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Effects of phosphorylation and cytoskeleton-affecting reagents on $GABA_A$ receptor recruitment into synaptosomes following acute stress

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Abstract

The [^{3}H]-flunitrazepam receptor density, measured ex vivo in synaptosomes at 4 °C, increased by about 30% because of acute stress in chicks. This increase was first reported to be a receptor recruitment due to the fact that the increase induced by subsolubilizing concentrations of Triton X-100 was not additive to the receptor increase induced by acute stress [J Neural Transm 87 (1992) 97]. In synaptosomal membranes from stressed chicks, the incorporation of alkaline phosphatase or ATP into the lumen abolished or increased, respectively, the receptor unmasking after incubation at 4 and 37 °C, suggesting that phosphorylation plays a role in the recruitment mechanism. Moreover, both colchicine and vinblastine, but not taxol, abolished the recruitment induced by stress at 37 °C only in synaptosomes, suggesting that micrutubule depolymerization plays a role in the masking of receptors. Furthermore, both cytochalasins C and D induced an increase of the receptor density, abolished by *N*-ethylmaleimide, in both the stressed and nonstressed conditions, suggesting that microfilament depolymerization induced the exposure to the radioligand of a cytosolic vesicular receptor pool, which had not fused yet with the postsynaptic membrane.

Keywords: Cytoskeleton and $GABA_A$ receptors; Stress and $GABA_A$ receptor; $GABA_A$ receptor recruitment and phosphorylation

1. Introduction

Gamma aminobutyric acid (GABA) exerts its physiological role through several GABA receptor subtypes (A, B and C) by modulating the pituitary hormone secretion, thermoregulation, ingestive behaviors and anxiety. The $GABA_A$ receptor ($GABA_AR$), also called the central BZD receptor (CBR), is the only subtype associated with the benzodiazepine (BZD) binding site (for review, see Deutsch et al., 1994).

The $GABA_ARs$ are ligand-gated chloride channels that mediate inhibitory neurotransmission. Each subunit of the pentameric receptor protein has ligand-binding sites in the amino-terminal extracellular domain and four membrane spanning regions, one of which forms the wall of the ion channel. Each subunit also has a large intracellular loop that may be a target for protein kinases (for review, see MacDonald and Olsen, 1994) and is required for subcellular targeting and membrane clustering of receptors by anchoring to the cytoskeleton (for review, see Whatley and Harris, 1996). Recently, a new cellular protein was identified, the $GABA_AR$-associated protein ($GABA_ARAP$), which can interact with the γ_2 subunit of the $GABA_ARs$, suggesting that this protein is a link between the $GABA_AR$ and microtubules (MTs) (Wang et al.,1999).

There is considerable evidence that environmental stressful conditions induce rapid changes on the CBR density, measured by the radioligand method, at 4 °C in synaptosomes obtained from the brain. However, the directions of the changes reported in rats have been inconsistent (for review, see Deutsch et al., 1994). However, it was demonstrated that the CBR density increased consistently in rats due to acute stress, and that the inconsistency mentioned above can be explained by the fact that the increase of the receptor was modulated by experience (Primus and Kellogg, 1991). Furthermore, we have reported that the CBR consistently increased in chick forebrain synaptosomes due to the stress accompanying a passive training task (Martijena and Arce, 1986, 1994), an imprinting task (Salvatierra et al., 1994), a food-discrimination task (Salvatierra et al., 1997) and an acute swimming stress (Salvatierra et al., 1994;

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Marín and Arce, 1996; Martijena et al., 1992). The CBR increase was transient, reaching a maximum value at 30 min and then disappearing at 60 min after the period of stressing (Martijena and Arce, 1986, 1994). We have also observed that the increase of the CBR induced by novelty stress in a food-discrimination task can be separated from the CBR increase induced by learning, and that both types of increase were nonrepetitive 24 h after testing the task (Salvatierra et al., 1997).

Furthermore, we have first reported that the increase in CBR density induced by stress is due to a rapid recruitment of a neurotransmitter receptor in the synaptosomal membranes (Martijena et al., 1992).The receptor increase was proposed to be a recruitment due to the fact that subsolubilizing concentrations of Triton X-100 on synaptosomal membranes induced an unmasking of hidden receptors which was not additive to the receptor increase induced by acute stress. The results suggested that stress induced a recruitment of receptors by unmasking hidden receptors and not by increasing their biosynthesis or diminishing degradation. More recently, it has been reported that insulin induces a rapid recruitment of the $GABA_AR$ in neuronal cultures (Wan et al., 1997).

In this paper, we have studied the effect of both phosphorylating and dephosphorylating reagents and the effects of cytoskeleton-affecting reagents on the CBR recruitment induced by acute stress in synaptosomes. Since the CBR is a marker of the GABAAR (Deutsch et al., 1994), the goal was to determine some aspects of the mechanism of the $GABA_AR$ recruitment induced by stress and eventually their masking or internalisation.

2. Materials and methods

2.1. Animals

Groups of 24 domestic chicks (Cobb hybrid of both sexes) were obtained immediately after hatching from a commercial hatchery INDACOR (Argentina) and housed in a white wooden box that measured $90 \times 40 \times 60$ cm (length $\times$ width $\times$ height).

2.2. Rearing of the chicks

The box was illuminated with a bright lamp (100 W) hanging immediately above it. Tap water and food were freely available. The box was kept in a small room (3×3 m) with constant temperature and humidity, in a 12/12-h. dark–light cycle (lights on at 7 a.m.). This way, chicks were socially reared until they reached 15 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. The population density increased to 16.7 kg of live weight per m^2 on Day 15, which is lower than the maximum recommended in most countries (Elwinger, 1995).

2.3. Induction of acute stress

The 15-day-old chicks were stressed as described (Martijena et al., 1992). Five chicks of the same batch were individually placed for 15 min in a basin ($22 \times 22 \times 22$ cm) which had been previously filled with clean water at 38 °C up to 15 cm deep. The birds did not show symptoms of exhaustion during this period. The chicks were sacrificed by decapitation immediately after the end of the stress time period. The forebrain hemispheres of each chick were removed and quickly dissected on ice. another five chicks of the same batch were simultaneously removed, sacrificed and the brain hemispheres dissected on ice. The tissue of each chick was used for one experiment.

