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Lysophosphatidic acid activates nociceptors and causes pain or itch depending on the application mode in human skin

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Abstract

Lysophosphatidic acid (LPA) is involved in the pathophysiology of cholestatic pruritus and neuropathic pain. Slowly conducting peripheral afferent C-nerve fibers are crucial in the sensations of itch and pain. In animal studies, specialized neurons ("pruriceptors") have been described, expressing specific receptors, eg, from the Mas-related G-protein-coupled receptor family. Human nerve fibers involved in pain signaling ("nociceptors") can elicit itch if activated by focalized stimuli such as cowhage spicules. In this study, we scrutinized the effects of LPA in humans by 2 different application modes on the level of psychophysics and single nerve fiber recordings (microneurography). In healthy human subjects, intracutaneous LPA microinjections elicited burning pain, whereas LPA application through inactivated cowhage spicules evoked a moderate itch sensation. Lysophosphatidic acid microinjections induced heat hyperalgesia and hypersensitivity to higher electrical stimulus frequencies. Pharmacological blockade of transient receptor potential channel A1 or transient receptor potential channel vanilloid 1 reduced heat hyperalgesia, but not acute chemical pain. Microneurography revealed an application mode–dependent differential activation of mechanosensitive (CM) and mechanoinsensitive C (CMi) fibers. Lysophosphatidic acid microinjections activated a greater proportion of CMi fibers and more strongly than CM fibers; spicule application of LPA activated CM and CMi fibers to a similar extent but excited CM fibers more and CMi fibers less intensely than microinjections. In conclusion, we show for the first time in humans that LPA can cause pain as well as itch dependent on the mode of application and activates afferent human C fibers. Itch may arise from focal activation of few nerve fibers with distinct spatial contrast to unexcited surrounding afferents and a specific combination of activated fiber subclasses might contribute.

Keywords: Lysophosphatidic acid, C- fibers, Microneurography, Psychophysics, TRPV1, TRPA1, Itch, Neuropathic pain, Cholestatic pruritus

1. Introduction

Increasing evidence suggests that lysophosphatidic acid (LPA) is involved in the pathophysiology of cholestatic pruritus and neuropathic pain.

Lysophosphatidic acid is a small bioactive glycerophospholipid, almost ubiquitously present in human organs and body fluids. It consists of subspecies differing in localization and composition of the acyl group[31,45] and affects physiological processes such as myelination and neuronal cell metabolism.[45,69]

Lysophosphatidic acid was revealed as a potential pruritogen in sera of patients with cholestatic liver diseases and pruritus.[28–30] The activity of the major LPA-producing enzyme autotaxin (ATX) correlated with itch intensity and response to therapeutic interventions.[30] In rodents, LPA elicited both itch-related[3,14,29] and acute pain-like behaviors.[18,38]

Six G-protein-coupled receptors pre-eminently mediate LPA effects (lysophosphatidic acid receptors 1-6, LPAR1-6).[69,70] Other potential targets of LPA in sensory neurons are the transient receptor potential channel vanilloid 1 (TRPV1),[19,23,38] voltage-gated sodium, voltage-gated calcium, and two-pore-domain potassium channels.[19] However, in calcium-imaging experiments on cultured dorsal root ganglia, LPA activated satellite glia cells, which do not express TRPV1, rather than sensory neurons.[45]

Although the understanding of generation and differentiation of itch and pain has expanded through the use of cell-based and rodent-based models,[48] the mechanisms in humans have not been unraveled. For histaminergic itch, a "labelled line" seems likely because histamine-responsive C fibers were detected with a specific pathway to the thalamus in primates,[6,52] which is sufficient to evoke itch in humans.[47] The peripheral histamine-sensitive neurons belong to the subclass of mechanoinsensitive C (CMi) fibers, which also play a major role in inflammatory and neuropathic pain as well as neurogenic inflammation.[24,35]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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However, chronic pruritus in patients with, eg, cholestatic liver diseases seems histamine independent because histamine levels do not correlate with itch intensity, and treatment with antihistamines is largely ineffective.[9,27,29]

In rodents, Mas-related G-protein-coupled receptors (Mrgprs) were shown to specifically mediate nonhistaminergic itch.[8] In human microneurography, cowhage, a tropical bean whose spicules evoke itch through the protease mucunain and protease-activated receptors,[43,60] activated all mechanosensitive C (CM) fibers strongly, but no CMi fibers.[34]

"Polymodal" CM fibers respond to painful heat stimuli and chemical mediators, considered to induce pain, but not itch.[15,53,54] An unspecific mechanism, according to the "spatial contrast theory," may provide distinction between the sensations of pain and itch: The disparity between strongly activated and surrounding silent C fibers from the same contiguous skin area creates a "spatial contrast" that may centrally be interpreted as itch.[11,36] Together with peripheral and spinal mechanisms of decoding itch, this creates a complex picture, further complicated by species differences.[41,59] The fiber classes of CM and CMi fibers have functionally been characterized in human, monkey, and pig, but CMi fibers are not clearly identified in mouse skin.[16]

Therefore, this study aims at exploring LPA effects on human C nociceptors (microneurography) and at psychophysically assessing the impact of LPA in healthy human subjects to gain insight into its involvement in itch and/or pain induction.

2. Methods

2.1. Subjects

Thirty-five healthy subjects (18 women, 17 men, age: 18-35 years) took part in the psychophysical study and 28 healthy subjects in the microneurography study (17 women, 11 men, age: 19-35 years). Subjects were recruited from the medical faculty of the University of Erlangen-Nürnberg by personal and online advertisement.

A questionnaire concerning health status and regular medication was collected from all participants, of whom none reported neurological, dermatological, or other chronic medical conditions. The subjects did not take any medication before the experiments (except for oral contraceptives). Participants received detailed information about the experimental set-up and proceedings, before handing in a written informed consent form according to the Declaration of Helsinki at least 24 hours before participating in the study. All experiments were conducted at the University of Erlangen-Nürnberg and approved by the local ethics committee (ethics approval number: 328_17B).

2.2. Psychophysical study

2.2.1. Substances and application

For intracutaneous microinjections, LPA 18:1 (oleoyl-LPA, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; Avanti Polar Lipids, Alabaster, AL) was first dissolved in phosphate-buffered saline containing human serum albumin 0.1% and further diluted with sterile synthetic interstitial fluid (SIF)[4] to concentrations of 0.01, 0.05, 0.1, and 0.5 mM. A stock solution of capsaicin (Sigma-Aldrich, Taufkirchen, Germany) dissolved in ethanol was diluted with SIF to a concentration of 0.0001%, corresponding to 3.4 μM.

Synthetic interstitial fluid contained (in mM) 107.8 NaCl, 3.5 KCl, 1.5 CaCl2, 0.7 MgSO4, 26.2 NaHCO3, 1.7 NaH2PO4, 9.6 sodium gluconate, 5.5 glucose, and 7.7 sucrose at a stable pH of 7.4. Sterile SIF solution represented the control to the application of substances through microinjection. Microinjections of 50 μL were performed on the volar forearms of the subjects using a 30-G insulin syringe (0.3 mL, Becton-Dickinson, Le Pont-de-Claix Cedex, France; for schematic depiction refer to **Fig. 1A**). Intracutaneous, just subepidermal, microinjection of 50 μL resulted in an injection bleb approximately 5 mm in diameter. Antagonists of transient receptor potential channel A1 (TRPA1) (A-967079, 10 μM in SIF; Tocris, Wiesbaden-Nordenstadt, Germany) and TRPV1 (BCTC, 1 μM in SIF; Sigma, Taufkirchen, Germany) were similarly applied using microinjection. Dosages of the antagonists were selected according to their previous validation of TRPA1 and TRPV1 agonists in humans.[57]

Cowhage was obtained from Odisha in eastern India. We used active and inactive cowhage spicules for focal applications. We inactivated the active protease mucunain in the spicules by autoclaving at 121°C for 20 minutes.[34] Owing to their hollow interior, heat-inactivated cowhage spicules can be loaded with dissolved molecules of interest.[64] Active and inactive cowhage spicules were applied to the skin using a cotton bud applicator. Under a binocular microscope, spicules were glued with their dull ends to the cotton bud, so 10 to 15 spicules were perpendicularly protruding from the top of the applicator. At the end of the respective experimental protocol, remaining cowhage spicules were removed from the skin using adhesive tapes. For focal application with inactivated cowhage spicules, LPA was diluted to 25 mM in $CHCl_3$ (chloroform) and the heat-inactivated spicules were loaded with this solution; they were then dried in a fume hood to allow evaporation of $CHCl_3$. Pure chloroform-treated inactivated cowhage spicules were used as control.

2.2.2. Pain and itch ratings

We used a numeric rating scale (NRS) from 0 to 10 with verbal statements for pain or itch. Zero was defined as no sensation, 1 as pain or itch threshold, and 10 as maximal imaginable pain or itch. The subjects could also address nonpainful or nonpruritic sensations between NRS 0.1 and 0.9, such as pressure, tingling, prickling, or a warmth feeling. Numeric rating scale 0 was defined as no sensation at all, 0.1 indicating a threshold perception defined as a minimal sensation when concentrating on it, and 0.9 being the strongest but still not clearly painful or pruritic sensation.

Subjects were asked about pain or itch intensity every 5 seconds for 30 seconds and thereafter every 10 seconds for the remaining time of the respective experiment (ranging from 3 to 7 minutes).

2.2.3. Psychophysical proceedings

Three separate experimental parts were performed in the psychophysical study that involved (1) application of LPA using microinjection vs focal application, including dose–response trials and laser Doppler imaging; (2) testing for potential sensitizing effects of LPA microinjections; and (3) modulation of LPA-induced effects by TRPA1 and TRPV1 antagonists (**Fig. 1A**).

The final doses of LPA were determined using results from intradermal injections in mice[29] and pilot psychophysical tests in a small number of subjects.

