Abstract The presynaptic P2X7 receptor (P2X7R) plays an important role in the modulation of neurotransmitter release. We recently demonstrated that, in nerve terminals of the adult rat cerebral cortex, P2X7-R activation induced Ca2+-dependent vesicular glutamate release and significant Ca2+-independent glutamate efflux through the P2X7-R itself. In the present study, we investigated the effect of the new selective P2X7-R competitive antagonist 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine (A-438079) on cerebrocortical terminal intracellular calcium (intrasyaptosomal calcium concentration; [Ca2+]i) signals and glutamate release, and evaluated whether P2X7-R immunoreactivity was consistent with these functional tests. A-438079 inhibited functional responses. P2X7-R immunoreactivity was found in about 45% of cerebrocortical terminals, including glutamatergic and non-glutamatergic terminals. This percentage was similar to that of synaptosomes showing P2X7-R-mediated [Ca2+]i signals. These findings provide compelling evidence of functional presynaptic P2X7-R in cortical nerve terminals.

Keywords: Presynaptic P2X7 receptors; Adult rat cortical terminals; Immunohistochemistry; Single synaptosomal calcium imaging; Glutamate release

1. Introduction

The P2X7 receptor (P2X7-R) is a non-selective cation channel activated by extracellular ATP. It is able to switch to a so-called pore conformation that is permeable to large organic molecules up to 900 Da, leading to lysis and cell death [1]. It has been shown to be expressed in immune cells and epithelia as an immunomodulatory receptor [1,2]. However, a growing amount of evidence indicates that the P2X7-R participates in distinct neuronal, astroglial and microglial functions associated with the modulation of synaptic neurotransmission, and that it is a potential target site in disorders of the nervous system [3–5]. We recently studied rat cerebrocortical synaptosomes, which constitute a useful model for the investigation of functional proteins at the presynaptic level; our findings suggested that the P2X7-R plays a significant role in ATP-evoked glutamate efflux, which involves both Ca2+-dependent vesicular release and Ca2+-independent efflux through the P2X7-R itself [6]. Although the immunoreactive P2X7 subunit has frequently been found on central and peripheral neurones [7–10], including rat cerebrocortical nerve terminals [11,12], the reliability of P2X7 antibody testing has been questioned [13,14]. Indeed, midbrain and cerebellar granule neurones in P2X7-R knockout mice seem to express a less efficient “P2X7-like” receptor which is recognized by commercial antibodies and exhibits some functional properties similar to those of the genuine P2X7-R [15,16]. However, on merging electrophysiological, molecular and microfluorometric assays, or pharmacological and immunocytochemical characterisations, other authors have provided compelling evidence of the existence of a genuine P2X7-R on different types of neurones [9–11,17]. The aim of the present study was to increase our knowledge of the P2X7-R receptor in synaptosomes of adult rat cerebral cortex by using different techniques. We explored the relationship between P2X7-R immunoreactivity and functional responses such as calcium (intrasyaptosomal calcium concentration; [Ca2+]i) increase and glutamate release evoked by the potent P2X7-R agonist 3’-O-(4-benzoyl)benzoyl ATP (BzATP). In addition, we evaluated whether these P2X7-R-mediated functional activities were inhibited by the selective antagonist 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine (A-438079), a new potential tool for identifying the presence of P2X7-R in the brain [18–20].

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley rats (200–250 g) were housed at constant temperature (22 ± 1 °C) and humidity under a regular light-dark schedule (light 7 a.m.–7 p.m.). Food and water were freely available. Experimental procedures were carried out in accordance with European legislation (European Communities Directive of 24 November 1986, 86/609/EEC). Every effort was made to minimize the number of animals used and to reduce suffering.
2.2. Preparation of purified synaptosomes

After decapitation, the cerebral cortex was rapidly removed and placed in ice-cold medium, and nerve terminals (purified synaptosomes) were prepared on a discontinuous Percoll gradient, as previously reported [6,21]. Protein determinations were carried out according to Bradford et al. [22]. For release experiments, synaptosomes were suspended in standard medium (mM): NaCl 135; KCl 2.4; MgSO4 1.2; CaCl2 1.2; H2PO4 1.2; NaHCO3 5 with glucose 10; pH 7.4. For immunofluorescence confocal microscopy or [Ca2+]i measurement, synaptosomal pellets containing 2 mg proteins were resuspended in 1 ml HEPES medium (mM): NaCl 128; KCl 2.4; MgSO4 1.2; CaCl2 1.0; HEPES 10 with glucose 10; pH 7.4. A aliquot (15 μl) of suspended synaptosomes was deposited onto 20-mm glass coverslips coated with Cell-Tack (BD Biosciences) and centrifuged at 3000 rpm (3 min.).

