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Characterization and possible function of adenosine 5′-triphosphate receptors in activated rat microglia

[1]W. Nörenberg, J.M. Langosch, *P.J. Gebicke-Haerter & [1]P. Illes

Department of Pharmacology, University of Freiburg, Hermann-Herder-Strasse 5, and *Department of Psychiatry, University of Freiburg, Hauptstrasse 5, D-79104 Freiburg, Germany

1 Purinoceptor agonist-induced currents in untreated (proliferating) and lipopolysaccharide (LPS; 100 ng ml^{-1})-treated (non-proliferating) rat microglial cells in culture were recorded by the whole-cell patch-clamp technique. These cells have two preferred resting membrane potentials, one at − 35 mV and another one at − 70 mV.

2 Most experiments were carried out in non-proliferating cells. ATP, ATP-γ-S and α,β-MeATP (1–1000 μM in all cases) evoked an inward current at a holding potential of − 70 mV, followed, in some experiments, by an outward current. At − 70 mV 2-methylthio ATP (1–1000 μM) evoked an inward current, whereas at − 35 mV it produced an outward current only.

3 When K^+ was replaced in the pipette solution by an equimolar concentration of Cs^+ (150 mM), the main outward component of the ATP-γ-S (10 μM) induced response disappeared. Instead, an inward current was obtained. Replacement of K^+ by Cs^+ did not affect the inward current evoked by 2-methylthio ATP (300 μM). 4-Aminopyridine (1–10 mM), however, almost abolished this current and unmasked a smaller outward current.

4 The rank order of agonist potency was 2-methylthio ATP > ATP > α,β-MeATP. Adenosine and UTP were inactive. Suramin (300 μM) and reactive blue 2 (50 μM) antagonized the effect of 2-methylthio ATP (300 μM).

5 *I–V* relations were determined by delivering fast voltage ramps before and during the application of 2-methylthio ATP (300 μM). In the presence of extra- (1 mM) and intracellular (150 mM) Cs^+, the 2-methylthio ATP-evoked current crossed the zero current level near 0 mV. When both Cs^+ (1 mM) and 4-aminopyridine (1 mM) were present in the bath medium, the intersection of the 2-methylthio ATP current with the zero current level was near − 75 mV.

6 2-Methylthio ATP (1–1000 μM) induced the same inward current both in proliferating and non-proliferating microglia. However, the depolarizing response to 2-methylthio ATP (300 μM) was larger and longer-lasting in the proliferating cells. When the free Ca^{2+} concentration in the pipettes was increased from the standard 0.01 to 1 μM, the amplitude and duration of this depolarization was increased in non-proliferating cells. 4-Aminopyridine (1 mM) enhanced the duration, but not the amplitude of responses.

7 ATP and its structural analogues stimulate microglial purinoceptors of the P_{2Y}-type. This leads to the opening of non-selective cationic channels and potassium channels. Depending on the resting membrane potential, depolarization or hyperpolarization prevails. Although the inward current produced by 2-methylthio ATP is of similar amplitude in proliferating and non-proliferating microglia, the resulting depolarization is smaller in the latter cell type because of the presence of voltage-sensitive, outwardly rectifying potassium channels.

Keywords: Microglia; lipopolysaccharide; P_2-purinoceptor; ATP; suramin; non-selective cationic channel; potassium channel

Introduction

Microglia originate from monocytes/macrophages entering the brain during the early embryonic development (Jordan & Thomas, 1988; Theele & Streit, 1993). These resident immunocytes are capable of regressive metamorphosis in pathological processes and thereby represent a major source of endogenous brain macrophages (Rieske *et al.*, 1989; Thomas, 1992). Resting (ramified) microglia differentiate into fully activated macrophages through a number of transitional states. In tissue culture systems, cells become initially activated by the isolation procedure and are capable of both proliferation and phagocytosis (Rieske *et al.*, 1989; Gebicke-Haerter *et al.*, 1989). They can be driven further into macrophage-like (amoeboid) microglia by stimuli such as bacterial lipopolysaccharide (LPS) (Adams & Hamilton, 1987). These cells present major histocompatibility antigen (MHC) type I and II, secrete cytokines (e.g. interleukin-1 and 6) and produce superoxide anions (Bignami, 1991; Dickson *et al.*, 1991). They no longer proliferate but are still involved in phagocytosis.

The predominant voltage-dependent current in proliferating rat microglia is potassium-selective and inwardly rectifying (Kettenmann *et al.*, 1990; Banati *et al.*, 1991). LPS-treatment leads to the expression of previously lacking outwardly rectifying potassium channels (Nörenberg *et al.*, 1992; 1993a,b). In addition to voltage-dependent currents, proliferating microglia were shown to exhibit an ATP-induced non-selective cationic conductance (Kettenmann *et al.*, 1993).

Three major types of adenosine 5′-triphosphate- (ATP) sensitive P_2-purinoceptors have been described, principally based on the rank order of agonist potencies (Gordon, 1986). The P_{2X}-(α,β-methylene ATP [α,β-MeATP] > ATP = 2-methylthio ATP) and P_{2Y}-(2-methylthio ATP > ATP > α,β-MeATP) subtypes occur in muscle and exocrine glands (Burnstock & Kennedy, 1985; Kennedy, 1990). Peripheral and central neurones are endowed with P_{2Y}-purinoceptors only (Illes & Nörbenberg, 1993). Receptors of mast cells and possibly lymphocytes are termed P_{2z}; they recognize the tet-

[1] Authors for correspondence.

rabasic form of ATP^{4-} (Gordon, 1986). ATP itself may be degraded to adenosine, which stimulates adenosine receptors, also named P_1-purinoceptors (Burnstock & Kennedy, 1985). Finally, separate pyrimidinoceptors have been described at which UTP is the prototypic agonist (Kügelgen *et al.*, 1987; Seifert & Schulz, 1989).

The aim of the present study was to characterize P_2-purinoceptors of rat microglial cells by the use of pharmacological agents, to investigate the ionic mode of ATP action, and to compare ATP effects in proliferating and non-proliferating microglia.

Methods

Cell cultures

Mixed astroglial-microglial cell cultures were prepared from cerebral hemispheres of newborn Wistar rats as described previously (Keller *et al.*, 1985). In brief, animals were killed by decapitation, meninges were removed, and forebrains were minced and gently dissociated by trituration in Hank's balanced salt solution. Cells were collected by centrifugation at 200 *g* for 10 min, resuspended in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), plated onto 35 mm Falcon culture dishes (5×10^5 cells/dish), and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Media were prepared taking extreme care to avoid all potential sources of LPS contaminations. After 14 days of culture under LPS-free conditions, floating microglial cells were harvested and reseeded into 35 mm Petri dishes to give pure microglial cultures (Gebicke-Haerter *et al.*, 1989). Small cells (diameter, 5–8 μm) with unipolar or bipolar processes were observed under phase contrast optics immediately after reseeding. When LPS (100 ng ml^{-1}) was added for 12–24 h, the microglial cells became circular in shape with ruffled edges (diameter, 15–23 μm).