In the first part, microinjections (50 μL) of LPA in varying concentrations (0.01, 0.05, 0.1, and 0.5 mM), capsaicin (3.4 μM), and SIF for control were performed double blinded on the forearms of the healthy subjects (**Fig. 1A**, n = 20). Then, at another skin site, LPA in heat-inactivated cowhage spicules and empty control

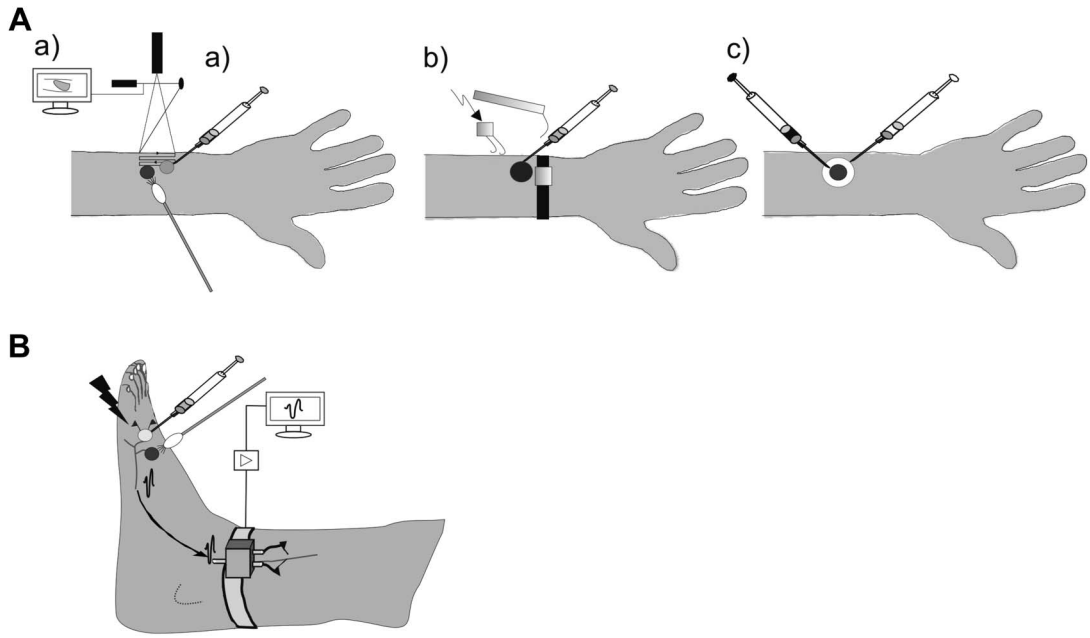

Figure 1. Schemes of the experimental proceedings. (A) Psychophysical testing. (a) Microinjections (50 μL) of LPA in varying concentrations (0.01, 0.05, 0.1, and 0.5 mM), capsaicin (3.4 μM), and SIF (control) were performed double blinded on the forearms of the healthy subjects (light gray circle). LPA (25 mM in $CHCl_3$) and $CHCl_3$ as control were soaked up in heat-inactivated cowhage spicules, evaporated, and then inserted using a cotton bud (dark gray circle). Laser Doppler imaging of superficial skin blood flow was conducted before or after each application. (b) Possible sensitizing effects of LPA microinjections 0.1 mM were evaluated by assessing different pain threshold modalities using a contact thermode (TSA 2001, MEDOC Ltd), pinprick stimulators, and bipolar surface electrodes for electrical stimulation. The different modalities were tried pre-injection or post-injection of LPA 0.1 mM and SIF in the same order of mechanical, thermal, and electrical testing. (c) TRPA1 and TRPV1 receptor involvement in LPA-mediated sensations was tested by pre-injection of 100 μL of TRPA1 (A-967079, 10 μM) and TRPV1 (BCTC, 1 μM) channel blockers (large white circle) before microinjection of 50 μL of LPA 0.1 mM (small black circle). Synthetic interstitial fluid served as control pre-injection. (B) Microneurography recordings. A tungsten recording electrode was inserted in the superficial peroneal nerve at ankle level and a reference electrode in the surrounding skin area. Low-frequency (0.25 Hz) ongoing electrical stimulation was used throughout the whole experiment in the receptive fields of the C nociceptors through 2 thin needles (black triangles on the dorsum of the foot). Recorded action potentials were then amplified, processed, and stored on a computer. Intracutaneous microinjections of 20 μL of LPA (0.05 and 0.1 mM) and SIF as well as application of LPA in inactivated cowhage spicules were performed into the receptive fields of the recorded C fibers (light and dark gray circles) during continuous electrical stimulation with 0.25 Hz. Abrupt increases in latency of the electrically evoked action potentials were considered as an indirect marker of chemical excitation of additional spikes causing activity-dependent slowing (ADS) of conduction velocity of C fibers. LPA, lysophosphatidic acid; TRPA1, transient receptor potential channel A1;TRPV1, transient receptor potential channel vanilloid 1; SIF, synthetic interstitial fluid.

spicules (see above) were applied for focal application; in addition, active cowhage spicules (not autoclaved) were used as positive control. After microinjection of LPA 0.1 mM and focal application of LPA and the respective controls (n = 20), laser Doppler imaging (moorLDI2-VR, 2001; Moor Instruments Ltd, Axminster, Devon, United Kingdom) was performed before and after each application at a distance of 35 cm from the skin site (**Fig. 1A**). The images of the superficial skin blood flow contained 256 × 256 pixel values with a scan resolution of 4 pixel/s. The areas of evoked axon reflex erythema ("flare") were analyzed off-line with MLDI 3.0 software (Moor Instruments, Axminster, United Kingdom).[25]

In another part of the study, potential sensitizing or desensitizing effects of LPA microinjections 0.1 mM were evaluated (n = 14). Heat and cold pain thresholds were assessed by temperature ramps (1°/s, baseline 34°C) applied by a contact thermode (diameter 1.6 cm; Thermal Sensory Analyzer: TSA 2001; MEDOC Ltd, Israel). The mechanical pain threshold was determined with 7 pinprick stimulators with strengths from 8 to 512 mN (MRC Systems GmbH, Heidelberg, Germany). The threshold was then calculated as the geometric mean of 3 descending and ascending courses of stimulation. The electrical pain threshold was assessed by the "method of limits" by the application of square pulses (2 Hz; pulse width 0.5 ms) through a constant current stimulator (Digitimer DS7A; Digitimer Ltd, Welwyn Garden City Hertfordshire, United Kingdom) using surface electrode pins consisting of 2 blunt steel threads (diameter 0.5 mm and distance 2 mm). The different modalities were tried before or after microinjections of LPA 0.1 mM and SIF in the same order of mechanical, thermal, and electrical testing.

In the third part of experimental proceedings (n = 10), TRPA1 and TRPV1 receptor involvement in LPA-evoked sensations was tested by pre-injection of 100 μL TRPA1 (A-967079, 10 μM) and TRPV1 (BCTC, 1 μM) channel blockers (diluted in SIF).[57] Fifty microliters of LPA 0.1 mM were then applied 2 minutes after microinjections of either a blocker or SIF at the same skin site in a random order and double-blind mode. The heat pain threshold was determined before and after both microinjections.

2.3. Microneurography

2.3.1. Microneurography recordings

Microneurography represents a method of extracellular recording of action potentials of single primary afferents in humans and has previously been described in detail[49,52] (**Fig. 1B**). In short, a tungsten recording needle (Frederick Haer Corp, Bowdoinham, ME) was inserted into the superficial peroneal nerve at the level of the ankle. Receptive fields of individual nociceptors were located by transcutaneous electrical stimulation with a pointed electrode (0.5 mm diameter), and C fibers were then identified by their low conduction velocity (<2 m/s). Two thin electrodes (0.15 mm diameter; Austerlitz, Australian Entomological Supplies, Australia)

were inserted in the epidermis of the receptive field of the recorded C fibers, and low-frequency electrical stimulation was maintained throughout the entire experiment (0.25 Hz, 0.5 ms, intensity at least 1.5 times fiber threshold) using a constant current stimulator (Digitimer DS7A; Digitimer Ltd). C-fiber action potentials were distinguished according to their individual conduction latency.

C fibers were then characterized by their responses to different stimuli (mechanical and chemical), identified by the "marking" method.[49] Additional action potentials, eg, evoked by mechanical, electrical, or chemical stimuli, induce an increase in C-fiber conduction latency due to activity-dependent conduction velocity slowing (ADS), which is referred to as "marking," and used to ensure the identity of the spike. The magnitude of latency shift during and after adequate stimulation correlates with the number of additional action potentials occurring in the interval between the electrical stimuli.[49]

The mechanical sensitivity of the recorded fibers was tested by prodding the skin of the receptive fields in an area of about 3 cm around the stimulating needles with a stiff von Frey filament (750 mN, Stoelting, Chicago, IL).

Microneurography data recordings were amplified, processed online, and stored on a PC using custom-written Spike2 software (CED, Cambridge, United Kingdom) or DAPSYS software (Brian Turnquist, Bethel University, St. Paul, MN) and a micro1401 DAC (CED). Data were analyzed off-line with DAPSYS and Microsoft Excel.

2.3.2. C-fiber classification

C fibers were differentiated into 2 main categories by the extent of ADS and sensitivity to mechanical stimuli. Fibers with below 5% ADS in a specific electrical stimulation pattern with low-rising frequencies (20 pulses at 0.125 Hz, 20 pulses at 0.25 Hz, and 30 pulses at 0.5 Hz)[58,67] and response to 750 mN von Frey stimulation were classified as CM fibers. C fibers that showed more than 5% ADS and did not respond to 750 mN von Frey stimulation were categorized as CMi fibers.

2.3.3. Activation intensity

A semiquantitative analysis of the activation strength of C nociceptors after substance application was performed using the marking method. The number of "activation periods" (the number of electrical stimulation intervals in which the conduction latency of an action potential was slowed down compared with the previous electrical stimulations at rest) and the cumulative ADS of the respective latency shifts were evaluated.[34] We additionally evaluated the time to onset of the activation periods and the duration of occurrence of activation periods after application of substances. According to a previous study assessing the response of C fibers after chemical activation and similar findings in this study, as shown in the Results section, the threshold of activation was defined as more than 5 activation periods and a cumulative latency increase of >2 ms.[10]

2.3.4. Axonal properties

The unconditioned latency of the single C fibers was determined as the first response latency after a 2-minute pause in electrical stimulation. Conduction velocity was calculated using the unconditioned latency and the distance between stimulation needles in the receptive field and the recording electrode in the nerve. Activity-dependent conduction velocity slowing during a low-frequency stimulation protocol was assessed at the end of the 0.125 Hz (20 pulses), 0.25 Hz (20 pulses), and 0.5 Hz (40 pulses) sequence and then normalized to the respective unconditioned latency. The total ADS was also determined at the end of the protocol as percentage of the unconditioned latency.

2.3.5. Substances and application

In microneurography experiments, intracutaneous microinjections of 20 μL LPA at concentrations of 0.05 and 0.1 mM and SIF for control as well as focal applications of LPA 25 mM in inactivated cowhage spicules were performed into the receptive fields of the recorded C fibers (**Fig. 1B**, light and dark gray circles) during continuous electrical stimulation with 0.25 Hz.

As part of the microneurography studies, histamine (Sigma-Aldrich; 1%, diluted in ultrapure water) was applied using iontophoresis.

We chose histamine iontophoresis over injection because it does not cause tissue damage and the skin depth as well as amount of histamine entering the skin is better controlled by concentration and electrical charge.

