

Bupivacaine Inhibits Activation of Neuronal Spinal Extracellular Receptor-activated Kinase through Selective Effects on Ionotropic Receptors

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Background: Central terminals of primary nociceptors release neurotransmitters glutamate and substance P, which bind to ionotropic or metabotropic receptors on spinal neurons to induce cellular responses. Extracellular signal-regulated kinases are activated by these receptors and are important modulators of pain at the dorsal horn. The authors investigated these pathways as potential targets for antinociceptive actions of local anesthetics.

Methods: The effects of bupivacaine on the activation of extracellular receptor-activated kinase (phosphorylation to pERK) in rat spinal cord slices, induced by presynaptic release (capsaicin), by presynaptic or postsynaptic ionotropic or metabotropic receptor activation, or by activation of intracellular protein kinase C or protein kinase A and also by a receptor-independent Ca^{2+} ionophore, were quantitated by immunohistochemistry, counting pERK-positive neurons in the superficial dorsal horn.

Results: Capsaicin (3 μM , 10 min)-stimulated pERK was reduced by bupivacaine (IC_{50} approximately 2 mM, approximately 0.05%), which similarly suppressed pERK induced by the ionotropic glutamate receptors for *N*-methyl-D-aspartate and (*S*)- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid but not that induced by the metabotropic receptors for glutamate, bradykinin, or substance P. Extracellular receptor-activated kinase activation by the Ca^{2+} ionophore ionomycin was also sensitive to bupivacaine, but direct activation by protein kinase A or protein kinase C was not.

Conclusions: Bupivacaine inhibits pERK activation resulting from different modes of Ca^{2+} influx through the plasma membrane. This represents a postsynaptic mechanism of analgesia that occurs in parallel with impulse inhibition during neuraxial blockade.

THE mechanism by which local anesthetics block impulses in peripheral nerves through inhibition of voltage-gated sodium channels is well established. In contrast, the overall mechanism of spinal and epidural anesthesia may be more complex than simply the blockade of impulses in nerve roots, involving presynaptic and postsynaptic receptors as well as intracellular pathways.¹ Local anesthetics may interact with many membrane phospholipids and proteins and thereby affect a variety of cellular activities. For example, local anesthetics can affect several subtypes of protein kinase C

(PKC),^{2,3} adenosine 3',5'-cyclic monophosphate-dependent kinase (PKA),⁴ guanosine triphosphate-binding proteins (G proteins),⁵ and many of the various receptors that activate them.⁶ Although there are many examples of these actions in isolated cells, few of them are spinal neurons, and none of them have been examined in spinal tissue.

Included in spinal responses to peripheral nerve input are the mitogen-activated protein kinases (MAPKs), a family of serine-threonine protein kinases that are activated in spinal cord by noxious peripheral stimuli and play important roles in modulating signal transduction, both acutely by protein phosphorylation and chronically by affecting gene transcription.⁷ Extracellular signal-regulated kinases (ERK 1 and 2) are members of the MAPK family that transduce extracellular stimuli into intracellular responses. ERK activation in spinal dorsal horn neurons has been demonstrated to be nociceptive activity dependent.⁸ An increase in excitation in the superficial dorsal horn induces the activation of PKA and PKC, eventually leading to the activation of ERK signaling. Increase of cytoplasmic Ca^{2+} also seems essential for noxious stimulation-induced ERK activation.⁹ Although PKA, PKC, and increased intracellular Ca^{2+} have all been shown as positive factors in modulating nociception-induced ERK activation at the spinal cord dorsal horn,¹⁰ and spinal local anesthetics are noteworthy for their antinociceptive action, almost nothing is known about the effects of local anesthetics on the responses of this MAPK.

Noxious stimulation causes the central terminals of primary nociceptor afferents to release neuropeptides such as substance P (SP) and the excitatory neurotransmitter glutamate in the spinal cord¹¹ (fig. 1). These chemicals bind to ionotropic and/or metabotropic receptors to induce cellular responses in postsynaptic dorsal horn neurons; SP acts on postsynaptic G protein-coupled neurokinin-1 (NK-1) receptors and stimulates G protein-mediated activation of phospholipase C, leading to ERK activation, and glutamate acts on ionotropic ligand-gated ion channels, such as (*S*)- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA/kainate) and *N*-methyl-D-aspartate (NMDA) receptors, and on G protein-coupled metabotropic glutamate receptors (mGluRs).¹² Activation of the ionotropic receptors causes a postsynaptic membrane depolarization and Ca^{2+} entry, whereas metabotropic receptors activate various kinases and also indirectly increase $[Ca^{2+}]_i$ in dorsal horn neurons of the spinal cord.¹² Capsaicin, by binding to the ligand-gated vanilloid-sensitive transient

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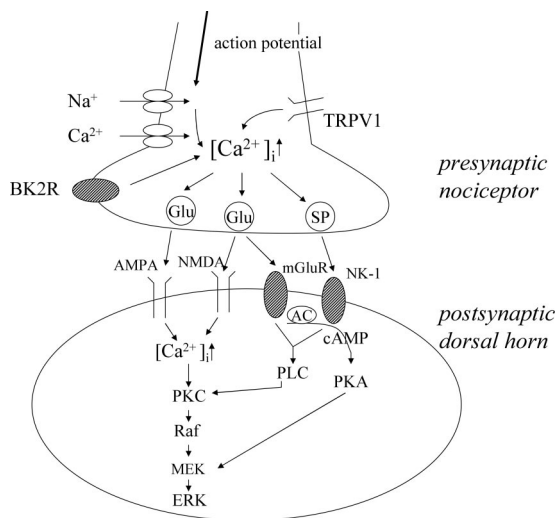


Fig. 1. A scheme of induction of extracellular signal-regulated kinase phosphorylation (pERK) at the central terminal of primary afferent. On presynaptic dorsal horn neurons, activation of vanilloid receptor (TRPV1) or action potential results in increased presynaptic $[Ca^{2+}]_i$ and subsequent release of glutamate (Glu) and substance P (SP); Glu binds to ionotropic *N*-methyl-D-aspartate (NMDA) and (*S*)- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and to metabotropic receptors mGluR; SP binds to G protein-coupled neurokinin 1 (NK-1) receptor. The activation of these postsynaptic receptors activates protein kinases such as PKA and PKC, and ERK pathway. AC = adenylate cyclase; BK2R = bradykinin 2 receptor; cAMP = cyclic adenosine monophosphate; MEK = MAP kinase kinase; PLC = phospholipase C.

