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Rapid communication

Calmidazolium selectively inhibits exocytotic glutamate release evoked by P2X7 receptor activation

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ABSTRACT

We previously observed that activation of presynaptic P2X7 receptors located on rat cerebrocortical nerve terminals induced the release of glutamate through different modes: the channel conformation allowing Ca^{2+} entry triggered exocytotic release, while the receptor itself functioned as a permeation pathway for the non-exocytotic glutamate release. Considering that exocytotic and non-exocytotic glutamate release evoked by the activation of P2X7 receptors might play a role in the control of glutamatergic synapses, we investigated whether calmidazolium (which has been found to inhibit small cation currents through recombinant P2X7 receptors, but not organic molecule permeation) could distinguish between P2X7-related exocytotic and non-exocytotic modes of glutamate release. We found that calmidazolium inhibited the intrasynaptosomal Ca^{2+} response to P2X7 receptor activation and the Ca^{2+}-dependent exocytotic glutamate release from rat cerebrocortical nerve terminals, but was ineffective against the Ca^{2+}-independent glutamate release. The P2X7 competitive antagonist A-438079 eliminated both exocytotic and non-exocytotic P2X7 receptor-evoked glutamate release. Selective inhibition of exocytotic glutamate release indicates that calmidazolium inhibits events dependent on the function of native rat P2X7 receptors as Ca^{2+} channels, and suggests that it can be used as a tool to dissociate P2X7-evoked exocytotic from non-exocytotic glutamate release.

1. Introduction

The P2X7 receptor (P2X7R) was initially discovered on cells of hematopoietic lineage and subsequently described on glial cells and neurons, primarily on glutamatergic neurons (Duan and Neary, 2006; Sperlagh et al., 2006; Burnstock, 2007; Bennett et al., 2009 and references therein). It became apparent that the activation of P2X7R located on rat cerebrocortical glutamatergic nerve terminals induced glutamate release through two different mechanisms. One was exocytotic: Ca^{2+} entered the nerve terminals through P2X7R and triggered Ca^{2+}-dependent exocytotic glutamate release (Leon et al., 2008; Marcoli et al., 2008; Alloisio et al., 2008). The other was non-exocytotic: Ca^{2+}-independent glutamate release appeared to occur through activated P2X7R (Marcoli et al., 2008). Evidence that P2X7R functioned as a permeation pathway for glutamate was also obtained in cultured cerebrocortical astrocytes (Duan and Neary, 2006) or by measuring [^{3}H]D-aspartate efflux from HEK293 cells transfected with rat P2X7R (Marcoli et al., 2008).

It has repeatedly been observed that, upon exposure to agonist, P2X7R can shift from a rapid gating channel conformation selective for small cations to "dilated pore" conformations permeable to large organic molecules and dyes. By acting at the external surface of the receptor, the calmodulin antagonist calmidazolium has been shown to block – in a non-competitive way compatible with direct ion channel block or with an allosteric action on the receptor – the currents through recombinant rat or human P2X7R expressed in HEK293 cells, while it had no effect on dye uptake (Virginio et al., 1997; Chessell et al., 1998). Recently, calmidazolium was shown to eliminate the currents through native rat P2X7R (Nörenberg et al., 2010; Oliveira et al., 2011). Calmidazolium could therefore be regarded as a tool to dissociate the rapid gated ion channel from the pore function of P2X7R (Virginio et al., 1997; Pelegrin and

Abbreviations: A-438079, 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)-methyl pyridine; BzATP, 2′-3′-O-(benzoylbenzoyl)ATP; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; P2X7R, P2X7 receptor; ROI, region of interest; VOCCs, voltage operated calcium channels.

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Surprenant, 2009 and references therein). In order to investigate whether calmidazolium could distinguish between P2X7R-evoked exocytotic and non-exocytotic glutamate release, we assessed its effects on presynaptic P2X7R function as Ca^{2+} channels coupled to exocytotic glutamate release and as a permeation pathway for glutamate.

2. Materials and methods

2.1. Animals

Adult male rats (Sprague–Dawley 200–250 g) were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light–dark schedule (lights on 7AM-7PM). Food and water were freely available. Experimental procedures and animal care complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by Italian legislation on animal experimentation (protocol number 29823–7). All efforts were made to minimize animal suffering and to limit the number of animals used.

2.2. Preparation of purified synaptosomes

After decapitation, the cerebral cortex was rapidly removed, and purified synaptosomes were prepared as previously reported (Marcoli et al., 2008). Briefly, the tissue was homogenized in 10 volumes of 0.32 mM sucrose, buffered at pH 7.4 with Tris–HCl. The homogenate was centrifuged (5 min, 1000 *g* at 4 °C) and the supernatant was stratified on a discontinuous Percoll gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500*g* for 5 min. The layer between 10% and 20% Percoll was collected and washed by centrifugation. Synaptosomes were then suspended in standard medium with the following composition (mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.0, and $NaHCO_3$ 22 with glucose 10 (gassed with 95%O_2-5%CO_2, pH 7.4). To measure intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), synaptosomal pellets containing 2 mg proteins were resuspended in 1 ml HEPES medium (mM: NaCl 128, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.0, and HEPES 10 with glucose 10, pH 7.4); 15 μl were deposited onto glass coverslips coated with poly-L-lysine.

