ATP stimulation of Ca^{2+}-dependent plasminogen release from cultured microglia

[1,4]Kazuhide Inoue, [2]Kazuyuki Nakajima, [2]Takako Morimoto, [2]Yoshiaki Kikuchi, [1]Schuichi Koizumi, [3]Peter Illes & [2]Shinichi Kohsaka

[1]Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158, [2]Department of Neurochemistry, National Institute of Neuroscience, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187, Japan and [3]Institute of Pharmacology and Toxicology, University of Leipzig, Härtelstraße 16/18, 04107 Leipzig, Germany

1 ATP (10–100 μM), but not glutamate (100 μM), stimulated the release of plasminogen from microglia in a concentration-dependent manner during a 10 min stimulation. However, neither ATP (100 μM) nor glutamate (100 μM) stimulated the release of NO. A one hour pretreatment with BAPTA-AM (200 μM), which is metabolized in the cytosol to BAPTA (an intracellular Ca^{2+} chelator), completely inhibited the plasminogen release evoked by ATP (100 μM). The Ca^{2+} ionophore A23187 induced plasminogen release in a concentration-dependent manner (0.3 μM to 10 μM).

2 ATP induced a transient increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner which was very similar to the ATP-evoked plasminogen release, whereas glutamate (100 μM) had no effect on $[Ca^{2+}]_i$ (70 out of 70 cells) in microglial cells. A second application of ATP (100 μM) stimulated an increase in $[Ca^{2+}]_i$ similar to that of the first application (21 out of 21 cells).

3 The ATP-evoked increase in $[Ca^{2+}]_i$ was totally dependent on extracellular Ca^{2+}, 2-Methylthio ATP was active (7 out of 7 cells), but α,β-methylene ATP was inactive (7 out of 7 cells) at inducing an increase in $[Ca^{2+}]_i$. Suramin (100 μM) was shown not to inhibit the ATP-evoked increase in $[Ca^{2+}]_i$ (20 out of 20 cells). 2'- and 3'-O-(4-Benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP), a selective agonist of $P2X_7$ receptors, evoked a long-lasting increase in $[Ca^{2+}]_i$ even at 1 μM, a concentration at which ATP did not evoke the increase. One hour pretreatment with adenosine 5'-triphosphate-2', 3'-dialdehyde (oxidized ATP, 100 μM), a selective antagonist of $P2X_7$ receptors, blocked the increase in $[Ca^{2+}]_i$ induced by ATP (10 and 100 μM).

4 These data suggest that ATP may transit information from neurones to microglia, resulting in an increase in $[Ca^{2+}]_i$ via the ionotropic $P2X_7$ receptor which stimulates the release of plasminogen from the microglia.

Keywords: Microglia; ATP receptors; internal Ca^{2+}; plasminogen release; NO release

Introduction

Recently, it has been suggested that microglia, a type of glial cell, play various important roles in the central nervous system (CNS) (Nakajima & Kohsaka, 1993; Verkhratsky & Kettenmann, 1996). The most characteristic feature of microglia is their rapid activation in response to pathological changes in the CNS (Kreutzberg, 1996). Activated microglia are mainly scavenger cells but also perform various other functions in tissue repair and neural regeneration. They can destroy invading micro-organisms, remove dangerous debris and induce tissue repair by secreting growth factors, thus facilitating the return to tissue homeostasis. They are able to release several cytotoxic substances *in vitro*, such as NO, as well as arachidonic acid derivatives, excitatory amino acids, quinolinic acid and cytokines (Colton & Gilbert, 1987; Banati *et al.*, 1993a, b). Free oxygen radicals released by microglia have a neurotoxic effect in co-cultures of neurones and microglia (Thery *et al.*, 1993). Activated microglia might also play a protective role. They produce the urokinase-type plasminogen activator and plasminogen, which promotes the development of mesencephalic dopaminergic neurones and enhances neurite outgrowth from explants of neocortical tissue (Nakajima *et al.*, 1992b, c). The activation and proliferation of microglia occurring after axotomy of the facial nerve are

accompanied by an increase in urokinase activity in the facial nucleus (Nakajima *et al.*, 1996).

These properties of microglia can be modulated by cytokines and neurotransmitters acting through receptors for CNS signalling molecules including adenosine 5'-triphosphate (ATP). It has been found that microglia express receptors for ATP, G-protein coupled-type P2 receptors such as P2Y and ionotropic P2 receptors such as P2Z (recently named $P2X_7$) (Neary *et al.*, 1996; Verkhratsky & Kettenmann, 1996). ATP induces the release of interleukin-1β (IL-1β) from mice brain microglia through P2Z (Ferrari *et al.*, 1996), evokes currents in rat brain (Walz *et al.*, 1993) or forebrain microglia (Nörenberg *et al.*, 1994; Langosch *et al.*, 1994) and stimulates an increase in intracellular Ca^{2+} in rat brain (Walz *et al.*, 1993). These data suggest that ATP mediates signals from neurones to microglia.

It has been shown that ATP is released from hippocampal slices by electrical stimulation of Schaffer collateral-comissural afferents (Wieraszko & Seyfried, 1989), and that ATP mediates synaptic transmission in rat cultured hippocampal neurones (Inoue *et al.*, 1992; 1995). We have previously found that exogenously applied plasminogen enhances synaptic transmission via NMDA-glutamate receptors in rat cultured hippocampal neurones (Inoue *et al.*, 1994). However, there have been few studies addressing the question of the mechanism of plasminogen release from microglia. Since microglia express P2-receptors but not any glutamate receptors (Verkhratsky & Kettenmann,

1996), it is possible that ATP stimulates the release of plasminogen from hippocampal microglia.

The aim of the present study was to examine the effect of ATP on the release of plasminogen from microglia and to speculate on the physiological significance of this release. We present evidence that ATP stimulates the release of plasminogen but not NO from cultured microglia following an increase in intracellular Ca^{2+} mediated via ionotropic $P2X_7$ receptors.

