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Integrin regulation of cytoplasmic calcium in excitatory neurons depends upon glutamate receptors and release from intracellular stores

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Abstract

Integrins regulate cytoplasmic calcium levels ($[Ca^{2+}]_i$) in various cell types but information on activities in neurons is limited. The issue is of current interest because of evidence that both integrins and changes in $[Ca^{2+}]_i$ are required for Long-Term Potentiation. Accordingly, the present studies evaluated integrin ligand effects in cortical neurons. Integrin ligands or $\alpha 5\beta 1$ integrin activating antisera rapidly increased $[Ca^{2+}]_i$ with effects greater in glutamatergic than GABAergic neurons, absent in astroglia, and blocked by $\beta 1$ integrin neutralizing antisera and the tyrosine kinase antagonist genistein. Increases depended upon extracellular calcium and intracellular store release. Ligand-induced effects were reduced by voltage-sensitive calcium channel and NMDA receptor antagonists, but blocked by tetrodotoxin or AMPA receptor antagonists. These results indicate that integrin ligation triggers AMPA receptor/depolarization-dependent calcium influx followed by intracellular store release and suggest the possibility that integrin modulation of activity-induced changes in $[Ca^{2+}]_i$ contributes importantly to lasting synaptic plasticity in forebrain neurons.

Keywords

AMPA receptor; NMDA receptor; adhesion; synaptic plasticity; $\beta 1$ integrin; fibronectin; calcium currents

Introduction

Integrins are transmembrane, heterodimeric ($\alpha\beta$) receptors that mediate cell-matrix and cell-cell adhesion throughout the body. Although integrins lack direct kinase activity, ligand binding initiates conformational changes and signaling through tyrosine kinase and Rho GTPase initiated cascades (Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002; DeMali et al., 2003; Bernard-Trifilo et al., 2005). Recent evidence indicates that brain integrins are concentrated at synapses, modulate excitatory transmission (Kramár et al., 2003), and play a critical role in the stabilization of Long-Term Potentiation (LTP) (Staubli et al., 1990; Staubli et al., 1998; Chun et al., 2001; Chan et al., 2006; Huang et al., 2006; Kramár et al., 2006; Nagy

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et al., 2006; Meighan et al., 2007; Wang et al., 2007), a form of synaptic plasticity widely thought to underlie learning (Morris, 2003; Lynch et al., 2007).

How brain integrins contribute to the production of stable LTP is poorly understood. Most of the integrins expressed in brain recognize an Arg-Gly-Asp (RGD) consensus sequence in their native ligands (e.g., fibronectin, vitronectin, others) (Pinkstaff et al., 1999; Plow et al., 2000). Treatment of mature hippocampal slices or synaptoneurosomes with ligand mimetic RGD-peptides or native ligands activates LTP-related kinases including calcium calmodulin protein kinase II (Kramár et al., 2003), focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2) and Src family kinases (Bernard-Trifilo et al., 2005). Signaling cascades in which these kinases participate mediate integrin-dependent increases in the phosphorylation and synaptic responses of AMPA- and NMDA- type glutamate receptors (Kramár et al., 2003; Bernard-Trifilo et al., 2005). Integrin signaling through these pathways also promotes spine elaboration and retention during development (Shi and Ethell, 2006; Webb et al., 2007) and is essential to the remodeling of the spine actin cytoskeleton during the production of stable LTP (Kramár et al., 2006).

Other work raises the possibility that integrins regulate activity-induced synaptic plasticity through effects on cytoplasmic calcium concentrations. Different integrins regulate intracellular calcium levels in non-neuronal cells through effects on influx and release from intracellular stores (Sjaastad et al., 1994; Tsao and Mousa, 1995; Schottelndreier et al., 2001; Wu et al., 2001). The few studies of neurons show that integrin ligands increase calcium influx through effects on voltage-sensitive calcium channels (VSCCs) (Wildering et al., 2002; Gui et al., 2006) and NMDA receptors (Watson et al., 2007), effects that could have a profound influence on the calcium-dependent LTP induction process (Lynch et al., 1983; Rose and Konnerth, 2001). However, the relative contributions of glutamate receptors and calcium channels, as well as potential release from intracellular stores, have not been studied. Whether the integrin driven increases vary between inhibitory vs. excitatory neurons, and occur in glia, is also unknown. The present study addressed these issues, and the interactions between integrins and glutamate receptors, using Flura2 imaging of cytoplasmic calcium levels in cultured cortical neurons.

Results

Integrin ligands increase $[Ca^{2+}]_i$ in cortical neurons

Cortical neuron-enriched cultures at 14 to 15 DIV were loaded with Fura2-AM for 30 min and ratiometric imaging was used to collect real time recordings of $[Ca^{2+}]_i$ within 8 to 12 individually identified cells in the visible field at 40X objective magnification. Baseline calcium levels were monitored for 5 to 10 min after which the integrin ligand peptide RGD or the non-ligand control peptide YIGSR was added. RGD (10 μ M) markedly increased $[Ca^{2+}]_i$ in most but not all cells in a given culture field (Fig. 1A,B) whereas YIGSR was without effect (Fig. 1C). Figure 1E shows plots of $[Ca^{2+}]_i$ of individual perikarya simultaneously recorded before, during and after two applications of the ligand. In this experiment, and as typically observed by ourselves and others (Numakawa et al., 2002), baseline recordings included occasional, synchronous $[Ca^{2+}]_i$ oscillations. RGD caused a rapid and synchronous increase in perikarya $[Ca^{2+}]_i$ that varied in magnitude across different cells. Calcium levels increased to an initial peak over 30 to 100 sec, then declined and returned to near control levels during a 30 min washout. Comparable results were obtained with a second RGD application (Fig. 1A).

