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Pharmacology, Biochemistry and Behavior 83 (2006) 302–306
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Expression of muscarinic and dopaminergic receptors and monoamine levels frontal cortex of epileptic rats

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Received 13 September 2005; received in revised form 21 January 2006; accepted 11 February 2006
Available online 6 March 2006

Abstract

Apart from stroke, epilepsy is the most common neurological disorder with 0.5% of prevalence. The present study was performed in order to determine the monoamine levels, (M_1-like) muscarinic and (D_1- and D_2-like) dopaminergic receptor changes in frontal cortex of adult rats after pilocarpine-induced status epilepticus (SE). Male Wistar rats were treated with a single dose of pilocarpine (400 mg/kg, s.c.) and the control group received 0.9% saline (s.c.). Both groups were sacrificed 1 h after treatment. The frontal cortex was dissected for neurochemical assays. The results show a downregulation of 27% in M_1 muscarinic receptor density, but in the dissociation constant (K_d) value remained unaltered. D_1 and D_2 dopaminergic receptor densities and their K_d values remained unaltered. Monoamine and metabolites levels presented decreases of 44%, 27%, 30% and 42% in dopamine (DA), homovanilic acid (HVA), norepinephrine (NE) and 5-hydroxyindoleacetic acid (5-HIAA) contents, respectively. Moreover, in serotonin (5-HT) level remained unaltered and the 4-hydroxy-3-methoxy-phenylacetic acid (DOPAC) concentration was augmented by 34%. The results suggest that dopaminergic system in this area studied may not be directly involved in the seizures and status epilepticus, but different monoamines and metabolites can be modified in this cerebral area during seizure process. In conclusion, the neurochemical alterations that occur in frontal cortex of adult rats observed during the establishment of the status epilepticus induced by pilocarpine are decrease in M_1 receptor density concentration and a reduction in DA and NE levels.

Keywords: Muscarinic; Dopaminergic; Monoamines; Pilocarpine; Seizures; Status epilepticus

1. Introduction

Epilepsies are complex neurobehavioural disorders resulting from increased excitability of cholinergic neurons in several brain regions that can be diffused to several neurotransmitters (Rauca et al., 2004). The cholinergic system (acetylcholine) plays an important role in generating electroencephalographic (EEG) activity as well as regulating the vigilance states. Pilocarpine is a cholinergic agonist with a moderate affinity for M_1 muscarinic receptors and higher for M_5 ones (Rauca et al., 2004).

The acute administration of a high dose of pilocarpine in rodents is an experimental model widely used to study the seizures and status epilepticus (SE) and can be important to demonstrate the involvement of monoamines, metabolites and receptor systems different during seizure activity in several cerebral structures. This seizure model influences differentially the transmission process of the central nervous system affecting many, if not all, of the known neurotransmitter systems (Al-Tajir et al., 1990a; Barone et al., 1991) and is used to study pathophysiology of seizures and SE (Kulkarni and George, 2000). This model also demonstrates the potent pro-convulsive and damaging effect of pilocarpine (Cavalheiro et al., 1994; Clifford et al., 1987).

The subcutaneous injection of pilocarpine (400 mg/kg) produces a sequence of behavioural alterations including peripheral

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0091-3057/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2006.02.011

cholinergic signs, tremors, staring spells, facial automatisms and motor limbic seizures which develop progressively within 1–2 h into SE (Costa-Lotufo et al., 2002; Freitas et al., 2003; Marinho et al., 1997; Turski et al., 1983a). Moreover, SE induced by pilocarpine administration can induce EEG alterations which are similar to those of human temporal lobe epilepsy (Clifford et al., 1987; Cavalheiro et al., 1994). SE in rats can contribute to the study of the acute period and neurochemical alterations involved in this phase of epilepsy model induced by pilocarpine (Cavalheiro et al., 1994; Freitas et al., 2003).

