Local anaesthetics inhibit signalling of human NMDA receptors recombinantly expressed in *Xenopus laevis* oocytes: role of protein kinase C

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**Background.** N-methyl-D-aspartate (NMDA)-receptor activation contributes to postoperative hyperalgesia. Studies in volunteers have shown that intravenous local anaesthetics (LAs) prevent the development of hyperalgesic pain states. One potential explanation for this beneficial effect is the inhibition of NMDA receptor activation. Therefore, we studied the effects of LA on NMDA receptor function.

**Methods.** The human NR1A/NR2A NMDA receptor was expressed recombinantly in *Xenopus laevis* oocytes. Peak currents were measured by voltage clamp in Mg- and Ca2+-free, Ba2+-containing Tyrode’s solution. Holding potential was −70 mV. Oocytes were stimulated with glutamate/glycine (at EC50) with or without 10 min prior incubation in bupivacaine, levobupivacaine, S-(-)-ropivacaine, or lidocaine (all at 10−9–10−4 M), procaine (10−4 M), R-(-)-ropivacaine (10−4 M), QX314 (permanently charged, 5×10−4 M) extracellularly or intracellularly or benzocaine (permanently uncharged, 5×10−3 M). We also determined the effect of the protein kinase C (PKC) inhibitors chelerythrine (5×10−5 M), calphostin C (3×10−6 M) and Ro 31-8220 (10−7 M), and the effect of PKC activation with phorbol ester (10−6 M).

**Results.** Non-injected oocytes were unresponsive to agonist application, but oocytes expressing NMDA receptors responded with inward currents (1.1±0.08 μA). All LA concentration-dependently inhibited agonist responses. The inhibition was reversible and stereoselective. Intracellular QX314 reduced responses to 59% of control, but extracellular QX314 was without effect. Benzocaine reduced responses to 33% of control. PKC inhibitors had no additional inhibitory effect beyond that of bupivacaine. The effect of PKC activation was abolished in the presence of bupivacaine.

**Conclusion.** All LA tested inhibited the activation of human NMDA receptors in a concentration dependent fashion. This effect may contribute to reduced hyperalgesia and opiate tolerance observed after systemic administration of LA. The effect is independent of the charge of LA; site of action is intracellular. The mechanism of action may be mediated by inhibition of PKC.

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Local anaesthetics (LAs) act beyond blocking Na channels. In addition to modulating processes like inflammation, coagulation, platelet aggregation and the microcirculation12 they also affect hyperalgesic pain states.3 Clinical studies demonstrate protection against hyperalgesia by systemic administration of LA.4–6 These clinical observations support preclinical studies demonstrating antihyperalgesic effects of LA in animal models. The mechanisms underlying these effects are unclear. Koppert et al.7 showed that low-dose systemic LAs are able to reduce secondary hyperalgesia by a
central mode of action. Since spinal N-methyl-D-aspartate (NMDA) receptors play a critical role in the development of secondary hyperalgesia,\textsuperscript{7–9} we hypothesized that the beneficial effects of LA on postoperative pain may be explained in part by the inhibition of NMDA receptor channel activation. To test this hypothesis we studied the influence of clinically relevant LA on human NMDA receptor function in an \textit{in vitro} model. In addition, we investigated the site and mechanism of action. Our results indicate that all LA studied inhibit NMDA signalling at clinically relevant concentrations. The mechanism appears to be indirect, via inhibition of protein kinase C (PKC).

**Materials and methods**

**Oocyte harvesting and preparation**

Procedures for \textit{Xenopus laevis} oocyte isolation, synthesis of NMDA receptor mRNA and microinjection technique were published previously.\textsuperscript{10–12} In brief, after approval by the local Animal Care and Use Committee, female \textit{Xenopus laevis} frogs were anesthetized by immersion in cold 0.2% 3-aminoazobenzoic-methyl-ester until unresponsive to a painful stimulus (toe pinching). Approximately 200 oocytes were surgically removed. The oocytes were defolliculated by treatment for 2 h with collagenase type 1A diluted in oocyte Ring er’s (OR2) solution (containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\) and 5 mM Hepes, pH adjusted to 7.4). Microscopic observation confirmed that the follicle cells had been removed.

