Abstract—The expression and functional responses of P2X receptors in bladder and cutaneous sensory neurons of adult rats and mice have been studied using immunohistochemistry and patch clamp techniques. Cell bodies of bladder pelvic afferents were identified in L6 and S1 dorsal root ganglia (DRG), following Fast Blue injection into the muscle wall of the urinary bladder. Similarly, cutaneous sensory neurons were identified in L3 and L4 DRG, following Fast Blue injection into the saphenous nerve innervating the skin. Bladder sensory neurons contained only weak to moderate P2X3-immunoreactivity (IR), in contrast to strong P2X3-IR observed in cutaneous sensory neurons containing IB4, isolectin B4 (IB4; Vulchanova et al., 1997, 1998; Bradbury et al., 1998). Whole-cell patch clamp recordings revealed that approximately 90% of bladder afferent neurons responded to αβ-methylene ATP (αβmeATP) and ATP (30 μM) with persistent currents, which were inhibited by 2',3'-O-dipherethoxyethyl-ATP (TNP-ATP) (0.3 μM) to 6.2±1.9% and 8.0±2.6% of control, respectively (n=8). The remaining bladder sensory neurons demonstrated biphasic, transient or no response to P2X agonists. In contrast, only 24% of cutaneous afferent neurons gave persistent currents to αβmeATP (30 μM), with 66% of cells giving transient or biphasic currents and the remaining 10% being non-responsive. Our results suggest that, in contrast to DRG neurons in general, bladder sensory neurons projecting via pelvic nerves express predominantly P2X2/3 heteromeric receptors, which are likely to mediate the important roles of ATP as a signaling molecule of urinary bladder filling and nociception. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: ATP, dorsal root ganglion, Fast Blue, pelvic nerve, saphenous nerve.

P2X receptors are ion channels activated by extracellular ATP. Seven subunits (P2X1–7) have been cloned in this family (North and Surprenant, 2000). Functional P2X receptors are probably trimers, assembled from either three identical (giving rise to homomers), or non-identical (heteromers) subunits (Nicke et al., 1998, 2003). Different P2X receptors display distinct yet overlapping phenotypes. For example, P2X3 homomers are activated by αβmeATP and desensitize rapidly, whereas P2X2 receptors do not respond to this ligand and their response to ATP desensitizes slowly (Brake et al., 1994; Chen et al., 1995). P2X2/3 heteromeric receptors display hybrid properties: they respond to αβmeATP, but desensitize slowly (Lewis et al., 1995).

In lumber dorsal root ganglia (DRG) of adult rats, intensive P2X3-IR is found predominantly in a subset of small- and medium-diameter neurons that bind isoleucin B4 (IB4; Vulchanova et al., 1997, 1998; Bradbury et al., 1998). The P2X3-IR is weaker in the peptidergic nociceptors that contain substance P and calcitonin gene-related peptide (CGRP), and undetectable in large DRG neurons that express neurofilament. In contrast, P2X3-IR has been reported in small as well as large DRG neurons, although the level is lower than that of P2X3 (Vulchanova et al., 1997; Labrakakis et al., 2000). Some neurons contain both P2X3- and P2X2-IR. Functional studies on acutely dissociated rat DRG neurons using patch clamp techniques revealed transient, sustained and biphasic ATP responses. The transient component appears to be mediated by P2X3 homomers, while the sustained component is mediated by heteromeric P2X2/3 receptors (with a small contribution from homomeric P2X2 receptors; for review, see Dunn et al., 2001). The heterogeneity of P2X responses cannot be fully correlated to the cell size, capsaicin sensitivity, or developmental stage.

