P2X purinoceptor-mediated excitation of trigeminal lingual nerve terminals in an *in vitro* intra-arterially perfused rat tongue preparation

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1. A novel *in vitro* intra-arterially perfused adult rat tongue–nerve preparation was used to explore the possible actions of P2X purinoceptor agonists (ATP and α,β-methylene ATP (α,β-meATP)) on sensory nerve terminals innervating the rat tongue. We made whole-nerve recordings of the trigeminal branch of the lingual nerve (LN), which conducts general sensory information (pain, temperature, touch, etc.), and the chorda tympani (CT), which conducts taste information. Changes in LN and CT activity following intra-arterial application of P2X agonists were compared.

2. In seven preparations, bolus close-arterial injection of ATP (30–3000 μM, 0·1 ml) or α,β-meATP (10–300 μM, 0·1 ml) induced a rapid (<1 s after injection), dose-related increase in LN activity that decayed within a few seconds. The minimal concentration of ATP (100 μM) required to elicit a response was about 10-fold higher than that of α,β-meATP (10 μM). Bolus injection of ATP or α,β-meATP induced a moderate decrease in firing frequency in three of seven CT preparations.

3. LN responses to P2X agonists showed signs of rapid desensitisation with the peak frequency of discharge being smaller when the agonists were applied at short intervals. Suramin (200 μM) or PPADS (200 μM) applied by intra-arterial perfusion each antagonised the rapid increase in LN activity following application of α,β-meATP (100 μM).

4. Capsaicin (10 μM, 0·1 ml, $n=5$ preparations) was injected intra-arterially to desensitise nociceptive fibres. This was found to block ($n=2$) or greatly reduce ($n=3$) the excitatory effects of α,β-meATP (100 μM, 0·1 ml) on LN activity, implying that only capsaicin-sensitive nociceptive fibres in LN were responsive to P2X agonists.

5. In contrast to the consistent excitatory responses in LN activity following fast application of P2X agonists as bolus, a variable and moderate change in discharge rate of LN and no change in CT activity ($n=5$) was observed after applying ATP (100–300 μM, $n=21$) or α,β-meATP (100–300 μM, $n=14$) by intra-arterial perfusion. The variable responses in LN activity to slow perfusion in contrast to close-arterial bolus injection are consistent with activation of the rapidly desensitising $P2X_3$ receptors.

6. In summary, ATP and α,β-meATP preferentially activate general sensory afferent fibres (LN) but not taste fibres (CT). We suggest that the increase in whole-nerve activity of LN following application of P2X agonists represents activation of nociceptive fibres which possess $P2X_3$ receptors. Our data indicate that ATP and $P2X_3$ receptors may play a role in nociception, rather than taste sensation in the tongue.

Painful physical or chemical stimuli are detected in the periphery by nociceptors that relay through C and A_δ fibres. The signal transduction mechanisms at the nociceptors have been extensively studied but still remain largely unresolved (for reviews see Wood & Docherty, 1997; McCleskey & Gold, 1999; Millan, 1999). There is now some evidence suggesting that adenosine-5′-triphosphate (ATP) may function as a chemical activator of nociceptors (Kennedy & Leff, 1995; Burnstock, 1996; Burnstock & Wood, 1996; Dowd *et al.* 1998; Hamilton *et al.* 1999). ATP is present at millimolar levels in cells (Fukuda *et al.* 1983) and may be released in large amounts in the vicinity of sensory nerve endings in

some conditions (Born & Kratzer, 1984; Burnstock, 1999). ATP causes pain when applied to a blister base in humans (Bleehen & Keele, 1977) and the nucleotide also depolarises sensory ganglion cell *in vitro* (Jahr & Jessell, 1983; Krishtal *et al.* 1988).

The extracellular effects of ATP are mediated via two families of membrane receptors, namely P2X receptors coupled with ligand-gated ion channels and P2Y receptors coupled with G-proteins (Abbracchio & Burnstock, 1994; Burnstock, 1997; North & Barnard, 1997; Ralevic & Burnstock, 1998). Among the seven P2X receptor subunits so far cloned, six ($P2X_{1\text{-}6}$) are present in sensory ganglia (Brake *et al.* 1994; Valera *et al.* 1994; Chen *et al.* 1995; Collo *et al.* 1996) and the $P2X_3$ subtype is selectively expressed in a subpopulation of small diameter sensory neurones (Chen *et al.* 1995; Lewis *et al.* 1995; Bradbury *et al.* 1998). In the rat trigeminal ganglia, immunoreactivity for $P2X_3$ receptor is localised to nociceptive neurones that innervate the tooth pulp but not non-nociceptive neurones (Cook *et al.* 1997).

However, despite overwhelming experimental data showing that ATP depolarises dissociated sensory neurones (Jahr & Jessell, 1983; Krishtal *et al.* 1988; Bean *et al.* 1990; Evans & Surprenant, 1996), there have been conflicting reports regarding the importance of ATP and P2X receptors in peripheral nociceptors. For example, whereas ATP depolarises dissociated rat trigeminal ganglion neurones and elicits action potentials in cultured tooth pulp nociceptive neurones (Cook *et al.* 1997), it failed to induce nociceptive responses when instilled into the cornea of the rat and cat (Dowd *et al.* 1997) or when applied to the tooth pulp (Matthews *et al.* 1997). A recent study claimed that very few cells in intact dorsal root ganglion (DRG) responded to purinergic agonists, whereas the majority of dissociated DRG neurones responded with an inward current (Stebbing *et al.* 1998). These observations raised the possibility that the expression and/or function of P2 receptors may alter under culture conditions as has been shown recently for glial cells (Jabs *et al.* 1997; Kimelberg *et al.* 1997). Therefore, further studies on purinergic actions at native sensory nerve endings are needed to clarify the importance of ATP and P2X receptors in peripheral sensory functions including nociception.

In the present report, P2X receptor-mediated actions on sensory nerve terminals were studied in the rat tongue. The tongue has a rich sensory innervation and serves both general (concerning pain, temperature, touch, etc.) and special (concerning taste) sensory functions. These two distinct classes of sensory information are conducted to the central nervous system by distinctive nerve bundles. Recently, an immunohistochemical study conducted in this laboratory demonstrated an abundance of $P2X_3$ receptor immunoreactivity on sensory nerve fibres in the circumvallate papillae in rat (Bo *et al.* 1999). However, the particular class of sensory fibres (general sensory or taste sensory) that possess $P2X_3$ receptors was not determined. The distribution of $P2X_3$ receptor immunoreactivity in the circumvallate papillae does not necessarily indicate the receptor to be primarily involved in taste sensation because the papillae are innervated by both taste and general sensory fibres. Therefore in the present study, we carried out whole-nerve recording of the general sensory trigeminal lingual nerve (LN) and taste sensory chorda tympani (CT) in an *in vitro* intra-arterially perfused rat tongue–nerve model. The effects of P2X agonists, ATP and α,β-methylene ATP (α,β-meATP) on the activity of these nerves were compared.