Neurochemical studies about neurotransmitters and receptors density (Costa-Lotufo et al., 2002; Erakovic et al., 2000; Freitas et al., 2004) have been performed after seizures and status epilepticus induced by pilocarpine to clarify the convulsive process. Seizure activity with a wide range of local biochemical changes that affect muscarinic, dopaminergic and serotonergic receptors densities in hippocampus (Fritschy et al., 1999; Marinho et al., 1997; Freitas et al., 2005) and striatum (Freitas et al., 2003, 2005) after seizures and SE induced by pilocarpine clearly demonstrated that seizures can be blocked by the prior use of atropine, thereby verifying the involvement of the cholinergic system in the activation of this epilepsy model (Marinho et al., 1998) and that other neurotransmitter systems may be connected with the propagation and/or maintenance of seizures and SE.

Nevertheless, it is not well established how other neurotransmitters play a role in the pilocarpine-induced seizures and SE. The role of cholinergic and dopaminergic receptors in the process of seizures is not clear and several authors have suggested that the activation of cholinergic receptors in seizure initiation is necessary and that other neurotransmitters (norepinephrine, dopamine, adenosine, serotonine, γ-aminobutyric (GABA) and glutamate, aspartate, tyrosine and glycine) can be related to installation, propagation and/or maintenance of seizures and SE (Cavalheiro et al., 1994; Fritschy et al., 1999; Hirsch et al., 1992; Khan et al., 2000; Kulkarni and George, 2000). It is likely that serotonergic and dopaminergic receptors may also participate in the SE, but when and how it happens has to be determined. Therefore, it is important to investigate monoamines levels and receptor density changes in frontal cortex, during the first hour of acute phase of seizures and status epilepticus induced by pilocarpine contributing to results for other areas (cerebellum, striatum and hippocampus), probably because these areas can be involved in the convulsive process during the propagation of epileptic activity. Therefore, it is important to investigate receptor density different after SE induced by pilocarpine.

Considering receptor density alterations and that the relation to other neurochemical changes, such as monoamines and metabolites contents involved in seizures and status epilepticus mechanism, are still unclear, this work was performed to determine what occurs in monoamine levels, muscarinic (M_1-like) and dopaminergic (D_1- and D_2-like) receptors density changes in frontal cortex of adult rats after pilocarpine-induced seizures and status epilepticus, contributing to the establishment of convulsive process. The hypothesis clearly of this study is to investigate if frontal cortex can be involved in SE process through neurochemical alterations during the acute phase of epileptic activity.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats (250–280 g; 2-month-old) were used. Animals were housed in cages with free access to food and water and were kept with standard light–dark cycle (lights on at 07:00 h a.m.). The experiments were performed according to the Guide for the care and use of laboratory the US of Department of Health and Human Services, Washington, DC (1985).

Pilocarpine hydrochloride was purchased from ICN (CA, USA) and atropine sulfate from Sigma (MO, USA). Radioligands, [^{3}H]-*N*-methylscopolamine methyl chloride ([^{3}H]-NMS, 85 Ci/mmol; Amersham Pharmacia Biotech NJ, USA), [^{3}H]-SCH-23390 (109 Ci/mmol, Amersham, Uppsala, Sweden), and [^{3}H]-spiroperidol ([^{3}H]-spiro, 114 Ci/mmol; and New England Nuclear, USA) were provided. All other drugs were of analytical grade.

Control animals received 0.9% saline subcutaneously (s.c.; $n=37$) and in the other group, the animals were treated with a single dose of pilocarpine hydrochloride (400 mg/kg, s.c., $n=38$). Behavioural changes were observed during 1 h after the treatment. The observed parameters were: number of peripheral cholinergic signs, tremors, stereotyped movements, seizures, SE and mortality. The SE was defined as continuous seizures for a period longer than 30 min. SE was induced by the method of Turski et al. (1983a). Mortality was recorded during 1 h after pilocarpine treatment.

