Guinea-pig sympathetic neurons express varying proportions of two distinct P2X receptors

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1. Characterization of P2X receptors on neurons of guinea-pig superior cervical ganglion (SCG) has been carried out using a whole-cell voltage-clamp technique.
2. Application of ATP and α,β-methylene ATP (αβ-MeATP) produced fast activating inward currents, which desensitized slowly. The maximum response to αβ-MeATP was 36 ± 23% (range 0-1-100%) of that evoked by ATP in the same cell.
3. Co-application of αβ-MeATP (300 μM) with ATP (300 μM) produced a response that was 97 ± 1% of that given by ATP alone. Following desensitization with αβ-MeATP, the decrease in response to ATP was equal to the absolute reduction in response to αβ-MeATP in the same cell.
4. The concentration-response curve for αβ-MeATP had an EC50 of 42 μM and a Hill coefficient of 1.17. For cells where the ratio of αβ-MeATP/ATP currents at 100 μM was <0.1, the ATP concentration-response curve had an EC50 of 56 μM and a Hill coefficient of 1.95. However, in cells where the ratio was >0.7, the curve had an EC50 of 60 μM and a Hill coefficient of 0.97.
5. The response to 100 μM αβ-MeATP was inhibited by 2’ (or 3’) O-trinitrophenyl ATP (TNP-ATP) with an IC50 of 70 nM. However, on cells where the ratio of αβ-MeATP/ATP currents was <0.1, ATP was inhibited by TNP-ATP with an IC50 of 522 nM.
6. Immunohistochemical staining with antibodies raised against rat P2X1 and P2X4 epitopes suggested that both subunits were expressed by guinea-pig SCG neurons.
7. We conclude that varying proportions of two distinct P2X receptors coexist on the cell bodies of individual guinea-pig SCG neurons, which may correspond to homomeric P2X1 and heteromeric P2X1/4 receptors.

It is now generally accepted that ATP can act as a fast excitatory neurotransmitter at the autonomic neuromuscular junction (for review, see Burnstock, 1997), in the central nervous system (Edwards et al. 1992; Bardoni et al. 1997; Neiber et al. 1997), myenteric neurons (LePard et al. 1997) and cultured coeliac ganglion neurons (Evans et al. 1992; Silinsky & Gerzanich, 1993), where it activates a class of ligand-gated cation channels, the P2X receptors. ATP also plays a role in presynaptic modulation of transmitter release (Gu & MacDermott, 1997; Khakh & Henderson, 1998).

To date seven P2X receptor subunits (P2X1-7) have been cloned. The functional homo-oligomeric receptors formed have different, but overlapping, biophysical and pharmacological properties (Brake et al. 1994; Valera et al. 1994; Bo et al. 1995; Chen et al. 1995; Collo et al. 1996; Suprenant et al. 1996; for review, see North & Barnard, 1997). Thus, P2X1 and P2X4 receptors are activated by α,β-methylene ATP (αβ-MeATP) and desensitize rapidly, whereas P2X2 receptors do not respond to this ligand and desensitize very slowly (Brake et al. 1994; Valera et al. 1994; Chen et al. 1995). In addition, some subunits can combine together to form hetero-oligomeric receptors with novel pharmacological and biophysical profiles (Lewis et al. 1995; Lé et al. 1998; Torres et al. 1998). For example, although homomeric P2X4 receptors give rise to fast desensitizing responses, hetero-multimeric P2X1/4 receptors respond to αβ-MeATP (a property of P2X4 receptors), but desensitize slowly (a property of P2X4 receptors). Furthermore, alternatively spliced variants of P2X receptor subunits have been reported for rat P2X receptors (Brändle et al. 1997; Simon et al. 1997), guinea-pig P2X4 receptors (Parker et al. 1998), mouse P2X4 receptors (Simon et al. 1999; Townsend-Nicholson et al. 1999) and human P2X4 receptors (Carpenter et al. 1999). The heterologously expressed rat P2X2δ splice
variant desensitized faster than P2X$_{4\alpha}$ receptors. The presence of splice variants may thus increase the variety of endogenous P2X receptors.

In the rat, sensory neurons from dorsal root (Robertson et al. 1996; Rae et al. 1998), nodose (Lewis et al. 1995) and trigeminal ganglia (Cook et al. 1997), all exhibit $\alpha\beta$-Me-ATP sensitivity. Pharmacological studies suggest that the P2X receptors present on these sensory neurons are mainly P2X$_1$ and/or P2X$_{1\gamma}$ subtypes (Lewis et al. 1995; Cook et al. 1997; Grubb & Evans, 1999; Ueno et al. 1999; Li et al. 1999). In contrast, the pharmacology of P2X receptors in rat superior cervical ganglion (SCG) neurons (Nakazawa, 1994) and the molecular and pharmacological properties of P2X receptors in rat pelvic ganglion neurons (Zhong et al. 1998) suggest them to be of the P2X$_2$ subtype. Interestingly, $\alpha\beta$-Me-ATP evoked responses from neurons in intact guinea-pig SCG (Reekie & Burnstock, 1994), and acted as a full agonist in guinea-pig cardiac ganglion neurons and rat nodose ganglion neurons (Khakh et al. 1995). This raises the possibility that, in the guinea-pig, the P2X$_2$ subunit may play a significant role in neurons other than sensory neurons. An alternative explanation for sensitivity to $\alpha\beta$-Me-ATP might be the expression of the P2X$_1$ subunit in these sympathetic neurons. In this study we have used electrophysiological recording and immunohistochemical techniques to characterize the P2X receptors present in neurons of the guinea-pig SCG. Part of the work has appeared in the form of an abstract (Zhong et al. 1999).

