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Differential expression of P2X receptors on neurons from different parasympathetic ganglia

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Abstract

Whole-cell patch clamp recording and immunohistochemistry were used to investigate the expression of P2X receptors on rat parasympathetic ganglion neurons of the otic, sphenopalatine, submandibular, intracardiac and paratracheal ganglia. Neurons from all five ganglia responded to ATP with a rapidly activating, sustained inward current. Neurons of intracardiac and paratracheal ganglia were insensitive to αβ-meATP, while all neurons in the otic and some neurons of sphenopalatine and submandibular ganglia responded. Lowering pH potentiated ATP responses in neurons from all five ganglia. Co-application of Zn^{2+} potentiated ATP responses in intracardiac, paratracheal and submandibular ganglion neurons. Immunohistochemistry revealed strong and specific staining for the $P2X_2$ subunit in all five ganglia and strong $P2X_3$ staining in otic, sphenopalatine and submandibular ganglia. In conclusion, there is heterogeneity in P2X receptor expression in different parasympathetic ganglia of the rat, but the predominant receptor subtypes involved appear to be homomeric $P2X_2$ and heteromeric $P2X_{2/3}$.

Keywords: P2X receptor; Otic; Submandibular; Sphenopalatine; Intracardiac; Paratracheal; Parasympathetic ganglion

1. Introduction

P2X receptors are ATP-gated ion channels found on many types of non-excitable and excitable cells including peripheral and central neurons (see Burnstock, 1997 for review). To date, seven P2X receptor subunit genes ($P2X_1$–$P2X_7$) and several splice variants have been cloned (for review, see North, 2002). When heterologously expressed, these subunits form functional homomeric and heteromeric channels (see North, 2002; Brown et al., 2002) with different but overlapping properties with regard to agonist and antagonist selectivity, desensitization and single channel characteristics. Over the last 5 years much effort has been devoted to understanding the identity of native P2X receptors (see Schwiebert, 2003).

Previous studies indicate that in the rat, sensory neurons are sensitive to αβ-methylene ATP (αβ-meATP) due to the presence of the $P2X_3$ subunit, while sympathetic neurons lack this subunit and do not respond to αβ-meATP (see Dunn et al., 2001). However, neurons from guinea-pig superior cervical ganglion (Reekie and Burnstock, 1994) and guinea-pig coeliac ganglion (Khakh et al., 1995) respond to αβ-meATP and the receptors present on guinea-pig coeliac ganglion neurons resemble the heteromeric $P2X_{2/3}$ receptor found on rat nodose ganglion neurons (Khakh et al., 1995; Lewis et al., 1995). These findings suggest the existence of inter-species and inter-ganglion variation.

Some studies have examined the actions of ATP on parasympathetic ganglia, but because of their small size

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and diffuse nature, they have so far received much less attention than sympathetic and sensory ganglia. Guinea-pig intracardiac neurons respond to ATP analogues with an agonist potency order of 2-methylthio ATP ≥ ATP > αβ-meATP, which was taken to indicate the involvement of a P2Y-like receptor (Allen and Burnstock, 1990). In rat submandibular ganglion neurons, ATP evoked an inward current, and acidification to pH 6.2 increased the response amplitude twofold, whereas alkalization to 8.2 and 9.2 markedly reduced current amplitude (Liu and Adams, 2001). Cell dialysis with anti-$P2X_2$ and/or anti-$P2X_4$ but not anti-$P2X_1$ antibodies attenuated the ATP-evoked current. These data might indicate the presence of homomeric and or heteromeric $P2X_2$ and $P2X_4$ receptor subtypes (Liu and Adams, 2001; Smith et al., 2001). Neurons of the parasympathetic ganglia share the same embryological origins as those of the sympathetic and sensory ganglia. However, each parasympathetic ganglion innervates a specific target organ. It is therefore of interest to determine what types of P2X receptors they express, and whether there is significant inter-ganglionic variation. Furthermore, exploitation of the potential therapeutic targets of purinergic signalling systems (Burnstock, 2002) will require the widest possible understanding of the variety of P2 receptors, and their roles in different tissues.

In this study, we have for the first time, compared directly the P2X receptors expressed in five different parasympathetic ganglia from a single species, namely the rat. The ganglia studied: otic, sphenopalatine, submandibular, paratracheal and intracardiac provide innervation to a variety of different tissues. The otic ganglion provides motor innervation to cranial blood vessels, the lacrimal and parotid glands. The submandibular ganglion innervates the submandibular and sublingual salivary glands; while the sphenopalatine ganglion, supplies the lacrimal gland and the cranial blood vessels. The paratracheal and intracardiac ganglia, provide motor innervation to airway and heart, respectively.

We have used whole-cell patch clamp recording from dissociated neurons to study the pharmacological properties of the P2X receptors. In addition, we used immunohistochemistry on both sections of ganglia and ganglion cell cultures to investigate the distribution of P2X receptor subunits.

2. Materials and methods

2.1. Cell culture

Sixteen to 18-day-old rats, weighing 30–40 g, were killed by inhalation of a rising concentration of CO_2 and death was confirmed by cardiac haemorrhage, in accordance with British Home Office and local ethics committee regulations. Otic (Suzuki and Hardebo, 1991), submandibular (Lichtman, 1977) and sphenopalatine (Spencer, 1990) ganglia were rapidly dissected out and placed in Leibovitz L-15 medium (Life Technologies, Paisley, UK). The ganglia were then desheathed and each ganglion was cut into 2–4 pieces. For preparation of intracardiac neurons, hearts were excised, the atria were separated and the medial region containing intracardiac ganglia was isolated and cut into pieces (Fieber and Adams, 1991). For preparation of paratracheal neurons, the trachea was removed and the superficial membranous layer containing paratracheal ganglia was rapidly isolated (Aibara et al., 1992; Ishibashi et al., 2001). The ganglia or tissues containing ganglia were 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, 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 200 μg ml^{-1} streptomycin. The ganglia were dissociated into single cells by gentle trituration. The cell suspension was diluted to 8 ml, and 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. Electrophysiological recording

Whole-cell voltage-clamp recording was carried out at room temperature (20–22 °C) using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) with membrane potential held at −60 mV. Data were acquired using pCLAMP software (Version 8, Axon Instruments). Signals were filtered at 2 kHz (−3 dB frequency, Bessel filter, 80 dB per decade), then digitized at 10–50 kHz (Digidata 1320A interface, Axon Instruments) and stored on the hard disk of a PC for viewing and analysis. Traces were acquired using Clampfit (pCLAMP software) and plotted using Origin 7 (Microcal, Northampton, MA, USA).

