P2X Receptor–Mediated Ionic Currents in Dorsal Root Ganglion Neurons

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INTRODUCTION

The dorsal root ganglion (DRG) contains the cell bodies of primary afferent sensory neurons that relay nociceptive and proprioceptive information from the periphery to the dorsal horn of the spinal cord. Pharmacological modulation of neurotransmitter receptors involved in the propagation of pain signals via the DRG should therefore shift the threshold for nociception. In this context, P2X receptors located on peripheral sensory neurons have been implicated in the initiation of pain (Bland-Ward and Humphrey 1997; Burnstock 1996; Cook et al. 1997). However, the P2X receptor subtypes underlying this phenomenon have not been elucidated, nor has their subcellular localization or activation characteristics.

P2X receptors belong to a family of ligand-gated ion channels that are activated by extracellular ATP (Ralevic and Burnstock 1998). Seven distinct P2X receptors (P2X 1–P2X 7 ) have been identified and cloned, and six of these (P2X 1–P2X 6 ) can be expressed on the plasma membrane of neurons (Collo et al. 1996; Lewis et al. 1995; Valera et al. 1994). Within the rat peripheral nervous system, localization of P2X 3 message was initially described in neurons of the DRG (Chen et al. 1995; Lewis et al. 1995). Although mRNA for other P2X receptors is present in these neurons, colocalization of both P2X 2 and P2X 3 receptor protein has been demonstrated in a subset of DRG neurons (Vulchanova et al. 1997). In rat nodose ganglia, coexpression of P2X 2 and P2X 3 receptor subtypes form functional heteromultimeric P2X 2/3 receptors (Radford et al. 1997; Thomas et al. 1998). From these data, it is clear that native P2X receptors can exist as either hetero- or homomultimeric complexes in sensory neurons.

Rat DRG are comprised of a variety of neuronal cell types that differ in their intrinsic electrophysiological (Caffrey et al. 1992; Harper and Lawson 1985b; Scroggs and Fox 1992; Scroggs et al. 1994) and cytochemical (Dodd et al. 1984; Schoenen et al. 1989) properties. Among these, nociceptive C-fiber neurons belong to a subset of small diameter cells located in the DRG (Harper and Lawson 1985a). The isolectin IB4 has been shown to selectively label small-diameter DRG nociceptors that are largely TrkA negative and nonpeptidergic (Molliver et al. 1995). IB4-positive neurons also express the P2X 3 receptor (Vulchanova et al. 1998), and expression patterns suggest that it is colocalized with the P2X 2 receptor in some DRG neurons (Vulchanova et al. 1997). A subset of capsaicin-sensitive, peripherin-positive C-fiber neurons also contains P2X 3 receptor mRNA (Chen et al. 1995). Taken together, it appears that P2X 3 receptors are expressed primarily on nociceptive DRG neurons.

It is known that P2X 2 and P2X 3 receptors are expressed on the surface of rat DRG neurons, but it is not known in what proportions functional homomeric P2X 2, P2X 3, or heteromeric P2X 2/3 receptors are expressed. Rapidly desensitizing ATP- or α,β-methylene ATP-evoked responses have been described in neonatal rat DRG (Jahr and Jessell 1983; Rae et al. 1998; Robertson et al. 1996). Although these response properties fit those for either P2X 1 or P2X 3 receptors, the pharmacological
profile of rat DRG responses suggests that the rapidly desensitizing response is mediated by P2X₂, and not P2X₃ receptors (Rae et al. 1998). However, there appear to be some species differences with respect to P2X receptor expression in DRG (Bean 1990). Rat DRG response properties resemble those of P2X₂ receptors (Chen et al. 1995), whereas the nondenaturing properties of bullfrog DRG neurons (Bean 1990; Li et al. 1993) resemble P2X₃ or P2X₂/₃ receptor activation (Brake et al. 1994; Lewis et al. 1995).

Many behavioral studies concerning the function of primary afferent neurons in various pain models are conducted in the adult rat. Because of possible species differences, potential developmental changes, or influences of long-term culture conditions on P2X receptor expression, the present study was designed to characterize P2X responses in acutely dissociated adult rat DRG neurons. Based on pharmacological and kinetic profiles, the data presented here support the existence of at least two distinct P2X receptor subtypes in adult DRG neurons, P2X₁ and P2X₂/₃. Preliminary results from these studies have appeared in abstract form (Burgard et al. 1998).

