Anesthesiology
76:617-623, 1992

Activation of C Fibers by Metabolic Perturbations Associated with Tourniquet Ischemia

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Peripheral A-delta and C fibers are activated during the production of ischemic or tourniquet pain; however, individual metabolic or molecular factors responsible for neural activation are not known. To elucidate these mechanisms the *in vitro* corneal nerve preparation was used. Electrophysiologic effects of individual metabolic perturbations associated with ischemia (hypoxia, hypoglycemia, lactic acid, and decreased *p*H) were investigated on A-delta and C fiber nociceptors. Increased tonic action potential activity occurred in C fibers but not in A-delta fibers after ischemia. The conduction velocity of C fibers was 0.85 ± 0.2 m/s (mean ± SD). Under control conditions (n = 43) there was very little fluctuation in the baseline action potential frequency (± 3.2%). Hypoxia (n = 12) resulted in a 213 ± 3.4% (mean ± SD) increase in C fiber action potential frequency relative to control ($P < 0.001$, ANOVA). L-glucose substitution for D-glucose (n = 8) increased C fiber discharge frequency by 653 ± 28% relative to control ($P < 0.001$) as did the combination of hypoxia and L-glucose substitution (n = 6) by 671 ± 14%. Comparison of hypoxia *versus* hypoxia and hypoglycemia conditions did not show them to be statistically different ($P > 0.5$). Lactate (10–1000 μg/ml) at a *p*H of 6.9 or 7.4 did not alter the action potential discharge frequency in corneal C fibers (n = 5, $P > 0.5$). A-delta fibers had conduction velocities of 2.35 ± 0.16 m/s (n = 10). Neither hypoxia nor hypoglycemia alone or in combination caused these nerves to become spontaneously active, and neither altered spike latency, amplitude, or spike width when electrically or mechanically stimulated. The *in vitro* corneal nerve preparation allows independent study of single metabolic perturbations on A-delta and C fiber electrophysiology and will allow further investigation into the molecular mechanisms of ischemic pain. (Key words: Eye, cornea: hypoglycemia; hypoxia; lactate. Nerve, peripheral: electrophysiology. Pain.)

PAIN IS ASSOCIATED WITH ISCHEMIA, which can result from tourniquet application,[1–4] thrombotic vascular occlusion,[5] or peripheral vascular disease.[6] This type of pain can be very intense and is often resistant to conventional analgesic and anesthetic agents.[2,7,8] The neural elements involved in ischemic pain have not been identified. Electrophysiologic studies have shown that tourniquet-induced limb ischemia can activate otherwise silent populations of myelinated and unmyelinated fibers in a reversible fashion.[9,10] The burning, aching pain present during tourniquet application may correspond to activation of peripheral C fibers, whereas tingling, buzzing sensations are consistent with myelinated fiber activation, which predominates after tourniquet deflation. The molecular mechanisms underlying ischemic neural activation are not known. *In vivo* studies have been limited by an inability independently to control different tissue factors (O_2, *p*H, glucose, and lactic acid) altered by limb ischemia. The *in vitro* corneal nerve preparation[11,12] is exclusively innervated by A-delta and C fiber nociceptors and allows independent evaluation of the electrophysiologic consequences of ischemia-induced metabolic alterations. The contributions of hypoxia, hypoglycemia, decreased *p*H, and increased lactate to peripheral nerve activation were investigated in the present study.

Materials and Methods

Experimental protocols were approved by the Institutional Animal Care Review Committee at Stanford University. Care was taken at all stages of handling to minimize stress and discomfort for the animals. Twenty albino rabbits (New Zealand White, Nittabell Inc.) weighing 2.5–3.0 kg were used in this study. Animals were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) and killed with pentobarbital. The methods used in this study have been described previously and therefore will be briefly presented.[11,12]

TISSUE ISOLATION

The cornea together with 3–5 mm of sclera was rapidly excised and placed over a small Plexiglas ring, attached to a tissue chamber, and continuously perfused with aqueous humor solution (AQH; 2 ml/min) maintained at a constant temperature (35° C) and intraocular pressure (18 mmHg; fig. 1). The external (epithelial) surface of the tissue was exposed to a warmed, humidified atmosphere of 95% O_2/5% CO_2 or the appropriate experimental gas mixture (see below). Nerves were visualized with a variable magnification dissecting microscope (Wild M5A).