The graphs in figures 1F and 1G summarize the effects of longer treatments with RGD and YIGSR as assessed at 30 sec intervals (Fig. 1F) and at the initial peak increase (Fig. 1G). As shown, RGD reliably produced prolonged increases in $[Ca^{2+}]_i$ whereas YIGSR had no

measurable effect. The RGD effects were dose-dependent: increases were evident at 1 μ M, minimally variable at 10 μ M, and greatly reduced at 1 mM (Fig. 1H) ($p < 0.001$ vs pretreatment control values at all three doses, TMT). This profile is consistent with evidence that RGD is a partial agonist which can have agonist or antagonist activities depending on dose (Tsao and Mousa, 1995).

Function-blocking antibodies were used to assess the relative contributions of $\beta 1$ or αv integrins to RGD-induced increases in $[Ca^{2+}]_i$. These subunits define the two major families of integrins expressed in brain (Milner and Campbell, 2002); $\beta 1$ -containing integrins are of particular interest because they modulate glutamatergic synaptic transmission (Kramár et al., 2003; Bernard-Trifilo et al., 2005) and plasticity (Kramár et al., 2002; Chan et al., 2003; Chan et al., 2006; Huang et al., 2006; Kramár et al., 2006). The effects of the integrin ligands and antisera on perikaryal $[Ca^{2+}]_i$ as assessed over time and at the initial peak RGD-induced increase for three experimental sets are summarized in figure 2. By either measure, the increase was blocked by each of two $\beta 1$ neutralizing antisera (Fig. 2A) and only partially reduced by antisera to αv integrin (Fig. 2B). Combining antisera to $\beta 1$ and αv completely eliminated the effect of RGD (Fig. 2B). These results demonstrate that both classes of receptors are involved, with the greater contribution coming from the $\beta 1$ class.

Following on these results, tests were made of whether activation of a specific member of the $\beta 1$ family is sufficient to trigger increases in cytoplasmic calcium levels. The $\alpha 5\beta 1$ integrin was selected because this receptor, and its preferred matrix ligand fibronectin (FN), are both expressed in neocortex (Hoffman et al., 1998; Bi et al., 2001), $\alpha 5\beta 1$ plays a role in stabilizing LTP (Chun et al., 2001; Chan et al., 2003), and it has been reported to modulate calcium influx in other studies (Wu et al., 2001; Gui et al., 2006). Treatment with FN (50 μ g/ml) or with $\alpha 5\beta 1$ activating antisera (20 μ g/ml) rapidly increased $[Ca^{2+}]_i$ (Fig 2C). Interestingly, the effects of FN, but not those elicited by anti- $\alpha 5\beta 1$, decayed to near-baseline levels over a period of 7 min; in both cases the increases in $[Ca^{2+}]_i$ were smaller than those induced by RGD (Fig. 2C, left). These results point to the conclusion that $\alpha 5\beta 1$ is one of several integrins that regulate intracellular calcium homeostasis.

Increases in $[Ca^{2+}]_i$ are neuron-specific and occur first in cell processes

Results presented above (Fig. 1) show that RGD treatment increases $[Ca^{2+}]_i$ in most, but not all, cells in a given field, thereby suggesting cell type-specific differences in response to the integrin ligand. To test responses by neurons and astroglia, cells plated on etched-grid coverslips were monitored for ligand effects on $[Ca^{2+}]_i$ and then processed for double labeling with antibodies against neuronal (MAP2) or astroglial (GFAP) markers. As shown for a representative field of cells in figure 3 (panels A-C), RGD increased $[Ca^{2+}]_i$ in MAP2 immunoreactive (ir) neurons (perikarya and neurites) but not in GFAP-ir cells. In other experiments, increases in $[Ca^{2+}]_i$ were observed in betaIII tubulin-ir neurons (data not shown). Among responsive cells, the magnitude of changes in $[Ca^{2+}]_i$ varied (Fig. 1A). To determine if increases in $[Ca^{2+}]_i$ differ with neuronal phenotype, responses to RGD were measured and then cells were processed for double immunostaining with antibodies for the glutamate transporter VGLUT1 and the gamma-aminobutyric acid (GABA) biosynthetic enzyme glutamic acid decarboxylase (GAD) 67 to identify glutamatergic and GABAergic neurons, respectively. As shown in figure 3 (D-F), RGD increased $[Ca^{2+}]_i$ in both GAD67-ir and VGLUT1-ir cells although responses appeared greater in the latter. Quantification confirmed that RGD effects on $[Ca^{2+}]_i$ were approximately 80% greater in VGLUT1-ir as compared to GAD67-ir neurons (across 3 experiments mean peak CF measures were 0.18 ± 0.03 and 0.33 ± 0.02 for GAD67-ir and VGLUT1-ir cells, respectively; $p = 0.002$, two-tailed t test).

To identify the locus of initial RGD effects on $[Ca^{2+}]_i$ and, in particular, to determine if first increases are present in neuronal somata or processes, target cells were continuously monitored

while RGD was applied via pressure (picospritzer) ejection from a drawn glass pipette into the media. Results from a representative experiment are shown in figure 3: panel G shows a large cortical neuron in differential interference contrast while panels H-L are pseudocolor Fura2 images of the same cell over time. RGD was applied at “0 sec”. Calcium levels in the neuronal processes were elevated modestly at 2 sec (Fig. 3I) and more robustly by 20 sec (Fig. 3D). At greater latencies, somal calcium levels increased markedly while those in the processes slowly declined (Figs. 3K and 3L). In a second experiment (not shown), first increases in $[Ca^{2+}]_i$ within the processes and soma were first detected at 12.5 and 22.5 sec, respectively.

