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Heterogeneity of P2X Receptors in Sympathetic Neurons: Contribution of Neuronal $P2X_1$ Receptors Revealed Using Knockout Mice

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ABSTRACT

P2X receptors are highly expressed throughout the nervous system, where ATP has been shown to be a neurotransmitter. The aim of this study was to characterize P2X receptor expression within sympathetic postganglionic neurons from the superior cervical ganglia. Reverse transcription-polymerase chain reaction showed the presence of mRNA for all P2X receptors, raising the possibility of multiple subunit expression within these ganglia. Whole-cell patch-clamp and calcium imaging studies revealed a heterogeneous population of P2X receptors in ~70% of neurons. We propose that the heterogeneity in properties could be caused by differential expression and/or subunit composition of the P2X receptor. The dominant phenotype was $P2X_2$-like; neurons showed slow desensitization, sensitivity to antagonists, and a profile of ionic modulation that is characteristic of $P2X_2$ receptors: potentiation by acidification and extracellular Zn^{2+} and attenuation by high extracellular Ca^{2+} and pH. A subpopulation of neurons (10–15%) were α,β-methylene ATP (α,β-meATP) sensitive, and in neurons from $P2X_1$ receptor-deficient mice the α,β-meATP response was reduced to 2% of all neurons, demonstrating a direct role for $P2X_1$ subunits. Control α,β-meATP responses were eliminated by high extracellular Ca^{2+} and pH, indicating the presence of heteromeric channels incorporating the properties of $P2X_1$ and $P2X_2$ receptors. This study demonstrates that in neurons, the $P2X_1$ receptor can contribute to the properties of heteromeric P2X receptors. Given the expression of $P2X_1$ receptors in a range of neurons, it seems likely that regulation of the properties of P2X receptors by this subunit is more widespread.

ATP is copackaged and coreleased with classic neurotransmitters throughout the nervous system (Bodin and Burnstock, 2001). Purinergic receptor-mediated currents were first described in sensory (Krishtal et al., 1983) and spinal cord neurons (Jahr and Jessell, 1983) and have subsequently been shown to mediate synaptic events and regulation of transmission in a range of both peripheral and central neurons (Dunn et al., 2001; Khakh, 2001; Robertson et al., 2001). In the peripheral nervous system, ATP is coreleased with noradrenaline from sympathetic nerves (Sneddon and Burnstock, 1984) and can stimulate noradrenaline release (von Kugelgen et al., 1997; Boehm, 1999) and mediate fast synaptic transmission (Evans et al., 1992; Silinsky et al., 1992). ATP is an agonist at P2X receptor ion channels (North, 2002) and some G-protein-coupled P2Y receptors (Abbracchio et al., 2003). Seven P2X receptor genes ($P2X_{1-7}$) have been identified; the functional receptors are thought to form as either homo- or heterotrimers, and a range of phenotypes has been described for recombinant receptors based on their subunit composition (North, 2002). In addition splice variants of P2X receptor subunits have also been described resulting in additional phenotypic variation (Brandle et al., 1997; Simon et al., 1997).

The superior cervical ganglia (SCG) are peripheral sympathetic ganglia involved in a range of activities, including innervation of the lacrimal gland, the salivary glands (submaxillary, sublingual, and parotid), and the dilator pupillae muscles in the eye. Previous studies suggested that rat peripheral sympathetic SCG neurons have a predominantly $P2X_2$-like receptor phenotype [α,β-methylene ATP (α,β-meATP)-insensitive and potentiated by zinc](Cloues et al., 1993; Khakh et al., 1995; Boehm, 1999; Dunn et al., 2001). In this study, RT-PCR analysis showed the presence of multiple P2X receptor subunits within the SCG, and patch-clamp and calcium imaging demonstrated a significant population (~10–15%) of α,β-meATP–sensitive neurons. α,β-meATP sensitivity is associated with the expression of $P2X_1$, $P2X_3$ (North, 2002), or $P2X_6$ (R. J. Evans, unpublished observa-

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ABBREVIATIONS: SCG, superior cervical ganglia; α,β-meATP, α,β-methylene ATP; RT-PCR, reverse transcription-polymerase chain reaction; WT, wild type; KO, knockout; TH, tyrosine hydroxylase; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate; Bp, base pair(s); VGCC, voltage-gated calcium channel; CPA, cyclopiazonic acid.

tions) receptor subunits. We have 1) used patch clamp electrophysiology and calcium imaging of acutely dissociated adult SCG neurons to determine the properties of SCG neuron P2X receptors and 2) compared the properties of SCG neurons from wild-type (WT) and $P2X_1$ receptor-deficient "knockout" (KO) mice (Mulryan et al., 2000). We have shown that the SCG has a heterogeneous population of P2X receptors and that the predominant isoform is most likely to be the $P2X_2$ receptor; we demonstrate a direct functional contribution of the $P2X_1$ receptor to receptor heteromers in ~10% of neurons.

Materials and Methods

Primary Cell Culture. Adult MF1 mice of either sex were given a lethal intraperitoneal injection of pentobarbitone, and death was confirmed by femoral exsanguination. For each culture, SCG from two animals were removed immediately, cut in half, and placed in ice-cold Hanks' buffered salt solution (HBSS). The tissue was placed in 0.1% L-cysteine and 0.05% papain (enzyme activity, 39.2 units/ml) in HBSS and digested for 15 min at 37°C. It was then transferred to 0.05% collagenase (enzyme activity, 215.5 units/ml) and 4% dispase (enzyme activity, 4.48 units/ml) in HBSS for 40 min at 37°C. The pieces of ganglia were washed with Leibovitz's 15 (L-15) media before being placed in 2.5 ml of feeding media (L-15 media supplemented with 1% fetal calf serum, 0.22% sodium bicarbonate, 0.54% glucose; 0.01 ng/ml nerve growth factor, 30 μg/ml penicillin, and 30 μg/ml streptomycin). Mechanical trituration was used to separate the cells, which were seeded onto poly-D-lysine–coated coverslips and incubated for 1.5 h before a further 1 ml of feeding media was added.

Electrophysiology. Electrophysiological recordings were made for 1 to 4 days after dissociation, using the whole-cell patch-clamp configuration, at room temperature. Microelectrodes were pulled from thin-walled, filamented borosilicate glass and had resistances of 3 to 7 MΩ when filled with internal solution: 140 mM potassium gluconate, 10 mM EGTA, 10 mM HEPES, and 5 mM NaCl, pH adjusted to 7.3 with KOH. The bath was continuously perfused with extracellular solution containing: 150 mM NaCl, 10 mM HEPES, 11 mM glucose, 2.5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$, pH adjusted to 7.3 with NaOH. Neurons were voltage-clamped at −70 mV (after correction for the tip potential) using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA). Data were collected with pClamp 8.1 software and analyzed using Origin 6 (OriginLab Corp, Northampton, MA).

