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AMPA receptor stimulation increases $\alpha 5\beta 1$ integrin surface expression, adhesive function and signaling

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Abstract

Integrin proteins are critical for stabilization of hippocampal long-term potentiation but the mechanisms by which integrin activities are involved in synaptic transmission are not known. The present study tested whether activation of α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) class glutamate receptors increases surface expression of $\alpha 5\beta 1$ integrin implicated in synaptic potentiation. Surface protein biotinylation assays demonstrated that AMPA treatment of COS7 cells expressing GluR1 homomeric AMPA receptors increased membrane insertion and steady-state surface levels of $\alpha 5$ and $\beta 1$ subunits. Treated cells exhibited increased adhesion to fibronectin- and anti- $\alpha 5$ -coated substrates and tyrosine kinase signaling elicited by fibronectin-substrate adhesion, as expected if new surface receptors are functional. Increased surface expression did not occur in calcium-free medium and was blocked by the protein kinase C inhibitor chelerythrine chloride and the exocytosis inhibitor brefeldin A. AMPA treatment similarly increased $\alpha 5$ and $\beta 1$ surface expression in dissociated neurons and cultured hippocampal slices. In both neuronal preparations AMPA-induced integrin trafficking was blocked by combined antagonism of NMDA receptor and L-type voltage-sensitive calcium channel activities but was not induced by NMDA treatment alone. These results provide the first evidence that glutamate receptor activation increases integrin surface expression and function, and suggest a novel mechanism by which synaptic activity can engage a volley of new integrin signaling in coordination with, and probably involved in, stabilization of synaptic potentiation.

Keywords

biotinylation; exocytosis; glutamate receptor; integrin; long-term potentiation; trafficking

Integrins are transmembrane, heterodimeric ($\alpha\beta$) receptors that mediate cell–extracellular matrix and cell–cell adhesion throughout the body (Alpin *et al.* 1998). The 18 α and nine β subunits so far identified combine in limited combinations to form over 20 different integrins with subunit composition determining both ligand specificity and signaling properties of the receptor (Hynes 1992). Integrins cross-link their extracellular matrix ligands (e.g. fibronectin, LI) (Plow *et al.* 2000; Previtali *et al.* 2001) with the actin cytoskeleton (Geiger *et al.* 2001) but also mediate what has been termed ‘outside in’ and ‘inside out’ signaling (Hynes 1992; Dedhar and Hannigan 1996). In the former instance, integrin ligand binding, or integrin clustering, leads to conformational changes that propagate from the extracellular to the cytoplasmic domain of the receptor and, by changing the spatial arrangements of associated signaling

proteins, initiate signaling activities including tyrosine kinase and Rho GTPase cascades (Howe *et al.* 1998; Vuori 1998; Giancotti and Ruoslahti 1999; Parsons *et al.* 2000). This integrin signaling influences an impressive array of basic cellular functions (e.g. gene expression, cell cycling, calcium influx, among others) (Yamada and Miyamoto 1995; Lafrenie and Yamada 1996). 'Inside out' signaling refers to the process of integrin activation. Transmembrane integrin receptors can exist in states of low or high affinity for ligand binding; intracellular events [e.g. changes in protein kinase C (PKC) activity or divalent cation concentrations] can increase ligand affinity and thereby 'activate' the receptor to enable ligand binding (Mould 1996; Hughes and Pfaff 1998).

Although studies of integrin function in neurons have largely focused on developmental phenomena (Milner and Campbell 2002), recent work has shown that integrins are broadly expressed in adult brain (Pinkstaff *et al.* 1999) and influence mature synaptic responses (Gall and Lynch 2004). In particular, manipulation of integrin function by treatment with soluble integrin ligands (Staubli *et al.* 1990, Staubli 1998; LeBaron *et al.* 2003), function blocking antibodies (Chun *et al.* 2001; Kramár *et al.* 2002) and high-affinity toxins (Chun *et al.* 2001) blocks the stabilization of long-term potentiation (LTP) at glutamatergic hippocampal synapses. Studies with antibodies have implicated specific integrins in the stabilization process and in particular suggested the involvement of $\alpha 5\beta 1$, $\alpha 3\beta 1$ and $\alpha v\beta 3$, all of which are expressed in hippocampus (Pinkstaff *et al.* 1999). Genetic suppression of $\alpha 5\beta 1$ and $\alpha 3\beta 1$ together perturbs hippocampal LTP and, in addition, produces severe impairments in hippocampus-dependent forms of learning (Chan *et al.* 2003). These findings accord with evidence that integrin ligand treatment blocks rapid kindling in rat hippocampal slices (Grooms and Jones 1997) and activity-induced synaptic plasticity in *Drosophila* (Rohrbough *et al.* 2000).

As best characterized in non-neural cells, integrin signaling regulates the activity of a variety of kinases (Vuori 1998; Avraham *et al.* 2000; Parsons *et al.* 2000) and a subset of these [e.g. focal adhesion kinase (FAK), Src, calcium-calmodulin-dependent kinase II] have otherwise been implicated in LTP (Malenka and Nicoll 1999; Ali and Salter 2001; Yang *et al.* 2003). Recent studies confirm that synaptic integrins regulate these same kinases as well as the phosphorylation of glutamate receptors (Kramár *et al.* 2002; Bernard *et al.* 2005) and the synaptic currents they mediate (Lin *et al.* 2003). These effects provide links between integrin signaling and the events that express and stabilize LTP.

Less progress has been made in understanding how integrin signaling is engaged by, or coordinated with, processes that induce activity-dependent synaptic plasticity. One possibility is that intense glutamatergic activity (the trigger for LTP) increases the abundance or availability of integrin ligands, as thought to occur with activity-regulated extracellular proteolysis (Davis *et al.* 2000). Alternatively, or in association with this, synaptic activity may increase integrin surface expression or activate latent transmembrane integrins, leading to new integrin binding to extant matrix ligands and a wave of increased integrin signaling. The latter idea seems reasonable given evidence that synaptic, or more specifically glutamatergic, activity drives other receptor types [e.g. α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, neurotrophin receptors] into the membrane at the region of the synapse (Du *et al.* 2000; Shi *et al.* 2001; Tomita *et al.* 2001). The present studies tested whether glutamate receptor stimulation increases integrin surface expression using surface protein biotinylation techniques and three experimental preparations. Initial studies employed COS7 cells transfected to express GluR1 homomeric AMPA receptors; COS7 cells naturally express high levels of the $\alpha 5$ and $\beta 1$ integrin subunits that together form the $\alpha 5\beta 1$ dimer implicated in LTP (Chun *et al.* 2001), and this and other clonal cell lines have proven valuable for studies of the parameters and mechanisms of receptor trafficking as occur in neurons (Liang and Haganir 2001; Passafaro *et al.* 2001; Shi *et al.* 2001). The GluR1 subunit was chosen for transfection because, among the AMPA receptor subunits, GluR1 has most convincingly been

demonstrated to play a critical role in LTP (Vanderklish *et al.* 1992; Lee *et al.* 2003; Meng *et al.* 2003; Schmitt *et al.* 2005). Subsequent experiments used cultured forebrain neurons and cultured hippocampal slices to test whether effects described in COS7 cells were similarly elicited in neurons. Together, the results from these studies demonstrate that stimulation of AMPA class glutamate receptors does indeed increase integrin surface expression in a time frame that is appropriate for a contributor to LTP consolidation.

Materials and methods

COS7 cell culture and transient transfection

Effects of AMPA receptor activation on integrin surface expression were evaluated in COS7 cells transfected to transiently express GluR1 giving rise to GluR1 homomeric AMPA receptors. COS7 cells (CRL-1651; American Type Culture Collection, Manassas VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Tech, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 100 units/mL each of penicillin and streptomycin in a humidified 5% CO₂ incubator at 37°C. Every 2–3 days the cells were subcultured by trypsinization (0.05% w/v trypsin, with 1% EDTA) followed by plating on to uncoated dishes (Hynes 1992). For experimental use, cells were transiently transfected with GluR1/pRK5 (Verdoorn *et al.* 1991) (a gift from Dr Kathy Partin and Dr Peter Seeburg) using Lipofectamine 2000 reagent (Invitrogen). At 2–3 days after transfection, GluR1 expression (total and cell surface) was verified by western blotting using techniques and antisera described below.

Cultured hippocampal slices

Hippocampal slices were cultured from Sprague-Dawley rats (9–10 days postnatal (P); Simonsen Laboratories, Gilroy, CA, USA) as described previously (Lauterborn *et al.* 2000). Slices were cut at a thickness of 400 µm perpendicular to the long axis of the hippocampus, and included hippocampus, entorhinal cortex and portions of adjacent neocortex. Slices were explanted on to Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA, USA) in a six-well culture plate (Corning Inc., Acton, MA, USA) (six slices/well) containing 1 mL/well medium consisting of minimum essential medium, 30 mM dextrose, 1 mg/L insulin, 20% horse serum (pH 7.2), 30 mM HEPES, 5 mM NaHCO₃, 3 mM glutamine, 0.5 mM ascorbic acid, 2 mM CaCl₂ and 2.5 mM MgSO₄ (all reagents from Sigma, St Louis, MO, USA unless otherwise noted); each pup generated four wells within one culture plate. Slices were maintained in a humidified, 5% CO₂ incubator at 37°C with medium changed every other day for 12–18 days *in vitro* before experimental use. For surface biotinylation studies described below, 18 slices from three separate culture plates were pooled after treatment for each experimental condition.

In all cases, experiments were performed in accordance with National Institutes of Health guidelines and protocols approved by the Institutional Animal Care and Use Committee, taking care to minimize distress to the animals and the number of animals used.

Primary neuronal culture

Dissociated neurons from P1 Sprague-Dawley rat hippocampus and neocortex were cultured on to six-well culture plates (Corning Inc.) coated with poly-D-lysine (Sigma) at 5–10 × 10⁶ cells/well as described by Li *et al.* (1997). Cells were fed twice per week with Neurobasal media containing B27 supplement (Invitrogen; medium preconditioned on astroglial feeder cells for 24 h) and the mitotic inhibitor 5-fluoro-2-deoxyuridine (to minimize non-neuronal cells in the cultures). Mature neurons (~ 20 days *in vitro*) from two culture wells were used for each experimental condition for integrin surface expression experiments.

