Kidins220/ARMS downregulation by excitotoxic activation of NMDARs reveals its involvement in neuronal survival and death pathways

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Summary

Functional and protein interactions between the N-methyl-D-aspartate type of glutamate receptor (NMDAR) and neurotrophin or ephrin receptors play essential roles in neuronal survival and differentiation. A shared downstream effector for neurotrophin- and ephrin-receptor signaling is kinase D-interacting substrate of 220 kDa (Kidins220), also known as ankyrin repeat-rich membrane spanning (ARMS). Because this molecule is obligatory for neurotrophin-induced differentiation, we investigated whether Kidins220/ARMS and NMDAR functions were related. Here, we identify an association between these proteins and discover that excitotoxicity, a specific form of neuronal death induced by NMDAR overstimulation, dramatically decreases Kidins220/ARMS levels in cortical neuronal death induced by NMDAR overstimulation, and subsequent Ca²⁺ influx, and involves a dual mechanism: rapid cleavage by the Ca²⁺-dependent protease calpain and calpain-independent silencing of Kidins220/Arms gene transcription. Additionally, Kidins220/ARMS knockdown decreases ERK activation and basal neuronal viability, and enhances neuronal death under excitotoxic conditions. Our results demonstrate Kidins220/ARMS participation in neuronal life and death pathways, and constitute the first report of its regulation under pathological conditions.

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Key words: Kidins220/ARMS (ARMS), NMDAR, Excitotoxicity, Ischemia, Neurotrophins, Trk receptors, Eph receptors, Calpain, ERK, Neuronal death, Survival

Introduction

Development and functioning of the central nervous system involves multiple processes, including neuronal differentiation, synaptogenesis and synaptic activity, as well as crucial death and survival choices. These activities require specialized signaling pathways generally initiated by activation of membrane receptors. Neurotrophin and ephrin receptors [tropomyosin-related kinase (Trk) and Eph, respectively], and those for the neurotransmitter glutamate, play pivotal roles. Their signaling cascades are supported by large and dynamic complexes formed by multiple proteins, such as those associated to the N-methyl-D-aspartate type of glutamate receptor (NMDAR) (Husi et al., 2000).

The interactions established among receptors and with their signaling proteins are challenged in several pathologies, for instance those related to excitotoxicity. This specific form of cell death, due to overstimulation of the NMDAR, contributes to neuronal degeneration in acute disorders such as hypoxia, ischemia, trauma and epilepsy, or chronic neurodegenerative pathologies such as Alzheimer’s, Parkinson’s or Huntington’s diseases (Choi, 1988).

The NMDAR is central to physiology, promoting neuronal survival and playing key functions in synaptic activity, plasticity, learning and memory, but is also involved in the etiology of human diseases and neuronal death. The basis for the dichotomy of NMDARs regulating survival and death decisions is beginning to be established. Physiological NMDAR stimulation is crucial for neuronal survival: it induces the activation of, among others, extracellular signal-regulated kinase (ERK; also known as mitogen-activated protein kinase) (Thomas and Huganir, 2004), and increases antioxidant defenses (Papadia et al., 2008). By contrast, activation of NMDARs under pathological conditions, for example in brain ischemia after massive glutamate release, opposes these neuroprotective effects and is coupled to cell death pathways (Hardingham et al., 2002). We have described an autoregulatory mechanism induced by excitotoxicity and ischemia; this mechanism rapidly decreases NMDAR function. It consists of transcriptional downregulation of the gene encoding the obligatory NR1 subunit (Gascon et al., 2005), and the processing of NR2A and NR2B C-terminal regions by calpain (Gascon et al., 2008), an important effector of Ca²⁺ overload resulting from NMDAR overactivation (Neumar et al., 2003). Calpain also cleaves several NMDAR-interacting and signaling proteins (reviewed by Vosler et al., 2008). This mechanism is central to neuronal death because it affects many...
components of NMDAR complexes, including the NR2A subunit, which is crucial for synaptic transmission and survival.

Interactions among NMDAR, Trk and Eph have been reported. TrkB coimmunoprecipitates with Fyn, a tyrosine kinase also associated to NR2B (Tezuka et al., 1999). Neural Shc (N-Shc), an adaptor protein transmitting neurotrophin signals from TrkB to ERK, also regulates NMDAR function (Miyamoto et al., 2005). Additionally, NMDAR association with HEB controls synapse formation (Dalva et al., 2000) and function (Henderson et al., 2001), and ephrin-B activation of EphB positively modulates NMDAR-dependent Ca²⁺ influx and gene expression (Takasu et al., 2002). A downstream effector for both Trk and Eph is kinase D-interacting substrate of 220 kDa (Kidins220) (Iglesias et al., 2000), also known as ankyrin repeat-rich membrane spanning (ARMS) (Kong et al., 2001). Kidins220/ARMS is an integral membrane protein originally identified as the first physiological substrate for protein kinase D (PKD) (Iglesias et al., 2000). This novel molecule bears multiple protein-interaction domains, including a binding motif for PDZ postsynaptic density 95 (PSD-95), discs large, zonula occludens-1 proteins at its C-terminus (Iglesias et al., 2000; Kong et al., 2001). This motif is responsible for Kidins220/ARMS localization at the neuromuscular junction, through association with ε-syntrophin, a PDZ protein that enhances EphA4 signaling in a Kidins220/ARMS-dependent manner (Luo et al., 2005). Notably, NR2A-C subunits, which present a PDZ ligand (Kornau et al., 1995), also interact with syntrophin in neurons (Cui et al., 2007). Additionally, Kidins220/ARMS forms a complex with neurotrophin receptors (Kong et al., 2001). The presence of Kidins220/ARMS within this complex is obligatory for prolonged ERK activation mediated by the GTPase Rap1, leading to neuronal differentiation in response to neurotrophins (Arevalo et al., 2006; Arevalo et al., 2004). The major component for sustained ERK stimulation relies on tetrameric complexes formed by Trk, Kidins220/ARMS, the PDZ protein synaptic scaffolding molecule (S-SCAM) and the activator of Rap1, PDZ-GEF1 (Hisata et al., 2007). It is noteworthy that S-SCAM was first cloned as an NMDAR-interacting protein (Hirao et al., 1998). All these observations evidence a possible, as-yet-unexplored, functional link between Kidins220/ARMS and NMDAR signaling pathways.

