British Journal of Pharmacology (2002) 135, 2047–2055

www.nature.com/bjp

Involvement of purinergic signalling in central mechanisms of body temperature regulation in rats

*,[1]Alexander V. Gourine, [2]Ekaterina V. Melenchuk, [2]Dmitry M. Poputnikov, [2]Valery N. Gourine & [1]K. Michael Spyer

[1]Department of Physiology, Royal Free and University College London Medical School, Rowland Hill Street, London NW3 2PF and [2]Institute of Physiology, National Academy of Sciences of Belarus, Minsk 220725, Belarus

1 P2 purinoreceptors are present in hypothalamic and brainstem nuclei that are involved in the regulation of body temperature (T_b). The role of ATP acting on these P2 receptors in thermoregulation was investigated by studying the effects of the stable ATP analogue α,β-methyleneATP (α,β-meATP) and P2 receptor antagonists suramin and pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) on T_b when injected intracerebroventricularly (i.c.v.) *via* a pre-implanted cannula in conscious rats at various ambient temperatures and during lipopolysaccharide (LPS)-induced fever.

2 Depending on ambient temperature, α,β-meATP (0.2 μmol, i.c.v.) induced a fall in T_b ($-3.3^{\circ}C$, $P<0.05$), no changes in T_b when compared to pre-injection levels, or an increase in T_b ($\sim 1.0^{\circ}C$, $P<0.05$) in rats maintained at 10°C, 25°C and 30°C ambient temperature, respectively.

3 Suramin (7 nmol, i.c.v.) induced a lasting (up to 6 h) increase in T_b (on average 1.2°C, $P<0.05$) in rats kept at 25°C or 30°C, but failed to induce any rise in T_b in rats at 10°C ambient temperature. An increase in T_b was also observed in rats (25°C ambient temperature) treated with PPADS (0.2 μmol, i.c.v.).

4 α,β-meATP (0.2 μmol) injected i.c.v. or directly into the anterior hypothalamus caused a profound fall in T_b (by 0.9°C and 1.0°C, respectively; $P<0.05$) during LPS (*E.coli*; 50 $\mu g\ kg^{-1}$)-induced fever in rats at 25°C ambient temperature. Fever was initiated more rapidly in rats treated with suramin (7 nmol) or PPADS (70 nmol), however its late phase was unaffected. Suramin (7 nmol) and PPADS (70 nmol) injected at the time when fever was already developed (2.5 h after LPS injections) did not alter febrile T_b.

5 These data indicate that purinergic signalling may play a significant role in central mechanisms of T_b regulation at various ambient temperatures and during fever.
British Journal of Pharmacology (2002) **135,** 2047–2055

Keywords: ATP; body temperature; fever; hypothalamus; α,β-methyleneATP; P2 receptors; purine; pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonic acid; suramin; thermoregulation

Abbreviations: α,β-meATP, α,β-methylene adenosine 5′-triphosphate; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; ATP, adenosine 5′-triphosphate; i.c.v., intracerebroventricular; IL-1β, interleukin-1β; i.p., intraperitoneal; LPS, lipopolysaccharide; NTS, nucleus tractus solitarius; PPADS, pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonic acid; s.e., standard error; T_b, body temperature

Introduction

There is growing evidence that extracellular adenosine 5′-triphosphate (ATP), known as an intracellular source of energy in metabolism, acting *via* P2X receptors, is a fast excitatory neurotransmitter in the central and peripheral nervous system (Edwards *et al*., 1992; Burnstock, 1999). P2X receptors are ATP-gated non-selective cation channels permeable to sodium, potassium and calcium ions (Ralevic & Burnstock, 1998). Seven subtypes of P2X receptors ($P2X_{1-7}$) have been cloned and characterized in terms of agonist/antagonist selectivity (Buell *et al*., 1996; Ralevic & Burnstock, 1998).

It has been shown recently that extracellular ATP, acting at P2X receptors, is involved in the brainstem mechanisms of cardiovascular and respiratory regulation (Phillis *et al*., 1997; Horiuchi *et al*., 1999; Ralevic *et al*., 1999; Ralevic, 2000; Spyer & Thomas, 2000). There is evidence that ATP, acting on P2X receptors, within the ventrolateral medulla may play a role in regulation of vasomotor tone and sympathetic activity (Horiuchi *et al*., 1999; Ralevic, 2000), and that P2X receptors in this area of the brainstem mediate hypercapnia-induced changes in respiration (Spyer & Thomas, 2000). There is evidence that purines may also play a role in cardiovascular and respiratory control through actions within the nucleus tractus solitarius (NTS) of the medulla oblongata (Phillis *et al*., 1997). The role for purinergic signalling in central cardiovascular and respiratory control is strongly supported by the results of immunohistochemical and *in situ* hybridization studies indicating that P2X receptors of different types are abundant in the brainstem (Kidd *et al*., 1995; Seguela *et al*., 1996; Vulchanova *et al*., 1996; Kanjhan *et al*., 1999; Yao *et al*., 2000).

It is well known that regulation of body heat exchange to the environment includes changes in peripheral blood flow,

*Author for correspondence; E-mail: a.gourine@rfc.ucl.ac.uk

blood redistribution and changes in respiratory rate. Although the role of extracellular ATP in brainstem mechanisms of cardiovascular and respiratory control has been demonstrated, the possibility that purinergic signalling may also be involved in central mechanisms of body temperature (T_b) regulation has not been addressed. This possibility is supported by the evidence that P2X receptors are also present in the hypothalamus (Shibuya *et al*., 1999; Xiang *et al*., 1998) – the primary area of the brain involved in regulation of body temperature and development of fever.

Recent evidence favours a role for extracellular ATP in the release of cytokines, such as interleukin (IL)-1β and tumour necrosis factor (Perregaux & Gabel, 1998; Hide *et al*., 2000; Mehta *et al*., 2001; Solle *et al*., 2001), essential for the development of fever (Kluger *et al*., 1995). Taking into account evidence that fever is initiated by IL-1β at the level of the anterior hypothalamus (Klir *et al*., 1994; Gourine *et al*., 1998), we suggest that changes in the level of extracellular ATP in this area of the brain play a part in the mechanisms underlying development of the febrile response.

In this pharmacological study, experiments have been designed to provide preliminary evidence on whether ATP acting on hypothalamic and (or) brainstem P2 receptors is involved in regulation of T_b at different ambient temperatures and during fever. The effects of the stable ATP analogue α,β-methyleneATP (α,β-meATP) and P2 receptor antagonists suramin and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) injected intracerebroventricularly (i.c.v.) on T_b in conscious rats at 10, 25 and 30°C ambient temperatures and during fever induced by bacterial lipopolysaccharide (LPS) were determined. α,β-meATP, suramin and PPADS that were injected into the third cerebral ventricle are believed to reach structures, containing different types of P2X receptors, in the hypothalamus that are concerned with thermoregulation as well as in the dorsal brainstem crucial for cardiovascular control (i.e. NTS). The effect of α,β-meATP microinjected directly into the anterior hypothalamus on T_b during fever in rats was also determined. Some of the results obtained have been reported previously in a preliminary abstract (Gourine *et al*., 2001).

