ATP is released from neurons and other cell types during several physiological and stress conditions under which it exerts various biological effects upon binding to purinoreceptors. A rather peculiar purinoreceptor called P2X7/P2Z is expressed on microglial and other myeloic cells. Although increasing evidence implicates an important role for P2Z in inflammatory processes, little information exists about underlying signaling pathways. Here, we report that in N9 microglial cells, extracellular ATP potently activates nuclear factor of activated T cells (NFAT), a central transcription factor involved in cytokine gene expression. ATP activated NFAT rapidly (within 1 min), whereas activation of nuclear factor κB was much delayed, with strikingly distinct kinetics. During ATP stimulation, both NFAT-1 and NFAT-2 were activated by a calcineurin-dependent pathway that required the influx of extracellular calcium ions. Based on the pharmacological profile, NFAT activation was specifically mediated by P2Z and not by other purinoreceptors. N9 cells that lacked P2Z but still expressed P2Y purinoreceptors failed to respond to NFAT activation. We conclude that P2Z-mediated NFAT activation may represent a novel mechanism by which extracellular ATP can modulate early inflammatory gene expression within the nervous and immune system.

Large amounts of ATP and other nucleotides can be rapidly released from different cellular sources such as nerve terminals, antigen-stimulated T cells, activated platelets, endothelial cells, and other cell types under either physiological and pathological conditions such as hypoxia, stress, and tissue damage. Particularly in the immune and nervous system, extracellular ATP serves as a mediator of cell-to-cell communication by triggering a variety of biological responses including excitatory transmitter function, mitogenic stimulation, or induction of cell death (reviewed in Refs. 1–3). These effects are not the result of nonspecific membrane alterations but rather are mediated through the activation of specific surface molecules called P2 purinoreceptors (reviewed in Refs. 1, 4, and 5). At least two mechanistically distinct subclasses of P2 purinoreceptors are currently known. The metabotropic P2Y receptors (formerly P2u, P2t, and P2y), which bind either UTP or ATP, initiate their biological effects through the G-protein-coupled activation of phospholipase C and subsequent Ca2+ mobilization from intracellular stores. The P2X receptors are a distinct subfamily of receptors that are related to glutamate receptors and function as ligand-gated ion channels. Engagement of P2X receptors by ATP causes an increase in Ca2+ permeability that is entirely dependent upon extracellular Ca2+ ions.

Recently, among the P2X subfamily, the molecular structure of the P2Z receptor, also called P2X7, has been elucidated (6, 7). The P2Z receptor contains two transmembrane domains and a large extracellular loop, structural features that are characteristic of members of the P2X family. Unlike other P2X receptors, the P2Z receptor has an unusually long C-terminal domain that does not contain any known signaling motifs. P2Z receptor expression appears to be rather restricted to myeloic cells such as dendritic cells, mature macrophages, mast cells, and microglial cells (reviewed in Refs. 8 and 9). The receptor is not expressed on monocytes but is induced by γ-interferon and during monocyte differentiation (10, 11). A unique response of the P2Z receptor is the formation of a large transmembrane pore permeable to hydrophilic molecules of up to 900 Da in size, which is formed by the ATP-induced aggregation of receptor subunits.

There is increasing evidence for a functional role of the P2Z receptor in immune reactions (8, 9). Continuous activation of the receptor and pore formation cause perturbations of ion homeostasis that may finally lead to cell death. Interestingly, it has been found that, in contrast to several other cell death inducers, only P2Z receptor ligation killed mycobacteria within BCG-infected macrophages (12, 13). It has been also postulated that P2Z receptor ligation is responsible for the formation of multinucleated giant cells during inflammatory granulomatous reactions (14). In addition, in lipopolysaccharide-primed macrophages and microglial cells, stimulation of the P2Z receptor induces the immediate release of interleukin (IL)1β, which is probably mediated by the activation of IL-1β-converting enzyme (15–18). Despite this accumulating evidence for an important function of the P2Z receptor in inflammatory processes, signaling events underlying these biological effects are almost entirely unknown.