receptor potential channel, TRPV1, on presynaptic terminals, directly catalyzes Ca^{2+} entry and also effects a depolarization that activates presynaptic calcium channels, as well as releasing Ca^{2+} from intracellular reticuli whose membranes contain TRPV1 channels,¹³ all of which result in the release of glutamate and SP from primary nociceptor terminals.^{13,14}

Therefore, to understand more fully the overall effects on cell activation signaling from the actions of local anesthetics in the spinal cord, we assessed the effects of bupivacaine on agonist-induced ERK activation in dorsal horn neurons *in vitro*, by immunohistochemical techniques, using transverse slices of the spinal cord in rats. We evaluated the action of bupivacaine on various modes of agonist-induced ERK activation, including the TRPV1 agonist capsaicin (presynaptic), G protein-coupled receptor (GPCR) agonists for the group 1 metabotropic glutamate receptors, mGluR1/5, the NK-1 receptor agonists SP, or bradykinin (presynaptic and postsynaptic), and ligand-gated receptor agonists such as NMDA and AMPA (postsynaptic) in dorsal horn neurons. Furthermore, to better know the subcellular site of action of bupivacaine, we investigated whether bupivacaine affected PKA-activated, PKC-activated, or Ca^{2+} ionophore-induced ERK phosphorylation (pERK) pathways in the spinal cord.

Materials and Methods

Experimental Animals

All the procedures were approved by the Standing Committee on Animals at Harvard Medical School, Boston, Massachusetts. The experiments were performed on male, adult Sprague-Dawley rats (age, 6–7 weeks; weight, 150–200 g; Charles River Laboratories, Cambridge, MA), which were kept in pairs inside a plastic cage with soft bedding. The rats were housed on a 12-h light-dark cycle with food and water provided *ad libitum*.

Preparation of Drugs

Drugs were ultimately dissolved in Krebs-Ringer's (K-R) solution containing 117 mM NaCl, 3.6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4 , 25 mM $NaHCO_3$, 11 mM glucose, and 20 mM HEPES, pH 7.4. A Ca^{2+} -free K-R solution had all the above ingredients save for added Ca^{2+} and included 4 mM EGTA to chelate any trace Ca^{2+} ions. Capsaicin, the phorbol ester 12-myristate-13-acetate (PMA), forskolin, bradykinin, H-89, and ionomycin (calcium salt) were first dissolved in pure dimethyl sulfoxide at 100–1,000 times the concentrations to be used and then diluted to the desired concentrations in K-R solution immediately before use. NMDA, SP, EGTA, AMPA, and (*R,S*)-3,5-dihydroxyphenylglycine (DHPG), an mGluR1 and mGluR5 selective agonist, were first dissolved in distilled water at 100 or 1,000 times the concentrations to be used and then diluted to the desired concentrations in K-R solution immediately before use. Bupivacaine-HCl was dissolved in K-R solution immediately before use to its final concentration. Racemic DHPG and ionomycin calcium salt were purchased from Tocris Bioscience (Ellison, MO), and capsaicin, bupivacaine, forskolin, bradykinin, (*S*)-AMPA, NMDA, SP, 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP), and EGTA were purchased from RBI/Sigma-Aldrich (St. Louis, MO). 8-Br-cAMP was first dissolved in NaOH (1N) at 1,000 times the concentrations to be used and then diluted to the desired concentrations in K-R solution immediately before use. PMA and H-89 hydrochloride were from CALBIOCHEM (San Diego, CA).

Preparation and Treatment of Spinal Cord Slices

Spinal cord slices were prepared as described previously.¹⁰ Rats were anesthetized with urethane (1.5 g/kg, intraperitoneal), a lumbosacral laminectomy was performed, and the spinal cord (L1–S3) was removed and placed in oxygenated (95% O_2 -5% CO_2) K-R solution at 1°–3°C. The rats were killed after removal of spinal cord by an overdose of pentobarbital (100–150 mg/kg, intraperitoneal) or urethane (4–5 g/kg, intraperitoneal). After all ventral and dorsal roots near the root entry site of the isolated cord were cut and the pia-arachnoid membrane was removed, the spinal cord was mounted on a vibratome (Series 1000®; Technical Products Interna-

tional, O'Fallon, MO), and several transverse slices (700 μm thickness) were cut. The slices were placed in a small superfusion chamber (RC-27L; Warner Instruments, Hamden, CT) and superfused in a preincubation period for 3 h at a flow rate of 8–10 ml/min with recirculated solution from an 80-ml reservoir containing K-R solution bubbled with 95% O_2 -5% CO_2 , maintained at pH 7.4. The perfusate was warmed so that the temperature in the superfusion bath was maintained at $36^\circ \pm 2^\circ\text{C}$.

For control experiments without bupivacaine, spinal slices were then exposed at the same superfusion condition to bath-applied stimulants: to capsaicin (3 μM) for 10 min and washout for 10 min with K-R solution; or to NMDA (100 μM) for 10 min, to AMPA (100 μM) for 20 min, to SP (100 μM) for 20 min, to bradykinin (100 nM) for 10 min, and to mGluR1/5 agonist (5 μM) for 10 min.

Agonist exposure times were chosen to be 10 or 20 min so that pERK levels would increase significantly. In preliminary experiments with capsaicin, we observed no difference in bupivacaine's relative inhibition of pERK activation after 10 compared with 20 min of exposure to the agonist (data not shown), indicating that the time of this agonist's exposure did not affect bupivacaine's effectiveness. Because insubstantial reversal of ERK activation by capsaicin occurred during the 10-min washout period (when no agonist was present; data not shown) we removed this step from the other drug exposure protocols. Bupivacaine (0.5, 2, or 5 mM) in K-R solution was continuously applied for 10 min before and also during stimulant application (and for 10 min after removal of capsaicin). After drug exposure, the slices were fixed in 4% paraformaldehyde for 60 min, replaced with sucrose (0.4 M, 15%) overnight at 8°C , and then cut in the cryostat to a thickness of 15 μm . Six to eight non-adjacent sections of these were randomly picked for analysis and processed for immunohistochemistry.