The P2X3 subunit is important in the voiding reflex of the urinary bladder as well as in the pain associated with nerve injury and chronic inflammation (Cockayne et al., 2000; Burnstock, 2001; Jarvis et al., 2002). P2X3 null mutant mice exhibit bladder hyporeflexia characterized by attenuated pelvic afferent responses to bladder distension, decreased voiding frequency and increased bladder capacity (Cockayne et al., 2000; Vlaskovska et al., 2001). However, it is not known whether the functional P2X receptors in bladder afferents are P2X3 homomers or heteromeric receptors containing the P2X3 subunit. It is also not clear whether or not the same functional P2X receptors are expressed in visceral and cutaneous afferents. The urinary bladder receives afferent innervation from the pelvic nerves, as well as from the hypogastric and the lumber splanchnic nerves (Jänig and Koltzenburg, 1990). Both the innocuous filling and painful sensation from the urinary...
bladder are conducted predominantly through the pelvic nerves. Retrograde tracing studies have shown that the bladder pelvic afferents arise from neurons in the L6 and S1 DRG, while those traveling via hypogastric nerves have cell bodies in L1 and L2 DRG (Sharkey et al., 1983). In the present study, we sought to determine which functional P2X receptors are present in cell bodies of bladder pelvic afferents (in L6 and S1 DRG) of the rat, using immunohistochemistry and patch clamp techniques. These bladder afferents included both thinly myelinated Aβ and unmyelinated C fibers. We also examined the phenotype of P2X receptors in cell bodies of cutaneous saphenous afferents (in L3 and L4 DRG), to test explicitly whether DRG neurons innervating the urinary bladder and skin may express different P2X receptors. Preliminary accounts of this work have appeared in the form of abstracts (Zhong et al., 2001a, 2002).

**EXPERIMENTAL PROCEDURES**

**Labeling of bladder and cutaneous afferents**

Adult Wistar rats (150–250 g, Charles River, Kent, UK) of either sex, adult female C57BL6 mice (20–30 g, Charles River) and P2Xβ, null mutant mice (generated by Roche Palo Alto, Palo Alto, CA, USA) were used. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. All efforts were made to minimize the number of animals used and their suffering. Dye injection was performed as described previously (Yoshimura et al., 1996; Bradbury et al., 1998). Briefly, animals were anesthetized with isoflurane (Rhône Mérieux Ltd., Harlow, UK). To label bladder afferents, the bladder was exposed under sterile conditions. Fast Blue (FB, 1%, EMS-Chemie, Germany) was injected into multiple sites (total volume 10–12 μl) in the muscle wall of the bladder using a Hamilton syringe. Injections were made in distal half of the dome and trigone regions, since suburothelial plexus is denser toward the trigone (Gabella and Davis, 1998). To label cutaneous afferents, the saphenous nerve was exposed and FB (1%, 1–2 μl) was injected directly into the nerve. Care was taken to ensure that surrounding tissues were not contaminated and any small leakage was blotted dry with cotton tips. The incision was closed and animals were allowed to recover from anesthesia under observation.

**Immunohistochemistry**

Three to 5 days after tracer injection, animals were deeply anesthetized with sodium pentobarbitone (60 mg/kg, i.p., Rhône Merieux Ltd.) and transcardially perfused with heparin saline (5 iu/ml in 0.9% saline) followed by 4% paraformaldehyde (in 0.1 M phosphate buffer). L6 and S1 DRG, or L3 and L4 DRG were dissected from animals in which the urinary bladder or saphenous nerves had been injected, respectively. In both cases, L5 DRG were taken out and served as the negative control. In some experiments, L1 and L2 DRG were also taken out from rats in which the urinary bladder had been injected with FB. L3, L4, L5 DRG were dissected from naive P2Xβ null mutant mice. Urinary bladders were also dissected from some naïve rats. The bladder pelvic afferents included both thinly myelinated Aβ and unmyelinated C fibers. Aβ and uncoupled C fibers.

Sections of 10 μm (DRG) and 12 μm (bladder) were cut on a cryostat. Double-staining protocols were performed as described previously (Bradbury et al., 1998). Primary antibodies used were rabbit anti-P2X3 (Neuromics, Minneapolis, MN, USA; 1:8000), mouse anti-βIII-tubulin (a pan-neuronal marker) (Promega, Southampton, UK; 1:2000), sheep anti-CGRP (Affinity, Exeter, UK; 1:4000), goat anti-Iβ4 (Vector, Burlingame, CA, USA; 1:2000). IB4 from Grifﬁonica simplicofolia was obtained from Sigma (Poole, UK) (5 μg/ml). Secondary FITC or TRITC-conjugated antisera (1:200) were obtained from Jackson (Luton, UK) or Sigma. In some control experiments, the primary antisera were omitted from the procedure. To check for cross-reactivity, single immunostaining for P2X3, CGRP, IB4 and βIII-tubulin was performed on some sections and the localization of each marker appeared identical to that observed with double-staining techniques. The P2X3 immunostaining was carried out on DRG sections from P2X3 knockout mouse, and no specific staining was found (see Results).

