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Coexistence of purino- and pyrimidinoceptors on activated rat microglial cells

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1 Nucleotide-induced currents in untreated (proliferating) and lipopolysaccharide (LPS; 100 ng ml^{-1}) treated (non-proliferating) rat microglial cells were recorded by the whole-cell patch-clamp technique. Most experiments were carried out on non-proliferating microglial cells. ATP (100 nM–1 mM), ADP (10 nM–10 mM) and UTP (1 μM–100 mM), but not uridine (100 μM–10 mM) produced a slow outward current at a holding potential of 0 mV. The effect of UTP (1 mM) did not depend on the presence of extracellular Mg^{2+} (1 mM). The outward current response to UTP (1 mM) was similar in non-proliferating and proliferating microglia.

2 In non-proliferating microglial cells, the ATP (10 μM)-induced outward current was antagonized by suramin (300 μM) or reactive blue 2 (50 μM), whereas 8-(p-sulphophenyl)-theophylline (8-SPT; 100 μM) was inactive. By contrast, the current induced by UTP (1 mM) was increased by suramin (300 μM) and was not altered by reactive blue 2 (50 μM) or 8-SPT (100 μM).

3 The current response to UTP (1 mM) disappeared when K^+ was replaced in the pipette solution by an equimolar concentration of Cs^+ (150 mM). However, the effect of UTP (1 mM) did not change when most Cl^- was replaced with an equimolar concentration of gluconate$^-$ (145 mM). The application of 4-aminopyridine (1 mM) or Cs^+ (1 mM) to the bath solution failed to alter the UTP (1 mM)-induced current. UTP (1 mM) had almost no effect in a nominally Ca^{2+}-free bath medium, or in the presence of charybdotoxin (0.1 μM); the inclusion of U-73122 (5 μM) or heparin (5 mg ml^{-1}) into the pipette solution also blocked the responses to UTP (1 mM). By contrast, the effect of ATP (10 μM) persisted under these conditions.

4 *I*-V relations were determined by delivering fast voltage ramps before and during the application of UTP (1 mM). In the presence of extracellular Cs^+ (1 mM) and 4-aminopyridine (1 mM) the UTP-evoked current crossed the zero current level near −75 mV. Omission of Ca^{2+} from the Cs^+ (1 mM)- and 4-aminopyridine (1 mM)-containing bath medium or replacement of K^+ by Cs^+ (150 mM) in the pipette solution abolished the UTP current.

5 Replacement of GTP (200 μM) by GDP-β-S (200 μM) in the pipette solution abolished the current evoked by UTP (1 mM).

6 When the pipette solution contained Cs^+ (150 mM) instead of K^+ and in addition inositol 1,4,5,-trisphosphate ($InsP_3$; 10 μM), an inward current absolutely dependent on extracellular Ca^{2+} was activated after the establishment of whole-cell recording conditions. This current had a typical delay, a rather slow time course and did not reverse its amplitude up to 100 mV, as measured by fast voltage ramps.

7 A rise of the internal free Ca^{2+} concentration from 0.01 to 0.5 μM on excised inside-out membrane patches produced single channel activity with a reversal potential of 0 mV in a symmetrical K^+ solution. The reversal potential was shifted to negative values, when the extracellular K^+ concentration was decreased from 144 to 32 mM. By contrast, a decrease of the extracellular Cl^- concentration from 164 to 38 mM did not change the reversal potential.

8 Purine and pyrimidine nucleotides act at separate receptors in rat microglial cells. Pyrimidinoceptors activate via a G protein the enzyme phospholipase C with the subsequent release of $InsP_3$. The depletion of the intracellular Ca^{2+} pool appears to initiate a capacitative entry of Ca^+ from the extracellular space. This Ca^{2+} then activates a Ca^{2+}-dependent K^+ current.

Keywords: Microglia; P_{2Y} purinoceptor; pyrimidinoceptor; ATP; UTP; Ca^{2+}-dependent potassium channel; G protein; inositol 1,4,5-trisphosphate

Introduction

The origin of microglial cells has been a major issue of debate for a number of years; it has been described alternatively as mesodermal, monocytic or neuroectodermal (Perry & Gordon, 1988). Recently, there is a growing concensus that microglia derive from monocytes/macrophages entering the brain during embryonic development (Thomas, 1992; Theele & Streit, 1993). Hence, microglia are resident immunocytes of the brain which are normally in the resting state in analogy to other tissue macrophages, e.g. in the bone marrow, liver and peritoneum (Jordan & Thomas, 1988). Neuronal damage leads to their activation, involving several steps and multiple factors such as colony stimulating factors, lipopolysaccharide (LPS) and interferon-γ (Adams & Hamilton, 1987; Thomas, 1992). Activated, macrophage-like microglia no longer proliferate, present major histocompatibility antigen (MHC) types I and II, secrete cytokines and produce superoxide anions (Dickson *et al*., 1991).

Adenosine 5′-triphosphate (ATP)-sensitive P_2 purinoceptors have been classified into the P_{2X} and P_{2Y} types, the former being a ligand-activated cationic channel, the latter a G protein-coupled receptor (Abbracchio & Burnstock, 1994; Fred-

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holm *et al*., 1994). P_{2U} receptors are also coupled to G proteins, but are activated both by ATP and the pyrimidine nucleotide uridine 5′-triphosphate (UTP; Harden *et al*., 1995). Whereas all these purinoceptor-types (P_{2X}, P_{2Y}, P_{2U}) are sensitive to $MgATP^{2-}$, P_{2Z} purinoceptors of macrophages and lymphocytes respond to tetraanionic ATP (ATP^{4-}) only (Gordon, 1986). It is noteworthy that a separate receptor recognizing UTP has been described in some tissues (von Kügelgen *et al*., 1987; Abbracchio & Burnstock, 1994).

ATP^{4-} permeabilizes the plasma membrane of mouse macrophages and the mouse macrophage-like cell line J774.2 (Steinberg *et al*., 1987). The resulting 800 Da pores allow not only the free passage of inorganic cations and anions, but also the entry of fluorescent dyes (Steinberg *et al*., 1987). The opening of the large non-selective pores inevitably leads to an enhanced entry of Ca^{2+} into macrophages (Sung *et al*., 1985), although the opening of smaller pores, sufficient for the passage of di- and monovalent organic cations only, may also subserve Ca^{2+} fluxes (Naumov *et al*., 1992). An additional release of Ca^{2+} from intracellular stores occurs due to the stimulation by ATP of a phospholipase C-coupled P_{2U} purinoceptor (Alonso-Torre & Trautmann, 1993; Murgia *et al*., 1993). The subsequent release of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate ($InsP_3$; Berridge, 1993) and the influx of Ca^{2+} via the plasma membrane presumably associated with the depletion of intracellular Ca^{2+} stores (Putney & Bird, 1993) may activate Ca^{2+}-dependent K^+ channels ($I_{K(Ca)}$; Alonso-Torre & Trautman, 1993).

Recently the presence of ATP-sensitive P_{2X} (Nörenberg *et al*., 1994b) and P_{2Y} purinoceptors (Nörenberg *et al*., 1994b; Langosch *et al*., 1994) has been shown in rat cultured microglial cells activated by lipopolysaccharide (LPS) (Illes *et al*., 1996). The purpose of the present study was three fold. Firstly, to investigate whether UTP-sensitive receptors are present in these cells. Secondly, it was attempted to clarify whether UTP acts at P_{2U} purinoceptors or a separate pyrimidinoceptor-type. Thirdly, the underlying ionic basis of the response to UTP was investigated and compared with that of ATP.

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 and 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, 13–23 μm).

Whole-cell recordings

Membrane currents of microglial cells were measured with the patch-clamp method in the whole-cell configuration. 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 Ca^{2+}- or Mg^{2+}-free bath solution was prepared by omitting $CaCl_2$ or $MgCl_2$. A high Ca^{2+} (10 mM) bath solution was obtained by adding 8 mM $CaCl_2$ and omitting 8 mM NaCl from the standard medium. 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. In some experiments the intracellular KCl was replaced by CsCl and the pH was set to 7.3 by adding CsOH. A low Cl^- pipette solution differed from the standard pipette solution in that it contained 5 mM KCl and 145 mM K-gluconate. 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 capacitative currents. The resistance of the electrodes was 2–5 MΩ.

Compensation of membrane time constant (τ_u) capacitance (C_m) and series resistance (R_s) was achieved with the inbuilt circuitry of the patch amplifier (List EPC-7, Darmstadt, Germany). These parameters were 380.5 ± 75.9 μs, 22.9 ± 1.5 pF and 18.3 ± 4.6 MΩ, respectively in 22 cells. Data were digitized at 1 kHz (Cambridge Electronic Devices 1401, Cambridge, U.K.) and then stored on and analysed with a laboratory computer (ESCOM/486, Heppenheim, Germany). 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 purinoceptor agonists, UTP and uridine were measured, the cells were held at 0 mV. All agonists were locally microperfused for 10 s by means of separate, wide-bore (3–5 μm), pressurized (10 kPa) puffer pipettes, placed about 50 μm away from the cell under examination.