Measures of integrin cell surface expression

Procedures for measuring new cell surface expression (i.e. membrane insertion and steady-state surface expression) were adapted from Liang and Huganir (2001). Similar procedures were applied to cultured GluR1-transfected COS7 cells (to be referred to as GluR1/COS7 cells), hippocampal slices and dissociated neurons. To measure proteins newly inserted into the membrane (membrane 'insertion' assay), cultures were treated with sulfo-N-hydroxysuccinimide (NHS)-acetate (4°C, three times each for 20 min; Pierce Biotechnology, Rockford IL, USA) to block pre-existing cell surface proteins from biotinylation. Cultures were washed twice with 0.1 M sodium phosphate buffer with 0.9% NaCl (phosphate-buffered saline, PBS) supplemented with 100 mM glycine and twice with PBS alone at 4°C, and then incubated with 50 µM AMPA (Tocris Cookson, Ellisville MO, USA) or vehicle at 37°C (10 min). In some experiments, parallel cultures were similarly treated but an antagonist was added to the medium 20 min before and during AMPA exposure; antagonists included the AMPA receptor antagonist 6,7 dinitroquinoxaline-2,3-dione (DNQX; 20 µM), the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV; 50 µM) and the L-type voltage-sensitive channel blocker nifedipine (Nif; 10 µM) (Tocris Cookson). To assess 'steady-state' integrin surface expression, cultures were similarly treated with AMPA (or AMPA + DNQX or AMPA + APV/Nif) alone; i.e. the sulfo-NHS-acetate blocking and glycine rinse steps were omitted. After AMPA treatment, cells/ explants were shifted to 4°C for surface biotinylation with 1 mg/mL sulfo-NHS-S-S-biotin (Pierce) for 60 min, washed twice with PBS supplemented with 100 mM glycine at 4°C (20 min), washed with PBS alone, and then lysed and tip sonicated within RIPA buffer containing 10 mM Tris, pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100, 1 mM Na₃VO₄ and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein levels in the lysate were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) and volumes were adjusted to normalize protein density. An aliquot of the normalized lysate was used for western blot assessment of total integrin protein levels. From the remaining samples biotinylated proteins were precipitated with NeutrAvidin beads (Pierce) and processed for western blot analysis of β1 and α5 integrin subunit immunoreactivities using antisera AB1952 and AB1921, respectively, and of actin immunoreactivity using MAB3128 (Chemicon, Temecula, CA, USA).

Immunoreactive bands were measured using enhanced chemiluminescence ECL Plus kit and film from Amersham Biosciences, Inc. (Arlington Heights, IL, USA) and densitometric analysis of films using the AIS image analysis system (Imaging Research, St Catherines, ON, Canada). For these and subsequent analyses, the significance of the effects of treatment was assessed using (1) the paired or unpaired *t*-test for comparison of two groups or (2) one- or two-way ANOVA followed by the Student–Newman–Keuls (SNK) *post hoc* test for paired comparisons for the analysis of three or more groups. The 95% confidence level ($p < 0.05$) was considered statistically significant.

Adhesion assays

Two different assay systems were used to evaluate GluR1/COS7 cell adhesion to fibronectin- and anti-α5 integrin-coated substrates. For the fibronectin adhesion assay (Dedhar and Hannigan 1996), tissue culture plates were coated with 10 µg/mL full-length human fibronectin (Invitrogen) in PBS overnight at 4°C and blocked with 1% bovine serum albumin before use. Near-confluent cells were serum starved overnight in DMEM containing 0.1% FBS. Cells were then incubated at 37°C in DMEM containing 0.1% FBS with or without AMPA (50 µM) for 10 min, washed with PBS, and then trypsinized in 0.01% trypsin–EDTA (Invitrogen) containing 5 mM EDTA for 5 min at 4°C. Trypsinization was terminated with 1 mg/mL soybean trypsin inhibitor (Invitrogen) in PBS containing 5 mM EDTA. Cells were collected, washed in PBS/5 mM EDTA, and resuspended in serum-free DMEM for at least 30 min at 4°C.

C. Cells were then placed on fibronectin-coated plates at a density of $3\text{--}4 \times 10^6$ cells per 10-cm plate at 37°C in a CO_2 incubator for 10 or 30 min. Cells were then washed once with DMEM to remove non-adherent cells and lysed on the plate in RIPA buffer (as above); lysate protein levels were assayed (Bio-Rad).

The second adhesion assay used the $\alpha 5$ Integrin-Mediated Cell Adhesion Kit from Oncogene Research Products (La Jolla, CA, USA). Briefly, near-confluent GluR1/COS7 cells were serum starved and treated with AMPA (or vehicle) for 10 min and then resuspended in serum-free DMEM (4°C for 30 min to 1 h) as above. The cell suspension was then distributed across wells of 96-well plates previously coated with murine monoclonal antiserum to the extracellular domain of $\alpha 5$ integrin (JA7673; Oncogene Research Products) as per kit instructions; negative control wells were similarly treated with 100 μL of assay diluent only. To each well, 150 μL of 1×10^5 cells per mL suspension was added and incubated at 37°C for 10 or 30 min. The medium and non-adherent cells were then removed and wells were washed gently with PBS containing 1 mM CaCl_2 and 1 mM MgCl_2 . The kit cell staining solution (100 μL /well) was then added for 15 min at room temperature ($22\text{--}24^\circ\text{C}$) and removed. Wells were washed with PBS containing calcium and magnesium (as above) and allowed to air dry. Finally, 100 μL /well of the kit stain extraction solution was then applied for 10–20 min to solubilize the cell-bound stain. The optical density of each well was determined at 595 nm using a microtiter plate reader and SoftMax Pro 4.0 software (Molecular Devices, Sunnyvale, CA, USA).

Adhesion-induced signaling

Evaluation of cellular mechanisms mediating treatment effects on adhesion-induced signaling employed the fibronectin substrate attachment preparation described above. In separate preparations, GluR1/COS7 cells were treated with 50 μM AMPA alone or in the presence of cycloheximide (10 $\mu\text{g}/\text{mL}$ for 30 min; Sigma), brefeldin A (BFA; 10 $\mu\text{g}/\text{mL}$ for 30 min; Sigma), or chelerythrine chloride (CCI; 500 nM for 30 min; Calbiochem, La Jolla, CA). Control cells were treated with normal medium for the same interval. Cells were washed and resuspended as described above and either left in suspension for 30 min or placed on fibronectin-coated plates at a density of $3\text{--}4 \times 10^6$ cells per 10-cm plate for 10 or 30 min. Suspended (non-adherent) cells were pelleted then lysed in RIPA. Adherent cells were washed once with DMEM and lysed in RIPA on the plate. Activation of integrin signaling intermediaries in suspended and adherent samples was examined by western blotting with tyrosine (Y) or threonine (T) phospho-specific antibodies to phosphorylated FAK (Y397) (Sanders and Basson 2000; Bongiorno-Borbone *et al.* 2002), Src (Y418) (Sanna *et al.* 2000), paxillin (Y31) (Schaller *et al.* 1999) (Biosource Int., Camarillo CA, USA) and p44/42 mitogen-activated protein kinase (MAPK) (T202/Y204) (Cell Signaling Technology, Beverly, MA, USA); all primary antisera were used at 1 : 1100 dilution in Tris-buffered saline supplemented with 5% bovine serum albumin. Western blot analysis of actin immunoreactivity used MAB3128 (Chemicon). Immunoreactive bands were detected using enhanced chemiluminescence techniques described above. Measures of protein levels reflect band relative optical density \times area.

Results

AMPA receptor activation increases $\alpha 5$ and $\beta 1$ integrin surface expression in GluR1/COS7 cells

COS7 cells transfected to express GluR1 homomeric receptors were used to measure the concentration of newly inserted copies of the $\alpha 5$ and $\beta 1$ integrin subunits. Preliminary studies using surface protein biotinylation followed by precipitation with NeutrAvidin beads and western blot analysis of the precipitates confirmed that GluR1/COS7 cells expressed GluR1 protein at the cell surface (data not shown). To measure stimulus-driven membrane insertion, the transfected cells were pretreated with membrane-impermeable sulfo-NHS-acetate to block

biotinylation of pre-existing cell surface proteins, and then treated for 10 min with AMPA (50 μ M, 37°C) followed by the membrane-impermeable sulfo-NHS-S-S-biotin. Under these conditions only newly inserted plasma membrane proteins are biotinylated. Biotinylated proteins were harvested by NeutrAvidin bead precipitation and then subjected to western blot analysis to assess levels of biotinylated α 5 and β 1 integrin protein. AMPA receptor stimulation dramatically increased insertion of α 5 and β 1 integrin relative to levels in cells processed without AMPA treatment. The immunoblots on the left and right side of Fig. 1(a) respectively show relative levels of (1) biotinylated protein, newly inserted into the cell membrane and (2) total protein within the lysate for one representative experiment. As shown, the agonist increased the insertion of both α 5 and β 1 proteins, and both effects were almost completely blocked by co-treatment with the AMPA receptor antagonist DNQX. In contrast, AMPA treatment did not detectably alter total levels of either α 5 or β 1 integrin protein (Fig. 1a, right side). There was no measurable biotinylation of the intracellular protein actin, which was readily labeled in cell lysates (Fig. 1a, bottom panel), indicating that the sulfo-NHS-S-S-biotin did not penetrate the plasma membrane and attached only to proteins on the cell surface. Figure 1(b) summarizes results from densitometric measurements of western blot band densities from four independent experiments. AMPA treatment caused an approximately seven-fold increase in membrane insertion of α 5 and an eight-fold increase in β 1, both effects being blocked by DNQX. Figures 1(c) and (d) show the effect of the AMPA receptor agonist on new membrane insertion of β 1 and α 5 integrin subunits in non-transfected COS7 cells; measurements from three independent experiments demonstrated that neither AMPA or AMPA + DNQX influenced β 1 or α 5 integrin membrane insertion in cells lacking GluR1 expression.

