GABA modulates presynaptic signalling mediated by dinucleotides on rat synaptic terminals.

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Abbreviations: adenylate cyclase, AC; bovine serum albumin, BSA; dibutyl cyclic AMP, DiBucAMP; fluorescein isothiocyanate, FITC; glial fibrillar acidic protein, GFAP; P¹,Pₙ-di(adenosine-5’) pentaphosphate, Ap₅A; P¹,Pₙ-di(guanosine-5’) pentaphosphate, Gp₅G; paraformaldehyde, PFA; postsynaptic density protein PSD-95; cyclic AMP-dependent protein kinase, PKA; PKA inhibitory peptide, PKA-IP; tetramethylrhodamine isothiocyanate, TRITC; \( \gamma \)-aminobutiric acid, GABA.

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Abstract

Diadenosine pentaphosphate (Ap5A) elicits Ca^{2+} transients in isolated rat midbrain synaptic terminals acting through specific ionotropic dinucleotide receptors. The activation of GABA_{B} receptors by baclofen changes the sigmoidal concentration-response curve for Ap5A (EC_{50} = 44 \, \mu{M}) into biphasic curves. Thus, when GABA_{B} receptors are activated, the curve shows a high affinity component in the picomolar range (EC_{50} = 77 \, p{M}) and a low affinity component in the micromolar range (EC_{50} = 17 \, \mu{M}). In addition, in the presence of GABA or baclofen, Ap5A calcium responses are increased up to 50% over the control values. Saclofen, an specific antagonist of GABA_{B} receptors, blocks the potentiatory effect of baclofen. As occurs with Ap5A, GABA_{B} receptors are also capable to modulate diguanosine pentaphosphate (Gp5G)-induced calcium responses. The combination of immunocytochemical and microfluorimetric techniques carried out on single synaptic terminals have shown that in the presence of baclofen, 64% of the terminals responding to 100 \, \mu{M} Ap5A are also able to respond to 100 \, n{M} Ap5A. This value is close to the percentage of synaptic terminals responding to Ap5A and labelled with the anti-GABA_{B} receptor antibody (69%). The activity of cyclic AMP-dependent protein kinase (PKA) seems to be involved in the potentiatory effect of GABA_{B} receptors on Ap5A calcium responses, since PKA activation by forskolin or dibutiryl cyclic AMP blocks the potentiatory effect of baclofen, while PKA inhibition facilitates calcium signalling mediated by Ap5A. These results demonstrate that the activation of presynaptic GABA_{B} receptors is able to modulate dinucleotide responses in synaptic terminals.
\(P^1, P^n-\text{Di(adenosine-5')}\) polyphosphates \(\text{(Ap}_n\text{A)}\) constitute a group of compounds structurally related to ATP. Members of this family are formed by two adenosine moieties linked by their 5’ ends through phosphate chains of variable length. Diadenosine polyphosphates are stored in secretory vesicles containing ATP and are released after synaptic terminal stimulation (Pintor et al., 1992; 1995). Other members of the dinucleotide family are the diguanosine polyphosphates \(\text{(Gp}_n\text{G)}\), a group of naturally occurring substances first isolated from platelets and more recently from neurosecretory vesicles (Schlüter et al., 1998; Jankowski et al., 2003). Once in the extracellular medium, dinucleotides are able to activate presynaptic purinergic receptors. Extracellular actions of \(\text{Ap}_n\text{A}\) in the central nervous system include the modulation of the firing rate and the inhibition/facilitation of the synaptic transmission in neurones (Klishin et al., 1994; Fröhlich et al., 1996). It has been demonstrated that rat midbrain synaptic terminals respond to the addition of ATP and \(\text{Ap}_n\text{A}\) with an increase in the intrasynaptosomal calcium concentration (Pintor y Miras-Portugal, 1995). The effect of ATP was mediated through \(\text{P2X}\) receptors with \(\text{P2X}_3\)-like pharmacology (Gómez Villafuertes et al., 2000). However, \(\text{Ap}_n\text{A}\) effect appeared to be mediated through different receptors, termed dinucleotide receptors, which are activated by dinucleotide polyphosphates but not by ATP or UTP. These receptors are insensitive to classical \(\text{P2}\) antagonists such as \(\text{PPADS}\) and suramin and can be selectively inhibited by nanomolar concentrations of diinosine pentaphosphate (Pintor et al., 1997a). The existence of dinucleotide receptors has been reported in rat, guinea pig and deermouse synaptosomes (reviewed by Pintor et al., 2000). Moreover, the \(\text{Ca}^{2+}\) signalling mediated by dinucleotide receptors is regulated by the action of protein kinases and phosphatases, and effectors increasing \(\text{PKA}\) or \(\text{PKC}\) activity result in a considerable reduction of the calcium response (Pintor et al, 1997b; Díaz-Hernández et al., 2000). The regulation of dinucleotide receptors by presynaptic G-protein coupled adenosine receptors, one of the most important regulators of synaptic transmission, has also been described. Thus, adenosine activating \(\text{A}_1\) or \(\text{A}_2\alpha\) receptors potentiate \(\text{Ap}_5\text{A}\) calcium responses in rat brain synaptic terminals (Díaz-Hernández et al., 2000; Díaz-Hernández et al., 2002a).

