α2A-Adrenoceptor Stimulation Reduces Capsaicin-Induced Glutamate Release from Spinal Cord Synaptosomes

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Received June 4, 2001; accepted August 28, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT
Glutamate (Glu) is involved in excitatory neurotransmission and nociception and plays an essential role in relaying noxious stimuli in the spinal cord. Intrathecal or epidural injection of α2-adrenergic agonists produces potent antinociceptive effects, alters spinal neurotransmitter release, and effectively treats acute nociceptive and chronic neuropathic pain. Although it is generally believed that α2-adrenergic receptor stimulation reduces excitatory neurotransmitter release from peripheral afferents, the subtype of receptor causing this effect and its specificity to nociceptive neurotransmission have been inadequately studied. We therefore examined the pharmacology of adrenergic agents to inhibit Glu release in spinal cord from stimulation with capsaicin, a specific agonist for nociceptive afferents. Capsaicin evoked Glu release in synaptosomes from normal rat dorsal spinal cord in a concentration-dependent manner. Glu release from 30 μM capsaicin was inhibited by adrenergic agonists with a relative potency of clonidine = dexmedetomidine > norepinephrine > ST91 > phenylephrine = 0, consistent with an action on α2A/D subtype receptors. Also consistent with this interpretation was the observation that inhibition of capsaicin-induced Glu release by clonidine or dexmedetomidine was blocked by the α2A/D antagonist BRL44408 but not by the α2B/C-preferring antagonist ARC239. Similar results were obtained in perfused spinal cord slices. These data suggest that capsaicin-evoked Glu release, likely reflecting stimulation of C fiber terminals, can be inhibited by activation of the α2A/D subtype, and this action of adrenergic agonists may reflect in part their efficacy in the treatment of acute pain.

α2-Adrenergic agonists, like opioids, are powerful analgesics and are considered to act at multiple sites. Both classes of analgesics are more potent after intrathecal than systemic administration, indicating a site of action in the spinal cord, where the receptors on which they act are concentrated (Yaksh et al., 1984). The mechanisms by which opioids and α2-adrenergic agonists act remain an active topic of investigation. For α2-adrenergic agonists, it has been suggested that they inhibit release of excitatory neurotransmitters from nociceptive afferents by a direct action on primary afferent terminals (Kuraishi et al., 1985). However, much of this work has been indirect, either examining inhibition of stimuli, such as depolarization with high concentrations of potassium, which excite all types of afferents (Kamisaki et al., 1993; Shinomura et al., 1999), or examining effects in complex systems, such as spinal cord slices, in which direct and indirect effects could occur (Ueda et al., 1995). One purpose of the current study was to examine the action of α2-adrenergic agonists using a specific activator or nociceptive afferents (capsaicin) and using a simplified system that primarily reflects direct actions on nerve terminals (synaptosomes).

α2-Adrenoceptors can be divided by either pharmacologic or molecular approaches into three major subtypes: α2A (or the D homolog in the rat), α2B, and α2C. Rat spinal cord dorsal horn contains primarily α2A/D and α2C subtypes, as defined by immunohistochemistry (Stone et al., 1998). There is strong evidence that antinociception from intrathecally administered α2-adrenergic agonists reflects actions on the α2A/D subtype in normal animals (Millan, 1992; Stone et al., 1997), although there is also some support for nonA subtypes causing antinociception in normal animals (Takano and Yaksh, 1983; Guo et al., 1999). We have previously demonstrated that the α2-adrenergic subtype mediating autoinhibition of norepinephrine release in the spinal cord was the α2A/D subtype (Li et al., 2000). A secondary purpose of the current study was to determine the α2-adrenergic subtype subserving inhibition of capsaicin-evoked glutamate release in the spinal cord in normal animals. A combination of methods was used, including both complex and simple systems (spinal cord slices and synaptosomes), specific activation of afferents with capsaicin, and determination of a structure-activity relationship for α2-adrenergic agonists and antagonists.

