The Journal of Pain, Vol 8, No 7 (July), 2007: pp 556-562
*Available online at www.sciencedirect.com*

# $\alpha_1$-Adrenergic Receptors Augment $P2X_3$ Receptor–Mediated Nociceptive Responses in the Uninjured State

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**Abstract:** **In the present study, the adrenergic receptor (AR) subtype mediating adrenergic augmentation of $P2X_3$ receptor–mediated nociceptive responses on sensory nerve endings was examined by using selective AR receptor agonists and antagonists in Sprague Dawley rats in the uninjured state. Local administration of $\alpha\beta$-methyleneATP (ligand for $P2X_3$/$P2X_{2/3}$ receptors) into the plantar hind paw produced few pain behaviors when given alone in this strain of rats; combination with adrenaline ($\alpha$1- and $\alpha$2-AR agonist) and phenylephrine ($\alpha$1-AR agonist) but not clonidine or UK 14,304 ($\alpha$2-AR agonists) increased flinching behaviors. Flinching produced by noradrenaline (NA)/$\alpha\beta$-methyleneATP was suppressed by low doses of prazosin ($\alpha$1-AR antagonist), and this reduction was selective compared with yohimbine ($\alpha$2-AR antagonist). Prazosin also reduced flinching produced by phenylephrine/$\alpha\beta$-methyleneATP. Using thermal threshold determinations, adrenaline and phenylephrine but not clonidine or UK 14,304, mimicked the action of NA in augmenting reductions in thermal thresholds produced by $\alpha\beta$-methyleneATP. Terazosin (another $\alpha$1-AR antagonist) inhibited hyperalgesia produced by NA/$\alpha\beta$-methyleneATP. These results provide evidence for $\alpha$1-AR involvement in adrenergic augmentation of $P2X_3$/$P2X_{2/3}$ receptor–mediated responses on sensory nerve endings in the uninjured state in Sprague Dawley rats.**

***Perspective:** This study indicates the $\alpha$1-adrenergic receptor subtype mediates adrenergic augmentation of the activation of sensory nerves by purinergic $P2X_3$ receptors (respond to ATP) in the periphery. Observations are potentially relevant to chronic pain conditions in which sympathetic nerves influence sensory nerves.*



***Key words:** $P2X_3$ receptors, $\alpha$1-adrenergic receptors, nociception.*

$P2X_3$ receptors are present on sensory afferent neurons and play a facilitatory role in nociception.[13,15,29] $P2X_3$ receptors in dorsal root ganglia (DRG) cell bodies (and presumably nerve endings of sensory afferents) are upregulated in inflammation,[47] whereas nerve injury produces complex changes in receptor mRNA and protein expression (upregulation in uninjured and downregulation in injured afferents,[4,14,19,42]; and increased translocation from cytoplasm to the membrane in injured neurons[6]). $P2X_3$ receptor activation leads to depolarization of isolated and cultured DRG cells[15,29] as well as activation of peripheral aspects of sensory nerves.[10] In the rat, local application of $P2X_3$ receptor agonists to the hind paw leads to spontaneous pain behaviors (flinching, biting/licking), thermal hyperalgesia, and mechanical allodynia; the former responses are sensitive to capsaicin and represent C-fiber–mediated responses, whereas mechanical allodynia is relatively insensitive to capsaicin and reflects A-fiber–mediated responses.[3,11,41]

Noradrenaline (NA) is coreleased with ATP from sympathetic nerves, and both agents contribute to sympathetically mediated autonomic responses.[5] The sympathetic nervous system is implicated in aspects of inflammatory and neuropathic pain, and these influences can occur at peripheral nerve endings, the site of injury, or on DRG cell bodies.[12] Facilitatory interactions between adrenergic and purinergic responses have been implicated in neuropathic pain.[26,31] NA augments flinching behaviors and thermal hyperalgesia produced by $\alpha\beta$-methyleneATP ($P2X_3$/$P2X_{2/3}$ receptor agonist) when coadministered in the rat hind paw; the former end point