We have recently shown that P2Z receptor ligation causes a rather unusual delayed and sustained activation of the transcription factor NFκB (19). Important transcriptional regulators, which weakly resemble NFκB in their DNA binding domain, comprise proteins of the nuclear factor of activated T cells (NFAT) family (reviewed in Refs. 20–22). NFAT proteins play an important role in inducible gene transcription by controlling the expression of several cytokines, such as IL-2, IL-4,
granulocyte macrophage colony-stimulating factor, tumor necrosis factor, CD40 ligand, and CD95 ligand (21, 22). To date, the cDNAs of four different genes belonging to the NFAT family have been described encoding NFAT-1 (NFATp) (23), NFAT-2 (NFATc) (24), NFAT-3 (25), and NFAT-4 (NFATx) (25–27). NFAT proteins show different tissue distribution and inducibility upon cell stimulation, raising the possibility that their functions may be distinct. In addition, some NFAT members are synthesized as multiple isoforms due to alternative splicing, translation initiation, and polyadenylation events (28–30).

The signaling mechanisms of NFAT have been most intensively studied in T- and B-lymphocytes, but there is a paucity of information on their role in other cell types. The NFAT complex is composed of a cytoplasmic subunit whose subcellular localization and DNA binding activity is controlled by Ca2+-mobilization. Nuclear translocation of NFAT is regulated by the Ca2+-dependent serine/threonine phosphatase calcineurin, which is the target for the immunosuppressive drugs cyclosporin A and FK506 (31–34). After nuclear translocation, NFAT may couple to activator protein 1 (AP-1) or other transcription factors, resulting in the coordinate induction of proinflammatory cytokine expression (35, 36).

In the present study, we investigated the expression and mechanism of NFAT activation in microglial cells, which are considered to be important immune effector cells of the brain (37). It is shown that extracellular ATP instantly induced the nuclear translocation of both NFAT-1 and NFAT-2 by a calcineurin-dependent mechanism. This effect required the influx of extracellular Ca2+ and was exclusively mediated by the P2Z and not by other P2 purinoreceptors. P2Z receptor-mediated NFAT activation may therefore represent a heretofore unappreciated mechanism by which extracellular ATP can modulate inflammatory processes within the nervous and other cellular systems.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—The mouse microglial cell line N9 has been described previously (38) and was kindly provided by Dr. P. Ricciardi-Castagnoli (University of Milan, Italy). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine and routinely passaged by trypsinization. ATP and other nucleotides were purchased from Roche Molecular Biochemicals. Periodate-oxidized ATP was a kind gift of Drs. S. Hanau and F. Di Virgilio (University of Ferrara, Ferrara, Italy) and was synthesized as described previously (39). FK506 and cyclosporin A (CsA) were obtained from the clinical pharmacy (Medical Clinics, Tübingen, Germany). The anti-mouse NFAT-1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY), and the anti-NFAT-2 antibody (clone 7A6) was obtained from Alexis Biochemicals (Grünenberg, Germany). For detection of NFAT-3, different rabbit antisera raised against residues 886–902 or residues 614–632 of human NFAT-3 (Ref. 29; kindly provided by Dr. N. R. Rice (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD)) and a serum directed against residues 387–406 of human NFAT-3 (Santa Cruz, Heidelberg, Germany) were used. For NFAT-4, an antisera raised against residues 130–149 of human NFAT-4 (Ref. 29; a kind gift from Dr. N. R. Rice) and a serum directed against mouse NFAT-4 (kindly provided by Drs. A. Avots and E. Serfling (University of Würzburg, Würzburg, Germany)) were used. An anti-c-Fos antiserum was obtained from Dianova (Ham burg, Germany).

**Preparation of Nuclear Extracts**—N9 cells (2 × 106) were plated in 6-well plates and allowed to adhere overnight. Cells were then treated with the indicated stimuli. Pretreatment with CsA was performed for 15 min, and pretreatment with FK506 was performed for 30 min. Nuclear extracts were prepared by resuspending phosphate-buffered saline-washed cells in 150 μl of Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of aprotinin and leupeptin). After a 20-min incubation on ice, cells were lysed by passing them three times through a G20 needle. The samples were then centrifuged, and the nuclear pellet was resuspended in 70 μl of Buffer C (20 mM Heps, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of aprotinin and leupeptin). After centrifugation at 4 °C for 20 min, nuclear extracts were used for electrophoretic mobility shift assays (EMSAs) and Western blot analysis.