**NMDA receptor expression**

The NMDA receptor combinations tested consist of NR1 and NR2 subunits. The human NR1 (\(\sim\)3000 bp) and NR2A (\(\sim\)5500 bp) subunits were obtained from Dr P. J. Whiting (Merck Sharp & Dohme Research Laboratories, Harlow, UK) as a complementary DNA in pcDNAI/Amp vectors. These constructs were linearized by either the nuclease \(Xba\)I (NR1A) or \(Eco\)RV (NR2A) and transcribed in the presence of capping analogue by bacteriophage RNA polymerase T7, using a commercial RNA preparation kit (mMESSAGE mACHINE TM T7 Kit, Ambion Inc., Austin, TX). Oocytes were injected, using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA, USA), with 6 ng of NR1/NR2A subunits in a 1:5 weight ratio in 30 nl RNase-free sterile water. Correct injection was confirmed by a slight increase in cell size. Oocytes were then stored for 48–72 h in MBS solution (MBS, containing 88 mM NaCl, 2.4 mM NaHCO\(_3\), 0.41 mM CaCl\(_2\), 0.82 mM MgSO\(_4\), 0.3 mM Ca\(_2\)\(_{3}\)\(_{4}\)O\(_7\), 10 \(\mu\)g ml\(^{-1}\) gentamicin, 10 \(\mu\)g ml\(^{-1}\) penicillin and 15 mM Hepes, pH adjusted to 7.4) at 16°C.\textsuperscript{13} Oocytes were transferred to fresh dishes with new MBS each day.

**Electrophysiology**

A single oocyte was positioned in a continuous-flow chamber with a volume of 0.5 ml and superfused (5 ml min\(^{-1}\)) containing Mg\(^{2+}\)/Ca\(^{2+}\)-free Tyrode’s solution containing Ba\(^{2+}\) (TyrBa, containing 150 mM NaCl, 5 mM KCl, 1.8 mM BaCl\(_2\), 10 mM dextrose and 10 mM Hepes, pH adjusted to 7.4). Microelectrodes were pulled in one stage from capillary glass on a vertical computer-controlled electrode puller (Model 773, Campden Instruments Ltd, Lafayette, IN, USA). Electrode tips were broken to a diameter of \(\sim\)10 \(\mu\)m, providing a resistance of 1–3 M\(\Omega\), and filled with 3 M KCl. The oocytes were voltage clamped using a two-electrode voltage clamp amplifier (OC725C; Warner Instruments Corp., New Haven, CT) connected to an IBM-compatible personal computer for data acquisition and analysis. All measurements were performed at a holding potential of \(-70\) mV; data were recorded for 120 s.

**Study protocols**

The physiological agonist glutamate (10\(^{-5}\) M), or the receptor-selective agonist NMDA (10\(^{-3}\) M), in combination with the obligatory co-agonist glycine (10\(^{-5}\) M), were diluted in TyrBa solution to the required concentrations and were delivered into the bath for 20 s. Responses were quantified by measurement of peak currents and are reported as \(\mu\)A. Substances used for incubation (LA, PKC activators and inhibitors) prior to measurements were diluted in MBS to the required concentrations. Oocytes were incubated in LA for 10 min. We tested bupivacaine, S-(−)-ropivacaine, lidocaine or levobupivacaine at 10\(^{-9}\)–10\(^{-4}\) M. Procaine and R+(+)-ropivacaine were tested at 10\(^{-4}\) M. QX314 (N-(2,6) dimethylphenylcarbamoylmethyl triethylammonium bromide, a quaternary LA, 99.9% permanently charged, which does not penetrate the cell membrane) was injected into the oocyte or applied outside the cell to identify an intracellular or extracellular site of action. For injection QX314 was diluted in 50 nl of 150 mM KCl to a concentration of 5 mM, resulting in an intracellular concentration of \(~5\times10^{-4}\) M QX314. However, the oocyte is heavily compartmentalized, which will lead to intracellular concentration differences. In addition it contains a large amount of yolk, which may bind various compounds with different affinities. Hence the concentration estimate may be prone to considerable variability. Control cells were injected with 150 mM KCl. Each cell was voltage clamped 10 min after injection. To differentiate the effects of charged and uncharged LA, we studied the effect of benzocaine (5\times10^{-3} M, permanently uncharged, freely membrane permeable). To study the role of PKC, cells were incubated with PKC inhibitors chelerythrine 5\times10^{-5} M, calphostin C 3\times10^{-6} M and Ro 31-8220 10^{-7} M for 50 min followed by 10 min bupivacaine 10^{-4} M in the continued presence of PKC inhibitor. To evaluate an inhibitory effect of LA on PKC activation in NMDA receptor-expressing oocytes, we tested two approaches: (i) cells were co-incubated in 10^{-6} M phorbolester (PMA, PKC activator) and bupivacaine for 5 min, followed by another 5 min in 10^{-4} M bupivacaine (co-administration); (ii) cells were incubated in 10^{-6} M PMA (5 min) followed by 10 min 10^{-4} M bupivacaine (subsequent application). Experiments
with PKC inhibitors and PMA were repeated with the ester-LA procaine $10^{-4}$ M to exclude a distinct mechanism for different types of binding.