2.3. HEK293 cells: cultures and stable transfection

Cultures of the human embryonic kidney cell line 293 (HEK293) were maintained in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham, supplemented with 10% foetal bovine serum and gentamicin/glutamine (5 mg/ml and 200 mM, respectively) in a humidified incubator at 37°C in a 5% CO2-enriched atmosphere. HEK293 cells were stably transplanted with the plasmid containing the full-length rat P2X7-green fluorescent protein (P2X7-GFP) cDNA in pcDNA3, as previously described [6,23].

2.4. Immunofluorescence confocal microscopy

Synaptosomes or HEK293 cells were fixed with 2% paraformaldehyde, permeabilized with 0.05% Triton X-100 (5 min.) and incubated for 30 min. with primary antibodies diluted in phosphate buffer saline (PBS) containing 3% albumin. The following antibodies were used: mouse anti-syntaxin1 (1:1000; Chemicon International), guinea pig anti-vesicular glutamate transporter 1 (VGLUT1; 1:1000; Chemicon International) and anti-extracellular epitope (aminoacids 136-152) of mouse anti-syntaxin1 (1:1000; Chemicon International), guinea pig anti-syntaxin1 (1:1000; Chemicon International), guinea pig anti-syntaxin1 (1:500, Alomone Labs) antibody. All the salts, the protease pore Corporation) membrane blocked in 5% dry fat milk in 50 mM Tris, 150 mM NaCl, and hybridised with the anti-extracellular epitope of P2X7-R (1:500, Alomone Labs) antibody. All the same, the protease inhibitor cocktail and the molecular mass marker used (wide molecular weight range) were obtained from Sigma.

3. Results

The effects of the potent P2X7R agonist, BzATP, on intrasynaptosomal calcium levels [Ca2+]i, and on [3H]p-aspartate efflux were studied simultaneously in sister preparations of freshly isolated adult rat cortical synaptosomes.

The Ca2+ imaging system was used in single cortical synaptosomes, and [Ca2+]i, was expressed as F340/F380 ratio (see Fig. 1A). In response to 100 μM BzATP, we identified a [Ca2+]i increase in about 45% of reactive synaptosomes tested by means of a depolarising event (35 mM K+] (n = 270 determinations from 16 independent preparations; Fig. 1B). To confirm the contribution of P2X7-R to the BzATP-induced [Ca2+]i increase, we used 3 μM of A-438079, a recently discovered selective P2X7 antagonist [26]. In the presence of A-438079, the [Ca2+]i increase was significantly blunted (n = 65 determinations from four independent preparations; Fig. 1C).

The effect of A-438079 on BzATP-evoked glutamate release was tested by measuring tritium efflux from purified synaptosomes pre-labeled with [3H]p-aspartate. The behavior of endogenous glutamate was indistinguishable from that of tritium after labeling with [3H]p-aspartate, which justified the use of [3H]p-aspartate. The fractional basal tritium outflow amounted to 0.28 ± 0.03%/min (n = 3); BzATP (100 μM)-evoked tritium release was inhibited by the selective P2X7-R antagonist A-438079 (3 μM; Fig. 1D). Addition of A-438079 at the concentration used did not affect basal tritium efflux (data not shown). These results unequivocally confirm the involvement of P2X7 in synaptosomal glutamate release, as already demonstrated in our previous study [6].