**Electrophoretic Mobility Shift Assay**—Equal amounts of the nuclear extracts (4 μg of protein) were incubated with the 32P-labeled NFAT-specific oligonucleotide. Binding reactions were performed in a 24 μl volume containing 4 μl of extract, 4 μl of 5× binding buffer (20 mM Heps, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl2, and 20% Ficoll), 1 μg poly(dI-dC) as nonspecific competitor DNA, 2 μg of bovine serum albumin, and 40,000 cpm (Cerenkov) of the labeled oligonucleotide. After a 20-min binding reaction at 4 °C, samples were loaded on a 4% non-denaturing polyacrylamide gel that was pre-run for 4 h at 4 °C in 0.5× TBE. After electrophoresis, gels were dried under a gel dryer and exposed to an x-ray film. The NFAT-binding oligonucleotide corresponding to the distal NFAT motif from the murine IL-2 promoter (5′-TCGACAAAAAGGAAAATTTGTTTCATACAGAAG-3′) was end-labeled using [γ-32P]ATP (3,000 Ci/mmol; Amersham-Buchler) and T4 polynucleotide kinase (Roche Molecular Biochemicals), followed by P-10 gel filtration (Bio-Rad) to remove nonincorporated radioactivity. A cold oligonucleotide mutated in the core NFAT recognition sequence (5′-TCGACAAAAAGGAAAATTTGTTTATACAGAAG-3′) was used for competition experiments (35). When supershift analysis was performed, nuclear extracts were preincubated with the antibodies for 30 min on ice.

**Western Blotting**—After the indicated treatments, nuclear extracts were prepared from 2 × 106 cells and loaded on an 8% SDS-PAGE under reducing conditions. Subsequently, separated proteins were electroblotted to a polyvinylidene difluoride membrane (Amersham-Buchler). The membranes were blocked for 1 h with 5% nonfat dry milk powder in Tris-buffered saline and incubated with different NFAT-specific antibodies. Membranes were washed four times with Tris-buffered saline and 0.05% Tween-20 and incubated with the respective peroxidase-conjugated affinity-purified secondary antibody for 1 h. After extensive washing, the reaction was developed by enhanced chemiluminescence staining using ECL reagents (Amersham-Buchler).

**RESULTS**

NFAT Is Activated upon Stimulation with Extracellular ATP—Incubation of N9 mouse microglial cells with extracellular ATP elicits a rapid increase in the intracellular Ca2+ concentration mediated by both the P2Z and P2Y receptors expressed in these cells (17). Whereas stimulation of P2Y receptors causes a transient Ca2+ increase mainly from intracellular stores, activation of the P2Z receptor involves a long-lasting Ca2+ influx across the plasma membrane. Because an increase in intracellular Ca2+ concentrations is required to activate the Ca2+-calmodulin-dependent phosphatase calcineurin that binds and dephosphorylates NFAT, we investigated the effect of extracellular ATP on NFAT activation in N9 cells. Until now, NFAT expression and activation have not been studied in microglial cells. In Fig. 1, we compared the effects of different agents leading to the elevation of intracellular Ca2+ ions. N9 cells were stimulated with ATP, the Ca2+-ionophore A23187, and thapsigargin, an inhibitor of the endoplasmic Ca2+-ATPase. After stimulation, nuclear extracts were prepared and analyzed for NFAT activation by EMSA. Trea...
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NFAT Activation Is Selectively Mediated by the P2Z Receptor—Because different members of the purinoreceptor superfamilies exhibit distinct affinities for ATP, we determined the dose dependence of ATP-induced NFAT activation. As shown in Fig. 3, NFAT activation became visible after cells were incubated with 1 mM ATP. Maximal NFAT activation was obtained with 3 mM ATP, whereas a higher concentration of 5 mM was less effective. This dose dependence corresponds to other effects of P2Z purinoreceptor signal transduction in N9 cells (17) and mouse macrophages (18).