**Image analysis**

Immunofluorescence was visualized under a Leica fluorescence microscope (Wetzlar, Germany) and all analysis was performed at ×20 objective magnification. Cell counts were performed for positively stained FB, CGRP and IB4 cells. For P2X3-IR analysis, since the staining intensity was continuous (see Results), we quantitatively analyzed the relative intensity of P2X3-immunostaining, as described by Ramer et al. (2001). Briefly, digital images were captured using a Hamamatsu video camera (Tokyo, Japan) and analyzed using Sigmascan Pro v4 (SPSS Inc., Chicago, IL, USA). All images were captured under identical brightness, contrast and exposure settings. All the tissues to be directly compared were immunohistochemically stained at the same time. Approximately 250–600 neurons from appropriate ganglia of each animal that were at least 4 sections apart were used. The profiles of FB positive cells in a ganglion section were outlined, creating a template that was overlaid onto the corresponding P2X3 staining image. Similarly, cell profiles for all neurons were outlined using βIII-tubulin staining images. The average level of immunohistochemical staining intensity and ferret diameter of each object identified by the overlay was then measured automatically. In each captured image, the threshold for immunopositivity was determined subjectively and subtracted to give the relative intensity. Recursive translation was performed to convert observed neuronal profiles to the cellular population from which they were drawn (Rose and Rohrlich, 1988; Ramer et al., 2001).

**Cell culture**

L6 and S1 DRG, or L3 and L4 DRG were dissected from rats received bladder or saphenous nerve injection. Cell cultures were prepared as described previously (Gavazzi et al., 1999). Briefly, the desheathed ganglia were incubated with 0.125% collagenase (Type XI; Sigma) for 2 h at 37 °C. Then they were mechanically dissociated in 1 ml modified Bottenstein and Sato’s culture medium containing 2% fetal calf serum (Life Technologies, Paisley, UK) in Ham’s F12 (Life Technologies). The resulting cell suspension was centrifuged at 600 r.p.m. for 8 min through a 2 ml cushion of 15% bovine serum albumin (BSA, Sigma) to remove cellular debris and enrich neurons. In some experiments the BSA cushion was omitted from the procedure. To check for cross-reactivity with FB-containing neurons were identified by brief exposure to UV light. Whole cell voltage-clamp recording was carried out at room temper-
ature using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Membrane potential was held at \(-60\) mV. The external solution contained (mM): NaCl \(154\), KCl \(4.7\), MgCl\(_2\) \(1.2\), CaCl\(_2\) \(2.5\), HEPES \(10\), glucose \(5.6\). The pH was adjusted to 7.4 using NaOH. Recording electrodes (resistance \(2\)–\(4\) M\(\Omega\)) were filled with internal solution which contained (mM): citric acid \(56\), MgCl\(_2\) \(3\), CsCl \(10\), NaCl \(10\), HEPES \(40\), EGTA \(0.1\), tetraethylammonium chloride \(10\), and the pH was adjusted to 7.2 using CsOH (total Cs concentration \(170\) mM). The composition of this solution was chosen to minimize complications arising from the secondary activation of any potassium or chloride currents. The threshold for minimum response detectable was set as \(20\) pA. Data were acquired using pCLAMP software (Axon Instruments). Signals were filtered at 2 kHz (\(3\) dB frequency, Bessel filter, \(80\) dB/decade).

Drugs were applied by gravity flow from independent reservoirs through a seven-barrel manifold (Dunn et al., 1996). The intervals between agonist application were 2 min for persistent responses, and 4 min for transient and biphasic responses, respectively, which were sufficient to achieve reproducible responses. Traces were acquired using Clampfit (pCLAMP8, Axon Instruments) and plotted using Origin (Microcal, Northampton, MA, USA). ATP and \(\alpha\beta\text{meATP}\) were from Sigma. 2',3'-O-trinitrophenyl-ATP (TNP-ATP) was obtained from Molecular Probes (Leiden, Netherlands).

