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PI3K is a key mediator of central sensitization in painful inflammatory conditions

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ABSTRACT

Here we show that phosphatidylinositol 3-kinase (PI3K) is a key player in the establishment of central sensitization, the spinal cord phenomenon associated with persistent afferent inputs and contributing to chronic pain states. We demonstrated electrophysiologically that PI3K is required for the full expression of spinal neuronal wind-up. In an inflammatory pain model, intrathecal administration of LY294002, a potent PI3K inhibitor, dose-dependently inhibited pain related behavior. This effect was correlated with a reduction of the phosphorylation of extracellular signal-regulated kinase (ERK) and CaMKinase II. In addition, we observed a significant decrease in the phosphorylation of the NMDA receptor subunit NR2B, decreased translocation to the plasma membrane of the GluR1 AMPA receptor subunit in the spinal cord and a reduction of evoked neuronal activity as measured using c-Fos immunohistochemistry. Our study suggests that PI3K is a major factor in the expression of central sensitization after noxious inflammatory stimuli.

INTRODUCTION

Central sensitization is a complex phenomenon of synaptic plasticity characterized by the rapid onset activity-dependent increase in the excitability of nociceptive spinal dorsal horn neurons (DHN). Physiologically, it is associated with a reduction in the activation threshold of DHNs, enhancement and enlargement of their responsiveness and receptive fields, respectively. It has recently become clear that there are distinct forms of central sensitization. Several other types of synaptic plasticity arise in response to noxious stimuli which modify nociceptive transmission by altering synaptic efficacy. These include other immediate-onset, transcription-independent phenomena, such as wind-up and long-term potentiation (LTP). Indeed, evidence suggests that central sensitization in the spinal cord and synaptic plasticity in the hippocampus (i.e., LTP) may share common signaling pathways (Ji et al., 2003; Malcangio and Lessmann, 2003; Sandkuhler, 2007). However, the intracellular mechanisms underlying this particular form of LTP at the spinal level are not well understood.

Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase which phosphorylates the inositol ring of phosphoinositides (PtdIns) (Whitman et al., 1988), generating three distinct membrane-associated second messengers (Toker and Newton, 2000). These second messengers are able to activate various effectors (e.g. Akt/PKB and ERK). Therefore, PI3K is involved in several signaling cascades and cellular processes (Cantley, 2002; Fresno Vara et al., 2004; Koyasu, 2003; Parcellier et al., 2007; Patel and Mohan, 2005; Patton et al., 2007). In particular, PI3K inhibitors suppress NMDA-receptor-mediated ERK activation in hippocampal neurons (Chandler and Sutton, 2005; Perkinton et al., 2002). Furthermore, several reports demonstrated interestingly a requirement of PI3K activity in different forms of synaptic plasticity including LTP (Man et al., 2003; van der Heide et al., 2005). An electrophysiological study in rat hippocampal slices showed that PI3K activity was induced during LTP and, conversely, LTP could be reduced by two PI3K specific inhibitors, LY294002 and wortmannin (Sanna et al.,

2002). However, after drug washout, LTP recovered suggesting a predominant role of PI3K in the expression rather than maintenance of LTP (Sanna et al., 2002). Further studies reinforced the role of PI3K in LTP through both ERK-dependent and ERK-independent mechanisms (Man et al., 2003;Opazo et al., 2003). In contrast to Sanna et al., Opazo et al., concluded that PI3K is only involved in LTP induction (Opazo et al., 2003;Sanna et al., 2002). Despite these discrepancies about the timing of activation of PI3K during LTP, it is clear that PI3K plays a crucial role. At spinal levels more physiological stimuli than the high frequency stimuli used to induce LTP in the forebrain can induce central sensitization and a number of correlates such as wind-up or temporal summation can be related to human psychophysical measures and pain complaints in patients (Koltzenburg et al., 1994;rendt-Nielsen et al., 1995). Therefore, we hypothesized that PI3K might be involved in early-onset phenomena of central sensitization occurring in the spinal cord. We investigated the effect of PI3K inhibition on spinal wind-up, the behavioral formalin test and subsequently examined the intracellular mechanisms involved. Our results suggest that PI3K is a key player in the early events of central sensitization.

METHODS

All experiments were undertaken with approval of the United Kingdom Home Office. The guidelines of the Committee for Research and Ethical Issues of the IASP (Zimmerman, 1983) were followed.

In vivo electrophysiology

Electrophysiological recordings were conducted in male Sprague-Dawley rats (200-250g, Central Biological Services, University College London, UK). Rats were anaesthetized using halothane and a laminectomy was performed at L1-L3 vertebral level. Extracellular recordings of single deep wide dynamic range (WDR) dorsal horn neurons were made using parylene coated tungsten electrodes (A-M Systems, USA). A train of 16 transcutaneous electrical stimuli (2ms wide pulses, 0.5Hz) was delivered to the receptive field at three times the threshold current for C-fiber via two stimulating needles inserted under the skin of the ipsilateral hindpaw. A post stimulus histogram was constructed and A β - (0-20ms), A δ - (20-90ms) and C-fiber (90-300ms) evoked responses were separated and quantified on a basis of latency. Responses occurring after the C-fiber latency band were taken to be the post-discharge (PD) of the cell (300-800ms). "Input" was calculated as the number of action potentials (AP) after the first stimulus x16. Wind-up was calculated as: (number of AP after 16 stimuli) – Input. Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design, UK; PSTH and rate functions).