Patch-clamp experiments

Membrane currents of microglial cells were measured with the patch-clamp method in the whole-cell configuration (Hamill *et al.*, 1981). The bath (extracellular) solution contained (mM): NaCl 160, KCl 4.5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) 5, glucose 11, pH 7.4 adjusted with NaOH. A nominally Mg^{2+}-free medium was prepared by omitting MgCl. A Na^+-free, high Ca^{2+} (100 mM) medium was obtained by replacement of NaCl by 98 mM $CaCl_2$ plus 60 mM tris (2-amino-2-[hydroxymethyl]-1,3-propanediol), and by setting the pH to 7.4 with HCl. The pipette (internal) solution contained (mM): KCl 150, $MgCl_2$ 2, $CaCl_2$ 1 (free Ca^{2+}, 0.01 μM), EGTA 11, HEPES 10, pH 7.3 adjusted with KOH. The free Ca^{2+} concentration was increased to 1 μM by decreasing the concentration of EGTA to 1.1 mM and leaving the other components of the solution unchanged. In some experiments the intracellular KCl was replaced by CsCl and the pH was set to 7.3 by adding CsOH. Pipettes pulled from borosilicate capillaries (Vitrex BRI/E, Poly-Labo-Strasbourg, France) were coated with beeswax and dipped in Sigmacote (Sigma, Deisenhofen, Germany) to minimize associated capacitive currents. The resistance of the electrodes was 2–5 MΩ.

Compensation of capacitance and series resistance was achieved with the inbuilt circuitry of the patch amplifier (List EPC-7, Darmstadt, Germany). Voltage-clamp protocols were generated with a laboratory computer (ESCOM/486, Heppenheim, Germany). Data were digitized at 1–7 kHz (Cambridge Electronic Devices 1401, Cambridge, UK) and then stored on and analyzed with the computer. Experiments were carried out at room temperature (20–24°C). Data illustrated are non-leak subtracted.

After whole-cell configuration was achieved, the membrane potential was determined in the current-clamp mode. Then the system was left for 5–10 min to allow stabilization of the voltage-dependency of various parameters. During this time the membrane potential of single cells alternated between two preferred values of approximately − 70 and − 35 mV. When currents induced by ATP and its structural analogues (I_{ATP}) were measured, the cells were held at − 70 mV. Purinoceptor agonists and uridine 5'-triphosphate (UTP) were locally microperfused for 10 s by means of separate, wide-bore, pressurized (10 kPa) puffer pipettes, placed about 50 μm away from the cell under examination.

Each agonist concentration was applied only once (T_1) to a cell, except in some cases when two subsequent applications (T_1,T_2) separated by 3 or 10 min intervals were made. During these intervals, the 3 ml bath was superfused with drug-free medium, or with medium containing purinoceptor antagonists. Puffer application of drug-free extracellular solution had no effect. All other compounds were superfused at a flow rate of 2 ml min^{-1}. Suramin and reactive blue 2 were present in the bath for 8 min before, and during T_2. When the effects of two consecutive administrations of purinoceptor agonists were compared, the current induced by T_2 was normalized with respect to the current induced by T_1. In some experiments, Cs^+ and/or 4-aminopyridine were added for 10 min before the pressure application of 2-methylthio ATP.

Voltage-dependent currents were evoked by voltage pulses of 300 ms duration delivered every 8 s from a holding potential of − 70 mV in 20 mV increments. Step range was from − 150 to + 50 mV. I–V relations were determined by using fast voltage ramps (− 70 mV holding potential, 1 s duration, from − 140 to + 40 mV). Ramps were generated both before the application of 2-methylthio ATP (300 μM) and during the peak response to the agonist. The 2-methylthio ATP current was obtained by digital subtraction.

Materials

The following materials and drugs were used: suramin hexasodium salt (Bayer, Leverkusen, Germany); reactive blue 2 trisodium salt (EGA-Chemie, Steinheim, Germany); Dulbecco's modified Eagle's medium, Hank's balanced salt solution, foetal calf serum (Gibco, Eggenstein, Germany); 2-methylthioadenosine 5'-triphosphate tetrasodium salt (RBI, Natick, MA, U.S.A.); lipopolysaccharide from *Salmonella typhimurium* (Sebak, Aidenbach, Germany); adenosine, adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-O-(3-thio)-triphosphate tetralithium salt (ATP-γ-S), 4-aminopyridine, α,β-methyleneadenosine 5'-triphosphate lithium salt (α,β-Me-ATP), uridine 5'-triphosphate sodium salt (UTP) (Sigma, Deisenhofen, Germany).

LPS, dissolved in distilled water (1 mg ml^{-1}), was further diluted into DMEM. 4-Aminopyridine was dissolved in the bath medium. Stock solutions (10–100 mM) of purinoceptor agonists and antagonists were prepared with distilled water and further diluted with medium.

Statistics

Means ± s.e.mean of *n* trials are shown. The Student's paired or unpaired *t* test was used to compare means as appropriate. Multiple comparisons were carried out by one way analysis of variance followed by the Student-Newman-Keuls test. A probability level of 0.05 or less was considered to be statistically significant.

Results

The present data were obtained from 41 proliferating and 247 non-proliferating microglial cells kept in primary culture.

Measurements of ionic currents

Effects of P_2-purinoceptor agonists Most of the experiments were carried out on proliferating microglia treated with LPS

(100 ng ml^{-1}) for 12–24 h. This treatment had driven the cells into a more activated, non-proliferating state. ATP (100 μM) was pressure-applied for 10 s every 3 min at a holding potential of − 70 mV (T_1–T_4: Figure 1a). In five out of seven cells, T_1 evoked an inward current only. The size of this response markedly decreased within the 10 s ejection period. In the residual two cells, the initial response rapidly disappeared and gave rise to a slowly developing and non-desensitizing outward current. Since the outward component was not present in all cells, only the inward component was statistically evaluated (Figure 5a).

The ATP-(100 μM) induced inward current appeared to decrease from T_1 to T_4 (Figure 1a), with a major and statistically significant decline from T_1 to T_2 only (see legend to Figure 1). When the interval between T_1 (− 46.2 ± 8.6 pA) and T_2 (− 33.0 ± 6.1 pA; $n = 8$; $P > 0.05$) was increased from 3 to 10 min, the responses became rather stable.