Herein, we demonstrate the association of Kidins220/ARMS and NMDARs in neurons and brain. We discover that NMDAR overstimulation provokes a dramatic downregulation of Kidins220/ARMS in cultured cortical neurons and in a model of transient cerebral ischemia, the first report of Kidins220/ARMS regulation under pathological conditions. This downregulation involves calpain-dependent and -independent mechanisms. Importantly, we show that Kidins220/ARMS knockdown decreases ERK activation and basal neuronal viability, and enhances neuronal death during excitotoxicity, demonstrating a key role for this protein in crucial life and death decisions.

**Results**

Kidins220/ARMS associates with NMDARs in cortical neurons and brain

We investigated the association between NMDARs and Kidins220/ARMS because they are targets and/or interact with neurotrophin and ephrin receptors (Dalva et al., 2000; Kong et al., 2001; Miyamoto et al., 2005; Takasu et al., 2002; Yamada and Nabeshima, 2004). Kidins220/ARMS and the NR2A, NR2B and NR1 subunits coimmunoprecipitated in rat cortical neurons grown for 14 days in vitro (DIV) (Fig. 1A) or brain (Fig. 1B).

Immunofluorescence and confocal microscopy showed that the NR1, and NR2A and NR2B (collectively referred to as NR2A/B) subunits mostly overlapped with Kidins220/ARMS at soma and along extensions (Fig. 1C; supplementary material Fig. S1). A pool of Kidins220/ARMS was enriched at tips of neuronal processes; this pool was especially detected when using the polyclonal antibody recognizing a Kidins220/ARMS C-terminal peptide as previously described (Iglesias et al., 2000). To analyze the coexistence of Kidins220/ARMS and NMDARs at the neuronal surface, we used lentiviral-infected neurons expressing recombinant NR2A with an N-terminal extracellular hemagglutinin epitope (HA-NR2A). We have previously demonstrated that this recombinant protein colocalizes with endogenous NR2A and NR2B subunits (Gascon et al., 2008). This approach was necessary owing to the lack of good antibodies for immunofluorescence that are able to recognize specifically the extracellular regions of each NR2 subunit. Neurons were stained for HA before permeabilization and Kidins220/ARMS labeling. Images showed HA-NR2A at the surface largely colocalizing with endogenous Kidins220/ARMS at certain plasma-membrane domains (Fig. 1D). Kidins220/ARMS immunostaining was very similar in infected and non-infected neurons, and alike to previous results (Sanchez-Ruiloba et al., 2006). These data establish an association of Kidins220/ARMS with NMDARs at the neuronal surface, suggesting that they could be functionally related.

Kidins220/ARMS is irreversibly downregulated by excitotoxic stimulation of NR2B-containing NMDARs in neurons

We next examined Kidins220/ARMS regulation by NMDAR overactivation, because other NMDAR-interacting proteins such as PSD-95 are downregulated in excitotoxicity (Gascon et al., 2008). Neurons were incubated for different times with high concentrations of NMDA (100 μM) and the co-agonist glycine (10 μM), conditions that overstimulate synaptic and extrasynaptic NMDARs. Excitotoxicity already decreased Kidins220/ARMS after 2 hours to levels of 46±10% (P<0.05) relative to untreated cells (Fig. 2A, upper panels). Stimulation over 4 and 6 hours further reduced Kidins220/ARMS levels to 27±4% and 16±3% (P<0.001), respectively (Fig. 2A, upper panels). NR2A analysis showed a slower kinetics for Kidins220/ARMS downregulation (Fig. 2A). Levels of the neuronal-specific enolase (NSE) were not significantly modified after NMDA treatment (Fig. 2A), suggesting that Kidins220/ARMS decrease was not a consequence of a massive neuronal death. Measurement of neuronal viability by MTT assays confirmed that excitotoxic death was a 35±5% (P<0.001) at 6 hours of NMDA treatment (Fig. 2B, lower panel), well below the reduction observed in Kidins220/ARMS levels at this time (84%) (Fig. 2B, upper panel). However, although viability was still high in this time window, these neurons were committed to die and a progressive increase in neuronal death was observed at later times of NMDA treatment (Fig. 2B, lower panel). Kidins220/ARMS immunofluorescence also decreased after 6 hours of NMDAR overstimulation, whereas levels of neuron-specific βIII-tubulin immunolabeling were not significantly modified (Fig. 2C). However, βIII-tubulin staining denoted morphological changes characteristic of the excitotoxic process, with dendritic focal swelling and varicosities preceding cell death as previously described (Park et al., 1996).
showed a dramatic reduction of Kidins220/ARMS (84% for 100 μM NMDA; \(P<0.001\)) only for concentrations higher than 10 μM (Fig. 2D). Excitotoxic neuronal death is coupled to stimulation of extrasynaptic NMDARs (Hardingham et al., 2002). Although some authors described NR2B as the main NR2 subunit in extrasynaptic positions (Li et al., 1998), others found receptors containing NR2A and NR2B subunits in either synaptic or extrasynaptic compartments (Thomas et al., 2006). We have previously shown using ifenprodil, a selective inhibitor for NR2B subunits (Williams, 1993), that downregulation of NR1 and NR2 subunits is irreversibly induced by brief agonist-overactivation of NR2B-containing NMDARs (Gascon et al., 2005; Gascon et al., 2008). Similarly, ifenprodil, or the generic NMDAR antagonist DL-AP5, prevented the Kidins220/ARMS decrease induced by NMDA (Fig. 2E). Because cultured neurons at this time express both NR2A and NR2B, we conclude that Kidins220/ARMS downregulation specifically requires overactivation of NMDARs containing NR2B subunits. Furthermore, stimulation of neurons with NMDA for 25 minutes was sufficient to induce a reduction in the levels detected 24 hours later, similar to that induced by 3 hours of treatment (Fig. 2F). This suggests that Kidins220/ARMS loss is triggered irreversibly by brief overstimulation of NMDARs, which cannot be blocked by antagonists, a similar mechanism to that operating on or over the NMDAR subunits.