2.3. Solutions and drugs

External solution contained (mM): NaCl 154, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, Hepes 10 and glucose 5.6; the pH was adjusted to 7.4 using NaOH. Recording

electrodes (resistance 2–4 MΩ) were filled with internal solution which contained (mM): KCl 120, Hepes 10, tripotassium citrate 10 and EGTA 10; 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. All responses were normalized to that evoked by 100 μM ATP in the same cell, unless otherwise stated.

ATP, αβ-meATP and ivermectin were obtained from Sigma Chemical Co. (Poole, UK). 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. They were applied rapidly through a manifold comprising seven 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 application. Agonists were applied for 4 s at 2 min intervals, a time sufficient for responses to be reproducible.

2.4. Immunohistochemistry

2.4.1. Sections

The rats were killed as described above. The otic, sphenopalatine, submandibular ganglia and the dorsal surfaces of the atria including intracardiac ganglia were dissected out. They were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 120 min, then rapidly frozen by immersion in isopentane at −70 °C for 2 min. Sections (10 μm) were cut using a cryostat, thaw-mounted on gelatin-coated poly-L-lysine-coated slides and air-dried at room temperature. Antibodies against C-terminal of the rat $P2X_{1-6}$ receptors obtained from Roche Palo Alto, USA (see Oglesby et al., 1999 for details) were used with an indirect three layer immunofluorescent method. Briefly, the sections were incubated overnight with the primary antibodies diluted to 3 μg/ml with 10% normal horse serum in phosphate buffered saline (PBS) containing 0.05% Merthiolate and 0.2% Triton X-100. Subsequently the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, PA, USA) diluted 1:500 in 1% normal horse serum 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 Citifluor (Citifluor, London, UK) 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.

2.4.2. Cultured neurons

Paratracheal, sphenopalatine, submandibular, intracardiac and paratracheal ganglion neurons were dissociated as above, plated in chamber slides and maintained in culture for 2–3 days. They were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 120 min, then washed with distilled water three times. Immunohistochemistry was then carried out as described above.

2.5. Data analysis

Data are expressed as mean ± SEM. Statistical comparisons were made using *t*-test for unpaired samples and analysis of variance (ANOVA; Tukey's Multiple Comparison Test), as appropriate, using Excel (Microsoft, USA), or Origin 7 (Microcal). Statistical significance was taken as $P < 0.05$. 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 ± SEM, determined by the fitting routine.

3. Results

3.1. Electrophysiology

3.1.1. Agonists

Fast application of ATP (100 μM) to isolated rat otic, sphenopalatine, submandibular, intracardiac and paratracheal ganglion neurons produced a rapidly activating, sustained inward current (Fig. 1). No rapidly desensitizing currents were detected in any of these ganglion neurons. The proportion of cells that responded to ATP varied from 64% in intracardiac to 98% in the otic ganglion (see Table 1). The amplitudes of the responses to 100 μM ATP were similar in otic, sphenopalatine, submandibular and paratracheal ganglia, with mean amplitudes of 0.49 ± 0.13 nA, 0.23 ± 0.05 nA, 0.38 ± 0.06 nA, 0.38 ± 0.19 nA ($n > 15$ cells for each ganglion). However, responses in intracardiac ganglion neurons were significantly smaller than in the other ganglion neurons ($P < 0.05$) with a mean value of 0.02 ± 0.004 nA ($n = 16$).

When αβ-meATP was tested as an agonist, the majority of neurons from the otic ganglion responded

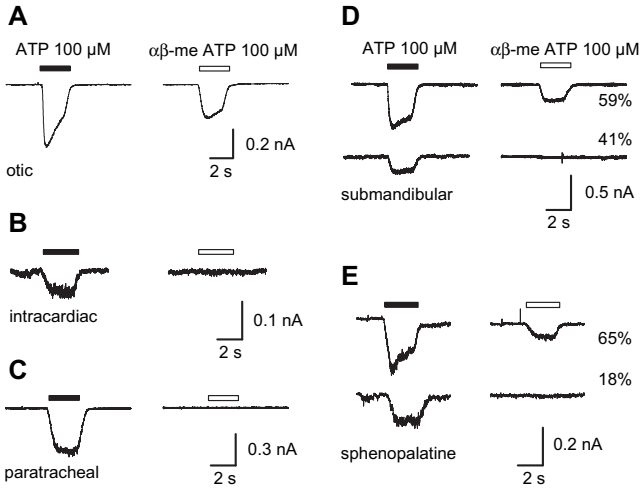

Fig. 1. Traces of currents evoked by ATP (100 μM) and αβ-meATP (100 μM) in rat otic (A), intracardiac (B), paratracheal (C), submandibular (D) and sphenopalatine (E) ganglion neurons. While almost all otic ganglion neurons responded to both ATP and αβ-meATP, cardiac and paratracheal ganglia responded only to ATP. In submandibular and sphenopalatine ganglia, some neurons responded to both agonists, while others failed to respond to αβ-meATP.

with a rapidly activating, sustained inward current (Fig. 1A). However, there were many neurons in submandibular and sphenopalatine ganglia that responded to ATP but not to αβ-meATP (Fig. 1D, E and Table 1). In contrast, in paratracheal and intracardiac ganglion neurons, αβ-meATP failed to evoke any significant response at concentrations up to 100 μM in any cell tested (Fig. 1B, C).

We investigated the concentration-dependence for the activation of the P2X receptors on these neurons by ATP and αβ-meATP. In sphenopalatine and submandibular ganglia, there are clearly two populations of neurons, expressing different types of P2X receptor (one activated by αβ-meATP, and one not). We therefore analysed these two populations of neurons separately. Concentration–response curves for ATP on neurons from all five ganglia are shown in Fig. 2A, B. On paratracheal ganglion neurons, the concentration–response curve gave an EC_{50} value of 158 μM. In sphenopalatine ganglion and submandibular ganglia, the αβ-meATP-insensitive neurons had a similar sensitivity to ATP as those in the paratracheal ganglion (Fig. 2 and Table 1). In contrast, neurons which responded to αβ-meATP were more sensitive to ATP with EC_{50} values similar to the 19 μM observed for otic ganglion neurons (see Table 1). Furthermore, concentration–response curves obtained in αβ-meATP-sensitive neurons from these ganglia were shallower than those in cells which did not respond to αβ-meATP. Surprisingly, the concentration–response curve for ATP on intracardiac neurons was also shallow, with a Hill coefficient of 0.6, although these cells did not respond to αβ-meATP. Concentration–response curves for αβ-meATP were obtained in neurons from otic, sphenopalatine and submandibular ganglia, and had EC_{50} values ranging from 47 μM to 137 μM and all had Hill coefficients ≥ 1 (Fig. 2C and Table 1).