2.4. Preparation of synaptosomes and synaptosomal membrane fractions

All the procedures for preparing the synaptosomes were carried out at 4 °C. The tissue was homogenised in 20 volumes of ice-cold 0.32 M sucrose/g original tissue, using a Potter glass–Teflon homogenizer and centrifuged at $1000 \times g$ for 5 min. The supernatant was then centrifuged at $20\,000 \times g$ for 20 min. The pellet was resuspended in 0.32 M sucrose and centrifuged at $20\,000 \times g$ for another 20 min to obtain a crude synaptosomal fraction. To obtain the membranes from the synaptosomal fraction, it was resuspended in 20 volumes of bidistilled water and centrifuged at $20\,000 \times g$ for 15 min to obtain a pellet (De Robertis et al., 1961). Then, the pellets were resuspended in a solution containing 50 mM Tris–HCl buffer, pH 7.4, 2 mM KCl and 5 mM $MgCl_2$, reaching a final concentration of 300 μg proteins/ml (Lowry et al., 1951). This was immediately used for the radioreceptor assay. However, when the pellets were kept at −15 °C for a week before being used, no difference was observed neither in the affinity nor in the density of the receptor compared to recently obtained pellets. In some experiments (Fig. 1a and b), the synaptosomal and membrane fractions were obtained from the tissue homogenate and resuspended as indicated above but in the presence of the following chemicals (Sigma): 80 μg/ml trypsin inhibitor from turkey egg, 2.5 mM benzamidine, 1 mM PMSF, 1 mM EDTA, 10 μg/ml pepstatin and 1 μg/ml aprotinin. No difference was observed in the results when compared to synaptosomes and synaptosomal membranes obtained in the absence of proteinase inhibitors.

2.5. Preparation of synaptosomal membranes with ATP and alkaline phosphatase

To obtain synaptosomal membranes with ATP (disodium salt, Sigma) in the lumen, the synaptosome fraction was submitted to a hyposmotic shock with 20 volumes of cold solutions containing 20, 40 and 60 mM ATP (Sigma) and 20 mM Tris–HCl buffer, pH 7.4. After the shock, the pre- and postsynaptic compartments spontaneously reseal

(De Robertis et al., 1961). To obtain the ATP solution, the nucleotide was dissolved in a NaOH (Sigma) solution concentrated enough to neutralise it in the presence of the Tris–HCl buffer.

To obtain synaptosomal membranes with alkaline phosphatase (Sigma, VII-L type) in the lumen, the synaptosome fraction was submitted to a hyposmotic shock with 20 volumes of cold solutions containing 10, 20 and 30 U/ml phosphatase and 20 mM Tris–HCl buffer, pH 7.4. In both cases (ATP or phosphatase containing synaptosomal membranes), the preparation was then centrifuged at $20\,000 \times g$ for 30 min. To obtain untreated synaptosomal membranes, the procedure was similar but the hyposmotic shock was done only with 50 mM Tris–HCl buffer. In every case, the pellets were resuspended in 50 mM Tris–HCl buffer, pH 7.4, reaching a final concentration of 300 μg proteins/ml.

2.6. Benzodiazepine radioreceptor assay

The specific binding of [^{3}H]-flunitrazepam ([^{3}H]-FNTZ) (85 Ci/mmol, New England Nuclear) was measured by a filtration technique at 4 or 37 °C. Binding was carried out in the presence of [^{3}H]-FNTZ at 0.5, 1, 2, 3, 4, 5, 8 and 9 nM final concentrations at 4 °C, or in the presence of 3, 6, 9, 18, 36 and 50 nM final concentrations at 37 °C. Each assay was performed in triplicate using 0.25-ml aliquots containing 0.25 mg of proteins from the synaptosomes or synaptosomal membranes. Nonspecific binding was measured in the presence of 10 μM diazepam (Hoffman LaRoche). After a 60-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-1219-RackBeta counter at 48% efficiency. B_{max} values were determined by a computer-aided nonlinear regression analysis of the experimental data.

2.7. Treatment of synaptosomes with MT-affecting alkaloids and microfilaments (MF)-affecting cytochalasins

Alkaloids were added to synaptosomes from stressed and nonstressed chicks during the radioreceptor assay to study their effect on the stress induced $GABA_AR$ recruitment. The alkaloids and cytochalasins were all obtained from ICN Biomedical. Colchicine and vinblastine were assayed at several final concentrations at both 4 and 37 °C during the radioreceptor assay. γ-lumicolchicine, an inactive isomer of colchicine, was assayed at 37 °C and 100 μM final concentration during the radioreceptor assay as a control for the colchicine treatment. During the radioreceptor assay, taxol (Schiff et al., 1979), cytochalasins D and C were assayed at 37 °C and at 10 μg/ml final concentration, as reported for neuronal cultures (Whatley and Harris, 1996).

Colchicine affects MT depolymerization depending on temperature and concentration. At 0 °C, colchicine does not depolymerize MTs for two reasons: (a) colchicine does not bind to tubulin at 0 °C and (b) labile MTs have already completely depolymerized at this temperature (Borisy et al., 1974). At 37 °C, colchicine binds to tubulin and depolymerizes MTs at a maximal rate. Furthermore, a short incubation with 100 μM colchicine not only inhibits the ability of disassembled tubulin to repolymerize but also depolymerizes assembled MTs (Wilson et al., 1974). Vinblastine binds to tubulin between 4 and 37 °C in a different place than colchicine but only depolymerizes MTs at 37 °C (Wilson et al., 1974). Taxol, unlike colchicine and vinblastine, binds to polymerised MTs and promotes MT assembly (Schiff et al., 1979), as well as protects them from depolymerization by cold or calcium (Wilson et al., 1974). Cytochalasins C and D bind specifically to the fast-growing end of MFs and prevent the addition of actin (Whatley and Harris, 1996).