Considering that dinucleotide receptors are receptor operated calcium channels, they might be involved in facilitatory presynaptic mechanisms in central neurones. In this way, the dinucleotide receptor activation induces the release of excitatory neurotransmitters such as acetylcholine or glutamate from cholinergic and glutamatergic synaptic terminals,
respectively (Díaz-Hernández et al., 2002b, Gualix et al., 2003). It is to notice that Ap₅A are also able to evoke γ-aminobutyric acid (GABA) release from GABAergic terminals (Gómez-Villafuertes et al., 2001). This fact together with the results reported previously by other groups suggests a close relationship between GABAergic and purinergic neurotransmitter systems in the CNS (Jo and Schlichter, 1999, Inoue et al., 1999; Hugel and Schlichter, 2000).

In the brain of vertebrates, GABA is the main inhibitory neurotransmitter, being able to activate two different mechanisms: one ionotropic, GABAₐ receptors, and other metabotropic, G-coupled GABAₐ receptors. Presynaptic GABAₐ receptors are widely distributed on brain nerve terminals where they act mediating the inhibition of neurotransmitter release by direct inhibition of voltage-dependent calcium channels. This effect is inhibited by pertussis toxin, indicating the involvement of a G₁/G₀-protein-coupled receptor. Additionally, GABAₐ receptor activation has been associated with the opening of inwardly rectifying K⁺ channels (GIRKs) and the modulation of adenylyl cyclase (AC) (Wu and Saggau, 1997; Filippov et al., 2000; Billinton et al., 2001). Keeping in view that diadenosine polyphosphates are able to induce GABA release form nerve terminals and, consequently, both transmitters can co-exist extracellularly in synaptosomal preparations, the aim of this work was to analyse the effect of presynaptic GABA receptor activation on the Ca²⁺ responses elicited by Ap₅A in rat synaptic terminals.
Methods

Chemicals

Ap5A, baclofen, bovine serum albumin (BSA), GABA, Gp5G, muscimol, paraformaldehyde (PFA), saclofen, mouse anti-synaptophysin, rabbit anti-GABA_A receptor α3 subunit, guinea-pig anti-GABA_B1 receptors, rabbit anti-glial fibrillar acidic protein (GFAP), rabbit anti-post-synaptic density marker protein (PSD-95), 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). Fura-2 acetoxyethyl ester (Fura-2 AM) and Fura-2 were obtained from Molecular Probes (Leiden, The Netherlands). Forskolin and PKA inhibitory peptide were supplied by Calbiochem (San Diego, CA, USA). Dibutyl cyclic AMP (DiBucAMP) was obtained from Research Biochemicals Inc. (Natick, MA, USA). SKF 89976A was from Tocris (Madrid, Spain). Other analytical grade reagents were purchased from Merck (Darmstadt, Germany).

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). The isolation procedure differed, depending on the proposed use of the synaptosomal preparation. Synaptosomes used for the determination of [Ca^{2+}]_i levels in synaptosomal populations were isolated following the method of Pintor et al. (1992). Briefly, freshly isolated midbrain was homogenised in 0.32 M sucrose containing 5 mM TES and 0.5 mM EDTA, pH 7.4, and centrifuged at 900xg for 5 min. The supernatant was centrifuged at 17000xg for 10 min and the resulting P2 fraction was resuspended in 0.25 M sucrose containing 5 mM TES pH 7.4. Protein determination was carried out by the Biuret’s method. Synaptosomes used to estimate [Ca^{2+}]_i in single synaptic terminals and those needed for immunocytochemical studies were purified on discontinuous Percoll gradients according to the procedure described by Dunkley et al. (1986). In brief, 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 DTT. Centrifugation was carried out at 25000xg for 10 min. Synaptosomes were collected from the 10%/23% Percoll interface, resuspended in
HBM (composition mM: NaCl 140, KCl 5, NaHCO₃ 5, NaH₂PO₄ 1.2, MgCl₂ 1, glucose 10, HEPES 10, pH 7.4) and centrifuged at 20000xg 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 [Ca²⁺]ᵢ increase.

**Determining intrasynaptosomal Ca²⁺ in synaptosomal populations**

Synaptosomal pellets containing 1 mg of protein were re-suspended in 1 mL incubation medium (composition mM: NaCl 122, KCl 3.1, KH₂PO₄ 0.4, NaHCO₃ 5, MgSO₄ 1.2, glucose 10 and TES buffer 20, pH 7.4). The cytosolic free calcium concentration was determined using Fura-2 as described by Grynkiewicz et al. (1985). Briefly, five minutes after re-suspension, 1.33 mM CaCl₂ and 5 µM Fura-2-AM were added to the synaptosomes. Following incubation for 25 min, the synaptosomes were pelleted (centrifuged at 15,700xg for 1 min), washed twice and re-suspended in fresh medium containing 1.33 mM CaCl₂. Fluorescence was measured in a Perkin Elmer Spectrofluorimeter LS-50, and monitored at λₑₓ 340 nm and λₑm 510 nm. Data were collected at 0.5 s intervals.

Synaptosomes were preincubated for different times (from 30 sec to 6 min) with 100 µM GABA, muscimol (GABAA receptor agonist) or baclofen (GABAB receptor agonist) before assaying Ap₅A. Once the optimal preincubation time was established, concentration-response curves for Ap₅A and Gp₅G ranging from 10⁻⁴ to 10⁻¹⁴ M were obtained in the presence of 100 µM baclofen. As controls, independent concentration-response curves (10⁻⁴ to 10⁻⁶ M) were prepared for Ap₅A and Gp₅G.

In some experiments, synaptosomes were preincubated for 2 min with 100 µM saclofen (a GABAB receptor antagonist), 10 µM forskolin (an adenylate cyclase activator) or 100 µM (DiBucAMP) before Ap₅A application. Since PKA inhibitory peptide (PKA-IP; 25 nM) does not cross the plasma membrane, it was added to the homogenisation buffer during synaptosomal preparation to allow its access to the intrasynaptosomal media (Pintor et al., 1997b). Finally, preincubation with 10 µM SKF-89976A (a specific GABA-uptake inhibitor) was performed for 2 min before Ap₅A addition in order to increase the extrasynaptosomal GABA concentration.

