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Multiple P2X receptors on guinea-pig pelvic ganglion neurons exhibit novel pharmacological properties

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1 Application of ATP and α,β-methylene ATP (αβmeATP) to voltage-clamped guinea-pig pelvic neurons produced three types of inward currents. A fast-desensitizing response was present in 5% (25/660) of neurons, 70% gave slowly-desensitizing currents, and the remainder had biphasic responses.

2 Slowly-desensitizing responses were characterized pharmacologically. The response to αβmeATP 100 μM was 46±27% (range 0–100%) of that evoked by ATP 100 μM in the same cell. Cross-desensitization indicated the presence of αβmeATP-sensitive and -insensitive receptors.

3 The concentration-response curve for αβmeATP had an EC_{50} of 55 μM, and a Hill coefficient of 0.99, while at the αβmeATP-insensitive receptor, ATP had an EC_{50} of 73 μM, with a Hill coefficient of 1.78.

4 The response to αβmeATP was blocked by pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), suramin and Cibacron blue. However, the αβmeATP-insensitive receptor was inhibited by PPADS, but not by the other two antagonists.

5 2′- (or 3′-) *O*-trinitrophenyl-ATP was 10 times more potent in inhibiting responses to αβmeATP than to ATP (at the αβmeATP-insensitive receptor).

6 Lowering extracellular pH potentiated responses to αβmeATP and ATP, while raising pH attenuated them.

7 Co-application of Zn^{2+} (3–300 μM) inhibited the responses to αβmeATP and ATP, with IC_{50} values of 286 and 60 μM, respectively.

8 In conclusion, unlike rat and mouse pelvic ganglion neurons, which only express $P2X_2$ homomers, at least three distinct P2X receptors are present in guinea-pig pelvic neurons, probably homomeric $P2X_2$, $P2X_3$ and heteromeric $P2X_{2/3}$ receptors. However, some of the novel pharmacological properties observed suggest that the guinea-pig P2X receptor subtypes may differ from their rat orthologues.

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Abbreviations: HBSS, Hanks' balanced salt solution with 10 mM HEPES buffer; Ip_5I, diinosine pentaphosphate; αβmeATP, α,β-methylene ATP; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; SCG, superior cervical ganglia; TNP-ATP, 2′-(or 3′-) *O*-trinitrophenyl-ATP

Introduction

ATP functions as a fast excitatory neurotransmitter by activating a class of ligand-gated cation channels, the P2X receptors (for review, see Ralevic & Burnstock, 1998). It also modulates fast synaptic transmission by acting presynaptically (Gu & MacDermott, 1997; Khakh & Henderson, 1998). Seven subunits, $P2X_{1-7}$, have been cloned in this family. When expressed heterologously, most of them can form functional homomeric receptors with different, yet overlapping phenotypes (Brake *et al*., 1994; Valera *et al*., 1994; Bo *et al*., 1995; Chen *et al*., 1995; Collo *et al*., 1996; Surprenant *et al*., 1996). In addition, they can form heteromeric channels with novel characteristics (Lewis *et al*., 1995; Lê *et al*., 1998; Torres *et al*., 1998; King *et al*., 2000). For example, $P2X_3$ receptors are activated by α,β-methylene ATP (αβmeATP) and desensitize rapidly, whereas $P2X_2$ receptors do not respond to this ligand and desensitize slowly (Brake *et al*., 1994; Chen *et al*., 1995). In contrast, $P2X_{2/3}$ heteromeric receptors respond to αβmeATP (a property of $P2X_3$ receptors), but desensitize slowly (a property of $P2X_2$ receptors) (Lewis *et al*., 1995).

A number of subtype-selective molecules have emerged in recent years, mainly from the studies on recombinant rat P2X (rP2X) receptors. While $P2X_2$ receptors are antagonized by suramin, Cibacron blue and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), $P2X_4$ receptors were relatively insensitive to them (Brake *et al*., 1994; Bo *et al*., 1995). 2′- (or 3′-) *O*-trinitrophenyl-ATP (TNP-ATP) is a potent antagonist on $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors, with 1000-fold less potency on $P2X_2$, $P2X_4$ and $P2X_7$ receptors (Virginio *et al*., 1998). Diinosine pentaphosphate (Ip_5I) selectively antagonizes $P2X_1$ and $P2X_3$ receptors, is inactive

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on $P2X_2$ and $P2X_{2/3}$ receptors, and potentiates $P2X_4$ receptors (King *et al*., 1999; Dunn *et al*., 2000). P2X receptors can also be influenced by allosteric modulators, thus extracellular acidification potentiates $P2X_2$ and $P2X_{2/3}$ receptors, while inhibiting $P2X_1$, $P2X_3$ and $P2X_4$ receptors (Stoop *et al*., 1997). Zn^{2+} potentiates $P2X_2$ and $P2X_{2/3}$ receptors (Li *et al*., 1993; Wildman *et al*., 1998), inhibits $P2X_1$ receptors (Wildman *et al*., 1999a), while producing a bell-shaped dose-response curve on $P2X_3$ and $P2X_4$ receptors (Wildman *et al*., 1999a,b). Ivermectin is a specific positive modulator of $P2X_4$ and $P2X_{4/6}$ receptors, while being inactive on $P2X_2$, $P2X_3$, $P2X_{2/3}$ and $P2X_7$ receptors (Khakh *et al*., 1999). These molecules may prove to be important pharmacological tools in characterizing endogenously expressed P2X receptors in native tissues.

In our previous studies, we have shown that the P2X receptors on rat pelvic ganglia neurons are of the $P2X_2$ subtype (Zhong *et al*., 1998), similar to those on rat superior cervical ganglia (SCG) (Nakazawa, 1994), and coeliac ganglia (Zhong *et al*., 2000a). In contrast, two distinct P2X receptors coexist on the same guinea-pig SCG neurons (Zhong *et al*., 2000b). While both desensitize slowly, only one is sensitive to $\alpha\beta$meATP. The proportions of these two receptors vary considerably from cell to cell. Guinea-pig coeliac neurons also demonstrated $\alpha\beta$meATP-sensitivity (Khakh *et al*., 1995). Therefore, in the present study, we sought to examine the P2X receptors on guinea-pig pelvic ganglia neurons, to find out whether they resemble those on rat pelvic, or those on guinea-pig SCG neurons. In addition, some experiments have been conducted on guinea-pig nodose neurons for comparison. Preliminary accounts of part of the work have appeared in the form of abstracts (Zhong *et al*., 1999, 2000c, 2000d).

Methods

Cell culture

Single neurons from the pelvic and nodose ganglia of male guinea-pigs (150–250 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 pelvic and nodose ganglia were rapidly dissected out, and placed in Leibovitz's L-15 medium (Life Technologies, Paisley, U.K.). 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, U.K.) and 6 mg ml^{-1} bovine serum albumin (Sigma, Poole, U.K.) at 37°C for 40 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 comprising of L-15 medium supplemented with 10% bovine serum, 50 ng ml^{-1} nerve growth factor, 2 mg ml^{-1} $NaHCO_3$, 5.5 mg ml^{-1} glucose, 200 IU 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, U.S.A.). Membrane potential was held at −60 mV. External solution contained (mM): NaCl 154, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10, 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): citric acid 56, $MgCl_2$ 3, CsCl 10, NaCl 10, HEPES 40, EGTA 0.1, tetraethylammonium chloride 10; the pH was adjusted to 7.2 using CsOH (total Cs^+ concentration 170 mM). Series resistance compensation of 70–75% 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).

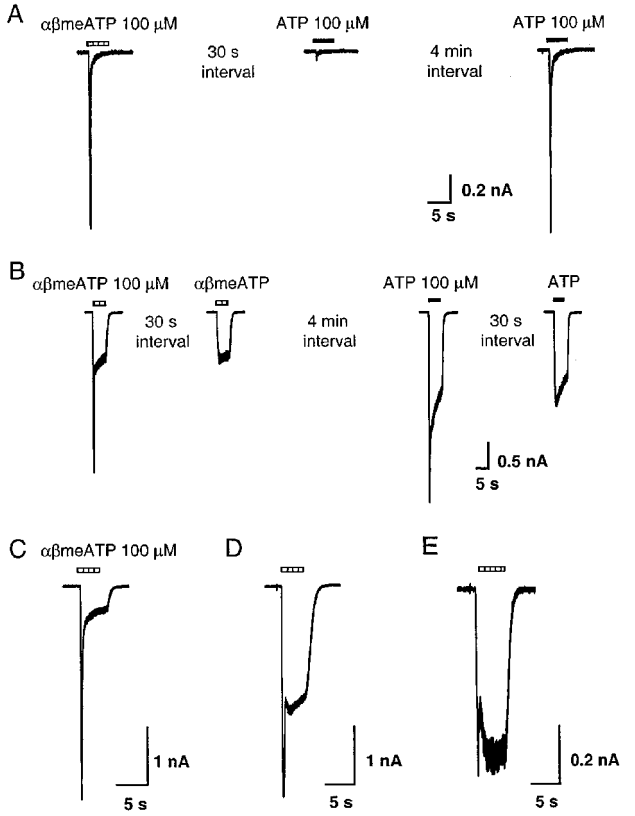

Figure 1 Heterogeneous responses to P2X agonists in isolated guinea-pig pelvic neurons. (A) Fast desensitizing inward current activated by $\alpha\beta$meATP and ATP (100 μM) in one neuron. Reapplying agonist 30 s after the first application evoked only a small response, but the response recovered fully after a 4 min interval. (B) In a different cell, application of $\alpha\beta$meATP and ATP evoked biphasic responses. Subsequent application of agonists 30 s later evoked only the slowly desensitizing response. (C–E) Examples of biphasic response to $\alpha\beta$meATP (100 μM) recorded from three guinea-pig pelvic neurons, illustrating the variation in the relative amplitude of the fast and slowly desensitizing components. All cells were voltage clamped at −60 mV. The horizontal bars above the traces indicate the duration of agonist application.

