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RESEARCH ARTICLE

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Distribution patterns of different P2x receptor phenotypes in acutely dissociated dorsal root ganglion neurons of adult rats

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Abstract P2x receptors may be used to detect ATP release from tissues during physiological and pathological conditions. We used whole-cell patch clamp recordings to study the expression of P2x receptor phenotypes, their distribution patterns, and their sensitivity to αβmATP and suramin in dorsal root ganglion (DRG) neurons acutely dissociated from adult rats. Based on the onset and decay rates of 10 μM ATP-evoked currents, we showed three types of P2x currents: fast, slow, and mixed. Each of these P2x receptor phenotypes had a distinct distribution pattern among DRG neurons. The fast P2x currents were predominantly expressed in small-diameter, isolectin-B4 (IB4)-positive, and capsaicin-sensitive DRG neurons. The slow P2x currents were expressed in both small and medium DRG neurons, and about half of them were IB4 positive. The mixed P2x currents were also expressed in both small and medium-sized DRG neurons, and most of these neurons were IB4-positive neurons. The slow and mixed P2x current groups had both capsaicin-sensitive and -insensitive DRG neurons. All phenotypes revealed with 10 μM ATP could be inhibited by 30 μM suramin. All DRG neurons with fast or mixed P2x currents were also sensitive to 10 μM αβmATP, and αβmATP evoked currents similar to those induced by ATP. The group expressing slow P2x currents could be further divided into αβmATP-sensitive and -insensitive groups. Thus, the relationships among P2x receptor phenotypes, cell sizes, IB4 positivity, and capsaicin sensitivity are more complicated than previously thought, and different P2x receptors may be involved in both nociceptive and non-nociceptive functions.

Key words Purinergic · P2x receptors · Nociceptor · Dorsal root ganglion

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Introduction

P2x receptors, a family of cation channels gated by ATP (North 1996), play significant roles in the peripheral and the central nervous systems (Evans et al. 1992; Edwards et al. 1992; Burnstock 1996; Bardoni et al. 1997). In the sensory system, P2x receptors can participate in impulse generation (Cook et al. 1997) and synaptic modulation (Gu and MacDermott 1997). Most dorsal root ganglion (DRG) neurons respond to ATP with strong inward currents due to the activation of P2x receptors (Krishtal et al. 1983; Jahr and Jessell 1983). P2x receptors may participate in the coding of a wide range of sensory modalities (Cook et al. 1997). Recent studies showed that P2x receptors participate in nociceptive impulse generation (Dowd et al. 1998) and in the induction of hyperalgesia (Tsuda et al. 1999).

DRG neurons are a heterogeneous population of somatic sensory neurons that are involved in nociceptive, mechanical, and chemical sensations. Different functional DRG neurons appear to have distinct properties, including cell size and electrophysiological as well as cytochemical characteristics (Harper and Lawson 1985; Scroggs et al. 1994; Dodd et al. 1984; Hall et al. 1997; Stucky and Lewin 1999). Isolectin-B4 (IB4) labels a subpopulation of small-diameter DRG neurons whose central terminals are located primarily in the inner layer of lamina II (Streit et al. 1985; Plenderleith et al. 1992; Molliver et al. 1995). Recently it has been shown that IB4-positive and -negative nociceptors are functionally distinct (Stucky and Lewin 1999). Many IB4-positive

neurons were found to be trkA negative, nonpeptidergic, GDNF dependent, and to express P2x3 receptor subunits (Silverman and Kruger 1990; Molliver et al. 1995, 1997; Bennett et al. 1998; Vulchanova et al. 1998), and were likely to be a subpopulation of nociceptive afferents (Stucky and Lewin 1999; Guo et al. 1999). Immunocytochemical studies showed that IB4 staining and capsaicin receptor expression largely overlapped among DRG neurons (Guo et al. 1999).

Recently, studies have demonstrated several different phenotypes of P2x currents in certain groups of DRG neurons (Grubb and Evans et al. 1999; Li et al. 1999; Burgard et al. 1999; Ueno et al. 1999). These studies showed a clear tendency for different phenotypes of P2x receptors to be preferentially expressed on some DRG neurons. Previous studies used electrophysiological approaches in conjunction with certain other phenotype classifications (Cook et al. 1997 used tracers to classify nociceptors and mechanoreceptors; Ueno et al. 1999 used capsaicin sensitivity). These studies suggested that the patterns of P2x receptor phenotype distribution among DRG neurons might be rather complicated. In addition, because the focus of P2x receptor research has been on nociceptive DRG neurons (Chen et al. 1995; Burgard et al. 1999), less attention has been given to the P2x receptors on other types of DRG neurons. However, P2x receptors on those normally non-nociceptive DRG neurons can be relevant to pain during pathological conditions if those afferents rearrange their innervation in dorsal horn (Woolf et al. 1992, 1995).

