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# Effect of Intrathecally Administered Local Anesthetics on Protein Phosphorylation in the Spinal Cord*

*Raju N. Nivarthi, Gilbert J. Grant, Herman Turndorf and Mylarrao Bansinath†*
Department of Anesthesiology, School of Medicine, New York University Medical Center, New York, NY 10016, U.S.A.

**ABSTRACT.** To elucidate the biochemical mechanisms of spinal anesthesia, we studied the effects of procaine and tetracaine on protein phosphorylation in the mouse spinal cord. Mice were injected intrathecally with either procaine, tetracaine (67 mM/~2%, 10 μL, N = 5/drug), or saline (N = 4/group). Five minutes after injection, animals were killed with a guillotine, and the spinal cord was removed. The caudal 3-cm cord segment was homogenized and centrifuged, and an aliquot of the supernatant was used for phosphorylation assays. Calcium-dependent phosphorylation was initiated by incubating the samples in buffer containing [$\gamma$-$^{32}$P]ATP at 37° for 30 min. The proteins were electrophoresed using slab gel and two-dimensional electrophoresis, and phosphorylated proteins were visualized by autoradiography. The data demonstrated that spinal anesthesia changes the phosphorylation state of five endogenous substrate proteins with apparent molecular masses of 130 (protein-a), 105 (protein-b), 55 (protein-c), 47 (protein-d), and 33 (protein-e) kDa. In two-dimensional electrophoresis, protein-a resolved into two proteins (a1 and a2). Analysis of variance of the densitometric data suggested a significant effect for the treatment ($F_{2,16}$ 735, $P < 0.00005$). Post hoc comparisons with the saline-treated controls, using the Newman-Keuls test, indicated that local anesthetics significantly affected phosphoproteins ($P < 0.05$) except for protein-a1 in the tetracaine-treated group. Further characterization of these phosphoproteins should aid in determining their role in the signal transduction cascade affected by spinal anesthesia. BIOCHEM PHARMACOL 53;7:979–986, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** spinal anesthesia; local anesthetics; procaine; tetracaine; protein phosphorylation; two-dimensional electrophoresis

Numerous electrophysiologic studies have investigated the effects of local anesthetics on the conduction of neural impulses *in vitro* [1, 2]. In most studies, local anesthetic-induced depression of action potential in isolated nerves is the endpoint. Collectively, these electrophysiological data suggest that the blockage of voltage-gated sodium channels may be an important mechanism for peripheral nerve block. However, *in vitro* effects of local anesthetics on isolated nerves do not always correlate with *in vivo* results [3]. Spinal anesthesia is a typical example of the disparity between *in vitro* and *in vivo* results. During surgical depths of spinal anesthesia, somatosensory evoked potentials persist, suggesting an incomplete block of neuronal impulses in the spinal cord [4, 5]. Local anesthetic-induced $Na^+$ channel inhibition may not be the sole mechanism involved in spinal anesthesia [1]. As yet, the biochemical basis underlying the electrophysiological changes in spinal anesthesia is not clear [1].

Phosphorylated proteins are among the most important molecular mechanisms by which extracellular signals produce their neuronal responses [6]. Phosphorylation serves to fine-tune the electrical properties of a neuron and allows it to alter its response to external stimulation. Neuronal depolarization is also a signal for protein phosphorylation [7]. In fact, some neuronal ion channels need to be phosphorylated in order to function as active channels [8]. Protein phosphorylation modulates the gating of the sodium channel, the principal site of local anesthetic action [9]. Protein phosphorylation *in vivo* is facilitated by a group of enzymes called kinases [10]. Recently, PKC‡, a family of phospholipid-dependent serine/threonine-specific protein kinases, was shown to be stimulated during spinal anesthesia [11, 12]. In this study, to better understand the biochemical mechanisms involved in spinal anesthesia, we assessed calcium-dependent phosphorylated proteins in the spinal cord after intrathecal injection of local anesthetics.

