RESEARCH ARTICLE

Developmental Changes in Heteromeric $P2X_{2/3}$ Receptor Expression in Rat Sympathetic Ganglion Neurons

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We have used whole cell patch clamp recording and immunohistochemistry to investigate the expression of $P2X_{2/3}$ receptors in rat superior cervical ganglion neurons during late embryonic and early post-natal development. Neurons from E18 and P1 animals responded to the nicotinic agonist dimethylphenylpiperazinium (DMPP), and the purinoceptor agonists ATP and α,β-meATP with sustained inward currents. Responsiveness to DMPP was maintained at P17, while that to ATP declined dramatically, and responses to α,β-meATP were rarely detected. Immunohistochemistry for the $P2X_3$ subunit revealed widespread staining in superior cervical ganglia from P1 rats, but little immunoreactivity in ganglia from P17 animals. In neurons from P1 animals, the response to α,β-meATP exhibited pharmacological properties of the heteromeric $P2X_{2/3}$ receptor. In conclusion, sympathetic neurons of the rat superior cervical ganglion are more responsive to ATP and α,β-meATP at birth and during the early post-natal period, due largely to the expression of the $P2X_3$ subunit, but these responses are much reduced in mature rats. *Developmental Dynamics 234:505–511, 2005.* © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Neuronal release of ATP was first demonstrated from the peripheral terminals of primary afferent fibres (Holton, 1959). Since then, co-storage and release of ATP from the terminals of both central and peripheral neurons has been widely described, and its role as a neurotransmitter is well established (see Burnstock, 1972, 1976, 2003). When released from nerve terminals, ATP can produce both rapid effects through the activation of a family of ligand gated ion channels (the P2X receptors), and slower and longer-lasting actions, which are usually mediated via the G protein–coupled P2Y receptors (Ralevic and Burnstock, 1998). To date, seven P2X receptor sub-units have been identified, which can assemble to form either homomeric or heteromeric receptors (North, 2002). In the periphery, the activation of P2X receptors is important for neuromuscular transmission in the vas deferens (Mulryan et al., 2000) and for the activation of primary afferent fibres in the urinary bladder (Cockayne et al., 2000; Vlaskovska et al., 2001) and carotid body (Prasad et al., 2001; Rong et al., 2003). P2X receptors are also found on most autonomic ganglion neurons (see Dunn et al., 2001). Since ATP is released from preganglionic nerve terminals (Vizi et al., 1997), these receptors may play a role in ganglionic neurotransmission.

In rat and mouse sympathetic ganglia, immunohistochemical and molecular biology suggest the presence of a variety of P2X subunits. However, most neurons respond to ATP, but not to α,β-meATP, which in combination with other pharmacological data indicates the presence of homomeric $P2X_2$ receptors (see Dunn et al., 2001).

Nevertheless, a few neurons in

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these ganglia do respond to α,β-meATP (Khakh et al., 1995; Schadlich et al., 2001; Calvert and Evans, 2004). This agonist is considered to be selective for P2X receptors containing $P2X_1$ or $P2X_3$ subunits (North, 2002), although some activation of receptors containing $P2X_5$ or $P2X_6$ subunits has been reported (Wildman et al., 2002; Jones et al, 2004). Studies using $P2X_1$ knockout mice have led to the suggestions that at least some α,β-meATP-evoked responses in mouse superior cervical ganglion neurones are mediated by receptors containing the $P2X_1$ subunit (Calvert and Evans, 2004). However, autonomic ganglion neurons share the same embryological origins as dorsal root ganglion neurones, which express predominantly $P2X_3$ homomeric or $P2X_{2/3}$ heteromeric receptors. It is therefore possible that some α,β-meATP responses in sympathetic neurons might be mediated by $P2X_3$-containing receptors.

There are many examples of changes in receptor expression during development due to the expression of different receptor subunits, for example, the γ to ϵ switch in nicotinic acetylcholine receptors at the neuromuscular junction (Mishina et al., 1986) and changes in GABA and NMDA receptors in the cerebellum (Gutierrez et al., 1997; Cathala et al., 2000). Changes in expression of P2 receptors also occur during embryonic and postnatal development (see Burnstock, 2001). For example, P2X receptors are expressed transiently in developing rat and chick skeletal muscle (Wells et al., 1995; Meyer et al., 1999; Ryten et al., 2001), and transient expression of the $P2X_3$ subunit has been observed in the brain and spinal cord during rat (Cheung and Burnstock, 2002) and mouse (Boldogkoi et al., 2002) embryogenesis. Dynamic expression of P2Y receptor subtypes has also been described in the embryonic rat brain (Cheung et al., 2003).

