P2Y1 Receptor-evoked Glutamate Exocytosis from Astrocytes

CONTROL BY TUMOR NECROSIS FACTOR-α AND PROSTAGLANDINS*

Maria Domercq1, Liliana Brambilla1, Ethel Pilati1, Julie Marchaland1, Andrea Volterra1,5, and Paola Bezzi1,2

From the 5Department of Cell Biology and Morphology, University of Lausanne, Rue du Bugnon 9, 1005 Lausanne, Switzerland and the 6Department of Pharmacological Sciences, Center of Excellence on Neurodegenerative Diseases, University of Milan, Via Balzaretti 9, 20133 Milan, Italy

ATP, released by both neurons and glia, is an important mediator of brain intercellular communication. We find that selective activation of purinergic P2Y1 receptors (P2Y1R) in cultured astrocytes triggers glutamate release. By total internal fluorescence reflection imaging of fluorescence-labeled glutamatergic vesicles, we document that such release occurs by regulated exocytosis. The stimulus-secretion coupling mechanism involves Ca2+ release from internal stores and is controlled by additional transductive events mediated by tumor necrosis factor-α (TNFα) and prostaglandins (PG). P2Y1R activation induces release of both TNFα and PGE2, and blocking either one significantly reduces glutamate release. Accordingly, astrocytes from TNFα-deficient (TNF−/−) or TNF type 1 receptor-deficient (TNFR1−/−) mice display altered P2Y1R-dependent Ca2+ signaling and deficient glutamate release. In mixed hippocampal cultures, the P2Y1R-evoked process occurs in astrocytes but not in neurons or microglia. P2Y1R stimulation induces Ca2+-dependent glutamate release also from acute hippocampal slices. The process in situ displays characteristics resembling those in cultured astrocytes and is distinct from synaptic glutamate release evoked by high K+ stimulation as follows: (a) it is sensitive to cyclooxygenase inhibitors; (b) it is deficient in preparations from TNF−/− and TNFR1−/− mice; and (c) it is inhibited by the exocytosis blocker bafilomycin A1 with a different time course. No glutamate release is evoked by P2Y1R-dependent stimulation of hippocampal synaptosomes. Taken together, our data identify the coupling of purinergic P2Y1R to glutamate exocytosis and its peculiar TNFα- and PG-dependent control, and we strongly suggest that this cascade operates selectively in astrocytes. The identified pathway may play physiological roles in glial-glial and glial-neuronal communication.

Studies of the last few years are revolutionizing our views on the functional interrelations between neurons and glia (reviewed in Refs. 1–5). Astrocytes are activated during synaptic transmission and typically respond by increasing their intracellular calcium concentration ([Ca2+]). Astrocyte [Ca2+], elevations start active signaling, in particular release of chemical gliotransmitters that propagate communication to other glial (6–8), blood vessel (9–12), or neuronal cells (for review see Ref. 4). Glutamate has been identified as a prominent gliotransmitter by which astrocytes modulate and synchronize the activity of neighboring neuronal circuits (13–24). In addition, glial cells control synaptic functions and neuronal excitability by releasing ATP (25), often rapidly hydrolyzed to adenosine (26–29) and D-serine, an agonist at the glycine-binding site of NMDA receptors (30, 31).

Astrocytes release glutamate by Ca2+-dependent and Ca2+-independent mechanisms. Release via the Ca2+-dependent pathway is generally triggered by activation of G-protein-coupled receptors with ensuing inositol 1,4,5-trisphosphate-induced Ca2+ release from the stores of the endoplasmic reticulum (13, 14, 32–36). Recently, a clear synaptic-like microvesicle compartment, equipped for uptake, storage, and release of glutamate, has been identified in adult hippocampal astrocytes, and Ca2+-dependent glutamate exocytosis in response to stimulation of metabotropic glutamate receptors has been documented in astrocyte cultures by TIRF imaging (4, 37). The existence of regulated glutamate exocytosis in astrocytes is further supported by studies using additional optical detection approaches (35, 38), membrane capacitance measurements (39, 40), electrochemical amperometry (41), and by studies of selective interference with the proteins of the synaptic vesicle fusion machinery (14, 33, 42–45).

Ca2+-independent glutamate release is less well characterized. It may take place by molecular transport of cytosolic glutamate across the plasma membrane through specialized proteins such as channels and transporters. Carrier-mediated release can occur by reversal of high affinity glutamate transmembrane transporters. This process is dependent on the availability of extracellular glutamate and is thought to be responsible for the large-amplitude fluctuations of glutamate concentration in the extracellular space. Control of glutamate transporters by G-protein-coupled receptors is probably the major way to modulate the release of glutamate by astrocytes (45). A positive modulating effect of dopamine on glutamate release has been reported (46).

3 The abbreviations used are: [Ca2+], intracellular calcium; TNFα, tumor necrosis factor-α; PGE2, prostaglandin E2; GFAP, glial fibrillary acidic protein; GDH, glutamate dehydrogenase; AO, acridine orange; BAPTA/AM, 1,2-bis(2-aminoethyl)iminooctane-N,N,N’,N’-tetraacetic acid tetrasodium (polyethyleneglycol) ester; Baf A1, bafilomycin A1; TeNT, tetanus neurotoxin; TNFR-1, TNF receptor-1; sTNFR1, soluble TNF receptor 1; VGLUTs, vesicular glutamate transporters; MeSAPD, α,β-methyleneadenosine 5’-triphosphate; EGFP, enhanced green fluorescent protein; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; TACE, TNF-α-converting enzyme; EPI, epifluorescence illumination; A3P5PS, adenosine 3’-phosphatase-5’-phosphosulfate; BzATP, 2’-3’-O-(4-benzoylbenzoyl) adenosine 5’-triphosphate; CPA, cyclopiazonic acid; INDO, indomethacin; ASA, aspirin.

* This work was supported in part by Swiss FNS Grant 3100A0-100850/1, OFES Grant 00.0553, Italian MIUR Cofin 2002 and FIRB 2003, and CARIPOLO (to A.V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Recipient of a postdoctoral fellowship from the Basque Government. Present address: Dept. of Neurosciences, Faculty of Medicine, University of the Basque Country, 48640 Leioa, Vizcaya, Spain.

2 To whom correspondence should be addressed. Tel.: 41-21-692-5284; Fax: 41-21-692-5105; E-mail: Paola.Bezzi@unil.ch.
Porter's, a process that depends on ionic membrane changes mostly occurring under ischemic/hypoxic conditions (46) or by cystine-glutamate exchange (47). Three types of large plasma membrane channels have been claimed to participate in the nonexocytotic release of glutamate as follows: (i) swelling-activated anion channels, whose identity is still unknown (48); (ii) purinergic P2X7 receptors, gated by high concentrations of extracellular ATP (49); (iii) gap-junction hemi-channels formed by hexamers of connexin 43 (50). Opening of connexin-hemichannels (51–53) or of P2X7 receptors (54) would contribute to release of ATP as well, although a Ca\(^{2+}\)-dependent ATP release process has also been described (55).

ATP and other purine nucleotides and nucleosides play an increasingly recognized role in rapid intercellular signaling in both the central and peripheral nervous system (reviewed in Refs. 4 and 56). Two large families of purinergic receptors mediate neural ATP functions, ionotropic P2XR and metabotropic P2YR, operating on different time scales; P2XR generally act within milliseconds and mediate ATP-dependent neurotransmission and other very fast ATP effects (57, 58). By contrast, P2YR act on longer time scales and, among other functions, mediate intercellular communication in glial networks (7, 8, 56, 59, 60). Here we show that selective activation of the purinergic P2Y1R subtype triggers glutamate exocytosis from cultured astrocytes as well as from acute hippocampal slices (but not from isolated synaptosomes). In both preparations P2Y1R-evoked Ca\(^{2+}\)-dependent glutamate release is controlled by TNF\(\alpha\) signaling via TNFR1 and by prostaglandins.

**MATERIALS AND METHODS**

**Pharmacological Agents**—Recombinant rat TNF\(\alpha\) and sTNFR1 were obtained from R&D Systems Europe (Oxon, UK); anti-TNF\(\alpha\) antibody (AbTNF\(\alpha\)) was from Genzyme (Cambridge, MA); PD98059 was from Bioworld (Plymouth Meeting, PA); tetanus neurotoxin (TeNT) was a gift from C. Montecucco; and BB3103 was a gift from British Biotech (Cambridge, MA); PD98059 was from Biomol (Plymouth, PA); tetanus neurotoxin (TeNT) was a gift from C. Montecucco; and BB3103 was a gift from British Biotech (Cambridge, MA). Activated microglia-pure cultures (>99% positive for the specific marker, isolectin B4) were prepared as described (33) and used in experiments within 12–24 h.

**Hippocampal Slices and Synaptosomes**—Transverse thin slices (200 \(\mu\)m) from the hippocampi of 6–8-week-old rats or mice were prepared using a vibratome (Campden Instruments Limited, UK) in oxygenated ice-cold artificial cerebrospinal fluid containing (in mM): NaCl 120, KCl 3.1, NaH\(_2\)PO\(_4\) 1.25, NaHCO\(_3\) 25, MgCl\(_2\) 2, CaCl\(_2\) 1, glucose 4, pyruvate 2, myoinositol 0.5, ascorbate 0.1 (pH 7.4). Hippocampal slices were carefully isolated from the cortical tissue and were subsequently incubated in artificial cerebrospinal fluid continuously bubbled with 95% O\(_2\), 5% CO\(_2\) at 37 \({\text{°C}}\) for at least 1 h before processing.