Immunohistochemistry

Immunofluorescence was quantitated as described previously.¹⁵ Briefly, spinal sections were blocked with 2% goat serum in 0.3% Triton X-100 for 1 h at room temperature (20° – 22°C) and incubated overnight at 4°C with anti-pERK primary antibody (anti-rabbit, 1:400; Cell Signaling Technology, Beverly, MA). The sections were incubated with Cy-3 conjugated second antibody (anti-rabbit, 1:300; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The stained sections were examined with a Nikon (Tokyo, Japan) fluorescence microscope, and images were captured with a charge-coupled device spot camera. Each section was counted under $10\times$ magnification. The numbers of immunoreactive neurons positive for pERK were counted in laminae 1 and 2.

Quantification and Statistics

Six to eight nonadjacent sections from the lumbar spinal cord (L4–L5) segments were randomly selected. The number of pERK-positive neurons in the superficial dorsal horn (laminae 1 and 2) from each of the six to eight sections incubated in identical conditions were averaged for each animal. The data are reported as mean \pm SEM of positive cells per section. Differences between the control (unstimulated) condition and stimulated slices were compared with or without bupivacaine to calculate percent inhibition for each separate experiment: % inhibition = $100\{1 - (\text{agonist with bupivacaine} - \text{control}) / (\text{agonist} - \text{control})\}$. These percent inhibitions were averaged over minimally three experiments, and the results were compared using multigroup analysis of variance with the Bonferroni correction (Statview Software, Cary, NC). The criterion for statistical significance was $P < 0.05$.

Results

Effects of Bupivacaine on Capsaicin-induced ERK Activation

Bath application of capsaicin (3 μM) for 10 min was sufficient to activate pERK in the superficial dorsal horn (figs. 2A and B). The number of activated neurons after capsaicin (3 μM) application for 10 min was increased by approximately fivefold compared with control. Activation of ERK by capsaicin was suppressed by bupivacaine (figs. 2B and C). Bupivacaine (0.5, 2, or 5 mM) continuously applied for 10 min before, during, and after capsaicin application decreased the number of pERK-positive cells in a concentration-dependent manner, with the highest bupivacaine concentration reducing pERK to the control level (fig. 3). There was no significant effect of bupivacaine (2 mM) on "control" pERK levels in the absence of stimulant ($114 \pm 19\%$, $P = 0.79$ compared with control). The stimulatory effects of all the agonists tested and their inhibition by bupivacaine are shown in the bar graphs (figs. 3–7) and the numerical values listed in table 1.

Effects of Bupivacaine on ERK Activation by Ligand-gated Receptor Agonists

Levels of pERK were significantly higher after spinal slice exposure to both NMDA (100 μM) and AMPA (100 μM) (307%, $P < 0.01$, and 401%, $P < 0.01$, respectively, compared with control). Bupivacaine (2 mM) reduced both NMDA- and AMPA-induced ERK activation, and by the same degree (59% inhibition for NMDA and 67% for AMPA) as that for the capsaicin response (fig. 4 and table 1). Comparison of the inhibitory effects of bupivacaine (2 mM) by analysis of variance among the three ligand-gated receptor agonists, capsaicin, NMDA, and AMPA, showed that the inhibitions were the same ($P = 0.65$).

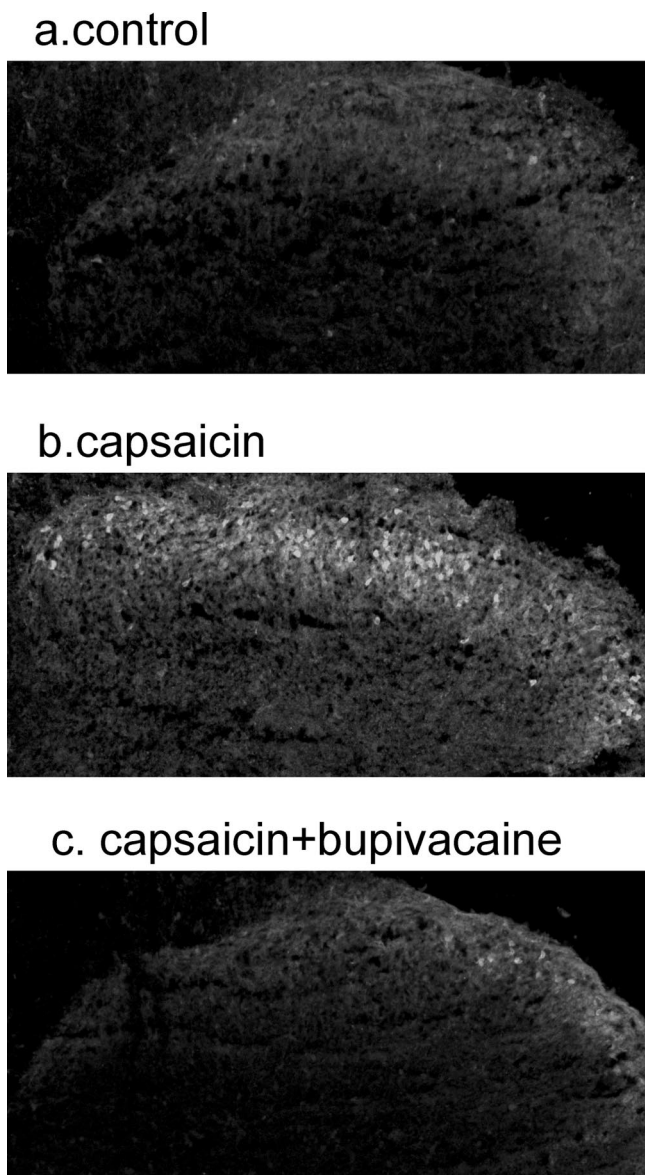


Fig. 2. Extracellular signal-regulated kinase phosphorylation (pERK) immunofluorescence in the superficial dorsal horn of spinal cord in control unstimulated (A), capsaicin (3 μM , 10 min)-stimulated (B), and capsaicin-stimulated with bupivacaine (2 mM) (C). Only the activated, phosphorylated form (pERK) binds the antibody.