For this purpose, a cotton disk was soaked with the histamine solution and put in the application chamber of the iontophoresis applicator. Intracutaneous application was achieved by anodal current and a charge transfer of 20 mC (1 mA, 20 seconds).[32]

2.3.6. Microneurography experimental protocol

After locating a nerve fascicle containing C fibers and the respective receptive field at the dorsum of the foot, fiber classification protocols were performed as described above. In addition, electrical thresholds and, for CM fibers, mechanical thresholds were determined using von Frey filaments. Afterwards, intracutaneous microinjections of LPA or SIF, application of LPA in heat-inactivated cowhage spicules, or histamine iontophoresis were performed directly between the stimulation needles or in the receptive field during continuous electrical stimulation with 0.25 Hz. Activation of the fibers by the different stimuli was detected using the marking technique, which requires ongoing electrical stimulation of C fibers with low frequency to evoke action potentials. After microinjections of LPA, the low-frequency protocol and mechanical and electrical threshold testing were repeated. After 5 minutes of observing a possible activation or 2 minutes after the last chemical activation, a new receptive field of other C fibers was searched at least 3 cm away from the first testing site and all protocols as well as applications of substances were repeated. In each subject, 1 to 3 different receptive fields were examined.

2.4. Data analyses and statistics

Statistical analyses were conducted with STATISTICA 7.0 software (StatSoft Inc, Tulsa, OK). Data were evaluated for normality by the Shapiro–Wilk W-test.

The nonparametric Wilcoxon matched pairs test or Mann–Whitney *U*-test was applied in case data were not normally distributed, and data are then given as median with 25% and 75% quartiles. In case of normal distribution, values are given as mean ± SEMs and were analyzed by repeated measures or multiway analysis of variance (ANOVA) with least significant difference (LSD) post hoc testing. The χ^2 test was used to compare numbers of events. The Spearman rank test was used to calculate correlations. Graphs and figures were generated with Origin 2019b, CorelDraw X7, and Microsoft Excel.

3. Results

3.1. Psychophysical study

3.1.1. Intracutaneous lysophosphatidic acid microinjections dose-dependently induce pain sensations in humans

Intracutaneous LPA microinjections (50 μL, 0.1 mM) induced a burning and/or stinging pain sensation in 100% of tested healthy subjects (n = 20, **Fig. 2A**). All subjects described a similar pain quality after capsaicin microinjections (50 μL, 3.4 μM; n = 20, **Fig. 2A**).

We assessed itch ratings simultaneously with pain ratings, but none of the subjects qualified the LPA or capsaicin microinjections as itchy. The median maximal pain rating after 0.1 mM LPA microinjections (NRS 3, quartile range 2-4) was lower compared with capsaicin microinjections (NRS 5.5, quartile range 4-7; $P < 0.001$, Mann–Whitney *U*-test). After LPA injection, the maximal pain sensation peaked later than after injection of capsaicin (LPA: median 30 seconds, quartile range 15 to 40 seconds; capsaicin: median 5 seconds, quartile range 5-10 seconds; $P < 0.0001$, Mann–Whitney *U*-test).

By contrast, the overall mean pain ratings (calculated as area under the curve [AUC] of pain ratings over time) after LPA 0.1 mM and capsaicin 3.4 μM microinjections were comparable (LPA: 28.2 ± 4.9; capsaicin: 31.7 ± 2.6; $F_{1,38} = 0.34$; ANOVA, with LSD post hoc test, $P = 0.6$). Microinjections of SIF did not induce a significant pain sensation in any of the subjects (**Fig. 2A**).

In addition, LPA microinjections were performed in increasing concentrations (0.01, 0.05, 0.1, and 0.5 mM, n = 7). Dose-dependent pain sensations were observed with an earlier onset at higher concentrations (**Fig. 2B**). Regardless of the applied concentration, none of the healthy subjects indicated the LPA microinjections to be pruritic. The subjects described either burning or stinging pain, an unspecific spurious sensation (NRS < 1, eg, tingling, prickling, or a warmth feeling), or no sensation at all.

The volunteers rated the mean maximum pain within the range of 20 to 40 seconds after microinjections. Area under the curve of NRS ratings over time increased with higher concentrations of LPA (**Fig. 2C**).

3.1.2. Focal application of lysophosphatidic acid causes itch sensations in humans

Application of LPA 25 mM soaked in heat-inactivated cowhage spicules caused a mild to moderate itch sensation in 100% of the tested human subjects (n = 20; mean maximal NRS itch rating: 2.2 ± 0.3), which was less pronounced compared with application of active cowhage spicules (mean maximal NRS itch rating 4.6 ± 0.3; **Fig. 2D**). None of the subjects qualified this focal LPA application as clearly painful.

A subgroup of subjects (n = 7) was additionally asked to specify the sensation during focal application of LPA in categories of "itching like a mosquito bite," "stinging," or "burning," of which itching was the dominating sensation because 100% of subjects reported on it with a mean maximal itch intensity of NRS 1.9 ± 0.5 (**Fig. 2E**). Only 50% of subjects experienced a stinging or burning background component, of which the mean maximal magnitude of NRS rating only reached a low extent (stinging: 0.7 ± 0.1; burning 0.7 ± 0.2).

3.1.3. Transient receptor potential channel blockade does not reduce lysophosphatidic acid–mediated pain sensations

To assess the involvement of TRP channels in acute chemical pain evoked by LPA microinjections, we used a pharmacological block of TRPV1 and TRPA1 by injection of BCTC and A-967079, respectively (**Fig. 2F**).

The microinjections of SIF, A-967079, or BCTC did not cause a pain sensation defined as NRS > 1 (n = 10). After pre-injection of SIF, LPA 0.1 mM microinjections (50 μL) resulted in a mean maximal pain rating of 3.6 ± 0.4 after a mean period of 35 seconds. Neither the pre-injection of TRPV1 channel blocker BCTC (1 μM; 100 μL) nor TRPA1 antagonist A-967079 (10 μM; 100 μL) significantly reduced the mean maximum pain rating after LPA microinjections (after BCTC: NRS 3.4 ± 0.4; after A-967079: NRS 3.8 ± 0.5, $F_{2,27} = 0.58$; ANOVA, $P = 0.6$ with LSD post hoc test; **Fig. 2F**). The overall pain ratings (calculated as AUC of pain ratings over time) also did not differ significantly for any combination of antagonist or SIF with LPA 0.1 mM ($F_{2,27} = 1.22$; ANOVA, $P = 0.3$ with LSD post hoc test).

3.1.4. Lysophosphatidic acid application evokes an axon reflex erythema in humans

Microinjections of 0.1 mM LPA (50 μL, n = 20) caused an axon reflex erythema with a mean maximal size of 8.9 ± 0.6 cm^2, ie, much larger than the injection bleb (for specimen refer to **Fig. 2G**). Thus, LPA most likely evoked action potentials conducted along the axonal tree within the skin, inducing calcitonin gene-related peptide release from the nerve fibers in the skin. Consequently, calcitonin gene-related peptide caused vasodilation that could be detected as blood flow increase around the injection site in LDI. The large area of flare reaction implies activation of mechanoinsensitive C fibers because only these fibers have correspondingly outspread receptive fields.[50]

Focal application of LPA in heat-inactivated cowhage spicules induced an erythema although with a smaller extension than on application by microinjection (2.7 ± 0.3 cm^2). The erythema did not exceed the spiked skin area. This can be interpreted as just a local vasodilation with a minimal axon reflex, if at all, indicating no or minor activation of CMi fibers. The respective controls, SIF microinjection and chloroform-treated spicules, induced only minor local erythemas at the application site (**Fig. 2G**).

3.1.5. Itch and pain ratings to pruritic and algogenic stimuli correlate positively

As there was a considerable interindividual variability in the NRS pain or itch ratings to different algogenic and pruritic stimuli, correlations were calculated to explore if subjects expressing higher pain ratings also reported higher pruritic intensity to respective stimuli (n = 20). We observed positive correlations between AUC of NRS pain or itch ratings over time for capsaicin (3.4 μM) microinjections and active cowhage spicules ($r_s = 0.59$) as wells as for LPA 0.1 mM microinjections and application of LPA in inactivated cowhage spicules ($r_s = 0.59$, data in suppl. Fig. 1, available as supplemental digital content at http://links.lww.com/PAIN/B414).

3.1.6. Lysophosphatidic acid microinjections sensitize the perception of painful heat and electrical stimuli

In all sensory tests, microinjections of control (SIF) did not significantly alter the respective thresholds or NRS pain ratings (n = 14, **Fig. 3**).

3.1.6.1. Thermal tests

The mean heat pain threshold decreased significantly by 1.5°C after LPA 0.1 mM microinjections from 46.5 ± 0.5°C to 45.0 ± 0.6°C (n = 14, $F_{1,13} = 9.53$; ANOVA repeated measures design, $P < 0.01$ with LSD post hoc test; **Fig. 3A**). By contrast, the mean

