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 ($\alpha\beta$-MeATP) produced fast activating inward currents, which desensitized slowly. The maximum response to $\alpha\beta$-MeATP was $36 \pm 23\,\%$ (range 0·1–100 %) of that evoked by ATP in the same cell.

3. Co-application of $\alpha\beta$-MeATP (300 μM) with ATP (300 μM) produced a response that was $97 \pm 1\,\%$ of that given by ATP alone. Following desensitization with $\alpha\beta$-MeATP, the decrease in response to ATP was equal to the absolute reduction in response to $\alpha\beta$-MeATP in the same cell.

4. The concentration–response curve for $\alpha\beta$-MeATP had an EC_{50} of 42 μM and a Hill coefficient of 1·17. For cells where the ratio of $\alpha\beta$-MeATP/ATP currents at 100 μM was $< 0{\cdot}1$, the ATP concentration–response curve had an EC_{50} of 56 μM and a Hill coefficient of 1·95. However, in cells where the ratio was $> 0{\cdot}7$, the curve had an EC_{50} of 60 μM and a Hill coefficient of 0·97.

5. The response to 100 μM $\alpha\beta$-MeATP was inhibited by 2′ (or 3′)-*O*-trinitrophenyl-ATP (TNP-ATP) with an IC_{50} of 70 nM. However, on cells where the ratio of $\alpha\beta$-MeATP/ATP currents was $< 0{\cdot}1$, ATP was inhibited by TNP-ATP with an IC_{50} of 522 nM.

6. Immunohistochemical staining with antibodies raised against rat $P2X_2$ and $P2X_3$ 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 $P2X_2$ and heteromeric $P2X_{2/3}$ 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; Nieber *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 ($P2X_{1\text{-}7}$) 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; Surprenant *et al.* 1996; for review, see North & Barnard, 1997). Thus, $P2X_1$ and $P2X_3$ receptors are activated by α,β-methyleneATP ($\alpha\beta$-MeATP) and desensitize rapidly, whereas $P2X_2$ 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; Lê *et al.* 1998; Torres *et al.* 1998). For example, although homomeric $P2X_3$ receptors give rise to fast desensitizing responses, hetero-multimeric $P2X_{2/3}$ receptors respond to $\alpha\beta$-MeATP (a property of $P2X_3$ receptors), but desensitize slowly (a property of $P2X_2$ receptors). Furthermore, alternatively spliced variants of P2X receptor subunits have been reported for rat $P2X_2$ receptors (Brändle *et al.* 1997; Simon *et al.* 1997), guinea-pig $P2X_2$ receptors (Parker *et al.* 1998), mouse $P2X_4$ receptors (Simon *et al.* 1999; Townsend-Nicholson *et al.* 1999) and human $P2X_4$ receptors (Carpenter *et al.* 1999). The heterologously expressed rat $P2X_{2(b)}$ splice

variant desensitized faster than $P2X_{2(a)}$ 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 $\alpha\beta$-MeATP sensitivity. Pharmacological studies suggest that the P2X receptors present on these sensory neurons are mainly $P2X_3$ and/or $P2X_{2/3}$ 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, $\alpha\beta$-MeATP evoked responses from neurons in intact guinea-pig SCG (Reekie & Burnstock, 1994), and acted as a full agonist in guinea-pig coeliac ganglion neurons and rat nodose ganglion neurons (Khakh *et al.* 1995). This raises the possibility that, in the guinea-pig, the $P2X_3$ subunit may play a significant role in neurons other than sensory neurons. An alternative explanation for sensitivity to $\alpha\beta$-MeATP 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 desheathed, cut and incubated in 4 ml Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution with 10 mM Hepes buffer (pH 7·4) (HBSS; Life Technologies) containing 1·5 mg ml^{-1} collagenase (Class 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 i.u. ml^{-1} penicillin and 200 μg ml^{-1} streptomycin. The ganglia were dissociated into single neurons by gentle trituration. 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 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, USA). Membrane potential was held at −70 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 which contained (mM): KCl 120, Hepes 10 and tripotassium citrate 10; the pH was adjusted to 7·2 using KOH, or a similar solution in which K^+ was replaced by Cs^+. No difference in response was observed between the two internal solutions. Series resistance compensation of 72–75% was used in all recordings. Data were acquired using pCLAMP software (Axon Instruments). Signals were filtered at 2 kHz (−3 dB frequency, Bessel filter, 80 dB per decade).

Drugs were applied rapidly through a 7-barrel manifold comprising fused glass capillaries inserted into a common outlet tube (tip diameter of ~200 μm) which was placed about 200 μm from the cell (Dunn *et al.* 1996). Solutions were delivered by gravity flow from independent reservoirs. One barrel was used to apply drug-free solution to enable rapid termination of drug application. Solution exchange measured by changes in open tip current was complete in 200 ms; however, complete exchange of solution around an intact cell was considerably slower ($\leqslant$ 1 s).

Data analysis

All responses were normalized to that evoked by 100 μM ATP in the same cell, unless otherwise stated. Except where indicated to the contrary, all data are expressed as the means ± S.E.M. Statistical analysis (Student's *t* test, *F* test and Pearson's correlation test) was performed using Prism v2 (Graphpad, San Diego, CA, USA).

Concentration–response data were fitted with the Hill equation: $Y = A/[1 + (K/X)^{n_H}]$, where A is the maximum effect, K is the EC_{50} and n_H is the Hill coefficient, using Prism v2. 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 (Microcal, Northampton, MA, USA). The desensitization traces were fitted using Clampfit (pCLAMP) software, to both the first and second order exponential decay. However, for the 2 min application of agonists, a significantly better fit was consistently found using the second order exponential decay (*F* test, $P < 0{\cdot}0001$).