Using electrophysiological, cytochemical, and pharmacological methods on DRG neurons acutely dissociated from adult rats, our study confirmed previous work on the phenotypes of P2x receptors in DRG neurons by others (Grubb and Evens et al. 1999; Li et al. 1999; Burgard et al. 1999; Ueno et al. 1999). Furthermore, we have extended this work by describing new and complex features of P2x phenotype expression patterns in DRG neurons that were potentially nociceptive and non-nociceptive.

Materials and methods

Acute dissociation of DRG neurons

"Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) were followed. Dorsal root ganglia were removed from adult rats weighing approximately 100–250 g, placed in a 35°C bath solution containing dispase (neutral protease, 5 mg/ml; Boehringer Mannheim) and collagenase (2 mg/ml; Sigma type 1). After 1 h incubation, DRGs were triturated to dissociate the neurons. The dissociated cells were plated on coverslips precoated with poly-L-lysine and allowed to adhere for 1 h before recording.

Electrophysiological recordings

The coverslips with dissociated DRG neurons were mounted in a 0.5-ml recording chamber and placed on the stage of an Olympus IX70 microscope. Cells were continuously perfused with bath solution (22°C) flowing at 1 ml/min. The bath solution contained (in mM): 150 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 hydroxyethylpiperazine ethanesulfonic acid (HEPES) (pH adjusted to 7.4 with NaOH; osmolarity adjusted to 320 mosmol with sucrose). Recordings were made with an Axopatch 200B amplifier (Axon Instruments) set at 2 MHz. Cells were voltage clamped at –70 mV in the whole-cell configuration. The recording electrode internal solution contained (in mM) 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 ethyleneglycoltetraacetic acid (EGTA), 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, pH of 7.4 adjusted by KOH and osmolarity of 315–325 mosmol. Total KCl after pH adjustment was 154 mM. Recording electrode resistance was between 2.0 and 5.0 Mohm.

Drug application

ATP, αβmATP, or capsaicin was rapidly applied to neurons through a glass tube (ID approximately 500 μm) positioned 1.0 mm away from the cell. The gravity-driven solution flow was electronically controlled by solenoid valves and triggered from a computer. Unless otherwise indicated, concentrations for both ATP and αβmATP were 10 μM, capsaicin was 1 μM, and each was applied for 2 s. In experiments to test P2x receptor sensitivity to the antagonist suramin, ATP (10 μM) was first applied for 2 s to obtain a control response. Then cells were perfused in normal bath solution for 15 min to allow recovery from desensitization. Subsequently, suramin (30 μM) was applied for 2 min and then coapplied with 10 μM ATP for 2 s.

IB4 staining

DRG neurons were incubated with 2 μg/ml fluorescein isothiocyanate (FITC)-conjugated IB4 (Sigma) in normal bath solution at 22°C for 30 min. Dishes were then perfused with normal bath solution. IB4-positive neurons were visualized with a fluorescence microscope. Electrophysiological studies were performed on DRG neurons that were preidentified as positive or negative for IB4 staining. In some cases, IB4 staining was performed after recordings were made and the cells were fixed (Petruska et al. 1997).

Data analysis

Rise time was measured from the digitized point of the trace from which all the subsequent points were continuously rising toward the peak amplitude. Decay time constants were determined after fitting the desensitizing phase (between 10% and 90%) of the inward currents with Clampfit 6 (Axon Instruments). Unless otherwise indicated, data are expressed as means ± SEM.