Agonists were applied for 1 s via a fast-flow U-tube (Evans and Kennedy, 1994). The off rate of agonist was dependent both on the U-tube used and the positioning relative to the cell, and changes in these parameters probably account for the variation in decay of P2X receptor current after removal of agonist. Antagonists and extracellular test solutions were perfused into the bath for 5 min before agonist application. Reproducible responses were obtainable with 5-min intervals between agonist applications. All experiments were performed on at least three separate neuronal cell cultures (2 mice per culture), except where stated in the text.

Fluorescent Calcium Imaging. SCG neurons were plated onto 16-mm coverslips and kept in culture for up to 3 days. Cells were loaded with Fluo-3 acetoxymethyl ester (final concentration, 1 μM) for 30 min at 37°C. The coverslips were washed in extracellular perfusion solution (as for electrophysiological experiments) for 30 min at 37°C. Argon laser excitation of the dye was at a wavelength of 488 nm with the filters set to capture emission at wavelengths greater than 510 nm. The neurons were imaged using a Fluoview FV300 confocal microscope (Olympus, Tokyo, Japan) at room temperature. Real-time fluorescence was captured by Olympus Fluoview ver. 4.2 software at a frequency of 0.5 Hz, for a continuous time period of ~360 s. Agonist application was via U-tube for 40 s after 30 s of baseline fluorescence was acquired. Agonist applications occurred at an interval of 15 min. Antagonists were applied for 8 min before test solution application. Extracellular solution changes were performed 2 min before agonist application to allow complete exchange of the bath solution.

Neurons were identified visually. When extracellular solution was applied to the neurons via the U-tube, 34.4% (142 of 413) of neurons gave an increase in fluorescence (puff response). In the presence of 100 μM suramin, 84.2% (16 of 19) of puff responses were abolished. The remaining 15.8% (3 of 19) of neurons had a response that was 85.7 ± 14.3% of the initial artifact. This indicates that ATP may be released from some cells (either neurons or glia) because of mechanical stimulation of the neurons, causing local activation. None of the neurons with puff artifacts was included in data analysis.

Neuronal viability/responsiveness was confirmed by Ca^{2+} influx in response to 60 mM K^+ perfusion solution; only cells that responded were included in the analysis (for both WT and KO mice). Average background fluorescence was measured from a cell-free area of the field of view. Baseline measurements were calculated as a mean of the first 10 s of recording. Neuronal traces were corrected for background and baseline and then expressed as a ratio of the baseline (referred to as self-ratio) to compensate for differential loading of the neurons. Comparisons were made between the normalized peak calcium changes under different agonist conditions. All experiments were performed on at least three separate neuronal cell cultures (2 mice per culture).

RT-PCR. Total RNA was isolated from six intact SCGs, and SCG cultures using the RNeasy Mini Kit (QIAGEN, Valencia, CA). The RNA was treated with DNase I (Sigma) to remove any DNA contamination. Half of the purified RNA was reverse-transcribed to cDNA using SuperScript II-reverse transcriptase (Invitrogen, Carlsbad, CA), and the remainder of the RNA was used as a negative control to ensure that there was no genomic contamination. Details of all the primers and the predicted size of the amplified products are given in Table 1. PCR reactions were carried out with 2 μl of cDNA and a final concentration of 500 nM of each primer, except for the $P2X_7$ primers, where 3 μl of cDNA was required. Amplification took place in a Techne Genius PCR machine (Techne, Cambridge, UK) using the following protocol: denaturation at 94°C for 5 min; repeated cycles of denaturation at 94°C, followed by annealing at 57°C and extension at 72°C, each for 30 s; final extension at 72°C for 10 min. Thirty-five cycles were used in most cases, except for tyrosine hydroxylase (TH) and β-actin amplification, for which 30 cycles were used. The amplification products were examined by electrophoresis on a 1% agarose gel and visualized using ethidium bromide under UV light. Positive controls used were TH for confirmation of the sympathetic nature of the tissue, and β-actin was used to confirm that there was no genomic DNA contamination. The primers for β-actin were designed such that they would amplify an intronic sequence of the gene, whereas mRNA would have spliced out this intron. Based on the size of the amplified product, any genomic contamination could therefore be detected. To confirm the identity of the two bands amplified for the $P2X_4$ receptor, the bands were sequenced using BigDye Terminator Version 1.0 sequencing kits (Applied Biosystems, Foster City, CA). Whole SCG were taken for RNA extraction; therefore, the sample contains RNA from non-neuronal cells (e.g., glia and blood cells).

Data Analysis. There was no significant difference between the amplitude of response (analysis of variance) or proportion of neurons responding (t test) over the 4-day test period for the electrophysiological data; therefore, the data have been pooled for all days. Statistical tests were performed using an unpaired t test, with a P value of <0.05 considered significant.

ATP was tested on each neuron. In responsive neurons, ATP was also applied after the test solution, to determine any run-down of responses. All electrophysiological data and fluorescent calcium imaging data are expressed as a percentage of the mean ATP responses measured before and after the test solution, except in the case of the antagonists, in which case it is the percentage of the initial ATP

response. Data are expressed as the mean ± S.E.M., unless otherwise stated.

Concentration-response data were collected from individual neurons that had three or more concentrations of ATP tested on them, and an average data set was also obtained. The response to 1 mM ATP was taken as 100%, and the rest of the data points were normalized. Curves were generated in Origin 6 using the Hill equation: $y = (V_{max} \times x^{nH})/(EC_{50}{}^{nH} + x^{nH})$ where V_{max} is the maximum response (100%), n_H is Hill coefficient, and EC_{50} is the concentration required to evoke a half-maximal response. pEC_{50} corresponds to $-\log_{10}$ of the EC_{50} concentration. The effects of the antagonists suramin and PPADS were calculated as the percentage inhibition of the peak response to ATP.

Results

Expression of P2X Receptor Subunit mRNA. To determine which P2X isoforms could be expressed by the mouse SCG, RT-PCR was performed on isolated total RNA (the samples corresponded to whole SCG and comprised a mixed population of cell types, including neurons, glia, and blood cells). No genomic DNA contamination of the RNA samples was found using the β-actin primers (data not shown), and primers for TH amplified a band of the expected size (~460 bp), confirming the sympathetic origin of the tissue. Using subtype-selective primers, bands of the appropriate size were amplified corresponding to $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_5$ receptors ($n = 3$ separate RNA extractions; Fig. 1) from reactions containing reverse transcriptase but not when it was absent, further confirming the lack of DNA contamination of the samples. Two bands were seen for the $P2X_4$ receptor, one at the expected ~560 bp and the other at ~630 bp. On sequencing, these were identified as two splice variants of the $P2X_4$ receptor, consistent with previous reports (Townsend-Nicholson et al., 1999). The product amplified using the $P2X_7$ primers was at the limit of detection and required 3 μl of cDNA template for a band to be evident (two of three separate RNA extractions). A band corresponding to $P2X_M$, which has been identified as the murine $P2X_6$ receptor (Robertson et al., 2001), was also just detectable (three of three). Dissociation of parasympathetic neurons from rat submandibular ganglia changes the profile of P2X receptor subunits as measured immunohistochemically. In intact ganglia, only $P2X_5$ receptors are detected; however after dissociation, high levels of $P2X_2$ and $P2X_4$ receptors were reported (Smith et al., 2001). In the present study, the same complement of P2X receptors was amplified from SCG neurons that had been in culture for 24 h (data not shown), indicating that there was no major change in the properties of P2X receptors in these neurons after short-term culture. These results indicate that multiple P2X receptor subunits may be expressed in the SCG and that individual neurons may express multiple P2X receptor subunits, findings that are consistent with those of previous studies (Collo et al., 1996; Dunn et al., 2001).