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 (⩽1 s).

Data analysis

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

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, Graphpad. 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, U.S.A.). 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, $P < 0.0001$).

Drugs

ATP, αβmeATP, Cibacron blue (Cibacron Blue 3GA, 65% pure) and ivermectin were obtained from Sigma Chemical Co. (Poole, U.K.). PPADS was obtained from Tocris Cookson (Bristol, U.K.). Suramin was a gift from Bayer plc (Newbury, U.K.). 2′- (or 3′-) *O*-trinitrophenyl-ATP was obtained from Molecular Probes (Leiden, Netherlands). Ip_5I was prepared by enzymatic degradation of diadenosine pentaphosphate (Ap_5A) (see King *et al*., 1999). Solutions (10–100 mM) of ATP and other drugs were prepared using deionized water and stored frozen, except for ivermectin, which was dissolved in dimethylsulphoxide to 1 mM. All drugs were then diluted in extracellular bathing solution to the final concentration.

Results

Three types of responses to αβmeATP and ATP

Rapid application of αβmeATP and ATP 100 μM on to isolated pelvic ganglia neurons (>600 cells) of guinea-pig, voltage clamped at −60 mV, induced three types of inward

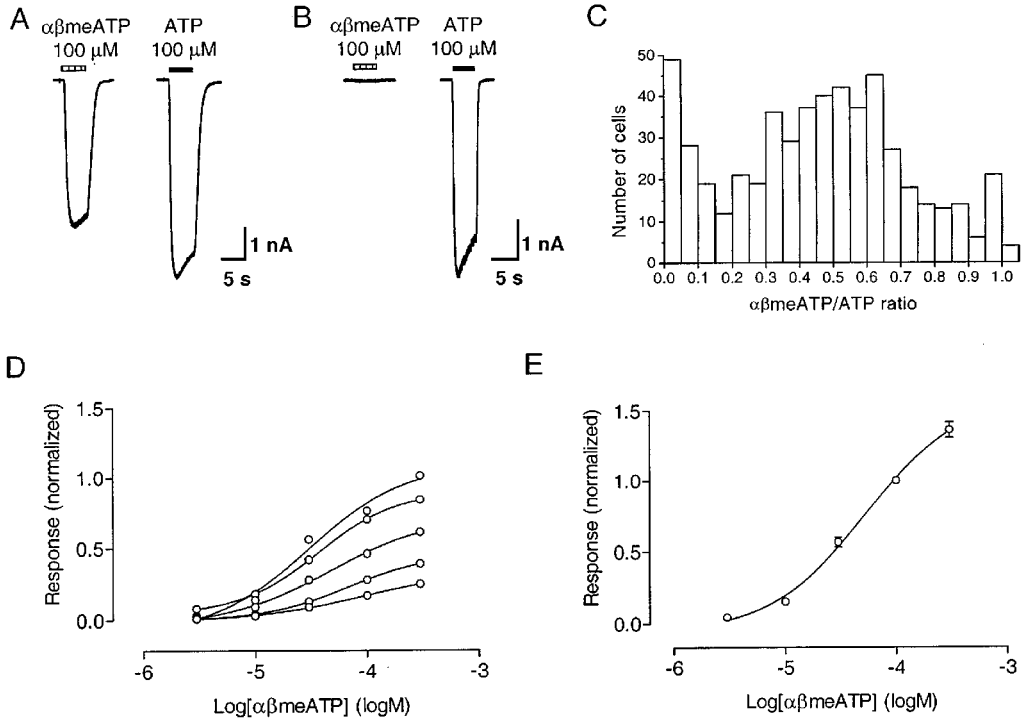

Figure 2 αβMeATP sensitivity of slowly-desensitizing responses in guinea-pig pelvic neurons. (A,B) Representative traces of slowly desensitizing responses evoked by αβmeATP (100 μM) and ATP (100 μM) from two neurons. Note the variation in the ratio of the slowly desensitizing responses to αβmeATP and ATP (αβmeATP/ATP ratio) in these two neurons. (C) The frequency distribution of the αβmeATP/ATP ratio for each of 531 cells. (D) Individual concentration-response curves for αβmeATP on six pelvic neurons, with responses normalized with respect to that obtained with 100 μM ATP on the same cell. (E) Concentration-response curves for αβmeATP, with responses normalized with respect to that obtained with 100 μM αβmeATP on the same cell. Points represent mean ± s.e.mean for 12 cells. When not visible, error bars lie within the symbol. Agonists were applied for 5 s at 2 min intervals, which was sufficient for responses to be reproducible.

currents. About 5% (25/660) of neurons showed predominantly fast-desensitizing response (Figure 1A), whereas 70% (471/660) of neurons showed slowly-desensitizing response (Figure 2A,B). The remaining 25% (164/660) showed a biphasic response, with both fast-desensitizing and slowly-desensitizing components being present (Figure 1B–E). The shape of the response for a given cell was always same for both agonists.

For cells demonstrating biphasic responses, the proportion of the fast-desensitizing and slowly-desensitizing components varied greatly from cell to cell (Figure 1C–E). Furthermore, in cells showing a fast-desensitizing inward current, reapplying agonist after an interval of 30 s evoked little response, but the current recovered fully after a 4 min interval (Figure 1A). In cells showing biphasic responses, subsequent application of agonists 30 s later evoked only the slowly-desensitizing component (Figure 1B).

The cells showing fast-desensitizing response had a capacitance of 17.5 ± 6.4 pF (mean $\pm$ s.d., $n=25$), which was significantly smaller than the capacitance of cells which demonstrated a slowly-desensitizing response (capacitance $= 33.4 \pm 17.6$ pF, mean $\pm$ s.d., $n=471$, $P<0.001$, Student's t-test) and those which demonstrated biphasic response (capacitance $= 26.6 \pm 13.4$ pF, mean $\pm$ s.d., $n=164$, $P<0.001$, Student's t-test). Because cells demonstrating a fast-desensitizing response comprised only 5% of the neurons, and it was difficult to isolate and study the transient component in cells showing biphasic responses, we have confined our pharmacological studies to the slowly-desensitizing responses.

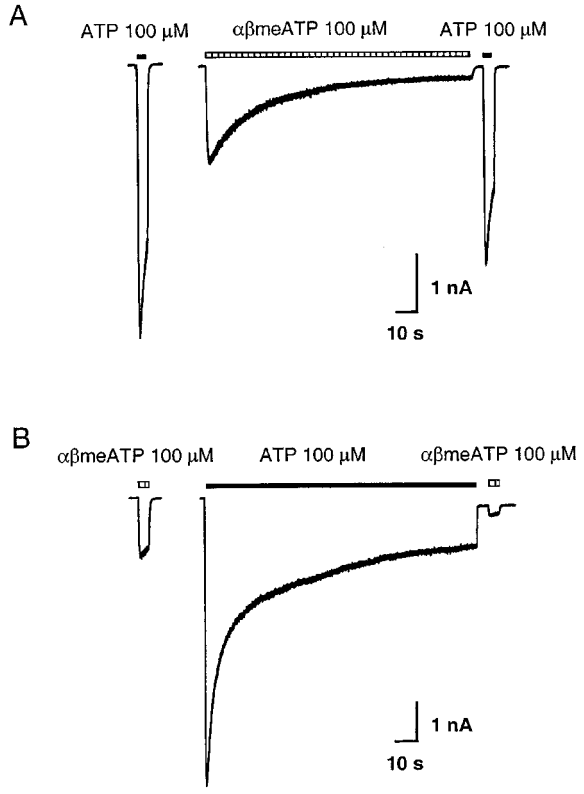

Figure 3 Cross-desensitization of P2X receptors on guinea-pig pelvic neurons by $\alpha\beta$meATP or ATP. (A) Traces of the membrane current recorded from one cell with a prolonged application of $\alpha\beta$meATP (100 μM). The control response evoked by 100 μM $\alpha\beta$meATP was 36% of that evoked by ATP (100 μM). A 2-min application of 100 μM $\alpha\beta$meATP produced a response which declined to 13% of the peak. In contrast, the response to 100 μM ATP was reduced to 73% of its own control. (B) Recordings from a different pelvic neuron with a prolonged application of 100 μM ATP. The control response evoked by 100 μM $\alpha\beta$meATP was 20% of that produced by 100 μM ATP. After 2 min desensitization by ATP 100 μM, the responses to $\alpha\beta$meATP and ATP were reduced to 18% and 14% of their respective controls. Cells were voltage clamped at -60 mV. The horizontal bars above the traces indicate the duration of agonist application.

