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Methyllycaconitine, α-bungarotoxin and (+)-tubocurarine block fast ATP-gated currents in rat dorsal root ganglion cells

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1 The effects of nicotinic acetylcholine receptor antagonists were studied on currents evoked by application of ATP to rat isolated dorsal root ganglion cells, and human embryonic kidney 293 cells expressing rat $P2X_3$ and $P2X_{2/3}$ receptors.

2 The rapidly desensitising (within 100 ms) current in dorsal root ganglion cells was inhibited by methyllycaconitine, α-bungarotoxin and (+)-tubocurarine (concentrations giving half-maximal inhibition were approximately 40, 60 and 800 nM, respectively), but not by hexamethonium (100 μM) or mecamylamine (100 μM). The sustained (>250 ms) current in dorsal root ganglion cells was inhibited by (+)-tubocurarine (80% by 10 μM), but not by methyllycaconitine (200 nM), α-bungarotoxin (200 nM), mecamylamine (100 μM) or hexamethonium (100 μM).

3 Rapidly desensitising currents evoked by α,βmethylene-ATP in human embryonic kidney cells expressing $P2X_3$ receptors were inhibited by methyllycaconitine and α-bungarotoxin, at concentrations similar to those effective in dorsal root ganglion cells.

4 The results indicate that some nicotinic acetylcholine receptor antagonists are potent blockers of P2X receptors on neurons, particularly the homo-oligomeric $P2X_3$ receptor. This finding suggests that these drugs should be used with care to discriminate between P2X and neuronal acetylcholine receptor types.

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Abbreviations: α, alpha; β, beta; αβmeATP, α,βmethylene-ATP; DRG, dorsal root ganglion; HEK, human embryonic kidney cells; NACh receptor, neuronal acetylcholine receptor; TNP-ATP, 2′,3′,-*O*-(2′,3′,4′)-trinitrophenyl-ATP

Introduction

The role of ATP as a peripheral neuroeffector transmitter is well established. It functions as the major sympathetic transmitter to smooth muscle in the vas deferens (Sneddon *et al*., 1982; French & Scott, 1983; Sneddon & Westfall, 1984) and mesenteric blood vessels (Ramme *et al*., 1987). It has been recognised more recently that it is also a key transmitter in the initiation of afferent nerve signals; it is released from epithelia to act on primary afferent nerve terminals (Ferguson *et al*., 1997). There is also growing evidence that it serves as a synaptic transmitter between neurons (Edwards *et al*., 1992; Evans *et al*., 1992; reviewed in Khakh, 2001; North, 2002). Other examples are in somatosensory cortex pyramidal cells (Pankratov *et al*., 2002), in lateral hypothalamus (Jo & Role, 2002), in CA1 (Pankratov *et al*., 1998) and CA3 (Mori *et al*., 2001) pyramidal cells of hippocampus, and in the dorsal horn of the spinal cord (Jo & Schlichter, 1999). One of the major difficulties in identifying cell–cell signaling mediated by ATP has been the limited selectivity of the available antagonists (North & Surprenant, 2000). This can be a particular problem in the nervous system where it may be important to discriminate between components of synaptic transmission mediated by ATP acting at P2X receptors and those mediated by acetylcholine acting at nicotinic receptors.

The acetylcholine receptor blocker (+)-tubocurarine is known to block P2X receptors on phaeochromocytoma cells at concentrations similar to those that block the nicotinic receptors (Nakazawa *et al*., 1991a, b), and it also blocks ATP responses in hair cells (Glowatzki *et al*., 1997). Moreover, some P2X receptors expressed in heterologous cells are also blocked by (+)-tubocurarine. The $P2X_2$ receptor in oocytes is 50% blocked by 1 μM (+)-tubocurarine (Brake *et al*., 1994), although much higher concentrations were required to block the $P2X_1$ or $P2X_2$ receptor in human embryonic kidney (HEK) cells (Evans *et al*., 1995). The effects of (+)-tubocurarine on other P2X receptors seem not to have been tested.

Methyllycaconitine is a blocker of the α-bungarotoxin-sensitive subclass of neuronal nicotinic receptors (Alkondon *et al*., 1992). In hippocampal CA1 cells, it blocks the actions of acetylcholine at concentrations of 1 nM (Alkondon *et al*., 1992), and similarly low concentrations can be used to block the homopentameric α7 nicotinic receptor when expressed in *Xenopus* oocytes (Briggs *et al*., 1995; Palma *et al*., 1996). Other central nicotinic receptors, such as α4β2, are also blocked but at 1000-fold higher concentrations (Buisson *et al*., 1996).

It is particularly important to be able to discriminate P2X receptors and nicotinic receptors because acetylcholine and ATP can be coreleased (Silinsky, 1975; Zhang *et al*., 2000) and the two receptors are widely coexpressed. Indeed, on phaeochromocytoma cells (Nakazawa *et al*., 1991b),

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autonomic neurons (Searl *et al*., 1998; Khakh *et al*., 2000) and in heterologous expression (Khakh *et al*., 2000), activation of one receptor inhibits the effectiveness of agonists at the other receptor. The molecular mechanism of this mutually occlusive interaction is not understood.