Each agonist concentration was applied only once to a cell in order to construct concentration-response curves. The curves were fitted to the data points by using the equation:

$$I/I_{max} = 1/\{1 + (K_D/[ATP])^{n_H}\}$$

where K_D is the apparent dissociation constant which equals the concentration evoking a half-maximal current (EC_{50}), and n_H is the Hill coefficient. Fitting was with the software package SigmaPlot (Jandel, Erkrath, Germany) by least-squares nonlinear regression according to the Marquardt-Levenberg algorithm.

From the concentration-response curves submaximal concentrations of ATP (100 μM) and UTP (1 mM) were selected for further experiments. These agonist concentrations were applied both under control conditions (standard bath and pipette solution; T_c) to one group of cells and under conditions of a modified bath and/or pipette solution (T_e) onto another group of cells kept in the same culture dish. Then the currents induced by ATP or UTP at T_e were normalized with respect to the mean control response to the same agonists at T_c. In some experiments two subsequent agonist applications (T_1, T_2) separated by 10 min intervals were made and the current at T_2 was normalized with respect to the current at T_1. During the interval between T_1 and T_2 the bath was superfused with drug-free medium or with medium containing purinoceptor antagonists at a flow rate of 2 ml min^{-1}. Charybdotoxin was locally microperfused immediately before T_2 for 1 min by means of separate puffer pipettes (see above). Puffer application of drug-free extracellular solution failed to induce a current response.

Current-voltage (*I*-V) relations were determined by using fast voltage ramps (0 mV holding potential, 1 s duration, from −130 to 50 mV). Ramps were generated both before the pressure application of UTP (1 mM) and during the peak re-

sponse to the agonist. The UTP current was obtained by digital subtraction.

The reversal potential of the calcium-release activated calcium current (I_{CRAC}) was measured by applying at a holding potential of 0 mV voltage ramps of 50 ms duration from −100 to 100 mV at a frequency of 1 Hz. Data were sampled at 10 kHz and filtered at 500 Hz.

Single-channel recordings

Single-channel currents were recorded from excised inside-out membrane patches of microglial cells. The (low Ca^{2+}) solutions bathing both sides of the membrane were identical (mM): KCl 144, NaCl 16, $MgCl_2$ 2, HEPES 5, EGTA 0.08, Ca^{2+} 0.0088 (free Ca^{2+}, 0.01 μM; Barrett *et al*., 1981), pH adjusted to 7.3 with KOH. Glucose 11 mM was present in the initial solution to maintain viability of microglia before the membrane patches were excised and was not present thereafter. In order to activate Ca^{2+}-dependent K^+ channels, the free Ca^{2+} at the intracellular membrane surface was increased to 0.5 μM by raising the Ca^{2+} concentration of the medium to 0.064 mM (Barrett *et al*., 1982). A low Ca^{2+}/low K^+/high Na^+ pipette solution contained 32 mM K^+, 128 mM Na^+ and 164 mM Cl^- (the concentration of KCl was reduced from 144 to 32 mM by replacement with NaCl). A low Ca^{2+}/low Cl^-/low Na^+ pipette solution contained 158 mM K^+, 2 mM Na^+ and 38 mM Cl^- (the standard 144 mM KCl was partially replaced by 126 mM K-gluconate and in addition the concentration of NaCl was reduced from 16 to 2 mM). Micropipettes of somewhat higher resistances (5–10 MΩ) than in whole-cell recordings were used. Data were digitized at 3 kHz and filtered at 1 kHz. Currents were amplified at a gain of 20 mV pA^{-1}.

Two different experimental approaches were used. In the first version excised inside-out patches were held at 40 mV in a low Ca^{2+} intracellular solution (free Ca^{2+}, 0.01 μM). Pressurized puffer pipettes filled with the same solution but containing elevated Ca^{2+} (free Ca^{2+}, 0.5 μM) were placed approximately 50 μm apart from the patch. Application was for brief periods (10–20 s) and was terminated by withdrawal of the puffer pipette allowing Ca^{2+} to diffuse away. In other experiments, first a set of data was sampled at low intracellular Ca^{2+} (free Ca^{2+}, 0.01 μM). Then the bath was superfused with a solution containing 0.5 μM free Ca^{2+} at a flow rate of 4 ml min^{-1} for 5 min. Then the superfusion was stopped and *I*-V relations of unitary current amplitudes were recorded at patch potentials of 40, 20, 0, −20, −40 and −60 mV. No attempts were made to terminate the effect of Ca^{2+}. Single channel conductance was estimated from *I*-V relations by fitting linear regressions to the experimental data.

Materials

The following materials and drugs were used: charybdotoxin (Bachem, Bubendorf, Switzerland); suramin hexasodium salt (Bayer, Wuppertal, Germany); Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution, foetal calf serum (FCS) (Gibco, Eggenstein, Germany); heparin sodium, 8-(p-sulphophenyl)-theophylline, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), reactive blue 2 (RBI, Natick, MA, U.S.A.); lipopolysaccharide from *Salmonella typhimurium* (Sebak, Aidenbach, Germany); adenosine 5′-diphosphate sodium salt (ADP), adenosine 5′-O-(2-thiodiphosphate) trilithium salt (ADP-β-S), adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-triphosphate magnesium salt (MgATP), 4-aminopyridine, guanosine 5′-O-(2-thiodiphosphate) tetralithium salt (GDP-β-S), guanosine 5′-triphosphate sodium salt (GTP), α, β-methyleneadenosine 5′-triphosphate dilithium salt (α,β-meATP), D-*myo*-inositol 1,4,5-trisphophate potassium salt, uridine, uridine 5′-triphosphate sodium salt (UTP) (Sigma, Deisenhofen, Germany).

LPS, dissolved in distilled water (1 mg ml^{-1}), was further diluted into DMEM. Stock solutions (1–100 mM) of all other drugs were prepared with distilled water with the exception of U-73122 which was dissolved in dimethyl sulphoxide. Aliquots were stored at −20°C. Further dilutions were made in the bath medium at respective experimental days.

Statistics

When appropriate, means ± s.e.mean of n trials are shown. Data were tested for normal distribution and equal variance by use of the Kolmogorov-Smirnov test with Lilifor's correction and the Levene Median test, respectively. Then, Student's unpaired t test was used to compare means of two independent experimental groups. Means obtained in the same cells before and after treatment were compared by Student's paired t test. Multiple comparisons between means were made by one way ANOVA followed by a t test with Bonferroni's correction. A probability level of 0.05 or less was considered to be statistically significant.

Results

Most of the experiments were carried out on proliferating microglia treated with LPS (100 ng ml^{-1}) for 12–24 h. This treatment has driven the cells into a more activated non-proliferating state.

Characterization of purinoceptors and pyrimidinoceptors with agonists

In previous studies, ATP and its structural analogue 2-methylthio ATP (2-MeSATP) caused at a holding potential of −70 mV a cationic inward current in non-proliferating microglia (Nörenberg *et al*., 1994b). By contrast, at a holding potential of 0 mV 2-MeSATP produced an outward potassium current, while the effect of ATP was not investigated (Langosch *et al*., 1994).

When ATP (10 μM) was pressure applied at 0 mV for 10 s, an outward current with a rather slow onset and offset developed (T_1; Figure 1a). However, when ATP (10 μM) was applied the second time (T_2) after a 10 min interval, there was almost no current response observed (Figure 1b). ATP (10 μM) had a reproducible effect in cells microdialysed with pipette solutions containing GTP (200 μM) or GTP (200 μM) plus MgATP (3 mM) (Figure 1b). Hence, in all further experiments GTP (200 μM) and MgATP (3 mM) were added to the pipette solution in order to avoid a time-dependent decrease of the ATP effect. The comparison of data was facilitated by normalizing the current at T_2 with respect to the current obtained at T_1 (see Methods).

UTP (1 mM) had no effect at a holding potential of −70 mV (−0.4 ± 1.6 pA; $n=7$), but at 0 mV produced an outward current (47.4 ± 3.2 pA; $n=94$) smaller in amplitude than that caused by ATP (10 μM; 68.3 ± 5.7 pA; $n=59$; $P<0.05$) (Figure 2a and b). It is interesting to note that the effect of ATP (10 μM) was rather consistent in all cells investigated, while the effect of UTP (1 mM) was more variable. This led us to normalize the UTP (1 mM)- and ATP (10 μM)-currents in each subsequent experimental group (see Methods).

