The Role of Piriform Cortex Adenosine A$_1$ Receptors on Hippocampal Kindling

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ABSTRACT: *Introduction:* The hippocampus and piriform cortex have a critical role in seizure propagation. In this study, the role of adenosine A$_1$ receptors of piriform cortex on CA1 hippocampal kindled seizures was studied in rats. *Methods:* Animals were implanted with a tripolar electrode in the right hippocampal CA1 region and two guide cannulae in the left and right piriform cortex. They were kindled by daily electrical stimulation of hippocampus. In fully kindled rats, N^6- cyclohexyladenosine (CHA; a selective adenosine A$_1$ receptors agonist) and 1,3-dimethyl-8-cyclopenthylxanthine (CPT a selective adenosine A$_1$ receptor antagonist) were microinfused into the piriform cortex. The animals were stimulated at 5, 15 and 90 minutes (min) after drug injection. *Results:* Obtained data showed that CHA (10 and 100 μM) reduced afterdischarge duration, stage 5 seizure duration, and total seizure duration at 5 and 15 min after drug injection. There was no significant change in latency to stage 4 seizure. CPT at concentration of 20 μM increased afterdischarge duration, stage 5 seizure duration, and total seizure duration and decreased latency to stage 4 seizure at 5 and 15 min post injection. Pretreatment of rats with CPT (10 μM), 5 min before CHA (100 μM), reduced the effect of CHA on seizure parameters. *Conclusion:* These results suggested that activity of adenosine A$_1$ receptors in the piriform cortex has an anticonvulsant effect on kindled seizures resulting from electrical stimulation of the CA$_1$ region of the hippocampus.

RÉSUMÉ: Le rôle des récepteurs A1 de l'adénosine du cortex piriforme dans l'embrasement de l'hippocampe. *Contexte :* L'hippocampe et le cortex piriforme jouent un rôle déterminant dans la propagation des crises d'épilepsie. Dans cette étude, nous avons évalué le rôle des récepteurs A1 de l'adénosine du cortex piriforme sur les crises provoquées par embrasement de la région CA1 de l'hippocampe chez des rats. *Méthodes :* Nous avons implanté une électrode tripolaire dans la région CA1 droite de l'hippocampe des rats et deux canules guides, dans le cortex piriforme gauche et droit. L'hippocampe était ensuite stimulé électriquement à tous les jours. Chez les rats complètement embrasés, la N6-cyclohexyladénosine (CHA; un agoniste sélectif des récepteurs A1 de l'adénosine) et la 1,3-diméthyl-8-cyclopenthylxanthine (CPT, un antagoniste sélectif des récepteurs A1 de l'adénosine) ont été microinfusés dans le cortex piriforme. Les animaux ont été stimulés 5, 15 et 90 minutes après l'injection de la substance. *Résultats :* Les données ainsi obtenues démontrent que la CHA (10 et 100 µM) a diminué la durée de la postdécharge, la durée du stade 5 des crises et la durée totale des crises, 5 minutes et 15 minutes après l'injection de la substance. On n'a pas observé de changement significatif de la latence au stade 4 des crises. La CPT à une concentration de 20 µM a augmenté significativement la durée de la postdécharge, la durée du stade 5 des crises et la durée totale des crises et a diminué la latence au stade 4 des crises 5 minutes et 15 minutes après l'injection. Le prétraitement des rats au moyen de la CPT (10 µM) 5 minutes avant l'injection de CHA (100 µM) a atténué l'effet de la CHA sur les paramètres des crises. *Conclusion :* Ceci laisse croire que l'activité des récepteurs A1 de l'adénosine du cortex piriforme a un effet anticonvulsivant sur les crises résultant d'un embrasement par stimulation électrique de la région CA1 de l'hippocampe.