Two receptors in the slowly-desensitizing response

As mentioned earlier, about 70% of guinea-pig pelvic ganglia neurons responded to both $\alpha\beta$meATP and ATP with slowly-desensitizing responses. However, the maximum response to $\alpha\beta$meATP was always smaller than that to ATP, and varied considerably from cell to cell (Figure 2A,B). In 531 cells where $\alpha\beta$meATP and ATP were tested on the same cells, the mean peak amplitude of the slowly-desensitizing current evoked by 100 μM $\alpha\beta$meATP was 2.2 ± 2.2 nA (mean $\pm$ s.d., $n=531$), while that evoked by 100 μM ATP was 5.2 ± 4.1 nA (mean $\pm$ s.d., $n=531$). 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. Thus the current elicited by 100 μM $\alpha\beta$meATP was $46 \pm 27\%$ (mean $\pm$ s.d., $n=531$) (range 0–100%) of that evoked by 100 μM ATP, although the EC_{50} values for these two agonists were similar (Figures 2 and 4). The frequency distribution of the $\alpha\beta$meATP/ATP ratio for each of 531 cells was clearly non-Gaussian (Figure 2C). While most neurons had a ratio about 0.5, there were substantial number of cells with a ratio of <0.1 or >0.9.

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 (Figure 2D). 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 very consistent (Figure 2E). Fitting the Hill equation to

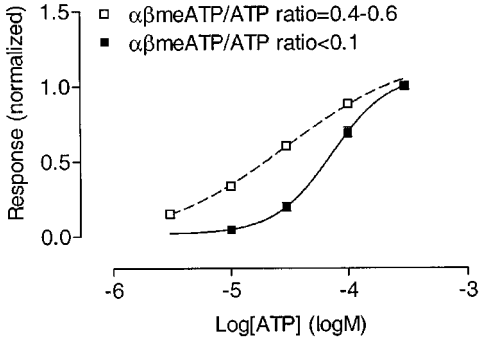

Figure 4 Concentration-response curves for the slowly-desensitizing responses to ATP on guinea-pig pelvic neurons. Concentration-response curves for ATP were constructed separately for guinea-pig pelvic neurons with an $\alpha\beta$meATP/ATP ratio <0.1, or in the range 0.4–0.6. For six cells possessing similar proportions of $\alpha\beta$meATP-sensitive and -insensitive receptors, (mean $\alpha\beta$meATP/ATP ratio $= 0.49$), the concentration-response curve for ATP was fitted with a two-component curve, assuming equal proportions of high and low affinity binding sites, with an EC_{50} value of 73 μM for the $\alpha\beta$meATP-insensitive site as previously determined. Responses were normalized with respect to that obtained with 300 μM ATP on the same cell. When not visible, error bars lie within the symbol.

the concentration-response curve for $\alpha\beta$meATP yielded an EC_{50} value of 55 μM (log $EC_{50} = -4.26 \pm 0.08$, $n = 12$), and a Hill coefficient of 0.99.

The variable $\alpha\beta$meATP/ATP ratio found on these guinea-pig pelvic neurons was similar to our previous findings on guinea-pig SCG neurons, which possess both $\alpha\beta$meATP-sensitive and -insensitive P2X receptors on the same cell (Zhong *et al*., 2000b). To examine whether this situation also occurs in guinea-pig pelvic neurons, we studied the effect of cross-desensitization.

When $\alpha\beta$meATP 100 μM was applied for 2 min, the time course of the decline in the $\alpha\beta$meATP-induced current fitted well to the sum of two exponentials, with the time constants of decay ($\tau 1$ and $\tau 2$) being 7.0 ± 1.0 s and 39.8 ± 4.9 s, respectively ($n = 6$). For six cells examined in this series of experiments, the peak response evoked by 100 μM $\alpha\beta$meATP was $41 \pm 6\%$ of that produced by 100 μM ATP. After a 2-min application of $\alpha\beta$meATP, the response to $\alpha\beta$meATP declined to $15 \pm 2\%$ of the peak (i.e. to 6% of the peak ATP response), while the response to 100 μM ATP was only reduced to $62 \pm 7\%$ of control (Figure 3A). Therefore, the fractional reduction in the $\alpha\beta$meATP response was significantly greater than that of the ATP response ($P < 0.001$, Student's *t*-test). On the other hand, when cells were desensitized by a prolonged exposure to 100 μM ATP, the responses to $\alpha\beta$meATP and ATP at the end of the 2-min desensitization were $15 \pm 6\%$ and $15 \pm 3\%$ of their respective controls ($n = 5$, Figure 3B). In another series of experiments, we examined the desensitization rate of the ATP response in cells showing a negligible $\alpha\beta$meATP response, i.e., an $\alpha\beta$meATP/ATP ratio < 0.1. In these neurons, the time course of the decline in the ATP-induced current was very similar to that of the $\alpha\beta$meATP response and fitted well to the sum of two exponential components, with time constants ($\tau 1$ and $\tau 2$) of 4.7 ± 0.7 s and 47.5 ± 9.6 s, respectively ($n = 6$).

These results suggested therefore, that in the 70% guinea-pig pelvic neurons that show slowly-desensitizing responses to agonists, two populations of P2X receptors are present. While both receptors are sensitive to ATP, only one can be activated by $\alpha\beta$meATP. To further characterize these two distinct P2X receptors pharmacologically, it was necessary to isolate each component of the response, and study them separately. To isolate the $\alpha\beta$meATP-sensitive slowly-desensitizing receptors, we used $\alpha\beta$meATP as a selective agonist. However, there is no selective agonist available for the $\alpha\beta$meATP-insensitive receptors. Thus, we selected neurons with an $\alpha\beta$meATP/ATP ratio < 0.1, and used ATP as the agonist. This type of cell made up to only approximately 5% of total (see Figure 2C). Since this population of cells showed negligible response to $\alpha\beta$meATP, the ATP current could be regarded as due only to the activation of the $\alpha\beta$meATP-insensitive receptors. This strategy was used throughout the pharmacological characterization.