ELECTROPHYSIOLOGY

Extracellular single and multiunit recordings of corneal nerve action potentials were obtained using standard electrophysiologic techniques. Suction electrodes (WPI

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Received from the Pain Research Laboratory, Department of Anesthesia, Stanford University School of Medicine, Stanford, California. Accepted for publication December 19, 1991. Supported by the Parker B. Francis Foundation and by National Institutes of Health grant 1RO1NS28646-01A1.

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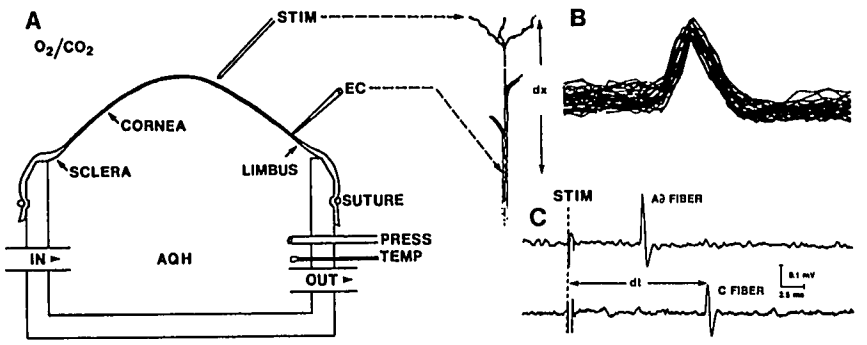

FIG. 1. Isolated cornea preparation and recordings of A-delta and C fiber action potentials for analysis of conduction velocity. *A:* The cornea is maintained at constant temperature (TEMP; 35° C) and intraocular pressure (PRESS; 18 mmHg) and perfused with artificial aqueous humor solution (AQH). A warm and humidified atmosphere of 95% O_2/5% CO_2 surrounds the outer surface to prevent drying of the epithelium, and extracellular recording electrodes (EC) are used to record discharge activity from corneal nerve bundles near the limbus. Nerve bundles can be clearly seen at the limbus, before entering the opaque sclera, and this point affords the maximal distance that can be reliably measured between stimulating and recording sites; this is important for conduction velocity determination (see below). *B:* Superimposed records (58 action potentials) of an isolated C fiber are shown following discrimination from a multiunit recording. Data were collected for 5 s every 20 min for 1 h to demonstrate stability of control discharge activity. *C:* Conduction velocities (dx/dt) for discriminated units were determined by measuring the time delay (dt) between stimulus artifact (STIM) and action potential peak amplitude; this was then divided into the measured distance between stimulating and recording sites (dx). A-delta fibers have a shorter time delay compared with C fibers because the larger axon diameter permits a higher conduction velocity.

Inc., thin wall 1.0 mm OD fiberfill glass, filled with AQH) were placed on corneal nerve bundles near the cornea–sclera border. Electrodes were DC-coupled to the active head stage of a cathode follower (WPI Model DAM 5A) and signals amplified (×20,000). Signals were then filtered (10–30 KHz, bandpass) and conditioned (DC offset) prior to being digitally stored (50 KHz) for computer analysis.

Continuous recordings of tonic discharge activity were analyzed for changes in spike frequency. This was accomplished using BrainWave software (BrainWave Systems, Broomfield, CO) to record digitally and discriminate individual single units. Single units were discriminated by the following parameters: 1) rate of rise of the action potential, 2) peak amplitude, 3) rise time, 4) rate of repolarization, and 5) peak negative (undershoot) amplitude. Discriminated units were then superimposed (fig. 1B) and visually inspected to confirm that only a single unit was contributing to the analysis. Units with amplitudes greater than three times the noise level were chosen to ensure consistent discrimination, even though the BrainWave software can discriminate units with a lower signal-to-noise ratio (*e.g.*, fig. 1B). Time *versus* frequency histograms with 30-s bin widths were used to monitor experimental effects on single discriminated unit discharge frequencies. Conduction velocities of single discriminated units were obtained by electrical stimulation using an optically isolated constant current source that produced square-wave stimuli of 10–100 μA that were delivered by bipolar tungsten microelectrodes (5–10 Mohm, Frederick Haer and Co.). The distance between stimulating and recording electrodes (6–10 mm) were measured as previously described and used in the calculation of conduction velocity.[11,12] Only units that exhibited spontaneous activity under control conditions were used for time–frequency analysis, since units which were silent prior to recruitment under experimental conditions lacked a suitable control measure. Recruitment of units following experimental conditions can be observed in the raw data records in figures 2 and 3. All data were analyzed for statistical significance using ANOVA, comparing drug effects to time-matched control recordings for each nerve fiber. Pooled data were normalized ([experimental/control] × 100) to compare single-unit discharge frequencies for fibers with different initial (control) discharge rates. Normalization reduced the variability within data sets to < 10%, well within the restriction limits for propagated errors of percentages.