## MATERIALS AND METHODS

Molecular weight markers, leupeptide, benzamidine, aprotinin, sodium orthovanadate, phenylmethylsulfonyl fluo-

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† Corresponding author: Dr. M. Bansinath, Department of Anesthesiology, New York University Medical Center, 550 First Ave., New York, NY 10016. Tel. (212) 263-5065; FAX: (212) 263-7254.

‡ *Abbreviations:* PKC, protein kinase C; and pI, isoelectric point.

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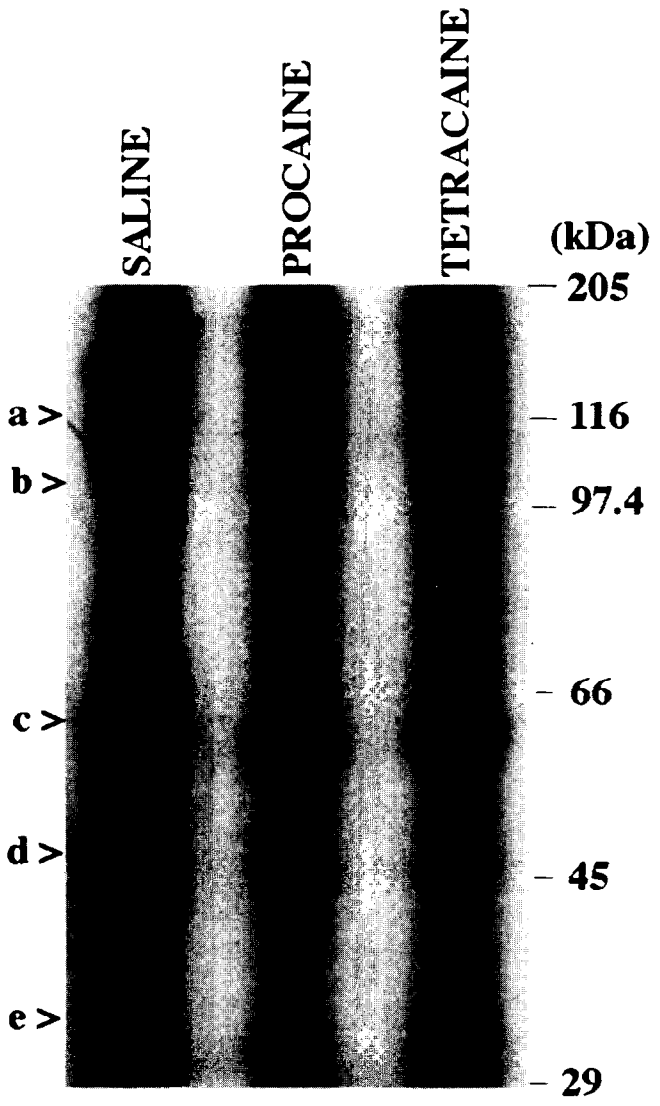


**FIG. 1. Autoradiogram of a one-dimensional gel illustrating the effects of procaine and tetracaine on phosphoproteins of 33–130 kDa in the spinal cord. Mice were given an intrathecal injection of either saline (10 μL), procaine or tetracaine (67 mM/~2%, in 10 μL) 5 min before they were killed. Three centimeters of the caudal portion of the spinal cord was homogenized in 2 mL of buffer, and 100 μg of total protein was subjected to *in vitro* phosphorylation reaction and to one-dimensional SDS–PAGE (with 10% acrylamide) as described in Materials and Methods. Resulting gels were dried and autoradiographed. The arrows indicate the positions of proteins a–e that show changes in local anesthetic-treated groups.**

ride, sodium dodecyl sulfate, and hydrochloride forms of procaine and tetracaine were obtained from the Sigma Chemical Co. (St. Louis, MO). [$\gamma$-$^{32}$P]ATP was procured from Amersham (Arlington Heights, IL).

The study protocol was approved by our institution's Animal Care and Use Committee. Male Swiss Webster mice (Germantown, NY) weighing 20–25 g were housed 5 per cage in a room with controlled temperature (22 ± 2°), humidity, and artificial light (6:30 a.m. to 7:00 p.m.). Animals had free access to food and water and were used after a minimum of 4 days acclimatization to the housing conditions.