Kidins220/ARMS is downregulated in an animal model of cerebral ischemia

Next, we examined Kidins220/ARMS protein after in vivo overstimulation of NMDARs. We selected an animal model of cerebral ischemia on the basis that excitotoxicity induced by overactivation of NMDARs is responsible for neuronal degeneration in this pathology (Choi, 1988). Transient focal cerebral ischemia was induced in rats by 1-hour occlusion of the middle cerebral artery (MCAO) followed by reperfusion from 0 to 48 hours. This is a highly reliable model that we have previously used, in which large infarcts detected by Nissl staining are produced in the right MCA territory after 24 hours of reperfusion (Gascon et al., 2005; Gascon et al., 2008). As shown in Fig. 3A, the ipsilateral neocortex presented a Nissl hypochromatic area indicative of neuronal injury, absent in the equivalent region of the contralateral cortex. Immunohistochemistry of adjacent sections showed a Nissl hypochromatic area indicative of neuronal injury, absent in the equivalent region of the contralateral cortex. Immunohistochemistry of adjacent sections showed Kidins220/ARMS in neuronal somas and fibers of the contralateral cortex and a dramatic signal decrease within ipsilateral infarcted tissue (Fig. 3A). The areas of staining reduction matched those of ischemic injury delimited by Nissl. Contiguous sections were immunolabeled for neuronal nuclei protein (NeuN) to show the presence of neurons in the infarcted region. The time course of Kidins220/ARMS downregulation was then analyzed by immunoblotting extracts from the infarced area and corresponding
Excitotoxicity regulates Kidins220/ARMS

regions of the contralateral hemisphere using animals subjected to MCAO and different durations of blood reperfusion (Fig. 3B). Occlusion induced a moderate decrease in Kidins220/ARMS (time 0; Fig. 3B). However, reperfusion extensively reduced Kidins220/ARMS levels (normalized to NSE levels) by 80-90% in the infarcted area of animals sacrificed after 24 or 48 hours. Similar results were obtained for NR2A, whereas NSE did not undergo major changes.

Kidins220/ARMS downregulation requires Ca\(^{2+}\) entry and calpain activation

NMDAR overstimulation leads to a Ca\(^{2+}\) influx that is required for the downregulation of its subunits (Gascon et al., 2005; Gascon et al., 2008). Buffering of extracellular Ca\(^{2+}\) with EGTA prevented Kidins220/ARMS decrease induced by NMDA (Fig. 4A), showing that Ca\(^{2+}\) entry also participates in Kidins220/ARMS reduction during excitotoxicity.

Calpain plays a major role in excitotoxicity and ischemia through processing different substrates (Hong et al., 1994; Siman and Noszek, 1988), including NR2A and NR2B together with their interacting protein PSD-95 (Gascon et al., 2008; Simpkins et al., 2003). We investigated calpain participation in Kidins220/ARMS decrease by using different inhibitors. Preincubation of cultures with the neutral cysteine protease inhibitors ALLN and ALLM partially prevented the decrease in Kidins220/ARMS levels induced by NMDA, whereas treatment with the lysosomotropic drug chloroquine did not (Fig. 4B). Pretreatment with calpain-specific inhibitor III (CiIII) almost completely hampered the downregulation of this protein (Fig. 4C). Other inhibitors, such as pan-caspase inhibitor zVAD or matrix-metalloproteinases inhibitor GM6001, exerted no effect on Kidins220/ARMS recovery (Fig. 4C). These results strongly support the requirement of calpain activity for Kidins220/ARMS downregulation stimulated by excitotoxicity and suggest Kidins220/ARMS as a novel calpain substrate.

The susceptibility of Kidins220/ARMS to in vitro processing by two concentrations of calpain I was analyzed in our neuronal protein extracts (Fig. 4D). The emergence of breakdown products (BDPs) from full-length (FL) brain spectrin, a widely used marker for calpain activity, confirmed activation of this protease. Kidins220/ARMS was found to be highly sensitive to calpain...
cleavage, and FL protein was not detected under conditions that only partially fragmented FL-spectrin. Notably, a band of approximately 70 kDa was recognized by anti-Kidins220/ARMS antibody at the lower calpain concentration, suggesting the generation of at least a transient C-terminal product. This finding is in contrast to results from the cellular and animal models, in which no C-terminal fragments were observed (data not shown). Two different calpain inhibitors (CiIII and calpeptin) prevented the in vitro processing of Kidins220/ARMS by calpain (supplementary material Fig. S2). The specificity of this assay was established by analyzing NSE, which is not a calpain substrate.

Next, we quantified the contribution of calpain to the mechanisms governing Kidins220/ARMS downregulation during excitotoxicity. Preincubation of cultures with CiIII prevented NMDA-induced spectrin proteolysis (Fig. 4E), although it was not able to completely restore Kidins220/ARMS to control levels, preserving only 86±11% (P<0.05) of this molecule (Fig. 4F). These data support the existence of other mechanisms contributing to Kidins220/ARMS disappearance during excitotoxicity. Collectively, these experiments define the mechanism of Kidins220/ARMS downregulation in excitotoxicity as a process triggered by Ca2+ entry, with one major component consisting of an extensive calpain-dependent processing and other component(s) to be determined.

Transcriptional inhibition of the Kidins220/Arms gene after excitotoxic activation of NMDARs

Contrary to NR2 subunits, levels of NR1 decrease during excitotoxicity, as a consequence of transcriptional repression of its promoter by calpain-independent mechanisms (Gascon et al., 2005). Because our results suggested a second component independent of calpain activity in Kidins220/ARMS downregulation, we explored a possible transcriptional control of the Kidins220/Arms gene under excitotoxic conditions. We analyzed, by northern blot, mRNA from neurons incubated for different times with excitotoxic concentrations of NMDA (Fig. 5A). Quantitation of steady-state Kidins220/Arms mRNA levels revealed a notable decrease (39%) after 2 hours of stimulation and a reduction that was more dramatic at later times. Real-time quantitative PCR analysis showed that transcripts were decreased by 54±10% (P<0.05) after 2 hours of NMDA stimulation, and further reduced at later times up to a 73±4% and 84±3% decrease (P<0.001) after 4 and 6 hours, respectively (Fig. 5B). Importantly, this decrease was independent of calpain activation, because preincubation with CiIII was not able to restore Kidins220/Arms mRNA to control levels (Fig. 5C), although it could largely prevent the protein decay induced by NMDA (Fig. 5C, inset). By contrast, and in accordance with protein data (Fig. 2E; Fig. 4A), treatment with EGTA (Fig. 5D) or ifenprodil (Fig. 5E) prevented the decrease of Kidins220/Arms mRNA. Furthermore, Kidins220/Arms mRNA decay was very similar in neurons treated with the transcriptional inhibitor actinomycin D alone or in combination with NMDA (Fig. 5F). These data indicate that Kidins220/Arms mRNA turnover does not change in excitotoxicity, and that its downregulation is independent of de novo transcription of transcriptional regulators.

