P2 Receptor Antagonist Trinitrophenyl-Adenosine-Triphosphate Protects Hippocampus from Oxygen and Glucose Deprivation Cell Death

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ABSTRACT

In this work, we mainly used the organotypic model of rat hippocampus to demonstrate the protective role of the P2 receptor antagonist trinitrophenyl-adenosine-triphosphate (TNP-ATP) during oxygen/glucose deprivation. Among the P2X receptors that TNP-ATP specifically blocks, mainly P2X1, seems to be involved in the processes of cell damage after oxygen/glucose deprivation. P2X1 receptor is strongly and transiently up-regulated in 24 h after an ischemic insult on structures likely corresponding to mossy fibers and Schaffer collaterals of CA1–3 and dentate gyrus. Furthermore, P2X1 receptor is down-regulated by pharmacological treatment with TNP-ATP, which is also found neuroprotective against ischemic cell death. Morphological studies conducted through immunofluorescence and confocal analysis in primary organotypic cultures, and in adult rat in vivo demonstrated the neuronal colocalization of P2X1 protein with neurofilament light chain and neuronal nuclei immunoreactivity in myelinated and unmyelinated fibers of both granular and pyramidal neurons. In conclusion, with this work, we proved the neuronal distribution of P2X1 receptor in hippocampus, and we presented evidence for a potential disadvantageous role of its expression during the path of in vitro ischemia.

Cerebral ischemia is one of the most common causes of death in aged people, being responsible for 10 to 12% of the world deaths per year (Hachinski, 2002). Ischemic injury involves a pronounced reduction in intracellular oxygen and glucose, which leads to rapid cell death associated with Ca2+ influx can directly control the activation of proteolytic enzymes and apoptotic genes and the production of reactive oxygen species (Dirnagl et al., 1999). One class of receptors that directly controls the intracellular calcium fluxes is the purinergic receptors (P2r) (Volonte et al., 2003; Franke and Illes, 2006) for extracellular ATP, a well known neurotransmitter instead characterized by fast excitatory neurotransmission, activation leading to cytosolic Ca2+ increase and cAMP modulation (Roberts et al., 2006). Ionotropic P2X receptors are divided in two subfamilies, ionotropic P2X and metabotropic P2Y receptors, which are distinct by molecular, pharmacological, and functional properties (Khakh et al., 2001; Gever et al., 2006; Volonté et al., 2006). Metabotropic P2Y are purine and pyrimidine receptors classified in eight different subtypes (P2Y1,2,4,6,11–14), whose activation displays slow excitatory responses, phospholipase C, and 1,4,5-trisphosphate activation leading to cytosolic Ca2+ increase and cAMP modulation (Roberts et al., 2006). Ionotropic P2X receptors are instead characterized by fast excitatory neurotransmission, and they are divided into seven different subtypes (P2X1–7) (North, 2002). Although each P2X receptor is defined by a specific nucleotide sequence, common to all the different subtypes is a direct influx of extracellular Ca2+ through the receptor channel. This leads to membrane depolarization, and consequently, to secondary activation of voltage-gated Ca2+ channels.

ABBREVIATIONS: P2r, purinergic receptor(s); CNS, central nervous system; PNS, peripheral nervous system; OGD, oxygen/glucose deprivation; TNP-ATP, trinitrophenyl-adenosine-triphosphate; PBS, phosphate-buffered saline; NFL, neurofilament light; GFM, glucose-free medium; Iba1, isolectin b4; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; PI, propidium iodide; DG, dentate gyrus; mf, mossy fibers; sc, Schaffer collateral fibers; NeuN, neuronal nuclei.
Both P2X and P2Y receptors are ubiquitously expressed in the CNS and PNS (Kucher and Neary, 2005). In particular, the P2X receptor is present on astrocytes of juvenile rats (Kukley et al., 2001), on rat cerebellar granule neurons (Amadio et al., 2002), and on purified synaptosomes from rat hippocampus (Rodrigues et al., 2005). Although the function of the P2X1 receptor is poorly described in the CNS (Brown et al., 2002; Aschner et al., 2004), more information is available for the PNS, where this receptor apparently participates to activation of pathological pain states (Chizh and Illes, 2001). Moreover, a role for various P2 receptors in the path of oxygen/glucose deprivation (OGD) is well established. In particular, we showed previously the presence and activation by OGD of microglial P2X1 in organotypic hippocampal slices (Cavaliere et al., 2003) and of neuronal P2X2 and P2X7 receptors (Cavaliere et al., 2002, 2004b; Melani et al., 2006); and in human neuroblastoma cells, we showed that heterologous expression of metabotropic P2Y4 receptor exacerbates cell death induced by metabolic impairment (Cavaliere et al., 2004a). Consistently, neurodegeneration induced by OGD is prevented by several different P2 antagonists (Cavaliere et al., 2001, 2003, 2004a,b, 2005).