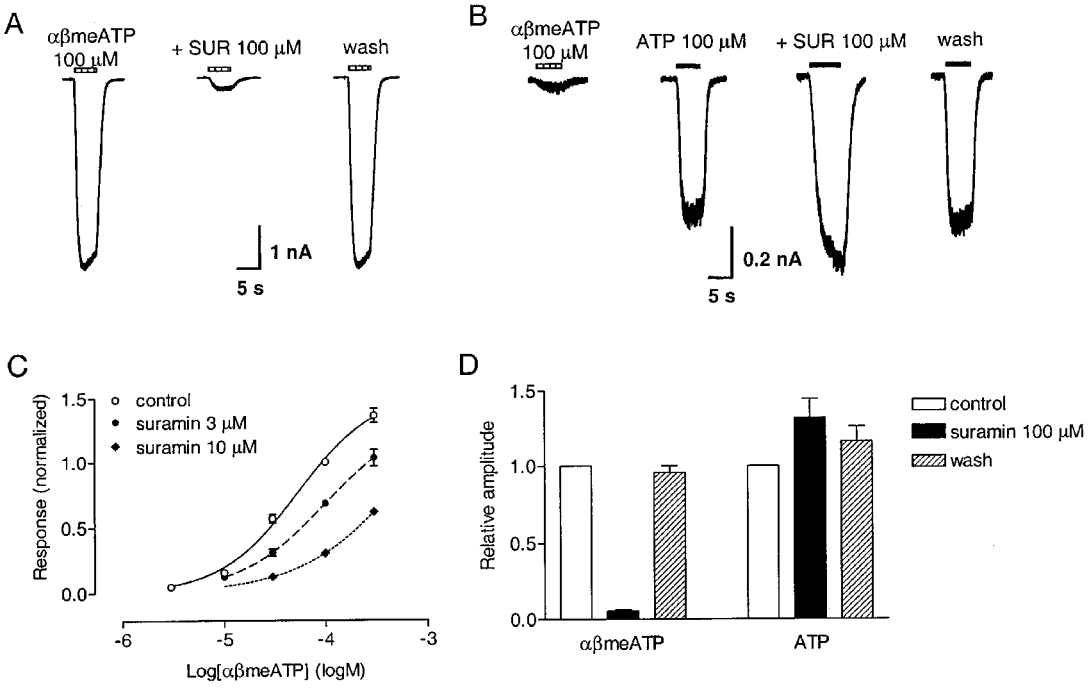

Figure 5 Effect of suramin on slowly-desensitizing responses evoked by $\alpha\beta$meATP and ATP in guinea-pig pelvic neurons. (A) Traces of three consecutive currents evoked from the same neuron by $\alpha\beta$meATP (100 μM) before, in the presence of suramin (100 μM) and 2 min after washing out the antagonist. Suramin was present for 2 min before and during the second application of $\alpha\beta$meATP. The horizontal bars above the traces indicate the duration of agonist application. (B) Traces from one cell ($\alpha\beta$meATP/ATP ratio < 0.1) showing the response to ATP (100 μM) in the presence and absence of suramin (100 μM). Suramin was present for 2 min before and during the reapplication of ATP. (C) Concentration-response curve for $\alpha\beta$meATP in the absence and presence of suramin, 3 and 10 μM. (D) Averaged peak currents induced by $\alpha\beta$meATP 100 μM or ATP (100 μM, on cells with an $\alpha\beta$meATP/ATP ratio < 0.1) in the presence and absence of suramin (100 μM). Bars represent responses in control condition, in the presence of suramin 100 μM and 2 min after washing out the antagonist. Responses were normalized with respect to that obtained with $\alpha\beta$meATP or ATP (100 μM) in the absence of suramin on the same cell. Each column represents the mean $\pm$ s.e.mean of 4–6 cells.

Further pharmacological characterization of the two slowly-desensitizing responses

The concentration-response relationship for ATP The concentration-response relationship for ATP of nine cells having an $\alpha\beta$meATP/ATP ratio <0.1 (mean ratio $=0.06$) is shown in Figure 4. Fitting the Hill equation to the data yielded an EC_{50} of 73 μM (log $EC_{50}=-4.14\pm0.07$, $n=5-9$ for each data point) and a Hill coefficient of 1.78. For six cells possessing similar proportions of $\alpha\beta$meATP-sensitive and -insensitive receptors ($\alpha\beta$meATP/ATP ratio of 0.4–0.6, mean $=0.49$), the concentration-response curve for ATP was fitted with a two-component curve, assuming equal proportions of high and low affinity binding sites, with an EC_{50} value of 73 μM for the $\alpha\beta$meATP-insensitive site as previously determined. This gave an EC_{50} value for the high affinity component of 13 μM.

Suramin On rat pelvic neurons, suramin (100 μM) practically abolishes the response to ATP 100 μM (Zhong *et al*., 1998). On guinea-pig pelvic neurons, a 2 min preincubation with suramin (100 μM) reversibly inhibited the response evoked by $\alpha\beta$meATP (100 μM) to $5\pm1\%$ ($n=6$) of control, with the response being $99\pm5\%$ of control 2 min after washing out suramin (Figure 5A). In the presence of suramin 3 μM, the concentration-response curve of $\alpha\beta$meATP was shifted to the right, giving an EC_{50} of 104 μM (log $EC_{50}=-3.98\pm0.18$, $n=5$) and a Hill coefficient of 1.00 (Figure 5C). From this increase in EC_{50}, the pA_2 value for suramin was estimated to be 5.45. Increasing suramin concentration to 10 μM shifted the concentration-response curve for $\alpha\beta$meATP further to the right, giving an estimated EC_{50} of 731 μM but with a Hill coefficient of only 0.83 ($n=5$). Thus, higher concentrations of suramin also appeared to have some non-competitive effects. In contrast, on cells showing an $\alpha\beta$meATP/ATP ratio <0.1 (mean ratio $=0.01$), after 2 min preincubation with suramin (100 μM), the response to ATP (100 μM) was $132\pm13\%$ ($n=4$) of control (Figure 5B,D, $P>0.05$, Student's t-test).

Cibacron blue On rat and mouse pelvic neurons, 2-min preincubation with 10 μM Cibacron blue abolished the response to 100 μM ATP (Zhong *et al*., 1998, 2000a). In contrast, on guinea-pig pelvic neurons, the response to $\alpha\beta$meATP (100 μM) was $106\pm7\%$ ($n=9$) of control after a 2 min preincubation with Cibacron blue (10 μM). Increasing the concentration of Cibacron blue to 50 μM inhibited the $\alpha\beta$meATP response to $35\pm1\%$ ($n=4$) of control (Figure 6A, C), and the response recovered to $86\pm6\%$ of control 2 min after washing out the antagonist. On cells showing an $\alpha\beta$meATP/ATP ratio <0.1 (mean ratio $=0.02$), the response to ATP (100 μM) was $113\pm8\%$ ($n=6$) and $122\pm8\%$ ($n=4$,

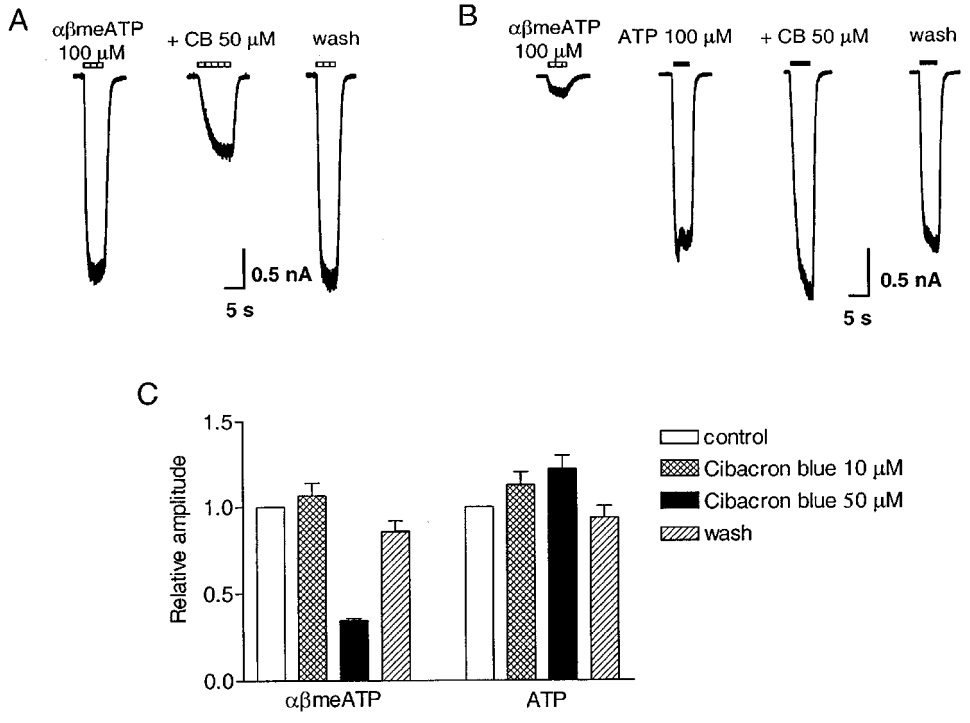

Figure 6 Effect of Cibacron blue on slowly-desensitizing responses evoked by $\alpha\beta$meATP and ATP on guinea-pig pelvic neurons. (A) Traces of three consecutive currents evoked from one cell by 100 μM $\alpha\beta$meATP before, in the presence of Cibacron blue (50 μM) and 2 min after washing out the antagonist. The horizontal bars above the traces indicate the duration of agonist application. (B) On another neuron with an $\alpha\beta$meATP/ATP ratio <0.1, four consecutive currents are shown to illustrate the control responses to $\alpha\beta$meATP (100 μM) and ATP (100 μM), and the responses to ATP in the presence of Cibacron blue (50 μM) and 2 min after washing out the antagonist. Cibacron blue was present for 2 min before and during the re-application of ATP. (C) Averaged peak currents induced by $\alpha\beta$meATP (100 μM) or ATP (100 μM, on cells with an $\alpha\beta$meATP/ATP ratio <0.1) in the presence and absence of Cibacron blue (10 and 50 μM). Bars represent responses in control condition, in the presence of 10 and 50 μM Cibacron blue and 2 min after washing out the antagonist. Responses were normalized with respect to that obtained with $\alpha\beta$meATP or ATP 100 μM in the absence of Cibacron blue on the same cell. Each column represents the mean $\pm$ s.e.mean of 4–9 cells.

