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Identification of P2X receptors in cultured mouse and rat parasympathetic otic ganglion neurones including P2X knockout studies

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Abstract

We have used patch-clamp recording from cultured neurones, immunohistochemistry and gene deletion techniques to characterize the P2X receptors present in mouse otic ganglion neurones, and demonstrated the presence of similar receptors in rat neurones. All neurones from wild-type (WT) mice responded to ATP (EC_{50} 109 μM), but only 38% also responded to αβ-meATP (EC_{50} 39 μM). The response to αβ-meATP was blocked by TNP-ATP with an IC_{50} of 38.6 nM. Lowering extracellular pH and co-application of Zn^{2+} potentiated responses to ATP and αβ-meATP. In $P2X_3^{-/-}$ mouse otic ganglion, all neurones tested responded to 100 μM ATP with a sustained current, but none responded to αβ-meATP. In $P2X_2^{-/-}$ mice, no sustained currents were observed, but 36% of neurones responded to both ATP and αβ-meATP with transient currents. In $P2X_2/P2X_3^{Dbl-/-}$ mice, no responses to ATP or αβ-meATP were detected, suggesting that other P2X subunits were not involved. In rat otic ganglia, 96% of neurones responded to both ATP and αβ-meATP with sustained currents, suggesting a greater proportion of neurones expressing $P2X_{2/3}$ receptors. The maximum response to αβ-meATP was 40–60% of that evoked by ATP in the same cell. Immunohistochemistry revealed staining for $P2X_2$ and $P2X_3$ subunits in WT mouse otic ganglion neurones, which was absent in knockout animals. In conclusion, we have shown for the first time that at least two distinct P2X receptors are present in mouse and rat otic neurones, probably homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors.

Keywords: Parasympathetic; Otic; Ganglion; P2X receptor

1. Introduction

It is now well established that ATP is an important intercellular signalling molecule and acts on two families of P2 nucleotide receptors (Burnstock, 1997), the G protein-coupled P2Y and ligand-gated P2X receptors. Seven P2X receptor subunits have been identified ($P2X_{1–7}$), which can assemble to form either homomeric or heteromeric receptors. Five functional heteromeric P2X receptors have so far been reported: $P2X_{2/3}$, $P2X_{4/6}$, $P2X_{1/5}$, $P2X_{2/6}$ and $P2X_{1/2}$ (see North, 2002; Brown et al., 2002). P2X receptors show considerable differences in their sensitivity to agonists, antagonists, and allosteric modulators and furthermore, show differences in kinetics of receptor activation and inactivation. Nevertheless, the overlap in physiological and pharmacological properties, combined with the ability of cells to express more than one subtype of receptor, makes the definitive classification of native receptors problematical.

Native P2X receptors have been studied in a number of neurones, including sensory, sympathetic, parasympathetic, myenteric and central neurones (for reviews, see Dunn et al., 2001; Robertson et al., 2001).

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While rat and mouse sympathetic ganglia express homomeric $P2X_2$ receptors, sensory neurones of the nodose ganglion express predominantly $P2X_{2/3}$ heteromers and dorsal root ganglion neurones express varying proportions of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors (see Dunn et al., 2001). Because of their small size and diffuse nature, parasympathetic ganglia have so far received much less attention. However, as they share the same embryological origins as sympathetic and sensory ganglia, it is interesting to see how the expression of P2X receptors compares between these different ganglia. Some properties of ATP responses in cardiac ganglion neurones have been described (Allen and Burnstock, 1990; Fieber and Adams, 1991). More recent studies on neurones from the submandibular ganglion suggest the presence of receptors involving $P2X_2$, $P2X_4$ and $P2X_5$ subunits (Liu and Adams, 2001; Smith et al., 2001), indicating that P2X receptors in parasympathetic ganglia may differ substantially from those in sympathetic ganglia. To further investigate P2X receptors in parasympathetic ganglia, we have characterized, for the first time, the P2X receptors present on neurones of the otic ganglion, which provide parasympathetic motor innervation to cranial blood vessels and the lacrimal and parotid glands. Because of the difficulties of pharmacological characterization of native P2X receptors, we have conducted these studies in the mouse, where we have been able to examine the receptors present in animals in which the genes for $P2X_2$, $P2X_3$ or both subunits have been deleted.

Previous studies from this laboratory have shown that the P2X receptors on rat pelvic ganglion neurones (Zhong et al., 1998) and coeliac ganglion neurones (Zhong et al., 2000b) are of the $P2X_2$ subtype. In contrast, two distinct P2X receptors ($P2X_2$ and $P2X_{2/3}$) co-exist on the same neurones in guinea-pig superior cervical ganglia (SCG) (Zhong et al., 2000a). Most studies on recombinant P2X receptors have been carried out on rat receptors. Although great similarity is usually observed between native mouse and rat P2X receptors (e.g. Zhong et al., 1998, 2000b), some caution is advisable in making direct comparison between the properties of native receptors in the mouse and recombinant rat P2X receptors, or in extrapolating from P2X gene deletion studies in mice to the situation in rat neurones.

2. Methods

Knockout mice were generated by selective deletion of genes encoding $P2X_2$ ($P2X_2^{-/-}$) and $P2X_3$ ($P2X_3^{-/-}$) as previously described (Cockayne et al., 2000). $P2X_2^{-/-}$ mice were generated by introducing a deletion that encompasses exons 2–11 of the mouse $P2X_2$ gene. Double knockout ($P2X_2/P2X_3^{Dbl-/-}$) mice were produced by conventional breeding of $P2X_2^{-/-}$ and $P2X_3^{-/-}$ animals to generate mice carrying a deletion of both genes.

