Differential mechanisms of glutamate receptor regulation of SynGAP in cortical neurones

Nichola Rockliffe*,1, Debra Gawler

The Physiological laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, UK

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Abstract One prime candidate linking N-methyl-D-aspartate (NMDA) receptors to the regulation of the MAP kinase cascade is SynGAP, a negative regulator of Ras. In order to assess how a physiological stimulus can alter SynGAP activity, an appropriate whole cell system must be used and SynGAP must be specifically extracted from membranes whilst preserving the catalytic activity of the protein. Here, we have achieved this and studied the effect of NMDA/α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate receptor stimulations on SynGAP activity in cortical neurones. Furthermore, we have examined the role of extracellular Ca²⁺, CaM kinase II and the PSD-95-NR2B subunit interaction in SynGAP activity regulation and propose a novel convergence of signalling between AMPA, kainate and PSD receptors.

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1. Introduction

Glutamatergic synapses are critical for excitatory synaptic transmission in the CNS. They are believed to play a crucial role in learning, memory and neuronal development processes [1]. Excitatory synapses contain two classes of glutamate receptors: metabotropic and ionotropic receptors. It is the ionotropic, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA), receptors that are specifically implicated as molecular components mediating neuronal development and synaptic plasticity [2,3]. In order to understand the underlying mechanisms by which these processes occur, it is important to investigate further the molecular components in the post synaptic density (PSD) fraction where these receptors are located and to identify the physiological roles played by the proteins co-complexing with these receptors. One such NMDA receptor co-complexing protein that has recently been identified is the Ras regulatory protein SynGAP [4,5]. This protein has been shown to be concentrated in excitatory synapses and directly interacts with the PSD protein, PSD-95 [4,5]. NMDA receptors and the Ras/MAPK cascade have been shown to be important in synaptic plasticity in a range of organisms [6–8] and this signalling pathway has been shown to be important in LTP; an apparent measure of synaptic plasticity [9–11]. Hence, it has been proposed that SynGAP could be a crucial signalling link between the NMDA receptor and induction and/or maintenance of synaptic plasticity [12]. Furthermore, SynGAP knock out studies have shown that SynGAP plays a critical role in neuronal development, induction of LTP, synapse formation and regulation of neuronal apoptosis [13–17]. Thus, whilst there is considerable evidence for the physiological function of SynGAP, there is still very little known about the cellular mechanisms regulating the activity of this important protein.

Previous work has shown that SynGAP is phosphorylated by CaM kinase II [18–20] and in vitro studies have shown that this phosphorylation increases the catalytic activity of SynGAP [21]. In addition, PSD-95 has been shown to promote this CaM kinase II dependent phosphorylation [22]. Whilst phosphorylation site mapping confirms that this phosphorylation does indeed occur in vivo, [21] the SynGAP activity assessment studies have so far only been performed on either recombinant SynGAP protein or on PSD preparations [21,23]. In order to assess more directly the effect a physiological stimulus has on SynGAP activity regulation, an appropriate cellular system must be used and SynGAP specifically extracted from membranes whilst preserving the catalytic activity of the protein. Here, we have successfully achieved this and using our assay system we have studied the effect ionotropic glutamate receptor stimulation has on SynGAP catalytic activity in neurones. Furthermore, we have studied the roles played by Ca²⁺, CaM kinase II and PSD-95 in these receptor triggered SynGAP activity regulation processes. Our results confirm some in vitro findings, modifying other assumptions made and demonstrate a novel convergence of signalling pathways between AMPA, kainate and NMDA receptors.

2. Materials and methods

2.1. Cortical neurone preparation

Neuronal cultures were prepared from 6- to 8-day-old Sprague–Dawley rats as previously described [24]. Cells were used after 14–19 days in culture.
2.2. Treatment and lysis of cells

Adherent cells were gently washed with phosphate-buffered saline (PBS) and incubated at 25 °C for 5 min in extracellular buffer containing 150 mM NaCl, 2.5 mM KCl, 10 mM Hepes (pH 7.5), 10 mM glucose, 5 mM EGTA and appropriate receptor agonists in the presence or absence of free Ca2+ concentrations as indicated. Cells were then washed with ice cold PBS and harvested by scraping in ice cold lysis buffer containing 20 mM Tris (pH 7.5), 1% (v/v) tergitol, 0.1% (w/v) sodium deoxycholate (SDS), 0.5 mM DTT, 100 mM NaCl, 1 mM MgCl2, 50 µg/ml leupeptin, 10 µg/ml aprotilin, 0.5 mM PMSF and 0.2 mM NaVO4. Cells lysates were homogenised on ice and then centrifuged at 2000 × gav for 5 min. Supernatants were collected for protein determination prior to SynGAP immunoprecipitation.