$P>0.05$, Student's t-test) of control, after 2 min preincubation with Cibacron blue 10 μM and 50 μM, respectively (Figure 6B,C). Two minutes after washing out Cibacron blue 50 μM, the response to ATP was $94\pm7\%$ of control.

PPADS The responses of guinea-pig pelvic neurons to both $\alpha\beta$meATP and ATP (on cells showing an $\alpha\beta$meATP/ATP ratio <0.1) were inhibited by PPADS (10 μM) with a slow time profile (Figure 7A). The decrease in response amplitude to $\alpha\beta$meATP (100 μM) could be described by a single exponential with a time constant of 1.7 min. The amplitude was $6\pm1\%$ ($n=9$) of control after 10-min preincubation with PPADS 10 μM, and it only partially recovered to $20\pm3\%$ of control 4 min after washing out the antagonist. Similarly, on cells showing an $\alpha\beta$meATP/ATP ratio <0.1 (mean ratio = 0.03), the response to ATP (100 μM) was inhibited by PPADS (10 μM) with a time constant of 2.0 min. The ATP response was $3\pm1\%$ ($n=4$) of control after 10-min preincubation with PPADS 10 μM, and recovered to $14\pm3\%$ of control 4 min after washing out the antagonist.

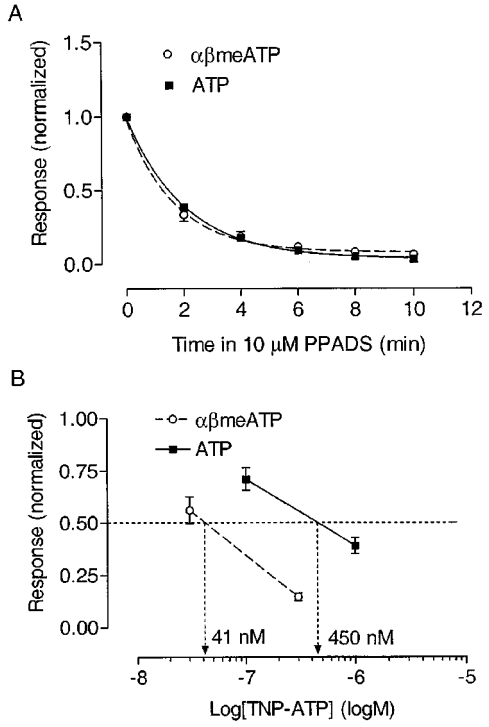

Figure 7 Inhibition of slowly-desensitizing responses to $\alpha\beta$meATP and ATP in guinea-pig pelvic neurons by PPADS and 2′- (or 3′-) *O*-trinitrophenyl-ATP (TNP-ATP). (A) Time profile of the inhibition by PPADS, using $\alpha\beta$meATP (100 μM) or ATP (100 μM) as the agonist. After determining the control responses to $\alpha\beta$meATP, or to ATP (on cells with an $\alpha\beta$meATP/ATP ratio <0.1), PPADS (10 μM) was applied to the cells and the response to agonists were redetermined every 2 min. Response was normalized with respect to that obtained with agonists in the absence of PPADS on the same neuron. Curves show single exponential fits to the data. Points represent mean$\pm$s.e.mean from 4–9 cells. (B) Inhibition by TNP-ATP of responses to $\alpha\beta$meATP (100 μM) or ATP (100 μM) (on cells with an $\alpha\beta$meATP/ATP ratio <0.1). Points represent mean$\pm$s.e.mean from four neurons. Responses were normalized with respect to that obtained with agonists in the absence of TNP-ATP on the same neuron. Each concentration of TNP-ATP was tested on a different sample of cells.

TNP-ATP TNP-ATP is a selective antagonist on $rP2X_1$, $rP2X_3$ and $rP2X_{2/3}$ receptors relative to $rP2X_2$ receptors (Virginio *et al*., 1998). In the present study, we investigated the antagonism by TNP-ATP on guinea-pig pelvic neurons. As shown in Figure 7B, against the response to $\alpha\beta$meATP 100 μM, TNP-ATP inhibited the response with an estimated IC_{50} of 41 nM ($n=4$ for each concentration). While on cells with an $\alpha\beta$meATP/ATP ratio <0.1 (mean ratio = 0.05), the response to ATP 100 μM was inhibited with an estimated IC_{50} of 450 nM ($n=4$ for each concentration). This is similar to the 8-fold difference in the potency of TNP-ATP in antagonizing the $\alpha\beta$meATP-sensitive and -insensitive receptors on guinea-pig SCG neurons (Zhong *et al*., 2000b).

pH Lowering extracellular pH from 7.4 to 6.8 increased the response to $\alpha\beta$meATP 30 μM to $120\pm2\%$ of control ($n=4$), whereas raising pH to 8.0 attenuated the response to $82\pm5\%$ of control ($n=4$) (Figure 8A,C). Acidification of the extracellular solution to pH 6.8 caused a leftward shift in the concentration-response curve of $\alpha\beta$meATP by 0.21 ± 0.03 log units ($n=4$, $P<0.01$, Student's t-test).

At the $\alpha\beta$meATP-insensitive receptor, lowering pH from 7.4 to 6.8 enhanced the response to ATP 100 μM to $126\pm5\%$ of control ($n=4$), while raising pH to 8.0 attenuated the response to $79\pm5\%$ of control ($n=4$) (Figure 8B,D). Change in pH from 7.4 to 6.8 shifted the concentration-response curve of ATP to the left by 0.15 ± 0.02 log units ($n=4$, $P<0.01$).

Zn^{2+} Co-application of Zn^{2+} (3–300 μM) produced a reversible concentration-dependent inhibition of the response evoked by $\alpha\beta$meATP on guinea-pig pelvic neurons (Figure 9). Inclusion of a 2-min preincubation of Zn^{2+} did not produce any additional inhibition (data not shown). The response to $\alpha\beta$meATP 100 μM was inhibited by Zn^{2+} with an IC_{50} of 286 μM ($\log IC_{50}=-3.54\pm0.10$, $n=8$), and a Hill coefficient of 0.59. In the presence of 300 μM Zn^{2+}, the EC_{50} for $\alpha\beta$meATP was estimated as 120 μM ($\log EC_{50}=-3.92\pm0.47$, $n=4$), with a Hill coefficient of 0.9 and an E_{max} of 0.85, compared with an EC_{50} of 55 μM, a Hill coefficient of 0.99 and an E_{max} of 1.62 in the control condition. Thus, the potency as well as the maximum response to $\alpha\beta$meATP appeared to have been reduced, indicating that the inhibition by Zn^{2+} (300 μM) is not competitive.

At the $\alpha\beta$meATP-insensitive receptor, co-application of Zn^{2+} inhibited the response to ATP 100 μM with an IC_{50} of 60 μM ($\log IC_{50}=-4.23\pm0.04$, $n=4$), and a Hill coefficient of 0.82. Thus, the potency of Zn^{2+} on these receptors was significantly higher than that on the $\alpha\beta$meATP-sensitive receptors ($P<0.001$).

Ip_5I and ivermectin The rat $P2X_4$ receptor is resistant to the antagonists suramin and Cibacron blue (Bo *et al*., 1995; Buell *et al*., 1996b). We therefore investigated the possible involvement of the $P2X_4$ subunit in the $\alpha\beta$meATP-insensitive receptor. Ip_5I is a P2X-subtype selective antagonist, which potently inhibits $rP2X_1$ and $rP2X_3$ receptors, but is inactive on $rP2X_2$ receptors at concentrations up to 30 μM, and enhances the action of ATP at $rP2X_4$ receptors (King *et al*., 1999). In four cells with a mean $\alpha\beta$meATP/ATP ratio of 0.03, after 2-min preincubation with Ip_5I 0.1 μM or 10 μM, the