In this study, we have used whole cell patch clamp recording and immunohistochemistry to investigate the expression of the $P2X_3$ receptor subunit and the formation of heteromeric $P2X_{2/3}$ receptors in rat superior cervical ganglion neurons during late embryonic and early post-natal development.

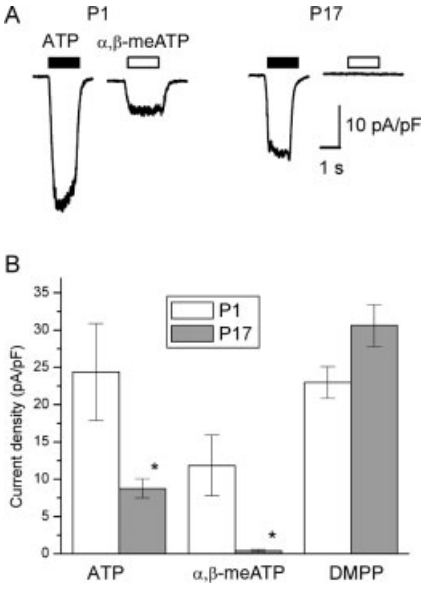

Fig. 1. Responsiveness of postnatal superior cervical ganglion neurons, from P1 and P17 rats. **A:** Representative whole cell patch clamp recordings (holding potential -60 mV) of responses to ATP and α,β-meATP (both at 100 μM). While P1 neurons respond to both agonists, neurons from older animals (P17) respond to ATP but not α,β-meATP. **B:** Histogram comparing chemosensitivity of P1 and P17 neurons to ATP (100 μM), α,β-meATP (100 μM), and the nicotinic agonist DMPP (10 μM). Columns represent the mean ± S.E. from 10 to 15 neurons, from three separate cultures.*Significantly different by unpaired Student's *t*-test.

RESULTS

Responses of P1 and P17 Superior Cervical Ganglion Neurons

We initially compared the responsiveness of superior cervical ganglion neurons from early (P1) and late (P17) post-natal rats to ATP and α,β-meATP. All neurons tested responded to the nicotinic agonist DMPP. All superior cervical ganglion neurons from P17 animals responded to 100 μM ATP with a sustained inward current, with current densities ranging from 3 to 19 pA/pF (Fig. 1A,B). In keeping with previous studies on adult neurons (Khakh et al., 1995; Calvert and Evans, 2004), 100 μM α,β-meATP failed to evoke any response in most neurons from P17 animals. However, in one culture, two neurons did give significant responses to α,β-meATP (see Fig. 2Bi). In superior cervical ganglion neurons from P1 animals, the responsiveness to ATP was significantly greater than that in P17 animals, and the majority of neurons also responded to α,β-meATP with an inward current (Fig. 1A,B)

Immunohistochemistry

In P17 ganglia, most neurons failed to show any significant staining for $P2X_3$. However a small sub-population of neurons did exhibit distinct immunoreactivity (Fig. 2Ai). In ganglia from P1 animals, many neurons were immunopositive for $P2X_3$, and the strongest staining was considerably more intense than that seen in P17 ganglia (Fig. 2Aii). This pattern of staining was consistent with the responses to α,β-meATP of neurons from P17 and P1 superior cervical ganglion (Fig. 2B). In contrast, neurons from both P1 (Fig. 2Ci) and P17 (Fig. 2Cii) superior cervical ganglia exhibited quite uniform immunoreactivity for the $P2X_2$ subunit.

Temporal Change in Agonist Responses

Having established that in contrast to adult neurons, the majority of neurons in P1 superior cervical ganglion respond to α,β-meATP, we investigated the time dependence of the change in agonist responsiveness, by looking at neurons from E18, P1, P7, and P17 animals. Neurons from E18 embryos all responded to 100 μM α,β-meATP with small inward currents, while more robust currents were produced by 100 μM ATP and 10 μM DMPP (Fig. 3). Response to all three agonists was maximal between birth and seven days. Following this, the current density to the nicotinic agonist DMPP was maintained, but there was a marked reduction in the response to ATP and few neurons responded to α,β-meATP (Fig. 3).

