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A Dominant Role of Acid pH in Inflammatory Excitation and Sensitization of Nociceptors in Rat Skin, *in vitro*

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A major role of local acidosis in long lasting excitation and sensitization of cutaneous nociceptors has recently been demonstrated. In inflamed tissue, acid pH meets with a mixture of inflammatory mediators which, by themselves, stimulate nociceptors though being subject to profound tachyphylaxis. We have mimicked this condition in a rat skin-saphenous nerve preparation *in vitro* which allows direct application of chemicals to the isolated receptive fields at the corium side. Stimulant solutions used were CO_2-saturated "synthetic interstitial fluid" (CO_2-SIF, pH 6.1), an "inflammatory soup" (IS) in submaximal concentration containing bradykinin, 5-HT, histamine, prostaglandin E_2 (all 10^{-6} M in SIF at 38.5°C and pH 7.0), and a combination made of CO_2-saturated IS (CO_2-IS, pH 6.1). Identified mechano-heat sensitive ("polymodal") C-fiber terminals (n = 36) were treated with these solutions for 5 min at 10 min intervals or for 30 min of sustained stimulation: 20 units responded to CO_2-SIF, 12 to IS, whereas 27 units (75%) were excited by CO_2-IS. Thus, 6 out of 15 units insensitive to either of the two basic solutions were stimulated by their combination. This enhanced effect of CO_2-IS was also expressed in shorter latencies (than with CO_2-SIF) and in a significantly larger mean response magnitude of the fiber population: 152 spikes with the combination versus 45 spikes evoked by IS and 93 spikes by CO_2-SIF (n = 25; $p < 0.002$ and < 0.02, respectively, Wilcoxon test). The synergistic interaction between CO_2 and IS also showed up during sustained nociceptor stimulation (30 min) by either CO_2-SIF (n = 7) or IS (n = 1) when, during the middle 10 min, CO_2-IS was applied which significantly increased the discharge.

There is a strong, potentially algogenic, interaction between acid pH and inflammatory mediators in terms of prevalence and magnitude of nociceptor excitation. At equal and pathophysiologically relevant concentration, however, hydrogen ions play a dominant role.

[Key words: pain, protons, inflammatory mediators, cutaneous, sensory nerve endings, primary afferents]

Since von Frey (1896) concluded that "pain receptors" ought to be chemoreceptive in nature, scientists went in search of mediators of inflammatory pain. Although a general chemical mediator of pain has not been identified, several endogenous agents have been found in inflammation and were shown to excite nociceptive nerve endings (Fjällbrand and Iggo, 1961). With *in vitro* techniques used for the study of afferent nerve endings in mammalian skin, it was shown that the hormonal mediators of inflammation could not excite all nociceptors and were subject to profound tachyphylaxis (Lang et al., 1990). Only a combination of these mediators (bradykinin, 5-HT, histamine, and prostaglandin) in very high concentration (10^{-5} M) was able to excite cutaneous nociceptors *in vitro* for a sustained period of 30 min (Reischl et al., unpublished observations). Considerably lower concentrations of bradykinin (2×10^{-8} M) and prostaglandin (10^{-7} M), at least, have been found in inflamed tissue (see Handwerker and Reeh, 1991).

High hydrogen ion concentrations have been found in inflamed tissue (down to pH 5.4), in fracture-related hematomas (down to pH 4.7), in cardiac ischemia (down to pH 5.7) and in and around malignant tumors (Häbler, 1929; Revici et al., 1949; Peer, 1955; Jacobus et al., 1977). Therefore, local acidosis has been suggested to provide the causal link between these diseases and their related pain (von Gaza and Brandi, 1926; Keele and Armstrong, 1964; Lindahl, 1974). Indeed, electrophysiological experiments in a rat skin nerve preparation *in vitro* showed that pathophysiologically relevant pH values (6.1–6.9 threshold) produced a selective nonadapting excitation of nociceptors and a significant sensitization to mechanical stimulation. Thus, it has been proposed that local acidosis plays a major role in cutaneous pain and hyperalgesia (Steen et al., 1992). However, previous psychophysiological work on the duration of pH-induced pain remained inconclusive since, methodically, the buffering capacity and counterregulations of the intact tissue were not taken into account. Only recently it was demonstrated that continuous intracutaneous infiltration of low pH buffer (pH 5.2) into human skin is able to induce sustained localized pain without adaptation (Steen and Reeh, 1993).

In inflamed or injured tissue several potent mediators meet in the interstitial fluid and form an inflammatory exudate. Bradykinin (BK) results from enzymatic actions on a plasma protein precursor, serotonin (5-HT) is released from mast cells (in the rat) and from activated thrombocytes and potentiates the excitatory action of BK on nociceptors (Fock and Mense, 1976; Lang et al., 1990). Histamine (HIS) is set free from mast cells and from invading basophilic leukocytes; its weak action on nociceptors is amplified by preceding BK (Koppert et al., 1993). The mediators cooperate in triggering synthesis of eicosanoids which, in turn, enhance the inflammatory symptoms vasodilatation and plasma extravasation and attract inflammatory blood

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cells. These leukocytes actively pump lactic acid into the exudate lowering the pH (McCarty et al., 1966) which, by itself, is a potent nociceptive stimulus. The open question in this respect is whether the different mediators would combine their effects in exciting nociceptors. Such synergism had previously been demonstrated for some of the above tissue hormones (Lang et al., 1990; Kessler et al., 1992) forming an experimental "inflammatory soup" which, however, did not contain high proton concentration (just pH 7.0). It was the aim of the present study to evaluate possible interactions between inflammatory mediators and low pH. The experiments were designed to quantify the role of equal and of pathophysiologically relevant concentrations of the compounds.