METHODS

Neuronal cultures

Adult male Sprague-Dawley rats (~8 wk old, 250–300 g) were deeply anesthetized with CO₂ anesthesia and killed by decapitation. Lumbar (L₅–L₆) DRG were dissected from the vertebral column and placed in Dulbecco’s modified Eagle medium (DMEM, Hyclone, Logan, UT) containing 0.3% collagenase B (Boehringer Mannheim, Indianapolis, IN) for 60 min at 37°C. The collagenase was replaced with 10% FBS (Hyclone), NGF (50 ng/ml, Boehringer Mannheim), and 100 U/ml Pen/Strep. Experimental procedures in- plated at a density of one DRG per coverslip in 1 ml DMEM supplemented with 10% FBS (Hyclone) and 100 U/ml Pen/Strep. Experimental procedures in-vitro were conducted under a protocol approved by an Institutional Animal Care and Use Committee.

To label cutaneous afferent neurons, the fluorescent carbocyanine dye fast DiI (DiI, Molecular Probes, Eugene, OR) was injected into the subplantar hindpaw region of 7- to 8-wk-old rats. Injections of 50 μl of a 20-MM solution in DMSO were administered 10–12 days before culture preparation. Labeled neurons were identified in vitro using fluorescence optics.

Neurons were used for electrophysiological recording within 4–48 h. In most experiments, the FITC-labeled plant lectin BSI-B₄ (IB4, 10 μg/ml) was added to the culture medium at 37°C for 5 min before recording. The coverslip was then washed with extracellular recording solution for 5 min before being placed in the recording chamber, which was mounted on the stage of an Olympus IX70 microscope equipped with fluorescence optics. Digital images were captured using a SPOT-2 camera system (Diagnostic Instruments, Sterling Hts, MI).

Cloning and expression of recombinant rat P2X receptors

Primers were designed to regions encompassing the initiation and termination codons of the rat P2X₂ and P2X₃ cDNAs (Genbank accession numbers; P2X₂, U14114; P2X₃, X90651). A consensus Kozak sequence was designed into the 5’ primer of each primer pair to facilitate optimal translation efficiency (Kozak 1984).

To clone the rat P2X₁ receptor, 100 ng of poly A+ RNA derived from rat total brain tissue (Clontech, Palo Alto, CA) was used in a first-strand cDNA synthesis reaction using Superscript II reverse transcriptase and reagents from GIBCO BRL. One-tenth of the reaction was used in a 50-μl amplification reaction with 10 picomoles of each of the P2X₂ primers (sense P2X₂, 5’CACCATTGTTCCCCGGCGCTTGGCCTGGGGCGC3’, antisense P2X₂, 5’TCAAAGTGTTGGCGCAACCTTGTGCTGTC3’). Additional components of the reaction included 200 μM dNTPs, 1 × Pfu buffer (Stratagene, La Jolla, CA). The reaction was incubated at 94°C for 1 min, then 80°C for 2 min during which 1.25 units Pfu polymerase (Stratagene) was added. The reaction was then cycled 35 times in a Perkin Elmer Model 9600 thermocycler (Perkin Elmer, Foster City, CA) under the following conditions: 94°C for 20 s, 65°C for 20 s, and 72°C for 4 min. Reaction products were separated by agarose gel electrophoresis; the major product of ~1.5 kilobases was isolated and cloned into the pcScript vector (Stratagene) according to the manufacturer’s instructions. The insert was sequenced by dye-terminator chemistry on a Perkin Elmer Model 310 genetic analyzer and found to be identical to the published sequence for the rat P2X₁ message (Brake et al. 1994). The cDNA was excised from the vector by digestion with the restriction enzymes Bam HI and Not I and ligated into the vector pIREs(hyg) vector (Clontech).

The P2X₁ receptor cDNA was cloned from rat brain poly A+ RNA essentially as described above. The primers used in the PCR amplification reaction were as follows: sense P2X₂, 5’TTCCACCATGAACTCTATATCAGACTTCTTC3’, antisense P2X₂, 5’AGAGGCCCTTAGTGACCATTATAGATGGCCGCC3’. Amplification thermopolymerase and buffers (Perkin Elmer) were used in this amplification. The reaction was cycled 35 times under the following conditions: 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min. The major product of 1.3 kilobases was cloned into the vector pCRII (Invitrogen, Carlsbad, CA) as per manufacturer’s instructions. The insert was sequenced and found to be identical to the published rat P2X₃ message (Chen et al. 1995). The cDNA was excised from the vector and ligated into the vector pIREs(neo) vector (Clontech).

Expression plasmids encoding rat P2X₂ and P2X₃ receptor cDNAs were transfected individually into 1321N1 human astrocytoma cells using Lipofectamine (GIBCO BRL). After transfection (48 h), cells were subcultured in growth medium containing 800 μg ml⁻¹ G418 (rP2X₂) or 100 μg ml⁻¹ hygromycin (rP2X₃). Surviving individual colonies were isolated, screened for P2 receptor activity using a fluorescence-based calcium imaging assay, and selected for further characterization. The cell line expressing homomeric rP2X₂ receptors was then transfected by transfection of the rP2X₂ cDNA into rP2X₃ expressing 1321N1 cells. Positive clones were isolated in growth medium containing 150 μg ml⁻¹ G418 and 75 μg ml⁻¹ hygromycin and selected based on their sensitivity to α,β-methoATP.