The method of intrathecal administration of local anesthetics was essentially similar to the protocol used in our earlier studies [13, 14]. Animals were lightly anesthetized with halothane, and the skin overlying the dorsal lumbar spine was opened using a transverse 10-mm incision. Animals were allowed to recover for 2 hr before inducing spinal anesthesia. Intrathecal injections were performed using an automatic syringe (Hamilton Co., Reno, NV) fitted with a 30-gauge needle. Isomolar concentrations (67 mM/~2%) of either procaine or tetracaine (in a fixed volume of 10 μL) were injected into experimental groups (N = 5 per group) of

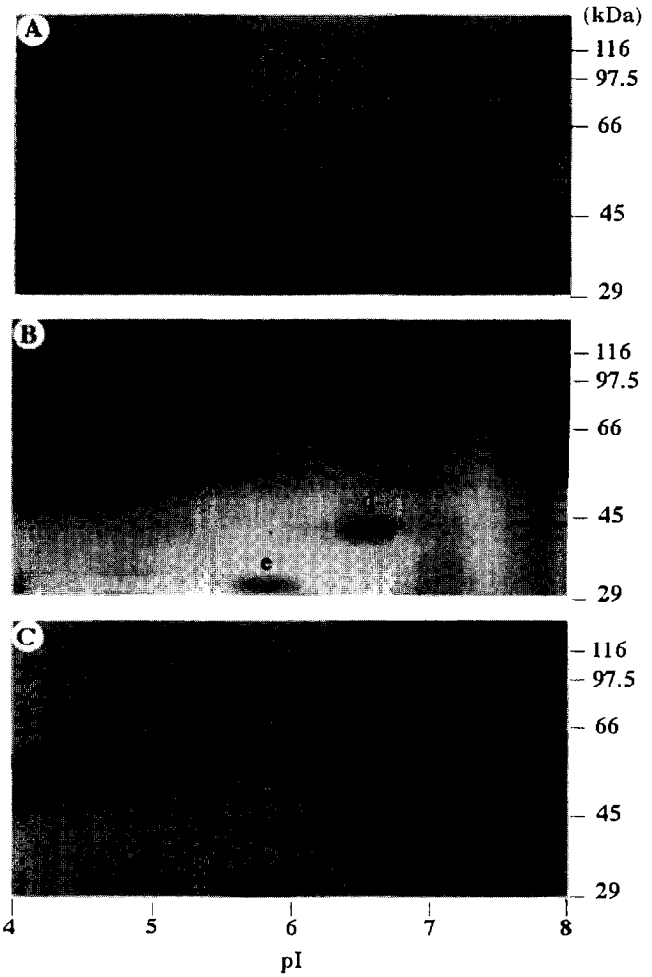


**FIG. 2. Representative autoradiograms of two-dimensional gels illustrating the effect of saline (A) procaine (B) and tetracaine (C) on phosphoproteins of 33–130 kDa in the spinal cord. Mice were given an intrathecal injection of either saline (10 μL), procaine or tetracaine (67 mM/~2%, in 10 μL) 5 min before they were killed. Three centimeters of the caudal portion of the spinal cord was homogenized in 2 mL of buffer, and 100 μg of total protein was subjected to *in vitro* phosphorylation reaction and to two-dimensional SDS–PAGE (with 10% acrylamide) as described in Materials and Methods. Resulting gels were dried and autoradiographed. Labels at the respective positions indicate the proteins of interest.**