2.8. Treatment of synaptosomes with N-ethylmaleimide (NEM)

Synaptosomes were incubated with 1 mM NEM (from a 50-mM stock solution in H_2O) for 5 min on ice, as described for neuronal cultures (Zheng and Bobich, 1998). This condition inactivates the NEM-sensitive fusion protein (NSF). Then, 2 mM glutathione was added to quench the unreacted NEM, and the incubations were allowed to stand on ice for another 5 min. In control experiments, 1 mM NEM was mixed with 2 mM glutathione before adding the synaptosomes.

2.9. Statistics

Results are expressed as the means ± S.E.M. of the B_{max} values of the CBR. Data were analysed using a two-way ANOVA followed by post hoc LSD test comparisons. A P value $\leq .05$ was considered to represent a significant difference between B_{max} values.

3. Results

3.1. Effects of ATP and phosphatase at 4 °C on the CBR density in synaptosomal membranes from nonstressed and stressed chicks

A two-way ANOVA of the B_{max} values (Table 1) revealed a significant interaction between the presence of a final concentration of 40 mM ATP and the stressed condition [$F(1,8) = 48.32$, $P < .001$]. The LSD test showed that in the absence of ATP, the B_{max} increased by about 30% ($P < 0.001$) in synaptosomal membranes from 925 fmol/mg protein in the nonstressed condition to 1226 fmol/mg protein in the stressed condition. This result confirms the reported recruitment of the CBR in synaptosomal membranes (Martijena et al., 1992). The LSD test also showed that the B_{max} increased by about 25% in the presence of ATP, in the nonstressed

Table 1
Effects of ATP and phosphatase on the CBR density in synaptosomal membranes from nonstressed and stressed chicks at 4 °C

	B_{max} (fmol/mg protein)	
Addition	Nonstressed	Stressed
–	925 ± 37 (4)	1226 ± 40 (4)[a]
ATP	1149 ± 47 (4)[a]	1202 ± 51 (4)
Phosphatase	978 ± 84 (4)	1009 ± 45 (4)[b]

Each value of B_{max} represents the mean ± S.E.M. of values obtained by nonlinear regression of experimental data from saturation curves. The number of separate experiments is indicated in parentheses. Two-way ANOVA of B_{max} values reveal a significant effect between stress and the phosphatase treatment.

[a] $P < .0001$ compared to nonstressed condition without any addition.

[b] $P < .001$ compared to stressed condition without any addition.

condition ($P < .001$) from 925 to 1149 fmol/mg protein. The receptor B_{max} did not significantly increase with ATP at 20 mM final concentration but it significantly increased with ATP at 60 mM final concentration (data not shown).

Two-way ANOVA of the B_{max} values (Table 1) also revealed significant interaction between the presence of 20 U/ml final concentration of alkaline phosphatase and the stressed condition [$F(1,8) = 27.44$, $P < .001$]. The LSD test showed that the B_{max} in the stressed condition decreased by about 20% ($P < .001$) from 1226 fmol/mg protein in the absence of phosphatase to 1009 fmol/mg protein in the presence of phosphatase. The receptor B_{max} did not significantly decrease at a final concentration of 10 U/ml but it did at 20 U/ml. (data not shown). The effects of ATP and phosphatase were not observed when these reagents were added outside the lumen of the synaptosomal membranes (data not shown).

3.2. Effects of ATP and phosphatase at 37 °C on the CBR density in synaptosomal membranes from nonstressed and stressed chicks

Two-way ANOVA of the B_{max} values (Table 2) revealed a significant interaction between the presence of phosphatase and the stressed condition [$F(1,8) = 27.40$, $P < .01$]. The LSD test showed that the B_{max} in stressed condition decreased by about 25% ($P < .001$) from 1252 fmol/mg protein in the absence of phosphatase to 930 fmol/mg protein in the presence of phosphatase. Table 2 also shows that the stress-induced receptor recruitment previously observed at 4 °C (Table 1) was not directly observed at 37 °C. In all cases of both ATP and phosphatase treatments, no effects were observed at 37 °C when the reagents were added outside the lumen of the synaptosomal membranes (data not shown).

Table 2
Effects of ATP and phosphatase on the CBR density in synaptosomal membranes from nonstressed and stressed chicks at 37 °C

	B_{max} (fmol/mg protein)	
Addition	Nonstressed	Stressed
–	1210 ± 45 (3)	1252 ± 49 (3)[a]
ATP	1292 ± 33 (3)	1236 ± 52 (3)
Phosphatase	1260 ± 48 (3)	930 ± 37 (3)

Each value of B_{max} represents the mean ± S.E.M. of values obtained by nonlinear regression of experimental data from saturation curves. The number of separate experiments is indicated in parentheses. Two-way ANOVA of B_{max} values reveal a significant effect between stress and the phosphatase treatment.

[a] $P < .0001$ compared to the phosphatase treatment in the stressed condition.

3.3. Effects of 100 μm colchicine at 37 °C on the CBR density of both synaptosomes and synaptosomal membranes from stressed chicks

Two-way ANOVA of the B_{max} values (Fig. 1) revealed a significant interaction between the presence of colchicine and the hypoosmotic treatment of synaptosomes to obtain synaptosomal membranes [$F(1,16) = 32.23$, $P < .001$]. The LSD test showed that the B_{max} in synaptosomes decreased by about 20% ($P < .001$) from 1956 fmol/mg protein in the absence of colchicine to 1573 fmol/mg protein in its presence (Fig. 1a). However, colchicine effects were not observed in the B_{max} of synaptosomal membranes (Fig. 1b). The LSD test also showed that the B_{max} in the absence of colchicine was about 30% lower ($P < .001$), 1368 fmol/mg protein in synaptosomal membranes (Fig. 1b) than in synaptosomes 1956 fmol/mg protein (Fig. 1a). Similar results were found (data not shown) when synaptosomes were obtained and resuspended in the presence of a mixture of proteinase inhibitors.