P2X Receptor-Mediated Currents. In 54.6% of neurons (318 of 582; Fig. 2A) ATP (30 μM-3 mM) evoked fast inward currents that were slowly desensitizing (peak current decayed to 83.6 ± 2.6% after 1-s application, $n = 20$). In 2.2% (13 of 582) of SCG neurons, the response to ATP had fast and slowly desensitizing components (Fig. 2A) and showed significantly greater decay in current amplitude during ATP application (the peak current decayed to 69.4 ± 7.2% of the peak after 1 s, $n = 9$). ATP was inactive at the remaining 43.1% (251 of 582) of SCG neurons. However, all of the mouse SCG neurons tested gave inward currents in response to ACh (30 μM-1 mM; $n = 113$; range for 100 μM ACh, 73–5880 pA). This demonstrates that there is some species variation in the proportion of SCG neurons expressing P2X receptors, because ATP-evoked currents were recorded from 100% of rat (Khakh et al., 1995) and guinea pig (Zhong et al., 2000) SCG neurons.

For the ATP-sensitive neurons, the mean amplitude of slowly desensitizing responses to ATP (100 μM) was 292.4 ± 36.5 pA ($n = 137$) with a range of 21 to 2060 pA. There was no correlation between amplitude of the current and the membrane capacitance (a measure of cell size; correlation coefficient, 0.1; membrane capacitance, 22.1 ± 1.1 pF; $n = 134$). The majority of neurons with slowly desensitizing P2X currents had small amplitudes; however, for 8% (11 of 137) of

TABLE 1

The mRNA primer sequences used for PCR amplification

Where available, murine sequences were used (m). For isoforms for which the murine homologue has yet to be sequenced, rat sequences were used (r). The $P2X_2$ primers are not in the region that would identify reported splice variants of the receptor.

Target Protein		Primer Sequence (5′ to 3′)	Product Size
			bp
$mP2X_1$	Fwd	aac cca gat ccc acc aac gaa c	344
	Rev	tca ccc aat gac gta gac cag aac	
$rP2X_2$	Fwd	cat ctt cag gct ggg ttt cat tg	477
	Rev	agg gtc aca ggc cat cta ctt g	
$mP2X_3$	Fwd	gct tcg gac gct atg cca aca a	470
	Rev	aac cac gtc ccc tac cct caa gat	
$mP2X_4$	Fwd	tcg gct cct cgg aca ccc aca g	559
	Rev	cct agg agc gcc aag cca gag c	
$mP2X_5$	Fwd	cct ccc act gca acc cac act att	429
	Rev	ggg cct gct cgt cct gat gaa c	
$mP2X_7$	Fwd	cgg cgt tgt aaa aag ggg tgg atg	480
	Rev	gtt gta gcc ggg gac gaa gga ctc	
$mP2X_M$	Fwd	cct ggc ctt gct ggg tgg tg	411
	Rev	aag gct ggc tcg gtc tat gaa ctg	
mTH	Fwd	ctc aac ctg ctc ttc tcc ttg agg	458
	Rev	tcc ttc cag gta gca att tcc tcc	
mβ-Actin	Fwd	atc cat gaa act aca ttc aat tcc at	199
	Rev	acc gat cca cac aga gta ctt gcg c	

Fwd, forward primer sequence; Rev, reverse primer sequence.

neurons, the ATP current was greater than 1 nA. The neurons that gave combined fast and slow desensitizing P2X currents had ATP (100 μM) evoked amplitudes that were significantly greater (2305.1 $\pm$ 457.9 pA; $n = 7$; $p < 0.001$) than slowly desensitizing responses and also had higher mean cell capacitance (32.3 $\pm$ 2.8; $n = 7$; $p < 0.05$).

For the SCG neurons that had a slowly desensitizing P2X receptor phenotype, ATP had a mean EC_{50} of ~35 μM (pEC_{50} of 4.7 $\pm$ 0.2, $n = 12$ neurons; Fig. 3, A and D). However, there was a considerable range in sensitivity, with EC_{50} values of 2.66 to 120.8 μM, with a mean Hill coefficient of 1.1 $\pm$ 0.1. This range of sensitivity is unlikely to result from variation in expression of the receptors (Clyne et al., 2003) because there was no correlation (correlation coefficient −0.03) between the amplitude of the response and the EC_{50} value.

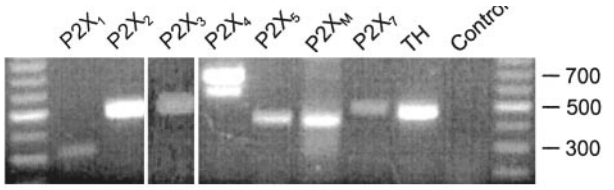

Fig. 1. RT-PCR products showed the presence of mRNA for all P2X isoforms. The control lane is TH primers tested on total RNA without any reverse transcriptase to ensure that there was no DNA contamination. The TH-positive control indicates the sympathetic nature of the tissue. The sample for $P2X_3$ was run on a separate gel.

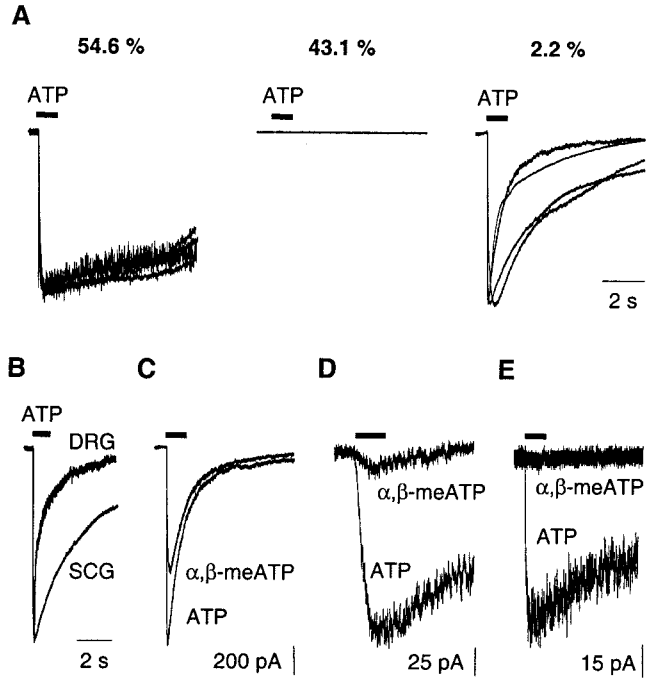

Fig. 2. Agonist sensitivity. A, example traces (normalized to peak current) of the different rates of desensitization seen within the neurons, with the percentage of cells displaying that characteristic. Multiple traces are shown for the slow and combined desensitization (first and third traces) to demonstrate the variability of time courses between neurons. B, a comparison of the time course of fast desensitization in DRG ($P2X_3$-expressing neurons) and SCG neurons (responses were normalized to the peak current). DRG currents desensitize faster in response to ATP than the SCG neurons. C, fast desensitizing neurons were α,β-meATP–sensitive (three of three), with a mean amplitude of 58.3 $\pm$ 9.8% of the response to ATP. D, slowly desensitizing neurons had a small current in response to α,β-meATP in two of eight neurons, with a mean amplitude of 15 $\pm$ 0.5% of the ATP response. E, no current was seen in response to α,β-meATP in six of eight neurons.