Experimental Procedures

Materials. ST91 was provided by Boehringer Ingelheim Pharmaceuticals USA (Ridgefield, CT). Dexmedetomidine was provided by Orion Pharmaceuticals, Inc. (Turku, Finland). ARC239 dihydrochlo-
ride and BRL44408 maleate were obtained from Tocris Cookson (St. Louis, MO). MgSO4, KCl, sodium bicarbonate, and glucose were obtained from Fisher Scientific (Fair Lawn, NJ). Capsaicin (8-methyl-N-vanillyl-6-nonenamide), glutamate, β-nicotinamide adenine dinucleotide (β-NAD), glutamate dehydrogenase, clonidine, and remaining chemicals were obtained from Sigma (St. Louis, MO).

**Synaptosome Preparation.** After obtaining Animal Care and Use Committee approval, male Sprague-Dawley rats (250 g) were used for all experiments. Animals were deeply anesthetized with 1.5 to 2.1% halothane and then decapitated. The spinal cord was quickly removed and placed in aerated (with 95% O2/5% CO2) modified Krebs-Ringer buffer containing 135 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3, 12.5 mM Hepes, and 10 mM glucose, at pH 7.4. The dorsal half of the lumbar spinal cord was dissected from two rats and homogenized in 14 ml of ice-cold 0.32 M sucrose, and a crude synaptosomal pellet (P2) was prepared by differential centrifugation with 1,000g for 5 min followed by 15,000g for 20 min as previously described (Lonart and Johnson, 1995).

**Glutamate Release in Synaptosomes.** In all synaptosomal experiments, the P2 pellet was resuspended in 8 ml of modified Krebs-Ringer buffer, aerated with 95% O2/5% CO2, and incubated at 37°C for 30 min. The suspension was then centrifuged at 12,000g at 37°C for 4 min, and the resultant pellet was resuspended in 4.5 ml of Krebs-Ringer buffer and aliquoted into a 96-well microplate with 100 μl in each well. This synaptosome suspension or standard concentrations of Glu (0, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, and 5 mM) were added to a buffer solution of 150 μl containing NAD (final concentration of 0.5 mM), glutamate dehydrogenase (final concentration of 1.3 units/well), and capsaicin with or without various agonists or antagonists. This assay relies on generation of NADH by glutamate dehydrogenase in the presence of glucose, with NADH being measured fluorometrically (Barrie et al., 1991). Plates were preheated to 37°C and fluorescence at 460 nm from excitation at 340 nm recorded on a 37°C surface using a commercial plate reader (FL 600 with KC4 software; BioTek Instruments, Inc., Winooski, VT). A kinetic analysis was performed, with readings every 30 s for 3 min. A standard curve was constructed, and glutamate generation from synaptosome suspensions was determined by linear regression. Values were normalized to protein concentration as determined by the method of Bradford (1976) with bovine serum albumin as a standard.

After determination of a capsaicin-concentration response for Glu release in this preparation, inhibition of Glu release induced by 30 μM capsaicin by morphine, phenylephrine, norepinephrine, clonidine, dexmedetomidine, and ST91 was examined. Antagonism of the effect of ST91 by the α2 antagonist was then examined. Finally, antagonism of the effect of 30 μM dexmedetomidine and clonidine on capsaicin-induced Glu release was examined by the α2A/D-prefering antagonist BRL44408 and the α2B/C-prefering antagonist AR239 (Bylund et al., 1988).