2.1. Cell culture

Mice, weighing 25–45 g, were killed by inhalation of a rising concentration of CO_2 and death was confirmed by cardiac haemorrhage. The otic ganglia (OTG) were rapidly dissected out (Suzuki and Hardebo, 1991), 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 (HBSS; Life Technologies) with 10 mM Hepes buffer (pH 7.4) containing 1.5 mg ml^{-1} collagenase (Class II, Worthington Biochemical Corporation, UK) and 6 mg ml^{-1} bovine serum albumin (Sigma, Poole, UK) at 37 °C for 45 min. The ganglia were then incubated in 4 ml HBSS containing 1 mg ml^{-1} trypsin (Sigma) at 37 °C for 15 min. The solution was replaced with 1 ml growth medium comprising L-15 medium supplemented with 10% bovine serum, 50 ng ml^{-1} nerve growth factor, 2 mg ml^{-1} $NaHCO_3$, 5.5 mg ml^{-1} glucose, 200 i.u. ml^{-1} penicillin and 2 mg ml^{-1} streptomycin. The ganglia were dissociated into single neurones by gentle trituration. The cell suspension was diluted to 8 ml, then centrifuged at 160 *g* for 5 min. The pellet was resuspended in 0.8 ml growth medium and plated onto 35 mm Petri dishes coated with 10 μg ml^{-1} laminin (Sigma). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and used on the following day.

2.2. 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 −60 mV. The 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 an internal solution which contained (mM): KCl 120, Hepes 10, tripotassium citrate 10 and EGTA 0.1; the pH was adjusted to 7.2 using KOH. In some experiments, a similar solution was used in which K^+ was replaced by Cs^+. No difference in response was observed between the two internal solutions. Data were acquired using pCLAMP software (Axon Instruments). Signals were filtered at 2 kHz (−3 dB frequency, Bessel filter, 80 dB per decade). Cells were confirmed as neurones by the presence of a fast rapidly inactivating inward current upon depolarization to 0 mV.

Drugs were applied rapidly through a manifold comprising six capillaries made of fused silica coated with polyimide, with 250 μm internal diameter (SGE, Milton Keynes, UK), connected to a single outlet made of the same tubing, which was placed about 200 μm from the cell. Solutions were delivered by gravity flow from independent reservoirs. One barrel was used to apply drug-free solution to enable rapid termination of drug applications. Solution exchange measured by changes in open tip current was complete in 200 ms; however, complete exchange of solution around an intact cell was considerably slower (1 s). Agonists were separately applied for 4 s at 2 min intervals, a time sufficient for responses to be reproducible. Antagonists were present for 2 min before and during the reapplication of agonists.

2.3. Immunohistochemistry

The mice were killed as described above and the otic ganglia were dissected out. These ganglia were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7·4) for 120 min, then they were rapidly frozen by immersion in isopentane at −70 °C for 2 min, cut into 10 μm sections using a cryostat, thaw-mounted on gelatin-coated poly-L-lysine-coated slides and air-dried at room temperature. Antibodies against rat $P2X_{1–6}$ receptors (see Oglesby et al., 1999) have been used in this study with an indirect three-layer immunofluorescent method. Primary antibodies to $P2X_{1–6}$ receptors raised in rabbit were detected with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, Pennsylvania, USA) and visualized with streptavidin–Texas Red (red fluorophore, Sigma) or streptavidin–fluorescein isothiocyanate (FITC—green fluorophore, Amersham, UK). Briefly, the sections were incubated overnight with the primary antibodies diluted to 3 μg/ml with 10% normal horse serum (NHS) in PBS containing 0.05% Merthiolate and 0.2% Triton X-100. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Pennsylvania, USA) diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate for 1 h, followed by incubation in streptavidin–FITC (or streptavidin–Texas Red) diluted 1:200 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. Slides were mounted with citiflour and examined with fluorescence microscopy. Control experiments were performed both by using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and by omission of the primary antibody to confirm the specificity of the immunoreaction.

In co-localization studies investigating the co-expression of $P2X_2$ and $P2X_3$ receptors, $P2X_2$ receptor immunoreactivity was enhanced with tyramide amplification, which allows high sensitivity and low background specificity (Renaissance, TSA indirect, NEN, USA). The use of TSA allows immunostaining with two rabbit antisera, as described previously (Bradbury et al., 1998; Ruan and Burnstock, 2003). Briefly, sections were incubated in 10% NHS in PBS for 30 min at room temperature, followed by incubation with the $P2X_2$ antibody (1 μg/ml) in 10% NHS and 0.2% Triton X-100 in PBS, overnight. Subsequently the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch; 1:500) for 1 h, with ExtrAvidin peroxidase (1:1500) for 1 h, with biotinylated tyramide for 8 min, and then in streptavidin–FITC (1:200) for 10 min. Polyclonal rabbit antibody against the $P2X_3$ receptor subtype (1:400) was applied as a second primary antibody and detected with Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). To check for non-cross-reactivity, $P2X_2$ receptor immunostaining using indirect TSA was performed alone on some sections, as was $P2X_3$ indirect immunofluorescence. The localization of each marker appeared identical to the localization observed with the double staining technique, with no apparent cross-reactivity.

2.4. Data analysis

All responses were normalized to that evoked by 100 μM ATP in the same cell, unless otherwise stated. All data are expressed as the means ± S.E.M. Statistical analysis (Student's t test, F test) was performed using Excel (Microsoft, USA). Concentration–response data were fitted with the Hill equation: $Y = A/[1 + (K/X)^{nH}]$, where A is the maximum effect, K is the EC_{50} and nH is the Hill coefficient. The combined data from the given number of cells were fitted, and the results are presented as values ± S.E., determined by the fitting routine. Traces were acquired using Fetchex (pCLAMP software) and plotted using Origin41 (Microcal, Northampton, MA, USA).

Cell counts for immunohistochemistry were performed at 20× objective magnification. $P2X_2$ receptor expression was determined by counting all $P2X_2$ receptor-positive cell profiles in every sixth section throughout the ganglia (a total of 500 cells). To calculate percentages of $P2X_2$ receptor co-localization with $P2X_3$, four randomly selected ganglion sections were chosen for each animal (200 cells). For each section, counts were made of the number of profiles positive for $P2X_2$ receptor, the number of profiles positive for the $P2X_3$ receptor and the number of profiles expressing both antigens, and percentages were calculated.