The pilocarpine group was constituted by rats that presented seizures, status epilepticus for a period longer than 30 min and that did not die after 1 h from the treatment. Pilocarpine and control group were killed by decapitation 1 h after the treatment and their brains were dissected on ice to remove frontal cortex for monoamine levels and muscarinic and dopaminergic receptor binding assays.

2.2. Monoamine level determinations

For determination of monoamine levels, control ($n=13$) and pilocarpine groups ($n=7$) were sacrificed after 1 h of observation, and the frontal cortex was dissected on ice for the preparation 10% homogenate (10% w/v) that were sonicated in 0.1 M $HClO_4$, for 30 s and centrifuged at 4 °C for 20 min at 26,000×*g*. A 20 μL sample of the supernatant was then analyzed by high performance liquid chromatography (HPLC). The mobile phase was 0.163 M citric acid (pH 3.0) containing 0.02 mM EDTA, with 0.69 mM sodium octanesulfonic acid (SOS), as ion pairing reagent, 4% v/v acetonitrile and 1.7% v/v tetrahydrofuran.

Frontal cortex concentrations of norepinephrine (NE), dopamine (DA), serotonin (5-hydroxytryptamine or 5-HT), and their metabolites 4-hydroxy-3-methoxy-phenylacetic acid (DOPAC), homovanilic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were detected electrochemically using an

amperometric detector Shimadzu, Japan by oxidation on a glassy carbon electrode at 0.85 V relative to the Ag–AgCl reference electrode and results expressed as ng/g wet tissue.

2.3. Binding assays

In binding assays, receptor density (B_{max}) and dissociation constant (K_d) were detected and expressed as fmol mg^{-1} of protein (fmol/mg protein) and nM, respectively. Control group (8 per group) and pilocarpine group (4 per group) were decapitated 1 h after treatment, and the cerebral area (frontal cortex) were dissected on ice for the preparation of a 10% (w/v) homogenate. Receptor numbers were measured through binding assays with 10% homogenate prepared (w\v) in 150 mM sodium phosphate buffer, pH 7.4, using [^{3}H]-NMS as the ligand (Dombrowski et al., 1983). This non-selective ligand binds to all subtypes of muscarinic receptors. The M_1-like receptors assay was performed with [^{3}H]-NMS in the presence of 100 μM carbachol for blocking M_2 sites. Total homogenates (0.05–0.10 mg protein) were incubated with 2.38 nM of [^{3}H]-NMS. Non-specific binding was determined in the presence of atropine (12.5 mM). The mixture was incubated for 30 min at 37 °C and the final volume was 0.2 ml.

Dopaminergic D_1- and D_2-like receptor assays. The methods of Meltzer et al. (1989) for D_1-like receptor and of Kessler et al. (1991) for D_2-like receptor were used. Control group (8 per group) and pilocarpine group (4 per group) were decapitated 1 h after treatment, and the cerebral area (frontal cortex) was dissected on ice for the preparation of a 10% (w/v) homogenate. The homogenate containing 60–120 μg of protein was incubated in 50 μM Tris–HCl buffer, pH 7.4, in the presence of 10 μM mianserin (Organon, São Paulo, Brazil), to block serotonergic receptors, in the case of D_2-like receptor binding assay. Several ligand concentrations of [^{3}H]-SCH-23390, from 0.135 to 6.75 nM, or [^{3}H]-spiroperidol, 0.09 to 4.76 nM, were used for D_1- and D_2-like receptors, respectively, in a final volume of 0.2 ml.

Reaction media were incubated at 37 °C for 60 min, and the reaction was terminated by filtration through Whatman GF/B filter paper on a cell harvester apparatus from Brandel (Gaitjersburg, MD, USA). Filters were washed 5 times with cold saline, dried in the oven for 2 h at 60 °C, and placed in vials containing 3 ml of a toluene (Vetec) based scintillation cocktail. Radioactivity was measured with a Beckman LS 100 counter with 62% efficiency. Specific binding was calculated as the total minus nonspecific binding in the presence of 5 μM dopamine (Sigma, St. Louis, MO, USA) containing 1 mg/ml ascorbic acid in order to prevent dopamine degradation.