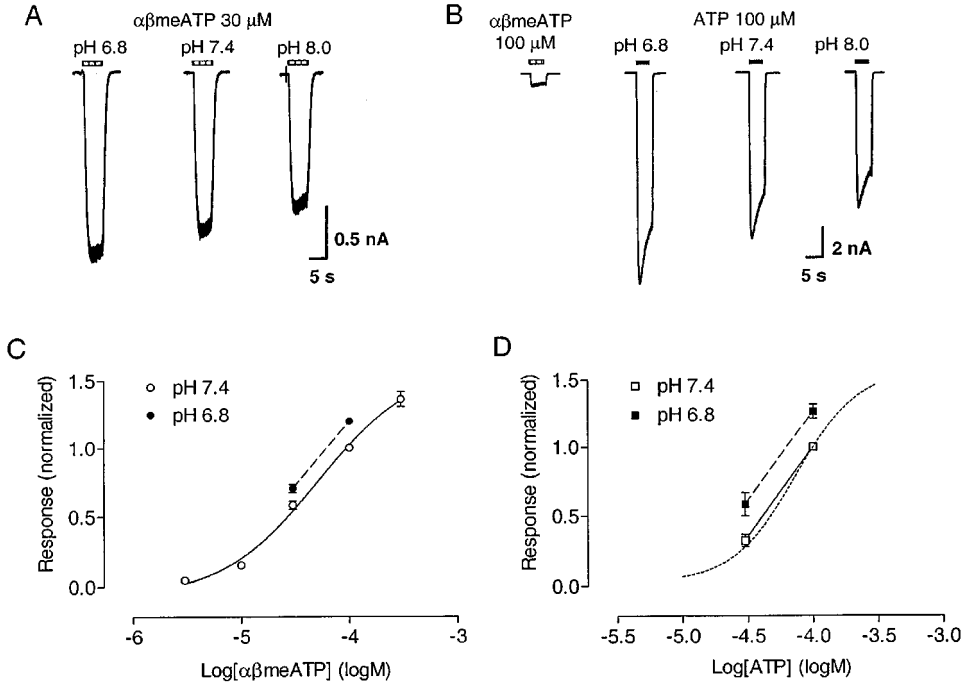

Figure 8 Effect of pH on slowly-desensitizing responses to αβmeATP and ATP on guinea-pig pelvic neurons. (A) Traces showing responses to αβmeATP (30 μM) of one neuron at pH 6.8, 7.4 and 8.0. (B) Traces showing responses to ATP (100 μM) of a neuron with an αβmeATP/ATP ratio <0.1 at pH 6.8, 7.4 and 8.0. The horizontal bars above the traces indicate the duration of agonist application. (C) Lowering extracellular pH from 7.4 to 6.8 displaced the αβmeATP concentration-response relationship to the left ($n=4$). (D) Changing extracellular pH from 7.4 to 6.8 shifted the ATP concentration-response relationship (for cells with αβmeATP/ATP ratio <0.1) to the left ($n=4$). The control concentration-response curve for ATP (pH 7.4) taken from Figure 4 has been superimposed (dotted curve) for comparison. Responses were normalized with respect to that evoked by αβmeATP or ATP (100 μM) at pH 7.4 on the same cell.

response to 100 μM ATP was 92±2% ($P<0.05$) and 80±1% ($P<0.001$) of control, respectively (Figure 10A,B).

Another subtype selective modulator of P2X receptors, ivermectin, selectively increases the potency and efficacy of ATP on recombinant $rP2X_4$ receptors (Khakh *et al.*, 1999), but has no effect on $rP2X_2$, $rP2X_{2/3}$ and $rP2X_3$ receptors. Therefore, we sought to test its effect on the slowly-desensitizing responses on guinea-pig pelvic neurons. After 2-min preincubation with 1 μM ivermectin, the response to 100 μM αβmeATP was 98±1% of control ($n=4$) (Figure 10D). In four cells with a mean αβmeATP/ATP ratio of 0.06, the response to 100 μM ATP after 2 min preincubation with ivermectin 1 μM was 101±5% of control ($n=4$, $P>0.05$, Figure 10C,D).

Effect of Zn^{2+} on guinea-pig nodose neurons

Slowly-desensitizing αβmeATP sensitive P2X receptors are present on rat nodose neurons, where responses are potentiated by Zn^{2+} (Li *et al.*, 1993). However, we observed inhibition by Zn^{2+} of the response to αβmeATP on guinea-pig pelvic neurons. To test whether this might be due to a species difference between rat and guinea-pig P2X receptors, we examined the effect of Zn^{2+} on αβmeATP response on guinea-pig nodose neurons.

On guinea-pig nodose neurons, αβmeATP evoked a slowly desensitizing response. Co-application of Zn^{2+} produced concentration-dependent inhibition of the αβmeATP response, similar to that seen on guinea-pig pelvic neurons (Figure 11). In the presence of 300 μM Zn^{2+}, the response to 100 μM αβmeATP was 39±3% of control ($n=6$).

Discussion

Two major findings have come out of this study. Firstly, we have demonstrated the presence of at least three distinct populations of P2X receptors on guinea-pig pelvic neurons. This is in contrast to the situations in rat and mouse pelvic neurons, where only a single population of $P2X_2$ homomeric receptors is present. Secondly, using currently available pharmacological tools, we have shown that these guinea-pig P2X receptors display novel pharmacological properties, which may reflect a species difference in the characteristics of P2X receptors, or may indicate the presence of novel P2X receptors.

Co-existence of three distinct P2X receptors

We have previously demonstrated that in guinea-pig SCG neurons two populations of P2X receptors are present, both desensitize slowly, but only one is activated by αβmeATP. The relative proportions of these receptors vary from cell to cell (Zhong *et al.*, 2000b). In the present study, we have observed two similar populations of receptors in neurons of guinea-pig pelvic ganglia. In addition, however, we also observed in some neurons, the presence of the third receptor type, which was activated by both ATP and αβmeATP,

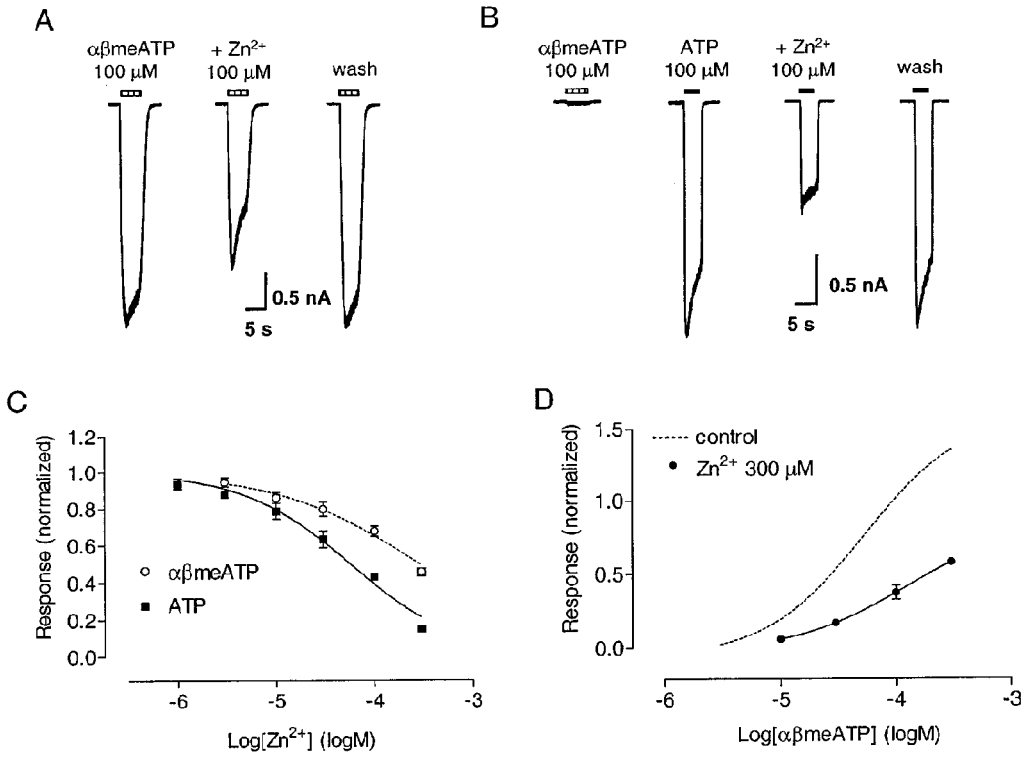

Figure 9 Effect of Zn^{2+} on slowly-desensitizing responses to $\alpha\beta$meATP and ATP on guinea-pig pelvic neurons. (A) Traces of three consecutive currents evoked from one neuron by 100 μM $\alpha\beta$meATP before, in the presence of Zn^{2+} 100 μM and 2 min after washing out Zn^{2+}. Zn^{2+} was co-applied with $\alpha\beta$meATP. (B) Traces from one cell ($\alpha\beta$meATP/ATP ratio <0.1) showing the response to 100 μM ATP alone, when co-applied in the presence of 100 μM Zn^{2+} and 2 min after washing out Zn^{2+}. The horizontal bars above the traces indicate the duration of agonist application. (C) Inhibition curve for Zn^{2+} when $\alpha\beta$meATP (100 μM) was used as the agonist, or on cells where the $\alpha\beta$meATP/ATP ratio was <0.1 with ATP (100 μM) as the agonist. Points represent mean $\pm$ s.e.mean from 4–8 neurons. (D) Concentration-response curve for $\alpha\beta$meATP in the presence of Zn^{2+} (300 μM). The control concentration-response curve taken from Figure 2E has been superimposed for comparison. Responses were normalized with respect to that obtained with $\alpha\beta$meATP or ATP 100 μM in the absence of Zn^{2+} on the same cell.

desensitized very rapidly, and required >3 min between agonist application to achieve reproducible responses. The presence of two populations of slowly desensitizing receptors was indicated by the variability in the relative maximum responses to $\alpha\beta$meATP and ATP, confirmed by cross-desensitization experiments, and by the pharmacological characterization of the receptors (see below and Table 1). Therefore, three distinct populations of P2X receptors can co-exist on the same guinea-pig pelvic neuron, one is activated by $\alpha\beta$meATP and rapidly-desensitizing, one is $\alpha\beta$meATP-insensitive and desensitizes slowly and the third is slowly-desensitizing but $\alpha\beta$meATP-sensitive.