Calcium influx and intracellular store release

Intracellular calcium levels are regulated through modulation of both calcium influx and release from intracellular stores (Rizzuto, 2001; Bardo et al., 2006). To identify the source(s) of RGD-induced increases in cytoplasmic calcium content, we examined ligand effects on cells (i) placed in calcium free medium or (ii) treated with agents that inhibit intracellular release. Figure 4 illustrates perikaryal $[Ca^{2+}]_i$ over time (Fig. 4A) and at the initial peak following RGD treatment (Fig. 4B). As shown, with cells in calcium-free medium RGD did not significantly increase $[Ca^{2+}]_i$. Depletion of intracellular calcium stores by 15 min preincubation with thapsigargin (1 μ M), a specific inhibitor of the sarcoendoplasmic reticulum Ca^{2+} -ATPase (Mintz and Guillain, 1997) substantially reduced, but did not eliminate, RGD-induced increases in $[Ca^{2+}]_i$ (Figs. 4A and 4B). Calcium release from the endoplasmic reticulum is controlled by ryanodine receptor (RyR) and inositol 1, 4, 5 triphosphate-gated receptor (IP3R) systems (Rizzuto, 2001; Bardo et al., 2006). Pretreatment with the RyR antagonist dantrolene (10 μ M) (Figs. 4A and 4B) or the IP3R antagonist 2-aminoethoxydiphenylborate (2-APB, 100 μ M) (Fig. 4B) (Krause et al., 2004) had no effect on $[Ca^{2+}]_i$ when applied alone but both significantly depressed responses to RGD treatment. Together with the thapsigargin results, these findings indicate that calcium influx from the extracellular environment is required for integrin-dependent increases in $[Ca^{2+}]_i$, with a further major contribution coming from intracellular stores.

Role of ionotropic glutamate receptors and voltage sensitive calcium channels

The origins of the ligand-induced Ca^{2+} -influx were investigated with antagonists for cyclic nucleotide-gated cation channels, voltage sensitive calcium channels (VSCCs) and the NMDA- and AMPA-class glutamate receptors. The cyclic GMP-dependent protein kinase activator beta-phenyl-1,N2-etheno-8-bromoguanosine-3',5'-cyclic monophosphorothioate (Sp-8-Br-PET-cGMPS) competitively inhibits cyclic nucleotide-gated cation channels and thereby reduces excitatory postsynaptic potentials (Wei et al., 2002). The inhibitor (500 μ M) caused a slight, transient increase in $[Ca^{2+}]_i$ ($\Delta F = 0.02$ at 200 sec) when applied alone but did not attenuate the effect of RGD treatment (data not shown). In contrast, both the broad spectrum VSCC antagonist Gd^{3+} (at 10 and 20 μ M) and the L-type VSCC antagonist nifedipine (at 1, 4, and 10 μ M) reduced ligand-induced increases in $[Ca^{2+}]_i$ by $\geq 50\%$ (Figs. 5A and 5C; Suppl. Fig. 1). The NMDA receptor (NMDAR) antagonist APV (20 μ M) had a somewhat larger effect but still did not fully block RGD-induced increases (Figs. 5A and 5C). In contrast, AMPA receptor (AMPA) antagonists (CNQX at 10 μ M; GYKI at 100 μ M) completely blocked the $[Ca^{2+}]_i$ increase induced by RGD treatment (Figs. 5B and 5C).

A small subset of AMPARs lacking the GluR2 subunit are known to flux calcium (Weiss and Sensi, 2000; Cull-Candy et al., 2006). Treatment with the Ca^{2+} -permeable AMPA/kainate receptor antagonist Joro spider toxin (JsTx; 6 μ M) (Blaschke et al., 1993) before and during RGD application only modestly attenuated RGD-induced increases in $[Ca^{2+}]_i$ at greater latencies (Figs. 5B and 5C). It thus appears that RGD-induced increases in $[Ca^{2+}]_i$ are dependent upon AMPA receptor activities without a disproportionately large contribution from the minority calcium-permeable variants of this receptor class.

The above pattern of results suggests that integrin ligation enhances AMPAR-mediated synaptic currents, and thereby depolarizes neurons to a degree sufficient for opening voltage sensitive channels (NMDARs, VSCCs) that flux calcium. If so, then blocking synaptic activity should seriously attenuate integrin ligand-induced changes in $[Ca^{2+}]_i$. In accord with this, the sodium channel poison tetrodotoxin (TTX) largely eliminated ligand-induced increases in $[Ca^{2+}]_i$ (Fig. 5C).

Increased calcium content is blocked by genistein

Prior studies have identified downstream signaling pathways that could account for the above findings. Integrin ligand induced increases in $[Ca^{2+}]_i$ are blocked by the broad spectrum tyrosine kinase inhibitor genistein (Xie et al., 1998; Schottelndreier et al., 1999; Wu et al., 2001) as well as by the Src family kinase inhibitor PP2 (Schottelndreier et al., 2001; Wu et al., 2001) in non-neural cells. Other work has shown that integrin signaling activates kinases (CamKII, Src, MAPK p42/44) that modulate glutamate neurotransmitter receptor and VSCC function in neurons (Kramár et al., 2003; Bernard-Trifilo et al., 2005; Watson et al., 2007) and receptor tyrosine kinases in other systems (Miranti and Brugge, 2002). Moreover, there is evidence that CamKII-mediated phosphorylation of ryanodine receptors potentiates calcium-induced release from intracellular stores (Zhang et al., 2004). The results from experiments testing for possible contributions of these signaling paths on RGD-induced increases in $[Ca^{2+}]_i$ are summarized in figure 6. Effects of RGD treatment on $[Ca^{2+}]_i$ were reduced by approximately 50% by the Src antagonist PP2, the CamKII antagonist KN93 (5 μ M) (Fig 6A), the Trk signaling antagonist K252a and the MEK inhibitor PD98059 (50 μ M) (not shown). Genistein has been reported to block ligand induced increases in intracellular calcium when applied in at doses of 80-100 μ M although with doses of ≥ 100 μ M it is known to influence activities of some serine-threonine kinases (e.g., protein kinase A) (Fleming et al., 1995). Therefore, to both replicate treatment effects of prior studies and to examine lower doses at this this antagonist is considered specific for tyrosine kinases genistein was applied at doses of 10, 50, and 200 μ M. All three doses fully blocked effects of RGD treatment on $[Ca^{2+}]_i$ (Figs. 6A,B).