Results

Fast application of 10 μM ATP to acutely dissociated rat DRG neurons produced four major types of response recorded from 81 DRG neurons (Fig. 1). These types were also observed in some cells tested with 100 μM ATP (data not shown). The first type of ATP response (n=28) was a fast inward current that had rapid onset and rapid decay (fast P2x current, Fig. 1A). During 2-s ATP application, the evoked current desensitized rapidly and approached baseline current level in less than 1/2 s. At the end of the 2-s ATP application, less than 5% of inward current remained. Rise time was 44±5 ms (range 16–88 ms, n=28). The responses in most cells showed a single decay phase with a decay time constant of 144±17 ms (range 20–279 ms, n=21). Of the 28 cells, responses from seven cells were best fitted with a two-

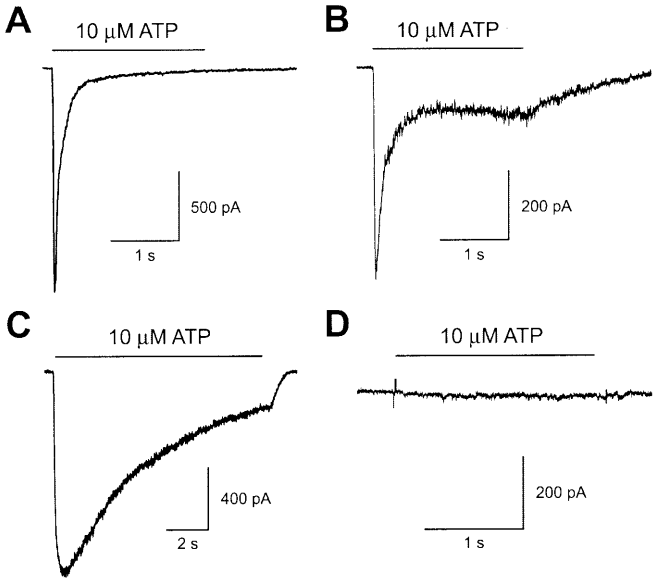

Fig. 1A–D Electrophysiological phenotypes of P2x receptors in acutely dissociated DRG neurons from adult rats. **A** A fast P2x current with rapid onset and decay was evoked by ATP. **B** A mixed P2x current with rapidly desensitizing and steady-state phases was evoked by ATP. **C** ATP evoked a slow P2x current with slow onset and weak desensitization. **D** ATP had little effect. Each recording was performed on different DRG neurons. ATP was applied at 10 μM for 2 s for **A**, **B**, and **D**. In **C**, 10 μM ATP was applied for 10 s

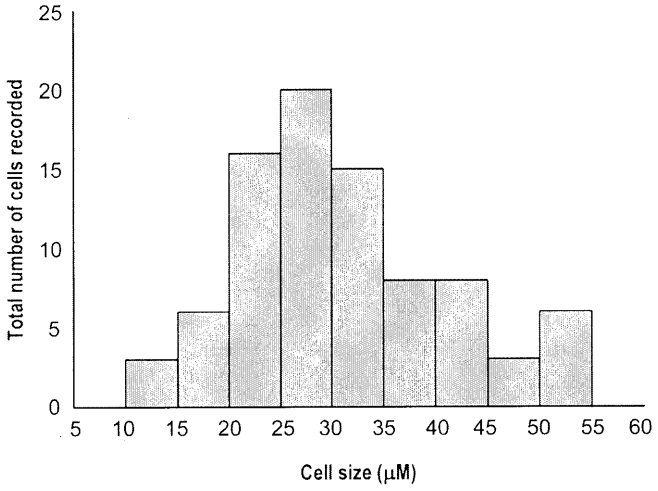

Fig. 2 Cell size distribution of DRG neurons included in the patch clamp recordings. A total of 81 DRG neurons were included. Cell size is expressed as average diameter of a cell

exponential equation, and the decay time constant was 39±7 ms for the first phase and 185±32 ms for the second phase. The second type of response (*n*=21) had a mixed inward current (mixed P2x current, Fig. 1B). It had an initially rapid onset and decay phase. However, the rapid decay phase did not approach the baseline current level. Instead, it was followed by a steady-state or sometimes slowly desensitizing current. This steady-state or slowly desensitizing current could last to the end of the 2-s ATP application. The mixed current had a rise time of 39±6 ms (*n*=21), similar to that of the fast P2x current. The decay phases could be best fitted with a two-exponential equation, with $\tau 1$=80±11 ms and $\tau 2$=1104±325 ms, for the majority of cells (*n*=12). For the rest of the other cells, curve fitting was poor with exponential equations. The third type of response (*n*=21) was an inward current that had slow onset and slow desensitization (slow P2x current, Fig. 1C). Rise time was 272±54 ms (*n*=21). Of the 21 cells, 13 showed very slow desensitization, and the decay time constant was 1746±362 ms (*n*=13). Even when ATP was applied for 10 s, a substantial inward current remained (Fig. 1C). Of the 21 cells, 8 of them had a plateau current with no desensitization. For the fourth type of cell, ATP did not evoke any detectable inward current (*n*=11).