Received September 7, 2006; Revised January 19, 2007; Accepted February 25, 2007.
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1526-5900/$32.00

doi:10.1016/j.jpain.2007.02.434

reflects direct interactions on sensory nerve endings, whereas the latter involves an indirect effect.[45] Both α1- and α2- adrenergic receptors have been implicated in sympathetic influences on nociception, and strain differences are prominent in these influences (see Discussion). Both adrenergic augmentation of flinching and intrinsic flinching induced by αβ-methyleneATP exhibit a strain difference, being more pronounced in Wistar than in Sprague Dawley rats.[45]

In the present study, we characterized adrenergic receptor (AR) subtypes involved in the adrenergic-purinergic facilitation of nociception at sensory nerve endings in Sprague Dawley rats. Drugs were coadministered into the hind paw, and observations are of particular relevance to adrenergic-sensory coupling that can occur at peripheral sensory nerve endings. Although Sprague Dawley rats do not exhibit the most prominent expression of the adrenergic-purinergic interaction, this is the most commonly used strain for neuropathic pain studies, and we wished the data generated to be relevant to a significant body of prior observations.[14,19,31,42,49] We examined the ability of selective AR agonists (α1, α2) to mimic the ability of NA to augment flinching behaviors and thermal hyperalgesia produced by αβ-methyleneATP and then effects of selective adrenergic receptor antagonists (α1, α2, β) on effects produced by NA or phenylephrine in combination with αβ-methyleneATP.

## Materials and Methods

### Animals

Experiments were performed with male Sprague Dawley rats (150 to 250 g) obtained from Charles River, Montreal. Experiments reported in this article were approved by the University Committee on Laboratory Animals and performed in accordance with Canadian Council on Animal Care guidelines. Rats were housed in pairs and maintained on a 12/12-hour light/dark cycle at 22 ± 2°C, with food and water freely available. Each hind paw was tested once, with an interval of 3 to 5 days between successive trials; a previous study indicated a high degree of reproducibility between such trials.[45]

### Drug

All locally administered drugs and combinations were injected in a total volume of 50 μL; injections were made subcutaneously into the plantar hind paw while rats were loosely restrained. The following selective agents were used to characterize ARs: phenylephrine (α1-AR agonist), clonidine and UK 14,304 (α2-AR agonists), prazosin and terazosin (α1-AR antagonists), yohimbine (α2-AR antagonist), and timolol and propranolol (β-AR antagonists). All drugs were dissolved in saline except for UK 14,304, yohimbine, and prazosin, which were dissolved in 10% DMSO/10% $H_2O$/80% saline; appropriate vehicle controls were used throughout. All drugs were purchased from Sigma (St. Louis, MO).

### Flinching Behaviors

Individual rats were acclimatized for a minimum of 15 minutes in a Plexiglas observation chamber (28 × 28 × 28 $cm^3$). Two rats were observed at a time in adjacent chambers in alternating 2-minute bins for 32 minutes, and spontaneous flinching (paw elevation or shaking of the hind paw) behaviors were recorded as a cumulative number of episodes. The time course for behaviors produced by adrenergic/purinergic combinations is continuous,[45] and this alternating method records one-half of the total incidence of behaviors. In that earlier study, behaviors were determined over a period of 60 minutes,[47] for doses of ≤25 nmol NA, where effects are local, behaviors occur primarily over a period of 30 minutes, and the shorter interval was used in the current study.

### Thermal Hyperalgesia

A thermal hyperalgesia test apparatus (Department of Anesthesiology, University of California San Diego) was used. Rats were placed in Plexiglas observation chambers positioned on a glass surface maintained at 30°C and acclimatized for 30 minutes, by which time spontaneous exploration had ceased. Radiant heat, in the form of a focused light beam, was directed through the glass at the plantar surface of the hind paw, and 3 baseline hind paw withdrawal latencies were determined at 10-minute intervals. After baseline determinations, rats were removed for injection of drugs; they were then returned to the chambers and latencies determined 5, 15, 30, 45, and 60 minutes after injections. Quiescent intervals between spontaneous episodes were selected for determining thermal latencies. The mean of 2 latency measurements taken at each time interval was used to calculate cumulative differences from baseline, and this approximates an area under the curve. A cutoff value of 20 seconds was imposed on the thermal stimulus to prevent tissue damage.