In those otic, submandibular and sphenopalatine ganglion neurons which responded to αβ-meATP, the response to ATP was greater than that to the same concentration of αβ-meATP. Thus the response to αβ-meATP 100 μM was $49 \pm 0.2\%$, $33 \pm 1.3\%$ and $66 \pm 2.0\%$ of that evoked by ATP 100 μM in the same cell in otic, submandibular and sphenopalatine ganglia, respectively. This difference may have been due to the different affinity of the receptors for the two agonists. Alternatively, if these neurons express a mixture of αβ-meATP-sensitive and -insensitive P2X receptors, ATP would be able to activate all the receptors and thus give a greater maximum response. To investigate this possibility, we carried out cross-desensitization experiments on otic ganglion neurons. When αβ-meATP 100 μM was applied for 2 min, the response desensitized.

Table 1
Summary of the properties of responses to ATP and αβ-meATP in different parasympathetic ganglion neurons

Ganglion	ATP					αβ-meATP				
	%[a]	EC_{50} (μM)	nH	% Of control		%[a]	EC_{50} (μM)	nH	% Of control	
				Zn^{2+} [b]	pH[c]				Zn^{2+}[b]	pH[c]
Otic	96	19 ± 5.1	0.8	89 ± 5	162 ± 30	96	72 ± 3.3	1.2	146 ± 16	142 ± 2.5
Sphenopalatine[d]	65	37 ± 11	1.0	95 ± 5	112 ± 4	52	47 ± 1.1	1.4	108 ± 10	172 ± 27
	18	134 ± 7	1.8	244 ± 76	185 ± 7	NR				
Submandibular[d]	59	43 ± 29	0.7	121 ± 15	137 ± 10	59	137 ± 57	1.0	131 ± 29	211 ± 23
	41	122 ± 62	0.9	260 ± 45	478 ± 55	NR				
Intracardiac	64	222 ± 27	0.6	252 ± 75	369 ± 86	NR				
Paratracheal	100	158 ± 72	1.3	1196 ± 75	220 ± 38	NR				

nH = Hill coefficient; NR = no response.
[a] Percentage of neurons responding to the agonist.
[b] Change in response amplitude in the presence of 10 μM Zn^{2+}.
[c] Change in response amplitude on lowering pH to 6.8.
[d] For sphenopalatine and submandibular ganglia, neurons were subdivided into those which responded to αβ-meATP and those which did not.

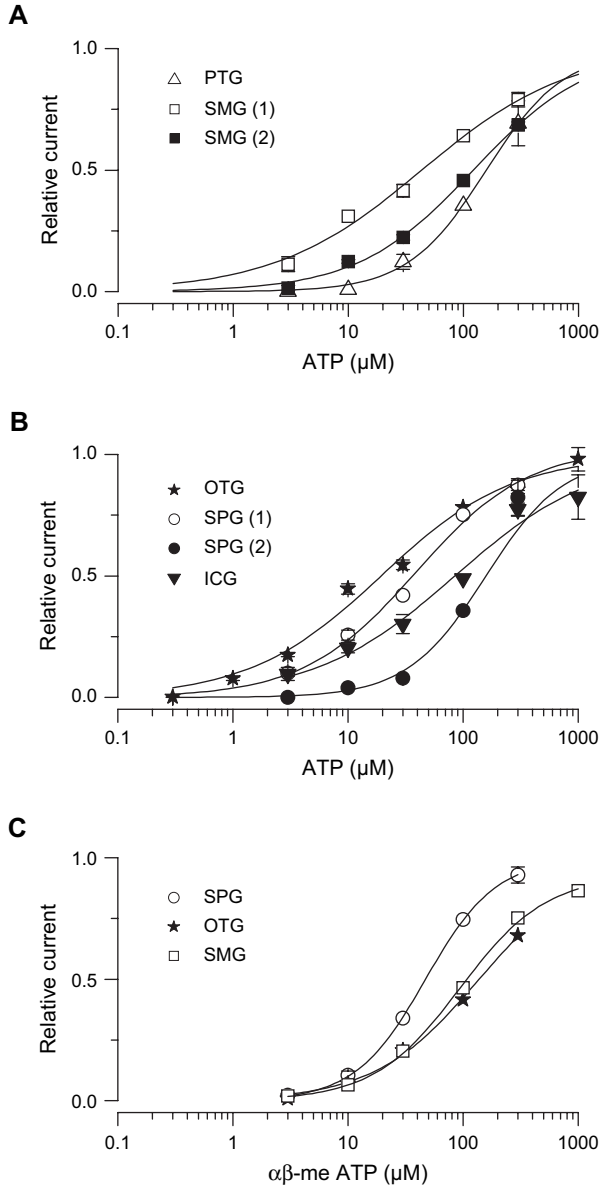

Fig. 2. Concentration-dependence of responses to ATP and αβ-meATP in parasympathetic ganglion neurons. (A) Concentration–response curves to ATP in paratracheal neurons (PTG, △), submandibular neurons which responded to αβ-meATP (SMG(1), □) and submandibular neurons with no response to αβ-meATP (SMG(2), ■). (B) Concentration–response curves for ATP in otic ganglion neurons (OTG, ★), intracardiac neurons (ICG, ▼), sphenopalatine neurons responding to αβ-meATP (SPG(1), ○) and sphenopalatine neurons with no response to αβ-meATP (SPG(2), ●). (C) Concentration–response curves for αβ-meATP in sphenopalatine (SPG, ○), otic (OTG, ★) and submandibular (SMG, □) ganglion neurons. Each point represents the mean ± SEM from 3 to 6 neurons. Responses were normalized with respect to that obtained with 100 μM ATP on the same cell, and fitted with the Hill equation. The data have then been rescaled to the fitted maximum.

The time course for the decay in the αβ-meATP-induced current fitted well to the sum of two exponentials, with the time constants of 6.6 ± 0.2 s and 37.8 ± 3.5 s, respectively ($n = 6$). After a 1-min application of αβ-meATP, the response to αβ-meATP declined to $4.1 \pm 0.3\%$ of the peak, while the response to 100 μM ATP was reduced to $7.4 \pm 0.6\%$ of control (Fig. 3). Following a 4-min recovery period, the response to ATP regained its control amplitude. The similar extent of the desensitization is in marked contrast to the situation in guinea-pig sympathetic ganglion neurons (Zhong et al., 2000) and indicates that, at least in the otic ganglion, few αβ-meATP-insensitive receptors are present. When ATP was used as the desensitizing agonist, there was again a comparable reduction in the response to both ATP and αβ-meATP (Fig. 3D–F).