We examined the relationship between cell size and P2x current phenotype to determine whether different P2x currents were preferentially expressed among DRG neurons. Figure 2 shows the cell size distribution of the 81 cells recorded in this study. We found that ATP responses had a distinct distribution pattern among different sizes of DRG neurons (Fig. 3). Fast P2x currents were often observed in small DRG neurons (20–30 μm in diameter, *n*=24, Fig. 3A). However, three large-size DRG neurons (~50 μm) also showed a rapid ATP response with fast kinetics. A fast P2x current appeared to be rare in medium-sized DRG neurons (30–50 μm). Cells displaying the mixed P2x current were in the small- to medium-sized range (*n*=21, Fig. 3B). The slow P2x current (*n*=21, Fig. 3C) was found to be expressed not only in medium neurons (see Li et al. 1999), but also in small DRG neurons. Finally, the cells that had no response to ATP (*n*=11, Fig. 3D) were found to be not only large cells (>50 μm, *n*=4) as previously reported (Li et al. 1999), but also some very small cells (<20 μm, *n*=6).

We further examined the P2x current phenotype distribution in relation to IB4 staining on DRG neurons. Figure 4 shows vital staining of DRG neurons using FITC-conjugated IB4. About 70% of the DRG neurons in our dishes were found to be IB4 positive (see also Burgard et al. 1999). This value is slightly higher than has been reported for DRG sections from intact animals. For example, Wang and colleagues (1994) reported approximately 50% for lumbar DRG, and Molliver and colleagues (1995) reported 67% for thoracic DRG. These differences are most likely due not to a change in binding properties, but to the fact that most larger diameter neurons (IB4 negative) are lost in the dissociation process, creating a neuronal population whose size/frequency distribution is skewed from that of tissue sections. Ninety-five percent (95%) of neurons that showed fast P2x current were IB4 positive (*n*=28, Fig. 5). However, these IB4-positive neurons with fast P2x currents only represented ~50% of the total number of DRG neurons that were labeled with IB4 in our experiments. The rest of the IB4-positive neurons (~50%) did not express fast P2x current, but instead expressed mixed P2x current,