Temporal lobe epilepsy is the most common disorder in adults and can be very difficult to manage in some patients.[1] Kindling is one of the most commonly used animal models of temporal lobe epilepsy which has been used for preclinical evaluation of antiepileptic drugs.[2] Kindling is the process by which repeatedly induced seizures result in an increasing seizure duration and enhanced behavioral involvement of those induced seizures. It is usually carried out by focal electrical stimulation of the brain. With repeated stimulation, seizure duration lengthens and behavior intensifies until these characteristics reach a plateau.[3]

Among different brain regions the piriform cortex and hippocampus are recognized as two important structures involved in the development and control of kindled seizures.[4,5] These two structures are interconnected by reciprocal pathways.[6,7] One of the most characteristic changes occurring during kindling is the increased propagation of the epileptic discharge from the site of stimulation e.g. the hippocampus, to other sites such as piriform cortex, and recruitment of those sites

into the discharge.[2,4] In fact, limbic and/or kindled seizure activity can spread through the hippocampus/piriform cortex circuit[4] and changes in neural activity of the hippocampus may alter the piriform cortex kindled seizures.

Adenosine is commonly accepted as a neuromodulator in the central nervous system,[8,9] and has been considered as an endogenous anticonvulsant agent.[10-12] Adenosine has anticonvulsant effects in different seizure models[11,13,14] and the levels of endogenous adenosine are dramatically elevated in the brain following seizures.[15] As the main anticonvulsant effects of adenosine are exerted through adenosine A_1 receptors,[11,16] it has been suggested that microinjection of selective A_1 receptor agonists into the regions containing these receptors should suppress kindled seizures elicited from other brain regions.

In previous studies, it has been shown that microinjection of selective A_1 receptor agonists into the amygdala,[17] hippocampus,[18] piriform cortex[19] and entorhinal cortex[20] suppresses amygdala kindled seizures. However, we have recently showed that intra-amygdala microinjection of a selective A_1 agonist had no effect on kindled seizures elicited from entorhinal[20] or piriform cortex,[21] suggesting, the anticonvulsant action of adenosine A_1 receptors is pathway specific. Figure 1 indicates a summary of our previous results (adapted from[22]). More studies are needed to provide a complete picture regarding the role of adenosine A_1 receptors in the interaction between different limbic structures during kindled seizures. As Figure 1 shows, activation of hippocampal adenosine A_1 receptors had an anticonvulsant effect on seizures elicited from piriform cortex kindling;[23] but the role of A_1 receptors of piriform cortex neurons on hippocampal kindled seizures, remained to be determined.

Therefore, given the critical role of the hippocampus and piriform cortex in seizure propagation, and the existence of adenosine A_1 receptors in the piriform cortex,[24] in this study we tried to determine the effects of microinjection of selected

adenosine A_1 receptor agonists and antagonists into the piriform cortex on kindled seizures elicited by electrical stimulation of hippocampal CA1 region.

METHODS

Animals

Male Sprague-Dawley rats weighing 300-350 g were housed under 12 hours light/12 hours dark conditions with ad libitum access to food and water. Procedures involving animals and their care were conducted in accordance with the "Guide to the care and use of experimental animals".[25] All experiments were done between 0900 to 1200 hours to avoid the bias of circadian rhythms.

