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THE ROLE OF ADENOSINE A_1 RECEPTORS IN MEDIATING THE INHIBITORY EFFECTS OF LOW FREQUENCY STIMULATION OF PERFORANT PATH ON KINDLING ACQUISITION IN RATS

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Abstract—Low frequency stimulation (LFS) has an inhibitory effect on rapid perforant path kindling acquisition. In the present study the role of adenosine A_1 and A_{2A} receptors in mediating this inhibitory effect was investigated. Rats were kindled by perforant path stimulation using rapid kindling procedures (12 stimulations per day). LFS (0.1 ms pulse duration at 1 Hz, 200 pulses, and 50–150 μA) was applied to the perforant path immediately after termination of each rapid kindling stimulation. 1,3-Dimethyl-8-cyclopenthylxanthine (CPT; 50 μM), a selective A_1 antagonist and ZM241385 (ZM, 200 μM), a selective A_{2A} antagonist were daily microinjected into the lateral ventricle 5 min before kindling stimulations. LFS had an inhibitory effect on kindling development. Pretreatment of animals with CPT reduced the inhibitory effect of LFS on kindling rate and suppressed the effects of LFS on potentiation of population EPSP during kindling acquisition. In addition, CPT was able to antagonize the effects of LFS on kindling-induced increase in early (10–50 ms intervals) and late (300–1000 ms intervals) paired pulse depression. ZM pretreatment had no effect on antiepileptogenic effects of LFS in kindling acquisition. In addition, LFS prevented the kindling-induced elevation of cyclic AMP (cAMP) levels in kindled animals. Based on these results, we suggest that the antiepileptogenic effects of LFS on perforant path kindling might be mediated through activation of adenosine A_1, but not A_{2A} receptors. Moreover, modulation of cAMP levels by LFS may potentially be an important mechanism which explains the anticonvulsant effects of LFS in kindled seizures.

Key words: seizure, brain stimulation, A_1 receptor, A_{2A} receptor, dentate gyrus.

Electrical stimulation for the treatment of pharmaco-resistant epilepsies is now being widely studied both clinically and experimentally (Velasco et al., 2001; Richardson et al., 2003; Goodman et al., 2005). There are a large number of studies showing the antiepileptic effects of low-frequency stimulation (LFS) applied in kindled animals and epileptic humans. Previous studies have demonstrated that in adult and immature animals (Velisek et al., 2002; Yamamoto et al., 2002; Goodman et al., 2005), as well as in patients with mesial temporal lobe epilepsy (Yamamoto et al., 2002), LFS (0.9–3 Hz) could produce inhibitory effects in epileptic activity (Weiss et al., 1995; Velisek et al., 2002; Yamamoto et al., 2002). On the other hand, it has also been shown that high-frequency stimulation exerts a potential efficacy in treatment of patients with epileptic seizures (Boon et al., 2007a,b; Van Roost et al., 2007). However, because of its less neural injury and a smaller amount of energy consumption, LFS has been suggested to be a better alternative therapy for epileptic disorders (Durand and Bikson, 2001).

LFS application depotentiates the synaptic potentiation in the amygdala efferent transmission induced by partial kindling and avoids the changes resulting from the kindling phenomenon in cats and rats (Adamec, 1999; Adamec and Young, 2000). Our previous study also showed that application of LFS during perforant path kindling retarded the kindled seizures development, inhibited the kindling-induced potentiation in perforant path–dentate gyrus synapses and prevented the increase in paired-pulse depression (Mohammad-Zadeh et al., 2007).

The precise antiepileptogenic mechanism of LFS is unknown. Application of LFS depotentiates the basal synaptic transmission after long-term potentiation (LTP) induction (Kulla et al., 1999; Mohammad-Zadeh et al., 2007). Since the kindling shares several features with LTP (Cain, 1989; Mohammad-Zadeh et al., 2007), similar mechanisms may account for the suppressing effect of LFS on LTP (depotentiation) and kindled seizures. In spite of many reports on the mechanism of LFS induced-depotentiation and/or long-term depression (LTD), there are few if any studies showing the mechanisms by which the LFS induces antiepileptic effects during kindling acquisition.

In hippocampal slices, LFS application changes the release of some neurotransmitters and neuromodulators including adenosine (Manzoni et al., 1994; Fujii et al., 2000b). In addition, it has been shown that adenosine derivatives are frequency dependently released as co-transmitters at hippocampal synapses (Schubert et al., 1976; Wieraszko et al., 1989; Cunha, 2001). Kindling and LFS-induced anticonvulsant effects are also frequency dependent phenomena (Gaito, 1980; Gaito et al., 1980; Sato et al., 1990). Thus, endogenous adenosine may contribute to LFS-induced antiepileptic effects in kindled seizures.

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Abbreviations: ACSF, artificial cerebrospinal fluid; cADD, cumulative afterdischarge duration; cAMP, cyclic AMP; CPT, 1,3-dimethyl-8-cyclopenthylxanthine; KLFS, kindled+low frequency stimulation; LFS, low frequency stimulation; LTD, long-term depression; LTP, long-term potentiation; pEPSP, population excitatory post-synaptic potential; PS, population spike; ZM, ZM241385.

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Adenosine is well known to play an important role in the modulation of central synaptic transmission and neuronal excitability (Ribeiro, 1995; de Mendonca and Ribeiro, 1997). Adenosine receptors are classified into A_1 and A_2 (including A_{2A} and A_{2B}) receptors (Daly et al., 1983; Schulte and Fredholm, 2003; van Calker et al., 1978, 1979) which have inhibitory and stimulatory effects on the levels of cyclic AMP (cAMP), respectively (Londos et al., 1980). There is a high concentration of A_1 adenosine receptors in the hippocampus (Fastbom et al., 1987). A_{2A} receptors have also been shown to be present in this region (Sebastiao and Ribeiro, 1992; Cunha et al., 1994).