In the experiments illustrated in Fig. 2, GluR1/COS7 cells were processed for analysis of the time course of AMPA effects on new membrane insertion and steady-state surface levels of the α 5 and β 1 integrin subunits. Cells were exposed to AMPA for 1 min (pulse treatment) or continuously until the time of harvest. For the membrane insertion assay cells were treated with sulfo-NHS-acetate to mask surface proteins to future labeling, treated with 50 μ M AMPA, and harvested 1, 3, 5, 8, 10 and 20 min after the onset of AMPA exposure for surface biotinylation. To assess AMPA treatment effects on steady-state α 5 and β 1 surface levels, cells were similarly processed but without the sulfo-NHS-acetate masking treatment before AMPA exposure. As illustrated in Fig. 2(a), with continuous AMPA treatment there was a clear, progressive increase in both newly inserted and steady-state levels of α 5 and β 1 immunoreactivity on the cell surface. For α 5, increased insertion was evident by 3 min and reached peak levels at 8–20 min; increases in steady-state levels (which should reflect both pre-existing and newly exposed surface immunoreactivities) were evident at 5 min and more markedly at 8 min after the start of treatment, although steady-state levels continued to increase up to the 20-min time point. Effects of continuous AMPA treatment on surface β 1 immunoreactivity were similar, with increases in both insertion and steady-state levels evident at 5 min and the rate of new insertion peaking at 8–10 min after treatment onset. Steady-state β 1 levels were dramatically increased at 8–20 min of treatment.

Results were similar with AMPA applied in a 1-min pulse and cell harvest at a range of time points thereafter. As shown in Fig. 2(b), with pulse treatment both newly inserted and steady-state surface β 1 immunoreactivity was abruptly increased between 3 and 5 min after treatment onset, peaked at 5–8 min, and then declined but remained above control levels at the 20-min time point. Pulse treatment increased α 5 insertion and steady-state levels most clearly by 5 and 8 min respectively, with a return to control values at the 20-min time point.

Effects of AMPA receptor activation on glutamate receptor surface expression are dependent upon calcium influx as demonstrated by the fact that they are not observed in calcium-free medium. To determine whether AMPA-induced increases in integrin surface expression involve similar mechanisms, GluR1/COS7 cells were treated with AMPA in calcium-free

medium and effects on integrin membrane insertion and steady-state surface expression were examined. As shown in Fig. 2(c), newly inserted and steady-state $\beta 1$ and $\alpha 5$ surface levels were unaffected by AMPA or AMPA + DNQX treatment under these conditions.

AMPA receptor activation increases cell-substrate adhesion

Although the above experiments indicate that stimulation of AMPA-type glutamate receptors increases the surface expression of $\alpha 5$ and $\beta 1$ integrins, they leave open the questions of whether the newly exposed subunits form functional fibronectin receptors and subserve increased adhesive function. To test this possibility, assays were conducted to determine whether the pronounced increases in membrane insertion are accompanied by equivalent changes in matrix adhesion by GluR1/COS7 cells.

Adhesion was assayed by plating cells on to substrates coated with either fibronectin or an antibody directed against the extracellular domain of $\alpha 5$ integrin. Figure 3(a) summarizes mean results for six independent experiments in which adhesion to fibronectin was compared in stimulated and yoked control populations of cells. In all cases cells were exposed to the glutamate receptor agonist AMPA (or control solution) for 10 min, washed and resuspended for 30 min, and then plated on to the test substrate for the indicated periods. At harvest the level of adhesion was assessed by measures of substrate adherent protein. Adhesion by control and experimental populations was highly correlated across experiments ($r = 0.98$) and, as shown in Fig. 3(a), the predicted difference was obtained in paired statistical comparisons. Adhesion measures for AMPA-treated cells were 26% greater than controls at 10 min after plating ($p = 0.029$, t -test) and were more prominent still (+ 60%; $p = 0.019$) after 30 min of attachment. Similar results, with less between-experiment variability in control binding, were obtained using immobilized antibodies directed to the $\alpha 5$ integrin as substrate (Fig. 3b). AMPA stimulation increased adhesion by 78% of paired control values at 10 min after plating ($p < 0.001$) and by 77% at 30 min ($p < 0.01$). The AMPA receptor antagonist DNQX had no effect on adhesion when applied alone but fully blocked AMPA effects on adhesion when applied 10 min before and during agonist treatment.

AMPA pretreatment enhances integrin-mediated signaling

Following binding to extracellular matrix ligands, the longer cytoplasmic tails of β integrin subunits directly associate with FAK and/or its homologue proline-rich tyrosine kinase 2 (Pyk2) (Schlaepfer and Hunter 1998; Schuster *et al.* 2001; Loeser *et al.* 2003). FAK and Pyk2 then become tyrosine phosphorylated, events that lead to binding and activation of Src family kinases as well as FAK phosphorylation at secondary sites (Vuori 1998; Parsons *et al.* 2000). Integrin ligation also results in tyrosine phosphorylation of paxillin, which binds both α and β integrin subunits (Liu *et al.* 2000; Parsons *et al.* 2000), and to downstream increases in activities of p44/42 MAPK (also known as ERK 1/2), Rho family GTPases and phosphatidylinositol 3-kinase (Vuori 1998; Giancotti and Ruoslahti 1999; Schwartz and Ginsberg 2002). These downstream events provide ample targets with which to determine whether increased insertion due to glutamatergic stimulation results in surface integrins that are not only competent for adhesion but are also capable of setting in motion the signaling sequences through which adhesion modifies cell structure and function. Accordingly, we tested whether AMPA stimulation of GluR1/COS7 cells potentiates key steps in the abovementioned integrin-driven phosphorylation cascades.

GluR1/COS7 cells were treated with 50 μ M AMPA for 10 min, resuspended, and then plated on to a fibronectin-coated substrate for 10 or 30 min, after which cells adhering to the substrate or remaining in suspension were harvested separately and processed for immunoblot analysis of levels of phospho (p)-FAK (Y397), p-Src (Y418), p-paxillin (Y31) and p-p44/42MAPK (T202/Y204) using phosphorylation site-specific antibodies. Results of a representative

experiment are shown in Fig. 4(a). Phospho-protein levels were low in control cells that had been plated for just 10 min, indicating that, although substantial adhesion had occurred by this point (see Fig. 3), signaling that follows from it was still in development. However, cells pretreated with AMPA had substantially higher levels of all four phospho-proteins even at 10 min (Fig. 4a). The difference between the two groups of cells was still very much in evidence at 30 min after plating, a time point at which signaling was well under way in control cells. Actin immunoreactivity was comparable at the two time points and was not detectably affected by AMPA treatment (Fig. 4a, bottom panel). The cells retained in suspension had negligible phospho-protein levels in the control group and, with the exception of very low levels of p-pMAPK, in the pretreated group; this confirms that activation of signaling is adhesion mediated and that AMPA receptor stimulation has little effect on the target proteins independent of adhesion. Quantification of treatment effects on p-FAK immunoreactivity across four independent experiments confirmed these observations (Fig. 4b). With AMPA pretreatment, levels of p-FAK (Y397) were significantly greater than the low levels found in yoked control cells at 10 min after plating on to the fibronectin substrate ($p = 0.014$; paired Mann-Whitney U -test); moreover, these large differences were again found at 30 min after plating, a point at which control levels were readily measured. Prior AMPA stimulation had no effect on p-FAK in cells in suspension (Fig. 4b, right bars). The same basic response profile was obtained with the protein synthesis inhibitor cycloheximide (10 μ M) applied at 30 min before and during AMPA treatment. Thus AMPA treatment potentiated adhesion-induced phosphorylation of all signaling intermediaries; among adherent cells p-FAK levels were significantly greater in AMPA-treated compared with non-pretreated cells at both 10 and 30 min after plating ($p < 0.05$; paired t -test, $n = 4$).

AMPA receptor-driven increases in $\alpha 5$ and $\beta 1$ surface expression involve PKC and BFA-sensitive exocytotic mechanisms

PKC has been implicated in both integrin trafficking to the membrane (Ng *et al.* 1999; Wang *et al.* 2002) and inside out integrin activation leading to a high-affinity ligand state (Hynes 1992; Mould *et al.* 1995; Hughes and Pfaff 1998; Parsons *et al.* 2000). We used the PKC inhibitor CCI to test whether the kinase also plays a role in AMPA-induced increases in integrin surface expression and adhesion-dependent signaling. As shown in Figs 5(a) and (b), AMPA treatment alone induced a robust increase in new membrane insertion of $\alpha 5$ and $\beta 1$ as indexed by surface protein biotinylation (as in above experiments); this increase was completely blocked by CCI which, by itself, had no measurable effect on surface (biotinylated) integrin levels.

Figures 6(a) and (b) illustrate the effect of CCI on AMPA modulation of integrin signaling activities in three experiments. GluR1/COS7 cells were treated with vehicle plus the inhibitor or with AMPA plus inhibitor, suspended and washed, and then plated for 10 or 30 min. As in the previous experiment (see Fig. 4b), levels of p-FAK increased dramatically from 10 to 30 min after plating and were higher in adherent cells than in cells in suspension (Fig. 6b), indicating that some level of adhesion-dependent signaling remained intact in the presence of the PKC antagonist. However, in marked contrast to the effects of AMPA treatment alone, AMPA treatment in the presence of CCI did not potentiate adhesion-induced signaling as assessed at 10 or 30 min after plating (Figs 6a and b).

BFA inhibits exocytosis by disruption of protein trafficking from the Golgi-endoplasmic reticulum (ER) network and recycling of endocytotic vesicles (Klausner *et al.* 1992; Shah and Klausner 1993). As a consequence, with BFA treatment newly synthesized proteins are retained in the ER and secretory activities are blocked (Shah and Klausner 1993). Recent work has shown that integrin-mediated adhesion and cellular migration can be controlled by proteins known to function in membrane trafficking and, in particular, involve processes sensitive to

the effects of BFA (Schultz and Armant 1995; Wang *et al.* 2002). As this type of rapid trafficking might account for the speed at which increased expression occurs in AMPA-treated cells, we tested the effects of BFA on surface concentrations of $\alpha 5$ and $\beta 1$ integrins. The blot in Fig. 5(c) shows that stimulation of AMPA receptors again increased levels of biotinylated (newly inserted) $\alpha 5$ and $\beta 1$ proteins exposed at the cell surface and that this effect was eliminated by 10 $\mu\text{g}/\text{mL}$ BFA co-treatment (Fig. 5c); BFA alone had no evident effects on surface expression. Figure 5(d) shows quantification of treatment effects on new insertion in two experiments. Tests of the effects of BFA on AMPA-driven increases in adhesion-induced kinase signaling (as in Fig. 6) demonstrated unanticipated responses to the antagonist applied alone: i.e. BFA increased p-FAK, p-Src and p-paxillin in both suspended and adherent cells. Hence, with BFA this assay was not considered useful as a secondary measure of AMPA effects on integrin surface function.

