Somatostatin-Induced Activation and Up-Regulation of N-Methyl-D-aspartate Receptor Function: Mediation through Calmodulin-Dependent Protein Kinase II, Phospholipase C, Protein Kinase C, and Tyrosine Kinase in Hippocampal Noradrenergic Nerve Endings

Anna Pittaluga, Marco Feligioni, Fabio Longordo, Marica Arvigo, and Maurizio Raiteri

Pharmacology and Toxicology Section, Department of Experimental Medicine (A.P., M.F., F.L., M.R.), Center of Excellence for Biomedical Research (A.P., M.R.), and Department of Endocrinological and Metabolic Sciences (M.A.), University of Genoa, Genoa, Italy

Received October 21, 2004; accepted December 15, 2004

ABSTRACT

Somatostatin receptors and glutamate N-methyl-D-aspartate (NMDA) receptors coexist on hippocampal noradrenergic axon terminals. Activation of somatostatin receptors was previously found to positively influence the function of NMDA receptors regulating norepinephrine release. The somatostatin receptors involved were pharmacologically characterized as sst5 type in experiments in Mg2+-free solutions. Here, we first confirm the pharmacology of these receptors using selective sst5 ligands and we show by Western blot that the sst5 protein exists on purified hippocampal synaptosomal membranes. We then investigated the pathways connecting the two receptors using as a functional response the release of norepinephrine from rat hippocampal synaptosomes in superfusion. The release of norepinephrine evoked by somatostatin-14 plus NMDA/glycine was partly prevented by the protein kinase C inhibitor U73122 [1-(6-[[17]-3-methoxyextra-1,3,5[10]-trien-17-y]amino]hexyl)-1H-pyrole-2,5-dione] and by the Ca2+/calmodulin-dependent protein kinase II (CaMkII) inhibitor KN93 [N-(2-[4-chlorocinnamyl]-N-methyl-amino-methyl[phenyl]-N-(2-hydroxyethyl)-4-methoxy-benzene-sulfonamide-phosphate salt], respectively; and it was unaffected by the protein kinase A inhibitor H89 [N-(2-[p-bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide hydrochloride]. The norepinephrine release evoked by somatostatin-14/NMDA/glycine was inhibited when anti-phosphotyrosine antibodies had been entrapped into synaptosomes. Entrapping the recombinant activated tyrosine kinase pp60-src strongly potentiated the release of norepinephrine elicited by NMDA/glycine in Mg2+-free medium but failed to permit NMDA receptor activation in presence of external Mg2+ ions. The results suggest the involvement of CaMkII in the sst5 receptor-mediated activation of NMDA receptors in presence of Mg2+ and of the PLC/PKC/Src pathway in the up-regulation of the ongoing NMDA receptor activity.

Glutamate N-methyl-D-aspartate (NMDA) receptors are ion channel-associated receptors expressed by coassembly of NR1 and NR2(A-D) subunits. These receptors display both pre- and postsynaptic localization and play relevant roles in development, neuroplasticity, and excitotoxicity (for review, see Engelman and MacDermott, 2004).

Interactions between NMDA receptors and G protein-coupled receptors (GPCRs) coexpressed on membranes have been reported by several laboratories (Lu et al., 1999; Pittaluga et al., 2000; Lan et al., 2001; Heidinger et al., 2002; see Engelman and MacDermott, 2004). ABBREVIATIONS: NMDA, N-methyl-D-aspartate; GPCR, G protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C; Src, tyrosine kinase; CaMkII, Ca2+/calmodulin-dependent protein kinase II; NE, norepinephrine; SRIF, somatostatin; IP3, inositoltrisphosphate; t-TBS, Tris-buffered saline-Tween; PP2, 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; GF109203X, dihydrochloride3-[1-(3-dimethylaminopyrrole-2,5-dione] and by the Ca2+/calmodulin-dependent protein kinase II (CaMkII) inhibitor KN93 [N-(2-[4-chlorocinnamyl]-N-methyl-amino-methyl[phenyl]-N-(2-hydroxyethyl)-4-methoxy-benzene-sulfonamide-phosphate salt]; MK801, dizocilpine; BIM-23056, D-Phe-Phe-Tyr-D-TRP-Lys-Val-Phe-D-Nal-NH2; L362,855, c[Aha-Phe-Trp-D-TRP-Lys-Thr-Phe]; PLC, phospholipase C; CAKβ/Pyk2, cell-adhesion kinase β/proline-rich tyrosine kinase 2.
Kotecha et al., 2003). In particular, activation of GPCRs can affect NMDA receptor function, representing a major mechanism of glutamate transmission modulation. The cross-talk between coexisting GPCRs and NMDA receptors involves intracellular kinase pathways that may differ among the different receptor-receptor interactions.