We finally examined a direct effect of excitotoxicity on the in vivo transcriptional rate of Kidins220/Arms by nuclear transcription run-on assay. Duplicate sets of filters containing cDNAs of Kidins220/Arms, NR2A, NR1, cyclophilin, γ-actin and NSE were hybridized to α-32P-UTP-labeled RNA probes prepared from nuclei purified from neurons, untreated or treated with NMDA for 4 hours (Fig. 5G). Quantitation showed a significant reduction of the Kidins220/Arms transcriptional rate under excitotoxic conditions, which was of 53%, 49% and 46% relative to untreated cells, using cyclophilin, γ-actin or NSE, respectively, for normalization. Similar results were obtained for NR1, although the decrease in its transcriptional rate was slightly higher (60-70%, depending on the gene used to normalize). By contrast, NR2A, a gene non-transcriptionally regulated by NMDAR overstimulation (Gascon et al., 2005), did not render variations (Fig. 5G). Thus,
Kidins220/Arms is a direct target transcriptionally downregulated by excitotoxicity.

Altogether, our data show a dual mechanism affecting Kidins220/ARMS at the protein and mRNA level. Both components are activated through NMDAR overstimulation leading to Ca\(^{2+}\) influx. Downstream, Kidins220/ARMS protein is rapidly proteolyzed by calpain and Kidins220/Arms gene transcription is directly downregulated by mechanisms independent of calpain activation (see Discussion).

Knockdown of Kidins220/ARMS decreases ERK activity and neuronal viability under basal and excitotoxic conditions

The results shown above demonstrating an association of Kidins220/ARMS with NMDARs, together with the unique role of this protein in neurotrophin- and ephrin-receptor signaling (Kong et al., 2001), strongly suggest an important role of Kidins220/ARMS in neuronal survival pathways. Therefore, the downregulation of Kidins220/ARMS induced by excitotoxicity could significantly contribute to neuronal death. To test the effect on neuronal viability of downregulating Kidins220/ARMS expression, we generated a lentiviral short hairpin (sh)RNA vector to knockdown Kidins220/ARMS expression (shK). An equivalent lentiviral vector containing a non-specific shRNA sequence was used as control (shC).

Immunoblot analysis showed that shK infection provoked an efficient and specific knockdown of Kidins220/ARMS expression compared to shC-transduced cells (Fig. 6A). When excitotoxicity was induced in shK-infected neurons, calpain-dependent (NR2A/B) and calpain-independent (NR1) downregulation mechanisms were similar to those in shC-transduced neurons (supplementary material Fig. S3). These data indicate that Kidins220/ARMS (and probably its association to NMDARs) is not obligatory for NMDA-induced excitotoxicity to occur.

It is known that Kidins220/Arms silencing hampers the sustained ERK activation elicited by neurotrophin-receptor stimulation (Arevalo et al., 2006; Arevalo et al., 2004). In addition, ERK signaling mediates synaptic NMDAR-dependent neuronal plasticity and survival (reviewed by Thomas and Huganir, 2004). We therefore first explored in our cortical cultures the effects of knocking down Kidins220/ARMS to different degrees on ERK activation (Fig. 6B).

The decline of Kidins220/ARMS levels using different amounts of shK virus was dose-dependent, and was accompanied by a progressive decrease of active phospho-ERK (ERK-P), whereas total ERK was unaffected (Fig. 6B, left panel). Quantitation of five experiments showed that, at the highest shk viral concentrations, ERK1-P and ERK2-P signals were reduced by 51±5% and 49±7% (P<0.01), respectively (Fig. 6B, right panel).

The results regarding activation-inactivation of the ERK signaling cascade during excitotoxicity are heterogeneous and depend on the particular experimental conditions used (Gouix et al., 2009; Ivanov et al., 2006; Leveille et al., 2008). Thus, we investigated ERK activation in response to excitotoxic concentrations of NMDA in our model, and in parallel the effects of knocking down Kidins220/ARMS to almost undetectable levels. In shC-infected cultures, we observed a progressive ERK activation that peaked at 2 hours of NMDA treatment, followed by a gradual reduction to basal levels after 6 hours (Fig. 6C, middle time points not shown). Overstimulation of NMDARs also evoked a moderate increase in ERK activity in shK-transduced neurons, but it was well below the one obtained with shC-expressing virus in all the NMDA-stimulation time-points studied. Furthermore, at 6 hours of NMDA treatment with NMDA requires Ca\(^{2+}\) entry and calpain activation. (A) Neurons were preincubated for 1 hour with the Ca\(^{2+}\) chelator EGTA (2 mM) before addition of NMDA and glycine (named as ‘NMDA’ here). To prevent toxic effects, cells were preincubated with EGTA for 1 hour before a short exposure to NMDA (1 hour) and, after elimination of EGTA and NMDA, culture proceeded for an additional 22 hours in the presence of DL-AP5. Kidins220/ARMS was detected by western blot (WB). (B,C) Cortical neurons were pretreated for 1 hour with inhibitors before the addition of NMDA for 4 hours. The inhibitors remained in the culture media for the duration of the experiment and were: chloroquine (Cloq; 200 \(\mu\)M), ALLM (5 \(\mu\)M), ALLN (5 \(\mu\)M), GM6001 (10 \(\mu\)M), ZvAD (100 \(\mu\)M) and CiIII (10 \(\mu\)M). Kidins220/ARMS was analyzed by WB. (D) Protein extracts from neuronal cultures were incubated for 30 minutes with 20 or 80 \(\mu\)M of purified calpain I (Calp I), or left untreated. Kidins220/ARMS signal was then analyzed by WB. A C-terminal fragment transiently produced is denoted by an asterisk. The electrophoretical mobility of full-length (FL) spectrin (240 kDa) or the breakdown products (BDPs) resulting from calpain activation (150 kDa and 145 kDa) are also indicated. (E,F) Cultures were pre-incubated for 1 hour with the calpain-specific inhibitor CiIII (10 \(\mu\)M) before a 4-hour treatment with NMDA. Kidins220/ARMS was detected by WB. (F) Quantitative analysis of Kidins220/ARMS WB signal was carried out by normalizing with NSE levels. Results are presented relative to data obtained for the untreated neurons, arbitrarily assigned a 100% value. The data shown are the means ± s.e.m. of four independent experiments, and statistical significance was evaluated by the Student’s unpaired t-test (*P<0.05, ***P<0.001). In all experiments, levels of NSE were used as control. Results are representative of three or four independent experiments.
treatment, levels of ERK1/2-\(P\) in shC neurons were similar to those detected in cultures transduced with shK-expressing lentivirus after only 2 hours of NMDA incubation. Our results support that Kidins220/ARMS contributes to the activation of ERK elicited by NMDA excitotoxicity in our model.

