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SELECTIVE BLOCKADE OF SPINAL P2X3 AND P2X2/3 RECEPTORS WITH A NOVEL AND SELECTIVE ANTAGONIST, AF-742, REVEALS CENTRAL ENDOGENOUS PURINERGIC REGULATION OF BLADDER ACTIVITY

Hypothesis / aims of study

P2X3 and P2X2/3 receptors are located on both peripheral and central terminals of primary afferents and implicated in various sensory functions, including those in the urinary bladder (1). Previous pharmacological studies using ligands of limited pharmacological and pharmacokinetic attractiveness have focused mostly on receptors located peripherally in regulating bladder activities (2) to extend observations from knockout mice (3). Here, we first characterized the selective potency of a novel P2X3 and P2X2/3 receptor antagonist, AF-792, followed by investigating whether the P2X3 and P2X2/3 receptors located specifically in the spinal cord are functional in regulating bladder reflex activities using AF-792 and other less selective purinergic compounds. In this study, whole-cell voltage clamp electrophysiology in nodose and dorsal root ganglion (DRG) neurons was used to determine the antagonistic potencies of AF-792. *In vivo* cystometry recordings were made to assess bladder reflex activities and spinal extracellular-signal regulated kinases (ERK) activation induced by acute noxious stimulation was used to correlate with bladder hyperactivity following drug treatments.

Study design, materials and methods

Standard giga-seal patch clamp technique was employed in acutely dissociated rat nodose ganglion and DRG neurons to study the activity of P2X2/3 and P2X3 receptors, respectively. To determine the antagonistic potencies of AF-792, 10 μM αbmeATP was applied to activate the receptors *in vitro* for constructing the inhibition curves..

For cystometry and ERK immunnohistochemical studies, intrathecal catheters were implanted into the lumbar subarachinoid space of female Sprague Dawley rats at least 1 day before experiments. Under urethane anaesthesia, the urinary bladder dome was cannulated and both the ureters and urethra were ligated to create an isovolumetric system.

For cystometry studies, saline was infused into the bladder dome at 100 μl/min until stable bladder contractions could be obtained and at which point the rate was lowered to 3-5 μl/min to maintain the contractions. Following observation of a stable baseline of contraction frequency by recording the intravesicular pressure, various doses of commercially available P2X3 and P2X2/3 inhibitors (in the forms of desensitising agonist: α,β-methylene ATP and antagonists: PPADS and TNP-ATP) and AF-792 were applied intrathecally. Recordings were made and analyzed to assess the effects of these compounds on bladder reflex contractions.

In separate experiments, ERK activation was studied in the spinal cord following 1% acetic acid acute stimulation in the bladder of naïve rats. At least one hour following bladder cannulation, AF-792 (300 nmol) or its vehicle was administered intrathecally. 1% acetic acid was then instilled and left in the bladder briefly for 2 min whereas sham animals did not receive any acetic acid stimulation. Spinal cord tissues were then harvested from animals following transcardial fixation. Following post-fixation, sucrose transfer, and embedment in OCT compound, L6 spinal cord segment sections were cut and immunostained for pERK. Number of pERK-positive cells in the spinal cord were counted and compared across treatment groups.

Results

Electrophysiological recordings obtained from acutely dissociated rat nodose ganglion and DRG neurons (predominantly P2X2/3 and P2X3, respectively) showed AF-792 blocks natively expressed P2X receptors with similar potency (pIC50 at nodose ganglion: 7.5; at DRG: 8.2) (Figure 1). All purinergic inhibitors tested inhibited bladder reflex activity significantly *in vivo*. In particular, we found AF-792 at the highest dose tested (300 nmol, i.t.) significantly increased baseline contraction intervals from 1.18 ± 0.07 min to 9.33 ± 2.50 min (Figure 2). In addition, inhibition of P2X3 and P2X2/3 receptors in the spinal cord significantly decreased acetic acid-induced spinal ERK activation by 46.4 ± 12.0% on average.