2.5. Drugs

ATP, αβ-meATP, suramin and ivermectin were obtained from Sigma Chemical Co. (Poole, UK). TNP-ATP was from Molecular Probes Europe (Leiden, The Netherlands). Solutions 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.

3. Results

3.1. Electrophysiology

3.1.1. Wild-type mice

All the otic ganglion neurones from wild-type (WT) mice responded to ATP (30–100 μM) with a rapidly activating and sustained inward current. Of these neurones, 38% (17/45) also responded with a similar sustained inward current to αβ-meATP, an agonist selective for receptors containing $P2X_1$ or $P2X_3$ subunits (Fig. 1A). The amplitudes of the responses to 100 μM ATP and 100 μM αβ-meATP were 1.61 ± 0.33 nA ($n = 45$) and 0.53 ± 0.22 nA ($n = 17$), respectively. Thus, neurones in the mouse otic ganglion can be divided into two groups, those that respond to αβ-meATP, and those that do not. The concentration dependence of the response to ATP was determined in those otic ganglion neurones that did not respond to αβ-meATP. Fitting the Hill equation to the data gave an EC_{50} for ATP of 109 μM, and a Hill coefficient of 2.0 (Fig. 1B). For those neurones that did respond to αβ-meATP, the concentration–response curve for this agonist had an EC_{50} of 39 μM, and a Hill coefficient of 1.75 (Fig. 1B).

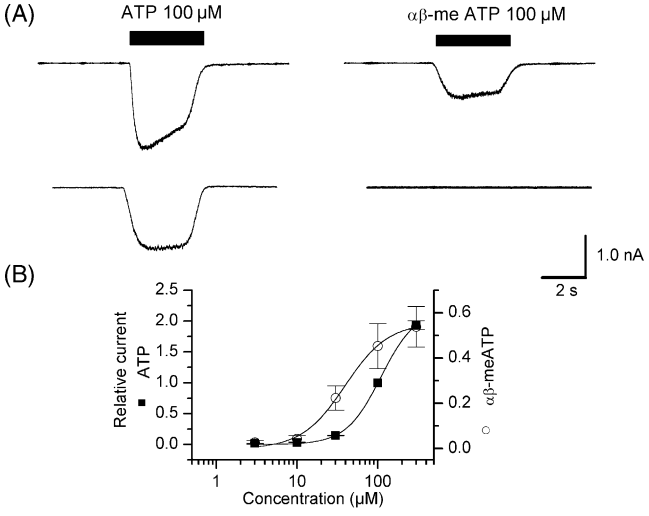

Fig. 1. Agonist responses in WT mouse otic ganglion neurones. (A) 38% of neurones responded to both ATP (100 μM) and αβ-meATP (100 μM) with a sustained current. 62% of neurones responded to ATP with sustained current but did not respond to αβ-meATP ($n = 45$). (B) Concentration–response curves for ATP in neurones with no response to αβ-meATP (■) and for αβ-meATP (○) on OTG neurones from WT mice. Responses have been normalized with respect to that obtained with 100 μM ATP on the same cell. Points represent mean ± S.E.M. from 4–7 cells.

Suramin is a relatively non-selective P2 receptor antagonist. We tested this drug against ATP response in those cells that did not respond to αβ-meATP, and against both ATP and αβ-meATP, in cells which did respond to the latter agonist. After 2 min pre-incubation with suramin, ATP responses were $17.1 \pm 0.7\%$ and $5.65 \pm 0.3\%$ of control, in cells with and without a response to αβ-meATP respectively (Fig. 2A). Suramin reduced the response to αβ-meATP to $2.27 \pm 0.4\%$ of control (Fig. 2A). Responses recovered to their control values within 4 min of washing out suramin.

TNP-ATP is a selective antagonist for $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors, being approximately 1000 times less potent at $P2X_2$ receptors (Virginio et al., 1998;

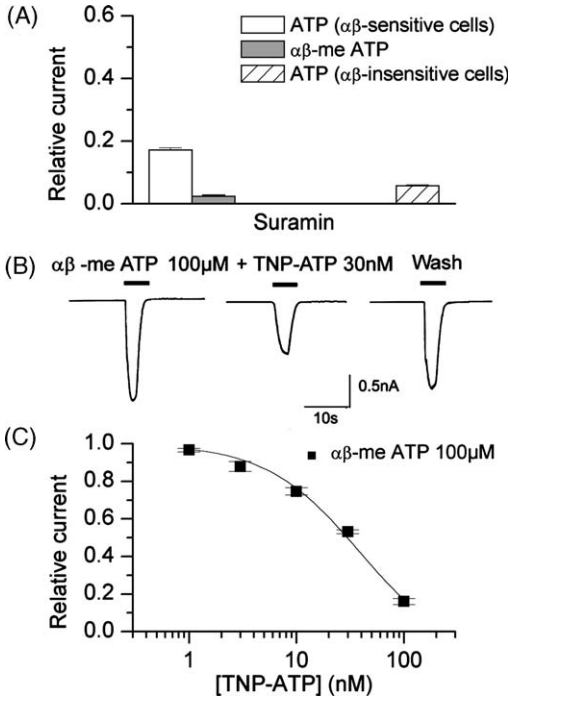

Fig. 2. Antagonist sensitivity of P2X receptors in otic ganglion neurones. (A) Inhibition by suramin (100 μM) of the response to ATP and αβ-meATP (30 μM) in cells responding to both agonists and of the response to ATP in cells with no response to αβ-meATP. Suramin was present for 2 min before and during the second application of agonists. Columns represent the mean ± S.E.M. from four cells. (B) Representative traces showing inhibition of αβ-meATP responses by TNP-ATP. (C) Concentration–effect curve for the inhibition of αβ-meATP by TNP-ATP. Points represent mean ± S.E.M. from 4–7 cells. Responses were normalized with respect to that obtained with agonists in the absence of TNP-ATP on the same neurone.