The dominant mouse SCG P2X receptor is most likely to correspond to the expression of $P2X_2$ receptor subunits (Cockayne et al., 2002). To corroborate this, other characteristics that are specific to the $P2X_2$ receptor were examined (Table 2). Suramin (100 μM) was an antagonist ($n = 20$ cells) and reduced the response to ATP (100 μM) by 78.9 $\pm$ 3.6% (Fig. 3B, $n = 5$ cells, from one culture, two animals), with recovery to 66.1 $\pm$ 7.7% of the initial ATP response after wash-off (10 min). The IC_{50} for suramin at these neurons was 18.4 μM ($n = 5$ cells). Iso-PPADS (30 μM) almost completely abolished (98.5 $\pm$ 1% inhibition, $n = 4$ cells from one culture, two animals; Fig. 3C) the response to 100 μM ATP. This effect was only slowly reversible, with a 46 $\pm$ 3.7% recovery after a 40-min wash period ($n = 3$). The $P2X_2$ receptor can also be modulated by extracellular ions including H^+, Zn^{2+} (King et al., 1996), and Ca^{2+} (Evans et al., 1996). Acidification of the extracellular solution to pH 6.3 potentiated the response to 30 μM ATP by 56 $\pm$ 45.5% ($n = 7$ cells from two cultures, four animals; Fig. 4A). In contrast an increase in extracellular pH to 8.3 attenuated the response to ATP to 24 $\pm$ 3.5% of the initial ATP response (30 μM; $n = 5$ cells from two cultures, four animals). Addition of zinc (10 μM) to the extracellular solution potentiated the P2X current to 233.3 $\pm$ 42.9% of the control ATP response (30 μM; $n = 6$ cells from two cultures, four animals; Fig. 4B). Increasing the

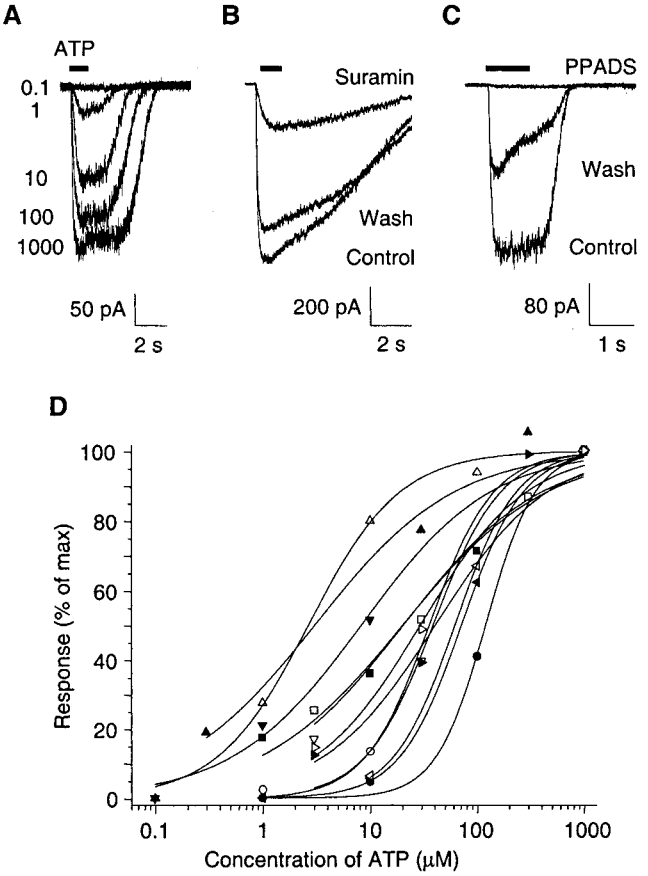

Fig. 3. The ATP current is concentration-dependant and antagonist sensitive. A, example traces of the response to varying concentrations of ATP in one neuron. B, 100 μM suramin caused 78.9 $\pm$ 3.6% inhibition of the ATP current (100 μM, $n = 5$). C, 30 μM iso-PPADS attenuated the ATP current by 98.5 $\pm$ 1% (100 μM, $n = 4$). D, concentration-response curves from individual neurons ($n = 12$). pEC_{50}, 4.7 $\pm$ 0.2; range of EC_{50} values, 2.66 − 120.8 μM; mean Hill coefficient, 1.1 $\pm$ 0.1.

extracellular Ca^{2+} concentration from 2.5 to 30 mM abolished the ATP evoked P2X current in SCG neurons ($n = 6$ cells, from three cultures, six animals; Fig. 4C; similar results were seen in calcium imaging studies—see below). Taken together, these results (31 cells from 7 cultures from 14 animals) support the conclusions of Cockayne et al. (2002) that the $P2X_2$ receptor phenotype dominates the properties of mouse SCG neurons.

The results from the RT-PCR suggest that receptor subunits other than $P2X_2$ may be expressed in the SCG and that they contribute to the P2X receptor phenotype. α,β-meATP is an agonist at receptors containing $P2X_1$, $P2X_3$, or $P2X_6$ receptor subunits. For neurons that showed slow desensitization to ATP, α,β-meATP (100 μM) evoked small currents (15.0 ± 0.5% of the response to ATP, 100 μM; Fig. 2D) in 25% of cells (two of eight) and was ineffective in the remainder of cells tested (six of eight; Fig. 2E). In contrast, all neurons that had a fast desensitizing component responded to α,β-meATP (three of three), with an inward current of 57.2 ± 10.4% of the response to ATP (100 μM; Fig. 2C). These results show that the P2X receptors in the SCG can be subdivided into at least three pharmacologically distinct P2X receptor phenotypes: 1) slowly desensitizing α,β-meATP insensitive (~41% of all neurons), 2) slowly desensitizing α,β-meATP–sensitive (~14% of all neurons), and 3) mixed fast/slow desensitizing α,β-meATP–sensitive neurons (~2% of all neurons). In the guinea pig SCG α,β-meATP sensitivity is thought to result from the coexpression of $P2X_{2/3}$ heteromeric channels (Zhong et al., 2000). However there is marked species variation in the properties of SCG P2X receptors; for example, in the rat SCG, α,β-meATP is ineffective as an agonist but produces a concentration-dependent attenuation of the response to ATP (Khakh et al., 1995). The α,β-meATP sensitivity in this study most probably suggests that $P2X_1$, $P2X_3$, and/or $P2X_6$ receptor subunits contribute to the P2X phenotype of ~10 to 15% of mouse SCG neurons.