Bupivacaine Does Not Attenuate ERK Activation by G Protein-coupled Receptor Agonists

Exposure of slices to SP (100 μM), to bradykinin (100 nM), or to an agonist of metabotropic glutamate (mGluR1, 5) receptors (DHPG, 5 μM) all induced ERK activation (fig. 5 and table 1). However, no significant inhibitory effects of bupivacaine (2 mM) were seen on ERK activation by any of these three GPCR agonists (fig. 5 and table 1). Although bupivacaine caused a small increase in SP-induced pERK activation (fig. 5A), this difference did not reach statistical significance.

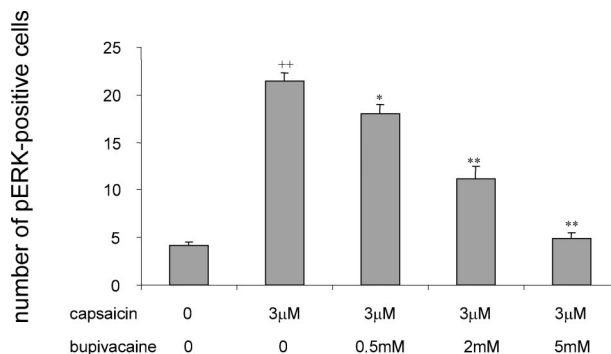


Fig. 3. Numbers of extracellular signal-regulated kinase phosphorylation (pERK)-stained neurons in the superficial dorsal horn (laminae 1 and 2) after bath-applied exposure of capsaicin (3 μM) with or without bupivacaine (0.5–5 mM). ++ $P < 0.01$ compared with control; * $P < 0.05$, ** $P < 0.01$ compared with capsaicin without bupivacaine.

Bupivacaine Does Not Accelerate Reversal of ERK Activation

To explore the possibility that bupivacaine might depress pERK levels by accelerating its dephosphorylation, we compared the levels of pERK between experiments where stimulation by agonist plus bupivacaine was followed by a 10-min washout period with bupivacaine present, to levels of pERK from the same stimulation protocol but without any washout. Specifically, bupivacaine's inhibition of NMDA-stimulated pERK was 66% after a bupivacaine-accompanied washout and 59% without washout ($P > 0.10$). Similarly, bupivacaine's insignificant inhibition of bradykinin-stimulated pERK was 3.4% with bupivacaine washout and 1.2% with no washout. Apparently, the reduction of pERK levels by bupivacaine during stimulation are due to an inhibition of pERK formation and not an acceleration of its dephosphorylation/deactivation.

Varied Effects of Bupivacaine on Intracellular Stimulant-induced ERK Activation

Ten minutes of bath application of the PKC activator PMA (5 μM), the PKA activator forskolin (10 μM), or the Ca^{2+} ionophore ionomycin (10 μM) also induced pERK activation (fig. 6 and table 1). Bupivacaine (2 mM) had no inhibitory effect on PMA-induced pERK but significantly suppressed ionomycin- and forskolin-induced pERK (but see the last section of Results).

Dependence of Agonist-induced pERK on Extracellular Ca^{2+}

Ionomycin causes a rapid increase in cytosolic Ca^{2+} that is due to both Ca^{2+} influx through the plasma membrane and a release of Ca^{2+} from cytosolic stores¹⁶ and activates MAPKs in neutrophils.¹⁷ To determine the contribution of extracellular Ca^{2+} influx on inotropic receptor agonist-induced pERK, spinal slices were pretreated for 10 min with EGTA containing Ca^{2+} -free K-R and then exposed to agonist in Ca^{2+} -free K-R. This pre-

Table 1. Agonist-induced pERK and Percent Inhibition of pERK with or without Bupivacaine

	Agonist/Control	P Value	n	% Inhibition	P Value	n
Ligand-gated receptor agonists						
Capsaicin	5.3 ± 0.6	< 0.01	6	60	< 0.01	6
NMDA	3.1 ± 0.3	< 0.01	8	59	< 0.01	8
AMPA	4.0 ± 0.8	< 0.01	7	67	< 0.01	7
GPCR agonists						
Bradykinin	2.0 ± 0.1	< 0.01	6	2	0.90	6
mGluR1/5	2.3 ± 0.2	< 0.01	6	6	0.75	6
Substance P	2.0 ± 0.2	< 0.01	7	(-) 29*	0.075	7
Intracellular stimulators						
PMA	2.0 ± 0.1	< 0.01	4	0.4	0.98	4
Forskolin	3.3 ± 0.4	< 0.01	6	39	0.026	6
Ionomycin	2.7 ± 0.4	< 0.01	4	61	< 0.01	4

Values are mean ± SEM.

* Stimulation of response by bupivacaine.

AMPA = (S)-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; GPCR = G protein-coupled receptor; mGluR = metabotropic glutamate receptor; NMDA = N-methyl-D-aspartate; pERK = extracellular signal-regulated kinase phosphorylation; PMA = phorbol ester 12-myristate-13-acetate.

treatment reduced ionotropic agonist-induced pERK activation by 93% (*P* < 0.01) for capsaicin, by 99% (*P* < 0.01) for NMDA, and by 93% (*P* < 0.01) for AMPA, resulting in levels of pERK indistinguishable from unstimulated controls. Such effects are consistent with the assignment of Ca²⁺ flux through the plasma membrane as the predominant source for driving ERK activation.

In contrast, Ca²⁺-free K-R treatment had no inhibitory effects on increase of pERK by SP (32% inhibition, *P* = 0.12), mGluR1, 5 (0.2% inhibition, *P* = 0.99), or bradykinin (-6% inhibition, *P* = 0.68) or on basal, unstimulated pERK levels. After Ca²⁺-free K-R treatment, pERK induced by these metabotropic receptors remained significantly higher than the unstimulated control (fig. 7).