In the series of experiments where $\alpha\beta$-MeATP and ATP were co-applied, the predicted curve assuming $\alpha\beta$-MeATP to be a partial agonist was calculated as follows. The decline of the current in the presence of 300 μM ATP was fitted using the first order exponential decay. $\alpha\beta$-MeATP and ATP were assumed to act on a single population of receptors, where $\alpha\beta$-MeATP was a partial agonist with similar affinity to ATP but lower efficacy (E, given by the ratio of the peak amplitude of the responses to the same near-maximal concentration of $\alpha\beta$-MeATP and ATP from the same neuron). Then, in the presence of $\alpha\beta$-MeATP (300 μM) and ATP (300 μM), half of the receptors would be occupied by $\alpha\beta$-MeATP and half by ATP. The fitted curve was then scaled by a factor of $0{\cdot}5(1 + E)$ to yield the predicted response.

Immunohistochemistry

Male guinea-pigs (200 g) were killed as described above and the nodose, pelvic and superior cervical ganglia were dissected out. These ganglia 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 slides and air-dried at room temperature. The slides were stored at −20 °C.

Sequence analysis has revealed that the carboxyl terminal region is one of the least conserved amongst members of the rat P2X

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: $P2X_2$, QQDSTSTDPKGLAQL; and $P2X_3$, VEKQSTDSGAYSIGH (see Xiang *et al.* 1998, for peptide sequences for other P2X receptor subtypes). The specificity of the antisera was verified by immunoblotting with the membrane preparation from CHO-K1 cells expressing the cloned $P2X_1$ to $P2X_6$ receptors (Oglesby *et al.* 1999). Immunoglobulin G (IgG) fractions were isolated from the preimmune and immune sera following the method of Harboe & Ingild (1973). The protein concentration was determined at 280 nm using an extinction factor of 1·43 for 1 mg ml^{-1}.

Antibodies against rat $P2X_{1-6}$ receptors have been used in this study, using the avidin–biotin (ABC) technique (Llewellyn-Smith *et al.* 1993; Zhong *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_2O_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_4Cl, 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 $\alpha\beta$-MeATP were obtained from Sigma Chemical Co. (Poole, UK). β,γ-Methylene-L-ATP was obtained from Tocris Cookson (Bristol, UK). 2′ (or 3′)-*O*-trinitrophenyl-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 $\alpha\beta$-MeATP (100 μM) was seen in > 95% of cells tested, but was always less than that to ATP (100 μM) (Fig. 1*A*). The mean peak amplitude of the currents evoked by 100 μM $\alpha\beta$-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 $\alpha\beta$-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 $\alpha\beta$-MeATP and ATP at 100 μM from the same neuron ($\alpha\beta$-MeATP/ATP ratio) varied considerably from cell to cell. The frequency distribution of the $\alpha\beta$-MeATP/ATP ratio for each of 367 cells was clearly non-Gaussian (Fig. 1*B*), 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 $\alpha\beta$-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. 1*C*). However, when the response to $\alpha\beta$-MeATP was normalized with respect to that produced by 100 μM $\alpha\beta$-MeATP from the same cell, the data were tight, with small error bars (Fig. 1*D*). Fitting the Hill equation to individual dose–response curves for $\alpha\beta$-MeATP gave a mean EC_{50} of 42 μM ($\log EC_{50} = -4{\cdot}38 \pm 0{\cdot}05$) and a Hill coefficient of 1·17 ± 0·07 ($n = 13$).

β,γ-Methylene-L-ATP ($\beta\gamma$-Me-L-ATP) was reported to be a selective agonist on $P2X_1$ receptors, with little activity on $P2X_3$ receptors (Trezise *et al.* 1995). On guinea-pig SCG neurons, $\beta\gamma$-Me-L-ATP was much less potent than $\alpha\beta$-MeATP, producing no response at concentrations less than 100 μM (Fig. 1*D*).

A larger maximum response to ATP than to $\alpha\beta$-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 $\alpha\beta$-MeATP at a near-maximum concentration (300 μM), and the effect of cross-desensitization.

Co-application of ATP and $\alpha\beta$-MeATP

If $\alpha\beta$-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*A* and *B*, co-application of $\alpha\beta$-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 $\alpha\beta$-MeATP was a partial agonist (see Methods). In nine cells tested in this series of experiments, co-application of $\alpha\beta$-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. 2*C*).

Cross-desensitization

The effect of long application of 100 μM αβ-MeATP is illustrated in Fig. 3*A* 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 (τ_1 and τ_2) 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 responses), while the response to 100 μM ATP was only reduced to 62 ± 6 % ($n = 10$) of control. Therefore, the fractional reduction in the αβ-MeATP response was much greater than that of the ATP response ($P < 0{\cdot}001$). Further examination revealed that the absolute reduction in αβ-MeATP response (d') following desensitization was similar to the reduction in the ATP response (d) ($d'/d = 99 \pm 3$ %, $n = 10$), while the absolute difference between the responses evoked by ATP and αβ-MeATP before (Δ) and after (Δ′) the desensitization remained unchanged ($\Delta'/\Delta = 99 \pm 4$ %) (neither d'/d nor Δ'/Δ was significantly different from 100 %, $P > 0{\cdot}1$).

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. 3*C*). 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 (τ_1 and τ_2) 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.