3.4. The CBR density at 37 and 4 °C in synaptosomes from stressed chicks as a function of colchicine concentration

Fig. 2 shows that colchicine at 37 °C induced a decrease in the B_{max} which was dependent on concentration, reaching a 40% of decrease at a concentration of 500 μM. The

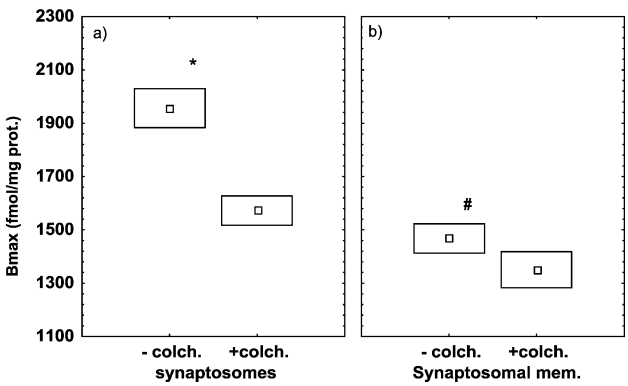

Fig. 1. Effects of 100 μM colchicine at 37 °C on the CBR density of synaptosomes and synaptosomal membranes from stressed chicks. Squares represent the mean of the B_{max} ± S.E.M. The number of separate experiments is $n = 5$. * $P < .0003$ compared to synaptosomes in the presence of colchicine. # $P < .0003$ compared to synaptosomes in the absence of colchicine.

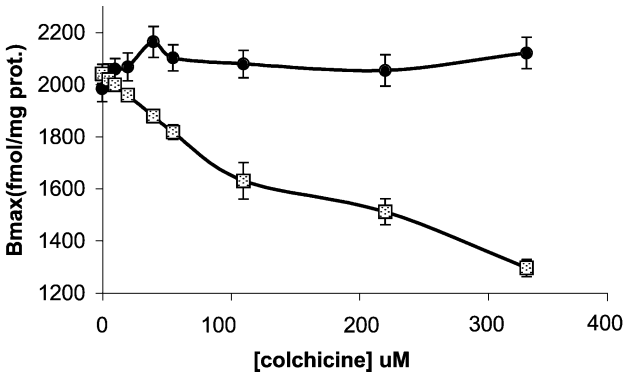

Fig. 2. The CBR density in synaptosomes from stressed chicks as a function of colchicine concentration at 4 °C (circles) and 37 °C(squares). Bars represent the mean of the $B_{max} \pm$ S.E.M. The number of separate experiments is $n = 6$.

presence of 100 μM γ-lumicolchicine at 37 °C did not affect the B_{max} values of the receptor (data not shown). Fig. 2 also shows that B_{max} values of the receptor were not affected by colchicine at 4 °C and concentrations ranging between 0 and 350 μM.

3.5. Effects of 100 μm colchicine, 10 μg/ml vinblastine and 10 μg/ml taxol at 37 °C on the CBR density of synaptosomes from nonstressed and stressed chicks

A two-way ANOVA of the B_{max} values (Fig. 3) revealed a significant interaction between the presence of colchicine and the stressed condition [$F(1,20) = 44.85$, $P < .001$]. The LSD test showed that the B_{max} in the stressed condition (Fig. 3a) decreased by about 30% ($P < .001$) from 2082 fmol/mg protein in the absence of colchicine to 1471 fmol/mg protein in its presence. Colchicine also caused a decrease of about 11% ($P < .01$) in the B_{max} of the nonstressed condition (Fig. 3b) from 1849 to 1651 fmol/mg protein.

Two-way ANOVA of the B_{max} values (Fig. 3) revealed a significant interaction between the presence of vinblastine and the stressed condition [$F(1,12) = 20.77$, $P < .001$]. The LSD test showed that the B_{max} in the stressed condition decreased by about 20% ($P < .001$) from 2002 fmol/mg protein in the absence of vinblastine to 1580 fmol/mg protein in its presence (Fig. 3c). No differences were observed in the nonstressed condition between the presence and absence of vinblastine (Fig. 3d).

Two-way ANOVA of the B_{max} values (Fig. 3) did not reveal significant interaction between the stressed condition and the presence of taxol at 10 μg/ml final concentration. Thus, taxol did not affect the B_{max} in neither the stressed (Fig. 3d) nor the nonstressed condition (Fig. 3e).

3.6. The CBR density at 37 °C in synaptosomes from stressed chicks as a function of vinblastine concentration

Fig. 4 shows that vinblastine induced the highest decrease of the B_{max} at concentrations ranging between 10 and 20 μg/ml. The vinblastine was less effective at lower and higher concentrations.

3.7. Effects of vinblastine at 4 °C on the CBR density of synaptosomes from nonstressed and stressed chicks

Two-way ANOVA of the B_{max} values (Fig. 5) did not reveal a significant interaction between the stressed condition and the presence of vinblastine at a final concentration of 10 μg/ml. However, the LSD test showed that in the