Depending on the subunits targeted and the kinases involved, phosphorylation can affect either negatively or positively the NMDA receptor function (see Kotecha and MacDonald, 2002). NR1 and NR2B subunits can undergo phosphorylation by several kinases including protein kinases A (PKA; Leonard and Hell, 1997), protein kinases C (PKC; Zheng et al., 1999; Liao et al., 2001), cytosolic tyrosine kinases of the Src family (Yu et al., 1997; Lu et al., 1999; Saltar and Kalia, 2004), and Ca2+/calmodulin-dependent kinase II (CaMKII; Leonard et al., 1999; Soderling et al., 2001). Although phosphorylation of NR1 subunits generally limits NMDA receptor function (Zukin and Bennett, 1995), phosphorylation of NR2B subunits enhances NMDA receptor-mediated effects (see MacDonald et al., 2001).

NMDA receptors exist on central nervous system noradrenergic nerve endings, where they mediate exocytotic release of norepinephrine (NE; Jones et al., 1987; Fink et al., 1992; Raiteri et al., 1992). In rat hippocampus, these receptors, which contain NR2B subunits (Pittaluga et al., 2001), colocalize with somatostatin (SRIF) receptors, which are positively coupled to phosphoinositide breakdown and, based on a pharmacological study with selective ligands, belong to the sst5 subtype (Pittaluga et al., 2000). Activation of SRIF receptors fails to affect NE release but seems to permit activation and up-regulation of NMDA receptors in presence of physiological concentrations of extracellular Mg2+ and without depolarization (Pittaluga et al., 2000).

The mechanisms underlying the positive effects of SRIF on NMDA receptor function are not known. We previously suggested the involvement of inositoltrisphosphate (IP3) and PKC (Pittaluga et al., 2001), colocalized with somatostatin (SRIF) receptors, which are positively coupled to phosphoinositide breakdown and, based on a pharmacological study with selective ligands, belong to the sst5 subtype (Pittaluga et al., 2000). Activation of SRIF receptors fails to affect NE release but seems to permit activation and up-regulation of NMDA receptors in presence of physiological concentrations of extracellular Mg2+ and without depolarization (Pittaluga et al., 2000).

The mechanisms underlying the positive effects of SRIF on NMDA receptor function are not known. We previously suggested the involvement of inositoltrisphosphate (IP3) and PKC (Pittaluga et al., 2001), colocalized with somatostatin (SRIF) receptors, which are positively coupled to phosphoinositide breakdown and, based on a pharmacological study with selective ligands, belong to the sst5 subtype (Pittaluga et al., 2000). Activation of SRIF receptors fails to affect NE release but seems to permit activation and up-regulation of NMDA receptors in presence of physiological concentrations of extracellular Mg2+ and without depolarization (Pittaluga et al., 2000).

The animals were killed by decapitation, and the hippocampi were homogenized in 40 volumes of 0.32 M sucrose buffered at pH 7.4 with Tris (final concentration, 0.01 M) and then purified by Percoll gradient. Briefly, the homogenate containing synaptic vesicles was homogenized in 40 volumes of 0.32 M sucrose buffered at pH 7.4 with Tris (final concentration, 0.01 M) and then purified by Percoll gradient. Briefly, the homogenate containing synaptic vesicles was centrifuged at 100,000 g for 20 min. Synaptosomes were then lysed in 1 ml of ice-cold water and pellets isolated by centrifugation at 7000 g.