Adenosine and its derivatives modulate several forms of synaptic plasticity including LTP and depotentiation (de Mendonca and Ribeiro, 1997; Fujii et al., 1999). Regarding the similarities between LTP and kindling-induced potentiation (Cain, 1989), it seems logical to assume a role for adenosine in anticonvulsant effects of LFS. However, the role of adenosine receptors in the depotentiation of LTP is not completely understood. Some investigators have demonstrated a facilitatory role (Fujii et al., 1997), and others showed an inhibitory role for adenosine A1 receptors in depotentiation (de Mendonca et al., 1997). There are also controversial reports on the role of A_2 adenosine receptors in the LFS-induced depotentiation (Fujii et al., 2000b; Huang et al., 1999).

In addition, there are many reports showing that A_1 receptor activation has antiepileptic effects in kindled seizures (Dragunow, 1988; Fredholm, 2003; Gouder et al., 2003; Mohammad-Zadeh et al., 2005). However, controversial results exist about the role of adenosine A_{2A} receptors in these seizures (Adami et al., 1995; Huber et al., 2002; Zeraati et al., 2006; Hosseinmardi et al., 2007). Changes in the concentration of cAMP, which occur following adenosine receptor activation, can also affect the seizure severity. The analogues of the nucleotide, db-cAMP, have been shown to be epileptogenic following intracerebral injection in rats (Kuriyama and Kakita, 1980; Itagaki, 1983).

Therefore, in the present study, we investigated the role of A_1 and A_{2A} receptors in mediating the anticonvulsant effect of LFS on perforant path-kindling acquisition by using selective antagonists of these receptors. Meanwhile, the changes in cAMP, as the main target second messenger of adenosine receptors, were also investigated following LFS application.

EXPERIMENTAL PROCEDURES

Animals

Eighty eight adult male Wistar rats (8–9 weeks old) obtained from Pasteur Institute of Tehran, Iran, were maintained in a colony room kept at a constant temperature on 12-h light/dark schedule. The light phase was started from 7:00 a.m. Animals were individually housed in plastic cages with woodchip bedding and permitted free access to food and water. Efforts were made to minimize the animal suffering and the number of animals used. All studies were performed in accordance with the ethical guidelines set by the "Ethical Committee of School of Medical Sciences, Tarbiat Modares University" that were completely coinciding with the "NIH Guide for the Care and Use of Laboratory Animals." All experiments were done at the same time (8:00 a.m. to 2:00 p.m.) to avoid the bias of circadian rhythms.

Surgical procedures

Under sodium pentobarbital anesthesia (50 mg/kg, i.p.) animals underwent stereotaxic implantation with a bipolar stimulating electrode in the perforant path (coordinates: A, −6.9 mm; L, 4.1 mm; and, V, 2–2.5 mm below dura) and a monopolar recording electrode in the dentate gyrus (coordinates: A, −2.8 mm; L, 1.8 mm; and, V, 2.5–3 mm below dura) of the right hemisphere (according to the atlas of Paxinos and Watson (1985)). Electrodes (stainless steel, Teflon coated, 127 μm in diameter, A.M. Systems, Inc., Carlsborg, WA, USA) were insulated except at their tips. The depth of the recording and stimulating electrodes was adjusted to maximize the population spike (PS) amplitude in the dentate gyrus in response to the perforant-path stimulation. Selective stimulation of the perforant path afferent fibers was confirmed by observing the paired pulse depression in response to paired pulses separated by 30–50 ms and recording the paired pulse facilitation in response to interpulse interval of 70 ms.

A 23-gauge guide cannula was also implanted in the right lateral ventricle (coordinates: A, −0.8 mm; L: ±1.4 mm and 2.6 mm below dura). The incisor bar was set 3.3 mm below interaural line. Stainless steel screws were positioned in the skull above the frontal and occipital cortices and served as reference and ground electrodes. All electrodes were connected to pins of a lightweight multichannel miniature socket as a head-stage and fixed on the skull with dental acrylic. Electrophysiological experiments were done after at least 10 days of recovery.

Stimulation and recording

All the recordings were performed after the rat had been transferred from the home cage to a recording box (30×30×30 cm) in the Faraday cage. The head-stage of the rat was connected to a flexible, shielded cable. The rat was allowed to move freely in the recording box. Evoked responses were collected while the rat was motionless and awake with its eyes open.

Input/output curves

Input/output curves were generated to establish the test intensity used in the subsequent experiments. For input/output tests, single 0.1 ms monophasic square wave pulses were delivered through Nihon Kohden stimulator (Japan, Tokyo) and Nihon Kohden SS-202J constant-current stimulus isolation unit every 10 s and applied at varying intensities (100 μA–800 μA) to the perforant path while the evoked field potentials were monitored in the dentate gyrus. For each time-point, 12 evoked responses were averaged. Both population excitatory post-synaptic potential (pEPSP) slope and PS amplitude were monitored. The PS amplitude was measured by averaging the height from the peak of the pEPSP to the base of PS, as shown in Fig. 4A. By means of input/output curve the maximum PS amplitude was determined for each animal and all potentials employed as baseline criteria were evoked at a stimulus intensity which produced 50% of this maximum response (i.e. test pulse). The measured test pulse for different animals was between 100 and 500 μA. Responses were evoked, amplified, digitized (at 10 kHz) using a PC-based data acquisition system (D3107, World of Science Instruments Co., Tehran, Iran) and custom-designed software, averaged and were continuously monitored and stored on disk.

