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Surfactant-induced sealing of electropermeabilized skeletal muscle membranes *in vivo*

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ABSTRACT **Victims of major electrical trauma frequently suffer extensive skeletal muscle and nerve damage, which is postulated to be principally mediated by electroporation and/or thermally driven cell membrane permeabilization. We have investigated the efficacy of two blood-compatible chemical surfactants for sealing electroporated muscle membranes. In studies using cultured skeletal muscle cells, poloxamer 188 (P188; an 8.4-kDa nonionic surfactant) blocks, and neutral dextran (10.1 kDa) substantially retards, carboxyfluorescein release from electropermeabilized cell membranes. To test whether P188 administered intravenously could have the same therapeutic effect *in vivo*, the rat biceps femoris muscle flap attached by its arteriovenous pedicle was electropermeabilized until its electrical resistivity dropped to 50% of the initial value. P188 (460 mg/kg) administered intravenously 20 min postshock restored the resistivity to 77% of the initial value. When P188 was administered intravenously 5 min before shock, a dose-dependent impedance recovery rate was observed. Neither neutral dextran (460 mg/kg) nor sterile saline was effective. Histopathologic studies indicated that postshock poloxamer administration reduced tissue inflammation and damage in comparison with dextran-treated or control tissues. Electrophysiologic evidence of membrane damage was not observed in flaps of animals pretreated with poloxamer. These results suggest that it may be possible to seal *in vivo* tissue membranes injured by electrical, thermal, or other membrane-damaging forces.**

Loss of structural integrity of the cell membrane is a central pathophysiologic event in many illnesses, including high-voltage electrical trauma (1–4). Despite improved life-support measures with increased victim survival, a high percentage of major electrical trauma victims still suffer extensive tissue necrosis, have high-level amputations, and become permanently disabled (3). Among electrical utility workers in the United States, the majority of shock victims experience hand-to-hand or hand-to-foot contacts between 6 and 10 kV. Electrical shock simulations by computer suggest that with perfect electrical contacts such circumstances can produce electric field strengths in upper extremity tissues ranging between 60 and 160 V/cm (4). Fields of this magnitude can produce skeletal muscle and peripheral nerve membrane damage through electroporation (2), Joule heating (1, 5), or a combination of both.

Membrane damage is often manifested clinically by release of intracellular contents into the intravascular space (5), one of the clinical hallmarks of major electrical trauma. Skeletal muscle and peripheral nerve necrosis appears to be the primary cause of the high amputation rates associated with electrical trauma. We have postulated that, in the majority of victims, cell membrane permeabilization is the most important pathophysiologic event leading to tissue death (4, 6, 7) and, therefore, effective therapy for victims of electric shock must reestablish cell membrane structural integrity.

Because membranes form spontaneously when surfactants (amphiphiles) are mixed in an aqueous solvent at sufficient concentration, we hypothesized that it may be possible to seal damaged cell membranes by exposing them to adequate concentrations of a noncytotoxic nonionic surfactant, possibly by incorporation of the surfactant into the membrane defects. In a preliminary test of this concept, we found that an 8.4-kDa nonionic synthetic surfactant, poloxamer 188 (P188), which has been clinically accepted for human intravenous administration, effectively sealed electroporated membranes of cultured skeletal muscle cells when used in concentrations >0.5 mg/ml (8, 9). We also noted that sealing the membrane enhanced cell survival as measured by vital dye [i.e., carboxyfluorescein (CF) and trypan blue] assays. The ability of P188 to bind to damaged membranes has been suggested in previous studies (10, 11).

The purposes of this investigation were to determine whether the observed membrane effects of P188 on isolated cells were relatively specific to its molecular properties by comparing P188 with a neutral polysaccharide known to adsorb on the lipid bilayer (12), to determine whether the P188 and neutral polysaccharide would also reach damaged cell membranes *in situ* via intravenous administration and seal them after electropermeabilization, and, most important, to determine whether membrane sealing could prevent tissue necrosis following electrical injury.

MATERIALS AND METHODS

***In Vitro* Muscle Cell Membrane Sealing.** We initially compared the efficacy of poloxamer to dextran or saline (controls) with isolated rat skeletal muscle cells. Skeletal muscle cells were harvested by 0.1% collagenase (type II; Sigma) in phosphate-buffered saline (PBS) digestion at 37°C, washed extensively with 10% horse serum-supplemented PBS, and maintained in culture as described (6, 13). The cells were maintained under physiologic conditions in Dulbecco's modified Eagles' medium (DMEM) supplemented with 20% calf serum and 2× penicillin/streptomycin until used experimentally after 48–72 hr.

