Integrins Regulate NMDA Receptor-Mediated Synaptic Currents

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Lin, Bin, Amy C. Arai, Gary Lynch, and Christine M. Gall. Integrins regulate NMDA receptor-mediated synaptic currents. J Neurophysiol 89: 2874–2878, 2003; 10.1152/jn.00783.2002. Synapses contain high concentrations of integrins, adhesion receptors known to influence the operation of neighboring transmembrane proteins. Evidence that integrins are important for consolidation of long-term potentiation suggests that these adhesion proteins may modulate activities of synaptic glutamate receptors. The present study provides a first test of the possibility that integrins modulate synaptic N-methyl-D-aspartate (NMDA)-type glutamate receptor activities. Excitatory postsynaptic currents (EPSCs) were recorded with whole cell clamp from hippocampal slices in which AMPA-type glutamate receptors and GABA\textsubscript{A} receptors were pharmacologically blocked. Microperfusion of the peptide integrin ligand gly-arg-gly-asp-ser-pro (GRGDSP) caused an approximately twofold increase in the amplitude and duration of NMDA receptor-gated synaptic currents. Control peptides had no effect. Paired-pulse facilitation was unchanged, indicating that the ligand did not modify neurotransmitter release probabilities. Infusion of the Src kinase antagonist PP2 but not the control drug 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine eliminated the enhancing effect of GRGDSP. Integrins regulate Src kinases that are known to phosphorylate NMDA receptors. It is concluded that integrins act through this route to exert potent modulatory effects on the operation of NMDA receptors.

INTRODUCTION

Integrins are transmembrane heterodimeric adhesion receptors that mediate both cell-cell and cell-matrix interactions throughout the body (Alpin et al. 1998; Hynes 1992; Schwartz et al. 1995). Recent studies have revealed novel roles for integrins in neural and glial development, neuropathology, and learning and memory (Bi et al. 2002; Grotewiel et al. 1998). At neuromuscular junctions, integrins not only provide for matrix attachment but also play important organizational and signaling roles (Burkin et al. 1998). Whether this also holds for synapses in adult brain is unclear, but evidence suggestive of such a regulatory function has been obtained in studies of long-term potentiation (LTP) (Bahr et al. 1997; Chun et al. 2001; Kramár et al. 2002; Staubli et al. 1998). Peptides and toxins that compete with matrix ligands for integrin binding interfere with the stabilization of hippocampal LTP, leaving potentiation to decay gradually over time and vulnerable to disruption. While these results demonstrate that integrins participate in changes that affect glutamate receptors, they do not address the possibility of direct relationships between integrins and glutamate receptor function although recent findings provide reasons to believe such interactions exist. The integrins are known to signal through tyrosine kinase intermediaries (Giancotti and Ruoslahti 1999; Miranti and Brugge 2002; Vuori 1998) and, in particular, to influence voltage-gated calcium channels in neurons (Wildering et al. 2002) and nonneuronal cells (Davis et al. 2002; Kwon et al. 2000; Wu et al. 1998) at least in part through Src tyrosine kinase (Wu et al. 2001). Other studies have shown that Src activation is necessary for LTP induction and may function by increasing N-methyl-D-aspartate (NMDA) receptor currents (Lu et al. 1998). Together these findings suggest that integrins may regulate NMDA receptor function through Src kinase. The present study tested this possibility for mature hippocampal synapses. Our results show for the first time that treatment with soluble integrin ligands enhances NMDA receptor currents and that this effect is dependent on Src activity.

METHODS

All animal procedures were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals and with protocols approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. This includes efforts to minimize animal suffering and numbers of rats used in the work described.

Hippocampal slices (450 μm) were prepared from 2- to 3-wk-old Sprague-Dawley rats (Charles River, Wilmington, MA) and placed in a holding chamber for ≥1 h before being transferred to a recording chamber (see Lin et al. 2002 for details). The slices were submerged in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 1.25 KH\textsubscript{2}PO\textsubscript{4}, 3.4 CaCl\textsubscript{2}, 2.5 MgSO\textsubscript{4}, 26 NaHCO\textsubscript{3}, and 10 D-glucose. In addition, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM picrotoxin were added to block AMPA and GABA currents, respectively. The ACSF was equilibrated with 95% O\textsubscript{2}-5%CO\textsubscript{2} (pH 7.3) and infused at 1.2 ml/min. All experiments were carried out at 32°C.

Field CA1b pyramidal neurons were visualized with an infrared microscope (Olympus BX50WI, Olympus, Melville, NY) with DIC configuration, and whole cell recordings were made with 3–5 MΩ recording pipettes containing (in mM) 130 Cs gluconate, 10 CsCl, 0.2 EGTA, 8 NaCl, 2 ATP, 0.3 GTP, 5 QX-314, and 10 HEPES (pH 7.35, 290–300 mosM). The liquid junction potential of the pipette solution was ~6 mV with respect to the external solution. Holding potentials were maintained at ~20 mV after correcting for the junction potential.