Drug administration: Stable control responses to electrical stimuli were established at 10 minutes intervals prior to drug administration. LY294002 (Tocris Biosciences, UK), a potent inhibitor of PI3K, was applied directly to the exposed surface of the spinal cord of rats (n=6), in a volume of 50 μ l using a Hamilton syringe as follows: 50 μ l of 50 μ M (0.8 μ g)

LY294002 was applied and the effects followed for one hour with tests carried out at 10, 20, 30, 40, 50 and 60 minutes. The remaining solution was then removed from the surface of the cord and replaced by 50µl of 100µM (1.6 µg) LY294002, with subsequent testing as above. Drug vehicle was 10% dimethyl sulfoxide (DMSO) and control experiments with this vehicle were also conducted (n=6, data not shown).

Statistical analysis: Data are presented as mean number of action potentials \pm SEM, unless otherwise stated. The effects of LY294002 versus control pre-drug values and assessed by repeated measures analysis of variance (one-way ANOVA), followed by Dunnett's multiple comparison test, using Graphpad Prism v.4. Levels of statistical significance were set at * $p < 0.05$ and ** $p < 0.01$.

Release of substance P from dorsal horn slices

Horizontal dorsal horn slices (400µm thick) with dorsal roots attached were obtained from the lumbar spinal cord of adult male Wistar rats (Harlan, UK) as previously described (Clark et al., 2006; Malcangio et al., 2000). Briefly, lumbo-sacral spinal cord was excised and longitudinally hemisected producing a horizontal slice with L4 and L5 dorsal roots attached. One slice was obtained from each rat, mounted in the central compartment of a three compartment chamber and continuously superfused (1ml/min) with oxygenated (95% O₂ + 5% CO₂) Krebs' solution (in mol/L: NaCl, 118; KCl, 4; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; CaCl₂, 2.5 and glucose, 11) containing 0.1% bovine serum albumin (BSA), 20 µg/ml bacitracin, 100 µM captopril, 1 µM phosphoramidon and 6 µM dithiothreitol (Sigma, UK). BSA and protease inhibitors were added to minimize loss of detectable substance P (SP)-like immunoreactivity (SP-LI) through surface adhesion and to prevent degradation. The dorsal roots were placed in the lateral compartments and immersed in mineral oil to avoid dehydration. Before, during and after dorsal root stimulation 8ml-fractions of superfusates, were collected from the central compartment in glass tubes containing acetic acid (0.1N final

concentration; VWR, UK) to stabilize SP. Three 8ml-fractions were collected before stimulation to measure basal levels of SP. The dorsal roots were then electrically stimulated (20V, 0.5ms, 10Hz for 8min). Three further fractions were collected following electrical stimulation to assess recovery to basal levels of SP (R1-3). LY294002 hydrochloride (50 μ M; Tocris Biosciences, UK) was added to the superfusion solution one fraction before and during electrical stimulation. To quantify SP-LI samples were partially purified and desalted using Sep-pak C₁₈ reverse-phase silica gel cartridges (Waters Associates, UK). The cartridges were conditioned with acetonitrile (100%; HPLC grade; VWR, UK) and trifluoroacetic acid (TFA) (0.1%; HPLC grade; VWR, UK). Samples were then loaded into the columns, and the peptide was eluted using acetonitrile/TFA (80:20) solution. The eluates were dried by evaporation under nitrogen. Dried samples were reconstituted in 300 μ l of phosphate buffer and 100 μ l assayed by radioimmunoassay in duplicate using scintillation proximity assay (Amersham, UK).

Surgery for intrathecal catheter and subsequent formalin test

One week before formalin experiments, adult male Wistar rats (Harlan, UK) were deeply anesthetized by intraperitoneal injection of a mixture of medetomidine (0.25mg/kg) and ketamine (60mg/kg). A flexible silastic catheter (external diameter 1.14 mm, Merck, Essex, UK) was inserted sub-durally in order to deliver LY294002 or vehicle at lumbar level. A small laminectomy was performed under sterile conditions at thoracic level and a thin catheter was inserted between the dura matter and the spinal cord. The tubing was secured to the atlanto-occipital bone with superglue and the external end guided under the skin and externalized over the head.

Formalin test experiments were performed one week after this surgery. Animals were placed in a Plexiglas box for 15 minutes to acclimatize. They then received intrathecal injections of 10 μ g, 50 μ g or 100 μ g of LY294002 in a volume of 10 μ l or vehicle (10%

DMSO) using the externalized catheter. Fifteen minutes later, rats received 50 μ l of 5% formalin injected subcutaneously (s.c.) into the plantar surface of the right hindpaw. Lifting, shaking, biting and licking of the injected paw were monitored by measuring the total duration of the response in seconds during 60 minutes following formalin administration. Data are presented in 5min bins. At the end of the test, animals were terminally anaesthetized using pentobarbitone and perfused intracardially with 100 ml saline followed by 400 ml 4% paraformaldehyde (PFA) with 15% of a saturated solution of picric acid for c-Fos immunohistochemistry.

For phospho-Akt, phospho-ERK and phospho-CAMKII immunohistochemistry, animals were first anaesthetized with urethane (1mg/kg), then pre-treated intrathecally with LY294002 (100 μ g/rat) or vehicle. Fifteen minutes later, rats were injected s.c. with 50 μ l of formalin into the plantar surface of the right hindpaw. Five minutes after formalin administration, rats were perfused transcardially with 100 ml of heparinized saline followed by 400 ml 4% paraformaldehyde with 15% of a saturated solution of picric acid.

For assessment of p-NR2A and p-NR2B NMDA receptor subunits and translocation of GluR1 AMPA receptor subunit, rats underwent the same process as above but were sacrificed by decapitation at 5, 10, 45 or 90 minutes after formalin administration. Fresh tissues were collected and immediately frozen in liquid nitrogen for further extraction and western-blotting.