METHODS

Intra-arterial perfusion and nerve recording

Adult Sprague-Dawley rats (200~350 g) were rendered unconscious by exposure to a rising concentration of CO_2 gas and killed by upper cervical dislocation. The head was removed to a bath containing cold Krebs-Hensleit (K-H) solution (contents (mM): KCl 4·8, NaCl 110·9, $CaCl_2$ 2·5, KH_2PO_4 1·2, $NaHCO_3$ 22·4, $MgSO_4$ 1·2) saturated with 95% O_2–5% CO_2. The tongue was dissected and placed in a recording chamber. The chamber comprised two pools separated by a Plexiglas wall (1 mm thick). The tongue was pinned in position in the test pool. The left lingual artery was catheterised with a 19G intravenous cannula and the tongue was then perfused with K-H solution (containing 1% dextran, MW 80 000, and 20 mM glucose, pH ~7·3) at a flow rate of 2–2·5 ml min^{-1} using a peristaltic pump (Watson, UK). A buffer cylinder made of two syringes (5 and 10 ml) was used to isolate the preparation electrically from the pump, and to buffer the perfusion pressure which otherwise tended to be pulsatile. The cannula had an additional port for direct injection of test solution close-arterially. Perfusion pressure was monitored continuously to record changes in vascular resistance. The LN and CT were isolated and de-sheathed before the point where they join together to form the mixed lingual nerve. Each nerve was pulled through an aperture (1 mm diameter) on the Plexiglas wall into the second pool that was filled with paraffin oil. The aperture was sealed with Vaseline. The nerves were placed individually onto pairs of Ag–AgCl wire electrodes connected to Neurolog headstages (NL100) and AC differential amplifiers (NL104). The signal was amplified (× 10 000), filtered (band-pass 100–4000 Hz) and monitored on an oscilloscope. Chamber temperature was kept around 30 °C during surgical dissection but was then raised to 34 °C for the recording session. The preparation was allowed to stabilise for at least 1 h before the actions of P2X agonists were to be tested.

At the start of recording, mechanical and chemical stimuli were delivered to the surface of the tongue to assess the viability of the preparation. To activate general sensory fibres, a mechanical stimulus was applied by probing the tongue with calibrated von Frey hairs (usually 76, 149 and 216 mN). To activate taste fibres, a drop of 0·2 M NaCl or 0·1 M HCl (room temperature, ~28 °C) was instilled onto the surface of the tongue; this was washed off with 1 ml distilled water ~10 s later. As will be described later, these stimuli gave rise to differential responses in LN and CT. Nerve preparations that failed to respond to the above stimuli were not studied further.

Purinergic agonists were administered intra-arterially. In initial studies, P2X agonists were applied by switching the perfusate to test solutions that contained ATP or α,β-meATP. The changeover time was ~40 s and the test solutions were applied usually for ~60 s. Most frequently, 100 μM ATP or 100 μM α,β-meATP were

first applied and the activity of LN and CT were monitored for at least 15 min. The test was repeated several times with 100 or 300 μM ATP or α,β-meATP. Later, test solutions were injected close-arterially through the side port on the arterial catheter, 0·1 ml test solution being injected within 1 s. To examine dose–response relationships, 0·1 ml of solutions that contained various concentrations of purinergic agonists were administered at intervals of 10 min in random order. P2X antagonists suramin or pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (both at 200 μM in K-H solution) were given by perfusion over 2 min. Capsaicin (1 or 10 μM, 0·1 ml) was applied by bolus injection.

Data analysis and statistics

Nerve signals from LN and CT were monitored on an oscilloscope and relayed to spike processors (Digitimer D130) which discriminated neural impulses from noise with a manually set amplitude and polarity window. The digitised signals, together with the perfusion pressure signal, were recorded on a computer equipped with a 1401 A/D interface and Spike 2 data capture and analysis software (Cambridge Electronic Design, Cambridge, UK). The ongoing activity of LN and CT was averaged (time constant 0·3 s) and estimated as mean firing frequency. The nerve activity in the 1 min period prior to commencement of drug administration was averaged and taken as the baseline firing frequency. Since the baseline firing frequency varied greatly from preparation to preparation and was influenced by the rather arbitrary setting of the window level, the percentage change in neural activity did not appear to be an appropriate measure of the neural responses. Effects on LN and CT activity were therefore calculated as maximal change in firing frequency, in other words the difference between the baseline firing frequency and the peak frequency following application of purinergic agonists. This provides an approximate estimation of the extent of changes in nerve activity; but it needs to be noted that there exists the possibility of action potentials being superimposed, and hence the number recorded could be an under-estimation of the true response. The latency of the response was measured from the start of drug administration to the time

