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Pharmacological properties of P2 receptors on rat otic parasympathetic ganglion neurons

Bei Ma [a,*], Li-hua Yu [a], Juan Fan [a], Xin Ni [a], Geoffrey Burnstock [b]

[a] Department of Physiology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, PR China
[b] Autonomic Neuroscience Centre, Royal Free and University College Medical School, Rowland Hill Street, London, NW3 2PF, U.K.

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ABSTRACT

To elucidate the pharmacological profile of P2X receptors and the probable expression of P2Y receptors in otic ganglion neurons from 17-day-old rats, single neurons were enzymatically isolated and maintained in tissue culture for up to 30 h. Whole-cell voltage-clamp recording was carried out at a holding potential of −60 mV. Most otic ganglion neurons responded to adenosine 5′-triphosphate (ATP), 2-methylthio ATP (2-MeSATP) and α,β-methylene ATP (αβ-meATP) with sustained currents and EC_{50} values of 19 µM, 47 µM and 94 µM, respectively. 2′,3′-*O*-trinitrophenyl-ATP (TNP-ATP) inhibited the response to αβ-meATP and ATP with an IC_{50} values of 3.9 nM and 18.3 nM, respectively, which was closed to that observed in nodose neurons. The response to ATP was antagonized by suramin and cibacron blue. The dose–response curve of suramin against ATP response at a pH of 6.5 was shifted to the left compared to that at a pH of 7.4. Diinosine pentaphosphate (Ip_5I), which blocks $P2X_3$, but not $P2X_{2/3}$-mediated responses, had no effect on the currents evoked by ATP or αβ-meATP. In some neurons, uridine 5′-triphosphate (UTP) induced a tiny, but long-lasting current with a mean amplitude of 0.034 ± 0.011 nA. Reverse transcriptase-polymerase chain reaction (RT-PCR) confirmed the expression of mRNAs for $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$ and $P2X_7$, but not for $P2X_1$ and $P2X_5$ receptors in otic ganglion. In conclusion, in rat otic ganglion neurons, $P2X_{2/3}$ heteromultimer receptors dominate, but $P2X_7$ and $P2Y_2$ or $P2Y_4$ receptors also play roles.

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Introduction

Adenosine 5′-triphosphate (ATP) plays an important role as an excitatory neurotransmitter/co-transmitter in both the peripheral and central nervous system (Burnstock, 2007a). Two classes of receptors for ATP have been identified, P2X receptor cation channels and P2Y metabotropic G protein-coupled receptors (Burnstock, 2007b; North, 2002). One area of current focus has been on the role of ionotropic P2X receptors in ATP-mediated nociception (Burnstock, 2001; Nakatsuka and Gu, 2006).

ATP is almost certainly co-released with acetylcholine from preganglionic 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 ganglion (OTG) neurons are attenuated, but not abolished, by high concentrations of mecamylamine or tubocurarine (Callister and Sah, 1997; Seabrook et al., 1990). It is tempting to speculate that the residual component is mediated by ATP. There is increasing evidence that ATP can also be released from surrounding glial cells and act at P2 receptors as part of a glial cell-neuron signalling system (Burnstock, 2007a). It is therefore of interest to identify the expression of P2X receptors in parasympathetic ganglion neurons. Some studies have shown that $P2X_2$ and $P2X_4$ receptor subunits are expressed in submandibular ganglion neurons (Liu et al., 2001). The P2X receptors on rat pelvic ganglion (Zhong et al., 1998, 2000a) and celiac ganglion (Zhong et al., 2000b) neurons are largely of the $P2X_2$ subtype. The $P2X_2$ subunit dominates in paratracheal and cardiac ganglia, whereas the $P2X_2$ and $P2X_{2/3}$ subunits are expressed in the submandibular and sphenopalatine ganglia (Ma et al., 2005). In a previous study, we found that there are two populations of neurons in the mouse parasympathetic OTG (Ma et al., 2004) that are either α,β-methylene ATP (αβ-meATP)-sensitive or αβ-meATP-insensitive, suggesting that $P2X_2$ and/or $P2X_{2/3}$ receptors are likely involved (Ma et al., 2005). In a preliminary study, in the rat parasympathetic OTG, however, most neurons responded to both ATP and αβ-meATP, suggesting that the $P2X_{2/3}$ heteromeric receptor probably predominates in this species (Ma et al., 2005).

Previous study also demonstrates the existence of inter-species and inter-ganglion variations. For example, rat sensory neurons demonstrate αβ-meATP sensitivity due to the presence of the $P2X_3$ subunit (Dunn et al., 2001; Lewis et al., 1995; Nakatsuka and Gu, 2006) while the neurons from sympathetic ganglion are αβ-meATP-insensitive, suggesting the absence of the $P2X_3$ subunit (Dunn et al., 2001; Xiang et al., 1998). However, sympathetic ganglia (such as the superior cervical ganglion and the coeliac ganglion) of the guinea pig

* Corresponding author.
E-mail address: mabei2004@yahoo.com.cn (B. Ma).