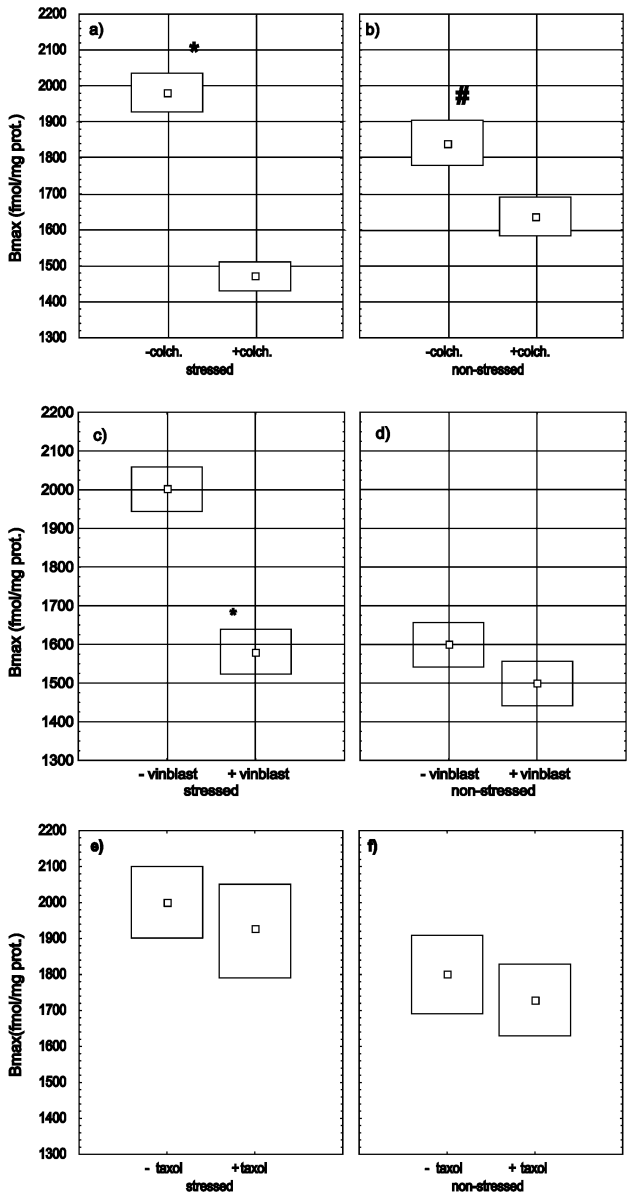

Fig. 3. Effects at 37 °C of 100 μM colchicine, 10 μg/ml vinblastine and 10 μg/ml taxol on the CBR density of synaptosomes from stressed and nonstressed chicks. Squares represent the mean of the $B_{max} \pm$ S.E.M. For colchicine, the number of separated experiments is $n = 6$. $^{*}P < .0001$ compared to the stressed condition in the presence of colchicine. $^{\#}P < .01$ compared to the nonstressed condition in the presence of colchicine. For vinblastine, the number of separate experiments is $n = 4$. $^{+}P < .0001$ compared to the stressed condition in the presence of vinblastine.

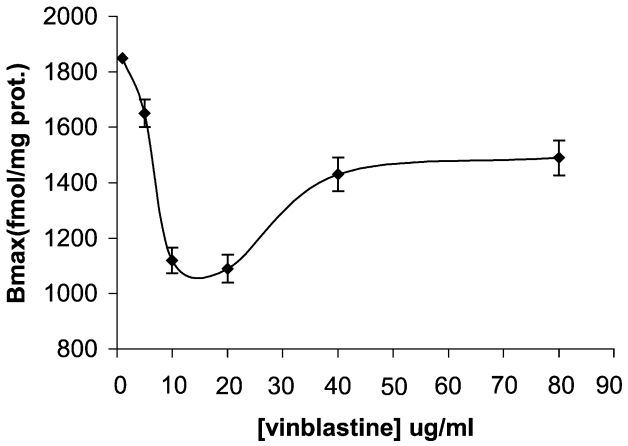

Fig. 4. The CBR density in synaptosomes from stressed chicks as a function of the vinblastine concentration at 37 °C. Bars represents the mean of the $B_{max} \pm$ S.E.M. The number of separate experiments is $n = 4$.

absence of vinblastine the B_{max} increased by about 19% ($P < .01$) from 1670 (Fig. 5b) to 1980 fmol/mg protein (Fig 5a), induced by stress. confirming the reported increase of the CBR in synaptosomes induced by acute stress (Salvatierra et al., 1994; Marín and Arce, 1996; Martijena et al., 1992). Similar results were observed in the presence of vinblastine.

3.8. Effects of cytochalasins D and C at 37 °C on the CBR density of synaptosomes from nonstressed and stressed chicks

Two-way ANOVA of the B_{max} values (Fig. 6) did not reveal a significant interaction between the presence of cytochalasin D and C, both at a final concentration of 10 μg/ml and the stressed condition. However, the LSD test showed that the B_{max} increased by about 23% ($P < .001$) due to the presence of cytochalasin D in the nonstressed condition from 2150 (Fig. 6a) to 2650 fmol/mg protein (Fig. 6b). Furthermore, the B_{max} increased by about 25% ($P < .001$) due to the presence of cytochalasin D in the stressed condition from 2250 (Fig. 6a) to 2810 fmol/mg protein (Fig. 6b).

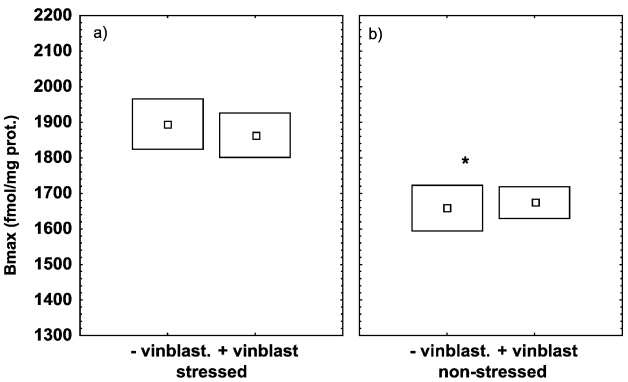

Fig. 5. Effects of vinblastine at 4 °C with a final concentration of 10 μg/ml on the CBR density of synaptosomes from nonstressed and stressed chicks. Bars represent the mean of the $B_{max} \pm$ S.E.M. The number of separate experiments is $n = 4$. $^{*}P < .001$ compared to stressed condition in absence of vinblastine.