### Data and Statistics

Data are presented as mean and SEM for the time course. Cumulative behaviors are depicted as the total number of flinches or cumulative difference from baseline during the indicated time interval. Data were analyzed by use of analysis of variance followed by the Student Newman Keuls test.

## Results

### Receptor Characterization of Flinching Behaviors Using AR Agonists

Local administration of 500 nmol αβ-methyleneATP into the plantar surface of the hind paw produces few flinching responses in Sprague Dawley rats (<10 to 15 flinches, as does saline); in combination with NA (which also produces minimal responses when given alone), αβ-methyleneATP leads to a marked expression of flinching over a period of 30 to 60 minutes.[45] These effects are locally mediated, as injection of NA into the contralateral hind paw does not augment flinches produced by

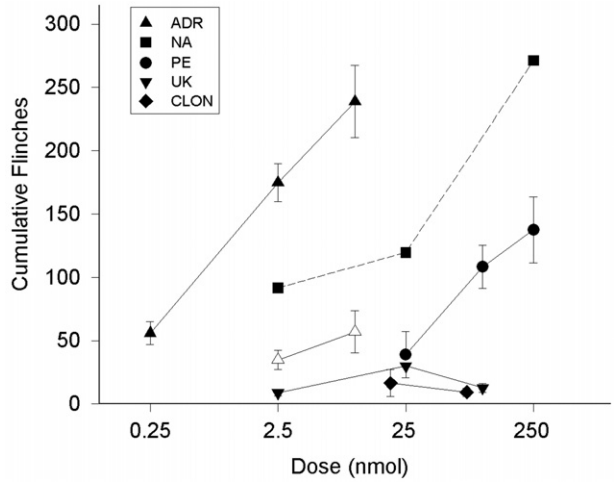


**Figure 1.** Dose-response curves for flinches produced by αβ-methyleneATP (500 nmol) coinjected in combination with AR agonists into the plantar surface of the hind paw of Sprague Dawley rats. Solid symbols indicate cumulative number of flinches over 32 minutes produced by combination with indicated drugs; hollow symbol indicates the intrinsic effect of adrenaline. [Dashed line indicates data for NA[47] redrawn for the shorter time course used in the present study and is provided for the purpose of direct comparison.] Data indicate mean ± SEM values; n = 5 to 7 per group. In all cases in which means are >60, values are increased ($P < .05$) compared with saline. ADR, Adrenaline; NA, noradrenaline; PE, phenylephrine; UK, UK14,304; CLON, clonidine.

αβ-methyleneATP (25/250 nmol respective drug doses; 135 ± 22 flinches over a period of 32 minutes for coinjection vs 7 ± 3 for contralateral injection; $P < .01$, n = 4). When adrenaline is combined with αβ-methyleneATP, there is also a marked expression of flinching behaviors (Fig 1). Although the dose-response curve lies to the left of the NA curve, adrenaline also produces some intrinsic flinching behaviors (hollow symbols) that contribute to the total effect of the drug combination. When selective α1-AR and α2-AR agonists are used, phenylephrine (α1-AR agonist) but not clonidine or UK14,304 (α2-AR agonists) mimics the effect of adrenaline and NA in augmenting αβ-methyleneATP responses (Fig 1). (Higher doses of α2-AR agonists were not examined, as these clearly lead to systemic sedative effects.) Apart from adrenaline, all other AR agonists lacked intrinsic activity compared with saline when tested at the maximal dose used in combination (data not shown).

## Receptor Characterization of Flinching Behaviors Using AR Antagonists

Prazosin, widely used as a selective α1-AR antagonist, leads to inhibition of flinches produced by NA/αβ-methyleneATP over a wide range of doses (0.03 to 3 nmol; Fig 2A). In contrast, yohimbine, a selective α2-AR antagonist, has no significant effect over this dose range, and there is a 1 to 2 order-of-magnitude difference in the action of these agents in reducing flinching (Fig 2A). Prazosin also inhibits flinches produced by phenylephrine/αβ-methyleneATP, and yohimbine appears to exhibit some activity at the highest dose (30 nmol; Fig 2B). It is of interest to note that prazosin was much more able to inhibit the NA/αβ-methyleneATP combination than the phenylephrine /αβ-methyleneATP combination. The β-AR antagonists timololol and propranolol, at 100 nmol, did not significantly alter flinches produced by NA/ αβ-methyleneATP (data not shown).