Highly purified synaptosomes were prepared from hippocampi of 6–8-week-old rats or mice as described with minor modifications (62). For obtaining highly pure synaptosomes, the crude P2 fraction was further purified by centrifugation (18,000 rpm, 5 min) on a discontinuous three-step 3–10–23% Percoll gradient (Amersham Biosciences), and the synaptosomes were collected at the second interface (23/10%). Synaptosomal pellets were resuspended (1 mg/ml) in phosphate buffer containing (in mM): Na\(_2\)HPO\(_4\) 300, NaH\(_2\)PO\(_4\) 100, NaCl 140, KCl 5, NaHCO\(_3\) 5, MgSO\(_4\) 1.3, CaCl\(_2\) 1, HEPES 10, glucose 10 (pH 7.4) and stored in suspension at 4 \({\text{°C}}\) before use (<6 h).

Just before the assay, synaptosomes were preincubated (30 min, 37 \({\text{°C}}\) with bovine serum albumin (16 \(\mu\)g/ml) to bind any free fatty acids released.

**TNF\(\alpha\)- and TNFR1-deficient Mice**—Mice homozygous for the null mutant TNF\(\alpha\) (TNF\(\alpha\)\(^{-/-}\)) (63) or TNF receptor 1 (TNFR1\(^{-/-}\)) allele, kindly provided by Dr. G. Kollias, were generated and maintained on a C57BL/6j background as described (63, 64). Knock-out animals were housed under specific pathogen-free conditions together with age- and sex-matched wild-type mice (TNF\(\alpha\)\(^{+/+}\) and TNFR1\(^{+/+}\), respectively). In our experiments we always utilized sister preparations made in parallel from transgenic and wild-type littermate animals.

**Enzymatic Assay of Endogenous Glutamate Release**—Efflux of endogenous glutamate from cell cultures, hippocampal slices, or synaptosomal suspensions was monitored by use of an enzymatic assay as described previously (33). Briefly, either type of preparation was lodged in a cuvette inside a computerized...
spectrofluorometer (LS50B, PerkinElmer Life Sciences) at 37 °C under stirring in a buffer containing (in mM) NaCl 120, KCl 3.1, NaH₂PO₄ 1.25, HEPES-Na 25, glucose 4, MgCl₂ 1, CaCl₂ 2 (pH 7.4), added with glutamate dehydrogenase (GDH, 40 units/ml; batch B27516; Calbiochem) and 1 mM NADP⁺

Glutamate released from the preparations was immediately oxidized by GDH to α-ketoglutarate with formation of NADPH and fluorescence emission at 430 nm (excitation light, 335 nm).

Release was quantified referring to standard curves constructed with exogenous glutamate and normalized for the protein content of each sample. Agents were added directly in the cuvette through a microsyringe, except those acting intracellularly, which required preincubation (30 min, unless otherwise specified). The effect of BaF A1 in hippocampal slices was assessed by exposing the slices to the drug for 2 h by having prestimulated the slices with agonists or saline at the beginning of the exposure period.

Astrocyte and INS-1 Cell Cultures for Imaging Experiments—

For TIRF and Ca²⁺ imaging experiments, we used high density astrocyte cultures (see above). To monitor glutamatergic vesicle fusions, astrocytes were transfected with the VGLUT2-EGFP construct as described (37) about 6 days after replating. For experiments using two color imaging with alternating TIRF and epifluorescence (EPI) illumination, we used low density co-cultures of astrocytes and INS-1 insulinoma cells (INS-1 cells; INS-1E cell line obtained from Dr. CB Wollheim). astrocytes were plated (2.5 × 10⁴ cells) on glass coverslips and used 2–3 days after transfection with the VGLUT2-EGFP construct, INS-1 cells were plated either alone (1.5 × 10⁵ cells) or on the transfected astrocytes and were used the next day. For experiments based on single cell [Ca²⁺]ᵢ measurements with EPI illumination, cells were loaded with 2.5 µg/ml X-rhod-1 AM (Molecular Probes) for 40 min at room temperature in a HEPES-KRH buffer containing (in mM) the following: NaCl 120, KCl 3.1, MgCl₂ 2, CaCl₂ 1.8, NaH₂PO₄ 1.25, HEPES-Na 25, glucose 4 (pH 7.4) and then allowed to de-esterify for 40 min before imaging. For experiments where we combined acridine orange (AO) and X-rhod-1 imaging under dual illumination (TIRF and EPI), X-rhod-1 loading was followed by staining with AO (2 µM, 10 min). After several washes, coverslips were mounted in an open perfusion micro-incubator (PDMI-2, Harvard Apparatus) set at 37 °C on the stage of the optical recording microscope. During experiments, cells were continuously perfused with HEPES-KRH (1 ml/min), and stimuli were rapidly (2 or 5 s) applied via a software-controlled micro-perfusion fast-step device (100 µl/min, Warner Instrument Corp.).

Optical Imaging—A Zeiss Axiovert 200 inverted fluorescence microscope was modified to allow both EPI and TIRF illumination (Visiion Systems). EPI illumination was utilized for single-cell [Ca²⁺]ᵢ measurements in either rat or mouse astrocytes or INS-1 cells. X-rhod 1 fluorescence was recorded through a 63× objective lens (Zeiss, Plan Apochromat) and directed through a 590 long pass filter (Zeiss filter set 15) at 50- or 100-ms (for INS-1 cells and astrocytes, respectively) intervals by imaging with excitation light at 568 nm generated by a polychromator illumination system (Visichrome, Visitrion, Germany). For TIRF illumination, the expanded beam (488/568 nm argon/krypton multiline laser, 20 milliwatts; Laserphysics) was passed through an AOIT laser wavelength selector (VisiTech International, UK) synchronized with a SNAP-HQ CCD camera (Roper Scientific) under Metafluor software (Universal Imaging) control and introduced from the high numerical aperture objective lens (Zeiss α-plan FLUAR 100×, 1.45 NA). Light entered the coverslip and underwent total internal reflection at the glass-cell interface. In our experimental conditions penetration depth of TIRF illumination was calculated to be 84 nm (37, 65). In experiments on high density astrocyte cultures, we imaged VGLUT2-EGFP and AO fluorescence under 488 nm TIRF illumination as described (37, 66). The images were acquired at 20 interlaced frames/s. In experiments on low density astrocyte-INS-1 co-cultures we imaged both X-rhod-1 and AO fluorescence with a dual shutter. In practice, we combined EPI illumination (568 nm xenon arc lamp) and TIRF illumination (488 nm laser) at 10 interlaced frames/s. Lights were filtered with a beam splitter (Zeiss filter set 24).

Image Analysis—Video images, digitized with MetaFluor, were analyzed with MetaMorph software (Universal Imaging). AO flashes were measured as fluorescence intensity changes (arbitrary units) in a circle (diameter, 895 nm) positioned on the spot, and in a concentric annulus (inner diameter, 895 nm; outer diameter, 1195 nm). Only when the fluorescence increased, spread, and then declined was the event counted as a flash (67). Temporal dynamics in X-rhod-1 fluorescence were expressed as background-subtracted ΔF/F₀ (%), where F₀ represents the fluorescence level of the cells before stimulation, and ΔF represents the change in fluorescence occurring during stimulation of the cell. Statistical differences were established using the Student’s t test at p < 0.01. Data are expressed as mean ± S.E.

Monitoring of TNFα Release and PGE₂ Formation—Cell-free supernatants from astrocyte cultures were collected after 3- or 5-min incubations with stimulants. Release of TNFα was monitored using a highly sensitive enzyme-linked immunosorbent assay kit (R&D Systems Europe, Oxon, UK). PGE₂ generation was detected using the sensitive prostaglandin E₂ EIA kit containing monoclonal antibody (Cayman Chemical, Ann Arbor, MI).

P2Y1 and TNFR1 Immunocytochemistry and Western Blotting—Double immunofluorescent labeling in astrocyte cultures was done sequentially with polyclonal antibodies directed against the following: (a) P2Y1R (2 µg/ml) (68); or (b) TNFR1 (1 µg/ml, R&D Systems) and with monoclonal antibodies against GFAP (1 µg/ml; Chemicon, Hofhein, Germany). In other cell culture models, MAP-2 (1:300; Sigma) or the lectin isolectin B4 (1:100; Vector Laboratories, Burlingame, CA) were used as selective neuronal and microglial markers, respectively. Primary antibodies were visualized with Alexa 488-conjugated anti-rabbit or anti-goat antibodies (1:300; Molecular Probes Europe, Leiden, The Netherlands) and with biotinylated antibodies to mouse IgGs (1:200; Vector Laboratories, Burlingame, CA) followed by streptavidin-Texas Red conjugate (1:100; Chemicon). Omission of the primary antibody or its replacement with nonimmune IgG yielded no labeling.
Western blot analysis of P2Y1R expression was performed in hippocampi from TNRα+/+ and TNRα−/− mice by conventional SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes by means of a semidyry electroblotter (BioRad). Nonspecific binding was blocked for 1 h at room temperature with 5% nonfat milk in 50 mM Tris-HCl, 200 mM NaCl, 0.1% Tween 20 (pH 7.4). Membranes were subsequently incubated overnight at 4 °C with antibodies to P2Y1R (5 ng/ml). Immunoreactive proteins were visualized with an enhanced chemiluminescence substrate (Amersham Pharmacia). Densitometric analysis was performed with the NIH Image program.