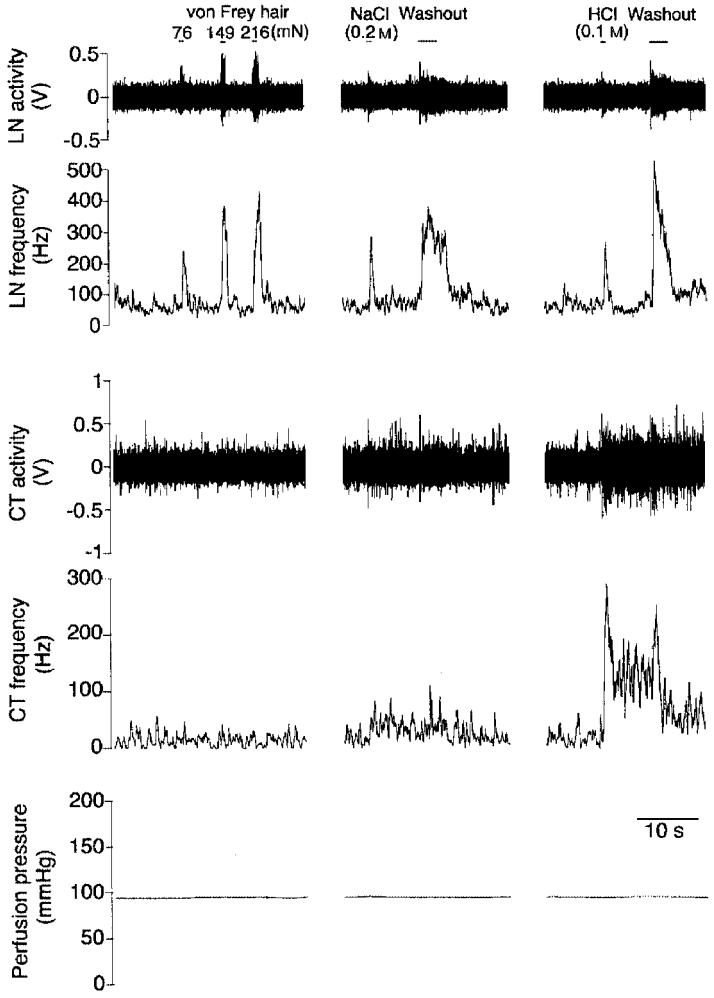

Figure 1. Differential responses of LN and CT to mechanical and chemical stimuli

From top to bottom the traces are: raw activity and mean firing frequency of LN, raw nerve activity and mean firing frequency of CT and perfusion pressure. Nerve activity is expressed in volts after amplification (× 10 000). Mechanical stimulation was effected by probing the tongue with calibrated von Frey hairs. Saline or acid was instilled onto the anterior surface of the tongue and washed out 10 s later with distilled water. These tests were carried out routinely as an assessment of the condition of the preparation. Note the saline- and acid-induced sustained increase in CT activity. The increase in LN activity during application of saline or acid (0·1 ml) and washout with distilled water (1 ml) may be due to mechanical stimulation. Time scale applies to all traces.

when nerve activity started to increase or decrease. The duration of response was measured from the time when nerve activity started to increase or decrease until the time it returned to baseline level. Values are expressed as means ± S.E.M. Student's paired t test was used for statistical analysis of the change in neural activity following drug treatment, with a P value of less than 0·05 indicating statistical significance.

Drugs

ATP (sodium salt, Sigma, USA), α,β-meATP (lithium salt, Sigma, USA), suramin (hexasodium salt, RBI, USA) and PPADS (RBI, USA) were diluted to final concentrations in K-H solution (pH adjusted to 7·3 with NaOH). Capsaicin (Tocris Cookson Ltd, UK) was dissolved in 80% ethanol as a 10 mM stock solution and diluted to final concentrations in K-H solution before use. The final concentration of ethanol was less than 0·1%.

RESULTS

Both LN and CT exhibited a variable but low level of ongoing activity under control conditions that ranged from a few to a few hundred impulses per second (Hz). However, mechanical and chemical stimulation over the surface of the tongue consistently produced differential responses in the two nerves (Fig. 1). Typically, probing the tongue with a calibrated von Frey hair (76–216 mN) induced graded increases in LN activity but left CT unaffected. Saline (0·2 M NaCl) or acid (0·1 M HCl) instilled onto the surface of the tongue led to a sustained increase in CT activity which returned to control levels after wash out with distilled water. These stimuli induced only a transient increase in firing frequency of LN that appeared to be a mechanical artefact resulting from the application of solutions.

Our studies indicated that CT was composed mainly of taste fibres, but there was usually an indication of contamination with general sensory fibres. By monitoring CT activity during mechanical stimulation and intravascular application of capsaicin, it was possible to examine the contributions of taste and general sensory activity.

Effects of P2X agonists administered by close-arterial injection

In seven preparations, we examined the effects of purinergic agonists administered by fast, close-arterial bolus injection through a side port on the arterial catheter. Bolus injection of ATP or α,β-meATP invariably resulted in a rapid increase (latency 0·5 s, peak after a further 0·6 s) in firing frequency of all 7 LN studied. Figure 2A and B illustrates

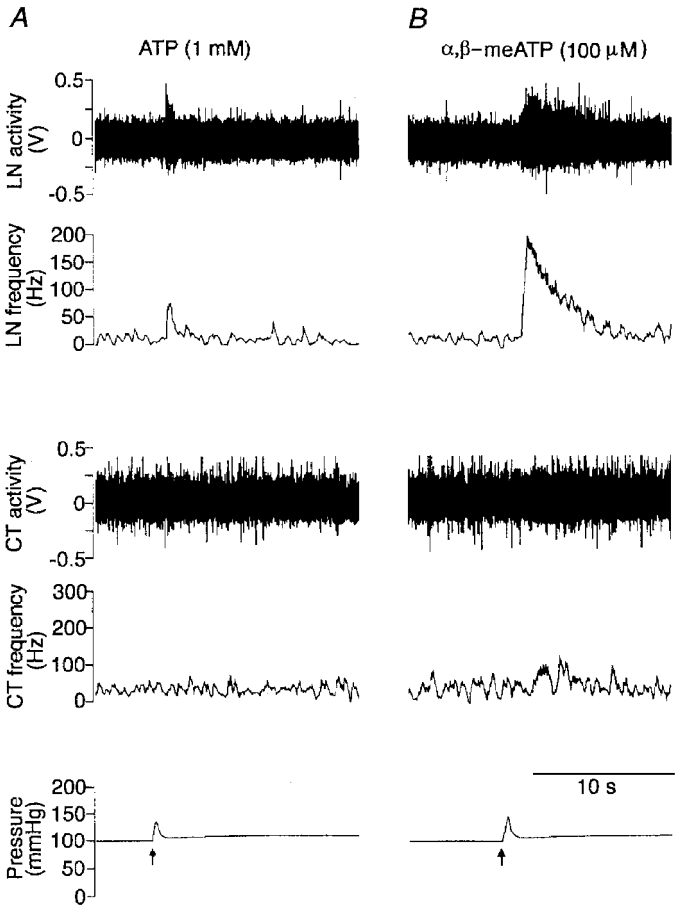

Figure 2. P2X agonists preferentially stimulate general sensory nerve activity

A and B are recordings from one preparation. From top to bottom the traces are: raw nerve activity and mean firing frequency of LN, raw nerve activity and mean firing frequency of CT and perfusion pressure. Nerve activity is expressed in volts after amplification (× 10 000). Arrows indicate the start of bolus injection. Time scale applies to all traces.

and compares the changes in LN activity after injection of ATP (1 mM) and α,β-meATP (100 μM) in the same preparation. The effects of ATP and α,β-meATP differed greatly with respect to the amplitude and duration of response. α,β-meATP (100 μM) evoked a much greater increase in LN activity than 1 mM ATP (297·4 ± 32·1 *versus* 176·7 ± 18·2 Hz, $n = 7$, $P < 0{\cdot}01$). These agonists also produced an increase in perfusion pressure (Figs 2 and 3), suggesting a P2X receptor-mediated vasoconstriction. Bolus injection of vehicle (K-H solution, 0·1 ml) did not produce any significant change in LN activity.