The above results with inhibitors of CamKII or Src suggest that phosphorylation of AMPA receptors could be a necessary, early step in the sequence of events responsible for RGD-induced increases in cytoplasmic calcium. Previous work with hippocampal slices (Kramár et al., 2003) showed that RGD increases phosphorylation of serine 831 on the AMPAR GluR1 subunit, a site known to (a) be a substrate for CamKII (Soderling and Derkach, 2000) and protein kinase C (Roche et al., 1996) and (b) potentiate AMPA receptor-mediated currents (Wang et al., 2005). To test for GluR1 phosphorylation here, cells were incubated with 10 μ M RGD for 3 or 10 min and harvested for western blot analysis of pGluR1 Ser831. As shown in Figure 7A (top row), RGD markedly increased pGluR1 levels at both time points; total GluR1 and actin immunoreactivities were comparable across groups (Fig. 7A, bottom rows). The RGD effects on pGluR1 were blocked by genistein (Figs. 7B and 7C).

Discussion

The above results indicate that matrix-ligand driven increases in neuronal calcium content reflect the activities of multiple integrins. Neutralizing antisera to $\beta 1$ and αv integrin subunits blocked and attenuated, respectively, effects of RGD on $[Ca^{2+}]_i$. The $\beta 1$ and αv subunits are broadly expressed by cortical neurons and are predicted components to most integrins expressed in brain (Pinkstaff et al., 1997; Pinkstaff et al., 1999; Milner and Campbell, 2002). $\beta 1$ forms dimers with $\alpha 1$ - $\alpha 11$ and αv ; the αv subunit additionally partners with $\beta 3$, $\beta 5$, $\beta 6$, and $\beta 8$ (Milner and Campbell, 2002). Previous studies have shown that integrin subunits $\beta 1$, $\beta 3$, $\beta 5$ (Cousin et al., 1997; Shi and Ethell 2006), $\alpha 3$ (Dulabon et al., 2000), $\alpha 5$ (Webb et al.,

2007) and $\alpha 8$ (Einheber et al., 1996) are expressed by immature and cultured forebrain neurons, and localized to spine synapses at the in vitro ages examined here. The $\alpha 5$ subunits is known to only form dimers with $\beta 1$ (Milner and Campbell, 2002). Thus, the simplest explanation is that ligand-induced increases in cytoplasmic calcium demonstrated here are mediated by multiple $\beta 1$ integrins, including $\alpha 5\beta 1$ and $\alpha v\beta 1$ although minor contributions by other αv integrins cannot be excluded. The RGD-binding $\beta 1$ integrins are $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$ and $\alpha v\beta 1$ (Plow et al., 2000). This list includes integrins directly implicated in calcium regulation by the present findings: treatment with activating anti- $\alpha 5\beta 1$ or with fibronectin, a ligand for all four integrins, increased $[Ca^{2+}]_i$. Interestingly, this list corresponds with that for integrins implicated in adult hippocampal LTP. Genetic (Chan et al., 2006; Huang et al., 2006) or immunochemical (Kramár et al., 2006) disruption of $\beta 1$ integrin function blocks the stabilization of hippocampal LTP whereas manipulation of the individual $\alpha 3$, $\alpha 5$, or $\alpha 8$ subunits has similar but more modest effects (Chun et al., 2001; Kramár et al., 2002; Chan et al., 2003). The fact that greater disruptions to LTP, and memory, are observed when the α subunits are manipulated in combination (i.e., in mutant mice underexpressing combinations of $\alpha 3$, $\alpha 5$ and $\alpha 8$, or in rat tissue treated with a cocktail of neutralizing antisera to $\alpha 3$, $\alpha 5$, and αv) (Chan et al., 2003; Kramár and Lynch, 2003) suggests that these integrins make additive contributions to processes supporting LTP, including increases in postsynaptic calcium content (Lynch et al., 1983; Rose and Konnerth, 2001).

The combinatorial effects of several integrins may account, in part, for differences in responses by different cell types. Calcium increases were twice as large in glutamatergic than in GABAergic neurons, and were not evident in astroglia. As best appreciated in studies of hippocampus, the majority of integrin transcripts (e.g., $\beta 1$, $\alpha 5$ and $\alpha 8$ mRNAs) are most highly expressed by the glutamatergic principal neurons (Pinkstaff et al., 1997; Pinkstaff et al., 1999). Moreover, glutamatergic but not GABAergic cells express CamKII (McDonald et al., 2002) which, as shown here, contributes to ligand-induced increases in $[Ca^{2+}]_i$. Beyond potential differences in integrin subunit expression profile, the absence of a detectable response in astrocytes could reflect the absence of a means for glial integrins to initiate an action sequence leading to the opening of voltage sensitive channels permeant to calcium.

The present results strongly suggest that integrin ligation in neurons leads to an initial Ca^{2+} -influx followed by release from intracellular stores. RGD had no effect on $[Ca^{2+}]_i$ in calcium free medium while pretreatment with thapsigargin, which blocks sarcoendoplasmic reticulum (SER) calcium-ATPase pumps and depletes intracellular stores (Verkhratsky and Shmigol, 1996; Bardo et al., 2006), reduced the perikaryal RGD response by 80%. Similarly, antagonists of the ryanodine or IP3 receptors controlling SER release markedly depressed ligand effects on $[Ca^{2+}]_i$. Together, the concentration of integrins at spine synapses (Nishimura et al., 1998; Huang et al., 2006; Shi and Ethell, 2006; Webb et al., 2007) and the contribution of SER stores to changes in $[Ca^{2+}]_i$ provide some understanding of the spatio-temporal pattern of the latter effect. RGD-induced increases in $[Ca^{2+}]_i$ appeared first in dendrites and tens of seconds later in somata. This is consistent with integrin ligation first elevating calcium influx and release from local (spine/dendritic) SER stores (Rose and Konnerth, 2001; Raymond and Redman, 2006) followed by a summation of local effects to elicit a second wave of calcium-induced calcium release from the larger SER network, including that in the perikaryon.