RESULTS

P2Y1R-evoked Glutamate Release from Cultured Astrocytes—Administration of ATP (100 μM) to high density astrocyte cultures elicited rapid release of endogenous glutamate (Fig. 1a). The effect of the nucleotide was dose-dependent (Fig. 1b); glutamate release responses were observed starting with 500 nM ATP (0.11 ± 0.02 nmol/mg protein, n = 5), had an EC50 of 6 μM, and were saturated with 100 μM ATP (0.78 ± 0.12 nmol/mg protein, n = 6). The nature of the receptors mediating this ATP effect was then investigated by use of pharmacological agents with known selectivity (69) (Fig. 1, a and c). The response to 100 μM ATP was abolished in the presence of either suramin (100 μM) or pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (100 μM), generic blockers of P2 purinergic receptors, but also with adenosine 3′-phosphate-5′-phosphosulfate (A3P5PS, 100 μM), a P2Y1R-selective antagonist (59). Conversely, the P2Y1R agonist, 2-(methylthio)adenosine 5′-diphosphate (2MeSADP) (59), potently stimulated glutamate release in astrocytes. At 10 μM, 2MeSADP induced glutamate release responses similar to those observed with 100 μM ATP (0.82 ± 0.11 nmol/mg protein; n = 6; Fig. 1, a and c). In contrast, UTP (100 μM), which activates several P2Y receptors but not the P2Y1R subtype, was unable to replicate the 2MeSADP-induced effect. Likewise, α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP; 100 μM) and 2′-3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (BzATP, 100 μM), two P2X-prefering agonists, acting at P2X1 and -3 receptors, respectively, induced no detectable release of endogenous glutamate (Fig. 1c).

Taken together, these results indicate that the identified glutamate release process is mediated specifi-
P2Y1-evoked Glutamate Exocytosis from Astrocytes

**FIGURE 2.** Astrocyte P2Y1R activation induces Ca^{2+}-dependent exocytosis of glutamatergic vesicles accompanied by NMDAR-dependent [Ca^{2+}]elevations in sniffer INS-1 cells. a, glutamate release induced by 2MeSADP (10 μM) is suppressed in astrocyte cultures pre-exposed to the following: (a) the intracellular Ca^{2+} chelator BAPTA/AM (50 μM, 30 min); or (b) the exocytotic fusion complex inhibitor TeNT (2 μg/mL, 20 h); or (c) the vesicular uptake inhibitor Baf A1 (100 nM, 2 h). Data (mean ± S.E.) are expressed as percentage of the 2MeSADP-evoked response (0.82 ± 0.11 nmol/mg protein; for each condition: n = 4–6 in triplicate). b, 2MeSADP elicits exocytosis of glutamatergic vesicles from VGLUT2-EGFP-expressing astrocytes in high density cultures. VGLUT^{−} and AO-loaded vesicles were monitored before, during, and after a 2-s pulse of 2MeSADP (10 μM, n = 5). The graph represents the time distribution of the fusion events; each individual histogram represents the number (mean ± S.E.) of AO flashes counted in a 50-ms-long frame in five different experiments. c, INS-1 cells respond to glutamate but not to 2MeSADP. INS-1 cells cultured with or without the astrocytes, and loaded with X-rhod-1 respond to glutamate (Gluc 100 μM, n = 12) with a [Ca^{2+}]elevation (ΔF/F0 (%), sensitive to the NMDAR antagonist D-AP5 (50 μM; n = 4; *, t test p < 0.001). INS-1 cells display a similar [Ca^{2+}]elevation in response to NMDA (50 μM, n = 5) but not to the P2Y1R agonist 2MeSADP (10 μM, n = 4). d, dual shutter TIRF and EPI fluorescence allows parallel monitoring of vesicle secretion in a VGLUT2-EGFP-expressing astrocyte and [Ca^{2+}]elevations in a INS-1 cell forming an isolated pair in low density astrocyte-INS-1 cell co-cultures. The figure shows one experiment, representative of 9, in which 2MeSADP induces fusion events (AO flashes) in the astrocyte followed by NMDAR-dependent (Ca^{2+})elevation, in the INS-1 cell. Black histograms represent the number of AO flashes attributed to VGLUT2-EGFP-positive vesicles in the astrocyte (left scale); the red dashed line represents [Ca^{2+}]elevation, in the INS-1 cell, expressed as ΔF/F0 (% of X-rhod-1 fluorescence (right scale). Cells were pulsed twice with 2MeSADP (10 μM, 2 s) with an interfused washing period (about 3 min). In the presence of the NMDAR antagonist D-AP5 (10 μM), 2MeSADP induces a burst of AO flashes in the astrocyte and no significant [Ca^{2+}]elevation, in the INS-1 cell. Upon washout of D-AP5, however, P2Y1R agonist-evoked AO flashes in the astrocyte are immediately followed by [Ca^{2+}]elevation. In the INS-1 cell, glutationate secretion is abolished by the exocytosis blocker TeNT and also by cyclopiazonic acid (CPA), a drug that depletes intracellular Ca^{2+}stores. 2MeSADP (10 μM) induces AO flashes from VGLUT2-EGFP-positive vesicles in astrocytes (black histograms, left scale) followed by [Ca^{2+}]elevation in INS-1 cells (red histograms, right scale). Preincubation of the cells with either TeNT (8 μM, 12 h, n = 4) or CPA (10 μM, 15 min; n = 5) abolished both AO flashes and [Ca^{2+}]elevation, in the INS-1 cells. **, t test p < 0.01.

tetanus neurotoxin (TeNT; 20 h, 2 μg/mL), an endopeptidase that cleaves specifically and inactivates VAMP, the v-SNARE protein essential for the formation of the vesicle fusion complex (71).

To directly demonstrate that the P2Y1R-evoked glutamate release occurs by regulated exocytosis, we utilized TIRF microscopy (72) and an experimental approach recently developed in our laboratory (4, 37, 65). Astrocytes in high density cultures were transfected with a chimeric vesicular glutamate transporter construct (VGLUT2-EGFP) and double-stained with acridine orange (AO), a cargo dye that accumulates in a self-quenched state inside acidic compartments (73), including VGLUT-EGFP-positive (VGLUT^{+}) vesicles (37). Therefore, EGFP fluorescence allows the identification of VGLUT^{+} vesicles, whereas abrupt changes in AO fluorescence signal their exocytic fusion. Thus, AO release in the extracellular space upon vesicle fusion with the plasma membrane leads to dequenching of the dye with a transient increase of fluorescent light (a “flash”) followed by diffusion and, eventually, disappearance of the fluorescence (37, 73, 74). Therefore, each fusion event can be detected and counted as a flash of AO fluorescent light (AO flash) (37, 74). A brief (2-s) localized pulse of 2MeSADP (10 μM) evoked numerous AO flashes from perfused astrocytes. Such flashes were counted in individual cells containing VGLUT^{+} vesicles. Histograms in Fig. 2b show the average time distribution of VGLUT^{+} vesicle fusions counted in 50-ms time frames (n = 5 different experiments). The number of fusion events rapidly increased to reach a peak within 350 ms from the start of 2MeSADP application to then gradually decrease and slowly return to background values after a total of about 5 s. Overall, 55.2 ± 9.2% of the VGLUT^{+} vesicles present in the TIRF field underwent exocytosis. When saline was applied instead of 2MeSADP, no increase above background number in the fusion of the cells was observed demonstrating the specificity of the 2MeSADP-dependent effect. In a second series of experiments INS-1 cells were co-cultured with astrocytes and used as biosensors of astrocytic glutamate release events in response to P2Y1R stimulation. INS-1 cells are well suited for this purpose because, when cultured in the absence of astrocytes, (a) they respond to glutamate with the NMDA receptor (NMDAR)-dependent [Ca^{2+}]elevations, blocked by D-AP5, duplicated by NMDA (37) (Fig. 2c), and (b) they do not show any detectable Ca^{2+} response to the P2Y1R agonist 2MeSADP (10 μM, see Fig. 2b).
were performed on isolated pairs of the two cell types, generally
arranged with the INS-1 cell and the astrocyte in direct lateral
contact (37). Cells were stimulated by two subsequent pulses (2 s)
of 2MeSADP (10 μM) separated by an interval (washing period)
of about 3 min (Fig. 2e). In nine experiments we always
noticed the same sequence of events as follows: (a) at first
2MeSADP induced AO flashes from VGLUT+ vesicles (33.4 ±
4.5% of those present in the TIRF field) in the astrocyte; such
flashes occurred in a burst that lasted about 1 s and peaked 350
ms from the start of drug application; (b) the AO flashes were
then rapidly followed by [Ca2+]i elevations (at peak: 35.4 ± 4.8
ΔF/Δt %) in the INS-1 cell (average peak AO-to-peak [Ca2+]i,
delay, 106 ± 52 ms). As shown in Fig. 2c, the [Ca2+]i elevations
in the INS-1 cell were glutamate-dependent because, in the
presence of the NMDAR antagonist D-AP5 (100 μM),
2MeSADP induced almost no [Ca2+]i, rise in the INS-1 cell,
while still producing multiple AO flashes in the attached astrocyte
(n = 4). Upon washout of D-AP5, however, the same pair of
cells responded to 2MeSADP with AO flashes in the astrocyte
followed by [Ca2+]i, elevation in the INS-1 cell. The existence of
a causal relation between exocytic fusions in the astrocyte and
glutamate release events detected in the INS-1 cell was sup-
ported by the observation that TeNT (8 μg/ml, 12 h) aboli-
shed both AO flashes in the astrocyte (−99 ± 1.5%; n = 4) and
[Ca2+]i, elevation in the INS-1 cell (−95 ± 1.2%; n = 4). Like-
wise, administration of cyclopiazonic acid (CPA, 10 μM, 15
min), a drug that depletes intracellular Ca2+ stores by inhib-
iting the endoplasmic reticulum Ca2+-ATPase, abolished not
only the AO flashes in the astrocytes (−98 ± 2%, n = 5) but also
the NMDAR-dependent [Ca2+]i, elevations (−92 ± 2.6%; n = 5)
in the INS-1 cells (Fig. 2d). Because D-serine is a gluta-
mate co-agonist at NMDAR and could be released by astrocytes
via Ca2+-dependent exocytosis (75), we verified whether NMDAR-
dependent [Ca2+]i, elevation in INS-1 cells contained a D-serine-
dependent component. To this end, 2MeSADP was applied in
the presence of the D-serine catalytic enzyme, D-amino acid
oxidase (0.5 units/mg protein, see Ref. 31). The NMDAR-de-
dependent [Ca2+]i, elevation was in this case 86.7 ± 12.4% of the
elevation observed in the absence of D-amino acid oxidase (n =
7, t test p > 0.85; not significant), suggesting that D-serine does
not contribute significantly to the response. Altogether these
observations demonstrate that activation of astrocytic P2Y1
receptors induces Ca2+-dependent glutamate exocytosis and
that this requires Ca2+ release from the intracellular stores.