**METHODS**

**Cell culture**

Single neurons from the SCG of male guinea-pigs (200 g) were enzymatically isolated as described previously (Zhong et al. 1998). Briefly, guinea-pigs were killed by inhalation of a rising concentration of CO$_2$ and death was confirmed by cardiac haemorrhage. The SCG were rapidly dissected out, and placed in Leibovitz L-15 medium (Life Technologies, Paisley, UK). The ganglia were then debrided, cut and incubated in 4 ml Ca$^{2+}$- and Mg$^{2+}$-free Hank's balanced salt solution with 10 mM Hepes buffer (pH 7.4) (HBSS; Life Technologies) containing 1.5 mg ml$^{-1}$ collagenase (Clat II, Worthington Biochemical Corporation, Reading, UK) and 6 mg ml$^{-1}$ bovine serum albumin (Sigma, Poole, UK) at 37°C for 60 min. The ganglia were then incubated in 4 ml HBSS containing 1 mg ml$^{-1}$ trypsin (Sigma) at 37°C for 20 min. The solution was replaced with 3 ml growth medium comprised of L-15 medium supplemented with 10% bovine serum, 50 ng ml$^{-1}$ nerve growth factor, 0.2% NaHCO$_3$, 5.5 mg ml$^{-1}$ glucose, 200 1 u ml$^{-1}$ penicillin and 200 $\mu$g ml$^{-1}$ streptomycin. The ganglia were dissociated into single neurons by gentle titration. The cells were then centrifuged at 160 g for 5 min, resuspended in 1 ml growth medium and plated onto 35 mm Petri dishes coated with 10 $\mu$g 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, USA). Membrane potential was held at $-70$ mV.
purinoceptor family. Peptides corresponding to 15 amino acid residues of the C-terminal region have been used to generate subtype-selective antibodies (Roche, BioScience). These peptides were covalently linked to keyhole limpet haemocyanin, and rabbits were immunized with the conjugated peptide by multiple monthly injection (performed by Research Genetics, Inc., Huntsville, AL, USA). The sequences of the synthetic peptides were: P2X$_4$, QQDSTSTDPKGLAQG; and P2X$_3$, VEQQSTDGSAYGISHG (see Xiang et al. 1998, for peptide sequences for other P2X receptor subtypes). The specificity of the antibodies was verified by immunoblotting with the membrane preparation from CHO-K1 cells expressing the cloned P2X$_4$ to P2X$_3$ receptors (Oglesby et al. 1999).

Antibodies against rat P2X$_4$ receptors have been used in this study, using the avidin–biotin (ABC) technique (Llewellyn-Smith et al. 1993; Zheng et al. 1998). Briefly, the sections were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 10 min. Endogenous peroxidase was blocked with 50% methanol containing 0.4% hydrogen peroxide (H$_2$O$_2$) for 10 min. Non-specific binding sites were blocked by a 20 min incubation with 10% normal horse serum (NHS) (Life Technologies) in phosphate-buffered saline (PBS) containing 0.05% merthiolate (Sigma). The sections were incubated with the primary antibodies diluted to 2.5 µg ml$^{-1}$ (determined as optimal by previous titrations) with 10% NHS in PBS containing 0.05% merthiolate overnight. Subsequently the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, PA, USA) diluted 1:500 in 1% NHS in PBS containing 0.05% merthiolate for 1 h, followed by incubation with ExtrAvidin–horseradish peroxidase (Sigma) diluted 1:1500 in PBS containing 0.05% merthiolate for 1 h. All incubations were held at room temperature and separated by three 5 min washes in PBS. Finally, a freshly prepared colour reaction mixture containing 0.5% 3,3′-diaminobenzidine, 0.1 M sodium phosphate, 0.004% NH$_4$Cl, 0.2% glucose, 0.04% nickel ammonium sulphate and 0.1% glucose oxidase was applied to the sections for 5–10 min or until colour product appeared. The sections were then washed, dehydrated, cleared in xylene and mounted using Eukitt (BDH, Poole, UK). Control experiments were performed using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and thus confirm a specific immunoreaction.

Drugs

ATP and αβ-MeATP were obtained from Sigma Chemical Co. (Poole, UK). βγ-Methylene-l-ATP was obtained from Torrie Cookson (Bristol, UK). 2′ (or 3′) -O-trinitropheryl-ATP was obtained from Molecular Probes (Leiden, Netherlands). Solutions (10–100 mM) 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.