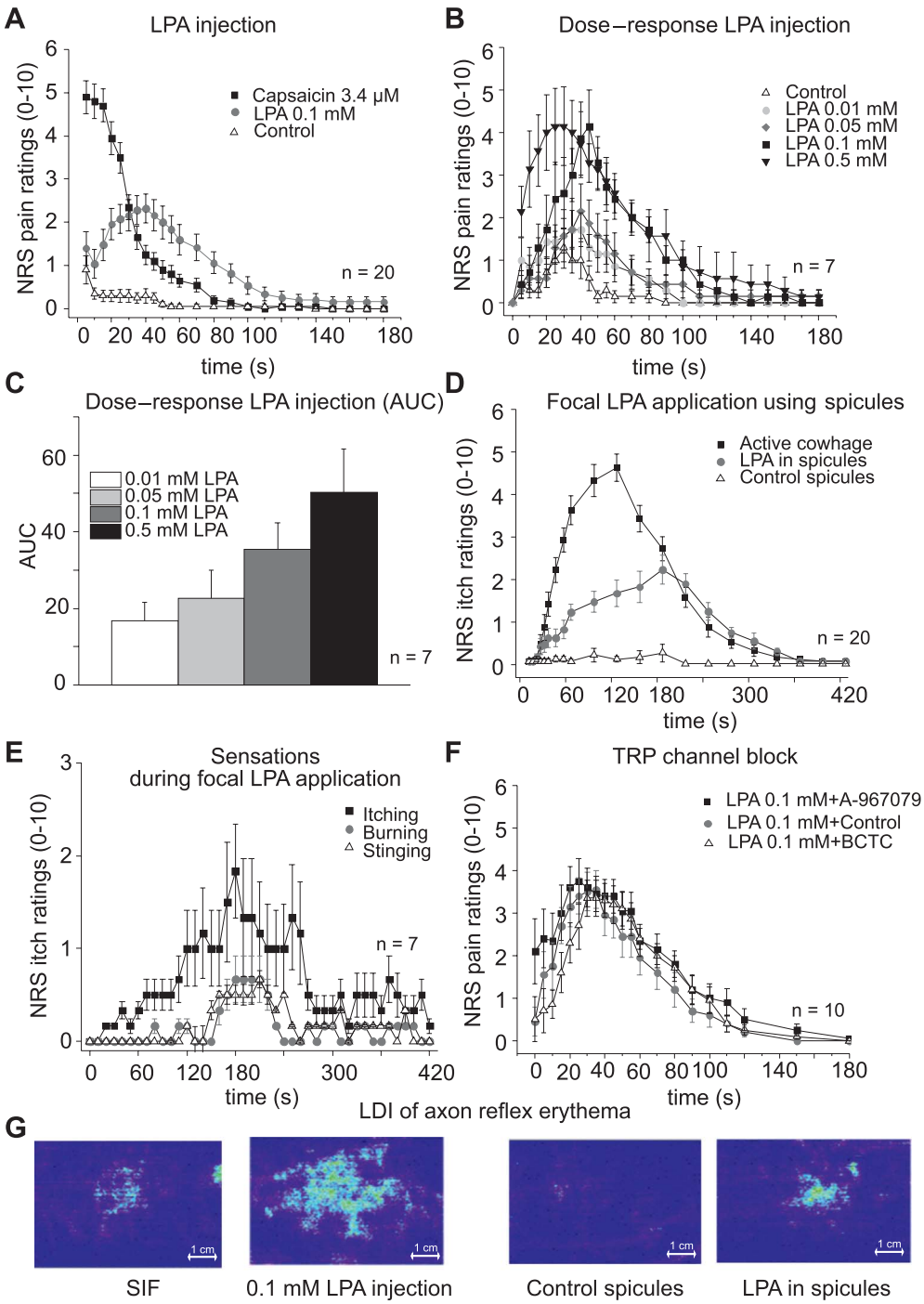

Figure 2. Psychophysical study on LPA application to the skin. Values are given as mean ± SEM. (A) Verbal ratings on a numeric rating scale (NRS) for pain of healthy subjects on intracutaneous LPA and capsaicin microinjections. Microinjections of 50 µL of LPA (0.01 mM, gray circles) and capsaicin (3.4 µM, black squares) caused a burning or stinging pain sensation in 100% of tested human subjects while SIF microinjections (control, open triangles) did not induce relevant sensations (n = 20). Synthetic interstitial fluid microinjections served as control in all psychophysical experiments. (B) Dose–response NRS pain ratings after LPA microinjections + C) AUC of NRS ratings over time of dose-dependent response to LPA microinjections. Lysophosphatidic acid microinjections (50 µL) in increasing concentrations (0.01, 0.05, 0.1, and 0.5 mM, n = 7) induced dose-dependent pain sensations in the subjects. (D) Numeric rating scale itch ratings of the subjects after focal application of LPA using heat-inactivated cowhage spicules. Application of LPA in heat-inactivated cowhage spicules (gray circles) caused itch sensation in all tested human subjects (n = 20), which was not as pronounced as after insertion of activate cowhage spicules (black squares). (E) Numeric rating scale ratings on application of LPA in heat-inactivated cowhage spicules presenting itch and other sensations. Subjects were asked to specify their sensation on focal LPA application in categories of "itching as an insect bite," "stinging," or "burning," of which itching was the dominating sensation in most subjects (black squares). Only 50% of subjects experienced a stinging (open triangles) or burning (gray circles) component, of which the mean maximum only reached a small magnitude. (F) Numeric rating scale pain ratings to LPA microinjections (0.1 mM) after pre-injection of TRPA1 and TRPV1 blockers. Lysophosphatidic acid (0.1 mM, 50 µL) was injected after the TRPA1 (A-967079) and TRPV1 (BCTC) channel blockers or SIF (100 µL) as control (n = 10). Neither the mean maximum pain rating nor the AUC of NRS pain ratings over time significantly differed between the 3 groups. (G) Laser Doppler imaging specimen after LPA vs control applications. Exemplarily, LDI results of one subject are displayed, in which after LPA, but not SIF, microinjection a widespread axon reflex flare can be observed. Lighter false colors from light green over yellow and red to white indicate increased skin blood flow. AUC, area under the curve; LPA, lysophosphatidic acid; TRPA1, transient receptor potential channel A1; TRP, transient receptor potential; TRPV1, transient receptor potential channel vanilloid 1; SIF, synthetic interstitial fluid.

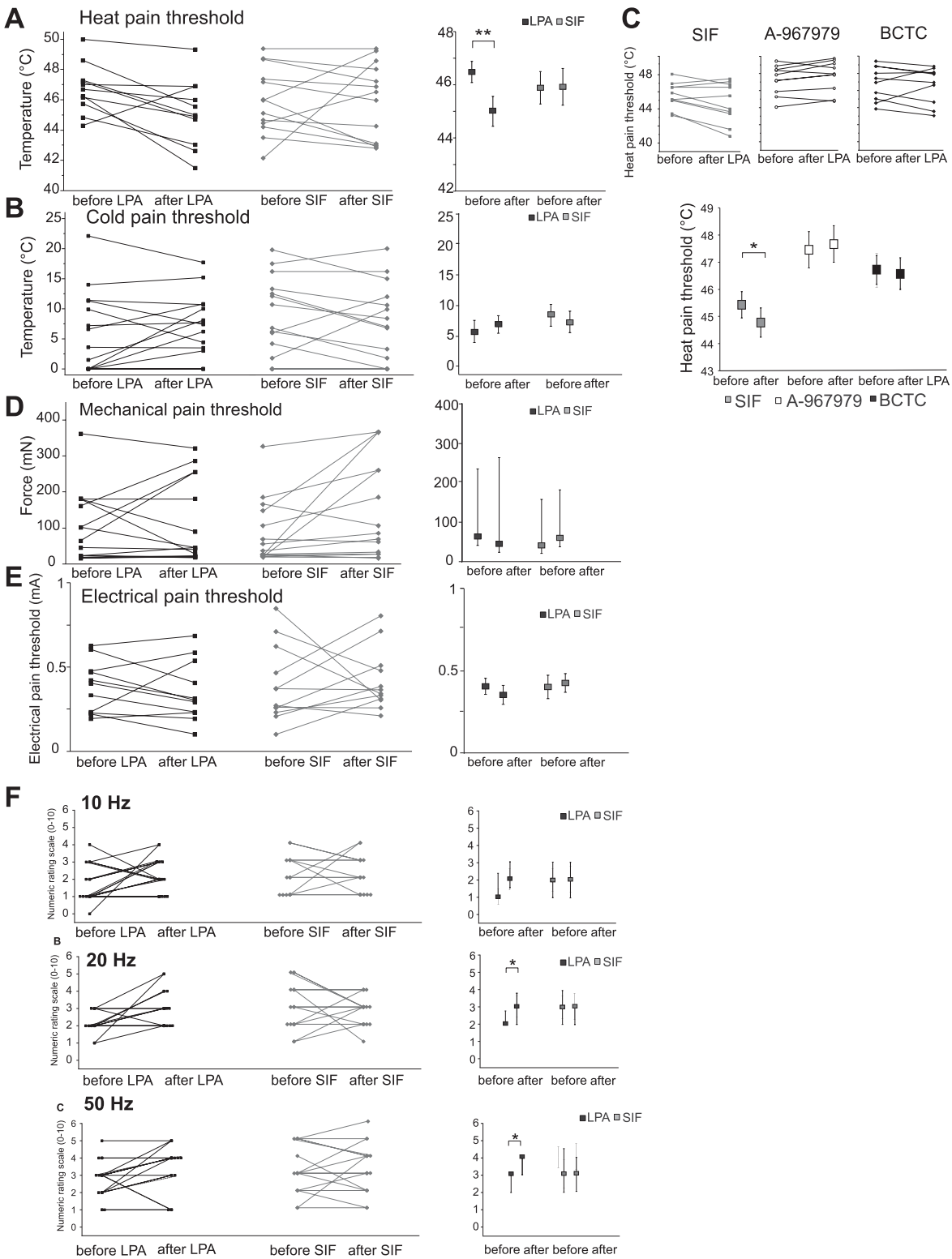

Figure 3. Pain thresholds to thermal, mechanical, and electrical stimuli and NRS pain ratings after LPA and TRP antagonist microinjections. A, B, D + F) The individual thresholds and NRS pain ratings of each subject are shown before and after LPA (0.1 mM) application in the first column (square, dark gray) and SIF microinjections in the second column (diamond, light gray). In the third column, mean values of thermal and electrical pain thresholds are displayed with SEM (A, B, E). Not normally distributed data are given as median with 25% and 75% quartiles (D + F). (A) The heat pain threshold was lowered after LPA in 12 of 14 subjects, and the mean was also significantly reduced ($P < 0.01$). B/D/E) The cold, mechanical, or electrical pain thresholds were not significantly changed after LPA. (C) In the first 3 panels on top, the individual values of heat pain thresholds of each subject (n = 10) before and after LPA microinjections are displayed separately for the pre-injection with SIF (light gray squares), A-967079 (open circles), and BCTC (black diamonds). The lower panel shows the significant heat sensitization after SIF pre-injection followed by LPA, which was prevented by both antagonists that, in addition, elevated the basal heat pain threshold. (F) An increase in individual and in median NRS pain ratings after suprathreshold electrical stimulation for 5 seconds was observed at higher frequencies after LPA 0.1 mM microinjections, which was significant for 20 and 50 Hz. Significant differences are indicated by an asterisk for $^*P < 0.05$. LPA, Lysophosphatidic acid; NRS, numeric rating scale; SIF, synthetic interstitial fluid.

cold pain threshold was not significantly altered after LPA microinjections (before LPA: 5.8 ± 1.8˚C, after LPA: 6.8 ± 1.4˚, $F_{1,13}$ = 1.07; ANOVA repeated measures design, P = 0.3, **Fig. 3B**).

3.1.6.2. Heat sensitization is prevented by transient receptor potential channel vanilloid 1 and transient receptor potential channel A1 blockade

To control for the additional volume of the pre-injection of TRP channel blockers, we used SIF pre-injection. Here, after the microinjections of LPA 0.1 mM (n = 10), the mean heat pain threshold was significantly lowered from 45.4 ± 0.5 to 44.7 ± 0.7˚C ($F_{1,9}$ = 5.46; ANOVA repeated measures design, P = 0.04, with LSD post hoc test; **Fig. 3C**). Both pre-injection of BCTC as TRPV1 blocker and A-967079 as TRPA1 blocker prevented the significant LPA-induced reduction of the heat pain threshold. These findings are in accordance with the established role of both TRPA1 and TRPV1 in noxious heat sensing in mice.[17,33]

3.1.6.3. Mechanical tests

The median mechanical pinprick thresholds were not significantly altered by 0.1 mM LPA microinjections (before LPA: 64.0 mN, quartile range 22.6-171.1 mN, after LPA: 45.3 mN, quartile range 21.4-218.5 mN, Wilcoxon matched pairs test, P = 0.9, **Fig. 3D**). The mean NRS pain rating to a suprathreshold mechanical pinprick stimulus (512 mN) was comparable before or after LPA microinjections (NRS 2.8 ± 0.2 to 3.2 ± 0.4, Wilcoxon matched pairs test, P = 0.2, data not shown).