Some of the results have previously been communicated as abstracts (Reeh et al., 1991; Steen et al., 1991).

Materials and Methods

Preparation. A rat skin nerve preparation *in vitro* was used which provided control over the chemical environment of nociceptive nerve terminals and allowed conventional single fiber recordings from identified primary afferents. This has previously been described in detail (Reeh, 1986). The preparation was taken from 28 male Wistar rats (350–550 gm body weight) anesthetized with sodium thiopental (120 mg/kg, i.p.) and killed by an intracardial injection of 2 ml of lidocaine after the dissection. The saphenous nerve, together with the complete skin flap of the dorsal hind paw and of the lower third of the leg, was subcutaneously excised and superfused in an organ bath with a "synthetic interstitial fluid" (SIF; Bretag, 1969), a solution consisting of 107 mM NaCl, 26.2 mM $NaHCO_3$, 9.64 mM sodium gluconate, 5.5 mM glucose, 7.6 mM sucrose, 3.48 mM KCl, 1.67 mM NaH_2PO_4, 1.53 mM $CaCl_2$, 0.69 mM $MgSO_4$ at 32°C (±0.5°C), continuously bubbled with carbogen (95% O_2 and 5% CO_2), which leads to pH 7.4. While one preparation was stored at 4°C in the electrolyte solution to be used later in the day, the other skin flap was mounted, epidermal side down, in the tissue chamber and which was perfused with oxygenated SIF (14 ml/min). The saphenous nerve was drawn through a hole into a second chamber where small filaments were split and further subdivided until single unit activity could be recorded monopolarly via gold wire electrodes. For that, the aqueous solution was overlaid by a layer of paraffin oil and a reference electrode located in the organ bath nearby.

Identification and characterization of C-fiber activity. The innervation of the skin was tested with a blunt glass rod mechanically searching for the receptive field of a nerve fiber. The nerve endings localized were electrically stimulated with Teflon-insulated steel microelectrodes to measure conduction velocity and to establish the identity of mechanically and electrically evoked action potentials with the "collision technique" (Iggo, 1958). The thresholds to punctuate mechanical stimulation were tested with a set of von Frey hairs made from nylon filaments with uniform tips (0.9 mm diameter) and calibrated in the form of a geometric series ($x_i = x_{i-1} \cdot \sqrt{2}$) ranging from 1 to 362 mN.

In order to test the heat sensitivity, a halogen bulb was posted below the translucent bottom of the skin chamber to apply radiant heat stimuli onto the epidermal surface of the receptive field. On the corium side, the temperature was feedback-controlled with a thermocouple and, for the purpose of stimulation, was raised in form of a ramp from 32°C to 46°C over a period of 20 sec which corresponds to a rise from 32°C to 52°C at the epidermal surface (Reeh, 1986). To prevent measuring errors due to thermal convection, a metal ring was placed over the receptive field and the fluid in the ring was sucked off. For cold stimulation, the same ring was filled with cold SIF (4°C), and the time course of the temperature was monitored.

Chemical stimulation and experimental protocol. To restrict the spread of agents during chemical stimulation, the receptive fields were isolated using metal rings with inner diameters of 6.6–9.6 mm (height 7.7 mm) which comprised volumes of 0.3–0.6 ml and were perfused at 38.5°C with a turbulent flow of 2.25 ml/min. When the perfusion was switched from normal SIF to stimulating solutions or vice versa, the ring chamber was emptied just prior to the arrival of the new fluid in order to provide an instantaneous change of the solutions. The chemical stimulations followed experimental protocols which are displayed along the abscissae of the figures and had proven suitable in previous works (Kessler et al., 1992; Steen et al., 1992). For short-term stimulation, a 5 min period of superfusion with acid pH (CO_2-saturated SIF; pH 6.1) was followed by a washout interval of 10 min, in which period the afterdischarge had faded away in all cases. A 5 min period of stimulation joined using a broad mixture of inflammatory mediators ("inflammatory soup," IS, containing bradykinin, 5-HT, histamine, and prostaglandin E_2, all 10^{-6} M) and this was followed by 10 min washout. Finally, a combination of low pH and the "inflammatory soup" (CO_2-IS; pH 6.1), was applied during 5 min and was followed by a final washout period (see Fig. 1 for examples). In a few cases the order of stimulation was changed by starting with IS, followed by CO_2-SIF, and finally CO_2-IS was applied. For long-term stimulation during a period of 30 min, CO_2-saturated SIF (pH 6.1) was applied and inflammatory mediators were added (CO_2-IS) during the middle 10 min period. In a reverse long-term protocol, a 30 min stimulus of IS was interrupted by CO_2-IS during the middle 10 min period (see Fig. 4).

The IS was made up of prostaglandin E_2 (SERVA, freshly prepared from an oxygen-free stock solution containing 0.3% ethanol), histamine triacetate (Sigma), 5-HT hydrochloride (Sigma), and bradykinin triacetate (Sigma) which were dissolved in SIF at concentrations of 10^{-6} M; the pH was titrated to 7.0 with HCl, and the potassium concentration was raised to 7 mM K^+ by adding KCl (for consistency with Kessler et al., 1992). This solution was applied at 38.5°C. For acidification, SIF or IS were continuously gassed with pure CO_2 which led to a constant pH of 6.1 (Steen et al., 1992).