The pattern of ligand-induced calcium influx followed by intracellular store release is typical of integrin effects on $[Ca^{2+}]_i$ in non-neuronal cells (Bhattacharya et al., 2000; Kwon et al., 2000; Schottelndreier et al., 2001). Moreover, when examined, influx is generally found to be mediated by VSCCs and dependent upon tyrosine kinase signaling (i.e., blocked by genistein) (Wu et al., 1998; Kwon et al., 2000; Wu et al., 2001). What distinguishes effects in neurons is the participation of neuron-specific receptors that produce depolarization (AMPA receptors) and voltage-dependent influx (e.g., NMDA receptors). In our studies, increases in $[Ca^{2+}]_i$ were

reduced, but not eliminated, by NMDA receptor and VSCC antagonists, indicating that both channels contribute to the initiating influx event. However, either AMPA receptor antagonists or the sodium channel blocker TTX, agents that prevent synaptic depolarization and radiation of synaptic activity through the largely glutamatergic cellular networks, caused a complete block. These results provide the first evidence for interactions between integrins and AMPA class glutamate receptors in controlling $[Ca^{2+}]_i$. The finding that the calcium permeable AMPAR antagonist JsTx did not block integrin-induced changes supports the interpretation that AMPAR - mediated synaptic responses (as opposed to calcium influx through the minority, calcium-permeable AMPA receptors) were critical for integrin ligand effects on $[Ca^{2+}]_i$ described here.

The relationship between integrins and AMPA receptors with regard to intracellular calcium could take several forms. First, AMPAR-mediated synaptic events may be permissive and simply provide the intermittent depolarization needed to allow integrin signaling influences on the otherwise voltage-blocked NMDARs and VSCCs to become manifest. Studies of non-neural cells have shown that integrins signal through FAK and Src kinases to phosphorylate and increase activities of plasma membrane calcium channels (Wu et al., 1998; Wu et al., 2001; Gui et al., 2006); we have shown similar integrin signaling effects on the NMDARs (Bernard-Trifilo et al., 2005). Second, direct integrin modulation of AMPA receptor function may be an important upstream event. Integrin ligation increases CamKII-mediated phosphorylation of AMPAR subunit GluR1 (Ser831) and initiates a slowly developing CamKII- and Src-dependent potentiation of AMPA receptor-mediated synaptic responses in hippocampal slices (Kramár et al., 2003). In the present studies, ligand effects on $[Ca^{2+}]_i$ were reduced by CamKII and Src antagonists whereas ligand effects on both $[Ca^{2+}]_i$ and GluR1 Ser831 phosphorylation were blocked by genistein. Appreciation of the potential contribution of these events to integrin-driven changes in $[Ca^{2+}]_i$ is limited by the paucity of information of the speed with which they occur: ligand-induced increases in $[Ca^{2+}]_i$ are extremely rapid in cultured neurons, emerging within a few seconds of treatment, whereas RGD effects on AMPA receptor-mediated currents in hippocampal slices require tens of minutes.

A third possibility is suggested by recent reports that integrins regulate properties of calcium channel proteins and that these same proteins form functional couplings with the AMPA receptors. The γ_2 calcium channel subunit 'stargazin' has been likened to an auxiliary subunit of the AMPAR and is importantly involved in AMPAR trafficking and function (Tomita et al., 2005; Vandenberghe et al., 2005; Tomita et al., 2006). More recently, neuronal calcium channels have been shown to reside in a multimolecular complex including the GluR1 and GluR2/3 AMPA receptor subunits which, in turn, co-immunoprecipitate with the pore forming α_1 subunit CAV2.1 (Kang et al., 2006). These findings point to the speculative possibility that integrin signaling may regulate AMPAR coupling with calcium channel proteins (γ_2 , CAV2.2, and maybe others) and thereby strengthen the influence of these glutamate receptors over calcium channel gating.

A minutes-long change in calcium dynamics initiated by integrin activation, as described here, is a plausible contributor to the post-synaptic modifications produced by brief periods of intense afferent activity. These include AMPA receptor trafficking (Maher et al., 2005), local protein synthesis (Dong et al., 2003; Smart et al., 2003; Kelleher et al., 2004) and regulation of calcium-activated proteases (Lynch et al., 1990; Lynch et al., 2007). Moreover, recent work suggests that calcium transients from different sources (i.e., NMDAR-, RyR-, IP3R-mediated) support different epochs in the consolidation of LTP (Rose and Konnerth, 2001; Raymond and Redman, 2006). It remains for future studies to test the hypothesis that these events can be manipulated in a predictable manner by treatments that affect integrin signaling.

Experimental Methods

Neuronal Cultures

Cortical neuron cultures were prepared from postnatal day 0 (P0) or P1 pups of International Cancer Research (ICR) mice as described previously (Hilgenberg and Smith, 2004). Cells were fed twice per week with Neurobasal media containing B27-supplement (Invitrogen, Carlsbad, CA) pre-conditioned on astroglial feeder cells for 24 hr. The mitotic inhibitor 5-fluoro-2-deoxyuridine (5 μ M) was added to the feeder media just once, for 3 days in vitro (DIV), to minimize non-neuronal cells in cultures. Calcium imaging and immunocytochemistry were performed on cells plated on poly-D-lysine coated glass-bottomed 35 mm dishes (MatTek Corp. Ashland, MA).