Cells expressing recombinant homomeric P2X₂ receptors were maintained at 37°C in DMEM (with 4.5 mg ml⁻¹ glucose and 4 mM L-glutamine), 10% FBS and 300 μg ml⁻¹ G418 or 100 μg ml⁻¹ hygromycin in a humidified 5% CO₂ atmosphere. Cells expressing rP2X₂/₃ receptors were maintained in growth medium containing 150 μg ml⁻¹ G418 and 75 μg ml⁻¹ hygromycin. Cells were plated onto PEI-treated glass coverslips and used for recording at ~50% confluency.

Electrophysiology

Whole cell patch-clamp recordings were obtained from both DRG neurons and stably transfected 1321N1 cells. Cells were maintained in an extracellular recording solution (pH 7.4, 325 mosM) consisting of (in mM) 155 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 12 glucose. Patch electrodes were pulled from borosilicate glass and fire polished to 3–10 MΩ tip resistance. Two internal pipette solutions (pH 7.3, 295 mosM) were used for recording. The first was used for the majority of recordings, and consisted of (in mM) 140 K-aspartate,
20 NaCl, 10 EGTA, and 5 HEPES. The second consisted of (in mM) 135 Cs-acetate, 10 CsCl, 0.5 EGTA, and 10 HEPES. No differences in P2X responses were observed when using either intracellular solution. Cells were typically voltage clamped at −60 mV, and series resistance was compensated 90–95%. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and digitized at 3 kHz for acquisition. For analysis, recordings were low-pass filtered at 1 kHz. Rise times of inward currents were measured between 10 and 90% peak. Exponential time constants of current desensitization (τ) were estimated using a Chebyshev curve-fitting algorithm. Most of the desensitizing currents in this study were best fitted by two exponential functions. For DRG neurons, resting membrane potential was measured immediately after rupture of the cell membrane in whole-cell patch mode. Neuronal input resistance was determined from the amplitude of the current response to a 10-mV hyperpolarizing pulse from a holding potential of −60 mV. A series of depolarizing voltage steps was applied to all neurons to determine the threshold for activation of a fast sodium current presumed to reflect firing of the action potential. For all cell types, baseline responses were recorded for a minimum of 10 min to ensure that the kinetics of the response were stable. A wash out or recovery period usually followed pharmacological manipulation of the response. Responses that exhibited long-lasting or irreversible changes in kinetics during the experiment were considered unstable and were not used for analysis. All data acquisition and analysis was performed using pClamp software (Axon Instruments).

Cells were constantly perfused with extracellular solution at a rate of 0.5 ml/min in the recording chamber. Agonists were applied to individual cells using a piezoelectric-driven rapid application system (Burleigh Instruments, Fishers, NY). Extracellular solution perfused the other barrel, and was applied by rapidly moving the solution interface across the cell. The time constant for solution exchange across the entire cell was 20 ms. This was measured by recording from 1321N1 cells expressing a nontropic P2X receptor (rP2X₂), eliciting a steady-state ATP (10 μM) current at −60 mV, rapidly switching from a low (2 mM) to high (55 mM) potassium extracellular ATP (10 μM) solution, and measuring the resulting current relaxation. This method gave a more realistic measure of total cell exchange time than measuring the exchange time across an open pipette tip. The pipette open tip solution exchange time was on the order of 1–2 ms and was checked after each cell recording to ensure proper agonist application. Agonist applications were 400–800 ms in duration and were typically given every 2–4 min. When the effects of pH were studied, cells were maintained at pH 7.4, and agonist (at pH 6.6) was applied. In this study, the P2X agonists ATP and α,β-methylene ATP (α,β-meATP), and antagonists suramin (RBI, Natick, MA) and trinitrophenyl-ATP (TNP-ATP, Molecular Probes, Eugene, OR) were used.

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. All data are expressed as means ± SE.

**RESULTS**

**Basic properties of DRG neurons**

The FITC-labeled lectin IB4 was used in initial experiments as a live cell marker to identify putative P2X₁ receptor-expressing nociceptive neurons (Vulchanova et al. 1998). Approximately 75% of all cultured DRG neurons were labeled by IB4 (Fig. 1), and 90% of these neurons responded to ATP. Once it was determined that these labeled cells would routinely respond to ATP, some subsequent experiments were performed on untreated neurons. No differences in neuronal membrane properties or P2X receptor–mediated responses were observed between IB4-labeled cells and small-diameter cells in cultures that were not treated with IB4.