To load the cytoplasm with a membrane-impermeant dye, the cells were preincubated in Ca^{2+}-free PBS with 1.5 mM $MgCl_2$/25 mM Hepes buffer/20 μM CF diacetate (CFDA) for 2 hr. They were then incubated in the same solution without CFDA for 30 min before transfer to a custom microscope stage chamber (6) containing the buffered Mg^{2+} PBS and aligned perpendicular to the direction of current passage. CF content in these 1000 × 30 μm (average length × diameter) rat flexor digitorum brevis skeletal muscle cells (n = 6) was measured by digital video data microfluorometry. The intracellular CF was excited at λ_{ex} = 480 nm. The emission at λ_{em}

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Abbreviations: P188, poloxamer 188; CF, carboxyfluorescein; CFDA, CF diacetate.

= 520 nm was converted to a standard RS170 video format (30 frames per s) with an intensified Hamamatsu Neuvicon camera and its output was digitized (512 × 512 × 16 bits) using a Hamamatsu C1966 image processor linked by DMA to a VAXstation II computer (Digital Equipment) (Fig. 1). Each data frame consisted of an average of 16 sequential frames. Gaussian filters were used to perform shading correction, after which the intracellular pixels were normalized to the mean background level; the background fluorescence value was then subtracted from the entire frame by digital image processing methods. A computer controlled shutter (AZI model) blocked the excitation beam between measurements to minimize cellular phototoxic effects. The excitation intensity was lowered with different neutral density filters to the level at which photobleaching did not produce detectable fluorescence emission loss over a 10-min interval.

Intracellular CF monitoring began at $t = 0$ min; the cell was then exposed to a single 200 V/cm, 4-ms duration field pulse (*E* in Fig. 2) at $t = 5$ min. Data frames were collected at 1-min intervals. This procedure was repeated with PBS supplemented with either 8 mg of neutral dextran per ml (10.1 kDa; Sigma) or 8 mg of P188 per ml (BASF, Parsippany, NJ) in the medium bathing the cells ($n > 5$).

***In Vivo* Muscle Tissue Impedance Responses.** Male Sprague–Dawley rats weighing 275–375 g were anesthetized using intraperitoneal ketamine (100 mg/kg) and xylazine (13 mg/kg). A biceps femoris muscle flap with an intact arteriovenous pedicle was developed (Fig. 3). The cells in this muscle range between 2 and 3 mm in length with diameters between 20 and 50 μm (7). Care was taken to keep the tissue moist and to avoid surgical damage to the muscle cells. Continuous blood flow through the vascular pedicle was confirmed throughout each experiment by microscopic examination.

The low-frequency (<1.0 kHz) electrical impedance of skeletal muscle is sensitive to cell membrane integrity (6, 14). The electrical impedance of the flap was measured as described (6). To measure membrane resistivity, the muscle flap was placed in an electrically insulating 2 (length) × 1 (width) × 1.5 (depth) cm chamber within a polysulfonate block constructed for making two-port electrical impedance measurements. Immediately before impedance measurement, excess buffer was blotted from the muscle surface, and the

