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RESEARCH PAPER

Bupivacaine-induced cellular entry of QX-314 and its contribution to differential nerve block

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BACKGROUND AND PURPOSE

Selective nociceptor fibre block is achieved by introducing the cell membrane impermeant sodium channel blocker lidocaine N-ethyl bromide (QX-314) through transient receptor potential V1 (TRPV1) channels into nociceptors. We screened local anaesthetics for their capacity to activate TRP channels, and characterized the nerve block obtained by combination with QX-314.

EXPERIMENTAL APPROACH

We investigated TRP channel activation in dorsal root ganglion (DRG) neurons by calcium imaging and patch-clamp recordings, and cellular QX-314 uptake by MS. To characterize nerve block, compound action potential (CAP) recordings from isolated nerves and behavioural responses were analysed.

KEY RESULTS

Of the 12 compounds tested, bupivacaine was the most potent activator of ruthenium red-sensitive calcium entry in DRG neurons and activated heterologously expressed TRPA1 channels. QX-314 permeated through TRPA1 channels and accumulated intracellularly after activation of these channels. Upon sciatic injections, QX-314 markedly prolonged bupivacaine's nociceptive block and also extended (to a lesser degree) its motor block. Bupivacaine's blockade of C-, but not A-fibre, CAPs in sciatic nerves was extended by co-application of QX-314. Surprisingly, however, this action was the same in wild-type, TRPA1-knockout and TRPV1/TRPA1-double knockout mice, suggesting a TRP-channel independent entry pathway. Consistent with this, high doses of bupivacaine promoted a non-selective, cellular uptake of QX-314.

CONCLUSIONS AND IMPLICATIONS

Bupivacaine, combined with QX-314, produced a long-lasting sensory nerve block. This did not require QX-314 permeation through TRPA1, although bupivacaine activated these channels. Regardless of entry pathway, the greatly extended duration of block produced by QX-314 and bupivacaine may be clinically useful.

Abbreviations

AITC, allyl isothiocyanate; CAP, compound action potential; DRG, dorsal root ganglion; QX-314, lidocaine N-ethyl bromide; TRP, transient receptor potential

Introduction

Local anaesthetics, such as lidocaine, are widely used to treat acute and chronic pain during surgery or dentistry (Ruetsch *et al.*, 2001). By inhibiting sodium channels, they reversibly attenuate action potential firing of all neurons, causing numbness and paralysis in addition to analgesia (Scholz *et al.*, 1998; Sheets *et al.*, 2011). To reach their binding site inside the pore, the compounds enter the phospholipid bilayer of the cell membrane in their neutral, non-protonated form (Chernoff and Strichartz, 1990; Hogberg *et al.*, 2007). The N-ethylated derivative of lidocaine, QX-314 is permanently charged and thus does not permeate the membrane passively (Blumberg, 2007). However, QX-314 can permeate through the pore of transient receptor potential V1 (TRPV1) channels following activation of these channels with capsaicin or protons (Binshtok *et al.*, 2007; Kim *et al.*, 2010; Liu *et al.*, 2011; Brenneis *et al.*, 2013; Puopolo *et al.*, 2013; channel nomenclature follows Alexander *et al.*, 2013). As a consequence, when applied with capsaicin, QX-314 accumulates intracellularly and blocks sodium channels only in nociceptors that express TRPV1 channels, producing analgesia with no impairment of TRPV1-negative neuronal populations, such as motor axons and low threshold mechanoreceptors.

Like TRPV1 (Meyers *et al.*, 2003; Chung *et al.*, 2008), permeability for large cations has also been reported for TRPA1 and P2X3, and under some conditions, for TRPM8 channels (Khakh *et al.*, 1999; Banke and Wickenden, 2009; Chen *et al.*, 2009), suggesting that these channels could also provide entry pathways for QX-314. Beyond these *in vitro* studies, several *in vivo* studies have found that at very high concentrations, QX-314 can, when applied alone, cause a long-lasting nerve block after a delayed onset (Lim *et al.*, 2007; Ries *et al.*, 2009; Roberson *et al.*, 2011). Under these particular conditions, the addition of an exogenous TRPV1 agonist was not required for QX-314's local anaesthetic efficacy, although capsazepine sensitivity suggested endogenous activation of TRPV1 channels under some conditions (Ries *et al.*, 2009). Also, motor neurons could be affected by high concentrations of QX-314 alone, implying a non-selective action on all axons.

The clinical utility of using a highly pungent TRP agonist such as capsaicin with QX-314 to produce peripheral nerve block is doubtful because of the painful irritation and neurotoxicity that results when capsaicin is directly applied to a peripheral nerve (Welk *et al.*, 1983). However, Leffler *et al.* discovered that lidocaine, at clinically relevant concentrations, shows the off-target effects of activating TRPV1 and TRPA1 channels (Leffler *et al.*, 2008; 2011). Using lidocaine as the TRP channel activator combined with QX-314, we found that local anaesthesia was markedly prolonged compared with lidocaine alone and showed a preference for sensory nerves without initial irritation (Binshtok *et al.*, 2009; Roberson *et al.*, 2011).

To explore whether other local anaesthetic compounds might be more efficacious than lidocaine to activate TRP channels and permit QX-314 entry, we have systematically screened a number of these compounds for their potential to activate TRP channels, promote neuronal QX-314 uptake and produce a sensory preferential blockade. In the experiments reported here, we have identified bupivacaine as an especially strong activator of both calcium entry into dorsal root ganglion (DRG) neurons and TRPA1 channels. When applied on isolated sciatic nerves, co-applied QX-314 and bupivacaine induced a prolonged block of C-fibre, but not A-fibre, compound action potentials (CAPs). However, the block of C-fibre CAP was not abolished in TRPA1$^{-/-}$/TRPV1$^{-/-}$ mice. Upon sciatic injection, the combination of bupivacaine with QX-314 not only produced a very long-lasting anaesthesia but also prolonged motor block, although much less than the sensory block. An explanation for the incomplete selectivity of nerve block and its independence from TRP channels came from a finding that high doses of bupivacaine promote cellular uptake of QX-314, independent of TRP channels. Regardless of what entry pathways are activated by bupivacaine, its combined application with QX-314 greatly extends the duration of bupivacaine action, and this combination may therefore have clinical utility where long-lasting regional block is beneficial.

Methods

Animals

All animal care and experimental procedures with mice were approved by the animal protection authorities of the local district government, Ansbach, Germany. All procedures involving rats were approved by the IACUC Committee of Children's Hospital Boston. Reporting of the *in vivo* experiments is carried out according to the ARRIVE guidelines (Kilkenny *et al.*, 2010). A total of 72 rats were used in the experiments described here.

For all behavioural experiments, we used 8–10 weeks old male Sprague-Dawley rats (200–250 g), purchased from Charles River Laboratories, Inc., Wilmington, MA, USA. All tests were performed with the experimenter unaware of the treatment. For CAP experiments, mice of both sexes with weights between 22 and 30 g were used. C57BL\6J mice were from an in-house breeding colony, the TRPA1 knockout (KO) strain was provided by D. P. Corey, and the TRPV1 KO mice by J. B. Davis (Davis *et al.*, 2000; Kwan *et al.*, 2006). TRPA1-V1 double KO mice were bred by cross-mating. All KO strains were continuously backcrossed to C57BL\6 and thus congenic.

Sciatic nerve injections and analysis of behaviour

Groups (randomized for treatments) of six to nine rats were lightly anaesthetized by inhalation of 2% isoflurane and injected with 0.2 mL of 0.5% QX-314 bromide salt with or without 0.5% bupivacaine hydrochloride or 2% procaine hydrochloride (all from Sigma, St. Louis, MO, USA) with its solvent (saline) into the left sciatic notch behind the hip bone, as described by Binshtok *et al.*, 2007. A description of methods used for behavioural phenotyping can be found in the Supporting Information.

Plantar incision model

Rats were randomly assigned to one of three treatment groups (n = 8 rats), then lightly anaesthetized with 2%

isoflurane. The plantar surface of the left hindpaw of each rat was incised at the junction of hairy and glabrous skin rather than at the mid-plantar line, as previously described (Brennan *et al.*, 1996), in order to place the surgical wound within the area of sciatic innervation. The surgical wound was closed with two mattress sutures, as described (Brennan *et al.*, 1996). Sciatic nerve injections were performed 24 h after lateral plantar surgery.

Primary DRG cultures

Rat DRGs from all spinal segments were dissociated with trypsin, collagenase and dispase II treatment and plated on poly-D-lysine and laminin-coated dishes, as described previously (Binshtok *et al.*, 2009). A detailed description of the method can be found in the Supporting Information.