In the previous work it has been shown, by using control stimuli (oxygenated phosphate puffer, pH 6.1), that low, probably intracellular pH is the specific stimulus for the excitation of nociceptors; the acid phosphate buffer excited an identical fiber-population and induced an equal magnitude of nociceptive response as the CO_2-saturated solutions of the same pH. A dose–response curve has been achieved by varying the pH of the phosphate buffered solution. The acidification by means of CO_2 led to a comparatively reduced latency of the response by easier penetration through the neuronal membrane, and the effect was effectively antagonized by using the carboanhydrase blocker acetazolamide (Steen et al., 1992).

Criteria of responsiveness of fibers. All units had to be silent in order to be included in this study. Therefore, an occasional spontaneously active fiber, occurring mostly as a result of heat stimulation, was not further investigated. A unit was regarded as responsive if the evoked discharge of the fiber could be washed out after chemical stimulation, and if the response was reproducible using the same agent (IS or CO_2-SIF) or the combination (CO_2-IS). In this study, we did not follow any arbitrary response criterion of a minimum discharge rate exceeded, since 10^{-6} M IS concentration had to be regarded as a threshold concentration for many units. Hence, in some cases the discharge rate was below the threshold to induce a pain sensation (1 spike/sec) in microneurography studies in human subjects (Handwerker et al., 1991). The magnitude of the responses to the different stimulations was assessed as the total number of spikes counted during a sampling period of 15 min after stimulus onset, that is, 5 min of stimulation and subsequent 10 min of the washout period. Therefore, "spikes/15 min" (in Figs. 2, 3) does not mean an average discharge rate but rather the total number of spikes per response; the actual duration of the response was usually much shorter. For the long-term stimulation, which required a period of 30 min and a subsequent washout phase of 10 min, we counted the total number of spikes during these 40 min.

Data analysis. The single nerve fiber activity was recorded, amplified, and passed through a window discriminator, and the normed pulses were recorded on line on an AT 386 type computer using the CED 1401 interface and SPIKE 2 software (CED, Cambridge, England). The SIGMAPLOT software package was used for supporting graphical presentation of the data (Jandel, Berkeley, CA). For statistical analyses, the Complete Statistical System (CSS, Statsoft, Tulsa, OK) was employed; to compare the different chemical responses within the fiber groups statistically the Wilcoxon matched pairs test was used, and its p values are presented in the figures.

Results

Population of afferent units

The categorization of the units was based on established criteria of sensory properties and of conduction velocities found in nerves to rat hairy skin (Lynn and Carpenter, 1982; Fleischer et al., 1983). In this study, receptive fields of 47 primary afferents were superfused with a combination of inflammatory mediators,

Table 1. Chemosensitivity of C-fibers

C-Fibers (n = 47)	MH	HTM	Others
Fibers tested	36	7	4
Resp. to CO_2 (pH 6.1)	20	2	0
Resp. to IS (10^{-6} M)	12	2	0
Resp. to CO_2-IS	27	2	0

MH, Mechano-heat sensitive, "polymodal"; HTM, high-threshold mechanosensitive; "Others" were mechano-cold and low-threshold mechanosensitive C-fibers. CO_2, "synthetic interstitial fluid" (SIF) gassed with pure CO_2. IS, "inflammatory soup": bradykinin, 5-HT, histamine, prostaglandin E_2 in SIF. Note that acidified "inflammatory soup" (CO_2-IS) excited a number of polymodal nociceptors (n = 7) that had previously been insensitive to both chemical stimuli (CO_2 and IS).

"inflammatory soup" (IS), CO_2-saturated "synthetic interstitial fluid" (CO_2-SIF, pH 6.1), and a combination of both (CO_2-IS, pH 6.1). The selection of single units was biased towards slow conducting nociceptive fibers. Therefore, the numbers of fibers shown in Table 1 are not representative for their relative frequency in rat skin. Since it has been shown before that rapidly adapting Aδ and all types of Aβ fibers are not excited or sensitized by acid pH (Steen et al., 1992) or inflammatory mediators (Kessler et al., 1992), they have not been included here. In pilot experiments, such fibers (n = 4) have proven to be also insensitive to the combination of substances (CO_2-IS).

We identified 36 of the 47 C-fibers as mechano-heat sensitive (C-MH, "polymodal") with conduction velocities (CV) ranging from 0.22 to 0.70 m/sec and mechanical (von Frey) thresholds from 8 mN to 90.5 mN. Seven C-fibers were named high-threshold mechanosensitive (HTM) since they were not sensitive to heat and cold stimulation and the von Frey thresholds were beyond 5.6 mN (up to 32 mN in our sample). The CV of these fibers ranged between 0.47 and 0.66 m/sec. One C-fiber was not sensitive to heat and cold-stimulation, had a von Frey threshold of 1 mN and was therefore categorized as a low-threshold-mechanoreceptor (LTM). Two mechanosensitive C units responded to mechanical and cooling but not heat stimuli (C-MC; von Frey thresholds, 1 and 64 mN) and one unit responded to cooling only (C-cold).

Chemosensitivity

None of the C-MC, C-cold, or C-LTM units responded to either CO_2-SIF or IS. Within the population of the seven C-HTM-fibers tested (Table 1), two units were excited by IS, by acid pH, and responded to the combination (CO_2-IS). Six C-fibers (MH and HTM) developed very low frequency irregular discharge (<12/min) during acid pH superfusion, which could hardly be washed out and which did not increase during CO_2-IS stimulation. This response pattern was previously referred to as "activated" (Steen et al., 1992). These fibers could not be identified by any other sensory properties. In this study those fibers were regarded as nonresponsive to chemical stimulation and were added to the respective group in Table 1.