Mechanically sensitive A-delta fibers were stimulated using a nylon filament coupled to an electromechanical actuator as previously described.[12] Stimulus intensity was adjusted to produce a suprathreshold response, such that each stimulus resulted in a single action potential. Stimuli were presented at 0.1 Hz throughout the course of the experiment and conduction velocities were determined for each fiber before and after each experiment (fig. 1).

EXPERIMENTAL CONDITIONS

Control

The control AQH solution had the following composition (millimolar): Na^+ 145, K^+ 4.5, Ca^{2+} 1.5, Mg^{2+} 0.6, Cl^- 126.3, SO_4^{2-} 0.6, HPO_4^- 0.6, HCO_3^- 25, and D-glucose 10. All chemicals were reagent grade or better and obtained from Sigma Chemical Company. Control AQH solutions were chilled to 5° C and saturated with 95% O_2/5% CO_2 (carbogen) prior to use and continuously bubbled with carbogen throughout the experiment. After warming to 35° C, solutions had a *p*H of 7.2 to 7.4. The control epithelial (external) environment consisted of a warmed, humidified, 95% O_2/5% CO_2 atmosphere, delivered at a flow rate of 1 l/min.

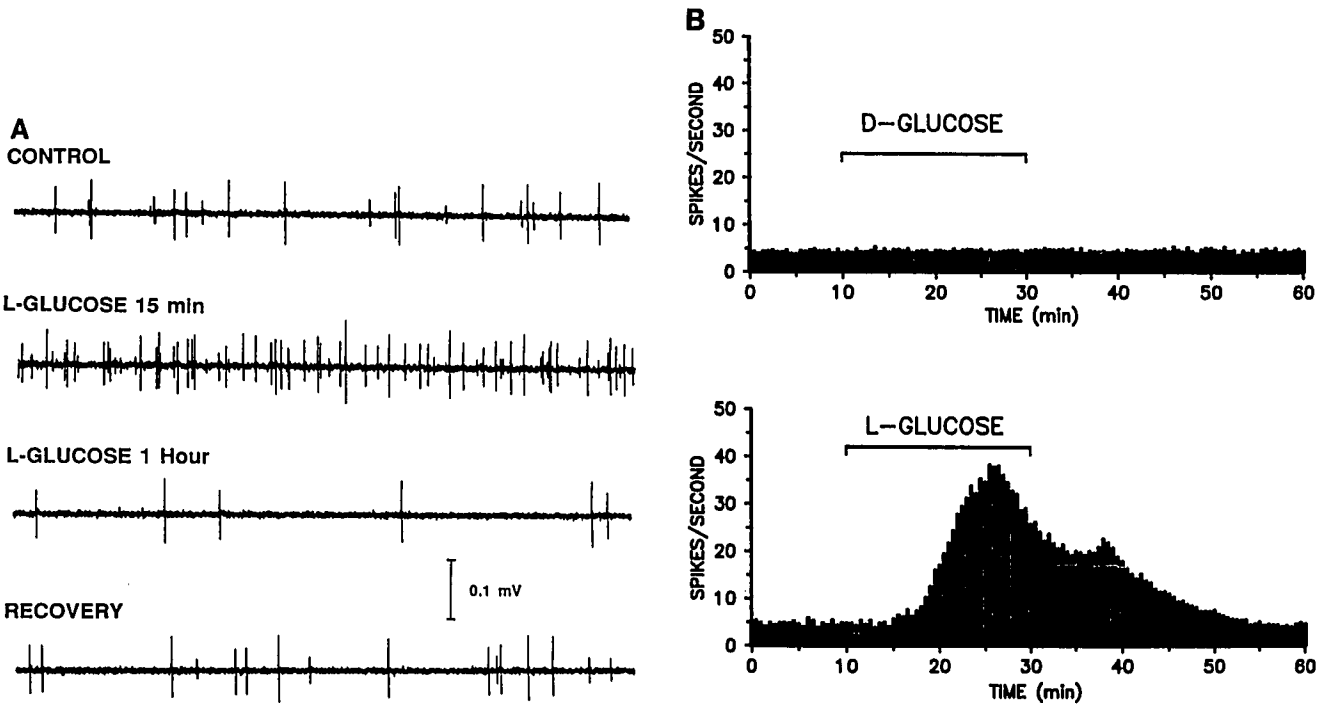

FIG. 2. Hypoglycemia, resulting from substitution of D-glucose with L-glucose, increases C fiber discharge activity. *A:* Recordings of 1.0-s data sweeps show an increase in multiple-unit discharge activity within 15 min following glucose substitution. The smallest fibers are first to exhibit hypoglycemic activation and are also first to be depressed (compare amplitudes of units in control and following 1 h of L-glucose). Recovery data were taken at 10 min following return to control discharge frequencies. *B:* Time *versus* discharge frequency plots show the time course of L-glucose induced excitation following a 20-min substitution. Control plot on top demonstrates no effect of switching perfusates when both contain D-glucose. Each bin represents the average discharge frequency (spikes per second) for a 30-s epoch from a single isolated unit. The units shown in the top and bottom plots were chosen on the basis of similar control discharge frequencies (approximately 5 spikes/s) and are from different preparations.