3.1.6.4. Electrical tests

Lysophosphatidic acid microinjections did not significantly decrease the mean electrical pain threshold from 0.4 ± 0.07 mA before to 0.35 ± 0.06 mA postinjection ($F_{1,13}$ = 0.44; ANOVA repeated measures design, P = 0.5, with LSD post hoc test, **Fig. 3E**). To test for suprathreshold electrical pain sensitivity and especially for higher frequency coding of nociceptors, suprathreshold electrical stimulation was applied at different frequencies (10, 20, and 50 Hz) for 5 seconds. Increased median NRS pain ratings for the whole 5 seconds pulse train were observed with higher stimulus frequencies after LPA 0.1 mM microinjections, which were significantly higher compared with SIF microinjections for 20 and 50 Hz (20 Hz before LPA: median NRS 2, quartile range 2-2.75; after LPA: median NRS 3, quartile range 2-3.75; Wilcoxon matched pairs test, $P < 0.05$; 50 Hz: before LPA: median NRS 3, quartile range 2-3; after LPA: median NRS 4, quartile range 3-4, Wilcoxon matched pairs test, $P < 0.05$, **Fig. 3F**), but not for 10 Hz (before LPA: median NRS 1, quartile range 1-2.5; after LPA: median NRS 2, quartile range 1.5-3; Wilcoxon matched pairs test, P = 0.15, **Fig. 3F**). Thus, after LPA microinjections, the rapidly successive electrical stimuli were perceived as more painful, suggesting less conduction failure of the excited C fibers.

3.2. Microneurography study

3.2.1. C-fiber classification

Recordings of 103 single C-fiber units over conduction distances between 30 and 220 mm were performed. Seventy-two fibers were categorized as CM fibers.[67] Twenty-eight fibers were classified as CMi fibers. Three units displayed a positive response to mechanical von Frey stimulation of 750 mN, but not to lower than 100 mN, and indicated axonal properties (ie, ADS behavior) similar to CMi fibers. These fibers were categorized as VHT (very high threshold) fibers, according to previous findings with single fiber recordings in pigs.[39]

Fifty-six C fibers were tested with LPA in the receptive fields at the back of the foot, and 47 C fibers were treated as controls with SIF (**Table 1**). No significant difference in pre-injection conduction velocity was found for the later SIF-treated or LPA-treated fibers.

3.2.2. Lysophosphatidic acid causes differential activation patterns in mechanosensitive C and mechanoinsensitive C fibers depending on the mode of application

In the psychophysical tests, we observed that LPA 0.05 and 0.1 mM evoked dose-dependent pain sensations, indicating activation of primary afferents at these concentrations. In the microneurography recordings, we found differences between CM and CMi nociceptors in the number of activated fibers as well as magnitude, onset, and duration of activation. These differences varied with the application mode of LPA. **Figure 4A** shows representative specimens of a CM and a CMi fiber during and after LPA microinjection. It can be observed that the CM fiber is only activated in the beginning with few activation periods, whereas the CMi fiber is repeatedly stronger activated (more and greater sum latency shifts) for a longer time.

When LPA was applied using inactivated cowhage spicules as "microcannulas" filled with LPA, these differences were far less pronounced.

Neither for CM nor CMi fibers there were significant differences between the 0.05 and 0.1 mM LPA-treated units for the evaluated activation parameters. Therefore, fibers activated by these 2 different concentrations of LPA microinjections were pooled for further analyses. Differences in the activation patterns were assessed not only between fiber classes but also between application modes, injection vs focal application using cowhage spicules (**Fig. 4B**).

After microinjections of LPA or application of LPA-loaded spicules, the activation of the fibers did not immediately occur but with a certain delay. After LPA microinjections, the activation started significantly earlier in mechanosensitive CM units (n = 24) after a mean of 4.8 ± 0.6 traces following removal of the injection needle, corresponding to about 20 seconds during ongoing electrical stimulation at 0.25 Hz. Mechanoinsensitive C fibers (n = 12) had a significantly later onset of activation with a mean of 8.2 ± 1.0 traces (approx. 32 seconds; $F_{1,34}$ = 4.87; ANOVA, $P <$ 0.05 with LSD post hoc test, **Fig. 4B**). By contrast, after

Table 1

Total number of C-fiber nociceptors recorded in microneurography experiments, treated with either lysophosphatidic acid or control (synthetic interstitial fluid) microinjections, or focal lysophosphatidic acid application by heat-inactivated cowhage spicules, shown as activated or total number out of fibers.

Substance	Fiber class		
	CM	CMi	VHT
LPA injection	24/40	12/15	1/1
0.05 mM	17/30	7/9	1/1
0.1 mM	7/10	5/6	/
LPA spicules	17/27	6/8	1/1
SIF	0/32	0/13	0/2

CM, mechanosensitive C-fibers; CMi, mechanoinsensitive C-fibers; LPA, lysophosphatidic acid; SIF, synthetic interstitial fluid; VHT, very high threshold C-fibers.

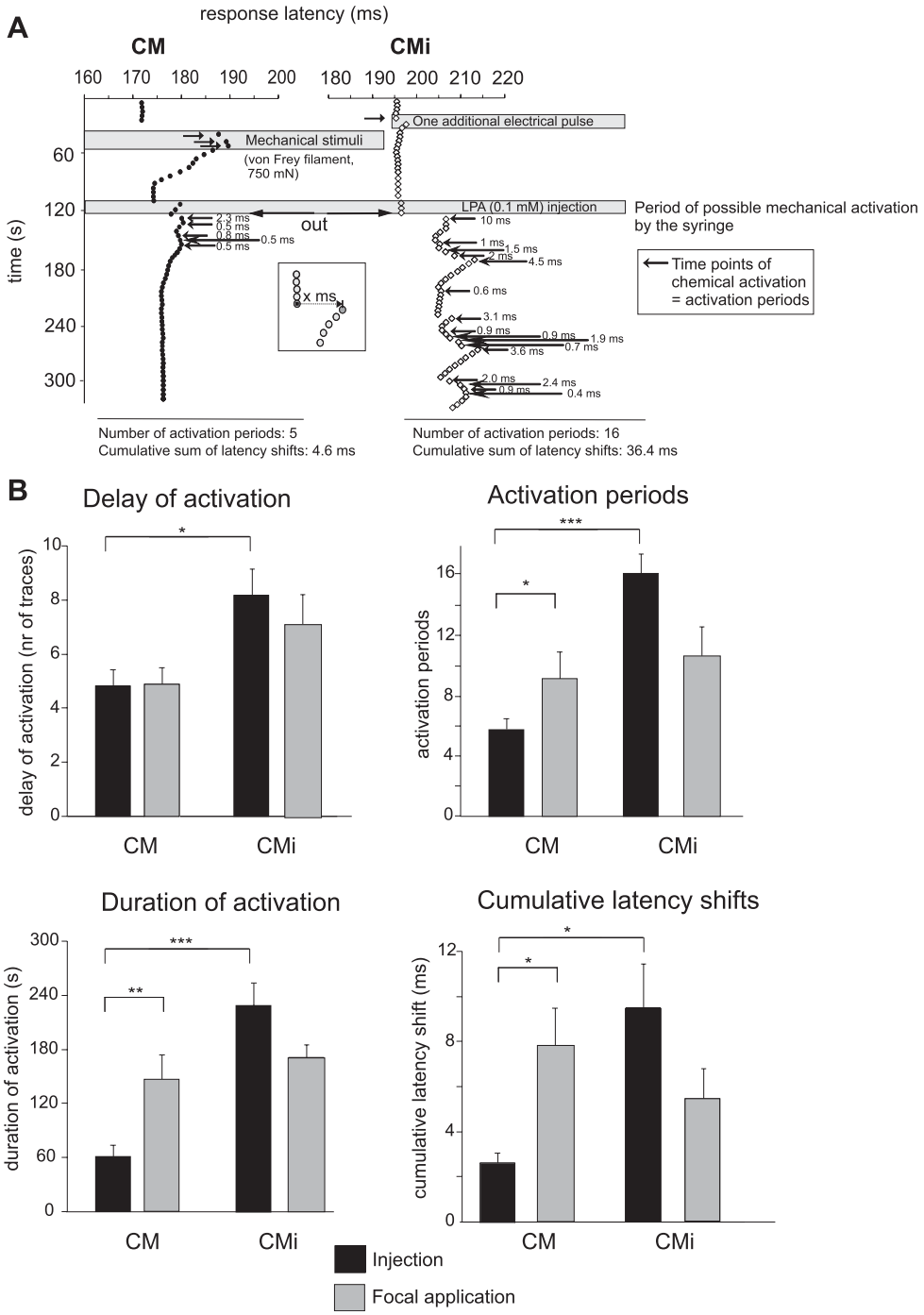

Figure 4. Microneurography data of single fiber responses to different LPA applications. (A) Latencies of electrically stimulated action potentials are shown for one CM fiber (black circles) and one CMi fiber (open diamonds) during ongoing stimulation at 0.25 Hz corresponding to a 4-second interstimulus interval. Before additional stimuli, ie, mechanical, electrical, or chemical (each marked with gray boxes), were applied, the latency did not change. (A shift of the action potential symbol to the right, reflecting a sudden increase in latency, occurs when the fiber has elicited additional action potentials before. The time point at which the LPA microinjection cannula was removed from the skin is marked with "out" and 2 black arrows. From this time point, our semiquantitative analyses of chemical activation started. During the time of insertion, injection of LPA and removal of the cannula (marked with a gray box and labelled LPA 0.1 mM injection), it was impossible to differentiate between mechanical and chemical activation of fibers. Please note, the CMi fiber was not activated during this period of mechanical manipulation within the receptive field. In both fibers, each latency shift to the right to longer latencies (= "activation period") was marked by a black arrow. The number of these activation periods was summed up and used for further comparisons. In this specimen, the CM unit was only activated in the beginning with 5 activation periods, whereas the CMi fiber exhibited 16 activation periods. Because the number of chemically induced action potentials closely correlated to the magnitude of the following latency shift of the electrically induced action potential, the latency shift in millisecond could be used as a semiquantitative analysis of activation magnitude. We calculated the individual latency shifts in millisecond by subtracting the latency of the slower action potential from the previous faster action potential (see inset). The individual latency shifts of each activation period are given in millisecond at the end of the arrows, marking activation periods. The CM fiber showed a cumulative latency shift (sum of all latency shifts) of only 4.6 ms. By contrast, the CMi fiber exhibited a larger cumulative latency shift of 36.4 ms. (B) Means and SEM of activation parameters (delay of activation, number of activation periods (each lasting 4 seconds), duration of activation, and cumulative latency increase) during activation. The 4 panels display the different quantified activation parameters. Black columns represent LPA microinjections and gray columns represent application of LPA-soaked cowhage spicules. Significant differences are indicated by asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CM, mechanosensitive C; CMi, mechanoinsensitive C; LPA, lysophosphatidic acid.

application of LPA using heat-inactivated cowhage spicules, the start of activation was not significantly different between CM and CMi fibers. Mechanosensitive C fiber (n = 17) activation after focal application was similar to activation after injection with a mean activation time of approximately 20 seconds, and CMi fibers (n = 6) took approximately 28 seconds (CM fibers: after 4.8 ± 0.9 traces; CMi fibers: after 6.9 ± 1.1 traces; $F_{1,22} = 2.15$, ANOVA, with LSD post hoc test, $P = 0.2$).