RESULTS

Responses to agonists

Fast application of ATP (3–300 µM) onto isolated guinea-pig SCG neurons, voltage clamped at −70 mV, evoked a rapidly activating inward current in all cells tested (>400 cells). The response to ATP desensitized slowly, with the current at the end of the 5 s application being 80 ± 2% (n = 5) of the peak amplitude. The mean peak amplitude of the response to 100 µM ATP was 7.9 ± 4.2 nA (mean ± s.d., n = 371).

A response to αβ-MeATP (100 µM) was seen in >95% of cells tested, but was always less than that to ATP (100 µM) (Fig. 1A). The mean peak amplitude of the currents evoked by 100 µM αβ-MeATP was 2.8 ± 3.4 nA (mean ± s.d., n = 371). When the two agonists were tested on the same cells, the current elicited by 100 µM αβ-MeATP was 36 ± 23% (range 0–1–100%) of that evoked by 100 µM ATP, although the EC$_{50}$ values for these two agonists were similar (see Figs 1 and 4). The ratio of currents to αβ-MeATP and ATP at 100 µM from the same neuron (αβ-MeATP/ATP ratio) varied considerably from cell to cell. The frequency distribution of the αβ-MeATP/ATP ratio for each of 387 cells was clearly non-Gaussian (Fig. 1B), with the probability of cells having a ratio between 0 and 0.5 being quite uniform. However, there were very few cells showing a ratio greater than 0.8.

When the αβ-MeATP response was normalized with respect to that produced by 100 µM ATP from the same cell, the individual concentration–response curves yielded variable maximum responses (Fig. 1C). However, when the response to αβ-MeATP was normalized with respect to that produced by 100 µM αβ-MeATP from the same cell, the data were tight, with small error bars (Fig. 1D). Fitting the Hill equation to individual dose–response curves for αβ-MeATP gave a mean EC$_{50}$ of 42 µM (log EC$_{50}$ = −4.38 ± 0.05) and a Hill coefficient of 1.17 ± 0.07 (n = 13).

βγ-Methylene-l-ATP (βγ-Me-1-ATP) was reported to be a selective agonist on P2X$_3$ receptors, with little activity on P2X$_4$ receptors (Trezise et al. 1995). On guinea-pig SCG neurons, βγ-Me-1-ATP was much less potent than αβ-MeATP, producing no response at concentrations less than 100 µM (Fig. 1D).

A larger maximum response to ATP than to αβ-MeATP might indicate that the latter is a partial agonist. Alternatively, this could result from the presence of a mixed population of receptors. To discriminate between these possibilities, we investigated the interaction between ATP and αβ-MeATP at a near-maximum concentration (300 µM), and the effect of cross-desensitization.

Co-application of ATP and αβ-MeATP

If αβ-MeATP is a partial agonist binding to the same sites as ATP, then co-application of both agonists at near-maximum concentrations will result in a reduction in the response to ATP. This is because a significant percentage of the receptors will be occupied by the partial agonist. As illustrated in Fig. 2 and B, co-application of αβ-MeATP (300 µM) with ATP (300 µM) produced a response that was very close to that given by ATP alone. Furthermore, the response in the presence of both agonists was very different from that predicted if αβ-MeATP was a partial agonist (see Methods). In nine cells tested in this series of experiments, co-application of αβ-MeATP (300 µM) with ATP (300 µM)
produced responses which were 97 ± 1% of those given by ATP alone. On the other hand, when ATP (300 μM) was applied in the presence of αβ-MeATP (300 μM), the response was similar to that evoked by ATP on its own (95 ± 4%, n = 5; Fig. 2C).

**Cross-desensitization**

The effect of long application of 100 μM αβ-MeATP is illustrated in Fig. 3A and B. The time course of the decline in the αβ-MeATP-induced current fitted well to the sum of two exponentials, with time constants (τ₁ and τ₂) of 5·2 ± 0·8 and 32·1 ± 2·6 s (n = 8). For 10 cells examined in this series of experiments, the peak response evoked by 100 μM αβ-MeATP was 45 ± 6% of that to 100 μM ATP. After a 2 min application of 100 μM αβ-MeATP, the response to αβ-MeATP had declined to 14 ± 3% of the peak (i.e. to 7 ± 2% of the peak ATP response), while the response to 100 μM ATP was only reduced to 62 ± 4% (n = 10) of control. Therefore, the fractional reduction in the αβ-MeATP response was much greater than that of the ATP response (P < 0·001). Further examination revealed that the absolute reduction in αβ-MeATP response (d') following desensitization was similar to the reduction in the ATP response (d) (d'/d = 99 ± 3%, n = 10), while the absolute difference between the responses evoked by ATP and αβ-MeATP before (Δ) and after (Δ') the desensitization remained unchanged (Δ'/Δ = 99 ± 4%) (neither d'/d nor Δ'/Δ was significantly different from 100%, P > 0·1).

On the other hand, when the cells were desensitized by a prolonged exposure to 100 μM ATP, the responses to αβ-MeATP and ATP were reduced proportionally (Fig. 3C). The responses to αβ-MeATP and ATP at the end of the 2 min desensitization were 18 ± 5 and 15 ± 3% (n = 10) of the control, respectively. The time course of the decline in the ATP-induced current also fitted well to the sum of two exponentials, with time constants (τ₁ and τ₂) of 5·0 ± 0·4 and 33·3 ± 3·8 s (n = 10).