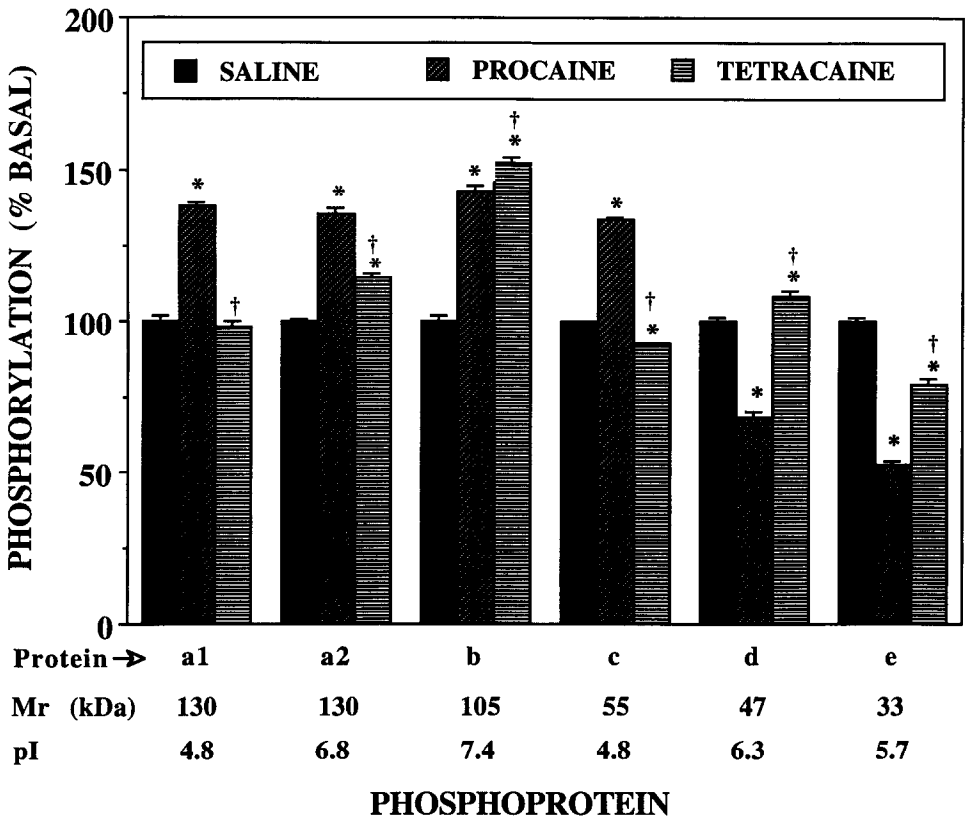


**FIG. 3. Effect of intrathecal injection of procaine and tetracaine on phosphoproteins of 33–130 kDa in the spinal cord. Data represent means ± SD of the densitometric analysis of two-dimensional gels obtained from three independent experiments. Analysis of variance indicated a significant effect for the treatment ($F_{2,16}$ 735, $P < 0.00005$). Key: $P < 0.05$, when compared with (*) saline-treated controls and (†) the procaine-treated group.**

mice. Control groups (N = 4 per group) were injected with 10 μL of saline. Five minutes after the injection, animals were killed with a guillotine, and the spinal cord was removed. Three centimeters of the caudal portion of the spinal cord was homogenized in 2 mL of buffer [50 mM Tris–HCl, pH 7.5, containing 10 mM EGTA, 5 mm EDTA, 0.3% (w/v) β-mercaptoethanol, 10 mM benzamidine, 100 μg/mL leupeptide, 100 μg/mL aprotinin, 30 μM sodium orthovanadate ($NaVO_3$) and 50 μg/mL phenylmethylsulfonyl fluoride], and centrifuged at 7000 *g* for 15 min. Protein concentration in the supernatant was measured using a colorimetric method that is immune to the presence of chelating agents (EDTA and EGTA) [15]. An aliquot equivalent of 100 μg (in 10 μL) of total protein was used for the *in vitro* phosphorylation reaction. The phosphorylation reaction was initiated by adding 100 μL of the reaction mixture [2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM NaCl and 5 μCi [$\gamma$-$^{32}$P]ATP (4500 Ci/mmol)] followed by incubation at 37° for 30 min. After the reaction was stopped (see below), the samples were subjected to gel electrophoresis and autoradiography.