Microinjections of LPA activated CMi fibers more strongly than CM fibers. Mechanoinsensitive C fibers exhibited a significantly higher count of markings, which are referred to as "activation periods" during time of activation, than CM fibers (CMi fibers: 16.2 ± 1.3; CM fibers: 5.8 ± 0.7; $F_{1,34} = 18.24$; ANOVA, $P < 0.001$ with LSD post hoc test, **Fig. 4B**). A higher count of activation periods indicates that during time of activation the respective fiber fired spikes in more intervals of the regular electrical stimulation at 0.25 Hz, causing a latency shift (ADS) of the next electrically induced action potential. A higher count of activation periods indicates more LPA-induced bursts of action potentials in CMi than CM fibers after LPA microinjections. In contrast to the microinjections of LPA, focal application using LPA-loaded spicules caused a similar number of activation periods in both fiber classes, CM and CMi fibers (CMi fibers: 10.4 ± 1.9 vs CM fibers: 8.8 ± 1.7; $F_{1,22} = 0.26$; ANOVA, with LSD post hoc test, $P = 0.6$), because CM fibers had a significantly higher mean amount of activation periods after focal LPA application using spicules than postinjection ($F_{1,39} = 5.37$; ANOVA, with LSD post hoc test, $P = 0.04$).

Another indirect measure of the number of action potentials evoked by a substance in microneurography is to sum up all latency shifts of electrically induced action potentials during 0.25 Hz stimulation. Assuming that the latency shifts roughly mirror the number of fired action potentials before the electrical stimulus response,[6] a higher cumulative latency shift correlates to more chemically elicited action potentials.[49] After LPA microinjections, the cumulative ADS of CMi fibers during activation (8.7 ± 1.8 ms) was again higher and equaled approximately 3 times the amount of the cumulative ADS of CM fibers (2.6 ± 0.4 ms; $F_{1,34} = 6.86$; ANOVA, $P < 0.05$ with LSD post hoc test, **Fig. 4B**).

In contrast to microinjections, the cumulative latency increase after LPA application in spicules was not different between both fiber classes (CMi fibers: 5.5 ± 1.3 ms; CM fibers: 6.5 ± 1.4 ms; $F_{1,22} = 0.42$; ANOVA, with LSD post hoc test, $P = 0.5$). Mechanosensitive C fibers increased their activity and displayed a significantly more pronounced mean cumulative latency shift after focal LPA application (6.5 ms) than LPA microinjection (2.5 ms; $F_{1,39} = 5.29$; ANOVA, $P < 0.05$ with LSD post hoc test). After LPA microinjections, the activation in CMi fibers lasted over 3 minutes, which is significantly longer than approximately 1 minute in CM fibers (CMi: 228.9 ± 25.9 seconds; CM: 61.5 ± 11.8 seconds; $F_{1,34} = 33.16$; ANOVA, $P < 0.00001$ with LSD post hoc test, **Fig. 4B**). After focal application of LPA, CMi fibers showed only a tendency to be activated for longer than CM fibers (CMi fibers: 168.0 ± 14.0 seconds vs CM fibers: 139.3 ± 25.2 seconds; $F_{1,22} = 0.51$; ANOVA, with LSD post hoc test, $P = 0.5$). Comparing the time of activation after LPA application by microinjections vs spicules, CM fibers were activated for double the time after focal application when compared with microinjections ($F_{1,39} = 9.91$, ANOVA, with LSD post hoc test, $P < 0.01$).

Microinjections of LPA (0.05 and 0.1 mM) activated a higher proportion of CMi (12 of 15 fibers, 80%) than CM fibers (24 of 40 fibers, 60%). If LPA was applied focally and superficially through inactivated LPA-soaked cowhage spicules, this difference was less pronounced between the 2 fiber classes: Focal LPA activated 6 of 8 CMi fibers (75%) compared with CM fibers (17 of 27 fibers, 63%).

In conclusion, LPA microinjections activated a larger number of CMi fibers more strongly and for a longer amount of time than CM fibers. LPA applied using spicules induced less pronounced activation in fewer CMi fibers, whereas the same proportion of CM fibers displayed more intense activation than after LPA microinjections.

3.2.3. Lysophosphatidic acid strongly activates a small subgroup of C nociceptors

We categorized the activated fibers according to the magnitude of activation on LPA microinjection or application in spicules. Three categories were differentiated by the number of activation periods and cumulative latency increase (**Fig. 5A**): Spurious reaction: below 5 activation periods and a cumulative latency increase <2 ms; strong activation: more than 15 activation periods and a cumulative latency increase >15 ms; and moderate response: a range between above mentioned values. CM and CMi fibers showed a different activation magnitude dependent on the application mode. Lysophosphatidic acid microinjections (0.05 mM) induced a moderate and strong activation in a significantly higher proportion of CMi than that in CM units (CM fibers: 6 of 30 fibers, CMi fibers: 5 of 9 fibers, $P < 0.05$, χ^2 test, **Fig. 5B**). Microinjections of 0.1 mM LPA did not reach a statistically significant difference in the occurrence of moderate or strong activation between both fiber classes (CM fibers: 3 of 10 fibers, CMi fibers: 3 of 6 fibers, $P = 0.4$, χ^2 test, **Fig. 5B**), but only CMi fibers were activated strongly at this concentration. When pooling all fibers treated with LPA microinjections irrespective of concentration, LPA microinjections caused again moderate and strong activation significantly more often in CMi than that in CM fibers (CM fibers: 9 of 40 fibers, CMi fibers: 8 of 15 fibers, $P < 0.05$, χ^2 test).

In contrast to that, focally applied LPA in inactivated cowhage spicules activated both fiber classes to a similar extent (CM fibers: 6 of 27 fibers, CMi fibers: 2 of 8 fibers, $P = 0.2$, χ^2 test, **Fig. 5B**). In the class of CM nociceptors, the percentage of moderately and strongly activated C fibers was similar by microinjections and focal application (22.5% microinjections vs 22.2% spicules, **Fig. 5C**), whereas the percentage of moderately and strongly activated CMi fibers decreased to less than half on LPA application in spicules (53.3% microinjections vs 25.0% spicules, **Fig. 5C**). One VHT fiber was tested with 0.05 mM LPA microinjection as well as LPA in spicules and was both times spuriously activated.

Nine CMi fibers that were activated by LPA microinjections were additionally tested with histamine (1%) iontophoresis over 20 seconds. Four fibers demonstrated a strong activation to histamine (His+) while 5 fibers only a weak response (His−). A strong activation on LPA microinjections, as defined by the categories mentioned above, was observed in CMi fibers also distinctly responsive to histamine (3 of 4 His+ fibers, **Fig. 5D**). Most CMi fibers with a weak response to histamine iontophoresis also showed spurious activation by LPA microinjection (4 of 5 His− fibers).

In summary, microinjections of LPA, which induce painful sensations, activated CMi fibers in larger numbers and more strongly than that in CM fibers, in particular the histamine-sensitive subgroup of CMi fibers. By contrast, both fiber classes were about equally activated by the itch-associated application of LPA in inactive cowhage spicules.