These results support the presence of two populations of P2X receptors, both sensitive to ATP but only one activated by αβ-MeATP.
Concentration–response curve for ATP

Although preliminary studies have shown that the concentration–response curve for ATP appeared to be monophasic, it must be assumed in the light of the foregoing results that there are in fact two components present (data not shown). We therefore attempted to look specifically for cells showing a small or large $\alpha\beta$-MeATP/ATP ratio, i.e. to select cells with predominantly one population of P2X

Figure 2. Co-application of ATP and $\alpha\beta$-MeATP to guinea-pig superior cervical ganglion neurons

A, response of one cell to $\alpha\beta$-MeATP (300 $\mu$M), ATP (300 $\mu$M) and the two agonists combined. First a response to $\alpha\beta$-MeATP was recorded. Then ATP was applied for a total of 9 s either alone (first 3 s and last 3 s) or in combination with $\alpha\beta$-MeATP (the middle 3 s). The current evoked by 300 $\mu$M $\alpha\beta$-MeATP was 1 % of that by 300 $\mu$M ATP. Co-application of $\alpha\beta$-MeATP and ATP evoked a current that was slightly bigger than that evoked by ATP alone. The decline of the ATP response was fitted to the single exponential decay (superimposed dashed line). The predicted current amplitude which would be evoked by co-application of $\alpha\beta$-MeATP and ATP, assuming $\alpha\beta$-MeATP to be a partial agonist, is shown by the dotted line (see Methods). The data were distinctly different from the prediction. B, recordings from another cell in which the response to $\alpha\beta$-MeATP was 65 % of that produced by ATP, co-application of $\alpha\beta$-MeATP and ATP resulted in a current that was slightly smaller than that by ATP itself. C, data from another cell in which a response to ATP was obtained first on its own, then when co-applied during the application of $\alpha\beta$-MeATP. The co-application evoked a current that was similar to that evoked by ATP itself. Neurons were voltage clamped at −70 mV. The bars above the traces indicate the duration of agonist application.
receptors on them, and to examine the concentration–response relationships for ATP on each population separately (Fig. 4). In eight cells showing an αβ-MeATP/ATP ratio < 0.1 (mean ratio = 0.07 ± 0.01, mean capacitance = 31.2 ± 5.7 pF), the ATP current could be regarded as due to the activation of a single population of αβ-MeATP-insensitive receptors. Fitting the Hill equation to the data gave an EC₅₀ of 56 μM (logEC₅₀ = −4.25 ± 0.11, data from 8 cells) and a Hill coefficient of 1.95.

Cells with a large αβ-MeATP/ATP ratio were encountered less frequently. From a total of 83 cells, we obtained 7 cells that had an αβ-MeATP/ATP ratio > 0.7 (mean ratio = 0.8 ± 0.02, mean capacitance = 49.6 ± 5.4 pF). For this group of cells, the concentration–response relationship for ATP could be regarded as being dominated by the αβ-MeATP-sensitive receptors. Fitting the Hill equation to the data gave an EC₅₀ of 60 μM (logEC₅₀ = −4.22 ± 0.26, data from 7 cells) and a Hill coefficient of 0.97.

Effects of TNP-ATP

Recently, the P₂X antagonist TNP-ATP (Mockett et al. 1994; King et al. 1997) has been described as a selective antagonist on P₂X₁, P₂X₃ and P₂X₅₄ forms relative to P₂X₂ receptors (Virgino et al. 1998). We sought to determine whether TNP-ATP would show different affinity for the two types of P₂X receptor present on guinea-pig SCG neurons.

TNP-ATP (0.001–10 μM) reversibly attenuated the response activated by 100 μM αβ-MeATP. This inhibition was fitted well by a single site model, giving an IC₅₀ of 70 nM (logIC₅₀ = −7.16 ± 0.08, data from 8 cells), and a Hill coefficient of 0.92 (Fig. 5). The inhibition by TNP-ATP of the response to 100 μM ATP varied substantially from cell to cell. Hence, we specifically looked for cells showing an αβ-MeATP/ATP ratio < 0.1, in which the ATP current was largely due to the activation of αβ-MeATP-insensitive receptors. A total of 25 such cells were studied in this series.

Figure 3. Cross-desensitization with αβ-MeATP or ATP on guinea-pig superior cervical ganglion neurons