In this work, we thus investigated the biological effect of the P2 receptor antagonist trinitrophenyl-adenosine-triphosphate (TNP-ATP) particularly during OGD and the potential role of the P2X1 receptor in this detrimental process in rat hippocampus. We found a direct time- and dose-dependent involvement of this receptor in the path of OGD-induced cell death.

Materials and Methods

Cell Culture

Hippocampal Organotypic Cultures. Slice cultures were prepared using a modification of the method by Stoppini et al. (1991). In brief, Wistar rat pups (8–10 days old) were sacrificed, and their brains were removed. Hippocampi were excised, cut at 400 µm in thickness on a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK), and separated into Hanks’ balanced salt solution (0.185 mg/ml CaCl2, 0.1 mg/ml MgSO4, 0.4 mg/ml KCl, 0.06 mg/ml KH2PO4, 8 mg/ml NaCl, 0.05 mg/ml Na2HPO4, 0.35 mg/ml NaHCO3, and 1 mg/ml glucose). Slices were plated on Millicell CM inserts (Millipore, Milan, Italy), and plates were maintained in 75% HME 03 (Cell Concept, Umkirch, Germany) and 25% horse serum (Invitrogen, Milan, Italy) for 3 days at 37°C, and then they were shifted to 33°C in Neurobasal medium supplemented with 0.5% B27 (both from Invitrogen). Experiments were performed after 12 to 14 days in vitro.

Primary Dissociated Hippocampal Cultures. Primary dissociated hippocampal cells were prepared from P2 rat pups. In brief, hippocampi from both hemispheres were dissected into small tissue blocks. Slices were then incubated in papain solution (116 mM NaCl, 5.4 mM KCl, 26 mM NaHCO3, 1 mM NaHPO4, 1.5 mM CaCl2, 1 mM MgSO4, 0.5 mM EDTA, 25 mM glucose, and 200 µU of papain 200) for 15 min, and then they were dissociated with a glass Pasteur pipette. Dissociated cells were washed in Earl’s balanced salt solution and plated in Eagle’s minimal essential medium, 10% dialyzed fetal bovine serum with 0.5 mM glutamine, 20 mM glucose, and gentamicin (all from Invitrogen). The cell density at plating was approximately 100–200 cells/mm2. Proliferation of non-neuronal cells was inhibited at 2 days after plating by the addition of 10 µM 1-ß-D-arabinofuranosyl cytosine (Sigma, Milan, Italy) to the growth medium. Experiments were performed after 8 to 10 days in vitro.

Histological Procedures

Adult Wistar rats weighing 200 to 250 g (Harlan, San Pietro al Natisone, Udine, Italy) were anesthetized by intraperitoneal injections of 60 mg/kg sodium pentobarbital, and they were perfused transcardially with 250 ml of saline at room temperature, followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, postfixed in the same fixative for 2 h, and then they were transferred to 30% sucrose in phosphate buffer at 4°C until they sank. In all preparations, animals were handled in accordance with the European Community Council Directive and in accordance with the Declaration of Helsinki. All possible efforts were made to minimize animal suffering and number of animals used.