Small (25–30 μm) neurons that were devoid of neurite extension had resting membrane potentials of −52 ± 3 (SE) mV and input resistances of 489 ± 109 MΩ (n = 9). All neurons generated an inward sodium current following membrane depolarization to 0 mV (spike threshold −18 ± 2 mV, n = 9). A typical fast inward current was recorded with K-aspartate–filled pipettes. Intracellular Cs-acetate produced a widening of the inward current duration and an appearance of a prolonged plateau current resembling the prolonged action potential duration of small nociceptive DRG neurons (Harper and Lawson 1985b; McLean et al. 1988). Other membrane properties or ATP responses recorded with intracellular pipette solutions containing Cs-acetate were not significantly different from those recorded with K-aspartate–filled pipettes.

**P2X responses in DRG neurons**

Three general types of P2X responses were recorded from DRG neurons after ATP application. The first was a slow, non-desensitizing response characterized by relatively slow activation and no desensitization of current (slow DRG, Fig. 2A). Current responses to 10 μM ATP in six neurons showed relatively slow rise times (113 ± 23 ms) to peak currents of 319 ± 80 pA. Slow DRG currents were nondesensitizing, because 97 ± 3% of peak current amplitude was still present at the end of ATP application. The second type of P2X response was fast, characterized by rapid current activation and fast desensitization (fast DRG, Fig. 3A). Current responses to 10 μM ATP in the fast DRG group (n = 5) had fast rise times (10 ± 2.0 ms) to peak currents of 536 ± 186 pA. In the presence of agonist, current desensitization followed biexponential kinetics. An initial fast component (τ₁ = 32 ± 2.7 ms) was followed by a smaller amplitude prolonged component (τ₂ = 339 ± 64 ms). The currents desensitized almost completely.

**FIG. 1.** IB4-positive neurons in acutely isolated dorsal root ganglion (DRG) cultures. A: phase-contrast image of neurons maintained in culture for 6 h. At this time, round, phase-bright neuronal somata were easily visible (arrows). No visible outgrowth of neurites was evident. B: after exposure to FITC-labeled IB4, a subpopulation of neurons showed membrane labeling with the fluorescent lectin. Calibration bar in A (25 μm) also applies to B.
because there was only 5.8 ± 2.1% of peak amplitude left as residual current at the end of agonist application. The third type of response was a mixed response (DRG-mixed, Fig. 3B) that also exhibited both fast and slow desensitization kinetics, but the slow component was much more prominent than that seen in fast DRG responses. Current responses to 10 μM ATP in this group (n = 5) had relatively fast rise times (38 ± 19 ms) to peak currents of 228 ± 88 pA. Both fast (τ₁ = 25 ± 2.0 ms) and slow (τ₂ = 424 ± 173 ms) desensitizing components were observed, with residual currents at the end of agonist application measuring 38 ± 4.8% of peak. Although the desensitization τ values were not different between fast and mixed DRG responses, mixed DRG responses had significantly larger residual slow component amplitudes than did fast DRG responses (P < 0.05, unpaired t-test). All three types of responses could be elicited by application of either ATP or the ATP analogue α,β-meATP. The three types of responses were observed with approximately equal frequency.

Activation of P2X receptors by ATP in DRG neurons was verified by the use of the P2X receptor antagonists suramin and TNP-ATP. Suramin (10 μM) produced a reversible inhibition of peak current to 48 ± 27% of control (n = 3) during coapplication with ATP (10 μM, mixed DRG responses). Likewise, TNP-ATP (0.1 μM), a P2X antagonist selective for P2X₂/₃, and P2X₁ receptors (Virginio et al. 1998), inhibited mixed DRG peak currents to 52 ± 7% of control when coapplied with ATP (10 μM, n = 3). However, when TNP-ATP was preapplied for at least 30 s before ATP application, both fast and slow P2X currents were completely and reversibly blocked (n = 3, Fig. 7B). These results suggest that preapplication of antagonist is necessary for optimal antagonism, and that the receptor subtypes underlying DRG P2X responses are either P2X₃, P2X₂/₃, or P2X₁.

**Comparison of DRG responses to recombinant rat P2X responses**

Because DRG P2X responses could be grouped as either fast (desensitizing), slow (nondesensitizing), or mixed (fast and slow), the P2X receptor subtype(s) underlying each type of response were investigated. To do this, DRG responses were compared with responses from recombinant P2X receptors expressed in 1321N1 cells that lack endogenous expression of either P2X or P2Y receptors (Schachter and Harden 1997; H. Yu, B. Bianchi, R. Metzger, K. J. Lynch, E. A. Kowaluk, M. F. Jarvis, and T. van Biesen, unpublished observations).