The pellets were solubilized in a lysis buffer containing 20 mM HEPES (pH 7.4), 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, and 4 mg/ml dodecyl-B-D-maltoside for 1 h at 4°C and then ultracentrifuged at 100,000 g for 1 h at 4°C. The supernatant containing solubilized synaptic membrane proteins was subjected to standardized colorimetric analysis to evaluate the protein content. Membrane protein (200 μg) was denatured and fractionated under reducing conditions on 10% SDS-PAGE, then transferred electrophoretically to Hybond C-Extra nitrocellulose membranes (Amersham Biosciences Inc., Piscataway, NJ). After transfer, nonspecific binding sites were blocked by Tris-buffered saline-Tween (t-TBS; 0.02 M Tris, 0.137 M NaCl, and 0.1% Tween 20) containing 5% nonfat dried milk. After three washes with t-TBS, membranes were incubated for 16 h at 4°C with a 1:150 dilution of goat anti-rat SST5r polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in t-TBS containing 3% bovine serum albumin. Membranes were washed three times with t-TBS, then incubated for 2 h at 22°C with a 1:5000 dilution of the antigen horseradish peroxidase-linked IgG. After three washes with t-TBS, immunoreactive bands were detected by the chemiluminescence detection system enhanced chemiluminescence Western blot analysis system (Amersham Biosciences Inc.). The immunoreactive bands were visualized by autoradiography after 0.5 min of exposure to Hyperfilm MP (Amersham Biosciences Inc.).

Calculations and Statistics. The radioactivity released into each superfusate sample was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected.
(fractional efflux). When the time course of the effect is reported, drug effects are expressed as percentage of effect and evaluated as the ratio between the percentage of tritium released into each fraction after the first and that in the first fraction collected. This ratio was compared with the corresponding ratio obtained under control conditions (no drug added).

Analysis of variance was performed by analysis of variance followed by Dunnett’s test or Newman-Keuls multiple comparisons test or Student’s t test as appropriate. Data were considered significant for \( p < 0.05 \) at least. Appropriate controls with antagonists and inhibitors were always run in parallel.

**Chemicals.** 1-[7,8-\textsuperscript{3}H]Norepinephrine (specific activity 39 Ci/mmol) RPN-800 prestained molecular mass marker, chemiluminescence detection system enhanced chemiluminescence Western blot analysis system, and Hyperfilm MP were from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Hydrobromic C-Extra nitrocellulose membranes were from Amersham Biosciences Inc. NMDA and PP2 were from Tocris Cookson Inc. (Bristol, UK). SRIF-14 was from Peninsula Laboratories (Belmont, CA). Glycine, GF109203X, U73122, lavendustin A [5-amino-[(N-2,5-dihydroxybenzyl)-N-2-hydroxybenzyl]salicylic acid], H89, and KN93 were purchased from Sigma/RBI (Natick, MA). Src (pp60\textsuperscript{(v-src)}) and mouse anti-phosphotyrosine (clone 4G10) were from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated anti-goat secondary antibody was purchased from Chemicon International (Temecula, CA). Goat anti-rat sst5R polyclonal antibody was from Santa Cruz Biotechnology, Inc. MK801 (dizocilpine) was from Merck Sharp and Dohme (Hoddesdon, UK), whereas 6-nitroquipazine maleate was from DuPhar (Amsterdam, The Netherlands). BIM-23056 and L362,855 were gifts from Drs. P. P. A. Humphrey (Glaxo Institute of Genetics, Research NOVARTIS Institute for Biomedical Research, Basel, Switzerland).

**Results**

The Function of NMDA Receptors in the Presence of Mg\textsuperscript{2+} Ions Is Facilitated by the Action of Somatostatin sst5 Receptors Present in the Hippocampus. As previously shown (Pittaluga et al., 2000), exposure of hippocampal synaptosomes to SRIF-14 plus NMDA/glycine evokes release of preloaded [\textsuperscript{3}H]NE in the presence of a physiological concentration of Mg\textsuperscript{2+} ions (1.2 mM). When added alone, neither SRIF-14 (1 nM) nor NMDA (100 \mu M) glycine (1 \mu M) can affect the release of [\textsuperscript{3}H]NE. The release induced by SRIF-14 plus NMDA/glycine depends on NMDA receptor activation since MK801, a selective NMDA channel blocker inactive on its own, almost totally prevented the releasing effect (1 \mu M MK-801 = 2.65 \pm 0.01\%, 1 nM SRIF-14 + 100 \mu M NMDA + 1 \mu M glycine = 73.08 \pm 6.56\%, + 1 \mu M MK-801 = 15.60 \pm 7.89\%, \( p < 0.05 \) at least; results expressed as percentage of increase over basal release).