Dunn et al., 2000). To test the possibility that the response to αβ-meATP was mediated via $P2X_{2/3}$ receptors, we tested the ability of TNP-ATP to antagonize this response. On WT otic neurones, TNP-ATP reversibly inhibited the response to αβ-meATP (100 μM) with an IC_{50} of 38.6 nM (Fig. 2B,C).

Zn^{2+} and H^+ have potent modulatory effects on some P2X receptors and are useful tools for identifying receptors containing $P2X_2$ subunits, where both agents increase agonist potency (for review, see North, 2002). In those neurones from WT mice which did not respond to αβ-meATP, the response to ATP (10 μM) was enhanced by co-application of Zn^{2+} (10 μM) to 585 ± 47% of the control ($n = 6$, Fig. 3A). However, in cells that responded to αβ-meATP, the enhancement of the ATP response was more modest (to 185 ± 12%; $n = 4$, Fig. 3A). Co-application of Zn^{2+} (10 μM) also enhanced the response to αβ-meATP (10 μM) to 124 ± 6% of the control ($n = 4$, Fig. 3A). Changing the extracellular pH had a profound effect on the response of otic neurones to ATP and αβ-meATP. In those cells which responded to αβ-meATP, lowering the extracellular pH from 7.4 to 6.8 potentiated the response to 30 μM ATP and 30 μM αβ-meATP to 163 ± 12% and 142 ± 15% of the control responses ($n = 6$, Fig. 3C), while raising the pH to 8.0 produced a dramatic reduction of the responses. In those cells which did not respond to αβ-meATP, reducing the pH from 7.4 to 6.8 potentiated the response to ATP to 248 ± 23% of the control, while increasing pH to 8.0 reduced the response to 11 ± 1% of control (Fig. 3B).

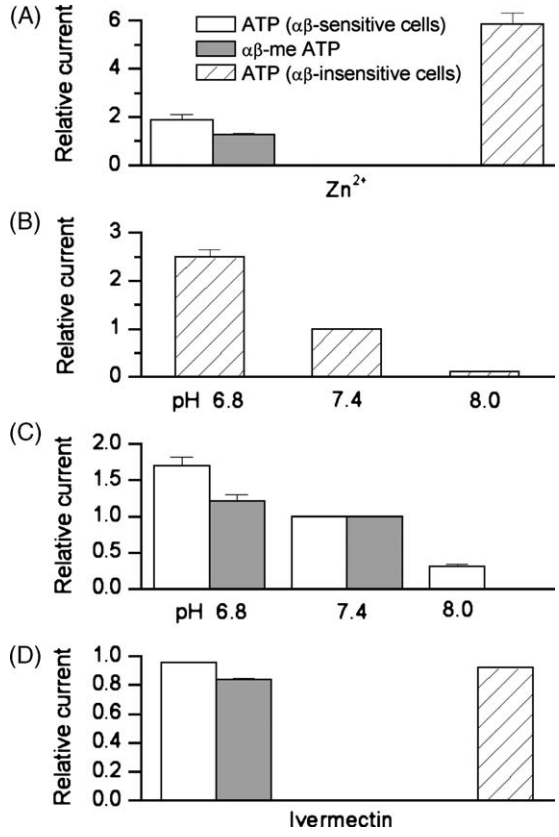

Fig. 3. Action of allosteric modulators. (A) Potentiation by Zn^{2+} (10 μM) of the currents activated by ATP (10 μM) and αβ-meATP (10 μM) on neurones from WT mice. Averaged peak currents induced by ATP and αβ-meATP in the presence and absence of Zn^{2+} on neurones with or without response to αβ-meATP in WT mice. (B, C) Effect of extracellular pH on the amplitude of currents activated by ATP and αβ-meATP. Averaged peak currents induced by ATP (30 μM) and αβ-meATP (30 μM) in the different extracellular pH, in those neurones without (B) or with (C) a response to αβ-meATP. Responses were normalized with respect to that obtained with agonists at pH 7.4 on the same neurone. (D) Effect of ivermectin on the amplitude of currents activated by ATP and αβ-meATP on WT mouse otic neurones. Averaged peak currents induced by ATP (30 μM) and αβ-meATP (30 μM) in the presence and absence of ivermectin on the neurones with or without a response to αβ-meATP.

It has been suggested that P2X receptors in submandibular ganglion neurones involve the $P2X_4$ subunit (Liu and Adams, 2001), and we detected some immunostaining for this protein in otic ganglion neurones (see below). Ivermectin is reported to be a subtype-selective modulator of $P2X_4$-containing receptors, which increases the potency and efficacy of ATP (Khakh et al., 1999). However, it has no effect on $P2X_2$, $P2X_{2/3}$ and $P2X_3$ receptors. To investigate the possible involvement of $P2X_4$ subunits in the response of otic ganglion neurones, we examined the effects of ivermectin. After a 2 min pre-incubation with 1 μM ivermectin, the response to 100 μM ATP, in cells which did not respond to αβ-meATP, was significantly reduced to 92 ± 0.3% of control ($P < 0.05$, $n = 3$, Fig. 3D). In four cells which responded to αβ-meATP, the response to 100 μM ATP in the presence of ivermectin 1 μM was significantly attenuated to 96 ± 0.6% of control ($P < 0.05$, $n = 4$, Fig. 3D), while the response to 100 μM αβ-meATP was 87 ± 2% of control ($P < 0.05$, $n = 4$, Fig. 3D).