For reversibility experiments a different study protocol with repeated measurements of the same cell was used. First the response to agonists were measured. The response serves as control value for the following measurements. After 10 min incubation in LA $10^{-4}$ M the response to agonists was recorded, followed by a measurement after 10 min wash out.

**Statistical analysis**

Unless stated otherwise, results are reported as mean±SEM. Differences between treatment groups were analysed using one-way ANOVA, corrected for multiple comparisons (Dunnet’s test). Differences between two groups were analysed using Student’s $t$-test. Because variability between batches of oocytes is common, at least 10 oocytes from at least 3 frogs were studied for each data point and responses were normalized to control values obtained on the same day in oocytes from the same batch. Differences between recordings were analysed using one-way repeated measures ANOVA, corrected for multiple comparisons (Dunnet’s test). $P<0.05$ was considered significant.

**Materials**

Levobupivacaine was obtained from Abbott, Eindhoven, The Netherlands, S-(-)-ropivacaine from AstraZeneca, Germany; R-(+)-ropivacaine from Astra USA Inc., Westborough, USA. All other chemicals were from Sigma Aldrich Chemie GmbH (Steinheim, Germany).

**Results**

**Functional expression of NMDA receptors in Xenopus oocytes**

Uninjected oocytes were unresponsive to either glutamate/glycine (Fig. 1A) or to NMDA/glycine (data not shown). In contrast, oocytes injected 48–72 h before experimentation with NR1A/2A receptor mRNA responded concentration-dependently with inward currents to glutamate/glycine (Fig. 1B). Glycine, as expected, evoked inward currents in the presence of glutamate only (Fig. 1B and D). The selective agonist NMDA ($10^{-3}$ M) was applied (in combination with $10^{-5}$ M glycine) to demonstrate that the receptors functioned appropriately as NMDA receptors. Responses were indistinguishable from those induced by glutamate/glycine (Fig. 1B and C). Therefore, for further experiments the physiologic agonist glutamate in combination with glycine was used. EC$_{50}$ concentrations as determined in a previous study from our group were employed: glutamate $8\times10^{-6}$ M/glycine $12\times10^{-6}$ M.$^{14}$

**LAs inhibit glutamate signalling**

We then tested the effects of LA on responses induced by glutamate/glycine at EC$_{50}$. S-(-)-ropivacaine or