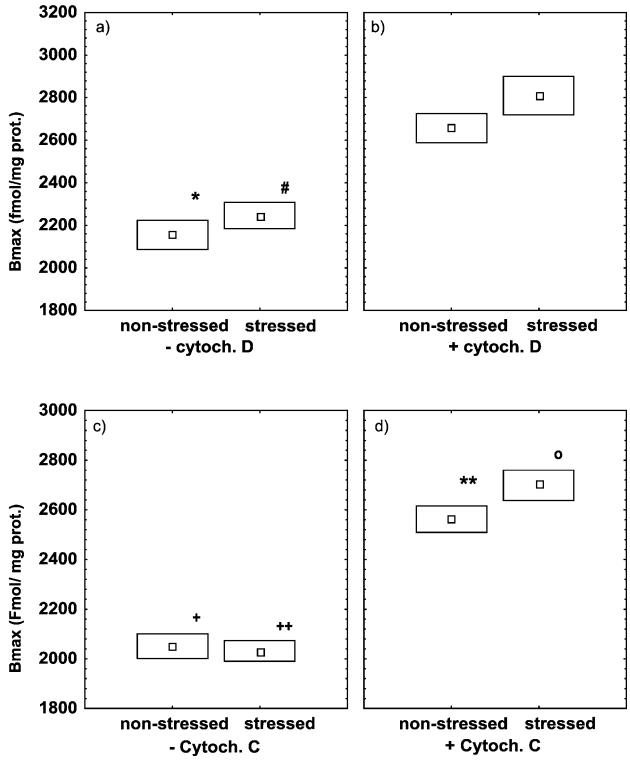

Fig. 6. Effects of cytochalasin D and C both at 37 °C and 10 μg/ml final concentration on the CBR density of synaptosomes from nonstressed and stressed chicks. Squares represent the mean of the $B_{max} \pm$ S.E.M. The number of separate experiments is $n = 5$. Cytochalasin D, $^{*}P < .0001$ compared to the nonstressed condition in the presence of cytochalasin D. $^{\#}P < .0001$ compared to the stressed condition in the presence of cytochalasin D. Cytochalasin C, $^{+}P < .0001$ compared to the nonstressed condition in the presence of cytochalasin C. $^{++}P < .0003$ compared with the stressed condition in the presence of cytochalasin C. $^{**}P < .001$ compared to cytochalasin C plus NEM (Fig. 7a) in the nonstressed condition. $^{\circ}P < .001$ compared to cytochalasin C plus NEM in the stressed condition (Fig. 7a).

The LSD test also showed that the B_{max} increased by about 25% ($P < .001$) in the presence of cytochalasin C in the nonstressed condition from 2052 (Fig. 6c) to 2563 fmol/mg protein (Fig. 6d). Furthermore the B_{max} increased by about 33% ($P < .001$) in the presence of cytochalasin C in the stressed condition from 2031 (Fig. 6c) to 2702 fmol/mg protein (Fig. 6d).

3.9. Effects of NEM on the CBR density increase induced by cytochalasin C at 37 °C in synaptosomes from nonstressed and stressed chicks

Two-way ANOVA of the B_{max} values did not reveal a significant interaction between both the absence of cytochalasin C (Fig. 6c) and the presence of NEM plus cytochalasin C (Fig. 7a) and the stressed condition. However, the LSD test showed that the presence of 1 mM NEM caused a B_{max}

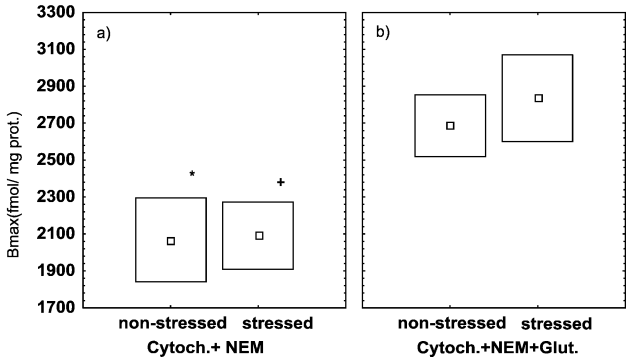

Fig. 7. Effects of *N*-ethylmaleimide (NEM) on the increase of CBR density induced by cytochalasin C at 37 °C in synaptosomes from nonstressed and stressed chicks. Squares represent the mean of the $B_{max} \pm$ S.E.M. The number of separate experiments is $n = 4$, $^{*}P < .02$ compared to cytochalasin C plus NEM plus glutathione in the nonstressed condition. $^{+}P < .005$ compared to cytochalasin C plus NEM plus glutathione in the stressed condition.

decrease of about 18% ($P < .001$) from 2531 (Fig. 6d) to 2075 fmol/mg protein (Fig. 7a) in the nonstressed condition. NEM also decreased the B_{max} about 22% ($P < .001$) from 2702 (Fig. 6d) to 2100 fmol mg protein in the stressed condition (Fig. 7a).

Two-way ANOVA of the B_{max} values (Fig. 6c) did not reveal a significant interaction between both the presence of NEM with (Fig. 7a) or without cytochalasin and the stressed condition. The LSD test did not show differences in B_{max} values between them at both the stressed and nonstressed conditions.

Two-way ANOVA on the B_{max} values did not reveal a significant interaction between both the presence of cytochalasin C with NEM (Fig. 7a) nor with NEM inactivated with glutathione (Fig. 7b) and the stressed condition. However, the LSD test showed that the inactivation of NEM with glutathione increased the B_{max} value about 30% ($P < .02$) from 2075 (Fig. 7a) to 2702 fmol/mg protein (Fig. 7b) in the nonstressed condition. A similar B_{max} increase about 36% ($P < .006$) was observed in the stressed condition from 2101 (Fig. 7a) to 2850 fmol/mg protein (Fig. 7b).

4. Discussion

In the present report, the CBR density was used to express the $GABA_AR$ density since the CBR is a FNTZ-binding site located in the $GABA_AR$ (Primus and Kellogg, 1991). [^{3}H]GABA or ligands at the GABA binding site of the $GABA_AR$ were not used for the radioreceptor assay due to the presence of relatively high concentrations of GABA in synaptosomes. Traditionally, and in order to eliminate the endogenous GABA and putative ligands, the synaptosomes are frozen, strongly homogenised and incubated at 37 °C for 30 min in the presence of 0.05% (v/v) of Triton X-100. Then they are centrifuged and washed (Enna et al., 1977).This procedure strongly affects the integrity of the synaptosomes and the synaptosomal membranes. The cytoskeleton components are lost and remaining membranes are affected by the caotropic detergent. Instead of the method abovementioned, we used synaptosomes and synaptosomal membranes both obtained through a mild procedure so that two different pools of receptors can be measured, masked and unmasked pools of receptors. Furthermore, [^{3}H]FNTZ was used to measure the CBR density in conditions that the endogenous GABA did not affect the FNTZ maximal binding.