3.1.2. Knockout mice

In $P2X_3^{-/-}$ mice, all cells ($n = 13$) gave a persistent response to 100 μM ATP but none of these neurones responded to 100 μM αβ-meATP (Fig. 4A). The mean amplitude of the response to 100 μM ATP was not significantly different from that of the WT mice ($P > 0.1$). We examined some of the pharmacological properties of this response. The concentration dependence of this response was investigated. Fitting the Hill equation to the data gave an EC_{50} of 47 μM, and a Hill coefficient of 1.91 (Fig. 4B). We determined the sensitivity of this response to the non-selective P2 antagonist suramin. After 2 min pre-incubation with this antagonist, the response to 30 μM ATP was reduced to 11.7 ± 6% of control (Fig. 4C). Co-application of the allosteric modulator Zn^{2+} (10 μM) enhanced the response to ATP (10 μM) to 542 ± 39% of the control ($n = 6$, Fig. 4C). This was not significantly different ($P > 0.1$)

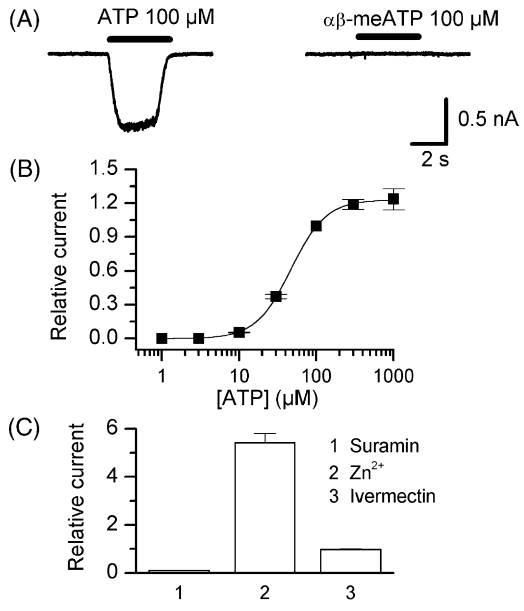

Fig. 4. Response in otic neurones from $P2X_3^{-/-}$ mice. (A) Representative traces of currents evoked by ATP and αβ-meATP. All cells responded to ATP but none responded to αβ-meATP. (B) Concentration–response curves of the inward currents produced by ATP. Responses have been normalized with respect to those obtained with 100 μM ATP in the same cell. Points represent mean ± S.E.M. from 4–7 cells. (C) Effect of suramin (100 μM), Zn^{2+} (10 μM) and ivermectin (1 μM) on the response to 30 μM ATP.

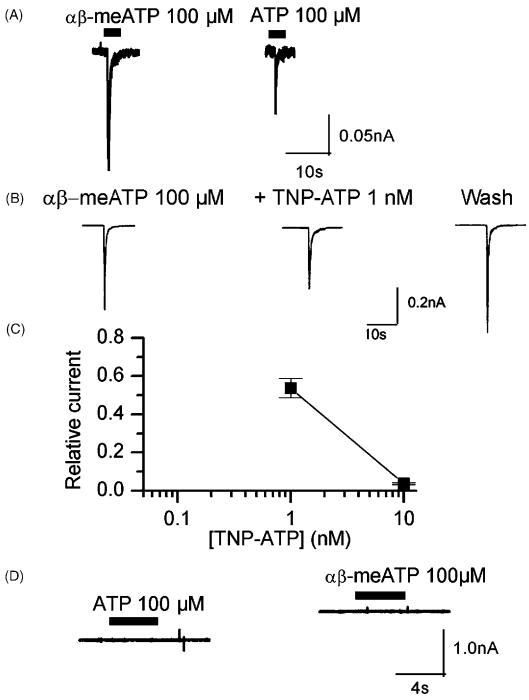

Fig. 5. Response in otic neurones from $P2X_2^{-/-}$ and $P2X_2/P2X_3^{Dbl-/-}$ mice. Representative traces of currents evoked by ATP and αβ-meATP. (A) 36% of neurones responded to both agonists with transient currents. (B) Representative traces of the inhibition by TNP-ATP (1 nM) of responses to αβ-meATP (100 μM). (C) Concentration dependence of the antagonism by TNP-ATP of the αβ-meATP (100 μM) response. (D) Neurones from $P2X_2/P2X_3^{Dbl-/-}$ mice failed to respond to either ATP or αβ-meATP.

from the potentiation seen in αβ-meATP-insensitive neurones from WT mice. Finally, we tested the effect of the $P2X_4$ selective modulator ivermectin on ATP responses in the $P2X_3^{-/-}$ mice. In the presence of 1 μM ivermectin, there was no significant effect, with the response to ATP being 98 ± 3% of the control current ($P > 0.1$, $n = 4$; Fig. 4C).

In otic ganglion neurones from $P2X_2^{-/-}$ mice, no sustained currents to either ATP or αβ-meATP were observed, confirming the absence of the $P2X_2$ subunit. However, 36% (14/39) neurones responded to both ATP and αβ-meATP with a transient response (Fig. 5A), suggesting the presence of homomeric $P2X_3$ receptors. The proportion of cells responding was not significantly different ($P > 0.1$) from the proportion of neurones responding to αβ-meATP in neurones from WT mice. The transient response in neurones from $P2X_2^{-/-}$ mice was potently inhibited by TNP-ATP, with an IC_{50} of less than 10 nM (Fig. 5B,C).

In $P2X_2/P2X_3^{Dbl-/-}$ mice, there was response neither to ATP (100 μM) nor to αβ-meATP (100 μM) in the 14 neurones tested (Fig. 5D). We did not investigate the activity of other P2X agonists on these neurones.

3.1.3. Rat otic ganglion neurones

We also investigated P2X receptors on rat otic ganglion neurones. Of the 67 cells tested, 64 (96%) responded to both ATP and αβ-meATP with a rapidly activating and persistent inward current (Fig. 6A). No rapidly desensitizing responses were observed. The amplitude of currents induced by ATP and αβ-meATP were 0.53 ± 0.05 and 0.27 ± 0.08 nA, respectively ($n = 56$). The response to αβ-meATP 100 μM was consistently between 40% and 60% of that evoked by ATP 100 μM in the same cell. The amplitude of the αβ-meATP response expressed as a fraction of that to ATP varied from cell to cell between 0 and 1. The relative amplitude appeared to be normally distributed with a mean value of 49 ± 0.2% (Fig. 6B).