Calcium Imaging

Cultures at 14-15 DIV were rinsed with HEPES-buffered Ca²⁺ salt solution (HCSS; 120mM NaCl, 5.4mM KCl, 1.8mM CaCl₂, 0.8mM MgCl₂, 15mM glucose, 20mM HEPES, 10mM NaOH, pH 7.4) and then loaded with 5 μ M Fura2-AM and 0.1% Pluronic F-127 (Molecular Probes, Eugene, OR) at 37 °C for 30 min (Neher, 1995). Cultures were then switched to growth media at 37 °C for 30 min to ensure completed hydrolysis of the Fura2-AM ester prior to imaging. Calcium concentration measurements were carried out using a standard ratiometric technique (Grynkiewicz et al., 1985). For optical recording, cultures were placed onto the stage of a Nikon TE300 inverted microscope and visualized using a 40X, 1.3 numerical aperture fluorite oil immersion objective. Fluorescence illumination was provided by a 150 W xenon arc lamp. Generally 8 to 12 cells in the image field were selected for monitoring. Excitation wavelengths (340 and 380 nm) were selected using band specific filters and then Pseudocolor Ratio images at alternating excitation wavelengths were acquired at 510 nm with a digital CCD camera (Orca-100, Hamamatsu, Bridgewater, NJ) and analyzed using Metafluor 4.0 software (Universal Imaging, West Chester, PA). Fluorescence increases over baseline (ΔF) were calculated as $F_x - F_0$, where F_x indicates the fluorescence at time x , and F_0 indicates the average baseline fluorescence for the same cells. The significance of effects of treatment was assessed using the one or two way ANOVA followed by Tukey's Multiple Comparison Test (TMT) for paired comparisons with $p \leq 0.05$ considered significant.

Cell treatments

For studies of putative ligand effects, baseline calcium levels were monitored (simultaneously for 8 to 12 cells in a field) for 5 to 10 min and then cells were treated with either the integrin ligand peptide GRGDSP (single amino acid code; hereafter referred to as RGD), the non-ligand control peptide YIGSR, fibronectin (FN) or integrin $\alpha 5\beta 1$ activating antibody. For studies of mechanisms mediating ligand effects, baseline [Ca²⁺]_i levels were recorded for 10 min, the different test antagonists for ion channels or glutamate neurotransmitter receptors were bath applied for 10 min, and then RGD combined with same test antagonist was bath applied for 10 min. For studies intended to impede release from intracellular calcium stores, cells were preincubated for 15 min with thapsigargin, 2-aminoethoxydiphenylborate (2-APB) or dantrolene prior to ligand application. In some preparations, RGD was applied by puffer using published techniques (Ogoshi et al., 2005). Briefly, RGD (5 mM pipette concentration) was applied through a micropipette (~15 μ m tip opening) attached to a pressure system including a "Spritzer-8" Cornerstone Series Pico-Spritzer (Dagan Corp, Minneapolis, MI) and an Isolated Pulse Stimulator (model 2100, A-M Systems, Inc, Sequim, WA): the peptide was ejected with pulses of 0.1 sec burst width (back pressure, 3psi) delivered at 0.3 pulse/sec. The tip of the puffer pipette was placed within the HCSS, 250 μ m above the substrate and at the outer edge of the microscope field, angled at 30° above the horizontal.

Reagents and antibodies

The following reagents and concentrations were used (unless otherwise noted, reagents were purchased from Sigma, St. Louis, Mo): 2-APB 100 μ M (Calbiochem, Temecula, CA); 1-(aminophenyl)-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466) 100 μ M (Tocris, Ellisville, MO); beta-phenyl-1,N2-etheno-8-bromoguanosine-3',5'-cyclic monophosphorothioate (Sp-8-Br-PET-cGMPS) 500 μ M; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) 10 μ M (Tocris); dantrolene 10 μ M; DL-2-amino-5-phosphonovaleric acid (APV) 20 μ M; fibronectin fragment (FN) 50 μ g/ml (F1904, 120 kDa; Chemicon); gadolinium III (Gd³⁺, G-7532) at 10 and 20 μ M; Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP or RGD) 10 μ M (Calbiochem); Joro spider toxin-3 (JsTx) 6 μ M (Calbiochem); nifedipine (Nif) at 1, 4, and 10 μ M (Alomone Labs., Israel); tetrodotoxin (TTX) 1 μ M (Alomone Labs.); thapsigargin 1 μ M; Tyr-Iso-Gly-Ser-Arg (YIGSR) 10 μ M (Calbiochem); kinase inhibitors (Calbiochem) including calcium calmodulin kinase II (CamKII) inhibitor, KN93 5 μ M; K252a, 200 nM; Src kinase inhibitor PP2 10 μ M; MAP kinase kinase (MEK) inhibitor PD98059 50 μ M; and genistein at 10, 50, and 200 μ M. Antisera used for tissue treatments were applied at 20 μ g/ml and included integrin β 1 neutralizing antisera (MAB1987Z from Chemicon and #555002 from BD Biosciences, San Diego, CA), integrin α v neutralizing antisera (AB553241, BD Biosciences) and α 5 β 1 activating antibody (MAB1969; Chemicon). Western blots were probed with rabbit anti-phospho (p) GluR1 Ser831 (Upstate Biotechnology; Lake Placid, NY), rabbit anti-GluR1 (AB1504; Chemicon) and mouse anti- β actin (A5441) all used at 1:1100.

Immunocytochemistry

After calcium imaging, cortical neurons were fixed immediately in 4% paraformaldehyde (1 hr, 4°C), permeabilized and blocked in 0.1M phosphate buffered saline (pH 7.4, PBS) containing 0.1% Triton X-100 and 5% bovine serum albumin (PBS/BSA) for 1 hr and then incubated at 4°C overnight in a primary antisera cocktail including (1) mouse monoclonal anti-microtubule associated protein-2 (MAP2) (1:500; SMI-52; Sigma) and rabbit polyclonal anti-astrocytic glial fibrillary acidic protein (GFAP) (1:1000; #Z0334, DAKO, Carpinteria CA) or (2) guinea pig polyclonal anti-glutamate transporter VGLUT1 (1: 4000; AB5905, Chemicon) and monoclonal anti-GAD67 (1: 1000; MAB5406, Chemicon). Cells were then washed in PBS, incubated in a cocktail of species appropriate secondary antisera conjugated with Alexa Fluor 594 or Alexa Fluor 488 (1:1000 each; Invitrogen/Molecular Probes, Seattle, WA) in PBS/BSA for 1 hr, washed in PBS, and coverslipped with Vectashield (Vector Laboratories Inc., Burlingame, CA). Cultures were photographed under epifluorescent illumination using a Nikon Optiphot-2 microscope.