2.3. Superfusion experiments

Synaptosomes were incubated (15 min at 37 °C) with [^{3}H]D-aspartate (0.03 μM), transferred to parallel superfusion chambers at 37 °C and superfused (0.5 ml/min) with standard medium (Marcoli et al., 2008). Briefly, after 33-min superfusion, superfusate fractions were collected in 3 min samples; after 38 min of superfusion, synaptosomes were exposed (120 s) to 2′-3′-O-(benzoylbenzoyl)ATP (BzATP) or high K^+ (KCl 15 mM substituting an equimolar concentration of NaCl). The effect of calmidazolium or 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)-methyl pyridine (A-438079) was evaluated by adding the drug 8 min before BzATP or high K^+. To evaluate the effect of external Ca^{2+} deprivation, synaptosomes were superfused with Ca^{2+}-free medium (supplemented with EGTA 0.5 mM) starting 18 min before the addition of BzATP or high K^+.

At the end of superfusion, the radioactivity in synaptosomes and superfusate samples was determined by liquid scintillation counting. The amount of endogenous glutamate in the fractions was measured by HPLC; protein determinations were carried out by using bovine serum albumin as standard (Marcoli et al., 2008).

The efflux of radioactivity in each fraction was calculated as a percentage of the total radioactivity present at the onset of the fraction considered (fractional release). The amount of endogenous glutamate released in the fractions was expressed as pmol/mg protein. The mean tritium fractional release or endogenous glutamate in the first two basal fractions was taken as the 100% control value for each chamber; tritium and glutamate efflux was evaluated as the percentage variation of tritium fractional release or glutamate with respect to the corresponding control value. The drug (or depolarization)-evoked tritium or endogenous glutamate efflux was measured by subtracting the area under the curve of percentage variations in tritium fractional release or in glutamate release in appropriate control chambers from the area under the curve of the percentage variations in drug (or high K^+)-treated chambers. In each experiment, at least one chamber was used as a control for each condition. Drugs were dissolved in distilled water or in physiological medium. Calmidazolium chloride was dissolved in DMSO; the final highest DMSO concentration used (0.02% v/v) did not affect basal, BzATP- or K^+-evoked tritium efflux (data not shown).

2.4. Calcium imaging

Intrasynaptosomal $[Ca^{2+}]_i$ was measured by the FURA-2AM microfluorimetric 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 fluorimetric Ca^{2+} imaging system (Hamamatsu). The ratio F340/F380 was used to indicate changes in $[Ca^{2+}]_i$ from selected regions of interest (ROI) covering a single synaptosome or macro-ROI consisting of 20–40 terminals. Experiments were performed as previously described (Alloisio et al., 2008). Each experiment was carried out on at least three independent synaptosomal preparations. A pulse of high K^+ (35 mM) at the end of each experiment verified the viability of synaptosomes.

2.5. Calculation and statistics

Log concentration–response relationships and IC_{50} values (half-maximum effective concentrations in inhibiting the BzATP-evoked efflux) were obtained through a four-parameter logistic function fitting routine (Sigma Plot software, Jandel-Scientific, San Rafael, CA, USA). Mean ± SEM of the numbers of experiments (n) are indicated throughout. Significance of the difference was analyzed by means of ANOVA followed by Student's *t*-test, with statistical significance taken at $p < 0.05$.

2.6. Materials

[^{3}H]D-aspartate (specific activity: 11.3 Ci/mmol) was from Amersham Radiochemical Centre (Buckinghamshire, UK); BzATP, calmidazolium chloride and FURA-2AM from Sigma–Aldrich (Milan, Italy); A-438079 from Tocris Cookson (Bristol, UK); poly-L-lysine from BD Biosciences (San Jose, CA, USA). All the salts and other reagents were from Sigma–Aldrich.

3. Results

3.1. BzATP-evoked [^{3}H]D-aspartate release

Glutamate release was studied by measuring tritium efflux from synaptosomes pre-labeled with [^{3}H]D-aspartate. In order to rule out the possibility that tritium release was an artifact resulting from the use of [^{3}H]D-aspartate, we monitored the release of endogenous glutamate; the behavior of endogenous glutamate proved to be indistinguishable from that of tritium after labeling with [^{3}H]D-aspartate, thus justifying the use of [^{3}H]D-aspartate. The basal fractional tritium outflow in the first two fractions collected from