Slow, nondesensitizing P2X currents were evoked in response to ATP application in six DRG neurons. α,β-meATP (10 μM) was also applied to four of these neurons, and it inhibited mixed DRG peak currents to 52 ± 7% of control when coapplied with ATP (10 μM, n = 3).

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**FIG. 2.** Nondesensitizing P2X responses activated by extracellular ATP or α,β-meATP. A: representative ATP response from a nondesensitizing DRG neuron. B: α,β-meATP response from the same neuron as in A. Note the similar kinetics and amplitude of responses to both agonists. C: response to α,β-meATP recorded in a 1321N1 cell expressing rP₂X₃ receptors. All cells were voltage clamped at −60 mV. Both agonists were applied at a concentration of 10 μM (denoted by the bar).

**FIG. 3.** Desensitizing P2X responses activated by extracellular ATP. A: representative response from a fast desensitizing DRG neuron. Note the complete loss of current in the presence of ATP. B: mixed DRG responses exhibited both fast and slow components of desensitization. The slow component resulted in a plateau current that did not desensitize completely. C: ATP response in a 1321N1 cell expressing rP₂X₃ receptors. These cells showed characteristic fast desensitization of ATP-induced current. All cells were voltage clamped at −60 mV. Desensitizing currents were fit with the sum of 2 exponential curves. ATP (10 μM) application is denoted by the bar.
evoked comparable currents that did not significantly differ in amplitude or kinetics from ATP responses (Fig. 2, A and B). These α,β-methyl-ATP–induced currents in neurons had peak amplitudes of $342 \pm 159 \text{ pA}$, rise times of $119 \pm 23 \text{ ms}$, and exhibited no desensitization. For comparison, the recombinant rP2X2/3 receptor exhibited similar α,β-methyl-ATP (10 μM) response kinetics to the slow DRG response (Fig. 2C). Recombinant rP2X2/3–expressing cells displayed peak amplitudes of $359 \pm 93 \text{ pA}$, rise times of $73 \pm 23 \text{ ms}$, and also did not desensitize. α,β-methyl-ATP was used to specifically activate P2X2/3 receptors in all cells, because we (Bianchi et al. 1999) and others (Brake et al. 1994) have observed that homomeric rP2X2 receptors are not activated by α,β-methyl-ATP. These results demonstrate that the slow DRG response is not mediated by a P2X2 receptor, but probably by a P2X2/3 heteromeric receptor.

Approximately 70% of P2X responses in DRG neurons were desensitizing. Fast DRG responses showed fast, almost complete desensitization (15% of peak current left at end of application) in the presence of agonist, whereas mixed DRG responses displayed fast desensitization, but did not desensitize completely (15% of peak current left at end of application, Fig. 3, A and B). For comparison, the recombinant rP2X3 receptor exhibited kinetics similar to both fast and mixed DRG responses (Fig. 3C). Responses from rP2X3-expressing cells activated rapidly (rise time $= 8.7 \pm 1.8 \text{ ms}$, $n = 6$) to peak currents of $1089 \pm 254 \text{ pA}$. Desensitization of these receptors was best characterized by the sum of two exponential functions ($\tau_1 = 39 \pm 4.7$, $\tau_2 = 239 \pm 39 \text{ ms}$), similar to fast DRG responses. In contrast to fast DRG responses, there was a significant residual inward current left at the end of ATP application ($23 \pm 4.7\%$ of peak amplitude, $P < 0.05$, unpaired $t$-test), indicating that desensitization in recombinant rP2X3 receptors was not complete. The relative ATP EC$_{50}$ values for rP2X3 and DRG responses were 0.3 and 1.6 μM ($n = 4$), respectively.

The residual current seen at the end of ATP application to cells expressing recombinant rP2X3 receptors prompted the question of whether or not the plateau current seen in mixed DRG responses was actually mediated by a separate non–desensitizing P2X receptor. To address this issue, the agonist sensitivity of mixed DRG responses was investigated. Both ATP and α,β-methyl-ATP were equally effective in activating mixed DRG responses (Fig. 4). In five neurons exposed to both ATP and α,β-methyl-ATP, both agonists produced peak amplitudes and desensitization kinetics that were not significantly different ($P > 0.05$, paired $t$-test). The observation that both fast and slow components could be activated by both agonists indicated that either P2X2/3 or incompletely desensitized P2X3 receptors were involved in the slow component of the mixed DRG response. The sensitivity to α,β-methyl-ATP indicated that P2X2 receptors were not involved, because P2X2 receptors are insensitive to α,β-methyl-ATP (Brake et al. 1994).