Methods

Cell culture

Microglia were obtained from primary cell cultures of cerebral hemispheres of neonatal rats as described previously (Nakajima *et al.*, 1989; 1992a). After 2 – 3 weeks in primary cell culture, microglia were prepared as floating cell suspensions by shaking growth flasks on an orbital shaker (80 r.p.m., 3 min). Aliquots of the cell suspension ($1.5 - 2.0 \times 10^5$ cells) were transferred to wells of a 12-well plate (Coster) and allowed to adhere at $37°C$. After 30 – 60 min, unattached cells were removed by rinsing 3 times with serum-free Dulbecco's modified Eagle's medium (DMEM). After the final rinse, 1 ml of the serum-free DMEM was added to each well, and the plate was kept in 10% CO_2 for 1 h before the measurement of plasminogen release. For the measurement of $[Ca^{2+}]_i$, the microglia were plated on poly-L-lysine-coated glass coverslips with a silicon rubber wall (Flexiperm, Heraeus Biotechnology, Hanau, Germany) at a density of 2×10^5 cells/well and maintained in 10% CO_2 for 2 – 3 h. Microglia prepared as above were estimated to be 99.8 – 100% pure based on their ED-1 immunoreactivity and the phagocytic activity of Latex-beads, as described previously (Nakajima *et al.*, 1989; 1992a).

Measurement of $[Ca^{2+}]_i$

The changes in $[Ca^{2+}]_i$ in single cells were measured by the fura-2 method as described by Grynkiewicz *et al.* (1985) with minor modifications (Koizumi *et al.*, 1994). The cells were washed with a balanced salt solution (BSS) of the following composition (mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.2, $MgCl_2$ 1.2, N - 2 - hydroxyethylpiperazine - N' - 2 - ethanesulphonic acid (HEPES) 25 and D-glucose 10, and incubated with 10 μM fura-2 acetoxymethylester (fura-2 AM) at $37°C$ in BSS. After 30 min incubation, the cells were washed with 0.2 ml of BSS. The coverslips were mounted on an inverted epifluorescence microscope (TMD-300, Nikon, Tokyo, Japan) equipped with a 75 W xenon-lamp and band-pass filters of 340 nm wavelength for measurement of the Ca^{2+}-dependent signal (F340), and 360 nm wavelength for measurement of the Ca^{2+}-independent signal (F360). Image data recorded by a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Hamamatsu, Japan), were processed by a Ca^{2+}-analysing system (Hamamatsu Photonics, Hamamatsu, Japan), and the ratio of emitted fluorescence (F340/F360) was calculated. Measurements were carried out at room temperature. Drugs were dissolved in BSS and applied by superfusion. For the Ca^{2+}-depleted experiments, a medium without Ca^{2+} and with 1 mM ethylenediaminetetraacetic acid (EGTA) was used (Ca^{2+}-free BSS).

Assay of plasminogen-secretion from microglia

ATP, glutamate or A23187 was added to the wells at a final concentration of 100 μM and the cultures were maintained in

10% CO_2. After 10 min, the conditioned medium was removed and centrifuged at 1500 r.p.m. for 10 min. The supernatant was concentrated with an Ultrafree C3-TKK (30 kDa cut, Millipore, Massachusetts, U.S.A.) and freeze-dried before determination of plasminogen by the Western blot method previously described (Nakajima *et al.*, 1992b). The freeze-dried conditioned medium was solubilized with 30 μl of non-reducing sodium dodecyl sulphate (SDS) sample solution (62.5 mM Tris HCl (pH 6.8), 2.3% SDS, 10% glycerol), and 20 μl was subjected to SDS-polyacrylamide gel electrophoresis (SDS – PAGE) on a 10 – 20% gradient gel (Daiichi Pure Chemicals, Tokyo, Japan) under non-reducing conditions. The proteins were transferred to Immobilon-P (Millipore, Massachusetts, U.S.A.) with a semi-dry type transblotting apparatus (ATTO, Tokyo, Japan). The Immobilon was incubated with a concentration of 2 μg ml^{-1} of anti- rat plasminogen antibody (IgG) in TNB solution (10 mM Tris HCl (pH 7.5), 150 mM NaCl, 3% bovine serum albumin (BSA)) for 1 h at room temperature. After washing with TN solution (10 mM Tris HCl (pH 7.5), 150 mM NaCl), the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (I : 200) for 1 h at room temperature. The antigen-antibody complex was detected by staining with 0.02% 3, 3'-
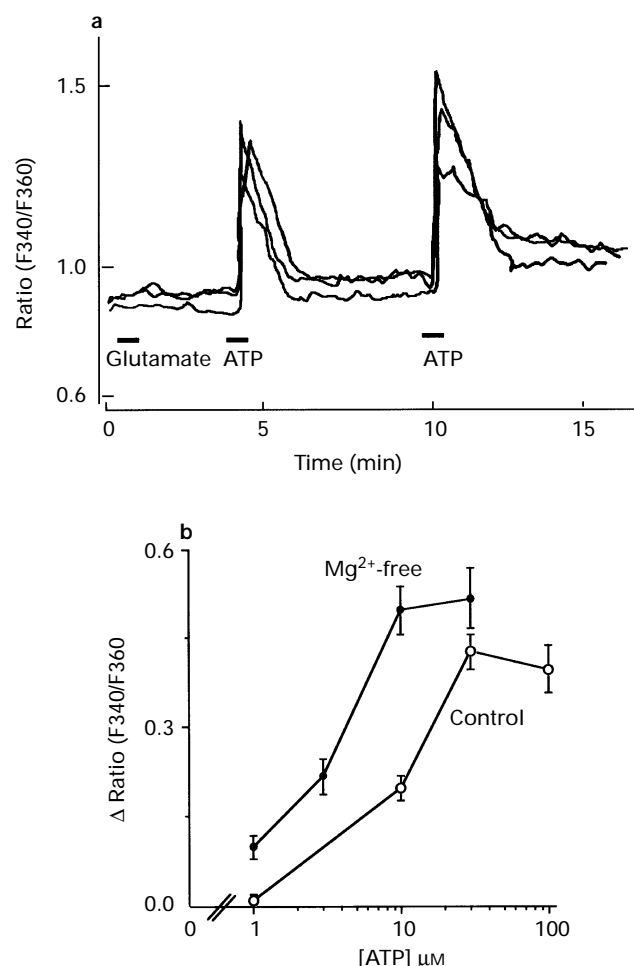

Figure 1 The effect of ATP and glutamate on $[Ca^{2+}]_i$ in microglia. (a) The effect of ATP and glutamate on $[Ca^{2+}]_i$ in individual microglial cells. Glutamate (100 μM) and ATP (100 μM) were applied by superfusion for 30 s as indicated by solid bars. The three cells shown are representative of 21 cells stimulated successively by drugs. (b) The concentration-dependence of the ATP-evoked increase in $[Ca^{2+}]_i$. Data are mean of the responses in 13 – 14 cells; vertical lines show s.e.mean. 'Mg^{2+}-free' means the responses were obtained in BSS lacking $MgCl_2$ (1.2 mM).

diaminobenzidine (DAB) and 0.01% H_2O_2. To quantify the amounts of plasminogen, blots were visualized with enhanced chemiluminescent (ECL, Amersham, Buckinghamshire, U.K.) and the intensity was measured by a densitometer. To examine the effect of BAPTA-AM, ATP was added 1 h after the addition of BAPTA-AM.