The purpose of the present experiments was to examine the effectiveness of methyllycaconitine and other acetylcholine receptor antagonists on neuronal P2X receptors. We used rat dorsal root ganglion cells, in which the fast-desensitising components of the current elicited by ATP and $\alpha\beta$methylene-ATP ($\alpha\beta$meATP) can be ascribed to activation of homomeric $P2X_3$ receptors, and the slow desensitising component to activation of heteromeric $P2X_{2/3}$ receptors (Lewis *et al*., 1995; Grubb & Evans, 1999; Ueno *et al*., 1999; North, 2002). In parallel experiments, we also examined effects of methyllycaconitine and α-bungarotoxin on currents mediated by rat $P2X_3$ and $P2X_{2/3}$ receptors in stably transfected HEK cells.

Methods

Tissue preparation

Thoracic dorsal root ganglia were removed from Wistar rats (9 or 10 days) killed by decapitation, and transferred to the extracellular solution containing (mM): 150 NaCl; 5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 HEPES, pH adjusted with NaOH to 7.3. They were gently proteolysed by 15 min exposure (34°C) to pronase E (1 mg ml^{-1}: calcium reduced to 0.5 mM), and single cells were prepared by successive trituration (fire polished pipettes with tip diameters from 500 to 200 μm). Recordings were made within 3 h of isolation.

The preparation of HEK 293 stably transfected with cDNAs encoding rat $P2X_3$ or $P2X_2$ and $P2X_3$ ($P2X_{2/3}$) receptors has been described previously (Kawashima *et al*., 1998); recordings were made 24–72 h after passage.

Electrophysiology and drug application

Currents were measured using the conventional whole-cell voltage-clamp technique. The patch pipette (1–2.5 MΩ) was filled with a solution composed of (mM): CsF 120 mM, Tris-Cl 20 mM, pH 7.3. The series and the input resistances were 3–8 and 300–900 MΩ, respectively. Currents were filtered at 1 kHz and digitised at 2 kHz. Agonists were applied using a modified 'square-pulse' concentration jump method (Lalo *et al*., 2001; Pankratov *et al*., 2001) or with the RSC 2000 system (Biological Science Instruments, Grenoble, France) of flow pipes as described (Spelta *et al*., 2002).

ATP (10 μM, typically for 250 ms) was applied repeatedly until the amplitude of the evoked current was stable. The interval between the applications was 7 min. Antagonists were applied for 2 min before the ATP, and then also during the application of ATP. After washout of antagonist, at least two further control responses to ATP were observed. All currents were measured −100 mV.

Data are presented as mean ± s.d. Concentration–response curves for antagonists were fitted to $I/I_0 = (A^*(1-A))/(1 + ([B]/IC_{50})^n)$, where I is the current in antagonist concentration $[B]$, I_0 is the current in control conditions, IC_{50} is the concentration of the substance producing half-maximal effect, A is an asymptotic level of maximal inhibitory effect and n is the Hill coefficient. Concentration–response curves for agonists were fitted to $I/I_S = 1/(1 + (EC_{50}/[C])^n)$, where I is the current for agonist concentration $[C]$, I_S is an asymptotic level of current in saturation conditions, EC_{50} is the half-activation concentration of the agonist and n is the Hill coefficient. The t-test and ANOVA were applied for testing of the statistical significance where appropriate. Drugs were purchased from Sigma Aldrich (Sigma Aldrich Company Ltd, U.K.)

Results

Dorsal root ganglion neurons

Recordings were made from freshly isolated rat dorsal root ganglion neurons of 10–25 μm diameter. ATP-evoked currents in dorsal root ganglion cells either showed very rapid desensitisation (within 250 ms) (Figure 1) or did not (Figure 2). $\alpha\beta$meATP induced inward current in all of the 43 neurons tested. The time course of the response to $\alpha\beta$meATP was similar to the kinetics of ATP-induced currents in all the cells tested. These two populations of cells and their relative proportions have been described before (Pankratov *et al*., 2001; see North, 2002). The rapidly desensitising component corresponds to current through homomeric $P2X_3$ receptors (Zhong *et al*., 2001). The rapidly desensitising current was completely blocked by α-bungarotoxin and (+)-tubocurarine