Traces of the membrane current recorded from three cells in response to a prolonged application of 100 μM αβ-MeATP or ATP A, on a neuron where the peak response evoked by 100 μM αβ-MeATP was 67% of that evoked by 100 μM ATP, 2 min application of 100 μM αβ-MeATP reduced the response to αβ-MeATP to 14% of control. In contrast, the response to 100 μM ATP was reduced to 44% of its own control. However, the absolute reduction in αβ-MeATP response (d') was comparable to that in the ATP response (d) (d'/d = 103%), and the absolute difference between the responses evoked by ATP and αβ-MeATP before (Δ) and after (Δ') desensitization was similar (Δ/Δ' = 110%). B, records from a different SCG neuron, where the response evoked by 100 μM αβ-MeATP was 31% of that produced by 100 μM ATP. A 2 min application of 100 μM αβ-MeATP desensitized the response to αβ-MeATP to 18% of the peak αβ-MeATP response, while that to 100 μM ATP was only reduced to 73% of control. Again, the absolute reduction in αβ-MeATP response was comparable to that in the ATP response (d'/d = 107%), and the absolute difference between the responses evoked by ATP and αβ-MeATP remained unchanged (Δ/Δ' = 99%). C, on a cell where the response evoked by 100 μM αβ-MeATP was 43% of that by 100 μM ATP, 2 min desensitization by 100 μM ATP reduced the response to αβ-MeATP and ATP to 27 and 30% of the control, respectively. Cells were voltage clamped at −70 mV. The bars above the traces indicate the duration of agonist application.
of experiments. Using 100 μM ATP as the agonist, the inhibition by TNP-ATP fitted well to a single component curve, yielding an IC₅₀ of 522 nM (logIC₅₀ = −6.28 ± 0.13, n = 3–6 for each data point), and a Hill coefficient of 0.79. This was significantly different from the IC₅₀ value of TNP-ATP on the αβ-MeATP response (P < 0.01). We also examined cells showing an αβ-MeATP/ATP ratio of 0.3–0.6. For these cells, the inhibition by TNP-ATP of the response to 100 μM ATP could be fitted with a single component curve, with an IC₅₀ of 195 nM (logIC₅₀ = −6.71 ± 0.06), and a Hill coefficient of 0.86 (n = 3–6 for each data point, pooled data from 33 cells, Fig. 5). Although these data could also be well fitted by a two-component curve using the IC₅₀ values previously determined (70 and 522 nM), with equal proportions of high and low affinity binding sites (see Fig. 5), the fit was not significantly better than that for the single site model (F-test, P > 0.1).

Variation of the αβ-MeATP/ATP ratio
As mentioned above, the maximum response of guinea-pig SCG neurons to αβ-MeATP was always less than that to ATP, but the αβ-MeATP/ATP ratio varied greatly from cell to cell. When we selected cells with very large or very small αβ-MeATP/ATP ratios, there was a marked difference in membrane capacitance (see above). However, among the 367 cells, the correlation between cell size (as determined by membrane capacitance) and the αβ-MeATP/ATP ratio was weak yet significant (Pearson’s r = 0.22, P < 0.0001, n = 367, data not shown).

Immunohistochemical evidence
To characterize further the P2X receptors on guinea-pig SCG, we carried out immunohistochemistry using the currently available antibodies raised against rat P2X₃ (rP2X₃) receptors. So far, the P2X₃ receptor is the only member of this family cloned from the guinea-pig (Parker et al. 1998). For this subunit, there are three splice variants which all share a common C-terminal peptide sequence, which differs by only one amino acid from that of the rat. Therefore, it is likely that the antibodies raised against rat P2X₃ receptors are also able to recognize the P2X receptors expressed in guinea-pigs.

To check this, we tested antibodies specific for rP2X₂ and rP2X₁ receptors on guinea-pig nodose ganglion sections (Fig. 6A and B). The small-diameter neurons showed strong P2X₂ immunoreactivity, while medium-diameter neurons showed less intense P2X₃ staining, with some large-diameter neurons being negative. In contrast, specific and strong P2X₃ immunoreactivity was only detected in a subpopulation of neurons. The staining pattern was similar to that observed on rat nodose ganglion for these two receptor subtypes (Xiang et al. 1998).

We then applied antibodies against rP2X₁ to guinea-pig SCG and pelvic ganglion sections. As shown in Fig. 6C and D, neurons in the guinea-pig pelvic ganglion showed specific

Figure 4. Concentration–response curves for ATP on guinea-pig superior cervical ganglion neurons
Concentration–response curves for ATP were constructed separately for guinea-pig SCG neurons with a small or large αβ-MeATP/ATP ratio (the ratio of αβ-MeATP/ATP currents at 100 μM from the same neuron). For 8 cells with a small αβ-MeATP/ATP ratio (mean ratio = 0.07 ± 0.01; ■), fitting the Hill equation to the data gave an EC₅₀ of 56 μM and a Hill coefficient of 1.95. For seven cells with a large αβ-MeATP/ATP ratio (mean ratio = 0.8 ± 0.02; □), fitting the data to the Hill equation gave an EC₅₀ of 60 μM and a Hill coefficient of 0.97. Responses were normalized with respect to that obtained with 100 μM ATP on the same cell.