We carried out immunocytochemistry and western blotting to identify P2X7-R expression in cortical synaptosomal terminals. To demonstrate the efficacy of the extracellular anti-P2X7 antibody, we immunostained HEK293 cells stably expressing rat P2X7-R-GFP (Fig. 2A–C). P2X7 immunostaining revealed evident cell-surface P2X7-R expression, which completely merged in GFP fluorescence. Total protein extracts from synaptosomes and HEK293 cells (native or transfected with rat P2X7-R) were probed with the anti-P2X7 antibody; western blot analysis revealed that native HEK293 cells did not show immunostaining in synaptosomal preparations. By contrast, the antibody displayed a distinct band of ~70 kDa corresponding to the rat P2X7 subunit, while the ~95 kDa band depicted in transfected HEK293 cells had the expected molecular weight of the P2X7-GFP protein complex (Fig. 2D).

Syntaxin1 was used as a marker for our purified cerebrocortical synaptosomal preparations, even though it has also been seen to label cerebrocortical astrocytes in culture [24] and golgi-somes obtained from adult rat brain [25]. However, in our previous study purified synaptosomes were labeled with anti-synaptophysin antibody, a marker of the synaptic terminal, and were negative for the glial marker GFAP (glial fibrillary

2.5. Synaptosome superfusion experiments

After incubation with [3H]p-aspartate (0.03 μmol/L; 15 min), synaptosomes were superfused (0.5 ml/min) in parallel superfusion chambers at 37°C with HEPES medium; tritium efflux in each fraction was calculated as a percentage of the total tritium present at the onset of the fraction considered (fractional release); the percent variation in fractional release, with respect to the basal control value, was evaluated in each fraction [6]. The drug-evoked tritium efflux was measured by subtracting the areas under the curves of the percent variations in tritium fractional release in control chambers from that seen in drug-treated chambers; in each experiment, at least one chamber was used as a control for each condition.

2.6. Calcium imaging in single synaptic terminals

Intrasynaptosomal [Ca2+], was measured by the FURA-2AM microfluorometric technique on single-glued terminals. Synaptosomes adhering to coverslips were loaded with 5 μM FURA-2AM (45 min at 37°C) and then mounted in a microperfusion chamber on the stage of a Nikon TE200 inverted fluorescence microscope equipped with a dual-excitation fluorometric Ca2+ imaging system (Hamamatsu). The ratio F340/F380 was used to indicate the changes in [Ca2+], from selected regions of interest (ROD) covering a single synaptic terminal. Experiments were performed as previously described [6,23].

2.7. Western blotting

HEK293 stably expressing rat GFP-tagged P2X7 in C-termine, native cells and synaptosomal pellets were crumbled, and the samples (10 mg) were separated by SDS-PAGE (10%), electrophoretically transferred to a polyvinylidene difluoride (Immobilon-P PVDF; Millipore Corporation) membrane blocked in 5% dry fat milk in 50 mM Tris, 150 mM NaCl, and hybridised with the anti-extracellular epitope of P2X7-R (1:500, Alomone Labs) antibody. All the same, the protease inhibitor cocktail and the molecular mass marker used (wide molecular weight range) were obtained from Sigma.
acidic protein), the microglia marker integrin-αM and the oligodendrocyte marker RIP, indicating the absence of non-neuronal contamination in our preparations [6].

Immunofluorescence confocal microscopy experiments showed that 66 ± 1.5% of rat cerebrocortical synaptosomes, which were positive for the presynaptic marker syntaxin1, were immunoreactive for VGLUT1 (Fig. 3A–D); 37 ± 3.7% of the VGLUT1-positive synaptosomes were also stained by the anti-P2X7 receptor antibody (Fig. 3E–H). VGLUT1-positive synaptosomes accounted for 55 ± 2.5% of the total number of P2X7-positive synaptosomes. Interestingly, the P2X7 antibody also labeled VGLUT1-negative synaptosomes (Fig. 3E–H). Collectively, these data indicate the presence of P2X7-R immunoreactivity in a sub-population amounting to about 45% of synaptosomes not restricted to glutamate-releasing nerve terminals (see illustration in Fig. 3I).

4. Discussion

The present study confirms the functional presence of the P2X7-R in presynaptic terminals from adult rat cortical neurons. The results add to our recent report, which demonstrated...
the involvement of presynaptic P2X\(_7\)R in ATP-induced glutamate release [6].