The CBR has been traditionally measured ex vivo in rat synaptosomes by the radioreceptor assay at 4 °C because at higher temperatures, the affinity of the receptor for one to four benzodiazepines diminished. However, without changing in density (Braestrup and Squires, 1977; Möhler and Okada, 1977). Afterwards, the dependence of affinity on the temperature and the independence of the density on the temperature have been widely studied in synaptosomes of rat brain (Salvatierra et al., 1994; Kochman and Hirsch, 1982). We confirmed the dependence of the affinity on the temperature (data not shown) but not the independence of the CBR density on the temperature. Thus, the CBR density in nonstressed chicks at 37 °C (Table 2) was about 30% higher than at 4 °C (Table 1). An explanation for the differences may be that in the previous reports (Braestrup and Squires, 1977; Möhler and Okada, 1977), the synaptosomes used were obtained from rats nonhabituated to the procedure of decapitation with the guillotine, as the importance of habituation had not been studied yet. Consequently, rats suffered the effects of the stressors as reported (Primus and Kellogg, 1991). To minimize the stressing effects that accompanied the procedure of chick sacrificing, they were decapitated with scissors, so the whole procedure lasted no more than 2 s.

Since an acute stressor induced a recruitment of about 30% of the $GABA_AR$ in chicks (Martijena et al., 1992) and in rats (Primus and Kellogg, 1991) when measured at 4 °C, although this recruitment was not observed in rats at 37 °C (Braestrup and Squires, 1977; Möhler and Okada, 1977), we suggest that the increase in the receptor density induced by temperature was not additive to the previous increase induced by stress. Thus, the $GABA_AR$ recruitment induced by stress can not be directly observed when measured at 37 °C. This suggestion is analyzed later in the discussion of the effect of alkaline phosphatase at 37 °C on the receptor density in the stressed and nonstressed condition (Table 2). In addition, we confirm (data not shown) that the effect of temperature on the receptor density was not reverted when the radioreceptor method was assayed at 4 °C (Marín, 1998).

Table 1 shows that the incubation at 4 °C of synaptosomal membranes containing ATP in the lumen, increased the $GABA_AR$ density in nonstressed chicks but not in stressed ones. Thus, the phosphorylation of membrane bound proteins, such as $GABA_AR$ and $GABA_ARAP$, may be the cause of the stress receptor recruitment. This possibility is in

accordance with the reported endogenous phosphorylation of the $GABA_AR$ in brain membranes when they are incubated with [γ-^{32}P]ATP (Minier et al., 2000). Table 1 also shows that the ATP effects on the $GABA_AR$ density were not observed in stressed chicks, indicating that ATP effects were not added to the in vivo stress recruitment. These results confirm the possibility that the stress receptor recruitment may occur by phosphorylation of the $GABA_AR$ and/or other membrane bound proteins. This suggestion is in accordance with the reported results (Spieler et al., 1993) showing that the stress accompanying a maze-learning task increased the protein kinase C (PKC) in rat synaptosomal membranes by translocation from the cytosol. As it has been reported (Whatley et al., 1994) that colchicine inhibited both GABA stimulated chloride uptake and the PKA activity in microsacs, it is possible that this enzyme is also involved in the stress $GABA_AR$ recruitment. Another report (Gyenes et al., 1994) that is in accordance with our results, is an electrophysiological study on cultured neurons, showing that the intracellular addition of ATP or alkaline phosphatase inhibited and stimulated respectively the run-down of the $GABA_AR$ by consecutive application of GABA to neurons. However, our results are not in accordance with reports (Leidenheimer et al., 1992) on *Xenopus* oocytes containing an expressed $GABA_AR$ combination, showing that the activation of the PKC induced a down-modulation of the GABA currents. However, the possibility that ATP itself or its derivatives can act modulating the activity of some enzyme and not through phosphorylation can not be discarded. Table 1 also shows that the incubation at 4 °C of synaptosomal membranes containing alkaline phosphatase, masked a number of receptors similar to that recruited by stress, perhaps by an internalisation process, suggesting that the recruitment and an eventual receptor internalisation occur through phosphorylation and dephosphorylation, respectively, of membrane bound proteins, like $GABA_AR$ and/or $GABA_ARAP$.

Table 2 shows that the receptor density was similar in both the stressed and the nonstressed condition when synaptosomal membranes were incubated at 37 °C without additions, indicating that the receptor increase induced by temperature was not additive to the stress recruitment, and that the receptor recruitment can not be observed straightforwardly at 37 °C. However, Table 2 also shows that the presence of alkaline phosphatase in the lumen of synaptosomal membranes abolished the stress receptor recruitment but not the receptor increase induced by temperature, indicating that the receptor recruitment was sensitive to phosphatase while the receptor increase induced by temperature was insensitive to it. The results suggest that dephosphorylation of the receptor induced its masking, but in a form that was free of bound radioligand. Table 2 also shows that when synaptosomal membranes containing ATP in the lumen were incubated at 37 °C, the receptor density did not increase in both the stressed and nonstressed condition, indicating that the effect of ATP was neither added to the stress receptor recruitment nor to the receptor increase induced by temperature. In all, the results of Tables 1 and 2 suggest that the receptor recruitment occurs by a different mechanism than the exposure of the receptor induced by temperature.