## Receptor Characterization of Thermal Hyperalgesia Responses

Peripheral administration of αβ-methyleneATP into the hind paw also leads to a transient thermal hyperalgesia in Sprague Dawley rats; this is increased by coadministration of NA, which augments both the magnitude and duration of hyperalgesia.[45] NA also leads to a prominent hyperalgesia in combination with an inactive dose of αβ-methyleneATP (150 nmol; Fig 3A). Thermal hyperalgesia is also elicited by phenylephrine given in combination with αβ-methyleneATP (Fig 3B), but not by clonidine or UK14,304 in combination with αβ-methyleneATP (Fig 3C). The time-response curves indicate that the effect of phenylephrine is less sustained (resolves by 30 minutes) than that observed with NA (still prominent at 45 min) (cf Figs 3B and 3A). With clonidine and UK 14,304, latencies at the end of the time course are elevated compared with corresponding time points in the αβ-methyleneATP group (Fig 3C), suggestive of intrinsic analgesic actions; however, these are not prominent in

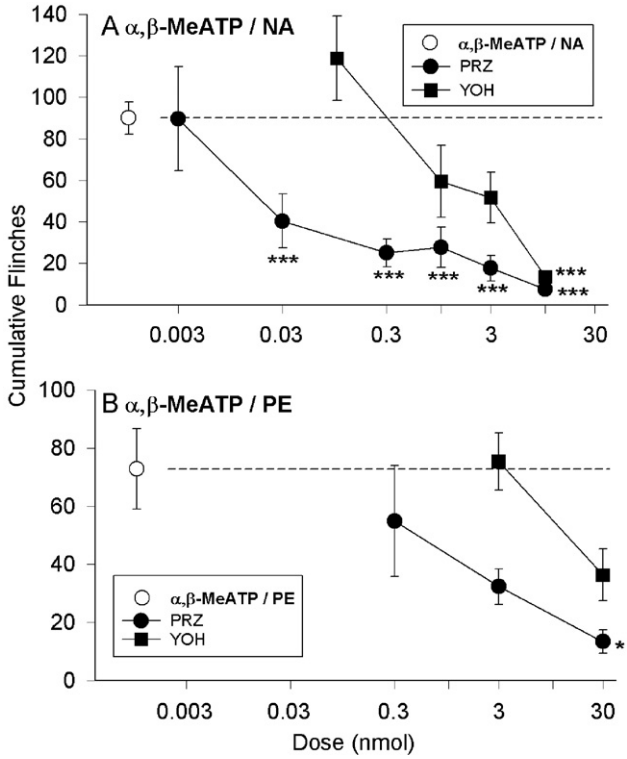


**Figure 2.** Inhibition of flinches produced by **(A),** NA with αβ-methyleneATP (25/300 nmol, respectively), and **(B)**, phenylephrine (PE) with αβ-methyleneATP (300/300 nmol, respectively) over a period of 32 minutes by prazosin (PRZ) and yohimbine (YOH). *$P < .05$, ***$P < .001$ compared with control group, indicated by hollow symbol in each panel (n = 6 per group).