Cell line culture and transient transfection

N1E-115 cells, RAW-264.7 macrophages, CHO cells and HEK-293 cells (all from Sigma) were cultured in DMEM containing 10% FBS and 1% penicillin–streptomycin (all Invitrogen, Carlsbad, CA, USA). HEK-293 cells stably transfected with a plasmid encoding for hTRPA1 were a generous gift from David Julius, UCSF. To maintain plasmids, cells were cultured in 15 $\mu g \cdot mL^{-1}$ blasticidin and 75 $\mu g \cdot mL^{-1}$ hygromycin, and to induce expression, 10 $ng \cdot mL^{-1}$ doxycycline was added (all from Sigma).

To screen for large-pore cation channels activated by bupivacaine, N1E-115 cells were plated on 3.5 cm plastic dishes and transiently transfected using Lipofectamine® 2000 reagent according to the manufacture's protocol (Invitrogen). The plasmids pIRES-EGFP-Flag-(m)TRPA1 and pcDNA3.1-(m)TRPV1 were kindly provided by Kelvin Kwan (Department of Neurobiology and Howard Hughes Medical Institute, Harvard Medical School, Boston, USA) and pcDNA3.1-YFP(h)TRPV4, pEGFP-Flag-(m)TRPV3, pEGFP-(r)TRPV2 or pEGFP-(m)TRPM8 by Grigory Krapivinsky (Department of Cardiology, Howard Hughes Medical Institute, Children's Hospital, Boston, MA, USA) and pcDNA3.1-(r)$P2X_3$ by Andreas Zimmer (Molecular Psychiatry, Rheinische Friedrich-Wilhelms University Bonn).

Calcium-imaging experiments

For calcium imaging of single cells, we used rat DRG cultures at 24–48 h after preparation or N1E-115 cells at 15–24 h after transient transfection. A description of loading and microscope settings for single-cell calcium imaging can be found in the Supporting Information.

For analysing Ca^{2+} flux in a high-throughput protocol, DRG neurons were dissected and purified from 8 week old C57Bl/6 mice, as described earlier (primary DRG cultures). After dissociation, cells were counted and seeded into laminin-coated 384-well plates (Greiner, Inc., Monroe, NC, USA) at 2000 cells in a volume of 20 µL per well. After 24 h incubation at 37°C and 5% CO_2, the plates were then assayed for calcium flux using the FLIPR calcium-5 kit (Molecular Devices, Downingtown, PA, USA) in an FDSS-7000 apparatus (Hamamatsu Photonics, Welwyn Garden City, UK) with various ligands. The response was measured as a ratio of change in well fluorescence ($\Delta Ratio = Ratio_{max} - Ratio_{min}/Ratio_{min}$) and plotted in Prism 5.0 analysis software (GraphPad, Inc., San Diego, CA, USA).

QX-314 cell-uptake assay

N1E-115 (CRL-2263) cells, RAW-264.7 macrophages or CHO cells were plated on poly-D-lysine (0.5 $mg \cdot mL^{-1}$; Sigma) coated 6-well dishes. When cells reached confluence, they were incubated with 0.5 mM QX-314 and the indicated concentrations of bupivacaine or lidocaine for 10 min. After washing cells carefully with 2 mL PBS (four times), 500 µL lysis buffer (0.1 M HCL with 1% Triton X-100) was added for 5 min to lysed cells. Next, the lysate was centrifuged at 10 000× *g* and the supernatant was analysed by LC-MS/MS.

A description of QX-314 uptake experiments in hTRPA1 inducible HEK-293 cells and DRG-neurons and the instrumentation for LC-MS/MS analysis can be found in the Supporting Information.

Patch clamp with heterologously expressed TRPA1 and TRPV1 channels

The concentration dependence of bupivacaine activation of TRPA1 channels was studied at 37°C using whole-cell recording from HEK 293 cells stably transfected with hTRPA1, with induction as already described. Effects on TRPV1 were assayed using HEK 293 cells transiently transfected with rTRPV1 (Supporting Information) and studied using whole-cell patch clamp at 37°C. In studies of QX-314 permeation through TRPA1, N1E-115 (CRL-2263) cells were co-transfected with human TRPA1, eGFP plasmids and used 24–56 h after transfection and studied by whole-cell patch clamp (Supporting Information). The ability of QX-314 to carry ionic current through TRPA1 channels was tested using an external solution containing QX-314 as the only external cation, consisting of 10 mM QX-314 hydroxide, 277 mM sucrose and 5 mM HEPES, with pH adjusted to 7.2 with HCl. The internal solution contained 135 mM N-methyl-D-glucamine (NMDG), 5 mM EGTA and 10 mM HEPES, with pH adjusted to 7.2 with HCl. The current–voltage relationship for TRPA1 current was determined by a 30 ms voltage ramp from +116 to –144 mV (preceded by a 30 ms voltage ramp from –144 to + 116 mV and using a steady holding potential of –64 mV). TRPA1 current was activated by 100 µM allyl isothiocyanate (AITC). These experiments were carried out at room temperature.

Electrophysiology from isolated peripheral nerves

Mice were killed in a pure CO_2 atmosphere. Sciatic nerves were exposed and excised from where they emerge out of the lumbar plexus to their trifurcation into the tibial, sural and peroneal nerves. The nerve was mounted in a three-compartment chamber and continuously superfused with carbogen-gassed physiological buffer (2 mM $CaCl_2$, 5.5 mM glucose, 10 mM HEPES, 3.5 mM KCl, 0.7 $MgSO_4$, 123 mM NaCl, 1.5 mM NaH_2PO_4, 7.4 mM saccharose, carbogene used for oxygenation during the experiment will set pH 7.4, SIF, Bretag, 1969) or different test solutions, respectively, at pH 7.4 and constant temperature of 37°C. Each end of the nerve was threaded into one of two rubber foil-sealed side chambers filled with fluorocarbon oil (FC-43, 3M Company, St. Paul, MN, USA), of which one contained gold wire electrodes for recording, whereas the other was used for electrical stimulation. CAPs from C- and A-fibres were recorded monopolarly and evoked by 25% supramaximal electrical stimulation with

constant voltage pulses (A395, WPI, Sarasota, FL, USA) of fixed duration (0.1 ms) at a rate of 1/20 s. C- and A-fibre CAPs were separately filtered and amplified but synchronously registered. Following a 20 min control period in SIF, the effects of the test substances applied for 5 min on both latency and amplitude of C- and A-fibre CAPs were assessed. Amplitude was taken as the difference between the maximum and minimum excursions of the recorded waveform (peak-to-peak). Latency was assessed as the delay after which a trigger level in the lower third of the CAP up-stroke was exceeded. Data were acquired and analysed using a CED Micro1401 and Spike2 software (Cambridge Electronic Design, Cambridge, UK). To assess the time lapse of the recovery from drug-induced nerve block during wash-out, the time constants (τ at 63% of maximal recovery) were calculated and displayed as means ± SEM.

Data analysis

Statistical differences in cell culture experiments were analysed by one-way ANOVA and Bonferroni correction using GraphPad Prism software. CAPs were analysed using Wilcoxon matched pairs test for intra-individual comparisons with Statistica 7 software (StatSoft, Tulsa, OK, USA). Temporal courses from animal behaviour data were analysed by two-way repeated measures ANOVA, followed by *post hoc* Bonferroni analysis using Sigmastat software. Differences were considered significant at $P < 0.05$.

Materials

The 12 local anaesthetics tested, isoflurane, capsaicin, AITC and Ruthenium red (all from Sigma).

Results

Screening of local anaesthetics that elicited a ruthenium red-sensitive calcium flux in TRPV1 + DRG neurons

To identify local anaesthetic compounds that activated nociceptive neurons, we performed a calcium-imaging screen on DRG neurons. To obtain a reference response, we first stimulated with lidocaine (3 mM, 10 s), which induces a robust, but submaximal TRPV1 activation (Leffler *et al.*, 2008) (Figure 1A). After a recovery period (25 min), we then applied the test compound at the same concentration and duration (3 mM, 10 s). At the end of the experiment, we applied capsaicin (300 nM, 10 s) to identify TRPV1+ nociceptors, and then treated the culture with ruthenium red (RR) (10 µM) followed by the test compound stimulation.