Immunohistochemistry for phospho-Akt, phospho-ERK, phospho-CaMKII and c-Fos

After perfusion (see above), spinal cords (L3-L6) were post-fixed overnight in the same fixative and cryoprotected in 30% sucrose solution overnight. Tissues were embedded in OCT and frozen using liquid nitrogen. Transverse spinal cord sections (30 μ m thickness) were cut using a cryostat and every section was collected and placed in PBS solution (Phosphate

buffer, NaCl 0.9%) for free-floating immunohistochemistry. Sections were washed with PBS and then incubated with primary antibody, either rabbit anti-phospho-Akt 1 S473 (1:300 in PBST-Azide (PBS with 0.3% Triton X100-Azide), New England Biolabs, Beverly, MA, USA), rabbit anti-phospho-ERK (1:300 in PBST-Azide, New England Biolabs, Beverly, MA, USA), rabbit anti-phospho-CaMKII T286 (1:300 in PBST-Azide, ref 5683, Abcam, UK) or rabbit anti-c-Fos (1:5000 in PBST-Azide, Calbiochem, UK) overnight at room temperature. Immunostaining against c-Fos and p-ERK were performed using direct fluorescence as follows: after several washes with PBS, sections were incubated for 2hrs at room temperature with secondary antibody (goat anti-rabbit IgG-conjugated Alexa Fluor™ 488, 1:1000 in PBST-Azide, Molecular Probes, Oregon, USA). Immunostaining against phospho-Akt S473 and phospho-CaMKII T286 required tyramide amplification: sections were incubated with the secondary antibody, goat anti-rabbit biotin (1:300 in PBST, Vector Laboratories, CA, USA) for one hour at room temperature. Following several washes, sections were incubated in Avidin : Biotinylated enzyme Complex (1:500 in PBS, Vector Laboratories, CA, USA) for one hour, followed by several washes in PBS. The sections were then incubated in biotinyl tyramide (1:75 in amplification buffer, Perkin Elmer, UK) for 10 min. After several washes in PBS, sections were finally incubated in Extravidin FITC (1:500, diluted in PBS, Sigma). For all immunostaining, sections were mounted onto slides and cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA). Slides were visualized under a Zeiss Axioplan 2 fluorescent microscope and L3, L4 and L5 sections were identified for quantification (5 sections per level per animal, randomly chosen). For p-ERK and c-Fos, labeled spinal cord neurons were counted in laminae I-II and V-VI, for both ipsilateral and contralateral sides by one blinded investigator. For assessment of p-Akt and p-CaMKII upregulation, images of dorsal horn (ipsilateral and contralateral at the level of lamina II, where changes are mostly observed) from 5 sections per animal, chosen randomly, were taken

at magnification x20, using the same set-up of acquisition for all animals. Densitometric analysis of medial p-Akt and p-CaMKII staining (since staining was too abundant to count individual neurons) was carried out using Scion Image software. Results for p-Akt and p-CaMKII expression are given as a percentage of increase in the ipsilateral side versus the contralateral side. Statistical significance was tested by the Mann-Whitney Rank Sum test using Sigma Stat software (USA).

Western immunoblotting for phospho-NR2A and phospho-NR2B

Spinal cord samples (L4-L5 levels) of LY294002-treated (n=4) and vehicle-treated (n=4) animals were homogenized in RIPA buffer (50mM Tris pH 8, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1mM sodium orthovanadate and Complete protease inhibitor cocktail). The protein concentrations of lysates were determined using a BCA Protein Assay Kit (Pierce, UK). Proteins (30µg/sample) were separated using 8% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then incubated with primary antibody, either rabbit phospho-NR2A Y1387 (1:500 in Tris 20mM, pH 7.5, NaCl 500mM, Tween 20 0.1% (TBST), ref 16647, Abcam, Cambridge, UK) or rabbit phospho-NR2B Y1472 (1:1000 in TBST, Imgenex, Servento Valley, CA, USA), overnight at 4°C. After several T-BST washes, membranes were incubated with donkey anti-rabbit HRP-linked secondary antibody (1:5000, Amersham, UK) for 1hr at room temperature, and revealed using ECL-plusTM reagent (5 minutes) for detection of phosphorylation by autoradiography. Gels were scanned and bands were quantified by densitometric analysis using Scion Image software. Results are expressed as percentage phosphorylation in the ipsilateral dorsal horn compared to the contralateral dorsal horn for each animal (both ipsilateral and contralateral samples were run on the same gel). Mann-Whitney Rank Sum tests were carried out using Sigma Stat software (USA) to test the statistical significance of

the results. Reprobe of the membranes with Ponceau red showed a lack of significant differences between the samples among the same gel.

Translocation of AMPA-R subunit GluR1

As above (see formalin test section), naïve animals (n=3 LY, n=3 vehicle) and animals injected with formalin were sacrificed by decapitation at 10 minutes (n=3 LY, n=3 vehicle), 45 minutes (n=3 LY, n=3 vehicle) and 90 minutes (n=3 LY, n=3 vehicle). Dorsal spinal cord, both ipsilateral and contralateral, at L4/L5 level, were dissected and frozen in liquid nitrogen for Western immunoblotting.