P2X Receptor-Mediated Changes in Intracellular Calcium. The functional activity of P2X receptors in mouse SCG neurons was also examined using fluorescent calcium imaging. This technique has the advantage that several neurons can be imaged at once and allows higher throughput for characterization of neuronal P2X receptors. Of the neurons that responded to 60 mM K^+ perfusion solution, 80.4% (701 of 872) gave an increase in fluorescence to ATP application (67.3% of all neurons, including those that failed to respond to 60 mM potassium, responded to ATP application). The mean self-ratio for the fluorescence increase to 100 μM ATP was 1.54 ± 0.03 ($n = 379$), which equates to a rise of 54% above the baseline levels of Ca^{2+} in the neurons. Removal of Ca^{2+} from the extracellular perfusion solution resulted in no increase in $[Ca^{2+}]_i$ when ATP was applied ($n = 26$; Fig. 5A), indicating that calcium influx is essential for the response and ruling out a role of ATP sensitive P2Y receptors (calcium responses to the P2Y receptor agonists UTP and UDP were still recorded in the absence of extracellular calcium; data not shown).

ATP evoked concentration-dependent changes in intracellular calcium with an EC_{50} of ~26 μM (pEC_{50} of 4.3 ± 0.1, with a range of 25.7–95 μM and a Hill coefficient of 2 ± 0.3, $n = 6$; Fig. 6, A and B). In common with P2X receptor-mediated currents, increasing extracellular calcium to 10 mM reduced the rise in intracellular calcium in response to ATP (100 μM) by 89 ± 6% ($n = 12$; Fig. 5A), indicating the contribution of $P2X_2$ receptor subunits. These results are essentially identical to those determined in patch-clamp studies (Fig. 6C) and indicate that calcium imaging is a robust and reliable method for characterization of P2X receptors in SCG neurons.

The data from both patch clamp and calcium imaging studies on ATP and ionic sensitivity, combined with the slowly desensitizing nature of the P2X response, suggests that the $P2X_2$ receptor subunit dominates the properties of SCG P2X receptors. This is consistent with an abstract on $P2X_2$ receptor knockout mice (Cockayne et al., 2002). However, Cockayne et al. (2002) indicated there was a small residual response to ATP application, suggesting that there were additional subunits contributing to the native P2X receptor phenotype in these neurons. We have confirmed the contribution of other P2X receptor subunits in this study by showing that the $P2X_1$, $P2X_3$, and $P2X_6$ receptor subunit agonist α,β-meATP (100 μM) evoked inward currents in patch-clamp studies and rises in intracellular calcium from ~10% of neurons (48 of 510 neurons, mean amplitude 28.3 ± 4.4% of the 100 μM ATP response; Fig. 7A). To determine

TABLE 2

A table summarizing the properties of P2X receptors in comparison to the properties seen in α,β-meATP sensitive, combined fast and slow desensitising neurons in the mouse SCG. No entry signifies that these conditions have not been studied for that receptor. The prefix 'r' indicates that the study was performed on rat P2X receptors. All data are from North and Suprenant (2000) and North (2002) except as indicated by footnotes.

	α,β-meATP Sensitivity	Rate of Current Decay	Effect of High Ca^{2+}	Effect of High pH	Effect of Zn^{2+} (10 μM)
$P2X_1$	Yes	Fast	No effect	No effect[a]	Decrease[b]
$P2X_2$	No	Slow	Decrease	Decrease	Increase
$P2X_3$	Yes	Fast	No effect	No effect[a]	Increase
$P2X_4$	No	Slow		No effect[a]	Increase
$rP2X_5$[b]	No	Slow	Decrease	No effect	Increase
$P2X_6$	Yes	Slow			
$P2X_7$	No	Slow	Decrease		Decrease
$rP2X_{1/2}$[c]	Yes	Fast/slow		Increase	
$P2X_{1/5}$	Yes	Fast/slow	No effect	Decrease	
$P2X_{2/3}$	Yes	Slow	Decrease	Decrease	Increase
$P2X_{4/6}$	Yes	Slow			Increase[d]
α,β-meATP–Sensitive SCG Neurons	Yes	Fast/slow	Decrease	Decrease	

[a] Stoop et al. (1997).
[b] Wildman et al. (2002).
[c] Brown et al. (2002).
[d] Le et al. (1998).

whether $P2X_1$ receptor subunits contribute to the α,β-meATP sensitivity of SCG neurons, we compared normal and $P2X_1$ receptor-deficient mice (Mulryan et al., 2000).

$P2X_1$ Receptor-Deficient Mice Reveal a Role for the $P2X_1$ Receptor in SCG Neurons. In calcium imaging studies, responses to ATP (100 μM) were seen in 44.3% (116 of 262) of neurons from $P2X_1$ receptor-deficient (KO) mice, which is significantly reduced compared with responses seen in WT mice. The magnitude of increase in $[Ca^{2+}]_i$ in response to ATP in KO neurons was not significantly different from that of WT, with a mean self-ratio of 1.6 ± 0.06 (n = 134). α,β-meATP (100 μM) caused a rise in $[Ca^{2+}]_i$ in only 1.7% (2 of 116) of the neurons that were responsive to ATP (the amplitude of the response to α,β-meATP had self-ratios of 1.16 and 1.47), compared with 9.4% of neurons from WT mice (Fig. 7B). The expression pattern of P2X receptor transcripts between WT and KO mice (data not shown) was the same except for the absence of the $P2X_1$ receptor transcript from SCG from $P2X_1$ receptor-deficient mice and suggests that there is no major compensatory change in P2X receptor expression associated with the $P2X_1$ receptor deficiency. These results demonstrate that the $P2X_1$ receptor is the predominant isoform responsible for ~80% of the α,β-meATP sensitivity seen in SCG neurons. The reduction in numbers of ATP-sensitive neurons in $P2X_1$ receptor-deficient mice may also indicate a role of the $P2X_1$ receptor in assembly and trafficking of functional P2X receptors in the SCG. The residual α,β-meATP–sensitive response in ~2% of SCG neu-

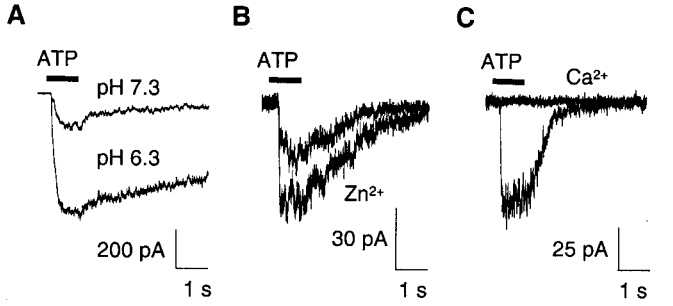

Fig. 4. Ionic modulation of the ATP response by pH, Zn^{2+}, and Ca^{2+}. A, a change in extracellular pH to 6.3 potentiates the ATP current (30 μM) by 156 ± 45.5% (n = 7). B, addition of extracellular Zn^{2+} (10 μM) causes potentiation of the ATP current (30 μM) by 233.3 ± 42.9% (n = 6). C, extracellular Ca^{2+} (30 mM) causes complete inhibition of the ATP current (n = 6).