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**Fig 1** Pharmacological characterization of NMDA (NR1/2A) receptors, recombinantly expressed in *Xenopus laevis* oocytes. Example traces of responses to 20 s agonist administration. (A) In oocytes which were not injected with NMDA mRNA. Cells were unresponsive to either glutamate/glycine or NMDA/glycine. (B) In oocytes injected with NMDA (NR1A/2A) mRNA 24–48 h prior to experiments. Glutamate/glycine at EC$_{50}$ concentration evoked inward currents. (c) The selective agonist NMDA ($10^{-3}$ M) in combination with glycine ($10^{-5}$ M) evoked inward currents in NMDA receptor expressing oocytes. Responses were indistinguishable from those stimulated by glutamate/glycine. (d) Glycine ($10^{-3}$ M) in the absence of glutamate did not evoke currents in oocytes expressing NMDA (NR1A/2A) receptors.
levobupivacaine at concentrations of 10^{-6} M significantly reduced NMDA signalling to 72\pm7.4\% (n=12) (levobupivacaine) and 66\pm2.9\% (n=11) [S-(-)-ropivacaine] (Fig. 2c and d). Responses to agonists were reduced to 62.2\pm5.4 (n=12) in the presence of 10^{-7} M or greater lidocaine and to 72\pm6.4\% (n=12) following 10^{-7} M or greater bupivacaine. Because inhibition of currents did not exceed 50% with the highest LA concentration tested (except for lidocaine and benzocaine) the IC_{50} was not calculated. Results are plotted as % of control. Black bars show control responses to EC_{50} glutamate/glycine after 10 min of incubation in different concentrations of local anesthetics. Responses were normalized to control values obtained on the same day in oocytes from the same batch. (*P<0.05 compared with control responses.). Ctrl, control.

To test if the effect of the LA was reversible and to exclude conclusively the possibility that the inhibitory effects of LA were due to variability among oocytes, we exposed oocytes to each of the LA at 10^{-4} M for 10 min and subsequently washed out the LA. After a 10-min wash-out in MBS the responses to agonists were similar to control values (P>0.05; t-test) (Fig. 3a and b), (n=6). These experiments yielded results similar to those shown in Figure 2 (Fig. 3a). Time matched blanks showed no significant differences.

**LA inhibition of NMDA signalling is stereoselective**

To investigate stereoselectivity of LA on NMDA receptors in oocytes, the effect of R-(+)-ropivacaine was compared with that of S-(-)-ropivacaine. Whereas R-(+)-ropivacaine was without effect, the clinically used S-(-)-ropivacaine inhibited the responses to glutamate/glycine to 61\pm9\% (n=14), indicating a stereoselective effect of LA on NMDA receptors, making protein interaction of LA with NMDA receptor signalling most likely (Fig. 4A). Since this degree of stereoselectivity is much greater than observed on other receptor systems we repeated these experiments three times with similar results.

**LA inhibit NMDA receptor signalling at an intracellular site**

We then determined if the site of action of the LA was intracellular or extracellular, using the permanently charged and therefore virtually non-membrane-permeable lidocaine analogue QX314. When applied extracellularly, QX314 did not inhibit agonist responses (responses were 91\pm19\% of control, P>0.05, n=10; Fig. 4b). In contrast, intracellular microinjection of the compound inhibited the responses to agonists effectively (by 41\%) to 59\pm8\% (n=10; Fig. 4b). To exclude an effect of the vehicle, 150 mM KCl was micro-injected into the cell and showed no effect (responses 99.6\pm11\% of control, n=10). This indicates that the site of
action of QX314 on NMDA receptor signalling is located intracellularly. The fact that the degree of inhibition is different from that of lidocaine may result from the approximate estimation of intracellular QX314 concentrations (see Materials and methods section).

**LA inhibition of NMDA signalling is not charge-dependent**

Our data with QX314 indicate that charged LA can block NMDA signalling. To clarify whether charge is required for NMDA signalling block by LA, the effects of the almost completely uncharged and highly membrane permanent LA benzocaine on NMDA signalling in oocytes were tested. The agonist response was inhibited to 33±4% of control when oocytes were exposed to benzocaine 5 mM (n=10). Therefore, both charged and uncharged LA are able to inhibit NMDA signalling (Fig. 4b). However, a conclusion whether the site of action of benzocaine is intra- or extracellular cannot be made.