Subcellular fractioning (isolation of cytoplasm and plasma membranes) was carried out as described by (Galan et al., 2004). In brief, spinal cord samples were homogenized in CLB buffer (10mM Tris pH 7.5, 300mM sucrose, 1mM EDTA, 1mM sodium orthovanadate and protease inhibitor cocktail), and then centrifuged at 7000g for 5mins. Supernatant containing cytoplasm (S1) was separated from the pellet containing nuclei and debris (P1), which was discarded. Supernatant (S1) was then centrifuged at 40,000g for 30mins to obtain pure cytoplasmic extract in the supernatant (S2) and crude membrane in the pellet (P2). This pellet (P2), which includes plasma and cellular organelles membranes, was resuspended in PBS with protease inhibitor cocktail (Complete, Roche, UK) and sodium orthovanadate. Protein titration was conducted using a BCA Protein Assay Kit (Pierce, UK) to determine protein concentrations in the cytoplasmic and membrane samples. 7.7µg and 6.0µg of proteins were loaded for each cytoplasmic (S2) and membrane (P2) samples, respectively. Proteins were separated using 7% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with primary antibody, rabbit anti-GluR2 (1:1000 in TBST, ref 1768, Chemicon, Temecula, CA, USA) or rabbit anti-GluR1 (1:1000 in TBST, ref 1504, Chemicon, Temecula, CA, USA) overnight at 4°C. After several T-BST washes,

membranes were incubated with donkey anti-rabbit HRP-linked secondary antibody (1:5000, Amersham, UK) for 1hr at room temperature and revealed using ECL-plusTM reagent (5 minutes) for detection by autoradiography. Gels were scanned and bands were quantified by densitometric analysis using Scion Image software. Results are expressed as percentage of expression at each time-point compared to naïve rats (100%) in each group. Mann-Whitney Rank Sum tests were carried out using Sigma Stat software to test the statistical significance of the results.

RESULTS

Spinal application of LY294002 reduces the ‘Wind-up’ of deep dorsal WDR neurons and inhibits electrically evoked responses

We first studied the potential involvement of PI3K on a form of short-term spinal plasticity, wind-up, which manifests as an increased response of spinal wide dynamic range (WDR) neurons in response to repetitive stimulation of primary afferent fibers. Application of the PI3K inhibitor LY294002 directly onto the spinal cord produced a significant reduction of ‘wind-up’ of deep dorsal horn WDR neurons with both 0.8 μ g (55% reduction, $p < 0.01$) and 1.6 μ g (50% reduction, $p < 0.01$) doses (Fig. 1A-B) compared to pre-drug control values. No change in ‘wind-up’ was seen with vehicle (10% DMSO) alone (data not shown). Electrically evoked responses of deep dorsal horn WDR neurons were also markedly reduced by spinal application of LY294002 (Fig. 2). Statistically significant reductions were seen of A δ -fiber (1.6 μ g, 24% reduction, $p < 0.05$), C-fiber evoked responses (0.8 μ g, 23% reduction $p < 0.01$; 1.6 μ g, 27% reduction, $p < 0.01$), and also of post-discharge of deep dorsal horn neurons, a direct measure of increased excitability (0.8 μ g, 39% reduction, $p < 0.05$; 1.6 μ g, 51% reduction, $p < 0.05$; Fig. 2) compared to pre-drug control values. In addition, A β -fiber evoked responses remained unaltered (Fig. 2). Importantly, neither LY294002, nor vehicle (10% DMSO), had any effect on input (number of action potentials after the first stimulus, which is an index of the effect of a drug on pre-synaptic inputs to the neuron), suggesting that effects of LY294002 were more likely due to a direct effect on dorsal horn neurons themselves rather than via pre-synaptic mechanisms. Administration of vehicle alone had no effect on any evoked response.

Electrically evoked Substance P release is not inhibited by LY294002

Using the isolated dorsal horn-with dorsal roots attached preparation we examined the contribution of PI3K to the release of primary afferent substance P (SP). We chose SP because it is a peptide contained in nociceptors and released by these fibers following increased activation of nociceptors *in vitro* (Malcangio and Bowery, 1994;Teoh et al., 1996) and painful stimulation *in vivo* (Lang and Hope, 1994;Yonehara et al., 1995). In dorsal horn superfusates, the levels of SP under basal conditions range from 15 to 23 pg/ml. Following electrical stimulation of the dorsal roots (Elec, white bar), in order to recruit both A and C-fibers, the levels of SP-LI in superfusates was significantly increased compared to basal levels ($p < 0.001$). This increase was still significant in the first recovery period (R1) following stimulation ($p = 0.009$). SP-LI levels returned to basal in the second and third recovery fractions (R2 and R3). Following superfusion of LY294002 (100 μ M) for a total of 16 minutes before and during electrical stimulation (Elec, black bar) SP-LI content in superfusates was also significantly enhanced compared to basal levels ($p = 0.042$). Furthermore, LY294002 did not inhibit electrically evoked SP-LI compared to control superfusates. SP-LI was elevated in the R1 fraction, although this increase failed to reach significance compared to basal levels, and returned to basal levels in the R2 and R3 fractions. These findings show that PI3K inhibition did not alter neurotransmitter release evoked by electrical stimulation of primary afferents, suggesting that LY294002 has post-synaptic effects on spinal neurons.

PI3K is involved in the development of central sensitization induced by intraplantar formalin

In order to investigate the role of PI3K in mechanisms of central sensitization *in vivo*, we investigated the ability of intrathecal administration of LY294002 to reduce pain like-behavior after intraplantar formalin injection. This test is typically characterized by two phases of pain behavior: a first phase mainly due to direct peripheral activation (0-5 min post-

injection) and a second phase due to spinal mechanisms of enhanced neuronal responsiveness, defined as central sensitization (15-40 min post-injection).

Intrathecal vehicle treatment (10% DMSO) did not affect pain-like behavior at any time point after intraplantar formalin. In contrast, intrathecal administration of LY294002 15 min before formalin injection induced a significant dose-dependent decrease (10 μ g 6% decrease $p>0.05$, 50 μ g 23% decrease $p=0.04$ and 100 μ g 31% decrease $p=0.025$) of the first phase of the formalin test (0-5 min, Fig. 3A). More interestingly, the second phase (20-40 min) is also reduced in a dose-dependent manner (4% reduction with 10 μ g, $p>0.05$, 21% reduction with 50 μ g, $p>0.05$; 50% reduction with 100 μ g, $p=0.017$, Fig. 3B-C), suggesting that PI3K is involved in the development of central sensitization induced by intraplantar formalin injection.