Puffer application of ATP-γ-S (100 μM) for 10 s every 3 min also resulted in a decrease of the inward current from T_1 to T_2 (Figure 1b). At the same time the outward current increased (Figure 1b). ATP-γ-S at 10 μM caused a transient inward current (which was sometimes missing; Figure 2a, left panel), followed by a more marked and slowly declining outward current (Figure 2a, right panel). The outward component tended to decrease when the concentration of ATP-γ-S was increased from 10 μM (10.6 ± 3.0 pA; $n = 8$) to 100 μM (3.9 ± 1.8 pA; $n = 7$; $P > 0.05$); at 1 mM ATP-γ-S only the inward component was present (Figure 5a). 2-Methylthio ATP (10–100 μM; $n = 5$–7) and α,β-MeATP (10–100 μM; $n = 6$) caused less outward current than ATP-γ-S (10–100 μM).

Ionic nature of P_2-purinoceptor agonist-induced currents When K^+ was replaced in the pipette solution by an equimolar concentration of Cs^+ (150 mM), the main outward component of the ATP-γ-S (10 μM)-induced response disappeared (Figure 2a). Instead, an inward current was obtained. By contrast, replacement of K^+ by Cs^+ did not affect the current evoked by 2-methylthio ATP (300 μM; $n = 6$). Apparently, intracellular Cs^+ selectively interfered with the outward component and, thereby, increased or even unmasked the inward component. 4-Aminopyridine (10 mM), however, almost abolished the inward current evoked by 2-methylthio ATP (300 μM) and unmasked a smaller outward current response (Figure 2b). A lower concentration of 4-aminopyridine (1 mM) also inhibited the 2-methylthio ATP (300 μM)-induced inward current (control, − 67.4 ± 12.8 pA; $n = 6$; 4-aminopyridine, − 9.7 ± 3.7 pA, $n = 5$; $P < 0.01$), but revealed only in some cells an outward current (17.9 ± 13.0 pA; $n = 5$).

At a holding potential of − 70 mV (the higher preferred membrane potential of microglia), 2-methylthio ATP (100 μM) caused an inward current (− 33.1 ± 6.5 pA; $n = 7$), whereas at a holding potential of − 35 mV (the lower preferred membrane potential of microglia) it caused an outward current (54.8 ± 8.9 pA; $n = 5$). This suggests that the inward and outward currents may be differentiated by their respective reversal potentials.

Therefore, I–V relations of non-proliferating microglial cells were determined by applying fast voltage ramps (− 70 mV holding potential, 1 s duration, from − 140 to + 40 mV) before and during the administration of 2-methylthio ATP (300 μM). The 2-methylthio ATP-evoked current was obtained by subtraction. The control I–V relation determined in a normal bath-medium with K^+-containing pipettes, exhibits both inward and outward rectification (Figure 3a, inset). This rectification is characteristic for non-proliferating microglia and can be elicited also by applying a series of hyperpolarizing and depolarizing voltage steps from a holding potential of − 70 mV (Figure 6a). The 2-methylthio ATP current crossed the zero current value at three points (− 76.4 ± 5.2 mV, − 28.8 ± 2.0 mV and − 18.6 ± 2.9 mV; $n = 8$ each), the two last points being rather similar (Figure 3a). Hence, the I–V curves of non-proliferating microglial cells appear to represent the sum of two ionic currents with two distinct

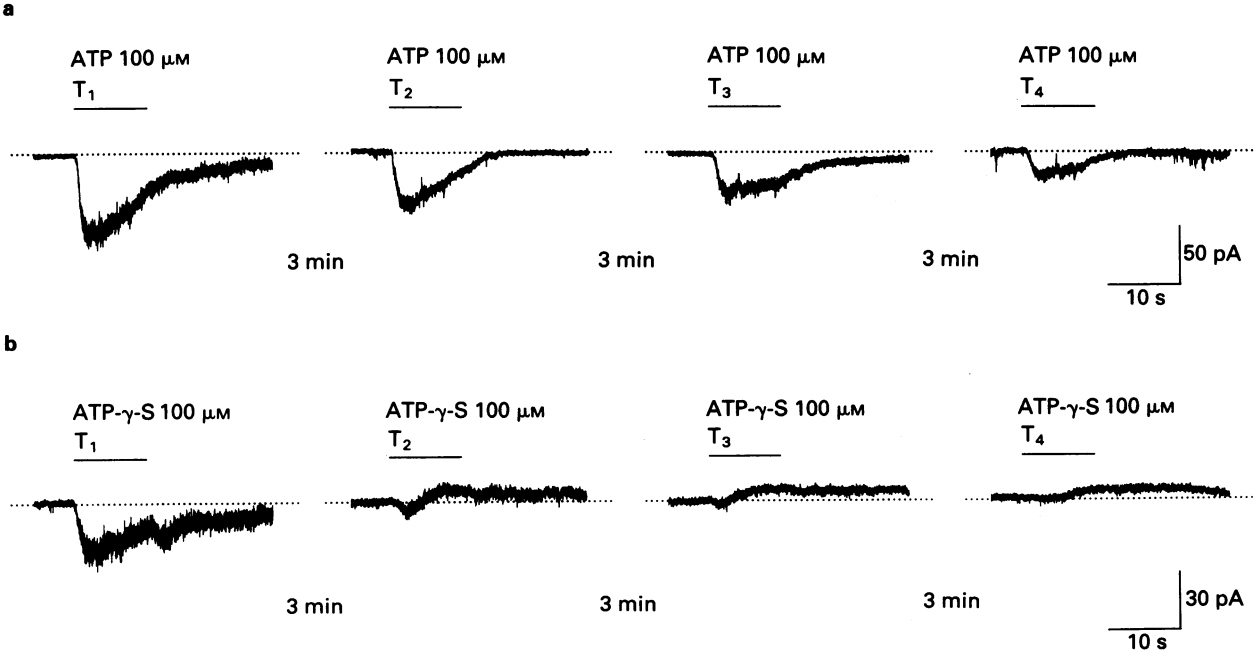

Figure 1 Inward currents activated by consecutive applications of ATP or ATP-γ-S in non-proliferating rat microglia. The holding potential was − 70 mV. Both ATP and ATP-γ-S were pressure-applied onto the same cell for 10 s and at 3 min intervals. (a) Currents evoked by 4 consecutive applications (T_1–T_4) of ATP (100 μM). The inward current appeared to decrease from T_1 to T_4, with a major and statistically significant decline from T_1 (− 26.7 ± 8.5 pA) to T_2 (− 11.9 ± 4.9 pA; $n = 7$; $P < 0.05$) only. (b) Currents evoked by 4 consecutive applications (T_1–T_4) of ATP-γ-S (100 μM). The inward current decreased from T_1 (− 16.8 ± 5.0 pA) to T_2 (− 6.9 ± 2.2 pA; $n = 7$; $P < 0.05$). At the same time the outward current increased (T_1, 3.9 ± 1.8 pA; T_2, 6.9 ± 1.7 pA; $n = 7$; $P < 0.05$). Representative tracings from 7 similar experiments in both (a) and (b). The dotted lines indicate the zero current levels.

reversal potentials. The slope conductance of the curve was determined, as in all similar experiments, between − 130 and − 110 mV and amounted to 1.7 ± 0.4 nS ($n = 8$).