With this experimental protocol, we tested 12 different local anaesthetics and found that bupivacaine induced the largest calcium increase, which was largely blocked by RR (Figure 1B). Most local anaesthetics, including bupivacaine,

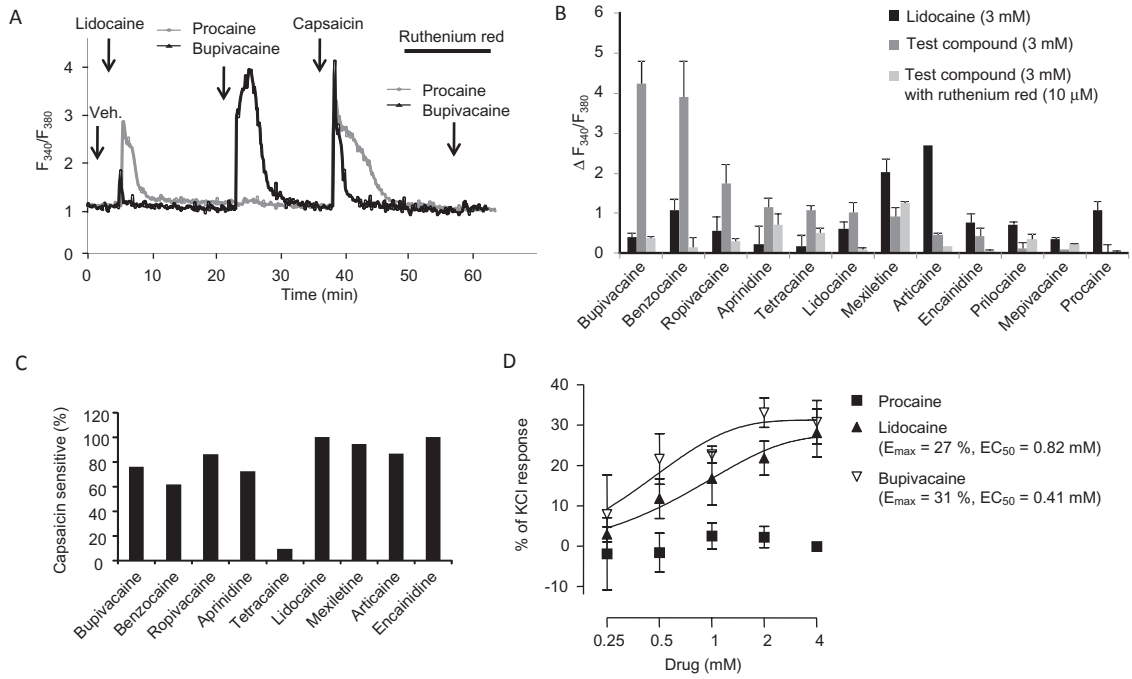

Figure 1

Identification of local anaesthetics that activate ruthenium red (RR)-sensitive calcium channels in dorsal root ganglia (DRG) neurons. (A) Representative recordings of neuronal calcium concentrations after stimulation with local anaesthetics. Cultured DRG neurons were stimulated for 10 s with lidocaine (3 mM), the test compound (3 mM), capsaicin (300 nM) and the test compound (3 mM) after wash of 10 µM RR. Intracellular calcium levels were determined by fura-2-based calcium imaging. (B) Statistical analysis of traces from neurons stimulated as in (A). The mean of [F_{340}/F_{380}] increases at peak amplitudes after stimulation is shown. n = 5–30 cells from three or more independent experiments ± SEM. (C) Capsaicin sensitivity for local anaesthetic responsive sensory neurons. Percentage of capsaicin sensitive cells within the local anaesthetic responsive group was calculated. (D) Concentration response analysis of procaine, bupivacaine and lidocaine-induced calcium flux in DRG neurons by a high-throughput protocol using an automated FLIPR system. n = 4 replicates from two independent experiments.

induced a calcium flux in neurons responsive to subsequent application of capsaicin (Figure 1C). After tetracaine application, however, almost no capsaicin-induced calcium flux could be observed in any neuron, suggesting a cytotoxic effect. Dibucaine and etidocaine could not be tested due to autofluorescence and weak solubility respectively.

To confirm the results and better describe the potency of the selected caines, we performed concentration response analysis in DRG neurons by a high-throughput automated calcium-imaging system. As expected, intracellular calcium concentrations did not increase after procaine application up to 4 mM, and bupivacaine (EC_{50} = 0.41 mM) was more potent and efficacious than lidocaine (Figure 1D). We conclude that in nociceptive neurons, bupivacaine directly or indirectly activates calcium-permeable channels. The sensitivity to RR is consistent with TRP channel mediation but is not diagnostic as RR is a non-selective blocker of other cation channels as well.

Bupivacaine activates heterologously expressed TRPA1

To investigate what channels can be activated by bupivacaine, we expressed a number of nociceptor-related calcium-permeable channels (P2X3, TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1) heterologously in N1E-115 cells and tested these for activation by bupivacaine (3 mM) with calcium imaging. We found that bupivacaine (3 mM) induced a strong response in TRPA1-expressing cells, but no response in cells expresssing P2X3, TRPV1, TRPV2, TRPV3 or TRPM8 channels or in control cells (Figure 2A and B). In every case, the standard agonist for each channel type produced a robust calcium response.

The selectivity of bupivacaine for TRPA1 over TRPV1 in Figure 2A and B differs from the previously reported action of lidocaine, which effectively activates both TRPV1 and TRPA1 (Leffler *et al.*, 2008; 2011). The calcium-imaging experiments with bupivacaine were carried out at room temperature, and given the temperature sensitivity of TRPV1, we considered the possibility that TRPV1 might be activated by bupivacaine at body temperature. We therefore tested the action of bupivacaine on both TRPV1 and TRPA1 at 37°C, making voltage clamp recordings from TRPV1 or TRPA1 channels heterologously expressed in HEK-293 cells. Bupivacaine effectively activated TRPA1 current (Figure 2C) but the concentration-dependence of bupivacaine activation varied from cell to cell. In most cells, currents were activated by concentrations as low as 0.1–0.3 mM bupivacaine, with maximal responses produced by 1–3 mM (Figure 2C and E). Typically, after maximal responses, further applications of either bupivacaine or AITC had no effect, as if the channel had inactivated or desensitized. Maximal activation followed by inactivation was seen in some cells even with 0.1 mM bupivacaine (Figure 2C, right panel). The inactivating behaviour is typical for TRPA1 activation in Ca-containing solutions and is likely to be due to a process of inactivation triggered by entry of calcium (Zurborg *et al.*, 2007; Wang *et al.*, 2008). In contrast to the results with TRPA1, application of bupivacaine produced very little activation of TRPV1 channels studied at 37°C (Figure 2D and E). Very small currents were sometimes evident with 1–3 mM bupivacaine, but these were always very small, with current activated by 3 mM bupivacaine averaging only 4 ± 2% of the current that could be activated by 1 μM capsaicin subsequently applied in the same cell (Figure 2E). These results show that bupivacaine is far more effective in activating TRPA1 channels compared with TRPV1 channels, even at physiological temperatures.

QX-314 can enter cells via TRPA1 channels

These results showed that bupivacaine activated TRPA1 channels. We next investigated whether QX-314 can permeate through TRPA1 channels. In experiments performed using TRPA1-expressing N1E-115 cells with an external solution in which QX-314 was the only extracellular cation, we found that AITC activated substantial inward currents (Figure 3A). Inward currents activated by AITC with QX-314 as the only external cation were seen in each of nine cells tested. In collected results, the reversal potential for QX-314-mediated current relative to an internal solution containing 135 mM NMDG was −61 ± 3 mV (n = 9), consistent with a relative permeability of $P_{QX\text{-}314}/P_{NMDG}$ = 1.2. Thus, QX-314 has a comparable permeability in TRPA1 channels as NMDG, one of several large cations previously shown to permeate through TRPA1 channels (Karashima *et al.*, 2010).

Next, we examined cellular drug uptake by measuring cellular QX-314 concentration with quantitative MS. Previously, we demonstrated that QX-314 can enter CHO cells selectively via TRPV1 channels when applied extracellularly at a concentration between 100 μM and 10 mM (Brenneis *et al.*, 2013). To detect uptake via TRPA1, we now incubated HEK-293 cells stably transfected with a plasmid encoding for doxycycline-inducible hTRPA1 with 0.5 mM QX-314 and determined QX-314 concentrations by LC-MS/MS. We found a significant increase of intracellular QX-314 concentration when AITC (100 μM) was added to TRPA1-expressing cells, but not when TRPA1 was expressed but not activated, or when AITC was added to cells with no TRPA1 expression (Figure 3B).