Immunofluorescence and Confocal Analysis

Organotypic Slices. Treated slices were fixed for 40 min in 4% paraformaldehyde, and slices were saturated at room temperature in 10% donkey normal serum (Alomone Labs, Jerusalem, Israel) in PBS. The slices were then incubated overnight at 4°C with different primary antisera in 1% donkey normal serum in PBS (rabbit anti-P2X1:1:300-399 from Alomone Labs was used at 1:500; goat anti-neurofilament light chain (NFL) (C15) from Santa Cruz Biotechnology, Inc. (Milan, Italy) was used at 1:100; goat anti-doublecortin (C18) (Santa Cruz Biotechnology) was used at 1:200; mouse anti-βIII tubulin from Promega (Milan, Italy) was used at 1:200; biotinylated isolectin b4 (IB4) from Griffonia simplicifolia seeds from Sigma was used at 10 µg/ml; mouse antigial fibrillary acidic protein (GFAP) (R&D, Milan, Italy) was used at 1:100; and mouse antimyelin basic protein (MBP) from Roche Diagnostics (Monza, Italy) was used at 1:200). After further washing, the cultures were incubated in a solution containing a mixture of secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA; Cy2-conjugated donkey anti-rabbit IgG and/or Cy3-conjugated donkey anti-mouse and anti-goat IgG; all used at 1:100). After further washing, the plates were coverslipped with Gel/Mount anti-fading mounting medium (Biomed, Foster City, CA).

Primary Dissociated Cultures. Cultures were fixed for 10 min in 4% paraformaldehyde and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Nonspecific sites were saturated in 10% donkey normal serum for 30 min at room temperature. Anti-P2X4 and anti-NFL primary antibodies were used at 1:1000 and 1:300, respectively, for 90 min at room temperature. Cells were washed three times, and then they were incubated with secondary antibodies (Cy2-conjugated donkey anti-rabbit IgG and/or Cy3-conjugated donkey anti-mouse and anti-goat IgG; all used at 1:100). After further washing, the cells were coverslipped, and then they were analyzed using an LSM 510 scanning confocal microscope (Carl Zeiss, Milan, Italy), equipped with an argon laser emitting at 488 nm and a helium/neon laser emitting at 543 nm.

Slides from Adult Rat Hippocampus. Transverse sections 40 µm in thickness were cut on a freezing microtome (Leitz, Oberkochen, Germany), and then they were processed for double-immunofluorescence studies. Nonspecific binding sites were blocked with 10% normal donkey serum in 0.3% Triton X-100 in PBS for 30 min at room temperature. The sections were incubated in a mixture of primary antisera for 24 h in 0.3% Triton X-100 in PBS. Rabbit anti-P2X4 (1:300) was used in combination with goat anti-NFL (1:100) or with mouse anti-MBP (1:200). The secondary antibodies used for double labeling were Cy3-conjugated donkey anti-rabbit IgG and/or Cy3-conjugated donkey anti-mouse IgG; all at 1:200). After further washes, the cells were coverslipped, and then they were analyzed using an LSM 510 scanning confocal microscope (Carl Zeiss, Milan, Italy), equipped with an argon laser emitting at 488 nm and a helium/neon laser emitting at 543 nm.
purified peptides (identity confirmed by mass spectrometry and amino acid analysis), corresponding to epitopes specific for each P2 receptor subtype and not present in any other known protein. The specificity of the labeling was confirmed by incubations performed with the secondary antibodies in the absence of the primary antisera and also by incubations with the primary antisera in the presence of the immunogenic neutralizing peptide. No immunoreactivity was observed under these conditions. Immunofluorescence was visualized by an LSM 510 scanning confocal microscope (Carl Zeiss).