ATP (100 nM–1 mM), ADP (10 nM–10 mM) and UTP (1 μM–100 mM), but not uridine (100 μM–10 mM) produced concentration-dependent responses (Figure 2b). Maximal currents were evoked by 100 μM ATP (121.7 ± 16.1 pA; $n=5$), 1 mM ADP (162.3 ± 24.5 pA; $n=7$) and 10 mM UTP (90.6 ± 10.3 pA; $n=7$). Further increases of the agonist concentrations resulted in bell-shaped concentration-response curves for ADP and UTP; the nucleotides caused at 10 mM (ADP; 114.4 ± 27.5 pA; $n=7$) and 100 mM (UTP; 23.3 ± 5.0 pA; $n=8$) less current than at 1 mM and 10 mM, respectively ($P<0.05$ each; Figure 2b). Subsequently, EC_{50} values and Hill coefficients were calculated for ATP (1.7 μM, 0.7),

ADP (8.6 μM, 0.4) and UTP (81.3 μM, 0.5) from the normalized concentration-response curves (Figure 2c).

A comparison of the normalized current amplitudes shows that the effect of UTP (1 mM) remained unchanged when Mg^{2+} (1 mM) was omitted from the bath medium (0.94 ± 0.04; $n=9$; $P>0.05$). Furthermore, outward current responses to UTP (1 mM) were similar in non-proliferating and proliferating (0.98 ± 0.26; $n=10$; $P>0.05$) microglial cells.

Characterization of purinoceptors and pyrimidinoceptors with antagonists

Since outward currents could be elicited both by ATP and UTP, further pharmacological studies were undertaken to discriminate between P_2 purinoceptor- (ATP), P_1 purinoceptor- (adenosine) and pyrimidinoceptor (UTP)-mediated responses. When ATP (10 μM) was applied twice with a 10 min interval and the pipette contained GTP (200 μM) plus MgATP (3 mM), the amplitudes of outward currents only slightly declined (Figure 3a). The two P_2 purinoceptor antagonists suramin (300 μM) and reactive blue 2 (50 μM) markedly inhibited the effect of ATP (10 μM), whereas 8-(p-sulphophenyl)-theophylline (8-SPT; 100 μM) a P_1 purinoceptor antagonist, was inactive (Figure 3a). In contrast to ATP (10 μM), the current induced by UTP (1 mM) markedly decreased from T_1 to T_2 (Figure 3b). Moreover, the effect of UTP (1 mM) was increased by suramin (300 μM) and was not altered by reactive blue 2 (50 μM) or 8-SPT (100 μM) (Figure 3b).

Transduction mechanisms of purinoceptors and pyrimidinoceptors

In the following experiments, the effect of UTP (1 mM) was tested with standard (K^+) pipette solution and with pipette solution in which all KCl was replaced with an equimolar concentration of CsCl (150 mM; Cs^+ pipette) or in which all but 5 mM KCl was replaced by an equimolar concentration of K-gluconate (145 mM; low Cl^- pipette). The UTP (1 mM)-induced normalized current almost disappeared when instead of a standard K^+ pipette a Cs^+ pipette was used (0.07 ± 0.04; $n=5$; $P<0.05$), indicating that the outward current is carried by K^+. The effect of UTP (1 mM) did not change when recording was with a low Cl^- pipette (1.11 ± 0.27; $n=6$; $P>0.05$), excluding a role of this ion as a charge carrier. It is interesting to note that the resting outward current observed at a holding potential of 0, but not -70 mV (compare upper and lower panels of Figure 2a) was also abolished after replacement of K^+ by Cs^+ in the pipette solution. The addition of 1 mM 4-aminopyridine (0.93 ± 0.03; $n=8$; $P>0.05$) or 1 mM

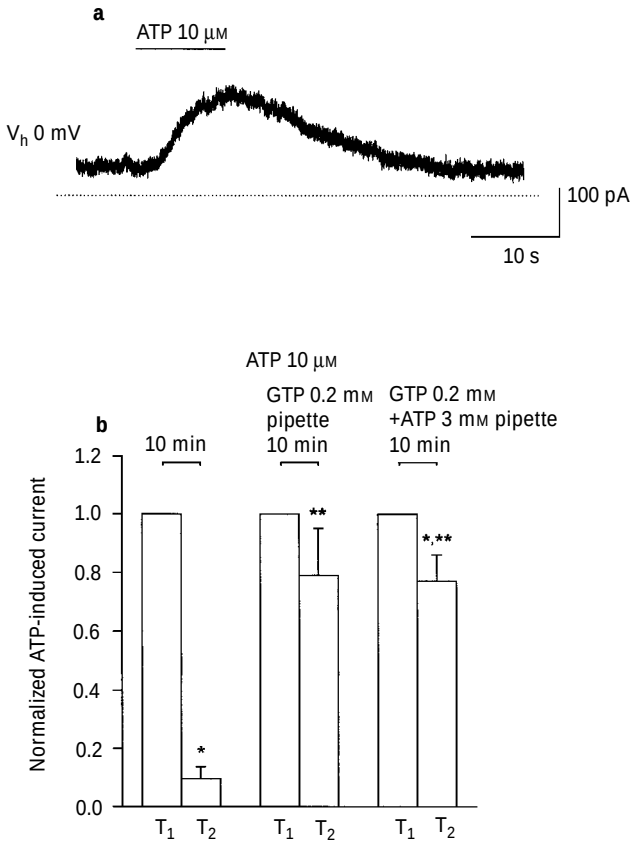

Figure 1 Outward current activated by ATP and its dependence on intracellular GTP. Cultivated non-proliferating rat microglial cells were used. Holding potential (V_h) was 0 mV. ATP (10 μM) was pressure applied twice for 10 s with 10 min interval (T_1, T_2). The effects of T_2 were normalized with respect to the effect of T_1. (a) ATP (10 μM)-induced current at T_1 with a pipette containing GTP (0.2 mM) and ATP (3 mM). Representative tracing in one out of 9 similar experiments. The dotted line indicates the zero current level. (b) Effects of two consecutive pressure applications of ATP (10 μM) with pipettes containing standard medium ($n=7$), GTP (0.2 mM; $n=12$) and GTP (0.2 mM) plus ATP (3 mM; $n=9$). $^*P<0.05$; significant differences from the respective T_1 $^{**}P<0.05$; significantly different from T_2 with standard medium only. Means $\pm$ s.e.mean from n experiments are shown.

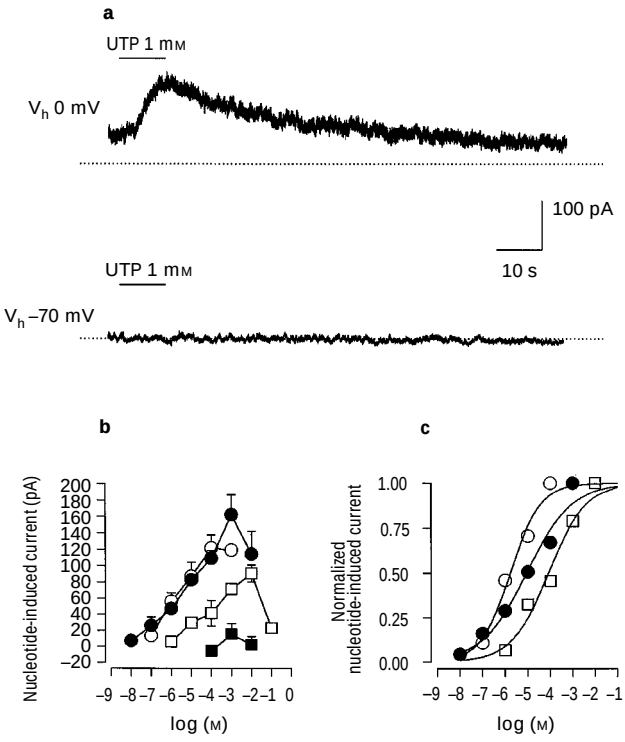

Figure 2 Outward currents activated by ATP, ADP and UTP. Cultivated non-proliferating rat microglial cells were used. Each concentration was pressure applied for 10 s, only once and to different cells. (a) UTP (1 mM)-evoked outward current at a holding potential (V_h) of 0 mV (upper panel) and no current response at a holding potential of -70 mV (lower panel). Representative tracings in one out of 84 and 7 similar experiments, respectively. The dotted lines indicate the zero current levels. Recordings from two different cells are shown. (b) Concentration-response curves for ATP (○; $n=5-11$), ADP (●; $n=7-9$), UTP (□; $n=4-8$) and uridine (■; $n=3-5$). Means from n experiments; vertical lines show s.e.mean. (c) Normalized concentration-response curves for ATP (○), ADP (●) and UTP (□). Mean currents at each nucleotide concentration were taken from (b) and were normalized with respect to the maximum current evoked by the same nucleotide. Continuous curves represent best fits to the equation $I/I_{max}=1/\{1+(K_D/[ATP])^{n_H}\}$, where K_D is the apparent dissociation constant which equals the concentration evoking a half-maximal current (EC_{50}), and n_H is the Hill coefficient. Fitting was by least-squares non-linear regression according to the Marquardt-Levenberg algorithm.

Cs^+ (0.85 ± 0.11; $n=6$; $P>0.05$) to the bath solution which block outwardly and inwardly rectifying K^+ conductances of microglia, respectively (see below), did not alter the normalized effect of UTP (1 mM).