Duration of nociceptive and motor block after perineural injection of QX-314 with procaine or bupivacaine

Previously, we demonstrated that after injection of 0.5 % QX-314 into the sciatic notch adjacent to the sciatic nerve, no behavioural deficits could be observed. However, when 0.5% QX-314 was combined with 2% lidocaine, it prolonged the nociceptive blockade produced by lidocaine alone, with a much shorter effect on motor function (Binshtok *et al.*, 2009; Roberson *et al.*, 2011). We now investigated whether bupivacaine, which is a more potent activator of nociceptive neurons than lidocaine, also produced a pain-preferential blockade when injected in combination with QX-314. In addition, we used procaine as a control as this compound blocks sodium channels but had no activating effect on DRG neurons or heterologously expressed TRP channels. To test the sensory selectivity of the nerve block, we analysed thermal nociceptive responsiveness by measuring the paw withdrawal latency induced by radiant heat stimulation, mechanical nociceptive threshold by evaluating the force needed to induce paw withdrawal after pinching with a calibrated forceps, and motor function by scoring the toe

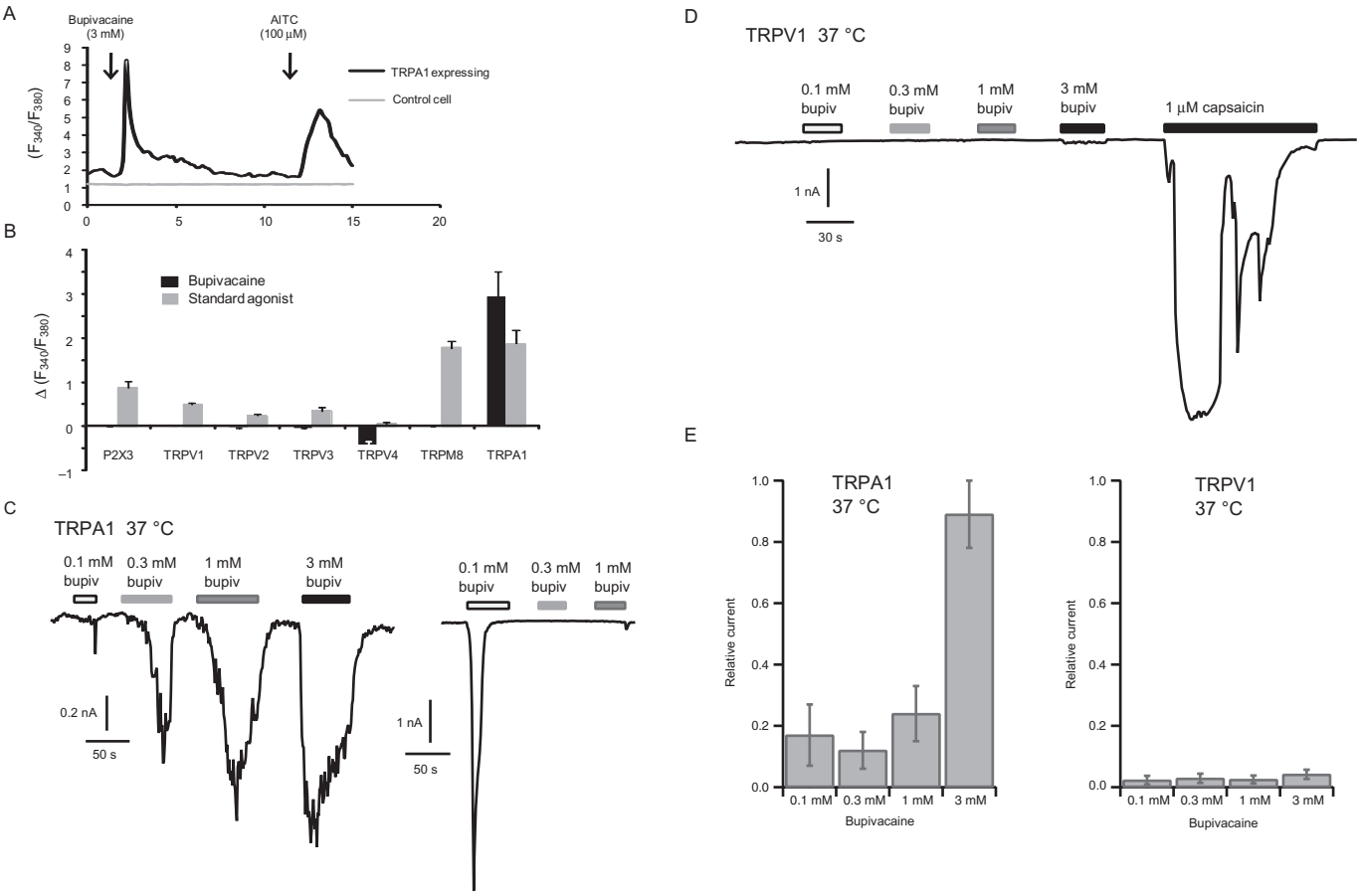

Figure 2

Bupivacaine activates heterologously expressed TRPA1 but not other relevant large-pore cation channels. (A) Representative recordings of calcium concentrations in N1E-115 neuroblastoma cells expressing mTRPA1 after stimulation with bupivacaine (3 mM, 10 s) and AITC (100 µM, 10 s). (B) Statistical analysis of $\Delta[F_{340}/F_{380}]$ at peak amplitudes in N1E-115 cells expressing mP2X3, mTRPV1, rTRPV2, mTRPV3, hTRPV4, mTRPM8 or mTRPA1 after stimulation with bupivacaine (3 mM) and the standard agonists α,β-methyl-ATP (300 µM), capsaicin (1 µM), 2-APB (100 µM), 2-APB (100 µM), 4αPDD (5 µM), menthol (200 µM) and AITC (100 µM) respectively. Shown is the mean of 14–27 cells ± SEM for each channel. (C) Representative current trace of recordings from two TRPA1-expressing HEK 293 cells during stimulation with increasing concentrations of bupivacaine. (D) Representative current trace of recording from a TRPV1-expressing HEK 293 cell during stimulation with increasing concentrations of bupivacaine (followed by capsaicin to verify expression of TRPV1). (E) Collected results (mean ± SEM) for bupivacaine-evoked currents from TRPA1- or TRPV1-expressing HEK 293 cells successively exposed to 0.1, 0.3, 1 and 3 mM bupivacaine, followed by either 100 µM AITC (for TRPA1, $n = 10$) or 1 µM capsaicin (for TRPV1, $n = 3$). Currents were normalized to the current evoked by 100 µM AITC or 1 µM capsaicin (except for TRPA1 cells in which bupivacaine produced a larger response than subsequent AITC as a result of TRPA1 inactivation, in which case normalization was to the largest current evoked by bupivacaine).

spreading reflex. We used 2% procaine or 0.5% bupivacaine combined with 0.5% QX-314.

As expected, procaine and bupivacaine alone both induced a short-lasting non-selective block of thermal and mechanical nociception and motor function (Figures 4A–F and 5A–D). A combination of QX-314 with bupivacaine very substantially prolonged the block of heat and mechanical nociception, and also, although to a much lesser extent, motor function (Figures 4D–F and 5D). Motor function deficits persisted for 6 h after the bupivacaine/QX-314 combination, whereas analgesia to noxious thermal and mechanical stimulation both persisted for longer than 12 h. In contrast, the effect of a procaine-QX-314 combination was much shorter and completely non-selective for motor and sensory functions (Figures 4A–C and 5C). In previous experiments, we found that RR applied perineurally at 1 mM did not modify nociception on its own but abolished the capsaicin-plus-QX-314-induced analgesia after co-injection, consistent with blocking TRPV1-mediated QX-314 uptake (Brenneis *et al.*, 2013). In contrast, however, the analgesia produced by bupivacaine/QX-314 co-application was not abolished by RR, with only a partial reduction in the mechanical and motor block (Supporting Information Figure S1). These data show that QX-314 prolongs bupivacaine-induced nerve block preferentially in sensory fibres but suggest that the mechanism is not confined to TRPA1/V1 activation. The increased duration of motor function block relative to bupivacaine alone also suggests an effect of the QX-314/bupivacaine combination not dependent on TRPA1 or TRPV1 channels, which are not expressed on motor axons. To investigate whether such a