2.3. SynGAP immunoprecipitation and activity assay

Cell lysates (100 µg solubilised protein) were incubated at 4 °C for 1.5 h with 1 µg anti-SynGAP antibodies (UBI) pre-conjugated to 100 µl 20% (v/v) protein A sepharose beads. Immune complexes were then washed three times with lysis buffer followed by gentle resuspension in 50 µl assay mix containing 25 mM Tris (pH 7.5), 6.25 mM MgCl2, 1 mM DTT, 62.5 µM GTP, 1.25 mg/ml BSA and 0.06% (v/v) NP-40. 1 µg recombinant GST-H-Ras (UBI) was pre-loaded with [γ-32P]GTP by incubation at 30 °C for exactly 10 min with 5 µCi [γ-32P]GTP (3000 Ci/mmol) in 20 mM Tris, pH 7.5 containing 5 mM EDTA. After loading, 0.1 µg of Ras was added to each SynGAP immunoprecipitation sample and incubated at 25 °C for exactly 10 min. Reactions were stopped with ice cold stop buffer containing 25 mM Tris (pH 7.5), 0.1 M NaCl and 5 mM MgCl2 and immediately filtered through 25 mM nitrocellulose filters under vacuum. Filters were washed three times with stop buffer before drying under vacuum and scintillation counting.

Quantification of SynGAP immunoprecipitated for GTPase activating protein (GAP) assays was undertaken using a modified Western blot probing procedure. Essentially, 50% of the SynGAP immunoprecipitate sample was subjected to SDS–PAGE, Western blotted, incubated with anti-SynGAP antibodies, followed by incubation with mouse anti-rabbit biotinylated secondary antibodies and anti-avidin HRP probing. The HRP activity was detected using a highly sensitive enhanced chemiluminescence detection kit (“Visualizer Western Blot Detection Kit”; U.B.I.) and images captured on BioMax XAR pre-flashed film.

2.4. Peptide sequences and loading of cells

All peptides used were synthesised at a purity of between 89% and 97% and were HPLC analysed (Sigma-Genosys). All peptides contained N-terminal fluorescein modification.

Peptide P1
RRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRR
system we are able to confirm that in cortical neurones, activation of the NMDA receptor can stimulate SynGAP activity in a Ca\(^{2+}\)/CaM kinase II dependent manner.

As a negative control we next incubated neuronal cultures with the competitive NMDA receptor antagonist D-(+/-)-2-amino-5-phosphono-pentanoic acid (APV) [27]. However, to our surprise, we found that this ligand actually stimulated SynGAP activity (Fig. 3A). In addition, this stimulation, unlike that triggered by aspartate, was not significantly altered by...
the presence of extracellular Ca^{2+} (44 ± 7% and 29 ± 8% for SynGAP activation with or without Ca^{2+}, respectively). Furthermore, the CaM kinase inhibitor KN-62 had no effect upon this APV triggered activation of SynGAP (42 ± 4% stimulation after inhibitor treatment of cells). This effect of a supposed receptor antagonist was highly reproducible and the levels of SynGAP activation were comparable to those observed with the receptor agonist (Fig. 2). So our data is consistent with this receptor ligand being a biological agonist in that it has post receptor effects and the classification of this drug as an antagonist was originally based on electrophysiological recordings which only reflect its inability to open the Ca^{2+} ion channel of the NMDA receptor. So, based on our observations we would suggest that APV upon binding to the NMDA receptor NR2 subunit, causes a conformational change which elicits a post-receptor biological effect (i.e., SynGAP activation). Thus, we have evidence to support both a Ca^{2+}-dependent and a Ca^{2+}-independent mechanism of NMDA receptor activation of SynGAP. Furthermore, this finding is very interesting in that others have shown differential effects on LTD and LTP using this NMDA receptor “antagonist” [30]. We suggest that maybe these differential effects are related to discrete biological post receptor effects of this drug. Thus, by using this drug it may be possible to examine in isolation Ca^{2+} influx and SynGAP-dependent events.

