BRAND ISCHEMIA INCREASED HIGH AFFINITY BINDING OF [³H]MUSCIMOL INTO SYNAPTIC PLASMA MEMBRANE RECEPTOR

M. SAMOCHOCKI and J. STROSZNAJDER

Department of Neurochemistry, Medical Research Centre, Polish Academy of Sciences, 3 Dworkowa St., 00-784 Warsaw, Poland

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Abstract. The authors investigated the high affinity binding of [³H]muscimol to the receptor of synaptic plasma membranes (SPM) isolated from a normoxic and ischemic brain. Brain ischemia enhanced the [³H]muscimol binding to the receptor, located in native (Triton X-100 untreated) membranes. Scatchard's analysis showed that the total number of binding sites (BMAX) and the KD value increased by about 60%. The higher KD value persisted during 20 min of the reperfusion period. Concomitantly, ischemia stimulated the activity of phospholipase C and phospholipase A₂, acting against phosphatidylinositol (PI). The degradation of PI and a transient accumulation of docosahexaenoic and arachidonic acids may be important factors involved in the modification of high affinity agonist binding to the GABA_A receptor of SPM isolated from the brain submitted to ischemia.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is established as a major inhibitory neurotransmitter in the mammalian central nervous system (CNS) (5). The specific binding of [³H]muscimol to SPM preparations, obtained from the rat brain, has been thought to reflect the association of this

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neuroactive amino acid with its physiologically relevant receptors (2, 6-8). Muscimol, isolated from Amanita Muscaria, is known to be a more potent agonist than GABA itself (9).

Our previous studies, indicated that brain ischemia decreased the GABA uptake into synaptosomes and simultaneously affected the lipid composition of nerve endings (4, 12, 16). It was suggested that ischemically induced lipid membrane changes were responsible for lower GABA uptake into synaptosomes. It has been shown that the lipid microenvironment of cell plasma membranes affect the system of neurotransmitter and hormone receptors (11).

The aim of our studies was to investigate the high affinity binding of \([3H]muscimol\) to the receptor of SPM isolated from the brain cortex immediately after ischemia. Moreover, the disturbances of the agonist binding to the GABA\(_\alpha\) receptor occurring during ischemia were correlated with the membrane lipid changes.

**MATERIAL AND METHODS**

**Materials.** \([3H]muscimol\) (23 Ci/mmol) and \([3H]inositol-phosphatidyl-inositol\) (6 Ci/mm mol) and 1-stearoyl-2-[\(^{14}\)C]arachidonyl-phosphatidylinositol (58 mCi/mmol) were purchased from Radiochemical Centre, Amersham, England. Unlabeled compounds were obtained from Sigma St. Louis, USA. TLC plates were from Merck, FRG.

**Experimental model of ischemia.** Ligation of both common carotid arteries has been carried out in adult gerbils (Meriones unquiculates) by means of surgical threads under light diethyl ether anesthesia, as described previously (4, 12). The duration of ischemia was 15 min. In some cases, 20 min recovery in the atmosphere of oxygen was applied after releasing the artery ligation. Sham surgery was done for all controls.

**Preparation of synaptic plasma membranes from animal brain.** Adult gerbils or Wistar rats were used for the experiments. After decapitation, the brain hemispheres were quickly removed (30 s) and homogenized in an ice-cold isolation medium (0.32 M sucrose, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA). The synaptic plasma membranes were isolated according to the procedure described by Costa et al. (3). A crude mitochondrial fraction (P2) was lysed in an ice bath with a 1 mM Tris-citrate buffer pH 7.0 for 30 min, following which the membranes were sedimented twice by centrifugation at 8,000 \(\times\) g for 10 min. Fresh SPM from combined supernatants were obtained by centrifugation at 48,000 \(\times\) g for 20 min and were washed 3 times by centrifugation. In some cases pellet was frozen overnight, and then thawed and incubated with a 50 mM
Tris-citrate buffer pH 7.1, containing 0.05%/ Triton X-100 at 37°C for 30 min. Subsequently, the membranes were sedimented and washed 3 times with the buffer by centrifugation.