We further examined the repeatability of LN responses by applying ATP (1 mM) or α,β-meATP (100 μM) at different intervals. As shown in Fig. 3*A* and *B*, the responses to ATP and α,β-meATP were much reduced when they were given

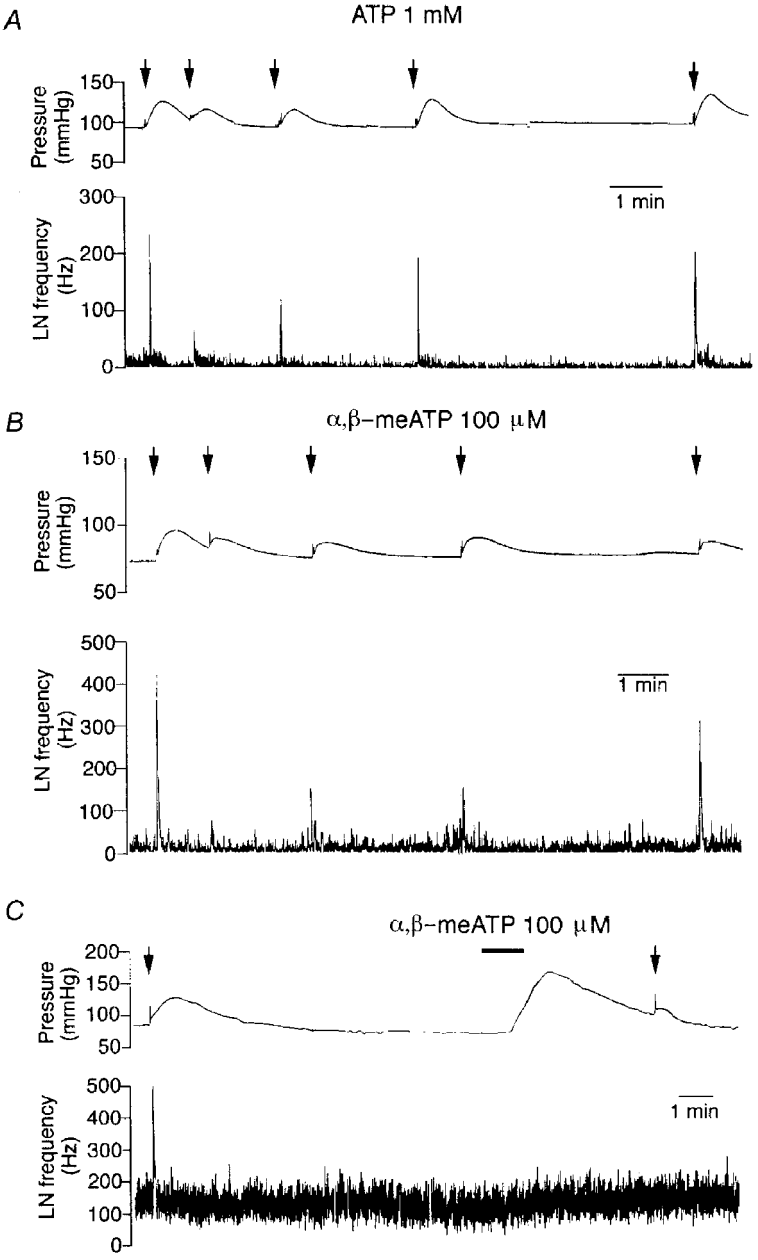

Figure 3. Sequential recordings of the LN response to P2X agonists applied at different intervals

A, bolus injections of ATP (1 mM, 0·1 ml) were administered at intervals of 1, 2, 3 and 5 min (arrows). Note that both pressure and LN responses to ATP were smaller at shorter intervals. *B*, in the same preparation as *A*, α,β-meATP (100 μM, 0·1 ml) was administered as bolus injections at intervals of 1, 2, 3 and 5 min (arrows). *C*, in another preparation, α,β-meATP (100 μM) was subsequently administered by bolus injection (0·1 ml) as indicated by arrows and perfusion (0·04 ml s^{-1} for 1 min) as indicated by horizontal bar. Note that α,β-meATP given by perfusion did not induce significant change in LN activity and blocked the effects of the subsequent bolus injection.

at short intervals. Not surprisingly, the response to ATP recovered faster than to α,β-meATP. About 90% of the control response could be obtained when the agonist was given at intervals of 7 min. In Fig. 3*C*, α,β-meATP (100 μM) given by bolus injection produced a brief increase in LN activity, but it did not evoke any change in LN activity when given by perfusion. Further, subsequent bolus injection of α,β-meATP failed to produce any change in LN activity.

The LN response to P2X agonists was dose related. In the experiments illustrated in Fig. 4*Aa* and *Ba*, α,β-meATP (30, 100 and 300 μM) or ATP (0·1, 0·3, 1 and 3 mM) were injected at intervals of 10 min; the higher doses produced consistently significantly greater increases in firing frequency of LN (Fig. 4*Ab* and *Bb*). The minimal concentrations of ATP and α,β-meATP required to evoke noticeable increase in LN activity were 100 and 10 μM, respectively.

ATP up to 300 μM (0·1 ml) or α,β-meATP up to 30 μM (0·1 ml) did not produce any noticeable change in CT activity ($n = 5$), however, ATP (1 and 3 mM) or α,β-ATP (100 and 300 μM) produced slow decreases in ongoing activity of CT in three of seven preparations (e.g. Figs 4*A* and 5*B*). In two of seven preparations, CT activity briefly increased (e.g. Fig. 5*A*) after application of ATP (1 and 3 mM) or α,β-meATP (100 and 300 μM). It is noteworthy that in these two preparations CT, like LN, could also be excited by mechanical stimulation and by intravascular administration of capsaicin, so the brief increases in CT