Intrasynaptosomal calcium levels are expressed as the mean ± SEM of at least three determinations performed in triplicate in different synaptosomal preparations. Concentration-response curves were fitted using Fig P version 6.0 software (Biosoft, Cambridge, UK). EC₅₀
and Hill values are expressed as EC$_{50}$ ± SD and $n_H$ ± SD respectively. Comparisons were performed by one-way ANOVA. Bonferroni post-test analysis was only applied when a significant ($p<0.05$) main effect was indicated by the ANOVA. Where appropriate, single experimental traces are shown in the figures; these represent at least three determinations performed in triplicate with equivalent results.

**Immunocytochemical procedures**

Synaptosomal pellets containing 0.5 mg of protein were resuspended in 1 mL PBS (composition mM: NaCl 137, KCl 2.6, KH$_2$PO$_4$ 1.5, Na$_2$HPO$_4$ 8.1, pH 7.4) and were allowed to attach to poly-L-lysine-coated coverslips for an hour. Then, the cover slips were fixed for 15 min in PBS containing 4% PFA (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: GABA$_A$ receptor (1:500), GABA$_B$ receptor (1:5000), synaptophysin (1:200), glial fibrillar acid protein (GFAP, 1:100) and postsynaptic density marker (PSD-95, 1:500). The commercial anti-GABA$_A$ receptor antibody was developed using a synthetic peptide (QGESRRQEPGDFVKQ) corresponding to amino acid sequence 1-15 of the extracellular N-terminus domain of the rat GABA$_A$ receptor $\alpha_3$ subunit. The commercial anti-GABA$_B$ receptor antibody was raised against an amino acid sequence (PSEPPDRLSCDGSRVHLLYK) that is common to both GABA$_B$R1a and GABA$_B$R1b receptors.

After washing in PBS/BSA, synaptosomes were incubated for 1 h at 37°C with the appropriate secondary antibodies (see Chemicals section) 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 100X 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 ± SEM corresponding to 4-6 visual fields from at least 2 different synaptosomal preparations.

**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-coted coverslip for 45 min at room temperature. This time period is sufficient for both sticking on the substrate and hydrolysis of the intrasynaptosomal Fura-2 AM. Next, the cover slips were washed with fresh HBM containing 1.33 mM CaCl$_2$, and mounted in a small superfusion chamber on the stage of a Nikon TE-200 microscope. For [Ca$^{2+}$]$_i$ measurements, synaptosomes were stimulated by 30-s pulses (indicated in each graph by a bar) of 100 nM Ap$_5$A in the absence and presence of 100 µM baclofen. Successive pulses of 100 µM Ap$_5$A and 30 mM KCl were applied at the end of each experiment to confirm the functionality and viability of the synaptosomes under study.

Synaptosomes were excited alternately at 340 and 380 nm through a 100X objective (oil, 1.3 NA) with the aid of a monochromator (12 nm bandwidth, Perkin Elmer Life Sciences, Cambridge, UK). These wavelengths correspond to the fluorescence peaks of Ca$^{2+}$-saturated and Ca$^{2+}$-free Fura-2 solutions. The fluorescence emitted from the synaptosomes was isolated by a dichroic mirror (430 nm) and was collected through a band-pass filter centered at 510 nm (Omega Optical). The video-images were obtained using an Ultrapix 2000 Mono CCD camera operating at 12-bit digitalisation (4095 levels) and controlled by Ultraview PC software (Perkin Elmer Life Sciences, Cambridge, UK). The exposure time was 50 ms and wavelength changed over time in less than 15 ms. Images were continuously acquired at 1.06 Hz and buffered on a fast SCSI disk. Time course data represent the average light intensity in a small elliptical region within each identified synaptic terminal. Background and autofluorescence components were subtracted at each wavelength and the ratio 340/380 was calculated by 32-bit float arithmetic (real numbers). Ratios were calibrated into [Ca$^{2+}$]$_i$ values using the Grynkewicz's equation (Grynkyevicz et al., 1985). The variables Rmax, Rmin and β were calculated "in vitro" from the spectra of small Fura-2 droplets in Ca$^{2+}$-saturated solution (composition mM: KCl 100, NaCl 10, MgCl$_2$ 1, Tris 10, MOPS 10, CaCl$_2$ 2 and Fura-2 100 µM) and Ca$^{2+}$-free solution (composition mM: KCl 100, NaCl 10, MgCl$_2$ 1, Tris 10, MOPS 10, CaCl$_2$ 2 and Fura-2 100 µM), both determined empirically in our system.
Results

Effect of GABA receptor agonists on Ap5A calcium responses in rat midbrain nerve terminals

Synaptosomal pellets from rat midbrain were preincubated with 100 µM GABA, baclofen (a selective GABA<sub>B</sub> receptor agonist) or muscimol (a specific GABA<sub>A</sub> receptor agonist) for 2 min before the addition of 100 µM Ap5A. As previously reported by Gómez-Villafuertes et al. (2003), synaptosomes stimulation with GABA, baclofen or muscimol in the absence of Ap5A evoked no intrasynaptosomal calcium transient (data not shown). However, GABA and baclofen produced around a 55% increase in the calcium response induced by Ap5A (Fig. 1A, second and third traces respectively), when compared to the control (Fig. 1A, upper trace). In contrast, muscimol failed to modify the intrasynaptosomal calcium increase induced by Ap5A (Fig. 1A, lower trace). To establish the optimal preincubation period leading to greatest potentiation of the response to Ap5A, synaptosomes were preincubated with 100 µM baclofen or muscimol for different time periods. Figure 1B shows the preincubation periods corresponding to 30 sec, 2 min, 4 min and 6 min. Potentiation of the Ap5A calcium responses evoked by baclofen reached their maximum after 2 min of incubation, whereas muscimol failed to significantly modify the Ap5A effect at each of the times assayed. Thus, a preincubation time of 2 min was fixed as the most appropriate to analyse baclofen effect on calcium responses induced by dinucleotides.