In the work by Pittaluga et al. (2000), the positive effect of SRIF-14 on the NMDA-evoked release of NE was related to activation of SRIF receptors exhibiting sst5 pharmacology. Since that pharmacological characterization was carried out in Mg\textsuperscript{2+}-free solutions, it was important to ascertain if sst5 receptor ligands behaved similarly in solutions containing a physiological concentration of Mg\textsuperscript{2+} ions. As shown in Fig. 1, BIM-23056, a selective sst5 receptor antagonist, totally prevented the release of [\textsuperscript{3}H]NE induced by SRIF-14/NMDA/glycine, suggesting that only sst5 receptors participate in the somatostatin-NMDA receptor-receptor interaction. The figure also shows that the sst5 partial agonist L362,855 mimicked in part SRIF-14.

The pharmacological characterization of the receptors involved as sst5 type may seem surprising. Based on morphological results, the presence of receptors of the sst5 type in some central nervous system regions is in fact controversial (Fehlmann et al., 2000; Rocheville et al., 2000a; Schulz et al., 2000; Kang et al., 2003). We therefore analyzed proteins from purified synaptosomal membranes for the presence of the sst5 receptor protein. As shown in Fig. 1, Western blot analysis with anti-sst5 antibodies recognized a component with an apparent mass of 60 to 70 kDa, suggesting that sst5 protein is expressed in nerve ending membranes. The prominent band with the apparent mass of 60 to 70 kDa should correspond to the monomeric form of the sst5 receptor (Rocheville et al., 2000b).

**Somatostatin-NMDA Receptor-Receptor Interaction: Involvement of PLC and PKC but Not PKA.** Table 1 shows that U73122 (0.1 \mu M), a selective inhibitor of PLC function, largely prevented the release evoked by SRIF-14/NMDA/glycine indicating the involvement of PLC present in noradrenergic nerve terminals. It is known that activated PLC promotes phosphoinositide breakdown followed by IP\textsubscript{3} and diacylglycerol production and possible consequent activation of intracellular PKCs. Accordingly, GF109203X, a selective PKC blocker, inhibited in part the release of [\textsuperscript{3}H]NE provoked by SRIF-14/NMDA/glycine in Mg\textsuperscript{2+}-containing medium (Table 1).

Little is known about the relations between PKA activity and NMDA receptor function. PKA-targeted sites were proposed to reside within the NR1 subunit, although PKA was shown to influence also the functional activity of NMDA receptors by modifying NR2 subunits (Leonard and Hell, 1997). These events, however, have been mainly related to inhibitory effects on NMDA receptor function. Under our experimental conditions, the PKA selective inhibitor H89, added at 0.5 \mu M, failed to affect the release of [\textsuperscript{3}H]NE induced by SRIF-14/NMDA/glycine (Table 1), suggesting that PKA-mediated phosphorylation of the NMDA receptor is unlikely to modulate NE release. Under very similar experimental conditions, H89 had been found to inhibit the upregulation of NMDA receptors provoked by nicotine receptor activation in noradrenergic axon terminals of the hippocampus (Risso et al., 2004). At the concentrations applied, the enzyme blockers used did not modify, on their own, the spontaneous release of tritium (see legend to Table 1).

**Involvement of Src.** It is known that PKC can phosphorylate NMDA NR2 subunits directly, on serine and threonine, as well as indirectly, on tyrosine, by activating cytosolic tyrosine kinases of the Src family (Yu et al., 1997; Lu et al., 1999; MacDonald et al., 2001; Saltar and Kaia, 2004). The possibility that the SRIF-NMDA receptor-receptor interaction involves the Src signaling was evaluated by studying the effects of the selective Src inhibitors PP2 and lavendustin A. As shown in Fig. 2, addition to the superfusion medium of PP2 (1 \mu M) or lavendustin A (5 \mu M) inhibited in part the [\textsuperscript{3}H]NE release induced by SRIF-14/NMDA/glycine from hippocampal synaptosomes. The spontaneous release of tritium was not affected by the kinase inhibitors used (see legend to Fig. 2A).

