Neurochem Res
DOI 10.1007/s11064-006-9261-1

ORIGINAL PAPER

Phosphatidylinositol 4,5-Bisphosphate Induced Flunitrazepam Sensitive-GABA$_A$ Receptor Increase in Synaptosomes from Chick Forebrain

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Accepted: 19 December 2006
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Abstract The flunitrazepam sensitive-GABA$_A$ receptor density was increased by cytochalasins C and D at 37°C suggesting that microfilament depolymerization induces exposure to the radioligand of a GABA$_A$ receptor in synaptosomes (Pharm Biochem Behav 72 (2002) 497). Similarly, phosphatidylinositol-4,5-bisphosphate (1–5 μM), but not a mixture of phospholipids, induced an increase of GABA$_A$ receptors in synaptosomes. Furthermore, N-ethyl maleimide, an inactivator of the sensitive fusion protein, which interacts with GABA$_A$ receptor, abolished the receptor increase induced by phosphatidylinositol-4,5-bisphosphate. Together, the results suggest that phosphatidylinositol-4,5-bisphosphate, acts via microfilament depolymerization increasing the binding of the radioligand to receptors possibly by modulation of their interaction with proteins involved in trafficking and docking mechanisms.

Keywords Phosphatidylinositol-4,5-bisphosphate · Microfilaments · GABA$_A$ receptors · Synaptosomes

Introduction

GABA is one of the most important inhibitory neurotransmitters in the CNS. It is estimated that, depending on brain region, 20–50% of all central synapses use GABA as their neurotransmitter [1]. The GABA$_A$ receptors (GABA$_A$ R) are ligand-gated chloride channels that mediate inhibitory neurotransmission. The receptors are pentameric hetero-oligomers, the subunits of which share a conserved structure that consists of a large extracellular amino-terminal, four transmembrane domains and a large intracellular loop between transmembrane domains III and IV [2]. Sixteen distinct GABA$_A$ R subunit genes have been identified to date, which are classified by sequence identity into seven subunit classes: α 1–6, β 1–3, γ 1–3, δ, ε, π and θ [3]. However, the current consensus of opinion, suggests that the majority of GABA$_A$ receptor subtypes in the brain are composed of heteropentameric assemblies of α, β and γ subunits [2, 3]. Functionally, the receptors containing α1–3 or α5 subunits represent the benzodiazepine-sensitive GABA$_A$ receptor subtypes known to mediate the diverse behavioral actions of diazepam [4].

Kneussel et al, [5], reviewed the dynamic regulation of GABA$_A$ Rs at synaptic sites. They indicated that the binding of GABA$_A$ R associate protein (GABARAP) with NSF (N-ethylmaleimide-sensitive factor) and SNARE (soluble NSF-attachment protein receptor) proteins, microtubules and gephyrin together with its localization at intracellular membranes suggested a role of GABA$_A$ R targeting and/or degradation.

Furthermore, regulation of GABA$_A$ R trafficking [6, 7] has been shown that GABA$_A$ Rs interact via receptor β subunits directly with NSF, a critical regulator of vesicular dependent protein trafficking and of receptor insertion into the plasma membrane, as measured by in vitro protein binding and co-immunoprecipitation assays [8].

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One major phosphoinositide, $PI(4,5)P_2$, had originally been associated with the generation of two second messengers. However, additional functions were later found, such as their roles in cytoskeletal reorganization and in membrane trafficking [8]. In response to extracellular stimuli of cells, activated events take place, such as the uncapping of the barbed end of actin filaments, causing an increase in local $PI(4,5)P_2$, the release of actin filament from focal adhesions and de novo actin polymerization, resulting in the organization of the filaments and ultimately initiating cell movement [9]. In this system, but not for postsynapsis, it was shown that PI(4,5)P2 provides signals for targeting vesicles to specific fusion sites.

In a different system from the above mentioned, the $GABA_A$ R density was measured in synaptosomes from chick forebrain. When both microfilament depolymerizating agents, cytochalasins C and D, were added in this system increased the $GABA_A$ R density at 37°C, suggesting that cytochalasins induced an increment of the flunitrazepam sensitive-$GABA_A$ R into synaptosomes [10]. [^{3}H]-Flunitrazepam is accessible to intracellular membranes of neurons, where it binds to sites on $GABA_A$ R in addition to those on cell surface [11]. However, the postsynapsis in synaptosomes is a small bit of the neuronal soma [12].

The aim of the present work was to investigate if $PI(4,5)P_2$, an agent that modulates the degree of polymerization of microfilaments, is able to induce changes in the ^{3}[H]-flunitrazepam sensitive-$GABA_A$ Rs density when it is added exogenously to synaptosomes from chick forebrain.