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respond to αβ-meATP, suggesting the existence of the $P2X_3$ subunit (Khakh et al., 1995; Reekie and Burnstock, 1994). The presence of alternatively spliced variants of P2X receptor subunits may impose additional complexity to the characterization of endogenous P2X receptors. In the rat, four splice variants of the $P2X_2$ receptor have been isolated: $P2X_{2(a)}$, $P2X_{2(b)}$, $P2X_{2(c)}$ and $P2X_{2(d)}$ (Simon et al., 1997). In the guinea pig, three splice variants of the $P2X_2$ receptor have been isolated, $P2X_{2\text{-}1}$, $P2X_{2\text{-}2}$ and $P2X_{2\text{-}3}$. The $P2X_{2\text{-}1}$ and $P2X_{2\text{-}2}$ variants are homologues to the rat $P2X_{2(a)}$ and $P2X_{2(b)}$ variants, while a homologue of the $P2X_{2\text{-}3}$ splice variant cannot be found in the rat (Parker et al., 1998). In the mouse, four splice variants of the mouse $P2X_4$ receptor, $P2X_{4(a)}$, $P2X_{4(b)}$, $P2X_{4(c)}$ and $P2X_{4(d)}$, have been reported (Townsend-Nicholson et al., 1999). A recent report demonstrated alternative splicing of the $P2X_{(6)}$ receptor subunit during postnatal development of the mouse brain, which is suggested to regulate $P2X_{(6)}$ receptor function during neuronal differentiation (Da Silva et al., 2007). Therefore, these spliced variants of P2X receptors may increase the number of subunits available for hetero-multimeric assembly.

To date, little is known about P2X and P2Y receptors distribution or their roles in parasympathetic signal transmission. Therefore, in this study, we have chosen the parasympathetic OTG, which innervates parotid glands, lacrimal glands and cranial vessels, to elucidate the pharmacological profile of P2X receptors and the probable expression of P2Y receptors in isolated and cultured OTG neurons in rats.

Materials and methods

Cell culture

Young rats (16–18 day old; 30–50 g) were killed by inhalation of a rising concentration of CO_2 and death was confirmed by cardiac hemorrhage. The OTG (Suzuki et al., 1991) were rapidly dissected out and placed in Leibovitz L-15 medium (Life Technologies, Paisley, UK). The ganglia were then de-sheathed, 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, UK) and 6 mg ml^{-1} bovine serum albumin (Sigma Chemical Co., 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 of L-15 medium supplemented with 10% bovine serum, 50 ng ml^{-1} nerve growth factor, 0.2% $NaHCO_3$, 5.5 mg ml^{-1} glucose, 200 i.u. ml^{-1} penicillin and 2 g ml^{-1} streptomycin. The ganglia were dissociated into single neurons by gentle trituration. The cell suspension was diluted to 8 ml, and 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.

Electrophysiological recording

Whole-cell voltage-clamp recording was carried out at room temperature 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 6.1. 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).

RNA extraction and RT-PCR

Total RNA was extracted using a RNeasy Mini Kit (QIAGEN; Clifton Hill, Australia). RNA purity was determined using a method of ultraviolet spectrophotometry at a wavelength of 260–280 nm. Two µg of total RNA was reversely transcribed to complementary DNA in a 20 µl reaction mixture containing 1× reverse transcriptase buffer (15 mM $MgCl_2$, 375 mM KCl, 50 mM DTT, 250 mM Tris–HCl, pH 8.3), 10 mM dNTP, 20 U RNase inhibitor, 200 U M-MLV reverse transcriptase, and 50 ng of oligo(deoxythymidine)$_{15}$ primer. The reaction time was at least 1 h at 42 °C. The cDNA was stored at −20 °C until real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. All reagents, with the exception of the RNeasy Mini Kit, were from Promega Corp. (Madison, WI).

Seven independent sense (S) and antisense (AS) primer pairs specific for $P2X_1$–$P2X_7$ were designed on the basis of the known cloned rat P2X receptor sequences deposited in GenBank (Shibuya et al., 1999). The nucleotide sequence and the lengths of the expected PCR products for each primer pair are shown in Table 1. The PCR solution consisted of 2.0 µl diluted cDNA, 0.4 µM of each paired primers, 2.5 mM Mg^{2+}, 250 µM deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Promega), and 1× PCR buffer. The PCR conditions were 94 °C for 5 min, then 40–60 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 40 s for $P2X_3$, $P2X_4$, $P2X_6$ and $P2X_7$; 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 40 s for $P2X_1$ and $P2X_2$; 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s for $P2X_5$. At the end of the PCR, samples were kept at 72 °C for 10 min for the final extension and then stored at 4 °C. Amplification products were separated by electrophoresis (1.5% agarose gel) and visualized by ethidium bromide staining using a 100-bp DNA ladder (Invitrogen) to estimate the band sizes. The lengths of the PCR products for $P2X_{1-7}$ mRNA were 452, 357, 440, 447, 418, 520 and 354 bp, respectively, as reported (Shibuya et al., 1999).