In contrast, from 36 mechano-heat sensitive ("polymodal") units tested (5 min and/or 30 min stimulation protocols) 20 responded to CO_2-SIF superfusion with a duration-dependent vigorous discharge. Twelve units of the 36 were excited by IS (10^{-6} M) and all but one of these were also driven by CO_2-SIF. Fifteen of the 36 C-MH fibers did not fire any spikes in response to either CO_2-SIF or IS, but a significant number of these (n = 6/15) were finally excited by the combination (CO_2-IS; pH 6.1). Altogether, 27 of the 36 units (75%) were driven by the acidified IS, including all those that had previously been responsive to CO_2-SIF, IS or both. We found a cross-sensitivity of polymodal (C-MH) nociceptors to acid pH and inflammatory mediators (IS) which was obviously restricted by the fact that the IS was used at 10^{-6} M in a submaximal concentration. Thus, the relatively small population of IS-sensitive C-MH fibers was almost completely contained in the larger population of the pH-sensitive ones, and this was part of the even larger group of units stimulated by acidic IS (Table 1). Out of 36 units, a total of 33 fibers underwent a protocol with 5 min stimulation periods; of the latter, five units received a long-term protocol in addition, and another three units were only treated with long-term stimulation for 30 min.

Comparison of excitatory potency

To compare the excitatory potency of protons and inflammatory mediators, both were applied in almost identical concentration, and we assessed the total discharge activity within the stimulation period of 5 min and the subsequent washout interval of 10 min irrespective of the actual duration of the response which was usually shorter. All 33 fibers were treated with each of the three test solutions (see Materials and Methods). In most experiments (29 CMH fibers) the chemical stimulation protocol started with 5 min CO_2-SIF superfusion, followed by IS and then by the combination, CO_2-IS, since CO_2 effects were known to be rapidly washed out (Steen et al., 1992). In four cases, however, the protocol was started with IS, but the order of stimulation did not obviously influence the outcome as afterdischarge following IS had always faded during the 10 min interval between the chemical superfusions. Figure 1 shows specimens of three individual fibers which demonstrate the three observed patterns of synergistic interaction between low pH and IS. (1) fibers which responded moderately to both solutions showed more than additive excitation by the combination. (2) In other fibers, a low frequency of discharge following pH stimulation was enhanced by the additional presence of the inflammatory mediators; however, the IS alone was not able to excite the units. (3) The mutual interaction was most impressive in cases in which the single chemicals were not able to excite the nociceptive unit, but a rather clear response was induced by the combination.

The full range of individual response magnitudes of the population of polymodal nociceptors can be seen in Figure 2. The acidified "inflammatory soup" showed the highest excitatory potency; the "gothic" appearance of the figure in general and of the median in particular illustrate the relative efficacy of the solutions in driving nociceptors: CO_2-IS > CO_2-SIF ≫ IS. Superfusion of the receptive fields with CO_2-IS was most effective in all (n = 19) but three cases, and CO_2-SIF was more effective than IS in all (n = 18) but one case.

The individual data displayed in Figure 2 has been pooled in two different groups of CMH-subpopulations in order to set up Figure 3. One category (Fig. 3*A*) encompassed the fibers responsive to CO_2-IS, the strongest stimulus, and the other category (Fig. 3*B*) included the particularly chemosensitive units responding to either of the superfusions. While Table 1 showed the higher prevalence of responsiveness upon CO_2-IS stimulation (including sustained-stimulation protocol), Figure 3 statistically demonstrates that the combined solution is more potent regarding the average magnitude of the excitatory effect. On average (Fig. 3*A*), we achieved 152 spikes with the combination (CO_2-IS) versus 45.5 spikes evoked by IS and 93 spikes by CO_2-

