo* phosphorylation state.

In summary, we found that 5-HT reuptake blockers (some of the most common antidepressant drugs) may have a remarkable effect on specific presynaptic effectors. To our knowledge, this is the first time that such a connection is demonstrated between psychotropic drugs and proteins of the presynaptic release apparatus. However, it should be kept in mind that currently no firm connection can be established between this effect and the therapeutic action of 5-HT reuptake blockers, a limitation that is common to other known long term biochemical changes induced by antidepressants. Also, further treatments with different antidepressants and with drugs that are devoid of an antidepressant effect are necessary to assess whether this effect is produced by other antidepressants or only by 5-HT reuptake blockers.

It is worth mentioning that this kind of effect on the presynapse may not be restricted to a single class of drugs (i.e., transmitter reuptake blockers). An 80% increase in CaMKII activity associated with an increase in glutamate release was recently found in the striatum of animals chronically treated with haloperidol, a dopamine D_2 receptor blocker (40). It will be interesting to assess whether the same mechanism of action affecting CaMKII and related presynaptic substrates applies to different drugs, targeted to different areas and physiopathological situations.

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