The application of Cs^+ (1 mM) to the bath-medium abolished the inward rectification of voltage-dependent control currents (Figure 3b, inset). When 4-aminopyridine (1 mM) was also applied to block outward rectification, the 2-methylthio ATP current crossed the zero current level at a single point only, in the range of the calculated K^+ equilibrium potential (− 89 mV) at − 75.0 ± 5.3 mV ($n = 6$) (Figure 3b). This may be due to the fact that 4-aminopyridine blocks in addition to the voltage-dependent outward rectification (Figure 3b, inset) also the 2-methylthio ATP-induced inward, cationic component (Figure 2b). The slope conductance of the curve was 3.6 ± 1.3 nS ($n = 6$).

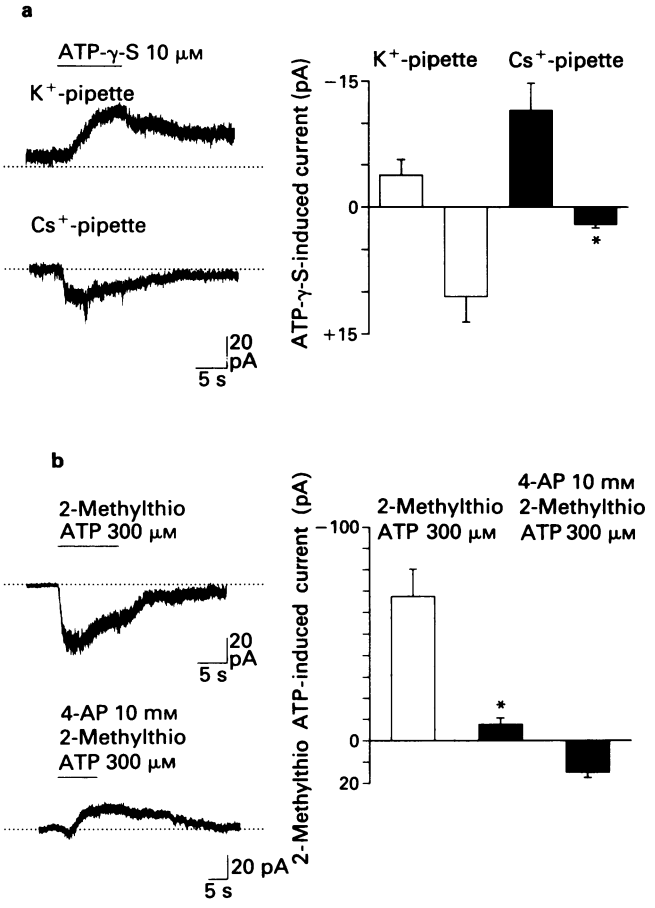

Figure 2 Selective blockade of the outward or inward components of the ATP-γ-S- and 2-methylthio ATP-evoked currents in non-proliferating rat microglia. Both purinoceptor agonists were pressure applied for 10 s at a holding potential of − 70 mV. Each agonist concentration was applied only once and to different cells. (a) Currents induced by ATP-γ-S (10 μM) were recorded with pipette solutions in which K^+ (150 mM; open columns) or Cs^+ (150 mM; solid columns) were the main intracellular cation, respectively. Representative tracings (left panel). Means ± s.e.mean from 7 experiments are shown in the right panel similar to those shown in the left panel. $*P < 0.05$; significant difference between the outward currents in the 1st and 2nd sets of columns. (b) Currents induced by 2-methylthio ATP (300 μM) were recorded either in the absence ($n = 6$; open columns) or presence ($n = 8$; solid columns) of 4-aminopyridine (4-AP; 10 mM). Representative tracings are shown in the left panel. Means ± s.e.mean from n experiments similar to those in the left panel are shown in the right panel. $*P < 0.05$; significant difference between the inward currents in the absence and presence of 4-aminopyridine. The dotted lines indicate the zero current levels both in (a) and (b). Note that inward currents are indicated by upward columns, while outward currents are indicated by downward columns.

Replacement of K^+ in the pipettes by Cs^+ (150 mM) and the simultaneous presence of Cs^+ (1 mM) in the bath medium were another means of blocking all voltage-dependent conductances (Figure 4a, inset). However, the 2-methylthio ATP current crossed now the zero current level near 0 mV (7.3 ± 1.8 mV; $n = 6$) and showed inward rectification (Figure 4a). Apparently extracellular Cs^+ abolished the outward, K^+-component of this current (Figure 2a). The slope conductance of the curve (2.2 ± 0.8 nS; $n = 6$; $P > 0.05$) did not differ from that measured in the combined presence of extracellular 4-aminopyridine (1 mM) and Cs^+ (1 mM).

Finally, the experimental conditions in Figure 4b are similar to those in Figure 4a in that Cs^+ is present both intra- and extracellularly. In a Na^+-free, high Ca^{2+} (100 mM) bath medium, the 2-methylthio ATP current crossed the zero current level at − 4.2 ± 2.6 mV ($n = 5$) and differed from I_{ATP} determined in a normal Na^+ (160 mM), normal Ca^{2+} (2 mM) bath medium by its failure to exhibit inward rectification (Figure 4b). Thus, Ca^{2+} was able to substitute for Na^+ as a charge carrier of the 2-methylthio ATP-induced inward, cationic component. The slope conductance (4.2 ± 2.1 nS; $n = 5$; $P > 0.05$) was similar to that of the reference curve in Figure 4a.