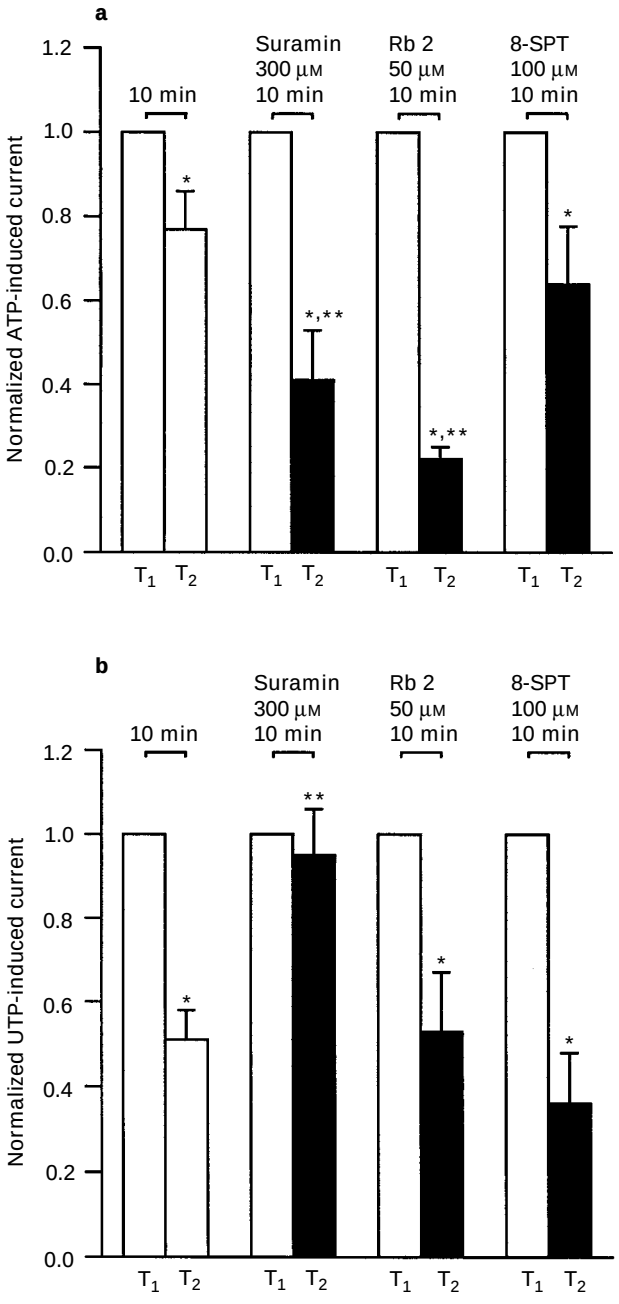

Figure 3 Interaction between ATP and UTP with purinoceptor antagonists. Cultivated non-proliferating rat microglial cells were used. Holding potential (V_h) was 0 mV. ATP (10 μM; a) and UTP (1 mM; b) were pressure applied twice for 10 s with 10 min interval (T_1, T_2). During this interval the bath was superfused with drug-free medium or with medium containing purinoceptor antagonists. The effects of T_2 were normalized with respect to the effect of T_1. (a) Antagonism of the ATP (10 μM)-induced outward current by suramin (300 μM; $n=7$) and reactive blue 2 (Rb 2; 50 μM; $n=8$) but not 8-(p-sulphophenyl)-theophylline (8-SPT; 100 μM; $n=6$). ATP (10 μM) was applied the second time either in the absence ($n=9$) or presence of the antagonists. (b) Potentiation of the UTP (1 mM)-induced outward current by suramin (300 μM; $n=7$) and no effect of reactive blue 2 (Rb 2; 50 μM; $n=7$) and 8-(p-sulphophenyl)-theophylline (8-SPT; 100 μM; $n=9$). UTP (1 mM) was applied the second time either in the absence ($n=9$) or presence of the antagonists. $*P<0.05$; significantly different from the respective T_1. $**P<0.05$; significantly different from T_2 with standard medium only. Means$\pm$s.e.mean from n experiments are shown.

I-V relations of microglial cells were determined by applying fast voltage ramps (0 mV holding potential, 1 s duration, from -130 to 50 mV) before and during the administration of UTP (1 mM). The control *I*-V relation determined in a standard bath medium with K^+-containing pipettes, exhibited both inward and outward rectification (Nörenberg *et al*., 1992; 1994a). This rectification of the voltage-dependent potassium current is characteristic for non-proliferating microglia (Illes *et al*., 1996). The UTP current determined by digital subtraction crossed the zero current value (77.6 ± 8.6 mV; $n=5$) in the range of the calculated K^+ equilibrium potential (-89 mV). When the voltage-dependent inwardly and outwardly rectifying conductances were blocked by Cs^+ (1 mM) and 4-aminopyridine (1 mM), respectively, the amplitude of the UTP current and its intersection with the zero current level did not change (-77.6 ± 3.1 mV; $n=11$; Figure 4a). However, omission of Ca^{2+} from the Cs^+ (1 mM) and 4-aminopyridine (1 mM)-containing bath medium ($n=5$; not shown) or replacement of K^+ by Cs^+ (150 mM) in the pipette solution ($n=6$) abolished the UTP current (Figure 4b). Under these conditions only small leak currents were measured which crossed the zero current level near 0 mV (i.e. Figure 4b). A likely explanation of

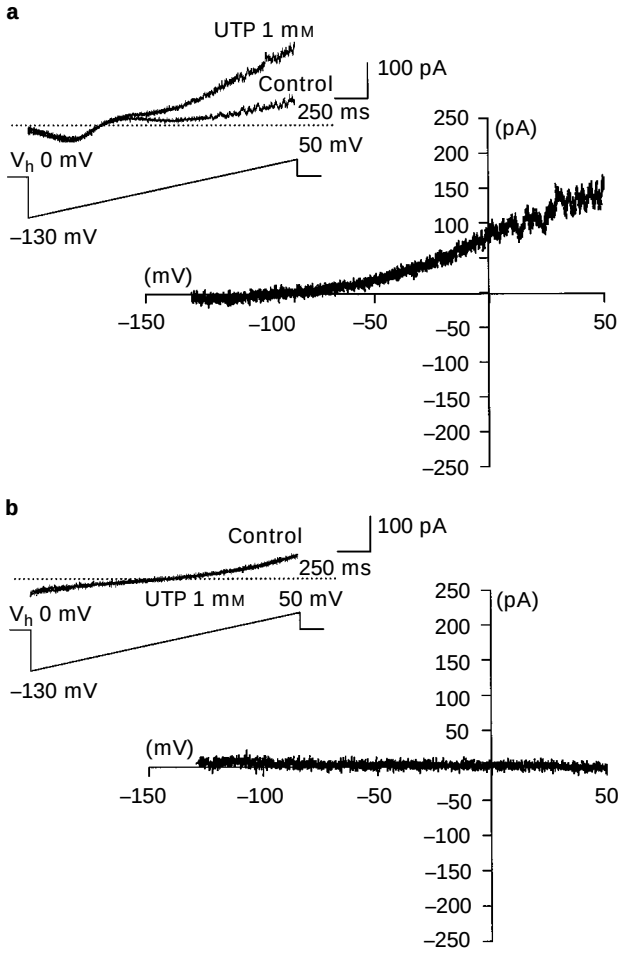

Figure 4 Current-voltage (*I*-V) relations of the UTP-induced conductance. Cultivated non-proliferating rat microglial cells were used. *I*-V relations of microglial cells were determined by applying depolarizing voltage ramps (0 mV holding potential (V_h), 1 s duration, from -130 to 50 mV) before a 10 s pressure application of UTP (1 mM) and during the peak response to the agonist (indicated in the insets of a and b). The UTP current was obtained by subtraction. Dotted lines indicate the zero current levels. (a) UTP current recorded with a K^+ pipette in the presence of extracellular 4-aminopyridine (4-AP; 1 mM) and Cs^+ (1 mM). (b) No UTP current was recorded with a Cs^+ pipette in the additional presence of extracellular Cs^+ (1 mM). Representative tracings in one out of 11 (a) and 6 (b) similar experiments.

these results is that a Ca^{2+}-dependent K^+ conductance is involved in the effect of UTP.

The response to ATP is dependent on the presence of GTP in the pipette solution (Figure 1b) and is abolished by inclusion of GDP-β-S (Langosch *et al*., 1994). The response to UTP (1 mM) was greatly attenuated by replacement of GTP by GDP-β-S in the pipette solution (Figure 5a). Measurements of *I*-V curves indicated that the UTP (1 mM)-induced current disappeared in a Ca^{2+}-free bath medium. These results were confirmed by the pressure application of UTP (1 mM) onto microglial cells held at 0 mV (Figure 5b). Moreover, in agreement with recordings with Cs^+ pipettes, the omission of Ca^{2+} from the bath medium abolished the voltage-dependent resting outward current observed at 0 mV holding potential when a standard pipette solution was used (Figure 5b; upper right panel). In contrast to the effect of UTP (1 mM), the effect of ATP (10 μM) was not inhibited in a Ca^{2+}-free medium (Figure 5b; lower panel).