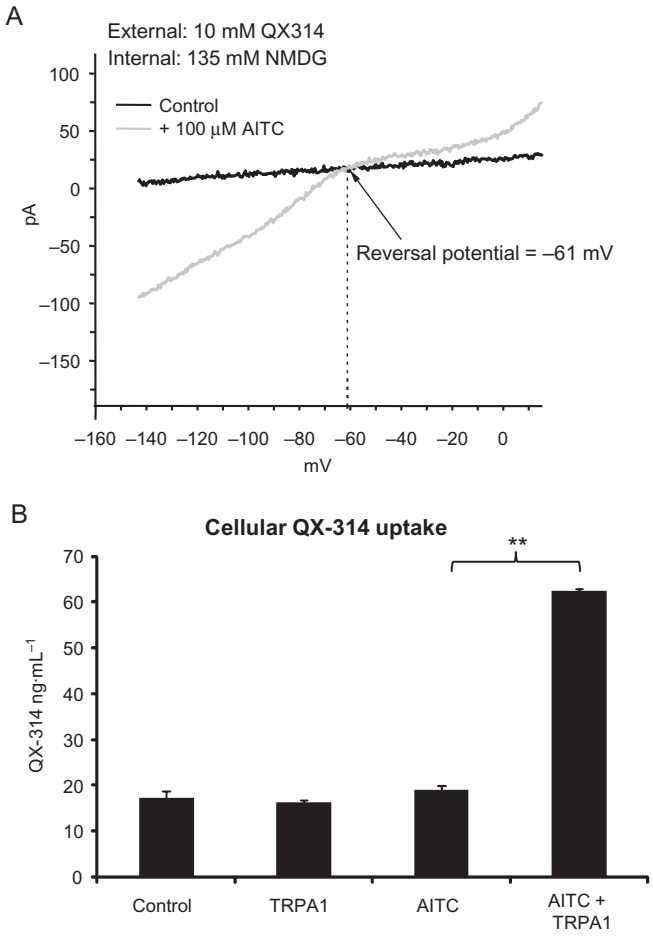

Figure 3

QX-314 can permeate into cells via TRPA1 channels. (A) QX-314 carries ionic current through TRPA1 channels heterologously expressed in N1E-115 cells. Current–voltage relation for current evoked by 100 μM AITC was determined using a 30 ms voltage ramp from +116 to –144 mV before and after application of 100 μM AITC with an external solution containing QX-314 as the sole cation (10 mM QX-314 hydroxide, 277 mM sucrose and 5 mM HEPES, pH adjusted to 7.4 with HCl). Internal solution was 135 mM N-methyl-D-glucamine, 5 mM EGTA and 10 mM HEPES, pH adjusted to 7.2 with HCl. Currents were averaged from 10 sweeps in control and then ~4 min after application of 100 μM AITC to activate TRPA1 current. (B) Cellular uptake of QX-314 into hTRPA1 inducible HEK-293t cells. Twenty-four hours after induction of TRPA1 expression with doxycycline, HEK-293t cells were incubated with QX-314 (0.5 mM) and AITC (100 μM) for 10 min. After washout, cellular QX-314 was determined from cytosolic fractions by LC-MS/MS analysis. Note that cells that are only induced for TRPA1 expression but received vehicle (0.1% DMSO) or cells which lack TRPA1 but are AITC treated do not show increased cellular QX-314 levels. Shown is the mean of 3 (for control) or 5 incubations (for AITC and/or TRPA1) ± SEM. One-way ANOVA and Bonferroni post-test compared with control: $^{**}P < 0.01$.

long sodium channel block by the bupivacaine and QX-314 combination may induce any cellular toxicity, we accessed apoptosis, necrosis and viability of DRG neurons or glia. However, incubation with 0.5 mM QX-314 and 1 mM bupivacaine (Supporting Information Figure S2) for 30 min did not increase water soluble tetrazolium or propidium iodide staining, whereas actinomycin D or H_2O_2 was highly effective.

To investigate the effects of bupivacaine with QX-314 in a more clinically relevant pain model, we analysed motor and sensory function in a rat model of post-surgical pain (Brennan *et al.*, 1996). Twenty-four hours after incision and closure of the lateral plantar surface of the rat left hindpaw, rats demonstrated significant mechanical hypersensitivity (Figure 6A–C). Co-injection of 0.5% QX-314, together with 0.5% bupivacaine, close to the left sciatic nerve inhibited the responses to noxious thermal stimuli for 9 h ($P < 0.05$; Figure 6C) and inhibited mechanical sensitivity for 12 h ($P < 0.05$; Figure 6B). This combination also generated motor functional motor deficits that were detectable for 6 h ($P < 0.01$; Figure 6A), resulting in 3 h of differential thermal block and a 6 h differential blockade of mechanical pain. Perisciatic injection of bupivacaine (0.5%) alone produced a non-selective motor and sensory nerve block that persisted for 2 h (Figure 6A–C).

Combined and selective effects of bupivacaine and QX-314 on CAPs of peripheral nerves ex vivo

The effect of a combination of bupivacaine with QX-314 on nerve conduction was examined by recording CAP responses from isolated mouse sciatic nerves. This approach relies on the functional expression of TRPA1 (and TRPV1) along peptidergic fibres in peripheral nerves (Brenneis *et al.*, 2011; Weller *et al.*, 2011). Applying bupivacaine (1 mM) to the nerve for 5 min immediately produced a robust decrease in the amplitude as well as an increase in the latency of the C- and A-fibre CAPs, which finally resulted in a complete conduction block within a few minutes. In A-fibres, the blocking effect was slightly slower to develop, and in several nerves, only an incomplete block was achieved. A representative example of C- and A-fibre CAP responses from one nerve is shown in Figure 7A. Apart from typical run-down of the amplitudes over time, more in A- than in C-fibres, the nerves recovered well from the bupivacaine block, with time constants $\tau = 18.8 \pm 5.5$ min for A- and $\tau = 31.9 \pm 5.0$ min for C-fibres. Subsequently, QX-314 was applied together with bupivacaine for 5 min, at a concentration (300 μM) that was without any effect when administered alone (data not shown). The nerve block produced by the combination of bupivacaine with QX-314 exhibited a slower recovery of both amplitude and latency of the CAP in C-fibres, with the time constant of recovery of CAP amplitude longer by 27.5% ($\tau = 40.7 \pm 5.0$ min, $P < 0.05$) and the time constant of CAP latency increased by 53.4% ($\tau = 27.3 \pm 7.6$ min, $P < 0.05$), compared with bupivacaine alone (Figure 7B, top). However, in A-fibres, there was no significant change in time constants of either amplitude or latency when QX-314 was co-applied with bupivacaine (Figure 7B, bottom). In addition to the slower time constant for recovery of CAP amplitude and latency, the duration of complete C-fibre block was increased by the combination of bupivacaine and QX-314. In contrast, CAPs of the A-fibres of the same nerve axon showed exactly the same block and recovery with the combination as with bupivacaine alone.

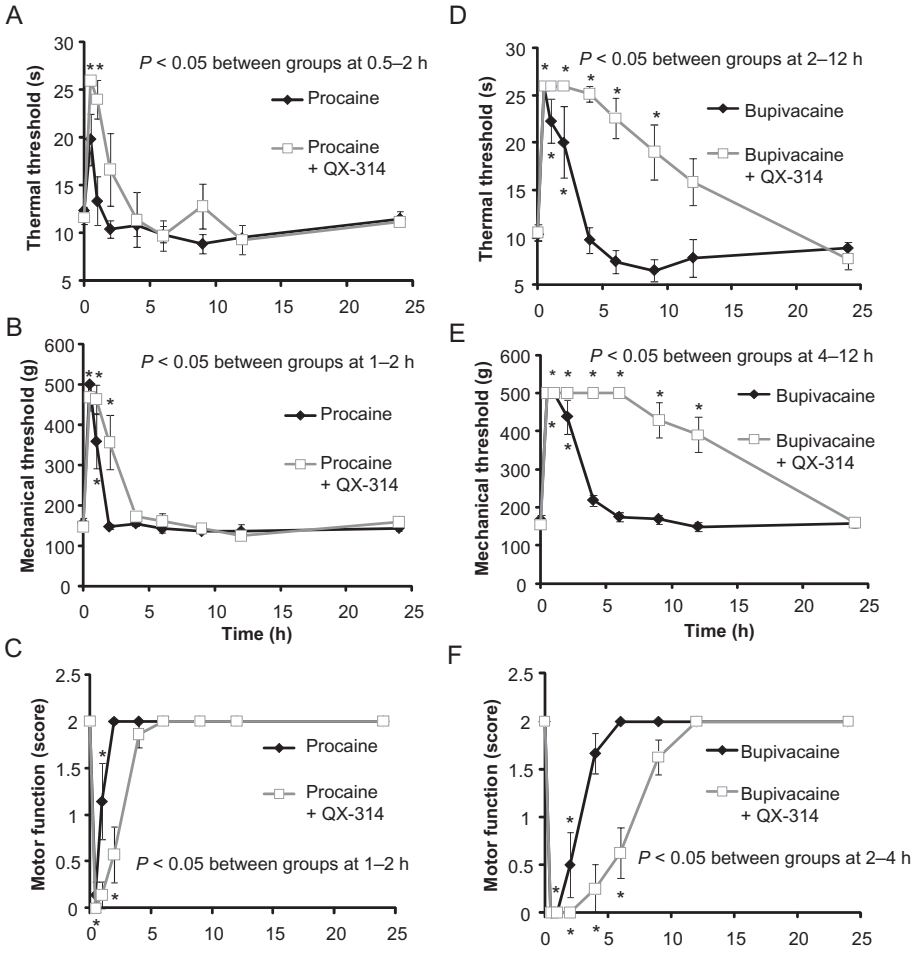

Figure 4

Nociceptive and motor function block after perineural injections of QX-314 and procaine or bupivacaine in naïve rats. (A–C) Local anaesthesia induced by procaine in combination with QX-314. Procaine (2%) was injected alone or in combination with QX-314 (0.5%) into the sciatic notch of adult rats and paw withdrawal latencies after radiant heat stimulation (A), mechanical thresholds after stimulation with a pincher (B) or toe spread reflexes ($n = 7$) (C), were determined at the indicated time points. (D–F) Anaesthesia induced by bupivacaine in combination with QX-314. Bupivacaine (0.5%) was injected alone or in combination with QX-314 (0.5%) into the sciatic notch and behaviour determined as in (A)–(C). Data represent the mean ± SEM ($n = 6$ rats for bupivacaine and $n = 8$ for bupivacaine and QX-314) per group. Significance was calculated by the two-way repeated measures ANOVA and Bonferroni post-test. *$P < 0.05$ compared with baseline.