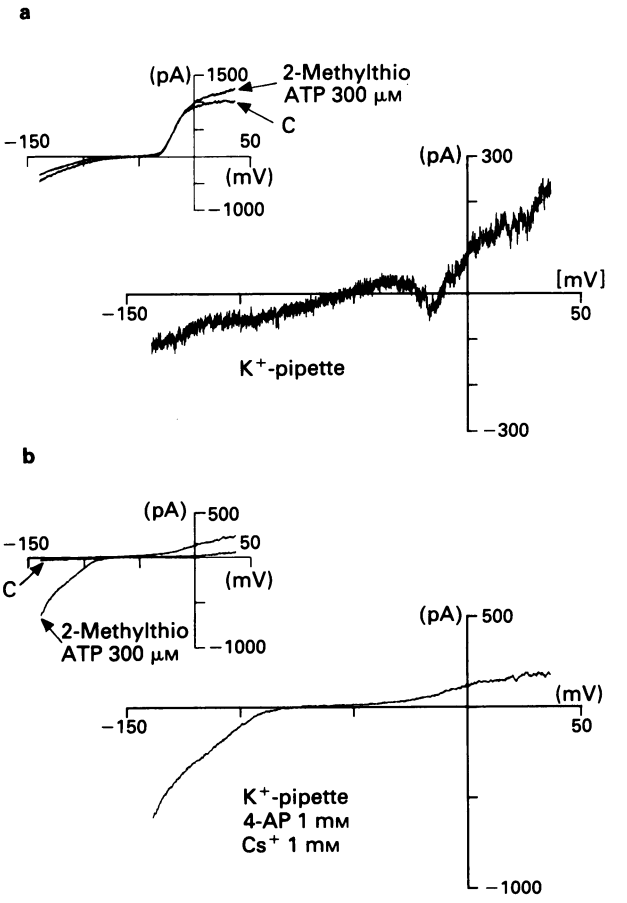

Figure 3 Current-voltage ($I-V$) relations of the 2-methylthio ATP-induced dual conductance (a) or potassium conductance (b) in non-proliferating microglia. $I-V$ relations of microglial cells were determined by applying depolarizing voltage ramps (− 70 mV holding potential, 1 s duration, from − 140 to + 40 mV) before a 10 s pressure application of 2-methylthio ATP (300 μM) and during the peak response to the agonist (indicated in the insets of a and b). The 2-methylthio ATP current was obtained by subtraction. (a) 2-Methylthio ATP current recorded with a K^+-pipette in a normal bath medium; (b) 2-methylthio ATP current recorded with a K^+-pipette in the presence of extracellular 4-aminopyridine (4-AP; 1 mM) and Cs^+ (1 mM). Representative tracings from 8 (a) and 6 (b) similar experiments.

Rank order of P_2-purinoceptor agonist potencies and blockade of agonist effects by P_2-purinoceptor antagonists Figure 5a shows concentration-response curves of various purinoceptor agonists and UTP in non-proliferating microglia. Of the compounds investigated, adenosine and UTP were inactive. At a concentration of 1 mM, 2-methylthio ATP induced the largest inward current (-100.5 ± 14.6 pA; $n = 8$), followed by ATP-γ-S (-30.7 ± 8.9 pA; $n = 7$) and ATP (-30.1 ± 6.5 pA; $n = 5$); α,β-MeATP had the lowest activity (-6.7 ± 3.1 pA; $n = 7$). Only the concentration-response curves of ATP and α,β-MeATP exhibited a clear maximum. The concentration causing a half maximal effect (EC_{50} value) was 25.1 μM for ATP. In the case of 2-methylthio ATP and ATP-γ-S there were no maximal effects and, therefore, EC_{50} values could not be determined. Finally, 2-methylthio ATP (300 μM) produced the same inward current both in the presence and absence of Mg^{2+} (1 mM) in the bath-medium (Figure 5a, inset).

When 2-methylthio ATP (300 μM) was applied twice, spaced 10 min apart, constant responses were obtained (Figure 5b). Incubation with suramin (300 μM) or reactive blue 2 (50 μM) for 8 min markedly reduced the effect of 2 methylthio ATP. Higher concentrations of the purinoceptor antagonists were not investigated.

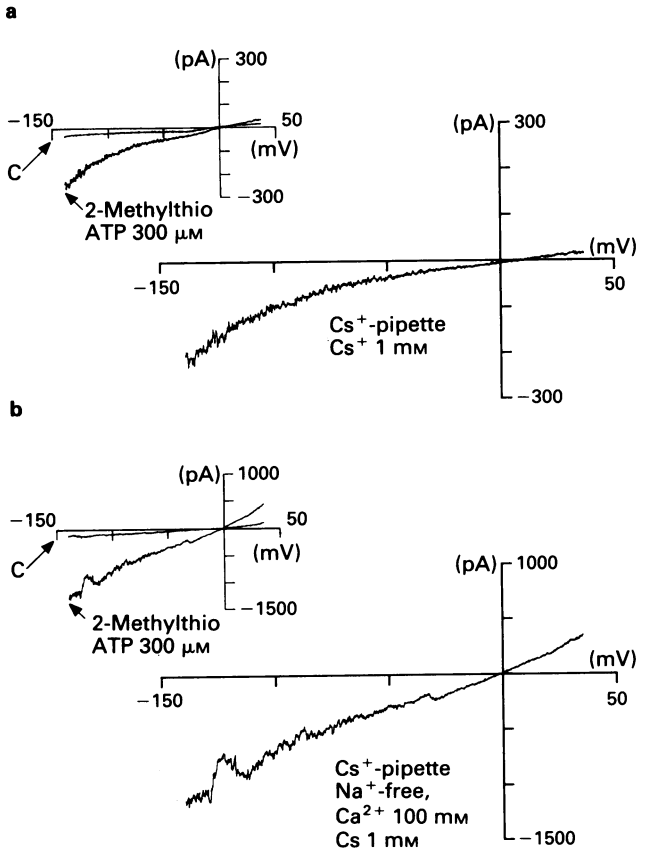

Figure 4 Current-voltage ($I-V$) relations of the 2-methylthio ATP-induced cationic conductance (a,b) in non-proliferating microglia. $I-V$ relations of microglial cells were determined by applying depolarizing voltage ramps (− 70 mV holding potential, 1 s duration, from − 140 to + 40 mV) before a 10 s pressure application of 2-methylthio ATP (300 μM) and during the peak response to the agonist (indicated in the insets of a and b). The 2-methylthio ATP current was obtained by subtraction. (a) 2-Methylthio ATP current recorded with a Cs^+ (150 mM) pipette and in the additional presence of extracellular Cs^+ (1 mM); (b) 2-methylthio ATP current recorded with a Cs^+-pipette and in the additional presence of extracellular Cs^+ (1 mM). A Na^+-free medium also containing Ca^{2+} (100 mM) was used. Representative tracings out of 6 (a) and 5 (b) similar experiments.