Pharmacological Properties

To further investigate the nature of the receptors responsible for the response to α,β-meATP in P1 sympathetic ganglia, we carried out pharmacological characterization of this receptor. The response to α,β-meATP was concentration dependent. Fitting the data with the Hill equation yielded an EC_{50} value of 29.9 ± 5 μM, with a Hill coefficient of 1.1. (Fig. 4A).

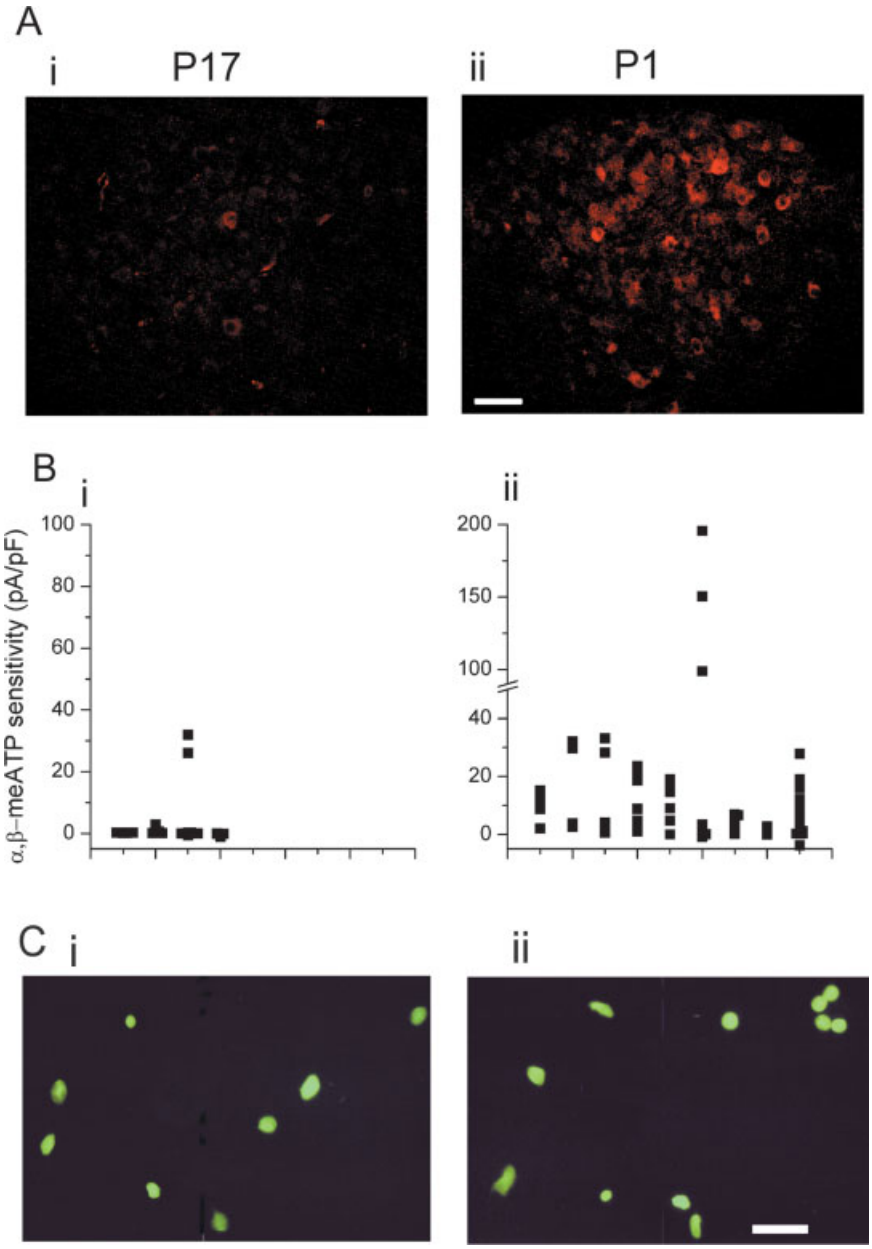

Fig. 2. Comparison of $P2X_2$ and $P2X_3$ immunoreactivity and α,β-meATP sensitivity in superior cervical ganglion neurons. **A:** Immunohistochemistry for the $P2X_3$ subunit in sections of superior cervical ganglia from P17 (**i**) and P1 (**ii**) rats. In P17 ganglia, few neurons are immunoreactive for the $P2X_3$ subunit, while in ganglia from newborn animals, the staining is widespread, and in some neurons intense. Calibration bar = 50 μm. **B:** Comparison of the responsiveness of neurons from P17 (**i**) and P1 (**ii**) superior cervical ganglia to α,β-meATP. Each point represents a single neuron, and each vertical column represents a single experiment. While few neurons from P17 ganglia gave any detectable response to α,β-meATP, most neurons from newborn animals responded, and in some cases the responses were very large. **C:** Immunohistochemistry for the $P2X_2$ subunit in cultured superior cervical ganglion neurons revealed strong and uniform staining in cells from both P1 (**i**) and P17 (**ii**) rats. Calibration bar = 50 μm.

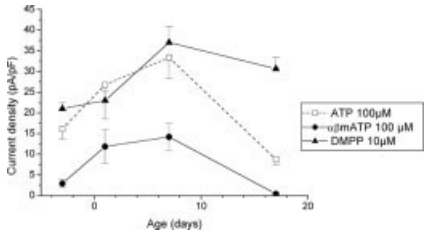

Fig. 3. Temporal change in chemosensitivity of rat superior cervical ganglia. The graph shows the sensitivity of neurons to ATP, α,β-meATP, and DMPP at 4 different developmental ages from late embryonic to weaning. Each point represents the mean ± S.E. for 6 to 34 neurons. Sensitivity to the nicotinic agonist DMPP increased from E18 to P7, and was then maintained at P17. In contrast, sensitivity to the purinergic agonists peaked at about P8 and then declined, so that by P17 responses to α,β-meATP were rarely detectable.