Statistics

Data were expressed as the mean \(\pm\) S.D. or mean \(\pm\) S.E.M. where appropriate. The relative staining intensity of P2X\(_3\)-IR was analyzed using the non-parametric Mann-Whitney test (Prism v4, Graphpad, San Diego, CA, USA).

RESULTS

Immunohistochemistry

Results from male and female rats were comparable and pooled. Following bladder injection, moderate number (approximately \(100\)–\(200\)) of neurons in bilateral L6 and S1 DRG and fewer in L1 and L2 DRG were labeled (Fig. 1). Saphenous nerve injection resulted in many FB-positive neurons in ipsilateral L3 and L4 DRG. Lightly labeled neurons in L5 DRG were rarely observed, suggesting no significant leakage of the dye. Most bladder sensory neurons of the rat were small to medium in cell size (here defined as having a cell radius \(<22.5\) \(\mu\)m; Ramer et al., 2001), with a mean radius of \(15.5 \pm 3.5\) \(\mu\)m (mean \(\pm\) S.D., 1243 cells from four rats, Fig. 2a). By comparison, all DRG neurons in L6 and S1 had cell radii ranging from 9 to 46 \(\mu\)m (1947 cells from four rats).

In L6 and S1 DRG, the relative intensity of P2X\(_3\)-IR showed a skewed distribution, with many neurons having weak or moderate P2X\(_3\)-IR, and a small proportion of cells showing strong P2X\(_3\)-IR, many of these were IB4-positive (Figs. 1, 2). Notably, none of the bladder sensory neurons contained strong P2X\(_3\)-IR, and none of the strongly P2X\(_3\)-positive neurons were FB-positive (i.e. those innervating bladder; Fig. 1a, b). The relative intensity of P2X\(_3\)-IR in bladder sensory neurons ranged from \(-25\) to \(85\) arbitrary units (1234 cells). This was compared with the range of \(-25\) to \(145\) arbitrary units obtained from all cells (1947

**FIG. 1.** Colocalization of P2X\(_3\)-IR with CGRP or IB4 in bladder and cutaneous sensory neurons of adult rats. FB-positive bladder sensory neurons (long arrows, A–C) displayed only weak to moderate P2X\(_3\)-IR, while strongly P2X\(_3\)-positive cells (short arrows, B) did not contain FB. Some bladder afferents were CGRP-positive (double long arrows, C). In contrast, the P2X\(_3\)-IR in cutaneous sensory neurons (long arrows, D–F) ranged from weak to intense. One intensely P2X\(_3\)-positive cutaneous afferent neuron was shown (E). Scale bar=\(100\) \(\mu\)m.
cells, Fig. 2c). Although the average level of staining was not significantly different between bladder sensory neurons and the whole population of L6 and S1 DRG neurons (P > 0.05, Mann-Whitney test), the intensely P2X3-positive neurons were clearly absent from bladder afferent population (Fig. 2c).

In contrast to bladder afferents, a sub-population of cutaneous sensory neurons displayed strong P2X3-IR (Fig. 1e). The distribution of P2X3-IR in cutaneous afferents (1981 cells) mirrored that in all L3 and L4 DRG neurons (2369 cells, Fig. 2d). In both populations, the relative intensity of P2X3-IR showed a skewed distribution, ranging from -25 to 165 arbitrary units, with majority of cells having little or low levels of P2X3-IR and fewer cells showing strong P2X3-IR. The mean intensity was not significantly different between these two groups (P > 0.05). Since intense P2X3-IR was present in some retrogradely labeled cutaneous afferents, the absence of strong P2X3-IR in bladder afferents was not due to the presence of FB.

Although P2X3-IR in bladder sensory neurons was fairly modest, it appeared to be higher than the background staining. To quantify this, we calculated the relative intensity of P2X3 staining in DRG from P2X3 null mutant mice (Cockayne et al., 2000), the relative intensity obtained represented the background. As shown in Fig. 2c, the relative intensity of P2X3 staining in lumbar DRG of P2X3 null mutant mice gave an average of -1.3 ± 3.3 arbitrary units (mean ± S.D., 383 cells) and a range of -25 to 15 (compared with -25–85 arbitrary units from rat bladder sensory neurons).