**LA inhibit NMDA receptor signalling by inhibiting PKC**

NMAD receptors are highly regulated by PKC, which enhances signalling by phosphorylating the C-terminal segments of NMDA receptors.\textsuperscript{15-16} Since interactions between LA and PKC have been shown previously,\textsuperscript{17} and since our data suggest an intracellular site of action of LA, we hypothesized that LA could affect NMDA signalling indirectly...
by inhibiting PKC. This could result either directly in a decreased phosphorylation state of the receptor or a decreased phosphorylation state of a signalling system downstream of PKC, which in turn would inhibit NMDA receptor function. To determine whether LA inhibit PKC and consequently inhibit NMDA signalling, the effect of PKC inhibition on the inhibitory effect of bupivacaine was studied. We compared the effects on NMDA signalling of (i) pre-treatment (incubation for 1 h) with the PKC inhibitors chelerythrine (5×10⁻⁵ M), Ro 31-8220 (10⁻⁷ M) or calphostin C (3×10⁻⁶ M); (ii) bupivacaine (10⁻⁴ M); and (iii) the combination of a PKC inhibitor and bupivacaine. Ro 31-8220 inhibited responses to agonists to 64±5% (n=12), chelerythrine to 66±7% (n=12) and calphostin C to 56±7% (n=11) of control cells. Bupivacaine inhibited responses to agonists to 57±9% (n=11) in chelerythrine experiments, 50±7% (n=12) in calphostin C experiments and 60±7% (n=12) in Ro-31-8220 experiments. The combination of the compounds did not further reduce responses to NMDA receptor agonists as compared with either compound alone to 62±10% in combination with Ro 31-8220 (Fig. 5c), to 62±9% in combination with chelerythrine (Fig. 5a) and to 36±6% in combination with calphostin C (Fig. 5b) [P>0.05; n=10]. This suggests that LA and the PKC inhibitors share a common PKC-dependent mechanism, and that LA block NMDA receptor signalling by inhibition of the PKC pathway.

LA should therefore be able to affect PKC activation of NMDA receptors. To test this hypothesis, we studied the effect of 10⁻⁴ M bupivacaine on phorbolester (PMA)-activated NMDA receptors. Bupivacaine alone inhibited responses to agonists to 55±10% (n=10) of control in this experiment.
PMA (10⁻⁶ M for 5 min) alone induced a significant increase to 188±10% (n=10) in glutamate/glycine activated cells expressing NR1A/2A NMDA receptors. Responses to glutamate/glycine were then elicited in cells (i) that had been incubated with PMA (10⁻⁶ M) for 5 min and subsequently with bupivacaine (BUP) for 10 min, or (ii) that had been exposed to a combination of PMA (10⁻⁶ M) and bupivacaine (10⁻⁴ M) for 5 min and subsequently to bupivacaine.
NMDA signalling have been proposed by Sugimoto et al. PKC signalling Ester and amide type LA similarly inhibit indirectly, via inhibition of a PKC dependent pathway.

Ester and amide type LA similarly inhibit PKC signalling

Different sites of action and mechanisms for LA effects on NMDA signalling have been proposed by Sugimoto et al. and an extracellularly located site of action closely related to those of Mg$^{2+}$ and ketamine has been suggested for procaine, an ester-type LA. Thus, to evaluate a potential difference in effect between amide- and ester-type LA we tested the effect of procaine on PKC activation by PMA (10$^{-6}$ M) and on PKC inhibition by chelerythrine (5x10$^{-7}$ M). Procaine (10$^{-4}$ M, 10 min incubation) inhibited responses to glutamate/glycine in NR1A/2A NMDA receptor expressing cells to 60±6% (n=10) compared with control responses. In these experiments PKC inhibition by chelerythrine (60 min incubation) induced a 47±5% (n=10) decrease in response. The combination of chelerythrine and procaine did not further inhibit responses to NMDA receptor agonists as compared with either compound itself. The combination of procaine and PKC activator PMA (93±12%; n=10) completely abolished the stimulatory effect of PMA on NMDA receptor currents (168±13%; n=10). These findings indicate that there is no difference in the site of action between amide- and ester-type LA.