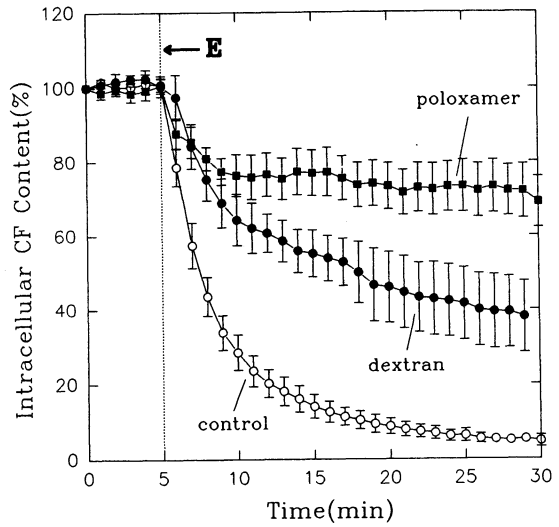

FIG. 2. CF release from isolated 1000 × 30 μm (average length × diameter) rat flexor digitorum brevis skeletal muscle cells ($n = 6$) after a single 200 V/cm, 4-ms-duration field pulse. The cells were preincubated in Ca^{2+}-free PBS with 1.5 mM $MgCl_2$/25 mM Hepes buffer/20 μM CFDA for 2 hr. The cells were then incubated in the same solution without CFDA for 30 min, transferred to a custom microscope stage chamber (6) containing the buffered Mg^{2+} PBS, and then exposed to field pulse. The field pulse (*E*) was delivered at $t = 5$ min. The intracellular CF was excited at 480 nm and the emission at 520 nm was quantified by digital imaging processing methods as described (9). The procedure was repeated with PBS supplemented with either 8 mg of neutral dextran per ml (10.1 kDa; Sigma) or 8 mg of P188 per ml (BASF).

cross-section of the muscle flap was measured. A Wavetek (San Diego) function generator (model 191) and a Kepco (Flushing, NY) bipolar operational amplifier (model BOP 200-1M) configured as a current source were used to establish a 100-Hz sinusoidal 1 mA (peak) current. Two Ag/AgCl electrodes mounted 5 mm apart in an epoxy block were centrally placed on the muscle surface to measure the voltage gradient. The potential difference between the Ag/AgCl electrodes was measured by a FET (field-effect transistor)

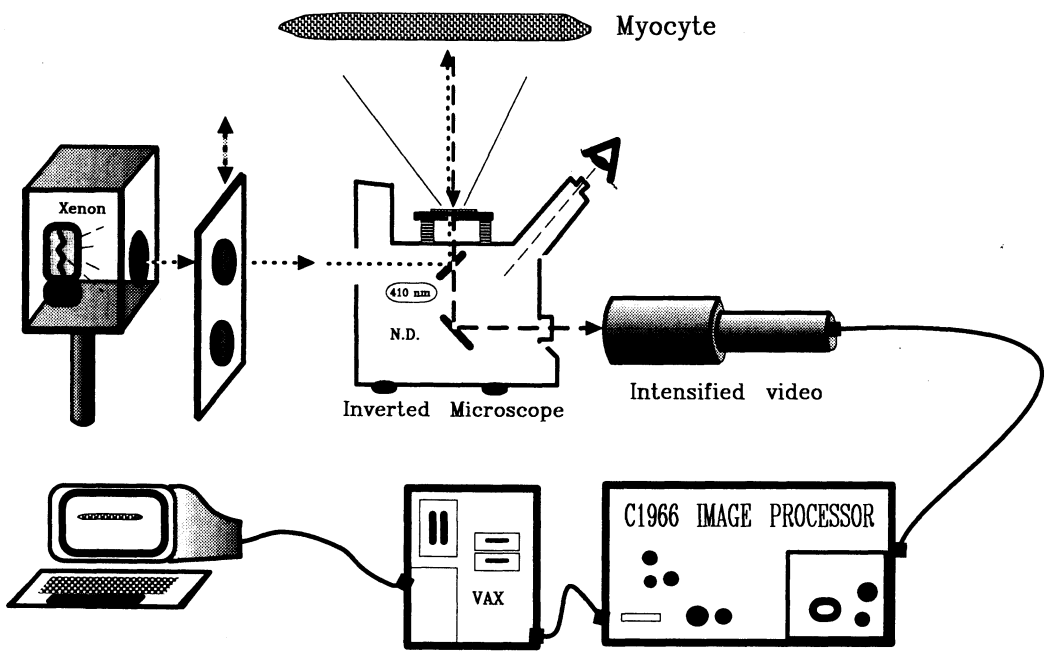

FIG. 1. Illustration of the experimental apparatus used to make membrane permeability measurements on isolated rat skeletal muscle cells. N.D., neutral density.

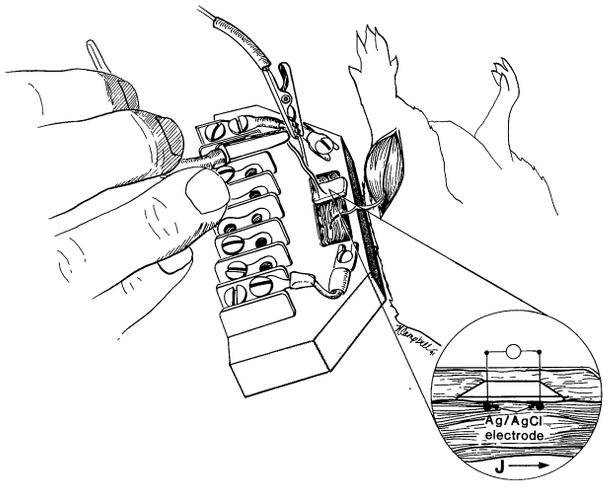

FIG. 3. Sketch of apparatus for measuring muscle impedance magnitude (100 Hz) with the rat biceps femoris island muscle flap in the apparatus.