Solutions and drugs

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 and tripotassium citrate 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.

ATP, αβ-meATP, 2-methylthio ATP (2-MeSATP), 2′,3′-*O*-trinitrophenyl-ATP (TNP-ATP), suramin, cibacron blue and diinosine pentaphosphate ($I_{P5}I$) were obtained from Sigma. Solutions of ATP and other drugs were prepared using deionized water and stored frozen. All drugs were then diluted in extracellular bathing solution to the final concentration. They were applied rapidly through a manifold comprising 7 capillaries made of fused silica coated with polyimide, with 250 µm internal diameter (SGE, Milton Kegnes, UK), connected to a single outlet made of the same tubing, which was placed about

Table 1
Individual P2X receptor primers for RT-PCR

Primer	Source[a]	Position	Sequence (5′ to 3′)	Predicted length (bp)
P2X1	X80447	776–801 (S)	GAAGTGTGATCTGGACTGGCACGT	452
		1203–1231 (AS)	GCGTCAAGTCCGGATCTCGACTAA	
P2X2	U14414	826–845 (S)	GAATCAGAGTGCAACCCCAA	357
		1183–1164 (AS)	TCACAGGCCATCTACTTGAG	
P2X3	X90651	708–731 (S)	TGGCGTTCTGGGTATTAAGATCGG	440
		1126–1147 (AS)	CAGTGGCCTGGTCACTGGCGA	
P2X4	X87763	749–774 (S)	GAGGCATCATGGGTATCCAGATCAAG	447
		1170–1195 (AS)	GAGCGGGGTGGAAATGTAACTTTAG	
P2X5	X92069	553–577 (S)	GCCGAAAGCTTCACCATTTCCATAA	418
		944–970 (AS)	CCTACGGCATCCGCTTTGATGTGATAG	
P2X6	X92070	444–468 (S)	AAAGACTGGTCAGTGTGTGGCGTTC	520
		938–963 (AS)	TGCCTGCCCAGTGACAAGAATGTCAA	
P2X7	X95882	384–410 (S)	GTGCCATTCTGACCAGGGTTGTATAAA	354
		711–737 (AS)	GCCACCTCTGTAAAGTTCTCTCCGATT	

S, sense; AS, antisense.
[a] Sources are described by the accession number in GenBank.

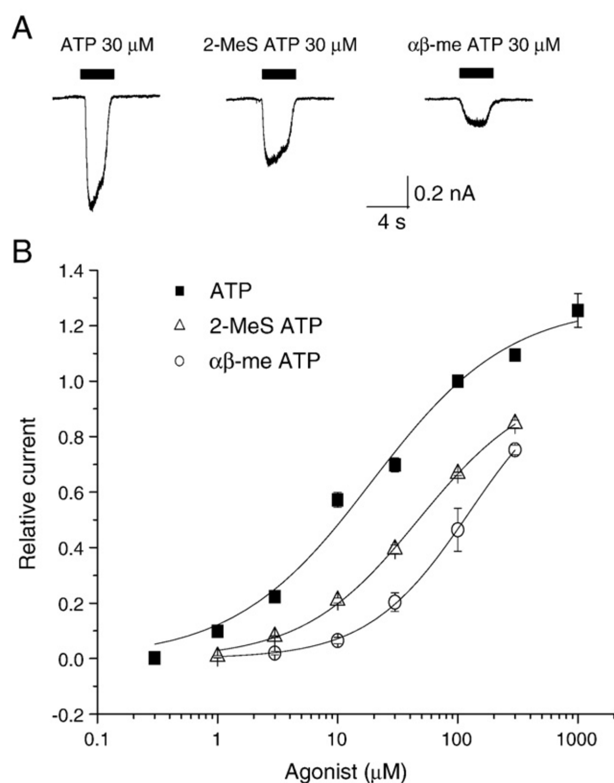

Fig. 1. (A) Traces of the slowly-desensitizing responses to adenosine 5′-triphosphate (ATP), α,β-methylene ATP (αβ-meATP) and 2-methylthio ATP (2-MeS ATP) in rat otic ganglion (OTG) neurons. (B) Concentration–response curves for agonists were constructed separately for rat OTG neurons. Responses were normalized with respect to that obtained with 100 μM ATP in the same cell.

200 μm from the cell. Solutions were delivered by gravity flow from independent reservoirs. One barrel was used to apply a drug-free solution to enable rapid termination of the drug application. Agonists were separately applied for 2 s at 2 min intervals, time which was sufficient for responses to be reproducible. Antagonists were present for 2 min before and during the reapplication of agonists.

Data analysis

Data are expressed as mean±SEM. Statistical comparisons were made using Student's *t*-test for paired samples, as appropriate, using Excel (Microsoft, USA), or Origin 7 (Microcal, USA). Statistical significance was set 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.