Because the recombinant rP2X3 receptor did not desensitize completely, the possibility remained that the residual current of mixed DRG responses was mediated by the same P2X3 receptors underlying the fast component. Desensitizing P2X receptors often enter a long-lasting desensitized state following activation, whereas non–desensitizing P2X receptors do not (Bean 1990; Krishit et al. 1983). The activation frequency dependence of the mixed DRG response was therefore examined to determine whether or not DRG responses exhibited long-lasting desensitization. In mixed DRG neurons, decreasing the interapplication interval from 4 min to 30 s produced a selective depression of the fast peak to 22% of control, but no depression of the slow component ($n = 3$, Fig. 5A). Similar results were seen when either ATP or α,β-methyl-ATP was used as an agonist (Fig. 7C). In contrast, the entire waveform of fast DRG responses was depressed when application frequencies were increased ($n = 4$, not shown). In these neurons, the peak response was depressed to 13% of control, and the plateau responses were already almost completely desensitized. Similar to fast DRG responses, the entire rP2X3 waveform was depressed during higher frequency applications (Fig. 5B), and responses required a 2- to 4-min interapplication interval to recover to control amplitude. Measurements taken at both peak and end of application revealed that increasing the stimulus frequency to every 15–30 s produced a depression of both peak ($57 \pm 7\%$ of control) and end of application amplitudes ($45 \pm 8\%$ of control, $n = 5$). In contrast, the non–desensitizing rP2X2/3 receptor could follow agonist application frequencies as fast as every 5 s without a decrease in amplitude or change in kinetics ($n = 3$, Fig. 5C). It is clear that mixed DRG responses have

![FIG. 4. Mixed fast and slow P2X responses in DRG neurons. A: ATP application elicited a response characterized by fast ($\tau_1 = 16 \text{ ms}$) and slow ($\tau_2 = 417 \text{ ms}$) desensitization kinetics. A large residual current was left at the end of ATP application. B: current response to α,β-methyl-ATP in the same neuron as A. Note similar desensitization kinetics. Cell was voltage clamped at −60 mV, and both agonists were applied at a concentration of 10 μM (denoted by the bar).

![A mixed DRG](image1.png) ![B mixed DRG](image2.png)
two components that show different long-lasting desensitization properties. The ability of the slow component of mixed DRG responses to follow increased application frequencies indicates the involvement of P2X$_{2/3}$ in the plateau (slow) phase.

To further differentiate the components of mixed DRG responses, the modulatory effects of extracellular protons were studied. Differential modulation of rP2X$_3$ and rP2X$_{2/3}$ receptors by pH has been previously demonstrated (Stoop et al. 1997), where recombinant rP2X$_3$-mediated currents were inhibited and rP2X$_{2/3}$ currents were potentiated by decreasing extracellular pH. In the present study, DRG neurons showed differential pH modulation of response amplitudes (Fig. 6A). Fast peak amplitudes were depressed by extracellular protons (52 ± 11% of control, $n = 4$), consistent with P2X$_3$ receptor activation. However, the plateau (end of application) amplitude was potentiated in mixed or slow DRG neurons (189 ± 15% of control, $n = 3$). In agreement with previous reports, decreasing the extracellular pH decreased both the peak (67% of control) and end of application amplitude (68%, $n = 2$) of recombinant rP2X$_3$ responses (Fig. 6B), so that the entire waveform was depressed. In contrast, the recombinant rP2X$_{2/3}$ response was potentiated by extracellular protons (Fig. 6C). Decreasing the pH of the agonist solution to 6.6 produced an increase in rP2X$_{2/3}$ peak amplitude to 153% of control ($n = 2$). Acidic extracellular recording solution alone had no effect on rP2X-transfected 1321N1 cells. DRG neurons have additional proton-activated inward currents, but the amplitudes of currents elicited by acidic solution alone were <25% of the proton-potentiated P2X currents in DRG neurons ($n = 3$).

FIG. 5. Frequency dependence of P2X receptor activation. A: a mixed DRG response showed both fast and slow components under control conditions (stimulation every 4 min). Decreasing the interstimulus interval to every 15 s resulted in the loss of only the fast component. B: recordings from a rP2X$_3$ cell under control conditions (stimulation every 2 min) and after an interstimulus interval of 15 s. Note that the entire current waveform was depressed, not only the peak. C: recordings from a rP2X$_{2/3}$ cell under control conditions (stimulation every 1 min) and after an interstimulus interval of 15 s. The rP2X$_{2/3}$ receptor was able to follow short interstimulus intervals. Cells were voltage clamped at −60 mV, and agonists (10 μM) were applied at the bar.