3.2. Immunohistochemistry

To provide further support for our pharmacological characterization, we carried out immunohistochemistry studies, using antibodies raised against the C-termini of rat $P2X_{1-6}$ subunits, to visualize protein expression in sections of mouse otic ganglia. Strong $P2X_2$ immunoreactivity was observed in most (92.6 ± 0.6%) neurones in otic ganglia of WT mice, while $P2X_3$ immunoreactivity was present in only a sub-population (34.3 ± 0.8%) of cells (Fig. 7). Double labelling experiments confirmed that both $P2X_2$ and $P2X_3$ subunits were co-expressed in a sub-population (37.1 ± 1.0%) of neurones (Fig. 7).

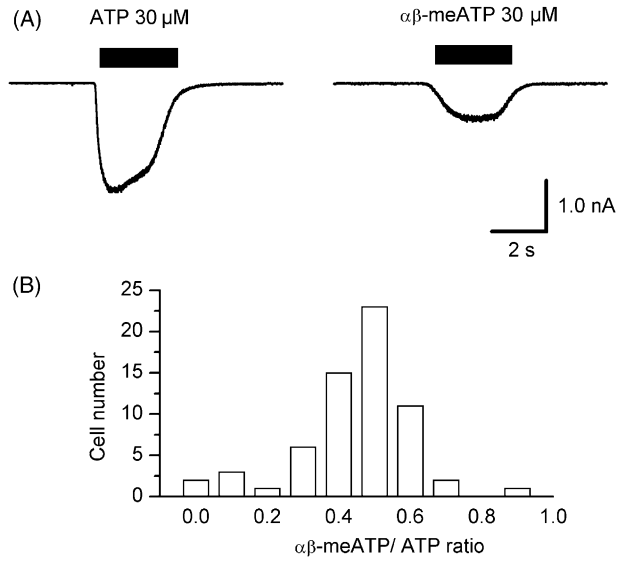

Fig. 6. Purinergic responses in *rat* otic ganglion neurones. (A) Representative traces of currents evoked by ATP (30 μM) and αβ-me ATP (30 μM) from a single neurone. (B) The amplitude distribution for the αβ-meATP (100 μM) response, expressed as a fraction of that to ATP (100 μM) in the same neurone.

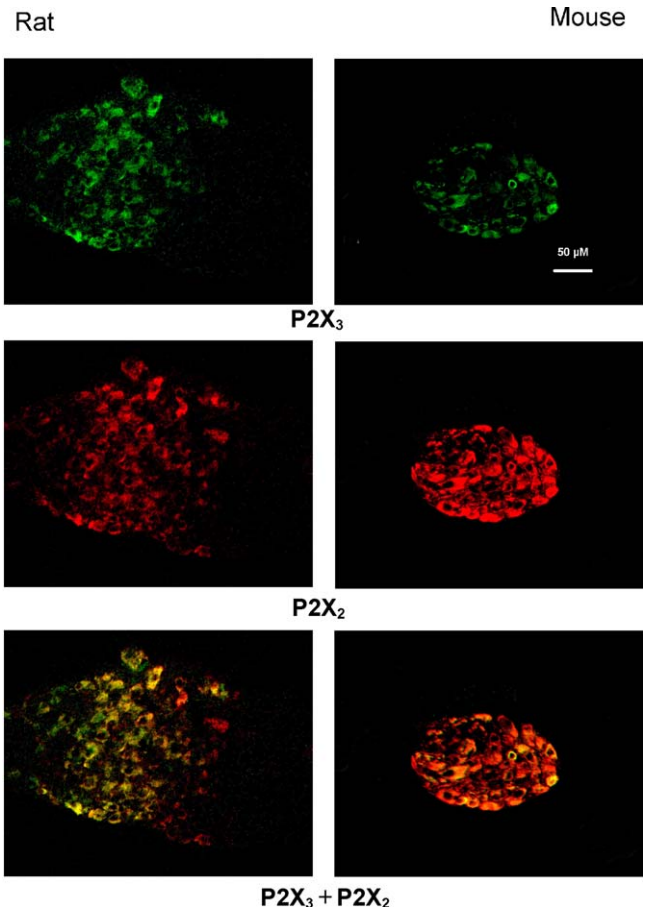

Fig. 7. Immunohistochemical staining of mouse and rat otic ganglia. Double labelling using polyclonal antibodies specific for $P2X_2$ and $P2X_3$ subunits shows high levels of $P2X_2$ and $P2X_3$ immunoreactivity in rat and WT mouse otic ganglion neurones. Overlay of the images shows much greater co-localization (yellow) in the rat otic ganglion compared with the mouse. Scale bar, 50 μm.

Immunostaining for $P2X_2$ and $P2X_3$ subunits was also present in rat otic ganglion neurones, and in agreement with our electrophysiological experiments, the majority of neurones exhibited co-localization of both proteins (Fig. 6).

In ganglia from $P2X_2^{-/-}$ mice, specific $P2X_2$ immunostaining was totally absent (Fig. 8A), while $P2X_3^{-/-}$ mice lacked any specific $P2X_3$ immunostaining (Fig. 8D). In ganglia from $P2X_2/P2X_3^{Dbl-/-}$ mice, specific $P2X_2$ and $P2X_3$ immunostaining were both absent (Fig. 8E,F). As with immunohistochemical studies of other autonomic ganglia (Xiang et al., 1998), some immunoreactivity for $P2X_1$, $P2X_4$, $P2X_5$, and $P2X_6$ protein was also detected; however, this was generally of low intensity, and occurred in a small sub-population of otic ganglion neurones (Fig. 9). The staining for these subunits was similar in both WT and knockout (KO) mice (not shown).