AMPA treatment increases neuronal $\alpha 5$ and $\beta 1$ surface expression

Results of the above studies demonstrate that stimulation of GluR1 homomeric AMPA receptors increases integrin surface expression in GluR1/COS7 cells. To test whether native AMPA receptors similarly regulate integrin surface expression in forebrain neurons, protein biotinylation techniques were used to examine the effects of glutamate receptor stimulation on $\alpha 5$ and $\beta 1$ surface expression in primary cultures of cortical/hippocampal neurons and in cultured hippocampal/entorhinal slices. Pilot studies indicated that the surface biotinylation assays could be used to measure surface proteins in either preparation with relatively modest changes from the protocols used in the GluR1/COS7 experiments.

Effects of AMPA treatment of cultured neurons and hippocampal slices are shown in Fig. 7. The blots in Fig. 7(a) (left panel) show that in primary neuronal cultures AMPA treatment for 10 min induced marked increases in both new $\alpha 5$ and $\beta 1$ membrane insertion (57–200% increase above paired control values in four independent measures across two experiments) and steady-state surface expression (64–171% increase above control values); both effects were absent when DNQX was applied before and during AMPA treatment. Blots in the right panel of Fig. 7(a) show there were no treatment effects on total $\beta 1$ and $\alpha 5$ protein levels. Results with cultured hippocampal slices were similar (Fig. 7b). AMPA treatment reliably increased $\beta 1$ and $\alpha 5$ membrane insertion and $\alpha 5$ steady-state membrane expression across three experiments. In this set, effects of treatment on steady-state $\beta 1$ surface expression were not reliably obtained. As with dissociated cell experiments, effects of AMPA treatment on $\alpha 5$ and $\beta 1$ surface expression were blocked or attenuated by DNQX.

In experiments using COS7 cells described above, AMPA effects on integrin surface expression were dependent on extracellular calcium. The GluR1 homomeric AMPA receptors expressed in the COS7 cell studies are known to pass calcium (Hollmann *et al.* 1991; Jonas and Burnashev 1995) and most probably mediated the calcium influx that supported integrin trafficking. The great majority of native, neuronal AMPA receptors contain a GluR2 subunit and, consequently, do not pass calcium (Jonas and Burnashev 1995). Thus, if neuronal integrin trafficking is similarly dependent on calcium influx, the activities of native AMPA receptors may regulate integrin trafficking, at least in part, through depolarization-induced increases in calcium influx via voltage-gated calcium channels and NMDA receptors. To test for involvement of these secondary channels in integrin trafficking, neurons were treated with AMPA alone or with AMPA in the presence of the NMDA receptor antagonist APV and the L-type voltage-sensitive calcium channel (VSCC) blocker Nif. As shown in blots from a representative experiment in Figs 8(a) and (b), treatment with AMPA alone induced clear increases in $\alpha 5$ and $\beta 1$ membrane insertion (Fig. 8a) and steady-state membrane levels (Fig. 8b). These increases were blocked with AMPA applied in the presence of APV and Nif, whereas treatment with APV and Nif alone did not alter either measure of surface expression.

Quantification of western blot band densities across three such experiments demonstrated that effects of treatment on $\alpha 5$ insertion were statistically significant ($p = 0.046$, $p < 0.05$ and $p = 0.053$ for effects of AMPA, APV/Nif, and an interaction between AMPA and APV/Nif respectively, two-way ANOVA; $p < 0.05$ for control vs. AMPA and for AMPA vs. AMPA + APV/Nif, SNK test). For $\beta 1$ there were significant interactions between effects of AMPA and APV/Nif treatment on membrane insertion ($p = 0.042$) although, with the small number of cases, *post hoc* tests did not identify significant group differences. Effects of treatments were more robust on measures of total, steady-state surface expression (Fig. 8d). There were significant effects of AMPA treatment on both $\alpha 5$ and $\beta 1$ ($p = 0.046$ and $p = 0.038$ respectively, two-way ANOVA; $p < 0.01$ and $p < 0.05$ for control vs. AMPA and for AMPA vs. AMPA + APV/Nif for $\alpha 5$ and $\beta 1$ respectively, SNK test). For $\alpha 5$, interactions between AMPA and APV/Nif treatment were also significant ($p = 0.012$ and $p = 0.06$ for interactions between AMPA and APV/Nif on $\alpha 5$ and $\beta 1$ steady-state membrane levels respectively).

To test whether NMDA receptor stimulation alone is sufficient to increase integrin trafficking to the membrane, NMDA (50 μM , 10 min) was applied in the presence of the AMPA receptor antagonist DNQX. These experiments were conducted in magnesium-free medium and with 200 μM glycine added to the NMDA treatment cocktail to alleviate the voltage-dependent magnesium block and to facilitate activation of the NMDA receptors respectively. As shown in the two righthand lanes of Figs 8(a) and (b), under these conditions NMDA application did not increase either new membrane insertion or steady-state membrane levels of the $\alpha 5$ or $\beta 1$ integrin subunits. Quantification of western blots found no significant effects of NMDA on either measure of $\alpha 5$ or $\beta 1$ surface expression (p -values ranged from 0.24 to 0.93 across comparisons, paired two-tailed t -test, $n = 3$ per group).

To determine whether AMPA treatment effects on integrin trafficking are dependent on NMDA receptor and VSCC activities in more mature cells embedded in an organotypic neuropil, the above studies were repeated using cultured hippocampal slices. Measures of western blots, evaluating AMPA treatment effects on new membrane insertion and steady-state membrane levels of $\alpha 5$ and $\beta 1$ integrin, are presented in Figs 8(e) and (f) respectively (values are mean \pm SEM for three independent experiments). As with cultured neurons, in hippocampal slices AMPA treatment increased membrane insertion of $\alpha 5$ and $\beta 1$ (Fig. 8e) and these increases were blocked by APV/Nif co-treatment (for $\beta 1$ $p = 0.001$ for AMPA treatment and interactions between AMPA and APV/Nif treatments, for $\alpha 5$ interactions between AMPA and APV/Nif were not significant, two-way ANOVA; $p < 0.05$ and $p < 0.01$ for $\alpha 5$ and $\beta 1$ respectively for *post hoc* comparisons of control vs. AMPA and for AMPA vs. AMPA + APV/Nif, SNK test). Treatment effects were also robust for effects on steady-state membrane expression: AMPA treatment significantly increased total $\alpha 5$ and $\beta 1$ surface expression and these effects were completely blocked by APV/Nif (for $\alpha 5$ and $\beta 1$ respectively $p = 0.028$ and $p = 0.008$ for effects of AMPA treatment, and $p = 0.03$ and $p = 0.0043$ for interactions between AMPA and APV/Nif treatment, two-way ANOVA; for $\alpha 5$ and $\beta 1$ $p < 0.01$ and $p < 0.001$ respectively for comparison of control vs. AMPA and of AMPA vs. AMPA + APV/Nif, SNK test) (Fig. 8f). As found with experiments using dissociated neurons, in cultured hippocampal slices NMDA treatment in the presence of DNQX did not increase new insertion or steady-state membrane levels of $\alpha 5$ or $\beta 1$ integrin (p -values ranged from 0.56 to 0.94 across comparisons, paired two-tailed t -test).

Discussion

Studies of synaptic plasticity in hippocampus indicate that integrins (Staubli *et al.* 1990, 1998; Bahr *et al.* 1997; LeBaron *et al.* 2003), particularly the $\alpha 5\beta 1$ integrin (Chun *et al.* 2001; Chan *et al.* 2003), are critical for stabilization of LTP at glutamatergic synapses. Although the mechanisms of integrin involvement in LTP are still unclear, recent work has

shown that integrin ligands activate kinases (Kramár *et al.* 2003; Bernard *et al.* 2005) implicated in LTP (Ali and Salter 2001; Huang *et al.* 2001; Poncer *et al.* 2002; Yang *et al.* 2003) and trigger phosphorylation of both AMPA and NMDA receptors (Kramár *et al.* 2003; Bernard *et al.* 2005), including phosphorylation of a GluR1 site (Kramár *et al.* 2003) that becomes phosphorylated with LTP (Barria *et al.* 1997). These phosphorylation events are accompanied by increases in AMPA and NMDA receptor-gated synaptic currents (Kramár *et al.* 2003; Lin *et al.* 2003; Bernard *et al.* 2005). Although these results provide evidence for an influence of synaptic integrins on neighboring glutamate receptors, they leave open the question of how intense stimulation of these neurotransmitter receptors (the induction event for LTP) might engage integrin activities. The present study examined one possibility, namely that AMPA receptor stimulation causes the insertion of additional LTP-related integrins into the cell membrane, resulting in new ligation and signaling. The results demonstrate that stimulation of GluR1 homomeric AMPA receptors in GluR1/COS7 cells increased (1) $\alpha 5$ and $\beta 1$ integrin membrane insertion, (2) adhesion to fibronectin- and anti- $\alpha 5$ -coated substrates, and (3) kinase signaling upon attachment to a fibronectin-coated substrate. Similar AMPA-induced increases in new membrane insertion and total surface expression of the $\alpha 5$ and $\beta 1$ integrin subunits were obtained in studies with dissociated neurons and cultured hippocampal slices. Other work in our laboratory and elsewhere has shown that in differentiated neurons integrin proteins are localized, and in the instance of $\alpha 5$ concentrated, at synapses (Nishimura *et al.* 1998; Rodriguez *et al.* 2000). Together these results provide evidence that glutamate receptor activation drives new integrins into the membrane and suggest that synaptic glutamate receptor activation is likely to elicit a similar increase in integrin exposure and function with the induction of LTP.

It should be noted that these stimulatory effects on integrin trafficking probably occur amidst other changes in synaptic adhesive chemistries that play a role in LTP (Benson *et al.* 2000). In addition to integrins, cadherin and immunoglobulin class adhesion receptors have been localized to the synaptic or parasynaptic region and implicated in LTP (Tang *et al.* 1998; Murase and Schuman 1999; Benson *et al.* 2000; Bukalo *et al.* 2004), and there is evidence that the functional representation of these receptors is modulated by intense glutamatergic and/or synaptic activity. The cadherins increase their adhesivity with *cis*-dimerization and the assumption of a rigid, rod-like structure (Tanaka *et al.* 2000). In neurons and hippocampal slices depolarization and the induction of LTP rapidly (within 1 min) increases cadherin dimerization, protease resistance and adhesive strength, suggesting the recruitment of pre-existing receptors to an activated state (Bozdagi *et al.* 2000; Tanaka *et al.* 2000; Huntley *et al.* 2002). Studies of the immunoglobulin class neural cell adhesion molecule (NCAM) have shown that LTP-inducing stimulation activates proteases that cleave the extracellular domain of the receptor (Hoffman *et al.* 1998a, 1998b; Endo *et al.* 1999). This may be linked with increased receptor turnover; depolarization of cultured neurons reportedly increases trafficking of polysialylated-NCAM to the membrane (Kiss *et al.* 1994), although studies of more mature neurons in cultured hypothalamic slices described constitutive polysialylated NCAM trafficking that was not modulated by glutamate receptor or calcium channel activities (Pierre *et al.* 2001).