The moderate level of P2X3-IR present in rat bladder sensory neurons was not expected given that P2X3 null mutant mice displayed bladder hyporeflexia (Cockayne et al., 2000). To find out whether this was due to the species difference, we examined P2X3-IR in bladder pelvic sensory neurons (in L6, S1 DRG) of mice. Similar to the rat, many mouse bladder pelvic afferents displayed varying low levels of P2X3-IR in their cell bodies (Fig. 3). The relative...
intensity did not go beyond 70 arbitrary units (356 cells from three mice), compared with the maximum of 115 arbitrary units obtained from the whole population of L6 and S1 DRG neurons (973 cells from three mice). In contrast to the rat, most mouse L6 and S1 DRG neurons were small to medium in cell size (cell radii $< 22.5 \mu m$). And mouse bladder sensory neurons were mainly small cells (cell radii $< 17.5 \mu m$), with a mean radius of 13.5 $\pm$ 3.1 $\mu m$ (mean $\pm$ S.D., derived from 356 cells).

A possible explanation for the modest P2X$_3$-IR in cell bodies of bladder pelvic afferents is that the P2X$_3$ protein was transported to nerve terminals. However, we did not find strong P2X$_3$-IR in afferent nerve endings in rat bladder either (data not shown). In addition, we examined P2X$_3$-IR in bladder sensory neurons in rat L1, L2 DRG, since these bladder afferents may be important in sensing chemical irritation (Mitsui et al., 2001). Similar to our findings in L6, S1 DRG, none of the bladder sensory neurons in L1, L2 DRG had intense P2X$_3$-IR (data not shown).

Patch clamp
In acutely dissociated DRG preparation, FB containing neurons were readily detectable with epifluorescence illumination (Fig. 4). Typically, approximately 50–100 bladder afferent neurons or over 200 cutaneous sensory neurons were harvested from each rat. Rapid application of $\alpha$-BTXmeATP (30 $\mu M$) evoked persistent currents in the majority of bladder sensory neurons (88%, 99/113 cells), with a mean amplitude of 0.89 $\pm$ 0.65 nA (mean $\pm$ S.D., $n=58$; Fig. 4). The remaining bladder sensory neurons demonstrated biphasic (5/113 cells), transient (5/113 cells) or no (4/113 cells) responses to $\alpha$-BTXmeATP. In contrast, rapid application of $\alpha$-BTXmeATP (30 $\mu M$) onto acutely dissociated cutaneous DRG neurons evoked transient and biphasic currents in approximately 34% (23/68 cells) and 32% (22/68 cells) of cells. Only 24% (16/68 cells) of cutaneous sensory neurons gave persistent currents and the remaining 10% (7/68 cells) were non-responsive (Fig. 4). The peak amplitude of currents to $\alpha$-BTXmeATP (30 $\mu M$) in re-
sponding cutaneous afferents was 0.87 ± 0.67 nA (mean ± S.D., n=53).

In bladder sensory neurons where αβmeATP evoked persistent currents, ATP also gave rise to the same type of currents. The amplitude of responses to 30 μM αβmeATP and ATP were 0.91 ± 0.68 nA and 1.80 ± 1.36 nA, respectively (mean ± S.D., n=44). We have previously observed larger ATP currents compared with αβmeATP currents in the same autonomic neurons of guinea-pig superior cervical and pelvic ganglia (Zhong et al., 2000, 2001c), where it can likely be accounted for by the presence of P2X2/3 heteromeric and P2X3 homomeric receptors in the same cell. To find out whether P2X2 homomers contributed to ATP-evoked currents in rat bladder afferents, we used the subtype selective antagonist TNP-ATP. TNP-ATP is 1000-fold more potent at inhibiting P2X2/3 receptors than P2X2 receptors (Virginio et al., 1998). Previously, we showed that TNP-ATP (0.3 μM) greatly inhibited P2X2/3 responses activated by αβmeATP (30 μM) on nodose neurons from P2X3 wild type mice (Zhong et al., 2001b). On rat bladder sensory neurons, TNP-ATP (0.3 μM) inhibited the persistent currents evoked by αβmeATP and ATP (30 μM) equally to 6.4 ± 1.9% and 8.0 ± 2.6% of control, respectively (mean ± S.E.M., n=8; Fig. 5).