Measurement of nitric oxide in the conditioned medium

The amounts of nitric oxide (NO) in microglial conditioned medium were monitored by the amount of NO_2, a breakdown product of NO, by use of an exclusive NO analysing system (ENO-10, EICOM Corp, Kyoto, Japan). At various times during culture, 10 μl of culture medium were directly applied to a column (NO-PAK; 9.6 mm $\times$ 50 mm, EICOM Corp, Kyoto, Japan) that separates NO_2 and NO_3. The NO_3 was further reduced to NO_2 in a reducing column (NO-RED, NORTIP, EICOM Corp, Kyoto, Japan). The original- and reduced-NO_2 were detected individually with Griess reagent (Green *et al.*, 1981) and the amount of NO_2 was calculated by use of sodium nitrite as the standard.

Chemicals

Drugs used were: adenosine 5'-triphosphate disodium salt (ATP; Yamasa Co., Choshi, Japan) and suramin monosodium salt (Bayer, Leverkusen, Germany). Fura-2 acetoxymethylester (fura-2 AM), HEPES and EGTA were purchased from Dojin (Kumamoto, Japan), α,β-methylene-adenosine 5'-triphosphate (α,β-methyleneATP) and 2-methyl-thio-adenosine 5'-triphosphate (2-MeSATP) were from Research Biochemicals International (Natick, MA, U.S.A.). Gluta-mate, 2'-and 3'-*O*-(4-benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP), and adenosine 5'-triphosphate-2', 3'-dialdehyde (oxidized ATP) were from Sigma (St. Louis, MO, U.S.A.). Other chemicals were from Wako Pure Chemical (Osaka, Japan). Drugs were directly dissolved in BSS or Ca^{2+} free BSS.

Statistics

Statistical differences in values were determined by analysis of variance and Dunnett's test for multiple comparisons.

Results

ATP evoked an increase in $[Ca^{2+}]_i$ in microglia in a concentration-dependent manner

Figure 1a shows the effects of ATP and glutamate on $[Ca^{2+}]_i$ in individual microglial cells. ATP (100 μM) induced an increase in $[Ca^{2+}]_i$ (41 out of 41 cells), whereas glutamate (100 μM) did not evoke any increase in $[Ca^{2+}]_i$ (70 out 70 cells). This is consistent with the finding that microglia express ATP receptors but not glutamate receptors (Verkhratsky & Kettenmann, 1996). The $[Ca^{2+}]_i$ increased quickly during a 30 s application of ATP and then decreased to control levels by 2 min after the ATP application. A second application of ATP (100 μM) induced an increase in $[Ca^{2+}]_i$ similar to that seen with the first application (21 out of 21 cells). The effect of ATP on $[Ca^{2+}]_i$ was concentration-dependent from 1 to 100 μM (Figure 1b). The estimated EC_{50} was near 12 μM.
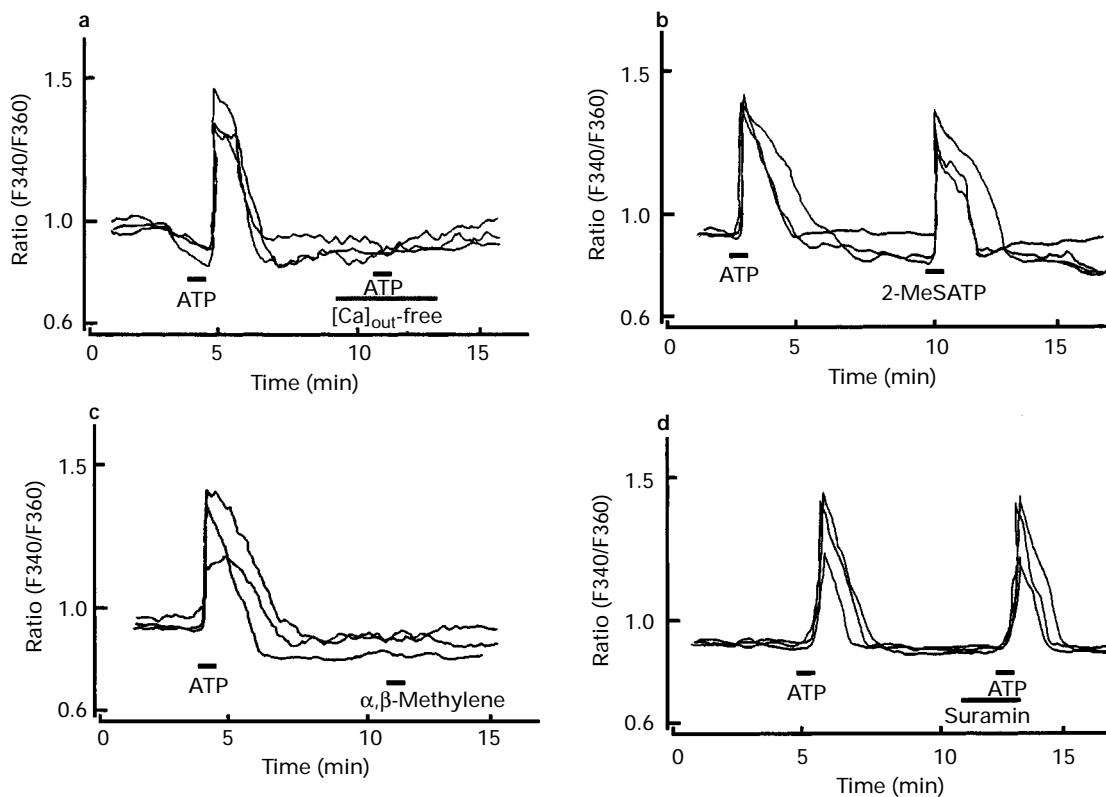

Figure 2 The effect of extracellular Ca^{2+}-free solution, ATP analogue and suramin on the increase in $[Ca^{2+}]_i$ by ATP in microglial cells. (a) The effect of extracellular Ca^{2+} free conditions. ATP (100 μM) was initially applied in the presence of extracellular Ca^{2+} (1.8 mM), and then applied in the absence of extracellular Ca^{2+} (100 nM) for 30 s as indicated by the solid bar. One minute before the second application of ATP, the Ca^{2+}-containing buffer was changed to Ca^{2+}-free buffer. (b) Typical $[Ca^{2+}]_i$ responses induced by 2-methylthio ATP (2-MeSATP). (c) Typical $[Ca^{2+}]_i$ responses induced by α,β-methylene ATP. (d) Effect of suramin (100 μM) on the ATP-evoked increase in $[Ca^{2+}]_i$. The three cells shown are representative of all the cells tested.