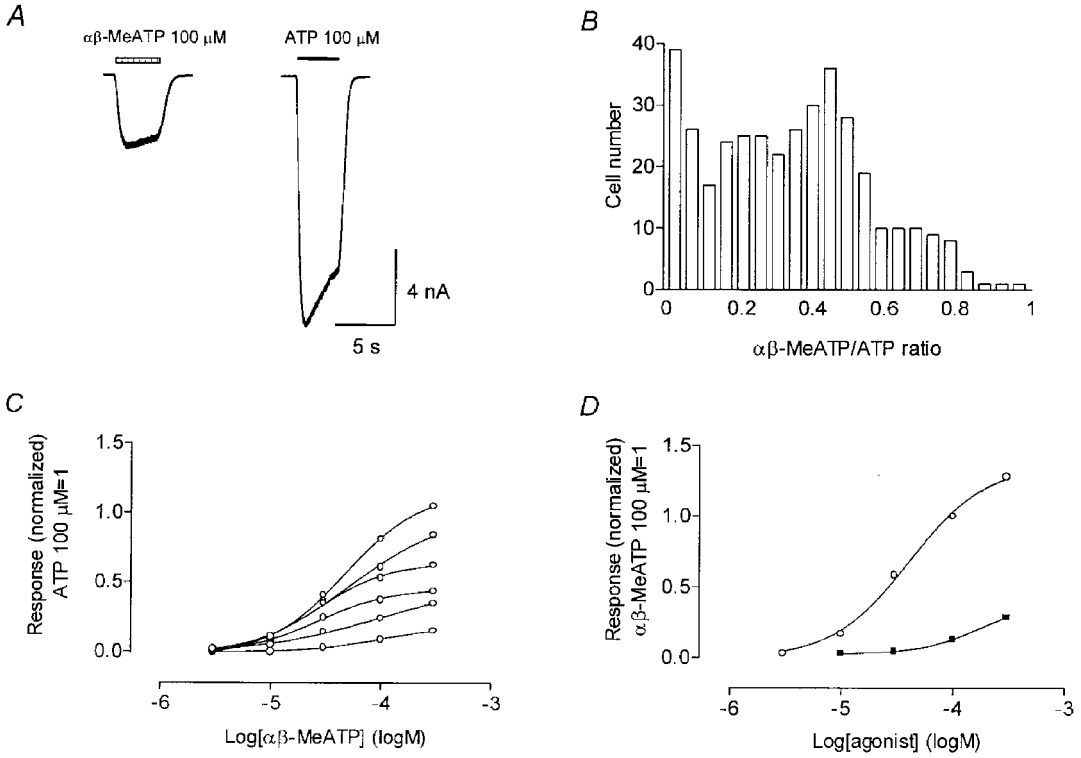

Figure 1. Agonist responses in isolated guinea-pig superior cervical ganglion (SCG) neurons

A, representative traces of the inward currents activated by 100 μM αβ-MeATP and ATP in a guinea-pig SCG neuron voltage clamped at −70 mV. The bars above the traces indicate the duration of agonist application. *B*, distribution of the ratio of responses to 100 μM αβ-MeATP and 100 μM ATP (αβ-MeATP/ATP ratio) on the same cell from each of 367 guinea-pig SCG neurons. *C*, individual concentration–response curves for αβ-MeATP on six SCG neurons, with responses normalized to that obtained with 100 μM ATP on the same cell. *D*, concentration–response curves for αβ-MeATP (○) and βγ-Me-L-ATP (■), with responses normalized to that obtained with 100 μM αβ-MeATP on the same cell. Points represent mean ± S.E.M. for 13 cells (○) and 4 cells (■), respectively. When not visible, error bars lie within the symbol. Fitting the Hill equation to individual concentration–response curves for αβ-MeATP gave a mean EC_{50} of 42 μM and a Hill coefficient of 1·17 ($n = 13$). Agonists were applied for 5 s at 2 min intervals, which was sufficient for responses to be reproducible.

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

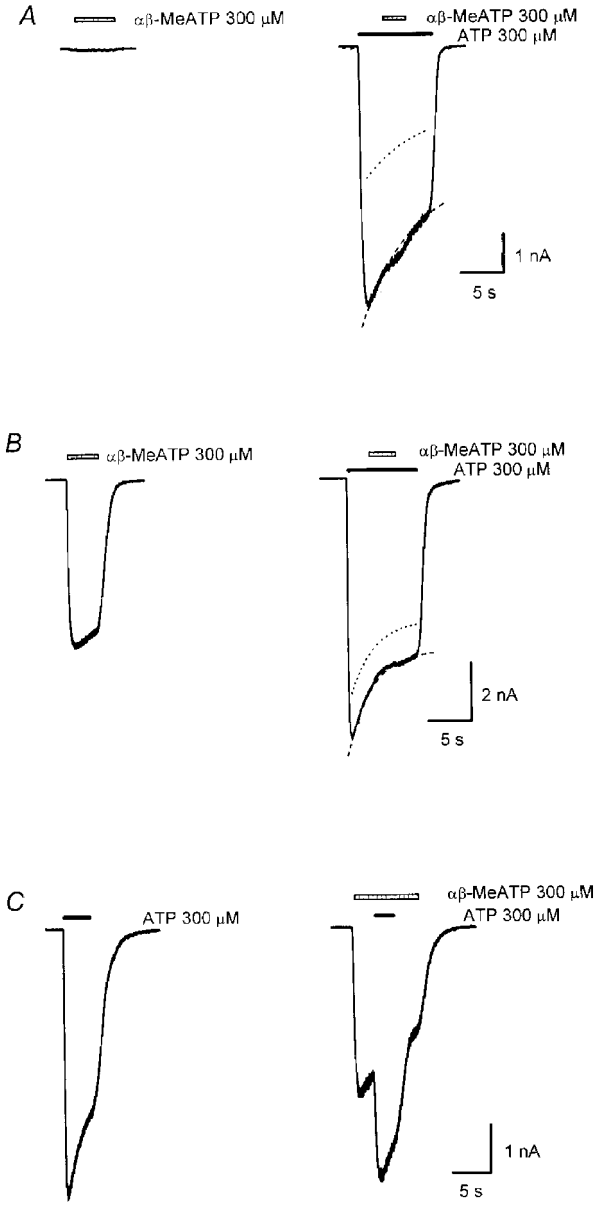

Figure 2. Co-application of ATP and $\alpha\beta$-MeATP to guinea-pig superior cervical ganglion neurons

A, response of one cell to $\alpha\beta$-MeATP (300 μM), ATP (300 μM) 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 μM $\alpha\beta$-MeATP was 1 % of that by 300 μM 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 $\alpha\beta$-MeATP/ATP ratio $< 0{\cdot}1$ (mean ratio $= 0{\cdot}07 \pm 0{\cdot}01$, mean capacitance $= 31{\cdot}2 \pm 5{\cdot}7$ pF), the ATP current could be regarded as due to the activation of a single population of $\alpha\beta$-MeATP-insensitive receptors. Fitting the Hill equation to the data gave an EC_{50} of 56 μM ($\log EC_{50} = -4{\cdot}25 \pm 0{\cdot}11$, data from 8 cells) and a Hill coefficient of 1·95.