**DISCUSSION**

**Identity of P2X receptors in bladder and cutaneous sensory neurons**

P2X3 and P2X2/3 receptors are the major P2X receptors in DRG neurons of the rat and mouse (see Dunn et al., 2001). These receptors are expressed either separately or together on individual neurons, giving rise to transient, persistent or biphasic responses to P2X agonists. In the present study, we have shown that in great majority (approximately 90%) of bladder sensory neurons, αβmeATP and ATP evoked persistent currents. Thus, P2X3 homomers are not the major functional P2X receptors in bladder afferents. In many bladder sensory neurons, ATP 30 μM evoked larger persistent currents compared with
In this series of experiments, the EC$_{50}$ value of $\alpha\beta$meATP on persistent currents was estimated to be around 45 $\mu$M (data not shown). This value was similar to that of 39 $\mu$M on rat nodose neurons (Dunn et al., 2000), but somewhat different from 6.5 $\mu$M on recombinant rat P2X$_{2/3}$ receptors (Spelletta et al., 2002), and 63 $\mu$M on persistent currents in rat DRG neurons (Ueno et al., 1999). In addition, 30 $\mu$M of $\alpha\beta$meATP activated a tiny current (about 5% of maximum) on recombinant P2X$_2$ receptors (Spelletta et al., 2002). But it was found inactive at concentrations up to 100 $\mu$M on endogenous P2X$_2$ homomers in rat pelvic ganglia (Zhong et al., 1998). The reason for the discrepancy is not clear, although possible explanations may include the existence of more than one form of P2X$_{2/3}$ heteromers and the modulation by host cells.

Heterogeneity of P2X receptors in rat DRG neurons

Rat DRG are comprised of many distinct subgroups of sensory neurons, which differ in their cell size, electrophysiological, cytochemical and functional properties. The relationship between P2X receptor phenotypes with cell size, IB4-binding and capsaicin-sensitivity was complex (Petruska et al., 2000a,b, 2002). Transient P2X currents were mainly found in small-diameter (<35 $\mu$M), IB4-positive and capsaicin-sensitive DRG neurons (Petruska et al., 2000b, 2002). However, persistent and biphasic ATP responses or non-responders were also encountered in other subtypes of small- to medium-diameter (<45 $\mu$M) neurons (Grubb and Evans, 1999; Petruska et al., 2000a,b, 2002). In addition, all four types of ATP responses were present in the large-diameter (>50 $\mu$M) population of DRG neurons, with higher proportion of non-responding cells (Ueno et al., 1999; Kage et al., 2002). Furthermore, P2X$_{3}$-IR varies in DRG from different spinal level. Thus, DRG from cervical levels have a greater proportion of P2X$_{3}$-positive neurons, many of which are large diameter and CGRP-positive (Ramer et al., 2001). In the present study, we examined the heterogeneity of P2X currents in DRG neurons from the functional perspective, to see whether bladder afferents (visceral) and cutaneous afferents may express different P2X receptors. We found that while bladder pelvic sensory neurons mainly express a homogeneous population of P2X$_{2/3}$ heteromers, those present on cutaneous afferents were more heterogeneous.

Retrograde labeling of sensory neurons

Bladder pelvic afferents are comprised of thinly myelinated (A$eta$) and unmyelinated (C) fibers (Yoshimura and De Groat, 1997). Our data suggest that both types of fibers express predominantly P2X$_{2/3}$ heteromers. However, although FB was injected into multiple sites in the bladder wall, it is certain that some bladder afferents remained unlabelled, which may express different P2X receptors. On the other hand, while bladder pelvic afferents are involved in micturition as well as nociception, those in hypogastric nerves may be more important in inflammatory pain (Mitsui et al., 2001). Further functional studies are needed to find out the functional P2X receptors in bladder sensory neurons in L1 and L2 DRG, although the P2X$_3$-IR was similar.