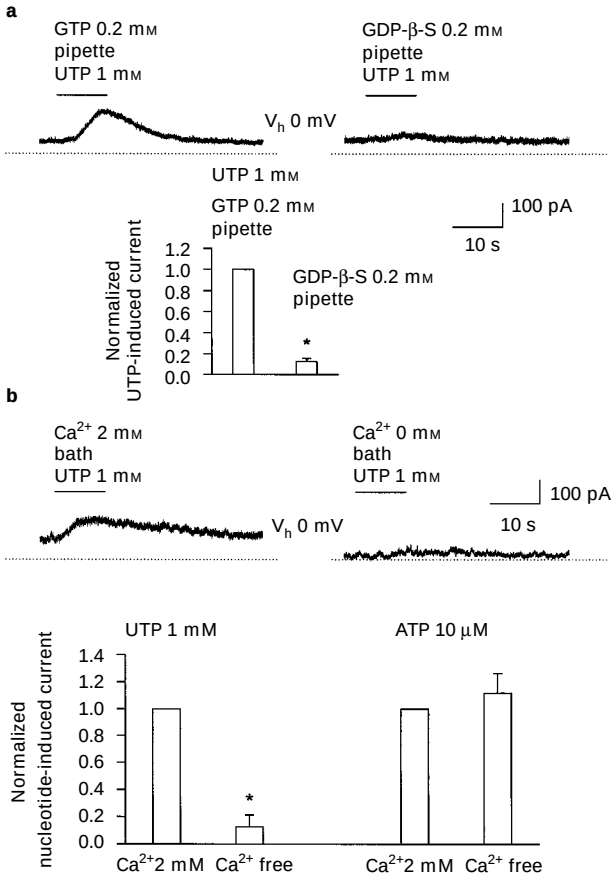

Figure 5 Inhibition of the UTP-induced current by intracellular GDP-β-S or the omission of extracellular Ca^{2+}. Cultivated non-proliferating rat microglial cells were used. Holding potential (V_h) was 0 mV. UTP (1 mM) was pressure applied for 10 s, only once and to different cells. (a) UTP (1 mM)-evoked outward current with pipette solution containing GTP (0.2 mM; upper left panel) and almost no current response with pipette solution in which GTP (0.2 mM) was replaced by GDP-β-S (0.2 mM; upper right panel). Representative tracings in one out of 10 similar experiments in both panels. The dotted lines indicate the zero current levels. Recordings from two different cells are shown. Means$\pm$s.e.mean of the current amplitudes obtained in 10 cells in each column are shown in the lower panel. The effect of T_e (GDP-β-S (0.2 mM) in the pipette solution) was normalized with respect to the effect of T_c (GTP (0.2 mM)) in the pipette solution). (b) UTP (1 mM)-evoked outward current in a bath solution containing Ca^{2+} (2 mM; upper left panel) and no current response in a nominally Ca^{2+}-free bath solution (upper right panel). Representative tracings in one out of 10 similar experiments in both panels. The dotted lines indicate the zero current levels. Recordings from two different cells are shown. Means$\pm$s.e. mean of the current amplitudes obtained in 10 cells in each column are shown in the lower panel. The effect of T_e (nominally Ca^{2+}-free bath medium) was normalized with respect to the effect of T_c (Ca^{2+} (2 mM)-containing bath medium). When in similar experiments ATP (10 μM) was used as an agonist both in the presence and absence of Ca^{2+} (2 mM) in the bath solution, there was no change at all. Means$\pm$s.e.mean of 7 experiments are shown in both columns. *$P<0.05$; significant difference from the respective T_c.

When ATP (10 μM) was applied twice with a 10 min interval, the usual decline of the normalized current amplitudes was observed (0.81 ± 0.06; $P<0.05$; compare with Figure 1b), which did not change ($P>0.05$) in the presence of charybdotoxin (0.1 μM), a blocker of Ca^{2+}-dependent K^+ channels (0.71 ± 0.04; $n=5$ each; $P<0.05$). In contrast, the UTP (1 mM)-evoked current amplitudes decreased to a larger extent ($P<0.05$) in the presence (0.27 ± 0.01; $P<0.05$) than in the absence (0.74 ± 0.04; $n=5$ each; $P<0.05$) of charybdotoxin (0.1 μM).

In the following series of experiments the involvement of the phospholipase C/$InsP_3$ pathway in the effects of ATP and UTP was tested. For this purpose a phospholipase C inhibitor (U-73122) and an antagonist of $InsP_3$ (heparin) were included into the pipette solution. Both U-73122 (5 μM) and heparin (5 mg ml^{-1}) almost abolished the response to UTP (1 mM), but did not alter the effect of ATP (10 μM) (Figure 6).

Inositol 1,4,5-trisphosphate ($InsP_3$)-activated whole-cell conductance

Patch pipettes were filled with a solution in which K^+ was replaced by Cs^+ in order to block outward K^+ currents; $InsP_3$ (10 μM) was also present in the solution (Figure 7a, upper panel). At a holding potential of 0 mV and in a high Ca^{2+} (10 mM) bath medium, soon after the solution gained access to the interior of microglial cells, an inward current developed (-10.5 ± 2.8 pA; $n=12$), which disappeared in the nominal absence of external Ca^{2+} (-0.57 ± 1.02 pA; $n=12$; $P<0.05$) (Figure 7a). The activation time-course of the inward current could be fitted by a single-exponential function, with a time constant (τ) of 25.1 ± 3.8 s ($n=12$). The current was preceded by a delay of 10.7 ± 1.8 s ($n=12$). All kinetic parameters are exemplified in Figure 7a (lower left panel). This inward current resembled the calcium-release activated Ca^{2+} current (I_{CRAC}) triggered by the depletion of intracellular Ca^{2+} stores in many non-excitable cells by $InsP_3$ (Putney & Bird, 1993).

The reversal potential of the $InsP_3$ (10 μM)-induced inward current was measured by fast voltage ramps from -100 to 100 mV (Figure 7b). These ramps were evoked both before and during current activation and were subtracted thereafter to yield the *I*-V curve of I_{CRAC}. I_{CRAC} was strongly inwardly rectifying and typically did not reverse up to 100 mV (Figure 7b; $n=5$). This finding once more favoured the involvement of a Ca^{2+} conductance since the calculated reversal potential for Ca^{2+} was 174.5 mV.

Ca^{2+}-activated single-channel conductance

Ca^{2+}-dependent K^+ channels are highly sensitive to micromolar concentrations of Ca^{2+}; it was earlier described that reversal of Ca^{2+}-induced effects is difficult to obtain, even by extensive washing periods (Barrett *et al*., 1982). To circumvent this problem, the Ca^{2+}-dependent activation of membrane channels as well as the reversal of this effect due to the withdrawal of internal Ca^{2+} were studied by means of pressure application techniques onto excised inside-out membrane patches. In these experiments, the patch potential was 40 mV, because depolarization increases the open probability and open time of large conductance Ca^{2+}-dependent K^+ channels (Marty, 1981; Pallotta *et al*., 1981). The sudden rise of the internal free Ca^{2+} concentration from 0.01 to 0.5 μM led to the immediate appearance of channel openings which disappeared after returning to the original low Ca^{2+} medium (Figure 8a; $n=4$).