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NMDA currents were recorded with a patch amplifier (AxoPatch-1D, Axon Instruments, Union City, CA) with a 4-pole low-pass Bessel filter at 2 kHz and digitized at 10 kHz with NAC program (Elecctek Enterprises). Synaptic responses were induced by stimulating the Schaffer collateral/commisural fibers every 2–3 s at a pressure of 2–4 psi and with a pulse duration of 56 ms using a Picospritzer (General Valve, Fairfield, NJ). Phenol Red dye was included in the pipette to monitor the spread of the solution. Drug application pipettes had a tip diameter of ~25 μm and were prepared with a conventional electrode puller. The drug solution was ejected once changes in these parameters were excluded from the analysis.

After stable recording for ≥10–20 min, the integrin ligand peptide gly-arg-gly-asp-ser-pro (GRGDSP; single amino acid code) (Ruoslahti 1996) or the control peptide gly-arg-ala-asp-ser-pro (GRADSP) (Pierschbacher and Ruoslahti 1984) was applied through a glass micropipette (pipette concentration, 30 mM) placed beneath the recording electrode (i.e., in stratum radiatum) and at the same distance from the cell body layer as the stimulation electrode. Drug application paired-pulse experiments were conducted under the same conditions using interpulse intervals of 50, 80, 100, and 200 ms. Six responses were collected at each interpulse interval to determine paired-pulse levels with a delay varying between 20–30 min (Fig. 1, A and B; n = 11). Normalizing the facilitated response to the amplitude of the baseline response confirmed that the integrin ligand had altered the waveform as well as the size of the EPSC (Fig. 1C). To verify that the GRGDSP effect on response size was due to enhancement of NMDA receptor currents, 50 μM d-APV, thereby demonstrating that they are NMDA receptor mediated (Fig. 1D).

**RESULTS**

Excitatory synaptic currents in response to Schaffer collateral activation were recorded at ~20 mV in the presence of CNQX and picrotoxin, antagonists of AMPA and GABA receptors, respectively; these currents are entirely and reversibly blocked by 50 μM d-APV, thereby demonstrating that they are NMDA receptor mediated (Fig. 1D). Treatment with the integrin ligand peptide, GRGDSP caused the amplitude and duration of NMDA receptor currents to increase to twice baseline levels with a delay varying between 20–30 min (Fig. 1, A and B; n = 11). Normalizing the facilitated response to the amplitude of the baseline response confirmed that the integrin ligand had altered the waveform as well as the size of the EPSC (Fig. 1C). To verify that the GRGDSP effect on response size was due to enhancement of NMDA receptor currents, 50 μM d-APV was applied to the bath over the same period as GRGDSP infusion; as observed for baseline responses (Fig. 1D), d-APV completely eliminated the EPSC and prevented

**FIG. 1.** The integrin receptor ligand gly-arg-gly-asp-ser-pro (GRGDSP) enhances N-methyl-d-aspartate (NMDA) receptor-mediated excitatory postsynaptic currents (EPSCs). Whole cell recordings were made from CA1 pyramidal cells in acute hippocampal slices in the presence of 10 mM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM picrotoxin (holding potential: ~20 mV). GRGDSP was ejected from a local micropipette (30 mM pipette concentration) during the interval indicated by the horizontal bars. Synaptic responses were induced every 20 s by stimulating Schaffer-commisural fibers. Graphs show the amplitude (A) and half-width (B) of NMDA receptor-mediated EPSCs expressed as a percent of preapplication baseline values. Each point corresponds to the mean ± SE of 11 experiments. As shown, both EPSC amplitude (A) and half-width (B) increased after ~20 min of GRGDSP treatment. C, 1 and 2: averages of 8 successive traces collected in the baseline period prior to GRGDSP treatment (I, control) and after 40 min of GRGDSP treatment (2). C3: 2C with amplitude normalized to that of C1 to demonstrate the effects of GRGDSP on response kinetics. C4: a superimposition of C1–C3 and illustrates the point that GRGDSP treatment alters both the amplitude and waveform of the NMDA EPSC response. D, 1 and 2: averages of 8 responses collected during the baseline period (1), 10 min into a period of 50 μM d-APV bath-infusion (2), and 10 min after d-APV washout (3). D4: a superimposition of D1–D3 and illustrates the point that EPSCs recorded under conditions used are reversibly abolished by d-APV and therefore NMDA receptor-mediated.
the emergence of additional conductances over 1 h of GRGDSP treatment (data not shown). Importantly, GRADSP, a control peptide with a markedly lower affinity for integrins (Pierschbacher and Ruoslahti 1984), did not increase the NMDA receptor-mediated synaptic currents in any of six slices tested (data not shown).