To examine this further, we looked to see if APV and Aspartate activation of SynGAP was additive. Triggering SynGAP activation with these receptor ligands under conditions where Aspartate activation of NMDA receptors is optimised (i.e., in the presence of glycine and extracellular Ca^{2+}) we found that these two ligands were indeed additive with respect to SynGAP activation (Fig. 3B). Alone APV activated SynGAP by 29 ± 2%, Aspartate activated SynGAP by 36 ± 4% and when these ligands were incubated together SynGAP was activated by 67 ± 2%. Hence, this finding provides further evidence that NMDA receptors are involved in two independent SynGAP activation pathways and is consistent with APV acting as a partial agonist.

Next, we reasoned that if APV was causing NMDA receptor conformational changes then, most probably, these would affect interactions between the receptor subunits and co-complexed signalling proteins. So, by disrupting receptor-signalling protein interactions we could probe further the molecular mechanisms required for each receptor ligand effect. One prime candidate for coupling SynGAP to the NR2 subunit of the NMDA receptor is the PSD protein PSD95. It is conceivable that SynGAP associates with the NMDA receptor via interaction with PSD95. Indeed, the C-terminal (QTRV) sequence found in SynGAP has been shown to interact directly with PSD-95 [4] and, in turn, PSD-95 interacts with the PDZ-binding motif (ESDV) located within the C-terminal region of the NR2 subunit [31,32,35]. In cortical neurones, both NR2A and NR2B subunits are believed to be expressed and associated with NR1 NMDA receptor subunits [33]. In common with the NR2B subunit, the NR2A subunit C-terminus also contains the ESDV motif. So, in order to disrupt the interaction of PSD95 with NR2 NMDA subunits we synthesised peptides containing an N-terminal fluorescein tag followed by an 11 arginine (R_{11}) protein transduction domain (which has been shown to allow the passage of peptides into cells) [34,35] and selective amino acid sequences corresponding to the NR2A and NR2B C-termini. These peptides were loaded into neurones as previously described [34,35] and loading efficiency assessed by fluorescence microscopic detection of the fluorescein tag. Under our loading conditions, 90–100% of adherent cells were routinely loaded with each peptide (data not shown). The effect of each peptide on aspartate (Ca^{2+}-CaM kinase II dependent) triggered activation of SynGAP was then studied (Fig. 4A). From these studies, we found that

![Fig. 3. Effect of the NMDA receptor antagonist, APV, on SynGAP activity in cortical neurones. Adherent cells in culture were treated at 25 °C for 5 min with 5 μM APV in extracellular incubation buffer in the presence or absence of 100 μM free Ca^{2+} as indicated. After treatment, cells were lysed, immunoprecipitated with anti-SynGAP antibodies and assayed for Ras GAP activity as detailed in Section 2. Data shown is representative of mean ± S.E.M values for n = 4 experiments performed on independent cell culture batches. Panel inserts show SynGAP immunoprecipitates representative of a batch of cells assayed; Panel A: lane 1, unstimulated; lane 2, APV-Ca^{2+}; lane 3, APV + Ca^{2+}; lane 4, APV + Ca^{2+} + KN-62 treated cells. Panel B: lane 1, unstimulated; lane 2, APV; lane 3, Asp; lane 4, APV + Asp stimulated cells.](image-url)
cells loaded with the control peptide P1, that contained only the fluorescein tag and the R11 protein transduction domain, was able to stimulate SynGAP to a similar extent as cells that were not peptide loaded (41 ± 9% and 53 ± 2% for peptide P1 and no peptide, respectively; indicating that the cellular loading of the tagged peptides did not interfere with the receptor-

**Fig. 4.** Effect of loading peptides corresponding to the C-terminal tail sequences of the NR2A and NR2B subunits on NMDA receptor triggered activation of SynGAP. Cells were pre-loaded with peptides as described in materials and methods section prior to incubation with 

![Graph A](image)

**Panel A:** lane 1, unloaded and unstimulated cells; lane 2, stimulated and unloaded cells; lane 3, stimulated and P1 loaded cells; lane 4, stimulated and P2 loaded cells; lane 5, stimulated and P3 loaded cells; lane 6, stimulated and P4 loaded cells. 

![Graph B](image)

**Panel B:** lane 1, unloaded and unstimulated cells; lane 2, stimulated and P1 loaded cells; lane 3, stimulated and P2 loaded cells; lane 4, stimulated and P3 loaded cells; lane 5, stimulated and P4 loaded cells. 