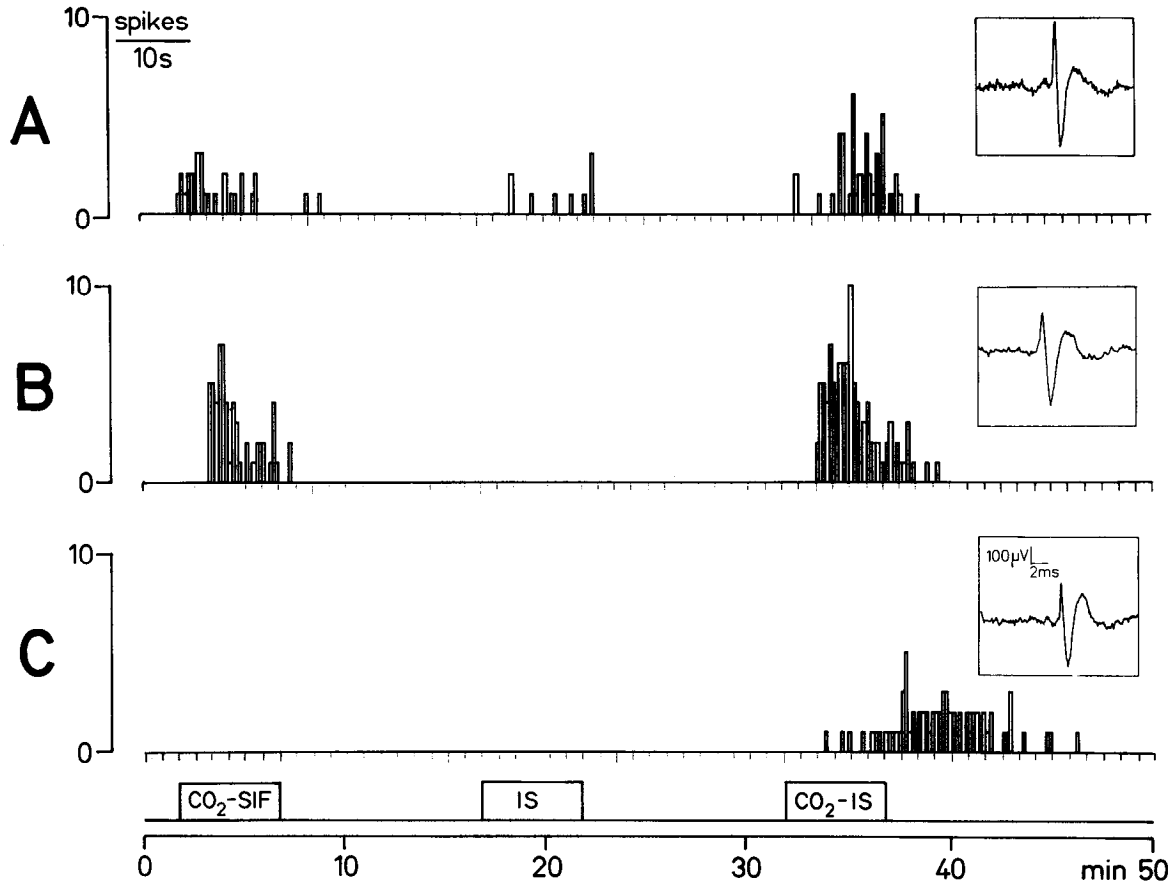

Figure 1. Specimen records (PST-histograms) from polymodal nociceptors displaying the three patterns of chemical responsiveness encountered. *A*, A unit responding to low pH (CO_2-SIF, pH 6.1) and to inflammatory mediators but showing strong response to the combination (CO_2-IS). *B*, A unit responding to low pH, not to inflammatory mediators but with an increased discharge to the combination. *C*, A unit insensitive to low pH and inflammatory mediators but responding to the combination. The *insets* display the action potentials as recorded from thin filaments of rat saphenous nerve, *in vitro*.

SIF. The differences in response magnitude (number of spikes) were significant. In contrast, in the constrained fiber population (Fig. 3*B*) the CO_2-SIF (192 spikes) and CO_2-IS (255 spikes) response magnitudes were not significantly different, but the difference in response magnitude between IS (74 spikes) and CO_2-IS became even more prominent. This again, indicates a relatively low weight of IS (10^{-6} M) in combination with acid pH. The acidification of IS increased the response magnitude of the particularly chemosensitive units by more than 300%, on average, and decreased the individual response latency in almost all cases [$\bar{x}$ = 49 sec (SEM 6.4) to 35 sec (SEM 7.2)].

Sustained stimulation

Five fibers which underwent the short-term protocol first and proved to provide particularly stable recordings were finally used for sustained chemical stimulations in order to demonstrate long-term effectiveness and lack of tachyphylaxis. In order to be selected, these fibers had to be mechanically excitable, had to provide stable spike amplitudes and had not to show ongoing activity. In three other fibers, long-term stimulation with acid pH for 30 min was employed without previous 5 min stimulations. In the long-term protocol, during the middle 10 min period, the receptive fields were superfused with CO_2-IS (Fig. 4*A*,*B*). One fiber was treated with 20 min IS stimulation and CO_2-IS during the middle 10 min period (Fig. 4*C*). The combination CO_2-IS increased the mean discharge during the ten-minutes period significantly [$n = 7$; $p < 0.02$ (Wilcoxon)]; there was no significant difference between the first (before CO_2-IS) and the second (after CO_2-IS) 10 min period of CO_2-SIF stimulation. Three typical patterns of responsiveness—essentially the same as in Figure 1—were observed in these experiments and are displayed in Figure 4.

As a specimen, Figure 4*A* shows a CMH-unit which had previously not responded to 5 min stimuli of low pH and of IS. Even during sustained application of pH 6.1 (CO_2-SIF) no activity evolved. However, during superfusion of the receptive field with acidified IS (CO_2-IS) a tonic low frequency discharge developed and later faded when the inflammatory mediators were again omitted from the superfusate. This unit is the same one as displayed in Figure 1*C*. Another unit, shown in Figure 4*B* had previously not responded to inflammatory mediators (5 min) but developed a low frequent discharge during sustained application of low pH. When inflammatory mediators were added to the acid solution (pH 6.1), the activity was greatly enhanced but subsided when the inflammatory mediators were washed out with CO_2-SIF. Figure 4*C* shows the response of a unit that had previously revealed a poor sensitivity to low pH (5 min). It responded to the sustained superfusion with inflammatory mediators but, due to the typical tachyphylaxis, the effect was lost within 5 min. However, acidification of the superfusate more than recovered the activity quickly, and later it subsided rapidly when the pH was neutralized again. It appears with the