Experimental procedure

Drugs

[^{3}H]-flunitrazepam ([^{3}H]-FNZ) (85 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA), diazepam was purchased from Hoffmann LaRoche, Switzerland. Phospholipids purified from bovine brain were purchased from Sigma Chemical Co, St Louis MO, USA (Brain extract type I; total diphosphoinositide and triphosphoinositide content 20–40%; the remainder being a mixture of phosphatidylinositol and phosphatidylserine). L-α-Phosphatidyl-D-myo inositol-4,5-bisphosphate was obtained from Roche Molecular Biochemicals. N-ethylmaleimide (NEM) was purchased from ICN, Ohio, USA. Other drugs and solvents were of analytical grade.

Animals

Chicks (*Gallus gallus domesticus*) of both sexes were obtained immediately after hatching from a commercial hatchery INDACOR (Argentina) when they were only a few hours old. On their arrival in the laboratory, chicks housed in a white wooden box that measured $90 \times 40 \times 60$ cm (length × width × height). All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee of the Universidad Nacional de Córdoba, and efforts were made to minimize animal suffering and to reduce the number of animals used.

Rearing of the chicks

The box was illuminated with a bright lamp (60 W) hanging just above it. Tap water and food were freely available. The box was kept in a small room (3×3 m) at constant temperature and humidity, in a 12–12 h dark-light cycle (lights on at 7 a.m.). These chicks were socially reared until they reached 10 days of age. Daily food replenishment (Cargill, broiler BB, and 20% minimum crude protein 12.34 MJ/kg) and maintenance chores were performed at 9 a.m.

Preparation of crude synaptosomal fraction

All chicks from a brooder were removed and immediately sacrificed by a procedure of decapitation lasting less than 5 s. Then, the brains were removed and forebrains quickly dissected on ice. The crude synaptosomal fraction was essentially obtained as described [12]. All the procedures were carried out at 4°C. Briefly, the tissue was homogenized in 20 volumes of ice-cold 0.32 M sucrose/g original tissue, using a Potter glass-Teflon homogenizer and centrifuged at $1{,}000 \times g$ for 10 min. The supernatant was then centrifuged at $10{,}000 \times g$ for 20 min. Then, the pellets were resuspended in a solution containing 50 mM Tris-HCl buffer pH 7.4, obtaining a final concentration of 0.3 mg proteins/ml [13], and were immediately used for the receptor assay.

[^{3}H]-flunitrazepam binding assay

The specific binding of [^{3}H]-FNZ (85 Ci/mmol,) was measured by a filtration technique. Binding was carried out in the presence of radioligand at final concentrations of 3, 6, 9, 18, 24, 30, 36 and 50 nM, at 37°C. Each assay was performed in triplicate using 1 ml aliquots containing 0.3 mg of proteins from the synaptosomal fraction. Non-specific binding was measured in the presence of

10 μM diazepam. After 15 min incubation, samples were filtered under vacuum through Whatman GF/B filters using a Brandel M-24 filtering manifold. Samples were washed three times with 4 ml of ice-cold Tris-HCl buffer (50 mM, pH 7.4) and the radioactivity was counted in an LKB-1214-RackBeta counter at 60% efficiency. The values Kd and Bmax were obtained by nonlinear regression using the equation for hyperbola (one binding site): $Y = \text{Bmax}*X/(\text{Kd} + X)$, where Bmax is the maximal binding, and Kd is the concentration of ligand required to reach half-maximal binding.

Treatment of crude synaptosomal fraction with phosphatidylinositol-4,5-bisphosphate or a mixture of Phospholipids

$PI(4,5)P_2$ and phospholipids (PLs) were added to a crude synaptosomal fraction obtained from chick forebrain in order to study their effect on the $GABA_AR$ density. PLs were a mixture of phospholipids purified from Bovine Brain. $PI(4,5)P_2$ was assayed at several final concentrations (0.25, 0.50, 1, 1.50, 2, 2.50, 3 and 5 μM) during the receptor assay. The mixture of PLs (control) was assayed at a final concentration of 2.70 μg/ml, being equal in weight to the $PI(4,5)P_2$, which in this case corresponded to 2.50 μM. PL mixture and $PI(4,5)P_2$ were resuspended as reported [14]. The phospholipids were dissolved in chloroform, and then the solvent was slowly evaporated by a stream of nitrogen gas. Then, both were resuspended in buffer (50 mM Tris-HCl, pH 7.4) by sonication at room temperature, using a bath sonicator for 15 min until the solution became visibly clear. Since exogenously added phosphoinositides vesicles and micelles are known to fuse with the plasma membrane of live cells [15], the crude synaptosomal fraction was incubated with $PI(4,5)P_2$ or the mixture of PLs for 15 min at 37°C, and then the receptor assay was performed.