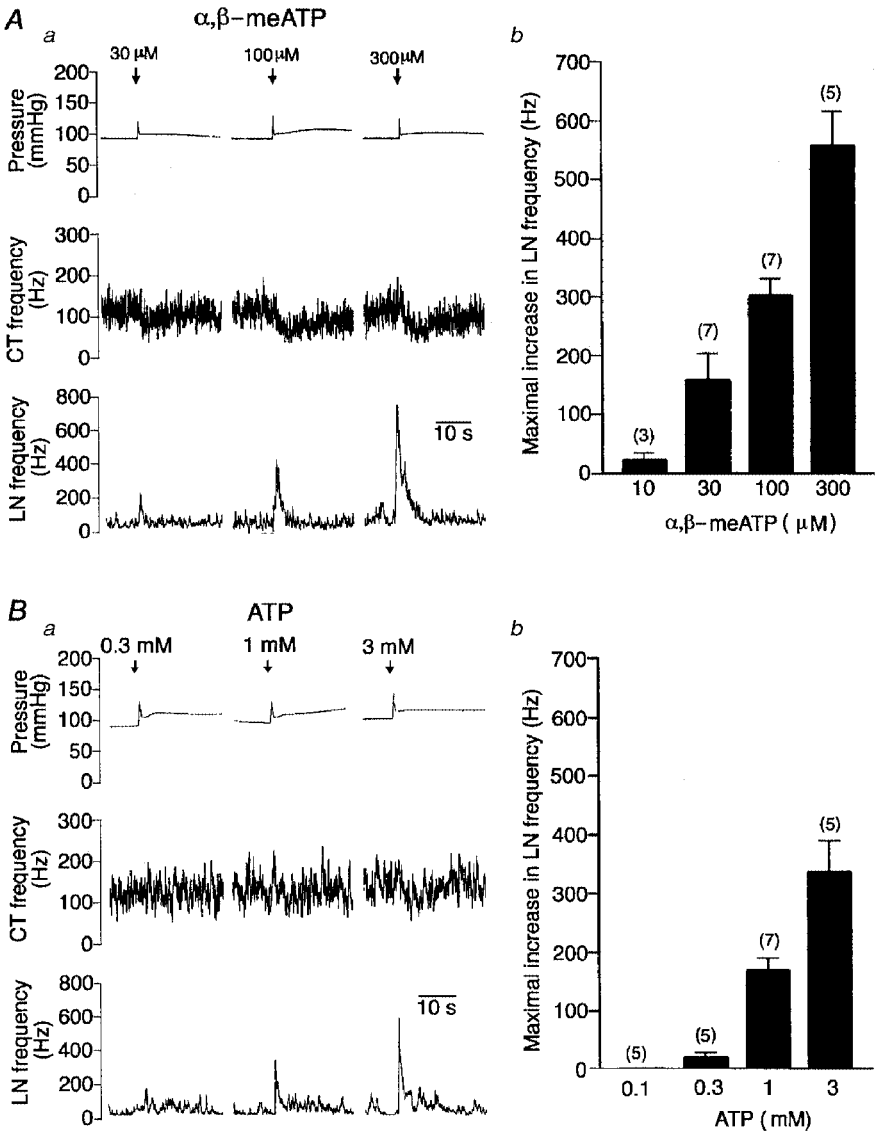

Figure 4. Dose-related increase in LN activity following bolus injection of P2X agonists

Aa and *Ba* are recordings from one preparation showing the changes in LN activity following bolus injection of different concentrations of α,β-meATP and ATP, respectively. The agonists were injected at 10 min intervals in random order. Note that CT activity started to decrease at the falling phase of the LN response. *Ab* and *Bb* are the averaged (mean ± S.E.M.) maximal increase in LN activity following different concentrations of α,β-meATP and ATP in seven preparations. Numbers of tests are shown in parentheses.

activity mediated by P2X agonists in these two particular cases appeared to be due to activation of general sensory fibres contained in the nerve. In one of the two preparations, the brief excitation of CT was followed by a slow inhibition. In the other two preparations of the seven examined, even a large dose of ATP (3 mM) or α,β-meATP (300 μM) did not induce a significant change in CT activity (e.g. Fig. 2).

Effects of P2X antagonists on LN responses to P2X agonists

Suramin (200 μM, $n = 2$) or PPADS (200 μM, $n = 3$) when given by intravascular perfusion for 2 min antagonised the action of α,β-meATP (100 μM, see Fig. 5). The effect of α,β-meATP was significantly reduced from an average increase of $290{\cdot}0 \pm 25{\cdot}9$ Hz to $46{\cdot}4 \pm 17{\cdot}4$ Hz after the P2X anatagonists ($P < 0{\cdot}01$). The responses to α,β-meATP recovered to control values in 30–90 min. In the preparation shown in Fig. 5*B*, the ongoing discharge of LN and CT were also reduced after application of suramin but in the other four preparations, the antagonists did not affect the baseline LN or CT activity but antagonised the effects of α,β-meATP.

Effects of capsaicin on LN and LN response to purinergic agonists

To test the hypothesis that P2X agonists can activate nociceptors in the tongue, the ability of capsaicin to selectively activate small diameter nociceptive neurones and at higher doses to produce prolonged depolarisation leading to desensitisation or tachyphylaxis was used as means of assessing purine actions on nociceptors (Heyman & Rang, 1985; Del Mar *et al.* 1996; Stebbing *et al.* 1998). An intra-arterial bolus injection of capsaicin (1 μM, 0·1 ml) gave rise to transient increases in LN activity in all seven preparations examined. Capsaicin produced little change in CT activity in

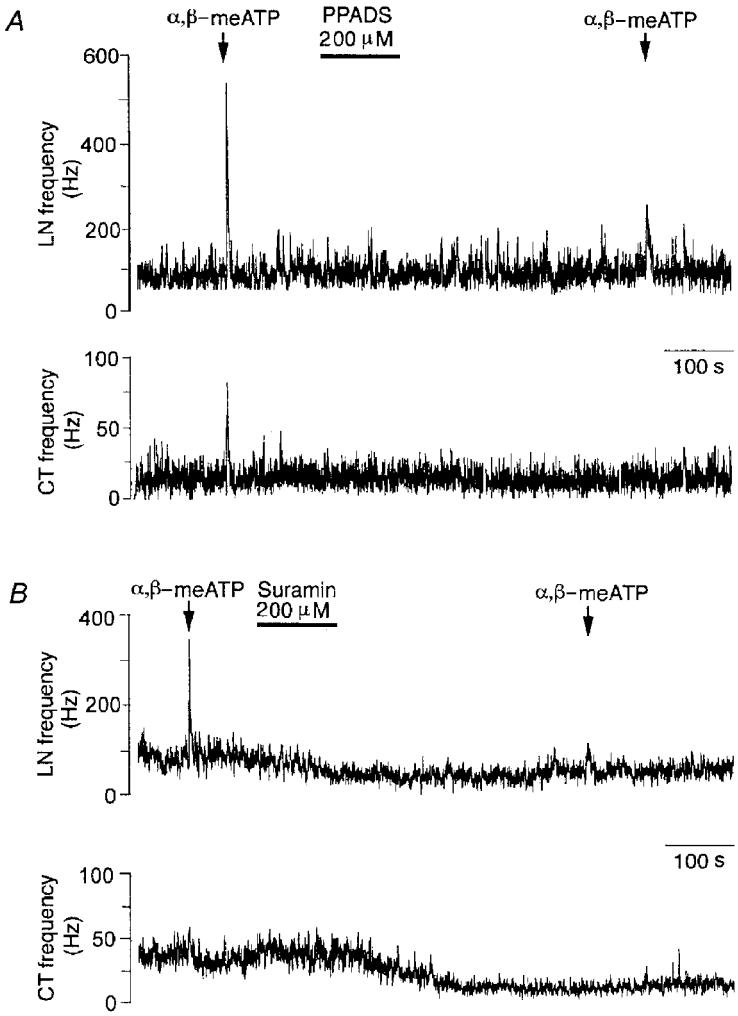

Figure 5. Effects of P2X antagonists on LN response to α,β-meATP

A, PPADS (200 μM) was given by intra-arterial perfusion for 2 min as indicated by horizontal bar. Bolus injection of α,β-meATP (100 μM) before application of PPADS led to a marked increase in LN activity and in this case CT activity as well. These effects were significantly reduced 5 min after application of PPADS. In this preparation, CT responded to mechanical stimulation and capsaicin in a pattern similar to the way LN responded to these tests (not shown in the figure). *B*, in another preparation, suramin (200 μM) given by intra-arterial perfusion blocked the effects of subsequent bolus injection of α,β-meATP (100 μM). In this particular case, suramin also inhibited the ongoing activity of LN and CT.