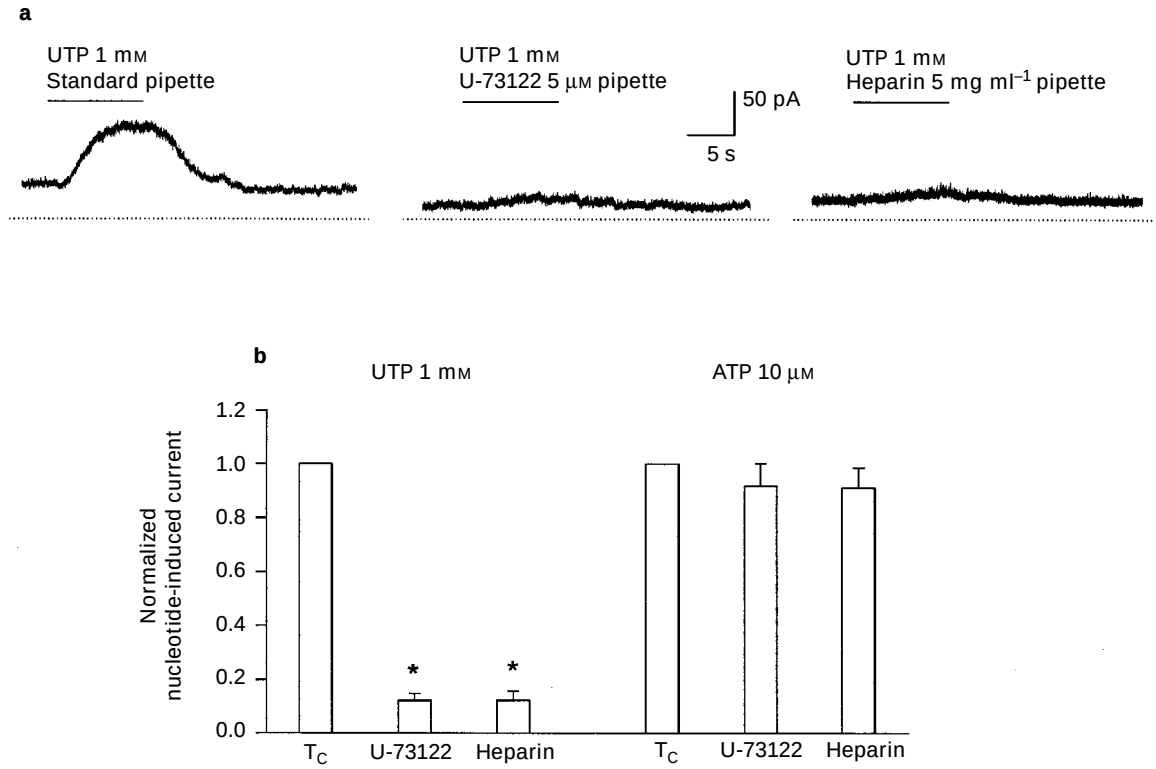

Figure 6 Inhibition of the UTP-induced current by intracellular U-73122 or heparin. Cultivated non-proliferating rat microglial cells were used. Holding potential (V_h) was 0 mV. UTP (1 mM) was pressure applied for 10 s, only once and to different cells. (a) UTP (1 mM)-evoked outward current with a standard pipette solution (left panel) and almost no current response with pipette solution containing U-73122 (5 μM; middle panel) or heparin (5 mg ml^{-1}, right panel). Representative tracings in one out of 6 similar experiments in each panel. The dotted lines indicate the zero current levels. Recordings from three different cells are shown. (b) Means$\pm$s.e.mean of current amplitudes obtained in 6 cells in each column. The effect of T_e (U-73122 (5 μM) or heparin (5 mg ml^{-1}) in the pipette solution) was normalized with respect to the effect of T_c (standard pipette solution). When in similar experiments ATP (10 μM) was used as an agonist both with a standard pipette solution and with a pipette solution containing U-73122 (5 μM) or heparin (5 mg ml^{-1}), there was no change at all. *$P<0.05$; significant difference from the respective T_c.

The activities of these Ca^{2+}-dependent channels were studied in the inside-out configuration at patch potentials of 40, 20, 0, -20, -40 and -60 mV in the presence of 0.5 μM free Ca^{2+} in the intracellular solution (Figure 8b). In symmetrical K^+, current amplitudes were outward at depolarized potentials, reversed at 0.92 ± 0.5 mV ($n=5$) and were inwardly directed at hyperpolarized potentials (8.0 ± 0.6 pA and -12.2 ± 0.6 pA at 40 mV and -60 mV, respectively; $n=5$). There were 2 to 4 Ca^{2+}-dependent channels in single patches as determined by the number of increasing step-wise changes in current. When the extracellular K^+ concentration was decreased from 144 to 32 mM, the reversal potential of the single-channel current was shifted to negative values (-33.8 ± 0.2 mV; $n=6$; $P<0.05$). By contrast, a decrease of the internal Cl^- concentration from 164 to 38 mM did not change the reversal potential (1.0 ± 0.5 mV; $n=3$; $P>0.05$). Hence, the currents were K^+ selective as indicated by their reversal potential of approximately 0 mV in symmetrical K^+ concentrations and its dependence on the extracellular K^+, but not the extracellular Cl^- concentration. The single channel conductances calculated from the *I*-V relations were 188.1 ± 15.5 pS ($n=5$) in symmetrical K^+, 182.9 ± 15.8 pS ($n=6$) when the extracellular K^+ concentration was decreased from 144 to 32 mM and 186.1 ± 27.1 pS ($n=3$) when the extracellular Cl^- concentration was decreased from 164 to 38 mM.

Discussion

Previous experiments showed that ATP evokes two different types of conductance changes in non-proliferating microglia mediated by P_{2X} (cationic conductance) and P_{2Y} (K^+ conductance) purinoceptors, respectively (Walz *et al*., 1993; Nörenberg *et al*., 1994b; Langosch *et al*., 1994; Illes *et al*., 1996). The two current components could be studied separately, by holding the cells at -70 mV close to the reversal potential of the K^+ current or at 0 mV near the reversal potential of cationic currents. UTP did not evoke an inward current at -70 mV, but caused an outward current at 0 mV. The rank order of agonist potencies for evoking outward currents by the purine and pyrimidine nucleotides was ATP = ADP > UTP; the pyrimidine nucleoside uridine was inactive. Since the concentration-response curves were bell-shaped and the slopes shallow which is an indication for receptor activation and desensitization going on simultaneously, no conclusions could be drawn about the stochiometry of the agonist-receptor interaction. Finally, the outward current amplitudes induced by UTP were similar in proliferating and non-proliferating microglia. This is in contradiction to the larger current amplitude evoked in proliferating than in non-proliferating cells by 2-MeSATP (Langosch *et al*., 1994). The most likely explanation for this finding is that 2-MeSATP and UTP act by different receptors. Furthermore, the effect of UTP is unlikely to involve extracellular phosphorylation, since the response is still present in the absence of extracellular Mg^{2+} (see e.g. Christie *et al*., 1992).

Two P_2 purinoceptor antagonists, suramin (Dunn & Blakeley, 1988) and reactive blue 2 (Burnstock & Warland, 1987; Kennedy, 1990) depressed the current response to ATP, while the P_1 purinoceptor antagonist 8-(p-sulphophenyl)-theophylline (8-SPT; Bruns *et al*., 1980) had no effect. By contrast, suramin increased the UTP-induced current, while reactive blue 2 and 8-SPT were inactive. Hence, ATP and UTP appear