Surgical and kindling procedure

For stereotaxic surgery, the rats were anesthetized using a combination of ketamine (100 mg/kg, [intraperitoneal (i.p.)]) and xylazine (12 mg/kg, i.p.). Animals were implanted with bipolar stimulating and monopolar recording electrodes (twisted into a tripolar configuration) terminating in the hippocampal CA1 region (coordinates: A, -3.6 mm; L: 2.3 mm and 2.1 mm below dura) of the right hemisphere. Two 22-gauge guide cannulae were also implanted in the right and left central piriform cortex (coordinates: A, +0.2 mm; L, 4.8 mm; and 7.6 mm below dura).[26] Electrodes (stainless steel, Teflon-coated, 127 μm in diameter, AM-Systems, USA) were insulated except at the cross section of their tips. Two other electrodes were connected to skull screws, placed above the left skull surface as ground and differential electrodes. One week after surgery, afterdischarge threshold was determined in hippocampus by a 2 seconds (s), 60 Hz monophasic square wave stimulus of 1 milisecond (ms) per wave. The stimulations were initially delivered at 10 μA and then at 5 minutes (min) intervals increasing stimulus intensity in increments of 10 μA until at least 5 s of afterdischarges were recorded as previously described.[18] The animals were then stimulated daily at the afterdischarge threshold intensity until five consecutive stage 5 seizure (fully kindled state) according to Racine scales[27] were elicited. The recorded parameters were: afterdischarge duration, the latency to the onset of stage 4 seizure, stage 5 seizure duration, total seizure duration (duration of convulsion) and seizure stage. Initiation of convulsive movement in the hind limbs or imbalance after rearing were considered the start of stage 5 seizure. When the animals regained hind limb balance again, it was considered the end of stage 5.

Drug administration

N^6-cyclohexyladenosine (CHA; Sigma, Germany), a selective adenosine A_1 receptor agonist and 1,3-dimethyl-8-cyclopentylxanthine (CPT; RBI, USA) a selective adenosine A_1 receptor antagonist were dissolved in artificial cerebrospinal fluid [ACSF (in mM):114 NaCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 1 $CaCl_2$, 3 KCl and 10 glucose]. pH of solutions was adjusted to 7.3-7.4 using NaOH (1 N). The solutions were then sterilized through microfilters (0.2 μm, Minisart, Sartorius, Germany). By means of a microsyringe pump (Stoelting, USA), drugs were infused bilaterally (1 μl over 2 min) via a 27 gauge cannula, which was 1 mm below the tip of a 22 gauge cannula.
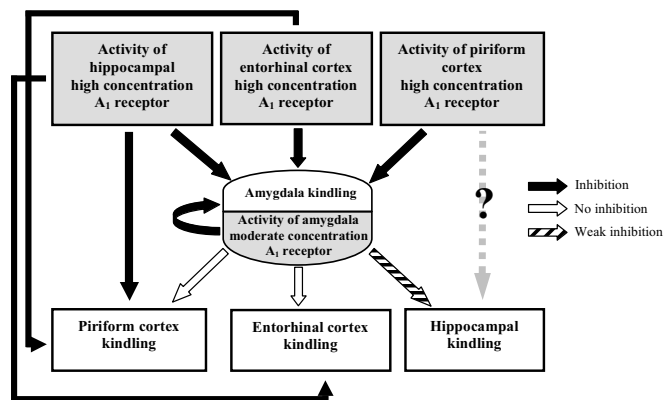

Figure 1: A block diagram drawing of the interaction between activity of A_1 receptors and kindled seizures in amygdala, hippocampus, entorhinal cortex and piriform cortex. As the figure shows, adenosine A_1 receptor anticonvulsant effect is pathway specific (from[22]). The question mark shows the pathway which is under investigation in this research. See text for more details.

A different group of rats was used for each of the drug doses employed and for the different time intervals after drug infusion. Six animals with correct position of electrodes and cannulae were used per group.

Microinjection of CHA or CPT

In different groups of fully kindled rats, CHA (1, 10 and 100 μM) or CPT (10 and 20 μM) was microinjected into the piriform cortex and 5, 15 and 90 min later, animals were stimulated at afterdischarge threshold. In each case, 24 hours before the experiment, animals received ACSF, were stimulated in the same manner and the results recorded as control values. Drug dosages and the stimulation time post-infusion were elected according to our previous studies[18,20,28] and preliminary experiments.

Microinjection of CHA with CPT

The effect of intra-piriform cortex CPT pretreatment upon administration of CHA was investigated. CPT (10 μM) was microinjected 5 min before CHA (10 μM) and the animals were stimulated at 5, 15 and 90 min after the agonist treatment.