Trinitrophenyl-ATP is a subtype selective antagonist with nanomolar affinity for P2X receptors containing the $P2X_1$ and $P2X_3$ subunits (Virginio et al., 1998). TNP-ATP produced a reversible concentration-dependent antagonism of the response to α,β-meATP. Fitting the Hill equation to this data gave an IC_{50} of 13 ± 8 nM, with a Hill coefficient close to unity (Fig. 4B).

A characteristic of P2X receptors involving the $P2X_2$ sub-unit is that they exhibit positive allosteric modulation by Zn^{2+} and H^+ ions (see North, 2002). We, therefore, investigated the effects of these two ions on response of P1 superior cervical ganglion neurons to α,β-meATP. Lowering the pH from 7.4 to 6.8 produced a dramatic increase in the response to 20 μM α,β-meATP (Fig. 4C). However co-application of 10 μM Zn^{2+} produced no significant change in the response to α,β-meATP. In contrast, this concentration of Zn^{2+} more than doubled the response to 20 μM ATP in these neurons (Fig. 4C). Adult nodose ganglion neurons respond to α,β-meATP with a sustained inward current due to the presence of heteromeric $P2X_{2/3}$ receptors (Lewis et al., 1995). We therefore investigated the effect of Zn^{2+} on responses to α,β-meATP on nodose ganglion neurons taken from newborn rats. On these neurons, Zn^{2+} produced a small but significant increase in the amplitude of the α,β-meATP response (Fig. 4C).

DISCUSSION

The main finding of this study is that there is a marked change in the expression of α,β-meATP-sensitive $P2X_{2/3}$ receptors in sympathetic neurons of the rat superior cervical ganglion. The levels of this receptor peak soon after birth, then decline to very low levels by the time animals are about 17 days old. The expression of P2X receptors may be altered by dissociation and cell culture (Smith et al., 2001), possibly as a result of ATP release due to metabolic stress, ischemia, or trauma (see Volonte et al.,