Methods

Animals

Two hundred and sixty-two adult male Wistar rats weighing 280–320 g were used in this study. They were housed in a room maintained at a constant temperature of 25±1°C, a temperature slightly below the thermoneutral zone of rats, and in a 12 : 12 h light–dark cycle with light onset at 0600 h. Drinking water and laboratory rodent chow were provided *ad libitum*. All studies on conscious rats were conducted in facilities of the Institute of Physiology, National Academy of Sciences of Belarus and were approved by the Institutional Animal Care and Use Committee.

Surgery

Rats were anaesthetized with a mixture of ketamine hydrochloride (87.0 mg kg^{-1}) and xylazine hydrochloride (13.0 mg kg^{-1}) injected intramuscularly. A miniature temperature-sensitive telemetry transmitter (model E-mitter, Minimitter, Sunriver, OR, U.S.A.) was implanted into the abdominal cavity of each rat for continuous monitoring of T_b. Then a 26-gauge guide injection cannula (Plastic Products Co., Roanoke, VA, U.S.A.) was stereotaxically implanted into the third cerebral ventricle or anterior hypothalamus (stereotaxic co-ordinates: 1.8 mm caudal to bregma, 0.5 mm lateral to midline and 8.6 mm ventral from the surface of the skull) according to the atlas of Paxinos & Watson (1986). Two small screws were placed into the skull, and the cannula was secured in place by dental acrylic. The guide cannula was closed with a dummy cannula that extended from the tip of the guide cannula by $\leqslant$0.2 mm. After the surgery animals were housed one per cage and were allowed to recover for at least 7 days before any experiment. At the end of the experiment the animals were killed humanely by an overdose (200 mg kg^{-1}) of pentobarbitone sodium injected intraperitoneally (i.p.), the brain was removed and the location of the cannula was confirmed histologically.

T_b measurements

Deep T_b ($\pm$0.1°C) was monitored with implanted telemetry units (Minimitter). Recordings were made at 1-min intervals by use of a peripheral processor (VitalView System, Minimitter) connected to an IBM PC.

Intracerebroventricular, intrahypothalamic and intraperitoneal injections

Animals were conditioned to handling for 5 min once a day for 6 days prior to the experiment. Microinjections into the third cerebral ventricle or into the anterior hypothalamus were made over a period of 1–2 min using an internal injection cannula connected to PE-50 tubing attached to a 50 μl or 10 μl syringe, respectively (Hamilton, Reno, NV, U.S.A.). The injection cannula was removed 2–3 min after the injection. The volumes of injections were 1 μl for the hypothalamus and 5 μl for the third ventricle. In the control experiments drugs were also injected i.p. in a volume of 0.1 ml.

Fever

Purified LPS (*Escherichia coli* endotoxin 0111 : B4, Sigma Chemical, St. Louis, MO, U.S.A.) was dissolved in pyrogen-free saline and injected i.p. at a dose of 50 μg kg^{-1}. Control rats received an equivalent volume of sterile pyrogen-free saline.

Drugs

α,β-methylene-adenosine 5′-triphosphate (SIGMA Chemical), suramin sodium salt (SIGMA Chemical) and PPADS (Tocris Cookson Ltd, Bristol, U.K.) were dissolved in artificial cerebrospinal fluid (aCSF) to the designed amount in 5 μl of aCSF. The aCSF used for injections consisted of (in mM): NaCl 145.0, KCl 3.3, $CaCl_2$ 1.3 and $MgCl_2$ 1.0 dissolved in sterile pyrogen-free water.

Experimental design

Experiment 1. Effect of i.c.v. injection of the ATP analogue α,β-meATP on T_b in rats maintained at 25°C α,β-meATP

(0.02 μmol and 0.2 μmol) was injected into the third cerebral ventricle of afebrile rats maintained at 25°C ambient temperature between 0900 and 1000 h. T_b was monitored for 3 h before and 9 h after the injections. Control animals were injected i.c.v. with aCSF (5 μl). To make sure that the effect of α,β-meATP on T_b was not due to its leakage into the circulation and action on the periphery, in control studies we investigated whether α,β-meATP affects thermoregulation when injected i.p. at the same amount (0.2 μmol) that was shown to influence T_b after i.c.v. injection.

Experiment 2. Effect of i.c.v. injection of the P2 receptor antagonists suramin and PPADS on T_b in rats maintained at 25°C Suramin (7 nmol), PPADS (7 nmol, 70 nmol and 0.2 μmol) or aCSF (5 μl) were injected into the third cerebral ventricle of afebrile rats kept at 25°C ambient temperature between 0900 and 1000 h. T_b was monitored for 3 h before and 9 h after the injections. To confirm that the effect of suramin on T_b is not due to its leakage into the circulation and action on the periphery, we investigated whether this P2 receptor antagonist induces hyperthermia when injected i.p. at the same amount (7 nmol) that was shown to influence T_b after i.c.v. injection.

Experiment 3. Effect of i.c.v. injection of the ATP analogue α,β-meATP or of the P2 receptor antagonist suramin on T_b in rats at low and high ambient temperatures Rats in their home cages (one rat per cage) were placed in a room at a constant temperature of either 10±1°C or 30±1°C at 0900 h. α,β-meATP (0.2 μmol), suramin (7 nmol), a mixture of α,β-meATP (0.2 μmol) with suramin (7 nmol), or aCSF (5 μl) were injected into the third cerebral ventricle of these rats between 0900 and 1000 h on the following day, i.e. after 24 h of exposure to the ambient temperature of 10°C or 30°C. T_b was monitored for 1.5 h before and 3 h after the injections.

Experiment 4. Effect of i.c.v. or intrahypothalamic injection of the ATP analogue α,β-meATP on T_b during LPS-induced fever in rats The results of the experiments 1 and 3 showed that the duration of α,β-meATP-induced changes in T_b is less than 2 h. Because in rats LPS induces delayed fever, α,β-meATP was injected at the time when a febrile response had developed but not prior to LPS treatment. α,β-meATP (0.2 μmol), a mixture of αβ-meATP (0.2 μmol) with suramin (7 nmol) or aCSF (5 μl) were injected into the third cerebral ventricle 2.5 h after i.p. injection of LPS. α,β-meATP (0.2 μmol) or aCSF (1 μl) were also injected into the anterior hypothalamus 2.5 h after injection of LPS. In the control experiment, to confirm that the effect of α,β-meATP on febrile T_b is not due to an action on the periphery, we investigated whether α,β-meATP affect fever when injected i.p. at the same amount (0.2 μmol) that was shown to influence febrile T_b after i.c.v. or intrahypothalamic injection. T_b was monitored for 3 h before and 9 h after LPS injections, which were performed between 0900 and 1000 h.