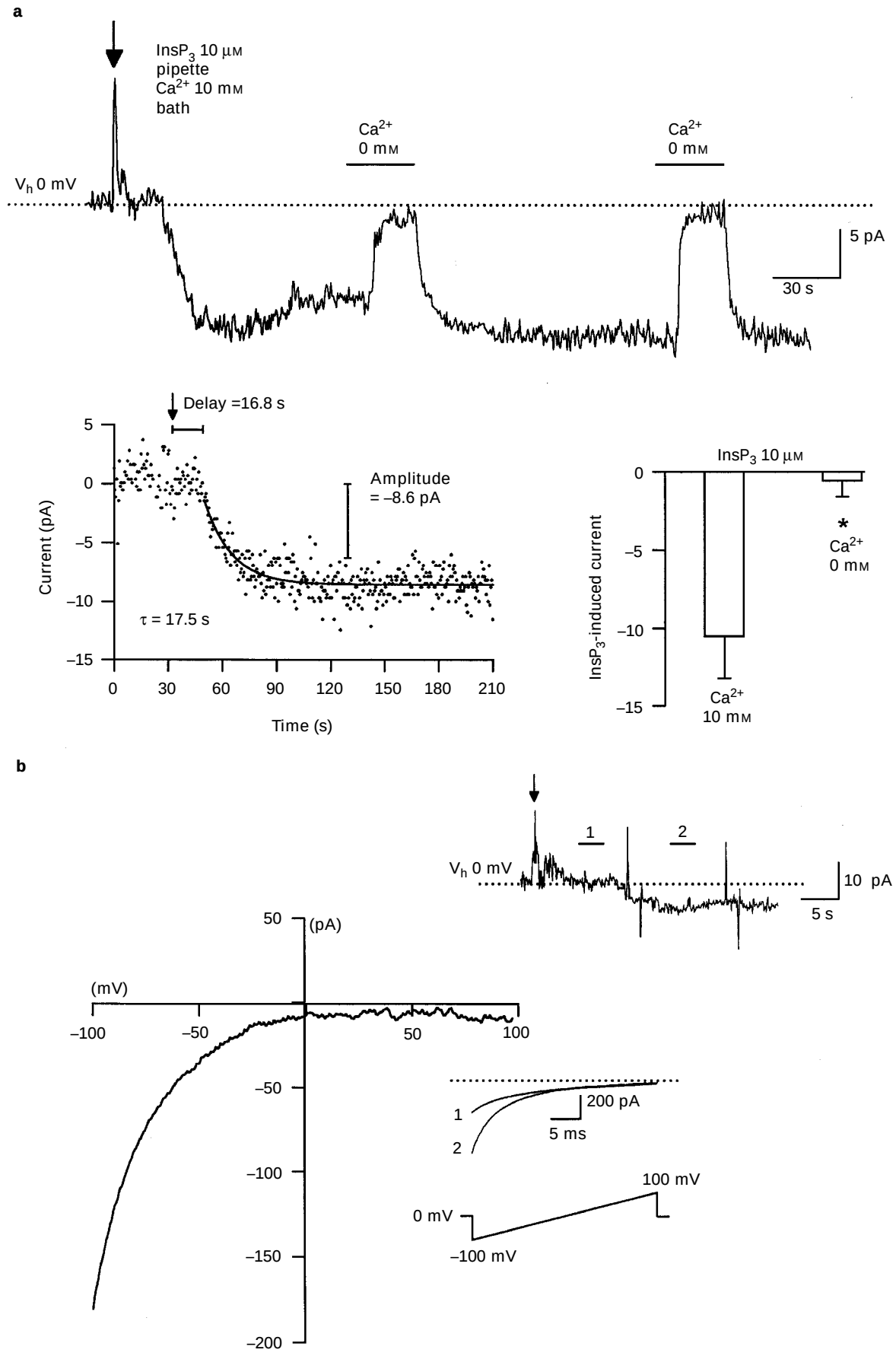

Figure 7 Inositol 1,4,5-trisphosphate ($InsP_3$)-induced current. Cultivated non-proliferating rat microglial cells were used. Holding potential (V_h) was 0 mV. (a) Ca^{2+}-dependence and time-course of $InsP_3$-induced current. K^+ was replaced by Cs^+ (150 mM) in the pipette solution and in addition $InsP_3$ (10 μM) was added. The bath medium contained Ca^{2+} (10 mM) except during the time indicated by horizontal bars, when a nominally Ca^{2+}-free bath medium was applied. The arrow indicates the time of establishing the whole-cell configuration. Representative tracing of the $InsP_3$-induced current in one out of 12 similar experiments (upper panel) and its activation kinetics (lower left panel). The dotted line indicates the zero current level. The current could be fitted with a

to act at separate receptors and neither of these agonists activates the adenosine-sensitive P_1 purinoceptors of microglial cells (Langosch *et al*., 1994). The reason for the potentiation of the UTP-induced current by suramin is unknown and probably not due to a blockade of ectonucleotidases (Crack *et al*., 1994). In cell culture systems, these enzymes fail to degrade considerably exogenous ATP or UTP during the short equilibration time necessary for current initiation (Evans & Kennedy, 1994).

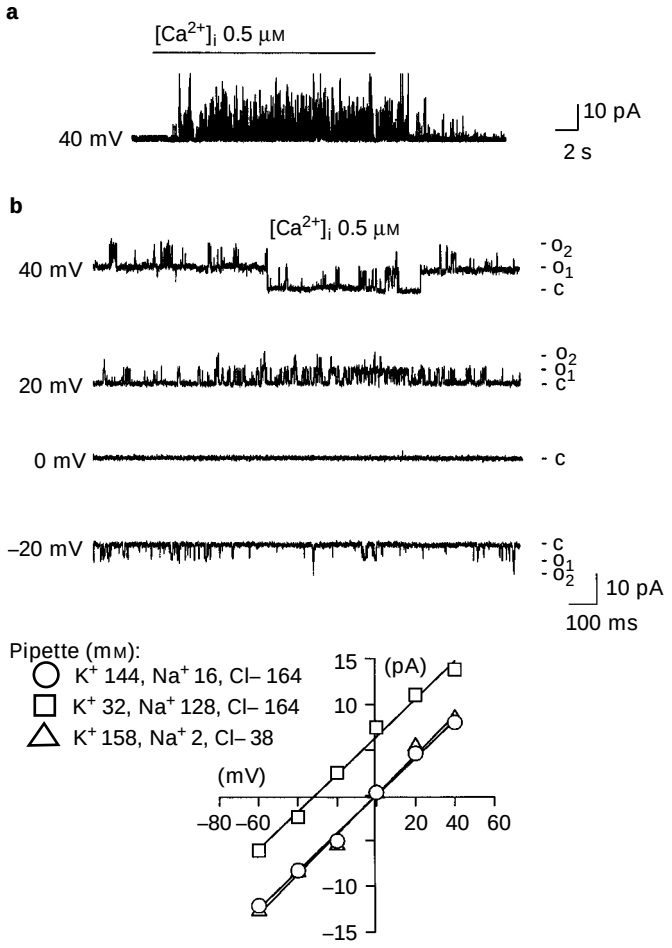

Figure 8 Ca^{2+}-activated single channel conductance. Excised inside-out membrane patches of cultivated non-proliferating rat microglial cells were used. The extracellular and intracellular solutions were identical and contained low Ca^{2+} (free Ca^{2+}, 0.01 μM). (a) Activation of the channel by Ca^{2+} on the intracellular surface of the membrane patch. Pressure application of an intracellular solution containing elevated Ca^{2+} (free Ca^{2+}, 0.5 μM) for 20 s evoked channel activity which declined thereafter. Patch potential was 40 mV. Representative tracing in one out of 4 similar experiments. (b) Effect of patch potential on single channel conductance. The patch potentials are indicated at the beginning of each tracing. Depolarization from 20 to 40 mV in an intracellular solution containing elevated Ca^{2+} (free Ca^{2+}, 0.5 μM) increased the frequency of channel openings (upper panel). At 40 mV one channel was open most of the time. The current levels with channels closed (c), with one channel open (o_1) and with two open (o_2) are indicated. Representative tracings in one out of 5 similar experiments. Plot of single channel current amplitude against patch potential with a low Ca^{2+}/standard pipette solution (K^+ 144, Na^+ 16, Cl^- 164 mM; o; $n=5$), with a low Ca^{2+}/low K^+/high Na^+ pipette solution (K^+ 32, Na^+ 128, Cl^- 164 mM; □; $n=6$) and with a low Ca^{2+}/low Cl^-/low Na^+ pipette solution (K^+ 158, Na^+ 2, Cl^- 38 mM; △; $n=3$) (lower panel). Means ± s.e.mean from n experiments are shown; the error bars were smaller than the size of the symbols.

The differential antagonism of ATP and UTP effects by suramin and reactive blue 2 excludes the involvement of P_{2U} purinoceptors on rat microglial cells, since these are usually suramin-sensitive (Pfeilschifter, 1990; Keppens *et al*., 1992). By contrast, UTP-selective pyrimidinoceptors may be clearly separated in various tissues both from coexisting P_{2X} (Connolly *et al*., 1993; Boehm *et al*., 1995) and P_{2Y} purinoceptors (Yang *et al*., 1996) by their suramin-insensitivity. Pyrimidinoceptors have recently been described in a rat glioma cell line (Lazarowski & Harden, 1994) and subsequently cloned from a human genomic library (Nguyen *et al*., 1995). The native receptor was coupled with a G protein and activated the enzyme phospholipase C which produced $InsP_3$ (Lazarowski & Harden, 1994); the same was true for the cloned receptor when expressed in a human astrocytoma cell line (Nguyen *et al*., 1995).

UTP appears to activate at 0 mV holding potential an outward K^+ current, since a replacement of K^+ with Cs^+ in the pipette solution inhibited the response to UTP. Extracellular Cs^+ and 4-aminopyridine, known to inhibit the microglial inwardly and outwardly rectifying K^+ conductances, respectively (Nörenberg *et al*., 1992; 1994a; Illes *et al*., 1996), did not interfere with the effect of UTP. Hence, the UTP-induced current differed from the voltage-dependent inward or outward K^+ conductances of microglial cells.

The current response to UTP at a holding potential of 0 mV disappeared when Ca^{2+} was omitted from the bath medium. Similarly, I_{UTP} obtained from the subtraction of *I*-V relations established in the presence and absence of UTP was abolished in a nominally Ca^{2+}-free bath solution. Both types of experiments are compatible with the activation of a Ca^{2+}-dependent K^+ current by UTP. It is interesting to note that the voltage-dependent outwardly rectifying K^+ current of rat microglia was inhibited by increasing intracellular Ca^{2+} (Nörenberg *et al*., 1994a). A similar inhibitory effect of high intracellular free Ca^{2+} was observed in T and B lymphocytes (Grissmer & Cahalan, 1989; Pahapill & Schlichter, 1992). However, in peripheral macrophages there is an additional outward current which is activated by intracellular Ca^{2+} (Gallin, 1984; 1991). A similar conductance appears to be present also in rat microglia, as indicated by single channel activity in excised inside-out patches in response to the intracellular application of Ca^{2+}. A reversal potential of about 0 mV in symmetrical K^+ and its shift to more negative potentials during a decrease of the extracellular K^+ concentration characterize the channels as K^+-selective. The single channel conductance of about 200 pS indicates the involvement of Ca^{2+}-dependent K^+ channels with a large unitary conductance ('BK channels'; Marty, 1983; Blatz & Magleby, 1987). BK channels are also present in human peripheral monocytes/macrophages (Gallin, 1984; 1991).