differential amplifier (model 11A33; Tektronic, Beaverton, OR) module in a Tektronix digital oscilloscope (model 11401) configured as an impedance spectrometer. The value of the flap impedance magnitude parallel to the muscle cell orientation, minus the impedance of PBS, was calculated, corrected for the cross-section, normalized to its initial value, and used as an index for membrane damage (6, 8). After three baseline impedance determinations, sufficient isotonic PBS with 50 mM Hepes buffer was added to cover the muscle and permit a uniform electric field. Sixty unipolar, rectangular, 1-A current pulses, each 4 ms in duration, induced a 150 V/cm electric field in the chamber. A 10-s duty cycle was set to minimize temperature elevation. The added PBS was then removed and postshock muscle impedance was monitored again. The cross-section of the muscle flap was measured before and after the electrical shock and was used to calculate resistivity.

Treatment and control intravenous injections were administered 20 min postshock into the contralateral femoral vein under microscopic visualization. Treated animals received bolus injections of either P188 (460 mg per kg of body weight) dissolved in 1.8 ml of isotonic saline or neutral dextran (460 mg/kg) dissolved in isotonic saline. Control animals received an equivalent volume of isotonic saline. Poloxamer and dextran injections were randomized and coded so that their identity was not known to the experimenter. All preparations were filter sterilized. For each group, the mean ± SEM of the normalized resistivity (i.e., impedance corrected for cross-section and normalized to the preshock value) was calculated. This protocol was carried out according to institutional guidelines for animal care. Statistical comparisons between sample means were made by using the Wilcoxon signed-rank test for nonparametric data with the null hypothesis that the means were identical.

Baseline tests were conducted to determine the effects of bath conditions and intravenous injections on resistivity. Resistivity measurements were made every 30 min for 2 hr on untreated animals and on animals that had received P188, neutral dextran, or isotonic saline. Dosages were designed to yield a peak blood level of 8 mg/ml.

Joule heating-related temperature changes were estimated by measuring bath temperature. A calibrated thermistor was centrally placed in the chamber, which was packed with surgical gauze (surgical gauze has a dc resistivity similar to skeletal muscle) and soaked with isotonic PBS with 50 mM Hepes buffer. The temperature was taken every 10 pulses for up to 60 pulses. The pH of the bath was determined with litmus paper precalibrated against a Corning pH meter.

***In Vivo* Response Kinetics.** To gain insight into the dose dependence of *in vivo* membrane sealing, a few experiments were performed in which the P188 at several different dosages was injected intravenously 10 min before electropermeabilization of the flap. The flap and the impedance measurement were set up as described above. To facilitate comparison with postshock therapy, several experiments ($n = 5$) were performed using P188 at 460 mg/kg. Impedance was monitored at 5-min intervals as described.

Postinjury Tissue Survival. Additional studies were conducted to examine the effect of P188 on muscle survival under electrical exposure conditions simulating accidental electrical shock. In these experiments, the skin overlying the flexor digitorum brevis muscle of the hind foot was raised as a flap to expose the muscle. The foot was immersed in the chamber described above, saline was added to cover the foot, and a 150 V/cm 60-Hz sinusoidal current was applied for 5 s. Either P188 or an equivalent volume of isotonic saline was injected intravenously 15 min postshock, using doses identical to those used in resistivity studies. The peak temperature of the bath immediately following current cessation was recorded with a calibrated thermistor. The flap was closed with sutures and cyanoacrylate glue and the foot was placed in a bulky dressing. At 4 and 24 hr postshock, each animal was again anesthetized and additional intravenous doses of either P188 or isotonic saline were given. Seventy-two hours later, the animal was sacrificed and the flexor digitorum brevis muscle was removed and fixed in neutral-buffered formalin.