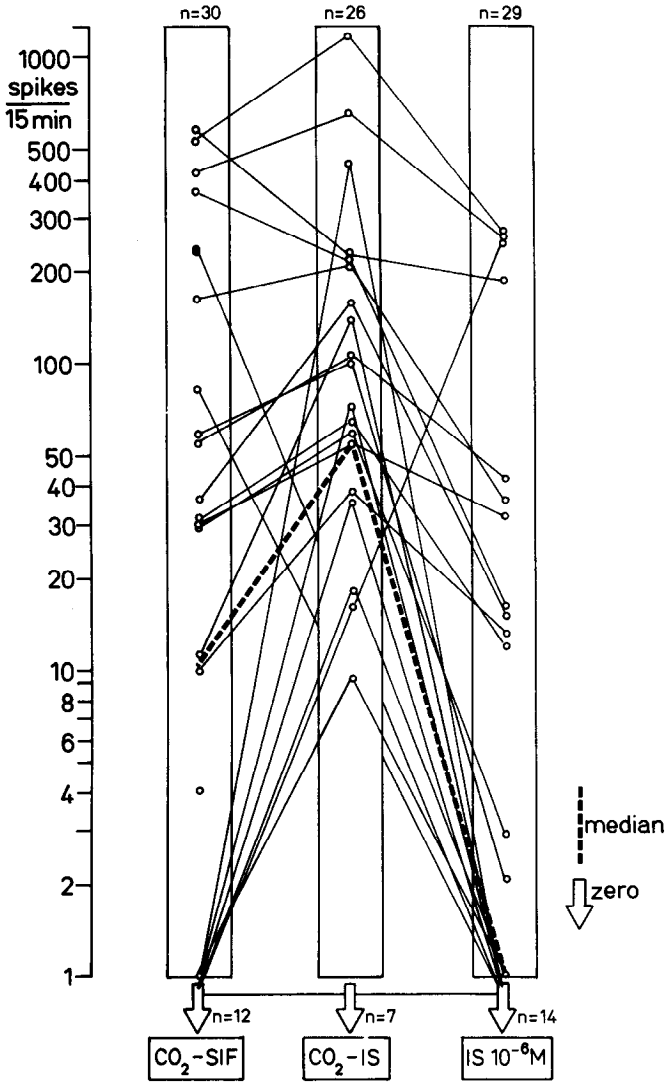

Figure 2. Individual response magnitudes of polymodal nociceptors (C-MH fibers) stimulated by superfusion of different chemical solutions for 5 min. The *open arrows* point to a number of units (*n*) not responding to the particular stimulus. The connecting lines refer to individual units receiving two or, as mostly, three superfusions applied, mostly, in the order CO_2-SIF/IS/CO_2IS at 10 min interval. The *dotted line* shows the median responses. Results from statistical analyses based on this individual data are given in Figure 3.

latter pattern of responsiveness that low pH was a necessary but not sufficient condition for excitation, becoming sufficient only through a sensitizing effect of the inflammatory mediators. In Figure 4*B* (and *A*), this sensitizing effect seems to outlast the presence of inflammatory mediators for several minutes and hence to maintain the pH-induced discharge for a few further minutes.

Discussion

It has previously been shown that pathophysiologically relevant hydrogen ion concentrations are able to excite and sensitize cutaneous nociceptors, *in vitro*, in a sustained manner (Steen et al., 1992). On the other hand, an ample combination of inflammatory mediators, bradykinin (BK), 5-HT, histamine (HIS), and prostaglandin E_2 (PGE_2), could also excite these nociceptors despite a certain tachyphylaxis or adaptation (Kessler et al., 1992). Thus, it was obvious to study whether protons interact with mediators of inflammation in increasing and/or prolonging the nociceptor discharge, which turned out to be the case.

The combination of substances used as "inflammatory soup" (IS) in this study is somewhat arbitrary since possibly important inflammatory agents (e.g., cytokines and nucleotides) or chemical properties (e.g., hyperosmolarity) were not taken into account. Also, the time courses of appearance and disappearance of mediators in different stages and types of inflammations were not accounted for. Nevertheless, the model had previously, using substance P, proven suitable in detecting synergistic interactions between single and combined mediators and in evaluating the relative significance of one additional mediator in the inflammatory exudate (Kessler et al., 1992).

Because of the well-established sensitivity of small afferent fibers to inflammatory mediators (Kumazawa et al., 1987; Neugebauer et al., 1989; Lang et al., 1990; Kessler et al., 1992; Khan et al., 1992; Steen et al., 1992), we exclusively examined (47) C-fiber units. According to a previous study *in vitro*, hydrogen ions selectively excite "polymodal" and "high-threshold mechanosensitive" C-fibers (Steen et al., 1992). The categorization into HTM and MH C-units was limited by the intensity of heat stimulation (52°C, epidermal) to the extent that some fibers, now categorized into the HTM group, might have responded to higher stimulus temperatures and were therefore MH nociceptors in reality (Fleischer et al., 1983). Since both groups were treated in the same way in the experiments (and responded about equally to chemicals), the results are not affected. In the present study, we investigated the particularly chemosensitive subpopulation of C-units, 36 "polymodals," and found 20 (55%) responsive to low pH (6.1) which was produced by CO_2 gassing of the superfusate. In terms of prevalence, that is, number of units excited, BK (10^{-5} M) and low pH (6.1) are the most effective single agents, each driving a similar percentage of the polymodals (Lang et al., 1990; Steen et al., 1992). Kessler et al. (1992) investigated 72 polymodal C-units and found 80% excited by IS in 10-fold higher concentration (10^{-5} M) than used in this study which found 33% of the CMH units to be excited with IS 10^{-6} M. The 10^{-5} M concentration of IS led to a sustained excitation of the responsive nociceptors, whereas IS in a 10^{-6} M concentration caused an adapting discharge of the units (see Fig. 4*C* and Reischl, Steen, and Reeh, unpublished observations). In this study, we used IS in this submaximal (10^{-6} M) concentration to avoid occlusion ("ceiling") of the effects which tend to be maximal at 10^{-5} M concentration (Lang et al., 1990). Beside that, a micromolar concentration of hydrogen ions is also the result of pH 6.1; protons and inflammatory mediators would thus be compared at about equimolar concentrations. This proton concentration had previously been shown to recruit all pH-sensitive polymodal C-fibers (Steen et al., 1992).