Discussion

Our results demonstrate that LA concentration-dependently and reversibly inhibit glutamate/glycine-induced NMDA receptor channel (NR1A/2A) activation. This effect is stereoselective and does not depend on the charge of the molecules. The site of action is located intracellularly. Inhibition of PKC seems to be involved. Several groups have demonstrated that LA are able to suppress hyperalgesic pain states, particularly secondary hyperalgesia. Our results might provide a partial explanation for the reduced hyperalgesia and opiate tolerance observed after administration of LA. Concentrations of LA that significantly inhibited NMDA receptor channel activation in our study

![Fig 6](https://example.com/fig6.png) (a) The effect of 10$^{-4}$ M bupivacaine (BUP) on phorbol ester (PMA) activated NMDA receptors. Bupivacaine alone inhibited responses to 55% (n=10) of control in this experiment. PMA (10$^{-6}$ M for 5 min) alone induced a significant increase of responses to 188% (n=10) in glutamate/glycine activated cells expressing NR1A/2A NMDA receptors. Responses to EC50 glutamate/glycine were then elicited in cells (i) that had been incubated with PMA (10$^{-6}$ M) for 5 min and subsequently to bupivacaine (10$^{-4}$ M) for 10 min and (ii) that had been exposed to a combination of PMA (10$^{-6}$ M) and bupivacaine (10$^{-4}$ M) for 5 min and subsequently to bupivacaine (10$^{-4}$ M) alone for 5 min. Whereas subsequent treatment with bupivacaine did not reverse the stimulatory effect of PMA (178%, n=12), bupivacaine completely inhibited the stimulatory effect of the PKC activator PMA on NMDA receptor currents (105%) (*P<0.05 vs control, **P<0.05 vs PMA and control). To evaluate a distinct mechanism between amide- and ester-type LA we tested the effect of procaine (Pro) on PKC activation by PMA (10$^{-6}$ M, 5 min) and PKC inhibition by chelerythrine (CHE, 5x10$^{-7}$ M, 60 min). Procaine (10$^{-4}$ M, 10 min incubation) inhibited responses to glutamate/glycine in NR1A/2A NMDA receptor expressing cells to 60% (n=10) compared with control responses. (n) PKC inhibition by chelerythrine induced a 47% (n=10) decrease in response sizes. Combination with procaine did not further inhibit responses to NMDA receptor agonists as compared with either compound itself. (c) The combination of procaine and PKC activator PMA (93%, n=10) completely abolished the stimulatory effect of PMA on NMDA receptor currents (168%, n=10) (*P<0.05 vs control). Ctrl, control.
are within the range as measured in blood during epidural anaesthesia.14

**NMDA receptor expression**

The NMDA receptor is a protein complex composed of two classes of subunits, the essential subunit NR1 and the modulating NR2 subunit (of which four different types exist: A–D). The NR2 subunits alone cannot form functional channels, but they amplify NR1 activity and induce functional variability in NMDA receptor signalling. These subunits co-assemble in various combinations to form functionally distinct NMDA receptors.20–22

In the present study, the NR1 subunit was co-expressed with the NR2A subunit. This combination is widely distributed in the brain and the dorsal horn of the spinal cord and is believed to play a relevant physiological role in the development of hyperalgesia and acute opioid tolerance.23 24

Although NMDA receptor expression in *Xenopus* oocytes is an established model11 some limitations of the model should be noted. First, our experiments were performed at room temperature, whereas the expressed receptor is derived from human sources and normally functions at 37°C. This might theoretically influence its behaviour. However, we found it more important to maintain the cell membrane in its normal state of fluidity. Second, NR2 subunits were co-expressed with NR1 subunits to enhance currents through expressed receptors and to provide a more physiological receptor configuration.25 In order to achieve heteromeric expression, the mRNA was injected into the oocytes in a weight ratio of 1:5 between NR1A and NR2A.25 We could not determine expression levels or stoichiometry for the subunit combinations. Therefore, although we used a defined mRNA weight ratio between NR1 and NR2 subunits, it is conceivable that the ratio of NR1 to NR2 varied during experimentation.

**Study protocol of electrophysiological measurements**

To exclude the possibility that results depend on variability of the oocytes (which is the main reservation towards this protocol), we also tested all LA at 10^{-4} M concentration with oocytes serving as their own control (protocol 2, shown in the reversibility experiments).

Results of both protocols yield comparable results, as had also been shown in preliminary experiments for the design of our study protocol. Both study protocols have been used successfully in this model in the past. As our experiments for the identification of intracellular mechanisms extend to 1 h incubation time, most experiments were performed with protocol 1. This protocol provides the advantage of longer incubation times. At least in our hands oocytes do not hold their membrane potential reliably for that time course. Two electrodes inside an oocyte for 1 h interfere with the membrane integrity. Therefore protocol 2 cannot be used for a second measurement of the same cell extending the time course of 40 min.