Because N9 cells express both P2Y and P2Z purinoreceptors, we further tried to specify the receptor subtype involved by using different nucleotides and pharmacological ATP analogues. In addition to ATP, benzoylbenzoic ATP, which is a more potent agonist for the P2Z receptor than ATP, was able to induce NFAT activation, even at a concentration of 1 mM (Fig. 5A). In contrast, oxidized ATP did not inhibit NFAT activation in response to the Ca\(^{2+}\) ionophore A23187, demonstrating that it did not interfere with the intracellular signaling of NFAT dephosphorylation and translocation. We also investigated the effect of NFAT activation in N9 derivative cells that lack the P2Z receptor but still express P2Y receptors (17, 41). As shown in Fig. 5B, NFAT activation was almost not inducible by ATP in clone N9R17, whereas a Ca\(^{2+}\) ionophore strongly activated the transcription factor.

Cyclosporin A and FK506 Inhibit P2Z Receptor-mediated NFAT Activation which Requires a Sustained Increase of Extracellular Ca\(^{2+}\)—The immunosuppressive drugs CsA and FK506 prevent NFAT activation by inhibiting the Ca\(^{2+}\)-calmodulin-dependent phosphatase calcineurin that dephosphorylates NFAT and allows the nuclear translocation of NFAT. As shown in Fig. 6A, pretreatment of N9 cells with CsA or FK506 was able to prevent NFAT binding to DNA in ATP-stimulated cells. Essentially the same results were demonstrated by the absence of an anti-NFAT-1 immunoreactive protein in the nuclear extracts of cells pretreated with immunosuppressive drugs (Fig. 6B). The results therefore indicate that ATP-triggered NFAT translocation and DNA binding are calcineurin-dependent processes.

A common intracellular event triggered by antigen-stimulated receptors such as the T- and B-cell receptors is an increase in the cytoplasmic free Ca\(^{2+}\) concentration, leading to calcineurin and subsequent NFAT activation. To investigate whether Ca\(^{2+}\) was essential for ATP-induced NFAT activation, N9 cells were stimulated with the nucleotide in the presence of the Ca\(^{2+}\) chelator EGTA. Pretreatment of cells with 2 mM oxidized ATP that covalently binds and inhibits the receptor (39). Pretreatment of cells with 300 \(\mu\)M oxidized ATP completely abolished ATP-induced NFAT activation (Fig. 5A). In contrast, oxidized ATP did not inhibit NFAT activation in response to the Ca\(^{2+}\) ionophore A23187, demonstrating that it did not interfere with the intracellular signaling of NFAT dephosphorylation and translocation. We also investigated the effect of NFAT activation in N9 derivative cells that lack the P2Z receptor but still express P2Y purinoreceptors (17, 41). As shown in Fig. 5B, NFAT activation was almost not inducible by ATP in clone N9R17, whereas a Ca\(^{2+}\) ionophore strongly activated the transcription factor.
In the present study, we demonstrate that extracellular ATP is a potent activator of the proinflammatory transcription factor NFAT in microglial cells. Using different pharmacological approaches and a receptor-deficient cell clone, evidence is pro-

EGTA completely prevented NFAT DNA binding as well as the nuclear translocation of NFAT-1, indicating that activation of the transcription factor required extracellular Ca\(^{2+}\). These data concur with the above experiments, demonstrating that P2Y receptors, which mediate a transient Ca\(^{2+}\) increase from intracellular stores, were unable to activate NFAT. Therefore, in microglial cells, ATP-induced activation of NFAT required a sustained increase in the intracellular Ca\(^{2+}\) concentration, which is exclusively triggered by the P2Z receptor.