Reassembly of lipids into Triton-treated SPM. The SPM protein was incubated with 700 μg of phosphatidylinositol (previously sonicated with the buffer during 1 min at low power L-4, with the use of MSE sonicator type PG 384) and 5%/ bovine serum albumin (free from fatty acids) at 37°C for 60 min. Subsequently, the membranes were centrifuged at 48,000 × g for 20 min. Control membranes were incubated under the same conditions without phospholipid.

Saturated and unsaturated fatty acids, 10-250 nmol/mg protein, were placed on the bottom of the assay tubes. The organic solvent was evaporated under nitrogen, then a 50 mM Tris-citrate buffer pH 7.1 was added and the samples were vigorously whirled for 1 min. In the case of stearic acid a small amount of ethanol (0.001%) was added.

Determination of the high affinity binding of [3H]muscimol to the GABA receptor. The kinetics of [3H]muscimol binding was obtained by the incubation of 0.2-0.3 mg of membrane protein and an appropriate amount of labeled ligand in a range of 1-10 nM in a 50 mM Tris-citrate buffer (Na+-free) pH 7.1 at 0-4°C for 30 min in a final volume of 1 ml, as described previously (15).

Assay of phospholipase A2 and phospholipase C acting against labeled phosphatidylinositol. The assay of phospholipase A2 was carried out as described by Jelsema (10), by using labeled phosphatidylinositol in the second position with the [14C]arachidonic acid, as a substrate.

The activity of phospholipase C upon PI labelled with the myo[3H]inositol was assayed by measuring the formation of radioactive inositol phosphate(s) as described by Strosznajder and Haefnner (14). The separation of [3H]inositol monophosphate, by column chromatography with the use of Dowex X-1 phase, was performed according to Berridge et al. (1).

RESULTS

Characterization of the [3H]muscimol binding to SPM. It was confirmed that frozen and thawed SPM indicated a significantly higher affinity binding of [3H]muscimol in comparison with fresh membranes. Subsequently, ionic and non-ionic detergent treatment produced a further marked increase of the receptor affinity. The high affinity binding of [3H]muscimol to frozen, thawed and trebly washed SPM from rat brain was characterized by the values: K_D = 26 ± 1 nM and B_MAX = 982 ± 26 fmol/mg protein. After 0.05%/ Triton treatment the K_D
decreased to 11 ± 0.6 nM and concomitantly the agonist binding sites 
(B_MAX) increased to 1455 ± 29 fmol/mg protein. After 0.1%/o sodium 
deoxycholate treatment the K_D value was 18.2 ± 0.7 nM and B_MAX 1032 ± 
31 fmol/mg protein.

**Effect of brain ischemia on [3H]muscimol binding to SPM.** In the 
studies of the ischemia effect on the [3H]muscimol binding fresh mem-
branes were used. Brain ischemia enhanced the [3H]muscimol binding 
to the receptor by about 60%/o. The value of K_D and the number of 
the binding sites (B_MAX) increased by about 60-66%/o. The higher K_D value 
persisted during 20 min of the reperfusion time. A Scatchard plot of the 
high affinity range of the [3H]muscimol binding to SPM isolated from 
the brain submitted to ischemia is presented in Fig. 1.

![Fig. 1. Scatchard plot of high affinity range of [3H]muscimol specific binding 
(S.B.) to GABA receptor of synaptic plasma membranes isolated from a gerbil 
brain submitted to ischemia and to ischemia followed by 20 min of recovery. The 
values of K_D and B_MAX were calculated from the [3H]muscimol binding in lower 
(1-30 nM) concentration range. Control: K_D = 154 ± 11 nM and B_MAX = 1344 ± 77 
fmol/mg protein; Ischemia: K_D = 271 ± 55 nM and B_MAX = 2388 ± 312 
fmol/mg protein; Recovery: K_D = 210 ± 11 nM and B_MAX = 1391 ± 100 
fmol/mg protein. Each 
of the values represents the mean value from three experiments.](image)

Concomitantly, investigations were made of the effect of ischemia 
on the degradation of labeled phosphatidylinositol (PI) by the action 
of phospholipase C and on the liberation of arachidonic acid from PI
by the membrane-bound enzyme(s). Ischemia stimulates the activity of phospholipase C by about 40% and the liberation of arachidonic acid by about 30% (see Table I).