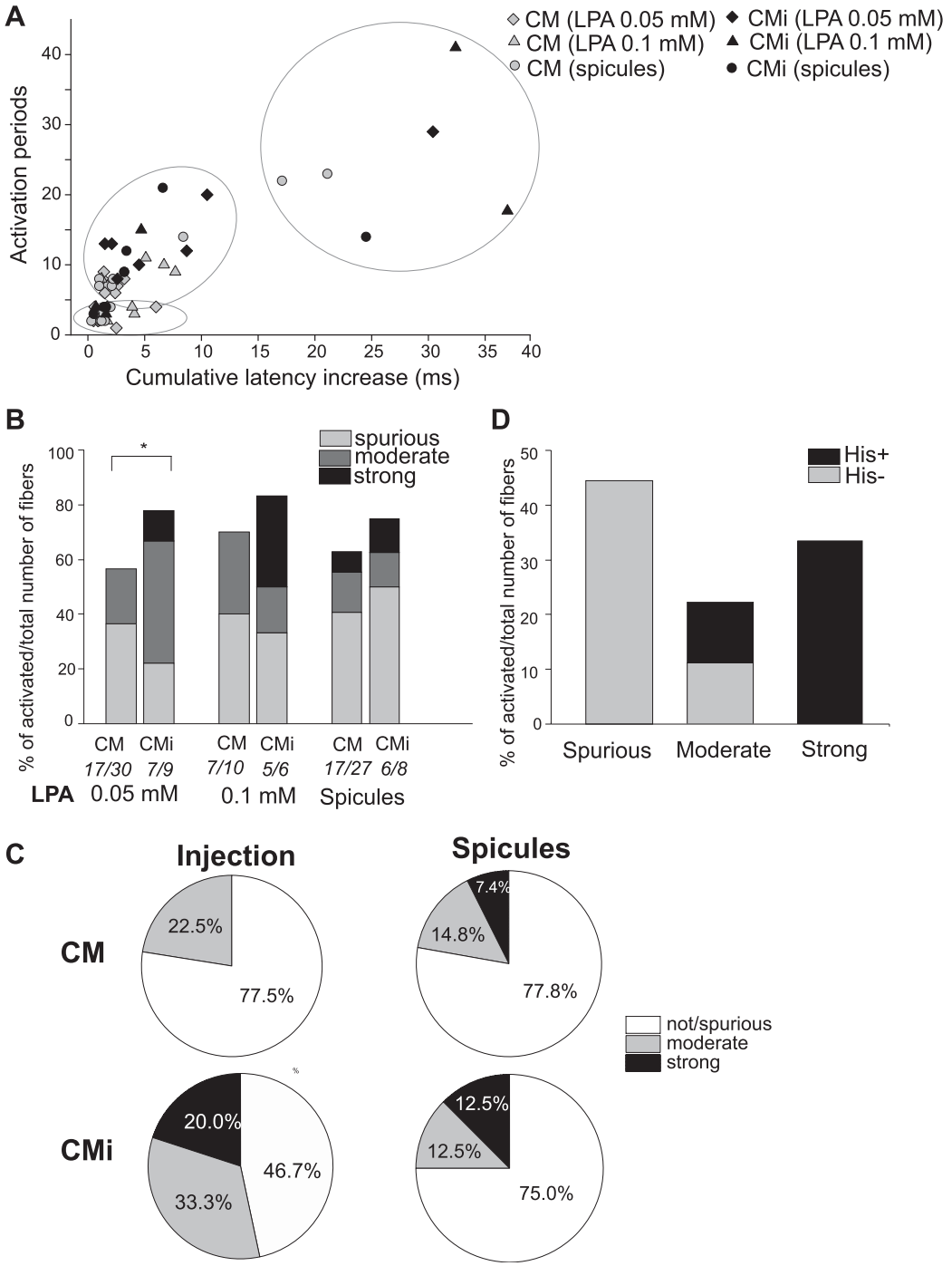

Figure 5. Differential activation of C-fiber classes by LPA: microinjection vs focal application in heat-inactivated cowhage spicules. (A) The number of activation periods and cumulative latency increase on microinjection of different LPA concentrations and on heat-inactivated cowhage spicules soaked with LPA. Both parameters correspond to the magnitude of response and correlate with each other. Three clusters (encircled) became apparent: Fibers with less than 5 activation periods and a cumulative latency increase <2 ms were categorized "spuriously" activated because this low magnitude of activation is not likely to induce an actual sensation in humans. More than 15 activation periods and a cumulative latency increase >15 ms were considered as "strong" activation. Fibers with parameters in between these limits were classified as "moderately" activated. On LPA microinjection, more CMi units (black symbols) exhibited higher values than CM fibers (light gray symbols) in each parameter, whereas CM fibers were more strongly activated by LPA application through spicules (light gray circles). (B) Percentages of LPA-activated C fibers are shown, color-coded according to the above defined activity clusters and sorted by fiber class (very high threshold C-fibers fibers not included) and concentration injected vs focal application. Lysophosphatidic acid microinjections (0.05 mM) induced moderate and strong activations in significantly more CMi than CM units. On 0.1 mM LPA microinjection, only CMi fibers were strongly activated. On LPA applied focally and superficially by inactivated cowhage spicules, no significant difference in moderate or strong activations between both fiber classes was found. (C) Percentages of LPA activation in CM and CMi fibers compared by the application mode. Comparing LPA microinjections and application through heat-inactivated cowhage spicules, the percentage of moderately and strongly activated C fibers was similar in CM fibers (22.5% injection vs 22.2% spicules), whereas in CMi fibers this percentage was twice as large on LPA microinjections as on focal LPA application (53.3% injection vs 25.0% spicules). (D) The percentage of histamine-sensitive and histamine-insensitive CMi fibers activated by LPA microinjections. Nine CMi fibers were tested with histamine (1%) iontophoresis (for 20 seconds). "Strong" activation by LPA was only observed in CMi fibers distinctly responsive to histamine (3 of 4 His+ fibers). Significant differences are indicated by an asterisk for $^*P < 0.05$. CM, mechanosensitive C; CMi, mechanoinsensitive C; LPA, lysophosphatidic acid.

3.2.4. Lysophosphatidic acid microinjections cause minor changes in action potential latency and activity-dependent conduction velocity slowing

The conduction velocities of the C-fiber nociceptors changed to a similar extent whether their receptive fields were injected with 0.05 or 0.1 mM LPA. Therefore, all data from both applied LPA concentrations were pooled for further analyses. After a 2-minute interval without electrical stimulation, we determined the unconditioned latency (ie, latency of the first electrical pulse after the pause). For CM fibers, there was a slight but significant increase in the normalized mean baseline latency after LPA microinjections (+2.4% for all fibers, +2.5% after no or spurious activation, and +2.0% after moderate or strong activation) compared with the mean normalized latency increase after SIF application (+0.6%, $F_{1,59} = 9.76$; ANOVA, with LSD post hoc test, $P < 0.05$; **Fig. 6A**). In CMi units, a more pronounced and significant prolongation in mean unconditioned latency after LPA microinjections was only observed in moderately and strongly activated fibers in comparison with the mean latency after SIF microinjections (+4.7% vs + 1.0%; $F_{1,14} = 11.82$, ANOVA, $P < 0.01$, with LSD post hoc test, **Fig. 6A**), indicating an outlasting effect of LPA-induced activation on the membrane properties, which slowed the conduction velocity.

In the subsequent experiments, ADS was provoked by electrical stimulation with increasing, still low, frequencies (**Fig. 6B**). Control microinjections (SIF) did not alter ADS neither in CM nor in CMi fibers. Lysophosphatidic acid microinjections did not significantly reduce the total amount of ADS (measured after 70 pulses). The latter parameter was reduced in CMi fibers but only relative to the prolonged baseline latency (shown in **Fig. 6A**). There was no significant correlation between prolongation of unconditioned baseline latency and changes of ADS in neither the LPA nor the control experiments.

In summary, LPA induced minor changes in axonal properties of C fibers, of which the most prominent was an increase in mean unconditioned response latency in CMi fibers, presumably because of more pronounced activation.

3.2.5. Lysophosphatidic acid microinjections do not significantly alter electrical and mechanical thresholds of C fibers

3.2.5.1. Electrical thresholds

Synthetic interstitial fluid microinjections did not significantly change electrical thresholds of 20 investigated CM and 9 CMi fibers. After LPA microinjections, 15 of 19 LPA-treated CM fibers displayed unchanged or slightly higher electrical thresholds, with no statistical median difference (before LPA: median 0.2 mA, quartile range 0.2-0.6 mA; after LPA: median 0.25 mA, quartile range 0.25-0.7 mA; Wilcoxon matched pairs test, $P = 0.06$). After LPA microinjections, 5 of 7 CMi units had higher electrical thresholds, but also not to a significant extent (0.7, 0.7-0.9 mA vs 0.7, 0.7-1.3 mA, Wilcoxon matched pairs test, $P = 0.3$). We observed no correlation in change of electrical thresholds with the concentration of applied LPA (0.05, 0.1 mM) or the degree of activation after LPA microinjections.

3.2.5.2. Mechanical thresholds

Neither SIF (n = 14) nor LPA (n = 8) microinjections induced a significant change in mechanical thresholds of the evaluated CM fibers. Slightly increased or unaltered mechanical thresholds were most frequently and comparably observed after LPA (7 of 8 CM fibers) and SIF application (11 of 14 CM fibers).

4. Discussion

In this study, we demonstrate for the first time that LPA activates human C nociceptors and causes, dependent on the application mode, pain and itch in healthy human subjects. Intracutaneous microinjections of LPA result in burning pain, whereas focal epidermal application in heat-inactivated cowhage spicules induces mild to moderate itch. In microneurography recordings of C fibers, we observed a differential activation and responsiveness of CM vs CMi fibers, determined by the application modes of microinjection vs focal administration.

Previous studies in rodents and patients with cholestatic pruritus suggested an involvement of LPA in both pain and itch.[14,23,28–30,44,65] In our human subjects, microinjections of LPA undoubtedly caused burning pain and never an itch sensation regardless of the applied LPA concentrations. This argues against the "intensity hypothesis" which proposed that weakly nociceptive stimuli induce itch. A similar application-dependent switch between pain and itch with the use of microinjections and spicule application was observed with capsaicin.[12, 63, 64]

In previous studies, using inactive cowhage spicules for application of capsaicin, histamine, and BAM8-22, human subjects reported predominant itch sensations with a subdominant component of stinging and burning[62–64]; we obtained similar results with subjects in our study after focal LPA application.

The spatial contrast theory may explain these findings (**Fig. 7**): Focal application of a substance selectively activates single or few nerve fibers in a skin area. The same effect could be achieved when only few nerve fibers express a specific receptor for pruritogens. The contrast between strongly activated and silent nerve fibers transmitting input from virtually the same skin site may centrally be interpreted as itch, possibly involving the mechanism of lateral inhibition.[11,36] The total amount of LPA delivered by spicules is most likely smaller than that by microinjections, but the local concentration around the spicules is higher because they were soaked with a 250-fold higher concentration. Thus, in case of LPA application through spicules, strong activation of few CM fibers in the epidermis may cause itch, whereas flooding larger and deeper skin areas with LPA through microinjections results in pain by activating more fibers, especially the deeper-lying CMi fibers. LPA microinjections cause less CM fiber activity than focal application, possibly because the injection is too deep or the small receptive fields of CM fibers desensitize too quickly.[51] However, not solely spatial contrast of activated vs silent fibers is likely responsible for itch but also other mechanisms may contribute. Our microneurography results in **Figure 5D** reveal a differential ratio of CM vs CMi activation: LPA microinjections activated a greater proportion of CMi fibers strongly (20%) but no CM fibers, whereas focal application of LPA activated 7% of CM strongly but clearly less CMi fibers (12.5%). This difference in specific fiber class input or lack thereof may contribute to the distinction of pain and itch. The observation of more intense activation of CMi fibers through microinjections is in line with our finding that by LPA microinjections a much larger axon reflex erythema was induced compared with focal LPA application, arguing for a more efficient activation of CMi fibers.[34,47,50] Interestingly, the area of erythema we observed with microinjections (~8.9 cm^2) is comparable with the innervation area of CMi fibers in Ref. 55 (median: 5.34 cm^2). However, we cannot exclude that LPA may spread beyond the