In this study, the recently discovered specific P2X\(_7\)R antagonist A-438079 induced a significant reduction in P2X\(_7\)-mediated Ca\(^{2+}\) responses and [\(3^H\)]D-aspartate-release, indicating for the first time the efficacy of this drug in the brain. The compound is reported to be essentially devoid of activity on other P2 receptors and to show little or no activity on a wide array of other cell-surface receptors and ion channels [19]. It has recently been demonstrated that this antagonist potently blocks both P2X\(_7\)-mediated ionic currents recorded in vivo in spinal neuronal subtypes [20] and rat or human non-neuronal P2X\(_7\)-R-induced Ca\(^{2+}\) increase [18,19]. As this drug has never been used on P2X\(_7\)-like protein in knockout mice, the efficacy of the antagonist does not exclude the possible masked presence of P2X\(_7\)-like protein in our native preparation. It is therefore very important to check the potency of this compound on the P2X\(_7\)-like receptor in knockout mice [15,16].

We found that a significant fraction of nerve terminals (about 45%) were stained by the anti-P2X\(_7\) subunit antibody. This finding is in line with observations made on other rodent brain areas (rat and mouse midbrain: 52% and 32%, respectively, and rat cerebellum: 60%) [15,27,28]. Notably, the percentage of immunoreactive terminals was comparable to that of synaptosomes showing P2X\(_7\)-mediated Ca\(^{2+}\) responses.

By using a quantitative immunohistochemical study, we demonstrated that about 66% of (syntaxin1-positive) cerebrocortical nerve terminals were glutamatergic, as indicated by expression of VGLUT1, and that a sub-population (about 37%) of the glutamatergic terminals was immunoreactive to

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**Figure 3.** P2X\(_7\)R localization in purified rat cerebrocortical synaptosomes. Immunofluorescence for syntaxin1 (A) and for VGLUT1 (B) and merge image showing co-localization (C). Immunofluorescence for P2X\(_7\)R (E) and for VGLUT1 (F) and merge image (G). (D and H) Bars indicate percentage of co-localization (% ± S.E.M. of 9–12 fields from three different preparations) of VGLUT1 with syntaxin1 (D, solid bar) or with P2X\(_7\)R (H, solid bar) and of syntaxin1 or P2X\(_7\)R with VGLUT1 (D or H, empty bars). (I) Schematic drawing of P2X\(_7\)-R localization on glutamatergic and non-glutamatergic nerve terminals of rat cerebral cortex.

**Figure 4.** Co-localization of P2X\(_7\)-R with WGA in single synaptosomes: the images represent WGA (A) and anti-P2X\(_7\) (B); merge image (C). (D) Overlay plot of fluorescence intensity profile along the white bar. Note the non-uniform P2X\(_7\)-R distribution on the synaptosomal membrane.
P2X_2 antibodies. A sub-population of non-glutamatergic nerve terminals was also labeled by the P2X_7 antibody, the VGLUT1-positive terminals representing only 55% of the P2X_2-positive synaptosomes. P2X_7 immunoreactivity on VGLUT1-negative terminals can most likely be assigned to non-glutamatergic terminals, as reported by Atkinson et al. [12]. Indeed, in rat cerebral cortex, P2X_7R co-localised only with VGLUT1 transporter on glutamatergic nerve terminals, whereas P2X_7-immunoreactive and VGLUT2-immunoreactive varicosities formed separate populations.

Confocal immunolocalization on single synaptic terminals confirmed the presence of P2X_7R on synaptosomes. The co-localization of P2X_7 immunoreactivity and the neuronal membrane marker WGA revealed a non-uniform receptor distribution on the synaptosomal membrane: such a distribution pattern is compatible with the hypothesis that P2X_7R could be localized near the active zones, as reported in cerebellar granules [29], and appears consistent with the involvement of P2X_7R in the control of synaptic vesicle trafficking.

In summary, the data reported in this study show a good correlation between P2X_7 immunoreactivity and functional assays, which is strongly supported by the antagonism of A-438079. Moreover, P2X_7R expression is associated with excitatory terminals (VGLUT1-positive), as well as the non-glutamatergic sub-population of synaptosomes, indicating a widespread presence of the receptor in the neocortex.

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References