Histology

Electrode and cannula locations were determined at the end of the procedure. Each animal was deeply anesthetized with urethane and sacrificed by perfusion-fixation with 10% paraformaldehyde by gravity feed through the left ventricle for 15 min. The brains were removed and sectioned. Coronal sections were cut and examined under microscope for electrode and cannula positions and the presence of any tissue damage. In the case of any abnormality, the data from that particular animal were not included in the results.

Statistical analysis

Results obtained are expressed as the means ± S.E.M. and accompanied by the number of observations. A two-way ANOVA was done to compare different groups of animals at different times post-different doses of A_1 agonist or antagonist injections. Comparison of data from animals receiving a drug with their respective controls was carried out by paired students' t-test. Normalized data (as % of control) were compared by unpaired students' t-test. In all experiments, P<0.05 was considered statistically significant.

RESULTS

All kindled rats responded to electrical stimulation with stable stage 5 seizures in either no infused condition or after vehicle infusion. Infused vehicles did not produce any significant changes in seizure parameters. At the doses employed, drugs did not exert any noticeable effect on behavioral or locomotor activity. Histological assessment indicated that the electrodes and infusion cannulae were positioned in the CA1 region of the hippocampus and piriform cortex.

Effects of intra-piriform cortex CHA or CPT on hippocampal kindled seizures

The inhibitory effects of CHA on seizure parameters were evident five minutes after drug infusion. Different doses of drugs had no significant effect on seizure stage at different time points post infusion. In addition, no significant effect was observed at the dose of 1 μM. At higher doses (10 and 100 μM), intra-piriform cortex CHA led to a significant decrease in the duration of afterdischarges generated from the hippocampus 5 and 15 min after microinjection (Figures 2A and 2B). Likewise, intra-piriform infusion of CHA (10 and 100 μM) resulted in the reduction of stage 5 seizure- and total seizure duration (Figure 2B). However, no significant effect was observed in the latency to the onset of forelimb clonus. All the measured parameters had returned to normal values (baseline) at 90 min post drug administration and thus the observed effects could not be related to tissue damage.

Bilateral microinjection of CPT at the dose of 20 μM, increased the duration of afterdischarge (Figures 3A and 3B), stage 5 seizure and total seizure and decreased the latency to stage 4 seizure significantly (Figure 3B). However, CPT had no effect on seizure parameters at the dose of 10 μM.

Effects of intra-piriform cortex CHA with CPT on hippocampal kindled seizures

CPT (10 μM) pretreatment, five min before CHA (10 μM), attenuated the CHA anticonvulsant effects, and antagonized the effect of CHA on afterdischarge duration, stage 5 seizure duration and total seizure duration (Figure 4). As shown in
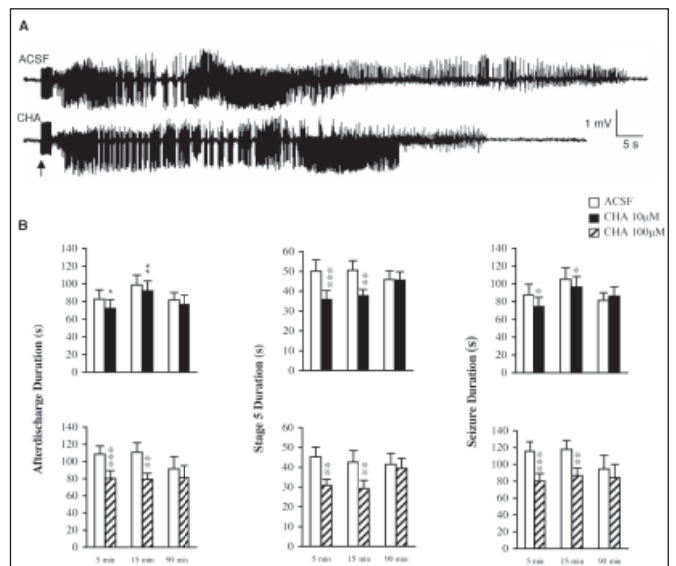