Western immunoblotting

Cultures were homogenized immediately following calcium imaging in ice-cold RIPA buffer containing 10 mM Tris, pH7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1X Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN), and Phosphatase Inhibitor Cocktails 1 and 2 (P2850 and 5726). Sample protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA), protein densities were normalized by volume adjustment and then equal amounts of protein were separated on 4 to 12% gradient SDS PAGE) with Rainbow molecular weight markers (RPN800, Amersham Biosciences, Piscataway, NJ) as described (Kramár et al., 2003; Lin et al., 2005). Proteins were transferred to polyvinylidene difluoride membranes (Hybond-P™, Amersham) which were blocked with 5% non-fat dry milk and 5% bovine serum albumin and then probed with rabbit anti-pGluR1 S831 (Upstate Biotechnology), rabbit anti-GluR1 (Chemicon) and rabbit anti- β actin (Sigma) at 1:1100 in Tris-buffered saline plus 5% non-fat dry milk. Immunoreactive bands were visualized using the ECL+Plus chemiluminescence detection system (Amersham); levels of immunoreactive proteins were determined by

densitometric analysis of ECL film using the AIS image system (Imaging Research; St. Catharines, Ontario, Canada).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The integrin ligand RGD stimulates rapid increases in $[Ca^{2+}]_i$

Dissociated cortical/hippocampal neurons were treated with RGD or the control YIGSR peptide and effects on cytoplasmic calcium levels were evaluated by Fura2 ratiometric imaging. (A-D) Photomicrographs of a group of cells showing (A-C) pseudocolor ratiometric imaging of $[Ca^{2+}]_i$ (colors denote calcium levels with red>orange>yellow>blue) and (D) the same field as seen under Differential Interference Contrast (DIC; Calibration bar = 10 μ m). Images show $[Ca^{2+}]_i$ in cells before treatment (A, CON) and after treatment with 10 μ M RGD (B) and 10 μ M YIGSR (C): in this set, RGD was applied 20 min after YIGSR washout. (A,B) RGD increased $[Ca^{2+}]_i$ in most cells (arrows) although some did not respond (arrowhead) whereas (C) YIGSR had no detectable effect on $[Ca^{2+}]_i$. (E) Graph shows the perikaryal $[Ca^{2+}]_i$ for 6 cells in a single microscopic field over time: 10 μ M RGD was applied twice during intervals indicated by horizontal lines at top; the fluorescence increase over baseline ($CF = F_x - F_0$) is plotted on the Y axis. (F, G) Graphs show quantification of $[Ca^{2+}]_i$ (F) at 30 sec intervals before and after 10 μ M RGD or YIGSR application at time "zero" and (G) mean levels during the baseline, pretreatment period (CON) and at the latency to the initial peak RGD response (mean $\pm$ SEM values shown for 36 RGD- and 22 YIGSR- treated cells over 4 experiments; ***p < 0.001 vs. CON, Tukey's Multiple Comparison Test, TMT). (H) Bar graph shows effects (mean $\pm$ SEM) of RGD dose on $[Ca^{2+}]_i$ as assessed at the latency to the initial peak increase (n $\geq$ 48 cells/group across $\geq$ 3 experiments). For all three doses, $[Ca^{2+}]_i$ were significantly different from control values (p < 0.001, TMT); *p < 0.05 for comparison of 10 μ M and 1 mM values, TMT.

Figure 2. RGD effects on $[Ca^{2+}]_i$ are mediated by $\beta 1$ integrins

Plots of $[Ca^{2+}]_i$ as determined by Fura2 imaging shows the effects of integrin ligand and neutralizing antibody treatment as assessed every 30 sec with 10 μ M RGD applied at time zero (at left) and at the latency to the initial peak RGD-alone effect (at right). For each panel, the grey tones on the bar graph, denoting the different treatment groups, corresponds the symbol fill tones on the corresponding time plot; e.g., in each results for the RGD treatment group are plotted with a black fill tone. Numbers over bars indicate the total number cells evaluated over ≥ 4 , 3 and ≥ 5 experiments in A, B and C, respectively: in each instance the CON group included ≥ 109 cells. In this and subsequent illustrations, control values represent baseline (pretreatment) $[Ca^{2+}]_i$ measures for the same cells later evaluated following drug treatment: mean control values were subtracted from all groups for bar graphs; statistical analyses were run on the raw

data. (A) Cultures were treated with RGD alone or in the presence of one of two $\beta 1$ integrin neutralizing antisera ($-\beta 1 = AB555002$; $-\beta 1' = AB1987Z$; applied 10 min before and during RGD treatment). Both $\beta 1$ antisera completely blocked RGD effects on $[Ca^{2+}]_i$ ($***p < 0.001$ vs. control values, TMT). (B) Cultures were treated with RGD alone, RGD plus neutralizing αv integrin antisera ($-\alpha v$), or RGD plus both anti- αv and anti- $\beta 1$ ($-\alpha v, -\beta 1$) antisera: anti- αv attenuated, and the combination of anti- αv and anti- $\beta 1$ neutralizing antisera blocked, effects of RGD treatment ($***p < 0.001$, $**p < 0.01$ vs. control, TMT). (C) Cultures were treated with RGD, fibronectin fragment (FN, 50 $\mu g/ml$) or $\alpha 5\beta 1$ integrin activating antibody ($\alpha 5\beta 1+$) at time zero; all three treatments significantly increased $[Ca^{2+}]_i$ ($***p < 0.001$ vs. control, TMT).