Treatment of crude synaptosomal fraction with N-ethylmaleimide in the presence of $PI(4,5)P_2$ at 2.50 μM concentration

Crude synaptosomal fraction was treated with $PI(4,5)P_2$ and incubated as described above, but using 1 mM of NEM from a 50 mM stock solution in H_2O for 5 min on ice, as described for neuronal cultures [16]. This treatment inactivates the NSF.

Statistic

Data of Bmax and Kd values for $GABA_A$ R density were analyzed by one-way analysis of variance (ANOVA). Whenever ANOVA indicated significant effects ($P < 0.05$), a pairwise comparison of means by Newman Keuls test was carried out.

Results

Increase of $GABA_A$ R density in synaptosomes as a function of the $PI(4,5)P_2$ concentration

Figure 1 shows the Bmax values of $GABA_A$ Rs in synaptosomes from chick forebrain as a function of the $PI(4,5)P_2$ concentration. One-way ANOVA revealed a significant effect (F (8,47) = 3,0157, $P < 0.008$) of different concentrations of $PI(4,5)P_2$ (0.25–5.00 μM) on Bmax values. The Newman–Keuls test showed that Bmax increased from 1,356 fmoles/mg protein in the absence of phospholipids (buffer only) to 1,661 fmoles/mg protein (22%, $P < 0.011$) for 1 μM; to 1,535 fmoles/mg protein (13%, $P < 0.019$) for 1.5 μM; to 1,677 fmoles/mg protein (24%, $P < 0.007$) for 2.0, μM; to 1,587 fmoles/mg protein (17%, $P < 0.001$) for 2.5 μM; to 1,572 fmoles/mg protein (16%, $P < 0.005$) for 3 μM; and to 1,533 fmoles/mg protein (13%, $P < 0.045$) for 5.0 μM concentration. The non-specific values were similar, both in the presence or in the absence of PLs or $PI(4,5)P_2$ (data not shown). No significant differences in Kd values were observed (F(8,47) = 1.753, $P = 0.12$) (Table 1).

Effect of NEM on the 2.5 μM $PI(4,5)P_2$-induced increase of $GABA_A$ R density in synaptosomes

As shown in Table 2, a one-way ANOVA of the Bmax values revealed a significant $PI(4,5)P_2$ effect

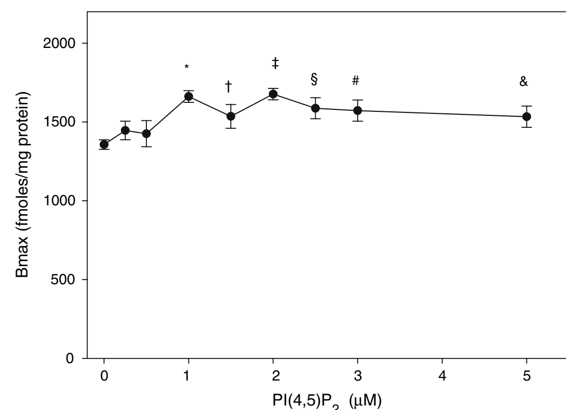

Fig. 1 The $GABA_AR$ density into synaptosomes from chicks as a function of the $PI(4,5)P_2$ concentration. Values are means ± S.E.M. The number of separate experiments is n = 6–8 chicks/group. $^*P < 0.011$, $^†P < 0.019$, $^‡P < 0.007$, $^§P < 0.001$, $^\#P < 0.005$, $^\&P < 0.045$ compared with control (without $PI(4,5)P_2$) (Newman–Keuls test)

Table 1 Effects of different concentrations of $PI(4,5)P_2$ on the Kd values of $GABA_AR$ into synaptosomes

$PI(4,5)P_2$ concentrations (μM)	Kd (nM)	% of control
0.00 (buffer)	10.16 ± 0.4	–
0.25	9.94 ± 0.6	–2
0.50	9.52 ± 0.7	–6
1.00	11.01 ± 0.5	8
1.50	10.69 ± 0.3	5
2.00	10.97 ± 0.2	8
2.50	11.29 ± 0.4	11
3.00	10.19 ± 0.3	1
5.00	10.03 ± 0.4	–1

Each value of Kd represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. The number of separate experiments is n = 6–8 chicks/group

(F (3,22) = 4.3442, $P < 0.01$). $PI(4,5)P_2$ induced an increase of 17% ($P < 0.01$) from 1,356 fmol/mg protein to 1,587 fmol/mg protein. The simultaneous treatment of NEM (1 mM) plus 2.5 μM $PI(4,5)P_2$ attenuated the $PI(4,5)P_2$-induced $GABA_A$ receptor increase ($P <$ 0.01) from 1,587 fmol/mg protein to 1,384 fmol/mg protein. The PLs mixture had no effect on $GABA_A$ receptor density (P = 0.79 vs control group). No significant differences in Kd values were observed (F(3,22) = 2.2916, P = 0.11) (Newman–Keuls test) (Table 2).