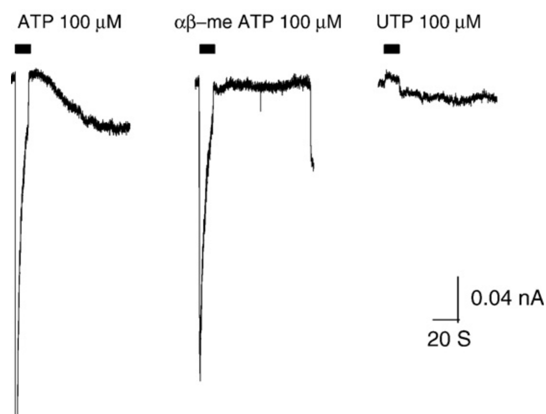

Fig. 2. Adenosine 5′-triphosphate (ATP; 100 μM) evoked a long-lasting current after a persistent current in otic ganglion (OTG) neurons. α,β-Methylene ATP (αβ-meATP; 100 μM) evoked a persistent current with a relatively smaller long-lasting current in OTG neurons. Uridine 5′-triphosphate (UTP; 100 μM) evoked a long-lasting current in OTG neurons.

Results

Agonists

According to the published data on recombinant receptors, only those containing the $P2X_1$, $P2X_3$, or to a lesser extent $P2X_5$ subunits respond to αβ-meATP (North, 2002; Wildman et al., 2002). As shown before, 96% of rat OTG neurons responded to both αβ-meATP and ATP with slowly-desensitizing currents. The maximum response to αβ-meATP was always smaller than that to ATP. The ratio of currents evoked by αβ-meATP and ATP at 100 μM from the same neuron (αβ-meATP/ATP ratio) was about 0.5 (Ma et al., 2004). The EC_{50} values of ATP and αβ-meATP were 19 μM and 94 μM, respectively, which were

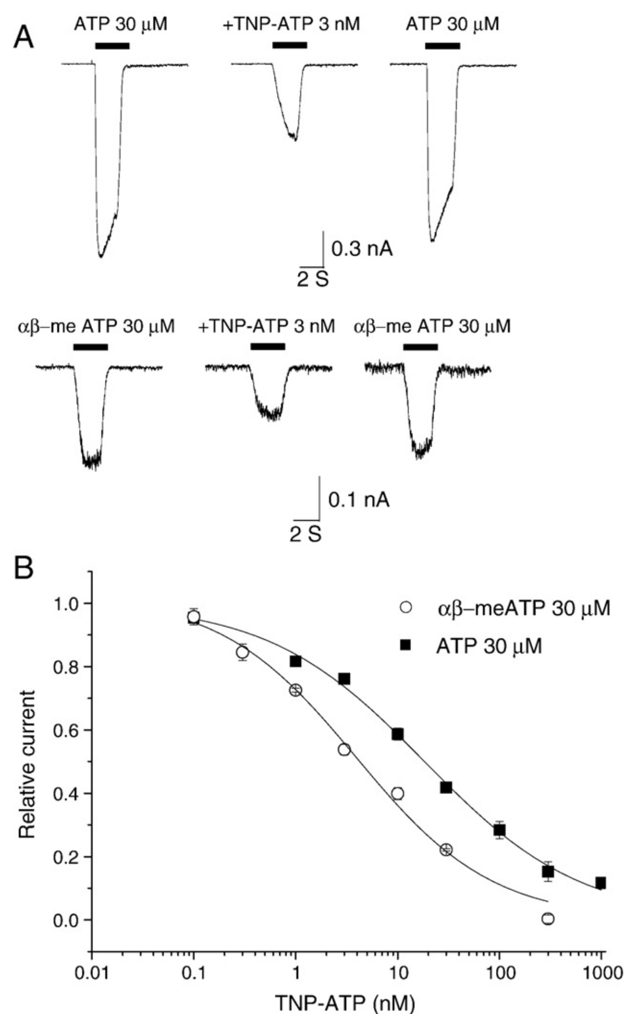

Fig. 3. Inhibition of slowly-desensitizing responses to α,β-methylene ATP (αβ-meATP) and adenosine 5′-triphosphate (ATP) in rat otic ganglion (OTG) neurons by 2′,3′-O-trinitrophenyl-ATP (TNP-ATP). (A) TNP-ATP (3 nM) was applied to the cells and the response to agonists were re-determined every 2 min. (B) Inhibition by TNP-ATP of responses to αβ-meATP (30 μM) or ATP (30 μM). Responses were normalized with respect to that obtained with agonists in the absence of TNP-ATP in the same neuron. Curves show single exponential fits to the data. Points represent mean±SEM. from 4–7 cells.

higher than those seen for recombinant $P2X_{2/3}$ receptors or in the rat nodose neurons. In further experiments where the sensitivity to 2-MeSATP was tested, 7 out of 8 cells responded to 2-MeSATP with a sustained current (Fig. 1A). Fitting the Hill equation to the concentration–response curve for 2-MeSATP yielded an EC_{50} value of 47 μM, and a Hill coefficient of 0.9 (Fig. 1B). The potency of the agonists was found to be: ATP>2-MeSATP>αβ-meATP. These results suggested that in 96% of the rat OTG neurons that show slowly-desensitizing responses to agonists, $P2X_{2/3}$ receptors are predominantly involved. We then investigated the desensitizing time constant (tau) of the sustained response. The desensitizing tau (t) of ATP response was 4.0±0.15 ms while that of αβ-meATP response was 6.6±0.19 ms, which were similar to those responses in recombinant $P2X_2$ and $P2X_{2/3}$ receptors (data not shown).