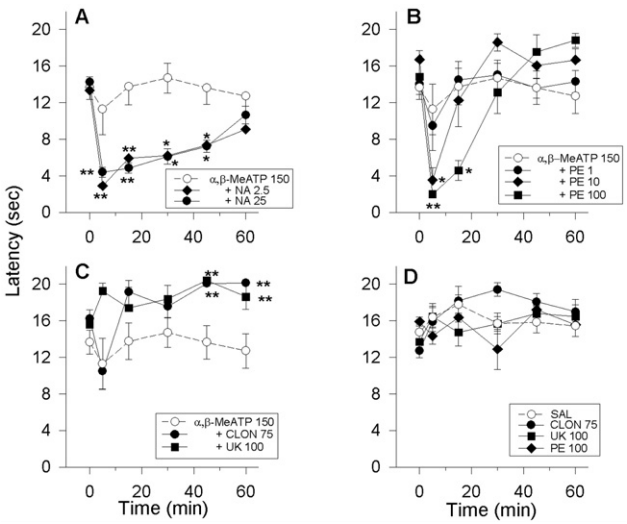


**Figure 3.** Time course of thermal hyperalgesia produced by αβ-methyleneATP injected into the plantar surface of the hind paw of Sprague Dawley rats combined with NA **(A),** phenylephrine **(B),** and clonidine or UK 14,304 **(C).** Indicated doses are in nmol. **(D),** Effects of adrenergic agonists are shown in comparison to saline (SAL). $*P < .05$, $**P < .01$ compared with group depicted by hollow symbols (n = 6 per group). NA, noradrenaline; PE, phenylephrine; CLON, clonidine; UK, UK14,304.

control experiments (Fig 3D). When hyperalgesia results are expressed as cumulative reductions in baseline, adrenaline augments thermal hyperalgesia by αβ-methyleneATP with an activity similar to NA, with phenylephrine producing a lesser cumulative effect due to the shorter time course of action (Fig 4).

In further thermal hyperalgesia experiments, terazosin, another selective α1-AR antagonist, produces a dose-related reduction in the sustained thermal hyperalgesia produced by NA in combination with a low dose of αβ-methyleneATP (150 nmol; Fig 5A), and this is dose-related (Fig 5B); however, it has no effect on the more transient hyperalgesia produced by a higher dose of αβ-methyleneATP (500 nmol) given alone (Fig 5C).

## Discussion

The present study used selective AR agonists and antagonists to characterize adrenergic receptors involved in augmenting peripheral pain signaling induced by $P2X_3/P2X_{2/3}$ receptor activation (via local hind paw administration of αβ-methyleneATP) in Sprague Dawley rats. α1-ARs, but not α2-ARs, are implicated in 2 different functional end points that reflect sensory nerve ending activation (flinching and thermal hyperalgesia) in the uninjured state. Both pain behaviors (flinching) and thermal hyperalgesia produced by plantar injections of αβ-methyleneATP reflect activation of receptors on C-fibers, based on sensitivity to capsaicin-pretreatment;[3,11,41] and both direct and indirect influences of NA on such activation can occur.[45] Recently, Maruo et al[26] published a study in which electrophysiologic interactions between adrenergic receptors and $P2X_3$ receptors were examined in Wistar rats after sciatic nerve transection (axotomy); they suggested that α1B-ARs are involved in potentiation of $P2X_3$-mediated responses in DRGs, based on mRNA analysis of adrenergic receptors. Although the α1-AR subtype involved in Sprague Dawley rats cannot be determined on the basis of the agonists and antagonists used in the present study, there is a clear consistency in the essential conclusion of implicating α1-ARs in augmenting $P2X_3$-mediated responses on sensory neurons in the two studies using quite different experimental approaches and 2 different strains of rats.

There has been little direct characterization of α1-ARs in DRG neurons. An early study noted an inability to observe mRNA for α1-ARs in sensory ganglia in Sprague Dawley rats.[33] In more recent studies, mRNA for multiple α1-ARs has been identified in sensory ganglia in Lewis rats[46] (this paper discussed possible reasons for the lack of detection in that earlier study) and Wistar rats,[26] and α1-ARs are implicated in functional studies of peripheral adrenergic influences on nociception using Lewis rats[22,46] and Wistar rats.[26] Intrinsic effects of NA on nociception after inflammation are more pronounced in Lewis rats compared with Sprague Dawley rats[2] and in the uninjured state in Wistar rats compared with Sprague Dawley rats.[45] Collectively, these observations suggest a prominent role of α1-ARs in mediating peripheral adrenergic nociceptive responses in both Lewis and Wistar strains of rats; there are fewer data implicating α1-ARs in such actions in Sprague Dawley rats.[30]