3.1.2. Allosteric modulators

In a previous study on mouse otic ganglion neurons, we demonstrated the expression of two populations of P2X receptors, namely $P2X_2$ homomers and $P2X_{2/3}$ heteromers (Ma et al., 2004). Because of the limited selectivity of the currently available P2X receptor agonists and antagonists, in this study we have used the allosteric modulators H^+, Zn^{2+} and ivermectin to investigate the types of P2X receptor expressed on rat parasympathetic ganglion neurons.

3.1.2.1. Low pH. Responses at most P2X receptors are inhibited by acidification. The exception to this are receptors containing the $P2X_2$ subunit, where reducing the pH produces a potentiation of the response by increasing the affinity for ATP (King et al., 1997; Stoop

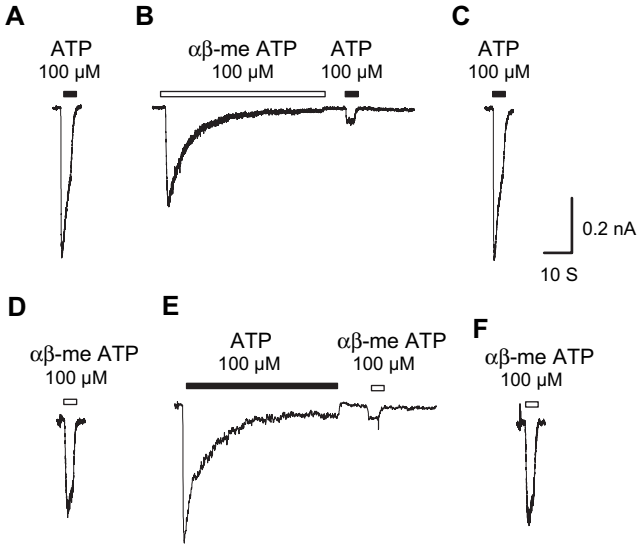

Fig. 3. Cross-desensitization of P2X receptors on rat otic ganglion neurons. Traces of the membrane current showing the response to ATP (100 μM) recorded from one cell before (A), after a prolonged application of αβ-meATP (100 μM; B) and after 4 min recovery. (C) Desensitization produced a comparable decrease in the amplitude of the response to both ATP and αβ-meATP. Panels D–F show the effect of desensitization with 100 μM ATP on the response to αβ-meATP. Again the response to both agonists is reduced by a similar proportion.

et al., 1997). Lowering pH from 7.4 to 6.8 increased the response to ATP (30 μM) in all neurons tested. The effect ranged from an increase to 112% of control in αβ-meATP-sensitive sphenopalatine neurons to 480% in αβ-meATP-insensitive neurons from the submandibular ganglion (see Fig. 4A, B and Table 1). The potentiation seen in αβ-meATP-insensitive submandibular neurons was significantly different ($P < 0.05$) to that in sphenopalatine neurons. When αβ-meATP (30 μM) was used as the agonist, potentiation was again observed in otic, sphenopalatine and submandibular ganglion neurons (Fig. 4A). However for submandibular neurons, there was a significant difference in the extent of the potentiation, depending on whether ATP or αβ-meATP was used as the agonist.

3.1.2.2. Zn^{2+}. Zn^{2+} increases the potency of ATP at $P2X_2$ and $P2X_4$ receptors (Wildman et al., 1998). The effect of Zn^{2+} on responses in parasympathetic ganglion neurons varied considerably between ganglia. In paratracheal ganglion neurons, co-application of Zn^{2+} (10 μM) potentiated ATP response to 1240 ± 40% of control, (Fig. 5A). A less dramatic potentiation of ATP responses was observed in intracardiac, and αβ-meATP insensitive submandibular ganglion neurons. In αβ-meATP insensitive sphenopalatine neurons, the increase in response amplitude did not reach statistical significance. In contrast, Zn^{2+} only significantly potentiated the response to αβ-meATP in otic ganglion neurons (see Fig. 5 and Table 1). The failure of 10 μM Zn^{2+} to ATP responses in otic ganglion neurons was unexpected. We therefore investigated the effect of Zn^{2+} over a wide range of concentrations (1–300 μM). However, we still failed to observe any potentiation (Fig. 6). When zinc (10 μM) was co-applied with a lower concentration of ATP (10 μM) to otic ganglion neurons, there was still little evidence of potentiation, with the responses in the presence of Zn^{2+} being 111 ± 6% of control ($n = 6$).

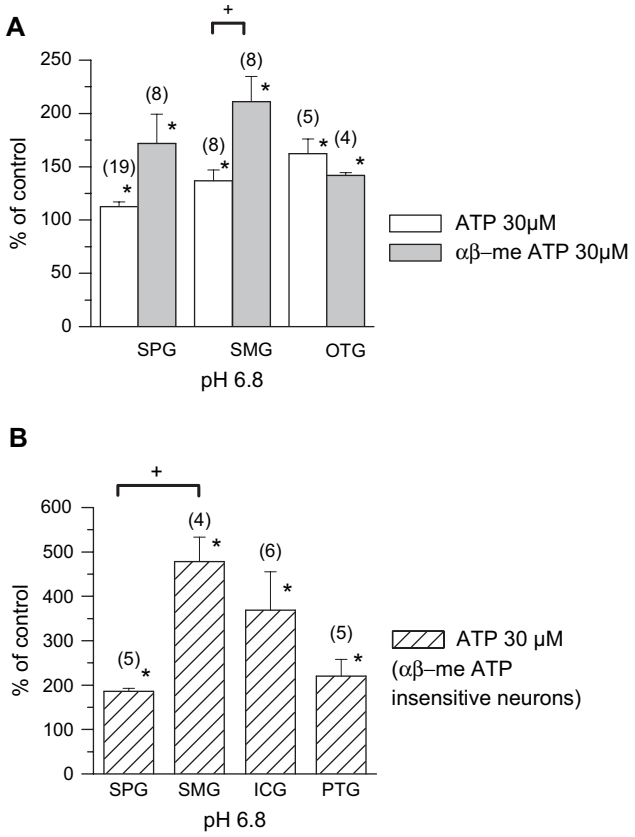

Fig. 4. (A) Effect of pH on the responses to ATP (30 μM) and αβ-meATP (30 μM) in rat sphenopalatine (SPG), submandibular (SMG), and otic (OTG) ganglion neurons which responded to αβ-meATP. (B) Effect of pH on responses to ATP (30 μM) on rat sphenopalatine, submandibular, intracardiac (ICG) and paratracheal (PTG) ganglion neurons which did not respond to αβ-meATP. Responses were normalized with respect to that evoked by αβ-meATP or ATP (100 μM) at pH 7.4 on the same cell. *Significantly different from unity ($P < 0.05$) by two-tailed t-test. + Significantly different ($P < 0.05$) by ANOVA with Tukey's multiple comparison test.