Comparison of ATP-induced inward currents in proliferating and non-proliferating microglia In a subsequent series of experiments currents were elicited by both negative and positive voltage pulses given in steps of 20 mV from a holding potential of − 70 mV (Figure 6a). Proliferating microglial cells exhibited only a hyperpolarization-evoked inward current; depolarizing steps were without effect ($n = 3$; Figure 6a, left panel). In contrast, non-proliferating cells responded to depolarizing pulses with an outward current, although the inwardly rectifying channels were also present ($n = 3$; Figure 6a, right panel). In spite of the different pattern of voltage-dependent current responses, 2-methylthio ATP (1–1000 μM) induced the same inward current in the two types of microglial cells (Figure 6b).

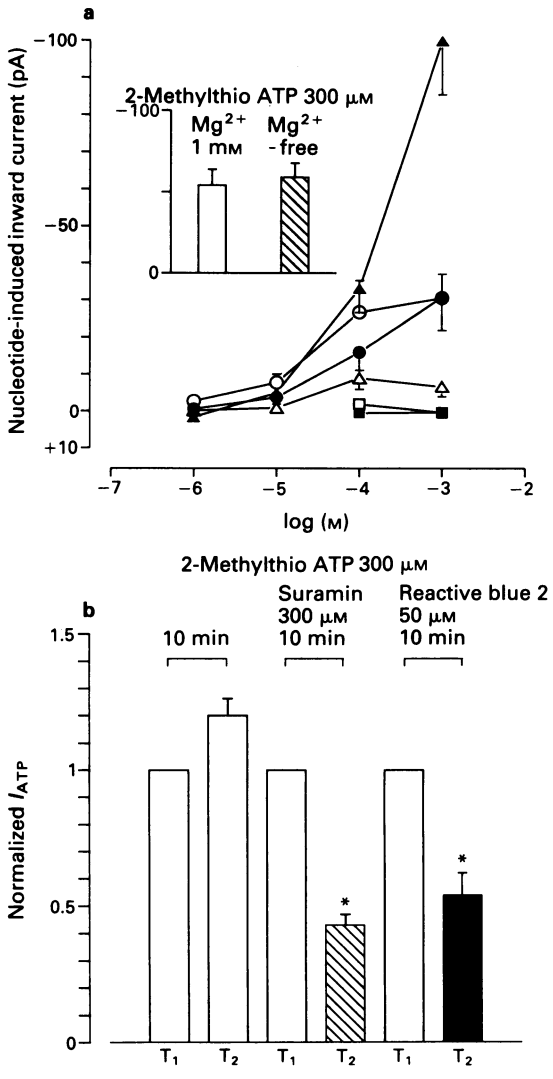

Figure 5 Pharmacological characterization of ATP-sensitive receptors in non-proliferating rat microglia. Inward currents obtained at a holding potential of − 70 mV were evaluated only. (a) Concentration-response curves to ATP (○), ATP-γ-S (●), α,β-methylene ATP (α,β-MeATP; △), 2-methylthio ATP (▲), adenosine (□) and UTP (■). The current induced by 2-methylthio ATP (300 μM) was the same both in the presence ($n = 6$) and absence of Mg^{2+} (2 mM; $n = 7$) (inset). Each concentration was pressure applied for 10 s, only once and to different cells. (b) Antagonism of the 2-methylthio ATP-(300 μM) induced current by suramin (300 μM; hatched column) or reactive blue 2 (50 μM; solid column). 2-Methylthio ATP (300 μM) was pressure applied twice for 10 s with 10 min interval (T_1-T_2), either in the absence (open columns) or presence of the antagonists. The effects of T_2 were normalized with respect to the effects of T_1. $^*P < 0.01$: significant difference between T_1 and T_2 in the 2nd and 3rd sets of columns.

Measurements of membrane potential changes

Comparison of ATP-induced depolarizing effects in proliferating and non-proliferating microglia The membrane potential of microglial cells, 5–10 min after gaining access to the cell interior was continuously recorded in the current-clamp mode of the patch-clamp amplifier. Random alterations of the membrane potential between two preferred values near − 35 and − 70 mV were observed. In most experiments, 2-methylthio ATP was pressure applied when the membrane potential was in the range of − 70 mV (see legend to Figure 7). 2-Methylthio ATP (300 μM) caused a large and long-lasting depolarization in proliferating microglia (Figures 6c, left panel and 7b). In contrast, the majority of non-proliferating cells responded to 2-methylthio ATP (300 μM) with a rapidly declining small depolarization (Figures 6c, right panel, 7a, upper panel and 7b). A few non-proliferating cells with a low membrane potential (− 39.4 ± 1.6 mV), were hyperpolarized by 2-methylthio ATP (300 μM) (− 10.1 ± 1.4 mV; $n = 5$).

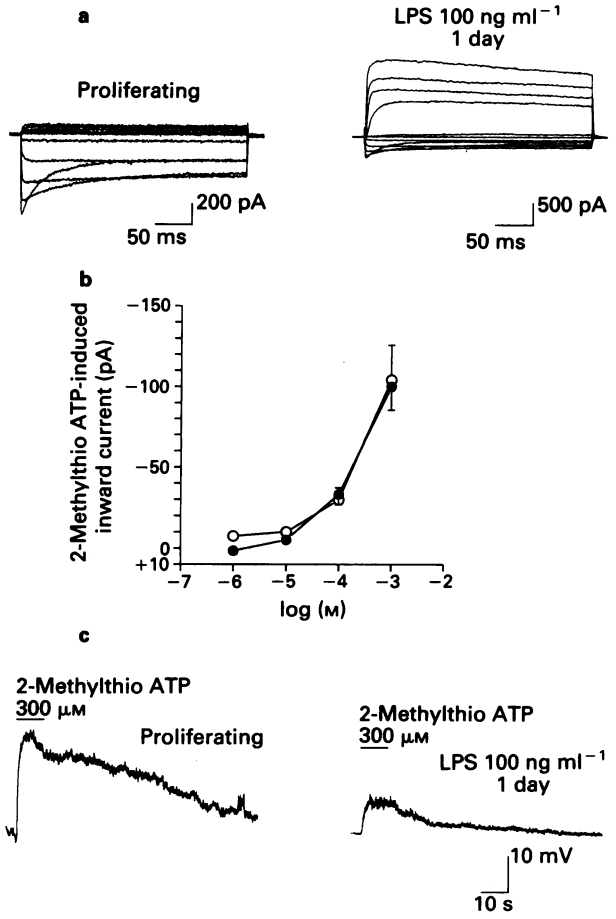

Figure 6 Voltage-sensitive and 2-methylthio ATP-evoked currents in proliferating and non-proliferating rat microglia. (a) Voltage pulses were applied every 8 s from a holding potential of − 70 mV in 20 mV increments. Step range was from − 170 to + 50 mV. Proliferating ($n = 3$; left panel) and lipopolysaccharide (LPS)- (100 ng ml^{-1} for 12–24 h) treated (non-proliferating; $n = 3$; right panel) cells were used. (b) Concentration-response curves to 2-methylthio ATP in proliferating (○) and non-proliferating (●) microglia. Inward currents obtained by 10 s pressure applications at a holding potential of − 70 mV were evaluated. Each concentration was applied only once and to different cells. Means ± s.e.mean from 5–8 (proliferating) and 5–7 (non-proliferating) cells. (c) Depolarization induced by a 10 s pressure application of 2-methylthio ATP (300 μM) to proliferating ($n = 8$) and non-proliferating ($n = 14$) rat microglial cells.