There is 1 data set in which α1-ARs do feature prominently in facilitating peripheral nociceptive responses in Sprague Dawley rats, and that is with interactions with TRPV1 receptors.[20,25,36] This response is maintained after sympathectomy, and the presence of α1-ARs on nocicep-

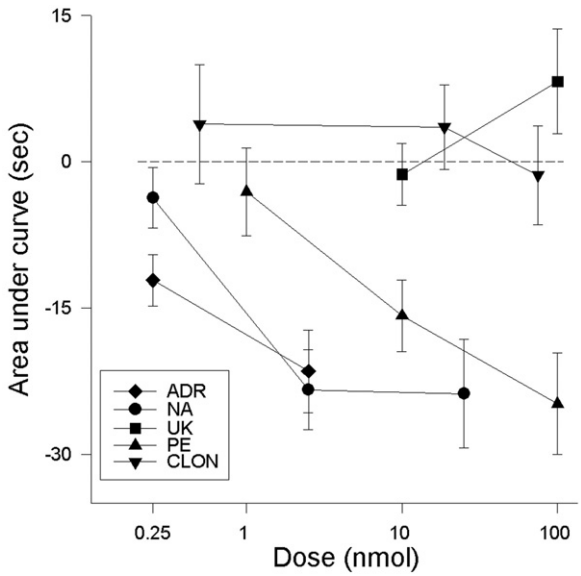


**Figure 4.** Cumulative changes from baseline, representing the area under the curve, for thermal hyperalgesia produced by αβ-methyleneATP (150 nmol) in combination with a number of AR agonists. Negative values indicate cumulative reductions in latency. n = 6 per group. ADR, Adrenaline; NA, noradrenaline; PE, phenylephrine; CLON, clonidine; UK, UK14,304.

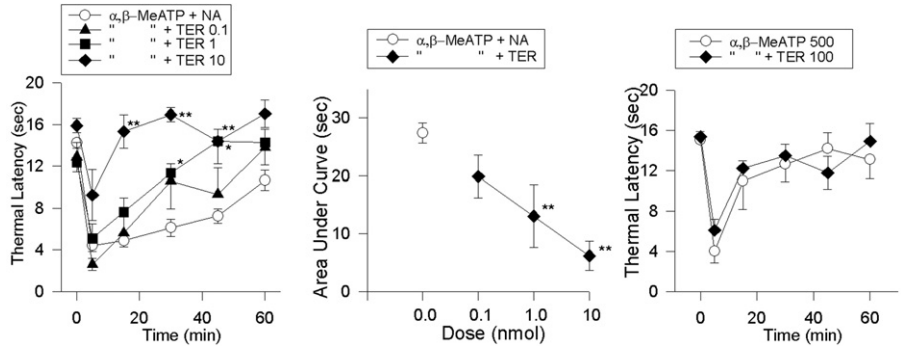


**Figure 5.** Effect of terazosin (TER) on thermal hyperalgesia produced by NA combined with αβ-methyleneATP (25/150 nmol, respectively). The left panel depicts the time course and the middle panel depicts the dose-response relation for terazosin in reducing sustained hyperalgesia produced by NA combined with αβ-methyleneATP. The right panel depicts the lack of effect of terazosin in reducing the transient hyperalgesia produced by a high dose of αβ-methyleneATP alone. Indicated doses are in nmol. $*P < .05$, $**P < .01$ compared with NA/αβ-methyleneATP (n = 6 per group).

tive afferent neurons has been emphasized in interpreting results.[25,36] Given that both $P2X_3$ and TRPV1 receptors colocalize in a significant population of sensory neurons[9] and that activation of both receptors allows for cation entry into sensory afferents and leads to enhanced nociception, a prominent involvement of α1-ARs in facilitating cation channel function on sensory afferents is plausible. Both TRPV1 and $P2X_3$ receptors are positively regulated by protein kinase C and other $Ca^{2+}$-dependent processes,[32,34,44,48] and these intracellular events can be activated as a result of α1-AR activation.[21]