**Oxygen/Glucose Deprivation**

Glucose-free medium (GFM; 120 mM NaCl, 4 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 2 mM KH₂PO₄, and 2 mg/ml mannitol, pH 7.4) was saturated with N₂. The inserts with organotypic slices were placed in 1 ml of saturated GFM, and they were maintained at 37°C for 40 min in an N₂-saturated hypoxic chamber (Billups-Rothenberg, Del Mar, CA). In the control conditions, medium consisted of GFM supplemented with 1 mg/ml glucose instead of mannitol. The GFM was replaced with the Neurobasal medium, and the cultures were kept under normoxic conditions for additional 24 h at 33°C.

**Total Protein Extraction**

Organotypic cultures were maintained under OGD conditions in the simultaneous presence or absence of 50 µM TNP-ATP (Sigma). After 24 h, four organotypic slices from each experimental condition were extracted in radioimmunoprecipitation assay buffer (PBS supplemented with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 µM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin, all from Sigma), and then homogenized. They were maintained for 1 h on ice, sonicated, and centrifuged at 4°C at 10,000 g for 10 min. Protein quantification was performed in the supernatants by Bradford colorimetric assay (Bio-Rad, Segrate, Milan, Italy).

**Western Blot**

An equal amount of total protein from each sample (50 µg) was separated by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel, and then protein was transferred overnight onto a nitrocellulose membrane (Hybond C; GE Healthcare, Cologno Monzese, Milan, Italy). The filters were pretreated in 5% nonfat milk in 10 mM Tris, pH 8, 150 mM NaCl, and 0.1% Tween 20, and then they were hybridized for 3 h with anti-P2X₁ antisera (1:200). All antisera were immunoneutralized with an anti-rabbit horseradish peroxidase conjugated antibody and after washing, with the secondary antibodies in the absence of the primary antisera to confirm specificity of the labeling. The immunogenic neutralizing peptide was added to each sample to confirm specificity and effectiveness. When we pretreated with TNP-ATP (1 h before OGD), the entire hippocampal slice tissue, with the exception of the CA2 region, showed cell damage reduced 50% with respect to the control condition. Nevertheless, TNP-ATP was inefficient when followed by post hoc test (Tukey’s honestly significant difference). All values reported are significant, with at least p < 0.05.

**Results**

With the aim to test the pharmacological outcome of inhibiting especially P2X₁ receptor during an OGD insult, we cultured hippocampal organotypic slices at 10 to 12 days in vitro for 40 min in OGD and with or without the specific P2X₁ receptor antagonist TNP-ATP. We performed a dose-response experiment with a concentration range of 10, 50, and 200 µM (Fig. 1A, samples 1, 2, and 3, respectively). PI incorporation during OGD revealed cell death or death in the CA1–3 neuronal layers, which was reduced by 30 to 40% and 70% in the presence of 50 and 200 µM TNP-ATP, respectively (Fig. 1B and C). The lower concentration of 10 µM reduced cell death by only 10 to 15%. For further experiments, we thus used the concentration of 50 µM to distinguish between specificity and effectiveness. When we pretreated with TNP-ATP for 1 h before OGD (Fig. 1, sample 4), the entire hippocampal slice tissue, with the exception of the CA2 region, showed cell damage reduced 50% with respect to the control condition. Nevertheless, TNP-ATP was inefficient when...
given 2 h after OGD. The pharmacological role of TNP-ATP is generally associated to the antagonistic block of purinergic P2X1 and P2X2/3 receptors (Lewis et al., 1998; Rodrigues et al., 2005). Because we already described the involvement of neuronal P2X2 in ischemic events in the hippocampus (Cavaliere et al., 2003), here we investigated the possible functional contribution of P2X1 and P2X3 receptors. Because we did not find evidence for P2X3 receptor modulation after OGD (data not shown), we concentrated our studies on the expression of P2X1 receptor on rat hippocampus, as analyzed by confocal immunofluorescence in organotypic slice cultures at 10 to 12 days in vitro. We found that P2X1 receptor protein is abundantly distributed on fibers in neuronal layers CA1–3 and in dentate gyrus (DG), probably corresponding to mossy fibers (mf) and Schaffer collaterals (sc) as shown by morphological confocal analysis (Fig. 2). In different hippocampal regions, we demonstrated colocalization of P2X1 protein with NFL both on dendritic fibers and around neuronal bodies (Fig. 3, A and B). Double immunofluorescence with NeuN confirmed the neuronal expression of P2X1 receptor (Fig. 3C). Negative colocalization with the neuronal markers doublecortin and βIII tubulin, which are mainly expressed on neuronal precursors in the neurogenic region of dentate gyrus, instead indicated a lack of P2X1 receptor expression in the early phases of neuronal maturation (data not shown). Immunofluorescence analysis of P2X1 and NFL on primary dissociated hippocampal neurons confirmed the neuronal localization of P2X1 protein on both pyramidal (Fig. 3D) and granular (Fig. 3E) phenotypes. Moreover, we excluded the concomitant presence of P2X1 receptor on microglia, astrocytes, and oligodendrocytes, by lack of colocalization with the microglial marker Ib4, the astrocitic GFAP, and the oligodendrocytic MBP (Fig. 4). The absence of colocalization with MBP further suggested that P2X1 receptor is localized on unmyelinated neuronal fibers, at least in hippocampal slices. We could not establish whether P2X1 receptor was also present on myelinated afferent and deferent fibers, since the organotypic slice preparation per se removes both myelinated fibers coming from the entorhinal cortex and those projecting to the subiculum. For this reason, we then analyzed directly in situ the presence of P2X1 receptor on hippocampal slices of adult rat. Colocalization with NFL was confirmed (Fig. 5A), and confocal double immunofluorescence of P2X1 receptor with MBP in the hilus (the hippocampal region receiving