**TNFa Signaling via TNFR1 Controls P2Y1R-dependent Glutamate Release**—The next intriguing observation was that puri-
nergic P2Y1R signaling in astrocytes induces release of TNFa.
Stimulation of astrocyte cultures with ATP (100 μM, 5 min)
resulted in a significant enhancement of the extracellular levels
of this cytokine, as measured by a sensitive enzyme-linked
immunosorbent assay (Fig. 3a). The effect of the nucleotide
was blocked by A3P5PS (100 μM) and reproduced by 2MeSADP (10
μM). In contrast, agonists of other P2 receptor subtypes, UTP
(100 μM) and BzATP (100 μM), were devoid of any effect (Fig.
3a). Given that BB3103 (3 μM), an inhibitor of the TNFa-con-
verting enzyme (TACE), abolished P2Y1R-dependent TNFa
release, the cytokine must be shed from its transmembrane pre-
cursor via the metalloprotease activity of this enzyme.

P2Y1R-evoked TNFa shedding involves Ca2+-dependent sig-
naling and activation of the MAPK/ERK pathway because it was
abolished in cultures pretreated with either the intracellular
Ca2+ chelator BAPTA/AM (50 μM, 30 min) or with PD98059
(30 μM), a MEK1/2 kinase inhibitor (Fig. 3a).

Release of TNFa controls the glutamate response to P2Y1R
stimulation. Indeed, 2MeSADP-evoked glutamate release in rat
cultures was significantly reduced in the presence of TNFa
blockers (Fig. 3b) as follows: (a) BB3103 (3 μM), inhibiting
TNFa shedding; (b) anti-TNFα antibody (anti-TNFα Ab, 100
ng/ml); and (c) soluble TNF receptor 1 (sTNFR1, 200 ng/ml),
acting as neutralizing acceptors of the soluble cytokine.
Consistent with the identified role of the ERK/MAPK kinase path-
way upstream of metalloprotease-dependent TNFa shed-
ing, blocking this pathway with PD98059 (30 μM) similarly
inhibited glutamate release.

In agreement with the pharmacological data, glutamate
release responses to 2MeSADP were about 7-fold lower in
astrocyte cultures prepared from TNFa-deficient (TNFa−/−)
mice (63) than in cultures from littermate TNFa+/+ mice (n =
6; Fig. 3c). The latter showed responses comparable with those
observed in cultures of rat astrocytes (Fig. 1a). Reduced glutama-
tic release was not associated with reduced expression of
P2Y1R receptors in TNFa−/− mice, as shown by Western blot
analysis of hippocampi of TNFa−/− and TNFa+/+ mice (Fig.
3c; P2Y1R receptor density, in arbitrary units 3.01 ± 0.10 and
2.93 ± 0.05, respectively; n = 6).

Experiments in mice null mutants for p55, type 1 TNF recep-
tors (TNFR1−/− mice) (64), show that TNFR1−/− astrocytes,
like TNFa−/− astrocytes, respond to 2MeSADP with low gluta-
amate release (Fig. 3d; n = 3). In contrast, they release TNFa to
an extent identical to that observed in astrocytes from
TNFR1+/+ littersmates (n = 3; Fig. 3d). Therefore, lack of
TNFR1 interferes with P2Y1R-dependent signaling down-
stream to the TNFa, directly implicating this receptor in the
TNFa-dependent control of glutamate release. Immunocyto-
chemistry experiments confirm that TNFR1 is constitutively
expressed in most GFAP-positive astrocytes in our cultures
(Fig. 3e).

**TNFa Mediates a Component of P2Y1R-dependent [Ca2+]i,
Elevation**—We next investigated by which mechanism(s)
TNFa could influence P2Y1R-dependent glutamate release.
Ca2+ signaling represents a converging pathway through which
different stimuli induce glutamate release from astrocytes (14,
33). Therefore, we verified whether TNFa-dependent signaling
has a role in the Ca2+ response evoked by P2Y1R activation.

Fast (2 s) localized application of 2MeSADP (10 μM) to high
density mouse or rat astrocyte cultures induced in the perfused
cells [Ca2+]i, elevations typically consisting of a single transient
(mean peak amplitude: mouse, 54.7 ± 11.2 ΔF/Δt; n = 18; rat,
47.8 ± 8.8 ΔF/Δt; n = 12; see Fig. 3f). Ca2+ responses were then
studied in sister cultures from TNFa−/− and TNFR1−/− mice
(Fig. 3f, left). In both TNFa−/− and TNFR1−/− astrocytes,
2MeSADP evoked [Ca2+]i, elevations. Their peak amplitudes
were, however, lower than that observed in TNFa+/+ mice
(−32.5 ± 5.7%, n = 9, and −30.6 ± 0.3%, n = 7, respectively).
To verify that reduced Ca2+ responses in knock-out animals
depended specifically on lack of TNFa signaling, we performed
FIGURE 3. TNFα release and signaling via TNFR1 controls P2Y1-evoked glutamate release and [Ca2+]i elevations. a, release of TNFα from astrocyte cultures in response to stimulation with ATP and purinergic agonists (5 min; for concentrations see legend to Fig. 1). Data are expressed as pg/ml above basal (1.8 ± 0.6 pg/ml; for each agent; n = 3 in triplicate). ATP-evoked TNFα release is prevented by the P2Y1R antagonist A3P5PS (100 μM) and reproduced by 2MeSADP (10 μM). 2MeSADP-evoked TNFα release is inhibited in cells preincubated with BAPTA/AM (50 μM), the MEK1/2 inhibitor PD98059 (30 μM), or the TACE inhibitor BB3103 (3 μM) (for each pharmacological condition; n = 3 in triplicate). b, 2MeSADP-evoked glutamate release is controlled by TNFα. Glutamate release in response to the P2Y1R agonist is strongly reduced in cultures pretreated with either (i) the MEK1/2 inhibitor PD98059 (30 μM) or (ii) TNFα blockers, including the TACE inhibitor BB3103 (3 μM) and the TNFα neutralizing agents sTNFR1 (200 ng/ml) and anti-TNFα antibody (100 ng/ml) (for each pharmacological condition n = 4–6 in triplicate). c, histograms show that astrocytes from TNFα-null mutant mice (TNFα−/−) display deficient glutamate release responses to 2MeSADP (10 μM), whereas those from wild-type (TNFα+/+) littermates respond as observed in rat astrocytes (Fig. 1b; n = 5–6 in triplicate). Inset, Western blot analysis of hippocampal homogenates showing that P2Y1R is expressed at similar levels in TNFα+/+ and TNFα−/− preparations. d, glutamate release (black bars) and TNFα release (white bars) elicited by 2MeSADP (10 μM) in astrocyte cultures from TNF receptor 1 null mutant mice (TNFα−/−) and wild-type (TNFα+/+) littermates (n = 3 in triplicate). e, double-labeling cytochemistry showing expression of TNF1 (green) in GFAP-positive cells (red). Scale bar = 10 μm. f, P2Y1R-evoked Ca2+ responses are partly controlled by TNFα. Left, graph represents distribution of [Ca2+]i peaks (expressed as ΔF/F0 % of X-rhod-1 fluorescence) induced by 2MeSADP (10 μM, 2 s) in astrocytes from TNFα+/+; TNFα−/−, and TNF1−/− mice. Gray symbols are data points representing the mean ± S.E. of Ca2+ responses of individual astrocytes to 2MeSADP in a field of about 40 cells in each experiment (n = 7–18 experiments). Open symbols represent the mean ± S.E. of all data points for each type of astrocyte culture. The mean amplitude of the [Ca2+]i peaks in the TNFα+/+ group is significantly higher than in TNFα−/− and TNF1−/− groups (*) p < 0.005, one-way analysis of variance). Right, traces ± S.E. of seven separate experiments illustrate the average kinetics of [Ca2+]i, changes induced by two sequential applications of 2MeSADP (10 μM, 2 s), named I and II, separated by a 20-min wash, in the presence (gray symbols) or absence of sTNFR1 (black symbols). Data points were acquired at 10 interlaced frames/s and represent mean ± S.E. of 230 cells. In the inset, a higher magnification of the boxed region in the average traces illustrates the kinetics of [Ca2+]i, in the first 2.3 s following the start of stimulus application (trace II has been positioned on top of the trace I). Black bar represents 2-s pulse of 2MeSADP.
pharmacological experiments in cultures of normal rat astrocytes exposed to the TNFα scavenger, sTNFR1 (200 ng/ml; Fig. 3f, right). In control experiments without sTNFR1, astrocytes stimulated by two sequential applications of 2MeSADP, separated by a 20-min interval, consistently responded with highly reproducible [Ca2+]i elevations (data not shown). When the first challenge with the P2Y1R agonist was performed in the presence of sTNFR1, the amplitude of the Ca2+ response was lower (60.6 ± 7.8%; n = 7; t test p < 0.01) than the amplitude observed at the second challenge, performed after washing out sTNFR1.