Fig. 5. Antagonism of P2X responses in bladder sensory neurons by TNP-ATP. (A) Representative traces showing six consecutive responses from a rat bladder sensory neuron: responses to $\alpha\beta$meATP (30 $\mu$M) alone, in the presence of TNP-ATP (0.3 $\mu$M), 2 min after washing out the antagonist; followed by responses to ATP (30 $\mu$M) alone, in the presence of TNP-ATP (0.3 $\mu$M), and 2 min after washing out TNP-ATP. Agonists were applied at a 2-min interval. TNP-ATP was present 2 min before and during the reapplication of the agonist. The horizontal bars above the traces indicate the duration of the agonist application. (B) Averaged data from eight cells. The membrane potential was held at -60 mV. $\alpha\beta$meATP 30 $\mu$M. This may indicate the presence of $\alpha\beta$meATP-insensitive receptors, e.g. P2X$_2$ homomers (Zhong et al., 2000). However, TNP-ATP (0.3 $\mu$M) nearly abolished persistent responses to both $\alpha\beta$meATP and ATP, suggesting that the contribution from P2X$_2$ homomers was minimal. Thus, the major functional P2X receptors in cell bodies of bladder afferents are P2X$_{2/3}$ heteromers. In contrast to visceral afferents projecting to the urinary bladder, the functional P2X currents in cell bodies of cutaneous afferents appeared to be more heterogeneous, with only 24% of neurons giving persistent currents to $\alpha\beta$meATP, while approximately 2/3 of neurons gave either transient or biphasic $\alpha\beta$meATP responses. Thus, cutaneous afferents expressed a greater proportion of P2X$_3$ homomers, in addition to P2X$_{2/3}$ receptors.
In both populations. Tracer injection into saphenous nerve labeled all types of cutaneous afferents, including myelinated (Aδ), thinly myelinated (Aδ) and unmyelinated (C) fibers, as evidenced by the cell size variation in their cell bodies. It will be interesting to find out whether sub-populations of cutaneous afferents, e.g. mechanosensors and nociceptors, may express different functional P2X receptors.

In a previous study, 30% of visceral afferents identified by tracer injection into the pelvic nerve of male rats were found to be P2X3-immunoreactive (Bradbury et al., 1998). Visceral afferents labeled this way included those innervating various pelvic organs, including urinary bladder, penis, prostate, colon, as well as perineal skin. Further studies are needed to determine the subtypes of P2X receptors in afferent pathway of each pelvic organ.

**Correlating findings from immunohistochemistry and patch clamp studies**

P2X3 receptors expressed by majority of bladder pelvic sensory neurons may be consistent with the weak to moderate P2X3-IR seen in them. This is because there are fewer P2X3 subunits in heteromeric versus homomeric receptors. Another possibility is that the subunit configuration in heteromers may hinder the binding of P2X3 antibody. For cutaneous afferents, although weak to moderate P2X3-IR was detected in many neurons, intense P2X3-IR was readily detectable in others. This may indicate higher levels of P2X3 homomer expression in some cutaneous afferents, consistent with our results from patch-clamp studies. In L6 and S1 DRG, a subset of small diameter afferents, consistent with our results from patch-clamp studies, were labeled by FB contained in- studies. In L6 and S1 DRG, a subset of small diameter DRG neurons that were not labeled by FB contained intense P2X3-IR. It is possible that at least some of these are cutaneous afferents that innervate perineal skin.

On the other hand, our results highlight a paradox in that while over 90% of bladder afferents responded with currents that suggest P2X3 subunit expression, only a minority of them displayed detectable P2X3-IR. One possible explanation is that patch clamp recording may be more sensitive than immunohistochemistry. Low levels of receptor expression that are sufficient to give measurable responses may not show up as “positive” staining. Labrakakis et al. (2000) found that while electrophysiological recordings revealed the heterogeneity in ATP current kinetics in cultured DRG neurons, immunohistochemical staining appeared to show a relatively homogeneous and widespread expression of the P2X2 and P2X3 subunits.

**Functional role for ATP in bladder afferent pathway**

There is much evidence to support a major functional role for ATP in mechanosensory transduction in the urinary bladder (Burnstock, 2001). P2X3-IR was found on afferent terminals in suburothelial plexus of mice (Cockayne et al., 2000; Vlaskovska et al., 2001) and human (Yiangou et al., 2001). Distension released ATP from the urothelium of rabbit (Ferguson et al., 1997), mouse (Vlaskovska et al., 2001) and human (Sun et al., 2001, 2002). Endogenously released ATP activated/sensitized bladder afferents, probably via P2X2 heteromers (Vlaskovska et al., 2001; Rong et al., 2002; this study). Thus, drugs that reduce ATP release, or block P2X2 (and P2X3) receptors may be potentially useful in relieving intractability and pain of the bladder.

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