Experiment 5. Effect of i.c.v. injection of the P2 receptor antagonists suramin and PPADS on T_b during LPS-induced fever in rats Suramin (7 nmol), PPADS (70 nmol) or aCSF (5 μl) were injected into the third cerebral ventricle immediately before, or 2.5 h after, an i.p. injection of LPS. Suramin (7 nmol) was also injected i.p. and the effect of this treatment on fever was determined in order to confirm that suramin effect on the development of the febrile response is not due to its leakage into circulation and action on the periphery. T_b was monitored for 3 h before and 9 h after LPS injections, which were performed between 0900 and 1000 h.

Statistical analysis

Data are reported as mean±standard error (s.e.). Comparisons between one experimental and one control group were made using one-way analysis of variance (ANOVA). For pairwise comparisons among more than two groups, data were examined for statistical significance using ANOVA followed by the method of Fischer least significant difference. A value of $P<0.05$ was considered to be significant.

Results

Experiment 1

Effect of i.c.v. injection of the ATP analogue α,β-meATP on T_b in rats maintained at 25°C There was an initial rise in T_b when an i.c.v. injection of aCSF was given, representing a stress-induced rise in T_b, caused by the handling and injection procedure (Figure 1). This stress-induced rise in T_b was attenuated by α,β-meATP injected at a dose of 0.02 μmol and was completely blocked by this ATP analogue at a dose of 0.2 μmol (Figure 1). Deep T_b of rats injected i.c.v. with α,β-meATP (0.2 μmol) was significantly ($P<0.05$) lower than in rats injected with aCSF 5–45 min after the injections. It is important to note that while this stress-induced rise in T_b was blocked by α,β-meATP, the T_b of rats injected with this ATP analogue was not different from pre-injection values (Figure 1). Since the mechanisms responsible for the stress-induced rise in T_b are believed to be similar to those responsible for fever (Kluger *et al*., 1987), these data are consistent with the results obtained in experiments in febrile rats that showed α,β-meATP to inhibit fever (Experiment 4). Although, there were no statistically significant differences in T_b between rats treated i.c.v. with aCSF and α,β-meATP 45 min after the

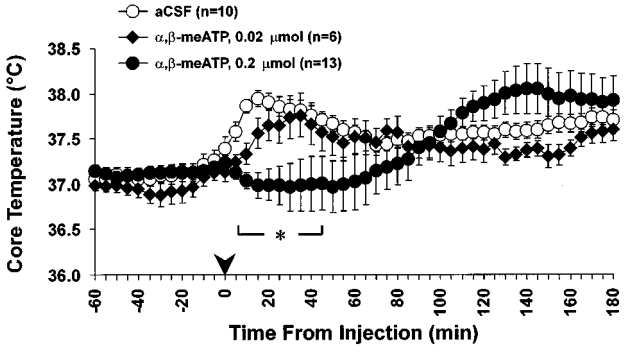

Figure 1 Effect of intracerebroventricular injection of the ATP analogue α,β-methyleneATP (α,β-meATP; 0.02 μmol and 0.2 μmol) or artificial cerebrospinal fluid (aCSF) on body temperature in rats at 25°C ambient temperature. Data are presented as mean±s.e. Numbers in parentheses indicate sample sizes. Arrowhead indicates time of injections. *Significant difference in body temperature between rats treated into the third cerebral ventricle with α,β-meATP (0.2 μmol) and rats treated with aCSF, $P<0.05$.

injection and thereafter, in five out of 13 rats the T_b notably increased 120–150 min after α,β-meATP administration (0.2 μmol). As shown in Figure 3, i.p. injection of α,β-meATP (0.2 μmol) had no effect on T_b.

Experiment 2

Effect of i.c.v. injection of the P2 receptor antagonists suramin and PPADS on T_b in rats maintained at 25°C Injection into the third cerebral ventricle of suramin (7 nmol) induced an immediate, long-lasting and profound rise in T_b (Figure 2A). T_b reached its maximal level of 39.17 ± 0.22°C 135 min after injection of suramin – a level of T_b some 1.6°C higher than T_b of rats injected i.c.v. with aCSF ($P<0.05$, Figure 2A). The T_b of rats treated with suramin was significantly ($P<0.05$) higher (on average by 1.2°C) than in rats treated with aCSF 60–360 min after the injection (Figure 2A). As shown in Figure 2B, i.c.v. injection of PPADS at doses of 7 nmol and 70 nmol had no significant effect on T_b. PPADS injected into the third cerebral ventricle at a dose of 0.2 μmol induced a short-lasting (1 h) but profound increase in T_b. Thirty min after injection of PPADS (0.2 μmol) the T_b peaked at 38.97 ± 0.29°C, which was 1.4°C higher than in rats treated with aCSF ($P<0.05$, Figure 2B). Suramin (7 nmol) injected i.p. had no significant effect on T_b in rats maintained at 25°C (Figure 3).

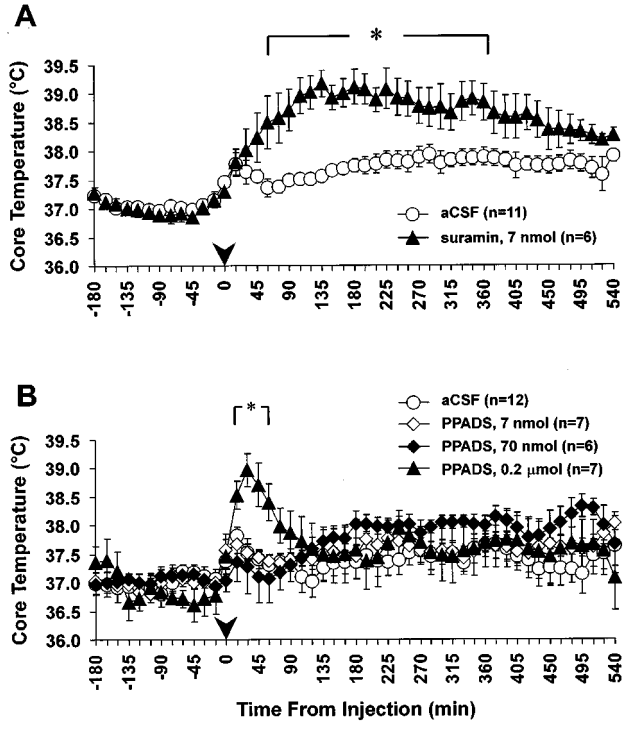

Figure 2 Effect of intracerebroventricular injection of the P2 receptor antagonists suramin (7 nmol (A)) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 7 nmol, 70 nmol and 0.2 μmol (B)) or artificial cerebrospinal fluid (aCSF) on body temperature in rats at 25°C ambient temperature. Data are presented as mean±s.e. Numbers in parentheses indicate sample sizes. Arrowhead indicates time of injections. *Significant difference in body temperature between rats treated into the third cerebral ventricle with suramin (A) or PPADS (B) and rats treated with aCSF, $P<0.05$.