Importantly, these variations on ERK activity correlate with changes in neuronal death. Kidin220/ARMS knockdown decreased neuronal viability under basal conditions, an effect that was enhanced by exposure to excitotoxic concentrations of NMDA for different durations (Fig. 6D). MTT assays performed in six

Fig. 5. Overstimulation of NR2B-containing NMDARs directly downregulates Kidins220/Arms gene transcription by a mechanism triggered by Ca\(^{2+}\) entry but independent of calpain activation. (A) Northern blot analysis of Kidins220/Arms in excitotoxicity. Total RNA was prepared from cortical neurons untreated or treated with NMDA and glycine (named as ‘NMDA’ here). A fragment of the rat Kidins220/Arms sequence was used as a DNA probe and \(\gamma\)-actin mRNA was detected as control. Quantitation of Kidins220/Arms radioactive signal, normalized to that of \(\gamma\)-actin in the same sample, is represented by a number below each band after assigning a value of 100% to untreated neurons. The autoradiography is representative of three independent experiments. (B) Quantitative real-time PCR analysis of Kidins220/Arms mRNA during excitotoxicity. Total RNA was amplified with Kidins220/Arms-specific primers. (C) Quantitative real-time PCR of Kidins220/Arms mRNA from neurons pretreated with the calpain inhibitor CiIII (10 \(\mu\)M) for 1 hour before addition of NMDA for 4 hours was performed. The inhibitor remained in the culture media for the duration of the experiment. The upper inset corresponds to a representative western blot (WB) performed with time-paired neurons subjected to the same treatments. (D) Cultures of neurons were preincubated for 1 hour with EGTA (2 mM) before addition of NMDA. After 1 hour of treatment, the medium was replaced by new conditioned medium containing the antagonist DL-AP5 (200 \(\mu\)M). Cultures were maintained up to 24 hours before determining Kidins220/Arms mRNA levels by quantitative real-time PCR. (E) Quantitative real-time PCR of Kidins220/Arms mRNA from neurons treated with NMDA alone or together with the NR2B-specific inhibitor ifenprodil (IFN, 10 \(\mu\)M) for 4 hours. (F) Kidins220/Arms mRNA decay in cortical neurons incubated with actinomycin D (ActD) alone or in combination with NMDA for 4 and 6 hours was determined by quantitative real-time PCR. Cells were pre-treated with ActD (2.5 \(\mu\)g/ml) for 1 hour, before adding the agonists. ActD was left in the medium for the duration of the experiment. Kidins220/Arms mRNA levels were normalized to those of L32. Values in arbitrary units are expressed relative to those found in untreated cells (assigned a value of 100%). For all quantitative real-time PCR results, the data shown are the means ± s.e.m. of three independent experiments performed in triplicate, and statistical significance was evaluated by the Student’s unpaired \(t\)-test (*\(P<0.05\), **\(P<0.01\), ***\(P<0.001\)). (G) The transcriptional rate of Kidins220/Arms gene was determined by in vivo transcriptional run-on assay. Nuclei were isolated from cortical neurons treated with NMDA for 4 hours or from time-paired untreated cells. Extending RNA was labeled using \(\alpha\)-\(32\)P-UTP, then extracted and used to hybridize membranes containing dots of plasmids encoding diverse cDNAs: cyclophilin (Cy), \(\gamma\)-actin (Ac), NSE, Kidins220/Arms (Kidins), NR1 and NR2A. Cyclophilin, \(\gamma\)-actin and NSE were used as normalizing genes. The table on the right was made after quantifying the radioactivity present in Kidins220/Arms, NR1 or NR2A dots, and represents the transcriptional rate of those genes after NMDA treatment (NMDA 4 h), compared with untreated neurons (C, control), assigned a value of one. Values in arbitrary units, obtained for Kidins220/Arms, NR1 and NR2A transcripts, were normalized separately with the signal for cyclophilin, \(\gamma\)-actin and NSE.
neuronal surface (Gascon et al., 2008). We investigated whether produces truncated N-terminal-NR2A/B subunits that remain at the C-terminal regions by calpain in excitotoxicity and ischemia length Kidins220/ARMS (supplementary material Fig. S4). excitotoxicity, have lost their C-termini can still associate with full-truncated forms of NR2A/B that, after short exposure to 2004). Here we have obtained data supporting this idea, because Kidins220/ARMS with all or some of the NMDAR subunits might exist. The transmembrane regions of these proteins could be several possibilities can be envisioned. A direct interaction of NR1 and NR2 subunits of the NMDAR in cultured cortical neurons. Association between Kidins220/ARMS and the NMDAR