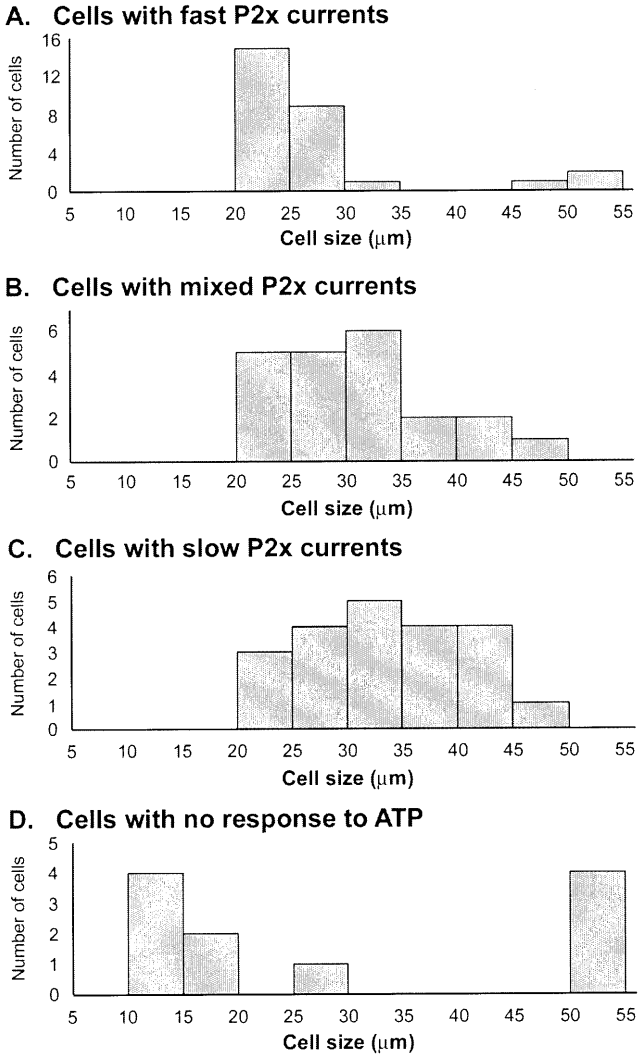

Fig. 3A–D Relationship between DRG neuron sizes and P2x receptor phenotypes. **A** Almost all DRG neurons that showed fast P2x currents were small cells with diameters of 20–30 μm. **B** Mixed P2x currents were observed in both small- and medium-sized DRG neurons (30–50 μm). **C** Slow P2x currents occurred in both small- and medium-sized DRG neurons. **D** Cells with little ATP response were usually either very small (<20 μm) or large DRG neurons (>50 μm)

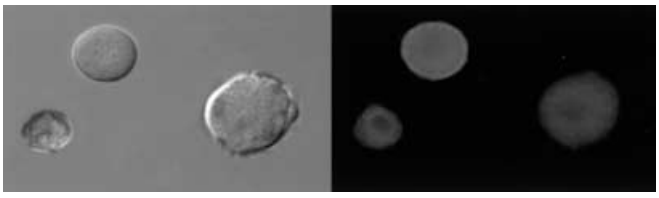

Fig. 4 Vital IB4 staining on acutely dissociated DRG neurons for the patch-clamp recordings. *The left panel* is a DIC image, and *the right panel* is a fluorescence image of the same cells. The top cell is IB4 positive. Two other cells are IB4-negative neurons. *Scale bars* 50 μm

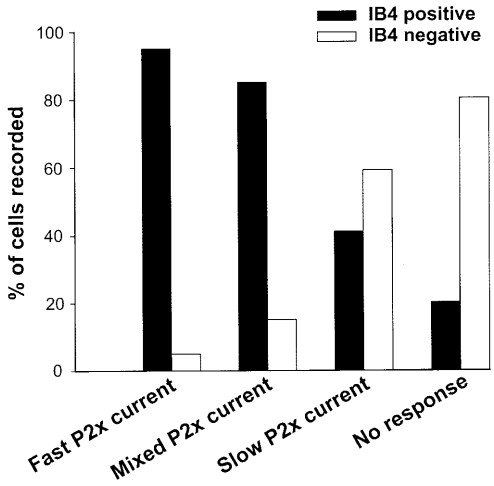

Fig. 5 Relationship between IB4 staining and phenotypes of P2x currents. Almost all cells with fast P2x currents were IB4 positive. Most cells with mixed P2x currents were IB4 positive. In DRG neurons with slow P2x currents, the total number of IB4-positive and IB4-negative cells were similar. Most cells with no response to ATP were IB4 negative

slow P2x current, or no response to ATP (Fig. 5). For cells showing mixed P2x currents (n=21), the majority (85%) were IB4-positive neurons, and only 15% were IB4-negative neurons. Slow P2x currents were found not only in IB4-negative neurons, but also in many IB4-positive neurons (Fig. 5). Of 21 cells with slow P2x currents, 9 were IB4-positive and the rest were IB4-negative neurons. Of the four cells that did not respond to ATP, three of them were found to be IB4-negative neurons and one was IB4 positive.

Capsaicin sensitivity was also examined in some cells (see also Ueno et al. 1999). Of ten neurons that showed fast P2x current induced by 10 μM ATP, nine also responded to 1 μM capsaicin (Fig. 6). Of the four cells with mixed P2x current, one was capsaicin sensitive and three were insensitive to capsaicin (Fig. 6). Of the nine cells that showed slow P2x currents, three were capsaicin sensitive and six were insensitive (Fig. 6). One cell that did not respond to ATP was also insensitive to capsaicin. In the above experiments, all the capsaicin-sensitive neurons were also IB4 positive. Further, with the exception of one cell, all capsaicin-insensitive neurons were also IB4 negative.

Different phenotypes of P2x currents were also examined for their sensitivity to the P2x receptor antagonist suramin and agonist αβmATP. Fast P2x currents were sensitive to suramin block. With 30 μM suramin, fast P2x current evoked by 10 μM ATP was inhibited by 84%, from –670±338 pA in control to –118±62 pA with suramin (n=3, Fig. 7A). In six cells tested with both ATP and αβmATP, αβmATP also evoked fast responses (rise time = 38±7, decay time constant, τ1=28±7 ms, τ2=124±44 ms).