**Glutamate Release in Slices.** To confirm clonidine inhibition of capsaicin-evoked Glu release, an in vitro spinal cord slice preparation was used as described previously (Xu et al., 1997). Rats were euthanized with pentobarbital (50 mg/kg, i.v.), and their spinal cords were removed. The spinal cord was divided between dorsal and ventral halves, and the dorsal half was chopped in 0.5-mm slices. Tissue sections from each hemi-spinal cord were put into an incubation chamber surrounded by a temperature-controlled water bath maintained at 37°C. Tissue slices were perfused continuously with a multichannel pump at 0.4 ml/min with oxygenated modified Krebs-Ringer solution gassed with 95% O2/5% CO2 at 37°C. The effluent from the spinal cord tissue chambers was collected on ice in 2-min aliquots. Experiments were started after spinal cord slices had incubated in the chamber for 60 min. To compare the clonidine effect on capsaicin-induced Glu release, spinal cord tissue chambers were infused with 30 μM capsaicin with or without 10 μM clonidine in modified Krebs-Ringer buffer, and the perfusates were analyzed for Glu. An equal number of chambers on the same day were perfused in modified Krebs-Ringer buffer with 30 μM capsaicin alone or with 10 μM clonidine. Samples were collected every 2 min. The concentration of Glu in each sample was determined using the fluorometric assay described above.

**Data Analysis.** Net Glu release in synaptosomes exposed to capsaicin with or without other agents was calculated by subtracting Glu release in wells on the same plate incubated in the absence of capsaicin. Data are presented as mean ± S.E. Synaptosome data were analyzed by one- or two-way analysis of variance followed by Dunnett’s or Student-Newman-Keuls test. Slice data were analyzed by two-way repeated measures analysis of variance. P < 0.05 was considered significant.

**Results**

**Capsaicin-Induced Glu Release.** In the absence of capsaicin stimulation, the basal release of Glu was 700 to 1300 pmol/mg of protein/3 min. Incubation with capsaicin resulted in a concentration-dependent release of Glu, with a threshold effect of 10 μM and release of Glu 3 orders of magnitude above baseline in the presence of 100 μM capsaicin (Fig. 1). Based on this concentration response, a probe concentration of 30 μM capsaicin was used in subsequent inhibition experiments.

**Inhibition of Capsaicin-Evoked Glu Release.** Both morphine and norepinephrine inhibited capsaicin-evoked Glu release in a concentration-dependent manner (Fig. 2). The ability to examine effects of these agents in concentrations greater than 1 μM was hindered by autofluorescence of these molecules, as determined in examination of solutions containing morphine and norepinephrine alone. This resulted in an artifactual increase in observed fluorescence in the synaptosomal suspensions at concentrations > 1 μM (Fig. 2). For this reason, a maximal effect, and hence the dose producing 50% of the maximal effect, could not be determined. The concentration producing a 25% reduction in capsaicin-evoked Glu release was less for morphine (10.6 ± 3.2 nM) than for norepinephrine (75.1 ± 15.5 nM; P < 0.05). In contrast, the selective α1-adrenoceptor agonist, phenyleph-
rine, had no effect on capsaicin-induced release of glutamate (Fig. 2).

In addition to norepinephrine, the effects of three selective α2-adrenergic agonists on capsaicin-evoked glutamate release were examined: clonidine and dexmedetomidine, with similar efficacies at all receptor subtypes, but dexmedetomidine exhibiting a greater α2-α1-adrenoceptor selectivity, and ST91, the diethyl analog of clonidine synthesized and described in the 1960s and demonstrated in some assays to exhibit greater specificity for the α2C adrenoceptor subtype (Takano and Yaksh, 1993; Graham et al., 2000). Clonidine and dexmedetomidine exhibited similar potencies (Fig. 3). ST91 also decreased capsaicin-evoked Glu release, but with a potency 4 to 5 orders of magnitude less than clonidine and dexmedetomidine (Fig. 3). The effect of ST91 was inhibited by the α2-adrenoceptor antagonist idazoxan (68 ± 12% inhibition by 1 μM idazoxan).

To further examine receptor subtype involved in the action of clonidine and dexmedetomidine, antagonist studies were performed with the α2A-prefering antagonist BRL44408 and the α2C-prefering antagonist ARC239. In the presence of 10 μM clonidine or dexmedetomidine, 10 μM BRL44408 significantly reversed inhibition of Glu release, whereas concentrations of up to 100 μM ARC239 were without effect (Fig. 4).