RESULTS

CF Membrane Permeation. Skeletal muscle cells isolated from the flexor digitorum brevis muscle ranged from 800 to 1200 μm long and 25 to 30 μm in diameter as described (5, 15). They maintained their normal sarcoplasmic striations and shape in culture. The viability of the cells was affirmed by the intracellular incorporation and catalysis of CFDA to its fluorescent form, CF. A single 4-ms-duration field pulse electropermeabilized 100% of the test cells. This was manifested by a loss of dye from the cell (Fig. 2). In control cells, the dye loss kinetics exhibited a single exponential decay with a characteristic time constant of $\approx$284 s. For cells bathed in either P188 or dextran, the step increase in membrane permeability observed immediately after the field pulse was followed by a smooth reduction in the slope of the dye loss curve. For the dextran-treated cells, the slope approached but never reached the preshock (near zero) level. However, the dye loss rate from P188-treated cells did return to the near-zero value. In effect, we observed that P188 or small molecular mass (11 kDa) neutral dextran retarded CF release from electropermeabilized rat muscle cells. Furthermore, while dextran retarded CF loss, P188 (8 mg/ml) completely arrested transmembrane CF flux.

Tissue Impedance Responses. Exposure to the electric field pulses used in this study caused an abrupt and substantial decrease in muscle tissue resistivity values (Fig. 4). The resistivity drop averaged 49.9% ± 3.6% (mean ± SEM) of initial values. Treatment with intravenous P188 restored flap resistivity to 77.4% ± 4.8% of initial values; this change was statistically significant ($P < 0.05$). Intravenous treatment with neutral dextran or isotonic saline had no effect on resistivity values (44.8% ± 8.3% and 39.0% ± 3.0%, respectively; $P > 0.1$ in both cases). The resistivity changes seen after P188 treatment occurred within 15 min of administration and resistivity was stable for at least 2 hr. No significant spontaneous changes in resistivity were observed in postshock control animals that were monitored for 2 hr ($n = 5$).

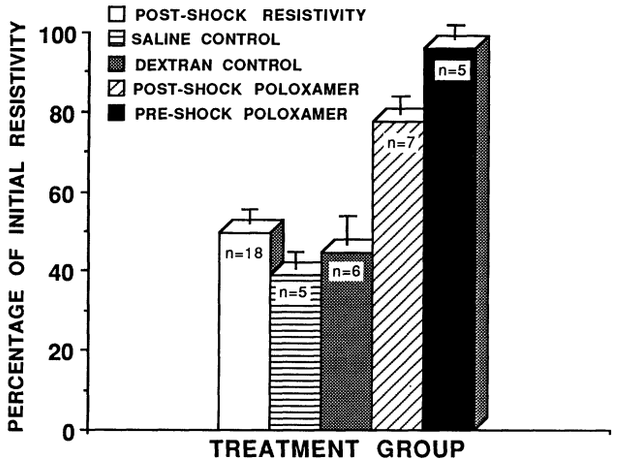

FIG. 4. Normalized resistivity for flaps immediately postelectroporation, 1 hr after intravenous saline fusion, 1 hr after intravenous dextran infusion, 1 hr after intravenous poloxamer infusion, and immediately after electroporation in animals pretreated with poloxamer.

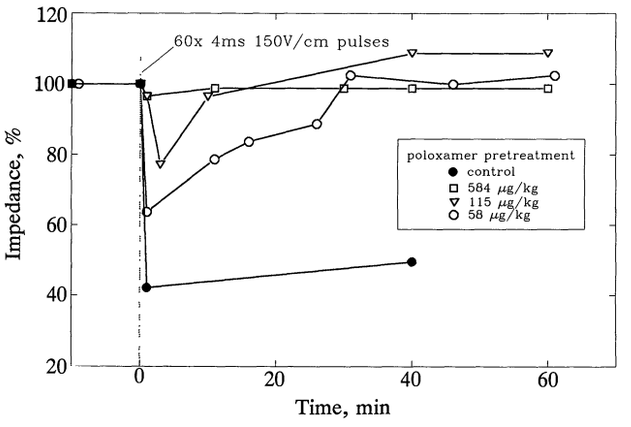

FIG. 5. Plot of membrane impedance versus time for four experiments at $t = 0$; 60 field pulses (shocks) were given, each pulse separated by 10 s. Muscle impedance was recorded before and after the shocks were administered.