five of these preparations, whilst in the other two there was a concomitant brief increase in CT discharge. The LN response lasted for 10–15 s and was repeatable at intervals of >10 min. As shown in Fig. 6*A*, the impulses in LN evoked by capsaicin had similar spike amplitudes to impulses induced by α,β-meATP. On the other hand, mechanical stimulation with a von Frey hair elicited a burst of discharges that consisted of much larger spikes. The effects of α,β-meATP (100 μM, 0·1 ml) were further compared before and after injection of a large dose of capsaicin (10 μM, 0·1 ml) which itself evoked a pronounced increase in LN activity that decayed over 30–45 s (Fig. 6*B*). This dose was sufficient to produce tachyphylaxis of nociceptive fibres in LN since application of capsaicin 10 min after the first injection failed to induce excitation of LN. Further in two preparations, a previously effective dose of α,β-meATP (100 μM) failed to produce noticeable change in LN activity after pre-treatment with capsaicin while the nerve still responded to mechanical stimulation with a von Frey hair. In three preparations, the effect of α,β-meATP (100 μM, 0·1 ml) was reduced greatly from an average increase of 281·3 ± 23·9 Hz in firing frequency of LN

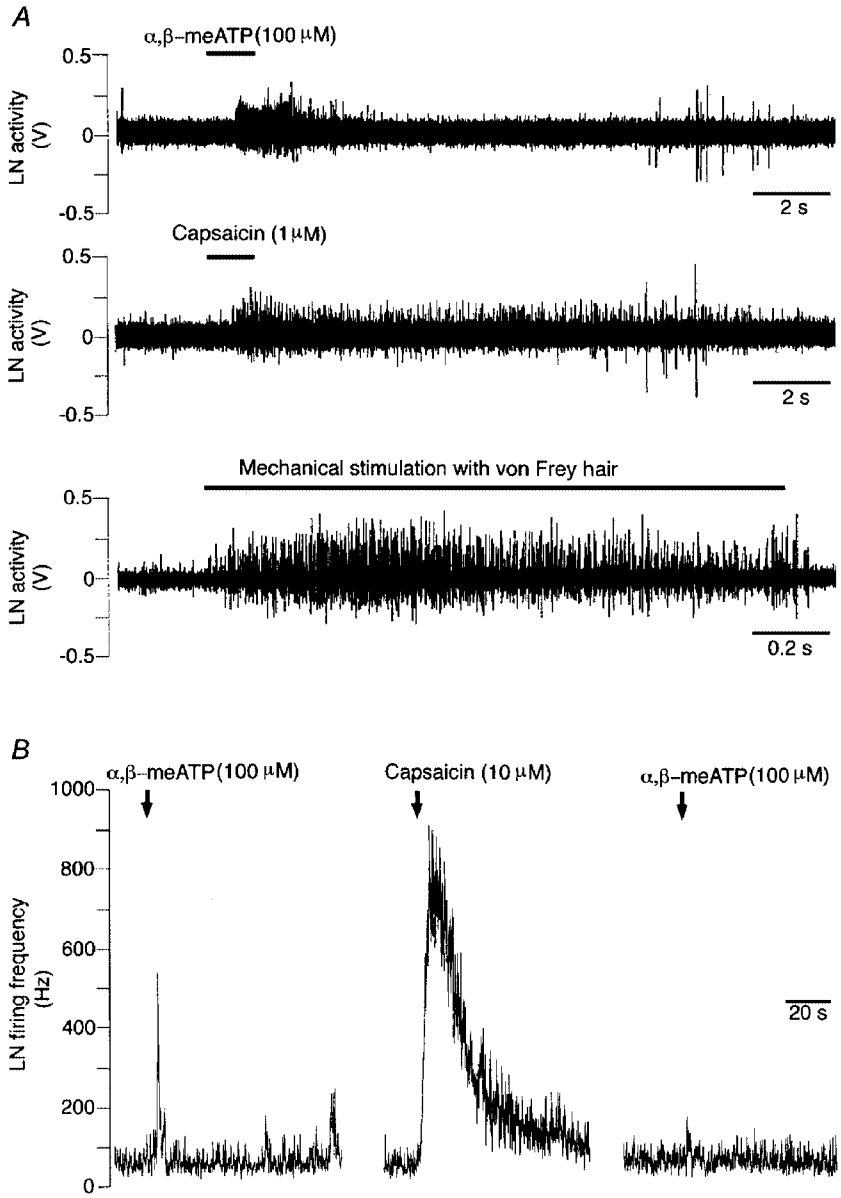

Figure 6. Effects of capsaicin on LN and its response to P2X agonist

A, all three traces are recordings from one preparation to compare afferent impulses evoked by bolus injection of α,β-meATP (100 μM, 0·1 ml), capsaicin (1 μM, 0·1 ml) and by mechanical stimulation with a von Frey hair (76 mN). The bottom trace is on a more expanded time scale than upper traces. Horizontal bars indicate the period of injection or mechanical stimulation. Notably, the impulses evoked by capsaicin and α,β-meATP are of similar amplitudes. *B*, in the same preparation as *A*, α,β-meATP (100 μM, 0·1 ml) was injected 10 min before (left trace) and 5 min after (right trace) a larger dose of capsaicin (10 μM, 0·1 ml). Arrows indicate when drugs were injected. Time scale applies to all three traces.

before application of capsaicin to an average increase of 67·7 ± 13·3 Hz at 15 min after ($P < 0·01$). We repeatedly injected α,β-meATP (100 or 300 μM, 0·1 ml) at intervals of ~30 min. Partial recovery of the LN response to α,β-meATP was observed in two of the five preparations at ~1·5 h after application of capsaicin. Recovery of response did not occur in the other three preparations.