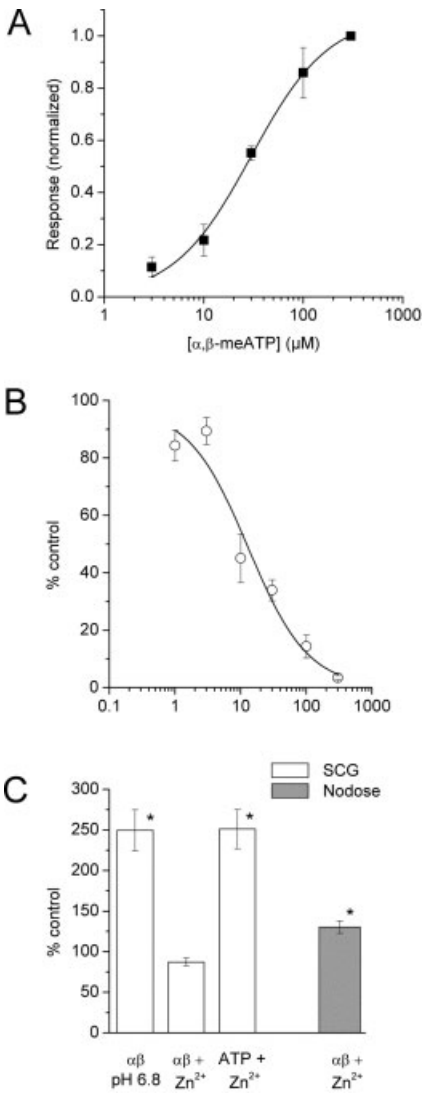

Fig. 4. Pharmacological properties of the α,β-meATP sensitive receptor in superior cervical ganglion neurons from newborn rats. **A:** Concentration response curve for the inward current evoked by α,β-meATP. Points represent mean ± SE from 5 neurons. The curve shows a least squares fit of the Hill equation to the data, with an EC_{50} of 29.9 ± 5 μM and a Hill coefficient of 1.1 ± 0.15. **B:** Concentration response curve for the inhibition of the response by TNP-ATP. Fitting the Hill coefficient to the data gave an IC_{50} of 13 ± 8 nM and a Hill coefficient of 0.95 ± 0.4. **C:** Effect of allosteric modulators on the response of P1 SCG neurons to purinergic agonists. While lowering the pH to 6.8 significantly potentiated the response to 20 μM α,β-meATP, the response was unaffected by 10 μM Zn^{2+}. In contrast, the response to 10 μM ATP was significantly increased. Similarly, the response of nodose ganglion neurons from P1 rats was significantly potentiated by 10 μM Zn^{2+}. Columns represent the mean ± S.E. from 7 neurons. *Significantly different from 100% by one sample *t*-test.

2003). Although our results show agreement between functional experiments and immunohistochemistry, we cannot rule out the possibility of changes resulting from the use of cell culture.

P2X receptors responding to α,β-meATP are believed to require the presence of either the $P2X_1$ or $P2X_3$ (North, 2002), although there is some evidence that $P2X_5$ or $P2X_6$ receptors may also respond to this agonist (Wildman et al., 2002; Jones et al., 2004). Much of our data suggests that the α,β-meATP-sensitive receptor expressed in sympathetic neurons from newborn rats is the heteromeric $P2X_{2/3}$ receptor. Firstly, we observed considerable levels of $P2X_3$ immunoreactivity in ganglia from P1 animals, which was virtually absent in ganglia from animals more than 17 days old. The sustained nature of the responses would argue against the involvement of homomeric $P2X_1$ or $P2X_3$ receptors, which give rapidly desensitizing responses (North, 2002). The EC_{50} value we obtained for α,β-meATP (30 μM) is similar to the value of 39 μM reported for nodose ganglion neurons (Dunn et al., 2000), but slightly greater than the value of 9 μM reported for heteromeric $P2X_{2/3}$ receptors expressed in Xenopus oocytes (Liu et al., 2001). The sensitivity of this receptor to the antagonist TNP-ATP, with an IC_{50} of 13 nM, is quite similar to the values of 7 and 11 nM reported for recombinant $P2X_{2/3}$ receptors (Virginio et al., 1998; Liu et al., 2001) and 21 nM for receptors in the rat nodose ganglion (Dunn et al., 2000). The potency of this antagonist is, however, considerably less than the low nanomolar values reported for the homomeric $P2X_3$ receptor (see North, 2002). The potentiation of α,β-meATP responses by low pH is also in keeping with the properties of the heteromeric $P2X_{2/3}$ receptor (Liu et al., 2001), and contrasts with the negative allosteric action of protons at the homomeric $P2X_3$ receptor (North, 2002). Responses at the $P2X_{2/3}$ receptor are also potentiated by Zn^{2+}, although this effect is less pronounced than at the homomeric $P2X_2$ receptor (Liu et al., 2001). In our experiments, Zn^{2+} enhanced responses to ATP in P1 superior cervical ganglion neurons, and also increased responses to α,β-meATP in nodose ganglion neurons from neonatal rats. However, we failed to observe potentiation of α,β-meATP responses in P1 superior cervical ganglion neurons. The reason for this is at present unclear, but might indicate the involvement of other P2X subunits or spliced variants. Studies using $P2X_1$ knockout mice have indicated that a small percentage of superior cervical ganglion neurons respond to α,β-meATP through activation of $P2X_1$ receptors (Calvert and Evans, 2004). Although we cannot exclude involvement of $P2X_1$ subunits in a heteromeric receptor, the kinetic and pharmacological properties of the response we observed do not match those of the homomeric $P2X_1$ receptor.