Discussion

Association between Kidins220/ARMS and the NMDAR

We have discovered an association of Kidins220/ARMS with the NR1 and NR2 subunits of the NMDAR in cultured cortical neurons and in brain. Although the nature of this association is unknown, several possibilities can be envisioned. A direct interaction of Kidins220/ARMS with all or some of the NMDAR subunits might exist. The transmembrane regions of these proteins could be mediating this interaction, in the same way it has been shown for the interaction between Kidins220/ARMS and Trk (Arevalo et al., 2004). Here we have obtained data supporting this idea, because truncated forms of NR2A/B that, after short exposure to excitotoxicity, have lost their C-termini can still associate with full-length Kidins220/ARMS (supplementary material Fig. S4). Recently, we showed that the processing of the NR2A and NR2B C-terminal regions by calpain in excitotoxicity and ischemia produces truncated N-terminal-NR2A/B subunits that remain at the neuronal surface (Gascon et al., 2008). We investigated whether cleavage of NR2 subunits interfered with their association with unprocessed Kidins220/ARMS molecules after brief NMDAR overstimulation (2 hours). Using an antibody against N-terminal regions of the NR2A/B subunits, we confirmed their extensive calpain cleavage to produce stable N-terminal fragments at this time point. Notably, intact Kidins220/ARMS still coimmunoprecipitated with shC- and shK-infected neurons were quantified and normalized to NSE and tot-ERK1/2 (right panel). (C) Cortical neurons transduced with shC or shK lentiviruses were infected with increasing doses (3×10⁵, 5×10⁵ and 8×10⁵ transduction units) of shC or shK lentiviruses. Kidins220/ARMS and ERK-P (p-ERK1/2) were detected by WB. NSE and total-ERK (tot-ERK1/2) levels were used as controls. Signals of ERK1-P and ERK2-P from five independent experiments from shC- and shK-infected neurons were quantified and normalized to NSE and tot-ERK1/2 (right panel). (C) Cortical neurons transduced with shC or shK lentiviruses were stimulated with NMDA and glycine (named as ‘NMDA’ here) for the indicated times and Kidins220/ARMS and ERK-P levels were analyzed by WB. (D) Quantitation of the decline of neuronal viability measured by MTT assays (expressed as the percentage of the value obtained in untreated shC-infected cells) in cultures infected with shC or shK lentiviral particles. Time of NMDA treatment is shown. The data shown are the means ± s.e.m. of six independent experiments, and statistical significance was evaluated by the Student’s unpaired t-test (*P<0.05, **P<0.01).
between cellular and animal models of excitotoxicity has been previously shown (Gascon et al., 2005; Gascon et al., 2008). It is important to highlight here that this is the first report on the regulation of Kidins220/ARMS in relation to a pathological condition. These results are not only meaningful for cerebral ischemia but might prove to be also important for other disorders in which excitotoxicity plays a central role, such as hypoxia, acute trauma and several neurodegenerative pathologies (Choi, 1988).

We have analyzed in detail the mechanism modulating Kidins220/ARMS after excitotoxic insults, finding two main contributions, one dependent on and the other independent of calpain activation (Fig. 7). Our results also reveal Kidins220/ARMS as a novel calpain substrate, because it is rapidly and efficiently cleaved by this protease. Calpains are important mediators of cellular toxicity and pathology (Vanderklish and Bahr, 2000) and are activated in processes of cerebral ischemia and excitotoxic degeneration, among others (Hong et al., 1994; Siman and Noszek, 1988). They contribute to neuronal degeneration by cleavage of specific substrates that have crucial roles in neuronal survival such as the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) (Bano et al., 2005), the major plasma-membrane Ca\(^{2+}\)-extruding system. Importantly, calpain processes NR2A and NR2B subunits as well as scaffolding and effector molecules associated to NMDARs (reviewed by Vosler et al., 2008), thus uncoupling them from downstream signaling pathways and the cytoskeleton. The degradation of Kidins220/ARMS by calpain in excitotoxic neurodegeneration constitutes an important finding given the involvement of this novel substrate in neuronal differentiation and survival. A transient C-terminal fragment of Kidins220/ARMS was only detectable after in vitro calpain digestion, suggesting that it is probably unstable. Excitotoxicity and calpain activation produces stable truncated NR2A/B subunits bearing their N-termini that interact with NR1 at the neuronal surface, whereas no NR2 C-terminal fragments are detected (Gascon et al., 2008). Whether proteolysis of Kidins220/ARMS after initial calpain processing is complete or only partial remains to be elucidated. Regardless of the existence of truncated forms of the protein, the loss of the C-terminal PDZ ligand of Kidins220/ARMS will hamper its association with PDZ.

![Fig. 7. Model of Kidins220/ARMS downregulation during excitotoxicity and ischemia. Kidins220/ARMS is associated with the NMDAR subunits NR1 and NR2A/B at the neuronal surface. Overactivation of NMDARs containing NR2B subunits by the co-agonists glutamate (Glu) and glycine (Gly) leads to high Ca\(^{2+}\) influx in postsynaptic neurons and excitotoxicity. Ca\(^{2+}\) entry downregulates Kidins220/ARMS by two mechanisms acting at the protein and mRNA levels, through calpain-dependent and -independent pathways, respectively. First, Ca\(^{2+}\) overloading results in the activation of the Ca\(^{2+}\)-dependent neutral cysteine protease calpain I. This protease rapidly cleaves the C-terminal region of Kidins220/ARMS, together with those of the NR2A and NR2B subunits. Note that the Kidins220/ARMS-specific cleavage site remains unknown, so it is arbitrary represented in the figure. A stable complex formed by the NR1 subunit and truncated N-terminal-NR2A/B subunits remains at the surface of overactivated neurons (Gascon et al., 2008). Kidins220/ARMS might be subjected to complete degradation after calpain activation. Alternatively, this protein might undergo cleavage to produce a C-terminal fragment of highly unstable nature, leaving a stable truncated form of the protein bearing N-terminal domains (similarly to the NR2A/B subunits), whose detection remains elusive with tools available at present. High Ca\(^{2+}\) influx during excitotoxicity also results in a rapid transcriptional inhibition of the Kidins220/Arms gene, similar to that observed for NR1 (Gascon et al., 2005). The mechanism responsible for this direct transcriptional blockage of the Kidins220/Arms gene is still unknown, although we have determined that it is calpain independent but requires Ca\(^{2+}\) entry and activation of NR2B-containing receptors. A general decrease of Kidins220/ARMS protein caused by these mechanisms would impact on signaling pathways and processes in which this protein participates, such as neurotrophin (TrkB)- and ephrin (Eph)-receptor cascades. Additionally, Kidins220/ARMS plays an important role in neuronal survival and might also have important implications for NMDAR functionality and molecular pathways (dashed line). Its downregulation during excitotoxicity could contribute to ERK inactivation and neuronal death.](image-url)
proteins and downstream signal transduction (Hisata et al., 2007; Luo et al., 2005).