For one-dimensional SDS–PAGE, the phosphorylation reaction was terminated by adding 2× Laemmli sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue) and boiling for 5 min. Samples were then subjected to one-dimensional SDS–PAGE on 10% acrylamide gel electrophoresis at 150 V for 4 hr using a Pharmacia Biotech electrophoresis power supply (EPS 3500 XL; Piscataway, NJ). The gels were stained and dried (Gel dryer, model 583, Bio-Rad Laboratories, Hercules, CA), and $^{32}$P-labeled proteins were visualized by autoradiography. The quantification of phosphorylated proteins was done by using an imaging densitometer (GC 670, Bio-Rad Laboratories).

For two-dimensional electrophoresis, the protein samples were solubilized in isoelectric focussing (IEF) sample lysis buffer [9.5 M urea, 2% ampholines (1.6% of pH 5–7, 0.4% of pH 3–10.5), 5% β-mercaptoethanol, and 2% SDS] by swirling at room temperature, followed by one freezing-and-thawing cycle. The protein mixture was centrifuged at 10,000 *g* for 5 min, and the clear supernatant was loaded onto the IEF gel. The method of two-dimensional electrophoresis, was based on the description of O'Farrell [16] with minor modifications [17]. In the first dimension, the IEF tube gel was prefocussed by applying the following voltage: 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. After prefocussing, the samples were loaded and the gels were run at 9000 V-hr (600 V for 12 hr, 700 V for 1.5 hr, and 750 V for 1 hr). The IEF gels were removed and equilibrated in an equilibration buffer (1% glycerol, 5 mM dithiothreitol, 2.3% SDS, and 0.625 M Tris–HCl, pH 6.8) for 0.5 hr to remove ampholines. The tube gels were transferred onto a second dimension run (SDS–PAGE) with the stacking gel (4.75% acrylamide and bis-acrylamide) over the resolving gel (10% acrylamide and bis-acrylamide). The contact between the IEF tube gel and the second dimension slab gel was established by embedding with a hot solution of 1% (w/v) agarose containing 0.0025% (w/v) bromophenol