In order to analyse in depth the modulatory effect of presynaptic GABA receptors on Ap5A calcium responses, the distribution of these receptors on synaptic terminals was studied using immunocytochemical techniques (Fig. 2). Antibodies raised against α3 subunit have been extensively employed as markers for GABA<sub>A</sub> receptors (Benke et al., 1991). In this way, the absence of immunolabelling against α3 subunit in rat midbrain synaptic terminals correlates well with the lack of effect of muscimol on Ap5A calcium responses (Fig. 2A-C). On the contrary, the analysis of roughly 1475 synaptosomes from six fields showed that 62.1 ± 6.9% of them exhibit GABA<sub>B</sub> receptors (Fig. 2D-F), supporting the modulatory role that these receptors are playing on Ap5A calcium responses at the presynaptic level. The same preparation did not show any GFAP or PSD-95 labelling, thus indicating the absence of glial and post-synaptic contamination in our synaptic terminal preparations (data not shown).
Ap5A increases intrasynaptosomal Ca\(^{2+}\) concentration in single synaptic terminals containing GABA\(_B\) receptors

To determine whether a co-localisation between GABA\(_B\) receptors and Ap5A calcium responses occurs, we combined microfluorimetric studies in single synaptic terminals with immunocytochemical assays. To carry out these studies it was necessary to employ microgrid cover slips (square size 55 \(\mu\)m) in order to relocate individual synaptosomes after immunostaining procedures. Thus, isolated rat midbrain synaptic terminals loaded with Fura-2 and sticked on microgrid cover slips were tested for their ability to mobilise Ca\(^{2+}\) after 100 \(\mu\)M Ap5A superfusion (Fig. 3A-C). The intrasynaptosomal Ca\(^{2+}\) increase after depolarisation with 30 mM KCl was analysed at the end of each experiment in order to confirm synaptosomal functionality. Thus, the number of synaptic terminals responding to KCl was always considered as 100% of the total functional synaptosomal population. Finally, synaptosomes were fixed and labelled with antibodies that recognise specifically the vesicular protein synaptophysin and the GABA\(_B\) receptor (Fig. 3D-E).

Terminals responding to 100 \(\mu\)M Ap5A represented 26.3 ± 3.3% of the total functional synaptosomal population (2275 synaptosomes from six fields). As expected for the widespread distribution of GABA\(_B\) receptors in our presynaptic model, 69.2 ± 10.8% of synaptic terminals responding to Ap5A were labelled with the anti-GABA\(_B\) antibody (Fig. 3E). This value is close to the distribution of GABA\(_B\) receptors in the total population of synaptosomes (62.1 ± 6.9%). In addition, 25.4 ± 5.2% of the total terminals expressing GABA\(_B\) receptors responded to Ap5A application, which is similar to the percentage of distribution shown for Ap5A calcium responses on the whole synaptosomal population (26.3 ± 3.3%). Both coincidences suggest that GABA\(_B\) receptors are not preferably distributed on Ap5A responding terminals from rat midbrain.

Presynaptic GABA\(_B\) receptor activation potentiates Ap5A and Gp5G calcium responses in rat midbrain nerve terminals

In order to corroborate the modulatory role of GABA\(_B\) receptors on Ap5A calcium responses, a concentration-response curve for the dinucleotide in the presence of 100 \(\mu\)M baclofen was performed. As a control, a concentration-response curve for Ap5A was obtained in the absence of additional compounds. The curve observed was monophasic with an EC\(_{50}\) value of 43.6 ± 7.0 \(\mu\)M and a maximal calcium increase of 28.1 ± 2.6 nM, which corresponds to 100% of the control maximal effect (Fig. 4A). When synaptosomes were preincubated with
100 µM baclofen, a substantial change in the concentration-response curve for Ap5A (from $10^{-14}$ to $10^{-4}$ M) was observed, since lower concentrations of Ap5A, ineffective in the absence of the GABA_B agonist, provoked a significant effect in its presence. Moreover, a biphasic concentration-response curve was obtained with a high affinity component in the picomolar range and a low affinity component in the micromolar range (Fig. 4A). The low potency component showed an increase of $52.0 \pm 6.2\%$ above the control curve value, changing the maximal Ap5A-induced calcium increment from $28.1 \pm 2.6$ to $42.7 \pm 3.4$ nM. These results obtained with baclofen, a selective agonist of GABA_B receptors, strongly support the involvement of these receptors in the potentiation of Ap5A calcium responses.