FIG. 6. pH dependence of P2X responses. A: a mixed DRG neuron responded to α,β-meATP at pH 7.4 with both fast and slow desensitization kinetics. At an agonist pH of 6.6, the fast peak was decreased, but the slow plateau amplitude was increased. B: rP2X$_3$ responses to α,β-meATP at either pH 7.4 or pH 6.6. Under acidic conditions, the entire waveform was depressed. C: rP2X$_{2/3}$ responses to α,β-meATP at either pH 7.4 or pH 6.6. Under acidic conditions, the entire waveform was increased in amplitude. All cells were voltage clamped at −60 mV, and α,β-meATP (10 μM at the indicated pH) was applied at the bar. Cells were maintained at pH 7.4 except during 400-ms drug applications.
proton currents appeared to be activated, they were very small and their amplitude contributed only a small proportion to the entire potentiation of the plateau current. This differential modulation of fast and slow P2X components in DRG neurons suggests activation of a mixed population of both P2X3 and P2X2/3 receptors.

To test whether or not cutaneous afferents that innervate the rat hindpaw express P2X3 or P2X2/3 receptors, subplantar injections of the fluorescent tracer DiI were performed in three rats to label sensory afferents projecting to the hindpaw. Corresponding DRG neurons were then selected in culture based on DiI fluorescence. In lumbar cultures prepared from DRG ipsilateral to the injection site, one to two percent of neurons were labeled with DiI. Figure 7 shows the P2X responses of a DRG nociceptor co-labeled with DiI and IB4. This mixed DRG response exhibited both fast and slow components. The entire P2X response was blocked by TNP-ATP (100 nM, Fig. 7B). However, increasing the agonist application frequency produced a selective inhibition of the fast component, leaving the slow component intact (Fig. 7C). Responses from six neurons co-labeled with DiI and IB4 indicated that slow (n = 1), fast (n = 2), and mixed (n = 3) P2X responses were present in cutaneous afferents. Based on response kinetics, TNP-ATP sensitivity and long-lasting desensitization, it appears that both P2X3 and P2X2/3 receptors can be expressed on these particular neurons. The subcellular distribution and physiological function of these receptor subtypes remains to be determined.

**DISCUSSION**

The present study has shown that identified rat DRG neurons respond to P2X receptor agonists with either slow non-desensitizing kinetics, fast desensitizing kinetics, or a combination of fast and slow kinetics. The rapidly desensitizing responses were similar to P2X3 receptor responses, and the non-desensitizing responses had properties indicative of P2X2/3 receptor activation.

Putative P2X3-containing nociceptors were identified by labeling neurons in culture with fluorescent isolectin IB4 (Vulchanova et al. 1998). Approximately 75% of neurons in culture were identified as expressing IB4 binding proteins. This is in general agreement with the percentage of IB4-labeled neurons in fixed rat DRG sections (67%) (Molliver et al. 1995). Of the IB4-positive neurons in culture, 90% exhibited a P2X3 or
P2X_{2,3}-like response to ATP. This relatively high percentage of cells that appear to have functional P2X_{3}-containing receptors contrasts to the smaller percentage (30–40%) of P2X_{3}-positive neurons in intact DRG identified by either in situ hybridization or immunohistochemical methods (Chen et al. 1995; Vulchanova et al. 1997). Although the reason for this is unclear, the possibility exists that the acute dissociation methods used here select for a high percentage of P2X_{3}-positive neurons in culture.

P2X responses in sensory ganglia are diverse, yet it appears that P2X_{2}, P2X_{3}, and P2X_{2/3} receptors are expressed on many sensory neurons. These receptors all function as nonspecific cation-selective channels, and most show strong inward rectification, yet they may vary in their agonist selectivity and desensitization kinetics. Trigeminal nociceptors appear to express fast P2X_{2}-like receptors, as well as slow nondesensitizing P2X_{2/3}-like receptors (Cook et al. 1997). In nodose ganglia (Lewis et al. 1995; Thomas et al. 1998), ATP responses are nondesensitizing and mediated by P2X_{2} and P2X_{2/3} receptors. In the DRG, early studies (Bean 1990; Jahr and Jessell 1983; Krishtal et al. 1983) reported the existence of both fast desensitizing and nondesensitizing responses to ATP. However, some of these responses may be species dependent. Amphibian DRG neurons routinely respond to ATP with nondesensitizing kinetics (Bean 1990; Li et al. 1993), whereas the response kinetics of mammalian DRG neurons are often rapidly desensitizing. Studies using cultured neonatal rat DRG neurons (Rae et al. 1998; Robertson et al. 1996) demonstrate that these neurons respond to P2X agonists exclusively with fast kinetics. The present data confirm previous reports demonstrating that many DRG neurons show fast desensitizing kinetics and extend these observations to include other neurons that respond with either mixed or nondesensitizing kinetics. The discrepancies between the present adult DRG responses and the neonatal responses of Robertson et al. (1996) could be explained by developmental differences in P2X receptor expression, although this possibility has not been investigated.