Figure 2: (A) Electrographic examples of afterdischarges taken from hippocampus after bilateral intra-piriform cortex microinjection of artificial cerebrospinal fluid (ACSF; upper trace) and N^6-cyclohexyladenosine (CHA, 100 μM; lower trace) in hippocampal kindled rats. As the records show, a reduction was observed in the duration of afterdischarges after CHA microinjection. The arrow shows stimulation time. (B) Effect of bilateral intra-piriform cortex micro-injection of CHA (10 and 100 μM) on afterdischarge duration, stage 5 seizure duration and seizure duration at five, 15 and 90 min post injection in hippocampal kindled rats (n=6). Values are mean+S.E.M. * P<0.05, ** P<0.01 and *** P<0.001 when compared to the respective artificial cerebrospinal fluid (ACSF) groups.
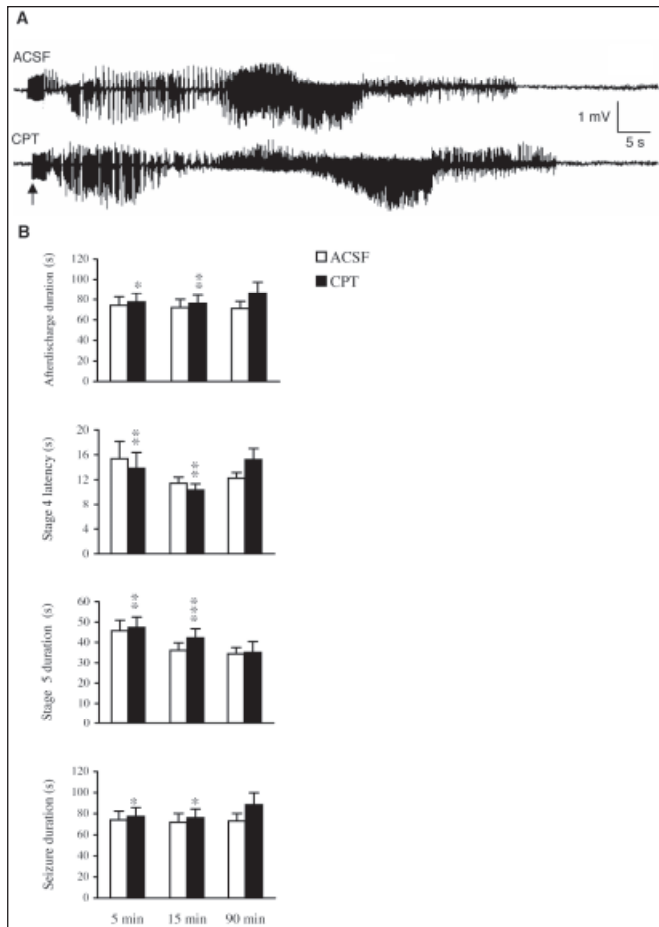
Figure 3: *. (A) Electrographic examples of afterdischarges taken from hippocampus after bilateral intra-piriform cortex microinjection of artificial cerebrospinal fluid (ACSF; upper trace) and 1,3-dimethyl-8-cyclopenthylxanthine (CPT; lower trace) in hippocampal kindled rats. As the records show, an increase was observed in the duration of afterdischarges after CPT microinjection. The arrow shows stimulation time. (B) Effect of bilateral intra-piriform cortex micro-injection of CPT (20 µM) on afterdischarge duration, stage 4 latency, stage 5 seizure duration and seizure duration at 5, 15 and 90 min post injection in hippocampal kindled rats (n=6). Values are mean+S.E.M. * P<0.05, ** P<0.01 and *** P<0.001 when compared to the respective artificial cerebrospinal fluid (ACSF) groups.*

Figure 4, in this group, there was no significant difference in seizure parameters with respect to their related controls.