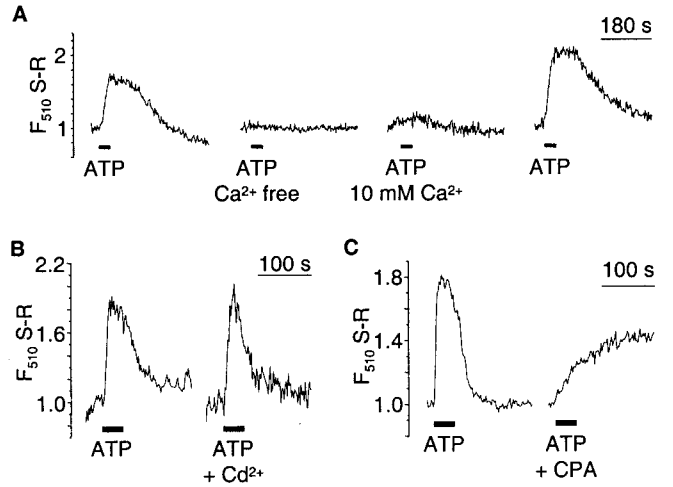

Fig. 5. A, ATP responses are sensitive to changes in the extracellular concentration of Ca^{2+}. ATP application in Ca^{2+}-free solution did not cause an increase in $[Ca^{2+}]_i$ (n = 26). 10 mM extracellular Ca^{2+} inhibited the ATP response by 89 ± 6% (n = 12). (S-R, self-ratio). B, the fluorescence change is not effected by VGCC block by 100 μM Cd^{2+}, with responses that were 102.3 ± 12.5% (n = 5) of the response to 100 μM ATP. C, depletion of the intracellular Ca^{2+} stores by preincubation with 30 μM CPA causes a slowing of the initial Ca^{2+} rise, prolongation of raised Ca^{2+} levels, and a reduction in the peak response (67.3 ± 10.4%; n = 32).

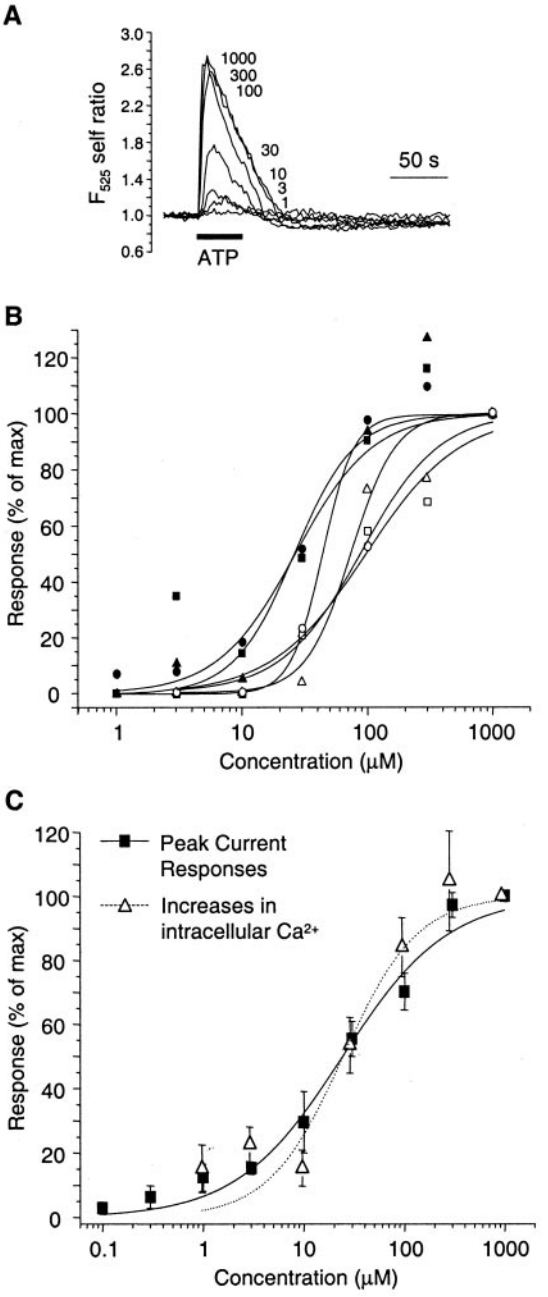

Fig. 6. The concentration-response data using the fluorescent calcium imaging technique closely correlates with electrophysiological data. A, example traces of the fluorescence increase in response to varying the concentration of ATP in a single neuron. B, concentration-response curves from individual neurons (n = 6). pEC_{50}, 4.3 ± 0.1; range of EC_{50} values, 25.7 to 95 μM; Hill coefficient, 2 ± 0.3. C, a comparison of the mean concentration response curves from electrophysiological experiments (solid line; EC_{50}, 24.3 μM; Hill coefficient, 0.8) and fluorescent calcium imaging experiments (dotted line; EC_{50}, 25.9 μM; Hill coefficient, 1.2).

rons from $P2X_1$ KO mice suggests that $P2X_3$ or $P2X_6$ receptor subunits may contribute to agonist sensitivity in these neurons.

$P2X_1$ Receptors Form Functional Heteromeric $P2X_{1/2}$ Channels in the SCG. The data with the $P2X_1$ receptor-deficient mice demonstrate that this receptor subunit gives the α,β-meATP sensitivity to ~8% of SCG neurons. The α,β-meATP–sensitive currents from the SCG in patch-clamp studies showed relatively slow desensitization (50% decay, >2 s). Slowly desensitizing recombinant $P2X_1$ receptor-mediated responses have been described previously (Parker, 1998). However, the predominant $P2X_1$ receptor phenotype reported for recombinant $P2X_1$ receptors, and for native mouse homomeric $P2X_1$ receptors in the bladder, vas deferens, and arteries, is of rapid desensitization [50% decay, <300 ms (Lewis and Evans, 2000; Mulryan et al., 2000; Vial and Evans, 2000, 2002; North, 2002)]. In addition, the ionic sensitivity of α,β-meATP evoked responses described in this study (see below) are inconsistent with the properties of homomeric $P2X_1$ receptors. Studies on recombinant receptors have indicated that $P2X_1$ receptors can heteropolymerize with other P2X receptor subunits, including the $P2X_2$ isoform (Torres et al., 1999; Brown et al., 2002). Homomeric $P2X_1$ receptors are unaffected by high extracellular calcium (Evans et al., 1996) and increasing pH (Stoop et al., 1997). In contrast, $P2X_2$ receptor subunits are inhibited by high extracellular calcium (Evans et al., 1996) and increase in pH (Stoop et al., 1997). When P2X receptor subunits come together to form heteromeric channels, the resultant properties are a composite of those of the constituent subunits; e.g., the $P2X_{2/3}$ heteromer shows the agonist sensitivity of the $P2X_3$ subunit and the time course of the $P2X_2$ subunit. The properties of heteromeric channels that have been characterized are detailed in Table 2. Knockout studies indicate that the $P2X_2$ receptor subunit predominates in the SCG (Cockayne et al., 2002). To determine whether heteromeric channels comprising the agonist sensitivity of $P2X_1$ receptors and the ionic sensitivity of $P2X_2$ receptors are functionally expressed in the SCG, we have tested the effects of high extracellular calcium and pH on α,β-meATP (100 μM)-evoked calcium responses. Ca^{2+} (10 mM) in the extracellular solution abolished the response to α,β-meATP in eight of nine cells; in the remaining cell, the response was unaffected and it is possible that this corresponds to the residual α,β-meATP–sensitive response recorded from $P2X_1$ receptor-deficient mouse cultures. Raising the pH to 8.3 reduced the response to α,β-meATP (100 μM) by 93.7 ± 6.3% (n = 9; Fig. 7C). These results, taken together with the ~80% loss of α,β-meATP–sensitive responses from $P2X_1$ receptor-deficient mouse SCG neurons, suggest that heteromeric channels incorporating the properties of $P2X_1$ and $P2X_2$ receptor subunits are expressed in the SCG.