Cells with a large $\alpha\beta$-MeATP/ATP ratio were encountered less frequently. From a total of 83 cells, we obtained 7 cells that had an $\alpha\beta$-MeATP/ATP ratio $> 0{\cdot}7$ (mean ratio $= 0{\cdot}8 \pm 0{\cdot}02$, mean capacitance $= 49{\cdot}6 \pm 5{\cdot}4$ pF). For this group of cells, the concentration–response relationship for ATP could be regarded as being dominated by the $\alpha\beta$-MeATP-sensitive receptors. Fitting the Hill equation to the data gave an EC_{50} of 60 μM ($\log EC_{50} = -4{\cdot}22 \pm 0{\cdot}26$, 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 $P2X_1$, $P2X_3$ and $P2X_{2/3}$ forms relative to $P2X_2$ receptors (Virginio *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 $\alpha\beta$-MeATP. This inhibition was fitted well by a single site model, giving an IC_{50} of 70 nM ($\log IC_{50} = -7{\cdot}16 \pm 0{\cdot}08$, 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 $\alpha\beta$-MeATP/ATP ratio $< 0{\cdot}1$, in which the ATP current was largely due to the activation of $\alpha\beta$-MeATP-insensitive receptors. A total of 25 such cells were studied in this series

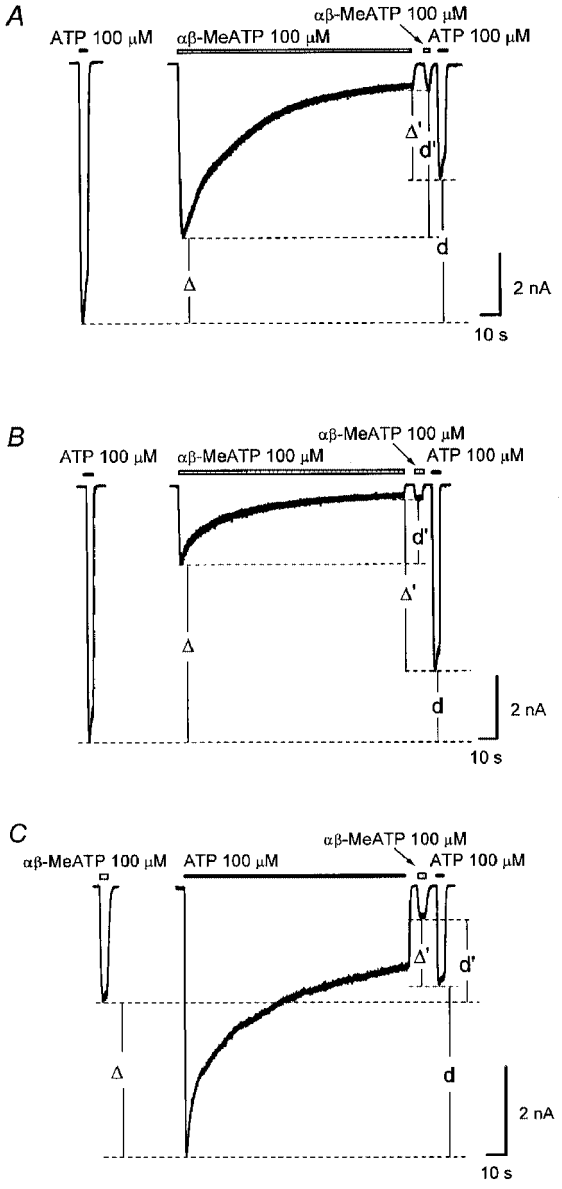

Figure 3. Cross-desensitization with $\alpha\beta$-MeATP or ATP on guinea-pig superior cervical ganglion neurons

Traces of the membrane current recorded from three cells in response to a prolonged application of 100 μM $\alpha\beta$-MeATP or ATP. *A*, on a neuron where the peak response evoked by 100 μM $\alpha\beta$-MeATP was 67 % of that evoked by 100 μM ATP, 2 min application of 100 μM $\alpha\beta$-MeATP reduced the response to $\alpha\beta$-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 $\alpha\beta$-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 $\alpha\beta$-MeATP before (Δ) and after (Δ') desensitization was similar ($\Delta'/\Delta = 110$ %). *B*, records from a different SCG neuron, where the response evoked by 100 μM $\alpha\beta$-MeATP was 31 % of that produced by 100 μM ATP. A 2 min application of 100 μM $\alpha\beta$-MeATP desensitized the response to $\alpha\beta$-MeATP to 18 % of the peak $\alpha\beta$-MeATP response, while that to 100 μM ATP was only reduced to 73 % of control. Again, the absolute reduction in $\alpha\beta$-MeATP response was comparable to that in the ATP response ($d'/d = 107$ %), and the absolute difference between the responses evoked by ATP and $\alpha\beta$-MeATP remained unchanged ($\Delta'/\Delta = 99$ %). *C*, on a cell where the response evoked by 100 μM $\alpha\beta$-MeATP was 43 % of that by 100 μM ATP, 2 min desensitization by 100 μM ATP reduced the responses to $\alpha\beta$-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 IC_{50} of 522 nM ($\log IC_{50} = -6{\cdot}28 \pm 0{\cdot}13$, $n = 3$–6 for each data point), and a Hill coefficient of 0·79. This was significantly different from the IC_{50} value of TNP-ATP on the $\alpha\beta$-MeATP response ($P < 0{\cdot}01$). We also examined cells showing an $\alpha\beta$-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 IC_{50} of 195 nM ($\log IC_{50} = -6{\cdot}71 \pm 0{\cdot}06$), 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 IC_{50} 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, $P > 0{\cdot}1$).