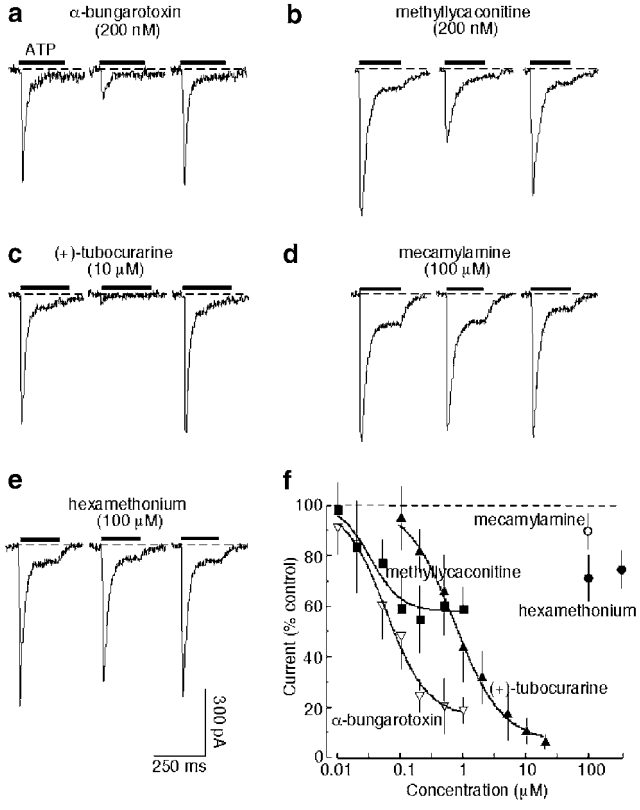

Figure 1 Nicotinic receptor antagonists inhibit ATP-evoked currents with fast desensitising kinetics in dorsal root ganglion neurons. Each panel (a–e) shows three responses to ATP (10 μM) before, during and after washout of the antagonist indicated. Each antagonist tested on a different cell: holding potential −100 mV. (f) Summarised data for the five antagonists; points are mean ± s.d. for 5–10 cells. Curve fits are as described in Methods.

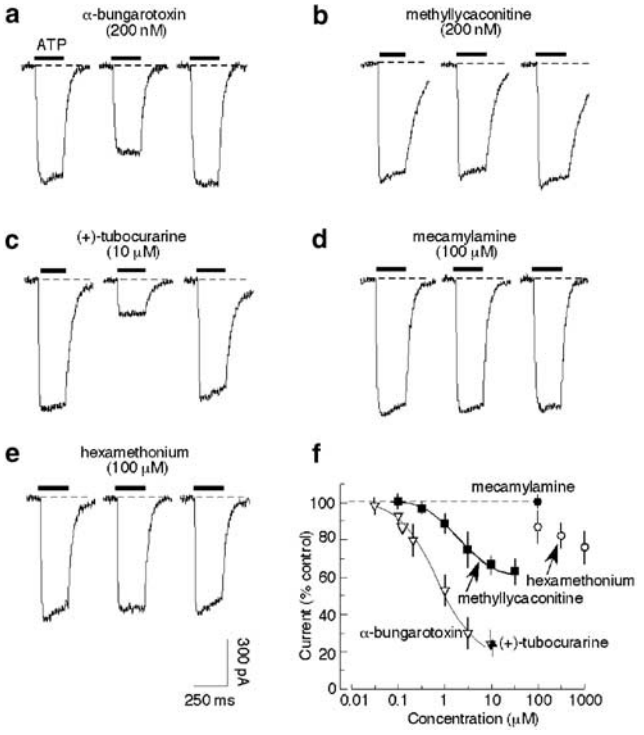

Figure 2 (+)-Tubocurarine inhibits ATP-evoked currents with slow desensitising kinetics in dorsal root ganglion neurons. Each panel (a–e) shows three responses to ATP (10 μM) before, during and after washout of the antagonist indicated. Each antagonist tested on a different cell: holding potential −100 mV. (f) Summarized data for the five antagonists; points are mean ± s.d. for 5–10 cells. Curve fits are as described in Methods.

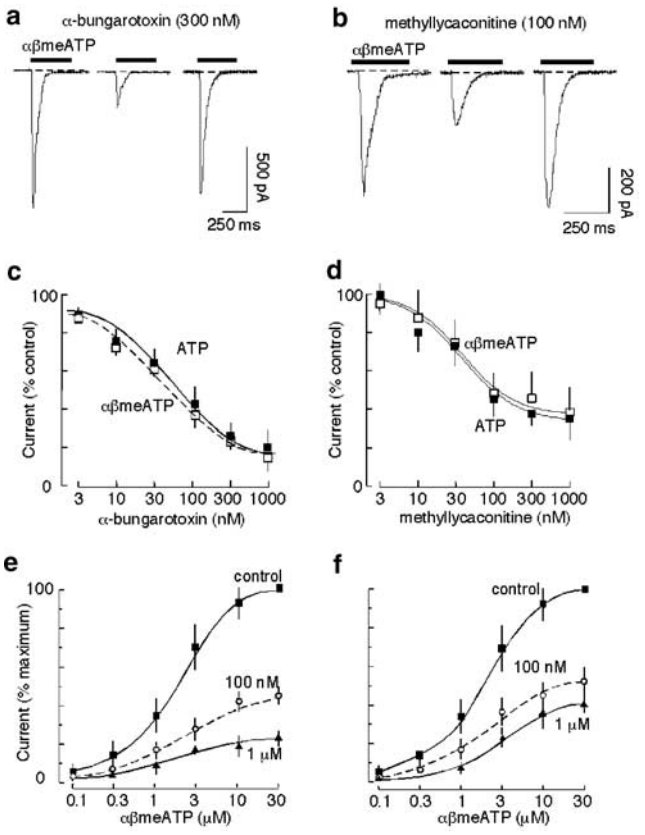

Figure 3 Methyllycaconitine and α-bungarotoxin inhibit ATP-evoked currents at $P2X_3$ receptors in HEK293 cells. Three responses to αβmeATP (10 μM) are shown, before, during and after washout of the α-bungarotoxin (a) or methyllycaconitine (b). (c, d) The antagonists are similarly effective whether ATP (10 μM) or αβmeATP (10 μM) is the agonist. (e, f) Summary dose–response curves for αβmeATP in the presence of two concentrations of α-bungarotoxin (e) and methyllycaconitine (f). Holding potential −100 mV. Points are mean ± s.d. for eight or nine cells.