For the cells with mixed P2x current, the fast components (–589±182 pA, n=4, Fig. 7B) were almost undetectable at the original peaking time, indicating a strong inhibition by suramin. The slow component was inhibited by 65%, from –247±166 pA in control to –102±78 pA

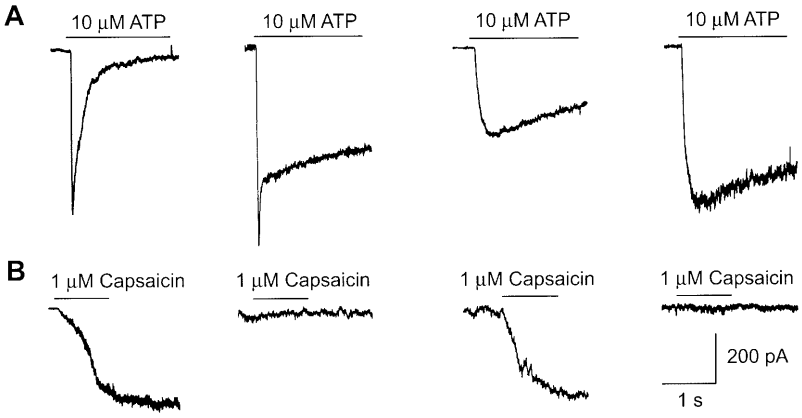

Fig. 6A, B Capsaicin sensitivity in DRG neurons with different phenotypes. **A** Three different types of P2x currents were evoked by 10 μM ATP in four DRG neurons. **B** Capsaicin sensitivity was tested on the same cell from **A** with 1 μM capsaicin

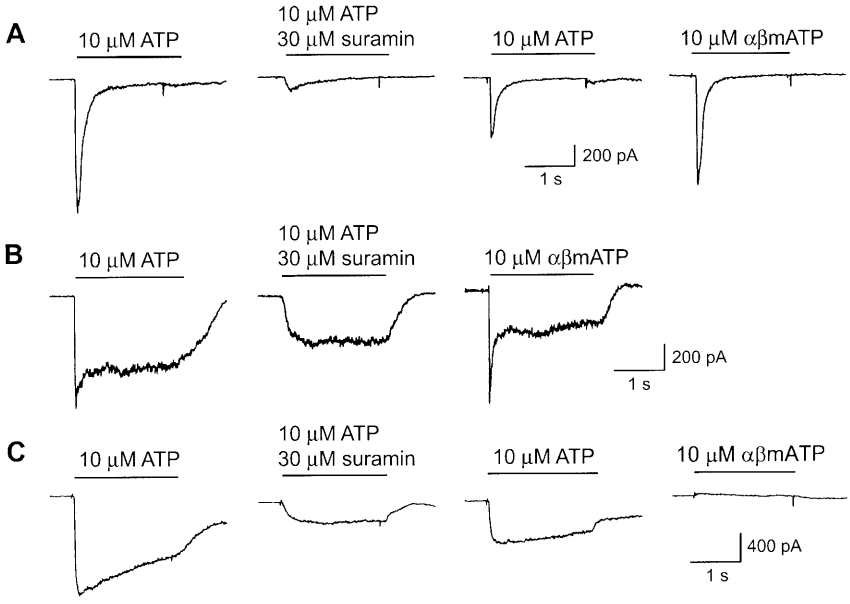

Fig. 7A–C Suramin and αβmATP sensitivity of different P2x receptors. **A** Fast P2x currents were sensitive to suramin block. αβmATP evoked a similar fast current. **B** In cells with mixed P2x current, suramin largely blocked the first phase of the current, and it was less effective for the steady-state current. αβmATP produced a similar mixed current. **C** Slow P2x currents were partially inhibited by suramin. In this cell, αβmATP did not evoke any inward currents. In all experiments, suramin was preapplied for 1 min before co-application of 10 μM ATP and 30 μM suramin

with suramin (*n*=4). For five cells tested with both ATP and αβmATP, αβmATP also evoked mixed currents (Fig. 7B).

For 21 cells with slow P2x current evoked by ATP, 17 of them were tested with αβmATP. Eight cells had no response to αβmATP (Fig. 7) and nine cells had slow current (current trace not shown). In the cells that showed ATP-evoked slow P2x current and were αβmATP insensitive, ATP-evoked currents were inhibited by 86%, from –952±232 pA in control to –154±61 pA with suramin (*n*=4). For the αβmATP-sensitive cells, αβmATP evoked a slow response with a rise time of 305±42 ms. The decay time constant was 1994±144 ms for six cells, and three cells had a plateau current without any desensitization. In these αβmATP-sensitive cells, suramin inhibited the ATP-evoked current by 66%, from –340±107 pA in control to –144±85 pA with suramin (*n*=4).

