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Integrin-binding RGD peptides induce rapid intracellular calcium increases and MAPK signaling in cortical neurons

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Integrins mediate cell adhesion to the extracellular matrix and initiate intracellular signaling. They play key roles in the central nervous system (CNS), participating in synaptogenesis, synaptic transmission and memory formation, but their precise mechanism of action remains unknown. Here we show that the integrin ligand-mimetic peptide GRGDSP induced NMDA receptor-dependent increases in intracellular calcium levels within seconds of presentation to primary cortical neurons. These were followed by transient activation and nuclear translocation of the ERK1/2 mitogen-activated protein kinase. RGD-induced effects were reduced by the NMDA receptor antagonist MK801, and ERK1/2 signaling was specifically inhibited by ifenprodil and PP2, indicating a functional connection between integrins, Src and NR2B-containing NMDA receptors. GRGDSP peptides were not significantly neuroprotective against excitotoxic insults. These results demonstrate a previously undescribed, extremely rapid effect of RGD peptide binding to integrins on cortical neurons that implies a close, functionally relevant connection between adhesion receptors and synaptic transmission.

Keywords: Integrin; NMDA receptor; MAP kinase; ERK; Calcium; GRGDSP; Cortical neuron

Introduction

Cell adhesion processes play important roles in the central nervous system (CNS) of developing and adult organisms (Milner and Campbell, 2002; Dityatev and Schachner, 2003). Receptors of the integrin family mediate extracellular adhesion and bidirectional signaling in many cell types, modulating cell shape, differentiation, migration and survival (Hynes, 2002; van der Flier and Sonnenberg, 2001; Humphries, 2000). Integrins are critical for the development of the nervous system (Clegg et al., 2003), and emerging evidence indicates multiple roles for integrins in adult neurons.

In the adult rodent brain, integrins are expressed differentially across brain regions and within individual neurons (Chan et al., 2003; Bi et al., 2001; Pinkstaff et al., 1999; Grooms et al., 1993). Integrins are particularly enriched in synaptic regions (e.g. Kramar et al., 2002; Einheber et al., 2001; Rodriguez et al., 2000; Nishimura et al., 1998; Bahr et al., 1997; Capaldi et al., 1997), where they participate in synaptic development, maintenance (Karanian et al., 2005; Hama et al., 2004; Nikonenko et al., 2003; Chavis and Westbrook, 2001) and the cytoskeletal rearrangements that accompany synaptic activity (Smart et al., 2004; Bahr, 2000). Integrins have neuromodulatory effects in mature neurons: ligand-mimetic peptides reversibly increase the strength and duration of fast AMPA receptor-dependent post-synaptic responses (Kramar et al., 2003) and modulate NMDA receptor subunit phosphorylation and currents (Bernard-Trifilo et al., 2005; Lin et al., 2003). AMPA receptor stimulation also increases surface expression and signaling downstream of integrin α5β1 (Lin et al., 2005). In the hippocampus, integrins regulate stabilization of long-term potentiation (LTP) (Kramar and Lynch, 2003; LeBaron et al., 2003; Kramar et al., 2002; Chun et al., 2001; Staubli et al., 1998; Bahr et al., 1997), while mice with altered expression of integrins show neurotransmission and memory defects (Chan et al., 2003, 2006).

Approximately half of all integrin heterodimers recognise the aspartate within the RGD peptide sequence (Hynes, 1992). Reagents based on the RGD motif have therefore been used as antagonists of integrin function *in vivo* (Humphries et al., 1986) and have therapeutic potential for treating many diseases (e.g. Leclerc, 2002). The ligand-mimetic nature of these peptides however also endows them with agonistic properties and the ability to stimulate integrin-dependent signaling (Humphries, 2000).

Soluble RGD peptides have been used to investigate the effects of integrin ligation on neuronal function. However, the immediate neuronal signaling responses to integrin ligation, or the consequences of antagonising integrin function, are currently unknown.

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Such information is crucial for understanding how integrins affect longer-term, stable alterations in neurons such as changes in synaptic efficiency or memory formation *in vivo*. We hypothesised that changes in integrin ligation may affect intracellular signaling events in neurons. The aim of this study was therefore to analyse integrin signaling in primary cortical neurons in response to addition of the integrin ligand GRGDSP.

Results

GRGDSP causes very rapid NMDA receptor-mediated intracellular calcium increases in cortical neurons

Real-time ratiometric calcium imaging was used to examine whether the integrin-binding ligand GRGDSP affects intracellular free calcium concentrations ($[Ca^{2+}]_i$) in primary cortical neurons. The authentic peptide GRGDSP or a negative control GRADSP, which binds to integrins with a much lower affinity than GRGDSP (both at 2.5 mM), was delivered by bath application. This concentration of peptide is consistent with the millimolar affinity of the peptide for isolated integrins (Akiyama et al., 1985) and it suppresses neurite outgrowth in cortical neurons cultured on fibronectin (J.D. Moore and R.M. Gibson, unpublished observations). GRGDSP peptide induced a rapid, spontaneous increase in intracellular calcium levels, peaking within 1–2 s, which then decayed to a steady-state level; $[Ca^{2+}]_i$ remained elevated throughout the remainder of the experiment (up to 1 h; Fig. 1a). The control GRADSP peptide induced a small change in intracellular calcium levels which was significantly different to the active peptide (Fig. 1a and d: peak change in $[Ca^{2+}]_i$: 21±7% (GRADSP) versus 65±6% (GRGDSP); $P<0.01$).