Characterization of ATP-evoked increase in [Ca²⁺]ᵢ in microglia

It has been shown that microglia express the P2Y receptor (a G-protein coupled-type P2 receptor) as well as the P2Z (P2X₇) receptor (a Ca^{2+}-permeable ligand-gated channel) (Waltz *et al*., 1993; Nörenberg *et al*., 1994; Langosch *et al*., 1994; Ferrari *et al*., 1996). ATP (100 μM) was shown to evoke an increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (1.8 mM), but the increase was not observed in the absence of extracellular Ca^{2+} (100 nM) (12 out of 12 cells) (Figure 2a). These data indicate that the ATP-evoked increase in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+}, suggesting that ionotropic ATP receptors are responsible for the Ca^{2+} response. We have classified the receptor subtypes responsible for these effects by measuring the relative potency of agonists such as ATP, 2-methylthio ATP (2-MeSATP) and α,β-methylene ATP. 2-MeSATP (100 μM, 7 out of 7 cells) as well as ATP (100 μM) evoked an increase in $[Ca^{2+}]_i$ in microglial cells (Figure 2b), whereas α,β-methylene ATP (100 μM, 7 out of 7 cells) did not (Figure 2c). The ATP-evoked increase in $[Ca^{2+}]_i$ was not inhibited by 100 μM suramin (20 out of 20 cells) (Figure 2d).

Quantitative data corresponding to Figure 1 and 2 are summarized in Figure 3. These data are consistent with the interpretation that P2X₇ is responsible for the observed increase in $[Ca^{2+}]_i$ in microglia in our experiments (see Ferrari *et al*., 1996; Surprenant *et al*., 1996). Next, we examined the effect of BzATP, a selective agonist of P2X₇ (Surprenant *et al*., 1996), and the effect of oxidized ATP, a selective antagonist of P2X₇ (Surprenant *et al*., 1996), on the ATP-evoked increase of $[Ca^{2+}]_i$. BzATP evoked a long-lasting increase in $[Ca^{2+}]_i$ even at 1 μM (Figure 4), a concentration at which ATP did not evoke the increase (Figure 1b). Moreover, 1 h pretreatment with oxidized ATP (100 μM) completely blocked the increase by ATP (10 μM). These data, taken together with the fact that the concentration-response curve for the increase in $[Ca^{2+}]_i$ by ATP was shifted to left in the Mg^{2+}-free condition (Figure 1b),

indicate that the ATP-evoked $[Ca^{2+}]_i$ increase is due to the activation of P2X₇ receptors.

ATP evoked the release of plasminogen in a concentration-dependent manner, but not NO

The primary aim of these experiments was to obtain evidence of the physiological significance of the observed ATP-evoked increase in $[Ca^{2+}]_i$. As mentioned in Introduction, activated microglia have opposing functions in the CNS, neurotoxic or protective. We measured the release of plasminogen from the ATP-activated microglia as an indication of the protective function, because plasminogen is thought to be a key molecule for the neuroprotective actions by microglia (Nakajima *et al*., 1992b, c; 1996; Kreutzberg, 1996). We also measured the release of NO as an indication of the toxic effect because NO is thought to be a neurotoxic substance (Kreutzberg, 1996).

Figure 5a shows the effect of ATP (10 – 100 μM) and glutamate (100 μM) on the release of plasminogen from microglia by use of an immunoblotting method with DAB staining. Figure 5b shows quantitative data from the immunoblotting experiments by use of a high sensitivity ECL method. Both figures demonstrate that ATP stimulated the release of plasminogen in a concentration-dependent manner
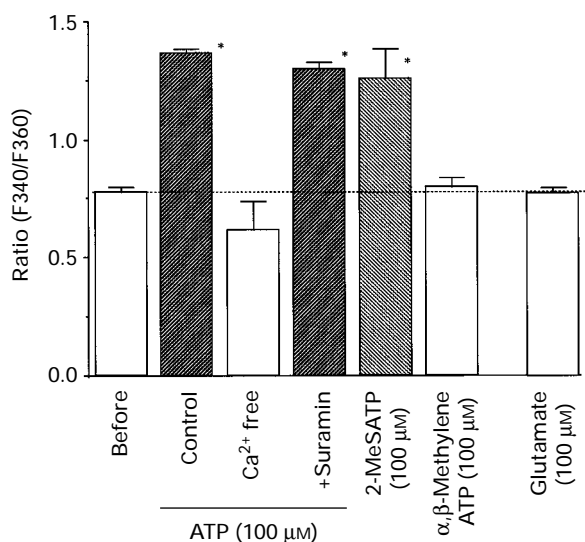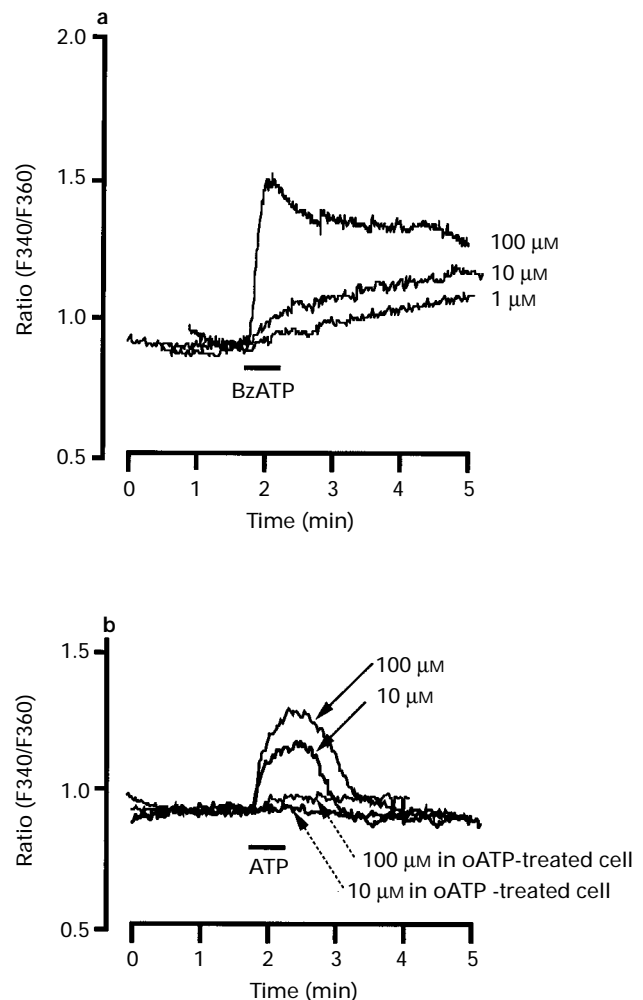