Of six cells with slow P2x current that were insensitive to αβmATP, half were IB4 positive and the others were IB4 negative. Of six cells with slow P2x current and sensitive to αβmATP, two cells were IB4 positive and the rest were IB4 negative.

In the above experiments, suramin inhibition was only partially recovered although each test had an interval of 15 min. The lack of full recovery could be due to either the slow washout of suramin from receptor-binding sites, or slow recovery of P2x currents from desensitization. If it was due to the slow recovery of P2x currents from desensitization with our test intervals, the percentage inhibition might have been overestimated.

Discussion

We have shown that, in adult rat DRG neurons, fast, mixed, and slow P2x currents are expressed in different DRG neurons with a complex distribution pattern. Consistent with previous studies (Li et al. 1999; Ueno et al. 1999), we found that many small-diameter DRG neurons expressed fast P2x currents. We have extended previous observations and demonstrated that slow P2x currents were not only expressed on medium-sized DRG neurons (Li et al. 1999), but also on some small-sized DRG neurons. Further, we demonstrated the expression of the

mixed P2x currents on small- to medium-sized DRG neurons. Unexpectedly, we also found that some very small DRG neurons had no response to ATP.

The presence of several phenotypes of P2x receptor currents within IB4-positive DRG neurons shown here is consistent with a very recent study (Burgard et al. 1999). Furthermore, we extended our studies on the patterns of P2x receptor distribution in these potentially nociceptive DRG neurons with the examination of the distribution, the pharmacological properties, and the capsaicin sensitivity of each phenotype. Interestingly, the previous study (Burgard et al. 1999) showed that all IB4-positive neurons with slow ATP responses were sensitive to αβmATP, and it was thought to be due to the heteromeric P2x2+3 receptors. Although there were some cells in our studies that fell into this category, we found that there were also some IB4-positive cells with slow P2x currents that were αβmATP insensitive. This indicates that other slow P2x receptors, e.g., homomeric P2x2, may contribute to the slow P2x current in IB4-positive DRG neurons as well. Consistent with this, we have immunocytochemical data showing that some numbers of IB4-positive DRG neurons did not express P2x3 receptors, but expressed P2x2 receptors (data not shown, but see also Cook et al. 1997).

Cells expressing fast P2x currents may be nociceptive DRG neurons, as has been strongly suggested from a previous study in nociceptive trigeminal neurons (Cook et al. 1997). Consistent with this, we found that almost all cells with fast P2x currents were both IB4 positive and capsaicin sensitive when tested. However, many cells that expressed slow and mixed P2x receptors may be nociceptive types as well. This is supported by our results that slow and mixed P2x currents were expressed on many IB4-positive DRG neurons. It is also supported by their capsaicin sensitivity.

The distribution pattern of P2x receptor phenotypes in the potentially nociceptive DRG neurons shown here suggests that different P2x receptors may be used to sense different degrees of tissue damage and produce different time courses of pain sensations. It is expected that fast P2x receptors will produce immediate, but very brief nociceptive signals because of their fast desensitization and slow recovery. Such responses could provide a warning about a harmful stimulation. The mixed and slow P2x receptors on nociceptive DRG neurons may sense ATP release from damaged tissues and produce longer periods of stimulation on nociceptive DRG neurons. Furthermore, the sustained P2x currents in nociceptive DRG neurons could provide a route for sustained Ca^{2+} entry (Taschenberger et al. 1999). This may result in the modulation of sensory neuron excitability (Kress and Guenther 1999) and induction of substance P release. Thus, the mixed and slow types of P2x currents are more likely to be involved in the induction of hyperalgesia following tissue damage.

Our data also showed that mixed and slow P2x receptors were expressed on IB4-negative neurons in the small to medium size range. If those cells are non-nociceptive DRG neurons, the normal physiological functions of these slow P2x receptors are difficult to interpret at present. However, a previous study showed that muscle stretch sensory neurons also expressed slow P2x receptors (Cook et al. 1997), indicating a potential role for slow P2x receptors in mechanical sensations.

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