### Table I

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<th>Effect of ischemia on phosphatidylinositol degradation by synaptic plasma membrane bound enzyme(s)</th>
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<tr>
<td>PhlC</td>
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<td>Control</td>
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<td>Ischemia</td>
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<td>% stimulation</td>
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The values are the means ±SD from 3-4 experiments. Phospholipase C activity was determined by using phosphatidyl [3H]inositol as a substrate. Arachidonic acid release was assayed with exogenous substrate 1-stearoyl -2-[1^4C]arachidonyl-glycerophosphoinositol (PI).

**Effect of lipids on the [3H]muscimol binding to SPM.** Endogenously activated phospholipase C, which mainly hydrolyzed phosphoinositides, enhanced the [3H]muscimol binding to SPM by about 70% (data not shown). On the other hand, reassembly of Triton-treated SPM with exogenously added PI resulted in decreasing the B\text{MAX} value by about 33% and the K\text{D} value by 20% (Fig. 2).

The effect of exogenously added fatty acids to a Triton-treated membrane is demonstrated in Fig. 3. In the case of saturated fatty acid as stearic acid, the [3H]muscimol binding remained unaffected. Polyunsaturated fatty acids, arachidonic and docosahexaenoic acids elevated the high affinity of the [3H]muscimol binding by about 30-50%.

**DISCUSSION**

The results of our studies indicate that brain ischemia enhances the high affinity [3H]muscimol binding to the GABA receptor. It seems that the degradation of PI and a transient accumulation of unsaturated fatty acids, arachidonic or docosahexaenoic acids may play an important role in the modulation of agonist binding to the GABA\text{A} receptor.

Toffano and his collaborators (17) concluded from their studies of the role of endogenous phospholipids that the GABA binding component of the receptor site is a lipoprotein or a lipid-dependent protein.
Fig. 2. Scatchard's analysis of high affinity range of $[^3]H$muscimol specific binding (S.B.) to GABA receptor of Triton treated rat brain synaptic plasma membranes and reassembled with phosphatidylinositol, as described in Material and methods. Control: $K_D = 11.0 \pm 0.6$ nM and $B_{MAX} = 1455 \pm 29$ fmol/mg protein; PI: $K_D = 9.0 \pm 0.5$ nM and $B_{MAX} = 979 \pm 65$ fmol/mg protein. Each of the values represents the mean value from three experiments.

Fig. 3. The effect of exogenously added free fatty acids on $[^3]H$muscimol high affinity specific binding (S.B.) to Triton-treated rat brain synaptic plasma membranes, as described in Material and methods. Control binding was about 650 ± 21.5 fmol/mg protein. The results are the means ± SD from three experiments. *$p < 0.01$. 
Our studies of high affinity $[^3]$H]muscimol binding to the GABA receptor suggested that the level of PI in the vicinity of the receptor may have a significant effect on agonist binding to the GABA$_A$ receptor. Loh and Low (11) suggested that the lipid milieu around the receptor modulates the binding of the hormones and neurotransmitters to the corresponding receptor(s). The degradation of membrane lipids by endogenous phospholipases and lipases may be important in the regulation of the agonist-receptor interaction. Several studies (13, 18) indicated that the action of phospholipase A$_2$ increased the affinity or density of the GABA/benzodiazepine recognition sites.

Our studies suggest that activation by ischemia of phospholipase C and also of phospholipase A$_2$, acting against phosphatidylinositol, may be important events responsible for the higher agonist binding to the GABA$_A$ receptor of SPM.

Polyunsaturated fatty acids liberated mainly from PI in synaptic plasma membranes may also be involved in the modification of high affinity binding of $[^3]$H]muscimol to the SPM receptor of an ischemic brain. Our previous studies (4, 12, 16) showed higher degradation of PI in synaptosomes after ischemia in comparison with the other phospholipids and a significant increase of the level of arachidonic acid and also of docosahexaenoic acid.

These results indicate that also agonist binding to the synaptic plasma membrane GABA$_A$ receptor is affected by ischemia and is modified by lipids or a lipid-protein interaction.

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