**Inhibition of Glu Release in Slices.** To examine the relevance of these observations to a more complex system, the effects of clonidine and capsaicin on Glu release from dorsal horn spinal cord slices perfused in vitro were determined. Ten micromolar capsaicin increased Glu in spinal cord slice perfusates by >50% (Fig. 5). Incubation of slices with 10 μM clonidine had no effect on basal Glu release in perfusates, but significantly reduced capsaicin-evoked Glu release (Fig. 5).

**Discussion**

α2-Adrenergic agonists are generally assumed to produce analgesia primarily by inhibiting release of excitatory neurotransmitters from afferents conveying nociceptive signals in the spinal cord. The current study supports this assumption in the normal condition, provides important novel information regarding the location and subtype of receptors responsible for this effect, and establishes testable hypotheses on the actions of α2-adrenergic actions in pathologic states.

α2-Adrenergic agonists have long been recognized to reduce excitatory neurotransmitter release in the spinal cord, including substance P (Kuraishi et al., 1985), calcitonin gene-related peptide and vasoactive intestinal peptide (Takano et al., 1993), and glutamate (Kamisaki et al., 1993). However, these studies were performed in slices, in which direct actions on terminals and indirect actions on local inhibitory circuits cannot be distinguished; used synaptic terminal preparations but used nonspecific stimulation with potassium, resulting in transmitter release from non-nociceptive afferents and spinal sources; or focused on distinguishing α1-versus α2-adrenergic receptor action rather than defining
subtypes of the 2-adrenergic receptor involved. The current study adds to these observations by using a preparation in which local circuits have been disrupted (synaptosomes), selectively stimulating with capsaicin, and distinguishing subtype identity by structure-activity relationships of agonists and antagonists.

Capsaicin selectively stimulates a subgroup of sensory afferents that express the VR-1 vanilloid receptor. VR-1 receptors are expressed on unmyelinated C fibers, can be demonstrated on small diameter cell bodies in the dorsal root ganglion that contain both peptides (especially calcitonin gene-related peptide, substance P) and glutamate, and are thought to transduce heat pain and underlie generation of some hypersensitivity states (Mantyh and Hunt, 1998). Stimulation of VR-1 receptors by capsaicin results in depolarization of dorsal root ganglion cell bodies (Petersen et al., 1996) and sensitization of dorsal horn neurons (Dougherty and Willis, 1992). Our result in spinal cord slices confirms previous work (Ueda et al., 1995) that capsaicin in low micromolar concentrations stimulated Glu release and that clonidine inhibits such release.

Synaptosomes, as prepared in the currently described method, contain a mixture of synaptic terminal structures from descending fibers, supraspinal projecting neurons, spinal interneurons, and afferent fibers. Given the nearly ubiquitous expression of glutamate by excitatory neurotransmitting elements in this mixture, interpretation of inhibition of glutamate release by generalized stimulation, either electrical or with potassium is problematic. Using capsaicin to selectively stimulate C fiber terminals, we observe inhibition by morphine and norepinephrine, similar to what others have observed using potassium (Kamisaki et al., 1993; Shinomura et al., 1999) and identifying for the first time existence of functional opioid and α2-adrenergic receptors on VR-1 expressing central afferent terminals.