To test if TRPA1 was the portal for entry of QX-314 into C-fibres, we repeated the same experiments using both TRPA1$^{-/-}$ mice. To our surprise, the selective prolongation of the C-fibre nerve block by the combination with QX-314 was the same in TRPA1$^{-/-}$ mice as in wild-type mice. To take account of the ability of bupivacaine to produce some (although small) activation of TRPV1, we also tested TRPV1/A1$^{-/-}$ double KO mice. We found that the selective prolongation of C-fibre block was still present in these mice (Figure 7C). To test whether compensatory gene regulation accounts for this surprising observation in KO mice, we did experiments in which TRPA1 and TRPV1 were instead blocked pharmacologically by RR. However, 10 μM RR, which was sufficient to abolish the bupivacaine-induced calcium flux in DRG neurons (Figure 1A and B), did not prevent the prolonged C-fibre CAP block by bupivacaine and QX-314 (Supporting Information Figure S3).

Thus, on peripheral nerves, QX-314 co-applied with bupivacaine has a selective action on C-fibres over A-fibres, but this effect does not depend on entry through TRPA1 or TRPV1. Bupivacaine must have some other action enabling QX-314 to exert a local anaesthetic action selectively on peripheral C-nerve fibres.

High concentrations of bupivacaine and lidocaine promote cellular QX-314 uptake independent of TRPV1 or TRPA1 activation

To investigate why QX-314, in combination with bupivacaine, produces a block of sensory and motor fibres independent of TRPV1 and TRPA1, we examined cellular QX-314 accumulation after stimulation of N1E-115 cells with different concentrations of bupivacaine and lidocaine.

We found that high doses of bupivacaine or lidocaine (3 or 10 mM, respectively) induced QX-314 accumulation in

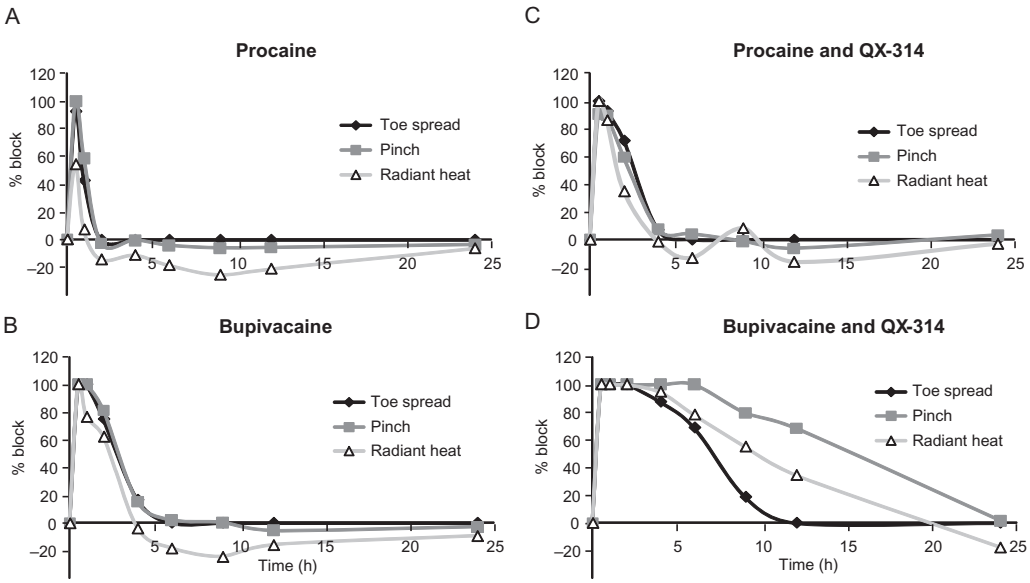

Figure 5

Differential nerve block after perineural injections of QX-314 and procaine, or bupivacaine. To illustrate the relative duration of nociceptive and motor block, the percentage of the initial anaesthesia at the different time points was calculated from data shown in Figure 4.

untransfected N1E-115 cells (Figure 8A) even though they produced no calcium flux in these cells. Lower doses, 3 mM lidocaine and 1 mM bupivacaine, which induce a robust calcium flux in DRG neurons (Figure 1E and F) and activate TRPA1 and TRPV1, did not affect QX-314 uptake in the untransfected N1E-115 cells. To investigate the efflux kinetics of the non-specific accumulation in N1E115 cells, we determined QX-314 concentrations in the cell lysate and culture medium after loading (5 min) with 0.5 mM QX-314 and 3 mM bupivacaine followed by incubation in fresh medium. The time course showed that after 0.5 h, most (86%) of the cellular bupivacaine has been released into the medium (Supporting Information Figure S4). We conclude that high concentrations of these two local anaesthetics (still in the clinical range used for local injection) can produce a transient QX-314 accumulation in cells independently of activating TRP channels or other calcium-permeable channels.

Next, we investigated whether the bupivacaine-induced cellular QX-314 uptake that is non-TRP mediated can also occur in other cell types. In both RAW-264.7 macrophages and CHO cells, we found a similar increase of cellular QX-314 uptake after adding a high concentration (3 mM) of bupivacaine (Figure 8B).

To analyse whether TRPA1 expression can elevate the already increased cellular QX-314 concentrations after 3 mM bupivacaine, we compared the QX-314 accumulation in control and TRPA1-expressing HEK-293t cells. In this same cell system, we demonstrated a TRPA1 selective uptake after stimulation with AITC (Figure 3B). However, the bupivacaine-stimulated QX-314 accumulation in the TRPA1-expressing HEK-293t cells was not statistically different from the increase in control cells (Figure 8C).

Finally, we tested if bupivacaine could also induce a non-TRP-mediated uptake of QX-314 in DRG neurons. Once again, 3 mM bupivacaine significantly increased cellular QX-314 uptake (Figure 8D), and this uptake was not prevented by pre-incubation with RR. We conclude that high concentrations of bupivacaine (3 mM) and lidocaine (10 mM) promote QX-314 uptake in both neuronal and non-neuronal cells by a mechanism independent of TRP channels (or other calcium-permeable channels).

Discussion and conclusion

Regional anaesthesia is often short in duration, and its non-selectivity leads to numbness and motor deficits that interfere with recovery. Previously, we found that when lidocaine was combined with QX-314, the resulting nerve block was much longer lasting than with lidocaine alone, and exhibited, moreover, a preference for pain, which we interpreted as being due to the selective QX-314 uptake into nociceptors (Binshtok *et al.*, 2009) resulting from lidocaine's activation of the large-pore TRPV1 and TRPA1 channels (Leffler *et al.*, 2008; 2011).

In a search for a drug combination with QX-314 that produces an even longer lasting differential local anaesthesia than lidocaine and QX-314, we screened clinically used local anaesthetics for their capacity to activate TRP channels in nociceptors and thus produce selective QX-314 uptake into these cells. We identified bupivacaine as the most potent inducer of an RR-sensitive calcium flux in nociceptor neurons, suggestive of activation of large-pore cation channels. Activation of nociceptors by local anaesthetics at the tested concentrations (up to 4 mM) is not universal as some, such as procaine, did not generate calcium fluxes in DRG neurons. By testing activation of heterologously expressed TRP channels, bupivacaine was found to activate TRPA1 channels, which are selectively expressed in a subset of C-fibre nociceptors (Jordt *et al.*, 2004). Consistent with activation of TRPA1 channels, only ~75% of neurons that