An increase in the duration and amplitude of NMDA currents could be accounted for by an increase in the probability of transmitter release. This possibility was evaluated using paired pulse facilitation, an effect that is sensitive to perturbations in release probability. Four different interpulse intervals of 50, 80, 100, and 200 ms were tested. As shown in Fig. 2, paired-pulse facilitation was not detectably changed by ≥50 min infusion of GRGDSP (n = 4).

After binding to extracellular matrix ligands, integrins activate at least two tyrosine kinases, focal adhesion kinase (FAK) and its homologue Pyk2, which in turn activate kinases within several signaling cascades (Giancotti and Ruoslahti 1999; Schlaepfer and Hunter 1998; Vuori 1998). Of particular interest in the present context is Src, a tyrosine kinase that is both activated by FAK/Pyk2 and phosphorylates the NR2 subunit of the NMDA receptor (Lau and Huganir 1995). Figure 3 (A and B) illustrates the effects of PP2, a Src kinase antagonist, on the interaction between GRGDSP and NMDA receptor-mediated synaptic responses. After pretreating slices with 2 μM PP2 for 10 min, GRGDSP was applied. As shown, GRGDSP had no effect on the NMDA receptor-mediated synaptic current in the presence of PP2 (n = 4). To control for potential side-effects of PP2, other slices were treated with the control compound PP3 (2 μM) that does not antagonize Src kinase activity. PP3 had modest effects on the NMDA receptor-mediated EPSC when applied alone but responses stabilized after 2 h of continuous infusion; after this point, treatment with GRGDSP induced increases in EPSC amplitude and half-width that were comparable to those seen in otherwise untreated slices (Fig. 3, C and D).

DISCUSSION

The present results constitute the first evidence that the level of integrin activation influences synaptic currents mediated by NMDA-type glutamate receptors. The soluble integrin ligand, GRGDSP, increased NMDA receptor currents, whereas the control peptide (GRADSP) with low affinity for integrins (Pierschbacher and Ruoslahti 1984) did not reproduce this effect. The enhanced currents are unlikely to be due to presynaptic actions because paired-pulse facilitation, a measure that is sensitive to changes in transmitter release probability, was unaffected by the ligand. In all, it appears that integrins regu-

![Figure 2](image-url)

**FIG. 2.** Paired-pulse facilitation is not affected by GRGDSP treatment. Pairs of stimulation pulses were applied at interpulse intervals indicated on the x axis. The plot shows the amplitude of the 2nd EPSC response expressed as a percentage of the amplitude of the 1st response (i.e., the degree of paired-pulse facilitation) as examined 5 min before (control) and 50 min after (GRGDSP-treated responses) GRGDSP application. Each point represents the mean ± SE of 4 experiments. As shown, GRGDSP treatment did not influence the degree of paired-pulse facilitation at intervals tested.

![Figure 3](image-url)