Rapid kindling procedures

Rapid kindling procedure was done as explained previously (Mohammad-Zadeh et al., 2007; Sadegh et al., 2007). Following 10 days' postsurgical recovery, afterdischarge threshold was deter-

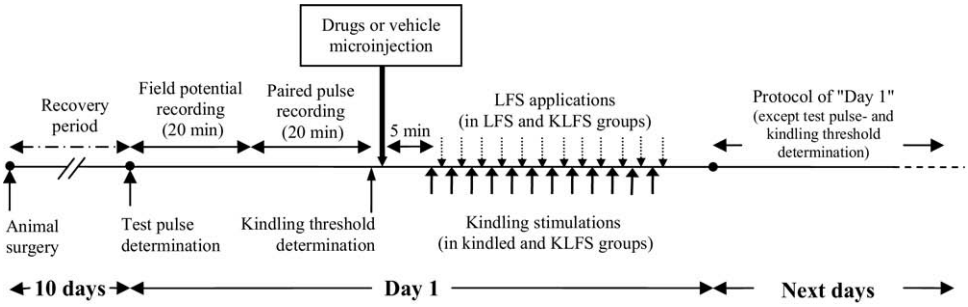

Fig. 1. Time-line diagram showing the experimental protocol used in kindled and KLFS groups.

mined by 1 ms monophasic square wave of 50 Hz with 5 s train duration. The stimulations were initially delivered at 30 μA and then at 5 min intervals increasing stimulus intensity in increments of 10 μA. The minimum intensity sufficient to induce afterdischarges for at least 15 s was designated as the afterdischarge threshold, and used for stimulation. The afterdischarge threshold intensity ranged from 50 to 150 μA. Rats were stimulated 12 times a day with 5 min intervals. The duration of epileptiform afterdischarges and the behavioral progression of kindling (stages 1–5; according to Racine scores (Racine, 1972)) were monitored.

Paired-pulse tests

Following the recovery period, post-treatment paired pulse tests were run every other day. Stimulation intensity (100–500 μA) for the paired pulse tests was set at test pulse and six sweeps were averaged at each of 10 interpulse intervals: 10, 20, 30, 40, 50, 70, 100, 300, 500 and 1000 ms. These intervals were tested randomly, and pulse pairs were separated by 10 s intervals (0.1 Hz). A paired pulse index was determined for each animal by calculating the percent ratio of the second PS (test) to the first (conditioning). Paired-pulse tests were done on the days 1, 3, 5 and 7 during kindling acquisition. On each day, the responses to paired-pulse stimulations were recorded before kindling stimulations.

Drug administration

1,3-Dimethyl-8-cyclopenthylxanthine (CPT; RBI, Natick, MA, USA), a selective A_1 receptor antagonist and ZM241385 (ZM; Tocris Cookson, Bristol, UK), a selective A_{2A} receptor antagonist were dissolved in artificial cerebrospinal fluid (ACSF). ACSF contained (in mM): 114 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 1 $CaCl_2$ and 10 glucose. ZM was dissolved in DMSO and then diluted in ACSF to the desired concentration so that the final concentration of DMSO was 0.1%. The pH of solutions was adjusted to 7.3–7.4 using 1 N NaOH. The solutions were then sterilized through microfilters (0.2 μm, Minisart, NML, Sartorius, Goettingen, Germany). The drugs were microinfused (1 μl over 2 min) 5 min before animal stimulation by means of a microsyringe pump (Stoelting, Keil, WI, USA) via a 30-gauge cannula, which was 1 mm below the tip of 23-gauge cannula.

Measurement of cAMP contents in the dentate gyrus

To prevent the loss of cAMP after decapitation, each rat was killed by cutter 5 min after the last stimulation (Schneider, 1984). Brains were removed rapidly and the dentate gyrus was dissected out according to the method of Glowinski and Iversen (1966) on an ice-cold plate. All brain tissues were stored at −80 °C until assayed. Each tissue was homogenized with cold 6% (w v^{-1}) trichloroacetic acid at two −8 °C to give a 10% (w v^{-1}) homogenate, and centrifuged at 2000×g for 15 min at 4 °C. The supernatant was washed with five volumes of water-saturated diethyl ether four times. The upper ether layer was discarded after each wash. The aqueous extract remaining was dried at 40 °C. Then the dried extract was dissolved in assay buffer. cAMP levels were determined using enzyme-immunoassay kit as described by the manufacturer (Pharmacia Amersham, NJ, USA).

Experimental design

Animals were divided into control, LFS, kindled and kindled+low frequency stimulation (KLFS) groups. In kindled groups animals were stimulated according to rapid kindling protocol and the kindling parameters were recorded. For baseline recording, field potential parameters were recorded every day just before kindling stimulations for 20 min. As mentioned above, paired pulse indices were also determined on days 1, 3, 5 and 7 after field potential recording (Fig. 1). In the KLFS group, animals followed the same protocol but they also received LFS (0.1 ms pulse duration at 1 Hz, 200 pulses, and 50–150 μA) during the intervals between kindling stimulations for 200 s (from 60 s after each kindling stimulation to 40 s before the next stimulation) (Fig. 1). LFS parameters were determined according to our preliminary experiments (Ghorbani et al., 2007; Mohammad-Zadeh et al., 2007). In a few situations LFS was applied during AD recording. However, in these situations ADs were completely recognizable and the LFS induced artifacts (because of their high amplitude and regular shape and frequency) had no interference with measuring the ADD. Animals received only LFS (without kindling stimulation) in the LFS group and received no stimulations in the control group. Both kindled and KLFS groups were then divided into four subgroups: (i) no injection groups (kindled and KLFS) which received no chemicals, (ii) vehicle groups (kindled+vehicle and KLFS+vehicle) that daily received vehicle 5 min before kindling stimulations; (iii) CPT groups (kindled+CPT (50 and 100 μM) and KLFS+CPT(50 μM)) that received CPT 5 min before kindling stimulations every day; (iv) ZM groups (kindled+ZM (200 and 500 μM) and KLFS+ZM (200 μM)) that received ZM 5 min before kindling stimulations daily. The experimental protocol has been shown as timeline in Fig. 1. The stimulation days were continued until the animals showed at least one stage 5 seizure after one of their daily 12 stimulations. The day that a given animal showed stage 5 seizure was considered as the end day of experiment for that animal. The mean number of stimulation days to achieve stage 5 seizure in kindled and kindled+vehicle groups was 7.1±0.6 and 7.8±0.3 respectively (and statistically there was no significant difference between the number of stimulations to reach stage 5 in kindled (10 2.5±14.5) and kindled+vehicle (103.2±12.7) groups). Thus, the 7th day was considered as the end day of experiment in the control and LFS groups. In addition, field potential parameters were also compared during the first 7 days of experiments and at the end day of experiment in different groups. At least six rats were used in each group.