Since the effects of PKC and Src inhibitors (used at the maximally effective concentration; data not shown) were only partial, we tested a combination of the two inhibitors. As reported in Table 2, addition of GF109203X together with...
PP2 did not produce additive inhibitory effect on the SRIF-14/NMDA/glycine-evoked release of [3H]NE. Involvement of CaMKII. NMDA receptor subunits have been identified as CaMKII anchoring proteins in postsynaptic densities. Upon kinase autophosphorylation, CaMKII can bind the cytosolic C-terminal region of NR2B subunits and affect the function of the NMDA-associated ionic channel (Leonard et al., 1999; Soderling et al., 2001). Therefore, we investigated the possibility that the SRIF receptor-NMDA receptor interaction that occurs presynaptically involves CaMKII-mediated phosphorylative processes by analyzing the effect of the selective CaMKII inhibitor KN93 on the release of [3H]NE induced by SRIF-14/NMDA/glycine. As shown in Fig. 2B, KN93, added at 1 μM, almost totally inhibited the evoked [3H]NE release. The spontaneous release of tritium was not affected by 1 μM KN93 (see legend to Fig. 2).

Synaptosomal Entrapping of Anti-Phosphotyrosine Antibodies Prevents the SRIF-14/NMDA/Glycine-Evoked Release of [3H]NE. The finding that kinases of the Src family participate in the interaction between SRIF and NMDA receptors implies that the cross talk between the two receptors includes phosphorylation of tyrosine residues. To evaluate the involvement of a tyrosine phosphorylative pathway, antibodies raised against phosphotyrosines were entrapped into synaptosomes. We had previously shown that synaptosomes isolated after homogenization of brain tissue in the presence of anti-syntaxin or anti-SNAP25 antibodies exhibited decreased transmitter release when exposed to depolarizing stimuli, indicating that the above antibodies had been entrapped into nerve endings (Raiteri et al., 2000). As shown in Fig. 3, entrapping anti-phosphotyrosine antibodies into hippocampal synaptosomes decreased by about 50% the

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>[3H]NE Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRIF-14 + NMDA + glycine</td>
<td>71.16 ± 8.95</td>
</tr>
<tr>
<td>SRIF-14 + NMDA + glycine + U73122 (0.1 μM)</td>
<td>18.77 ± 7.33</td>
</tr>
<tr>
<td>SRIF-14 + NMDA + glycine + GF109203X (0.1 μM)</td>
<td>24.36 ± 3.09</td>
</tr>
<tr>
<td>SRIF-14 + NMDA + glycine + H89 (0.5 μM)</td>
<td>66.01 ± 15.34</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. control.
The main findings of the present study are that activation of sst5 receptors present on hippocampal noradrenergic terminals positively affects the function of coexisting NMDA receptors through pathways involving CaMKII, PLC, PKC, and Src; somatostatin seems to trigger NMDA receptor activation through CaMKII; and the PLC/PKC/Src pathway up-regulates NMDA receptor function once the receptor is gated to an open state.

Noradrenergic axon terminals in the hippocampus possess NMDA receptors mediating exocytotic-like NE release (Jones...
The release of NE elicited by SRIF-14/NMDA/glycine, in the presence of extraterminal Mg\(^{2+}\), was completely prevented by the sst5 receptor antagonist BIM-23056 and by the NMDA receptor antagonist MK-801. Furthermore, it was largely reduced when PLC was inhibited by U73122. It was reported that SRIF receptors of the sst5 type can couple to PLC and to enhancement of phosphoinositide metabolism (Wilkinson et al., 1997), with consequent production of IP\(_3\) and diacylglycerol. Thus, it seems that PLC activation by SRIF acting at sst5 receptors plays an important role in the function of NMDA receptors in the presence of extraterminal Mg\(^{2+}\). The mechanism is probably indirect and mediated by downstream signaling molecules of PLC, in particular PKC. Accordingly, the release of NE provoked by SRIF/NMDA/glycine was in part reduced by selective PKC inhibitors (Table 1; see also Pittaluga et al., 2000). PKC can phosphorylate NR1 or NR2 subunits leading to inhibition or potentiation of NMDA receptor responses, respectively (MacDonald et al., 2001; Kotecha and MacDonald, 2002). The finding that PKC blockade inhibited the evoked NE release is compatible with the view that PKC participates in the phosphorylation of NR2 subunits of the NMDA receptor. A number of PKA phosphorylation sites exist on NMDA receptors (Leonard and Hell, 1997). Here, we found that inhibition of PKA by H89 had no effect on the release of NE evoked by SRIF-14/NMDA/glycine, excluding the involvement of the enzyme.