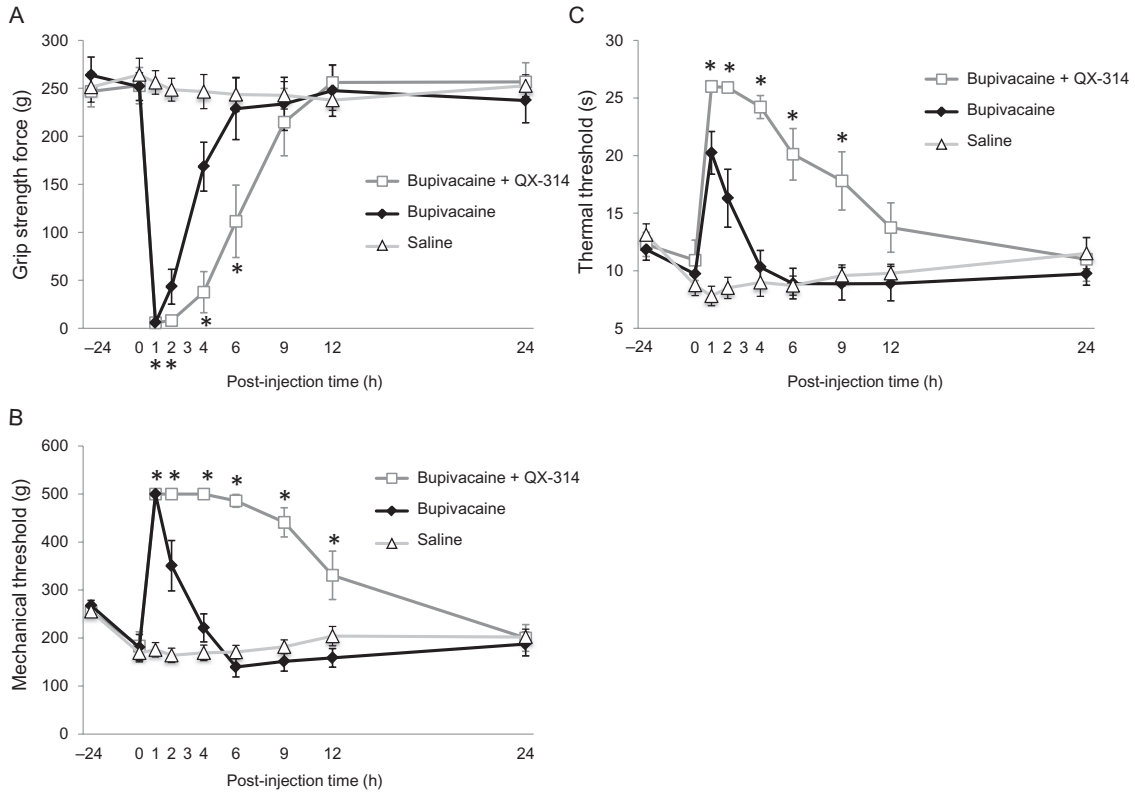

Figure 6

Nociceptive and motor function after plantar incision and subsequent differential nerve block by perineural injections of QX-314 with bupivacaine 24 h after lateral plantar incision, motor and sensory function was assayed by testing grip strength (A), pinch tolerance threshold (B) and thermal response latency ('Hargreaves' test', C). Thereafter, rats were injected nearby the left sciatic nerve with 200 μL of vehicle (0.9% saline), bupivacaine (0.5%) or a combination of bupivacaine (0.5%) with QX-314 (0.5%). Motor and sensory measures were again assayed at 1, 2, 4, 6, 9, 12 and 24 h after perineural injections. All injections administered under isoflurane-induced general anaesthesia at time 0. Significance for the QX-314 + bupivacaine group was calculated by the two-way repeated measures ANOVA and Bonferroni post-test. *$P < 0.05$ compared with post-injury baseline; $n = 8$ rats for all groups.

responded to bupivacaine also responded to capsaicin. This is unlike lidocaine, which activates both TRPV1 and TRPA1 channels equally (Leffler *et al.*, 2008; 2011).

Capsaicin stimulation of TRPV1 channels allows permeation of large cationic molecules, including NMDG, FM1-43 Yo-Pro and QX-314 (Meyers *et al.*, 2003; Hellwig *et al.*, 2004; Chung *et al.*, 2008; Banke *et al.*, 2010; Puopolo *et al.*, 2013). AITC-induced activation of TRPA1 channels also allows Yo-Pro uptake (Chen *et al.*, 2009; Banke *et al.*, 2010), and our data show that QX-314 also permeates rapidly through TRPA1 channels, carrying sizable macroscopic currents. Accordingly, we found, by MS, that activation of TRPA1 channels by AITC produces QX-314 accumulation intracellularly, with a three-fold increase in intracellular QX-314 levels compared with controls with TRPA1 activation. Although this increase is significant, it is very low compared with the 52-fold cellular increase in QX-314 levels observed on capsaicin stimulation of TRPV1 channels (Brenneis *et al.*, 2013 and data not shown). One factor may be the fast inactivation of TRPA1 channels by calcium, which may limit the time these channels stay open, preventing sustained QX-314 permeation (Zurborg *et al.*, 2007; Wang *et al.*, 2008; Banke *et al.*, 2010).

Perineural co-injection of bupivacaine with QX-314 produced a long-lasting behavioural blockade *in vivo*, with a preference for sensory nerves at later times. However, the TRP channel blocker RR did not fully prevent the effects of QX-314 on bupivacaine actions. Therefore, in addition to potential TRPA1-mediated entry of QX-314 into neurons, further mechanisms may be participating on these effects. As TRPA1 channels are expressed by C-fibres on membranes along nerve axons (Jordt *et al.*, 2004; Brenneis *et al.*, 2011; Weller *et al.*, 2011), we examined if bupivacaine and QX-314 exhibited a combined effect on sodium channel block and action potential propagation in sensory nerve fibres and if this was TRPA1 channel-dependent. Bupivacaine and QX-314 prolonged the local anaesthetic effect on C-fibres CAPs by ~30% compared with bupivacaine alone, but not on A-fibres. Surprisingly, however, the prolonged combined effects were no different in mice lacking TRPA1 and TRPV1 channels or after blockade of the channels by RR. Collectively, the data indicate that while bupivacaine does activate TRPA1 channels, this alone cannot be responsible for the prolongation of local anaesthesia produced by combination of QX-314 and bupivacaine. Again, a possible factor reducing QX-314 entry

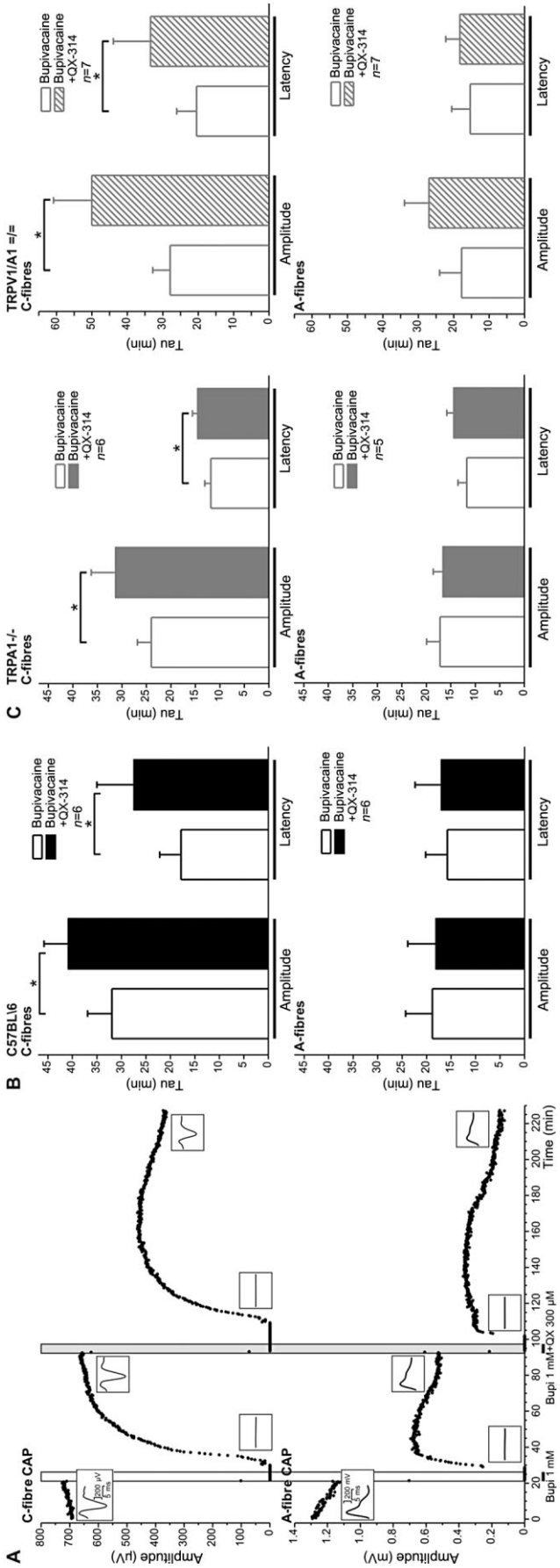

Figure 7

Prolonged and selective effects of the combination of bupivacaine and QX-314 on C-fibre CAPs in C57BL\6, TRPA1$^{-/-}$ and TRPV1/A1$^{=/=}$ mice. Isolated mouse sciatic nerves were superfused with bupivacaine (1 mM) and its combination with QX-314 (300 μM), and CAPs were recorded upon electrical stimulation. (A) Representative traces of CAP amplitudes of C- (upper) and A-fibres (lower) after application of bupivacaine and the combination with QX-314; insets show CAPs registered at critical time points. (B) Time constants (τ) of recovery from nerve block during wash-out of drugs in C57BL\6, with amplitudes and latencies of CAPs separately evaluated ($n = 6$). (C) Same as in (B) but nerves from TRPA1$^{-/-}$ (left panels, $n = 6/5$) and TRPV1/A1$^{=/=}$ mice (right panels, $n = 7$). Data represent means ± SEM; *$P < 0.05$ calculated by Wilcoxon matched pairs test for intra-individual comparisons.