Figure 3 Quantitative data for $[Ca^{2+}]_i$ responses shown in Figures 1 and 2. Data are mean±s.e.mean of the responses in 41 cells by ATP (100 μM) in the presence of extracellular Ca^{2+}, 12 cells by ATP (100 μM) in the absence of extracellular Ca^{2+}, 20 cells by ATP (100 μM) with suramin (100 μM), 7 cells by 2-MeSATP (100 μM), 7 cells by α,β-methylene ATP (100 μM), or 70 cells by glutamate (100 μM). An asterisk indicates significant difference from the value of 'before' the stimulation ($P<0.05$).

Figure 4 Effects of BzATP and oxidized ATP in microglia. (a) Effect of BzATP on $[Ca^{2+}]_i$ in microglia. Data represent the mean of the responses in 14 cells. (b) Effect of 1 h pretreatment with oxidized ATP (oATP) on the ATP-evoked increase in $[Ca^{2+}]_i$ in microglia. Data represent the mean of the responses in 13 – 14 cells.
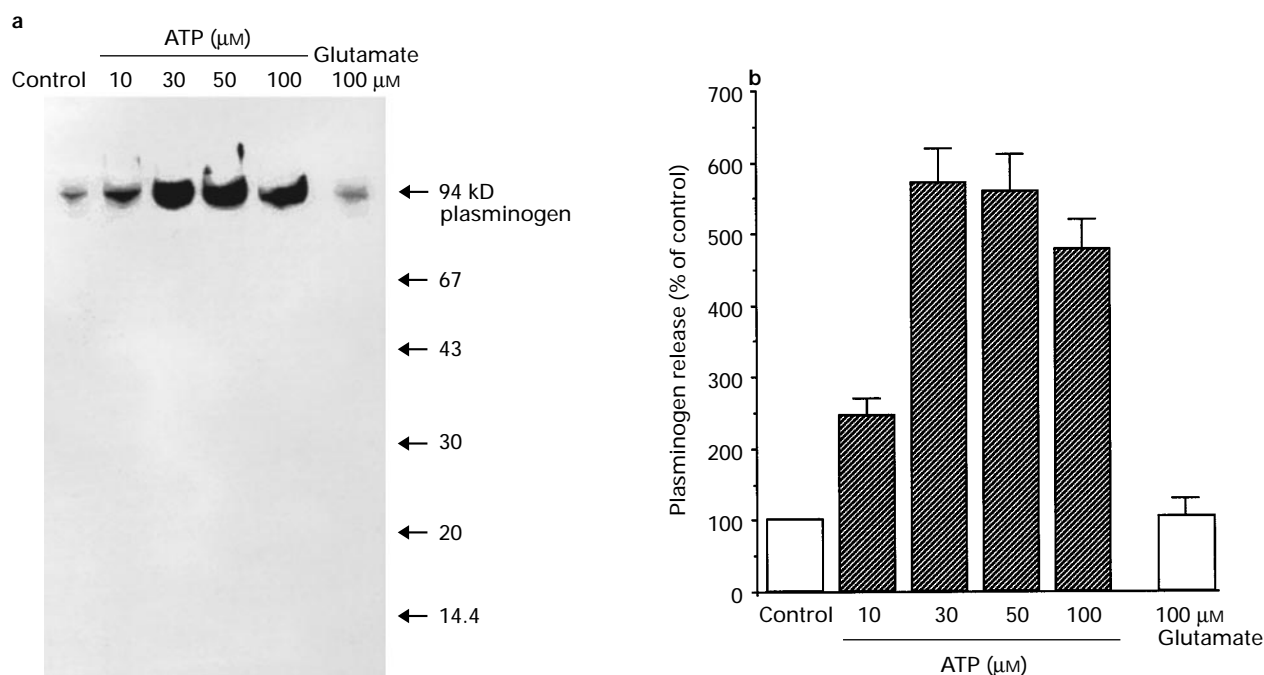
Figure 5 The effect of ATP (10–100 μM) and glutamate (100 μM) on the release of plasminogen from microglia 10 min after stimulation. (a) Immunoblot with DAB staining. (b) Quantitative data obtained from immunoblotting with ECL staining. Data are means$\pm$s.e.mean of measurements from 3–5 blots.

(the estimated EC_{50} was near 15 μM), whereas glutamate (100 μM) did not evoke the release of plasminogen 10 min after the stimulation. The result is in accord with the data of the internal Ca^{2+} response shown in Figure 1.

NO has been shown to be released from various cells following the cascade of Ca^{2+} influx, activation of Ca^{2+} calmoduline-dependent protein kinase and activation of NO synthetase (Palmer *et al.*, 1987). It has also been found that ATP stimulates NO release from endothelial cells, presumably through an increase in $[Ca^{2+}]_i$ (see review of Olsson & Pearson, 1990). However, we could not detect a stimulating effect of ATP (10 to 100 μM) on NO release from our microglia (Figure 6). Lipopolysaccaride (LPS, 0.5 μg ml^{-1}), a positive control, stimulated NO release from the microglia.