A second mechanism of Kidins220/ARMS regulation induced by excitotoxicity consists of a rapid suppression of Kidins220/Arms gene transcription in a process independent of calpain activation (Fig. 7). This mechanism resembles that described for NR1 (Gascon et al., 2005) (and herein Fig. 5), suggesting the existence of a common process controlling the transcriptional blockage of both genes probably mediated by Ca^{2+}-dependent transcription factors. A good candidate is CREB (cAMP/calcium response element binding protein), whose signaling pathway undergoes a shut-off coupled to cell death under excitotoxic circumstances (Hardingham et al., 2002). CREB inactivation has been also described in stroke conditions (Walton and Dragunow, 2000). Studies with Kidins220/Arms and NRI promoters will help us to identify the mechanisms governing their transcriptional regulation.

Between the two mechanisms contributing to Kidins220/ARMS downregulation, calpain cleavage is very fast and probably operates from early stages after exposure to excitotoxic insults, such as neuronal stimulation with high concentration of NMDA or cerebral ischemia. Our previous results obtained in both models probe a very early activation of this protease after NMDAR overstimulation (Gascon et al., 2008). The data herein also indicate that calpain processing will be the first and preferential mechanism to downregulate Kidins220/ARMS, because we have observed it to be responsible for an almost 80% decrease of Kidins220/ARMS levels after 4 hours of NMDA treatment (see Fig. 4F). In accordance with this data, the kinetics of Kidins220/ARMS regulation is very fast in cerebral ischemia and MCAO is sufficient to induce an important decrease in the levels of this protein as demonstrated in animals sacrificed immediately after 1 hour of occlusion. Downregulation of Kidins220/ARMS is similar to that of NR2A, a protein we have previously shown to be processed very early after ischemic injury, in parallel to calpain activation (Gascon et al., 2008). We propose this rapid calpain-dependent mechanism as the main contributor to Kidins220/ARMS modulation by ischemic insults. The downregulation of Kidins220/Arms gene transcription would result in a progressive decrease of Kidins220/ARMS levels after long-term excitotoxic damage.

What might be the functional implications of Kidins220/ARMS downregulation induced by excitotoxicity?

Altogether, our findings open the perspective of Kidins220/ARMS as being a common target in the glutamate, neurotrophin and ephrin signaling pathways. A general decrease of Kidins220/ARMS will undoubtedly impact on signaling pathways in which this protein participates. Kidins220/ARMS is a unique downstream target for neurotrophin and ephrin receptors (Kong et al., 2001), acting as an early effector for prolonged neurotrophin signaling (Arevalo et al., 2006; Arevalo et al., 2004; Hisata et al., 2007) and a modulator of ephrin-receptor cascades (Luo et al., 2005). The downregulation of Kidins220/ARMS might also have important implications not only in neuronal excitotoxicity pathways but also in NMDAR physiological function because the latter is regulated by interaction with Trk (Yamada and Nabeshima, 2004) and Eph (Henderson et al., 2001) receptors. In this context, Kidins220/ARMS has been found to present an inverse relationship with the level of synaptic activity, being suggested as a modulator of NMDAR basal activity under some conditions (Cortes et al., 2007). Identifying an association of Kidins220/ARMS with NMDAR further supports a role of this protein in neurotransmission and signaling events triggered by activation of this type of glutamate receptor.

The stimulation of Trk receptors by neurotrophins provokes a sustained activation of the ERK cascade, crucial for neuronal differentiation and survival. The formation of a tetramer complex Trk–Kidins220/ARMS–S-SCAM–PDZ-GEF1 has been identified as the major contributor to this prolonged ERK activation, the presence of Kidins220/ARMS being obligatory (Hisata et al., 2007). ERK signaling also mediates NMDAR-dependent neuronal plasticity and survival (reviewed by Thomas and Huganir, 2004). However, there is still some controversy regarding the regulation of ERK activity during excitotoxicity because the results are heterogeneous and depend on the experimental conditions and models employed (Gouix et al., 2009; Ivanov et al., 2006; Leveille et al., 2008). In our cellular model of excitotoxicity, we found an early activation of ERK that peaks at 2 hours and is gradually shut-off up to 6 hours of NMDAR overstimulation. Considering that NMDA treatment in our model would stimulate synaptic and extrasynaptic NMDARs, this initial increase in ERK-P levels might be due to activation of the synaptic pool of receptors. At later times, calpain-dependent processing of NR2 subunits and transcriptional downregulation of NR1 will hamper NMDAR function, which, together with the previous overstimulation of extrasynaptic receptors, would result in the shut-off of the ERK pro-survival cascade.

After knocking down Kidins220/ARMS, basal ERK activity decreases and, during excitotoxicity, ERK-stimulation values are far below those obtained in control cultures. Importantly, this variation on ERK activity correlates with decreases in neuronal viability that occur already under basal conditions but that are enhanced during excitotoxicity. Therefore, in this model, ERK activation induced by NMDA treatment might be regulated by two different pathways, one of them being dependent on Kidins220/ARMS. The sh-RNA-interference of this signaling component leads to a reduction of ERK activity registered at early time points of NMDA incubation. These low ERK-P values might be under the threshold required to preserve neuronal viability in untreated cultures, leading to a faster induction of neuronal death in excitotoxic conditions. Similarly, a decrease in the levels of Kidins220/ARMS protein induced by excitotoxicity through calpain cleavage and downregulation of transcription could make neurons more vulnerable to excitotoxic damage and death by a mechanism that leads to an abnormal and lower activation of the ERK signaling pathway. Our results demonstrate an important role of Kidins220/ARMS in neuronal survival pathways and strongly support that its downregulation during excitotoxicity could significantly contribute to ERK inactivation and neuronal death.