In animals preloaded with P188 (8 mg/kg) ($n = 5$), postshock flap resistivity averaged 96.3% ± 1.8% of initial values ($P > 0.1$) (Fig. 4). Baseline tests were conducted to determine the effects of bath conditions and intravenous injections on resistivity. Resistivity measurements were made every 30 min for 2 hr on untreated animals and on animals that had received P188, neutral dextran, or isotonic saline. Dosages were designed to yield a peak blood level of 8 mg/ml. No significant spontaneous changes in flap resistivity were observed in unshocked, untreated animals or in unshocked animals receiving intravenous P188, neutral dextran, or isotonic saline.

Bath temperatures increased by <1.5°C, and no pH changes in the bath medium were detected during or after the electric shock series of 60 pulses. Temperature studies made during actual muscle flap electric field exposure yielded identical results.

Fig. 5 shows the kinetic response for a range of P188 dosages. Animals preloaded with saline did not demonstrate spontaneous recovery over a 1-hr period (Fig. 5). All P188 preloaded animals manifested complete membrane sealing

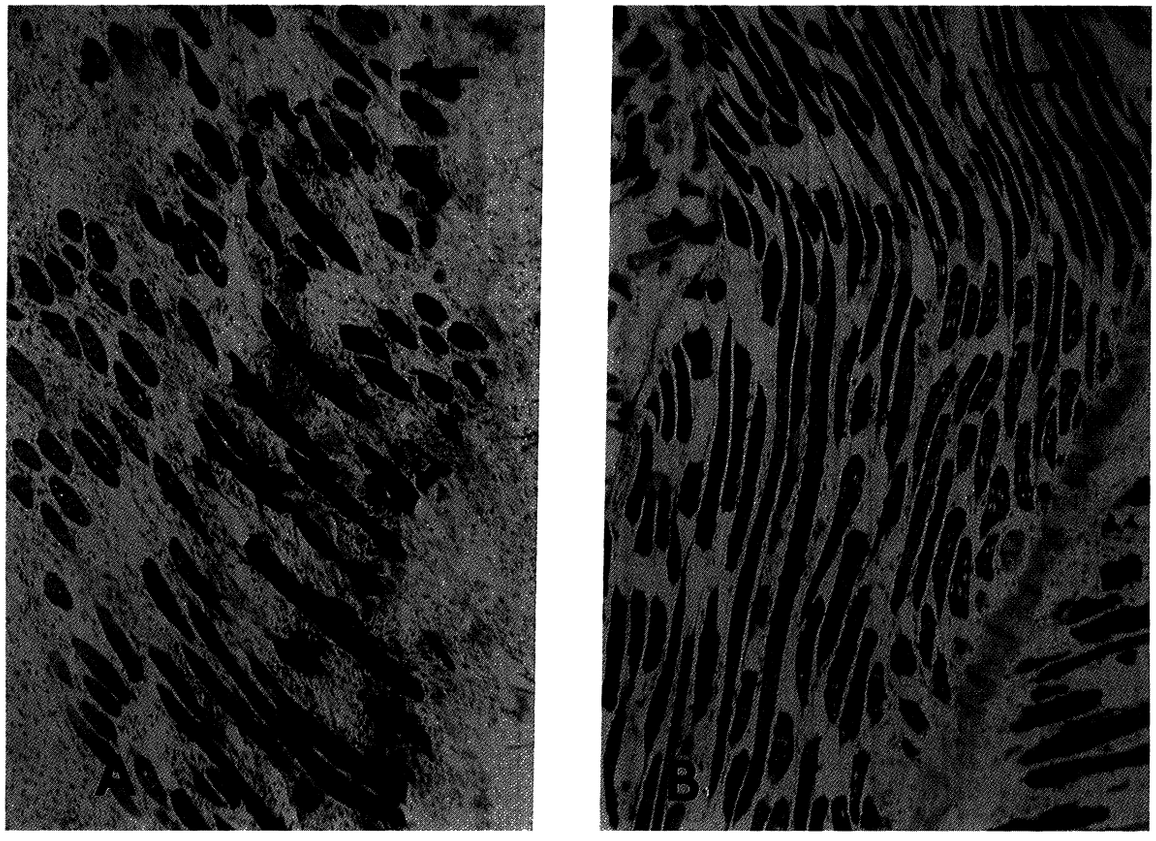

FIG. 6. (*A*) Histophotomicrograph of skeletal muscle that received electrical shock and that was then treated by intravenous saline injection. (*B*) Muscle in animals that received P188 manifested much less inflammation and edema. (Bars = 100 μm.)

after electropermeabilization. As shown in Fig. 5, the rate of membrane sealing was dose dependent.