Increases in neuronal $[Ca^{2+}]_i$ can be generated via entry through the calcium-permeable NMDA subtype of glutamate receptor (Mori and Mishina, 1995). To test whether the intracellular calcium changes observed on peptide addition were due to opening of NMDA receptor channels, GRGDSP was applied in the presence of the NMDA receptor antagonist MK-801 (Huettner and Bean, 1998). MK-801 abolished the GRGDSP-induced calcium increase (Fig. 1b and d: peak change in $[Ca^{2+}]_i$: 7±2% (GRGDSP+MK801) versus 65±6% (GRGDSP); $P<0.001$), indicating that calcium influx depends on the activity of NMDA receptors. NMDA receptors are multimeric channels, containing the obligate NR1 subunit with combinations of NR2A-D and NR3A/B subunits (Cull-Candy et al., 2001). Antagonists such as ifenprodil that bind to the polyamine site on the NR2B subunit can be used to analyse the contribution of NMDA receptors containing this subunit. The peak change in $[Ca^{2+}]_i$ induced by GRGDSP in the presence of ifenprodil was not significantly different to that with peptide alone (Fig. 1c and d: peak change in $[Ca^{2+}]_i$: 40±5% (GRGDSP+ifenprodil) versus 65±6% (GRGDSP)). GRGDSP-induced increases in $[Ca^{2+}]_i$ were nonetheless altered by ifenprodil, suggesting that these receptors may partially contribute to the calcium changes observed.

GRGDSP peptides induce rapid phosphorylation and nuclear translocation of ERK1/2 MAP kinase

Neurons were exposed to 2.5 mM GRGDSP or GRADSP peptide and phosphorylation of ERK1/2 and other key signaling

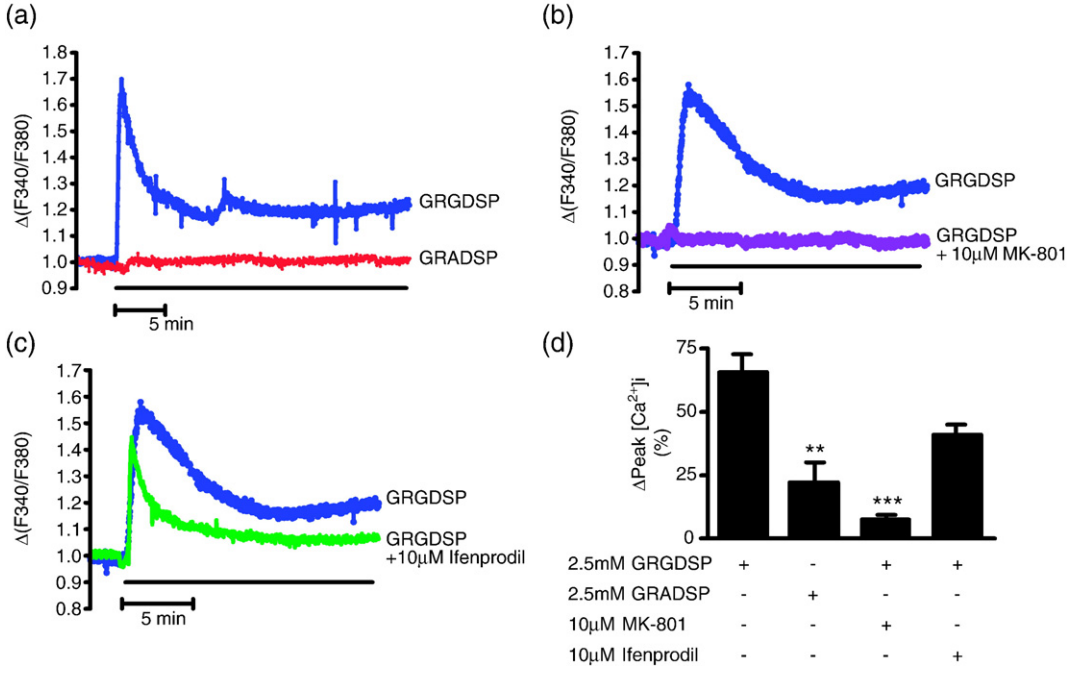

Fig. 1. Integrin-binding GRGDSP peptides induce rapid increases in intracellular calcium in cortical neurons. Intracellular calcium changes in cortical neurons (12–14 DIV) were analysed by ratiometric fura-2 imaging after treatment with (a) 2.5 mM GRGDSP or GRADSP; (b) 2.5 mM GRGDSP or GRGDSP plus 10 μM MK-801; (c) 2.5 mM GRGDSP or GRGDSP plus 10 μM ifenprodil. NMDA antagonists were added to the bath 10 min before the peptide. Horizontal black lines on each trace represent the duration of peptide treatment. Representative traces showing the average responses of at least 50 individual neurons per experiment are shown and superimposed traces are averages from the same sister culture. (d) Calcium responses from all experiments were quantified as the difference between the average normalized baseline (100%) fluorescence values subtracted from the average peak fluorescence value, for each stimulation (each experiment measuring at least 50 cells). Data are presented as mean±SEM ($n=3$). Statistical differences were calculated using one-way ANOVA with Tukey *post hoc* test: $^{**}P<0.01$, $^{***}P<0.001$ versus GRGDSP alone.