**TABLE 1. Local anesthetic effects on membrane-associated receptors, enzymes and other proteins**

| Parameter | Type | Tissue source | Agent (LA) | Dose (mM) | Effect | Ref. |
|---|---|---|---|---|---|---|
| Enzyme (activity) | PKC | Mouse spinal cord | Procaine | 67 | ↑ | 11 |
| | | | Tetracaine | 67 | | |
| | | Rat brain | Mepivacaine | 1.0–3 | ↓ | 19 |
| | | | Bupivacaine | | | |
| | | | Procaine | 1.0–10 | ↓ | 20 |
| | | | Tetracaine | 0.02–2 | | |
| | | | Dibucaine | 0.35 | ↓ | 21 |
| | | | Tetracaine | 1.1 | | |
| | | Rabbit platelets | Dibucaine | | ↓ | 22 |
| | | | Tetracaine | | | |
| | | Neuroblastoma cells (Neuro-2A and SK-N-MC) | Bupivacaine | 1 | ↔ | 23 |
| | | | Ropivacaine | 0.1–1 | | |
| | | | Lidocaine | 1.0–3 | | |
| | Adenyl cyclase | Rat liver plasma membrane | Dibucaine | 0.01–10 | ↓ | 24 |
| | | | Mepivacaine | 0.10–100 | | |
| | | Frog erythrocyte membrane | Dibucaine | 1.0–2 | ↓ | 25 |
| | | | Tetracaine | 1.0–2 | | |
| | | | Bupivacaine | 1.0–2 | | |
| | | | Lidocaine | 2.0–5 | | |
| | Guanylate cyclase | Mouse neuroblastoma clone N1E-115 cells | Tetracaine | 0.0–0.1 | ↓ | 26 |
| | | | Butacaine | | | |
| | | | Procaine | | | |
| | | | Dibucaine | | | |
| | | | Lidocaine | | | |
| | $Ca^{2+}$-ATPase | Canine cardiac microsomes (fragmented SR) | Dibucaine | 0.01–10 | ↓ | 27 |
| | | | Tetracaine | | | |
| | | | Lidocaine | | | |
| | | Rabbit skeletal muscle SR | Lidocaine | 0.0–200 | ↓ | 28 |
| | | Canine cardiac SR | Lidocaine | 0.0–32 | ↓ | 29 |
| | | | Benzocaine | 1 | ↔ | |
| | $Na^+/K^+$-ATPase | Cultured heart cell membrane | Tetracaine | 10 | ↓ | 30 |
| | | Human red cells | Procaine | 0.37–37 | ↓ | 31 |
| | | | Lidocaine | 1.7–14 | | |
| | | | Dibucaine | 0.0079–0.79 | | |
| | | | Tetracaine | 0.35–1.3 | | |
| | | | Cocaine | 1.5–5.9 | | |
| | $Mg^{2+}/Ca^{2+}$-ATPase | Bovine brain cortex | Tetracaine | 3.3 | ↓ | 32 |
| | | | Procaine | | | |
| | Phospholipase $A_1$ and $A_2$ | Rat liver mitochondria | Lidocaine | 0.0–0.4 | ↓ | 33 |
| | | | Procaine | | | |
| | | | Dibucaine | | | |
| | | | Butacaine | | | |
| | Phospholipase C | Rat brain and liver | Tetracaine | 0.0–4 | ↓ | 34 |
| | GTPase (GTP hydrolysis) | HL-60 cells | Tetracaine | 0.001–10 | ↑ | 35 |
| | | | Bupivacaine and Lidocaine | | ↑ | |
| | | | Procaine | | ↓ | |
| | Phosphodiesterase and Myosine light chain kinase | Bovine brain | Dibucaine | 0.0–1 | ↓ | 36 |
| | | | Tetracaine | 0.0–2 | | |
| | | | Lidocaine | 0.0–8 | | |
| Receptor (ligand binding) | β-Adrenergic | Frog erythrocyte membrane | Dibucaine | 0.5–1 | Agonist and antagonist | 25 |
| | | | Tetracaine | 1.0–2 | | |
| | | | Bupivacaine | 1.0–2 | ↓ | |
| | | | Lidocaine | 2.0–5 | | |
| | Ryanodine | Sheep brain microsomal membrane | Tetracaine | 0.0–2 | Agonist | 37 |
| | | | Dibucaine | 0.0–1 | ↓ | |
| | | | Lidocaine | 0.0–6 | ↓ | |
| | | | | | ↑ | |

**TABLE 1. Continued**

| Parameter | Type | Tissue source | Agent (LA) | Dose (mM) | Effect | Ref. |
|---|---|---|---|---|---|---|
| | Muscarinic | Mouse neuroblastoma clone N1E-115 cells | Tetracaine<br>Butacaine<br>Procaine<br>Dibucaine<br>Lidocaine | 0.0–0.1 | Agonist<br>↓ | 26 |
| | | Guinea pig cerebral cortex synaptosomes | Tetracaine<br>Procaine<br>Bupivacaine<br>Lidocaine<br>Prilocaine | 0.01–100 | Antagonist<br>↓ | 38 |
| | NK1 (substance P) tachykinin | Chick brain membrane | Bupivacaine<br>Tetracaine<br>Benzocaine<br>Lidocaine | 0.0–3 | Antagonist<br>↓ | 39 |
| | Tachykinin | Rabbit iris sphincter muscle | Bupivacaine<br>Oxybuprocaine | 0.0001–0.3 | Antagonist<br>↓ | 40 |
| | κ-Opioid | Guinea pig cerebellum and Rat spinal cord | Bupivacaine<br>Tetracaine | 0–0.000005 | ↔ | 41 |
| Protein (expression) | DnaK and GroEL (heat shock) proteins | *Escherichia coli* | Dibucaine<br>Lidocaine<br>Tetracaine<br>Procaine | 2<br>100<br>4<br>400 | ↑ | 42 |
| | G-protein | Rat brain synaptosomes | Tetracaine | 0.001 | ↓ | 43 |
| Others | $Na^+/Ca^{2+}$ exchange | Human glial cells-U1242MG | Lidocaine<br>Procaine | 0.5–2<br>0.5 | ↓ | 44 |
| | $Ca^{2+}$ stores | Rabbit corneal epithelial cells | Tetracaine<br>Proparacine<br>Cocaine | 0.5–2.5<br>1.0–5<br>4.0–10 | ↑ | 45 |
| | | Murine cell-P388D1 | Bupivacaine<br>Tetracaine<br>Benzocaine<br>Lidocaine | 0.0–3 | ↓ | 39 |
| | $PIP_2$ breakdown | Rat cerebral miniprisms | Bupivacaine<br>Lidocaine<br>Etidocaine<br>Tetracaine<br>Prilocaine | 0–0.1 | ↓ | 46 |
| | | Pig cerebral cortical synaptoneurosomes | Dibucaine<br>Tetracaine<br>Bupivacaine<br>Lidocaine<br>Piperocaine<br>Prilocaine<br>Cocaine<br>Etidocaine | 0.0001–1 | ↓ | 47 |