(Figure 1a and c). It was also blocked by methyllycaconitine, but maximal inhibition in this case was about 50% (Figure 1). Concentrations giving half-maximal block (IC_{50}) were: for α-bungarotoxin 59 ± 7 nM ($n = 7$), for (+)-tubocurarine 800 ± 70 nM ($n = 9$) and for methyllycaconitine 34 ± 11 nM ($n = 8$). Hexamethonium (100 and 300 μM) and mecamylamine (100 μM) had little or no effect on these currents (Figure 1d–f).

The nondesensitising current evoked by ATP or αβmeATP was much less sensitive to inhibition by α-bungarotoxin and methyllycaconitine (Figure 2). α-Bungarotoxin and methyllycaconitine inhibited the sustained currents in a concentration-dependent manner with half-maximal block concentrations of 1.1 ± 0.11 μM ($n = 5$) and 3.0 ± 0.18 μM ($n = 7$), respectively (Figure 2). The inhibitory effect of methyllycaconitine was not complete (maximum 40%) even at high concentration. (+)-Tubocurarine (10 μM) blocked this current by 72 ± 10% ($n = 5$) (Figure 2c). Hexamethonium had essentially no effect (Figure 2) at a concentration up to 1 mM that completely blocks neuronal nicotinic receptors (e.g. Fieber & Adams, 1991). Mecamylamine also had no effect (Figure 2).

$P2X_3$ receptors in HEK293 cells

ATP and αβmeATP evoked currents in these cells that desensitised rapidly. The peak amplitude of the current was substantially decreased by α-bungarotoxin (Figure 3a) and methyllycaconitine (Figure 3b), with no marked change in rise and decay time constants. Figure 3c and d shows that the inhibition did not reach 100%; it was similar whether ATP or αβmeATP was used as the agonist. In the latter case, the concentrations giving half-maximal inhibition (α-bungarotoxin: 52 ± 5 nM, $n = 6$; methyllycaconitine 44 ± 12 nM, $n = 8$) were not different from those determined for the rapidly desensitising current in dorsal root ganglion cells. The dose–response relationships for αβmeATP were somewhat right-shifted by methyllycaconitine (Figure 3f). The control EC_{50} was 1.6 ± 0.10 μM ($n = 9$); this value was 1.8 ± 0.12 μM ($n = 6$) in 100 nM methyllycaconitine, and 3.7 ± 0.35 μM ($n = 7$) in 1 μM methyllycaconitine. At the same time, the inhibition by α-bungarotoxin was nonsurmountable (Figure 3e).

$P2X_{2/3}$ receptors in HEK293 cells

We avoided possible involvement of homomeric $P2X_2$ receptors (Spelta *et al*., 2002) by using αβmeATP as the agonist at concentrations not exceeding 30 μM. These currents were also sensitive to α-bungarotoxin and methyllycaconitine (Figure 4), with half-maximal inhibition occurring with 1.2 ± 0.14 μM ($n = 5$) and 3.3 ± 0.3 μM ($n = 6$) for α-bungarotoxin and methyllycaconitine, respectively. The maximal