P_{2U} purinoceptors are coupled via a G protein to phospholipase C, which releases intracellular Ca^{2+} via the generation of $InsP_3$ and thereby opens Ca^{2+}-dependent K^+ channels (Alonso-Torre & Trautmann, 1993; Murgia *et al*., 1993) in murine peritoneal macrophages and the macrophage-like cell line J774. Hence, in these cells ATP and UTP possibly stimulate the same purinoceptor-type and initiate a common signalling cascade. In rat microglia, separate P_{2Y} purinoceptors and pyrimidinoceptors are activated by ATP and UTP, respectively, for the following reasons. Firstly, only the ATP-induced outward current was inhibited by the P_2 purinoceptor

single-exponential function, yielding the time-constant (τ) and the current amplitude. The delay represents the time from break in until activation. Means ± s.e.mean of the current amplitudes obtained in 10 cells are shown in the lower right panel. $^*P<0.05$; significant difference between the two columns. (b) *I*-V relation of the $InsP_3$-induced current. Voltage ramps of 50 ms duration from −100 to 100 mV at a frequency of 1 Hz were applied from a holding potential (V_h) of 0 mV (upper and lower insets). The ramps in the period indicated by the 1st horizontal line were subtracted as leaks from the ramps in the period indicated by the 2nd horizontal line. Representative tracings in one out of 5 similar experiments.

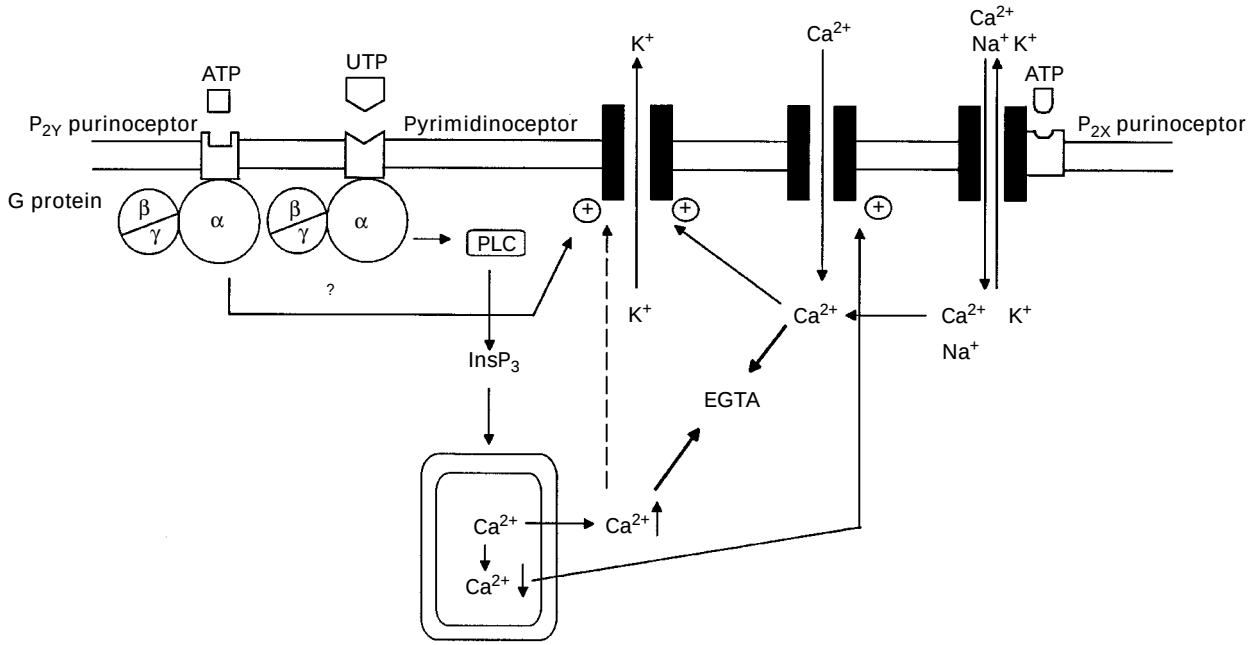

Figure 9 Transduction mechanisms of coexisting purinoceptors (P_{2X}, P_{2Y}) and pyrimidinoceptors in rat microglial cells. The depletion of the intracellular Ca^{2+} pool by pyrimidinoceptor activation (via the G protein, phospholipase C and inositol 1,4,5-trisphosphate cascades) leads to the opening of Ca^{2+}-selective membrane channels (capacitative entry of Ca^{2+}; I_{CRAC}). This Ca^{2+}, rather than Ca^{2+} released from the intracellular pool (which is chelated by EGTA) activates the Ca^{2+}-dependent K^+ channel. Extracellular Ca^{2+} may enter the cells also via ligand-activated cationic channels (P_{2X} purinoceptor). The P_{2Y} purinoceptor is coupled to a G protein which mediates opening of K^+ channels with or without the involvement of further protein kinases. It is unclear whether this K^+ channel is Ca^{2+}-sensitive or belongs to one of the Ca^{2+}-insensitive channel-types present in microglia. α, β, γ, subunits of G proteins; PLC, phospholipase C; $InsP_3$, inositol 1,4,5-trisphosphate; Ca^{2+} ↑, increase in the concentration of Ca^{2+}; Ca^{2+} ↓, decrease in the concentration of Ca^{2+}; +, opening of channels.

antagonists suramin and reactive blue 2 (see above). Secondly, the current response to ATP persisted in the absence of external Ca^{2+}, while a similar response to UTP was abolished in a Ca^{2+}-free bath medium. Thirdly, charybdotoxin, a blocker of BK channels in monocytes (Gallin, 1984) did not interfere with the effect of ATP, but strongly inhibited the effect of UTP. Although charybdotoxin inhibits the voltage-dependent outwardly rectifying K^+ current of rat microglia as well, the resistance of the UTP-induced current to 4-aminopyridine excludes the involvement of this latter channel type (Nörenberg *et al*., 1994a). Fourthly, interference with the phospholipase C/$InsP_3$ pathway by the phospholipase C inhibitor U-73122 (Smith *et al*., 1990) or the $InsP_3$ antagonist heparin (Salter & Hicks, 1995) almost abolished the response to UTP, but did not alter the effect of ATP.

Since $InsP_3$ releases Ca^{2+} from its intracellular pools, the absolute dependence of the UTP effect on extracellular Ca^{2+} needs further explanation. It was shown that in various non-excitable cells the depletion of the $InsP_3$-sensitive intracellular Ca^{2+} pool signals to the plasma membrane and opens Ca^{2+}-selective channels (Clementi *et al*., 1992; Hoth & Penner, 1993). The signalling to the membrane occurs either by direct coupling, or via the cytoskeleton or by a small diffusible messenger (Putney & Bird, 1993). The current induced was also named the capacitative Ca^{2+} entry I_{CRAC}. Our experiments unequivocally demonstrate the presence of I_{CRAC} in rat microglia after the intracellular application of $InsP_3$. The typical delay and exponential activation of this current, its dependence on extracellular Ca^{2+} and its failure to reverse at amplitude between −100 and 100 mV are characteristic of the I_{CRAC} recorded in i.e. rat mast cells (Hoth & Penner, 1993).

In addition, we showed that the signalling cascade of the pyrimidinoceptors involves a G protein. When cells were microdialysed with an enzymatically stable GDP analogue GDP-β-S, the outward current evoked by UTP markedly decreased. GDP-β-S is known to block G protein-mediated effects of agonists (Dunlap *et al*., 1987). Previous experiments showed that both the intracellular application of GDP-β-S and incubation with pertussis toxin abolished the 2-MeSATP-induced outward current (Langosch *et al*., 1994). Pertussis toxin selectively ADP-ribosylates α-subunits of G proteins (Dolphin, 1987). Both these experiments and the marked rundown of the current response to ATP in the absence of GTP in the pipette solution suggest a coupling of P_{2Y} purinoceptors to a G protein.

Hence, P_{2Y} purinoceptors are probably coupled to a G protein which mediates opening of K^+ channels with or without the involvement of further protein kinases (Figure 9; Pfaffinger & Sieglebaum, 1990). It is unclear whether this K^+ channel is Ca^{2+}-sensitive or belongs to one of the Ca^{2+}-insensitive channel-types present in microglia. The transduction mechanism of the pyrimidinoceptor is more complex (Figure 9). Receptor stimulation most likely leads via a G protein to the activation of the enzyme phospholipase C with the subsequent release of Ca^{2+} by the generated $InsP_3$. Since the pipette solution is buffered by EGTA, the intracellular Ca^{2+} is rapidly chelated and is probably not a major signal for the opening of Ca^{2+}-dependent K^+ channels. However, the depletion of the intracellular Ca^{2+} pool initiates the capacitative entry of Ca^{2+} from the extracellular space. This Ca^{2+} then activates $I_{K(Ca)}$. Of course an alternative entry of Ca^{2+} is via P_{2X} purinoceptors, which are markedly permeable to Ca^{2+} in microglial cells (Figure 9; Nörenberg *et al*., 1994b).

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