3.1.2.3. Ivermectin. Another subtype-selective modulator of P2X receptors, ivermectin, selectively increases the potency and efficacy of ATP on recombinant $rP2X_4$, but has no effect on $rP2X_2$, $rP2X_{2/3}$ and $rP2X_3$ receptors (Khakh et al., 1999). We tested the effect of ivermectin on the responses of rat otic, sphenopalatine and submandibular ganglion neurons to P2 receptor agonists. In otic ganglion neurons, after a 2-min preincubation with 1 μM ivermectin, the response to ATP (30 μM) and αβ-meATP (30 μM) were 90 ± 0.8% ($n = 5$) and 95 ± 1.4% ($n = 4$) of control, respectively (Fig. 5C). A similar lack of potentiation was also seen in sphenopalatine and submandibular ganglion neurons (Fig. 5C).

3.2. Immunohistochemistry

Antibodies raised against the C-terminus of rat $P2X_{1-6}$ subunits were used to visualise protein expression in cultured neurons from otic, submandibular, sphenopalatine, paratracheal and intracardiac ganglia. In addition, we also examined sections of all except paratracheal ganglia to rule out changes in receptor expression due to dissociation and culturing the neurons. Strong and specific $P2X_2$ immunoreactivity was observed in most intracardiac, submandibular, sphenopalatine, paratracheal and otic ganglion neurons (Fig. 7A, C, E, G, I). The majority of otic ganglion neurons showed strong staining for $P2X_3$, and double staining experiments demonstrated a high level of co-localization with $P2X_2$ immunoreactivity (Fig. 7K). In sphenopalatine and submandibular ganglia, $P2X_3$ staining was observed in a subpopulation of neurons (Fig. 7F, D). In contrast, no $P2X_3$ immunoreactivity was observed in paratracheal ganglion neurons (Fig. 7H) while weak staining was observed in a small subpopulation of

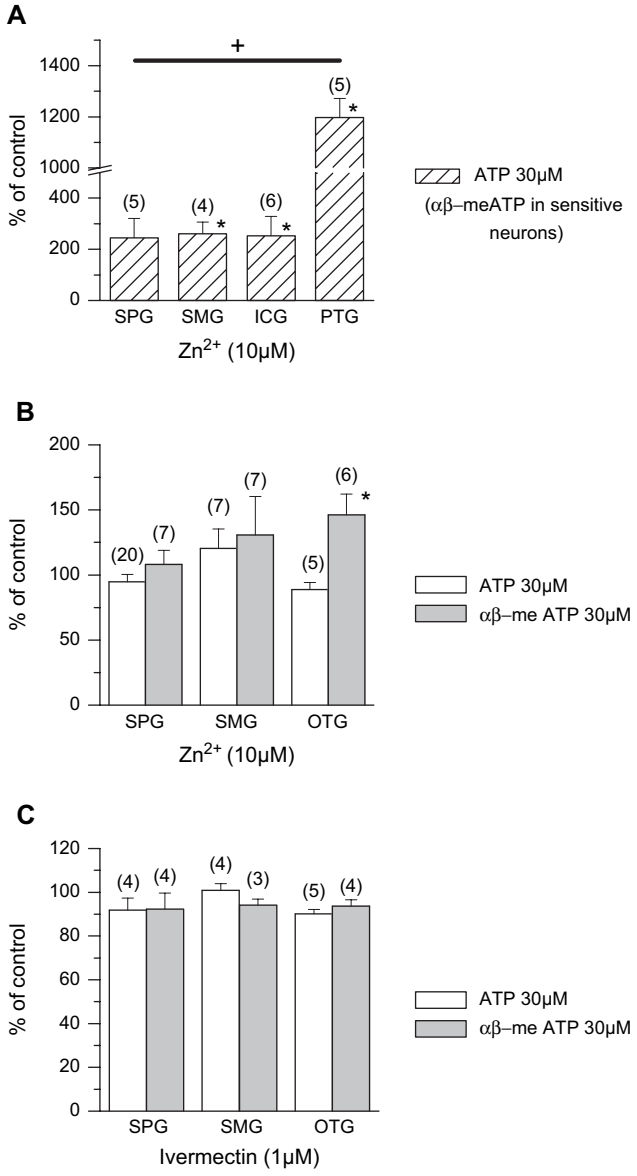

Fig. 5. (A) Effect of Zn^{2+} on the response to ATP (30 μM) on rat sphenopalatine (SPG), submandibular (SMG), intracardiac (ICG) and paratracheal (PTG) ganglion neurons which did not respond to αβ-meATP. (B) Effect of Zn^{2+} on the responses to ATP (30 μM) and αβ-meATP (30 μM) on rat sphenopalatine, submandibular, and otic ganglion neurons with a response to αβ-meATP. Responses were normalized with respect to those evoked by αβ-meATP or ATP (30 μM) in the same cell in the absence of Zn^{2+}. (C) Averaged peak amplitudes of the current induced by ATP (30 μM) and αβ-meATP (30 μM) in the presence of 1 μM ivermectin in rat sphenopalatine, submandibular and otic ganglion neurons. Responses were normalized with respect to those evoked by ATP (30 μM) or αβ-meATP (30 μM) in the same cell, in the absence of ivermectin. The numbers of neurons tested are shown in parentheses. *Significantly different from unity ($P < 0.05$) by two-tailed t-test. + Significantly different ($P < 0.05$) by ANOVA with Tukey's multiple comparison test.