Our results suggest that Src-mediated tyrosine phosphorylation plays a role. This is supported by the inhibition of the evoked NE release observed in the presence of two Src inhibitors as well as by the finding that anti-phosphotyrosine antibodies prevented the effect of SRIF-14/NMDA/glycine.

The release-enhancing NMDA receptor present on hippocampal noradrenergic terminals is an NR2B-containing receptor (Pittaluga et al., 2001). NMDA receptor subunit NR2B is the major tyrosine phosphorylated protein in the postsynaptic density (Moon et al., 1994). Src is associated with NMDA receptors, and phosphorylation by Src up-regulates NMDA receptor currents (Yu et al., 1997; Lu et al., 1999). Several studies have addressed the signaling mechanisms controlling Src family kinase activation during glutamatergic transmission. One likely signal is the cell adhesion kinase β/proline-rich tyrosine kinase 2 (CAKβ/Pyk2; Huang et al., 2001), which can be stimulated by PKC; in turn, CAKβ binds and activates Src family kinases (Dikic et al., 1996). Thus, the PLC/PKC/CAKβ/Src pathway may well couple sst5 receptor activation and NMDA receptor function in hippocampal noradrenergic neurons. This cascade has been proposed to mediate up-regulation of NMDA currents by various GPCRs (Lu et al., 1999; Heidinger et al., 2002; Kotecha et al., 2003; Salter and Kalia, 2004).

The sst5-NMDA receptor-receptor interaction seems to strictly depend on CaMKII activity since KN93 completely abolished the evoked NE release. Increased cytosolic Ca\(^{2+}\) availability from various sources, including stimulation of IP\_3 receptors consequent to PLC activation, could trigger rapid CaMKII autophosphorylation and translocation to NR2B subunits of the NMDA receptor (Leonard et al., 1999; see Soderling et al., 2001). The interaction between NR2B and CaMKII was reported to lock the enzyme in an active conformation (Bayer et al., 2001); according to the authors, binding of CaMKII to NR2B and enzyme autophosphorylation can function synergistically, thus constituting a feedforward pathway able to positively affect NMDA-mediated transmission.

Antagonists at sst5 and NMDA receptors completely abolished the SRIF-14/NMDA/glycine-evoked NE release. Inhibition of CaMKII also totally blocked the evoked release of the catecholamines. In contrast, PKC and Src inhibitors prevented only in part the SRIF-14/NMDA/glycine effect. The finding that PKC and Src inhibitors, when combined, did not elicit additive effect is consistent with PKC and Src working in series.

At this point, it seems important to recall that NMDA and glycine, added in the presence of Mg\(^{2+}\) ions and without depolarization, are unable to elicit NE release, whereas release occurs when SRIF-14 is added with the NMDA receptor agonists. Therefore, one could distinguish two aspects of the SRIF action: a permissive role on the activation of the NMDA receptors, followed by up-regulation of the receptor ongoing activity.

To shed light on the question, we entrapped into synaptosomes pp60\(^{\text{src}}\)-Bac, a recombinant Src in the activated form (Yu et al., 1997; Lu et al., 1999), having assumed that Src comes last in the PLC/PKC/CAKβ/Src sequence. Should Src permit NMDA receptor activation, NE release would be observed when pp60\(^{\text{src}}\)-staffed synaptosomes are exposed to NMDA/glycine in the presence of Mg\(^{2+}\). However, if Src only potentiates the ongoing NMDA activity, NMDA/glycine would release NE from pp60\(^{\text{src}}\)-staffed synaptosomes only in Mg\(^{2+}\)-free medium. Entrapped pp60\(^{\text{src}}\) failed to permit NMDA receptor activation in Mg\(^{2+}\)-containing medium but potentiated the ongoing activity of NMDA, suggesting that the PKC/CAKβ/Src cascade mediates up-regulation of the function of NMDA receptors already activated. This idea does not exclude the participation of other agents, considering the impressive number of proteins constituting the NMDA receptor complex (Husi et al., 2000).

