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Existence of high and low affinity dinucleotides pentaphosphate-induced calcium responses in individual synaptic terminals and lack of correlation with the distribution of $P2X_{1-7}$ subunits

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Abstract

Individual analysis of synaptic terminals calcium responses, induced by dinucleotides pentaphosphate, Ap_5A or Gp_5G, demonstrates the presence of two main groups considering the concentration required for stimulation. The first group corresponds to those responding to Ap_5A or Gp_5G at nanomolar concentration, representing 16% and 12%, respectively, and the second one responds to micromolar concentration and represents, respectively, 17% and 14%, of the total functional synaptosomal population in rat midbrain.

Dose–response curves in single terminals showed an Ap_5A EC_{50} values of 0.9 ± 0.2 nM and 11.8 ± 0.9 μM, being the maximal intrasynaptosomal calcium increase of 200 ± 0.3 and 125 ± 0.2 nM for the high and low affinity responding terminals, respectively.

Combination of microfluorimetric and immunocytochemical studies showed lack of correlation between dinucleotides pentaphosphate responses and P2X receptor subunits expression, in spite of the abundance of $P2X_2$, $P2X_3$ and $P2X_7$ at the presynaptic level in rat midbrain synaptosomes.

Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), a P2X receptors antagonist, showed no effect on low affinity dinucleotides receptors population, and partial inhibition on the high affinity one. On the other hand, diinosine pentaphosphate (Ip_5I) completely abolished the low affinity dinucleotides responses, and 60% inhibition of the high affinity ones.

Keywords: Dinucleotides pentaphosphate; P2X subunits; Microfluorimetric studies; Ip_5I; PPADS; Individual synaptic terminals; Rat midbrain

1. Introduction

Diadenosine polyphosphates (Ap_nA) are naturally occurring molecules formed by two adenosine moieties bridged via their 5′ position by a phosphate chain of variable length, where *n* represents the number of phosphates ($n = 2–6$) (for review, see Pintor et al., 2000). Ap_4A and Ap_5A are present in high concentrations in rat brain synaptic terminals and, after nerve stimulation, Ap_nA are released in a Ca^{2+}-dependent process (Pintor et al., 1995).

Some diadenosine polyphosphates actions are mediated by P2 receptors activation, a family of nucleotide-sensitive receptors subdivided into ligand-gated cation channels permeable to Na^+, K^+ and Ca^{2+} (P2X) and G protein-coupled receptors (P2Y). Until the moment, seven distinct members of the P2X family ($P2X_{1-7}$) have been characterised in a variety of tissues. All P2X subunits have been found in central nervous system (CNS), but with a differential distribution between CNS areas (Xiang and Burnstock, 2005; Franke and Illes, 2006; Roberts et al., 2006).

It has been described that diadenosine polyphosphates can activate recombinant rat homomeric $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ receptors expressed in *Xenopus* oocytes. In addition, some diadenosine polyphosphates can act as agonist of some P2X receptors (Pintor et al., 1996, 2003; Wildman et al., 1999). In contrast, diguanosine polyphosphates (Gp_nG), another group of naturally occurring dinucleotides, are not active at $P2X_1$ and $P2X_3$ receptors (Cinkilic et al., 2001).

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Due to the evident structural analogies between diadenosine polyphosphates and adenosine 5′-triphosphate (ATP), it is hard to understand the existence of a selective dinucleotides receptor. However, Pintor and Miras-Portugal (1995) reported that diadenosine polyphosphates, specially Ap_5A, increases the intrasynaptosomal calcium concentration in synaptic terminals from rat midbrain acting through specific ionotropic dinucleotides receptors distinct from P2X receptors. It is to notice that Gp_nG appears to be as good an agonist as Ap_nA activating these dinucleotides receptors (Pintor et al., 2001). Similar results were obtained when studying synaptosomal preparations from guinea pig, deer mouse or human, although the molecular structure of this dinucleotides receptor remains unknown (reviewed by Pintor et al., 2000). In addition, microfluorimetric studies in single synaptic terminals have shown the presence of specific and independent ionotropic receptors for nucleotides and dinucleotides. These receptors may or may not coexist at the same terminal and can be located on GABAergic, cholinergic and glutamatergic terminals, being able to induce neurotransmitter release once activated (Gómez-Villafuertes et al., 2001; Díaz-Hernández et al., 2002a; Gualix et al., 2003). The modulation of dinucleotides receptor activity by presynaptic G_i/G_0-protein coupled receptors has been reported for adenosine A_1 and $GABA_B$ receptors, both producing the appearance of biphasic Ap_5A concentration–response curves with a high affinity component when activated (Díaz-Hernández et al., 2000, 2002b; Gómez-Villafuertes et al., 2004). The biphasic behaviour of Ap_5A curve makes it necessary to study the calcium responses evoked by low and high concentrations of dinucleotides. Specific metabotropic receptors with signalling cascades showing homology with those of growth factors, such as EGF and NGF, have been described for dinucleotides compounds (Delicado et al., 2006).

In the present study, the correlation between P2X subunits and the existence of Ap_5A/Gp_5G calcium responses is analysed in order to clarify the nature of the dinucleotides-sensitive receptor or receptors present in single synaptic terminals from rat midbrain. Using microfluorimetric techniques we have found the presence of two synaptosomal populations responding to dinucleotides polyphosphate. Immunocytochemical studies show no clear correlation between these responses and P2X subunits, suggesting the existence of specific dinucleotides receptors in midbrain synaptic terminals.