In control experiments, diguanosine polyphosphates (Gp_nG) appears to be as good agonist as Ap_nA acting through presynaptic specific dinucleotide receptors in rat midbrain synaptic terminals (Pintor et al., 2000). To determine whether baclofen could enhance diguanosine polyphosphates effect, Gp_5G was assayed. The control curve obtained was monophasic with an EC$_{50}$ value of $20.8 \pm 3.1$ µM and a maximal calcium increase of $32.3 \pm 3.2$ nM, which corresponds to 100% of the control maximal effect (Fig. 4B). Moreover, the preincubation of synaptosomes with 100 µM baclofen also induced the appearance of a biphasic Gp_5G concentration-response curve (Fig. 4B). It is to notice that Hill values observed for the high potency component of Ap5A or Gp_5G concentration-response curves were < 1, whereas Hill numbers obtained for the low potency component were always > 1. However, the biphasic curve for Gp_5G differed from the two-stepped Ap5A concentration-response curve in both, the maximal calcium responses and the affinity of the high potency step. Table 1 provides the EC$_{50}$ values, maximal responses and Hill numbers obtained in each experimental condition.

**Effect of baclofen on Ap5A induced-intrasynaptosomal Ca$^{2+}$ concentrations determined in single synaptic terminals**

After analysing the potentiatory effect of baclofen in synaptosomal populations, we sought to study the effect of GABA_B receptor activation on Ap5A calcium responses in single synaptic terminals from rat midbrain. To carry on this assay, synaptosomes were tested for their ability to mobilise Ca$^{2+}$ after 100 nM Ap5A superfusion in the absence or presence of 100 µM baclofen. The Ap5A concentration 100 nM was chosen since in synaptosomal populations it induced no calcium response in the absence of baclofen but evoked a significant effect when synaptosomes were preincubated with the GABA_B agonist (Fig. 4A).
Thus, synaptic terminals mounted in a superfusion chamber, were first challenged with 100 nM Ap5A, and then superfused with 100 µM baclofen for 2 min. Successive stimulation was achieved with 100 nM Ap5A in the presence of baclofen immediately followed by 100 µM Ap5A. As usually, a pulse of 30 mM KCl was applied at the end of each experiment.

The analysis of roughly 2450 synaptosomes from six fields showed that four different populations could be identified according to their response to Ap5A. The first group was formed by a significant percentage of synaptosomes that failed to respond to 100 nM Ap5A in the absence of baclofen, but responded to this stimulus in its presence (Fig. 5A). This group represented 16.8 ± 2.9% of the synaptic terminals responding to 30 mM K+, which were always considered as 100% of the total functional synaptosomal population. A second synaptosomal population, accounting for 9.5 ± 3.9% of the total, showed no response to 100 nM Ap5A in the presence of baclofen, only responding to 100 µM Ap5A (Fig. 5B). These results indicated that baclofen is able to modulate the effect of low Ap5A concentrations in approximately 64% of the synaptosomes responding to high, but not to low, dinucleotide concentrations. It is no notice the presence of a third group of synaptic terminals that showed an intense response to low Ap5A concentrations (100 nM) in the absence of baclofen but did not enhance calcium entrance when further stimulated with Ap5A (Fig. 5C). This group represented 16.1 ± 3.1% of the total rat midbrain synaptic terminals. Finally, the fourth population corresponded to synaptic terminals that do not respond to Ap5A (data not shown).

*The potentiatory effect of baclofen on Ap5A calcium responses is mediated by PKA inhibition*

In order to corroborate the involvement of GABA$_B$ receptors in the potentiation of Ap$_5$A calcium responses, we analysed the effect of 100 µM saclofen, a GABA$_B$ receptor antagonist. This compound blocked the potentiatory effect mediated by baclofen, producing an approximate calcium increase of 24.2 nM that corresponds to a percentage of 86.0 ± 12.3% when compared to the control value (Fig. 6). The incubation of the synaptosomal preparation with the GABA-uptake inhibitor SKF 89976A (10 µM), which increase the extrasynaptosomal GABA concentration, was able to enhance the Ap$_5$A calcium response to 36.2 nM, corresponding to 28.9 ± 7.7% over the control response (Fig. 6).

Previous results have shown that the activation of GABA$_B$ receptors by baclofen mediates a decrease in intracellular cAMP levels in a variety of cell systems, including rat midbrain synaptic terminals (Gómez-Villafuertes et al., 2003). In this way, we adopted two experimental approaches to analyse the involvement of PKA in modulating the Ap$_5$A...
receptors in our model: one was designed to activate and the other to inhibit the activity of this enzyme (Fig. 6). PKA activation was indirectly induced using 10 µM forskolin and directly achieved with 100 µM DiBucAMP. As described for saclofen, preincubation of synaptosomes with forskolin or DiBucAMP completely inhibited the potentiatory effect mediated by baclofen, producing calcium increases of 24.5 nM and 26.1 nM respectively, which correspond to values of 87.2 ± 8.5% and 93.1 ± 10.9% when compared to the control (100%). PKA-IP (25 nM) was also assayed to check whether this kinase was implicated in the potentiation observed. This inhibitory peptide enhanced by its own the A_{p5}A calcium response to 38.6 nM, corresponding to an increase of 37.5 ± 6.1% over the control value. Collectively, these findings are consistent with an inhibitory action of PKA on dinucleotide receptors.
Discussion

Presynaptic terminals have a large variety of receptors, including G-protein coupled receptors and multisubunit ion channels, which can be activated by the transmitters released from the same or adjacent terminals. There are many reports of presynaptic G\(_i/G_0\)-coupled receptors able to modulate the activity of ion channels, including metabotropic GABA\(_B\) receptors (Boehm and Kubista, 2002). GABA\(_B\) receptor activation decreases neurotransmitter release at GABAergic and other synapses via the inhibition of Ca\(^{2+}\) conductance, presumably at N-type channels (Takahashi et al., 1998; Filippov et al., 2000). The present work provides the first demonstration of a presynaptic interaction between GABA\(_B\) receptors and ligand-gated calcium channels activated by dinucleotides. This result is supported by the broad distribution of metabotropic GABA\(_B\) receptors in synaptic terminals, in agreement with previous studies (Billinton et al., 2001; Möhler et al., 2001). On the contrary, synaptosomes did not show positive immunostaining against α3 subunit, which is among the most strongly expressed GABA\(_A\) receptor subunit in the CNS (Benke et al., 1991), and muscimol has no effect on Ap\(_5\)A calcium responses. Indeed, a presynaptic localisation of GABA\(_A\) receptors has been only reported in rat suprachiasmatic nucleus (Belenky et al., 2003).