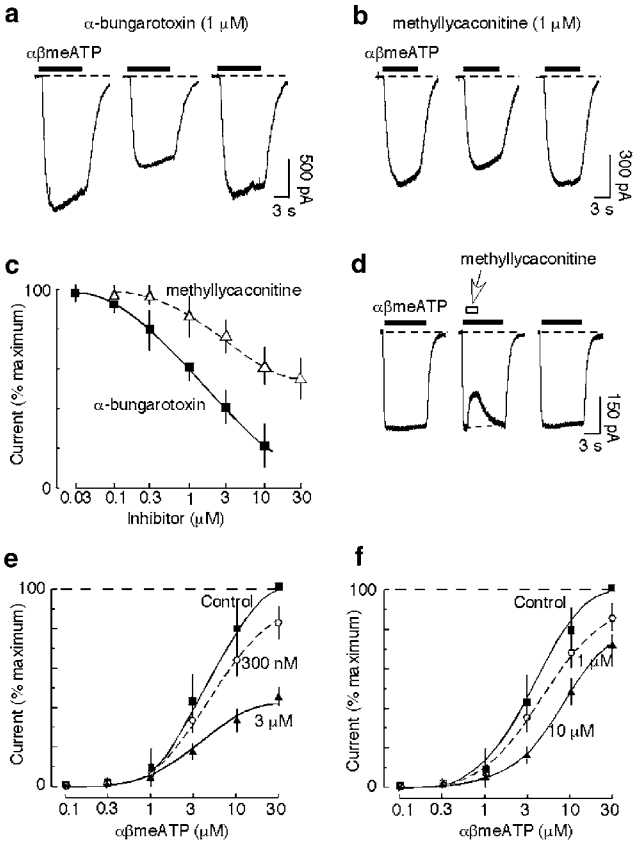

Figure 4 Methyllycaconitine and α-bungarotoxin inhibit ATP-evoked currents at $P2X_{2/3}$ receptors in HEK293 cells. Three responses to αβmeATP (10 μM) are shown, before, during and after washout of the α-bungarotoxin (a) or methyllycaconitine (b). (c) Inhibition of currents evoked by αβmeATP (10 μM) by α-bungarotoxin and methyllycaconitine. (d) Kinetics of onset and offset of methyllycaconitine (MLA open bar: 3 μM) applied during the application of αβmeATP (10 μM). (e, f) Summary dose–response curves for αβmeATP in the presence of two concentrations of α-bungarotoxin (e) and methyllycaconitine (f). Holding potential −100 mV. Points are mean ± s.d. for five or six cells.

inhibition was approximately 85% for α-bungarotoxin and 50% for methyllycaconitine. The inhibition by α-bungarotoxin was not obviously surmountable when the αβmeATP concentration was raised in the range 1–30 μM (Figure 3). The dose–response curves for αβmeATP were right-shifted by methyllycaconitine (Figure 4f): the control EC_{50} was 4.3 ± 0.33 μM ($n = 10$); this value was 4.9 ± 0.26 μM ($n = 7$) in 1 μM methyllycaconitine, and 7.4 ± 0.32 μM ($n = 6$) in 10 μM methyllycaconitine.

The slow desensitisation of the $P2X_{2/3}$ current allowed us to measure the onset and offset of block by methyllycaconitine (see Spelta *et al*., 2002). In experiments of the type illustrated in Figure 4d, we fit the onset and offset of inhibition with exponentials of time constant τ_{on} and τ_{off}. For 3 μM methyllycaconitine, these values were 0.65 ± 0.12 s and 1.9 ± 0.13 ($n = 4$). These correspond to estimates of k_{-1} ($= 1/\tau_{off}$) of 0.53 ± 0.04 s^{-1}, k_{+1} ($= 1/3\tau_{off}$) of 0.34 ± 0.13 $\mu M^{-1} s^{-1}$ and K_D ($= k_{-1}/k_{+1}$) of 1.6 ± 0.26 μM. Similar experiments were not possible with α-bungarotoxin because of a much slower (> 10 times) onset of action.

The summary results on the inhibitory action of acetylcholine receptor antagonists on native and recombinant $P2X_{2/3}$ and $P2X_3$ receptors are present in Table 1.

Discussion

It is generally thought that the rapidly desensitising currents in dorsal root ganglion cells result from activation of homomeric $P2X_3$ receptors. This is because there is good agreement in the time course of the responses seen in dorsal root ganglion cells and HEK293 cells expressing only the $P2X_3$ subunit (Lewis *et al*., 1995; also Figures 1 and 3), because both responses are similarly sensitive to P2X antagonists (2′,4′,6′)-trinitro-phenyl-ATP (Virginio *et al*., 1998; Burgard *et al*., 1999; Grubb & Evans, 1999) and A-3174914 (Jarvis *et al*., 2002), and because mice with a disrupted $P2X_3$ gene lack this response (Souslova *et al*., 2000; Zhong *et al*., 2001). Our principal finding is that these responses to ATP are also remarkably sensitive to the nicotinic receptor antagonists α-bungarotoxin, methyllycaconitine and (+)-tubocurarine (Figure 1). In the case of methyllycaconitine and α-bungarotoxin, we extended these findings by experiments on $P2X_3$ and $P2X_{2/3}$ subunits expressed in HEK293 cells.

The effective concentrations of α-bungarotoxin (EC_{50} 60 nM) and (+)-tubocurarine (EC_{50} 800 nM) are not greatly dissimilar from those required to block certain nicotinic acetylcholine receptors. For example, 10 nM α-bungarotoxin completely blocks the homomeric α7 receptor expressed in *Xenopus* oocytes (Couturier *et al*., 1990), and the dissociation equilibrium constant for (+)-tubocurarine as a competitive blocker at the frog neuromuscular junction is about 300 nM (Colquhoun *et al*., 1979). The effective concentration of methyllycaconitine (EC_{50} about 200 nM) is much higher than that required to block α-bungarotoxin-sensitive neuronal nicotinic receptors (such as homomeric α7), but similar to that observed at the α-bungarotoxin-insensitive class of neuronal nicotinic receptor, such as that comprised of α4 and β2 subunits

Table 1 IC_{50} values of acetylcholine receptor antagonists for native $P2X_3$ and $P2X_{2/3}$ receptors in DRG neurons and recombinant $P2X_3$ and $P2X_{2/3}$ receptors in HEK293 cells