Figure 5. Antagonism of P2X receptors in guinea-pig superior cervical ganglion neurons by 2' (or 3')-O-trinitrophenyl-ATP (TNP-ATP)
Inhibition by TNP-ATP was studied using 100 μM αβ-MeATP (●) or 100 μM ATP (▲, △) as the agonist on cells with an αβ-MeATP/ATP ratio < 0.1 (▲) or between 0.3–0.6 (△). With 100 μM αβ-MeATP as the agonist, the inhibition by TNP-ATP fitted well to a single component curve, having an IC₅₀ of 70 nM (data from 8 cells). When 100 μM ATP was the agonist, on cells showing an αβ-MeATP/ATP ratio < 0.1, fitting the Hill equation to the pooled data from 25 cells gave an IC₅₀ of 522 nM (n = 3–6 for each data point). On cells showing an αβ-MeATP/ATP ratio of 0.3–0.6, the degree of inhibition of the ATP response by TNP-ATP was in between the above two. Although this inhibition could be fitted by a two-component curve with IC₅₀ values of 70 and 522 nM (superimposed dashed line), this was not significantly better than that obtained with a single component curve (continuous line). The responses were normalized to that obtained with agonist (100 μM) in the absence of TNP-ATP on the same cell. TNP-ATP was present for 2 min before and during the re-application of agonist.
Figure 6. Immunohistochemical staining in guinea-pig sensory and autonomic ganglia using polyclonal antibodies specific for rat P2X$_2$ and P2X$_3$ receptors

P2X$_2$ (A) and P2X$_3$ (B) immunoreactivity in guinea-pig nodose ganglion. P2X$_2$ (C) and P2X$_3$ (D) immunoreactivity in guinea-pig pelvic ganglion. In the same pelvic ganglion, the immunoreactivity is abolished after absorption of rP2X$_2$ antibody with P2X$_2$ peptide (E) and absorption of rP2X$_3$ antibody with P2X$_3$ peptide (F). P2X$_2$ (G) and P2X$_3$ (H) immunoreactivity in guinea-pig superior cervical ganglion. Scale bar, 100 μm.
immunoreactivity to both P2X$_3$ and P2X$_4$ antibodies. The specificity of the immunoreaction was ascertained with the peptide pre-absorption. Thus, incubation of the antibodies with an excess of corresponding peptides used for immunization abolished the immunoreactivity (Fig. 6E and F). In sections of guinea-pig SCG (Fig. 6G and H), immunoreactivity for P2X$_3$ and P2X$_4$ was clearly present. The staining for P2X$_4$ appeared to be cell membrane-associated, and was present in most neurons, while specific P2X$_3$ immunoreactivity was detected in a sub-population of neurons. Antibodies to rP2X$_3$ and rP2X$_{4,6}$ receptors failed to detect any immunoreactivity in guinea-pig SCG and pelvic ganglion neurons (data not shown). However, these antibodies did produce specific staining in guinea-pig blood vessel smooth muscle (P2X$_4$), spinous and granular cell layers of epithelium (P2X$_3$) and cerebellar Purkinje cells (P2X$_4$ and P2X$_{4,6}$)(data not shown), suggesting that they do recognize the corresponding guinea-pig receptors.

DISCUSSION

Co-expression of two P2X receptors in the same guinea-pig SCG neuron

The major finding in this study is the demonstration of the presence of mixed populations of P2X receptors on guinea-pig SCG neurons. Several lines of evidence are in support of this. 1) The response to $\alpha$-$\beta$-MeATP (100 $\mu$M) was always smaller than that to ATP (100 $\mu$M) from the same neuron, and the ratio of the $\alpha$-$\beta$-MeATP/ATP currents varied greatly from cell to cell. This phenomenon could not be explained by the assumption that $\alpha$-$\beta$-MeATP behaved as a partial agonist. 2) Co-application of the near-maximum concentration of $\alpha$-$\beta$-MeATP with ATP did not significantly inhibit the response to ATP as would be predicted if $\alpha$-$\beta$-MeATP was a partial agonist. 3) On desensitizing these neurons with $\alpha$-$\beta$-MeATP, the response to ATP was not reduced proportionally, but by the same absolute amount as the response to $\alpha$-$\beta$-MeATP. 4) Responses to $\alpha$-$\beta$-MeATP and ATP had different sensitivity to the antagonist TNP-ATP. Furthermore, when ATP was the agonist, the sensitivity to TNP-ATP depended on the $\alpha$-$\beta$-MeATP/ATP ratio. Thus, with $\alpha$-$\beta$-MeATP as the agonist, the $IC_{50}$ for TNP-ATP was 70 nM, while for cells expressing predominantly $\alpha$-$\beta$-MeATP-insensitive receptors, the $IC_{50}$ against ATP was 522 nM. All this evidence strongly suggests that there are two distinct populations of P2X receptors co-existing on the same guinea-pig SCG neuron, one is sensitive to $\alpha$-$\beta$-MeATP whilst the other one is not, with the proportion of each receptor subtype varying from cell to cell. This is very similar to the situation in rat nodose neurons (Thomas et al. 1998).

Is $\alpha$-$\beta$-MeATP a partial agonist?