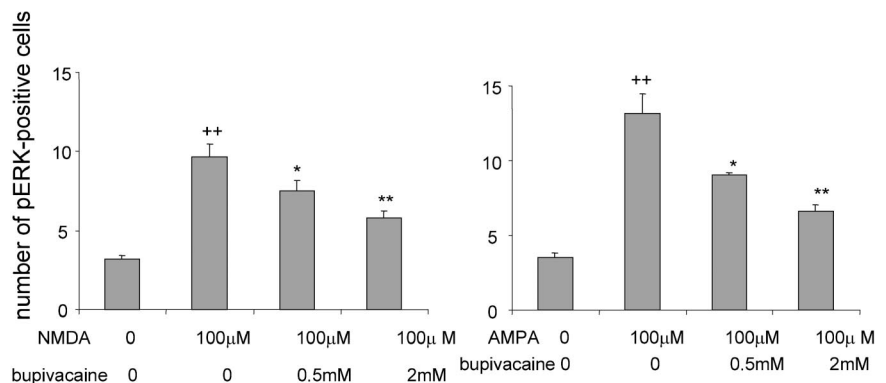
Interestingly, in Ca²⁺-free K-R, the stimulatory action of forskolin in increasing pERK was also reduced (by 84%), consistent with the mechanism of forskolin involving the entry of extracellular Ca²⁺ (probably resulting from forskolin's blocking K⁺ channels and resultant neuron depolarization) rather than the traditional action of stimulating adenylate cyclase and thus activating PKA.¹⁸ To confirm this hypothesis, we used the cell membrane-permeable cAMP analog, 8-Br-cAMP, to directly activate PKA and thereby increase pERK (206% of control, *P* < 0.01). Bupivacaine had no significant inhibitory effect on

this 8-Br-cAMP-induced increase of pERK (26% inhibition, *P* = 0.089; fig. 8). In addition, use of a specific inhibitor of PKA, H-89 (1 μM), resulted in only 7% inhibition of forskolin stimulation of pERK (*P* > 0.25 compared with uninhibited forskolin) independently confirming that PKA was not involved in forskolin-induced pERK increase (fig. 8). Both results support the model of forskolin-activated pERK activation by Ca²⁺ entry through depolarized plasma membrane rather than by direct activation of PKA.

Discussion

Agonists to both ionotropic receptors (capsaicin, NMDA, and AMPA) and metabotropic receptors (SP, bradykinin, and mGluR1/5) are known to induce pERK activation in superficial dorsal horn neurons of spinal cord slices from rats.¹⁰ Receptors for capsaicin (TRPV1) and bradykinin (B2) are assumed to be present exclusively on presynaptic terminals of nociceptive afferents (fig. 1), whereas both types of the glutamate and the SP (NK-1) receptors may be on both postsynaptic dorsal horn and presynaptic neuronal elements.^{19,20} The work here shows that bupivacaine selectively and equipo-

Fig. 4. Numbers of extracellular signal-regulated kinase phosphorylation-stained neurons in the superficial dorsal horn (laminae 1 and 2) after bath-applied exposure of ionotropic glutamate receptor agonists N-methyl-D-aspartate (NMDA, 100 μM; left) and (S)-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA, 100 μM; right) in the presence of bupivacaine (0.5–2 mM). ++ *P* < 0.01 compared with control; * *P* < 0.05, ** *P* < 0.01 compared with NMDA without bupivacaine or AMPA without bupivacaine.



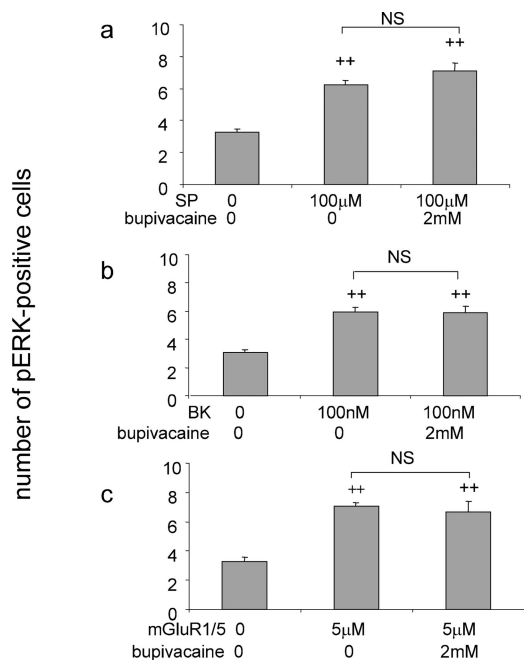


Fig. 5. Numbers of extracellular signal-regulated kinase phosphorylation-stained neurons in the superficial dorsal horn (laminae 1 and 2) after bath-applied exposure of metabotropic receptor agonists, substance P (SP, 100 μM; A), bradykinin (BK, 100 nM; B), and agonists for metabotropic glutamate receptors 1/5 (mGluR1/5, 5 μM; C) with or without bupivacaine (2 mM). ++ $P < 0.01$ compared with control. NS = not significant.

tently inhibits ERK activation by all the ionotropic receptor agonists. In contrast, bupivacaine was without effect on metabotropic receptor-induced pERK and on unstimulated pERK levels in control slices. The Ca^{2+} ionophore ionomycin and the PKC activator PMA also increased pERK, with ionomycin sensitive to but PMA insensitive to bupivacaine. Forskolin, probably acting not through its traditional mode of PKA activation but instead probably by depolarizing membranes to allow Ca^{2+} entry, also activated ERK, an action also inhibited by bupivacaine. In all cases where pERK induction involved Ca^{2+} flux through the plasma membrane, it was suppressed by bupivacaine, but if pERK was activated by processes downstream of these steps, such as release of Ca^{2+} from intracellular stores or by direct activation of PKC, there was no effect of bupivacaine.

These differential effects raise several fundamental questions: Is this action of bupivacaine primarily presynaptic or postsynaptic? What is the molecular basis for the sensitivity of Ca^{2+} entry pathways? How does this action of bupivacaine compare with its effects on ionotropic receptors and GPCRs in other systems?