Experiment 3

Effect of i.c.v. injection of the ATP analogue α,β-meATP or of the P2 receptor antagonist suramin on T_b in rats at low and high ambient temperatures These studies indicate that the effects of ATP analogue (α,β-meATP) and P2 receptor antagonist (suramin) on T_b depend on ambient temperature (Figure 4). As described above, at 25°C ambient temperature α,β-meATP (0.2 μmol) blocks the stress-induced rise in T_b (i.e. that elicited by the injection procedure), without inducing any significant changes in T_b relative to the pre-injection values (Figures 1 and 4B). At 10°C ambient temperature rats treated i.c.v. with α,β-meATP (0.2 μmol) developed a marked and lasting (90 min) decrease in T_b (Figure 4A). At this ambient temperature the T_b of rats treated i.c.v. with α,β-meATP was 36.33 ± 0.30°C ($P<0.05$), 34.69 ± 0.52°C ($P<0.05$) and 35.90 ± 0.69°C ($P<0.05$) 30, 60 and 90 min after the injections, respectively. This effect of α,β-meATP on T_b was completely blocked by suramin (Figure 4A). At 10°C ambient temperature the T_b of rats injected with both α,β-meATP (0.2 μmol) and suramin (7 nmol) was similar to that observed in rats treated with aCSF. At 30°C ambient temperature, rats showed an increase in T_b after i.c.v. administration of α,β-meATP at a dose of 0.2 μmol (Figure 4C). The T_b of rats kept at 30°C reached a maximum of 38.86 ± 0.36°C 75 min after i.c.v. injection of α,β-meATP, some 1.1°C higher than T_b of rats injected i.c.v. with aCSF (37.77 ± 0.22°C, $P<0.05$; Figure 4C).

As shown already, at a 25°C ambient temperature suramin (7 nmol) induced a long-lasting increase in T_b after injection into the third cerebral ventricle (Figures 2 and 4E). At 30°C ambient temperature rats treated i.c.v. with suramin (7 nmol) also developed hyperthermia (Figure 4F), which was similar to that observed in rats injected with this P2 receptor antagonist at 25°C. However, i.c.v. administration of suramin (7 nmol) did not induce any significant T_b rise in rats maintained at 10°C ambient temperature (Figure 4D). At this ambient temperature suramin blocked completely the stress-induced rise in T_b (caused by injection procedure), without inducing a significant T_b change compared to the pre-injection values (Figure 4D). At 10°C ambient temperature, the T_b of rats treated i.c.v. with suramin was significantly

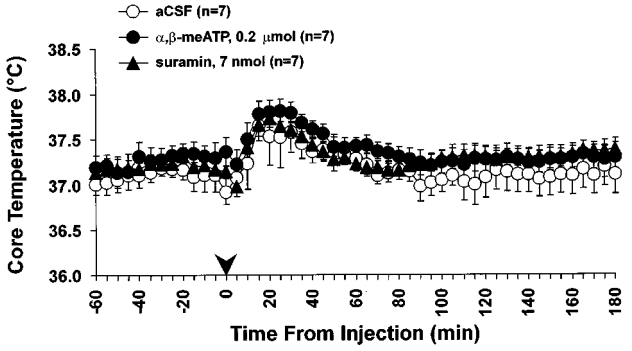

Figure 3 Effect of intraperitoneal injection of the ATP analogue α,β-methyleneATP (α,β-meATP; 0.2 μmol), P2 receptor antagonist suramin (7 nmol) or artificial cerebrospinal fluid (aCSF) on body temperature in rats at 25°C ambient temperature. Data are presented as mean±s.e. Numbers in parentheses indicate sample sizes. Arrowhead indicates time of injections.

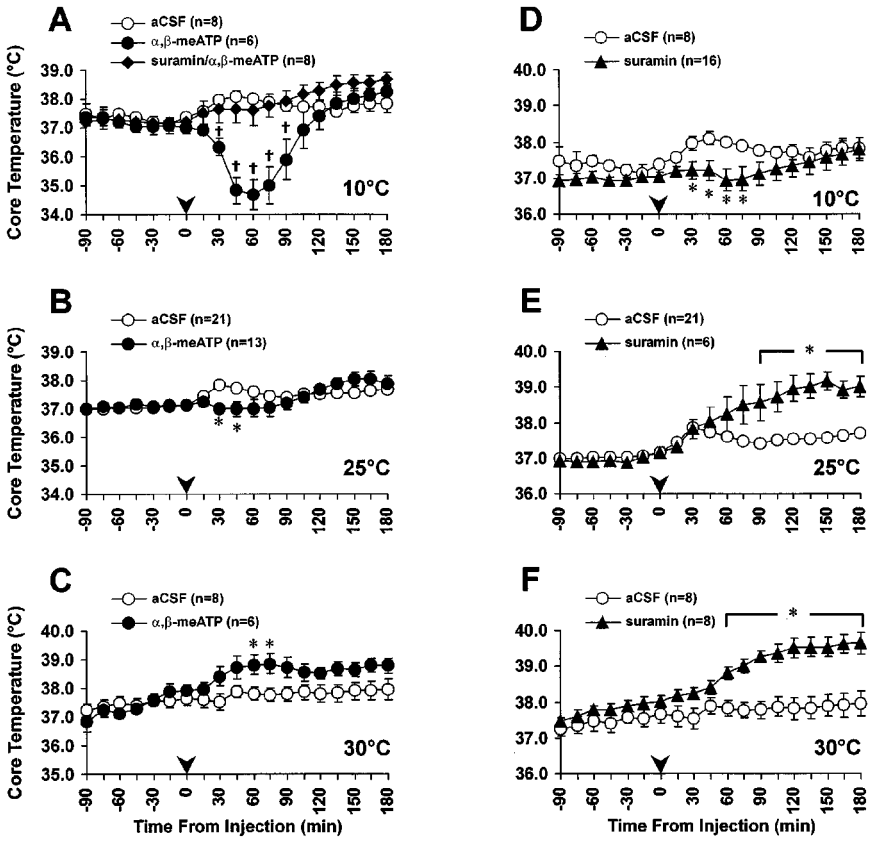

Figure 4 Effect of intracerebroventricular injection of the ATP analogue α,β-methyleneATP (α,β-meATP; 0.2 μmol (A, B, C)), P2 receptor antagonist suramin (7 nmol (D, E, F)), mixture of α,β-meATP (0.2 μmol) with suramin (7 nmol (A)) or artificial cerebrospinal fluid (aCSF) on body temperature in rats at 10°C (A, D), 25°C (B, E) and 30°C (C, F) ambient temperatures. Data are presented as mean ± s.e. Numbers in parentheses indicate sample sizes. Arrowhead indicates time of injections. *Significant difference in body temperature between rats treated into the third cerebral ventricle with α,β-meATP (B, C) or suramin (D, E, F) and rats treated with aCSF, $P<0.05$. †Significant difference in body temperature between rats treated into the third cerebral ventricle with α,β-meATP and rats treated with aCSF or rats treated with mixture of α,β-meATP with suramin, $P<0.05$.

($P<0.05$) lower (on average by 0.9°C) than in rats treated with aCSF 30–75 min after the injection (Figure 4D).