At the end day of experiment in control, LFS, kindled+vehicle, KLFS+vehicle, KLFS+CPT and KLFS+ZM groups, brain sampling was done for cAMP measuring.

Statistical analysis

Data were averaged and expressed as mean±standard error of the mean (S.E.M.) and accompanied by the number of observations. For each time point during the experiment, average and S.E.M. were calculated from the data on 12 (for basal synaptic response experiments) or six (for input/output curve recordings and paired-pulse experiments) successive evoked responses. A mean value of responses at 10 time points on day 1 was defined as the baseline (100%). Subsequent data were expressed as the percent change from the baseline. Two-way repeated measures ANOVA was used to determine changes in cumulative afterdischarge duration (cADD) (the sum of afterdischarges recorded after 12 daily stimulations), pEPSP slope and PS amplitude in experimental groups during different days. Statistically significant differences were evaluated further by a Tukey's post hoc test. Comparing the changes in data of two independent groups was done using a Student unpaired *t*-test. The probability level interpreted as statistically significant was $P<0.05$.

RESULTS

Sixteen rats were eliminated from the study because of incorrect position of electrodes and thus, disruption of their electrophysiological responses during freely-moving records. There was no significant difference between test pulse intensity of different groups on the first day of stimulation. It means that there was no difference in synaptic sensitivity of animals at the beginning of the experiments. In addition, statistical analysis showed that microinjection of vehicles (ACSF or ACSF+DMSO) produced no significant difference in measured parameters. Thus, the effect of CPT- or ZM-receiving animals was compared with their respective vehicle-receiving groups. Of course, as no significant difference was observed between the data after microinjection of these two vehicles, we only showed the data of ACSF as the vehicle in Figs. 3 and 5 to avoid the figure complexity.

Effect of adenosine receptor blockade on the inhibitory effect of LFS on perforant path kindling rate

At the first day of experiments there was no significant difference between afterdischarge thresholds of experimental groups. Thus, there was no variation in seizure susceptibility of different groups at the beginning of the experiments. In kindled+CPT group, microinjection of CPT at the dose of 100 μM decreased cADD during the first 7 days of kindling acquisition [$F_{(6,90)}=7.1$; $P<0.01$] compared with kindled+vehicle group. However, CPT had no significant effect on seizure parameters in kindled+CPT animals at the dose of 50 μM. Thus, in the KLFS+CPT group, CPT was microinjected only at the dose of 50 μM.

As reported previously (Mohammad-Zadeh et al., 2007), LFS application resulted in a significant retardation of kindling acquisition. It prevented the progress in behavioral seizure stages, so that after 7 days the animals of the KLFS+vehicle group still showed only stages 1 and 2 seizure. Statistical analysis showed a significant decrease in seizure stage from the third- to seventh-day of stimulations (i.e. from 36 to 84 stimulations in Fig. 2). CPT microinjection in the KLFS+CPT group significantly decreased the inhibitory effect of LFS on kindling rate and increased the progress in seizure stages compared with the KLFS+vehicle group (Fig. 2).

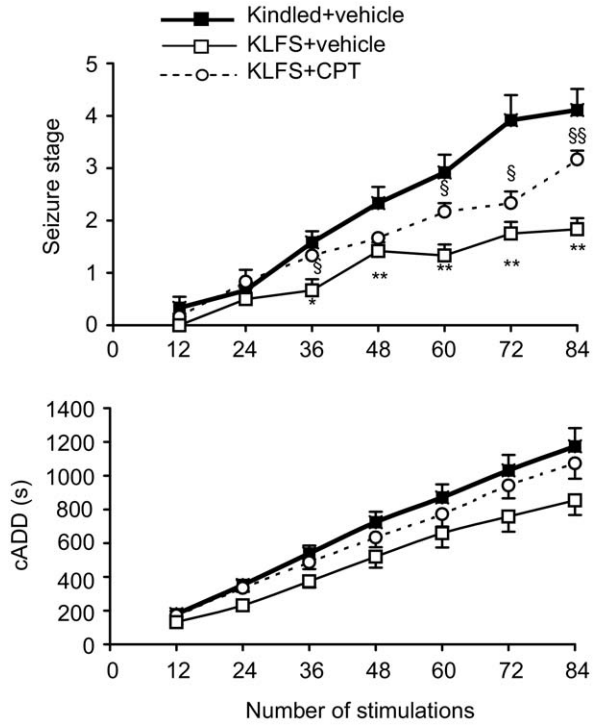

Fig. 2. Changes in seizure stages (upper graph) and cADD (lower graph) during the first 7 days of kindling development. There is a significant decrease in seizure stages from 3rd day (36 stimulations) to 7th days (84 stimulations) of kindling procedure. No significant changes were observed in cADD. Values are mean±S.E.M. ($n=6$). * $P<0.05$ and ** $P<0.01$ when compared with the kindled+vehicle group; § $P<0.05$ and §§ $P<0.01$ when compared with the KLFS+vehicle group.