Phosphorylation of Akt1 S473 in spinal neurons following intraplantar formalin

We used the phosphorylation of downstream kinase Akt1 (S473) as an indicator of the activation of PI3K pathway. We examined the effect of intrathecal administration of LY294002 (100 μ g) on Akt1 S473 phosphorylation 5 minutes after formalin injection using immunohistochemistry. Formalin injection produced a large increase of p-Akt in the medial part of the superficial (I-II) layers of the ipsilateral dorsal horn (L4-L5 level; Fig. 4B, average increase of 82.2% \pm 22.1) compared to the contralateral side (vehicle treated) rats (Fig. 4A). Inhibition of PI3K by intrathecal injection of LY294002 caused a reduction in Akt phosphorylation in the ipsilateral dorsal horn (17.8% increased \pm 21.5) compared to vehicle treated rats (i.e. 78% reduction, $p=0.079$, $n=5$). However, this effect failed to reach statistical significance.

PI3K is implicated in the phosphorylation of ERK and CaMKII in spinal neurons following intraplantar formalin

In order to define the molecular mechanism by which PI3K is involved in the development of central sensitization, we studied the effect of PI3K inhibition on the activation of several intracellular signaling pathways previously involved in synaptic plasticity in the hippocampus and activated in the spinal cord following an inflammatory stimulus. Therefore, LY294002 (100 µg) was injected intrathecally 15 min prior to intraplantar injection of formalin and tissues were processed to study the activation of receptors (NMDA receptor subunits, GluR1) or ERK and CaMKII.

Using immunohistochemical methods, we observed an increase in the number of phospho-ERK (p-ERK) positive neurons in the superficial layers (I-II) of the L4-L5 ipsilateral dorsal horn in vehicle-treated rats (Fig. 5B) compared to the contralateral side, 5 minutes following formalin injection (Fig. 5B versus 5A). We also observed an increase in deeper laminae (V-VI), although to a lesser extent than in the superficial laminae (Fig. 5E). In contrast, intrathecal administration of LY294002 (100µg) significantly attenuated this ERK phosphorylation in both superficial (31% reduction, $p=0.006$) and deep laminae of the ipsilateral dorsal horn (63% reduction, $p=0.018$) compared to vehicle treated rats (Fig. 5D-E), 5 minutes after formalin injection.

We also examined the effect of intrathecal administration of LY294002 on CaMKII phosphorylation 5 minutes after formalin injection using immunohistochemistry. Phosphorylation of CaMKII was quantified medially in the superficial (I-II) layers of the dorsal horn of L4-L5 region (Fig 6B). Formalin injection produced a large increase in p-CaMKII in the ipsilateral dorsal horn of vehicle treated rats (Fig. 6B, C & E) compared to the contralateral side (Fig. 6A). Inhibition of PI3K by intrathecal injection of LY294002 resulted in a significant reduction in CaMKII phosphorylation in the ipsilateral dorsal horn compared

to vehicle treated rats (70% reduction, $p=0.013$, Fig. 6D-E), suggesting that the phosphorylation of both ERK and CaMKII induced by peripheral inflammation are PI3K-dependent mechanisms.

Results are expressed as mean percentage of increase in the CaMKII staining in the medial part of lamina II in the ipsilateral side versus the contralateral side \pm SEM. $*p<0.05$ compared to the vehicle group, student *t*-test. $n=5$ in each group.

PI3K is implicated in the phosphorylation of NR2B subunits of the NMDA receptor

We performed Western blot analysis to assess the role of PI3K in the phosphorylation of NMDA receptor subunits NR2A and NR2B in spinal dorsal horn of the L4-L5 spinal cord, 5 minutes after formalin injection. Vehicle treated rats showed a marked increase in both NR2A and NR2B phosphorylation compared to the contralateral dorsal horn (100%) (Fig. 7A-D). Intrathecal administration of LY294002 significantly attenuated p-NR2B expression compared to vehicle treated rats (Fig. 7C-D). Although a reduction in p-NR2A expression was also seen with LY294002, this effect did not reach statistical significance (Fig. 7A-B).

PI3K is involved in the trafficking of AMPA-R GluR1 subunit after formalin injection

Using Western blot analysis, we tested whether inhibition of PI3K affects the trafficking of AMPA receptor GluR1 subunit from the cytoplasm to the plasma membrane in the dorsal horn of the spinal cord after intraplantar formalin injection. As previously demonstrated following visceral inflammatory stimulation (Galan et al., 2004), intraplantar injection of formalin (in vehicle treated animals) induced an initial reduction of the relative amount of GluR1 receptor in the cytoplasm (10 minutes post-formalin injection, 39% decrease, $p=0.029$, Fig. 7E, 7G), followed by an increase at 45 min and a significant increase at 90 min post injection in the amount of cytoplasmic GluR1 (42% increase, $p=0.039$, Fig. 7E, 7G). This was paralleled by increased GluR1 in the membrane fraction at 45 and 90 min post injection (Fig.

7F, 7H). PI3K inhibition by LY294002 (100 μ g) prevented both the reduction of cytoplasmic GluR1 (10 minutes, Fig. 7E, 7G) and the increase in both the cytoplasm (90 minutes, Fig. 7E, 7G) and the membrane (45 and 90 minutes, Fig. 7F, 7H). The level of GluR1 expression in the membrane after LY294002 was statistically not different from the control group ($p>0.05$).