**Effects of LA on NMDA signalling in other models**

The charge of LA influences its pharmacological properties. Since charged fraction varies greatly among clinically used LA it is important to know whether the charge of LA might influence the inhibition of NMDA receptors. Our results indicate that both a completely uncharged and also a completely charged LA were able to inhibit NMDA receptor activation. Interestingly, QX314 only inhibits activation of the NMDA receptor when applied intracellularly, but not when applied extracellularly. Therefore the site of action of charged LA seems to be located intracellularly. The uncharged benzocaine is freely membrane permeable, therefore no site of action can be determined. These findings are in agreement with previous studies. In whole cell patch clamp experiments in the electrosensory lateral line lobe of the fish *Apteronotus leptorhynchus*, the amplitudes of NMDA-evoked excitatory postsynaptic potentials (EPSPs) have been shown to be inhibited in the presence of intracellular QX314. The authors suggest an indirect inhibitory effect of QX314 acting through a block of persistent sodium channels.26 Our results indicate that QX314 may also affect NMDA signalling in the absence of sodium channel signalling.

In contrast to our results Nishizawa and colleagues27 could not find an inhibitory effect on NMDA-induced currents of clinically relevant concentrations of lidocaine or bupivacaine using the whole cell patch clamp technique in CA1 mouse pyramidal neurons. Bupivacaine did inhibit NMDA-induced currents but only at 1 mM concentration. In addition to model differences and a possible difference in NMDA receptor subtypes studied (which were not defined in the study of Nishizawa and colleagues), a potential explanation for these different results might be the choice of agonists. Whereas in our model the influence of LA on stimulation with the physiological agonists glutamate and glycine were tested, the specific but non-physiological agonist NMDA was used in the study of Nishizawa and colleagues. A potential inhibition induced by greater concentrations of LA could not be tested in their model because the neurons became leaky at high LA concentrations.

**Effects of ester- and amide-type LAs**

Sodium channel blocking potency of LA depends upon the type of linkage in the intermediate chain. Ester-linked LA produce more potent Na channel blockade. Sugimoto and colleagues18 found that ester-type LA inhibit NMDA signalling more potently than did amide-type LA and propose two distinct extracellularly located mechanisms of action. Our data cannot confirm these findings. In contrast, we found that there is no difference in potency concerning the inhibitory effect of LA on NMDA receptor signalling. The site of
action is intracellular and no mechanistic difference was observed between amide- and ester-type LA. In contrast to our study, Sugimoto and colleagues only observed inhibition of NMDA responses at concentrations of LA greater than $10^{-4}$ M; responses were blocked almost completely at $10^{-3}$ M. At these high concentrations of LA unspecific membrane effects cannot be excluded and mechanistic conclusions cannot probably be drawn.

**Role of PKC**

PKC-dependent phosphorylation of NMDA receptor subunits amplifies NMDA receptor responses, resulting in enhanced currents through NMDA receptor channels. Inhibition of PKC results in reduced responses to NMDA receptor agonists in trigeminal and spinal cord dorsal horn neurones as well as in oocytes expressing recombinant NMDA receptors. In our study, no additive effect was observed when oocytes were incubated simultaneously in LA and one of three structurally distinct PKC inhibitors, suggesting that LA and PKC antagonists share a common mechanism. Therefore it appears that the inhibitory effects of LA on NMDA receptor signalling are indirect and mediated by inhibition of PKC. Despite truncation and point mutation experiments it remains unclear whether PKC-mediated action on NMDA receptors is entirely direct (via direct phosphorylation of NMDA receptors), indirect (via a downstream located signalling peptide of the non-receptor tyrosine kinase Src) or a mixture of both. Our results do not address this issue but show that inhibition of PKC is most likely the mechanism involved in LA inhibitory action.

In summary, clinically relevant LA concentration-dependently inhibit the activation of human NMDA receptors. The effect is intracellularly located and seems to be mediated by PKC inhibition.

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