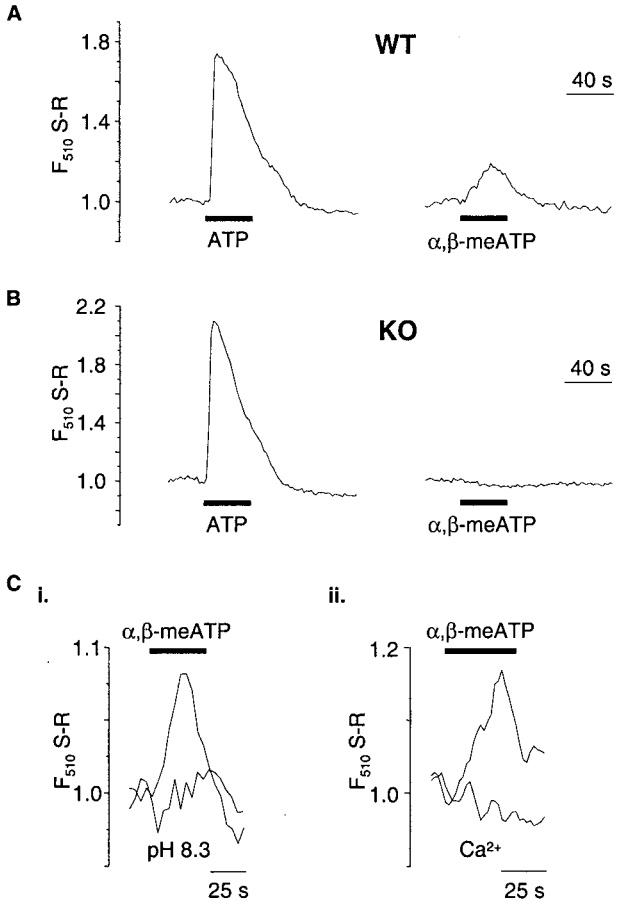

Fig. 7. α,β-meATP-sensitivity in neurons from WT and $P2X_1$ KO mice. A, in WT neurons responses to α,β-meATP were seen in 9.4% of neurons. B, α,β-meATP–evoked responses from $P2X_1$ KO were recorded from only 1.4% of neurons. However, the ATP response was still present in the KO neurons. C, the α,β-meATP response is modulated by extracellular ions, as shown by example traces of α,β-meATP responses at pH 8.3 (i) and in the presence of 10 mM Ca^{2+} (ii).

P2X Receptors and Calcium Signaling in Neurons. Changes in intracellular calcium can have profound effects on neuronal signaling. We have investigated the mechanisms associated with changes in intracellular calcium levels after P2X receptor activation. Calcium influx could be caused by conductance through the P2X receptor, and/or from activation of voltage-gated calcium channels (VGCCs). Extracellular Cd^{2+} (100 μM) blocks VGCCs and abolished the K^+ induced increases in $[Ca^{2+}]_i$ in SCG neurons (n = 10). Application of 100 μM Cd^{2+} did not affect the ATP-induced $[Ca^{2+}]_i$ increase (100.3 ± 9.1% of the response to 100 μM ATP; n = 8; Fig. 5B), indicating that the calcium rise is caused by the calcium permeability of the P2X receptor. The calcium influx through the P2X receptor could be amplified by calcium-induced calcium release from intracellular stores. Cyclopiazonic acid (CPA) depletes intracellular Ca^{2+} stores by blocking the endoplasmic reticulum Ca^{2+} pump. CPA (30 μM with a 15-min preincubation) caused a small increase in the background fluorescence (10.3 ± 2.8%, n = 37) and a change in the time course of the fluorescence signal (n = 37; Fig. 5C). There was a slowing of the initial phase of calcium rise and a reduction in the plateau (60.9 ± 9.6% of control response, n = 37), and this level was maintained until the CPA was washed off. The continued elevation of intracellular Ca^{2+} was probably caused by block of the neuron's normal calcium sequestration mechanisms. The block was reversible, and ATP responses returned to normal after a 20-min wash period. These results indicate that calcium-induced calcium release contributes to P2X receptor signaling in neurons. This amplification of the response (which would be expected to improve the signal-to-noise ratio) may help to explain the slightly larger proportion of P2X receptor mediated responses recorded from SCG neurons in calcium imaging studies. These studies demonstrate that P2X receptors can act as

a direct source for calcium influx into SCG neurons, independent of the activation of voltage-dependent calcium channels. Similar direct influx has been reported in other neurons [e.g., spinal horn interneurons (Rhee et al., 2000)] and is associated with regulation of transmitter release. However, this study also reveals that calcium influx is not the sole source of the rise in intracellular calcium, that calcium-induced calcium release from intracellular stores contributes to the response, and that the ER sequestration of calcium plays a significant role in the termination of the calcium signal after P2X receptor activation.

Discussion

Pharmacological profiles and knockout mice have contributed significantly to our understanding of the physiological roles of some P2X receptor isoforms in the nervous system. For example, a role for $P2X_3$ receptors in sensory perception was initially proposed because of pharmacological studies (Chen et al., 1995; Lewis et al., 1995). This work was confirmed using $P2X_3$ knockout mice, which had reduced nociceptive responses to tissue damage and urinary bladder hyporeflexia (Cockayne et al., 2000; Souslova et al., 2000). In this study, we have characterized P2X receptor-mediated responses in mouse SCG and used $P2X_1$ receptor-deficient mice to demonstrate a contribution of these receptors to neuronal function.