Time courses of LTP stabilization and increases in surface integrins are comparable

The idea that AMPA receptor-induced increases in integrin surface expression are involved in LTP stabilization is reinforced by the good alignment between the temporal parameters of these effects (Chun *et al.* 2001; Gall and Lynch 2004). After induction by afferent stimulation, LTP can be reversed by various manipulations (e.g. transient anoxia, adenosine, low-frequency stimulation) for about 30 min, with the degree of LTP reversal declining with greater poststimulus latencies during this interval (Arai *et al.* 1990; Staubli and Chun 1996; Huang *et al.* 1999). Studies in our laboratories using ligand peptides, neutralizing antibodies and potent

disintegrins antagonists to manipulate integrin function (Bahr *et al.* 1997; Staubli *et al.* 1998; Chun *et al.* 2001; Kramár *et al.* 2002) have shown that integrins are important for LTP stabilization for about 20–30 min after induction, with the magnitude of effects on potentiation declining over this interval. Applying integrin antagonists before induction does not increase the level of disruption and applying them 30 min or more after induction does not disturb potentiation. However, using lower doses of integrin ligands, LeBaron *et al.* (2003) described integrin involvement in LTP extending only a few minutes after stabilization. Our results lead to the suggestion that integrins become involved in the potentiation phenomenon after the initial induction phase, and are engaged, or initiate processes that are engaged, in LTP stabilization through a period of about 30 min. In GluR1/COS7 cells, AMPA-induced increases in integrin surface expression became evident within 3–5 min; with pulse treatment increases were maximal at 5–8 min and declined slowly thereafter. Thus, increases in surface expression are maximal at latencies of predicted maximal integrin involvement in LTP stabilization and decline slowly over a time period in which there is clear but diminishing requirement for integrins in the consolidation of synaptic plasticity.

In our studies, continuous AMPA treatment led to protracted increases in integrin surface expression, whereas pulse treatment led to a more transient response particularly for the $\alpha 5$ subunit. The fact that increases in insertion and steady-state levels followed each other quite closely suggests that AMPA treatment did not stimulate comparable increases in integrin endocytosis which, had they been present, could have maintained surface integrin immunoreactivities closer to control levels despite new insertion. It is noteworthy that the two subunits showed somewhat different time courses of response, most particularly with pulse treatment. This is not unexpected given that $\beta 1$ integrin is predicted to be included in several different integrin dimers ($\beta 1$ pairs with $\alpha 1$ –8 and v) that might have different responses to AMPA treatment. In contrast, the $\alpha 5$ subunit pairs with $\beta 1$ alone (Hynes 1992, 1999). Integrin heterodimers form in the ER before export to the plasma membrane (Cheresh and Spiro 1987); thus trafficking of $\alpha 5$ to the membrane provides an index for movements of the $\alpha 5\beta 1$ dimer specifically.

AMPA pretreatment increased integrin adhesive and signaling functions

Ligands for $\alpha 5\beta 1$ include fibronectin, fibrinogen and LI (Ruppert *et al.* 1995; Plow *et al.* 2000; Milner and Campbell 2002). Tests of AMPA treatment effects on $\alpha 5\beta 1$ integrin function focused on responses to fibronectin- or anti- $\alpha 5$ -coated substrates. AMPA stimulation of GluR1/COS7 cells increased their adhesion to both substrates; this result is consistent with there being greater numbers of functional (i.e. activated) $\alpha 5\beta 1$ integrins at the cell surface of AMPA pretreated compared with control cells. AMPA pretreatment effects were greater for adhesion to anti- $\alpha 5$ than to fibronectin. This difference may be due to the involvement of integrins other than $\alpha 5\beta 1$ in adhesion to fibronectin. Although $\alpha 5\beta 1$ is known as the ‘fibronectin receptor’, this matrix protein is a ligand for several other integrins including $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 8\beta 1$ and $\alpha v\beta 1$ (Plow *et al.* 2000; Milner and Campbell 2002). It is possible that one or more of these other integrin dimers contributed to fibronectin adhesion of GluR1/COS7 cells but were unaffected by AMPA treatment. Alternatively, differences in the magnitude of AMPA effects on adhesion to the different substrates may be related to the strength of attachment to anti- $\alpha 5$ compared with fibronectin and hence to the efficiency of the assay with use of different attachment substrates.

Measures of AMPA treatment effects on levels of phosphoproteins known to be influenced by integrin signaling reinforced the conclusion that AMPA treatment increased numbers of functional integrins at the cell surface. Although integrins lack kinase activity, upon ligand binding or clustering these receptors stimulate increases in tyrosine and serine kinase signaling (Zhu and Assoian 1995; Vuori 1998; Giancotti and Ruoslahti 1999; Yamada and Even-Ram

2002). These cascades typically include increases in phosphorylation of FAK and paxillin that bind integrin proteins directly (Schaller *et al.* 1995), Src family kinases associated with activated FAK and the FAK homologue Pyk2 (Parsons *et al.* 2000) and, further downstream, p44/42MAPK (Zhu and Assoian 1995). Therefore, to test whether AMPA-induced increases in $\alpha 5\beta 1$ integrin surface expression subserve increased integrin signaling function, the effects of fibronectin attachment on levels of p-FAK, p-Src, p-paxillin and p-p44/42MAPK were evaluated using phospho-specific antisera to the activation sites for FAK and Src, to a tyrosine within the SH2 binding domain for paxillin, and to the Thr-Glu-Tyr motif for p44/42MAPK (Hanks and Hunter 1995). The results demonstrated that adhesion to fibronectin elicited significantly larger increases in relative levels of each of these phosphoproteins in AMPA-pretreated compared with naive cells; similar AMPA pretreatment effects were not evident in cells remaining in suspension. These results support the conclusion that integrins, driven into the membrane by AMPA treatment, are active and mediate increased kinase signaling upon contact with ligand (i.e. the fibronectin substrate). The generally low phosphoprotein levels in suspended cells, either pretreated with AMPA or not, indicates that these phosphorylation events are adhesion dependent and not induced by AMPA treatment alone.

AMPA treatment effects on surface integrins depend on exocytotic and PKC activities

The most likely explanation for the present results is that AMPA stimulation resulted in translocation of integrins from the ER and/or endocytotic vesicles into the plasma membrane. Although relatively little is known of the precise mechanisms regulating integrin membrane insertion, results from other systems suggest that conventional BFA-sensitive exocytotic processes and PKC are likely to be involved. For example, studies of blastocyst implantation have shown that ligand-induced up-regulation of integrin-mediated adhesion to fibronectin is blocked by both BEA and antagonists of PKC but is unaffected by the protein synthesis inhibitor cycloheximide (Schultz and Armant 1995; Wang *et al.* 2002); in this system increased adhesion is also associated with a redistribution of integrins to the membrane and a BFA-sensitive clearance of vesicles from below the plasma membrane surface (Schultz and Armant 1995). These combined results suggest that rapid increases in integrin adhesion can be mediated by PKC- and BFA-sensitive mobilization of receptors from ER-derived transport vesicle fusion. Studies of migrating cells indicate that integrins are also trafficked to and from the surface membrane via an endocytotic recycling pathway (Maxfield 1995; Pierini *et al.* 2000); these studies have implicated PKC in both membrane insertion and internalization (Ng *et al.* 1999; Ivaska *et al.* 2002) although, in some cases, this endocytotic integrin cycling was found to be insensitive to BFA (Ng *et al.* 1999). To test whether similar mechanisms mediated increased integrin surface expression and signaling in the present studies, the effects of the PKC antagonist CCI and BFA were examined. CCI blocked both AMPA-induced increases in new $\alpha 5$ and $\beta 1$ integrin membrane insertion, as determined by the surface protein biotinylation assay, and enhancement of fibronectin adhesion-induced signaling by AMPA pretreatment. The first of these observations provides the most direct evidence for PKC involvement in $\alpha 5$ and $\beta 1$ integrin trafficking whereas the latter probably reflects increased membrane insertion as well as potential PKC-mediated increases in integrin ligand affinity (Kolanus and Seed 1997; Hughes and Pfaff 1998).

BFA also blocked AMPA effects on new membrane insertion of the $\alpha 5$ and $\beta 1$ integrin proteins, indicating that conventional mechanisms of exocytosis of products from the Golgi-ER network, which are blocked by BFA (Klausner *et al.* 1992; Shah and Klausner 1993), are required for the AMPA-induced mobilization of $\alpha 5\beta 1$ integrin to the cell surface.

The observation that AMPA-induced increases in integrin surface expression depend on extracellular calcium in GluR1/COS7 cells accords with evidence from studies of integrin ligand effects on adhesion, i.e. that increases in intracellular calcium provide the effective

trigger for PKC- and BEA-dependent increases in integrin trafficking to the membrane (Maxfield 1995; Pierini *et al.* 2000). Indeed, in trophoblasts treatment with the calcium ionophore ionomycin mimics the effects of fibronectin contact in stimulating integrin trafficking and increased adhesive function (Wang *et al.* 2002). Similar calcium-dependent exocytotic processes may have mediated increased integrin surface expression and function in both GluR1/COS7 and neuronal preparations in the present studies. The GluR1/COS7 cells used here expressed GluR1 homomeric receptors (Verdoorn *et al.* 1991) that flux calcium upon activation (Hollmann *et al.* 1991; Jonas and Burnashev 1995). Therefore, AMPA treatment of the GluR1/COS7 cells would be expected to have increased calcium influx and intracellular calcium content, and the latter might have been sufficient to trigger integrin trafficking to the membrane. Mechanisms subserving the observed AMPA-induced increases in integrin surface expression in neurons and in cultured hippocampal slices were undoubtedly somewhat different because the great majority of native, neuronal AMPA receptors contain a GluR2 subunit that prevents calcium influx (Hollmann *et al.* 1991; Jonas and Burnashev 1995). However, in neurons AMPA receptor-mediated depolarization would be expected to alleviate the voltage block from NMDA receptors and VSCCs that, in turn, could support calcium influx and mobilization of integrins to the synaptic membrane. Tests of this possibility verified that in both neuronal preparations AMPA-induced increases in $\alpha 5$ and $\beta 1$ surface expression are indeed dependent on NMDA receptor and/or L-type VSCC activities. The fact that NMDA receptor activation alone did not increase membrane insertion or surface expression suggests that AMPA effects on trafficking depend (1) primarily on VSCC-mediated calcium influx or (2) on a coordination of events including calcium influx and other, yet to be identified, consequences of AMPA receptor activation. Future studies will test these possibilities.