On the other hand, there was not any significant change in cADD during the first 7 days of kindling development in kindled+vehicle and KLFS+vehicle groups [$F_{(12,162)}=1.7$; $P=0.06$]. When animals exhibited the stage 5 seizure, cADD was increased in KLFS+vehicle-treated rats compared with the kindled+vehicle group. This was evident by showing that these animals needed more stimulations to show stage 5 seizure. However, we were not able to show a statistically significant difference between the groups using one-way ANOVA ($F_{(2,27)}=3.2$, $P=0.06$) (Fig. 3A).

We also compared the average number of stimulations required to reach different seizure stages in different groups. A two-way ANOVA showed that it was significantly increased in the KLFS+vehicle group compare with the kindled+vehicle group ($P<0.001$) (Fig. 3B). Here, CPT significantly decreased the inhibitory effect of LFS on kindling rate and reduced the average number of stimulations required to reach seizure stages 2–5 compared with the KLFS+vehicle group (Fig. 3B).

ZM had a significant inhibitory effect on kindling rate when microinjected at the dose of 500 μM in the kindled+ZM group. It decreased cADD during the first 7 days of kindling acquisition [$F_{(6,\ 96)}=2.8$; $P<0.05$] and increased the average number of stimulations required to reach different seizure stages [$F_{(4,\ 64)}=39.6$; $P<0.001$]

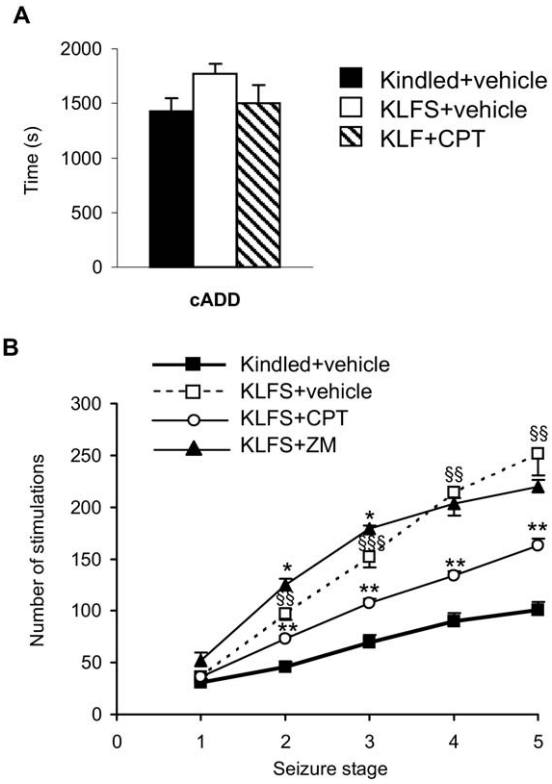

Fig. 3. (A) Changes in cADD after achieving a stage 5 seizure. Although cADD was greater in the KLFS+vehicle than the kindled+vehicle group and microinjection of th CPT (KLFS+CPT) group removed the effect of LFS, statistical analysis showed no significant difference between the three groups (n=6). (B) Effect of CPT and ZM microinjection on the anticonvulsant effect of LFS on the number of stimulations needed to achieve different seizure stages. There is a significant increase in the number of stimulations to seizure stages 2–5 in the KLFS+vehicle compared with the kindled+vehicle group. While the microinjection of CPT (KLFS+CPT group) decreased the effect of LFS on the number of stimulations, microinjection of ZM (KLFS+ZM group) increased the LFS effect. (n=6). Values are mean±S.E.M. §§ $P<0.01$ and §§§ $P<0.001$ when compared with the kindled+vehicle group and * $P<0.05$ and ** $P<0.01$ when compared with the KLFS+vehicle group (KLFS+CPT and KLFS+ZM groups did not compare with the kindled+vehicle group).

compared with its related control group (kindled+vehicle). However, ZM had no significant effect on seizure parameters when microinjected at the dose of 200 μM. Therefore, to investigate the effect of A_{2A} receptor blockade on anticonvulsant effects of LFS, ZM was microinjected in the KLFS+ZM group at the dose of 200 μM. Here, ZM not only had no decreasing effect on LFS anticonvulsant action, but also increased the average number of stimulations required to reach the seizure stages 2 and 3 compared with the KLFS+vehicle group (Fig. 3B). It had also no significant effect on other parameters of kindling rate.

Effect of adenosine receptors blockade on inhibitory effect of LFS on field potential recordings

The basal synaptic transmission was potentiated during kindling acquisition. In the kindled+vehicle group, there was a potentiation in synaptic transmission during the first 7 days of kindling procedure. There was a 72.3±2.7% increase in pEPSP and 74.9±1.8% increase in PS amplitude (Fig. 4A, B). The potentiation effect of kindling on basal synaptic transmission was significantly suppressed by LFS application. In the KLFS+vehicle group, no significant change was observed in field potential recorded from the dentate gyrus during7 days of kindling procedure (there was a 4.8±2.4% and 3.4±2.3% increase in pEPSP and PS amplitude respectively) (Fig. 4A, B).

CPT (50 μM) microinjection significantly suppressed the inhibitory effect of LFS on pEPSP slope [$F_{(12,\ 162)}$=32.6; $P<0.001$] and PS amplitude [$F_{(12,\ 162)}$=41.9; $P<0.001$]. As Fig. 4B shows, during the first 7 days of kindling procedure there was 35.4±1.1% increase in pEPSP and 31.3±1.1% increase in PS amplitude in KLFS+CPT group. (Compare with the respective data in the KLFS+vehicle group.)