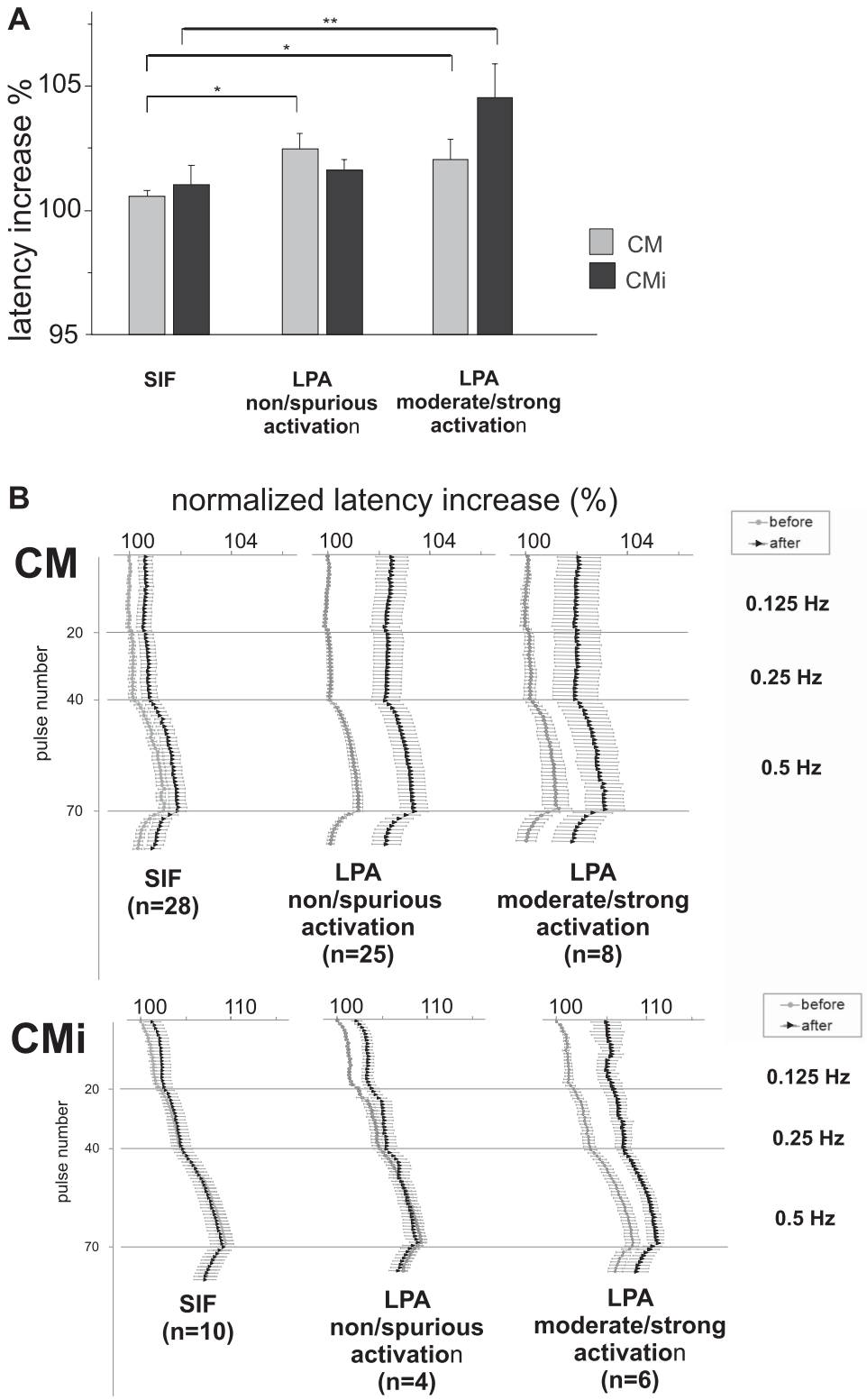

Figure 6. Change in action potential latencies on electrical stimulation after LPA and control microinjections. (A) Baseline latencies are displayed as percentage of their pre-injection value. Compared with SIF microinjection, LPA left slightly increased baseline latencies of CM fibers (gray columns). In CMi fibers (black columns), after LPA vs SIF microinjections, a significant prolongation in mean unconditioned latency was observed if the units had moderately or strongly been activated. Significant differences are indicated by asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$. (B) Mean latencies of CM and CMi fibers during low frequency electrical stimulation at 0.125, 0.25, and 0.5 Hz are shown with time lapse from top to bottom; data are normalized to the latency of the first action potential pre-injection, and gray symbols indicate testing before LPA and black symbols after LPA. Of note, although the relative value of activity-dependent conduction velocity slowing (ADS) after 70 pulses was reduced in CMi fibers, especially in moderately and strongly activated units, no absolute latency reduction was observed because of the significant delay in baseline latency after LPA (shown in Fig. 6A). CM, mechanosensitive C; CMi, mechanoinsensitive C; LPA, lysophosphatidic acid; SIF, synthetic interstitial fluid

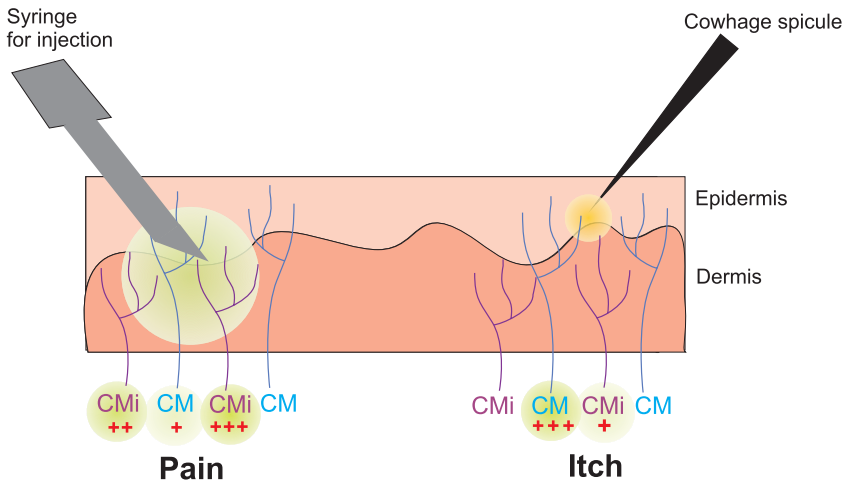

Figure 7. Application mode–dependent C-fiber activation by LPA—a hypothesis. The application of LPA (and other excitatory compounds) through heat-inactivated cowhage spicules deposits a high focal concentration with a steep centrifugal gradient, exciting only few C fibers, preferably mechanosensitive, mostly polymodal ones, at the border of epidermis and dermis. Surrounding and deeper-lying C fibers such as CMi fibers remain silent, causing a high spatial contrast of focal activation decoded downstream as an itch signal. On the other hand, microinjection of LPA excites many C fibers including CMi fibers in a contiguous area of skin with blurred boundaries, which is decoded downstream as a pain signal. CMi, mechanoinsensitive C; LPA, lysophosphatidic acid.

bleb site and activate mast cells, which by degranulation of histamine may contribute to the axon reflex erythema.

When comparing the response magnitude of C fibers on LPA application with other chemical stimuli, ie, active cowhage spicules, we observed a strong activation in very few fibers.[34] Low activation rates, as observed in CM fibers in response to histamine, do not substantially influence conscious sensations of itch.[47,68] The fibers classified as "spuriously" or "moderately" responding likely do not contribute to perceived itch sensation. Strong activation of CM units was exclusively observed after focal LPA application.

The quantitative differences are small, despite statistically significant, and therefore, the interpretation is limited.

Whether pattern coding, eg, bursting in strongly activated CM, contributes to itch or pain distinction cannot be made because of our limited analyzing techniques in microneurography. Indication for spatiotemporal coding comes from a recent study on a genetically defined intraepidermal C-fiber subpopulation expressing MrgprA3 Gq-protein-coupled receptors. Their metabotropic activation by the pruritogen chloroquine, probably evoking low-frequency asynchronous discharges, induced hind paw scratching in the nape, whereas optogenetic (ionotropic) activation, presumably evoking high-frequency synchronous discharges in the very same fibers resulted in various pain-like nocifensive behaviors.[56] Like CM fibers, these MrgprA3$^+$ fibers respond to capsaicin as well as to inserted cowhage spicules, supporting the classical view that nociceptors can mediate itch, and vice versa, that pruriceptors can signal pain. On the other hand, CMi fibers are not a homogenous group: A small subgroup of CMi fibers heavily activated by histamine are believed to form a labelled line for itch,[52] and LPA can activate mast cells releasing histamine.[13] We found that LPA microinjections activated predominantly these histamine-responsive CMi fibers. However, the number of tested fibers was low, limiting the power of our conclusion. We speculate that activation of histamine-responsive CMi fibers alone would induce itch, but additional recruitment of histamine-irresponsive CMi and CM fibers results in pain,[53] as also observed with histamine injections into deeper skin layers and after bradykinin-induced sensitization to histamine.[21,26,53]

One factor not investigated here is the involvement of Aδ fibers, on which little data are available regarding chemical responsiveness or termination site in human skin.

The involvement of TRPV1 and TRPA1 in LPA-mediated effects in the nociceptive system is still under debate.[19,23,38,45] In humans, we observed that neither pharmacological TRPV1 nor TRPA1 blockade, which were recently validated in human skin,[57] reduced LPA-induced pain. This supports the hypothesis that neither TRPV1 nor TRPA1 alone play a crucial role in acute LPA-evoked pain. By contrast, we found that heat hyperalgesia evoked by LPA was prevented by TRPV1 and TRPA1 blockers. Similarly, a mismatch between PGE_2-mediated sensitization of proton-induced pain and heat sensitization has been observed.[37] Intracellular signal transduction in nerve endings, actuated by LPA, could sensitize TRP channels to heat,[23] causing a drop in heat activation threshold, independently of induction of acute chemical pain through, eg, the LPA1 receptor or indirectly through Schwann cells.[45,65]

Interestingly, in a recent study on mouse dorsal root ganglia, extremely few neurons were activated by LPA, but calcium imaging revealed vast activation of satellite glia and Schwann cells.[45] C fibers are unmyelinated, but their terminal axon is embedded in a Schwann glia cell, which forms a fine process joining the axon through the epidermal junction up into superficial skin layers.[5] It has recently been shown that these specialized "nociceptive Schwann cells" as well as keratinocytes play an active role in transducing noxious thermal and mechanical stimuli, transmitting an excitatory signal, perhaps ATP, to the joined axon.[1,46] Intriguingly, in the dermo–epidermal juncture, where these Schwann cell axon formations are most prevalent, cowhage spicule insertion caused maximal itch sensation.[1,21,61] It may be hypothesized that these Schwann cells, which express G-protein-coupled LPA receptors,[42,66] also play an active role in LPA-induced pain and itch in humans.

In psychophysics and microneurography, we found discrete hints for an LPA-induced increase in nerve fiber excitability, which outlasted the overt excitatory effects. The augmented painfulness of higher-frequency electrical stimulation may indicate an improved conduction safety across the branch points of the C-fiber arborizations in the skin. The voltage-gated sodium channel $Na_v1.8$, perhaps a target of LPA,

could sustain higher firing frequencies.[22] The slight reduction found in ADS of C fibers could be interpreted as a sign of hyperexcitability, leading to enhanced synaptic transmission[7]; but this effect was minor compared with studies using lidocaine microinjections or nerve growth factor.[20,40]

7.1. Implications for cholestatic itch and neuropathic pain

The question arises, why would cholestatic patients not suffer from pain through LPA? The LPA concentration required for contiguous nociceptor activation may not be reached; it might just suffice to activate clusters of CM or CMi fibers anywhere in the body. In addition, synergistic effects of LPA with other accumulating mediators, ie, bile acids might play a role. Altered fiber properties caused by chronic cholestatic conditions could enhance spatial contrast. Chronic scratching can cause loss of intraepidermal nerve fibers in lesional skin[56] so that spatial contrast between activated and lost nerve terminals becomes even more probable. No painful human disease is known to be associated with increased LPA levels. The nature of the excitatory effect of LPA—relevant for neuropathic pain—could not be elucidated because TRPV1 and TRPA1 channels seem only to be responsible for a mild heat hyperalgesia.[18]

However, LPA is present in the cerebrospinal fluid, and in rodents, intrathecal injection of LPA induces neuropathic pain-like symptoms, indicating a profound reorganization of the spinal dorsal horn circuitry called spinal or central sensitization.[18] Whether this spinal sensitization circuitry exists in cholestatic patients with chronic pruritus remains to be investigated.[2]

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B414.

Supplemental video content

A video abstract associated with this article can be found at http://links.lww.com/PAIN/B415.

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