Hypoxia

AQH solution was used after it was degassed and saturated with 95% N_2/5% CO_2. Care was taken to minimize O_2 absorption into the perfusate by using glass or Teflon vessels, tubing, and valves. The control epithelial (external) environment consisted of a warmed, humidified, 95% N_2/5%CO_2 atmosphere, delivered at a rate of 1 l/min. A POET II (model 684, Criticare Systems Inc.) gas analyzer continuously monitored the concentration of O_2 in the surrounding atmosphere.

Hypoglycemia

For these experiments D-glucose in the control AQH solution was substituted with the cellular impermeant isomer L-glucose (10 mM). The other environmental conditions remained the same.

Hypoxia and Hypoglycemia

AQH solution with L-glucose was used and saturated with 95% N_2/5% CO_2. Additionally, the control epithelial (external) environment consisted of a warmed, humidified, 95% N_2/5% CO_2 atmosphere, delivered at a rate of 1 l/min.

Lactate

Lactate (10–1,000 μg/ml) was added to the AQH solution with a resultant decrease in *p*H (6.9 at 1,000 μg/ml). In some experiments the lactate AQH solution was buffered to the normal AQH *p*H of 7.4, using NaOH.

Results

Effects of Hypoglycemia on C Fibers

Corneal C fibers normally exhibited a low level (two to seven spikes/s) of spontaneous (unstimulated) discharge at 35° C. Control recordings from individual C fibers remained stable for periods of 5–7 h; varying < 5% per hour in their discharge frequency. The conduction velocity of corneal C fibers recorded in this study were 0.85 ± 0.2 m/s (mean ± SD, n = 28). The stability of one such single unit is shown by the time *versus* frequency histogram in figure 2B, where under control conditions the cornea was perfused with AQH solution containing 10 mM D-