Experiment 4

Effect of i.c.v. or intrahypothalamic injection of the ATP analogue α,β-meATP on T_b during LPS-induced fever in rats Following intraperitoneal injection of LPS a fever reached a maximal T_b (around 39°C) 2.5 h after injection (Figure 5). α,β-meATP (0.2 μmol) injected i.c.v. or into the anterior hypothalamus at the peak of the LPS-induced fever caused profound decrease in febrile T_b (Figure 5A, B). Ten min after i.c.v. injection of α,β-meATP T_b of febrile rats decreased to 37.84 ± 0.19°C, some 0.9°C lower that the T_b of febrile rats 10 min after i.c.v. treatment with aCSF (38.74 ± 0.19°C; $P<0.05$). There were no differences in T_b between febrile rats treated with either α,β-meATP or aCSF 25 min or later after the injections (Figure 5A). The decrease in T_b of febrile rats induced by intrahypothalamic injection of α,β-meATP developed slower and lasted longer (Figure 5B) compared to the response evoked by this ATP analogue injected into the third cerebral ventricle. Forty min after injection of α,β-meATP into the anterior hypothalamus T_b of febrile rats decreased to 37.43 ± 0.38°C, some 1.0°C lower than the T_b of febrile rats 40 min after intrahypothalamic administration of aCSF (38.42 ± 0.10°C; $P<0.05$). As shown in Figure 5C, i.p. injection of α,β-meATP (0.2 μmol) at the peak of the LPS-induced fever had no significant effect on T_b.

The effect of α,β-meATP injected i.c.v. on T_b of febrile rats was attenuated significantly by the P2 receptor antagonist suramin (Figure 6). Ten min after i.c.v. injections, T_b of rats treated with α,β-meATP was 37.85 ± 0.16°C, whereas the T_b of rats treated with aCSF was 38.67 ± 0.15°C ($P<0.05$), and the T_b of rats injected with mixture of α,β-meATP and suramin was 38.42 ± 0.17°C ($P<0.05$).

Experiment 5

Effect of i.c.v. injection of the P2 receptor antagonists suramin and PPADS on T_b during LPS-induced fever in rats Treatment with suramin (7 nmol) or PPADS (70 nmol) resulted in an enhanced initiation of LPS-induced fever in rats (Figure 7A,B). The T_b of rats treated with LPS and aCSF (i.c.v.) were 37.69 ± 0.15°C, 37.32 ± 0.15°C and 37.29 ± 0.11°C, 20, 40 and 60 min after injection, respectively. At these time points, T_b of LPS-treated rats given suramin (i.c.v.) were 38.33 ± 0.13°C ($P<0.05$), 38.27 ± 0.14°C ($P<0.05$) and

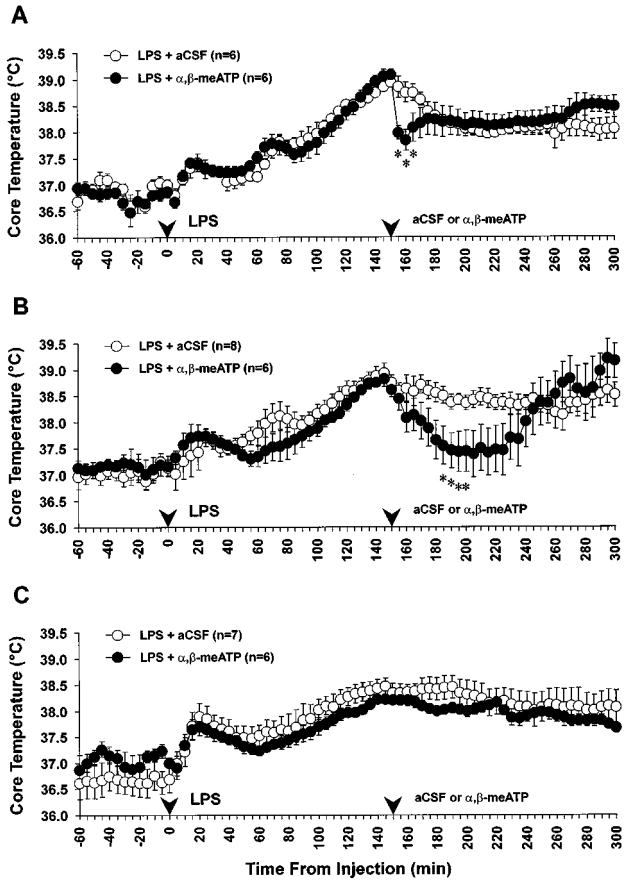

Figure 5 Effect of intracerebroventricular (A), intrahypothalamic (B) and intraperitoneal (C) injection of the ATP analogue α,β-methyleneATP (α,β-meATP; 0.2 μmol) or artificial cerebrospinal fluid (aCSF) on body temperature at the peak of fever induced by *E.coli* lipopolysaccharide (LPS, 50 $\mu g\,kg^{-1}$) in rats (25°C ambient temperature). Data are presented as mean$\pm$s.e. Numbers in parentheses indicate sample sizes. α,β-meATP or aCSF were injected 2.5 h after intraperitoneal injection of LPS. Arrowheads indicate time of injections. *Significant difference in body temperature between LPS-treated rats injected into the third cerebral ventricle (A) or into the anterior hypothalamus (B) with α,β-meATP and rats injected with aCSF, $P<0.05$.

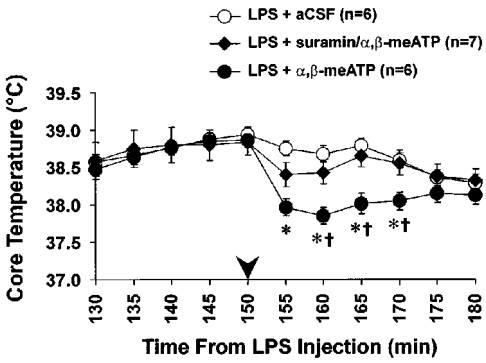

Figure 6 Effect of intracerebroventricular injection of the ATP analogue α,β-methyleneATP (α,β-meATP; 0.2 μmol), mixture of α,β-meATP (0.2 μmol) with P2 receptor antagonist suramin (7 nmol) or artificial cerebrospinal fluid (aCSF) on body temperature at the peak of fever induced by *E.coli* lipopolysaccharide (LPS, 50 $\mu g\,kg^{-1}$) in rats (25°C ambient temperature). Data are presented as mean$\pm$s.e. Numbers in parentheses indicate sample sizes. α,β-meATP, mixture of α,β-meATP with suramin or aCSF were injected 2.5 h after intraperitoneal injection of LPS (the time of LPS injection not shown). Arrowhead indicates time of injections. *Significant difference in body temperature between LPS-treated rats injected into the third cerebral ventricle with α,β-meATP and rats injected with aCSF, $P<0.05$. †Significant difference in body temperature between LPS-treated rats injected into the third cerebral ventricle with α,β-meATP and rats injected with mixture of α,β-meATP with suramin, $P<0.05$.