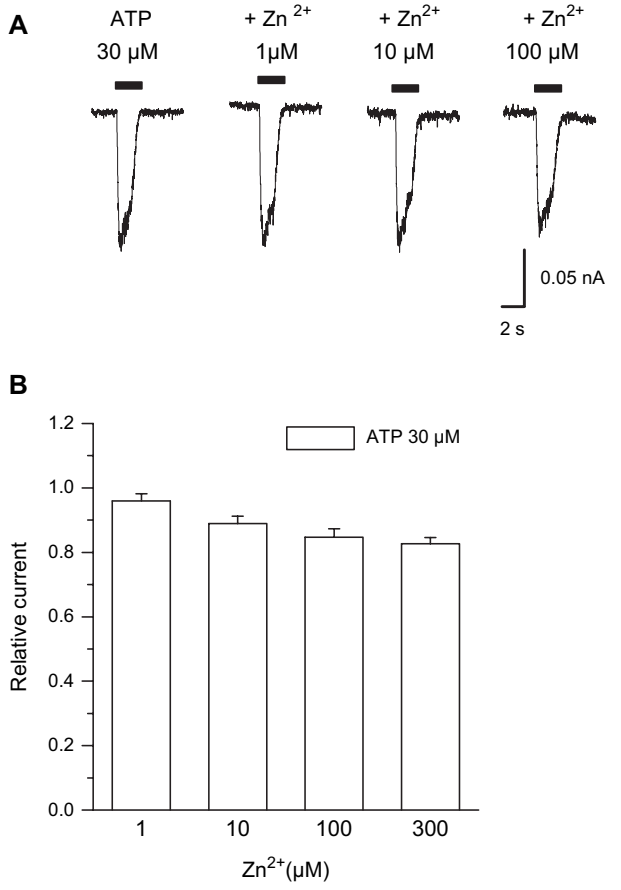

Fig. 6. (A) Traces showing the response to ATP (30 μM) in otic ganglion neurons, when co-applied with different concentrations of Zn^{2+}. (B) Averaged peak amplitudes of the current induced by ATP (30 μM) in the presence of increasing concentrations of Zn^{2+} in rat otic ganglion neurons. Responses were normalized with respect to those evoked by ATP (30 μM) on the same cell, in the absence of Zn^{2+} ($n = 4$).

intracardiac ganglion neurons (Fig. 7B). Some, $P2X_4$ and $P2X_6$ immunoreactivity was observed in all above ganglion neurons while $P2X_5$ immunoreactivity was not detected (Table 2). The results of immunoreactivity in cultured neurons of otic, sphenopalatine, submandibular and intracardiac ganglia were similar with those in ganglion sections (Table 3, Fig. 8). The main difference was that although moderate $P2X_4$ immunoreactivity was observed in sections, the only strong $P2X_4$ staining present in cultures could be seen in glial cells rather than neurons. These results suggest that some of the stronger $P2X_4$ immunoreactivity detected in sections of ganglia was in fact in non-neuronal cells.

4. Discussion

In this study, we have used immunohistochemistry and pharmacological experiments to investigate the P2X receptors present in five different parasympathetic ganglia of the rat. Based on the sensitivity of neurons to αβ-meATP, the ganglia can be divided into three groups. Neurons in the paratracheal and intracardiac

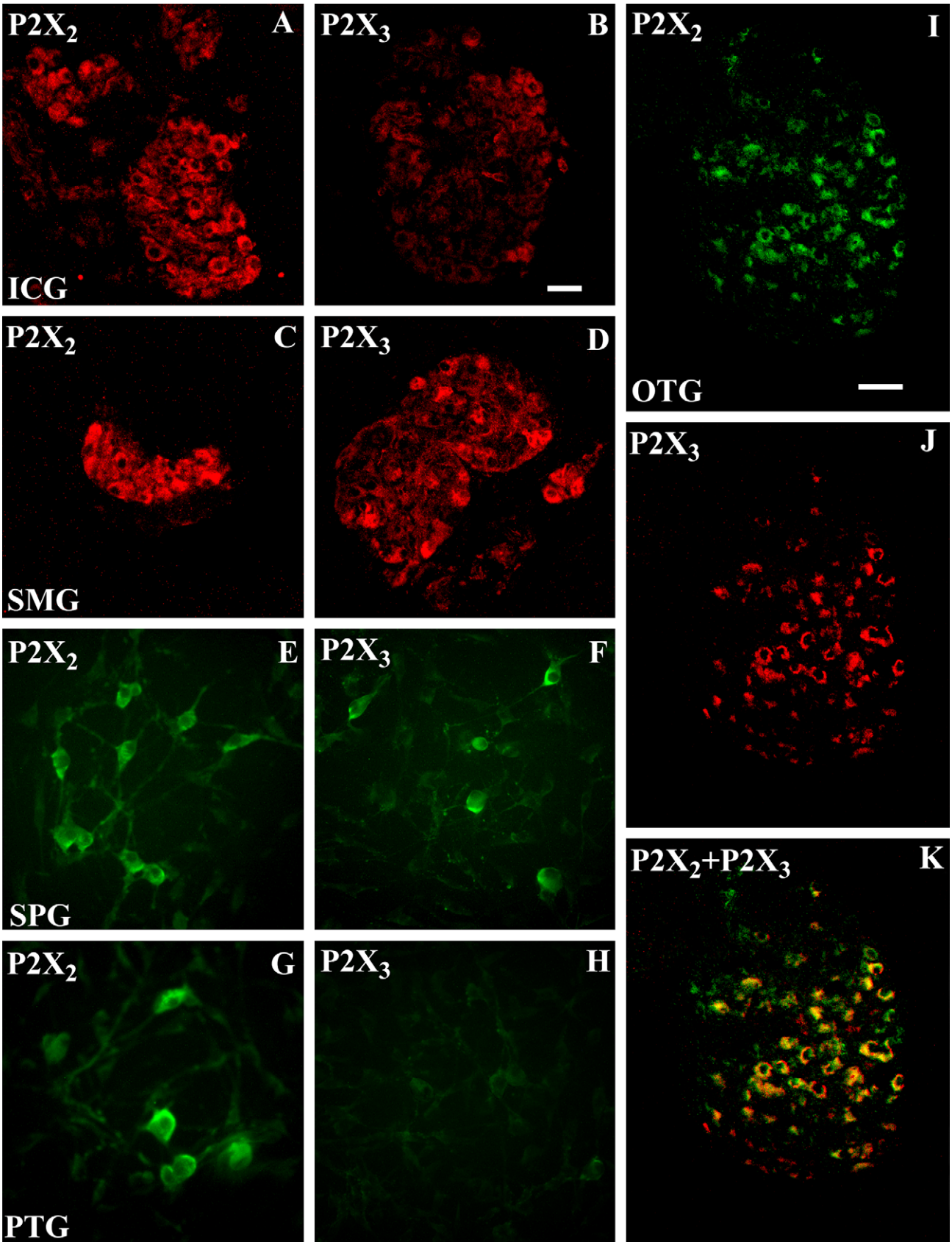

Fig. 7. Immunostaining for $P2X_2$ and $P2X_3$ subunits in neurons from different parasympathetic ganglia. $P2X_2$ (A) and $P2X_3$ (B) immunoreactivity in sections of an intracardiac ganglion. $P2X_2$ (C) and $P2X_3$ (D) immunoreactivity in sections of a submandibular ganglion. $P2X_2$ (E) and $P2X_3$ (F) immunoreactivity in cultured neurons from sphenopalatine ganglia. $P2X_2$ (G) and $P2X_3$ (H) immunoreactivity in cultured neurons from paratracheal ganglia. Double labelling for $P2X_2$ and $P2X_3$ in a single section of otic ganglion are shown in I and J. The overlay of the images (J) shows a high degree of co-localization (yellow). Calibration bar = 50 μm.