Variation of the $\alpha\beta$-MeATP/ATP ratio

As mentioned above, the maximum response of guinea-pig SCG neurons to $\alpha\beta$-MeATP was always less than that to ATP, but the $\alpha\beta$-MeATP/ATP ratio varied greatly from cell to cell. When we selected cells with very large or very small $\alpha\beta$-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 $\alpha\beta$-MeATP/ATP ratio was weak yet significant (Pearson's $r = 0{\cdot}22$, $P < 0{\cdot}0001$, $n = 367$, 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 $P2X_{1\text{-}6}$ ($rP2X_{1\text{-}6}$) receptors. So far, the $P2X_2$ 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 P2X receptors are also able to recognize the P2X receptors expressed in guinea-pigs.

To check this, we tested antibodies specific for $rP2X_2$ and $rP2X_3$ receptors on guinea-pig nodose ganglion sections (Fig. 6*A* and *B*). The small-diameter neurons showed strong $P2X_3$ immunoreactivity, while medium-diameter neurons showed less intense $P2X_3$ staining, with some large-diameter neurons being negative. In contrast, specific and strong $P2X_2$ immunoreactivity was only detected in a sub-population 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 $rP2X_{1\text{-}6}$ to guinea-pig SCG and pelvic ganglion sections. As shown in Fig. 6*C* and *D*, neurons in the guinea-pig pelvic ganglion showed specific

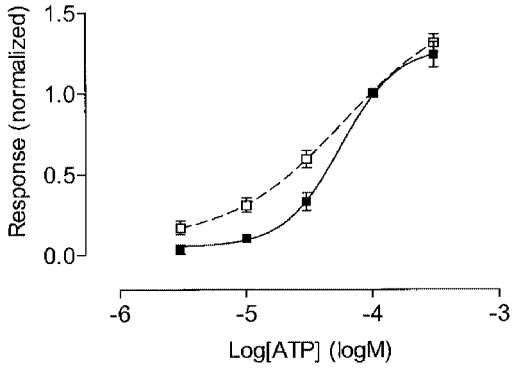

Figure 4. Concentration–response curves for ATP on guinea-pig superior cervical ganglion neurons

Concentration–response curves for ATP were constructed separately for guinea-pig SCG neurons with a small or large $\alpha\beta$-MeATP/ATP ratio (the ratio of $\alpha\beta$-MeATP/ATP currents at 100 μM from the same neuron). For 8 cells with a small $\alpha\beta$-MeATP/ATP ratio (mean ratio = 0·07 ± 0·01; ■), fitting the Hill equation to the data gave an EC_{50} of 56 μM and a Hill coefficient of 1·95. For seven cells with a large $\alpha\beta$-MeATP/ATP ratio (mean ratio = 0·8 ± 0·02; □), fitting the data to the Hill equation gave an EC_{50} 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.

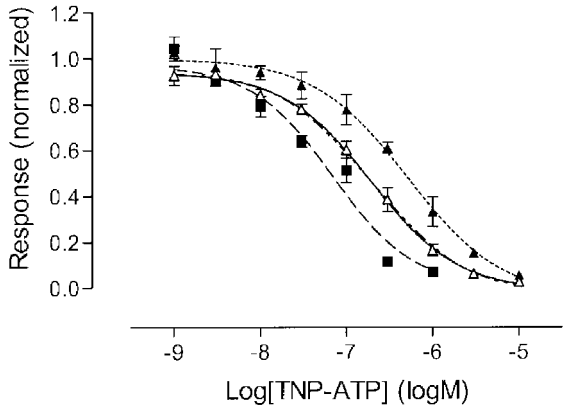

Figure 5. Antagonism of P2X receptors in guinea-pig superior cervical ganglion neurons by 2′ (or 3′)-*O*-trinitrophenyl-ATP (TNP-ATP)

Inhibition by TNP-ATP was studied using 100 μM $\alpha\beta$-MeATP (■) or 100 μM ATP (▲, △) as the agonist on cells with an $\alpha\beta$-MeATP/ATP ratio < 0·1 (▲) or between 0·3–0·6 (△). With 100 μM $\alpha\beta$-MeATP as the agonist, the inhibition by TNP-ATP fitted well to a single component curve, having an IC_{50} of 70 nM (data from 8 cells). When 100 μM ATP was the agonist, on cells showing an $\alpha\beta$-MeATP/ATP ratio < 0·1, fitting the Hill equation to the pooled data from 25 cells gave an IC_{50} of 522 nM ($n = 3$–6 for each data point). On cells showing an $\alpha\beta$-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 IC_{50} 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 agonists.