Uridine 5′-triphosphate (UTP) is a selective agonist of $P2Y_2$ and $P2Y_4$ receptors. Fifty percent of (14/28) OTG neurons responded to UTP (100–300 μM) with a slowly activating and long-lasting current. The amplitude of the response to 100 μM UTP was 0.034±0.011 nA (Fig. 2). When ATP (100 μM) was applied for 10 s, OTG neurons first responded with a P2X receptor-mediated slowly desensitizing current, followed by a delayed current which lasted for 60–100 s and had a mean amplitude as 0.045±0.012 nA. The application of αβ-meATP (100–300 μM) evoked similar biphasic responses (Fig. 2).

Antagonists

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). To confirm that the response to αβ-meATP was mediated via $P2X_{2/3}$ receptors, we tested the ability of TNP-ATP to antagonize this response. As shown in Fig. 3, TNP-ATP inhibited the response to αβ-meATP (30 μM) with an estimated IC_{50} of 3.9 nM (Fig. 3, n=4–7 for each concentration), which is close to that seen in nodose neurons (IC_{50} IC_{50}=3 nM). TNP-ATP also inhibited the response to ATP with an estimated IC_{50} of 18.3 nM (Fig. 3).

Both suramin and cibacron blue are non-selective P2 receptor antagonists that have no effect on the rat $P2X_4$ receptor (Bo et al., 1995; Buell et al., 1996). In rat and mouse pelvic neurons, a 2-min pre-incubation with 10 μM cibacron blue abolished the response to 100 μM ATP (Zhong et al., 1998, 2000a). In contrast, in rat OTG neurons, the response to ATP (30 μM) and αβ-meATP (30 μM) was 75±7% (n=6) and 25±7% of the control (n=6), respectively, after a 2 min pre-incubation with cibacron blue (10 μM) (Fig. 4).

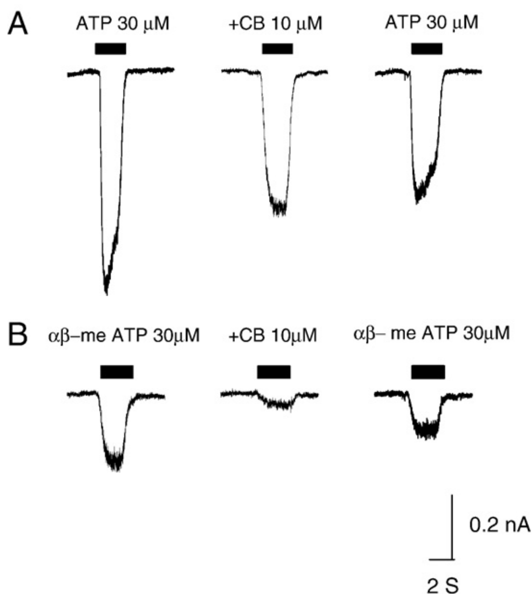

Fig. 4. Effect of cibacron blue (CB) on slowly-desensitizing responses evoked by α,β-methylene ATP (αβ-meATP) and adenosine 5′-triphosphate (ATP) in rat otic ganglion (OTG) neurons. Traces of three consecutive currents evoked from the same neuron by ATP (30 μM) (A) and αβ-meATP (30 μM) (B) before, in the presence of CB (10 μM) and 2 min after washing out the antagonist.