The Locus of Bupivacaine's Action: Presynaptic or Postsynaptic

What does the selective effect of bupivacaine tell us about its locus of action? Receptors for capsaicin (TRPV1) are exclusively located at presynaptic terminals of nociceptive afferents, but their opening leads to re-

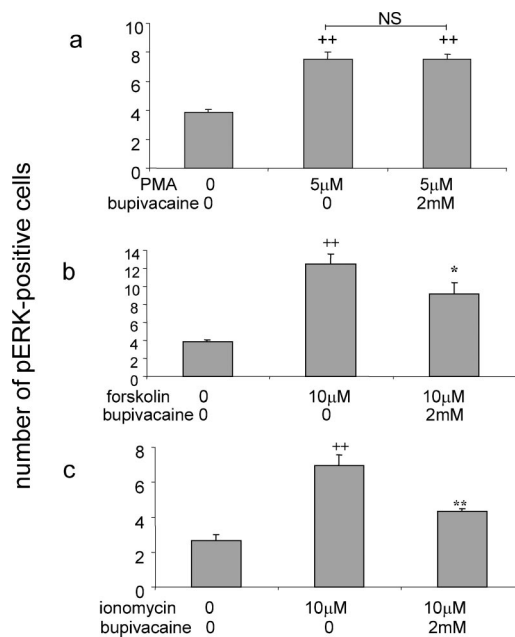
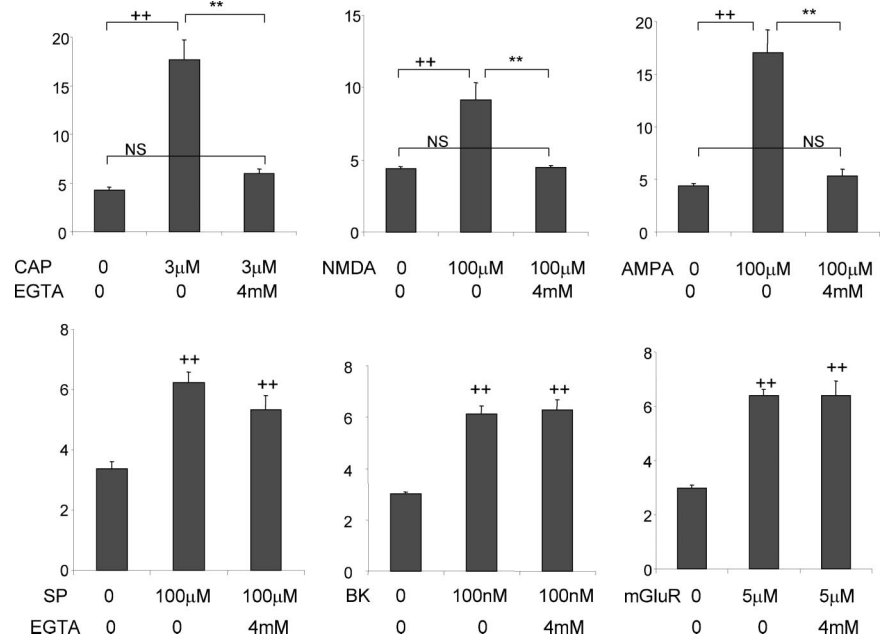


Fig. 6. Numbers of extracellular signal-regulated kinase phosphorylation-stained neurons in the superficial dorsal horn (laminae 1 and 2) after bath application of intracellular stimulants, the protein kinase C activator phorbol ester 12-myristate-13-acetate (PMA, 5 μM; A), the protein kinase A activator forskolin (10 μM; B), and the Ca^{2+} ionophore ionomycin (10 μM; C), each with or without bupivacaine (2 mM). ++ $P < 0.01$ compared with control; * $P < 0.05$, ** $P < 0.01$ compared with forskolin without bupivacaine or ionomycin without bupivacaine. NS = not significant.

lease of glutamate¹³ into the synaptic cleft and the subsequent occupancy and activation of ionotropic receptors on dorsal horn neurons, a process also shown here to be bupivacaine sensitive. On the other hand, AMPA and NMDA receptors are classically located on postsynaptic sites but also can be found on presynaptic endings. Presynaptic NMDA autoreceptors seem to facilitate the release of SP and glutamate in a positive-feedback network.¹⁹ Therefore, the inhibitory effects of bupivacaine on NMDA-induced pERK might be at presynaptic and/or postsynaptic sites. In contrast, AMPA receptors have an excitatory postsynaptic action but also a strong *inhibitory* presynaptic action on glutamate release from primary afferent terminals in the superficial dorsal horn.²⁰ Therefore, AMPA's activation of pERK must be occurring predominantly through postsynaptic receptors, in which case the inhibitory site of bupivacaine for this action also must be postsynaptic. So bupivacaine unquestionably has presynaptic actions, blocking Na^{+} channels, Ca^{+2} channels, and TRPV1 receptor channels (as well as some ionotropic receptors) but also seems to have actions at sites on the postsynaptic structure. Blockade of the presynaptic Na^{+} and Ca^{+2} channels is probably an insignificant mechanism for bupivacaine's suppression of ERK activation by the agents studied here, because the reported IC_{50} values for inhibition of these voltage-gated ion channels are in the range of 10^{-5} and 10^{-4} M,

Fig. 7. Numbers of extracellular signal-regulated kinase phosphorylation-stained neurons in the superficial dorsal horn (laminae 1 and 2) after bath-applied exposure of ionotropic receptor agonists, capsaicin (CAP, 3 μ M), *N*-methyl-D-aspartate (NMDA, 100 μ M), and (*S*)- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA, 100 μ M), metabotropic receptor agonists, substance P (SP, 100 μ M), bradykinin (BK, 100 nM), and metabotropic glutamate receptor agonists 1/5 (mGluR, 5 μ M), in the presence of EGTA (4 mM) with Ca²⁺-free Krebs-Ringer's solution. ++ *P* < 0.01 compared with control; ** *P* < 0.01 compared with capsaicin, NMDA, AMPA, or BK. NS = not significant.