PI3K is involved in the increased synthesis of c-Fos after formalin injection

We examined the effect of intrathecal administration of LY294002 (100 μ g) on the increased synthesis of c-Fos induced by formalin injection. As previously described (Presley et al., 1990), formalin injection induced an up-regulation of c-Fos in superficial (laminae I-II) and deep laminae (V-VI) of the dorsal horn (Fig. 8A-B) in the lumbar cord L3 to L4. Inhibition of PI3K by intrathecal injection of LY294002 resulted in a significant reduction of c-Fos expression in the superficial (54% reduction in L3 $p=0.027$, 61% reduction in L4, $p=0.049$, compared to vehicle treated group, Fig. 8B-E) and to a smaller extent in deep laminae (in L3: 85% reduction in laminae V-VI, $p=0.15$; 100% reduction in lamina X, $p=0.23$; 100% reduction in lamina VII, $p=0.23$, compared to vehicle treated group, Fig. 8B-E).

DISCUSSION

This study aimed at determining the potential involvement of PI3K in mechanisms of enhanced synaptic strength, such as wind-up and central sensitization, in the spinal dorsal horn, that contribute to pain hypersensitivity following peripheral inflammation. We have shown that PI3K is involved in a transcription-independent and short-term form of spinal plasticity, termed “wind-up”, which may underlie central sensitization. We demonstrated a dose-dependent inhibitory effect of a specific PI3K inhibitor, LY294002, on A-delta- and, more potently, on C-fiber-mediated evoked responses. We also found an inhibitory dose-dependent effect of intrathecal LY294002 administration on the first phase and most interestingly on the second phase of the formalin test. This inhibitor also reduced the phosphorylation of NR2B subunits of NMDA receptor and long-term phenomenon such as c-Fos expression. This inhibitory effect of LY294002 was also correlated with a reduction of the phosphorylation of ERK and CaMKII as early as 5 min after formalin injection, as well as the abolishment of the translocation of GluR1 AMPA receptor subunit to the plasma membrane. This mechanism is an essential mediator of the establishment of LTP in hippocampus {Man, 2003 168 /id} but has never been demonstrated before at the spinal cord level in a model of somatic inflammatory pain.

Development of wind-up is PI3K dependent

“Wind-up” in the spinal cord is a phenomenon whereby repetitive stimulation of deep dorsal horn WDR neurons induces an increase of their evoked responses and post-discharge with each stimulus (Dickenson and Sullivan, 1987). This form of spinal plasticity is NMDA-dependent, as is LTP, and seems to be the neuronal correlate of pain hypersensitivity. Despite being a fast-onset and short-lasting phenomenon, wind-up may contribute to long-term changes in the spinal cord leading to central sensitization as is produced by peripheral formalin injection (Haley et al., 1990). Our results indicate that PI3K is required for the full expression of wind-up, as the potent PI3K inhibitor, LY294002, was able to reliably reduce wind-up by 50%. In addition, we observed a more profound dose-dependent reduction of the A δ - and C-fiber-mediated responses rather than A β -fiber-mediated responses, suggesting that PI3K blockade mainly interferes with nociceptive signaling. The ability to

apply the drug locally to a restricted cord zone in this static system explains the low effective doses as compared to the behavioral study where CSF flow and dilution and the smaller volume make higher doses necessary. Nevertheless, the biochemical data establishes the selectivity of even the highest dose of LY294002 for PI3K.

We found that the “input” to the dorsal horn was unaltered with both LY294002 and vehicle, indicating that the reductions observed in evoked neuronal response and wind-up were probably not due to an inhibition of primary afferent signaling, but rather a direct effect on the post-synaptic dorsal horn neurons themselves. This hypothesis was partially confirmed by the observation that the PI3K inhibitor did not alter SP-LI released from primary afferents after electrical stimulation of the dorsal roots compared to the control preparation. We cannot entirely exclude that LY 294004 might affect glutamate release from primary afferent fibers. However, it seems more likely that PI3K is essentially involved at the post-synaptic level in the dorsal horn.

Spinal involvement of PI3K in an animal model of NMDA-dependent sustained neuronal activation

Despite differences between studies in the involvement of PI3K in the induction and/or expression of LTP, it is clear that PI3K has an important role to play in this phenomenon (Man et al., 2003; Opozo et al., 2003). Because of some similarities between the mechanisms of enhanced synaptic strength in the hippocampus and the spinal cord (Malcangio and Lessmann, 2003) and a study demonstrating an involvement of spinal PI3K on pain following peripheral capsaicin injection (Sun et al., 2006), we hypothesized that PI3K could be an important intracellular mediator for the development of central sensitization, and proceeded to reveal the mechanisms involved. We employed an inflammatory pain model induced by intraplantar formalin injection to investigate intracellular mechanisms contributing to central sensitization. We found an activation of PI3K pathway after formalin administration reflected by increased phosphorylation of downstream p-Akt, like Sun et al., (2006) with the capsaicin model. Importantly, we observed a dose-dependent inhibitory effect of PI3K blockade on both the acute (1st phase) and “tonic” pain (2nd phase) phases of the formalin test. The first phase is considered to be mainly A δ and C-fiber mediated and activates second order neurons in

the superficial laminae of the dorsal horn. Inhibition of this acute phase could reflect the decreases in A δ -fiber and C-fiber-mediated responses, as seen in our electrophysiological results. The second phase is known to be due to spinal mechanisms of enhanced synaptic strength, such as post-discharge and wind-up, reflecting central sensitization and requires spinal NMDA receptor activation (Haley et al., 1990). As PI3K is a protein kinase and NMDA phosphorylation is associated with wind-up and central sensitization, we assessed the effect of LY294002 on NR2A and NR2B subunit phosphorylation after formalin administration. We found a significant inhibition of NR2B subunit phosphorylation in rats pre-treated with LY294002. NMDA receptor subunit phosphorylation, especially of the NR2B subunit, has been correlated with pain behaviors in several inflammatory pain models and inhibition of phosphorylation of this particular subunit has produced a potent antinociceptive effect (Boyce et al., 1999;Malmberg et al., 2003;Tan et al., 2005). Finally, specific inhibition of phosphorylated NR2B subunit potentially reversed electrophysiological wind-up activity (Kovacs et al., 2004). Therefore, it is reasonable to speculate that PI3K promotes the phosphorylation of NR2B subunits, directly or indirectly via ERK and/or CAMKII activation, and, is involved in the establishment of central sensitization.