	P2X₃		*P2X₂/₃*	
	DRG	*HEK293*	*DRG*	*HEK293*
Methyllycaconitine	40 nM max. inhibition 50%	44 nM max. inhibition 65%	3 μM max. inhibition 40%	3.3 μM max. inhibition 50%
α-Bungarotoxin	60 nM max. inhibition 85%	52 nM max. inhibition 85%	1.1 μM max. inhibition 80%	1.2 μM max. inhibition 85%
(+)-Tubocurarine	800 nM	ND	> 2 μM	ND
Hexamethonium	> 300 μM	ND	> 1000 μM	ND
Mecamylamine	> 100 μM	ND	> 100 μM	ND

*ND = not determined.

(Buisson *et al*., 1996). Hexamethonium and mecamylamine had little or no effect on ATP-evoked currents, even at 300 μM. Taking into account that they are open-channel blockers, we tested their action during repetitive activation of P2X receptors by ATP, but found that they remained ineffective (unpublished observations).

There was a clear difference in sensitivity to the nicotinic blockers between the fast-desensitising currents of homomeric $P2X_3$ receptors and the slow-desensitising currents. The subunit composition of the receptors that underlie these slow-desensitising currents cannot be known without further studies (e.g. with $\alpha\beta$meATP, or with 2′,3′,-*O*-(2′,3′,4′)-trinitrophenyl-ATP (TNP-ATP)), although it is known that a predominate form in the dorsal root ganglion cells is the heteromeric $P2X_{2/3}$ receptor (see North, 2002). These currents (Figure 2) were significantly blocked by α-bungarotoxin and (+)-tubocurarine but their effective concentrations (10 μM) considerably exceed that required to block most nicotinic receptors. The range of (+)-tubocurarine concentrations required to block other P2X receptors extends from 1 μM ($P2X_2$ in oocytes: Brake *et al*., 1994) to about 100 μM ($P2X_2$ in HEK293 cells: Evans *et al*., 1995; P2X in hair cells: Glowatzki *et al*., 1997).

We do not know the mechanism by which acetylcholine receptor antagonists block P2X receptors. Given the evidence for direct interaction between nicotinic and P2X receptors (see Introduction), it is possible that antagonist binding to the former might inhibit currents through the latter. Our studies on dorsal root ganglion cells show that currents through $P2X_3$ receptors are most strongly inhibited by nicotinic antagonists, whereas it is the currents through $P2X_2$ receptors that have been most extensively studied with respect to the interaction (Nakazawa *et al*., 1991b; Khakh *et al*., 2000). The simplest interpretation is that the antagonists bind directly to one or more sites on the P2X protein ectodomain. Of the antagonists tested, α-bungarotoxin blocked $P2X_3$ receptors with the highest potency. There is detailed structural information regarding the binding α-bungarotoxin to nicotinic receptor subunits (Samson *et al*., 2002); however, there is no region in the P2X receptor with obvious sequence relatedness to the part of the acetylcholine receptor subunits that interact with α-bungarotoxin. The block by α-bungarotoxin that we observed was quite rapidly reversible (Figure 1), which is in contrast to its long-lasting action at the neuromuscular junction. This parallels the observations that we have previously reported for ω-conotoxin GVIA. This 27-amino acid *Conus* snail toxin also selectively blocks the rapidly desensitising componenent of the ATP-evoked current in rat dorsal root ganglion cells (Lalo *et al*., 2001), and the effective concentrations of ω-conotoxin GVIA are very close to those observed for block of N-type calcium channels in the same cells (EC_{50} 20 nM: Regan *et al*., 1991). Whereas the block of P2X receptors by ω-conotoxin GVIA is quickly reversible, the inhibition of calcium currents is not. Taken together, these observations suggest that the toxins have a relatively rapid association to the $P2X_3$ receptor, as recently argued for TNP-ATP (Spelta *et al*., 2002). It seems unlikely that open channel block contributes directly to inhibition of the currents. The observation that there was no change in the kinetics of these relatively fast currents (e.g. Figure 1) suggest that the channel does not need to be opened to be blocked by methyllycaconitine or α-bungarotoxin.

In conclusion, we find that some P2X receptors, particularly the homo-oligomeric $P2X_3$ receptor, are readily blocked by molecules in general use as nicotinic receptor antagonists. The results suggest that mecamylamine and hexamethonium, although less potent, are more selective in this regard than methyllycaconitine, α-bungarotoxin and (+)-tubocurarine.

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References

ALKONDON, M., PEREIRA, E.F., WONNACOTT, S. & ALBUQUERQUE, E.X. (1992). Blockade of nicotinic currents in hippocampal neurons defines methyllycaconitine as a potent and specific receptor antagonist. *Mol. Pharmacol.,* **41,** 802–808.

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

BRIGGS, C.A., MCKENNA, D.G. & PIATTONI-KAPLAN, M. (1995). Human alpha 7 nicotinic acetylcholine receptor responses to novel ligands. *Neuropharmacology,* **34,** 583–589.