$\alpha$-$\beta$-MeATP has been reported to be a full agonist on guinea-pig coeliac ganglion, rat nodose and rat dorsal root ganglion (DRG) neurons (Khakh et al. 1995; Lewis et al. 1995; Robertson et al. 1996). On recombinant heteromeric P2X$_{4,6}$ receptors (Lo et al. 1998), guinea-pig intracardiac neurons (Allen & Burnstock, 1990) and rat cardiac neurons (Fiebier & Adams, 1991), $\alpha$-$\beta$-MeATP has been proposed to act as a partial agonist since it was less potent than ATP and produced a smaller maximum response. However, on guinea-pig myenteric neurons (Zhou & Gallega, 1996), $\alpha$-$\beta$-MeATP clearly evoked variable maximum responses in different cells. We found that co-application of near-maximal concentrations of $\alpha$-$\beta$-MeATP and ATP to guinea-pig SCG neurons did not affect the ATP-induced inward currents. A similar effect has been reported on guinea-pig myenteric neurons (Barajas-Lopez et al. 1996). Thus, our results are not consistent with the suggestion that $\alpha$-$\beta$-MeATP is a partial agonist, but rather that both $\alpha$-$\beta$-MeATP-sensitive and -insensitive receptors are co-expressed on the same cell, with $\alpha$-$\beta$-MeATP being a full agonist at the former. In the light of our findings and the work of Thomas et al. (1998), it is clear that $\alpha$-$\beta$-MeATP can produce a reduced maximum response compared with ATP, with the relative amplitudes varying from cell to cell, if mixed P2X receptor populations coexist on the same neuron. Analysis at the single channel level may be required to confirm or refute the suggestion that $\alpha$-$\beta$-MeATP is a partial agonist on other neurons.

Possible identity of the P2X receptors on guinea-pig SCG neurons

The slowly desensitizing response to $\alpha$-$\beta$-MeATP indicates that the $\alpha$-$\beta$-MeATP-sensitive receptors on these neurons may be of P2X$_{4,6}$ phenotype, like those found in rat nodose ganglion (Lewis et al. 1995), trigeminal ganglion (Cook et al. 1997) and capsaicin-insensitive DRG neurons (Ueno et al. 1999). We demonstrated, using the polyclonal antibodies raised against rat P2X receptors, the presence of the P2X$_4$ and P2X$_6$ immunoreactivity in SCG and pelvic neurons of the guinea-pig. This is in contrast to the situation in the rat pelvic ganglion, where only the P2X$_4$ was identified (Zhong et al. 1998). Apart from P2X$_4$ (Parker et al. 1998), all the other guinea-pig P2X receptor subtypes have yet to be cloned, so the degree of homology between rat and guinea-pig P2X receptors is at present unknown, and some caution must be used in the interpretation of these results. Nevertheless, the C-terminal peptide sequences used to raise these antibodies are well conserved between P2X$_2$ receptors from rat and guinea-pig, and between other P2X receptors from rat and human. Furthermore, although we cannot completely exclude the possibility that our antibodies raised against the rat sequences do not recognize the corresponding guinea-pig receptors, a similar staining pattern was observed for P2X$_{4,6}$ antibodies in appropriate guinea-pig tissues, compared with that in rat tissues. This suggests that these antibodies may correctly recognize guinea-pig P2X receptors and that the receptors on guinea-pig SCG neurons, like those on rat nodose neurons, may be P2X$_4$ and P2X$_{4,6}$.

The presence of varying proportions of two types of P2X receptors on guinea-pig SCG neurons greatly complicated their pharmacological characterization. For $\alpha$-$\beta$-MeATP,
presumed to be acting on a single population of receptors, we
obtained an EC$_{50}$ value of 42 nM. This is lower than the
EC$_{50}$ values found on recombinant rP2X$_{4/3}$ receptors (Lewis
et al. 1995), rat nodose neurons and guinea-pig coeliac
neurons (Khakh et al. 1995). However, the EC$_{50}$ value we
obtained was similar to those found on capsaicin-insensitive
rat DRG neurons (Ueno et al. 1999), and on rP2X$_{4/3}$
receptors co-expressed in C6BU-1 gloma cells (Ueno et al.
1998). It is possible that the coexistence of P2X$_2$ and P2X$_3$
subunits could result in several subtypes of heteromeric
receptors of unknown stoichiometry, with different affinity
for agonists. Furthermore, we cannot exclude the possibility
of the involvement of other P2X subunits, or of splice
variants. Of the recombinant homomeric receptors, only
P2X$_2$ and P2X$_3$ receptors respond to $\alpha\beta$-MeATP. However,
the P2X$_{1/3}$-selective agonist $\beta\gamma$-Me-1-ATP (Trezise et al.
1995) was much less potent than $\alpha\beta$-MeATP on guinea-pig
SCG neurons, suggesting that the P2X$_1$ subunit is unlikely
to be involved.

There is at present no selective agonist for the $\alpha\beta$-MeATP-
insensitive receptor. We therefore selected cells with a
$\alpha\beta$-MeATP/ATP ratio < 0.1, where there were
predominantly $\alpha\beta$-MeATP-insensitive receptors and obtained
an EC$_{50}$ value for ATP of 56 nM and a Hill
coefficient of 1.95. As cells with a very large $\alpha\beta$-MeATP/
ATP ratio (> 0.9) were encountered very rarely (~1% of
cells), it was not possible to obtain a precise value for the
affinity of ATP at the $\alpha\beta$-MeATP-sensitive receptor. However,
analysis of cells where the $\alpha\beta$-MeATP/ATP ratio was
> 0.7 revealed an EC$_{50}$ value for ATP similar to that at
$\alpha\beta$-MeATP-insensitive receptors. Interestingly, the Hill
coefficient was considerably less (0.97). This might indicate
the presence of multiple receptor subtypes, or that there is
positive co-operativity at the $\alpha\beta$-MeATP-insensitive receptor,
but not at the $\alpha\beta$-MeATP-sensitive ones. Irrespective of the
reason, a similar difference in Hill coefficients was observed
between $\alpha\beta$-MeATP-insensitive receptors in rat nodose
and $\alpha\beta$-MeATP-insensitive receptors in rat SCG
neurons (Khakh et al. 1995).