B_{max} and K_d were calculated by the Instat Program for PC computers. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.4. Statistics

The Student–Newman–Keuls test was used for the multiple comparison of means of two groups of data whose differences were considered statistically significant at $p<0.05$. Differences in experimental groups were determined by analysis of variance (ANOVA) two-tailed.

3. Results

Immediately after pilocarpine administration, all of the 2-month-old animals persistently had behavioural changes, including initial akinesia, ataxic lurching, peripheral cholinergic signs (miosis, piloerection, chromodacriorrhea, diarrhea and masticatory automatisms), stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes that persisted for 10–15 min), clonic movements of forelimbs and head bobbing.

The rate of 50% of adult (19/38) animals presented tremors. Fifty-eight percent of the animals presented motor limbic seizures which progressed and persisted for 30–50 min evolving to SE (22 out of 38 animals) as previously described by Turski et al. (1983a). The mortality rate was 0% during the behavioural study. In the control group, no behavioural alterations were noticed.

Frontal cortex monoamine concentrations and their metabolites are presented in Fig. 1. After pilocarpine-induced status epilepticus, DA [$T(17)=8.980$; $p<0.0001$] and NE [$T(14)=6.585$; $p<0.0001$] concentrations had 44% and 27% decreases, respectively. In the metabolite DOPAC content [$T(17)=4.768$; $p<0.0002$] was verified to have a significant 34% increase, however, the 5-HT level [$T(17)=0.3936$; $p=$N.S.] remained unaltered. In addition, in the metabolites HVA [$T(17)=2.129$; $p<0.0482$] and 5-HIAA content [$T(17)=7.741$; $p<0.0001$] there were decreases of 30% and 42%, respectively as compared to control group (Fig. 1).

M_1-like receptor density (B_{max}) studies in adult rat after status epilepticus have shown a significant 31% decrease [$T(10)=2.360$; $p<0.0400$], but the K_d values was not altered [$T(10)=1.337$; $p=$N.S.] as compared to control group (Table 1). No alteration was observed in the D_1-like receptor density [$T(10)=1.838$; $p=$N.S.] and in K_d values [$T(10)=0.1861$; $p=$N.S.]. Similarly, no change

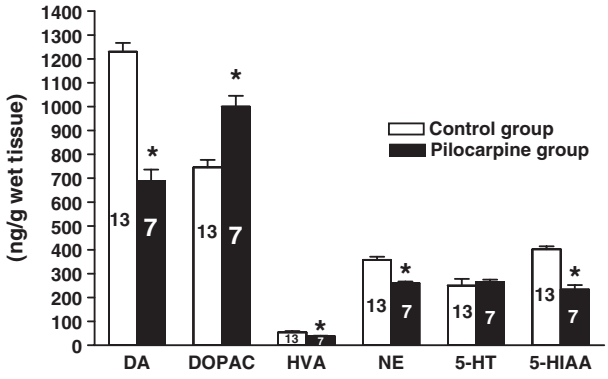

Fig. 1. Determination of monoamine and metabolite levels in frontal cortex of adult rats after pilocarpine-induced status epilepticus. Each bar represents mean± S.E.M. of the number of animals shown in parentheses. *$p<0.05$ as compared to control animals (ANOVA) and the differences in experimental groups were determined by Student–Newman–Keuls test. Abbreviations: Dopamine (DA), serotonin (5-HT), norepinephrine (NE) and their metabolites: 3.4-hydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), 5-Hydroxyindoleacetic acid (5-HIAA).