Regardless of precise mechanism, the effects of AMPA stimulation on integrin trafficking described here suggest that intense, activity-driven AMPA receptor stimulation will similarly mobilize integrins to the synaptic membrane. Considered more broadly, these results suggest that with high-frequency or repeated-burst afferent stimulation, as used to elicit LTP, AMPA receptor activation will lead to local increases in intracellular calcium that may, in turn, drive synapse-specific insertion of integrins into the synaptic membrane and recruitment of new integrin signaling into processes mediating the consolidation of LTP.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionate
APV	DL-2-Amino-5-phosphonovaleric acid
BFA	brefeldin A
CCI	chelerythrine chloride
DMEM	Dulbecco's modified Eagle's medium

DNQX	6,7 dinitroquinoxaline-2,3-dione
ER	endoplasmic reticulum
FAK	focal adhesion kinase
FBS	fetal bovine serum
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase p42/44
NCAM	neural cell adhesion molecule
NHS	N-hydroxy-succinimide
Nif	nifedipine
NMDA	<i>N</i> -methyl-D-aspartate
PBS	phosphate-buffered saline
PKC	protein kinase C
Pyk2	proline-rich tyrosine kinase 2
SNK	Student-Newman-Keuls
VSCC	voltage-sensitive calcium channel

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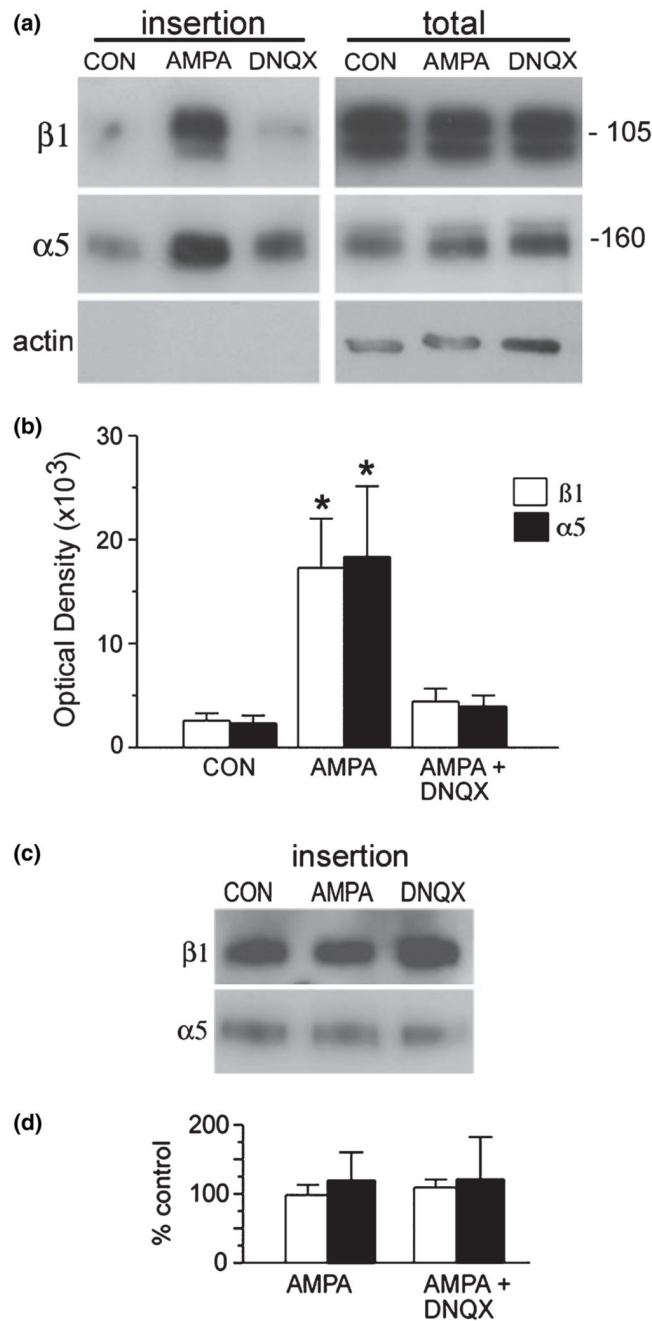
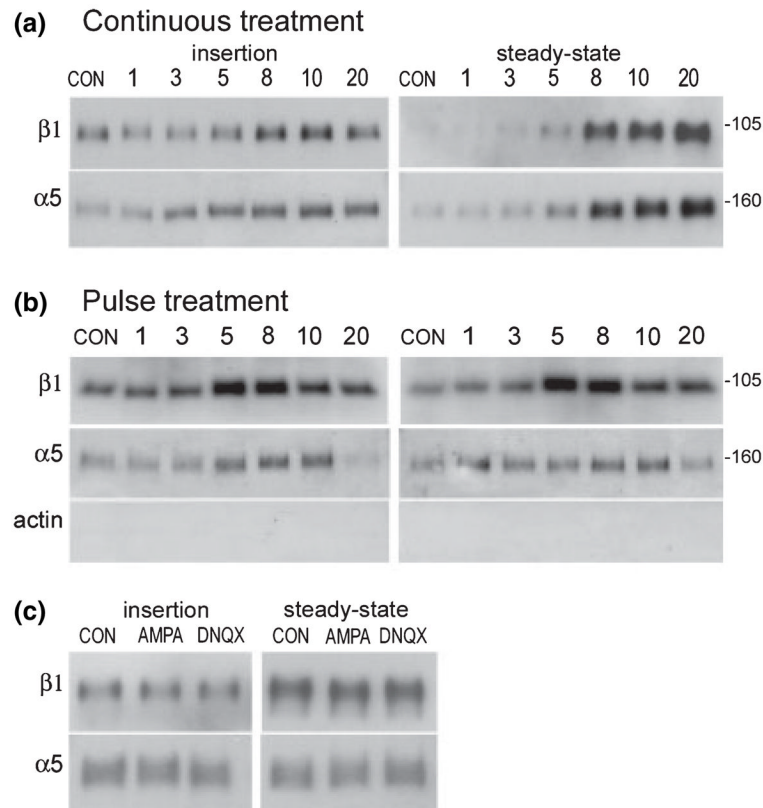
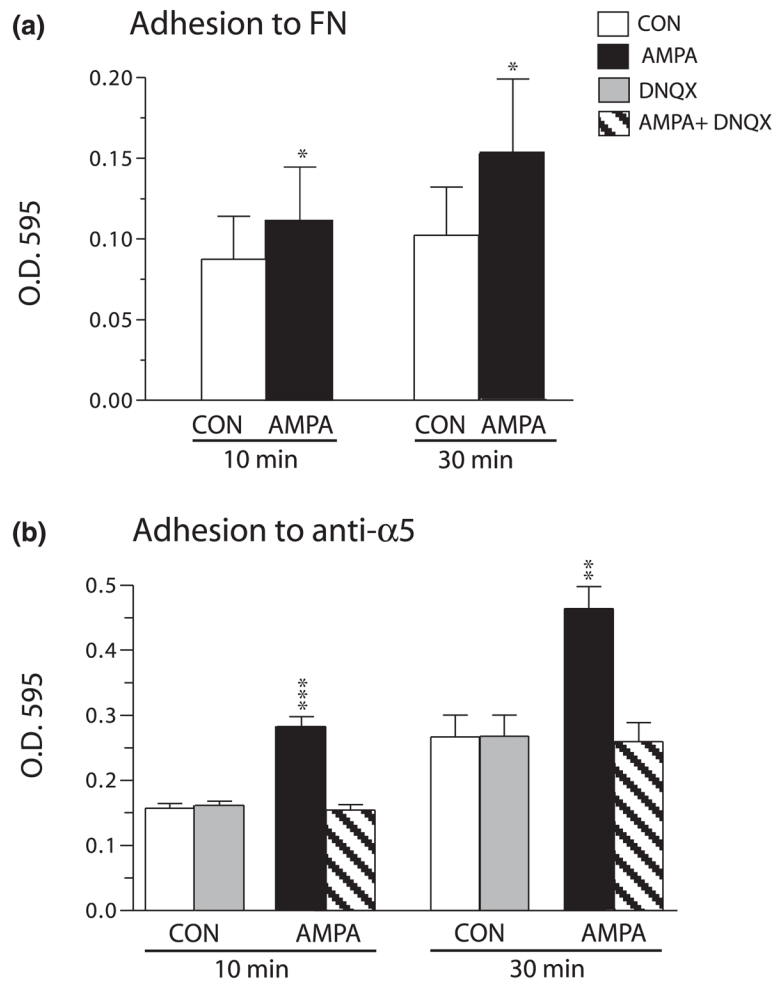


Fig. 1. AMPA stimulation increases surface expression of $\beta 1$ and $\alpha 5$ in GluR1/COS7 cells. GluR1-transfected COS7 cells were pretreated with sulfo-NHS-acetate then incubated for 10 min in the absence (control; CON) or presence (AMPA) of 50 mM AMPA. Parallel sets of cells were treated with DNQX before and during AMPA treatment. Newly exposed cell surface proteins were then biotinylated and precipitated, and western blots were used to determine $\alpha 5$ and $\beta 1$ levels in the biotinylated fraction ('insertion' samples) and the total cell lysate ('total' samples), (a) Western blots show immunoreactivity within the insertion (left) and total lysate (right) pools for a single experiment. AMPA induced large increases in newly inserted $\alpha 5$ and $\beta 1$ immunoreactivities that were blocked by DNQX co-treatment. Blots on the right show that

AMPA and AMPA + DNQX (DNQX lane) had no obvious effect on total $\alpha 5$ or $\beta 1$ protein levels. Lower blots in each column show levels of actin immunoreactivity. (b) Bar graph shows quantification of newly inserted $\alpha 5$ and $\beta 1$ immunoreactivities as determined from densitometric analysis of western blots (mean \pm SEM optical density values; $n = 4$). AMPA treatment reliably increased $\alpha 5$ and $\beta 1$ membrane insertion ($p = 0.01$, one-way ANOVA; $*p < 0.05$ versus CON, SNK test) that was blocked by DNQX. (c) Western blot shows levels of newly inserted $\beta 1$ and $\alpha 5$ immunoreactivities in non-transfected COS7 cells treated with normal medium (CON), 50 μM AMPA or AMPA + DNQX. AMPA treatment did not increase surface expression in non-transfected cells, (d) Bar graph shows quantification of newly inserted $\alpha 5$ and $\beta 1$ immunoreactivities in non-transfected cells treated with AMPA or AMPA + DNQX (expressed as percentage of paired CON values; mean \pm SEM; $n = 3$).