Recently, the coexistence of homomeric P2X$_2$ and
heteromeric P2X$_{4/3}$ receptors has been revealed on rat
nodose ganglion neurons using a selective antagonist TNP-ATP
(Thomas et al. 1998), which has 1000-fold higher potency on
recombinant rP2X$_{4/3}$ than rP2X$_2$ receptors. On guinea-pig
SCG neurons, the IC$_{50}$ values for TNP-ATP on $\alpha\beta$-MeATP-
sensitive and $\alpha\beta$-MeATP-insensitive receptors were 70 and
522 nM, respectively. This 8-fold difference was smaller than
that reported between rP2X$_2$ and rP2X$_{4/3}$ receptors, and
the IC$_{50}$ value for TNP-ATP against $\alpha\beta$-MeATP is greater
than that reported for the heterologously expressed rP2X$_{4/3}$
receptors (Virgilio et al. 1998). One possible explanation for
this is that TNP-ATP may be unstable in aqueous solution.
However, the greater than expected potency of the
$\alpha\beta$-MeATP-insensitive receptors would argue against this.
Furthermore, we have observed inhibition by TNP-ATP of
a rapidly desensitizing ATP response in rat DRG neurons,
with an IC$_{50}$ of approximately 1 nM under identical
conditions (P. Dunn, Y. Zhong & G. Burnstock, unpublished
observations). In a study on rat nodose neurons, the inhibition of $\alpha\beta$-MeATP by TNP-ATP was biphasic, with
the lower affinity component having an IC$_{50}$ of 50 nM
(Thomas et al. 1998), which is quite similar to the value we
obtained. Because of the small difference in affinity of TNP-
ATP for $\alpha\beta$-MeATP-sensitive and -insensitive receptors, the
inhibition curve for cells having approximately equal
numbers of both receptors was not clearly biphasic. Although
we cannot rule out the possibility that guinea-pig SCG
neurons possess a novel P2X receptor, a more likely
explanation is that guinea-pig P2X$_2$ and P2X$_{4/3}$ receptors
may exhibit slightly different pharmacological properties
compared with those of the rat.

**Inter-species variation**

Another finding in this study is the species difference in the
expression of P2X receptors between rat and guinea-pig.
Previous work by Khakh et al. (1995) demonstrated that
neurons in the guinea-pig coeliac ganglion, like those in the
rat nodose ganglion, respond to $\alpha\beta$-MeATP, while those in
the rat SCG do not. On the basis of those observations the
authors suggested that neurons of the SCG may be
anomalous. An alternative explanation, and one which we
favour, is that expression of P2X receptor subtypes is
different in rat and guinea-pig. Thus, neurons from guinea-
pig SCG (this study), coeliac (Khakh et al. 1995) and pelvic
ganglia (Y. Zhong, P. M. Dunn & G. Burnstock, unpublished
observations) all respond to $\alpha\beta$-MeATP, while those in
the rat SCG (Nakazawa, 1994), coeliac (Zhong et al. 2000) and
pelvic ganglia (Zhong et al. 1998) do not. In addition, the
properties of the P2X$_2$ receptors are different in the outer
hair cells of rat and guinea-pig (Chen et al. 1997). There
seems, therefore, to be inter-ganglion (e.g. autonomic vs.
sensory ganglion) as well as inter-species (e.g. rat vs.
guinea-pig) differences in the expression of P2X receptors.

In the rat, high levels of P2X$_2$ subunit expression appear to
be localized exclusively in sensory neurons (Buell et al.
1996), with a low level expression in sympathetic neurons
detectable by immunohistochemistry and in situ
hybridization (Xiang et al. 1998). However, immuno-
histochemical and pharmacological data indicate that the
expression of P2X$_2$ subunits may be more widespread in
the guinea-pig.

The presence of multiple receptor subtypes occurring in the
same neuron has been observed previously for nicotinic
acetylcholine receptors (Connolly et al. 1995; Poth et al.
1997), and more recently for P2X receptors (Thomas et al.
1998; Grubb & Evans, 1999; this study). At present, it is not
clear what factors may control the expression of these mixed
populations of P2X receptors. Whether the proportions of
them are determined simply by the relative amounts of the
subunits synthesized and remains constant in individual cells,
or whether the proportions can change in developmental or
pathological conditions remains to be determined.
In conclusion, in the present study, we have characterized P2X receptors on single neurons of guinea-pig SCG, using subtype-selective agonists, antagonists and immunohistochemistry. Our results suggest that varying proportions of two distinct P2X receptors coexist on the same neuron, which may correspond to homomeric P2X<sub>1</sub> and heteromeric P2X<sub>1/4</sub> receptors. Thus, there is an inter-species difference in the expression of P2X receptors in sympathetic neurons.


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