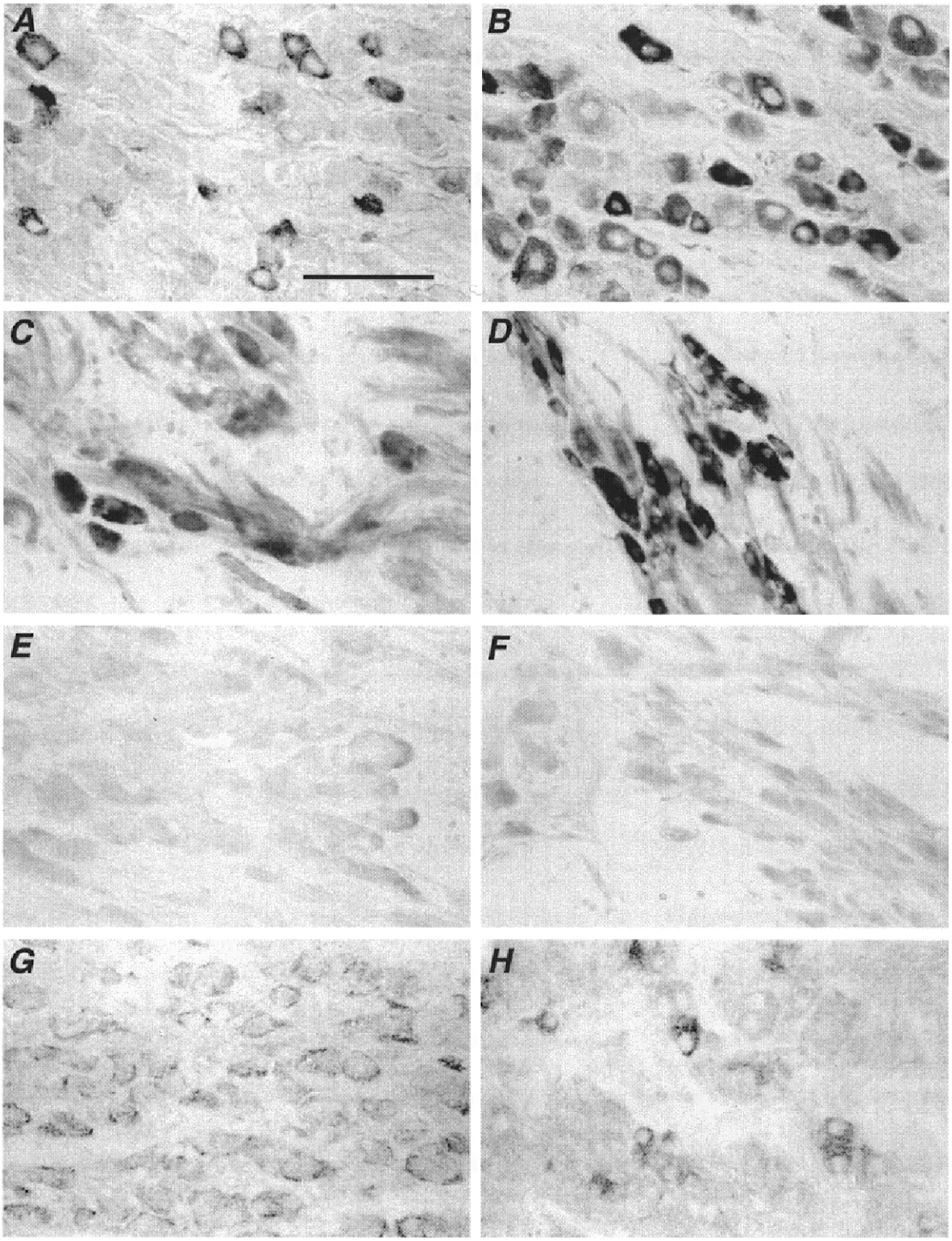

Figure 6. Immunohistochemical staining in guinea-pig sensory and autonomic ganglia using polyclonal antibodies specific for rat $P2X_2$ and $P2X_3$ receptors

$P2X_2$ (*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 $P2X_2$ and $P2X_3$ 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. 6*E* and *F*). In sections of guinea-pig SCG (Fig. 6*G* and *H*), immunoreactivity for $P2X_2$ and $P2X_3$ was clearly present. The staining for $P2X_2$ 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 $rP2X_1$ and $rP2X_{4\text{-}6}$ 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 ($P2X_1$), spinous and granular cell layers of epithelium ($P2X_5$) and cerebellar Purkinje cells ($P2X_4$ and $P2X_6$) (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 μM) was always smaller than that to ATP (100 μM) 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 (Lê *et al.* 1998), guinea-pig intracardiac neurons (Allen & Burnstock, 1990) and rat cardiac neurons (Fieber & 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 & Galligan, 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-López *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 $P2X_{2/3}$ 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 $P2X_2$ and $P2X_3$ 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 $P2X_2$ immunoreactivity was identified (Zhong *et al.* 1998). Apart from $P2X_2$ (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 $P2X_{1\text{-}6}$ 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 $P2X_2$ and $P2X_{2/3}$.

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 μM. This is lower than the EC_{50} values found on recombinant $rP2X_{2/3}$ 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 $rP2X_{2+3}$ receptors co-expressed in C6BU-1 glioma cells (Ueno *et al.* 1998). It is possible that the coexistence of $P2X_2$ and $P2X_3$ subunits could result in several subsets 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 $P2X_3$ and $P2X_1$ receptors respond to $\alpha\beta$-MeATP. However, the $P2X_1$-selective agonist $\beta\gamma$-Me-L-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 $< 0{\cdot}1$, where there were predominantly $\alpha\beta$-MeATP-insensitive receptors and obtained an EC_{50} value for ATP of 56 μM and a Hill coefficient of 1·95. As cells with a very large $\alpha\beta$-MeATP/ATP ratio ($> 0{\cdot}9$) 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 $> 0{\cdot}7$ 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-sensitive receptors in rat nodose neurons and $\alpha\beta$-MeATP-insensitive receptors in rat SCG neurons (Khakh *et al.* 1995).

Recently, the coexistence of homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ 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 $rP2X_{2/3}$ 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 $rP2X_{2/3}$ receptors, and the IC_{50} value for TNP-ATP against $\alpha\beta$-MeATP is greater than that reported for the heterologously expressed $rP2X_{2/3}$ receptors (Virginio *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 $P2X_{2/3}$ 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 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 $P2X_3$ 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, immunohistochemical and pharmacological data indicate that the expression of $P2X_3$ 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 $P2X_2$ and heteromeric $P2X_{2/3}$ receptors. Thus, there is an inter-species difference in the expression of P2X receptors in sympathetic neurons.

Allen, T. G. J. & Burnstock, G. (1990). The action of adenosine 5′-triphosphate on guinea-pig intracardiac neurons in culture. *British Journal of Pharmacology* **100**, 269–276.

Barajas-López, C., Huizinga, J. D., Collins, S. M., Gerzanich, V., Espinosa-Luna, R. & Peres, A. (1996). P2X-purinoceptors of myenteric neurons from the guinea-pig ileum and their unusual pharmacological properties. *British Journal of Pharmacology* **119**, 1541–1548.

Bardoni, R., Goldstein, P. A., Lee, C. J., Gu, J. G. & MacDermott, A. B. (1997). ATP P2X receptors mediate fast synaptic transmission in the dorsal horn of the rat spinal cord. *Journal of Neuroscience* **17**, 5297–5304.