Effect on Postshock Survival. For the flexor digitorum brevis muscle survival experiments, bath temperature peaked at 5 s (when the current was turned off), with an average change of 16°C (reaching a maximum of 37°C). Within 24 hr after electrical shock, there was a readily detectable difference in tissue swelling between control and P188-treated animals. P188 treatment substantially reduced tissue edema, suggesting a reduction in inflammation. The photomicrographs in Fig. 6 represent typical histomorphology of poloxamer-exposed and control hematoxylin and eosin-stained muscle. The architecture of the P188-exposed muscles (Fig. 6*B*) was significantly less edematous and has fewer inflammatory changes in comparison with saline-exposed tissue (Fig. 6*A*). The morphology and extent of changes in the P188-treated group were similar to those observed in tissue adjacent to a surgically closed wound. The intracellular matrix of the poloxamer-treated group of muscle cells appeared to be intact, while the cells in saline-treated tissue were fewer in number and showed evidence of extensive damage.

DISCUSSION

It has been postulated that the appearance of surfactant molecules on earth several billion years ago was one of the critical events that made life possible (16). In an aqueous environment, surfactants possess the ability to self-assemble into electrically insulating membranes, membranes that permit ionic compartmentalization. Several common, serious illnesses are related to the loss of cell membrane integrity, among them postischemic tissue reperfusion injuries, ionizing irradiation injuries, and "burns" of a chemical or thermal nature. Electrical shock injury is the clinical paradigm for these disorders. Advances in clinical medicine over the past two decades have focused on correction of the physiologic consequences of these injuries at the tissue and organ system level; these measures have enhanced patient survival. The next important step is correction of the underlying cytopathology. Progress toward more effective clinical intervention depends on development of an effective approach to cell membrane repair.

The results of this study suggest that effective sealing of electropermeabilized cells can be achieved with the use of a mild nonionic surfactant, P188. P188 is an industrial surfactant with a critical micelle concentration of ≈1 mg/ml (10). It has been used for a range of purposes including as a mild detergent to cleanse wounds (17), as an emulsifier for nonpolar lipids, and as a laxative; it was also used to coat the surface of early (1960s) membrane oxygenators in order to reduce erythrocyte lysis during cardiopulmonary bypass (10). With the exception, at high concentrations, of retarding thrombosis, there have been no reports of any toxic effects. It is excreted in the urine unmetabolized (10).

In experiments with isolated, cultured skeletal muscle cells loaded with CF dye, dextran exposure caused a substantial slowing of the rate of postshock dye loss, indicating that the membrane was less permeable to CF in the presence of neutral dextran. It is widely known that neutral polysaccharides chemically interact with cell membranes (12). They have been shown to induce cell aggregation (18). Minetti *et al.* (12) suggested that a direct adsorptive binding between dextrans and phospholipids occurs that induces an altered phospholipid packing order. The exact molecular mechanisms of these effects are unknown. However, the nature of the interaction is such that a complete seal of the electropores was not achieved in this study of isolated cells. The ineffectiveness of dextran *in vivo* to restore muscle impedance toward baseline may be due to insufficient tissue concentrations, binding in the extracellular matrix, or perhaps the inability of dextran to alter the transmembrane transport of mobile ions to which the impedance measurement is sensitive.

Intravenous administration of P188 was found to be effective for delivering the surfactant to the muscle cell membrane. It restored membrane integrity to isolated muscle cells and to 77% of preshock values within intact tissue. Loss of intracellular material with concomitant cell shrinkage (3) and disruption of the histoarchitecture (7) may explain the incomplete recovery *in vivo*. This explanation is consistent with the absence of a permanent resistivity drop in animals that were preloaded with P188 because membrane sealing would have occurred rapidly, preventing any loss of intracellular content.

P188 administration was quite effective in reducing tissue damage resulting from electropermeabilization of flexor digitorum brevis cell membranes. Histologic evidence of muscle inflammation and damage was still evident, although substantially less than that seen in control animals. The presence of inflammation despite P188 administration may be the result of surgical injury during surgical manipulation of the skin flaps or perhaps because of nonuniformity in delivery of P188 to the muscle.

The present results indicate that a direct approach may exist for the treatment of illnesses that are primarily related to cell membrane damage, setting the stage for development of effective medical therapy for victims of high-voltage electrical shock.

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