α2-Adrenergic receptor subtypes can be defined pharmacologically in tissues and in cells transfected to express only one subtype (Bylund et al., 1992). Of the available agonists, clonidine and dexmedetomidine are subtype nonselective (Bylund et al., 1992) or slightly prefer α2A over nonA subtypes (Jasper et al., 1998). Depending on the assay, in vitro compared with in vivo administration, endogenous compared with transfected receptors, and species, ST91 may be equipotent at all three receptors or 10- to 100-fold more selective for nonA subtypes, most likely α2C (Nagasaka and Yaksh, 1990; Graham et al., 2000; Millan et al., 1994). ST91 is approximately one-tenth as potent as clonidine to produce antinociception after intrathecal administration in normal rats (Nagasaka and Yaksh, 1990) and approximately one-tenth as potent as clonidine to reduce capsaicin-evoked Glu release in rat spinal cord slices (Ueda et al., 1995). In contrast, ST91 was less than 1/10,000 as potent as clonidine or dexmedetomidine to reduce capsaicin-evoked Glu release from a preparation of synaptic terminals in the current study, suggesting that the antinociceptive effects of ST91 in vivo are unlikely to reflect a primary action on inhibition of
afferent release of Glu. The effect of ST91 is unlikely to be due to actions on α1-adrenergic receptors, because it was reversed by a selective α2-adrenergic receptor antagonist, and because the selective α1-adrenergic receptor agonist phenylephrine did not reverse capsaicin-induced glutamate release.

ST91 is much less potent than clonidine or dexmedetomidine to reduce glutamate release in this synaptic terminal preparation, whereas it is more similar in potency to the other agents in the more complete slice preparation. These results suggest that the α2-adrenergic receptor subtype responsible for inhibition of Glu release is most likely α2A/D. This interpretation of α2A/D mediation of inhibition of Glu release is further supported by the antagonist study. Of available α2-adrenergic antagonists, BRL44408 and AR(C)239 most effectively separate α2A from α2-nonα receptors and have been used to make this distinction in cell culture, isolated tissues, and central nervous system in vivo (Bylund et al., 1992; Kiss et al., 1995; Callado and Stanford, 1999).

The current data suggesting a primary role for α2A-adrenergic receptors in reducing capsaicin-evoked Glu release from C fiber terminals in rats are consistent with immunocytochemical studies that demonstrate α2A-adrenergic receptors concentrated on fibers in the superficial dorsal horn (Stone et al., 1998). α2A-Immunostained fibers colocalize with substance P, are reduced after neonatal capsaicin treatment to destroy C fibers, and are greatly reduced following dorsal rhizotomy, whereas α2C-Immunostained fibers are present on fibers in both superficial and deep dorsal horn, do not colocalize with substance P, and are minimally affected by neonatal capsaicin or dorsal rhizotomy (Stone et al., 1998).

Although these data agree with studies in genetically altered mice that suggest the antinoceptive action of intrathecally administered α2-adrenergic actions occurs by stimulation of α2A-adrenergic receptors (Lakhlani et al., 1997), they raise interesting questions regarding efficacy of α2-adrenergic agonists in the treatment of neuropathic pain. Intrathecal clonidine is more potent to treat hypersensitivity states, including neuropathic pain in humans, than to reduce responses to acute noxious stimuli or provide analgesia in acute pain settings such as the postoperative state (Eisenach et al., 1995, 1996, 1998). Animal models of hypersensitivity after peripheral nerve injury resulted in C fiber degeneration or destruction and subsequent loss of α2A-adrenergic immunostaining in the spinal cord ipsilateral to nerve lesion (Stone et al., 1999), yet increased potency of intrathecally administered α2-adrenergic agonists (Pule and Wiesenfeld-Hallin, 1993). This paradox between increased efficacy and reduced targets on afferent terminals suggests that other mechanisms and perhaps other α2-adrenergic subtypes are responsible for anti-hypersensitivity than for acute antinoceception. This is further underscored by the observation that spinal circuitry activated by α2-adrenergic agonists differs in normal and hyperpathic animals—intrathecal atropine has no effect on α2-adrenergic antinocepcion in normal rats but completely antagonizes the effect of intrathecal clonidine after spinal nerve ligation (Xu et al., 2000).

In summary, capsaicin evokes Glu release in slices and synaptosomes from spinal cord dorsal horn from normal rats, and this release is inhibited by clonidine. Agonist and antagonist series activity suggest that this action in normal ani-

mals is due to stimulation of α2A/D receptor subtypes. These data suggest that acute analgesia from intrathecally administered α2-adrenergic agonists reflects in part inhibition of C fiber-evoked Glu release.


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