**Microglial Cells Express and Activate NFAT-1 and NFAT-2**—Among the NFAT family, four different genes encoding NFAT-1, NFAT-2, NFAT-3, and NFAT-4 have been identified (reviewed in Ref. 22). Although all members exhibit similar DNA binding specificity, they differ in their tissue distribution and inducibility upon cell stimulation, suggesting that each protein may serve specific functions. Whereas NFAT-1 is constitutively expressed in lymphocytes and in several non-lymphoid cells, NFAT-2 expression has been reported to be induced in activated T and B cells (24, 42). To analyze specificity and investigate which NFAT member contributes to the ATP-induced DNA complex, we performed competition and supershift analyses. In these experiments, higher resolution gels were used that could separate two ATP-induced DNA complexes. Both DNA complexes were efficiently competed by an excess of the NFAT-binding oligonucleotide, whereas an oligonucleotide mutated in the NFAT recognition sequence did not markedly affect NFAT DNA binding (Fig. 7A). As shown in Fig. 7B, an antibody against NFAT-1 reduced and supershifted the ATP-triggered DNA-protein complexes. Formation of the NFAT-specific complex, in particular, the lower DNA complex, was also inhibited by anti-NFAT-2, whereas no immunoreactivity was observed using a panel of antisera directed against different epitopes of either NFAT-3 or NFAT-4. In addition, most of the induced DNA complexes were inhibited and supershifted by a combination of anti-NFAT-1 and anti-NFAT-2. Because transcription factor AP-1 can associate with NFAT, we also used an anti-c-Fos antibody that supershifted the upper ATP-induced DNA complex (Fig. 7B).

The activation of NFAT-1 and NFAT-2 by ATP was confirmed by Western blot analysis of nuclear extracts from ATP-stimulated cells. After ATP stimulation, anti-NFAT-1 detected a nuclear protein of approximately 120 kDa that was absent in nuclear extracts from control cells (Fig. 7C). Anti-NFAT-2 recognized a prominent protein doublet band of approximately 90 kDa, similar in size to the short NFAT-2 isoform described previously (24, 29, 30). In contrast, we could not detect the expression and nuclear translocation of NFAT-3 or NFAT-4 in immunoblot analyses using different antisera (data not shown). Together, these results suggest that microglial cells express and activate NFAT by the P2Z purinoreceptor that consists mainly of NFAT-1 and NFAT-2.

**DISCUSSION**

In the present study, we demonstrate that extracellular ATP is a potent activator of the proinflammatory transcription factor NFAT in microglial cells. Using different pharmacological approaches and a receptor-deficient cell clone, evidence is pro-
vided that ATP-induced NFAT activation is selectively triggered by the P2Z receptor and not by other purinoreceptor subtypes. Because the P2Z receptor, but not P2Y receptors, induces a long-lasting transmembrane Ca\textsuperscript{2+} influx, the data concur with our observation that ATP-induced NFAT activation is dependent on the cytoplasmic increase of extracellular Ca\textsuperscript{2+}. The results therefore demonstrate a novel pathway by which extracellular ATP can modulate the activation of NFAT and subsequent proinflammatory processes.

The P2Z receptor is attracting increasing interest due to its role in inflammatory reactions and the induction of cell death. Several reports have recently demonstrated that the P2Z receptor is a potent mediator of IL-1β. Several reports have recently demonstrated that the P2Z receptor may also be involved in gene-regulatory events by activating transcription factor NF\textsuperscript{kB} (19). In addition, it has been found that extracellular ATP is involved in mitogenic stimulation of T-lymphocytes (44). Although the purinoreceptor subtype involved in this effect has not been clearly defined, its pharmacological profile resembles that of the P2Z receptor. It is therefore tempting to speculate that ATP-triggered T-cell mitogenicity may involve NFAT activation and subsequent IL-2 expression.