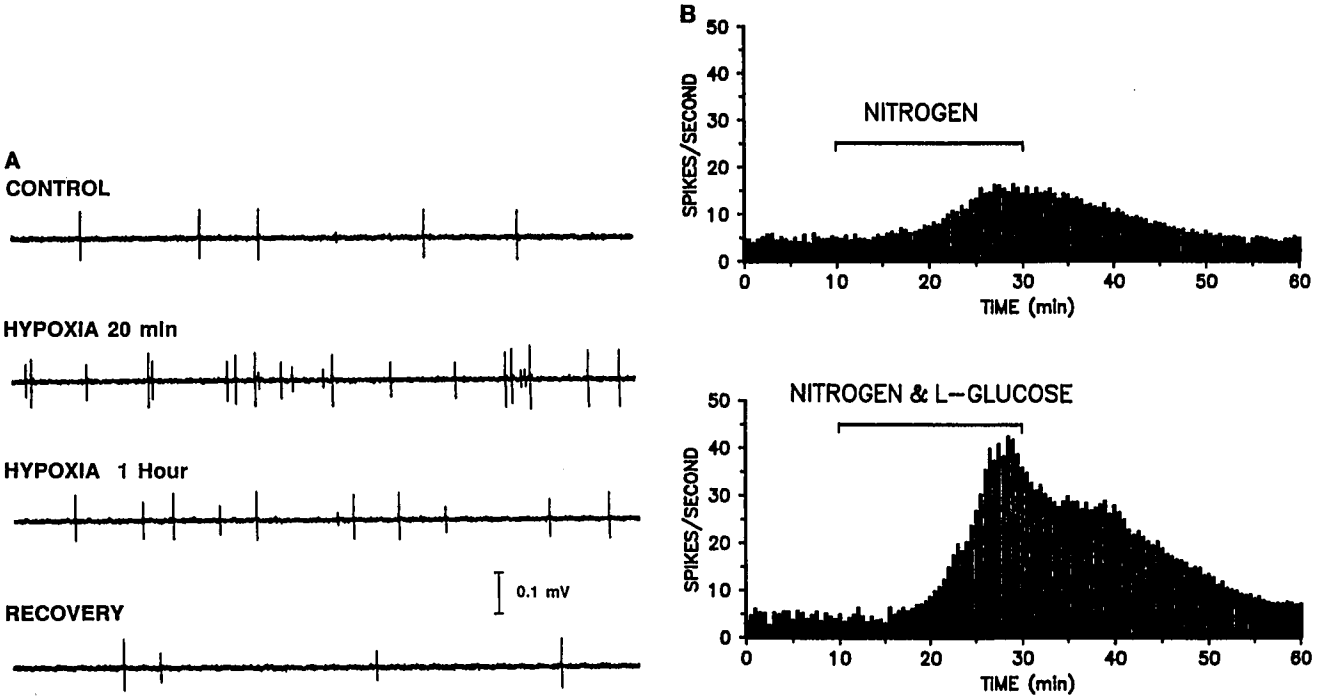

FIG. 3. *A:* Hypoxia resulting from substitution of oxygen by nitrogen (95% O_2 or N_2/5% CO_2) increased C fiber discharge activity of smaller amplitude units. *B:* Time *versus* frequency plots demonstrate the time course of increased discharge frequency of single discriminated C fiber following hypoxia. Combined hypoxia and hypoglycemia produced a greater increase in discharge activity than hypoxia alone. Data on top and bottom plots are from different preparations; each bin = 30 s.

glucose and saturated with 95% O_2/5% CO_2. Figure 2A displays a 1.0-s recording of multiple unit C fiber discharge activity. After 15 min of hypoglycemia produced by L-glucose substitution, there was an increase in the C fiber discharge frequency from 4.9 to 38 Hz. Prolonged hypoglycemia eventually resulted in a decrease in tonic activity, which returned to control values after reperfusion with D-glucose-containing solution. The time–frequency relationship of an individual C fiber subjected to L-glucose substitution is shown in figure 2B. After approximately 5 min, discharge frequency began to increase, from a baseline of 5.1 ± 0.2 spikes/s to a maximum of 37.5 spikes/s after 15 min. The tonic discharge frequency then began to decline, and a transient increase in frequency occurred after reperfusion with D-glucose, before discharge activity returned to control values. The transient increase in frequency was statistically significant ($P < 0.05$; ANOVA n = 8) when compared to the discharge activity 5 min before the peak increase in reperfusion frequency.

Effects of Hypoxia on C Fibers

The effect of hypoxia resulting from the substitution of O_2 by nitrogen (95% [N_2 or O_2]/5% CO_2), was investigated on C fiber discharge. Hypoxia produced an increase in tonic C fiber discharge similar to that observed with hypoglycemia (fig. 3A). However, even after 1 h of hypoxia, the discharge rate remained increased above control values. The time–frequency histogram in figure 3B shows that the onset time and magnitude of hypoxic frequency increase was less than that for hypoglycemia alone. Combining both hypoglycemia and hypoxia resulted in a frequency increase comparable to the hypoglycemic condition alone (fig. 3B). Pooled data from several discriminated single unit experiments are summarized by the bar graphs in figure 4. Under control conditions (n = 43) there is very little fluctuation in the baseline frequency (± 3.2%). Exposure to hypoxia (n = 12) resulted in a 213 ± 3.4% (mean ± SD) increase in C fiber action potential frequency relative to control ($P < 0.001$). L-glucose substitution (n = 8) increased C fiber discharge frequency by 653 ± 28% relative to control ($P < 0.001$), as did the combination of hypoxia and L-glucose substitution (n = 6; 671 ± 14%). Comparison of the hypoxia *versus* the hypoxia and hypoglycemia conditions did not show them to be statistically different ($P > 0.5$).