A major finding was that the acidification of the "inflammatory soup" (CO_2-IS) caused 75% of the nociceptive nerve fibers to discharge, and these responses were sustained for as long as the solution was superfused. Relative to the concentration, CO_2-IS was the most potent of all chemical combinations so far tested in the skin-nerve preparation. A significant number of units which were insensitive to acid pH as well as to IS (6 out of 15 units) were excited by the combination. The acidification of IS significantly increased the response magnitude by more than 300% on average.

Which component of the IS is the most likely agent to interact with acid pH? It has been shown that PGE_2 and HIS do not play

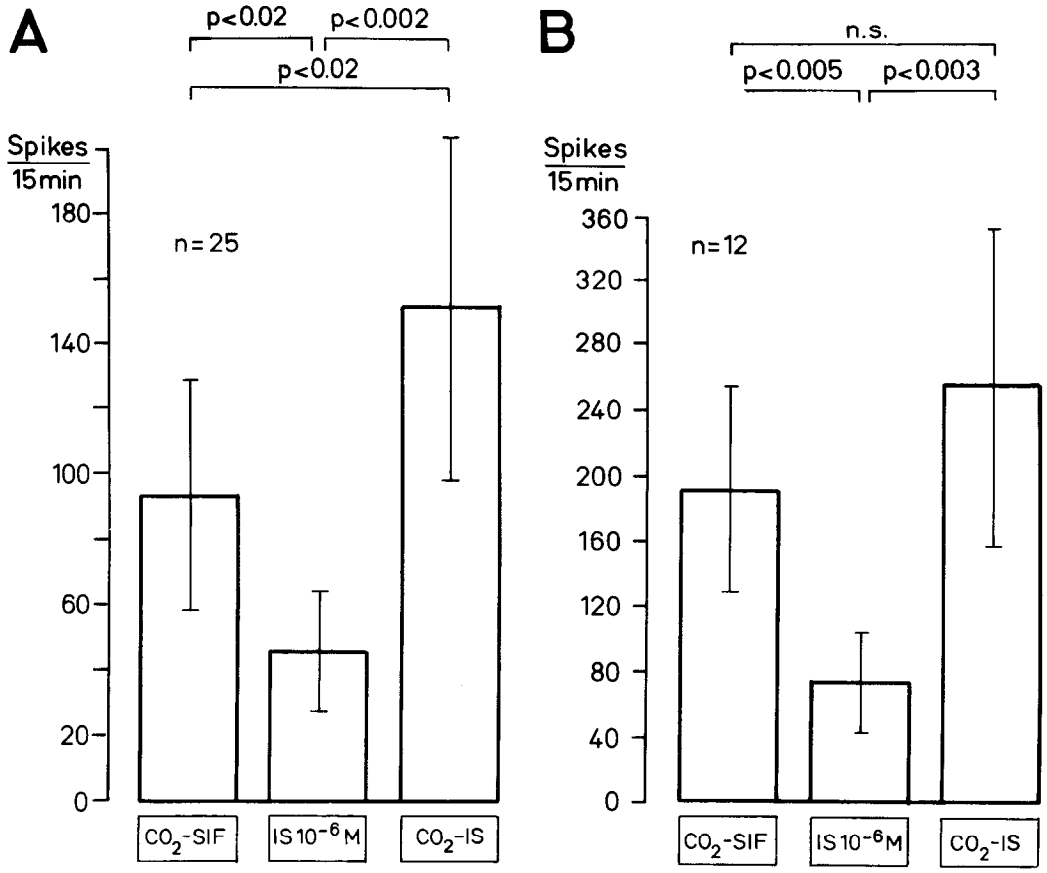

Figure 3. *A* shows the population of C-MH fibers which were sensitive to CO_2-IS (*third column*). It compares the response of these fibers with their responses to CO_2 (*first column*) and to IS (*second column*). The ordinate reproduces "spikes/15 min," which means total number of spikes counted during 15 min after stimulus onset including 5 min of stimulation and 10 min washout. *B* shows the subpopulation of particularly chemosensitive C-MH fibers which responded to CO_2 (*first column*), IS (*second column*), and CO_2-IS (*third column*), leaving out all the fibers which did not respond to any one of the stimulus solutions. *p* values are derived from Wilcoxon test.

an important role in the IS (Lang et al., 1990). In the skin-nerve preparation, PGE_2 neither excites nor sensitizes nociceptors, in contrast to the testicular preparation *in vitro* (Mizumura et al., 1987) and to the cat knee joint *in vivo* (Schaible and Schmidt, 1988). HIS can activate only a small population of polymodal C-fibers in our preparation. It did excite these fibers significantly more after BK pretreatment, but its effect did not add to an ongoing BK-induced discharge (Koppert et al., 1993). BK and 5-HT, each excite a proportion of nociceptors, and 5-HT is able to sensitize the nociceptors for the action of BK (Lang et al., 1990). The excitatory effect of IS seems to depend strongly on this sensitization, since the 5-HT_3 antagonist metoclopramide can suppress the IS response reversibly (Handwerker et al., 1990). Thus, there are indications that BK and 5-HT are the most important compounds in the IS and the most likely candidates for an interaction with hydrogen ions.

There are a few, yet vague indications from our data that the interaction of low pH and inflammatory mediators is not really reciprocal:

(1) Only one fiber was found that responded to IS but not to CO_2. Thus, an increase of the IS response by a possible sensitizing (not excitatory) effect of CO_2 could hardly be demonstrated, whereas the reciprocal—increase of CO_2 response by a sensitizing (not excitatory) effect of IS—was a regular finding.