Internal Ca^{2+} dependence of the release of plasminogen from microglia

It is already known that neurotransmitter release from synaptic vesicles is triggered by a transient increase in the internal Ca^{2+} concentration through voltage-gated Ca^{2+} channels after priming (Sudhof, 1995). Since there are few data on the relationship between internal Ca^{2+} and the release of plasminogen, we examined the intracellular Ca^{2+} dependence of the release by use of BAPTA-AM, which is converted in the cytosol to BAPTA, an intracellular Ca^{2+} chelator (Negulescu *et al.*, 1989). Figure 7a shows the effect of a 1 h pretreatment of cells with BAPTA-AM (200 μM) on the plasminogen release evoked by 10 min of ATP (100 μM) stimulation. BAPTA-AM treatment inhibited the plasminogen release completely. Figure 7b shows that the ionophore A23187, which evokes an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Foder *et al.*, 1989; Mills & Kater, 1990), stimulated plasminogen release from microglia in a concentration-dependent manner. These data suggest that ATP evokes an increase in $[Ca^{2+}]_i$ resulting in plasminogen release from microglia.
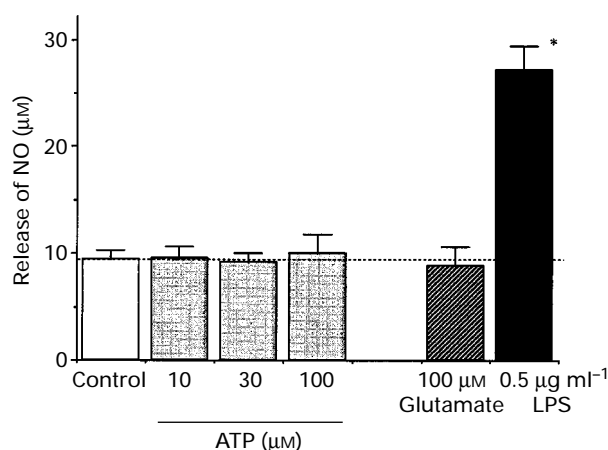

Figure 6 The effect of ATP (10–100 μM), glutamate (100 μM) and lipopolysaccharide (LPS, 0.5 μg ml^{-1}) on the release of NO from microglia. LPS, a positive control, stimulated NO release from the microglia, whereas neither ATP nor glutamate stimulated the NO release. Data are mean$\pm$s.e.mean of 3 determinations (μM). An asterisk indicates significant difference from control ($P<0.05$).

Discussion

It has been shown that activated microglia can display both neuroprotective and neurotoxic functions in the CNS. Plasminogen is thought to be a key molecule for the neurotrophic effect, because it promotes the development of mesencephalic dopaminergic neurones and enhances neurite outgrowth from explants of neocortical tissue (Nakajima *et al.*, 1992b, c). NO is thought to be neurotoxic via a mechanism involving free oxygen radicals and it has been demonstrated that NO release from microglia has a neurotoxic effect in co-cultures of neurones and microglia (Thery *et al.*, 1993). In this
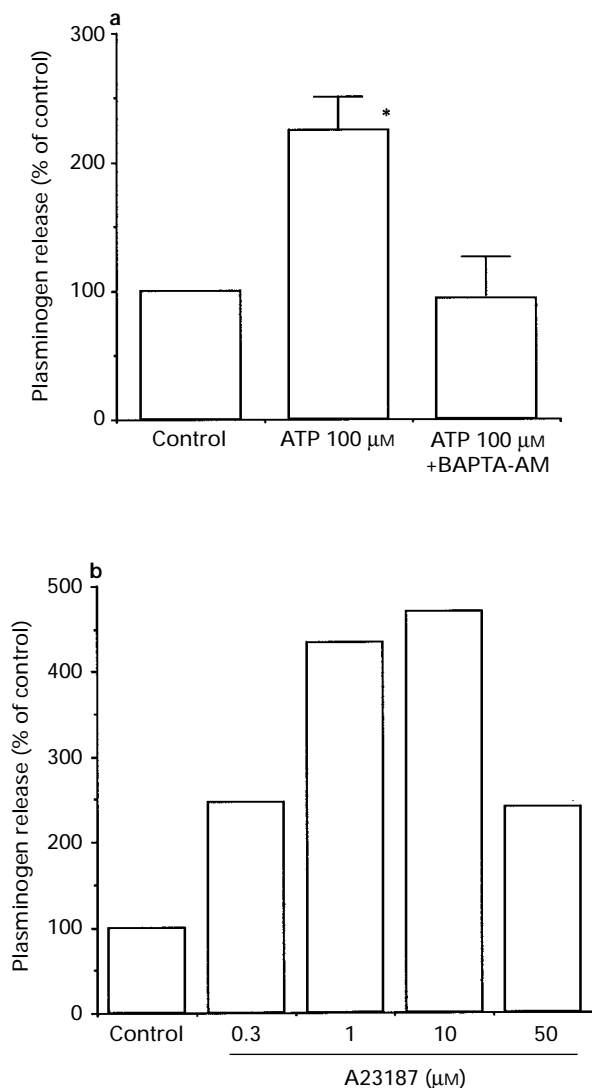
Figure 7 Dependence on Ca^{2+} of the release of plasminogen from microglia. (a) The effect of 1 h pretreatment of cells with BAPTA-AM (200 μM) on plasminogen release evoked by ATP (100 μM). Data are mean$\pm$s.e.mean from measurements of 3–5 blots. An asterisk indicates significant difference from the BAPTA-AM-treated group ($P<0.05$). (b) The Ca^{2+} ionophore A23187 stimulated plasminogen release from microglia. A23187 was added to the cell for 10 min.

paper we present evidence that ATP evokes an increase in $[Ca^{2+}]_i$ which stimulates plasminogen release from cultured microglia. On the other hand, ATP did not stimulate NO secretion from the same preparations.

It is well known that neurotransmitter release from synaptic vesicles is triggered by a transient increase in $[Ca^{2+}]_i$ through voltage-gated Ca^{2+} channels (mostly N-type Ca^{2+} channel in neurone) (Sudhof, 1995). A similar relationship was obtained for glutamate release from astroglia and IL-aβ release from microglia (Parpura *et al.*, 1994; Ferrari *et al.*, 1996). However, no data were obtained suggesting a relationship between an increase in $[Ca^{2+}]_i$ and the release of plasminogen from microglia. We examined the intracellular Ca^{2+} dependence of the release of plasminogen by using BAPTA-AM (an intracellular Ca^{2+} chelator) and A23187 (a Ca^{2+} ionophore). A 1 h pretreatment with BAPTA-AM completely inhibited the plasminogen release stimulated by ATP (Figure 7a). A23187, which can increase $[Ca^{2+}]_i$ also evoked plasminogen release in a concentration-dependent manner (Figure 7b). These data suggest that the ATP-evoked

plasminogen release is triggered by an increase in $[Ca^{2+}]_i$ in microglia.