When analyzing the kinetics of the 2MeSADP-evoked [Ca2+]i elevations, we noticed that sTNFR1 caused a significant delay in the time-to-peak (mean value, 1.34 ± 0.64 s in control condition versus 2.24 ± 0.98 s in the presence of sTNFR1; n = 7; t test p < 0.01; Fig. 3f, right, inset). Overall, these data suggest that TNFα signaling contributes to the amplitude and kinetics of the astrocyte [Ca2+]i elevations in response to P2Y1R stimulation.

**Prostaglandins Are Involved Downstream to the TNFα**—Prostaglandins can be rapidly formed and released in association with astrocytic [Ca2+]i rises (9, 14, 76). In addition to their effects on the vasculature (9), they play a key role in the control of glutamate release in response to glutamatergic stimulation (14, 43). So we checked whether prostaglandins are involved in the P2Y1R-dependent signaling pathway. Indeed, by measuring PGE2 with a sensitive EIA, we found that both ATP (100 μM) and 2MeSADP (10 μM) increased the extracellular levels of this prostaglandin (Fig. 4a). The TACE inhibitor BB3103 (3 μM) strongly inhibited the 2MeSADP-dependent effect (Fig. 4a), indicating that PGE2 formation largely depends on TNFα shedding. Prostaglandins contribute to P2Y1R-evoked glutamate release, because the phenomenon is strongly inhibited in the presence of cyclooxygenase blockers, either indomethacin (INDO, 1 μM) or aspirin (ASA, 10 μM) (Fig. 4, b and c).

**P2Y1R Coupling to Glutamate Release Is Restricted to Astrocytes**—We next conducted studies in mixed hippocampal cultures that stain positively for neuronal, astrocytic, and microglial markers (33). Administration of 2MeSADP (10 μM) to these mixed cultures induced a rapid glutamate release response (Table 1). Interestingly, the extent of such a response was identical to that of the response evoked in astrocyte cultures plated at the same density (see Fig. 1b). This observation suggests that the neuronal cells do not contribute significantly to P2Y1R-evoked glutamate release. In agreement, no detectable glutamate release was observed in neuron-enriched cultures stimulated with 2MeSADP; moreover, in mixed cultures depleted of their neuronal component (61), P2Y1R-evoked glutamate release was not reduced (Table 1). A possible contribution of microglia was also excluded because the glutamate release response evoked by 2MeSADP in astrocyte cultures was not amplified by addition of microglial cells (10–12%) to the cultures, even when the microglia had been preactivated by exposure to lipopolysaccharide (24 h). In agreement, lipopolysaccharide-activated microglia-pure cultures did not show any detectable glutamate release in response to 2MeSADP (Table 1). Therefore, in our hippocampal cultures, P2Y1R-dependent glutamate release appears to be astrocyte-specific.

**FIGURE 4. Involvement of prostaglandins in P2Y1R-induced glutamate release.** a, accumulation of PGE2 in the extracellular medium of astrocyte cultures during 3-min incubations with either ATP (100 μM) or 2MeSADP (10 μM). Data are expressed as pg/ml above the levels of PGE2 in unstimulated cultures (n = 3 in triplicate). Both agents induce a significant increase in extracellular PGE2. The effect of 2MeSADP is inhibited in the presence of the TACE inhibitor BB3103 (3 μM, n = 4 in triplicate). b, representative fluorescence traces showing the inhibitory effect of two cyclooxygenase blockers, INDO (1 μM) and ASA (10 μM), on glutamate release induced by ATP (100 μM). c, histograms represent glutamate release evoked by 2MeSADP (10 μM) in the absence or presence of cyclooxygenase blockers INDO or ASA (for each condition, n = 5 in triplicate).

**TABLE 1**

| Glutamate release induced by P2Y1R stimulation in different types of hippocampal cell cultures |
|---|
| **Cell culture type** | **Glutamate release (nmol/mg protein)** |
| Astrocytes | 0.82 ± 0.11 |
| Neurons | ND |
| Astrocyte-neuron co-culture | 0.76 ± 0.10 |
| Astrocyte-neuron co-culture depleted of neuronal cells | 0.72 ± 0.05 |
| Microglia | ND |
| Astrocyte-microglia co-culture | 0.77 ± 0.08 |

**P2Y1R Stimulation Induces Ca2+-dependent Glutamate Release from Hippocampal Slices; Roles for TNFα and Prostaglandins**—Next, we addressed whether the P2Y1R-dependent signaling system identified in cultured astrocytes is operant also in tissue preparations. At first, we tested whether purinergic stimulation induces glutamate release from acute hippocampal slices prepared from adult rats. Indeed, ATP (100 μM) evoked rapid glutamate release responses from the slices (n = 7; Fig. 5a). Like in astrocyte cultures, 10 μM 2MeSADP mimicked the effect of 100 μM ATP (n = 5), and the responses to both the P2Y1R agonist and the endogenous nucleotide were blocked by the P2Y1R antagonist, A3P5PS (100 μM, n = 5), indicating that the observed glutamate release in situ is mediated by the P2Y1 subtype of purinergic receptors. Preincuba-
tion of the slices with the Ca\textsuperscript{2+} chelator, BAPTA/AM (50 μM, n = 4) (Fig. 5a), abolished 2MeSADP-evoked release, revealing the Ca\textsuperscript{2+} dependence of the process. In the hippocampus, two distinct Ca\textsuperscript{2+}-dependent processes that may be responsible for glutamate release have been described to date, i.e. classical exocytosis from neuronal terminals and release from astrocytes (14, 33, 37). To distinguish between them, we compared the characteristics of 2MeSADP-evoked glutamate release with those of synaptic glutamate exocytosis evoked by high K\textsuperscript{+} stimulation (14, 33). The two processes were found to differ fundamentally in several aspects. First of all, when utilizing hippocampal slices from TNF\textalpha\textsuperscript{-/-} mice (Fig. 5b), we could not see appreciable glutamate release responses to 2MeSADP (10 μM) stimulation, whereas responses to high K\textsuperscript{+} (20 mM) were similar to those observed in slices of TNF\textalpha\textsuperscript{+/+} mice (TNF\textalpha\textsuperscript{+/+}, 3.32 ± 0.10 nmol/mg protein, n = 7; TNF\textalpha\textsuperscript{-/-}, 3.17 ± 0.07 nmol/mg protein, n = 6). The same dichotomy was observed using slices from TNFR1\textsuperscript{-/-} animals (Fig. 5b). Likewise, blocking cyclooxygenases with INDO (1 μM) reduced by 6-fold the 2-MeSADP-induced glutamate release from rat hippocampal slices (2MeSADP, 1.25 ± 0.1 nmol/mg protein; 2MeSAADP + indomethacin, 0.2 ± 0.05 nmol/mg protein; n = 4) but left intact high K\textsuperscript{+}-induced glutamate release (high K\textsuperscript{+}, 1.93 ± 0.1 nmol/mg protein; high K\textsuperscript{+} + indomethacin, 1.95 ± 0.2 nmol/mg protein; n = 4). Finally, although both processes were found to be sensitive to the vesicular exocytosis blocker Baf A1, the time course of inhibition was different in the two cases. High K\textsuperscript{+}-evoked glutamate release was blocked within 2 h of exposure of the slices to Baf A1 (1 μM), whereas 2MeSADP-evoked release was blocked by about 50% within 6 h (n = 3; *; t test, p < 0.05; Fig. 5c). However, by changing the experimental protocol, we accelerated the inhibitory effect of Baf A1 and also strongly blocked the 2MeSADP-evoked process within 2 h. Because Baf A1 prevents refilling of transmitter-empty vesicles, although it is without effect on transmitter-filled vesicles (77), we reasoned that an initial stimulation of the release leading to discharge of the vesicles contents would result in an enhanced inhibitory efficacy of the drug (43). So, we decided to challenge the slices with 2MeSADP (10 μM) at the beginning of the incubation period with Baf A1 (1 μM); this procedure was sufficient to suppress the release in response to a second challenge with the P2Y1R agonist after 2 h of exposure to Baf A1 (n = 7; p < 0.05; see Fig. 5d). Inhibition was because of the specific action of Baf A1, because repeated challenges with 2MeSADP at a 2-h