BUISSON, B., GOPALAKRISHNAN, M., ARNERIC, S.P., SULLIVAN, J.P. & BERTRAND, D. (1996). Human $\alpha4\beta2$ neuronal nicotinic acetylcholine receptor in HEK 293 cells: a patch-clamp study. *J. Neurosci.,* **16,** 7880–7891.

BURGARD, E.C., NIFORATOS, W., VAN BIESEN, T., LYNCH, K.J., TOUMA, E., METZGER, R.E., KOWALUK, E.A. & JARVIS, M.F. (1999). P2X receptor-mediated ionic currents in dorsal root ganglion neurons. *J. Neurophysiol.,* **82,** 1590–1598.

COLQUHOUN, D., DREYER, F. & SHERIDAN, R.E. (1979). The actions of tubocurarine at the frog neuromuscular junction. *J. Physiol.,* **293,** 247–284.

COUTURIER, S., BERTRAND, D., MATTER, J.M., HERNANDEZ, M.C., BERTRAND, S., MILLAR, N., VALERA, S., BARKAS, T. & BALLIVET, M.A. (1990). Neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-bungarotoxin. *Neuron,* **5,** 847–856.

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

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

EVANS, R.J., LEWIS, C., BUELL, G., VALERA, S., NORTH, R.A. & SURPRENANT, A. (1995). Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2X purinoceptors). *Mol. Pharmacol.,* **48,** 178–183.

FERGUSON, D.R., KENNEDY, I. & BURTON, T.J. (1997). ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes – a possible sensory mechanism? *J. Physiol.,* **505,** 503–511.

FIEBER, L.A. & ADAMS, D.J. (1991). Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J. Physiol.,* **434,** 215–237.

FRENCH, A.M. & SCOTT, N.C. (1983). Evidence to support the hypothesis that ATP is a co-transmitter in rat vas deferens. *Experientia,* **39,** 264–266.

GLOWATZKI, E., RUPPERSBERG, J.P., ZENNER, H.P. & RUSCH, A. (1997). Mechanically and ATP-induced currents of mouse outer hair cells are independent and differentially blocked by (+)-tubocurarine. *Neuropharmacology,* **36,** 1269–1275.

GRUBB, B.D. & EVANS, R.J. (1999). Characterization of cultured dorsal root ganglion neuron P2X receptors. *Eur. J. Neurosci.,* **11,** 149–154.

JARVIS, M.F., BURGARD, E.C., MCGARAUGHTY, S., HONORE, P., LYNCH, K., BRENNAN, T.J., SUBIETA, A., VAN BIESEN, T., CARTMELL, J., BIANCHI, B., NIFORATOS, W., KAGE, K., YU, H., MIKUSA, J., WISMER, C.T., ZHU, C.Z., CHU, K., LEE, C.H., STEWART, A.O., POLAKOWSKI, J., COX, B.F., KOWALUK, E., WILLIAMS, M., SULLIVAN, J. & FALTYNEK, C. (2002). A-317491, a novel potent and selective non-nucleotide antagonist of $P2X_3$ and $P2X_{2/3}$ receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proc. Natl. Acad. Sci. U.S.A.,* **99,** 17179–17184.

JO, Y.H. & ROLE, L.W. (2002). Coordinate release of ATP and GABA at *in vitro* synapses of lateral hypothalamic neurons. *J. Neurosci.,* **22,** 4794–4804.

JO, Y.H. & SCHLICHTER, R. (1999). Synaptic co-release of ATP and GABA in cultured spinal neurons. *Nat. Neurosci.,* **2,** 241–245.

KAWASHIMA, E., ESTOPPEY, D., VIRGINIO, C., FAHMI, D., REES, S., SURPRENANT, A. & NORTH, R.A. (1998). A novel and efficient method for the stable expression of heteromeric ion channels in mammalian cells. *Receptors Channels,* **5,** 53–60.

KHAKH, B.S. (2001). Molecular physiology of P2X receptors and ATP signalling at synapses. *Nat. Rev. Neurosci.,* **2,** 165–174.

KHAKH, B.S., ZHOU, X., SYDES, J., GALLIGAN, J.J. & LESTER, H.A. (2000). State-dependent cross-inhibition between transmitter-gated cation channels. *Nature,* **406,** 405–410.

LALO, U.V., PANKRATOV, Y., ARNDS, D. & KRISHTAL, O.A. (2001). Ω-conotoxin GVIA potently inhibits the currents mediated by P2X receptors in rat DRG neurons. *Brain Res. Bull.,* **54,** 507–512.

LEWIS, C., NEIDHART, S., HOLY, C., NORTH, R.A., BUELL, G. & SURPRENANT, A. (1995). Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature,* **377,** 432–435.

MORI, M., HEUSS, C., GAHWILER, B.H. & GERBER, U. (2001). Fast synaptic transmission mediated by P2X receptors in CA3 pyramidal cells of rat hippocampal slice cultures. *J. Physiol.,* **535,** 115–123.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1991a). Comparison of adenosine triphosphate- and nicotine-activated inward currents in rat phaeochromocytoma cells. *J. Physiol.,* **434,** 647–660.