The relevance of GABA\(_B\) receptors on Ap\(_5\)A calcium responses can be inferred from the abundance of single terminals that respond to Ap\(_5\)A and exhibit positive immunostaining against GABA\(_B\) receptors. Besides, GABA\(_B\) receptors activation in synaptosomal populations results in a dramatic change on the Ap\(_5\)A concentration-response curve. Thus, the initially sigmoidal curve, with micromolar EC\(_{50}\) value, becomes biphasic with two saturation steps and EC\(_{50}\) values in the picomolar and micromolar range. Moreover, maximal calcium response induced by Ap\(_5\)A is significantly increased when compared to the control. The two-stepped curve could be analysed taking into account that about 2/3 of the terminals responding to Ap\(_5\)A contain GABA\(_B\) receptors. In this population, the pre-stimulation with baclofen induces an increase in the affinity and maximal calcium response of the dinucleotide receptor. The remaining population (approximately 1/3 of the total) keeps the dinucleotide receptor in its low affinity state, which also corresponds to 1/3 of the Ap\(_5\)A control curve.

Recently, it has been described that diguanosine polyphosphates are, together with diadenosine polyphosphates, natural occurring substances in chromaffin granules (Jankowski et al., 2003). The present work shows that, as occurs with Ap\(_5\)A, GABA\(_B\) receptors are also capable to modulate Gp\(_5\)G calcium responses in synaptosomal populations, inducing the appearance of a two-stepped concentration-response curve. Since P2X are not activated by
GTP or GDP (Pintor et al., 2000), the existence of specific dinucleotide receptors equally sensitive to both adenine and guanine dinucleotides is required. The high potency step of Gp5G curve shows a significant reduction in the affinity and maximal calcium response when compared to the biphasic curve obtained for Ap5A. Although these data could be analysed under the scope of the existence of various dinucleotide receptors, it is also possible that in control situation Ap5A and Gp5G behave as full agonists and induce similar effects on the dinucleotide receptor, but after GABA modulation they do not keep that behaviour, being Gp5G a partial agonist with lower affinity than Ap5A. It is to notice that the two steps of Ap5A or Gp5G concentration-response curves exhibit cooperativity. The negative cooperativity showed by the high potency component (nH<1) indicates that the binding of one agonist molecule decreases the intrinsic affinity of another vacant binding site. This behaviour results in a broad concentration range around the EC50 value before reaching the maximal response and could be a way to control dinucleotides-induced presynaptic events. It is also possible that the low Hill slopes reveal the appearance of additional binding sites or affinity states of the dinucleotide receptor when GABA_B receptors are activated. Concerning the low potency component of the curves, micromolar dinucleotide concentrations, which probably are only reached in the borderline of physiological/pathological conditions, are required. In this situation, a positive cooperativity (nH>1) could be a way to face excitotoxic events in neurones.

Microfluorimetric experiments carried out on single synaptic terminals confirm that baclofen is able to potentiate the effect of low Ap5A concentrations in approximately 64% of the synaptosomes responding to high, but not to low, dinucleotide concentrations. This value is closely related to the widespread distribution of GABA_B receptors in synaptic terminals (62%), as well as to the percentage of synaptic terminals responding to Ap5A and labelled with the anti-GABA_B antibody (69%). In addition, 25% of the terminals expressing GABA_B receptors responded to Ap5A application, which is similar to the percentage of distribution shown for Ap5A calcium responses on the whole synaptosomal population (26%). Both coincidences indicate that GABA_B receptors are not preferably distributed on Ap5A responding terminals from rat midbrain. As reported previously in synaptosomal populations, these results do not exclude the presence of more than one type of receptor responding to Ap5A, but the biphasic curves obtained could be explained by the presence or absence of GABA_B and dinucleotide receptors in the same terminal (Fig. 7). The modulation of dinucleotide receptor activity by presynaptic G-protein coupled receptors has also been described for adenosine A1 and A2A receptors, both producing the appearance of biphasic
concentration-response curves for Ap$_5$A when activated (Díaz-Hernández et al., 2002a). It is to notice that microfluorimetric studies on single synaptic terminals require a continuous superfusion with fresh buffer, which takes out from the synaptosomes surrounding area a large variety of compounds able to negatively modulate presynaptic receptors. This situation can explain that, even in the absence of baclofen, the application of 100 nM Ap$_5$A induces an increase in the [Ca$^{2+}$]$_i$ in a significant percentage of synaptosomes. This type of receptor activated by Ap$_5$A, which is not susceptible of study in synaptosomal populations, has been reported here for the first time and will require a deeper functional approach in the future.