Lactate and *p*H Effects on C Fibers

The effect of lactic acid on C fiber tonic discharge was investigated at concentrations of 10, 100, and 1000 μg/ml at a *p*H of 6.9 and 7.4. These values represent the

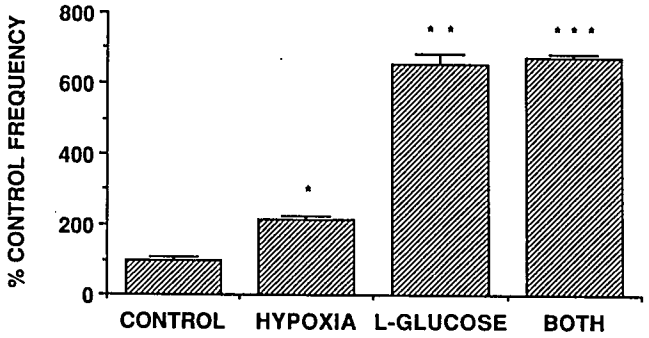

FIG. 4. Bar graph comparing the cumulative effects of hypoxia and hypoglycemia (L-glucose) on C fiber discharge frequency. Each bar represents the mean ± SD for data from individual preparations measured at the time of maximal effect. Hypoxia (n = 12) produced a statistically significant increase in discharge frequency compared with control (n = 43; for time-matched ANOVA, $P < 0.001$). Hypoglycemia (n = 8) produced a significant increase relative to both control and hypoxia ($P < 0.001$). Combined hypoxia and hypoglycemia (n = 6) did not produce a significantly greater increase in discharge frequency compared with hypoglycemia alone ($P < 0.05$).

ranges of clinical alteration of lactate and *p*H in ischemic tissues after 1 h.[13–15] In figure 5, it is apparent that 1000 μg/ml lactate whether at a *p*H of 6.9 or 7.4 does not alter the action potential discharge frequency in corneal C fibers (n = 5, $P > 0.05$). Lactate at concentrations of 10 and 100 μg/ml also did not produce any effect.

EFFECTS OF HYPOXIA AND HYPOGLYCEMIA ON A-DELTA FIBERS

In addition to C fibers, the corneal nerves also contain A-delta fibers, which are mechanically sensitive.[11] The conduction velocity of these A-delta fibers was 2.35 ± 0.16 m/s, n = 10 (fig. 1). Neither hypoxia nor hypoglycemia alone or in combination caused A-delta fibers to become spontaneously active. The effect of hypoxia and hypoglycemia on electrical or mechanical activation of the A-delta fibers was investigated using bipolar electrical stimulation or an electromagnetic mechanical stimulator. Figure 6 shows a single-unit electrically evoked A-delta fiber action potential before and after 1 h of hypoxic and hypoglycemic conditions. The spike latency, amplitude, and spike width were not altered. The same was true for all A-delta (n = 10) and C fibers (n = 15) responding to electrical stimulation. Similarly, hypoxia and hypoglycemia did not alter A-delta action potential parameters evoked by natural mechanical stimulation (fig. 7).

Discussion

During tourniquet application patients report pain at the tourniquet site as well as distal to the tourniquet.[1–4,9,10] This pain has been characterized as burning, cramping, tingling, and as a heavy sensation. The nerve fiber types

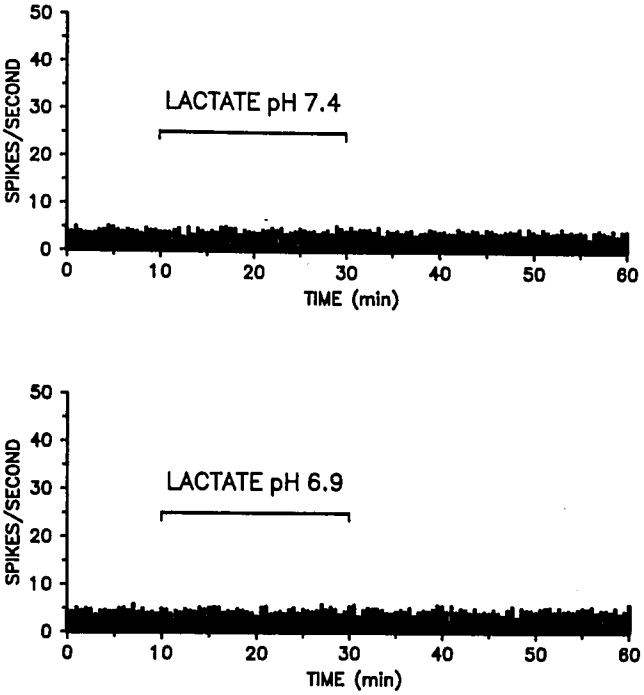

FIG. 5. Lactic acid accumulation did not alter C fiber discharge frequency. The top time *versus* frequency plot shows the lack of effect in the presence of 1,000 μg/ml of lactate in the perfusate (20 min) when the *p*H is corrected to control levels, *p*H 7.4. The bottom plot shows lack of effect in the presence of 1,000 μg/ml of lactate with the *p*H not adjusted. Data on the top and bottom plots are from the same discriminated C fiber; each bin = 30 s.