DISCUSSION

Present results indicated that activation of adenosine A_1 receptors of the piriform cortex is effective in attenuating the seizure activity in hippocampal kindled rats. Anticonvulsant effects elicited from intra-piriform cortex microinjection of CHA are consistent with the previous studies which showed its anticonvulsant effects in different models of epilepsy including kindling.[11,18,20,29] The existence of adenosine A_1 receptors in the

piriform cortex[24] and the antagonism of anticonvulsant effects of intra-piriform cortex CHA by CPT pretreatment, confirmed that in our experiments, the anticonvulsant effects of CHA were mediated through activation of adenosine A_1 receptors.

As previously discussed,[21] the anticonvulsant effect of adenosine A_1 receptors is pathway-specific. For example, intra-amygdala CHA has no significant effect on entorhinal cortex-,[21] piriform cortex-[21] and hippocampal (CA1)- kindled seizure parameters (with the exception of secondary afterdischarges)[29] (Figure 1). Of course, as discussed previously,[22] the absence of anticonvulsant effects of intra-amygdala CHA on kindled seizures cannot be attributed to weak adenosine A_1 receptor activity or low concentration of these receptors in the amygdala, because it has previously been shown that intra-amygdala microinjection of 2-chloroadenosine (an adenosine receptor agonist) at doses as low as 0.25 nM could significantly reduce amygdala afterdischarge duration and other seizure parameters in amygdala-kindled rats.[17,30]

Obtained data could supplement our previous research in which the role of hippocampal A_1 receptors on piriform kindled
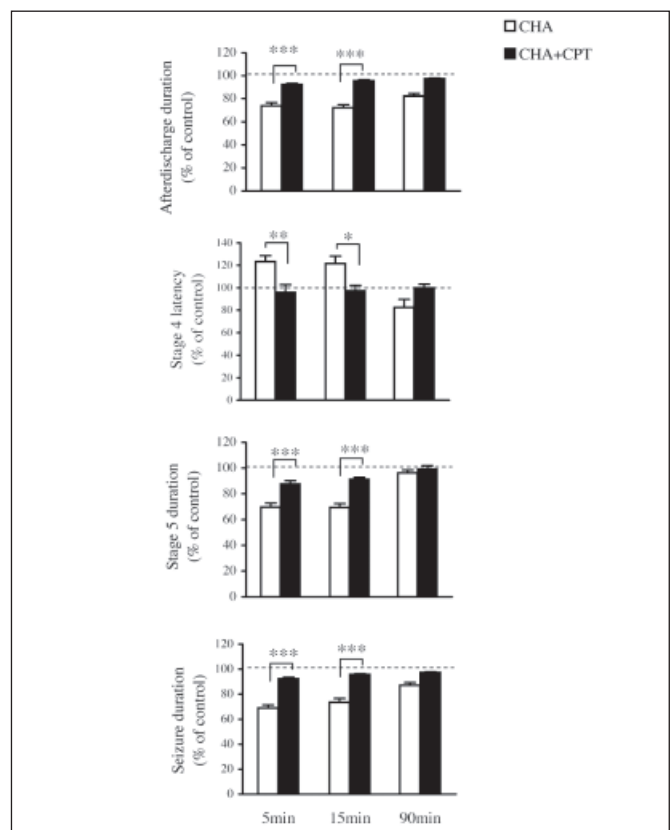

Figure 4: *Effect of bilateral CPT pretreatment on reduction effects of CHA on afterdischarge duration, stage 5 seizure duration and seizure duration. CPT (10µM) was microinjected 5 min before CHA (10 µM) (n=6). Values are mean ± S.E.M. * P<0.05, ** P<0.01 and *** P<0.001 when compared with respective control.*

seizures was investigated.[23] These two studies showed that there is a reciprocal interaction between the hippocampus and piriform cortex in exerting the anticonvulsant effect of adenosine A_1 receptors. However, intra-hippocampal CHA administration had a greater anticonvulsant effect than intra-piriform.