(2) The abrupt time course of the CO_2 effects, in contrast to the slowly developing and outlasting action of the inflammatory mediators in the long-term stimulation experiments, strongly suggest an excitatory role for low pH (CO_2) and a sensitizing one for IS (see Fig. 4).

Indeed, BK is known to have a transient sensitizing action on nociceptors with respect to heat sensitivity (Kumazawa et al., 1991; Khan et al., 1992; Koltzenburg et al., 1992). Further evidence for the above distinction of the roles of pH and IS comes from a recently introduced psychophysiological model of pH-induced pain (Steen and Reeh, 1993). Intracutaneous injection of IS at hardly perceivable concentration (10^{-7} M) left with a marked increase in painfulness of subsequent injections of acidic buffer (Steen et al., 1993). Thus, we tend to conclude that it is the inflammatory mediators that potentiate the excitatory effects of low pH rather than vice versa.

The transduction mechanism of proton induced nociceptor excitation is not yet perfectly clear. It has been suggested that intracellular acidification may be the basis for the excitatory effect of CO_2 because of the nociceptor and lingual nerve responses being inhibited by acetazolamide (Steen et al., 1992; Komai and Bryant, 1993). In patch-clamp experiments on DRG cells, a sustained ionic inward current activated by protons has been demonstrated (Bevan and Yeats, 1991). The same authors suggested that common mechanisms of protons and of capsaicin induce excitation of sensory neurons; however, a complete cross-sensitivity to capsaicin and protons could not be confirmed in saphenous nerve endings (Steen et al., 1992). Petersen and

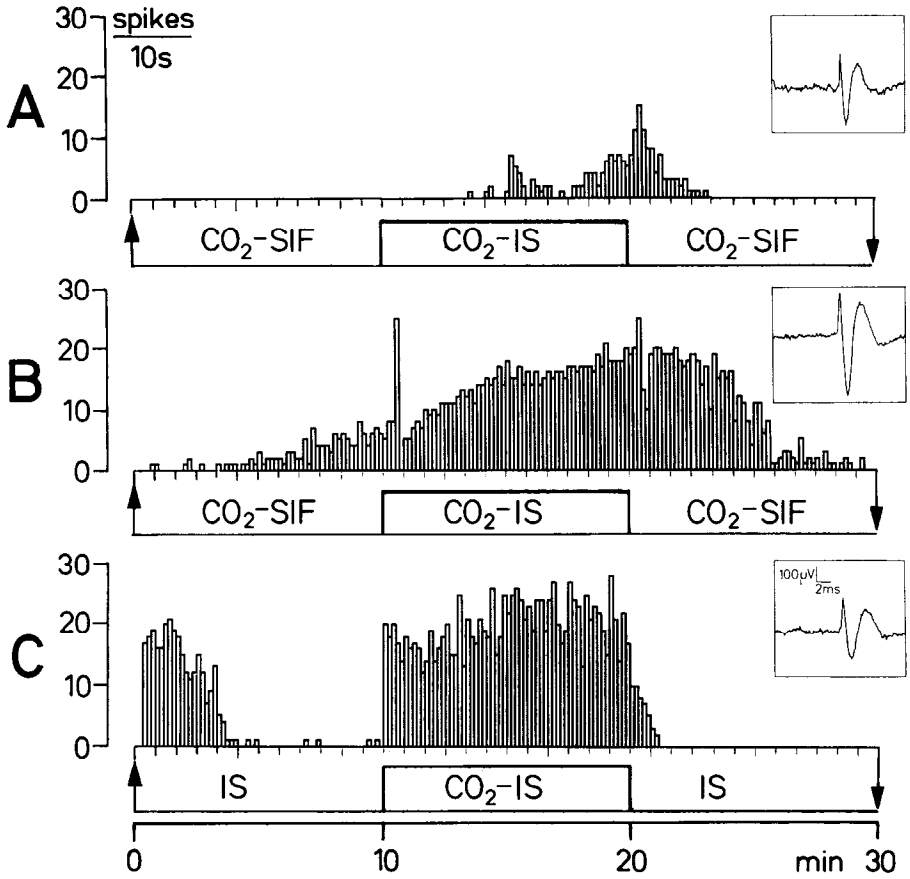

Figure 4. Specimens of prolonged records (PST-histograms) from polymodal nociceptors under sustained chemical stimulation. The *insets* display the action potentials of the particular saphenous nerve fibers. See text for description of the response patterns.

LaMotte (1993) showed an interaction in DRG cells in such a way that acidification of the extracellular medium increased the capsaicin-evoked inward current. BK also evokes an unspecific cationic inward current in these cells, which may relate to its excitatory action on nociceptive afferents (Burgess et al., 1989). On the other hand, BK also acts through G-protein dependent activation of protein kinase C which may convey its sensitizing properties (Dray et al., 1988). This may provide a future working hypothesis to explain the inflammatory potentiation of the pH-induced nociceptor discharge.

The recognition of the excitatory and the sensitizing actions of inflammatory mediators onto nociceptors did not really help to explain ongoing "spontaneous" and recurrent pain from inflamed tissues, since tachyphylaxis turned out to restrict the effects during prolonged or repeated exposure (Kanaka et al., 1985, Kumazawa et al., 1987; Handwerker et al., 1990; Lang et al., 1990; Kessler et al., 1992). Our finding of the sustained nociceptor discharge induced by the local acidosis typical to inflamed tissue filled a logical gap (Steen et al., 1992). However, to cause pain, nociceptor activity must exceed a certain, yet undefined quantity. The prominent synergism between inflammatory mediators and tissue acidosis, concluded from our results, may provide this critical quantity by increasing temporal as well as spatial summation of nociceptive input.

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