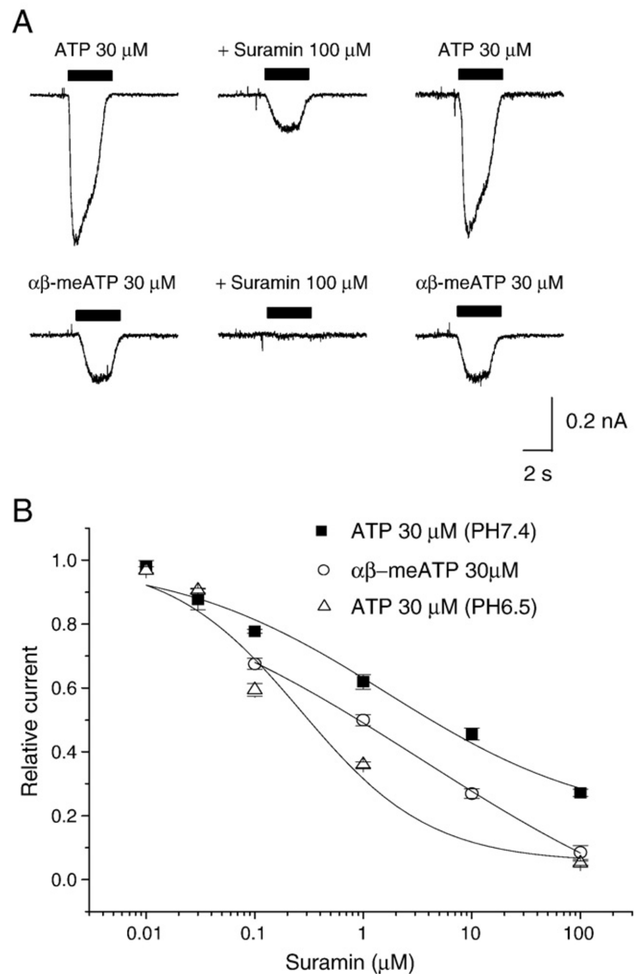

Fig. 5. Effect of suramin on slowly-desensitizing responses evoked by α,β-methylene ATP (αβ-meATP) and adenosine 5′-triphosphate (ATP) in rat otic ganglion (OTG) neurons. (A) Traces of three consecutive currents evoked from the same neuron by ATP (30 μM) and αβ-meATP (30 μM) before, in the presence of suramin (100 μM) and 2 min after washing out the antagonist. (B) Concentration–response curve for ATP (30 μM) in pH 7.4 or pH 6.5 and αβ-meATP (30 μM) in the presence of suramin. Responses were normalized with respect to those obtained with αβ-meATP (30 μM) or ATP (30 μM) in the absence of suramin in the same cell.

In rat pelvic neurons, suramin (100 μM) practically abolishes the response to ATP 100 μM (Zhong et al., 1998). Here, in rat OTG neurons, a 2 min pre-incubation with suramin (100 μM) reversibly inhibited the response evoked by αβ-meATP (30 μM) to 8.5±2% (n=6) of the control, while the response evoked by ATP was reduced to 27±1.2% of the control (Fig. 5A). Suramin inhibited the ATP (30 μM) and αβ-meATP (30 μM) responses with estimated IC_{50} of 3.5 μM and 1.6 μM, respectively (Fig. 5B). This property suggested the possible involvement of the $P2X_4$ or $P2X_{4/6}$ receptor subunits.

To analyze the P2X receptor subtype in the OTG neurons, we examined the inhibitory effect of suramin under different pH conditions. The concentration–response curve of suramin against ATP (30 μM) was shifted to the left at the lower pH (IC_{50}=0.2 μM at pH 6.5 vs. 3.5 μM at pH 7.4; Fig. 5B). The calculated IC_{50} in OTG neurons is closer to the reported IC_{50} of recombinant $P2X_2$ than $P2X_{2/6}$ receptors (King et al., 1997, 2000).

Allosteric modulator

The rat $P2X_4$ receptor is resistant to the antagonists, suramin and cibacron blue (Bo et al., 1995; Buell et al., 1996). In our experiment, about 27% and 75% of the ATP response remained after 100 μM suramin or 10 μM cibacron blue, respectively. We therefore investigated the possible involvement of the $P2X_4$ subunit in OTG neurons. Ip_5I is a P2X subtype-selective antagonist that potently inhibits $rP2X_1$ and $rP2X_3$ receptors, is inactive on $rP2X_2$ and $P2X_{2/3}$ receptors at concentrations up to 30 μM (Dunn et al., 2000), and enhances the action of ATP at $rP2X_4$ receptors (King et al., 1999). In four cells, after a 2-min pre-incubation with Ip_5I (1 μM), the response to 30 μM ATP and 30 μM αβ-me ATP was 97±3.1% and 95±1.4% of the control, respectively (n=4, p>0.05, p>0.05, Fig. 6A, B). Therefore, it was unlikely that the $P2X_4$ receptor involved as there was no potentiation of ATP response by Ip_5I.

RT-PCR analysis of P2X receptors in the OTG

To identify the subtypes of P2X receptors expressed in the OTG, RT-PCR was performed using seven pairs of primers. The OTG expressed mRNA for the $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$ and $P2X_7$ receptors, but not for the $P2X_1$ and $P2X_5$ receptors (Fig. 7). Of the seven P2X receptor mRNAs, those for $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_6$ receptors were dominant, which is consistent with our previous study (Ma et al., 2004). $P2X_7$ mRNA was also expressed in abundance in the rat OTG.