37.94 ± 0.16°C ($P<0.05$) 20, 40 and 60 min after injections, respectively (Figure 7A). Similarly, 40 min after LPS injection T_b of rats pretreated i.c.v. with PPADS increased to 38.48 ± 0.20°C, some 1.0°C higher than the T_b of LPS-injected rats 40 min after i.c.v. administration of aCSF (37.47 ± 0.14°C, $P<0.05$; Figure 7B). These differences in T_b between rats treated with suramin and aCSF and between rats treated with PPADS and aCSF disappeared 80–100 min and 55–65 min following injection of LPS, respectively, indicating that the late phase of fever was unaffected by these P2 receptor antagonists (Figure 7A,B). The changes in T_b in response to LPS followed at 2.5 h by i.c.v. administration of suramin are shown in Figure 7C. Suramin (Figure 7C) and also PPADS (data not shown) injected when LPS-induced fever was already developed (i.e. 2.5 h after LPS injections) did not induce significant changes in febrile T_b. Intraperitoneal injection of suramin (7 nmol) at the peak of the LPS-induced fever also had no significant effect on T_b (data not shown).

Discussion

In the present study, the role of extracellular ATP, acting on P2 receptors, in central mechanisms of thermoregulation was investigated by studying the effects of both a stable ATP analogue and two P2 receptor antagonists injected into the third cerebral ventricle on T_b in conscious rats at various ambient temperatures and during fever.

It was found that the effect of activating or antagonizing brain P2 receptors on T_b in rats was dependent on the ambient temperature, and also on whether or not the animals were febrile. Since deep T_b is similar at 10°, 25° and 30°C ambient temperatures in rats, the effects of the ATP analogue and P2 receptor antagonists, appears to depend on the level of afferent information received by the thermoregulatory centres of the hypothalamus. The magnitude of the changes of T_b evoked by activation and blockade of P2 receptors during fever (when T_b is elevated) was significantly different from that observed in afebrile rats at the same ambient temperature, suggesting that the effects of α,β-meATP and P2 receptor antagonists on T_b are also influenced by pyrogenic stimulation.

As a brief summary of all the results, it was shown that the stable ATP analogue α,β-meATP after injection into the third cerebral ventricle decreases T_b in rats only when the activity of mechanisms responsible for heat production is enhanced and the activity of mechanisms responsible for heat loss is suppressed (i.e. at low ambient temperature and during fever). On the other hand, P2 receptor antagonists induce an increase in T_b in conditions at or above thermoneutrality, when heat production is minimal and the mechanisms responsible for heat loss are active or facilitated. In addition, suramin and PPADS injected at the time when fever was already developed did not alter febrile T_b, suggesting that

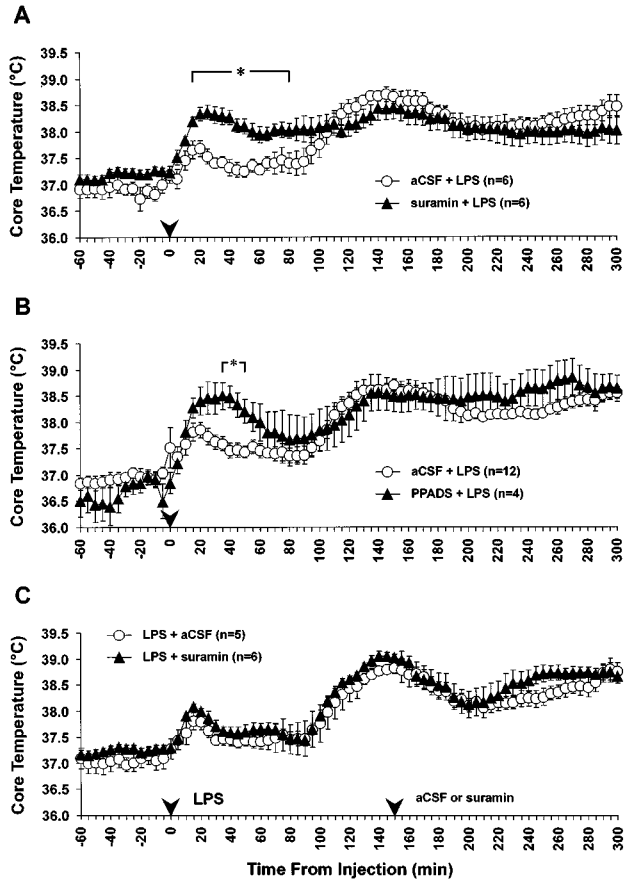

Figure 7 Effect of intracerebroventricular injection of the P2 receptor antagonists suramin (7 nmol (A)) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 70 nmol (B)) or artificial cerebrospinal fluid (aCSF) on body temperature during fever induced by *E.coli* lipopolysaccharide (LPS, 50 μg kg^{-1}) in rats (25°C ambient temperature). Suramin was injected immediately before (A) or 2.5 h after (C) intraperitoneal injection of LPS. PPADS was injected immediately before (B) intraperitoneal injection of LPS. Data are presented as mean ± s.e. Numbers in parentheses indicate sample sizes. Arrowhead indicates time of injections. *Significant difference in body temperature between LPS-treated rats injected into the third cerebral ventricle with suramin (A) or PPADS (B) and rats injected with aCSF, $P<0.05$.

development of the febrile response prevents the effects of P2 receptor antagonists on thermoregulation. Potentiation of the initial phase of fever when P2 receptor antagonists were given i.c.v. before LPS injection was rather minor and changes in T_b of rats treated with suramin or PPADS followed by LPS did not exceed changes observed in rats treated with either of these antagonists alone.

These data are consistent, if it is assumed that ATP, acting on P2 receptors, modulates transmission in a hypothalamic neuronal pathway relaying afferent information from skin warm-receptors to the neurones responsible for activation of heat loss. Analysis of these results allow us to suggest, that hypothalamic warm-sensitive neurones are the likely targets for the action of ATP revealed by the use of the ATP analogue and P2 receptor antagonists in our experiments. About 30% of the anterior hypothalamic neurones are warm-sensitive, many receiving input from afferent pathways relaying information from skin thermoreceptors (Boulant & Hardy, 1974). Hypothalamic warm-sensitive neurones also provide descending control of thermoregulatory effectors and are believed to be the site of action of pyrogens, which cause fever by reducing their discharge (for recent reviews, see Boulant, 1999; 2000). The above hypothesis is supported strongly by the results showing that during fever α,β-meATP induces significant decrease in febrile T_b when injected not only into the third ventricle but also directly into the anterior hypothalamus. Although, most of the data from this study fit well within this hypothesis, it is clear that further studies are needed to determine whether hypothalamic warm-sensitive neurones are indeed the site of action of ATP analogue and P2 receptor antagonists on T_b regulation.

When α,β-meATP and suramin were injected i.p. at the same amounts that were shown to influence T_b after i.c.v. injection, they failed to induce any changes in T_b in either afebrile or febrile rats. These data suggest that the effects of the ATP analogue and P2 receptor antagonist on T_b are evoked within the central nervous system and not on the periphery (due to a possible leakage into the circulation). There is evidence that $P2X_2$ receptors are dense in the hypothalamus (Xiang *et al*., 1998; Kanjhan *et al*., 1999). Using *in situ* hybridization and RT–PCR analysis $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$ and $P2X_7$ subunits, but not $P2X_1$ subunits, have been also shown to be expressed in neurones of the hypothalamic supraoptic and paraventricular nuclei (Shibuya *et al*., 1999). These data indicate that the effects of α,β-meATP and P2 receptor antagonists on T_b may be a result of their direct action on the hypothalamic neurones.