Fig. 1a shows that the addition of colchicine at 100 μM final concentration to synaptosomes at 37 °C during the radioreceptor assay masked or internalized a number of receptors in the stressed condition similar to the number exposed by stress at 4 °C (Table 1), suggesting that the stress recruitment was abolished by MT depolymerization. Fig. 1b shows that colchicine did not affect the receptor density in synaptosomal membranes, suggesting that cytoplasmatic systems, as MTs that were lost by hyposmotic washing were needed for the receptor masking. Fig. 1 also shows that the receptor density in the absence of colchicine, was about 30% lower in synaptosomal membranes (b) than in synaptosomes (a), suggesting that a unknown number of receptors, possibly a cytosolic vesiculated form, was lost by hiposmotic washing of synaptosomes. In the synaptosomal membrane system we proposed, the existence of two pools of $GABA_AR$ bound to the postsynaptic membrane: one of them exposed to the extracellular radioligand in the basal condition of nonstressed chicks, and another pool of hidden receptors that are unmasked by stress which is about a quarter of the total membrane bound $GABA_AR$ (Martijena et al., 1992). In accordance to this report, it is probable that MT depolymerization induced a masking of those receptors free of radioligand. In addition, there may exist of a third $GABA_AR$ pool in the synaptosome system, located in the cytoplasm in a vesicular form which could be similar to the one described for glutamate receptors before the fusion of receptors containing vesicles to the postsynaptic membrane (Hass, 1998).

An important question is, if colchicine, a depolymerizing MT agent, abolished the stress receptor recruitment in synaptosomes at 37 °C (Fig. 2), why was the $GABA_AR$ density higher in the stressed (Fig. 5a) than in the nonstressed condition at 4 °C (Fig. 5b), a temperature that induced MT depolymerization? (Borisy et al., 1974). It could be because the polymerisation of MTs is not needed for the recruitment, but a previous anchoring of the recruited receptors to MTs is needed for the colchicine to induce their masking or internalization. This possibility is in accordance with reports (Whatley et al., 1994) indicating that colchicine at 37 °C did not affect the CBR density in nonstressed chicks.

Fig 2 shows the $GABA_AR$ masking in synaptosomes from stressed chicks, measured at 37 °C as a function of colchicine concentration. The decrease in receptor density reached its maximum at a 300-μM concentration. Fig. 2 also shows that when incubations were performed in the presence of colchicine at 4 °C, a non-MT-depolymerizing condition for the colchicine action, the receptor density almost did not change, suggesting that colchicine exerted its action through MT depolymerization but not through

nonspecific effects. These results are not in accordance with electrophysiological studies on neuronal cultures, indicating that MT depolymerization was not related to inhibition of GABA-gated chloride currents, and that colchicine was an antagonist at the $GABA_AR$ (Bueno and Leidenheimer, 1998). However, results reporting that colchicine, vinblastine and taxol all inhibited the chloride uptake in brain microsacs (Whatley and Harris, 1996) are in accordance with our results. Fig. 3 shows that colchicine at 37 °C decreased the receptor density about 30% in the stressed condition (a), but only about 11% in the nonstressed condition (b), suggesting that the receptor recruitment induced by stress was more sensitive to colchicine than the receptor increase induced by temperature. Fig. 3 also shows that, vinblastine at 10 μg/ml, also a MT depolymerizing agent at 37 °C, decreased the receptor density about 22% (Fig. 3c) and about 40% (Fig. 4) in the stressed condition but did not affect the receptor density in the nonstressed condition (Fig. 3d). Together, the results obtained with colchicine (Fig. 3a) and vinblastine (Figs. 3c, 3d and 4) suggest that the receptor recruitment induced by stress was sensitive to MT depolymerizing agents while the $GABA_AR$ increase induced by temperature was insensitive to them. These results can be compared with the results showing that the receptor increase induced by temperature in synaptosomal membranes was also insensitive to alkaline phosphatase (Table 2). The results also confirm that in the absence of colchicine and at 37 °C there were no differences in the $GABA_AR$ density between the stressed and the nonstressed condition similarly to what was observed in synaptosomal membranes (Table 2). Taxol, a stabilising MT agent, at a concentration of 10 μg/ml (Fig. 3d and e) did not affect the $GABA_AR$ density in synaptosomes in both the stressed and nonstressed condition. Together, the results of colchicine, vinblastine and taxol suggested that MT depolymerization was needed for the masking or internalisation of $GABA_ARs$ exposed by stress.

In addition, vinblastine at 37 °C and concentrations higher than 20 μg/ml did not affect the stress-induced receptor recruitment (Fig. 4). Furthermore, vinblastine, a nondepolymerizing MT agent at 4 °C, did not affect the stress receptor recruitment with the B_{max} about 20% higher in the stressed condition (Fig. 5a) than in the nonstressed condition (Fig. 5b).

Both cytochalasins D and C (Fig. 6) increased the $GABA_AR$ density at 37 °C in both the stressed and nonstressed condition, suggesting that cytochalasins induced the in vitro fusion of a vesicular receptor pool located in the cytoplasm to the postsynaptic membrane through the MF depolymerization independently of the stressed condition. Then, the effect of temperature may have induced an unmasking of these hidden receptors. It is possible that a fusion mechanism could be physiological for the $GABA_AR$ exposure to the synaptic cleft similarly to the model proposed for the glutamate receptor (Hass, 1998). This possibility is in accordance with the results shown in Fig. 7, where NEM, an inhibitor of the NSF (Hass, 1998), inhibited the $GABA_AR$ exposure induced by cytochalasin C. However, exposure of the $GABA_AR$ through MF depolymerization is not in accordance with reports (Allison et al., 1998) showing that $GABA_AR$ was not dependent on F-actin for the maintenance or the synaptic localisation of receptor clusters in neuronal cultures.

Even if the stress recruitment of receptors or their masking or internalisation described in the present paper are not direct measures of the $GABA_A$ function, they can be compared to results reporting (Whatley and Harris, 1996) that colchicine, vinblastine and taxol, which disrupt MT through different mechanisms, all inhibited the chloride uptake by brain microsacs, while cytochalasin D did not affect this GABA function. It is possible that stressors induced a generalised increase of the synaptic strength at both pre-and post-synaptic sites in brain. Thus, postsynaptic receptors are likely to be saturated by released GABA and a rapid increase or recruitment of the postsynaptic $GABA_AR$ may be a more efficient way of strengthening synaptic efficacy of the GABA function. Thus, this conjecture could explain the reported CBR recruitment induced by stress (Martijena et al., 1992).

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