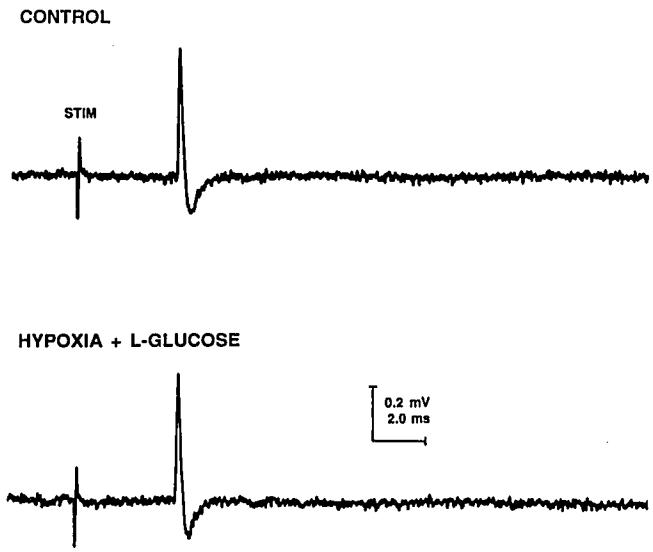

FIG. 6. Neither hypoxia nor hypoglycemia altered A-delta fiber responses to electrical stimulation. Single isolated unit recordings demonstrate that there was no effect on discharge threshold (12 μA; STIM), spike latency (3.9 ms), or spike amplitude (460 μV) following 1 h of hypoxia and hypoglycemia.

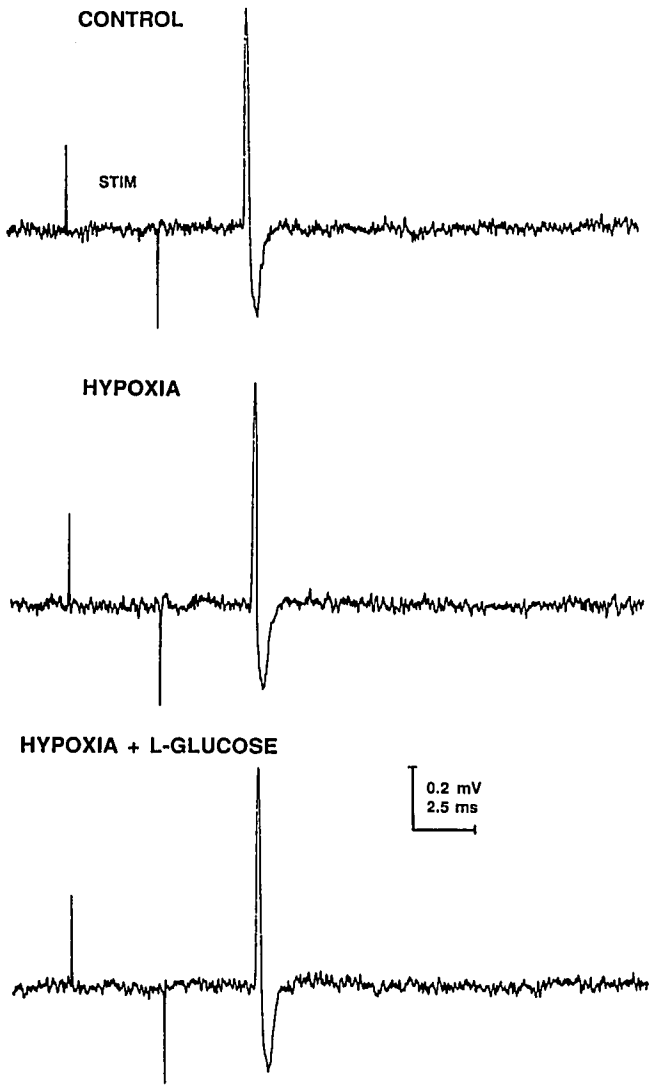

FIG. 7. Neither hypoxia nor hypoglycemia altered A-delta fiber responses to physiologic mechanical stimulation. Single isolated unit recordings show no change in threshold (350 dyne–3 ms; STIM), spike latency (7.5 ms), or spike amplitude (700 μV) following 1 h of hypoxia and hypoglycemia.