Desensitization kinetics and agonist sensitivity are often used to broadly discriminate between P2X receptor subtypes. In this context, two distinct forms of desensitization, acute and long-lasting, were examined. Acute desensitization refers to the decrease in response amplitude in the presence of agonist, and long-lasting desensitization refers to the decrease in amplitude of the second of a pair of responses. In the latter case, channels have entered a desensitized state from which they cannot be activated for periods of up to minutes. While the rP2X_{3} receptor responds to both ATP and a,b-meATP and responds to ATP with nondesensitizing kinetics (Brake et al. 1994; Chen et al. 1995). The rP2X_{2/3} receptor has a novel profile, being a,b-meATP-sensitive, yet having nondesensitizing kinetics (Lewis et al. 1995). The present study found that both rP2X_{3} and fast DRG responses were activated by either ATP or a,b-meATP, displayed biphasic acute desensitization kinetics, and required prolonged interapplication intervals (minutes) to recover from long-lasting desensitization. Although the responses always desensitized in the presence of agonist, acute desensitization was not always complete, and a plateau current was often present at the end of the application. Shorter interapplication intervals produced a subsequent depression of the entire rP2X_{3} or fast DRG waveform, not just the peak. Similarly, both rP2X_{2,3} and slow DRG responses were activated by either ATP or a,b-meATP. However, there was no acute desensitization, and responses could easily follow interapplication intervals as fast as 5 s without a decrease in response amplitude. The differences in long-lasting desensitization were used as discriminators when interpreting the receptor subtypes underlying mixed DRG responses. Applications of either ATP or a,b-meATP elicited mixed DRG responses characterized by both fast and slow response kinetics. When short interapplication intervals were used, only the fast peak of mixed DRG responses was depressed, and the slow plateau amplitude was left unaffected. This response profile indicates activation of P2X_{3} in the fast phase, and P2X_{2/3} receptors in the slow plateau phase.

The fast DRG components responded to all pharmacological manipulations similarly to rP2X_{3} receptor responses. ATP and a,b-meATP were relatively equipotent at eliciting this response. The fast response was also blocked by the nonspecific P2X receptor antagonist suramin, as well as the more selective antagonist TNP-ATP. At nanomolar concentrations, TNP-ATP has been shown to be a selective antagonist for P2X_{3}, P2X_{2}, and P2X_{2/3} receptors (Virgino et al. 1998). The entire waveform of the fast DRG response was also depressed by lowering external pH, an effect attributed to proton modulation of the P2X_{3} receptor (Stoop et al. 1997). Interestingly, P2X_{1} receptors also share this pharmacological profile. However, previous results based on selectivity of b,γ-meATP isomers have indicated that fast DRG responses are P2X_{3}, and not P2X_{1} mediated (Rae et al. 1998). The pharmacological similarities between fast DRG responses in the present study and those of Rae et al. (1998) also implicate the involvement of P2X_{3} receptors.

The fact that both ATP and a,b-meATP elicited slow DRG responses implicates P2X_{2/3} and not P2X_{2} receptor activation. Although the present study did not specifically address the existence of P2X_{2}, homomeric receptors, a consistent observation was that comparable slow DRG currents were elicited by either agonist in the same neuron, indicating that homomeric P2X_{2} receptors were not activated. The P2X antagonist TNP-ATP is relatively insensitive at P2X_{2} receptors, yet it completely blocked slow DRG responses, consistent with a preferential expression of P2X_{2,3} over P2X_{2}. The slow component of mixed DRG responses was increased in amplitude under acidic extracellular conditions and could follow short interapplication intervals. These effects are opposite to those seen with fast DRG responses under identical conditions, further emphasizing that fast and slow components of mixed DRG responses are mediated by separate P2X receptors.

The precise role of P2X receptors on nociceptive afferents is not known. However, increasing evidence supports the idea that P2X receptors can increase sensory neuron excitability. Subplantar administration of a,b-meATP produces a nociceptive response in rats (Bland-Ward and Humphrey 1997), consistent with functional involvement of P2X receptors in the periphery (Cook et al. 1997). Gu and MacDermott (1997) have demonstrated that presynaptic P2X receptors located on DRG terminals can increase glutamatergic neurotransmission at DRG–dorsal horn synapses in culture. Similar results have been obtained from recordings in brain stem slices (Khakh and Henderson 1998). A presynaptic facilitatory role for P2X receptors at central dorsal horn synapses.
could enhance neurotransmission, leading to an increase in pain sensation. The present study has demonstrated that two P2X receptors could mediate this effect, a fast, transient P2X receptor as well as a slow, sustained P2X2/3 receptor. The purpose of each receptor subtype in this pathway remains unknown, but it appears that there exist two distinct P2 receptor subtypes at which pharmacological agents could potentially be targeted for the treatment of pain.

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