NAKAZAWA, K., INOUE, K., FUJIMORI, K. & TAKANAKA, A. (1991b). Effects of ATP antagonists on purinoceptor-operated inward currents in rat phaeochromocytoma cells. *Pflugers Arch.,* **418,** 214–219.

NORTH, R.A. (2002). Molecular physiology of P2X receptors. *Physiol. Rev.,* **82,** 1013–1067.

NORTH, R.A. & SURPRENANT, A. (2000). Pharmacology of cloned P2X receptors. *Annu. Rev. Pharmacol. Toxicol.,* **40,** 563–580.

PALMA, E., BERTRAND, S., BINZONI, T. & BERTRAND, D. (1996). Neuronal nicotinic alpha 7 receptor expressed in Xenopus oocytes presents five putative binding sites for methyllycaconitine. *J. Physiol.,* **491,** 151–161.

PANKRATOV, Y., CASTRO, E., MIRAS-PORTUGAL, M.T. & KRISHTAL, O.A. (1998). Purinergic component of the excitatory postsynaptic current mediated by P2X receptors in the CA1 neurons of the rat hippocampus. *Eur. J. Neurosci.,* **10,** 3898–3902.

PANKRATOV, Y., LALO, U., DASHKIN, A. & KRISHTAL, O. (2001). Heterogeneity of the functional expression of $P2X_3$ and $P2X_{2/3}$ receptors in the primary nociceptive neurons of rat. *Neurochem. Res.,* **26,** 993–1000.

PANKRATOV, Y., LALO, U., KRISHTAL, O. & VERKHRATSKY, A. (2002). Ionotropic P2X purinoreceptors mediate synaptic transmission in pyramidal neurons of layer II/III of somato-sensory cortex. *J. Physiol.,* **542,** 529–536.

RAMME, D., REGENOLD, J.T., STARKE, K., BUSSE, R. & ILLES, P. (1987). Identification of the neuroeffector transmitter in jejunal branches of the rabbit mesenteric artery. *Naunyn Schmiedebergs Arch. Pharmacol.,* **336,** 267–273.

REGAN, L.J., SAH, D.W. & BEAN, B.P. (1991). Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and omega-conotoxin. *Neuron,* **6,** 269–280.

SAMSON, A., SCHERF, T., EISENSTEIN, M., CHILL, J. & ANGLISTER, J. (2002). The mechanism for acetylcholine receptor inhibition by alpha-neurotoxins and species-specific resistance to alpha-bungarotoxin revealed by NMR. *Neuron,* **35,** 319–332.

SEARL, T.J., REDMAN, R.S. & SILINSKY, E.M. (1998). Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. *J. Physiol.,* **510,** 783–791.

SILINSKY, E.M. (1975). On the association between transmitter secretion and the release of adenine nucleotides from mammalian motor nerve terminals. *J. Physiol.,* **247,** 145–162.

SNEDDON, P. & WESTFALL, D.P. (1984). Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens. *J. Physiol.,* **347,** 561–580.

SNEDDON, P., WESTFALL, D.P. & FEDAN, J.S. (1982). Cotransmitters in the motor nerves of the guinea pig vas deferens: electrophysiological evidence. *Science,* **218,** 693–695.

SOUSLOVA, V., CESARE, P., DING, Y., AKOPIAN, A.N., STANFA, L., SUZUKI, R., CARPENTER, K., DICKENSON, A., BOYCE, S., HILL, R., NEBENUIS-OOSTHUIZEN, D., SMITH, A.J., KIDD, E.J. & WOOD, J.N. (2000). Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. *Nature,* **407,** 1015–1017.

SPELTA, V., JIANG, L.H., SURPRENANT, A. & NORTH, R.A. (2002). Kinetics of antagonist actions at rat $P2X_{2/3}$ heteromeric receptors. *Br. J. Pharmacol.,* **135,** 524–530.

UENO, S., TSUDA, M., IWANAGA, T. & INOUE, K. (1999). Cell type-specific ATP-activated responses in rat dorsal root ganglion neurons. *Br. J. Pharmacol.,* **126,** 429–436.

VIRGINIO, C., ROBERTSON, G., SURPRENANT, A. & NORTH, R.A. (1998). Trinitrophenyl-substituted nucleotides are potent antagonists selective for $P2X_1$, $P2X_3$, and heteromeric $P2X_{2/3}$ receptors. *Mol. Pharmacol.,* **53,** 969–973.

ZHANG, M., ZHONG, H., VOLLMER, C. & NURSE, C.A. (2000). Co-release of ATP and ACh mediates hypoxic signalling at rat carotid body chemoreceptors. *J. Physiol.,* **525,** 143–158.

ZHONG, Y., DUNN, P.M., BARDINI, M., FORD, A.P., COCKAYNE, D.A. & BURNSTOCK, G. (2001). Changes in P2X receptor responses of sensory neurons from $P2X_3$-deficient mice. *Eur. J. Neurosci.,* **14,** 1784–1792.

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