Direct phosphorylation processes have been proposed as a regulatory mechanism for different ionotropic neurotransmitter receptors such as nicotinic, GABA$_A$, NMDA, AMPA, P2X and dinucleotide receptors (Swope et al., 1999; Chen and Bobin, 1998; Paukert et al., 2001; Pintor et al., 1997b). GABA$_B$ receptors are negatively coupled to adenylate cyclase (AC), decreasing intracellular cAMP levels when activated. Previous studies in synaptic terminals have demonstrated that the activation of presynaptic GABA$_B$ receptors potentiates ATP responses acting through P2X receptors and blocks forskolin-stimulated cAMP formation (Gómez-Villafuertes et al., 2003). Based on these results, it seems reasonable to suggest that a decreased cAMP concentration reduces PKA activity, leading to a lower degree of phosphorylation of the dinucleotide receptor that could modify its affinity for Ap$_5$A. It is to notice that the dinucleotide receptor has not been cloned yet, but ionotropic nucleotide receptors, such as P2X$_2$, contain phosphorylation sites for PKA in the C-terminal region (Chow and Wang, 1998; Chen and Bobbin, 1998). To verify the phosphorylation hypothesis, we analysed the effect of activators or inhibitors of the AC-PKA cascade on the potentiation of Ap$_5$A responses induced by GABA. Thus, PKA activation significantly inhibits the potentiation induced by baclofen, whereas inhibition of PKA mimics the effect of baclofen on the Ap$_5$A-induced Ca$^{2+}$ responses. The inhibition of GABA uptake inhibitor enhances the Ca$^{2+}$ influx evoked by Ap$_5$A, indicating that endogenous GABA is able to provoke the potentiation observed. This potentiation is dependent on GABA$_B$ receptor activation, since the antagonist saclofen causes a return to control values.

These studies expand on the variety of interactions that occur between GABAergic and other neurotransmitter systems. Recently, it has been proposed that GABA$_B$ receptors are playing an important role in pain perception modulation at the cerebral cortex level (Jasmin et al., 2003). Moreover, GABAergic pathways and interneurones are highly abundant in the midbrain and have been related to the physiological control of sleep induction in the anterior hypothalamus and voluntary movement in basal ganglia (Kim, 1997; Kriem et al., 1998). The
mentioned physiological effects carried out by GABA_B receptors, are postulated to occur mainly via G_i/G_0 protein through the modulation of voltage operated Ca^{2+} and K^+ channels, which exhibit a broad presence in neural cells (Filippov et al., 2000; Billinton et al., 2001). The relevance of the findings here reported relies on the effect of GABA_B receptor activation on other variety of ion channels, but this time being ligand-operated Ca^{2+}/Na^+ channels that exhibit a much more restricted distribution. It is particularly relevant the location of ionotropic dinucleotide receptors in aminergic terminals from rat basal ganglia and in midbrain cholinergic and GABAergic terminals (Giraldez et al., 2001; Díaz-Hernández et al., 2002b; Gómez-Villafuertes et al., 2001). It is to notice that in brain perfusates from neostriatum of conscious rat submitted to strong brain stimulation, the extracellular amounts of dinucleotides increases dramatically, reaching nanomolar concentrations (Pintor et al., 1995). The longer half-life of dinucleotides compared to ATP made these compounds to be able to exert their effects in more distant areas, inducing presynaptic stimulation when GABA_B receptors are activated (Mateo et al., 1997). It is too early to fully understand the physio-pathological implications of these findings, but they should be taken into account when considering the modulatory actions of GABA_B receptors and the regulation of presynaptic ligand-gated ion channels.
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References


Footnotes

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Legends for figures

Fig. 1: Effects of GABA receptor agonists on calcium responses induced by 100 µM Ap5A. (A) Single experimental traces of the effect of Ap5A in the absence (upper trace) and presence of GABA (second trace), baclofen (third trace) or muscimol (lower trace), all added at 100 µM. The traces presented in the figure represent three determinations performed in triplicate with equivalent results. (B) Determination of the effects of baclofen and muscimol on Ap5A-induced calcium at different incubation periods. Each bar represents the mean ± SEM of three determinations performed in triplicate. *p<0.05 vs. control; ** p <0.01 vs. control.

Fig. 2: Co-localisation of synaptophysin and GABA receptors in nerve terminals from rat midbrain. Synaptic terminals were fixed onto poly-L-lysine-coated coverslips and double-stained using antibodies against synaptophysin (A and D) and GABA A receptor α3 subunit (B) or GABA B receptor (E). Merged panels are shown in C and F, respectively. Antibody localisation was visualised with fluorescein filters for synaptophysin and rhodamine filter for GABA receptors. Scale bar, 5µm.

Fig. 3: Responses to 100 µM Ap5A in single nerve terminals from rat midbrain containing GABA B receptors. Synaptic terminals glued onto a microgrid coverslip mounted in a superfusion chamber were loaded with Fura-2 AM to follow the changes [Ca 2+]i induced by stimulation with 100 µM Ap5A. After these studies, synaptosomes were fixed in 4% PFA and labelled with anti-GABA B receptor and anti-synaptophysin antibodies. (A) Phase contrast image of synaptosomes where a detail of the grid is shown. (B) Fluorescence image of the same field reveals synaptosomes challenged with the calcium dye Fura-2. (C) Time course of Fura-2 intrasynaptosomal calcium transients recorded for the terminal labelled with an arrow in B, D and E. (D) Same field viewed with fluorescein optics reveals anti-synaptophysin-FICTC immunolabelling. (E) Rhodamine optics shows anti-GABA B-TRITC-immunostained synaptosomes. Scale bar, 5 µm.

Fig. 4: Concentration-response curves for Ap5A and Gp5G in the presence of baclofen. Before the addition of Ap5A (A) or Gp5G (B), baclofen was preincubated at a concentration of 100 µM for 2 min as described in “Methods” section. The 100% effect corresponds to the maximal calcium transient elicited by 100 µM Ap5A or Gp5G in the absence of any other compound. Values are the mean ± SEM of at least four experiments performed in triplicate (n=4-8).
Fig. 5: Potentiation of Ap₅A calcium responses by baclofen in single synaptosomes from rat midbrain. Synaptic terminals glued into a coverslip mounted in a superfusion chamber were loaded with Fura-2 AM to monitor the changes in intrasynaptosomal Ca²⁺ concentration induced by 100 nM and 100 µM Ap₅A, in the absence and presence of 100 µM baclofen. Representative traces of the intrasynaptosomal calcium transient time course are shown. Bars indicate the time of application of each substance.