2. Experimental procedures

2.1. Chemicals

Ap_5A, bovine serum albumin (BSA), Gp_5G, paraformaldehyde (PFA), mouse anti-synaptophysin antibody, fluorescein isothiocyanate (FITC)-coupled goat anti-mouse IgG, tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG and TRITC-conjugated rabbit anti-guinea pig IgG were all purchased from SIGMA Chemicals (St. Louis, MO, USA). Guinea-pig anti-$P2X_2$ receptor and guinea-pig anti-$P2X_3$ receptor antibodies were purchased from Neuromics (Northfield, MN, USA). Rabbit anti-$P2X_1$ receptor and rabbit anti-$P2X_7$ receptor antibodies were obtained from Chemicon (Temecula, CA, USA). Rabbit anti-$P2X_4$ receptor and rabbit anti-$P2X_6$ receptor antibodies were from Alomone Labs. (Jerusalem, Israel). Rabbit anti-$P2X_5$ receptor antibody was kindly donated by Dr. M. Voigt (Cox et al., 2001). Fura-2 acetoxymethyl ester (Fura-2 AM) was obtained from Molecular Probes (Leiden, The Netherlands). Pyridoxalphosphate-6-azophenyl-2′,4′-disulfhonic acid (PPADS) was from RBI (Natick, MA, USA). P^1,P^5-di(inosine-5′) pentaphosphate (Ip_5I), was synthesized in our laboratory, as indicated in a previous work (Pintor et al., 1997a). Other analytical grade reagents were purchased from Merck (Darmstadt, Germany).

2.2. Synaptosomal preparation

Synaptosomes were obtained from the rat midbrain of cervically dislocated and decapitated adult male Wistar rats as indicated previously (Gómez-Villafuertes et al., 2001). All the experiments carried out at the Universidad Complutense de Madrid were performed according to the guidelines of the International Council for Laboratory Animal Science (ICLAS). Synaptosomes were isolated following the method of Dunkley et al. (1986). Briefly, freshly isolated midbrain was homogenised in 0.32 M sucrose containing 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic (TES) and 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, and centrifuged at $900 \times g$ for 5 min. The supernatant was centrifuged at $17{,}000 \times g$ for 10 min and the resulting P2 fraction was layered on top of a Percoll density gradient composed of 3%, 10% and 23% Percoll prepared in a 1.6 M sucrose medium containing 0.5 M EDTA and 0.1 M dithiothreitol (DTT). Centrifugation was carried out at $25{,}000 \times g$ for 10 min. Synaptosomes were collected from the 10%/23% Percoll interface, resuspended in HBM [composition mM: NaCl 140, KCl 5, $NaHCO_3$ 5, NaH_2PO_4 1.2, $MgCl_2$ 1, glucose 10, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, pH 7.4] and centrifuged at $20{,}000 \times g$ for 3 min. Following the last centrifugation step, the supernatant was discarded and the pellet containing the synaptosomes was stored on ice. Under this conditions the synaptosomes remain fully viable for at least 4–6 h as judged by the extent of the KCl-evoked $[Ca^{2+}]_i$ increase. From a single synaptosomal preparation (1 animal) synaptosomes are obtained to prepare 4-6 coverslips as described in Sections 2.3 and 2.4.

2.3. Immunocytochemical procedures

Synaptosomal pellets containing 0.5 mg of protein were resuspended in 1 mL phosphate buffer saline (PBS) (composition mM: NaCl 137, KCl 2.6, KH_2PO_4 1.5, Na_2HPO_4 8.1, pH 7.4) and were allowed to attach to poly-L-lysine-coated coverslips for 1 h. Then, the coverslips were fixed for 15 min with 4% PFA in PBS (w/v). Following several washes in PBS, synaptosomes were incubated for 1 h in PBS containing 5% normal goat serum (v/v), 3% BSA (w/v) and 0.1% Triton X-100 (v/v). They were then incubated for 1 h at 37 °C with the appropriate primary antibody diluted in PBS containing 3% BSA (PBS/BSA) as follows: $P2X_1$ receptor (1:500), $P2X_2$ receptor (1:500), $P2X_3$ receptor (1:500), $P2X_4$ receptor (1:200), $P2X_5$ receptor (primary antiserum, 1:500), $P2X_6$ receptor (1:200), $P2X_7$ receptor (1:200), synaptophysin (1:200). After washing in PBS/BSA, synaptosomes were incubated for 1 h at 37 °C with the appropriate secondary antibodies: FITC-goat anti-mouse IgG, TRITC-goat anti-rabbit IgG or TRITC-rabbit anti-guinea pig IgG, all diluted 1:200 in PBS/BSA. Finally, synaptosomes were washed in PBS and mounted with Prolong Antifade Kit from Molecular Probes (Leiden, The Netherlands). As controls for the immunochemical reactions, primary antibodies were omitted from the staining procedure.

Synaptosomes were viewed with a Nikon TE-200 microscope equipped with a 100× objective (oil, 1.3 NA), a mercury lamp light source and fluorescein-rhodamine Nikon filter sets. In order to quantify the number of terminals showing co-localisation of two markers, FITC- and TRITC-fluorescent images were subsequently obtained using an Ultrapix 2000 Mono CCD camera controlled by Ultraview PC software (Perkin Elmer Life Sciences). Images were analysed using Lucida 3.0 software (Kinetic Imaging). Data are presented as the mean ± S.E.M. corresponding to 10-12 visual fields from 3 different synaptosomal preparations.

2.4. Calcium imaging in single synaptosomes

Synaptosomal pellets containing 0.5 mg of protein were resuspended in 1 mL HBM and loaded with 5 μM Fura-2 AM and 1.33 mM $CaCl_2$ for 45 min at 37 °C. The synaptosomal suspension was attached to a poly-L-lysine-coated