Materials and Methods

Materials and chemicals
N-Methyl-D-aspartate (NMDA), glycine, cytosine β-D-arabinofuranoside (AraC), poly-L-lysine, L-laminin, N-acetyl-L-lysyl-L-lysyl-L-norleucinal (ALLN), N-acetyl-L-lysyl-L-lysyl-L-phenylalanine (ALLM), actinomycin D and chloroquine were obtained from Sigma (St Louis, MO). Antagonists 2-amino-phosphonopentanoic acid (DL-AP5) and ifenprodil were obtained from Tocris-Cookson (Bristol, UK). Calpain 1 (μ-calpain), carbenoxoxy-valinyl-phenalenalamin (Ci III), calpeptin, GM6001 and z-VAD-FMK were obtained from Calbiochem (Merck Bioscience, Darmstadt, Germany). Oligonucleotide primers were from Invitrogen (Carlsbad, CA).

Rabbits

Rabbit polyclonal and mouse monoclonal antibodies against Kidins220/ARMS were used as described (Cabrera-Poch et al., 2004; Iglesias et al., 2000). Mouse monoclonal antibodies for NR1, hemagglutinin (HA) and neuronal nuclei protein (NeuN) were from Pharmingen (San Diego, CA), Covance (Berkeley, CA) and Abcam (Cambridge, MA).
Neurons grown for 7 DIV were infected with lentivirus directly added to the growing sequence corresponds to rat mRNA positions 4890-4912. All novel constructs were and p44/p42 Map kinase (herein active ERK1/2—Horseradish peroxidase-conjugated and Texas-red- and Alexa-Fluor-488-conjugated minutes at 4°C. After centrifugation for 5 minutes at 1000 Rabbit polyclonal antibodies recognizing phospho-p44/p42 Map kinase (Thr202/Tyr204) bands were visualized by ECL (General Electric, Fairfield, CT). Protein loading equivalent volumes of immunoprecipitates were analyzed by western blot (WB). Membranes were incubated with different primary antibodies and the immunoprecipitation of Kidins220/ARMS and NR1 was performed as described previously (Gascon et al., 2005). NR2A and NR2B subunits were immunoprecipitated similarly to NR1. Equal amounts of total lysates or previously (Gascon et al., 2008; Iglesias et al., 2000). NR2A and NR2B subunits encoding ribosomal protein L32. In this case, the forward primerspanned nucleotides 5011-5030 of its cDNA (5’-CGCGATGTCCATTTGGCAACTT-3’), and the reverse primerspanned nucleotides 5112-5131 (5’-TGGCAGGTGCCTGGTTAGA-3’). These primers amplified a fragment of 121 bp. The amplification of Kidins220/Arms transcripts was normalized against the rat gene encoding ribosomal protein L3. In this case, 25-273 (5’-CTGGAAGTCTGCTGCTGAGC-3’) and the reverse primer spanned nucleotides 359-379 (5’-CTTTGCGAGATGTGACGATCTGA-3’), giving a PCR product of 127 bp.

Run-on assay for in vivo transcription analysis

Intact nuclei were obtained from 11 DIV cultured neurons untreated or treated with NMDA and glycine for 4 hours. Nuclear isolation and transcriptional nuclear run-on assays were carried out as before (Iglesias et al., 1996). Linearized plasmids (5 μg) containing Kidins220/Arms, NR1, NR2A, NSE, γ-actin or cyclophilin cDNAs were denaturation in 0.3 mM NaOH for 10 minutes at 95°C. After neutralization, cDNAs were immobilized on duplicate nylon membranes. RNAs produced and 32P-labeled in nuclei from untreated or treated neurons were used as probes to hybridize each membrane, using standard hybridization protocols. Radioactivity was quantified using a Typhoon Trio Variable Modo Imager (General Electrics HealthCare Life Sciences, Uppsala, Sweden).

Animal model of cerebral ischemia

All animal procedures were performed in compliance with European Community law 86/609/EEC and were approved by the ‘Comasso Superior de Investigacions Cientificas’ ethical committee. Male Sprague-Dawley adult rats (275-300 g) were anesthetized, maintained and monitored during the surgical procedure for MCAO, as we have previously performed (Gascon et al., 2005; Gascon et al., 2008). To prepare protein extracts, brain was sectioned into 2-mm slices and stained with a 2% solution of triphenyltetrazolium chloride (TTC) (Merck Biosciences, Darmstadt, Germany). The unstained area of the cerebral cortex (right hemisphere), defined as infarcted tissue, was dissected, as well as the corresponding region in the left hemisphere. For immunohistochemistry, 24 hours after blood reperfusion, rats were anesthetized and perfused intracardially with cold 4% paraformaldehyde in PBS. Brains were removed immediately and post-fixed in the same fixative at 4°C for 6 hours. Then, they were cryoprotected by serial immersion for at least 6 hours in increasing concentrations of sucrose (10, 15 and 20%) in PBS at 4°C. After that, coronal frozen sections (25 μm thick) were prepared using a cryostat (Leica, Heidelberg, Germany).

Immunohistochemistry

Identification of the infarcted tissue in the neocortex was performed by Nissl [0.1% (v/v) Cresyl Violet] staining of slide-mounted coronal sections. Adjacent sections were then processed for immunofluorescence staining by permeabilization and blocking with 10% (v/v) sheep serum, 0.4% (v/v) Triton X-100 in TBS for 3 hours at room temperature. Sections were incubated overnight at 4°C with anti-Kidins220/Arms or -NeuN antibodies prepared in 4% (v/v) sheep serum, 0.2% (v/v) Triton X-100 in TBS. Then, samples were incubated for 2 hours with biotinylated anti-rabbit or anti-mouse secondary antibodies, respectively, before incubation with streptavidin peroxidase-conjugate diluted in 4% (v/v) sheep serum prepared in TBS for 30 minutes at room temperature. Antigen was visualized by incubation with diamino-benzidine for 10 minutes in the dark. Once the color developed, sections were washed and dehydrated before mounting on PDX (Sigma, St Louis, MO). Controls without primary antibodies showed very low levels of nonspecific staining. Images were obtained using a Leica DM IL inverted microscope with a 4× objective and photographed with an Olympus DP12 digital camera.
Statistical analysis
All data are expressed as mean ± standard error of the mean (s.e.m.) of at least three independent experiments. Statistical significance was determined by Student’s t-test. A P-value smaller than 0.05 was considered statistically significant.

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