**Fig. 2.**

AMPA-induced increases in integrin surface expression: time course and calcium dependence. (a,b) GluR1/COS7 cells were treated with 50 μ M AMPA continuously for 1–20 min (a) or for 1 min and then harvested from 1 to 20 min after treatment onset (b) and processed for surface biotinylation/western blot analysis of new membrane ‘insertion’ (left panel) and ‘steady-state’ surface expression (right panel) of $\beta 1$ and $\alpha 5$ immunoreactivities present as bands at 105 kDa and 160 kDa respectively. (Note, blot for the continuous-treatment steady-state assay was exposed to film more briefly than the others to avoid saturation of labeling at the later time points. Consequently low levels of surface $\beta 1$ in control samples are not evident in the panel shown). With both continuous and pulse AMPA treatment, increases in newly inserted and steady-state $\alpha 5$ and $\beta 1$ surface expression were evident in 5–8 min; with pulse AMPA application, new insertion and steady-state levels then declined, (c) GluR1/COS7 cells in calcium-free medium were treated with 50 μ M AMPA for 10 min and processed for either the new membrane insertion or steady-state surface protein assay. In the absence of extracellular calcium AMPA treatment had no effect on $\alpha 5$ or $\beta 1$ membrane insertion or steady-state surface levels.

**Fig. 3.**

AMPA treatment increases GluR1/COS7 cell adhesion to fibronectin- and anti- $\alpha 5$ -coated substrates. GluR1/COS7 cells were treated for 10 min, plated on to either fibronectin (FN)-coated (a) or anti- $\alpha 5$ integrin-coated (b) substrates, and adherent protein levels were assessed at 10 and 30 min after plating. Graphs show mean \pm SEM values for six and three experiments in (a) and (b) respectively, (a) AMPA treatment increased GluR1/COS7 adhesion to FN at both 10 and 30 min after plating [at 10 min: $*p < 0.03$ versus control (CON) (one-tailed paired *t* test; correlation coefficient = 0.98, effectiveness of pairing $p = 0.004$); at 30 min: $*p = 0.019$ versus CON (correlation coefficient = 0.98, effectiveness of pairing $p = 0.0002$)]. (b) AMPA treatment increased adhesion to anti- $\alpha 5$ -coated substrate ($p < 0.05$ and $p < 0.001$ for main effect of treatment, separate one-way ANOVA analyses for 10- and 30-min time points respectively; $**p < 0.01$ and $***p < 0.001$ versus yoked control values, SNK *post hoc* test).

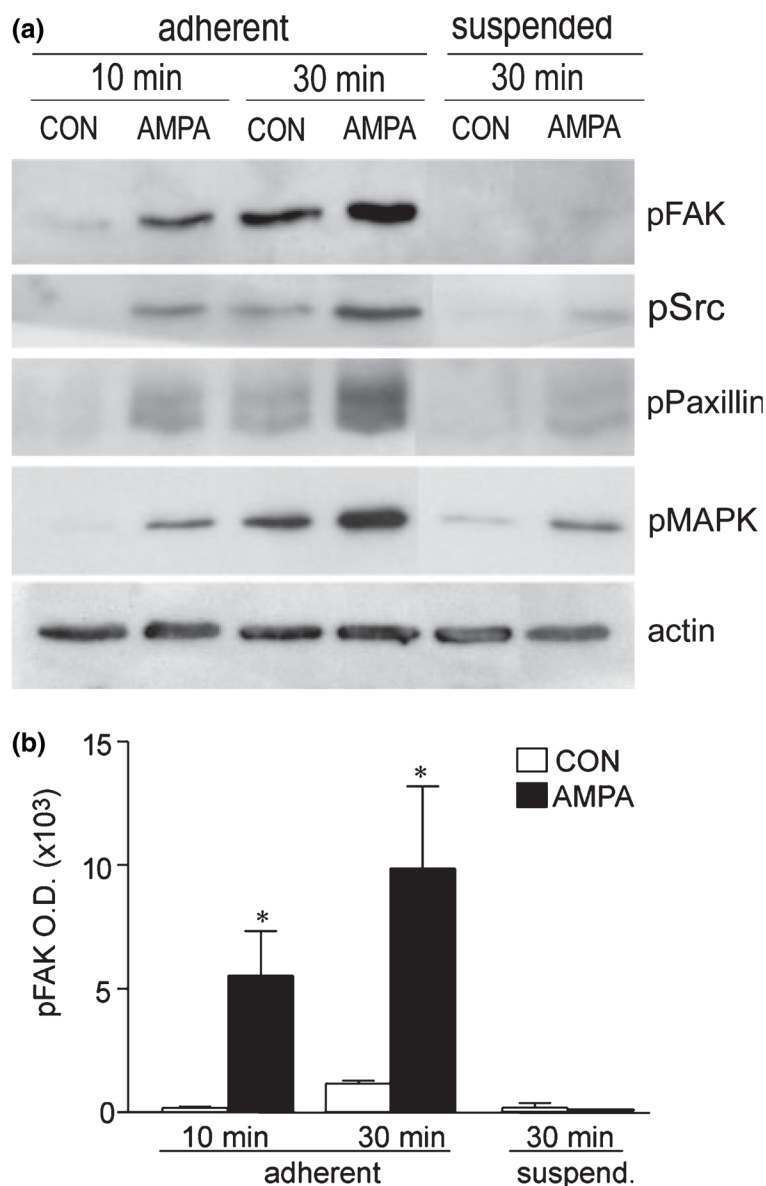


Fig. 4. AMPA pretreatment increases adhesion-induced signaling. GluR1/COS7 cells were treated with normal medium (CON) or 50 μ M AMPA for 10 min then retained in suspension or plated on to fibronectin for 10 or 30 min. Cell lysates were processed for western blot analyses using phospho (p)-specific antibodies to p-FAK (Y397), p-Src (Y418), p-paxillin (Y31) and p-p44/42MAPK (T202/Y204). Aliquots were also assessed for actin immunoreactivity to verify comparable sample protein content, (a) Blots show results for one experiment (aliquots from the same samples, all rows). For adherent cells, levels of p-FAK, p-Src, p-paxillin and p-pMAPK were all higher in AMPA-pretreated (AMPA) than in control (CON) samples at 10 and 30 min after plating. In contrast, phospho-protein levels were extremely low in suspended cells although p-p44/42MAPK was more abundant in AMPA-pretreated than CON cells, (b) The same pattern of results was obtained across four experiments as illustrated by the quantification of p-FAK band densities (mean \pm SEM; $n = 4$ per group). AMPA pretreatment significantly increased adhesion-induced p-FAK levels at 10 and 30 min after plating ($*p < 0.05$).

0.05 vs. CON, one-tailed paired Mest). ANOVA of 30-min values (among adherent and suspended cells) demonstrated significant effects of AMPA ($p < 0.05$) and adhesion ($p < 0.01$), and significant interactions between the two groups ($p < 0.05$).

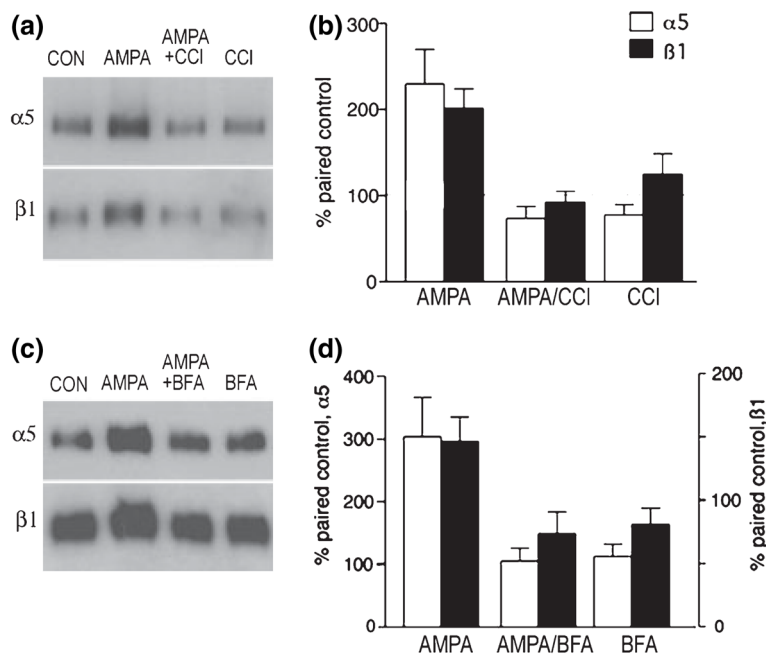


Fig. 5.

CCI and BFA block AMPA-induced increases in $\alpha 5$ and $\beta 1$ integrin membrane insertion. GluR1/COS7 cells were pretreated with membrane-impermeable sulfo-NHS-acetate and then treated with normal medium (CON), 50 μ M AMPA for 10 min, 500 nM CCI or 10 ng/mL BFA for 30 min before and during AMPA treatment, or CCI or BFA alone for 40 min. Newly exposed cell surface proteins were then biotinylated, precipitated, and processed for western blot analysis of $\alpha 5$ and $\beta 1$ levels, (a) Blots from one experiment using CCI demonstrate that AMPA alone induced marked increases in biotinylated (i.e. newly inserted) $\alpha 5$ and $\beta 1$ levels that were blocked by CCI co-treatment, (b) Quantification of band densities for the four treatment groups in three experiments (mean \pm SEM) demonstrates that CCI co-treatment blocked AMPA-induced increases in newly inserted $\alpha 5$ and $\beta 1$ levels (AMPA vs. CON, $p < 0.01$ for both $\alpha 5$ and $\beta 1$; other groups not significantly different; SNK test), (c) Blot from one experiment showing that AMPA treatment increased levels of newly inserted $\alpha 5$ and $\beta 1$ levels that were blocked by BFA co-treatment, (d) Graph showing mean band densities in two experiments using BFA.