In previous studies, α2-ARs (particularly α2A and α2C) have been examined directly in DRG neurons in Sprague Dawley rats.[7,8,38] This emphasis was based on the many previous functional studies that had implicated α2-ARs in pronociceptive responses after various forms of nerve or tissue injury.[17,23,28,37,40] The present study does not provide clear evidence for α2-AR involvement in augmenting flinching or thermal hyperalgesia responses produced by $P2X_3$ receptor activation, as α1-AR (but not α2-AR) agonists clearly mimic the effect of NA, and α1-AR antagonists show a clear selectivity in blocking the effects of NA combined with αβ-methyleneATP. The ability of a high dose of yohimbine to block flinching produced by NA/αβ-methyleneATP may reflect loss of receptor selectivity at higher doses, as responses to phenylephrine, a selective α1-AR agonist, in combination with αβ-methyleneATP, also was reduced at a high dose. It is interesting to note that prazosin is much more potent in blocking the effect of NA than phenylephrine in combination with αβ-methyleneATP, and it is possible that there could be an α1/α2-AR interaction expressed with the less selective agonist (NA). With thermal hyperalgesia responses, there is the additional possibility that intrinsic antinociceptive effects of α2-AR agonists[1,17] could, in some instances, confound increased nociception produced by α2-ARs in combination with αβ-methyleneATP (cf Fig 3C). Whereas β-ARs are implicated in facilitation of some nociceptive responses in Sprague Dawley rats,[18] selective β-AR antagonists (propranolol and timolol) did not inhibit responses produced by NA/αβ-methyleneATP, and it is unlikely that these receptors contribute to effects observed in the present study.

The involvement of sympathetic nerves in adrenergic augmentation of $P2X_3$/$P2X_{2/3}$ receptor–mediated thermal hyperalgesia provides a model of neuronal-neuronal (sympathetic-sensory) interactions that occur at the level of the sensory nerve terminal. Other potential indirect mechanisms, including non-neurogenic ones, need to be considered as perhaps contributing to the present observations. (1) NA could lead to vascular constriction via α1-ARs, which leads to retention of αβ-methyleneATP in the tissue for longer, allowing for a pharmacokinetic, rather than a pharmacodynamic, mechanism to contribute to observed interactions. In Sprague Dawley rats, this is unlikely to be the only mechanism involved, as NA clearly potentiates αβ-methyleneATP responses even when there is no intrinsic effect produced by αβ-methyleneATP (eg, Fig 3A). In Wistar and Long Evans rats, in which intrinsic effects of both αβ-methyleneATP and NA are observed with flinching, there is no potentiation of the immediate peak effect but a progressive augmentation with time and a marked prolongation of action[45]; although this pattern could reflect a pharmacokinetic interaction, the progressive nature of development of the peak effect (doubling of the initial effect) clearly suggests a pharmacodynamic influence in which time is required for full expression of peak action. (2) NA could act on other cell types to release chemical mediators that then act on sensory neurons to interact with the $P2X_3$ receptor–mediated responses; this represents a non-neurogenic indirect mechanism. For example, phenylephrine (α1-AR agonist) has been shown to release nerve growth factor from cultured vascular smooth muscle cells,[43] and this mediator could contribute to acute facilitatory effects on nociception by actions on sensory afferents,[24] although its more prominent role is in regulation of longer-term events.[35] Further clarification of the nature of the marked positive interaction observed between α1-ARs and $P2X_3$ receptors on peripheral nociception will require the use of additional experimental approaches.

In summary, the present study reveals a prominent positive interaction between $\alpha$1-ARs and $P2X_3/P2X_{2/3}$ receptors on sensory nerve endings. Direct effects on sensory nerves do appear to occur, but indirect effects on sympathetic neurons and/or vascular smooth muscle, involving release of intermediary agents which then act on sensory afferents, also may contribute to such effects. Given that peripheral $\alpha$1-AR mechanisms are implicated in human neuropathic pain states[39] and that antagonists for $P2X_3$ receptors are receiving considerable attention as a potential novel analgesic,[16,27] this interaction may be of importance to address using further in vivo and in vitro techniques. Such studies will need to attend to the prominent strain difference that occurs in rats with this interaction.

## Acknowledgment

We thank Allison Reid for technical assistance.

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