Role of voltage-sensitive potassium channels in ATP-induced depolarizations The normal pipette solution contained 0.01 μM free Ca^{2+}. Elevation of the internal free Ca^{2+} concentration to 1 μM, increased the amplitude and duration of 2-methylthio ATP- (300 μM) induced depolarizations (Figures 7a, lower panel and 7b). Recordings with 0.01 and 1 μM internal Ca^{2+} were carried out alternately on cells from the same culture dishes. The application of 4-aminopyridine (1 mM) increased the duration, but not the amplitude of the depolarizing response to 2-methylthio ATP (300 μM) (Figure 7b).

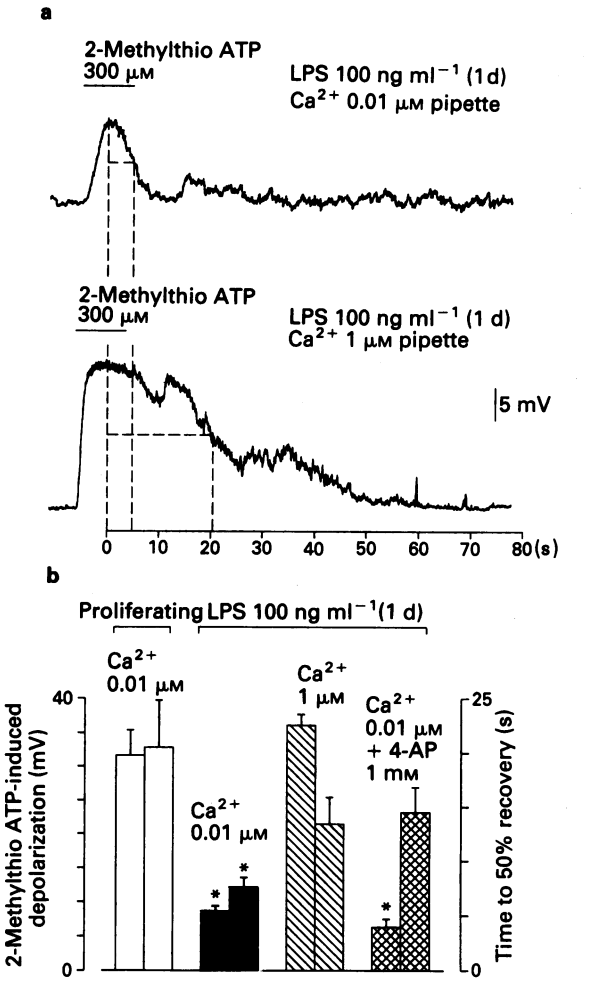

Figure 7 Role of voltage-sensitive potassium channels in the 2-methylthio ATP-induced depolarization. 2-Methylthio ATP (300 μM) was pressure applied for 10 s to proliferating and lipopolysaccharide (LPS)- (100 ng ml^{-1} for 12–24 h) treated (non-proliferating) rat microglial cells. (a) Representative depolarizations recorded with pipettes containing 0.01 μM (upper panel) or 1 μM (lower panel) Ca^{2+} in non-proliferating cells. The time to 50% recovery of depolarization was calculated as indicated. (b) Amplitude (left column of each pair) and time to 50% recovery (right column of each pair) of the 2-methylthio ATP-(300 μM) induced depolarization. Recordings were with 0.01 μM free Ca^{2+}-containing pipettes in proliferating (open column; membrane potential, − 61.0 ± 5.4 mV; $n = 8$) or non-proliferating microglia (solid column; membrane potential, − 61.7 ± 4.6 mV; $n = 14$). Other recordings were with 0.01 μM free Ca^{2+}-containing pipettes in the presence of 4-aminopyridine (4-AP; hatched columns; membrane potential, − 79.3 ± 1.2 mV; $n = 8$) or with 1 μM free Ca^{2+}-containing pipettes (cross-hatched columns: membrane potential, − 69.5 ± 4.9 mV; $n = 8$) in non-proliferating microglia. $^*P < 0.05$; significant difference from the amplitude and time to 50% recovery of 2-methylthio ATP-induced depolarization in proliferating microglia, respectively.

Discussion

The membrane potential of proliferating and non-proliferating rat microglial cells exhibits two stable values resulting in random switching between approximately − 35 mV and − 70 mV (Nörenberg *et al.*, 1993b). The reason for this behaviour is a negative slope region of the *I–V* curves reported also for mouse macrophages (Gallin, 1981) and human T-lymphocytes (Maltsev, 1993). In most experiments of this study, microglial cells were held at the preferred membrane potential of − 70 mV.

The predominant membrane current induced by ATP and its structural analogues was inward at − 70 mV. However, intermediate concentrations of ATP-γ-S and, to a minor extent, of 2-methylthio ATP and ATP, evoked an additional outward current, which followed the rapidly desensitizing inward current. Moreover, the repetitive application of ATP-γ-S, which appears to cause a stronger desensitization of P_2-purinoceptors than 2-methylthio ATP, led to the disappearance of the inward component and, thereby, to the enhancement or even unmasking of a non-desensitizing outward component. This outward component was inhibited when Cs^+ instead of K^+ was the main intracellular cation. Furthermore, with Cs^+-containing pipettes, the inward component became larger or, in some cases, a previously absent inward component emerged. The likely explanation for these changes is the blockade of K^+ outward currents by occlusion of the inner channel mouth by Cs^+ (Bezanilla & Armstrong, 1972).

In contrast, Cs^+ was unable to alter 2-methylthio ATP-elicited inward currents. However, in the presence of 4-aminopyridine, the inward current was nearly abolished and an outward component became unmasked. Apparently, in addition to the well-known ability of 4-aminopyridine to block both delayed rectifier and transient potassium channels (Adams & Nonner, 1990), this compound may inhibit ATP-activated cation channels as well. This novel effect of 4-aminopyridine was unexpected, but firmly established in two independent series of experiments. Hence, P_2-agonists may initiate a conductance increase both towards cations and potassium ions, which predominate at − 70 mV and − 35 mV, respectively. The present findings agree with the reported occurrence of an ATP-induced non-selective cationic conductance in proliferating microglia (Kettenmann *et al.*, 1993). A recent study of the same group, published during the preparation of this manuscript, demonstrated an additional increase of K^+ conductance by ATP in proliferating microglia (Walz *et al.*, 1993). We observed both types of conductances also in non-proliferating cells.