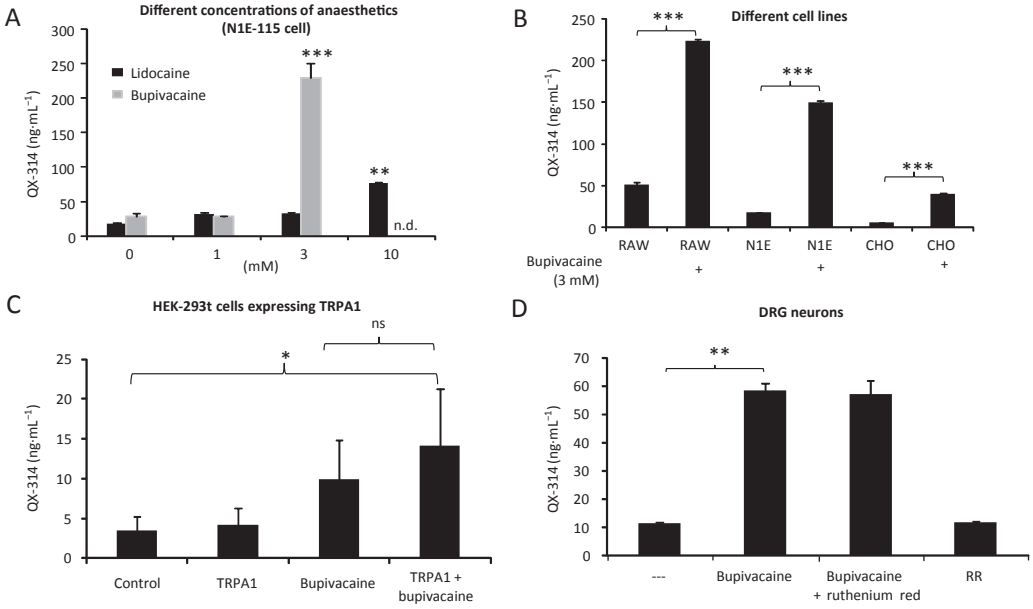

Figure 8

Higher concentrations of lidocaine and bupivacaine induce QX-314 uptake into cell lines and DRG neurons independently of calcium-permeable channels. Cells were incubated with 0.5 mM QX-314 and different local anaesthetics for 10 min. After washout, QX-314 concentrations were determined in cell lysates by LC-MS/MS analysis. (A) QX-314 uptake in N1E-115 cells induced by different concentrations of lidocaine and bupivacaine. (B) 3 mM bupivacaine increases QX-314 concentrations in different cell lines. RAW macrophages, N1E-115 neuroblastoma cells and CHO cells were incubated as in (A). (C) Bupivacaine-induced cellular uptake of QX-314 does not increase significantly after TRPA1 expression. Twenty-four hours after induction of hTRPA1 expression with doxycycline, HEK-293t cells were incubated as in (A). (D) Bupivacaine increases QX-314 concentrations in DRG neurons. Primary DRG cultures from adult rats were incubated with QX-314 (0.5 mM) and bupivacaine (3 mM) ± ruthenium red (20 μM) as in (A). Data from all panels represent the mean conc. ± SEM in lysates of 3–5 culture dishes. One-way ANOVA and Bonferroni post-test compared with control. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

through TRPA1 channels is the rapid inactivation or desensitization of these channels, which was also evident for activation by bupivacaine (Figure 2C). Apparently, bupivacaine can engage a TRP-independent uptake mechanism to get QX-314 into axons. This mechanism was not functional for all local anaesthetics as procaine, which does not activate TRP channels, also failed to increase the duration of block when co-administered with QX-314. Furthermore, this mechanism seems more prominent on C than A fibres because the combination only had an action on C-fibre CAPs in isolated nerves and the prolongation of motor block was less than that of sensory block.

Relatively high concentrations of bupivacaine and lidocaine (3 and 10 mM) (within clinical range but higher than EC_{50} for TRP channel activation) caused QX-314 accumulation in cells that did not express TRPV1 or TRPA1 channels, including non-neuronal cells. In DRG neurons, QX-314 uptake in response to these high concentrations of bupivacaine could not be blocked by the TRP-channel pore blocker RR. What mechanism of uptake may be involved? Lidocaine decreases lipid molecule density in membranes and lowers membrane thickness in model membranes (Yi *et al.*, 2011). Most local anaesthetics can affect the physical structure of membrane bilayers by interaction with cholesterol (Koo *et al.*, 2008; Mizogami *et al.*, 2008) and interestingly even QX-314 fluidizes phosphatidylserine-containing nerve cell model membranes (Tsuchiya and Mizogami, 2008). Further experiments are needed to clarify what mechanisms underlie the non-specific uptake of QX-314 induced by high concentrations of bupivacaine.

Increasingly, bupivacaine alone is applied in surgical wounds to prolong post-surgery analgesia (Gorfine *et al.*, 2011). Our data show that co-applying QX-314 with bupivacaine greatly prolongs local anaesthesia in naïve rats and during post-surgical pain after plantar incision compared with bupivacaine alone, with preferential prolongation of sensory versus motor block at late time points. The prolongation of nerve block apparently reflects bupivacaine-induced entry of QX-314 into nerve fibres through pathways not confined to entry through TRP channels. Whatever the mechanism of QX-314 entry is, the long-lasting nerve block produced by the combination of QX-314 and bupivacaine could be therapeutically useful, particularly in clinical situations, such as surgery, where initial immobilization is acceptable.

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Conflict of interest

Endo Pharmaceuticals have licensed technology for silencing fibres from Harvard University invented by BB and CJW.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Nociceptive and motor function block after perineural injections of QX-314, bupivacaine and ruthenium red (RR). (A–C) Anaesthesia was induced by sciatic injections of bupivacaine (0.5%) in combination with QX-314 (0.5%) and RR (1 mM). Toe spread reflexes (A), paw withdrawal latencies after radiant heat stimulation (B) or mechanical thresholds after stimulation with a pincher (C) were determined at the indicated time points. (D) To illustrate the relative duration of nociceptive and motor blockade, the percentage of the initial complete blockade at the different time points was calculated from data shown in (A)–(C). Data represent the average ± SEM form 6 rats per group. Significance was calculated by the two-way repeated measures ANOVA and Bonferroni correction. $*P < 0.05$ compared with baseline.

Figure S2 Viability of rat DRG cultures after incubation with QX-314 and bupivacaine. Rat DRG cultures were stimulated with vehicle (H_2O), 0.5 mM QX-314, 0.5 mM QX-314 with 1 mM bupivacaine, actinomycin D [1 $\mu g \cdot mL^{-1}$ (A)] or H_2O_2 [300 μM (B)] for 30 min. (A) Viability was assessed using a water soluble tetrazolium-assay 0–72 h post-stimulation. (B) Propidium iodide staining of DRG cultures 0–48 h post-stimulation. One-way ANOVA with Bonferroni post-correction: $*P < 0.05$, $**P < 0.01$.

Figure S3 Prolonged effects of the combination of bupivacaine and QX-314 on C-fibre CAPs are not affected by co-application of ruthenium red (RR). Isolated mouse sciatic nerves were superfused with bupivacaine (1 mM) and QX-314 (300 μM) and their combination with RR (10 μM) and CAPs were recorded upon electrical stimulation. (A) Representative traces of CAP amplitudes of C- (upper) and A-fibres (lower) after application of bupivacaine and QX-314 without or with RR. (B) Time constants (τ) of recovery from nerve block during wash-out of drugs, with amplitudes (left) and latencies (right) of C- and A-fibre CAPs separately evaluated ($n = 3$ and 2 respectively).

Figure S4 Time course of QX-314 release from bupivacaine activated cells. (A) N1E-115 cells were incubated with QX-314 (0.5 mM) and bupivacaine (3 mM) for 10 min. After washing and incubation in fresh medium for the indicated durations, cells were lysed and QX-314 conc. determined. (B) Same conditions as in (A) but QX-314 concentrations were analysed in culture supernatant. Data represent the average conc. and SEM from three culture dishes.