Intracellular mechanisms involved in PI3K-mediated central sensitization

Activation and phosphorylation of the NMDA receptor provokes increased calcium influx which may induce sensitization-like mechanisms in the dorsal horn of the spinal cord. CAMKII, as well as having a crucial role in LTP, contributes to central sensitization in the spinal cord in several pain models (Choi et al., 2006;Choi et al., 2005;Fang et al., 2002). Influx of calcium via NMDA receptors leads to the phosphorylation and activation of CaMKII, and its inhibition prevents LTP as well as central sensitization using electrophysiological and behavioral methods (Fang et al., 2002;Malinow et al., 1989;Nagy et al., 2004;Silva et al., 1992). In our study, intrathecal administration of LY294002 produced a significant reduction in CaMKII phosphorylation in the dorsal horn induced by formalin injection. Our results suggest that the role of CaMKII in central sensitization and subsequently pain behavior might be dependent on upstream PI3K activity. Furthermore, the effect of CaMKII on pain transmission may be due to an alteration of the trafficking

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and/or activation of GluR1 subunit as previously observed following visceral inflammatory stimulation (Galan et al., 2004). Phosphorylation and trafficking of GluR1 by CaMKII is an essential component of the early phase of LTP (Derkach et al., 2007). We, indeed, observed changes in the trafficking of AMPA receptors (GluR1 but not GluR2/3, data not shown) from the cytoplasm to the plasma membrane. Finally, CAMKII activation may also affect phosphorylation of NR2B subunit.

We also observed a reduced number of phospho-ERK positive cells after intrathecal LY294002 injection. The phosphorylation of ERK has long been known to be involved in inflammatory pain (Obata and Noguchi, 2004; Silva et al., 1992). For example, ERK activation occurs rapidly in neurons of the dorsal horn after formalin injection and its inhibition blocks the resulting pain behavior (Ji et al., 1999; Ji et al., 2002). Interestingly, studies on LTP describe the role of PI3K through both ERK-dependent and ERK-independent mechanisms (Chen et al., 2005; Man et al., 2003; Opazo et al., 2003; Perkinton et al., 2002; Zhuang et al., 2004). In inflammatory pain conditions, Zhuang et al., (2004) demonstrated that PI3K and ERK signaling pathways were intricately associated at the periphery. ERK activation may participate in central sensitization through the phosphorylation of NR2B subunit and/or GluR1 trafficking. As c-Fos up-regulation is due to early increased activity of various intracellular pathways, the robust reductions of two important pathways, CaMKII and ERK, via PI3K inhibition, were also likely to contribute to the decreased incidence of c-Fos up-regulation.

In conclusion, this study reinforces the idea of similarities between LTP in the hippocampus or amygdala and central sensitization in the spinal cord. However, even though these two phenomena could be equivalent, they are not strictly similar and this may relate to the functional sequelae of prolonged painful inputs into spinal circuits as opposed to synaptic inputs that may induce memory in higher centers. For example, the effect of PI3K on CAMKII is intriguing as it has never been shown before in LTP in the hippocampus. Here, we show a direct and/or indirect relationship between these two pathways in spinal plasticity. The precise order, if any, by which PI3K activates different intracellular pathways that ultimately lead to central sensitization remains to be further clarified. Indeed, the effects on NR2B, CAMKII and ERK after PI3K inhibition were observed as soon as 5 minutes after formalin injection. Thus, PI3K seems to sit at the junction of different pathways which

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interact and work in concert to induce the early phase of central sensitization (see the supplementary figure for diagram).

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Figures Legend

Fig. 1 Spinal application of PI3K inhibitor (LY294002) inhibits wind-up of deep dorsal horn WDR neurons in naïve rats

(A) Example of the effect of spinal application of LY294002 (0.8 and 1.6 μ g) on the wind-up of a single deep dorsal horn wide dynamic range (WDR) neuron. Number of action potentials (AP) is plotted against the stimulus number. (B) Both 0.8 and 1.6 μ g doses of LY294002 significantly reduced wind-up of dorsal horn neurons compared to pre-drug control values. Data are presented as mean number of action potentials (spikes) \pm SEM, ** p <0.01, One-way ANOVA followed by Dunnett's multiple comparison test. n =6 in each group

Fig. 2 Spinal LY294002 does not modify the initial dorsal horn response or the release of substance P, but reduces the C and A δ -fiber evoked responses of deep dorsal horn WDR neurons in naïve rats.

A-D: LY294002 significantly reduced A δ - (B) and C-fiber mediated responses (C) and post-discharge of dorsal horn neurons (D) compared to pre-drug control values. No effect was seen on A β -fiber mediated responses (A). PI3K inhibition did not affect input (E). Data are presented as mean number of action potentials (spikes) \pm SEM, * p <0.05, ** p <0.01, One-way ANOVA followed by Dunnett's multiple comparison test. F: LY294002 (100 μ M) did not modify release of substance P induced by electrical stimulation of primary afferent fibers (20V, 0.5ms, 10Hz for 8min). * p <0.05, ** p <0.01 compared to basal level, One-way ANOVA followed by Tukey post-hoc test. n =5 in each group.