The presence of both rapidly- and slowly-desensitizing responses in the same neuron gave rise to biphasic currents, which were encountered in about 25% of guinea-pig pelvic neurons. Biphasic P2X-mediated currents have previously been described in a sub-population of rat trigeminal and dorsal root ganglia (DRG) neurons, and have been attributed to the presence of homomeric $P2X_3$ receptors and heteromeric $P2X_{2/3}$ receptors (Cook *et al*., 1997; Burgard *et al*., 1999; Grubb & Evans 1999). Fast- and slowly-desensitizing ATP currents were previously observed in guinea-pig myenteric neurons, although no biphasic current was reported in these cells (Zhou & Galligan, 1996).

Possible identity of P2X receptors

Of the recombinant homomeric P2X receptors, only $P2X_1$ and $P2X_3$ respond significantly to $\alpha\beta$meATP. These receptors, however, desensitize very rapidly. In contrast, heteromeric $P2X_{2/3}$ receptors respond to $\alpha\beta$meATP with a slowly-desensitizing profile. We have previously demonstrated the presence of $P2X_2$ and $P2X_3$ immunoreactivity in neurons of the guinea-pig pelvic ganglia (Zhong *et al*., 2000b). Thus, the simplest explanation of our results is that these neurons express varying proportions of $P2X_2$ and $P2X_3$ subunits which assemble to produce three different receptors, namely homomeric $P2X_2$ and $P2X_3$, and heteromeric $P2X_{2/3}$.

This explanation would account for the temporal profile of the responses, and the presence of $\alpha\beta$meATP-sensitive and -insensitive components. Due to the infrequency of encountering cells showing transient response, we have been unable to characterize the pharmacological properties of the rapidly desensitizing receptors. Another possible explanation for a rapidly desensitizing $\alpha\beta$meATP sensitive receptor is the involvement of $P2X_1$. However, since we were unable to detect the $P2X_1$ immunoreactivity (Zhong *et al*., 2000b), this hypothesis is unlikely.

Of the slowly-desensitizing responses, the properties of the $\alpha\beta$meATP-sensitive receptors are most consistent with those

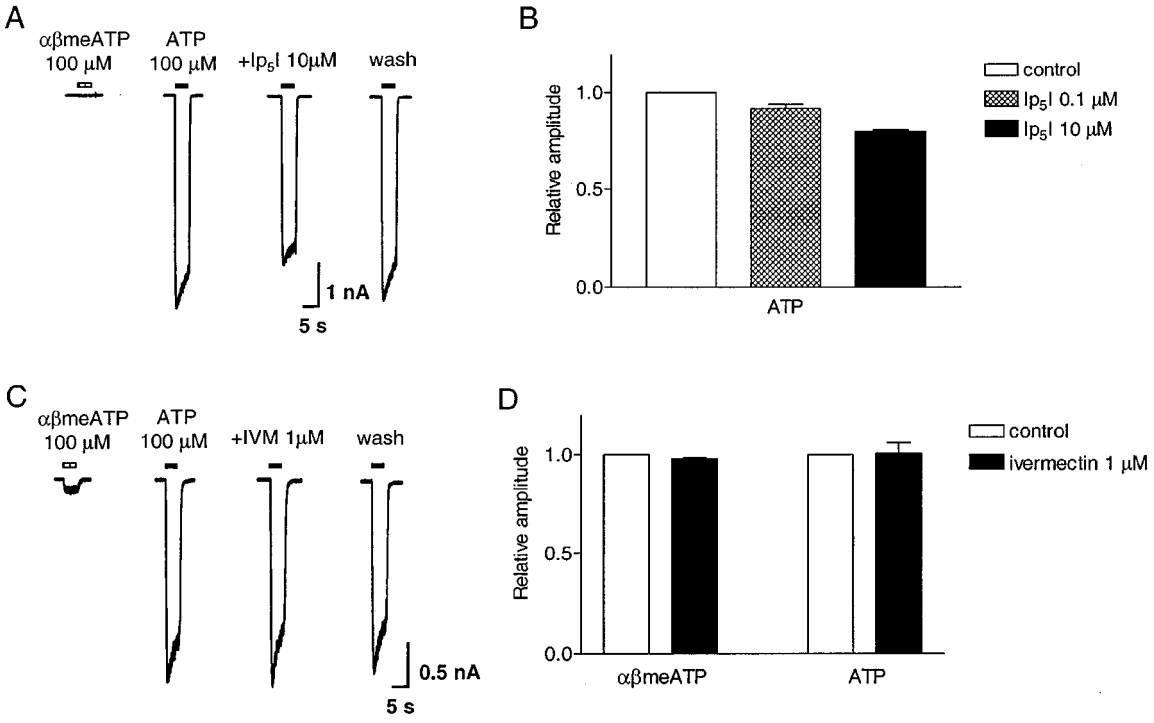

Figure 10 Effect of diinosine pentaphosphate (Ip_5I) and ivermectin on slowly-desensitizing responses to $\alpha\beta$meATP and ATP on guinea-pig pelvic neurons. (A) Traces of responses to ATP (100 μM) in the absence and presence of Ip_5I (10 μM) in a cell with an $\alpha\beta$meATP/ATP ratio <0.1. Ip_5I was present 2 min before and during reapplication of ATP. The horizontal bars above the traces indicate the duration of agonist application. (B) Averaged peak amplitudes of the current induced by ATP (100 μM) in the absence and presence of Ip_5I, 0.1 and 10 μM. (C) Traces of responses to ATP (100 μM) in the absence and presence of ivermectin (1 μM) in a cell with an $\alpha\beta$meATP/ATP ratio <0.1. Ivermectin was present 2 min before and during reapplication of ATP. (D) Averaged peak responses to $\alpha\beta$meATP or ATP (100 μM). ($\alpha\beta$meATP/ATP ratio <0.1), in the absence and presence of ivermectin (1 μM). Responses were normalized to that in the absence of Ip_5I or ivermectin from the same cell. Each column represents the mean$\pm$s.e.mean of four cells.

of $P2X_{2/3}$ receptors, e.g., the slowly desensitizing profile of the $\alpha\beta$meATP response, the sensitivity to antagonists suramin, Cibacron blue and PPADS and the potentiation by acidification (Lewis *et al*., 1995; Stoop *et al*., 1997; Liu & King, personal communication). The sensitivity to TNP-ATP of these receptors was close to that found previously on guinea-pig SCG neurons (Zhong *et al*., 2000b), and may suggest a slightly lower sensitivity to TNP-ATP of guinea-pig $P2X_{2/3}$ receptors compared with those of the rat. The modulation by Zn^{2+} on guinea-pig neurons however, appeared to be different from that on rat. While the slowly-desensitizing $\alpha\beta$meATP-sensitive responses on rat nodose neurons were potentiated by Zn^{2+} (Li *et al*., 1993), those on guinea-pig pelvic neurons were inhibited. Although a novel P2X receptor might be involved here, it is also possible that guinea-pig $P2X_{2/3}$ receptors differ from $rP2X_{2/3}$ in their modulation by Zn^{2+}. We found that on guinea-pig nodose neurons, which showed specific immunoreactivity for $rP2X_2$ and $rP2X_3$ antibodies in a pattern similar to that on rat nodose ganglia (Xiang *et al*., 1998; Zhong *et al*., 2000b), Zn^{2+} also inhibited the responses to $\alpha\beta$meATP. Similar inhibition by Zn^{2+} has been reported for the persistent response to ATP on bullfrog DRG neurons, which cannot be accounted for by the decrease in the concentration of one or more active forms of ATP (Li *et al*., 1997). This indicates that the difference in the modulation by Zn^{2+} seen here may reflect a species difference, i.e. the $rP2X_{2/3}$ receptors are potentiated, while those of guinea-pig and bullfrog are inhibited.