Bo, X., Zhang, Y., Nassar, M., Burnstock, G. & Schoepfer, R. (1995). A P2X purinoceptor cDNA conferring a novel pharmacological profile. *FEBS Letters* **375**, 129–133.

Brake, A. J., Wagenbach, M. J. & Julius, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371**, 519–523.

Brändle, U., Spielmanns, P., Osteroth, R., Sim, J., Surprenant, A., Buell, G., Ruppersberg, J. P., Plinkert, P. K., Zenner, H.-P. & Glowatzki, E. (1997). Desensitization of the $P2X_2$ receptor controlled by alternative splicing. *FEBS Letters* **404**, 294–298.

Buell, G., Collo, G. & Rassendrn, F. (1996). P2X receptors: an emerging channel family. *European Journal of Pharmacology* **8**, 2221–2228.

Burnstock, G. (1997). The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* **36**, 1127–1139.

Carpenter, D., Meadows, H. J., Brough, S., Chapman, G., Clarke, C., Coldwell, M., Davis, R., Harrison, D., Meakin, J., McHale, M., Rice, S. Q., Tomlinson, W. J., Wood, M. & Sanger, G. J. (1999). Site-specific splice variation of the human P2X4 receptor. *Neuroscience Letters* **273**, 183–186.

Chen, C., LeBlanc, C. & Bobbin, R. P. (1997). Differences in the distribution of responses to ATP and acetylcholine between outer hair cells of rat and guinea-pig. *Hearing Research* **110**, 87–94.

Chen, C.-C., Akopian, A. N., Sivilotti, L., Colquhoun, D., Burnstock, G. & Wood, J. N. (1995). A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* **377**, 428–431.

Collo, G., North, R. A., Kawashima, E., Merlo-Pich, E., Neidhart, S., Surprenant, A. & Buell, G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels. *Journal of Neuroscience* **16**, 2495–2507.

Connolly, J. G., Gibb, A. J. & Colquhoun, D. (1995). Heterogeneity of neuronal nicotinic acetylcholine receptors in thin slices of rat medial habenula. *Journal of Physiology* **484**, 87–105.

Cook, S. P., Vulchanova, L., Hargreaves, K. M., Elde, R. & McCleskey, E. W. (1997). Distinct ATP receptors on pain-sensing and stretch-sensing neurons. *Nature* **387**, 505–508.

Dunn, P. M., Benton, D. C., Campos-Rosa, J., Ganellin, C. R. & Jenkinson, D. H. (1996). Discrimination between subtypes of apamin-sensitive Ca^{2+}-activated K^+ channels by gallamine and a novel bis-quaternary quinolinium cyclophane, UCL 1530. *British Journal of Pharmacology* **117**, 35–42.

Edwards, F. A., Gibb, A. J. & Colquhoun, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature* **359**, 144–147.

Evans, R. J., Derkach, V. & Surprenant, A. (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature* **357**, 503–505.

Fieber, L. A. & Adams, D. (1991). Adenosine triphosphate-evoked currents in cultured neurons dissociated from rat parasympathetic cardiac ganglia. *Journal of Physiology* **434**, 239–256.

Grubb, B. D. & Evans, R. J. (1999). Characterization of cultured dorsal root ganglion neuron P2X receptors. *European Journal of Neuroscience* **11**, 149–154.

Gu, J. G. & MacDermott, A. B. (1997). Activation of ATP P2X receptors elicits glutamate release from sensory neuron synapses. *Nature* **389**, 749–753.

Harboe, N. & Ingild, A. (1973). Immunization, isolation of immunoglobulins, estimation of antibody titre. *Scandinavian Journal of Immunology Supplement* **1**, 161–164.

Khakh, B. S. & Henderson, G. (1998). ATP receptor-mediated enhancement of fast excitatory neurotransmitter release in the brain. *Molecular Pharmacology* **54**, 372–378.

Khakh, B. S., Humphrey, P. P. A. & Surprenant, A. (1995). Electrophysiological properties of P2X-purinoceptors in rat superior cervical, nodose and guinea-pig coeliac neurons. *Journal of Physiology* **484**, 385–395.

King, B. F., Wildman, S. S., Ziganshina, L. E., Pintor, J. & Burnstock, G. (1997). Effects of extracellular pH on agonism and antagonism at a recombinant $P2X_2$ receptor. *British Journal of Pharmacology* **121**, 1445–1453.

Lê, K.-T., Babinski, K. & Séguéla, P. (1998). Central $P2X_4$ and $P2X_6$ channel subunits coassemble into a novel heteromeric ATP receptor. *Journal of Neuroscience* **18**, 7152–7159.

LePard, K. J., Messori, E. & Galligan, J. J. (1997). Purinergic fast excitatory postsynaptic potentials in myenteric neurons of guinea-pig: distribution and pharmacology. *Gastroenterology* **113**, 1522–1534.

Lewis, C., Neidhart, S., Holy, C., North, R. A., Buell, G. & Surprenant, A. (1995). Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* **377**, 432–435.

Li, C., Peoples, R. W., Lanthorn, T. H., Li, Z.-W. & Weight, F. (1999). Distinct ATP-activated currents in different types of neurons dissociated from rat dorsal root ganglion. *Neuroscience Letters* **263**, 57–60.

Llewellyn-Smith, I. J., Pilowsky, P. & Minson, J. B. (1993). The tungstate-stabilized tetramethylbenzidine reaction for light and electron microscopic immunocytochemistry and for revealing biocytin-filled neurons. *Journal of Neuroscience Methods* **46**, 27–40.

Mockett, B. G., Housley, G. D. & Thorne, P. R. (1994). Fluorescence imaging of extracellular purinergic receptor sites and putative ecto-ATPase sites on isolated cochlear hair cells. *Journal of Neuroscience* **14**, 6992–7007.