Table 2
Summary of the P2X receptor subunit immunoreactivity in sections of parasympathetic ganglia

Ganglion	Antibody					
	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$
Otic	–	+++	+++	+	–	++
Submandibular	–	+++	++	+	–	+
Sphenopalatine	–	+++	++	+	–	+
Intracardiac	–	++	±	+	–	+

+++, very strong signal; ++, strong signal; +, moderate signal; ±, just detectable; –, undetectable.

ganglia are virtually devoid of any response to αβ-meATP. In contrast, all neurons in the otic ganglion respond to this agonist, while sphenopalatine and submandibular ganglia contain a mixture of αβ-meATP-sensitive and insensitive neurons. For technical reasons, we used juvenile animals in this study. If there are developmental changes in P2X receptor subunit expression in these neurons, as occurs elsewhere in the nervous system (see Dunn et al., 2001; Cheung and Burnstock, 2002), a different expression profile may be present in adult rats.

4.1. Nature of the receptors

The ganglia we investigated all stained strongly for the $P2X_2$ subunit, suggesting that it is heavily involved in the receptors we were studying. From published data on recombinant receptors, only those containing the $P2X_1$, $P2X_3$, or $P2X_5$ subunits respond to αβ-meATP (see North, 2002; Wildman et al., 2002). The lack of any detectable $P2X_1$ or $P2X_5$ immunostaining would argue against the involvement of these subunits. In contrast, immunoreactivity for $P2X_3$ was present in otic, submandibular and sphenopalatine ganglia, which all contain neurons sensitive to αβ-meATP, while intracardiac and paratracheal ganglion neurons which did not stain for $P2X_3$ failed to respond to this agonist. The absence of any rapidly desensitizing responses, even when low concentrations of agonist were used, would argue against the presence of homomeric $P2X_3$ receptors

Table 3
Summary of the P2X receptor subunit immunoreactivity in cultured neurons of parasympathetic ganglia

Ganglion	Antibody					
	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$
Otic	–	+++	+++	±	–	++
Submandibular	–	+++	++	±	–	+
Sphenopalatine	–	+++	++	±	–	+
Intracardiac	–	++	–	±	–	+
Paratracheal	–	++	–	±	–	+

+++, very strong signal; ++, strong signal; +, moderate signal; ±, just detectable; –, undetectable.

on otic, submandibular and sphenopalatine ganglion neurons, and would suggest that the responses to αβ-meATP were mediated by heteromeric $P2X_{2/3}$ receptors (see Lewis et al., 1995).

The data we have obtained are in general consistent with the notion that parasympathetic ganglion neurons contain either homomeric $P2X_2$ and/or heteromeric $P2X_{2/3}$ receptors. Thus, responses to ATP and αβ-meATP were potentiated by lowering the pH, which is a characteristic of $P2X_2$ containing receptors (see North, 2002). However, responses in cells sensitive to αβ-meATP were not potentiated as much, which would be consistent with data for the $P2X_{2/3}$ heteromer (Liu et al., 2001). Furthermore, the potency of ATP was always greater in cells which responded to αβ-meATP, which is again in keeping with the presence of $P2X_{2/3}$ receptors in these neurons. The mechanisms for regulating the assembly of P2X receptors are still unclear. However, the lack of any rapidly desensitizing homomeric $P2X_3$ receptors might occur if these neurons always express an excess of $P2X_2$ receptor subunits.

The effects of zinc were, however, less consistent with the general hypothesis. Zinc is an allosteric modulator, potentiating the response to ATP at receptors containing $P2X_2$ and $P2X_4$ subunits, by increasing the affinity for the agonist (Wildman et al., 1998; Miller et al., 1998; Xiong et al., 1999). Potentiation of responses in those neurons which failed to respond to αβ-meATP in paratracheal, intracardiac, and submandibular ganglia, would agree with the presence of the $P2X_2$ subunit in these neurons, but the much more dramatic effect on paratracheal ganglion neurons might suggest that the receptors present in these ganglia are not identical. What is harder to account for is the failure of 10 μM Zn^{2+} to potentiate ATP responses in the αβ-meATP-sensitive neurons in otic, sphenopalatine and submandibular ganglias, yet it did potentiate responses to αβ-meATP in otic ganglion neurons. The extent of potentiation by Zn^{2+} will depend on the concentration of agonist used relative to its EC_{50}. The agonist concentration (30 μM) used in our experiments with Zn^{2+} was between the EC_{10} and EC_{60} values (for paratracheal and otic neurons, respectively) so this alone cannot account for our results. Other possible explanations include the involvement of additional subunits, or splice variants in the P2X receptors in these ganglia. Although the presence of splice variants of the $P2X_2$ subunit has been described in sympathetic neurons (Schadlich et al., 2001), this is associated with changes in the kinetics of desensitization, rather than changes in pharmacological profile. Further studies will be required to test these hypotheses.

In a previous study of submandibular ganglion neurons, it was suggested that the main P2X receptor involved is the $P2X_4$ subunit (Liu and Adams, 2001). Although we observed some immunoreactivity for the