Field potential parameters were also compared after animals achieved a stage 5 seizure (i.e. the day that the given animals showed stage 5 seizure at least one time). In the kindled+vehicle group, the increased percentage of pEPSP slope and PS amplitude (compared with day 1) was 74.1±5.3 and 81.4±4.0 respectively. However, in the KLFS+vehicle group the percent of increase in the pEPSP slope was 47.0±3.9 and in PS amplitude was 60.9±19.7 (Fig. 4C). Although both groups showed a significant increase in these parameters, statistical analysis of the data using one-way ANOVA and Tukey's post hoc test showed that the increase in percentage of pEPSP slope was significantly lower in the KLFS+vehicle group compared with the kindled+vehicle group ($F_{(2,\ 27)}$=7.5; $P<0.05$). There was no significant difference in the PS amplitude between the groups ($F_{(2,\ 27)}$=0.2; P=0.58)). Here again the CPT decreased the prevention effect of LFS after achieving the stage 5 seizure. The increased percentages of the field potential parameters in KLFS+CPT (54.3±3.3% in pEPSP and 76.7±4.2% in PS amplitude) were greater than in the KLFS+vehicle group and there was no significant difference between KLFS+CPT and kindled+vehicle groups (Fig. 4C).

On the other hand, a two-way ANOVA showed that ZM (200 μM) did not change the inhibitory effect of LFS on pEPSP slope and PS amplitude during the first 7 days of stimulations. There was no significant change in field potential parameters between KLFS+vehicle and KLFS+ZM groups. In addition, in the kindled+ZM group ZM (200 μM) had no effect on seizure and field potential parameters compared with the kindled+vehicle group.

Effect of adenosine receptors blockade on inhibitory effect of LFS on paired pulse measurements

There was not any significant variation between the conditioning responses in different experiments. Paired pulse indices were measured based upon PS amplitude. As it has been reported previously (de Jonge and Racine, 1987; Maru and Goddard, 1987; Gilbert, 1991; Mohammad-Zadeh et al., 2007) kindling produced an increase in early and late paired pulse depression and a decrease in paired pulse facilitation during the first 7 days of kindling acquisition (Fig. 5A). These effects were prevented by LFS application in KLFS+vehicle (Fig. 5B).