**FIG. 3.** GRGDSP effects on NMDA receptor-mediated synaptic currents depend on Src kinase. **A and B:** hippocampal slices were equilibrated with 2 μM 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine (PP2) for 10 min (thicker horizontal bar) and then GRGDSP (pipette concentration, 30 mM; thinner horizontal bar) was applied. Plots show mean EPSC amplitude (A) and half-width (B) values expressed as a percentage of pre-GRGDSP baseline values (means ± SE, n = 4). C and D: hippocampal slices were equilibrated with 2 μM 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3) for 2 h (prior to the interval plotted) after which baseline responses were collected for 10 min and then GRGDSP was applied as above (means ± SE, n = 4). As shown, in the presence of PP2, NMDA receptor currents were unchanged by GRGDSP treatment (A and B). In contrast, in the presence of PP3, GRGDSP induced increases in EPSC amplitude and half-width that were of the same magnitude as seen in otherwise untreated slices (as in Fig. 1, A and B).
Integrin ligand binding activates associated protein tyrosine kinases (e.g., FAK and Pyk2) that, in turn, trigger additional intracellular signaling kinases (Giancotti and Ruoslahti 1999; Miranti and Brugge 2002; Vuori 1998). Src is one of these kinases and is of particular interest in the present context because it is known to phosphorylate NMDA receptors (Grosshans et al. 2001; Hisatsune et al. 1999), an event that is reported to enhance NMDA receptor-gated currents (Ali and Salter 2001; Chen and Leonard 1996; Kohr and Seeburg 1996; Wildering et al. 2002). Although soluble peptides containing the matrix RGD integrin binding sequence act as antagonists for many measures of integrin function (e.g., adhesion) (Ruoslahti 1996), short matrix sequences, and soluble RGD-containing peptides in particular, can mimic the effects of native matrix ligands for some cell types and measures (Davis et al. 2002; Mogford et al. 1997; Tsao and Mousa 1995; Wildering et al. 2002; Wu et al. 1998). In particular, both RGD-peptides and native matrix proteins can activate integrin signaling including Src phosphorylation and Src-mediated events (Kwon et al. 2000; Wu et al. 1996, 2001). In agreement with this, ongoing studies in our laboratories have shown that GRGDSP and fibronectin similarly stimulate increases in Src (Y418) phosphorylation in synaptoneurosomes from adult rat forebrain (C. M. Gall and J. A. Bernard, unpublished observations). The present results show that PP2, a potent inhibitor of Src kinases, completely blocks GRGDSP effects on NMDA currents while PP3, which does not block Src kinase activity, did not inhibit these GRGDSP effects. Together, these findings suggest that in mature hippocampal neurons integrin-ligand binding activates Src that, in turn, phosphorylates NMDA receptors, thereby increasing their function. It is not known if this particular integrin-NMDA receptor interaction contributes to previously described integrin effects on LTP stabilization (Chun et al. 2001; Kramár et al. 2002; Staubli et al. 1998): it is possible that the various integrins expressed by CA1 pyramidal cells are differentially involved in regulating receptor currents and use-dependent synaptic plasticity. However, it is intriguing that LTP stabilization, during which potentiation becomes progressively less vulnerable to disruption or reversal (Martin 1998; Staubli and Lynch 1990), occurs over 30–60 min after induction and, in our studies, GRGDSP application increased NMDA currents with a latency of ~20–30 min.

Studies in other laboratories have shown that both matrix proteins and soluble RGD-peptides can stimulate increased calcium influx via voltage-gated calcium channels in invertebrate neurons (Wildering et al. 2002) and in nonneuronal cells (Davis et al. 2002; Mogford et al. 1997; Wu et al. 1998, 2001). The present results indicate that new integrin binding enhances NMDA receptor currents in mature forebrain neurons and raise questions as to the nature of integrin effects on NMDA receptor function in situ. It is possible that in brain integrin binding is dynamic and changes in coordination with neuronal activity. Recent studies have shown that neuronal activity regulates surface expression of multiple receptor types (Broutman and Baudry 2001; Du et al. 2000; Lin and Gall 2002; Moro et al. 2002) as well as extracellular proteolytic activity (Gualandris et al. 1996; Okabe et al. 1996) that targets adhesion and matrix proteins (Basbaum and Werb 1996; Endo et al. 1999; Hoffman et al. 1998; Pittman and Buettner 1989; Tsirka et al. 1997; Wu et al. 2000) and could expose new integrin ligands (Davis et al. 2000). Thus it is possible that through new integrin or ligand exposure, episodes of increased synaptic activity could lead to a volley of integrin binding and transient effects on NMDA receptor function. However, it is also possible that stable integrin binding tonically influences NMDA receptor properties. Tests for chronic influences are in principle possible by reducing the baseline level of binding with blocking agents that have no agonist properties; e.g., neutralizing antibodies or disintegrins.

The preceding possibility raises the question of which integrin is responsible for the observed changes in NMDA receptor function. As reviewed elsewhere (Gall and Lynch 2003; Kramár et al. 2002), adult rat CA1 pyramidal cells express moderate to high concentrations of the α3, α5, α8, αv, β1, and β5 integrin subunits, whereas other subunits are not detected (Pinkstaff et al. 1998, 1999) or present at very low levels. These data suggest that mature CA1 pyramidal cells express at least seven different RGD-binding integrins that could account for effects observed in the present study. The list of candidates involved in the regulation of NMDA receptor function can be shortened by considering only those in or near synaptic junctions. Immunoelectron microscopic experiments have shown that hippocampal spines contain concentrations of α8 (Einer-Jensen et al. 1996), β8 (Nishimura et al. 1998), and β1 (Schuster et al. 2001) immunoreactivities, whereas α3 has been localized to spine synapses in cerebral cortex (Rodriguez et al. 2000) and synaptic membrane fractions from hippocampus (Kramár et al. 2002). The α5 subunit is also present in high concentrations proximal to spines in hippocampus (Bi et al. 2001). This leaves four RGD-binding integrins (α3β1, α5β1, α8β1, αvβ8) as the strongest candidates for the integrin that regulates NMDA receptor currents. Important goals of future studies will be to refine this list and to test the integrin-signaling cascade proposed above.

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REFERENCES


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