4. Discussion

4.1. Pharmacological study of P2X receptors in mouse otic ganglion

In otic ganglia from WT mice, all neurones respond to ATP with slowly desensitizing currents while 38% cells also respond to αβ-meATP with similar, sustained currents. These results suggest the presence of neurones with homomeric $P2X_2$ receptors and a sub-population of neurones with heteromeric $P2X_{2/3}$ receptors in the otic ganglion. The sensitivity to αβ-meATP, the sustained nature of the response, and the sensitivity to the antagonist TNP-ATP are all consistent with the involvement of the heteromeric $P2X_{2/3}$ receptor. The ATP response is potentiated by Zn^{2+} and by lowering pH, which are characteristics of the recombinant $rP2X_2$ receptor (King et al., 1997; Wildman et al., 1998). Thus, our data suggest that some mouse otic neurones express $P2X_2$ receptors, while others contain predominantly heteromeric $P2X_{2/3}$ receptors. Since the otic ganglion provides motor innervation to cranial blood vessels and the lacrimal and parotid glands, it will be interesting to determine whether there is any correlation between the types of P2X receptors a neurone expresses and the target organ which it innervates.

4.2. Pharmacological study in P2X subunit knockout mice

In $P2X_3^{-/-}$ mice, ATP induces a slowly desensitizing inward current with an EC_{50} of 47 μM, while αβ-meATP

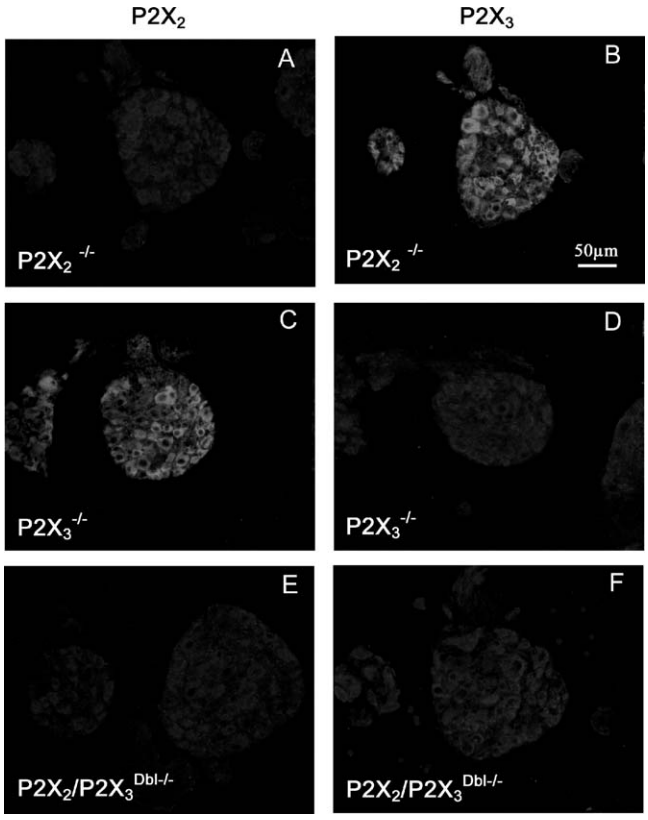

Fig. 8. Immunohistochemical staining in otic ganglia from P2X knockout mice. Staining using $P2X_2$ (A, C, E) and $P2X_3$ (B, D, F) antibodies was examined in sections of otic ganglia from $P2X_2^{-/-}$ (A, B), $P2X_3^{-/-}$ (C, D) and $P2X_2/P2X_3^{Dbl-/-}$ (E, F) mice. While specific $P2X_3$ staining was observed in the $P2X_2^{-/-}$ ganglia and $P2X_2$ staining was observed in $P2X_3^{-/-}$ animals, no staining was observed in sections from double knockout animals.

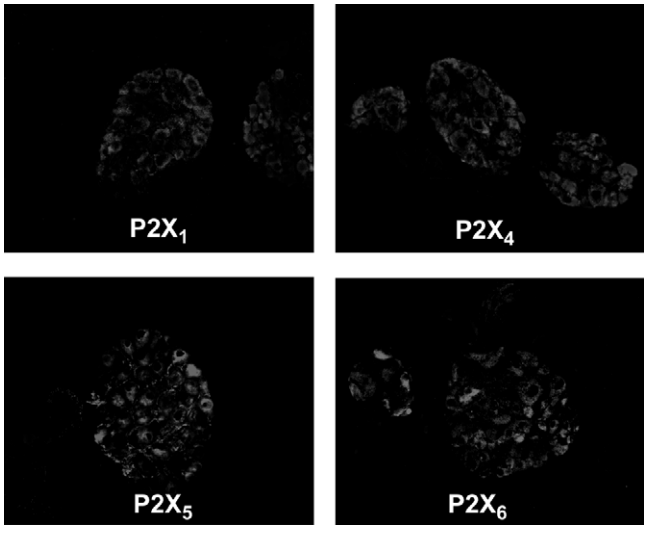

Fig. 9. Immunohistochemical staining for other P2X subunits in otic ganglia from WT mice. Low levels of staining for $P2X_1$ and $P2X_4$ protein were detected in many cells. In contrast, a very small number of cells showed stronger immunoreactivity for $P2X_5$ and $P2X_6$.

is inactive. The response to ATP was inhibited by suramin but potentiated by Zn^{2+} and low pH. These properties are similar to those observed in sympathetic neurones (see Dunn et al., 2001) and fit the profile of the recombinant $P2X_2$ receptor (Brake et al.,1994; Wildman et al., 1998, 1999; King et al., 2000).

In $P2X_2^{-/-}$ mice, ATP and αβ-meATP evoke inward currents in only 38% neurones; these responses were transient in nature. However, these responses were very sensitive to TNP-ATP, with an IC_{50} of 1.4 nM. These properties are consistent with those of the homomeric $rP2X_3$ receptor (Virginio et al., 1998).

In otic ganglion neurones from $P2X_2/P2X_3^{Dbl-/-}$ mice, no obvious responses to either ATP or αβ-meATP were observed. This suggests that P2X receptors present in WT neurones may be composed of only $P2X_2$ and $P2X_3$ subunits. Furthermore, there appears to be no compensatory up-regulation of other subunits in otic ganglion neurones in KO mice.