Nakazawa, K. (1994). ATP-activated current and its interaction with acetylcholine-activated current in rat sympathetic neurons. *Journal of Neuroscience* **14**, 740–750.

Nieber, K., Poelchen, W. & Illes, P. (1997). Role of ATP in fast excitatory synaptic potentials in locus coeruleus neurons of the rat. *British Journal of Pharmacology* **122**, 423–430.

North, A. & Barnard, E. A. (1997). Nucleotide receptors. *Current Opinion in Neurobiology* **7**, 346–357.

Oglesby, I. B., Lachnit, W. G., Burnstock, G. & Ford, A. P. D. W. (1999). Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, $P2X_1$ through $P2X_7$. *Drug Development Research* **47**, 189–195.

Parker, M. S., Larroque, M. L., Campbell, J. M., Bobbin, R. P. & Deininger, P. L. (1998) Novel variant of the $P2X_2$ ATP receptor from the guinea-pig organ of Corti. *Hearing Research* **121**, 62–70.

Poth, K., Nutter, T. J., Cuevas, J., Parker, M. J., Adams, D. J. & Luetje, C. W. (1997). Heterogeneity of nicotinic receptor class and subunit mRNA expression among individual parasympathetic neurons from rat intracardiac ganglia. *Journal of Neuroscience* **17**, 586–596.

Rae, M. G., Rowan, E. G. & Kennedy, C. (1998). Pharmacological properties of $P2X_3$-receptors present in neurons of the rat dorsal root ganglia. *British Journal of Pharmacology* **124**, 176–180.

Reekie, F. M. & Burnstock, G. (1994). Some effects of purines on neurons of guinea-pig superior cervical ganglia. *General Pharmacology* **25**, 143–148.

Robertson, S. J., Rae, M. G., Rowan, E. G. & Kennedy, C. (1996). Characterization of a P2X-purinoceptor in cultured neurons of the rat dorsal root ganglia. *British Journal of Pharmacology* **118**, 951–956.

Silinsky, E. M. & Gerzanich, V. (1993). On the excitatory effects of ATP and its role as a neurotransmitter in coeliac neurons of the guinea-pig. *Journal of Physiology* **464**, 197–212.

Simon, J., Chessell, I. P., Jones, C. A., Michel, A. D., Barnard, E. A. & Humphrey, P. P. A. (1999). Molecular cloning and characterisation of variants of the mouse $P2X_4$ receptor. *British Journal of Pharmacology* **126** (suppl.), 127P.

Simon, J., Kidd, E. J., Smith, F. M., Chessell, I. P., Murrell-Lagnado, R., Humphrey, P. P. A. & Barnard, E. A. (1997). Localization and functional expression of splice variants of the $P2X_2$ receptor. *Molecular Pharmacology* **52**, 237–248.

Surprenant, A., Rassendren, F., Kawashima., E., North, R. A. & Buell, G. (1996). The cytolytic P_{2Z} receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science* **272**, 735–738.

Thomas, S., Virginio, C., North, R. A. & Surprenant, A. (1998). The antagonist trinitrophenyl-ATP reveals coexistence of distinct P2X receptor channels in rat nodose neurons. *Journal of Physiology* **509**, 411–417.

Torres, G. E., Haines, W. R., Egan, T. M. & Voigt, M. M. (1998). Co-expression of $P2X_1$ and $P2X_5$ receptor subunits reveals a novel ATP-gated ion channel. *Molecular Pharmacology* **54**, 989–993.

Townsend-Nicholson, A., King, B. F., Wildman, S. S. & Burnstock, G. (1999). Molecular cloning, functional characterization and possible cooperativity between the murine $P2X_4$ and $P2X_{4a}$ receptors. *Molecular Brain Research* **64**, 246–254.

Trezise, D. J., Michel, A. D., Grahames, C. B. A., Khakh, B. S., Surprenant, A. & Humphrey, P. P. A. (1995). The selective P2X purinoceptor agonist, β,γ-methylene-L-adenosine 5′-triphosphate, discriminates between smooth muscle and neuronal P2X purinoceptors. *Naunyn-Schmiedeberg's Archives of Pharmacology* **351**, 603–609.

Ueno, S., Koizumi, S. & Inoue, K. (1998). Characterization of Ca^{2+} influx through recombinant P2X receptor in C6BU-1 cells. *British Journal of Pharmacology* **124**, 1484–1490.

Ueno, S., Tsuda, M., Iwanaga, T. & Inoue, K. (1999). Cell type-specific ATP-activated responses in rat dorsal root ganglion neurons. *British Journal of Pharmacology* **126**, 429–436.

Valera, S., Hussy, N., Evans, R. J., Adami, N., North, R. A., Surprenant, A. & Buell, G. (1994). A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature* **371**, 516–519.

Virginio, C., Robertson, G., Surprenant, A. & North, R. A. (1998). Trinitrophenyl-substituted nucleotides are potent antagonists selective for $P2X_1$, $P2X_3$ and heteromeric $P2X_{2,3}$ receptors. *Molecular Pharmacology* **53**, 969–973.

Xiang, Z., Bo, X. & Burnstock, G. (1998). Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. *Neuroscience Letters* **256**, 105–108.

Zhong, Y., Dunn, P. M. & Burnstock, G. (1999). Guinea-pig SCG neurons express varying proportions of two P2X receptors. *Journal of Physiology* **518.P**, 121*P*.

Zhong, Y., Dunn, P. M. & Burnstock, G. (2000). Pharmacological comparison of P2X receptors on rat coeliac, mouse coeliac and mouse pelvic ganglion neurons. *Neuropharmacology* (in the Press).

Zhong, Y., Dunn, P. M., Xiang, Z., Bo, X. & Burnstock, G. (1998). Pharmacological and molecular characterization of P2X receptors in rat pelvic ganglion neurons. *British Journal of Pharmacology* **125**, 771–781.

Zhou, X. & Galligan, J. J. (1996). P2X purinoceptors in cultured myenteric neurons of guinea-pig small intestine. *Journal of Physiology* **496**, 719–729.

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