However, at present we can only speculate about which P2 receptors are affected by α,β-meATP, suramin and PPADS and responsible for changes in T_b when drugs are administered into the third cerebral ventricle. α,β-meATP was used in this study because, in contrast to the endogenous ligand ATP, it is relatively stable to hydrolysis by ecto-ATPases (Welford *et al*., 1986). This stability of the agonist was important for our study, since changes in T_b develop relatively slow. The effects we observed with α,β-meATP, suramin and PPADS may suggest the involvement of $P2X_1$ and/or $P2X_3$ receptors (for recent review see North & Surprenant, 2000). On the other hand, these receptors are known to be desensitized rapidly by α,β-meATP (for reviews, see Ralevic & Burnstock, 1998; North & Surprenant, 2000). However, in the majority of our experiments the effects of α,β-meATP on T_b were opposite to the effects of suramin (and PPADS) and at low ambient temperature and during fever were blocked by suramin, suggesting that these effects are due to α,β-meATP-induced activation of certain P2 receptors, rather than desensitisation. On the other hand, in rats maintained at 30°C α,β-meATP induced changes in T_b which were similar in direction (i.e. an increase) to that observed after injection of suramin, suggesting that this effect of ATP analogue may be a result of desensitization of certain P2 receptors, rather then their activation. Also, it was found that in five out of 13 rats maintained at 25°C the T_b notably increased 120–150 min after α,β-meATP (0.2 μmol) administration (although the mean difference was not statistically significant). It is possible that this late response is mediated by P2 receptors with different pharmacology than those mediating the initial response (perhaps by the receptors from the P2Y family) or by the P2 receptors located in distant brain regions (e.g. caudally), or both. Interestingly, it was

also found that the time course of the hyperthermia induced by suramin in afebrile rats was markedly different from that induced by PPADS (Figure 2). This could be due to several factors such as the rate of antagonist diffusion to the target structure, stability of the antagonist, profile of P2 receptors affected, or degree of suppression of the nucleotidase activity induced by these compounds (Hourani & Chown, 1989; Ziganshin *et al*., 1995). It is clear that the development of new highly selective P2X receptor agonists and antagonists is needed to investigate further the effects of purines on T_b.

We suggested initially that extracellular ATP acts to induce the release of cytokines to play an important role in the mechanisms for the development of fever. This was based on the evidence that fever is initiated by IL-1β (and probably other cytokines) at the level of the anterior hypothalamus (Klir *et al*., 1994; Gourine *et al*., 1998) and that ATP is a potent inducer of IL-1α, IL-1β and tumour necrosis factor release (Perregaux & Gabel, 1998; Hide *et al*., 2000; Mehta *et al*., 2001; Solle *et al*., 2001). The effect of ATP on cytokine release is thought to be mediated *via* $P2X_7$ receptors (Hide *et al*., 2000; Mehta *et al*., 2001; Solle *et al*., 2001) which are present in the hypothalamus (Shibuya *et al*., 1999). $P2X_7$ receptors are sensitive to the antagonists suramin and PPADS (North & Surprenant, 2000). If our hypothesis is correct, and ATP, *via* cytokine release, plays a role in fever development, an attenuation of fever by suramin and PPADS would have been expected. However, fever was virtually unaffected, or even exaggerated, following administration of these P2 receptor antagonists into the brain, suggesting that at the hypothalamic level either ATP is not involved in the mechanisms of cytokine release, or alternatively that ATP-induced cytokine production is not essential for the development of fever following LPS challenge.

It is possible that $P2X_7$ receptors in the peripheral tissues may play an important role in mediating LPS-induced responses, including cytokine production and fever. It has been found recently, that $P2X_7$ receptor contains a conserved LPS-binding domain (Denlinger *et al*., 2001), suggesting that LPS may have a direct effect on $P2X_7$ function and therefore on ATP-induced responses mediated by this receptor, including cytokine release. Study of the potential role of peripheral P2 receptors in fever was not a subject of the current study. However, exciting new data on the role of purinergic signalling in the febrile response could be obtained in experiments, investigating the development of fever in conditions when specific $P2X_7$ receptor agonists or antagonists are applied peripherally in amounts sufficient to affect function of these receptors.

In conclusion, this report describes the involvement of purinergic signalling in central mechanisms of thermoregulation. Since regulation of T_b is severely disrupted under anaesthesia, this initial pharmacological study was conducted in conscious rats investigating the effects of a stable ATP analogue and P2 receptor antagonists injected into the third cerebral ventricle on T_b at various ambient temperatures and during fever. Analysis of the differences in T_b responses observed following administration of α,β-meATP, suramin and PPADS into the third cerebral ventricle at different ambient temperatures and during fever, allow us to suggest that extracellular ATP, acting on P2 receptors is involved in central mechanisms of T_b regulation, perhaps by modulating transmission in the hypothalamic neural pathway from peripheral warm-receptors to the neurones responsible for the stimulation of heat loss. We speculate, that hypothalamic warm-sensitive neurones are the likely site of action of ATP analogue and P2 receptor antagonists in relation to regulation of T_b.

For further understanding of the role of purinergic signalling in the central mechanisms of thermoregulation, the effects of P2 receptor agonists and antagonists applied into the cerebral ventricles on the peripheral mechanisms of heat loss (e.g. peripheral (tail) blood flow) and heat production (e.g. thermogenesis in the brown fat) could be investigated in anaesthetized animals. The hypothesis that population of hypothalamic warm-sensitive neurones is the site of action of ATP analogue and P2 receptor antagonists on T_b, can be tested effectively only in a more reduced preparation, such as hypothalamic slice, where P2X receptor profile as well as the effects of ATP, selective P2X receptor agonists and antagonists on activity and thermosensitivity of the hypothalamic neurones could be determined.

We thank Drs Brian King and Andrew J. Lawrence for critical reviews of the manuscript.

References

BOULANT, J.A. (1999). Cellular mechanisms of neuronal thermosensitivity. *J. Therm. Biol.,* **24,** 333–338.

BOULANT, J.A. (2000). Role of the preoptic-anterior hypothalamus in thermoregulation and fever. *Clin. Infect. Dis.,* **31,** (Suppl 5): S157–S161.

BOULANT, J.A. & HARDY, J.D. (1974). The effect of spinal and skin temperatures on the firing rate and thermosensitivity of preoptic neurones. *J. Physiol.,* **240,** 639–660.

BUELL, G., COLLO, G. & RASSENDREN, F. (1996). P2X receptors: an emerging channel family. *Eur. J. Neurosci.,* **8,** 2221–2228.

BURNSTOCK, G. (1999). Current status of purinergic signalling in the nervous system. *Prog. Brain Res.,* **120,** 3–10.