4.3. Confirmation by immunohistochemistry of the existence of $P2X_2$ and $P2X_3$ subunits in otic ganglion neurones

In WT mice, both $P2X_2$ and $P2X_3$ immunoreactivity were detected in otic ganglion neurones, and co-localization was observed in a sub-population of cells. Similarly, in $P2X_2^{-/-}$ mice, only a few neurones showed specific $P2X_3$ receptor immunoreactivity. In $P2X_3^{-/-}$ mice, many otic neurones show specific $P2X_2$ receptor immunoreactivity, whilst in $P2X_2/P2X_3^{Dbl-/-}$ mice, neither $P2X_2$ nor $P2X_3$ immunoreactivity were detectable. Our immunostaining results demonstrated that $P2X_2$ and $P2X_3$ are dominant P2X receptor proteins expressed in mouse otic ganglion neurones. Selective gene deletion resulted in loss of appropriate proteins, with no obvious up-regulation of expression of the other P2X subunits, such as $P2X_1$ and $P2X_6$, which showed low levels of immunoreactivity in a sub-population of neurones. In rat otic ganglia, $P2X_2$ and $P2X_3$ immunoreactivity were also dominant. In contrast to the situation in mouse ganglia, where co-localization was detected in a sub-population of neurones, these two subunits were co-localized in most neurones in the rat ganglia.

It has been suggested that the expression of P2X receptors may be modified by dissociation/cell culture (Smith et al., 2001). Furthermore, in the gerbil hippocampus, P2X receptor expression is modified by activation of $GABA_A$ receptors, possibly as a consequence of changes in neuronal activity (Kang et al., 2003). However, our immunohistochemical findings on sections of intact ganglia were in good agreement with our functional studies on dissociated neurones in short term culture.

4.4. Are any other P2X subunits involved?

It is unlikely that homomeric $P2X_1$ or $P2X_3$ receptors exist in WT otic ganglion neurones because no rapidly desensitizing responses to ATP were observed. The absence of any response in $P2X_2/P2X_3^{Dbl-/-}$ mouse otic neurones also argues against a role for $P2X_1$ receptors. Our immunohistochemistry results show that $P2X_4$, $P2X_5$, and $P2X_6$ immunoreactivity was present in similar low levels in otic ganglion neurones from WT and knockout mice. The absence of any ATP response in the neurones from $P2X_2/P2X_3^{Dbl-/-}$ mice suggests that no homomeric receptors are formed from these subunits. However, this does not rule out the possibility that they are involved in the formation of heteromeric receptors in the WT neurones. Ivermectin is a selective potentiator of receptors containing the $P2X_4$ subunit (with an EC_{50} close to 1 μM), while it is inactive on $P2X_2$ receptors at concentrations up to 10 μM (Khakh et al., 1999). On neurones from WT mice, ivermectin produced not an enhancement of the ATP response but a small, though significant, reduction in the response. Thus, the native receptors on otic ganglion neurones are unlikely to contain the $P2X_4$ subunit. Although we cannot rule out the possible involvement of $P2X_5$ or $P2X_6$ subunits in some heteromeric complexes, the simplest explanation that our data would support is that the response of WT mouse otic ganglion neurones is mediated by homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors.

4.5. Are $P2X_2$ and $P2X_{2/3}$ receptors present in the rat otic ganglion?

In this study, we have investigated P2X receptors in mouse otic ganglion neurones. This has enabled us to use not only classical pharmacology but also gene deletion techniques. However, most studies of recombinant receptors have used rat receptors and the rat is still the laboratory animal of choice for many studies. It was therefore important to confirm the similarity between mouse and rat. Previous studies show that $P2X_2$ homomeric receptors are dominant in rat sympathetic ganglia, while homomeric $P2X_2$, $P2X_3$ and heteromeric $P2X_{2/3}$ receptors are present in rat sensory ganglia. Our results show that $P2X_2$ and $P2X_{2/3}$ receptors are present in mouse otic ganglion neurones, and that similar receptors are expressed by these neurones in the rat. However, a significantly greater proportion of neurones express $P2X_{2/3}$ receptors in the rat (96%) compared with the mouse (38%). In a study of rat submandibular neurones, it was suggested, on the basis of pH sensitivity and inhibition of the ATP-evoked current by specific anti-P2X antibodies, that homomeric and/or heteromeric $P2X_2$ and $P2X_4$ receptor subtypes are present (Liu and Adams, 2001). Further studies are required to determine the extent of the variation in P2X receptor expression between different parasympathetic ganglia and between different species.

It is interesting to note that, in terms of the P2X receptors which they express, parasympathetic motor neurones of the otic ganglion are more similar to sensory neurones of the nodose ganglion than postganglionic motor neurones of the sympathetic nervous system.

4.6. Physiological role

ATP is stored with acetylcholine in synaptic vesicles of cholinergic nerve terminals and is co-released with ACh from motor nerve terminals (Redman and Silinsky, 1994) and sympathetic pre-ganglionic fibres (Vizi et al., 1997). ATP is almost certainly co-released with ACh from pre-ganglionic nerve terminals in the otic ganglion, and thus P2X receptors on these neurones are likely to be activated during synaptic transmission. The fast EPSP in rat otic ganglion neurones is greatly attenuated but not abolished by high concentrations of mecamylamine or tubocurarine (Callister et al., 1997), and it is tempting to speculate that the residual component is mediated by ATP. The significance of a small purinergic component to the EPSP remains to be determined. However, P2X channels have a high permeability to calcium. Consequently, their opening could activate various biochemical pathways, including those regulating post-synaptic architecture. A second possibility is that these receptors respond to ATP released from surrounding glial cells as part of a glial cell–neurone signalling system. A third possibility for a physiological role for P2X receptors present on parasympathetic ganglion neurones is that they are involved in the regulation of transmitter release at the nerve terminal. Such a role has recently been demonstrated for P2X receptors on the sympathetic nerve terminals in the vas deferens (Queiroz et al., 2003).

In conclusion, unlike rat and mouse sympathetic ganglion neurones, parasympathetic ganglion neurones of the otic ganglion express two distinct P2X receptors. The simplest explanation, with which our data are consistent, is that these are homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors. The physiological significance of these receptors has yet to be determined.

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