Both the NFAT and NF\textsuperscript{kB} transcription factors are Ca\textsuperscript{2+}-sensitive. Interestingly, activation of the two transcription factors occurred with strikingly different kinetics. NFAT DNA binding was visible within 1 min after ATP stimulation, whereas significant NF\textsuperscript{kB} activation was not detectable before 3 h of stimulation, when NFAT activation had already declined. It can be presumed that the required amplitude and duration of Ca\textsuperscript{2+} signals differ for the activation of NF\textsuperscript{kB} and NFAT and may therefore contribute to this temporal transcriptional specificity of ATP stimulation. In this respect, it has been demonstrated that in B cells, a low sustained Ca\textsuperscript{2+} increase is sufficient for NFAT activation upon B-cell receptor ligation, whereas high levels of Ca\textsuperscript{2+} are required to activate NF\textsuperscript{kB} (45, 46). Another reason for the different kinetic of activation of the two transcription factors could be that NF\textsuperscript{kB} may not be activated by Ca\textsuperscript{2+} alone but may require additional second messenger systems. Likely candidates in this respect are reactive oxygen intermediates, which are generated upon Ca\textsuperscript{2+} overload and other stress conditions (47, 48). Indeed, ATP-induced NF\textsuperscript{kB} activation could be abolished by antioxidants in microglial cells (19).

Currently, there is very little information about the expression and role of NFAT within the brain. Our study shows for the first time that microglial cells, which are regarded as the principal immune effector cells of the brain (37), may express both NFAT-1 and NFAT-2. Initially, it was reported that NFAT DNA binding activity could not be detected in crude brain extracts (49). More recently, however, NFAT has been found in PC12 pheochromocytoma cells as well as in neurons within the olfactory bulb (50). It will be interesting to investigate whether extracellular ATP, which exerts excitatory transmitter function, activates NFAT in these cells.

In lymphocytes, the expression of NFAT proteins is differentially regulated. Resting cells express only NFAT-1, whereas expression of NFAT-2 is induced to significant levels upon stimulation by Ca\textsuperscript{2+}-dependent signaling (24, 42). In addition, in normal lymphocytes, NFAT-3 is not expressed, and NFAT-4 is only detectable at low levels (29). In microglial cells, we observed that NFAT-1 and NFAT-2 were activated and translocated to the nucleus with a similar rapid kinetic, suggesting that both proteins are constitutively expressed in these cells. Because we demonstrated NFAT activation in the N9 cell line, future experiments will have to investigate the regulation of NFAT proteins in primary microglial cells. We could not detect expression and activation of NFAT-3 or NFAT-4, whereas, similar to previous reports (32, 35, 36), part of the ATP-induced NFAT complex was obviously associated with AP-1. The anti-serum against murine NFAT-4 used in this study recognizes NFATx, the longest isoform of NFAT-4, and detects all isoforms of the protein. In addition, we used several antisera raised against different epitopes of human NFAT-3. Although we cannot completely exclude that the failure to detect NFAT-3 may be due to a lack of cross-reactivity with the murine protein, we consider it rather unlikely that NFAT-3 was activated in N9 cells. A combination of anti-NFAT-1 and anti-NFAT-2 strongly prevented DNA binding, indicating that the ATP-induced DNA complex predominantly consisted of NFAT-1 and NFAT-2.

It can be presumed that microglial NFAT is involved in the inducible expression of proinflammatory cytokines. In this respect, it is known that activated microglia in inflammatory processes, Alzheimer’s disease, and other forms of neurodegeneration are capable of producing high amounts of cytokines that have been implicated in disease progression (reviewed in Ref. 37). Recent data further suggested that certain NFAT proteins play an important role exceeding their established immunoregulatory function in cytokine secretion. NFAT-2 and NFAT-3 have been implicated in heart development and cardiac hypertrophy (51, 52), indicating a morphogenetic and developmental role of NFAT in some tissues. Future studies are required to investigate the functional role of NFAT expressed in microglial cells or other cells within the brain.

In this study, we used microglial cells that have been previously characterized in detail for purinoreceptor expression (18, 40). These cells are a good model, because they may be stimulated to produce cytokines by ATP from adjacent neurons. ATP has been convincingly shown to be physiologically released from neurons, where it is co-accumulated with acetylcholine and noradrenalin in adrenergic and cholinergic nerve terminals (53, 54). Collectively, the specific activation of NFAT through P2Z receptor ligation may represent a novel mechanism of how extracellular ATP may trigger inflammatory processes and exert neuroimmunomodulatory functions.

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