Table 1
Muscarinic and dopaminergic receptors density concentration in frontal cortex of adult rats after pilocarpine-induced status epilepticus

Groups	M_1-like		D_1-like		D_2-like	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
Control group	199.00±18.69 (8)	1.30±0.09 (8)	209.78±11.09 (8)	1.45±0.06 (8)	377.51±8.22 (8)	1.48±0.01 (8)
Pilocarpine group	145.00±5.78 (4)*	1.37±0.05 (4)	244.00±14.00 (4)	1.48±0.12 (4)	401.75±2.17 (4)	1.39±0.06 (4)

Male Wistar rats (250–280 g, 2-month-old) were treated with a single dose of pilocarpine (400 mg/kg, s.c.) and the control group with 0.9% saline. Animals were submitted to a 1 h observation and afterwards sacrificed. The values represent mean±S.E.M of the number of animals shown in parentheses. *$p<0.05$ as compared to control animals (ANOVA) and the differences in experimental groups were determined by Student–Newman–Keuls test.

was verified in B_{max} [$T(10)=2.020$; p=N.S.] and in K_d values [$T(10)=1.752$; p=N.S.] of D_2 receptor after status epilepticus as compared to control group (Table 1).

4. Discussion

Frontal cortex M_1 receptors, DA and NE can be directly involved in the convulsive process during seizures and after SE in adult rats. Other studies indicate that hippocampal and striatal several neurotransmitters have been implicated in the mechanism of pilocarpine-induced seizures and SE (Freitas et al., 2003, 2005). While activation of muscarinic receptors is the first step for seizure activity, dopaminergic and other systems (GABAergic, glutamatergic, adenosine and serotonergic) appear to mediate seizure propagation and/or maintenance in rodent epilepsy models (Turski et al., 1987c, 1989).

Our results have shown a downregulation in (M_1-like) muscarinic receptor. Interestingly, K_d value remained the same in frontal cortex after pilocarpine-induced SE, indicating that the ligand affinity in this area was altered during epileptic phenomenon. The M_1 downregulation verified in this area is an immediate compensatory mechanism due to the increase in acetylcholine concentration produced by pilocarpina during the acute phase of seizures. In addition, in the present study, B_{max} and K_d of D_1 and D_2 receptors were not significantly altered after status epilepticus induced by pilocarpine, suggesting that this system in frontal cortex can not be contributing for the establishment of convulsive process in relation the ligand affinity by receptors.

Our results suggest that during the first hour of acute phase of seizures, in the area studied, D_1 and D_2 receptor cannot be directly involved in the convulsive process, however, dopaminergic receptor mediates opposite functions in the regulation of epileptic activity as previously described by Khan et al. (2000). Thus, D_1 but not D_2 receptor stimulation reduces the threshold for pilocarpine-induced epileptic activity in rats. This effect is prevented by D_1 receptor blockade. Conceivably, D_1 receptor are localized in brain structures that control the spread of the convulsive activity which might be inhibited by D_2 receptor stimulation (Hirsch et al., 1992).

Nevertheless, the role of dopamine in the development of seizure activity remains an unresolved issue. It is known that dopamine exert effects pro- and anticonvulsive by D_1, and D_2 receptor stimulation, respectively (Turski et al., 1983a). It has been demonstrated that the activation of dopaminergic system via D_2 receptor in the hippocampus is able to protect the animal against limbic motor seizures produced by excessive muscarinic stimulation of the limbic system areas (Al-Tajir et al., 1990a, Persinger et al., 1993). There is evidences that the effects of acetylcholine on other modulatory neurotransmitters such as adenosine, glutamate, noradrenaline, dopamine and serotonine may be involved in the propagation of limbic cholinergic seizures for several cerebral areas (Freitas et al., 2004; Hirsch et al., 1992).