involved in this phenomenon have been shown to include both small myelinated and unmyelinated nerve fibers.[8,9] In addition, during initial tourniquet compression and after decompression, larger myelinated nerve fibers may contribute to the paresthetic sensations, but these fibers rapidly undergo conduction block.[10,16] The predominant sensations experienced during ischemia are aching and burning, which are believed to be associated with unmyelinated nerve fibers.[3,13] In humans, microneurography has been used to record postischemic myelinated nerve activity associated with sensations of tingling, pricking, and buzzing.[10,16] In the rat model of tourniquet ischemia, Chabel *et al.*[9] demonstrated that after tourniquet application, spontaneous myelinated fiber activity decreases while previously inactive C fibers begin to spontaneously discharge. Results of the present study agree with these observations and suggest that C fiber activation produces the major contribution to ischemic pain in this model.

Ischemia-induced alterations that are responsible for the generation of tonic C fiber activity are not known. Following limb or tissue ischemia, changes in venous and tissue lactate, O_2, glucose, and energy substrates occur.[13–15,17] After an average tourniquet application of 36 min in humans, venous blood lactate increases from a baseline of 90 μg/ml to 280 μg/ml while venous *p*H is reduced from 7.37 to 7.30.[13] In skin flaps subjected to 1 h of vascular occlusion, the glucose content decreases 70% while tissue lactate increases 3-fold.[14] Using the *in vitro* corneal nerve preparation, the relative contribution of these ischemia-induced metabolic perturbations on A-delta and C fiber electrophysiology were examined. Hypoglycemia, induced by substitution of D-glucose by the L isomer resulted in the greatest increase in C fiber tonic action potential activity. Hypoxia also increased tonic C fiber discharge, but to a lesser extent. Combined hypoglycemia and hypoxia did not produce an increase in C fiber action potential frequency beyond hypoglycemia alone; this may represent a saturation effect due to approaching the maximal physiologic C fiber firing rate. Changes in lactate concentration and *p*H, equal to and greater than those reported during ischemia, did not increase C fiber activity. A-delta fiber activity was not altered by any of the substitutions. The neural activity induced in C fibers correlates with human sensations of burning and aching pain that occur during tourniquet application. It is interesting that A-delta fibers, believed to be responsible for "pin prick" or sharp pain, did not become spontaneously active when exposed to ischemic changes; neither is a sensation of sharp pain reported during tourniquet inflation in humans. Corneal tissue is not innervated by larger myelinated fibers, which could also contribute to postischemic sensations such as tingling or buzzing.

The mechanism whereby hypoglycemia or hypoxia result in C fiber activation can be answered by further study. However, it is known that depletion of glucose and O_2 result in reduction of high-energy substrates, such as ATP, necessary for enzymatic processes. During anaerobic metabolism, the breakdown of one molecule of glucose to lactate results in the production of two molecules of ATP as compared to 38 molecules of ATP during complete aerobic oxidation of glucose.[18] Anoxia *in vitro* results in a progressive depolarization of peripheral nerve axons, which leads to hyperexcitability.[19] Inhibition of the axonal Na^+–K^+ ATPase by a reduction of the ATP/ADP ratio secondary to either anoxia or hypoglycemia will then depolarize C fiber axons resulting in spontaneous activity.

Because smaller axons generally have higher membrane resistances, a large surface-to-volume ratio, and a proportionally larger contribution of the NA^+–K^+ pump to their resting potential, it is not surprising that C fibers are preferentially activated rather than the larger diameter A-delta nerve fibers.[20]

It is interesting that tourniquet pain develops under conditions of regional anesthesia adequate to perform surgery.[2,7,8] The quality of pain, dull and aching, is the same as in unanesthetized individuals and is abolished by tourniquet deflation. The peripheral mechanism for generation of this pain is most likely similar in both cases. It has been suggested that C fibers activated by ischemia may enter the sympathetic chain and join the spinal cord at a level above the local anesthetic somatic nerve block.[9] Understanding the peripheral mechanisms of ischemic C fiber activation may provide insight into interventions for reduction of tourniquet pain.

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