Recently we showed that CHA had an inhibitory effect on synaptic transmission in piriform cortex pyramidal cells *in vivo*.[19] The piriform cortex may contribute to the excitatory mechanisms that underlie the propagation of kindled seizures from the hippocampus[4] and A_1 receptor activation has an inhibitory effect on seizure propagation through this pathway. In this regard, our previous work also showed that intra-hippocampal microinjection of CHA reduces the severity of piriform cortex kindled seizures.[23] In addition, intra-piriform cortex microinjection of CHA significantly reduced the duration of stage 5 seizures used as an index of generalized seizures. The decrease in stage 5 seizure reveals that the piriform cortex may play a role in spreading of epileptic spikes from the hippocampal CA1 region to other brain region(s) and/or relaying of these spikes.

The anticonvulsant effects of CHA were evident at 5 and 15 minutes post infusion. In our previous report,[18] we showed that a lipid insoluble dye (pontamine sky blue) spread over 0.8 mm (diameters) at 5 minutes and 1.2 mm diameter at 15 minutes after microinjection (1 μl/2 min) and thus was restricted to the site of injection. As CHA has low lipid solubility, its effects can be attributed to the activation of adenosine A_1 receptors at the injected site (piriform cortex). At 90 minutes post injection, the drug had no significant effects probably because of its diffusion.

Several mechanisms may account for the anticonvulsant effects of CHA. It has been reported that activation of adenosine A_1 receptors can lead to a reduction in release of glutamate[31,32] which is the excitatory neurotransmitter in piriform cortex afferents.[4,33] Glutamatergic transmission has been shown to be important in kindling and antagonists of the N-methyl D-aspartate (NMDA) receptor are known to suppress kindled seizures.[34,35] In addition, the adenosine A_1 receptor has also been shown to be coupled to specific K^+ channels[36] and the anticonvulsant effects of adenosine could be mediated through the A_1 receptor activation of K^+ channels on postsynaptic membranes.[37] Therefore, the mentioned mechanisms might collectively participate in the expression of anticonvulsant activity of CHA through adenosine A_1 receptors located in the piriform cortex.

CHA was microinjected into the central part of the piriform cortex. It has been shown that among different piriform cortex sub-regions, the central part seems to be involved in the generation of paroxysmal activity produced by kindling. According to previous studies, lesions in the central- but not in the anterior or posterior- piriform cortex retards the development of generalized kindled seizures.[38] In addition, bilateral microinjection of vigabatrin (an irreversible inhibitor of GABA transaminase) into the central piriform cortex retards kindled seizures in rats.[39] Recently, it has also been shown that low frequency stimulation of the central piriform cortex has an anticonvulsant effect on kindling rate.[40] Therefore, our results confirmed the role of central piriform cortex on seizures induced by this kindling model.

In our experiments, intra-piriform cortex CPT increased seizure parameters. This effect has been shown in many other brain areas *in vivo*[11,23,41] and *in vitro*.[42] Similarly, Burdette and Dyer (1987) reported that intrapretoneal administration of caffeine (a non selective adenosine receptors antagonist) prolonged the hippocampal rebound ADs without affecting the primary ADs in hippocampal kindled rats.[43] (This was consistent with our previous study which showed that intra-amygdala microinjection of CHA had an anticonvulsant effect on secondary but not primary hippocampal ADs in hippocampal kindled rats[29]). In the present study intra-piriform cortex microinjection of adenosine receptor antagonist (CPT) also reduced primary hippocampal ADD. (Of course, we did not measure the secondary or rebound ADs). This study suggests that the piriform cortex is among the most effective regions for adenosine anticonvulsant action in hippocampal kindled rats. This indicates that endogenous adenosine may have an antiepileptic activity in the piriform cortex.

On the basis of obtained results, it may be concluded that activation of adenosine A_1 receptors of the piriform cortex has anticonvulsant effects on hippocampal CA1 region kindled seizures. Further work is warranted to determine the role of adenosine A_1 receptors of different brain structures and their interactions in kindled seizures.

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