Abbreviations: $PIP_2$, phosphatidylinositol 4,5-biphosphate; PKC, protein kinase C; SR, sarcoplasmic reticulum; LA, local anesthetic; ↓ = decrease or inhibition; ↑ = increase or stimulation; and ↔ = no effect.

blue (BPB). The second dimension run was performed at 15 mA until the tracking BPB dye entered into the separating gel, and then at 20 mA until the dye reached to the bottom.

After completion of the electrophoresis, the stacking gel was removed and the separating gel was fixed in a fixing solution (50% methanol, 12% glacial acetic acid) for 1 hr on a shaker with gentle shaking and transferred into 50% ethanol for 1 hr. The gels were treated with sodium thiosulfate (0.2 g/L) for 1 min and washed with distilled water for 1 min, including three changes for every 20 sec. The gels were then impregnated with silver nitrate (1 g/L) containing 0.75 mL of 37% formaldehyde for 30 min [18]. After washing the gels with distilled water for 1 min, the protein spots were developed with sodium carbonate (60 g/L) containing 0.5 mL of 37% formaldehyde/L. After this, gels were washed with distilled water and placed in fixing solution for 1 hr. The gels were photographed immediately and stored in 50% ethanol. For phosphorylated proteins, the gels were dried and then autoradiographed (Kodak, X-omat, Sigma). Quantitative analysis of proteins was done using an imaging densitometer.

The molecular mass of the resolved proteins in SDS–

PAGE was determined using standards that were co-electrophoresed with the samples (myosin—205,000; β-galactosidase—116,000; phosphorylase *b*—97,400; bovine albumin—66,000; egg albumin—45,000; carbonic anhydrase—29,000). In two-dimensional electrophoresis, the proteins were identified based on their molecular mass ($M_r$) and isoelectric point (pI). The pI of a specific protein was determined using the pH gradient of the IEF tube gel (gel extrusion method), which is co-run with ampholines alone. The IEF gel was cut into equal size (0.5-cm length) pieces, and each gel piece was placed in 1 mL of distilled water overnight with gentle shaking. The pH extruded from the gel into the distilled water was measured. A standard graph was drawn by plotting gel length on the X-axis and pH on the Y-axis.

To assess the cross-reactivity of protein-c with c-Fos antibody, Western blot analysis was used as detailed earlier [11].