Discussion

In the present report, the central benzodiazepine receptor density was used to express the $GABA_A$ Rs density, due to the fact that the flunitrazepam-binding site is located in the subunit α of $GABA_A$ R [17]. To study the trafficking and the insertion of receptors in the membrane experiments, cell-biology techniques in a culture system are usually performed. However, it is of our interest to study the last steps of trafficking and docking of $GABA_A$ Rs into synaptosomes. Since a previous report [7, 18] indicated that $PI(4,5)P_2$ acts as a modulating agent in the degree of polymerization of microfilaments, we investigated whether this exogenously added phospholipid could induce a change in the $GABA_A$ Rs density in synaptosomes in a similar way to cytochalasins C and D described elsewhere [11]. Synaptosomes are fortuitous artifacts created by the pinching-off and self-selling of the synaptic contact between two nerve cells during homogenization of brain tissue [12]. Furthermore, the synaptosome is the simplest preparation that possesses all machinery for synaptic vesicle exocytosis and endocytosis [19] and contains cytoskeleton and organelles in addition to synaptic vesicles with neurotransmitters and receptors for reuptake at the presynaptic zone [20]. Furthermore, synaptic $GABA_A$ Rs are mainly localized in the postsynapsis of synaptosomes which is a small bit of the neuronal soma [12].

Figure 1 shows that $PI(4,5)P_2$ was able to induce a higher density of $GABA_A$ Rs into synaptosomes, when incubated at concentrations ranging, from 1 to 5 μM. At these concentrations assayed, the aqueous dispersions of $PI(4,5)P_2$ form small micelles, which can rapidly diffuse to the inner membrane of the crude synaptosomal fraction [15]. The micelles are of small relative molecular mass (82–93,000), in contrast to the large bilayer vesicles characteristically formed by other phospholipids with two acyl chains [21].

Similarly, the results in Table 2 show that $PI(4,5)P_2$ at a concentration of 2.50 μM significantly increased the Bmax values of $GABA_A$ R in synaptosomes at 37°C compared to control (buffer alone) or a brain PLs mixture resuspended in buffer. Crude synaptosomes were used to study the thermodynamics of binding of FNZ and other benzodiazepines with its receptors at temperatures ranging between 4 and 35°C [22]. More recently, García et al. [23] reported an increase in Kd values, but not in Bmax, in crude synaptosomes upon temperature elevation, indicating a higher tendency of the ligand to dissociate from the receptor. Our Kd values were similar to ones described [23], however, we no observed differences in the Kd values between different conditions of receptor assay (Tables 1 and 2).

Table 2 Effects of PLs mixture, 2.5 μM $PI(4,5)P_2$ and $PI(4,5)P_2$ + NEM on the Bmax and Kd values of $GABA_AR$ into synaptosomes

	Bmax (fmol/mg protein)	Increase (%)	Kd (nM)	Increase (%)
Control (buffer)	1,356 ± 28 (8)	–	10.16 ± 0.4	–
PLs mixture	1,375 ± 47 (6)	1	10.99 ± 0.2	8
$PI(4,5)P_2$	1,587 ± 67 (6)*	17	11.29 ± 0.3	11
$PI(4,5)P_2$ + NEM	1,384 ± 64 (6)	2	10.55 ± 0.3	4

Each value of Bmax or Kd represents the mean ± SEM of values obtained by non-linear regression of experimental data from saturation curves. The number of separate experiments is indicated in parentheses. *$P < 0.01$, $P < 0.02$ and $P < 0.01$ compared to Bmax in buffer, PLs mixture, and $PI(4,5)P_2$ plus NEM groups respectively (Newman–Keuls test)

Table 2 also shows that NEM, an inhibitor of the NSF [24], which co-localized with $GABA_A$ Rs [8], abolished the effect of PI(4,5)P_2 on $GABA_A$ R increase.

Together the results suggest that PI(4,5)P_2 acts via microfilament depolymerization increasing the binding of the radioligand to $GABA_A$ Rs, possibly by modulating the interaction with proteins involved in trafficking and docking mechanisms.

Acknowledgments This research was supported by grants from FONCYT, CONICET, Agencia Córdoba Ciencia and SECyT-UNC (Argentina). M.P.C. holds a research fellowship from CONICET. N.A.S. is a scientific career researcher of CONICET. We would like to thank native speaker Dr. Paul Hobson for revision of the manuscript.

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