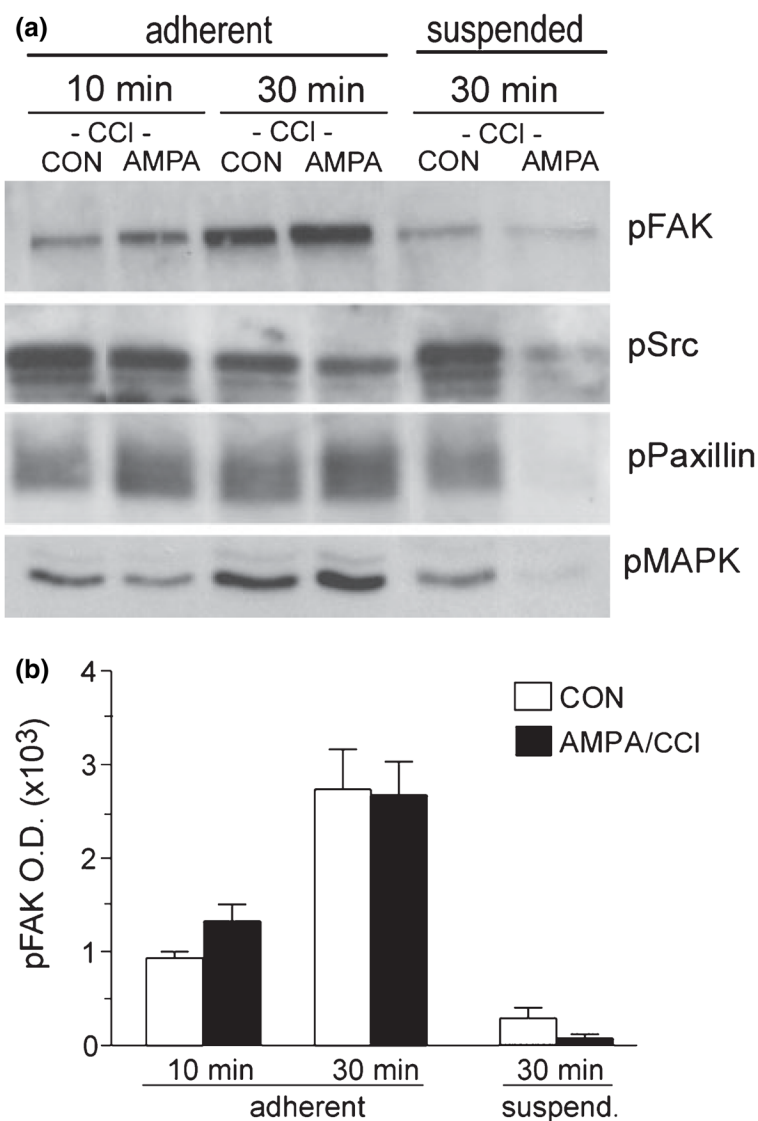


Fig. 6. CCI blocks AMPA enhancement of adhesion-induced signaling. GluR1/COS7 cells were treated with CCI (500 nM) 30 min before and during treatment with AMPA (50 μ M, 10 min) or normal medium (CON), and then processed for western blot analysis of adhesion-induced increases in phosphoprotein levels at 10 and 30 min after plating on a fibronectin substrate ('adherent' groups) or after 30 min in suspension ('suspended' groups), (a) Western blots show levels of phospho (p)-FAK (Y397), p-Src (Y418), p-paxillin (Y31) and p-p44/42MAPK (T202/Y204) in samples from an individual experiment. In the presence of CCI, AMPA treatment only very modestly enhanced the adhesion-induced increase in p-FAK and p-paxillin at 10 and 30 min after plating. CCI eliminated AMPA enhancement of adherent cell p-Src and p-p44/42MAPK levels, (b) Bar graph shows quantification of p-FAK band densities across four experiments (mean \pm SEM). CCI blocked the effects of AMPA but not of substrate adhesion on levels of this phosphoprotein (for 30-min groups, $p < 0.001$ for effects of adhesion; effects of AMPA not significant, interaction between AMPA and adhesion not significant; two-way ANOVA).

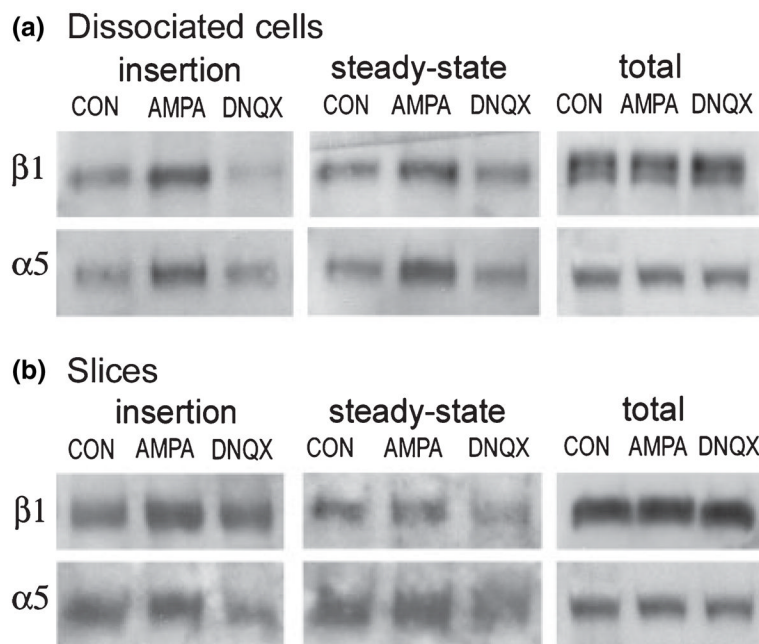
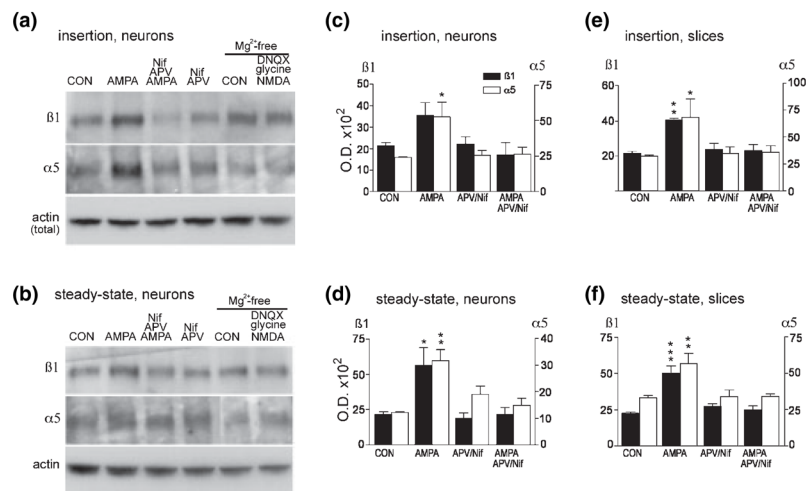


Fig. 7. AMPA stimulation increases neuronal $\beta 1$ and $\alpha 5$ integrin surface expression. Dissociated cortical/hippocampal neurons (a) and cultured hippocampal slices (b) were treated for 10 min with normal medium (CON), 100 μM AMPA, or AMPA plus 40 μM DNQX (DNQX lanes), and $\alpha 5$ and $\beta 1$ immunoreactivities were measured in the newly inserted membrane protein (left panels), steady-state surface protein (middle panels) and total cell lysate (right panels), (a) Western blots from a representative dissociated cell experiment show that, relative to control levels, AMPA treatment induced increases in new membrane insertion and steady-state membrane levels of $\alpha 5$ and $\beta 1$ integrin immunoreactivities that were blocked by DNQX co-treatment. Blots in the 'total' column show that AMPA and AMPA + DNQX had no obvious effect on total integrin protein levels, (b) Western blots show that in cultured hippocampal slices AMPA treatment increased $\beta 1$ and $\alpha 5$ membrane insertion and $\alpha 5$ steady-state surface expression. For both subunits, DNQX attenuated or blocked increases in insertion and steady-state surface expression. AMPA and AMPA + DNQX had no effect on total $\beta 1$ and $\alpha 5$ protein levels in cultured slices.

**Fig. 8.**

Activity of AMPA receptors, NMDA receptors and L-type VSCCs is required for full AMPA-induced increases in neuronal $\beta 1$ and $\alpha 5$ integrin surface expression. Dissociated cortical/hippocampal neurons were treated for 10 min with normal medium (CON), 100 μM AMPA, AMPA plus 50 μM APV and 10 μM Nif, or APV plus Nif only. $\alpha 5$ and $\beta 1$ immunoreactivities in the newly inserted and steady-state surface protein pools were evaluated. Western blots from a representative dissociated cell experiment show that, relative to control levels, AMPA increased $\alpha 5$ and $\beta 1$ integrin protein levels in both new membrane insertion (a) and steady-state (b) surface protein pools and that these increases were blocked by APV and Nif co-treatment. APV/Nif treatment alone did not clearly influence surface $\alpha 5$ and $\beta 1$ levels. Right two lanes in (a) and (b) show samples from parallel sets of cells placed in Mg^{2+} -free medium and then treated with normal medium (CON) or with a cocktail of 50 μM NMDA, 200 μM glycine and 40 μM DNQX for 10 min, and processed for new membrane insertion and steady-state surface protein assays. NMDA treatment did not increase integrin protein levels in either new insertion or steady-state surface pools. In (a) and (b) lower panels show that levels of actin immunoreactivity were comparable in aliquots removed from total cell lysates before execution of insertion and steady-state assays, (c–f) Quantification of AMPA effects on $\alpha 5$ and $\beta 1$ new membrane insertion (c,e) and steady-state surface levels (d,f) in primary neuronal cultures (c,d) and cultured hippocampal slices (e,f). Values were obtained by den-sitometric analysis of western blots and are mean \pm SEM of $n = 3$ per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus CON (SNK test).