Figure 3. RGD increases $[Ca^{2+}]_i$ in glutamatergic and GABAergic neurons

(A-F) Double labeling immunocytochemistry was used to identify cells with RGD-induced increases in $[Ca^{2+}]_i$. Panels A-C show calcium imaging before (A, CON) and after (B, RGD) RGD treatment for cells subsequently processed for localization of MAP2 and GFAP immunoreactivities. As shown, RGD increased calcium levels of MAP2-ir neurons (arrows, red label in C) but not GFAP-ir astroglial cells (open arrows, green label in C). (D-F) Panels D-E show $[Ca^{2+}]_i$ before (D) and after (E) RGD treatment for cells subsequently processed for localization of VGLUT1-ir and GAD67-ir (F): RGD increased $[Ca^{2+}]_i$ in both VGLUT1-ir glutamatergic neurons (arrows, red label in F) and, to lesser extent, in GAD67-ir GABAergic cells (open arrows, green label in F) (Calibration bar in F = 20 μm for A-F). (G-L) Photomicrographs H-I show Fura2 imaging of $[Ca^{2+}]_i$ in the same microscopic field over time after local (microspritzer) application of RGD into the bath slightly to the right of the central neuron (seen in DIC in G); RGD was applied at time zero, the latency to each photograph is

indicated in the lower right. As shown with the shift to yellow and then red labeling, $[Ca^{2+}]_i$ levels are increased in the processes of this cell at 2 sec (I) and in the soma at 60 sec (K) after RGD treatment. At 300 sec (L) calcium levels are still highly elevated in the soma but have declined in the processes. Calibration bar = 10 μm in G for G-L.

Figure 4. RGD effects on $[\text{Ca}^{2+}]_i$ depend upon Ca^{2+} influx, but include release from intracellular stores

Plots show Fura2 determination of perikaryal $[\text{Ca}^{2+}]_i$ in dissociated cortical/hippocampal neurons (A) over time (RGD applied at time zero; measures at 30 sec intervals) and (B) at the latency to the initial peak RGD-induced increase for cells treated with 10 μM RGD in normal calcium containing medium (RGD), in calcium free medium ($-\text{Ca}^{2+}$) and in the presence of 10 μM thapsigargin (THP), 10 μM dantrolene (DAN) or 200 μM 2-APB (APB). As shown, dantrolene, 2-APB and thapsigargin attenuated, and calcium free medium blocked, RGD effects on $[\text{Ca}^{2+}]_i$ (mean $\pm$ SEM values; $n \geq 40$ /experimental group and $n=171$ controls over

5 experiments; *** $p < 0.001$ and ** $p < 0.01$ vs. CON, TMT; $p < 0.001$ for RGD vs. RGD with Ca^{2+} -free medium, DAN, APB or THP, TMT).

Figure 5. RGD effects on $[Ca^{2+}]_i$ depend on glutamate receptor and VSCC activities

Panels A and B show effects of RGD on $[Ca^{2+}]_i$ over time, alone and in the presence of (A) voltage sensitive calcium channel (VSCC) blockers and the NMDAR antagonist APV and (B) AMPAR antagonists. (A) Nifedipine (10 μ M, Nif/RGD), Gd³⁺ (20 μ M, Gd³⁺/RGD) and APV (20 μ M) attenuated RGD effects on $[Ca^{2+}]_i$. (B) CNQX (10 μ M) and GYKI (100 μ M) completely blocked RGD-induced increase in $[Ca^{2+}]_i$ while the Ca²⁺ permeable AMPAR antagonist JsTx (10 μ M) had a modest effect. (C) Bar graph shows effects of all of the above antagonists, and TTX, on RGD-induced increases in $[Ca^{2+}]_i$ as assessed at the latency to the initial peak RGD effect (from the same experiments illustrated in A and B); numbers over bars denote number of cells evaluated over 4-5 experiments for each group; CON group mean

represents mean baseline measures for cells prior to RGD application (mean $\pm$ SEM shown; *** $p < 0.001$ vs. CON, TMT; values for RGD treatment alone were significantly greater than those for all other drug treatment groups at $p < 0.001$ excepting the JsTx/RGD group which was not significantly different, TMT).

Figure 6. RGD-induced changes in $[Ca^{2+}]_i$ are kinase-dependent

Plots show Fura2 determination of perikaryal $[Ca^{2+}]_i$ in dissociated cortical/hippocampal neurons over time with 10 μM RGD (RGD) applied alone or the presence of kinase antagonists including, in A, PP2 (10 μM), KN93 (5 μM), PD98059 (50 μM) and K252a (200 nM), and genistein (200 μM) and, in B, genistein at 10 μM . In each instance the antagonist was applied to the bath 10 min prior to the addition of RGD at time zero. Control measures (CON) were obtained during the baseline recording period prior to drug treatment: values plotted are group means $\pm$ SEM for (A) $n \leq 23$ cells/RGD group and $n=140$ for CON and (B) $n \leq 13$ cells/group. When applied with RGD, each antagonist significantly reduced initial peak $[Ca^{2+}]_i$ levels as compared to those attained with RGD treatment alone ($p < 0.001$, TMT). All RGD+antagonist

treatment group measures were significantly different from CON values ($p < 0.001$, TMT) with the exception of the RGD+genistein groups ($p > 0.05$ vs CON in A and B).

Figure 7. RGD-induced increases in pGluR1 are blocked by genistein

Dissociated cortical/hippocampal neurons were treated with normal media ("CON"), 10 μ M RGD alone, or 10 μ M RGD in combination with genistein (GN; 200 μ M) and were then processed for western blot analysis of pGluR1 Ser831 levels using phospho-specific antisera. (A) Western blots from a representative experiment show that, relative to control levels, 3 and 10 min RGD treatments increased pGluR1 Ser831-ir (top) but not total GluR1 (middle) levels; levels of actin-ir (bottom) show relatively equal amounts of protein were loaded on each lane. (B) Western blot shows that increases in pGluR1 Ser831 induced by 10 min RGD treatment were blocked by genistein. (C) Bar graph shows quantification of pGluR1 Ser831 band densities across from 4 experiments as illustrated in B (mean $\pm$ SEM optical density values shown): RGD reliably increased pGluR1 ($p = 0.029$, one way ANOVA; $*p < 0.05$ for comparison to CON, TMT post hoc test) and that this effect was blocked by genistein cotreatment.