Effects of P2X agonists administered by intra-arterial perfusion

In the initial set of experiments performed on 21 preparations, we applied P2X agonists by perfusing the tongue intra-arterially for 1 min with solutions that contained ATP or α,β-meATP. Both agonists consistently produced a gradual increase in perfusion pressure but resulted in variable changes in LN activity and no effect on CT ($n = 5$) activity. Four patterns of change in LN activity were observed following application of ATP ($n = 21$) and these were exemplified in Fig. 7. Briefly, primarily excitatory responses were seen in 12 of 21 LN, but the latency and duration of responses varied from preparation to preparation. In three of these 12 preparations, there was a brief increase in LN activity (Fig. 7*A*) with a maximal increase in firing frequency of 69·7 ± 14·7 Hz ($P < 0·05$) after 100 μM ATP and 90·0 ± 13·2 Hz ($P < 0·05$) after 300 μM ATP. This pattern of response is consistent with ATP acting at rapidly desensitising $P2X_3$ receptors. In the other nine cases, the response was characterised by a slower increase in LN activity (Fig. 7*B*) and this was followed in three preparations by a delayed fall in discharge frequency (Fig. 7*C*). In four preparations (4/21), ATP evoked a slow decrease in LN activity followed by prolonged excitation (Fig. 7*D*). In a further five preparations (5/21), LN activity remained unchanged after application of up to 300 μM ATP.

LN responses to intra-arterial perfusion of α,β-meATP ($n = 14$) comprised two patterns: excitation or inhibition. Excitatory responses occurred in five preparations (5/14) with the average maximal increase in firing frequency being 63·7 ± 5·4 Hz ($P < 0·05$) after 100 μM and 104·3 ± 7·8 Hz ($P < 0·05$) after 300 μM α,β-meATP. Inhibition of LN activity occurred in three preparations (3/14) with a maximal decreases in firing frequency of 41·6 ± 13·2 Hz ($P < 0·05$) and 68·7 ± 10·4 Hz ($P < 0·05$) after 100 μM and 300 μM α,β-meATP, respectively. No delayed effects were observed. The firing frequency of six LN (6/14) remained unchanged following application of α,β-meATP (up to 300 μM).

In eleven preparations, we applied ATP (up to 10 mM) or α,β-meATP (up to 1 mM) directly to the surface of the tongue by superfusion or instillation but failed to elicit significant changes in either LN or CT activity.

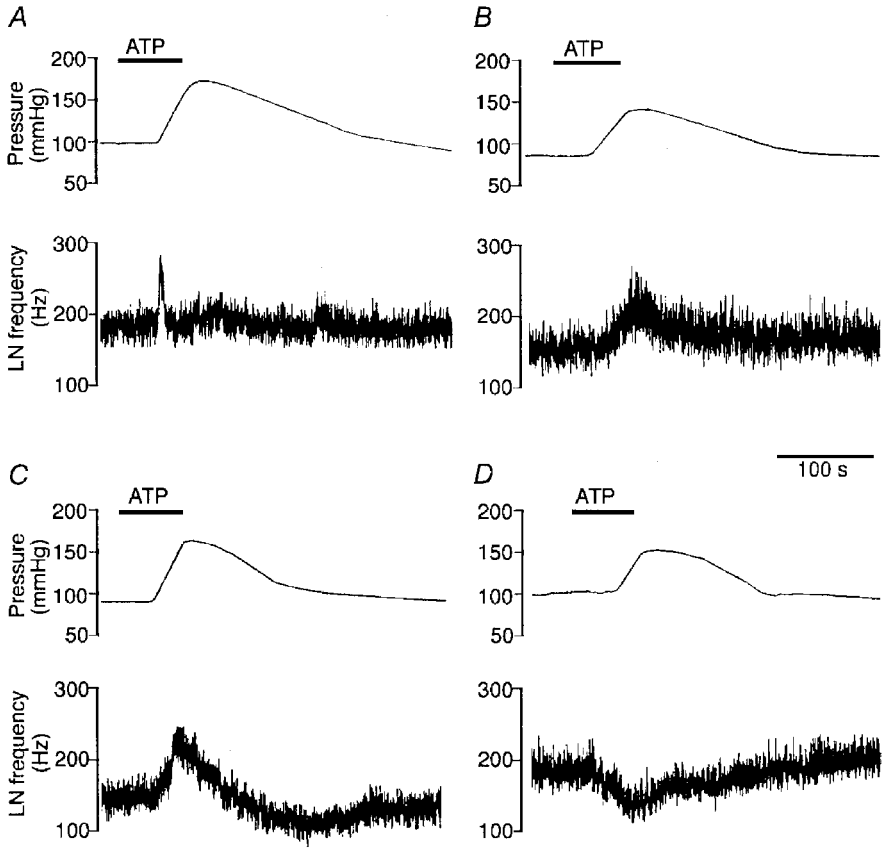

Figure 7. Patterns of LN responses to ATP applied by intra-arterial perfusion

A–D are recordings from four different preparations. The horizontal bar above each panel indicates the period of ATP (100 μM) perfusion. Note that solution changeover time is ~40 s. Time scale applies to all panels.

DISCUSSION

Significance of P2X receptors in sensory functions of the tongue

The most significant finding of the present study is that P2X agonists activate preferentially the trigeminal branch of the lingual nerve rather than the chorda tympani. LN consists of afferent fibres that carry general sensory information concerning pain, temperature, touch, etc., while the majority of afferent fibres in CT carry information concerning taste. Therefore, our findings indicate that ATP and P2X receptors may play a role in general rather than taste sensation in the tongue. LN responses to ATP and α,β-meATP could be prevented or reduced by pretreatment with a large dose of capsaicin which presumably desensitises most nociceptive fibres. This suggests that ATP may be involved in peripheral nociception by interacting with P2X receptors.

Actions of P2X agonists at sensory nerve terminals have been explored in some recent studies. It was reported that ATP analogues evoked excitation of visceral (Pelleg & Hurt, 1996) and knee-joint afferent nerves (Dowd *et al.* 1998) and induced cutaneous pain in some animal pain models (Bland-Ward & Humphrey, 1997; Sawynok & Reid, 1997; Hamilton *et al.* 1999). However, ATP analogues did not induce afferent activity in trigeminal nerves when applied to the tooth pulp of rat or onto the cornea of the rat and cat (Dowd *et al.* 1997; Matthews *et al.* 1997). The present study provides the first evidence that P2X agonists are able to activate a branch of the trigeminal nerve by acting on receptors at nerve terminals. Further, we demonstrated contrasting results of P2X agonists when given by slow perfusion, which produced variable or no effects on LN (e.g. Fig. 3*C*), and by fast bolus injection, which consistently led to excitation of LN. The discrepancy is explained by the rapid desensitisation of certain P2X receptors. We also found that the concentration of ATP required to elicit a neural response was greater than that of α,β-meATP. This implies that ATPase is present in the tongue leading to a rapid degradation of ATP. Indeed, ATPase has been shown to be present in many tissues (Knowles *et al.* 1983; Connolly *et al.* 1998). The combination of receptor desensitisation and rapid degradation of ATP by ATPase will have major influences on the efficacy of ATP analogues when applied onto tissue blocks in, for example, isolated intact sensory ganglia (e.g. Stebbing *et al.* 1998) or applied topically to tissues in *in vivo* experiments.