How sst5 receptors mediate activation of NMDA receptors on noradrenergic neurons remains to be established. Multiple mechanisms can be envisaged. Based on our results, a

**TABLE 3**

Effects of entrapped pp60\(^{\text{src}}\) on the NMDA-evoked [\(^{3}H\)]NE release in the presence or absence of extraterminal Mg\(^{2+}\) ions

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>pp60(^{\text{src}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA (100 (\mu M)) + glycine (1 (\mu M))</td>
<td>6.34 ± 3.25</td>
<td>0.57 ± 4.86</td>
</tr>
<tr>
<td>Mg(^{2+}) (1.2 mM)</td>
<td>64.22 ± 5.81</td>
<td>142.24 ± 8.65</td>
</tr>
</tbody>
</table>

\(p < 0.05\) at least vs. respective control.
likely possibility is that phosphorylation of NR2B subunits by CaMKII leads to removal of Mg$^{2+}$ from the NMDA receptor channel and to activation of the receptor in absence of depolarization. Another possibility stems from the increasing evidence that GPCRs can interact with NMDA receptors by physically associating with their subunits (Liu et al., 2000; Fiorentini et al., 2003; Salter, 2003). SRIF receptors of the sst2 type were found to physically associate with a scaffolding protein which can also bind to NR subunits (Peineau et al., 2003). One may speculate that also sst5 can perform a similar interaction, resulting in NMDA receptor activation.

Kotecha et al. (2003) have found that, in cultured hippocampal neurons, activation of mGluR5 and NMDA receptors up-regulate NMDA receptor currents through the PKC/CaMKII/Src cascade. Interestingly, NMDA channels must be gated to an open state during stimulation of mGluR5 for the up-regulation to occur; in fact, no potentiation could be observed in presence of Mg$^{2+}$. Differently, coapplication of SRIF-14 and NMDA/glycine elicited NE release in presence of external Mg$^{2+}$, compatible with the idea that somatostatin initially permits NMDA receptor activation in the presence of physiological concentrations of Mg$^{2+}$ and without depolarization and subsequently mediates up-regulation of receptor function through the PKC/CaMKII/Src pathway. Together with previous reports (see Kotecha and MacDonald, 2002 and refs. therein), the present results confirm the multiplicity of the pathways that can be implicated in GPCR-NMDA receptor interactions, indicating the importance of their careful characterization.

To our knowledge, a clear distinction between activation and up-regulation of NMDA receptor function by GPCRs has not been previously considered. The reason may be that, in the experimental systems generally used, one can measure a basal channel activity that may be decreased or, more frequently, augmented by GPCR activation. In our system, the endogenous agonists released are immediately removed by the medium up-down superfusing the synaptosomal thin layer, so that NMDA and SRIF receptors remain virtually ligand-free and functionally silent. Addition of NMDA/glycine cannot elicit any NE release if Mg$^{2+}$ is present, but release occurs if SRIF and NMDA/glycine are added concomitantly. Thus, our technique permits to identify conditions leading to NMDA receptor activation or to potentiation of the receptors already activated in absence of Mg$^{2+}$.

Considering the involvement of NMDA receptors, somatostatin, and norepinephrine in memory and learning (Olias et al., 1998) to be employed in conditions of cognitive impairments. We thank Maura Agate for excellent assistance in preparing the manuscript. Acknowledgments We thank Wort Maga for excellent assistance in preparing the manuscript. References Bayer K-U, De Koninck P, Leonard AS, Heli JW, and Schulman H (2001) Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature (Lond) 411:891–895.


Address correspondence to: Dr. Anna Pittaluga, Dipartimento di Medicina Sperimentale, Sezione Farmacologia e Tossicologia, Viale Cembrano 4, 16148 Genova, Italy. E-mail: pittalug@pharmatox.unige.it