ATP is co-released with acetylcholine from pre-ganglionic nerve terminals (Vizi et al., 1997), and may thus play a role in synaptic transmission. This notion is supported by observation of synaptic responses, which are resistant to nicotinic receptor antagonists in some ganglia (Seabrook et al., 1990; Callister et al., 1997). P2X receptors are also present on the terminals of postganglionic sympathetic neurons, where they can modulate the release of noradrenaline (Sperlagh, et al., 2000; Queiroz et al., 2003). In the central nervous system, many $P2X_3$-containing receptors are localized to presynaptic terminals. Thus, the loss of the $P2X_3$ subunit in P17 SCG neurons may reflect the targeting of these subunits to the nerve terminal.

$P2X_{2/3}$ receptors exhibit a higher affinity for ATP than the homomeric $P2X_2$ receptors present on adult SCG neurons. This is likely to account for the high responsiveness to ATP, which we observed in embryonic and P1 ganglion neurons. Interestingly, this change in P2X receptor expression occurs at a time when synaptogenesis is taking place in the superior cervical ganglion (Smolen and Raisman, 1980; Mills and Smith, 1983), which might indicate a role for purinergic receptors in this process.

In conclusion, we have shown that sympathetic neurons of the rat superior cervical ganglion exhibit larger responses to ATP and α,β-meATP at birth and during the early post-natal period. This appears to be due, at least in part, to the expression of the $P2X_3$ subunit, giving rise to the presence of heteromeric $P2X_{2/3}$ receptors. Sensitivity to purinergic agonists then de-

clines. It is tempting to speculate that the role of these receptors may be in some way related to synapse formation, which occurs during the early post-natal period.

EXPERIMENTAL PROCEDURES

Cell culture

Superior cervical ganglion neurons were cultured from E18, P1, P7, and P17 rats. Post-natal rats and pregnant females were killed by inhalation of a rising concentration of CO_2 and death was confirmed by cardiac haemorrhage. Embryos were removed from pregnant females and placed in Leibovitz L-15 medium (Life Technologies, Paisley, UK). Neonatal animals were killed by cervical dislocation followed by decapitation. Superior cervical ganglia were rapidly dissected out, and placed in L-15 medium. The ganglia were then desheathed, cut, and incubated in 4 ml Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS; Life Technologies, Bethesda, MD) with 10 mM Hepes buffer (pH 7.4) containing 1.5 mg ml^{-1} collagenase (Class II, Worthington Biochemical Corporation, UK) and 6 mg ml^{-1} bovine serum albumin (Sigma Chemical Co., Poole, UK) at 37°C for 45 min. The ganglia were then incubated in 4 ml HBSS containing 1 mg ml^{-1} trypsin (Sigma) at 37°C for 15 min. The solution was replaced with 1 ml growth medium comprising L-15 medium supplemented with 10% bovine serum, 50 ng ml^{-1} nerve growth factor, 2 mg ml^{-1} $NaHCO_3$, 5.5 mg ml^{-1} glucose, 200 i.u. ml^{-1} penicillin, and 200 μg ml^{-1} streptomycin. The ganglia were dissociated into single neurons by gentle trituration. The cell suspension was diluted to 8 ml, and centrifuged at 160*g* for 5 min. The pellet was resuspended in 0.8 ml growth medium and plated onto 35-mm Petri dishes coated with 10 μg ml^{-1} laminin (Sigma). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and used on the following day.