It has been found that ATP activates non-selective cation conductance or increases permeability changes by creating non-specific large pores in the plasma membranes of microglia, suggesting the expression of P2X and P2Z as ionotropic P2 receptors in these cells (Walz *et al.*, 1993; Nörenberg *et al.*, 1994; Langosch *et al.*, 1994; Ferrari *et al.*, 1996). Recently, a new nomenclature of the P2 receptors based on the cloning and expression of cDNAs of receptor subclasses was published (Burnstock & King, 1996). According to the new nomenclature, there are 7 identified subclasses of ionotropic P2 receptors (P2X$_1$ to P2X$_7$. P2X$_7$ is thought to be a candidate for P2Z (Surprenant *et al.*, 1996). In our experiment, the ATP-evoked increase in $[Ca^{2+}]_i$ was totally dependent on extracellular Ca^{2+} (Figure 2a). These data suggest that an ionotropic P2 receptor(s) is responsible for the increase in $[Ca^{2+}]_i$. The ATP-evoked increase in $[Ca^{2+}]_i$ was insensitive to suramin (Figure 2d). Interestingly, P2X$_4$, P2X$_6$ and P2X$_7$ are known to be suramin-insensitive (Buell *et al.*, 1996). 2-MeSATP was active and α,β-methylene ATP was inactive in evoking the $[Ca^{2+}]_i$ increase (Figure 2b and c). These characteristics are consistent with those of P2X$_4$, P2X$_6$ and P2X$_7$. Many previous studies have suggested that P2Z might be involved in IL-1β release from microglia (Ferrari *et al.*, 1996). We also examined the effects of BzATP, a selective agonist (Ferrari *et al.*, 1996), and oxidized ATP, a selective antagonist of P2X$_7$ (Surprenant *et al.*, 1996). BzATP evoked an increase in $[Ca^{2+}]_i$ more efficiently than ATP, and oxidized ATP (100 μM) inhibited the effect of ATP. Moreover, the concentration-response curve of the increase in $[Ca^{2+}]_i$ induced by ATP was shifted to the left in the Mg^{2+}-free condition (Figure 1b), suggesting that the active form of ATP that stimulates the responses may be ATP^{4-} which is a specific endogenous agonist of P2X$_7$. All the data indicate that the ATP-evoked $[Ca^{2+}]_i$ increase is due to the activation of P2X$_7$ receptors.

Several lines of evidence support the role of ATP as a neurotransmitter in the hippocampus (Inoue *et al.*, 1996). Messenger RNAs of ionotropic and metabotoropic ATP receptors have been detected by *in situ* hybridization in hippocampus (Collo *et al.*, 1996). However, the physiological significance of ATP in the hippocampus is still unknown. We have previously demonstrated that exogeneously applied plasminogen enhances synaptic transmission through NMDA-glutamate receptors in cultured hippocampal neuronal networks (Inoue *et al.*, 1994). In this study we found that ATP, but not glutamate (which is an ubiquitous excitatory neurotransmitter), stimulated plasminogen release from microglial cells. Therefore, it is possible that ATP released from nerve endings transmits information to microglia and stimulates them to release plasminogen in the hippocampus. As mentioned above, the released plasminogen may modulate the function of the neurones. Thus, ATP from purinergic neurones can transmit information to glutamate-related neurones via microglia. In other words, a triangular connection of neurone–microglia–neurone may be completed by ATP and plasminogen in the hippocampus. ATP also exists in cytosol at a concentration of several mM and is able to come out of cells damaged by ischaemia or trauma. It has been found that microglia respond to ischaemia in the CA1 area of hippocampus (Yamashita *et al.*, 1994). ATP leaking from damaged cells as well as ATP from nerve endings may be able to activate hippocampal microglia.

The activation and proliferation of microglia occurring after axotomy of the facial nerve are accompanied by an increase in the activity of urokinases such as plasminogen in

the facial nucleus (Nakajima *et al.*, 1996). Proliferating microglia detach afferent synaptic terminals from the motoneurone surface within a few days after the transection of facial nerve (Kreutzberg, 1996). This so-called 'synaptic stripping' is a well-known phenomenon but the mechanism is still unknown. It is supposed that ATP from the damaged facial nerve may play some role in triggering microglia to commence 'synaptic stripping'.

In conclusion, we have found that ATP stimulates plasminogen release from cultured microglia. This is due to the activation of ionotropic $P2X_7$ receptors, leading to an increase in $[Ca^{2+}]_i$.

References

BANATI, R.B., GEHRMANN, J., SCHUBERT, P. & KREUTZBERG, G.W. (1993a). Cytotoxicity of microglia. *Glia,* **7,** 111 – 118.

BANATI, R.B., ROTHE, G., VALET, G. & KREUTZBERG, G.W. (1993b). Detection of lysosomal cysteine proteinases in microglia: flow cytometric measurement and histochemical localization of cathepsin B and L. *Glia, 7,* 183 – 191.

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

BURNSTOCK, G. & KING, B.F. (1996). Numbering of cloned P2 purinoceptors. *Drug. Dev. Res.,* **38,** 67 – 71.

COLLO, G., NORTH, R.A., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, S., SURPRENANT, A. & BUELL, G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J. Neurosci.,* **16,** 2495 – 2507.

COLTON, C.A. & GILBERT, D.L. (1987). Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett.,* **223,** 284 – 288.

FERRARI, D., VILLALBA, M., CHIOZZI, P., FALZONI, S., RICCIARDI-CASTAGNOLI, P. & DI VIRGILIO, F. (1996). Mouse microglial cells express a plasma membrane pore gated by extracellular ATP. *J. Immunol.,* **156,** 1531 – 1539.

FODER, B., SCHARFF, O. & THASTRUP, O. (1989). Ca^{2+} transients and Mn^{2+} entry in human neutrophils induced by thapsigargin. *Cell Calcium, 10,* 477 – 490.

GREEN, L.C., RUIZ DE LUZURIAGA, K., WAGNER, D.A., RAND, W., ISTFAN, N., YOUNG, V.R. & TANNENBAUM, S.R. (1981). Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. U.S.A.,* **78,** 7764 – 7768.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440 – 3450.