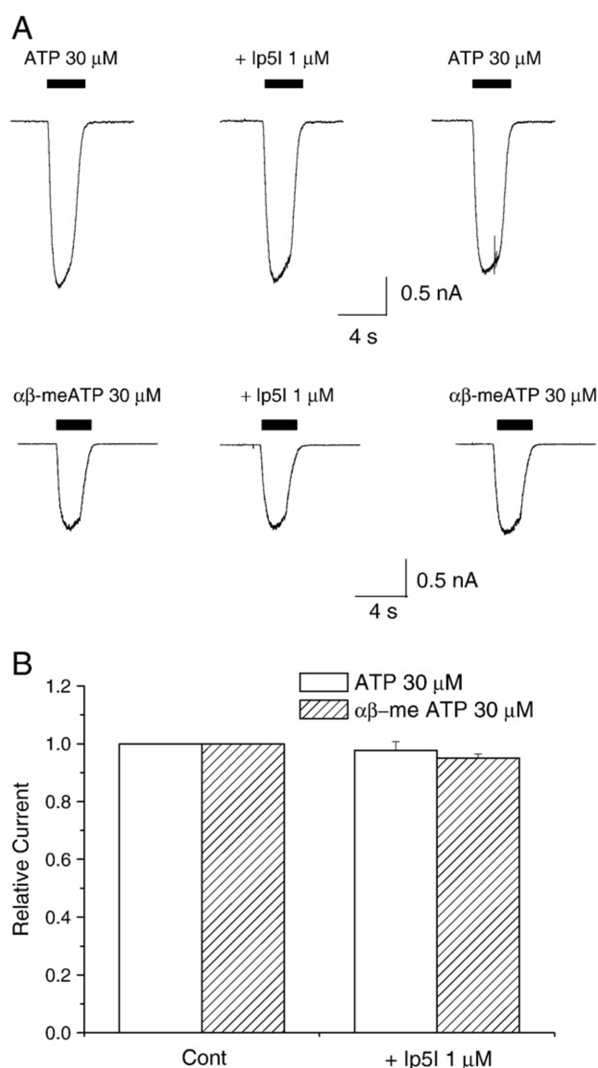

Fig. 6. Effect of diinosine pentaphosphate (Ip_5I) on slowly-desensitizing responses to α,β-methylene ATP (αβ-meATP) and adenosine 5′-triphosphate (ATP) on rat otic ganglion (OTG) neurons. (A) Traces of responses to ATP (30 μM) and αβ-meATP (30 μM) in the absence and presence of Ip_5I (1 μM) in a cell with a αβ-meATP/ATP ratio <0.1. Ip_5I was present 2 min before and during reapplication of agonists. (B) Averaged peak amplitudes of the current induced by ATP (30 μM) and αβ-meATP (30 μM) in the absence and presence of Ip_5I (1 μM).

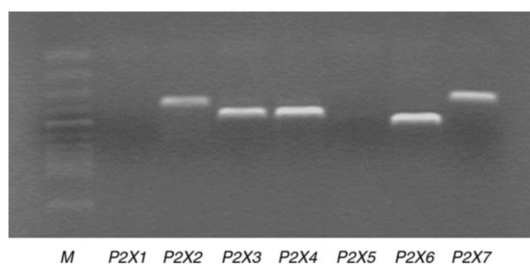

Fig. 7. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of P2X purinoceptor mRNAs expressed in the otic ganglion (OTG). The total RNAs from the OTG were reverse transcribed, and amplified by PCR with each primer pair described in Table 1. Amplification products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. Lane M, DNA marker. When PCR was performed with each sample without prior reverse transcription, there was no amplification product, indicating that the bands appearing on the gels were not derived from genomic DNA.

Discussion

It is unlikely that OTG neurons express functional homomeric $P2X_1$ or $P2X_3$ receptors because αβ-meATP, which activates $P2X_1$ and $P2X_3$ receptors (Lewis et al., 1998) was ineffective at evoking rapidly rapidly-desensitizing inward currents in these cells. We have shown that the currents evoked in OTG neurons are not rapidly desensitizing, whereas currents evoked through $P2X_1$ and $P2X_3$ receptors desensitize within milliseconds (Virginio et al., 1998). Further evidence comes from the relatively low potency of αβ-meATP on OTG neurons (EC_{50}=94 μM), the potentiation of ATP and αβ-meATP responses by lowered pH (data not shown), and the lack of inhibition of the ATP-activated response by Ip_5I. Ip_5I is a non-competitive antagonist that interacts with the desensitized state of the $P2X_1$ or $P2X_3$ receptor while having much less potency at slow-desensitizing $P2X_2$ and $P2X_{2/3}$ subtypes that lack the fast desensitized conformational state (Dunn et al., 2000; Ford et al., 2005).

To further investigate whether other possible subunits were involved, we examined the effects of TNP-ATP. TNP-ATP is a potent antagonist (IC_{50} values close to 1 nM) at $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors, and a low potency antagonist (IC_{50} values in the μM concentration range) at $P2X_2$, $P2X_4$, $P2X_7$ (Virginio et al., 1998), $P2X_5$ and $P2X_{1/5}$ receptors (Haines et al., 1999; Lê et al., 1998; Wildmann et al., 2002). It was reported that TNP-ATP inhibited the current evoked by 1 μM αβ-meATP significantly more than the one evoked by 10 μM αβ-meATP and significantly slowed down the desensitization rate constant of the current, suggesting that TNP-ATP acts as a competitive antagonist and competes with αβ-meATP at the $P2X_1$ or $P2X_3$ agonist binding site (Ford et al., 2005). In the present study, as shown in Fig. 3, TNP-ATP inhibited the response to αβ-me ATP (30 μM) with an estimated IC_{50} of 3.9 nM, which is close to that in rat nodose neurons (Dunn et al., 2001). These results suggested that $P2X_{2/3}$ receptors probably dominate in the cultured OTG neurons.