**FIGURE 5.** P2Y1R-evoked glutamate release from hippocampal slices: a Ca\textsuperscript{2+}-dependent phenomenon distinct from high K\textsuperscript{+}-evoked release from nerve terminals. a, application of ATP (100 μM) or the P2Y1R agonist 2MeSADP (10 μM) induces glutamate release from rat hippocampal slices. Either response is prevented in the presence of the P2Y1R antagonist A3P5PS (100 μM) or in slices preincubated with the intracellular Ca\textsuperscript{2+} chelator BAPTA/AM (50 μM, 30 min). For each condition n = 4–7. b, left, representative fluorescence traces of the glutamate release responses to sequential application of 2MeSADP (10 μM) and high K\textsuperscript{+} (KCl, 20 mM) in hippocampal slices from TNF\textalpha\textsuperscript{+/+} and TNF\textalpha\textsuperscript{-/-} mice. Notice in TNF\textalpha\textsuperscript{-/-} mice a selective defect in the response to the P2Y1 R agonist, but not in those induced by high K\textsuperscript{+}. Right, histograms compare the glutamate release response to 2MeSADP (10 μM) and high K\textsuperscript{+} (KCl, 20 mM) in hippocampal slices from TNF\textalpha\textsuperscript{+/+} and TNF\textalpha\textsuperscript{-/-} mice as well as from TNFR1\textsuperscript{+/+} and TNFR1\textsuperscript{-/-} mice (n = 6–7 for each animal group). Responses to 2MeSADP are selectively defective in TNF\textalpha\textsuperscript{-/-} and TNFR1\textsuperscript{-/-} mice, whereas responses to high K\textsuperscript{+} are identical in the four groups of mice. c, glutamate release responses to 2MeSADP (10 μM, black bars) and high K\textsuperscript{+} (20 mM, white bars) in rat hippocampal slices preincubated with Baf A1 (1 μM) for 2 or 6 h (n = 3 for each time). Data are expressed as percentage of the effect observed at identical times in sister slices not exposed to Baf A1. Baf A1 maximally inhibits high K\textsuperscript{+}-induced release within 2 h, whereas it partially inhibits 2MeSADP-evoked release after 6 h (*, p < 0.05 versus control; t test). d, prestimulation of the slices with 2MeSADP speeds Baf A1 inhibition. Histograms represent glutamate release responses to two sequential stimulations with 2MeSADP, the first performed at the beginning of the experiment (1st: 0 h) and the second 2 h later (2nd: 2 h). Responses in naive slices (Ctrl, n = 6) and in slices exposed to Baf A1 (Baf A1, n = 7) are compared; in the latter, the 2nd response to 2MeSADP is abolished, whereas the former is identical to the 1st one. Therefore, the inhibitory potency of Baf A1 is greatly enhanced (compare with c) by the initial stimulation with 2MeSADP. e, glutamate release responses in rat hippocampal synaptosomes; the representative fluorescence traces show that the same synaptosomal preparation responds with massive glutamate release to high K\textsuperscript{+} (KCl, 20 mM, n = 12) but does not show any release in response to 2MeSADP (10 μM, n = 6) or to TNF\textalpha (30 ng/ml, n = 6).
interval in slices not incubated with Baf A1 always gave identical glutamate release responses (n = 6; see Fig. 5d). This result supports the exocytotic nature of the glutamate release process induced by P2Y1R stimulation in situ. Overall, the 2MeSADP-induced glutamate release from hippocampal slices has features corresponding to those of the P2Y1R-dependent effect in cultured astrocytes and distinct from exocytosis from neuronal terminals.

P2Y1R Stimulation Does Not Induce Glutamate Release from Hippocampal Synaptosomes—To more directly exclude involvement of synaptic exocytosis, we tested whether hippocampal synaptosomal preparations, enriched in isolated neuronal terminals, released glutamate in response to activation of the P2Y1R-dependent cascade (Fig. 5e). Stimulation of nerve terminals with high concentrations of either 2MeSADP (10–100 μM) or TNFα (30–100 ng/ml) did not induce detectable glutamate release. The same nerve terminal preparations, however, promptly responded with massive glutamate release to depolarization with high K⁺ (20 mM, 3.17 ± 0.2 nmol/mg protein, n = 6).

**DISCUSSION**

This study identifies the coupling of purinergic P2Y1 receptors to glutamate release in astrocytes. The stimulus-secretion coupling is Ca²⁺-dependent, mediated by Ca²⁺ release from internal stores, and occurs via regulated exocytosis, as demonstrated by real time imaging of VGLUT-expressing vesicle fusions in response to P2Y1R stimulation. Both [Ca²⁺]ᵢ elevation and glutamate release appear to be under the control of TNFα signaling, acting via its p55 receptor, TNFR1, and downstream prostaglandins. Finally, we provide strong evidence that the P2Y1R-glutamate release coupling is restricted to astrocytes, both in hippocampal cell cultures and in acute hippocampal slices.

Glutamate release from astrocytes in response to ATP has been reported in several studies (4). The underlying mechanisms remain controversial, however, and may actually be multiple, mediated by distinct purinergic receptor subtypes. Astrocytes express several pharmacologically distinguishable P2YR (59, 79) as well as several ionotropic P2XR subunits, including P2X7 (49). The glutamate release process identified here in response to the nucleotide appears to be specifically mediated by P2Y1R. It is faithfully reproduced by 2-MeSADP, a rather selective P2Y1R agonist (59), and it is blocked by A3P5PS, a specific P2Y1R antagonist (80). Roles for other P2YR subtypes, such as P2Y2, -4 and -6, are excluded because UTP, a potent activator of these receptors, was unable to mimic the effect of 2MeSADP. Moreover, the ATP-evoked glutamate release is sensitive to pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt, whereas P2Y4R and P2Y6R are largely insensitive to this purinergic antagonist (69). A previous report identified ionotropic P2X7R as source of glutamate release from astrocytes (49). In many cell types, P2X7R form homomorphic complexes that, after prolonged exposure to high concentrations of ATP, open large ion channels permeable to molecules up to 900 Da in size, including glutamate and aspartate (81). The characteristics of P2X7R-dependent glutamate release in astrocytes are fully distinct from those of the P2Y1R-dependent process identified here: P2X7R-induced release is Ca²⁺-independent, nonvesicular, and catalyzed by reducing the Ca²⁺ and Mg²⁺ ion concentration in the cell-bathing medium, a procedure that increases the affinity of P2X7R for their ligands (58). However, in normal extracellular medium, as used in our experiments, we could not observe significant glutamate release in response to the P2X7R agonist BzATP (100 μM). This is perhaps not surprising given that astrocyte P2X7R are only weakly activated in physiological media (49). Therefore, in view of the peculiar gating properties of P2X7R channels and the weak agonist potency of ATP at these receptors (81), it remains to be demonstrated that P2X7R-dependent glutamate release occurs under physiological conditions. However, this pathway could be an important player in pathological states such as spinal cord injury and could contribute to excitotoxic neuronal death (82). ATP-evoked glutamate release through a Ca²⁺-dependent pathway has been ascribed to metabotropic P2YR acting via Ca²⁺ release from internal stores. The downstream events remain controversial, however. Kang et al. (35) have shown that inositol 1,4,5-trisphosphate formation induces exocytotic release of glutamate from hippocampal astrocytes, corroborating previous findings in cultured cells (37, 39). However, another study by the same authors (36) reported that ATP or UTP evokes a glutamate release process in cultured astrocytes that is, at the same time, dependent on Ca²⁺ release from internal stores and insensitive to exocytosis inhibitors. The authors propose that the release occurs via volume-regulated anion channels, as they find that these channels can permeate high millimolar glutamate concentrations (36). Whether the channels would drive significant glutamate release at the physiological cytosolic concentrations of the amino acid, 50-fold lower than those used in the study (83), is not yet known. The P2Y1R-mediated release identified here is also clearly distinct from the above process, not just because it occurs by vesicular exocytosis but also because it displays distinct purinergic pharmacology (i.e., preferential activation by nucleoside diphosphates and unresponsiveness to UTP), and because it is inhibited by cyclooxygenase blockers, although the process reported by Takano et al. (36) is not. We cannot easily explain the different properties of ATP-evoked Ca²⁺-dependent glutamate release in the two studies. One possibility is that different culture conditions lead to the expression of different P2YR subtypes in the cultures and that these subtypes activate distinct signaling pathways. Importantly, all the properties of the P2Y1R-dependent pathway observed in cell cultures were seen also in hippocampal slices, indicating that this pathway cannot be an artifact of the culture conditions.

The characteristics of P2Y1R-induced glutamate release, i.e., its activation by low ATP concentrations (EC₅₀ 6 μM) and probably much lower ADP concentrations, in view of the preferential affinity of P2Y1R for nucleoside diphosphates (56), its sophisticated Ca²⁺ regulation involving TNFα and prostag-

---

4 P. Jourdain, L. H. Bergersen, K. Bhaukaurally, P. Bezzi, M. Domercq, C. Matute, F. Tonello, V. Gundersen, and A. Volterra, submitted for publication.