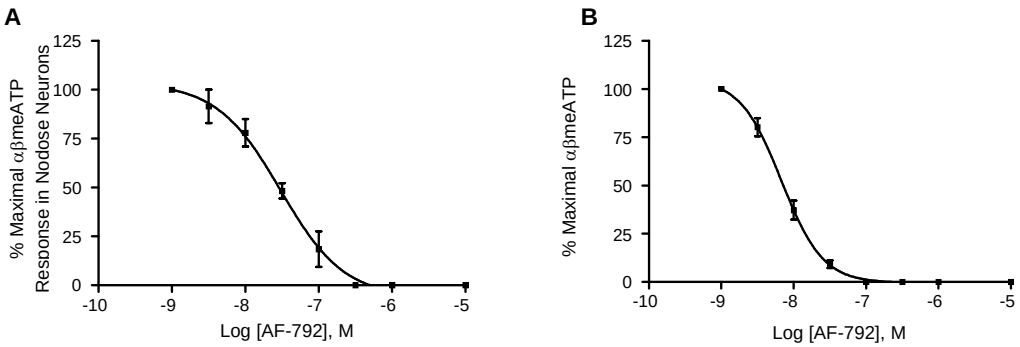

Figure 1. Inhibition curves of AF-792 constructed in the presence of 10 μM αbmeATP using whole-cell patch clamping in rat nodose (**A**) and dorsal root (**B**) ganglion neurons *in vitro*.

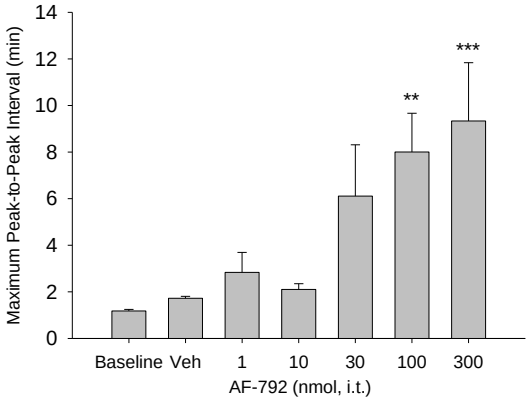

Figure 2. Intrathecal administration of AF-792 inhibited micturition reflex bladder contraction activity by significantly increasing the peak-to-peak interval between contractions following blockade of P2X3 and P2X2/3 receptors in the spinal cord. Statistical comparison was done using one-way repeated measures ANOVA followed by the Bonferroni *post-hoc* test (**: $p < 0.01$ and ***: $p < 0.001$ against vehicle).

Interpretation of results

AF-792 was demonstrated to be a selective and potent P2X3 and P2X2/3 receptor antagonist. Selective blockade of the receptors in the spinal cord functionally decreased micturition reflex contraction frequency significantly. AF-792 produced longer-lasting inhibitory effects than PPADS and TNP-ATP, likely reflecting both increased selectivity for P2X3 and P2X2/3 receptors and metabolic stability. By using spinal ERK activation as another measure for afferent input into the spinal cord following acute bladder stimulation, AF-792 (i.t.) significantly attenuated the number of pERK-positive cells in the spinal cord. Together, these results indicate that presynaptic P2X3 and P2X2/3 receptors in the dorsal horn facilitate afferent limb of the micturition reflex.

Concluding message

P2X3 containing channels have surfaced prominently as novel medicinal targets to treat sensory dysfunction in a variety of somatic and visceral systems, with indication potential in painful bladder syndrome and overactive bladder. Our data indicate that as selective medicinal candidates are developed, clinical exploration of the effect of P2X3 antagonism in urologic indications will require consideration of activity at spinal cord as well as peripheral sites. An endogenous purinergic central regulation in the spinal cord for the micturition reflex was also established.

References

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What were the subjects in the study?	**ANIMAL**
Were guidelines for care and use of laboratory animals followed or ethical committee approval obtained?	**Yes**
Name of ethics committee	**All procedures were performed in accordance with the United Kingdom Home Office regulations (Animals Scientific Procedures Act, 1986).**