respectively, well below the 2×10^{-3} M value reported here.²¹⁻²³

Interaction of Bupivacaine and Ionotropic Receptors

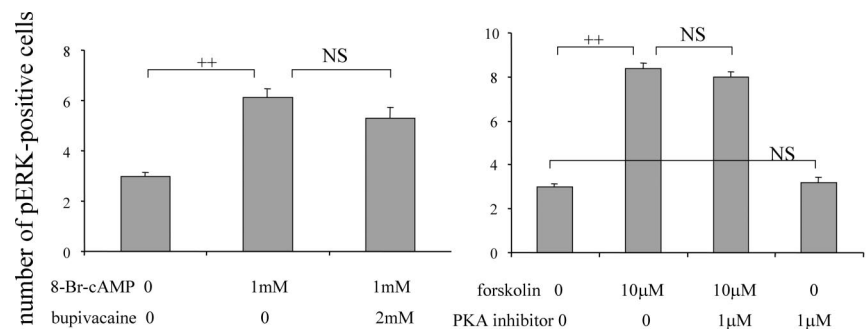
There is ample evidence that synaptic transmission in the spinal cord is inhibited by local anesthetics (LAs) through modification of presynaptic and postsynaptic ligand-gated channels as well as the traditional targets of presynaptic voltage-gated Na⁺ and Ca²⁺ channels that are essential to stimulate transmitter release (reviewed in Gokin and Strichartz²⁴). Hirota *et al.*²⁵ have reported that LAs are direct antagonists of TRPV1 receptors expressed in transfected human embryonic kidney cells. Similarly, a recent study showed that 1 mM bupivacaine reduces capsaicin-activated TRPV1 currents by approximately 50%, consistent with a direction inhibition by bupivacaine of capsaicin's stimulation of pERK in the current study.²⁶

The NMDA receptors are one of the major receptor channels mediating rapid excitatory neurotransmission in the central nervous system and also play a key role in central sensitization regarding long-term pain.²⁷ The in-

hibition of NMDA receptors by LAs in the spinal cord could therefore be important in preventing the development of pain and might play an important role in the prevention of pain by spinal and epidural anesthesia. However, there is little information about the interaction of LAs and NMDA receptors in the spinal cord. Previous electrophysiologic studies demonstrated that LAs inhibited NMDA-induced current from receptors expressed in *Xenopus* oocytes²⁸ and in mouse CA1 pyramidal neurons.²⁹ The slow ventral root potentials recorded from isolated hemisectioned spinal cord, and known to be driven by NMDA and NK-1 receptors, were markedly attenuated by low concentrations of lidocaine,³⁰ and the NMDA receptor-dependent windup observed in superficial dorsal horn neurons *in vivo* during repetitive electrical stimulation at C-fiber intensity was also strongly suppressed by lidocaine, albeit from intrathecal application of high "clinical concentrations" (2%, approximately 75 mM).³¹ These effects are consistent with our observations that NMDA-induced pERK activation was inhibited by bupivacaine.

The role of AMPA receptors in pain processing in the spinal cord dorsal horn is complex. The Ca²⁺-imperme-

Fig. 8. Both the cyclic adenosine monophosphate (cAMP) homologue 8-Br-cAMP (*left*) and forskolin (*right*) increase the number of pERK-positive neurons in the dorsal horn, but the stimulation by 8-Br-cAMP is insensitive to bupivacaine (at concentrations that suppress the stimulation by forskolin), and the stimulation by forskolin is not reduced by H-89, an inhibitor of protein kinase A (PKA), together showing that the cAMP-PKA pathway for extracellular signal-related kinase activation is not inhibited by bupivacaine. NS = not significant. ++ *P* < 0.01.



able species of AMPA receptors supports a fast transient depolarization that drives the membrane potential toward impulse threshold and also, if sufficiently prolonged, facilitates release of Mg^{2+} from its blocking site in the pore of the "open" conformation of the NMDA receptor. Calcium entry thus occurs only indirectly through the actions of these AMPA receptors. The Ca^{2+} -permeable AMPA receptors are not essential for acute nociceptive processing but are critically involved in the spinal plasticity that underlies hyperalgesia in inflammatory pain states, including the phosphorylation of ERK.^{20,32} The dorsal horn of the spinal cord shows a high density of these Ca^{2+} -permeable AMPA receptors, particularly in the superficial spinal laminae (laminae 1 and 2).³³ Our finding that AMPA-induced pERK activation was reduced by pretreatment with Ca^{2+} -free K-R confirms that Ca^{2+} -permeable AMPA receptors are involved here and is consistent with the participation of spinal ERK in hyperalgesia from peripheral nerve injury or inflammation. Specifically, inhibition of ERK responses to AMPA and NMDA predict that postinjury responses in dorsal horn through these receptors will be effectively suppressed by this local anesthetic. We also show that the inhibitory potency of bupivacaine (2 mM) is same among the three ionotropic receptors, TRPV1, NMDA, and AMPA, and thus suggest that similar mechanisms for blocking Ca^{2+} influx might be involved in the actions on all ligand-gated receptors.

It is noteworthy that bupivacaine similarly inhibited pERK activation by ionomycin, a Ca^{2+} ionophore whose molecular mechanism involves the reversible chelation of calcium ions, that decreases their energy for diffusing through lipid bilayer regions of membranes, independent of any proteinaceous receptor.¹⁶ This broad pharmacology for bupivacaine suggests that it is acting at a locus on the membrane phospholipids and not at any defined sites on receptors, a mechanism consistent with its low potency and lack of specificity among ionotropic receptors.