Fig. 2. P2X1 receptor is expressed on fibers in CA1–3/DG hippocampal region. Hippocampal slices at 12 days in vitro were fixed and immunostained with anti-P2X1 antiserum. Images were taken at 10× magnification on different regions (CA1–3 and DG). The scheme represents neuronal circuits of hippocampus. Pyramidal neurons on CA1 and CA3 are shown as triangles, and open circles represent granular neurons. Afferent fibers on perforant pathway from entorhinal cortex (pp) and deferent fibers from CA1 to subiculum (sb) are represented as dotted lines; mf from DG to CA3 and sc from CA3 to CA1 are represented as lines.

Fig. 3. P2X1 receptor expression on neuronal fibers. Hippocampal slices at 12 days in vitro (A–C) and primary dissociated hippocampal neurons at 8 days in vitro (D and E) were fixed and double immunostained with anti-P2X1 receptor antiserum (green), NFL (A, B, D, and E), and NeuN (C), both red. Images were taken from dentate gyrus (A and B) and CA1 (C) regions. Neuronal bodies in C are marked with an asterisk, and arrows show the P2X1-positive fibers. Colocalization of P2X1 receptor with NFL was also demonstrated on pyramidal (D) and granular (E) dissociated neurons, as shown on merged pictures at 40× magnification.
myelinated afferent fibers) showed that, in transversally sectioned myelinated fibers, MBP immunoreactivity partially overlapped with P2X1 receptor signal (Fig. 5, B and C). The merged field conferred only apparent colocalization between P2X1 and MBP, due to close location of the two immunoreactive structures. Double immunofluorescence of P2X1 with microglial and astroglial markers (Ib4 and GFAP, respectively) provided negative results also in adult hippocampal slices in vivo (data not shown).

Moreover, immunofluorescence confocal analysis revealed a significant increase in P2X1 receptor expression occurring 24 h after OGD on fibers of the stratum oriens and radiatum and on fibers that cross the granular layer (Fig. 6, A and B). Pharmacological treatment with 50 μM TNP-ATP during and after OGD reduced P2X1 receptor expression in all these regions. Different doses of TNP-ATP used during OGD demonstrated that the concentration-response curves for prevention of both cell death and modulation of P2X1 receptor were...
very similar (data not shown). The results were confirmed by Western blot analysis showing that P2X1 receptor expression was induced of approximately 10-fold with respect to control at 24 h after OGD, whereas TNP-ATP reduced P2X1 protein expression by approximately 35% with respect to OGD (Fig. 6, C and D). We next measured the damage produced at 24 or 48 h after OGD. We found that cell damage or death visualized by PI gradually increased up to 48 h, whereas P2X1 receptor protein was maximally induced at 24 h and it decreased at 48 h, therefore, preceding the maximal extent of cell death (Fig. 7, A and B).