Fig. 6: Modulation Ap₅A-evoked Ca²⁺ responses by PKA and GABA_B receptor effectors in rat midbrain synaptic terminals. The GABA_B receptor antagonist saclofen (100 µM) inhibited the potentiation evoked by baclofen on the Ap₅A calcium response. The specific GABA-uptake inhibitor SKF-89976A (10 µM) caused a significant enhancement of the Ca²⁺ response elicited by Ap₅A. The preincubation of synaptosomes for 2 min with the adenylate cyclase activator forskolin (10 µM) and the PKA activator DiBucAMP (100 µM) significantly blocked the potentiation induced by baclofen on the calcium response elicited by Ap₅A. PKA-IP on its own enhanced the response induced by Ap₅A. Each bar represents the mean ± SEM of at least three determinations performed in triplicate (n=3-6). *p<0.05 vs. control; ** p<0.01 vs. control; †† p<0.01 vs. baclofen.

Fig. 7: Schematic diagram of synaptic terminals exhibiting dinucleotide receptors. Diadenosine polyphosphates, GABA and other neurotransmitter are stored within secretory vesicles and are released to the extracellular medium, activating presynaptic receptors. Extrasynaptic or non-exocytotic release of Ap₅A and GABA is also possible. (A) In nerve terminals coexpressing GABA_B and dinucleotide receptors, GABA is able to facilitate Ap₅A calcium signalling increasing the maximal calcium entry and the affinity of dinucleotide receptors. (B) In synaptic terminals that do not co-express both receptors, dinucleotide receptors are in a state of low affinity for Ap₅A and the calcium influx through the channel is reduced. The absence of GABA_B receptors in this synaptosomal population does not exclude the existence of other G_i/G_o-protein coupled receptors, such as A₁ or A₂A adenosine receptors, able to modulate Ap₅A calcium responses. Nts; neurotransmitters.
Table 1.- EC\textsubscript{50} values and Hill numbers for Ap\textsubscript{5}A and Gp\textsubscript{5}G concentration-response curves in the absence or presence of baclofen.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Ap\textsubscript{5}A</th>
<th></th>
<th>Gp\textsubscript{5}G</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Baclofen</td>
<td>Control</td>
<td>Baclofen</td>
</tr>
<tr>
<td>EC\textsubscript{50} high affinity component</td>
<td>------</td>
<td>77.30 ± 6.03 pM</td>
<td>------</td>
<td>326.0 ± 16.4 pM</td>
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<tr>
<td>EC\textsubscript{50} low affinity component</td>
<td>43.57 ± 7.04 µM</td>
<td>16.51 ± 1.66 µM</td>
<td>20.78 ± 3.09 µM</td>
<td>19.09 ± 2.52 µM</td>
</tr>
<tr>
<td>Maximal response (% Control effect)</td>
<td>100</td>
<td>152.03</td>
<td>100</td>
<td>130.56</td>
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<tr>
<td>n\textsubscript{H} high potency component</td>
<td>------</td>
<td>0.40 ± 0.13</td>
<td>------</td>
<td>0.40 ± 0.08</td>
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<tr>
<td>n\textsubscript{H} low potency component</td>
<td>1.60 ± 0.40</td>
<td>2.11 ± 0.39</td>
<td>2.31 ± 0.69</td>
<td>3.03 ± 0.33</td>
</tr>
</tbody>
</table>

EC\textsubscript{50} values and Hill numbers for Ap\textsubscript{5}A and Gp\textsubscript{5}G concentration-response curves obtained in the presence of baclofen. Data are expressed as the EC\textsubscript{50} values (concentration of agonist producing 50% of the maximum response) and maximal responses induced by Ap\textsubscript{5}A and Gp\textsubscript{5}G in the different experimental conditions. n\textsubscript{H} is the Hill coefficient. Data represent mean ± SD (n=3-8).
Figure 1

A

ΔCa$^{2+}$
30 nM

Control

GABA

Baclofen

Muscimol

Ap$_5$A 100 µM (preinc. 2 min)

Time (25 sec/div)

B

Baclofen 100 µM

Muscimol 100 µM

Ca$^{2+}$ response induced by Ap$_5$A 100 µM (%)

Preincubation Time

Control 30 sec 2 min 4 min 6 min

Figure 1
Figure 2
Figure 3
Figure 4

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A

B

\( \Delta Ca^{2+} \) induced by Ap5A

% control 100 \(\mu\)M

\[ [\text{Ap5A}] \ M \]

\( 10^{-15} \ 10^{-13} \ 10^{-11} \ 10^{-9} \ 10^{-7} \ 10^{-5} \ 10^{-3} \)

\( \Delta Ca^{2+} \) induced by Gp5G

% control 100 \(\mu\)M

\[ [\text{Gp5G}] \ M \]

\( 10^{-15} \ 10^{-13} \ 10^{-11} \ 10^{-9} \ 10^{-7} \ 10^{-5} \ 10^{-3} \)

Figure 4
Figure 5
Figure 6

$Ca^{2+}$ response induced by $Ap_5A$ 100 µM (%)
GABA<sub>B</sub> modulable population

GABA<sub>B</sub> non-modulable population

Figure 7