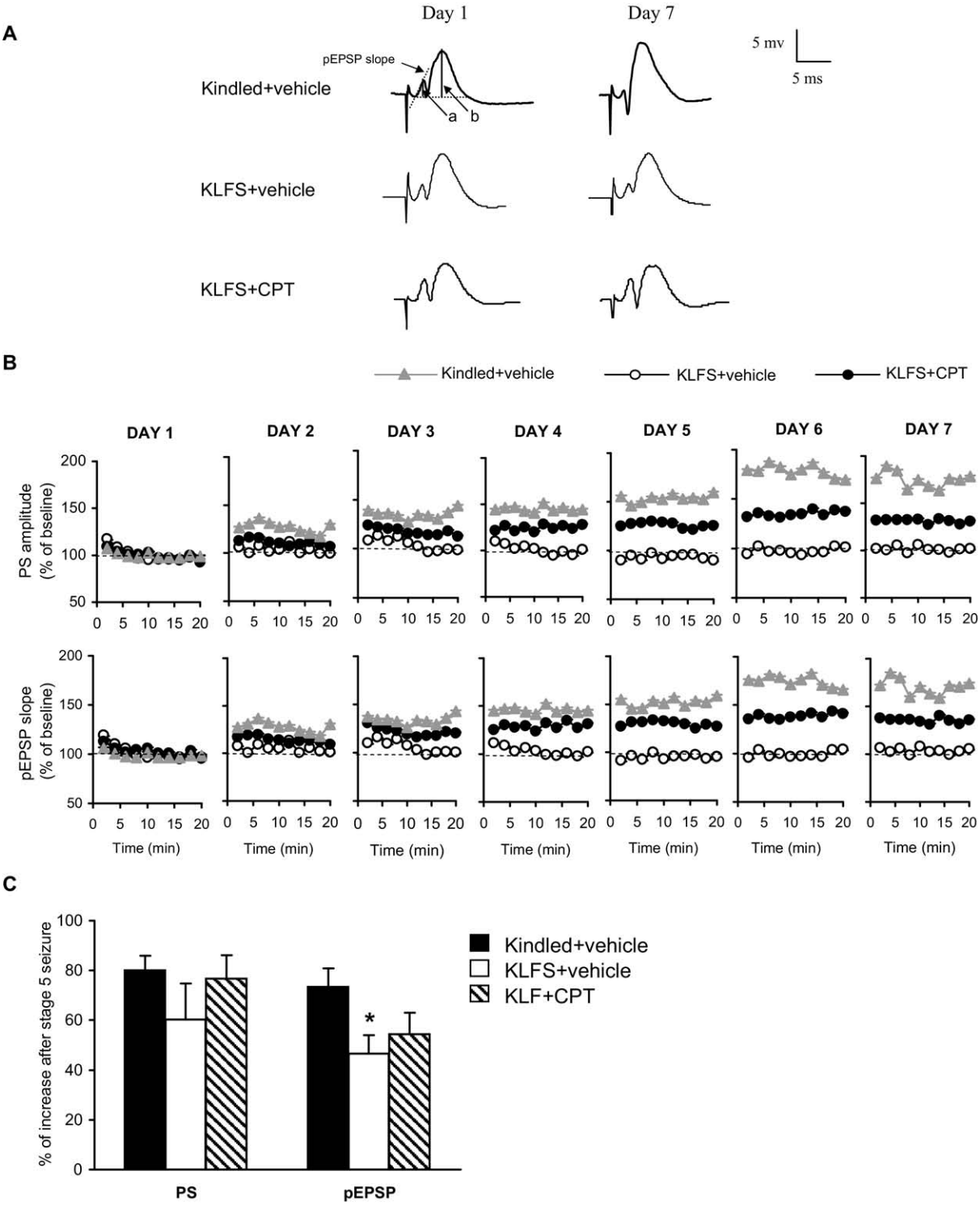

Fig. 4. (A) Sample averages of 12 wave-forms, taken on days 1 and 7 in the kindled+vehicle, KLFS+vehicle and KLFS+CPT groups. Note the changes in PS amplitude in different groups. The calculation of pEPSP slope and PS amplitude is depicted. The slope of pEPSP was determined at a fixed interval beginning approximately 1.0 ms after response onset and continuing within approximately 0.5 ms before PS onset. PS amplitude was calculated by averaging the height from the peak of the pEPSP to maximum downward deflection of the PS. In the figure the PS amplitude is (a+b)/2. (B) Time-course diagrams showing the changes in PS amplitude and pEPSP slope in the kindled+vehicle (n=8), KLFS+vehicle (n=6) and KLFS+CPT (n=6) groups during the first 7 days of kindling acquisition. Each graph shows 20 min baseline perforant path–evoked responses recorded before kindling stimulation on different days. Application of LFS inhibited kindling induced potentiation (reveals as increase in PS amplitude and pEPSP slope in the kindled+vehicle group). Microinjection of CPT in the KLFS+CPT group reduced the inhibitory effect of LFS on kindling-induced potentiation. (C) Effect of CPT (50 μM) microinjection on the inhibitory effect of LFS on PS amplitude and pEPSP slope after achieving a stage 5 seizure. The % of increase in parameters in the kindled+vehicle was greater than in the KLFS+vehicle group (n=6). Microinjection of CPT in the KLFS+CPT group (n=6) decreased the inhibitory effect of LFS. Values are mean±S.E.M. * P<0.05 when compared with the kindled+vehicle group (n=6).