PKA and PKC

Both PKA and PKC contribute to the activation of ERK in dorsal horn neurons. Kawasaki *et al.*¹⁰ reported that there were additive effects of PKA and PKC on ERK activation, indicating that PKA and PKC are independent pathways to activate ERK in dorsal horn neurons. Forskolin, acting as a classic stimulator of adenylyl cyclase,³⁴ can increase cAMP, activating PKA, and thus coupling to ERK through the Raf/Ras pathway. Although bupivacaine had no effects on pERK induced by nociceptor-associated GPCR agonists, which might also act through Ras/Raf, bupivacaine still reduced forskolin-induced pERK. Several studies have demonstrated, however, that forskolin has diverse sites of action including not only adenylyl cyclase, but also the glucose transporter and ligand- and voltage-gated ion channels.³⁵ Forskolin mod-

ulates voltage-dependent K^+ conductance through cAMP-dependent and cAMP-independent mechanisms,³⁵ inhibiting voltage-dependent K^+ channels in *Helix* neurons,³⁶ nudibranch neurons,¹⁸ and PC12 cells³⁷ in a cAMP-independent manner. Potassium channel activity is a major determinant of resting and action potentials. Inhibition of some of these K^+ channels results in a steady depolarization that can support Ca^{2+} entry through non- or slowly inactivating Ca^{2+} channels and thus activate ERK. Because our data show that bupivacaine had no significant effects on 8-Br-cAMP-induced pERK and that a PKA inhibitor had no effects on forskolin-induced pERK, the mechanism by which bupivacaine inhibits forskolin-induced pERK seems to be independent of adenylyl cyclase and PKA and likely involves voltage-gated Ca^{2+} channels. Our results also showed that increase of pERK by the PKC activator PMA was insensitive to bupivacaine, indicating the action of bupivacaine occurs neither at PKC itself nor at sites downstream. This finding is at variance with reports of the LA sensitivity of a number of PKCs^{38,39} and implies that the PKC isoforms involved in ERK activation will be relatively insensitive to bupivacaine.

Bupivacaine and G Protein-coupled Receptors

There are some contradictory results between our findings and the literature on LA actions on GPCRs. Local anesthetics inhibit certain GPCRs (LPA, TXA2, PAF, and m1 muscarinic receptors) expressed in *Xenopus* oocytes^{6,40} but were ineffective on others, *e.g.*, angiotensin receptor signaling⁴¹ and, in general, GPCRs that require inositol trisphosphate-induced Ca^{2+} release, mediated by inositol trisphosphate that is liberated by phospholipase C.⁴² It seems that GPCRs working through the G_q α subunit are the types that are susceptible to LAs.⁴³ The LAs seem to be acting at a common intracellular site, at a locus downstream of the guanosine diphosphate-guanosine triphosphate exchange reaction⁶ and of the extracellular agonist binding site.⁴¹ An exception is the m1 muscarinic receptor, which seems to have both intracellular and extracellular inhibitory sites for LAs,⁴³ but almost all other GPCRs are affected by LAs at an intracellular site alone.

Bradykinin is a potent inflammatory mediator that causes pain and hyperalgesia. Intrathecal injection of bradykinin in mice causes pain-related behavior⁴⁴ and thermal hyperalgesia.⁴⁵ At the cellular level, bradykinin activation of the B2 receptor in sensory neurons leads to activation of an otherwise uncharacterized nonselective cation (inward) current,⁴⁶ perhaps by activating phospholipase C⁴⁷ through a pathway potentiated by other proalgesic agents, some of which activate PKA.^{47,48} No inhibitory effects of bupivacaine were seen on bradykinin-induced pERK in dorsal horn neurons in the current study.

Although there is little published about local anesthet-

ics and SP, Li *et al.*⁴⁹ reported that bupivacaine (1–3 mM) inhibited both SP binding and the SP-evoked increase in intracellular Ca^{2+} in clonal macrophages (P388D₁ cells) expressing NK-1 receptors. Our current results show that SP activated pERK in the spinal slices, but without inhibition by bupivacaine (2 mM). One reason for the discrepancy might be the difference in the type of G-protein coupling. Both G_q and G_{11} α subunits have been reported to be coupled to NK-1 receptors, even in the same tissue⁵⁰; G_q might be the predominant subunit in the cloned macrophages studied by Li *et al.*, whereas G_{11} might be the one in spinal cord.

Subsequent to activation of sensory neurons, glutamate is released in the spinal dorsal horn, where it acts on ionotropic and metabotropic glutamate receptors. The eight cloned mGluRs (mGluR1–8) are classified into three groups—group 1 (mGluR1, 5), group 2 (mGluR2, 3), and group 3 (mGluR4, 6, 7, 8)—based on their sequence homology, pharmacology, and association with intracellular effector systems.¹² Group 1 subtypes, which were specifically activated in the current study, are reported to induce spontaneous nociceptive behavior when delivered intrathecally⁵¹ and lead to activation of ERK in the spinal cord in mice.⁵² Like the other GPCRs, mGluR1/5 stimulation also induced pERK and also was unaffected by bupivacaine. Exposure of spinal slices to GPCR agonists in Ca^{2+} -free K-R had no effect on their pERK-activating effect, supporting the concept that there is a selective action of bupivacaine on those processes that involve Ca^{2+} entry through the plasmalemma rather than on a general increase in intracellular Ca^{+2} .

Relation to Spinal Anesthesia

The usual concentration of bupivacaine applied for peripheral and neuraxial anesthesia is 0.25–0.75% (approximately 8–23 mM), with lower concentrations often used for obstetric anesthesia. Although bupivacaine itself has not been examined directly, soon after intrathecal injection of other LAs at similar doses, their concentrations in CSF can reach millimolar levels.^{24,53–55} Based on these observations, it is possible that the concentrations of bupivacaine (0.5–5 mM) in our study are well within the relevant range that occurs during spinal anesthesia. Although it is unlikely that any equilibrium of drug with tissue is reached after a bolus injection,²⁴ the more superficial parts of the dorsal horn where pERK-positive neurons were detected probably receive a relatively higher amount of the total dose. Studies using intrathecal application of LAs *in vivo* will be required to accurately determine how effectively MAPKs are inhibited during peripheral nerve stimulation, but the current study on isolated slices clearly demonstrates the potential of bupivacaine on ERK.

In conclusion, the activation of excitatory ionotropic and metabotropic receptor agonists results in pERK activation in superficial dorsal horn neurons in rats. Bupivacaine blocked ionotropic but not metabotropic receptor-induced pERK, apparently by a blocking Ca^{2+} influx in the spinal cord, at a location upstream of PKC. The action sites of bupivacaine may be both presynaptic, at nociceptor afferent terminals, and postsynaptic, at dorsal horn neurons containing AMPA and NMDA receptors.

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