We have shown using a combination of electrophysiology and calcium imaging that functional P2X receptors are expressed on ~60 to 70% of mouse SCG neurons. In contrast, nicotinic acetylcholine receptor-mediated responses were recorded from all neurons tested. This raises the possibility that P2X receptor expression is coded and corresponds to the target of particular SCG neurons. This has been demonstrated for neuropeptide expression in the rat SCG (Bergner et al., 2000) and for 5-HT_3 receptors in superior cervical, dorsal root, and nodose ganglia (Morales and Wang, 2002). The marked heterogeneity in properties of P2X receptors (e.g., the α,β-meATP sensitivity of ~10 to 15% of neurons in the SCG) also indicates that there is further specialization in receptor expression and function.

The predominant phenotype of P2X receptors in the mouse SCG, based on the time course and antagonist/ionic sensitivity, indicates a substantial contribution of $P2X_2$ receptor subunits; however, our studies did reveal some heterogeneity in the responses. These results are consistent with an abstract on $P2X_2$ receptor knockout mice that showed the $P2X_2$ receptor isoform dominates the properties of SCG P2X receptors (Cockayne et al., 2002). However, the fact that small residual P2X receptor-mediated responses were recorded in SCG neurons from $P2X_2/P2X_3$ double-knockout mice (Cockayne et al., 2002) indicates that additional P2X receptor subunits contribute to receptor expression in sympathetic neurons. This is supported by three pieces of evidence: 1) RT-PCR, in situ hybridization, and immunohistochemical studies (Collo et al., 1996; Dunn et al., 2001) indicate that multiple P2X receptor subunits are present in the SCG, and coimmunoprecipitation studies have demonstrated that $P2X_2$ receptor subunits may associate with $P2X_{1,3,5\&6}$ receptor subunits (Torres et al., 1999), 2) patch clamp and calcium imaging studies showed that there was marked variation in ATP sensitivity of P2X receptor-mediated responses (EC_{50} values ranged from 2.5 to 120 μM); and 3) $P2X_1$ receptor subunit-dependent α,β-meATP sensitivity was demonstrated in a subpopulation of neurons. It is likely therefore that the variation in agonist sensitivity of mouse SCG neuron P2X receptors results, at least in part, from the expression of a range of different homomeric and/or heteromeric subunit combinations within a single neuron, as has been demonstrated for guinea pig SCG neurons (Zhong et al., 2000) and sensory neurons (Grubb and Evans, 1998; Thomas et al., 1998; Burgard et al., 1999). In this study, we have focused on the contribution of $P2X_1$ receptors to the phenotype of SCG neurons, and the contribution of other P2X receptor subunits to P2X receptor-mediated responses in SCG remains to be determined. It is also possible that the properties of SCG P2X receptors may be subject to additional modification by phosphorylation (Boue-Grabot et al., 2000), splice variants (Brandle et al., 1997; Simon et al., 1997), or additional regulatory factors.

The functional characterization of heteromeric P2X receptor channels and their contribution to native phenotypes has relied on distinct properties of heteromeric channels [e.g., α,β-meATP–sensitive, nondesensitizing $P2X_{2/3}$ receptors (Lewis et al., 1995)]. α,β-meATP sensitivity is associated with the expression of $P2X_1$, $P2X_3$, and $P2X_6$ receptor subunits (Table 2). Previous studies have demonstrated a functional role of α,β-meATP–sensitive $P2X_1$ receptors in smooth muscle and blood cells (Mulryan et al., 2000; Vial et al., 2002; Oury et al., 2003). $P2X_1$ receptor expression in neurons has been shown by in situ hybridization (Collo et al., 1996) and immunohistochemical studies (Vulchanova et al., 1996), and it has been suggested, based on electrophysiological and immunohistochemical studies, that $P2X_1$ receptors contribute to the rapidly desensitizing response in a subset of rat dorsal root ganglion neurons in which $P2X_3$ receptor immunoreactivity was below the limit of detection (Petruska et al., 2000a,b). Once again, this suggests that there may be species variation in P2X receptor-mediated responses in neurons, because α,β-meATP–sensitive P2X receptor currents were abolished in sensory neurons from $P2X_3$ receptor-deficient mice (Cockayne et al., 2000; Souslova et al., 2000; Zhong et al., 2001). In this study, the 80% reduction of α,β-meATP–sensitive responses in neurons from $P2X_1$ receptor-deficient mice shows directly that $P2X_1$ receptor subunits can be functionally expressed in neurons and results in α,β-meATP sensitivity in ~10% of SCG neurons.

This study demonstrates that $P2X_1$ receptor subunits contribute to the heteromeric assembly of P2X receptors in neurons, because the time course and ionic modulation of $P2X_1$ receptor subunit-dependent α,β-meATP–evoked responses were different from homomeric $P2X_1$ receptor channels (Evans et al., 1996; Stoop et al., 1997; Mulryan et al., 2000). The reason for this preferential incorporation of $P2X_1$ receptor subunits into heteromeric rather than homomeric channels remains to be determined but could reflect the relative abundance of different P2X receptor subunits being processed in the neuron. In calcium imaging studies, we demonstrated that the α,β-meATP–evoked responses show sensitivity to ionic modulation (inhibition by high extracellular calcium and increasing pH) that bear the hallmark of the contribution of $P2X_2$ receptors and not that of homomeric $P2X_1$ receptors (Evans et al., 1996; Stoop et al., 1997). Given the preponderance of functional of $P2X_2$ receptors in the SCG

(Cockayne et al., 2002), it seems likely that the α,β-meATP–sensitive receptors described in this study incorporate both $P2X_1$ and $P2X_2$ receptor subunits. Recombinant heteromeric rat $P2X_{1/2}$ receptors have been described previously in which the phenotype has the rapidly inactivating component of the $P2X_1$ receptor and responses are increased by raised pH (Brown et al., 2002). However, in our study, the abolition of the response to high pH and the relatively slow time course of the α,β-meATP–sensitive response contrast with those of Brown et al. (2002) and suggest that a heteromeric channel incorporating only $P2X_1$ and $P2X_2$ receptor subunits is unlikely to account for the responses observed (unless the responses possibly reflect different subunit assembly stoichiometry or species differences). Because P2X receptors are thought to form as trimers, this suggests that, in addition to $P2X_1$ and $P2X_2$ receptor subunits, an additional P2X receptor subunit contributes to α,β-meATP–sensitive heterotrimeric channels in the SCG.

In summary we have shown there are at least three broad categories of P2X receptor expression in SCG neurons: 1) neurons with a phenotype dominated by the properties of $P2X_2$ receptors, 2) α,β-meATP–sensitive neurons that reflect the expression of $P2X_1$ receptor subunits, most probably as part of heteromeric channels, and 3) neurons that have no detectable functional P2X receptor expression. This study shows a role of $P2X_1$ receptors in neurons. However, in contrast to homomeric $P2X_1$ channel expression in smooth muscle and platelets, we propose that it forms heteromeric P2X receptors, containing $P2X_1$ and $P2X_2$ receptor subunits, in SCG neurons. $P2X_1$ receptors are thought to be expressed widely in the nervous system, and a contribution of this receptor subunit to functional P2X receptors in other neuronal preparations seems likely.

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