DENLINGER, L.C., FISETTE, P.L., SOMMER, J.A., WATTERS, J.J., PRABHU, U., DUBYAK, G.R., PROCTOR, R.A. & BERTICS, P.J. (2001). Cutting edge: the nucleotide receptor $P2X_7$ contains multiple protein- and lipid-interaction motifs including a potential binding site for bacterial lipopolysaccharide. *J. Immunol.,* **167,** 1871–1876.

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

GOURINE, A.V., RUDOLPH, K., TESFAIGZI, J. & KLUGER, M.J. (1998). Role of hypothalamic interleukin-1β in fever induced by cecal ligation and puncture in rats. *Am. J. Physiol.,* **275,** R754–R761.

GOURINE, V.N., MELENCHUK, E.V., POPUTNIKOV, D.M., GOURINE, A.V. & SPYER, K.M. (2001). Evidence for involvement of ATP in regulation of body temperature during fever in rats. *J. Physiol.,* **533,** 91P.

HIDE, I., TANAKA, M., INOUE, A., NAKAJIMA, K., KOHSAKA, S., INOUE, K. & NAKATA, Y. (2000). Extracellular ATP triggers tumor necrosis factor-α release from rat microglia. *J. Neurochem.,* **75,** 965–972.

HORIUCHI, J., POTTS, P.D., TAGAWA, T. & DAMPNEY, R.A. (1999). Effects of activation and blockade of P2X receptors in the ventrolateral medulla on arterial pressure and sympathetic activity. *J. Auton. Nerv. Syst.,* **76,** 118–126.

HOURANI, S.M. & CHOWN, J.A. (1989). The effects of some possible inhibitors of ectonucleotidases on the breakdown and pharmacological effects of ATP in the guinea-pig urinary bladder. *Gen. Pharmacol.,* **20,** 413–416.

KANJHAN, R., HOUSLEY, G.D., BURTON, L.D., CHRISTIE, D.L., KIPPENBERGER, A., THORNE, P.R., LUO, L. & RYAN, A.F. (1999). Distribution of the $P2X_2$ receptor subunit of the ATP-gated ion channels in the rat central nervous system. *J. Comp. Neurol.,* **407,** 11–32.

KIDD, E.J., GRAHAMES, C.B., SIMON, J., MICHEL, A.D., BARNARD, E.A. & HUMPHREY, P.P. (1995). Localization of P2X purinoceptor transcripts in the rat nervous system. *Mol. Pharmacol.,* **48,** 569–573.

KLIR, J.J., MCCLELLAN, J.L. & KLUGER, M.J. (1994). Interleukin-1β causes the increase in anterior hypothalamic interleukin-6 during LPS-induced fever in rats. *Am. J. Phsiol.,* **266,** R1845–1848.

KLUGER, M.J., KOZAK, W., LEON, L.R., SOSZYNSKI, D. & CONN, C.A. (1995). Cytokines and fever. *Neuroimmunomodulation,* **2,** 216–223.

KLUGER, M.J., O'REILLY, B., SHOPE, T.R. & VANDER, A.J. (1987). Further evidence that stress hyperthermia is a fever. *Physiol. Behav.,* **39,** 763–766.

MEHTA, V.B., HART, J. & WEWERS, M.D. (2001). ATP-stimulated release of interleukin (IL)-1β and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. *J. Biol. Chem.,* **276,** 3820–3826.

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

PAXINOS, G. & WATSON, C. (1986). *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press.

PERREGAUX, D.G. & GABEL, C.A. (1998). Post-translational processing of murine IL-1: evidence that ATP-induced release of IL-1α and IL-1β occurs via a similar mechanism. *J. Immunol.,* **160,** 2469–2477.

PHILLIS, J.W., SCISLO, T.J. & O'LEARY, D.S. (1997). Purines and the nucleus tractus solitarius: effects on cardiovascular and respiratory function. *Clin. Exp. Pharmacol. Physiol.,* **24,** 738–742.

RALEVIC, V. (2000). P2 receptors in the central and peripheral nervous systems modulating sympathetic vasomotor tone. *J. Auton. Nerv. Syst.,* **81,** 205–211.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

RALEVIC, V., THOMAS, T., BURNSTOCK, G. & SPYER, K.M. (1999). Characterization of P2 receptors modulating neural activity in rat rostral ventrolateral medulla. *Neuroscience,* **94,** 867–878.

SEGUELA, P., HAGHIGHI, A., SOGHOMONIAN, J.J. & COOPER, E. (1996). A novel neuronal P2X ATP receptor ion channel with widespread distribution in the brain. *J. Neurosci.,* **16,** 448–455.

SHIBUYA, I., TANAKA, K., HATTORI, Y., UEZONO, Y., HARAYAMA, N., NOGUCHI, J., UETA, Y., IZUMI, F. & YAMASHITA, H. (1999). Evidence that multiple P2X purinoceptors are functionally expressed in rat supraoptic neurones. *J. Physiol.,* **514,** 351–367.

SOLLE, M., LABASI, J., PERREGAUX, D.G., STAM, E., PETRUSHOVA, N., KOLLER, B.H., GRIFFITHS, R.J. & GABEL, C.A. (2001). Altered cytokine production in mice lacking $P2X_7$ receptors. *J. Biol. Chem.,* **276,** 125–132.

SPYER, K.M. & THOMAS, T. (2000). Sensing arterial CO_2 levels: a role for medullary P2X receptors. *J. Auton. Nerv. Syst.,* **81,** 228–235.

VULCHANOVA, L., ARVIDSSON, U., RIEDL, M., WANG, J., BUELL, G., SURPRENANT, A., NORTH, R.A. & ELDE, R. (1996). Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 8063–8067.

WELFORD, L.A., CUSACK, N.J. & HOURANI, S.M. (1986). ATP analogues and the guinea-pig taenia coli: a comparison of the structure-activity relationships of ectonucleotidases with those of the P2-purinoreceptor. *Eur. J. Pharmacol.,* **129,** 217–224.

XIANG, Z., BO, X., OGLESBY, I., FORD, A. & BURNSTOCK, G. (1998). Localization of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamus. *Brain Res.,* **813,** 390–397.

YAO, S.T., BARDEN, J.A., FINKELSTEIN, D.I., BENNETT, M.R. & LAWRENCE, A.J. (2000). Comparative study on the distribution patterns of $P2X_1$-$P2X_6$ receptor immunoreactivity in the brainstem of the rat and the common marmoset (*Callithrix jacchus*): association with catecholamine cell groups. *J. Comp. Neurol.,* **427,** 485–507.

ZIGANSHIN, A.U., ZIGANSHINA, L.E., BODIN, P., BAILEY, D. & BURNSTOCK, G. (1995). Effects of P2-purinoceptor antagonists on ecto-nucleotidase activity of guinea-pig vas deferens cultured smooth muscle cells. *Biochem. Mol. Biol. Int.,* **36,** 863–869.

(Received October 18, 2001
Revised December 21, 2001
Accepted February 14, 2002)