5 V. Gundersen, personal communication.
P2Y1-evoked Glutamate Exocytosis from Astrocytes

landin signaling (see below), and the quantal nature of the release indicate that this pathway is potentially implicated in physiological processes. Indeed, we now have direct evidence that astrocyte P2Y1R receptors are activated during normal synaptic transmission in the hippocampal dentate gyrus and control synaptic strength via glutamate release.4

The use of TIRF microscopy allowed us to directly document P2Y1R-dependent exocytosis of glutamatergic vesicles and to define several properties of this process. The limited penetration of the evanescent wave of excitation beyond the astrocyte plasma membrane (about 80 nm in our experiments) selectively visualized a population of double-fluorescent vesicles loaded with the dye AO and expressing fluorescent vesicular glutamate transporters (VGLUT2-EGFP). Such vesicles were therefore docked or immediately adjacent to the astrocyte plasma membrane and most likely represented the release-ready pool. The fluorescence spots had a diameter of 325 ± 96 nm, comparable with that of classical synaptic microvesicles seen under TIRF illumination (67). This observation is in line with previous colocalization studies (38) that concluded that the spots represent the population of VGLUT2- and cellubrevin-bearing clear synaptic-like microvesicles identified at the electron microscopic level (37, 38).

Single fusion events were monitored as “AO flashes,” a sequence of stereotyped fluorescence changes (brightening, spreading, and eventually disappearing) that signal discharge and then diffusion of the AO cargo in the extracellular medium upon fusion of the vesicle membrane with the plasma membrane (4, 37, 73, 74). By this approach we directly counted in individual astrocytes the number of VGLUT-expressing vesicles undergoing exocytosis in response to application of 2MeSADP. About 55% of the vesicles present in the TIRF field (a total of 550 vesicles) were exocytosed within 5 s. Interestingly, in experiments on isolated astrocyte-INS-1 cell pairs, a lower proportion (30%) of vesicles was exocytosed and on a shorter time scale (about 1 s). In both high density astrocyte cultures and in isolated astrocyte-INS-1 cell pairs the maximal fusion rate was achieved within 350 ms from the start of 2MeSADP application. Therefore, the density of the cultures seems to influence the entity and duration of the exocytotic process, particularly its slower phase. One possible explanation is that late exocytic events in high density cultures are caused by intercellular cross-talk and paracrine amplification of the primary stimulus.

The secretion of VGLUT-expressing vesicles was accompanied by glutamate release as detected by temporally correlated NMDAR-dependent [Ca2+]i elevations in co-cultured INS-1 cells (37). We verified the correspondence between the amount of glutamate released in response to 2MeSADP or ATP as measured by the GDH assay (Fig. 1b; 0.2 fmol/cell in our high density cultures) and the amount of glutamate released by vesicular exocytosis. Because the region of an astrocyte monitored by TIRF illumination represents about 1/30 of its total surface (calculated with Imaris software), and assuming that the fusion events in this region are representative of the events occurring in the rest of the cell, we estimate that each astrocyte secretes about 9000 vesicles in response to 2MeSADP. The reported diameter of VGLUT2-expressing vesicles in cultured astrocytes is varied, ranging between 30 and 80 nm (37, 38). Taking an average diameter of 55 nm and assuming that the glutamate concentration in astrocytic vesicles is 100–400 mM as in synaptic vesicles (84, 85), the amino acid content of each vesicle will be 8.7–34.8 × 10−6 fmol, corresponding to a P2Y1R-evoked release of 0.078–0.312 fmol/cell. This estimation is in good agreement with the value obtained in GDH assays. It differs, however, from the value calculated by Takano et al. (36). One of the problems with those calculations is that the authors did not take into account that the region monitored under TIRF illumination represents a small part of the astrocyte surface in three dimensions.

AO flashes from VGLUT-expressing vesicles as well as NMDAR-dependent responses in INS-1 cells were abolished in the presence of TeNT, confirming that both depend on a SNARE-dependent process. They were similarly abolished in the presence of CPA, a blocker of the endoplasmic reticulum Ca2+-ATPase, indicating that the secretory process is triggered by Ca2+ release from the internal stores, consistent with the reported coupling of P2Y1R with inositol 1,4,5-trisphosphate-dependent [Ca2+]i elevations (86). Additional transductive events, notably those depending on TNFα and PGs, exert a control on P2Y1R-dependent glutamate release. At least three lines of evidence support the implication of these mediators as follows: 1) both TNFα and PGE2 are released from astrocytes in response to ATP-dependent stimulation of P2Y1R (PGE2 release is TACE-dependent and therefore occurs downstream to the TNFα release (see also Ref. 33); 2) P2Y1R-evoked glutamate release is strongly reduced in astrocyte cultures and in acute hippocampal slices treated with pharmacological blockers of either TNFα or PG production; and 3) the release is similarly reduced in preparations from TNF−/− and TNFR1−/− mice. Although the above observations highlight a critical control of TNFα and PGs on P2Y1R-dependent glutamate release, we cannot conclude that these mediators are indispensable for glutamate release because conditions abolishing TNFα or PG signaling strongly reduced but did not completely block glutamate release. The residual release accounts for 10–25% of the normal release in astrocyte cultures and for an even smaller proportion in acute slices. Incomplete penetration of GDH in the slices and competing uptake could, however, explain the latter result.

The specific roles of TNFα and PGs in the stimulus-secretion coupling mechanism remain to be established. Signaling by these mediators could contribute to one or several of the following processes: (a) Ca2+ release from the internal stores triggering vesicle fusion; (b) priming of the secretory process itself; and (c) amplification of the whole cascade by autocrine/paracrine loops. For instance, PGE2 is known to induce [Ca2+]i elevation in astrocytes, possibly by binding to surface E-series prostaglandin (EP) receptors (14, 32, 76).

By comparing 2MeSADP-evoked [Ca2+]i elevations in normal astrocytes and in astrocytes from TNF−/− and TNFR1−/− mice or in astrocytes where sTNFR1 neutralized released TNFα, we observed specific changes in the initial phase of the Ca2+ transients, notably a 30–40% reduction of the Ca2+ peak accompanied by a slowing down of the time-to-peak. These observations suggest that TNFα signaling, although not indispensable for the generation of P2Y1R-dependent [Ca2+]i eleva-
tions, contributes to their shaping. The impact of this contribution to the overall glutamate release process remains to be established with approaches offering higher temporal-spatial resolution.

Another aspect that needs future clarification is the time course of P2Y1R-evoked TNFα and PG release. In order for these agents to control glutamate secretion, their release must occur in the time scale of milliseconds. Unfortunately, the assays used in this study lack adequate temporal resolution and cannot confirm this possibility. A recent study using appropriate sniffer cells, detected PGE2 release from astrocytes within 2 s from stimulation (76). As for TNFα, the soluble ectodomain of the protein can be rapidly released from its transmembrane precursor by the action of TACE, a specific metalloproteinase constitutively expressed at the surface of the astrocytes (87). Based on the present evidence, however, we cannot exclude the possibility that tonic basal release of TNFα and PGs sets the appropriate conditions for efficient [Ca2+], elevations and glutamate release in response to 2MeSADP stimulation. Indeed, evidence for tonic TNFα production in astrocyte cultures exists (88, 89). In this perspective, P2Y1R-stimulated release of the cytokine could act to reinforce the tonic effect.

Another relevant observation of this study is that P2Y1R are coupled to glutamate release selectively in astrocytes. By applying 2MeSADP to different types of hippocampal cultures, enriched in neurons, microglia, or astrocytes, we could detect glutamate release only from the astrocyte cultures. Moreover, by applying the P2Y1R agonist to mixed neuron-glia cultures or to co-cultures of astrocytes and microglia, we never observed higher glutamate release than in cultures containing only astrocytes. In the in situ experiments we found that P2Y1R-evoked glutamate release had properties analogous to the process in cultured astrocytes and different from synaptic glutamate release evoked by high K+ stimulation, despite that both processes were Ca2+-dependent. Indeed, 1) high K+ but not 2MeSADP induced glutamate release from hippocampal synaptosomes, despite that this preparation apparently contains P2Y1R (90); 2) P2Y1R-evoked glutamate release from hippocampal slices, but not high K+-evoked release, was sensitive to inhibition of TNFα and PG signaling; and 3) both processes were inhibited by Baf A1 but with a different time course. The sensitivity to Baf A1 of the P2Y1R-evoked release in situ is a relevant finding as it corroborates the evidence that the process is vesicular in nature. The slower rate of inhibition with respect to the high K+ -evoked process highlights functional differences with synaptic release, and possibly the fact that spontaneous exocytosis of astrocytic vesicles occurs less frequently than spontaneous exocytic events at the synapses.

In the hippocampus, P2Y1R are expressed in both neurons and astrocytes, although not homogeneously. In the area CA1, they are present in astrocytes of the stratum radiatum, pyramidal neurons, and GABAergic interneurons (8, 68, 91), whereas in the dentate gyrus they are expressed mostly, if not exclusively, in astrocytes. At the ultrastructural level, P2Y1R in the dentate molecular layer are observed in astrocytic processes surrounding asymmetric synapses.4 Such localization strongly suggests that they can be activated by ATP released during synaptic transmission. There is increasing evidence that ATP participates in the excitatory transmission in the hippocampus; for example, a P2X2-dependent component of excitatory postsynaptic currents (EPSCs) was identified at various hippocampal synapses (92, 93). Moreover, work in hippocampal slices shows that ATP released as result of Schaffer collateral stimulation induces [Ca2+]i increases in astrocytes through P2Y1R activation (8). As we demonstrate here that P2Y1R trigger glutamate exocytosis from astrocytes, activation of this astrocyte receptor may serve important physiological regulatory functions. Indeed, we now have evidence that this pathway is involved in a positive control of perforant path-to-granule cell synapses.4 In addition, astrocyte P2Y1R can be activated by ATP released from the astrocytes during propagation of intercellular Ca2+ waves (6, 7, 59). The resulting glutamate release may be part of the mechanism sustaining signal propagation in the glial network (78) while at the same time providing an interface with the neuronal circuitry.