It appears that the sensitivity of muscarinic and dopaminergic receptors in the frontal cortex is not modified during the seizures and SE. On the other hand, in the striatum an increase in ligand affinity of these receptors during seizure activity and SE induced by pilocarpine was noticed (Freitas et al., 2003). Therefore, our results are important once they lead us to know neurochemical alterations in frontal cortex and, moreover, can be compared to other changes already described with regard to other cerebral areas (hippocampus and striatum), contributing to the clarify of seizure and SE mechanisms of each cerebral area in the epileptic phenomenon consenting the investigation of news drugs and strategies of treatment for human refractory epilepsy.

Significant differences in monoamines content were evident in striatum during development of seizures and SE after pilocarpine treatment (Freitas et al., 2003). Our work showed that in the cerebral area studied, DA and NE levels decreased in adult rats, suggesting that these monoamines have a function in seizures and SE induced by pilocarpine. There is evidence of an inhibitory role of dopamine, mediated by D_2 receptors in reduce the hyperexcitability of hippocampal and striatal neurons involved in the seizures and SE (Benardo and Prince, 1982; Clifford et al., 1987; Costa-Lotufo et al., 2002; Mc Donald et al., 1991; Freitas et al., 2004). In contrast, endogenous dopamine release by D_1 receptors was implicated in the initiation and spread of limbic seizure (Turski et al., 1983a). It seems that in the pilocarpine model of seizures D_2 agonists exert a powerful anticonvulsant effect which is mediated by D_2 receptors in striatum, but not by D_2 receptors in substantia nigra (Marinho et al., 1998).

In addition, 5-HT level did not change, but its metabolite 5-HIAA content was reduced. It was also observed a significant increase in DOPAC level and a decrease in HVA content. During the subsequent SE, monoamine levels were modified in different ways in frontal cortex, suggesting that there is a higher function for neurotransmitters NE, DA and metabolites (5-HIAA, DOPAC and HVA) in the convulsive process as compared to other neurotransmitters such as 5-HT that was not modified after the first hour of the acute period of seizures. The data for frontal cortex, with regard to monoamine content, is similar to DA, NE and HVA when compared to striatum after seizures and SE induced by high doses of pilocarpine in adult rats observed during 1 h (Freitas et al., 2003) showed a similar participation in seizures independently of cerebral area investigated of epileptic rats.

The neurochemical changes in frontal cortex detected after SE induced by pilocarpine in high dose can be associated with other pathologies, such as depression, acute mania, schizophrenia and psychosis (Nadkarni and Devinsky, 2005), due to the participation of several neurotransmitter systems (amino acid, monoamine and metabolites) and receptors different (adenosine, muscarinic, dopaminergic, serotonergic, GABAergic and glutamatergic) that can participle of neurochemical mechanisms of this pathologies. In summary, the neurochemical changes that occur in frontal cortex of adult rats observed during the establishment of the status epilepticus induced by pilocarpine are decrease in M_1 receptor density concentration, a reduction in DA, NE, 5-HIAA, HVA levels and an increase in DOPAC content.

In summary, the 5-HT in frontal cortex was altered indicating that this monoamine can not influence the propagation of seizures and that other mechanisms can be involved in this epilepsy model. Studies concerning dopaminergic, serotonergic, GABAergic, glutamatergic, adenosine and noradrenergic systems are relevant and can permit the identification of modulators of epileptogenesis.

The results obtained in our experiments indicate that the frontal cortex can be brain structure responsible for propagation and/or maintenance of the acute phase of epileptic activity due the neurochemical alterations in monoamines levels and receptor density detected in this study, and other areas not yet studied (cerebellum, motor cortex, amygdale) in this epilepsy model also can be involved during the acute phase of seizures induced by pilocarpine. Further studies in rodents models of epilepsy are needed to better clarify if cerebral area is involved during the establishment of the epileptic phenomenon.

Acknowledgments

This work was supported by a research grant from the Brazilian National Research Council (CNPq). R.M.F. is a fellow from CNPq. The technical assistance of Maria Vilani Rodrigues Bastos and Stênio Gardel Maia are gratefully acknowledged.

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