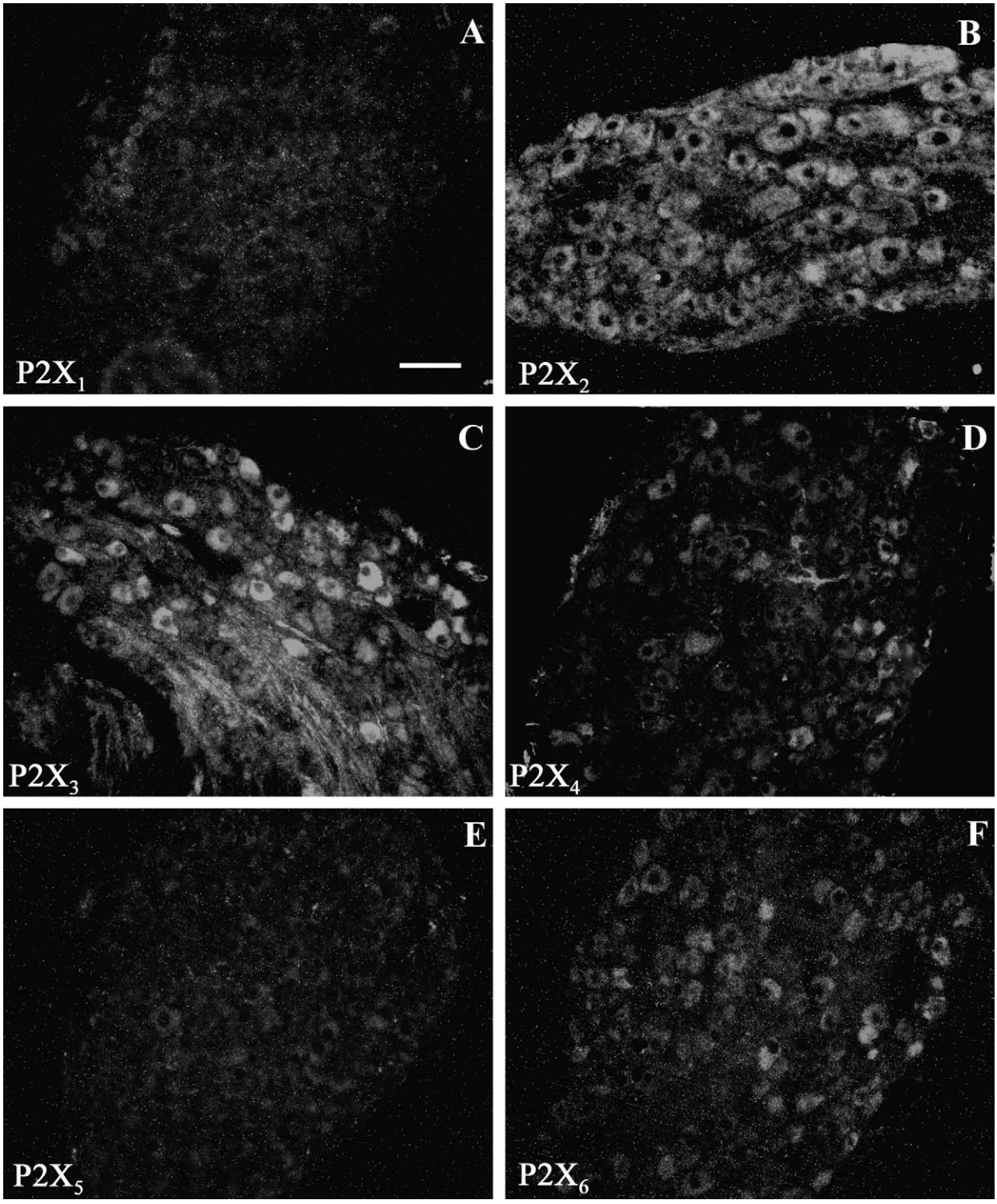

Fig. 8. Immunostaining for $P2X_{1-6}$ subunits in sections of rat sphenopalatine ganglion. While strong staining for $P2X_2$ (B) and $P2X_3$ (C) subunits was observed, there was no detectable staining for $P2X_1$ (A) and $P2X_5$ (E). Low levels of staining for $P2X_4$ (D) and $P2X_6$ (F) were present in some cells. Calibration bar = 50 μm.

$P2X_4$ subunit in both sections and cultured cells, the majority of this staining appeared to be in non-neuronal cells. Furthermore, we did not observe any potentiation by ivermectin, an allosteric modulator of $P2X_4$-containing receptors (Khakh et al., 1999), on submandibular, otic or sphenopalatine ganglion neurons. Thus our results provide little support for the involvement of $P2X_4$ subunits. However, the differences in the EC_{50} for ATP between neurons from different ganglia may be further evidence of heterogeneity.

4.2. Comparison with sympathetic and sensory ganglia

Parasympathetic ganglion neurons, like those of the sympathetic and most sensory ganglia are derived from the neural crest. While sensory neurons express $P2X_2$ and $P2X_3$ subunits in varying proportions, giving rise to either homomeric $P2X_3$ receptors or heteromeric $P2X_{2/3}$ receptors, sympathetic ganglion neurons in the rat appear to express almost exclusively $P2X_2$ receptors (see Dunn et al., 2001). In contrast, chromaffin cells of the rat adrenal medulla, which are also of neural crest origin, are devoid of P2X receptors (Liu et al., 1999). We have now shown that there is considerable heterogeneity in the expression of P2X receptors in rat parasympathetic ganglia. While neurons in some ganglia such as intracardiac and paratracheal express only $P2X_2$ receptors, these otic ganglion neurons express predominantly heteromeric $P2X_{2/3}$ receptors. In other ganglia such as the sphenopalatine and submandibular, neurons express either $P2X_2$ or $P2X_{2/3}$ receptors. The basis of this heterogeneity remains to be determined, but possible mechanisms include the regulation of subunit expression by either target tissues, or pre-ganglionic axons.

4.3. Inter-species variation

In the mouse otic ganglion, all neurons respond to ATP, but less than 50% respond to αβ-meATP (Ma et al., 2004). In contrast, we have observed that all neurons in rat otic ganglion respond to both agonists. Furthermore, in mouse otic ganglion neurons, responses to ATP and αβ-meATP are potentiated both by lowering the extracellular pH, and by the co-application of zinc. Immunohistochemistry and gene deletion experiments suggest that in the mouse otic ganglion, the receptors present are $P2X_2$ and $P2X_{2/3}$ (Ma et al., 2004). The underlying basis for the novel pharmacological properties we have observed in rat otic ganglion neurons remains to be determined, but the differences we have observed between rat and mouse otic ganglion neurons highlight the need for caution in extrapolating from knockout studies carried out in the mouse.

4.4. Physiological role

ATP is stored with acetylcholine in synaptic vesicles of cholinergic nerve terminals and is co-released with acetylcholine from motor nerve terminals (Silinsky, 1975) and sympathetic pre-ganglionic fibres (Vizi et al., 1997). ATP is almost certainly co-released with acetylcholine from pre-ganglionic nerve terminals in parasympathetic ganglia, thus P2X receptors on these neurons are likely to be activated during synaptic transmission. The fast excitatory postsynaptic potentials in some otic and cardiac ganglion neurons are attenuated but not abolished by high concentrations of mecamylamine or tubocurarine (Callister and Sah, 1997; Seabrook et al., 1990), and it is tempting to speculate that the residual component is mediated by ATP. A second possibility is that these receptors respond to ATP released from surrounding glial cells as part of a glial cell-neuron signalling system. A third possibility is that these receptors 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).

5. Conclusion

We have shown that neurons in different parasympathetic ganglia of the rat express P2X receptors with different properties. Based on immunohistochemistry and preliminary pharmacological characterization, homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors appear to be involved. However, differences in agonist potency, and the modulatory effect of Zn^{2+} suggest that the receptors expressed in different ganglia are not identical. Better pharmacological tools are required for a more detailed characterization of the P2X receptor subtypes present in these ganglia.

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