Fig. 3 Spinal blockade of PI3K activity reduced the development of central sensitization induced by peripheral inflammation.

Intrathecal administered LY294002 statistically reduced nocifensive behavior (time spent licking and lifting of the injected paw), both during the first (0-5 minutes post-injection, A) and the second phase (20-40 minutes, B) of the formalin test. C: Time-course of nocifensive behavior induced by

intraplantar formalin in animals receiving intrathecal vehicle (DMSO) or LY294002 (50 or 100 µg).

The dose 10 µg had no effect on both phases. Data are presented as the mean time spent by the animal licking and/or lifting his injected paw. * $p < 0.05$ compared to the vehicle group, student *t*-test. $n = 8$ in each group.

Fig. 4 Effect of inhibition of PI3K on Akt phosphorylation induced by intraplantar formalin.

Injection of formalin into the right hind paw induced a significant increased density of p-Akt in the medial part of superficial laminae (I-II) in the ipsilateral spinal dorsal horn (B) compared to the controlateral side (A) 5 minutes after formalin injection. n rats pre-treated with vehicle (DMSO). Pre-treatment with the PI3K inhibitor LY294002 (100 µg) attenuated this increase in p-Akt staining in lamina I-II (C, ($p = 0.079$)). Scale bars: A-B: 80 µm.

Fig. 5 ERK phosphorylation induced by intraplantar formalin is reduced by inhibition of PI3K activity.

Injection of formalin into the right hind paw induced a significant increase in the number of p-ERK positive neurons in the ipsilateral spinal dorsal horn 5 minutes after formalin injection (B) when compared to the contralateral side (A) in rats pre-treated with vehicle (DMSO). This increase of ERK phosphorylation was most abundantly seen in laminae I-II but also in laminae V-VI (B, E). Pre-treatment with the PI3K inhibitor LY294002 (100 µg) caused a significant reduction in the magnitude of ERK phosphorylation (D, E), both in superficial and deep laminae of the dorsal horn. Results are expressed as the mean number of p-ERK positive neurons per section \pm SEM. * $p < 0.05$ compared to the vehicle group, student *t*-test. $n = 5$ in each group. Scale bars: A-B: 125 µm, C-D: 32 µm.

Fig. 6 Increased CaMKII phosphorylation induced by intraplantar formalin is mediated by PI3K.

Injection of formalin into the hind paw induced an increase in the density of CaMKII phosphorylation in the medial part of laminae I-II in the ipsilateral spinal dorsal horn 5 minutes after formalin injection (B) compared to the contralateral side (A) in rats pre-treated with vehicle (DMSO).

Pre-treatment with the PI3K inhibitor LY294002 (100 μ g) caused a significant reduction in the density of p-CaMKII staining in lamina II (D). Scale bars: A-B: 130 μ m, C-D: 32 μ m.

Fig. 7 Increased phosphorylation of NMDA receptor 2B and translocation of AMPA receptor GluR1 induced by inflammatory stimulation (intraplantar formalin) are PI3K-mediated mechanisms. A-B: Phosphorylation of NMDA receptor subunits 2A and 2B. Intraplantar injection of formalin in the ipsilateral hindpaw (A, C, Ipsi) induced an increased phosphorylation of NMDA receptor 2A and 2B compared to the contralateral side. Intrathecal injection of LY294002 (100 μ g) significantly abolished the increased phosphorylation of NR2B (A, bottom panel and B) but not NR2A subunits (A, top panel and B). Results are expressed as the mean ratio of the densitometric analysis ipsilateral/contralateral \pm SEM. The scattered line shows 100%, i.e. the lack of changes ipsi versus the contralateral side. C-F: Translocation of AMPA receptors GluR1 from the cytoplasm to the plasma membrane induced by peripheral inflammation is mediated by PI3K. Intraplantar injection of formalin in the right hindpaw induced a decrease in the relative amount of GluR1 in the cytoplasm 10 minutes post-formalin injection (C, E) and an increase in the relative amount of GluR1 in the plasma membrane 45 and 90 minutes post formalin injection (not statistically significant, D, F). Intrathecal injection of LY294002 (100 μ g) abolished significantly both the decrease of GluR1 in the cytoplasm (C, E) and its increase in the membrane (D, F). Results are expressed as the mean ratio of the densitometric analysis ipsilateral/contralateral \pm SEM. The scattered line shows 100%, i.e. the lack of changes ipsilateral versus the contralateral side. Mann-Whitney Rank Sum tests, n=4 per group and time points for p-NR2A and p-NR2B and n=3 for analysis of cytoplasmic GluR1.

Fig. 8 Long-term effects of PI3K inhibition following intraplantar formalin injection: reduction of c-Fos up-regulation in spinal neurons. Intraplantar injection of formalin induced an up-regulation of c-Fos in both superficial (I-II) and deep layers (V, VI, VII, X) of the spinal cord (A, C). This up-regulation was significantly attenuated by pre-treatment with the PI3K inhibitor LY294002 (100 μ g, B, D). Results are expressed as mean number of c-Fos positive profiles per section in the different

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groups of laminae \pm SEM. * $p < 0.05$ compared to the vehicle group, student *t*-test. $n=5$ in each group.

Scale bars: A-B: 155 μ m, C-D: 78 μ m.

Supplementary figure: Diagram summarizing some of the mechanisms involved in central sensitization and their relationship with PI3K. Arrows mean activation.

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