Acknowledgment—We thank H. Stubbe for technical support.

REFERENCES

1. Volterra, A., Magistretti, P. J., and Haydon, P. G. (eds) (2002) The Tripar- tite Synapse: Glia in Synaptic Transmission, Oxford University Press, Oxford, UK
2. Newman, E. A. (2003) Trends Neurosci. 26, 536–542
3. Auld, D. S., and Robitaille, R. (2003) Neuron 40, 389–400
4. Volterra, A., and Meldolesi, J. (2005) Nat. Rev. Neurosci. 6, 626–640
5. Fellin, T., Pascual, O., and Haydon, P. G. (2006) Physiol. (Bethesda) 21, 208–215
6. Newman, E. A. (2001) J. Neurosci. 21, 2215–2223
7. Schipke, C. G., Boucsein, C., Ohlemeyer, C., Kirchhoff, F., and Kettenmann, H. (2002) FASEB J. 16, 255–257
8. Bowser, D. N., and Khakh, B. S. (2004) J. Neurosci. 24, 8606–8620
9. Zonta, M., Angulo, M. C., Gobbo, S., Rosengarten, B., Hossmann, K. A., Pozzan, T., and Carmignoto, G. (2003) Nat. Neurosci. 6, 43–50
10. Mulligan, S. I., and MacVicar, B. A. (2004) Nature 431, 195–199
11. Metea, M. R., and Newman, E. A. (2006) J. Neurosci. 26, 2862–2870
12. Takano, T., Tian, G. F., Peng, W., Lou, N., Libionka, W., Han, X., and Nedergaard, M. (2006) Nat. Neurosci. 9, 260–267
13. Parpura, V., Basarsky, T. A., Liu, F., Jef tinija, K., Jef tinija, S., and Haydon, P. G. (1994) Nature 369, 744–747
14. Beazzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Rizzini, B. L., Pozzan, T., and Volterra, A. (1998) Nature 391, 281–285
15. Araque, A., Sanzgiri, R., Parpura, V., and Haydon, P. G. (1998) J. Neurosci. 18, 6822–6829
16. Newman, E. A., and Zahn, K. R. J. (1998) Neuroscience 18, 4022–4028
17. Kang, J., Jiang, L., Goldmann, S. A., and Nedergaard, M. (1998) Nat. Neurosci. 1, 683–692
18. Parri, R. H., Gould, T. M., and Crunelli, V. (2001) Nat. Neurosci. 4, 803–812
19. Liu, Q. S., Xu, Q., Arcuinno, G., Kang, J., and Nedergaard, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 3172–3177
20. Fiacco, T. A., and McCarthy, K. D. (2004) J. Neurosci. 24, 722–732
21. Fellin, T., Pascual, O., Gobbo, S., Pozzan, T., Haydon, P. G., and Carmignoto, G. (2004) Neuron 43, 729–743
22. Angulo, M. C., Kozlov, A. S., Chappar, S., and Audinat, E. (2004) J. Neuro- sci. 24, 6920–6927
23. Perea, G., and Araque, A. (2005) J. Neurosci. 25, 2192–2203
24. Tian, G. F., Azmi, H., Takano, T., Xu, Q., Peng, W., Lin, J., Oberheim, N., Lou, N., Wang, X., Zielke, H. R., Kang, J., and Nedergaard, M. (2005) Nat. Med. 11, 973–981
25. Gordon, G. R., Baimoukhametova, D. V., Hewitt, S. A., Rajapaksha, W. R., Fisher, T. E., and Bains, J. S. (2005) Nat. Neurosci. 8, 1078–1086
P2Y1-evoked Glutamate Exocytosis from Astrocytes

26. Zhang, J. M., Wang, H. K., Ye, C. Q., Ge, W., Chen, Y., Jiang, Z. L., Wu, C. P., Poo, M. M., and Duan, S. (2003) *Neurosci.* 20, 2800–2808
27. Newman, E. A. (2003) *J. Neurosci.* 23, 1659–1666
28. Pascual, O., Casper, K. B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J. Y., Takano, H., Moss, S. J., McCarthy, K., and Haydon, P. G. (2005) *Science* 310, 113–116
29. Serrano, A., Haddjeri, N., Lacaille, J. C., and Robitaille, R. (2006) *J. Neurosci.* 26, 5370–5382
30. Yang, Y., Ge, W., Chen, Y., Zhang, Z., Shen, W. W., Cao, P., and Duan, S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 15194–15199
31. Panatier, A., Theodosis, D. T., Motter, J. P., Touquet, B., Pollegioni, L., Poulain, D. A., and Oliet, S. H. (2006) Cell 125, 775–784
32. Sanzgiri, R. P., Araque, A., and Haydon, P. G. (1999) *J. Neurobiol.* 41, 221–229
33. Bezzi, P., Domercq, M., Brambilla, L., Galli, R., Schols, D., De Clercq, E., Vescovi, A., Bagetta, G., Collias, L., and Volterra, A. (2001) Nat. Neurosci. 4, 702–710
34. Jeremic, A., Jefinjia, K., Stevanović, J., Glavaski, A., and Jefinjia, S. (2001) *J. Neurochem.* 77, 664–675
35. Kang, N., Jiang, L., He, W., Xu, J., Naus, C. C., and Nedergaard, M. (1998) *J. Neurosci.* 21, 15735–15740
36. Zhang, Q., Fukuda, M., Van Bockstaele, E., Pascual, O., and Haydon, P. G. (2004) *J. Biol. Chem.* 279, 12724–12733
37. Kreft, M., Stenovec, M., Rutnik, M., Grilc, S., Krzan, M., Pangršic, T., Kreft, M., Krzan, M., Li, N., Sul, J. Y., Halassa, M., and Nedergaard, M. (2002) *J. Neurosci.* 22, 9134–9141
38. Chimelli, H. K., Goderie, S. K., Higman, S., Shen, W., and Storm-Mathisen, J. (1998) *J. Neurosci.* 18, 268–275
39. Franks, K. M., Stevens, C. F., and Sejnowski, T. J. (2003) *J. Neurosci.* 23, 3168–3195
40. Fam, S. R., Gallagher, C. J., and Salter, M. W. (2000) *J. Neurosci.* 20, 2800–2808
41. D'Arcangelo, G., Tancredi, V., Onofri, F., D'Antuono, M., Gobbo, S., Carmignoto, G., and Volterra, A. (2001) Nat. Rev. Neurosci. 2, 1573–1586
42. Zhang, Q., Paus, T., Vicini, S., and Carmignoto, G. (2001) *J. Neurosci.* 21, 477–484
43. Montana, V., Ni, Y., Sunjara, V., Hua, X., and Parpura, V. (2004) *J. Neurosci.* 24, 2633–2642
44. Wang, Y., Xu, J., Lin, S. Y., Sauer, M., and Walz, M. (2002) *J. Neurosci.* 22, 3588–3596
45. Cotrina, M. L., Lin, J. H., Alves-Rodrigues, A., Liu, S., Li, J., Azmi-Ghadimi, H., Kang, J., Naus, C. C., and Nedergaard, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 15735–15740
46. Stout, C. E., Costantin, J. L., Naus, C. C., and Charles, A. C. (2002) *J. Biol. Chem.* 277, 10482–10488
47. Aracuino, G., Lin, J. H., Takano, T., Liu, C., Jiang, L., Gao, Q., Kang, J., and Nedergaard, M. (2002) *J. Biol. Chem.* 277, 3588–3596
48. Fields, R. D., and Burnstock, G. (2006) *Nat. Rev. Neurosci.* 7, 423–436
49. Khard, B. S. (2001) *Nat. Rev. Neurosci.* 2, 165–174
50. North, R. A. (2002) *Physiol. Rev.* 82, 1013–1067
51. Fam, S. R., Gallagher, C. J., and Salter, M. W. (2000) *J. Neurosci.* 20, 2800–2808
52. D'Arcangelo, G., Tancredi, V., Onofri, F., D'Antuono, M., Gobbo, S., and Benfenati, F. (2000) *Eur. J. Neurosci.* 12, 1241–1252
53. Pascarakis, M., Alexopoulos, I., Papakostas, G., and Kollas, G. (1996) *Exp. Med.* 184, 1397–1411
54. Pfeiffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P. S., Kronke, M., and Mak, T. W. (1993) Cell 73, 457–467
55. Cheviet, S., Bezzi, P., Ivanov, R., Renstrom, E., Vierlt, D., Kasas, S., Catisca, S., and Regazzi, R. (2006) *J. Cell Sci.* 119, 2912–2920
56. Boyer, J. L., Romero-Avila, T., Schachter, J. B., and Harden, T. K. (1996) *Mol. Pharmacol.* 49, 262–275