The *I–V* relation of non-proliferating microglial cells exhibited strong inward and outward rectification (Nörenberg *et al.*, 1992; 1993b). The ATP-evoked current obtained by subtraction crossed the zero current level in the range of the potassium equilibrium potential (− 89 mV) and additionally near the equilibrium potential for non-selective cationic conductances (0 mV). A combination of intra- and extracellular Cs^+ appears to inhibit voltage-sensitive, inwardly and outwardly rectifying K^+ channels of microglia (Nörenberg *et al.*, 1993b). Furthermore, intracellular Cs^+ has been shown to block 2-methylthio ATP-induced outward K^+ currents (see above). In fact, only when Cs^+ was present both intra- and extracellularly did the ATP current cross the zero current level near 0 mV and exhibited inward rectification similar to that described for I_{ATP} in neurones and smooth muscle cells (Bean & Friel, 1990).

It is interesting to note that when K^+ channels were blocked by Cs^+, and a Na^+-free, high Ca^{2+} bath medium was used, the ATP current persisted, but its inward rectification disappeared. Hence, the channels were permeable not only to Na^+, but also to Ca^{2+}, as reported previously for various cell types (Bean & Friel, 1990; Bean, 1992). The permeability of Ca^{2+} relative to that of Na^+ varies widely from 3:1 in ear artery smooth muscle (Benham & Tsien, 1987) to 0.3:1 in sensory neurones (Bean *et al.*, 1990). No attempt was undertaken in the present study to determine the permeability ratio of these two cations. In contrast to conditions which block the potassium component of the ATP current, extracellular application of 4-aminopyridine abolished the cationic component. In the additional presence of extracellular Cs^+, an increased K^+ conductance was left behind with the expected intersection of the zero current level in the range of the K^+ equilibrium potential.

In view of the monocytic origin of microglia it is interesting to note that ATP is able to permeabilize to cations the plasma membranes of rat (Naumov *et al.*, 1992) and mouse peritoneal macrophages (Steinberg *et al.*, 1987), and of the murine macrophage-like cell-line J774 (Sung *et al.*, 1985; Steinberg *et al.*, 1987). This effect is associated with depolarization (Sung *et al.*, 1985; Buisman *et al.*, 1988) and an increase in membrane conductance (Buisman *et al.*, 1988). Membrane permeabilization also leads to an enhanced entry of Ca^{2+} into macrophages (Sung *et al.*, 1985), although an additional release of Ca^{2+} from intracellular stores may be also involved (Greenberg *et al.*, 1988). ATP has been shown to increase the intracellular Ca^{2+} concentration of mouse peritoneal macrophages and, in consequence, may activate Ca^{2+}-dependent K^+ channels (Hara *et al.*, 1990). All these effects are due to the stimulation of P_{2z}-purinoceptors by ATP^{4-} (Steinberg *et al.*, 1987; Greenberg *et al.*, 1988).

The present experiments were principally aimed at elucidating the receptors involved in the microglial I_{ATP}. The lack of effect of adenosine and UTP excluded the presence of P_1-purinoceptors and pyrimidinoceptors. In contrast, ATP and its analogues were effective and appeared to increase the permeability of the membrane by receptor-stimulation, rather than by phosphorylation of extracellular proteins. ATP-induced phosphorylation is Mg^{2+}-dependent (see e.g. Christie *et al.*, 1992) and, therefore, the similar amplitudes of inward currents induced by 2-methylthio ATP both in the presence and absence of extracellular Mg^{2+} (1 mM) favour a receptor-mediated effect. Additional arguments also support this conclusion: (1) The ATP-induced inward current strongly desensitized both during contact with the agonists and on repetitive agonist applications; this phenomenon was observed previously in muscle preparations and neurones (Bean & Friel, 1990; Illes & Nörenberg, 1993). (2) Suramin and reactive blue 2 antagonized the effect of 2-methylthio ATP. Suramin is an antagonist at both P_{2X}- and P_{2Y}-purinoceptors, while reactive blue 2 appears to display some preference for the latter receptor-type (Kennedy, 1990). (3) The rank order of agonist potency (2-methylthio ATP > ATP > α,β-MeATP) was also compatible with the presence of P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985; Kennedy, 1990). This strongly suggests that peripheral macrophages (P_{2Z}) and resident brain macrophages (microglia; P_{2Y}) are endowed with a distinct population of purinoceptors.

ATP has been reported to cause a long-lasting depolarization in proliferating rat microglia, because voltage-dependent, outwardly rectifying K^+ channels are absent and there are no efficient means of repolarizing the membrane (Kettenmann *et al.*, 1993). Cellular damage may both activate microglia (Dickson *et al.*, 1991), and allow leakage of ATP from neurones and glial cells into the extracellular space (White & Hoehn, 1991). In the present study, proliferating microglia were incubated with LPS in order to drive them into a more activated state (Adams & Hamilton, 1987). It was confirmed that non-proliferating microglial cells express previously missing, outwardly rectifying K^+ channels (Nörenberg *et al.*, 1992; 1993b) which are able to restore efficiently the resting membrane potential after depolarization. Thus, although in proliferating and non-proliferating cells 2-methylthio ATP produced identical inward current amplitudes, the depolarizing response to 2-methylthio ATP was much larger in proliferating than in non-proliferating cells. Moreover, manipulations known to block this type of K^+ channel, such as an enhanced intracellular Ca^{2+} concentration and extracellular

4-aminopyridine (Nörenberg *et al.*, 1993b), increased the extent and/or duration of the ATP-induced depolarization. The amplitude of the depolarization did not increase after the application of 4-aminopyridine, because this compound has a dual effect. It blocks voltage-dependent potassium conductances but seems to inhibit the cationic current initiated by P_2-purinoceptors as well.

In conclusion, ATP and its structural analogues stimulate P_{2Y}-purinoceptors in rat microglia. The mode of action of these receptors is the opening of cationic channels, which causes depolarization at the preferred membrane potential of − 70 mV. The concomitant opening of potassium channels causes hyperpolarization at the other preferred membrane potential of − 35 mV. The expression of outwardly rectifying K^+ channels in activated microglia decreases the sensitivity of these cells towards the depolarizing, excitatory effect of ATP.

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