P2X subtypes mediating transmembrane currents, or changes in membrane potential of single sensory neurones are designated on the basis of two criteria: whether they are sensitive to α,β-meATP and whether and how fast the response desensitises (Evans & Surprenant, 1996). In principle, an α,β-meATP-sensitive and rapidly desensitising response (within 100–300 ms) indicates the activation of $P2X_1$ or $P2X_3$ subtypes whereas an α,β-meATP-insensitive and slowly or non-desensitising response indicates involvement of $P2X_2$ or $P2X_4$ subtypes. In our experiments, both ATP and α,β-meATP produced rapid increase in LN activity when injected as a bolus into the lingual artery. The response decayed rapidly with the response lasting only a few seconds. This suggests that the receptors involved in activation of LN fibres desensitise rapidly and may be of $P2X_1$ or $P2X_3$ subtype. Given the fact that $P2X_3$ rather than $P2X_1$ receptors have been localised immunohistochemically on nerve terminals in the rat tongue, we consider the effects of the purinergic agonists on LN were most probably mediated by $P2X_3$ receptors. However, this does not exclude the possibility that other slowly desensitising P2X subtypes like $P2X_2$ or heteromultimeric $P2X_{2/3}$ (Lewis *et al.* 1995) were also involved.

In the present study, we have carried out whole-nerve recording of LN and CT. We took advantage of the fact that general sensory and taste fibres form distinct bundles, allowing us to compare the effects of purinergic agonists on these two distinct classes of sensory modalities by whole nerve recording. Our data demonstrate that P2X agonists preferentially activate LN rather than CT. However, this technique does not discriminate which functional types of afferent fibres in LN are sensitive to P2X agonists. LN consists of four physiological classes of afferent fibres: mechanoreceptors, thermoreceptors, nociceptors and proprioceptors. Presumably, not all these types of fibre are sensitive to purinergic agonists and it needs to be stressed that an increase in whole-nerve discharge rate may be due to increased discharge rate of all types of fibres, or due to an increase in discharge rate of one (or more) type of fibre and a smaller decrease in discharge rate of the other types of fibre. We attempted to dissect out purinergic actions on nociceptive fibres by using capsaicin. This is justified as capsaicin depolarises selectively small diameter sensory neurones (Heyman & Rang, 1985; Del Mar *et al.* 1996; Liu & Simon, 1996; Stebbing *et al.* 1998) and at large doses produces prolonged depolarisation and lowered excitability (desensitisation or tachyphylaxis). We noticed that the spikes evoked by purinergic agonists were of similar amplitudes to those evoked by capsaicin, whereas some spikes evoked by mechanical stimuli (von Frey hair) were of much greater amplitudes (presumably action potentials of larger diameter fibres). Further, it was found that P2X receptor-mediated changes in LN activity were diminished by pretreatment with a large dose of capsaicin (Fig. 6*B*) while the responses to mechanical stimuli (von Frey hair) were largely retained. These observations suggest that capsaicin-sensitive fibres were activated by purinergic agonists. The results therefore suggest that P2X receptors may play a role in nociception in the tongue, although single fibre recording is needed to confirm this.

Our data suggest that P2X receptors play an insignificant role in the transmission of taste information from taste buds to taste fibres in CT. In contrast to the consistent excitation of LN, the most frequent (3/7) response in CT following bolus injection of α,β-meATP and ATP (1 or 3 mM) was a relatively slow decrease in firing frequency. Only two of

seven CT showed a brief excitation (similar to the responses in LN) after bolus injection of purinergic agonists. This brief excitatory response in CT was likely to be due to the excitation of general sensory fibres that are intermixed with taste fibres; as both mechanical stimuli and capsaicin (applied intra-arterially) also led to rapid increase in CT firing frequency. As yet there is no simple explanation for the slow inhibition of CT activity by P2X agonists. A direct inhibitory action of purinergic agonists on taste fibres could not be excluded, but we favour an alternative explanation. In the fungiform papillae, LN fibre terminals are in close proximity to both taste cells and CT fibres and there is evidence that LN fibres may exert considerable modulatory actions on taste fibre terminals and taste cells. Most notably, CT fibre activity decreases during electrical stimulation of LN (Wang *et al.* 1995). Hence, in our experiments the robust excitation of LN fibres evoked by P2X agonists could lead indirectly to these inhibitory responses in CT.

P2X agonists applied intra-arterially consistently produced increased perfusion pressure through vasoconstriction. This effect is probably mediated by $P2X_1$ receptors, which have been shown immunohistochemically to be present on smooth muscles of the lingual vessels (Bo *et al.* 1999).

Potential applications of the *in vitro* tongue–nerve preparation

The tongue is involved in important sensory functions that are critical for the survival of animals including humans. Extensive studies of the sensory mechanisms in the tongue have been carried out in *in vivo* animal preparations. However, in *in vivo* experiments it is difficult to achieve precise control over the intra-oral environment (such as temperature, circulation, etc.) which presumably affect the properties of sensory receptors and sensory nerve terminals. It also requires considerable skill to deliver quantitatively and accurately mechanical, chemical or thermal stimulation over the tongue because of its location. We describe here a novel *in vitro* intra-arterially perfused tongue–nerve model that seems to overcome many of these problems. This model does not involve complex surgical procedures and avoids major technical pitfalls although intravascular perfusion must be started as soon as possible after the animal is killed. The preparation appears to survive well for a reasonable length of time, providing adequate perfusion is maintained. In our experiments, LN responses to purinergic agonists and capsaicin to mechanical stimulation of the tongue and CT responses to chemical stimulation could be recorded for as long as 7–9 h. Although some deterioration of the neural responses did occur after several hours, the model seems suitable for many different experimental protocols. Given the obvious advantage of easy manipulation of the extracellular environment, this model seems particularly suitable for investigating neurotransmitters and modulators involved in transmission of signals from taste cells to taste fibres, and for studying endogenous and exogenous chemical modulators of nociceptors. The preparation may also prove useful in identifying selective antagonists for native $P2X_1$ and $P2X_3$ receptors since they seem to be expressed differentially on lingual vascular smooth muscles and lingual nerve terminals (Bo *et al.* 1999).

In conclusion, using a novel *in vitro* tongue–nerve preparation, we have found that ATP and α,β-meATP preferentially activate general sensory afferent fibres but not taste fibres, which indicates that ATP and $P2X_3$ receptors may play a role in nociception.

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