**Discussion**

In this work, we described the expression of purinergic P2X1 receptor on rat hippocampal postmitotic neurons, and we discussed its participation particularly in OGD-induced neuronal death. In contrast to P2X2 receptor localized mainly at synaptic terminals (Cavaliere et al., 2004b) but similarly to P2X2 receptor present on neurons (Cavaliere et al., 2003), P2X1 protein is localized on neuronal fibers and cell bodies in both primary dissociated pyramidal and granular neurons in CA1–3 and DG layers of organotypic slices and also in adult rat hippocampus. Specificity of the P2X1 receptor signal is proved by the result that although double immunofluorescence with NeuN confirms the neuronal expression of P2X1 receptor on myelinated and unmyelinated fibers of CA1–3 and dentate gyrus, the P2X1-positive signal also present in CA1–2 pyramidal cell layer throughout the strata oriens and radiatum colocalizes neither with NeuN nor with MBP (Cavaliere et al., 2004b). Thus, this difference in localization would indicate a different expression of these two receptor subtypes in the same hippocampus. Moreover, neither P2X1 nor P2X2 receptor signals overlap, in view of the fact that,
under identical ischemic conditions (40 min of OGD followed by 20–24 h post-treatment), the two proteins elicit different levels of induction (10- and 2.5-fold, respectively; also see Cavaliere et al., 2003). Finally, the possibility of cross-reactivity of P2X1 receptor is excluded with either P2X3 subtype (not apparently modulated by ischemic conditions) or P2X4 subtype (expressed on microglia cells and not on neurons; Cavaliere et al., 2003). All this evidence demonstrates the high degree of heterogeneity, and, simultaneously, the specificity of the cell and tissue distribution of different P2 receptor subtypes in the hippocampus, as plausible indications of a diverse functional role of these receptors in this specific brain area. Because of its topographic distribution and by the lack of colocalization with various glial markers in both organotypic cultures and adult rat hippocampus, we assumed that the P2X1 receptor is expressed on mossy fibers and Schaffer collaterals. In particular, the absence of colocalization with MBP in organotypic cultures sustained the presence of P2X1 receptor on unmyelinated fibers of the internal circuit, which are described in the hippocampal CA1–3 areas as thin axons with no myelin sheath (Shepherd and Harris, 1998). Nonetheless, studies performed on adult rat hippocampus demonstrated the simultaneous expression of P2X1 receptor also on afferent myelinated fibers, suggesting that this receptor as a general marker for the entire hippocampal circuitry, independently from the granular versus pyramidal neuronal phenotypes, or from the different electrical conductance of myelinated versus unmyelinated fibers. In this regard, our work establishing the presence of P2X1 receptor on neurons of CA1–3 and DG rat hippocampus extends previous results obtained for astrocytes of stratum oriens, radiatum, and lacunosum (Kukley et al., 2001).