- SynGAP pathway. Next, we looked at the effect of tagged peptides containing specific NR2 subunit sequences on the NMDA receptor activation of SynGAP and interestingly found that peptide P2 which contained the C-terminal amino acid sequence of the NR2B subunit not only blocked the receptor triggered activation, but in fact inhibited SynGAP activity when compared to the basal (unstimulated) SynGAP activity approximately two fold (Fig. 4A). Furthermore, peptide P3, which corresponds to the C-terminal amino acid sequence of the NR2A subunit, had no significant effect on the NMDA receptor triggered activation of SynGAP (33 ± 7% stimulation). Since both these peptides contain the ESDV motif, we reasoned that the selective inhibitory effect of the NR2B sequence may correspond to the unique YEKLSSI sequence found in the NR2B but not the NR2A subunit C-terminal tail. So we loaded cells with a peptide corresponding to this sequence (herein denoted peptide P4) and assessed its effect on NMDA receptor triggered activation of SynGAP. We found that this peptide similarly inhibited the receptor triggered activation of SynGAP (150 ± 44%). From these data we conclude firstly that the NR2B subunit and not the NR2A subunit selectively couples to the (aspartate-mediated) NMDA receptor triggered activation of SynGAP. In addition, this subunit and SynGAP activation selectivity is conferred by the YEKLSSI sequence since both peptides P2 and P3 contained the ESDV motif, but only peptide P2 disrupted the SynGAP activation. These findings are intriguing because very recently the NR2B subunit of the NMDA receptor has been shown to selectively associate with SynGAP and appears to be required for inhibition of the NMDA receptor dependent Erk activation. This, in turn, correlates with the inhibition of the surface expression of the GluR1 (AMPA) receptor [36]. Our findings would be consistent with this subunit selectivity for SynGAP and, since CaM kinase II is believed to be critical for GluR1 recruitment at the synapse, this would also agree with our finding of this CaM kinase II dependent mechanism being mediated selectively via the NR2B receptor. Indeed, CaM kinase II does bind directly to the NR2B subunit [37].

Next we probed the molecular mechanism involved in the Ca^2+ | CaM kinase II independent SynGAP activation process which we observed when triggering NMDA receptor activation with APV (Fig. 4B). Using the same peptide loading procedure as previously employed and triggering SynGAP activation with APV (in the absence of extracellular Ca^2+), we firstly looked at the effect of peptides P2 and P3 (corresponding to the NR2B and NR2A subunit sequences, respectively) and control peptide P1 on this process. We found that both peptides P2 and P3, but not peptide P1 blocked SynGAP activation, suggesting that this time the ESDV motif may be important for this activation process. Using a peptide whose sequence corresponded to the ESDL motif alone (peptide P5) we observed the same blocking effect, whereas the peptide P4, which corresponded to the “YEKLSSI” NR2B specific sequence did not block the receptor activation of SynGAP. From these data we conclude that the Ca^2+/CaM kinase II-independent receptor activation of SynGAP may not be NR2B subunit selective and is mediated via a mechanism which requires this ESDL sequence found in both NR2A and B subunits.

In order to evaluate the effect of these peptides on the PSD-95, SynGAP and NR2B subunit co-association in our system, we immunoprecipitated PSD-95 from peptide loaded cells.
and Western blot probed to detect for each protein (Fig. 5). We found (in agreement with others using slightly longer C-terminal peptides in brain slice experiments [35]) that peptides P2 and P3 inhibited the PSD-95 co-association with NR2B subunits. In addition, by using fragments of the NR2B tail sequence, we found that peptide P5 was also sufficient to inhibit this interaction whilst peptides P1 and P4 had no significant effect. From these data we conclude that the APV-mediated stimulation of SynGAP correlates well with the loss of PSD-95 interaction with the NR2B subunit, but there is no correlation between these proteins interacting and the ability of aspartate (via CaM kinase II) to stimulate SynGAP activity. In addition, SynGAP levels found associated with PSD-95 were not significantly found to alter with peptide treatments (Fig. 5).