## RESULTS

Intrathecal administration of local anesthetics resulted in reversible bilateral hind limb paralysis. This behavioral effect was accompanied by calcium-dependent phosphorylation of five spinal cord proteins. These proteins had apparent molecular masses of 130 (protein-a), 105 (protein-b), 55 (protein-c), 47 (protein-d), and 33 (protein-e) kDa (Fig. 1). Since the molecular weight of protein-c correlated with c-Fos, cross-reactivity was checked with c-Fos antibody. The results indicated that protein-c did not react with the antibody for c-Fos. On two dimensional electrophoresis, protein-a resolved into two acidic proteins (protein-a1 and a2 with pI values of 4.8 and 6.8, respectively) (Fig. 2, A–C). Statistical analysis of the densitometric data, using analysis of variance, indicated a significant effect for the treatment ($F_{2,16}$ 735, $P < 0.00005$). Post hoc comparisons with the saline-treated controls, using the Newman-Keuls test, indicated that local anesthetics significantly affected all phosphoproteins ($P < 0.05$) except for protein-a1 in the tetracaine-treated group. Moreover, comparison of the procaine- and the tetracaine-treated groups suggested significant ($P < 0.05$) differences in phosphoproteins (Fig. 3).

## DISCUSSION

In the past, local anesthetic-induced modulation of many cellular events mediated through membrane-associated receptors, enzymes, and other proteins has been assessed (Table 1). By identifying five specific phosphorylated proteins affected by spinal anesthesia, the present study has provided some markers for the signaling molecules affected during spinal anesthesia. Spinal anesthesia is known to stimulate PKC activity in the spinal cord [11, 12]. PKC activation is associated with several phosphorylated proteins that have electrophoretic characteristics similar to those we observed for proteins a–e. For example, neuromodulin (GAP-43, p57), an acidic phosphoprotein with an approximate molecular mass of 43–57 kDa, is associated with PKC activation [7]. Similarly, phosphoproteins with a molecular mass of 30–36 kDa, designated as RACKS (receptors for activated C-kinase), are associated with PKC activation [48]. Moreover, phosphorylation of the 48-kDa subunit of the glycine receptor is associated with PKC activation [49]. There is evidence that the phosphorylation of phospholipase C (PLC, the enzyme that cleaves inositol phospholipids to diacylglycerol and inositol phosphates), is mediated by PKC [50].

Among the proteins that had altered phosphorylation state during spinal anesthesia in the present study, the electrophoretic characteristics of protein-c are consistent with those of neuromodulin. The electrophoretic characteristics of protein-e are consistent with those of RACKS and cdc-2-like kinase [48, 51]. The electrophoretic characteristics of protein-d are consistent with those of the glycine receptor subunit [49]. The electrophoretic characteristics of protein-a are consistent with those of PLC-β [20]. Interestingly, PLC-β, as we observed in two-dimensional electrophoresis in our study, can resolve into two bands [52]. Therefore, further studies using western blot analysis to confirm the identity of the phosphoproteins a–e will be of value.

PKC is one among several kinases involved in phosphorylation. Thus, the proteins phosphorylated during spinal anesthesia may not necessarily reflect the effect of the anesthetics on PKC activity. Our data showed that the molecular weight of protein-c correlated with that of c-Fos; however, protein-c did not react with the antibody for c-Fos. In any case, based on the present results, it would be difficult to exclude the role of kinases other than PKC in the mechanism of spinal anesthesia.

Local anesthetic potency correlates with physicochemical properties. Using a test paradigm similar to the one used in the present study, we have shown that the lipid/water partition coefficient predicts *in vivo* potency of local anesthetics [13]. In an effort to assess the relative potency of local anesthetics on phosphoproteins, isomolar concentrations of the less lipid-soluble procaine and the more lipid-soluble tetracaine were used in this study. Drug-induced quantitative and qualitative differences in phosphoproteins may thus reflect differential sensitivity of these phosphoprotein-mediated signal transduction pathways to the lipid solubility of the compound being tested. Assessment of time–course and dose–response of many local anesthetics with different lipid-water partition coefficients is warranted to determine the effects of anesthetic potency on specific phosphoproteins.

In conclusion, the present *in vivo* data demonstrated that spinal anesthesia changes the phosphorylation state of five endogenous substrate proteins. Further characterization of these phosphoproteins affected during spinal anesthesia is indicated to confirm their identity. Such an identification should aid in a better understanding of the role of these

specific proteins in the signal transduction cascade affected by spinal anesthesia.

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