As we have shown before, immunohistochemical studies in the OTG suggest that there are high expression levels of $P2X_2$ and $P2X_3$, low expression levels of $P2X_1$, $P2X_4$ and $P2X_6$ and no detectable expression of $P2X_5$ receptors (Ma et al., 2004). $P2X_1$ homomeric receptors have been excluded as described above since there are no rapidly-desensitizing currents evoked by either ATP or αβ-meATP. Homomeric $P2X_4$ receptors are insensitive to blockade by suramin and cibacron blue (Bo et al., 1995; Buell et al., 1996) while potentiated by Ip_5I (King et al., 1999). In our study of rat OTG neurons, we found that after a 2 min pre-incubation with suramin (100 μM) or cibacron blue

(10 μM), 73% or 25% of the ATP response was inhibited, respectively, and that neither the ATP nor the αβ-meATP response was potentiated by Ip_5I. Thus, these results do not support the involvement of the hemomeric $P2X_4$ receptor. The other subunit insensitive to suramin is $P2X_6$, which has been shown immunohistochemically to be expressed in OTG neurons. It has been suggested that the $P2X_6$ subunit may not form functional homomeric channels (Lê et al., 1998; Torres et al., 1999), so the heteromeric receptors involved in the OTG could be $P2X_{1/6}$, $P2X_{2/6}$ or $P2X_{5/6}$ heteromer. $P2X_{5/6}$ can be excluded since there was no evidence of either $P2X_5$ immunoreactivity or mRNA in OTG neurons.

The most convincing difference between $P2X_2$ and $P2X_{2/6}$ receptors is that (at pH 6.5) the inhibition of the current by suramin is clearly biphasic; one component has the high sensitivity of homomeric $P2X_2$ receptors (IC_{50}~80 nM), whereas the other component is less sensitive (IC_{50}~2 nM) (King et al., 1997, 2000). In OTG neurons, the concentration–response curve of suramin against ATP (30 μM) was shifted to the left at lower pH (IC_{50}=0.2 μM at pH 6.5 vs. 3.5 μM at pH 7.4; Fig. 5B). The calculated IC_{50} in the study is closer to the reported IC_{50} of recombinant $P2X_2$ than $P2X_{2/6}$ receptors, suggesting that the $P2X_{2/6}$ heteromeric receptors are not the major receptor subtype in OTG neurons.

Although the IC_{50} value of TNP-ATP of about 4 nM in OTG neurons is clearly in the range of heteromeric $P2X_{2/3}$ receptors, the EC_{50} values for ATP, 2-MeSATP and αβ-meATP are much higher than the heterologously expressed $P2X_{2/3}$ receptors (Gever et al., 2006; Khakh, 2001). Differences in agonist potency and the modulatory effect of Zn^{2+} (Ma et al., 2005) suggest that the pharmacology of P2X receptor subtypes expressed in OTG neurons is different from that of either the recombinant or natural P2X receptors. Firstly, this is partly because heteromultimers as well as homomultimers are involved in forming the trimer ion pores. In this study, we found for the first time that mRNA for the $P2X_7$ receptor subunit was expressed in the OTG, which suggests the possible existence of homomeric $P2X_7$ receptors or heteromeric $P2X_{4/7}$ receptors (Guo et al., 2007) in OTG neurons. Secondly, since the $P2X_2$ subunit is involved in OTG neurons, splice variants of $P2X_2$ receptor subtypes might play a part (Chen et al., 2000; Simon et al., 1997). It has been reported that all types of $P2X_2$ mRNA was seen in the nodose ganglion neurons, although the signal for the $P2X_{2(d)}$ transcript was weaker than the others. $P2X_{2(b)}$ and $P2X_{2(c)}$ splice variants mRNAs, but not $P2X_{2(d)}$, were also detected in the superior cervical ganglion neurons, while they were expressed at the lowest levels in dorsal root ganglion (Simon et al., 1997). Better pharmacological tools are required for a more detailed characterization of the P2X receptor subtypes present in the OTG.

In our study, ATP application evoked a long-lasting current followed by a slowly activated and slowly desensitizing current mediated by P2X receptors and probable involvement of P2Y receptors. UTP is a selective agonist of $P2Y_2$ and $P2Y_4$ receptors. OTG neurons responded to UTP with a slowly activating and long-lasting current, suggesting the probable involvement of $P2Y_2$ or $P2Y_4$ receptors.

In summary, we conclude that the homomeric $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors do not contribute significantly to the P2X receptor-mediated currents recorded in OTG neurons. The dominant receptor subunit is the $P2X_{2/3}$ heteromultimer. The $P2X_7$ and $P2Y_2$ or $P2Y_4$ receptors are also involved.

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