We used $\alpha\beta$meATP as a selective agonist to isolate the $\alpha\beta$meATP-sensitive slowly-desensitizing receptors. To study the $\alpha\beta$meATP-insensitive receptors, we had to select neurons that express exclusively this type of receptor and use ATP as the agonist. Unfortunately, this type of cell made up only 5% of the total number (see Figure 2C), which added considerable difficulty to the study. While these receptors had many pharmacological properties observed for recombinant $rP2X_2$ receptors, there were some clear differences. Thus, responses were potentiated by acidification and antagonized by PPADS–$rP2X_2$-like (Zhong *et al*., 1998). However, these $\alpha\beta$meATP-insensitive responses were not blockable by Cibacron blue (50 μM) or suramin (100 μM). In view of the antagonist resistance observed, we considered the possible involvement of $P2X_4$ subunits, although we have been unable to detect this protein using immunohistochemistry (Zhong *et al*., 2000b). Ip_5I and ivermectin both potentiate $P2X_4$ receptors (King *et al*., 1999; Khakh *et al*., 1999). However, responses in guinea-pig pelvic neurons were not potentiated by these modulators, which argues against the involvement of this subunit.

So far, $P2X_2$ is the only subunit cloned from the guinea-pig, and three spliced variants have been identified (Parker *et al*., 1998). When expressed heterologously, homomeric guinea-pig $P2X_{2\text{-}1}$ and $P2X_{2\text{-}2}$ receptors gave rise to large

currents which desensitized quite fast in a double exponential fashion, whereas homomeric $P2X_{2-3}$ receptors gave rise to small, but sustained, currents (Chen *et al*., 2000). ATP was a potent full agonist on all of them and $\alpha\beta$meATP evoked little response at concentrations up to 100 μM. Interestingly however, co-application of ATP with suramin or PPADS produced variable degrees of inhibition on these recombinant guinea-pig receptors. Partial inhibition by suramin, Cibacron blue and PPADS at high concentrations was previously reported for guinea-pig chromaffin cells (Liu *et al*., 1999). In contrast, ATP responses on guinea-pig myenteric neurons were enhanced by suramin and Cibacron blue, but inhibited by PPADS (Barajas-López *et al*., 1996). Thus, the phenotype of $\alpha\beta$meATP-insensitive receptors on guinea-pig pelvic neurons was very similar to that on guinea-pig myenteric neurons. The discrepancy in the antagonist sensitivity seen here may result from the co-assembly of different spliced variants of guinea-pig $P2X_2$ receptors in various native cells. Alternatively, this may be due to hetero-multimeric receptors containing $P2X_2$ as well as other subunits. The third possibility is that novel P2X receptors are yet to be cloned from guinea-pig pelvic and myenteric neurons.

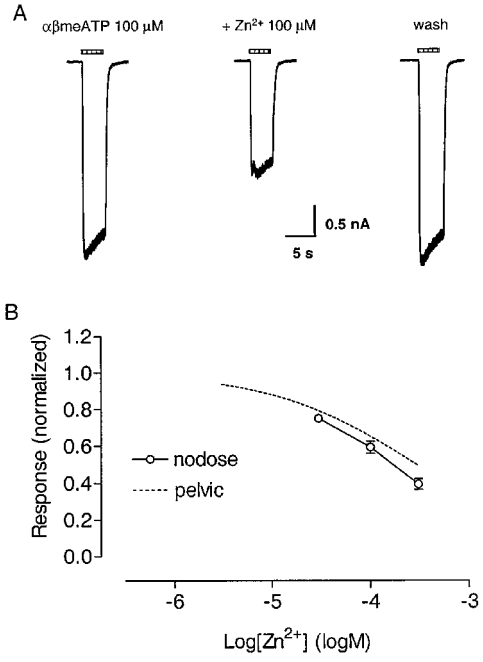

Figure 11 Effect of Zn^{2+} on slowly-desensitizing responses to $\alpha\beta$meATP on guinea-pig nodose neurons. (A) Traces showing responses to $\alpha\beta$meATP (100 μM) in the absence and presence of Zn^{2+} (100 μM) on one guinea-pig nodose neuron. Zn^{2+} was co-applied with $\alpha\beta$meATP. The horizontal bars above the traces indicate the duration of agonist application. (B) Concentration-response relationship for the inhibition by Zn^{2+} of responses to $\alpha\beta$meATP (100 μM) on guinea-pig nodose neurons. Zn^{2+} was co-applied with the agonist. Responses were normalized with respect to that obtained with $\alpha\beta$meATP (100 μM) in the absence of Zn^{2+} on the same cell. The inhibition curve for Zn^{2+} against $\alpha\beta$meATP on guinea-pig pelvic neurons (taken from Figure 9C) has been superimposed for comparison.

Inter-species variation

The findings in the present study support and extend our previous suggestion that the expression of P2X receptors is different in rat and guinea-pig. Thus, homomeric $P2X_2$ receptors may be the dominant subtype in neurons of rat SCG (Nakazawa, 1994), coeliac (Zhong *et al*., 2000a) and pelvic ganglia (Zhong *et al*., 1998). In contrast, neurons in guinea-pig SCG express two distinct P2X receptors (Zhong *et al*., 2000b), and guinea-pig pelvic neurons express three P2X receptors, with different desensitizing kinetics and pharmacological properties (present study). Neurons in guinea-pig coeliac ganglia have also been reported to be $\alpha\beta$meATP-sensitive (Khakh *et al*., 1995). It remains to be seen whether in fact multiple P2X receptors are also present in guinea-pig coeliac neurons. In addition, while rat chromaffin cells lack P2X receptors, those of the guinea-pig are sensitive to ATP (Liu *et al*., 1999). Similarly, most outer hair cells of rat do not appear to possess P2X receptors, while those of guinea-pig do (Chen *et al*., 1997). The pharmacological and immunohistochemical evidence suggests that, in guinea-pig, $P2X_3$ subunits may be present in autonomic as well as sensory neurons (Zhong *et al*., 2000b). However, in the rat, high levels of $P2X_3$ subunit expression can only be detected in sensory neurons (Buell *et al*., 1996a; Xiang *et al*., 1998).

Inter-species difference is also evident from the differences in pharmacological properties between P2X receptor orthologues. For example, recombinant $hP2X_4$ receptors displayed notably higher sensitivity to antagonists suramin and PPADS compared with the rat homologue. A single-point mutation on $rP2X_4$ (Q78K) was sufficient to account for this increase in suramin sensitivity (Garcia-Guzman *et al*., 1997). Similarly, a lysine residue at position 249 on $rP2X_1$ and

Table 1 Comparison of the pharmacological properties of the $\alpha\beta$meATP-sensitive and -insensitive slowly-desensitizing receptors on guinea-pig pelvic neurons

	αβmeATP-sensitive	*αβmeATP-insensitive*
EC_{50} of ATP	13 μM	73 μM
EC_{50} of $\alpha\beta$meATP	55 μM	–
Inhibition by Suramin*	pA_2 5.45	No antagonism at 100 μM
Inhibition by Cibacron blue 50 μM*	65%	No block
Inhibition by PPADS 10 μM†	94%	97%
IC_{50} of TNP-ATP*	41 nM	450 nM
pH 7.4 to 6.8	Leftward shift by 0.21 ± 0.03 log units	Leftward shift by 0.15 ± 0.02 log units
IC_{50} of Zn^{2+}‡	286 μM	60 μM
Response in the presence of Ip_5I 10 μM*	nd	80%
Response in the presence of Ivermectin 1 μM*	98%	101%

*With 2-min preincubation; †with 10-min preincubation; ‡co-application; nd = not determined.

$rP2X_2$ seems to be crucial for the potency and kinetics of the antagonism by PPADS (Buell *et al*., 1996b). In the light of these findings, it is possible that the difference in the amino acids sequences between rat and guinea-pig P2X receptors at certain positions may confer distinct pharmacological phenotypes. The three spliced variants of guinea-pig $P2X_2$ receptors share 80–83% homology with those of the rat (Brändle *et al*., 1997; Parker *et al*., 1998). Thus, the construction of chimeric rat/guinea-pig receptors should enable the amino acids involved in the Zn^{2+} binding site to be identified.

Neurons in the guinea-pig pelvic plexus provide motor innervation to urogenital organs and the lower digestive tracts. We found that neurons expressing only the fast-desensitizing P2X receptors are significantly smaller than the others. It remains to be seen whether there is a correlation between the property of the neuron, their target organ, and the type of P2X receptors expressed. On the other hand, it will be interesting to determine whether the proportions of multiple P2X receptors within a given cell remain constant, or change during development or under pathological conditions.

To conclude, we have identified three different P2X receptors on guinea-pig pelvic neurons. The proportion and combination of each type vary greatly from cell to cell. The novel pharmacological properties demonstrated by these neurons suggest that guinea-pig P2X receptors have different properties compared with those of the rat orthologues. Alternatively, a novel P2X receptor or heteromultimer with novel subunit combinations may be present in these neurons.

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