Finally, we went on to look at whether AMPA and kainate receptor activation could regulate SynGAP activity. Ras/MAPK regulation is believed to play an important role in both the AMPA receptor expression and the localisation/clustering of receptors in the post synaptic membrane [36]. Hence, cross-talk between post synaptic glutamate receptors could be an important link in Ras regulation pathways. Therefore, we activated both AMPA and kainate receptors with AMPA and in the presence or absence of extracellular Ca²⁺ and looked to see what effect these treatments had on SynGAP activity (Fig. 6). We found that AMPA treatment of cells whether in the presence or absence of extracellular Ca²⁺ inhibited SynGAP activity (by 74 ± 8% and 44 ± 8%, respectively). However, we consistently found that the presence of extracellular Ca²⁺ during the AMPA treatment of cells reduced the inhibition of SynGAP inhibition by approximately 30% (Fig. 6A). This suggested that there may be an AMPA inhibitory mechanism which was attenuated by a Ca²⁺-dependent stimulatory mechanism. We reasoned that the dependent Ca²⁺ mechanism could be a Ca²⁺/CaM kinase II dependent effect since AMPA and kainate receptor activation would cause membrane depolarisation which in turn could conceivably activate voltage operated Ca²⁺ channels to allow Ca²⁺ influx. However, when we pre-treated cells with KN-62 we found no significant effect on this Ca²⁺ mediated attenuation of SynGAP inhibition (data not shown). Thus indicating that CaM kinase II plays no role in this Ca²⁺ mediated event. Furthermore, when we looked at the effect of our control (peptide P1) or NR2 subunit peptides (P2 and P3) on this Ca²⁺ and AMPA triggered inhibition of SynGAP (Fig. 6B). We found that none of these peptides had any significant effect on the Ca²⁺ mediated attenuation of the AMPA response; indicating that this Ca²⁺ mediated mechanism was not significant.

Fig. 5. Effect of loading peptides corresponding to the C-terminal tail sequences of the NR2A and NR2B subunits on the Co-immunoprecipitation of PSD-95, NR2B and SynGAP from cortical neurones. Cells were pre-loaded with peptides as described in materials and methods section. Cells were then lysed and incubated with anti-PSD-95 antibodies, followed by Western blot analysis probing for the presence of PSD-95, SynGAP and NR2B subunits. Data shown is representative of n = 3 independent experiments performed.

Fig. 6. AMPA treatment inhibits SynGAP activity in cortical neurones. Adherent cells in culture were treated at 25 °C for 5 min with 5 μM AMPA in extracellular incubation buffer in the presence or absence of 100 μM free Ca²⁺ as indicated. After treatment, cells were lysed, immunoprecipitated with anti-SynGAP antibodies and assayed for Ras GAP activity as detailed in the methods section. (A) effect of extracellular Ca²⁺ on AMPA inhibition of SynGAP. (B) Pre-loading cells with peptides has no effect on the Ca²⁺ mediated relief of SynGAP inhibition triggered by AMPA treatment. Data shown is representative of mean ± S.E.M values for n = 3 experiments performed on independent culture batches. Panel inserts show SynGAP immunoprecipitates representative of a batch of cells assayed. Panel A: lane 1, untreated cells; lane 2, AMPA stimulated – Ca²⁺; lane 3, AMPA stimulated + Ca²⁺. Panel B: lane 1, AMPA stimulated – Ca²⁺; lane 2, AMPA stimulated + Ca²⁺ and no peptide loaded; lane 3, AMPA stimulated + Ca²⁺ and P1 loaded; lane 4, AMPA stimulated + Ca²⁺ and P2 loaded; lane 5, AMPA stimulated + Ca²⁺ and P3 loaded cells.
related to those observed for the APV or aspartate triggered activation of SynGAP.

From this study we conclude firstly, that APV is not a true “antagonist” of the NMDA receptor and, as such, studies using this compound and assuming antagonistic biological function may need some re-evaluation. Secondly, we have detected both a Ca\(^{2+}\)/CaM kinase II dependent and a Ca\(^{2+}\)/CaM kinase II independent (but PSD-95/NR2 subunit dependent) mechanism for SynGAP stimulation. These data suggest that SynGAP activity-dependent cellular effects may not necessarily be related to PSD-95 interaction with the NR2B subunit. Instead, the CaM kinase II dependent mechanism of SynGAP activation does not appear to be linked with the PSD-95/NR2B subunit interaction but does appear to involve the “YELKSL” sequence in the NR2B subunit. The molecular components involved in this mechanism require further elucidation.

In addition, it is also possible that specific isoforms of SynGAP may exhibit differential mechanisms of signalling via NMDA receptors. Such mechanisms, in turn, may be dependent upon the stage of neuronal development and the expression levels of each molecular component in specific locations within the brain. All of these possibilities can be further studied using this SynGAP assay system in combination with immunoprecipitating antibodies recognising selective epitopes found in specific SynGAP isoforms. Further work in this area may lead to a better understanding of the physiological functions of this protein.

Finally, since not only NMDA receptors but also AMPA/kainate receptors appear to regulate SynGAP activity, it is conceivable that the combination of the two events, (which would be expected to occur upon glutamate release from pre-synaptic sites) may serve to finely tune SynGAP activity. Hence it may be subtle changes towards critical threshold levels which in turn result in MAPK-dependent effects leading to synaptic plasticity events.

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Appendix A Supplementary data


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