INOUE, K., KOIZUMI, S., NAKAJIMA, K., HAMANOUE, M. & KOHSAKA, S. (1994). Modulatory effect of plasminogen on NMDA-induced increase in intracellular free calcium concentration in rat cultured hippocampal neurons. *Neurosci. Lett.,* **179,** 87 – 90.

INOUE, K., KOIZUMI, S. & NAKAZAWA, K. (1995). Glutamate-evoked release of adenosine 5′-triphosphate causing an increase in intracellular calcium in hippocampal neurons. *NeuroReport,* **6,** 437 – 440.

INOUE, K., KOIZUMI, S. & UENO, S. (1996). Implication of ATP receptors in brain functions. *Prog. Neurobiol.,* **50,** 483 – 492.

INOUE, K., NAKAZAWA, K., FUJIMORI, K., WATANO, T. & TAKANAKA, A. (1992). Extracellular adenosine 5′-triphosphate-evoked glutamate release in cultured rat hippocampal neurons. *Neurosci. Lett.,* **134,** 215 – 218.

KOIZUMI, S., NAKAZAWA, K. & INOUE, K. (1995). Inhibition by Zn^{2+} of uridine 5′-triphosphate-induced Ca^{2+}-influx but not Ca^{2+}-mobilization in rat phaeochromocytoma cells. *Br. J. Pharmacol.,* **115,** 1502 – 1508.

KOIZUMI, S., WATANO, T., NAKAZAWA, K. & INOUE, K. (1994). Potentiation by adenosine of ATP-evoked dopamine release via a pertussis toxin-sensitive mechanism in rat phaeochromocytoma PC12 cells. *Br. J. Pharmacol.,* **112,** 992 – 997.

KREUTZBERG, G.W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends. Neurosci.,* **19,** 312 – 318.

LANGOSCH, J.M., GEBICKE-HAERTER, P.J., NÖRENBERG, W. & ILLES, P. (1994). Characterization and transduction mechanisms of purinoceptors in activated rat microglia. *Br. J. Pharmacol.,* **113,** 29 – 34.

MILLS, L.R. & KATER, S.B. (1990). Neuron-specific and state-specific differences in calcium homeostasis regulate the generation and degeneration of neuronal architecture. *Neuron, 4,* 149 – 163.

NAKAJIMA, K., HAMANOUE, M., SHIMOJO, M., TAKEI, N. & KOHSAKA, S. (1989). Characterization of microglia isolated from a primary culture of embryonic rat brain by a simplified method. *Biomed. Res., 10,* 411 – 423.

NAKAJIMA, K. & KOHSAKA, S. (1993). Functional roles of microglia in the brain. *Neurosci. Res., 17,* 187 – 203.

NAKAJIMA, K., REDDINGTON, M., KOHSAKA, S. & KREUTZBERG, G.W. (1996). Induction of urokinase-type plasminogen activator in rat facial nucleus by axotomy of the facial nerve. *J. Neurochem., 66,* 2500 – 2505.

NAKAJIMA, K., SHIMOJO, M., HAMANOUE, M., ISHIURA, S., SUGITA, H. & KOHSAKA, S. (1992a). Identification of elastase as a secretory protease from cultured rat microglia. *J. Neurochem., 58,* 1401 – 1408.

NAKAJIMA, K., TSUZAKI, N., NAGATA, K., TAKEMOTO, N. & KOHSAKA, S. (1992b). Production and secretion of plasminogen in cultured rat brain microglia. *FEBS Lett., 308,* 179 – 182.

NAKAJIMA, K., TSUZAKI, N., SHIMOJO, M., HAMANOUE, M. & KOHSAKA, S. (1992c). Microglia isolated from rat brain secrete a urokinase-type plasminogen activator. *Brain Res., 577,* 285 – 292.

NEARY, J.T., RATHBONE, M.P., CATTABENI, F., ABBRACCHIO, M.P. & BURNSTOCK, G. (1996). Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci., 19,* 13 – 18.

NEGULESCU, P.A., REENSTRA, W.W. & MACHEN, T.E. (1989). Intracellular Ca requirements for stimulus-secretion coupling in parietal cell. *Am. J. Physiol., 256,* C241 – C251.

NÖRENBERG, W., LANGOSCH, J.M., GEBICKE-HAERTER, P.J. & ILLES, P. (1994). Characterization and possible function of adenosine 5′-triphosphate receptors in activated rat microglia. *Br. J. Pharmacol., 111,* 942 – 950.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Physiol. Rev., 70,* 761 – 845.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature, 327,* 524 – 526.

PARPURA, V., BASARSKY, T.A., LIU, F., JEFTINIJA, K., JEFTINIJA, S. & HAYDON, P.G. (1994). Glutamate-mediated astrocyte-neuron signalling. *Nature, 369,* 744 – 747.

SUDHOF, T.C. 1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature, 375,* 645 – 653.

SURPRENANT, A., RASSENDREN, F., KAWASHIMA, E., NORTH, R.A. & BUELL, G. (1996). The cytolytic P_{2Z} receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science,* **272,** 735 – 738.

THERY, C., CHAMAK, B. & MALLAT, M. (1993). Neurotoxicity of brain macrophages. *Clin. Neuropathol., 12,* 288 – 290.

VERKHRATSKY, A. & KETTENMANN, H. (1996). Calcium signalling in glial cells. *Trends Neurosci., 19,* 346 – 352.

WALZ, W., ILSCHNER, S., OHLEMEYER, C., BANATI, R. & KETTENMANN, H. (1993). Extracellular ATP activates a cation conductance and a K^+ conductance in cultured microglial cells from mouse brain. *Neuroscience, 13,* 4403 – 4411.

WIERASZKO, A. & SEYFRIED, T.N. (1989). ATP-induced synaptic potentiation in hippocampal slices. *Brain Res., 491,* 356 – 359.

YAMASHITA, K., NIWA, M., KATAOKA, Y., SHIGEMATSU, K., HIMENO, A., TSUTSUMI, K., NAKANO-NAKASHIMA, M., SAKURAI-YAMASHITA, Y., SHIBATA, S. & TANIYAMA, K. (1994). Microglia with an endothelium ETB receptor aggregate in rat hippocampus CA1 subfields following transient forebrain ischemia. *J. Neurochem., 63,* 1042 – 1051.