The general mechanistic role played by purinergic receptors in cell loss induced by various noxious insults such as ATP, glutamate excitotoxicity, growth factor deprivation, and hypoglycemia and chemical hypoxia is well known (Volonté et al., 2003, 2006). For example, P2X4 receptor is up-regulated in hippocampus on microglia cells during OGD-induced gliosis (Cavaliere et al., 2003); P2X7 receptor is expressed, on neuronal synapses in hippocampus (Cavaliere et al., 2004a) or on microglia in cerebral cortex and striatum only after in vitro or in vivo ischemia (Melani et al., 2006); and P2X4 present on neuronal fibers in hippocampus is directly involved in ischemic cell death (Cavaliere et al., 2003). We now strengthened and extended the finding of those studies to the P2X1 receptor, proving that P2X1 protein expression also is highly augmented in hippocampus by OGD, although exclusively on postmitotic mature neuronal cells, and that the selective ATP analog and nearly specific P2X1 receptor antagonist TNP-ATP can reduce both ischemic damage and P2X1 protein expression. The functional down-regulation of P2X receptors protein by antagonists is already well discussed (Amadio et al., 2002; Cavaliere et al., 2003), even if down-regulation of receptors may also occur in the presence of specific agonists, as in the case of norepinephrine (Hillman et al., 2005). For any given antagonist, the rank order potency for inhibition of P2 receptor actions strictly depends on conditions such as the use of endogenous versus transfected receptors, the cell type or tissue adopted, the in vitro or in vivo experimental conditions, the presence of divalent cations, and the ionic strength of the buffer in use, to name a few. Although TNP-ATP inhibits P2X2 receptor at a concentration that is 1000-fold higher than that inhibiting P2X1 subtype (Khakh et al., 2001; Virginio et al., 1998), in our work we cannot exclude the participation, at least in part and at least in principle, of P2X2 receptors in the actions inhibited by TNP-ATP. The involvement of P2X4 or P2X2/3 receptors (inhibited by TNP-ATP at concentrations comparable with those working on P2X1) is instead probably excluded, because P2X4 is apparently not up-regulated (Cavaliere et al., 2002), but eventually inhibited during an ischemic insult. Nevertheless, we performed experiments using lower doses of TNP-ATP, demonstrating that the concentration-response curves for prevention of both cell death and

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**Fig. 7.** P2X1 receptor expression precedes in time the maximal extent of cell death. Organotypic cultures were subjected to OGD for 40 min, and they were subsequently stained with PI (B; red). Twenty-four and 48 h after the insult, P2X1 expression (A; green) and cell damage (B; red) were evaluated by immunofluorescence and PI incorporation in CA3 hippocampal region.
modulation of P2X1 receptor were very similar, thereby reinforcing the involvement of the P2X1 subtype in ischemic actions. In addition, TNP-ATP only weakly prevents the up-regulation of P2X2 receptors by ischemia. Moreover, our results are consistent with the general pharmacological neuroprotective effect evoked by several antagonists of P2 receptors particularly against OGD-induced cell death (Cavaliere et al., 2001, 2003, 2004b; Melani et al., 2006).

Because we also proved here that maximal P2X1 protein expression anticipated maximal neuronal loss, we do not exclude that expression and function of P2X1 receptor might potentially trigger and/or boost cell death; consequently, the time window connecting these events might become fundamental for neuroprotective strategies. This hypothesis is in accordance with data by Rodrigues et al. (2005), establishing that activation especially of P2X1 protein among P2X receptors modulates the release, for example, of glutamate, inducing further membrane damage and neuronal death. Our results suggest that induced expression of P2X1 during OGD might take place as part of a wider purinergic network up-regulation. First, the abundant ATP overflow occurring during an ischemic event is compatible with P2 receptor activation (Neary and Kang, 2005); second, a defined hierarchy in ligand binding affinity subsists in P2X receptor activation by extracellular ATP (the EC50 for ATP is 7.0 on P2X1 receptor, 5.4 on P2X2 receptor, 5.3 on P2X3 receptor, and 3.4 on P2X7 receptor); and third, P2X2, P2X3, and P2X6 are also induced by OGD, although with diverse cellular and/or subcellular localization. Although within different binding affinities, time frames, and cellular specializations, different receptor subtypes (making up at least the P2X2,4,7 receptors) would be simultaneously be recruited and combined on the same cell membrane in a sort of receptor web (Volonte et al., 2006) to extend to the P2X1 subtype the repertoire of ionotropic neurotransmitters known to be involved in ischemic cells regulation of P2X2 receptors by ischemia. Moreover, our results suggest that expression and function of P2X1 receptor might participate in oxygen/glucose deprivation-induced cortical damage. Neuroscience 136:615–623.


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