Electrophysiology

Whole-cell voltage-clamp recording was carried out at room temperature using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Membrane potential was held at −60 mV. External solution contained (mM): NaCl 154, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, Hepes 10, and glucose 5.6; the pH was adjusted to 7.4 using NaOH. Recording electrodes (resistance 2–4 MΩ) were filled with internal solution that contained (mM): KCl 120, Hepes 10 and tripotassium citrate 10, EGTA 0.1; the pH was adjusted to 7.2 using KOH. In some experiments, a similar solution was used in which K^+ was replaced by Cs^+. No difference in response was observed between the two internal solutions. Data were acquired using pCLAMP software (Axon Instruments). Signals were filtered at 2 kHz (−3 dB frequency, Bessel filter, 80 dB per decade). Cells were confirmed as neurones by the presence of a fast rapidly inactivating inward current upon depolarization to 0 mV.

Drugs were applied rapidly through a manifold comprising 6 capillaries made of fused silica coated with polyimide, with a 250-μm internal diameter (SGE, Milton Keynes, UK), connected to a single outlet made of the same tubing, which was placed about 200 μm from the cell. Solutions were delivered by gravity flow from independent reservoirs. One barrel was used to apply drug-free solution to enable rapid termination of drug applications. Solution exchange measured by changes in open tip current was complete in 200 msec; however, complete exchange of solution around an intact cell was considerably slower (1 sec). Nevertheless, solution exchange using this system is fast enough to observe rapidly desensitising responses in dorsal root ganglion neurons (see Dunn et al., 2000). Agonists were separately applied for 1–5 sec at 2-min intervals, a time sufficient for responses to be reproducible. Antagonists were present for 2 min before and during the reapplication of agonists.

Immunohistochemistry

Rats were killed as described above and the superior cervical ganglia were dissected out. For immunohistochemistry on sections, the ganglia were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 120 min, then they were rapidly frozen by immersion in isopentane at −70°C for 2 min, cut into 10-μm sections using a cryostat, thaw-mounted on gelatin-coated poly-L-lysine-coated slides, and air-dried at room temperature. For immunohistochemstry on cultured neurons, ganglia were dissociated as above, plated in chamber slides and maintained in culture for 24 hr. They were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 120 min, then washed with distilled water three times.

Antibodies against rat $P2X_2$ and $P2X_3$ subunits (see Oglesby et al., 1999) were used in this study with an indirect three-layer immunofluorescent method. Primary antibody to P2X subunits were raised in rabbits, detected with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) and visualised with Streptavidin-Texas Red (red fluorophore, Sigma). Briefly, the sections or cells were incubated overnight with the primary antibodies diluted to 3 μg/ml with 10% normal horse serum (NHS) in PBS containing 0.05% Merthiolate and 0.2% Triton X-100. Subsequently, the slides were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch) diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate for 1 hr, followed by incubation in Streptavidin-Texas Red diluted 1:200 in PBS containing 0.05% Merthiolate for 1 hr. All incubations were held at room temperature and separated by three 5-min washes in PBS. Slides were mounted with citiflour and examined with fluorescence microscopy. Control experiments were performed both by using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and by omission of the primary antibody to confirm the specificity of the immunoreaction.

Data Analysis

All responses were normalized with respect to cell capacitance, to give a current density in pA/pF, unless oth-

erwise stated. All data are expressed as the means ± S.E.M. Statistical analysis (Student's *t*-test) was performed using Origin 4.1 (Microcal, Northampton, MA). Concentration-response data were fitted with the Hill equation:

$$Y = A/[1 + (K/X)^{nH}]$$

where A is the maximum effect, K is the EC_{50}, and nH is the Hill coefficient. The combined data from the given number of cells were fitted, and the results are presented as values ± S.E., determined by the fitting routine. Traces were acquired using Fetchex (pCLAMP software) and plotted using Origin 4.1.

Drugs

ATP, αβ-meATP, and 1,1-Dimethyl-4-phenylpiperazinium (DMPP) were obtained from Sigma. TNP-ATP was from Molecular Probes Europe (Leiden, The Netherlands). Solutions of ATP and other drugs were prepared using deionized water and stored frozen. All drugs were then diluted in extracellular bathing solution to the final concentration.

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