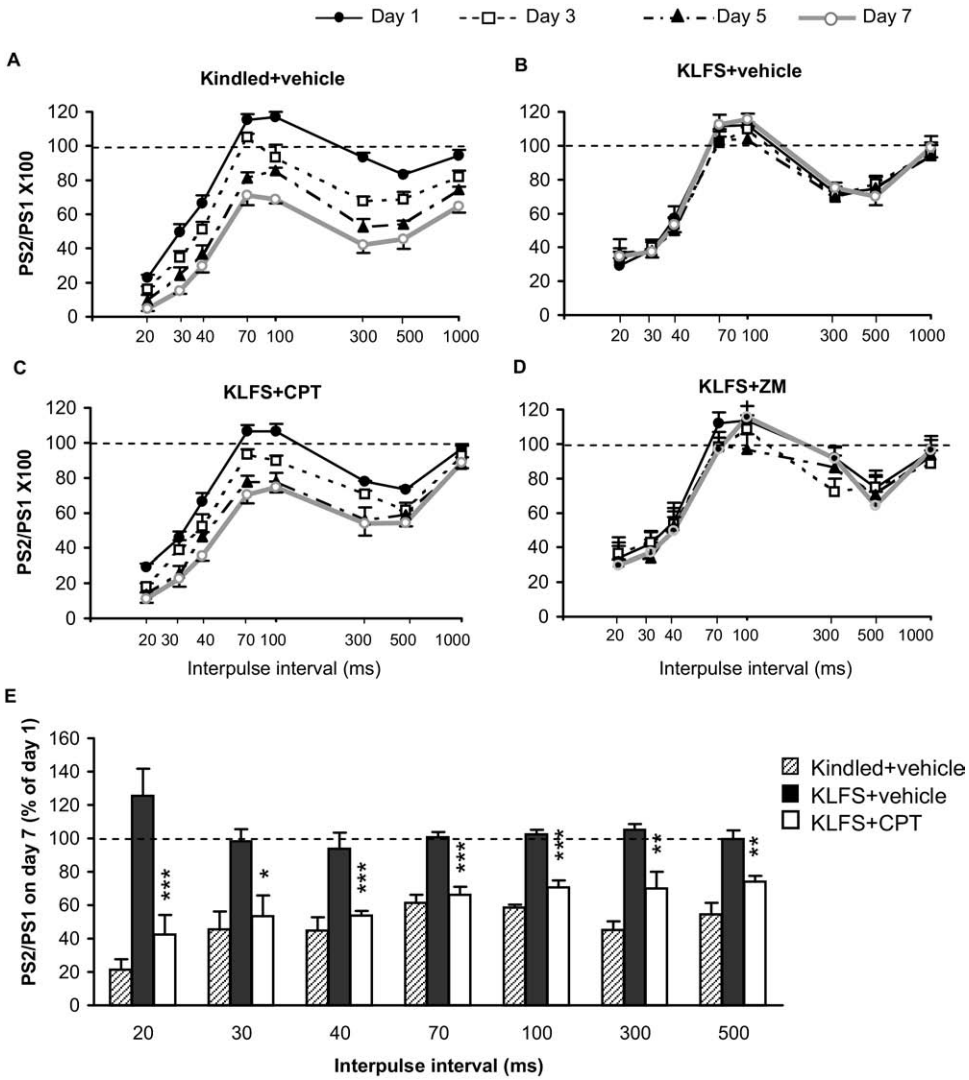

Fig. 5. Plot of paired pulse index (second PS/first PS×100) values obtained for different interpulse intervals in the kindled+vehicle (A; $n=6$), KLFS+vehicle (B; $n=6$), KLFS+CPT (C; $n=6$) and KLFS+ZM (D; $n=6$) groups. LFS application prevented an increase in early and late paired pulse depression and also a decrease in paired pulse facilitation (compare A and B). Microinjection of CPT in the KLFS+CPT group decreased the inhibitory effect of LFS (C). Microinjection of ZM in the KLFS+ZM group had no effect on the inhibitory effect of LFS (D). The percentage changes of the paired pulse indices on day 7 (relative to day 1), at six selected interpulse intervals in the kindled+vehicle, KLFS+vehicle and KLFS+CPT groups have been shown in part E. As the figure shows, a significant decrease was observed in paired pulse indices in the kindled+vehicle group. LFS application (KLFS+vehicle group) prevented this change, but microinjection of CPT (KLFS+CPT group) reduced the LFS effects; thus, a significant difference was observed between KLFS+vehicle and KLFS+CPT groups at all interpulse intervals. Values are mean±S.E.M., * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ when compared with the KLFS+vehicle group.

Microinjection of CPT (50 μM) in the KLFS+CPT group suppressed the inhibitory effect of LFS on potentiating of both early and late paired pulse depression during the first 7 days of the kindling procedure. CPT also suppressed the inhibitory effect of LFS on the kindling-induced decrease in paired pulse facilitation (Fig. 5C). Fig. 5E shows the percentage changes of paired pulse indices at the 7th day with respect to the 1st day. In the kindled+vehicle group, this parameter was decreased at all interpulse intervals significantly. At interpulse intervals equal to 20, 30, 40, 300 and 500 ms this decrease is interpreted as potentiation of paired pulse depression, however, at interpulse intervals of 70 and 100 ms this decrease shows a reduction in paired pulse facilitation. At the day 7, this parameter was near 100% of the 1st day in the KLFS+vehicle group. It means that no change was produced in paired pulse indices at different interpulse intervals during 7 days and LFS prevented the kindling-induced changes in paired pulse indices. In the KLFS+CPT group the data were similar to those of the kindled+vehicle group and LFS could not prevent the changes in paired pulse indices. Of course, CPT application had no significant effect on paired pulse indices in the kindled+CPT group.

Microinjection of ZM (200 μM) in the KLFS+ZM group did not alter the effects of LFS on kindling-induced

Table 1. Changes in cAMP content of the dentate gyrus in different groups

Groups (n=4)	cAMP (pmol/g)
Control	130±8.1
LFS	87±4.2**
Kindled+vehicle	254.6±6.3***
KLFS+vehicle	171.8±8.4**†
KLFS+CPT	197.7±7.1§
KLFS+ZM	101.0±4.3§§§

Values are mean±S.E.M.
** $P<0.01$ and *** $P<0.001$ compared to control group.
§ $P<0.05$ and §§§ $P<0.001$ compared to KLFS+vehicle.
† $P<0.001$ compared to kindled+vehicle.

changes on paired pulse indices during the first 7 days of the kindling procedure (Fig. 5D). It had also no significant effect in the kindled+ZM group.

When the animals of the KLFS+vehicle group reached stage 5 seizure, paired pulse indices were changed similar to those of the kindled+vehicle group (i.e. paired pulse depression increased and paired pulse facilitation decreased). In this situation, there was no significant difference between the kindled+vehicle and KLFS+vehicle group.

cAMP assay

In the kindled+vehicle and KLFS+vehicle groups, the cyclic AMP content of the dentate gyrus was increased after achieving a fully kindled state compared with the control group. However, in the KLFS+vehicle group this increase was significantly lower than that of kindled+vehicle animals (Table 1). It means that LFS prevented the kindling-induced increase in cAMP. It has to be noted that application of LFS alone had also a decreasing effect on cAMP content.

The cAMP content was also measured in KLFS+CPT and KLFS+ZM groups to show that effects of CPT and ZM may be explained according to changes induced in cAMP concentration. As was expected previously, microinjection of CPT increased while ZM decreased the cAMP in the mentioned groups compared with the KLFS+vehicle group (Table 1). However, because no significant changes were observed in kindled groups after application of these drugs, cAMP did not measure in these groups.

DISCUSSION

Results of the present study showed that activation of adenosine A_1 receptors has a role in mediating the anticonvulsant effects of LFS on perforant path kindling acquisition so that microinfusion of an adenosine A_1 receptor antagonist (CPT) suppressed this inhibitory effect of LFS. A summary of obtained results has been shown as a block diagram in Fig. 6.

There are many studies showing the anticonvulsant effects of adenosine A_1 receptors in different laboratory models of epilepsy including kindling (Dragunow, 1988; Fredholm, 2003; Gouder et al., 2003; Mohammad-Zadeh et al., 2005). In addition, adenosine and its derivatives modulate several forms of short-term and long-term activity dependent synaptic plasticity (de Mendonca et al., 1997). For example, it has been shown that an adenosine receptors agonist, 2-chloroadenosine, decreases LTP at Schaffer collateral–CA1 synapses (Arai et al., 1990; Dolphin, 1983). Activation of adenosine receptors also has a role in suppressing effect of LFS on LTP (depotentiation). Considering the similarities between the kindling phenomenon and LTP (Cain, 1989; Mohammad-Zadeh et al., 2007), similar mechanisms may account for LFS induction of depotentiation and the anticonvulsant mechanisms of LFS on kindled seizures. However, the precise role of adenosine in depotentiation of LTP is controversial at present time. de Mendonca et al. (1997) have reported that endogenous adenosine, acting through A_1 adenosine receptors, is able to limit depotentiation in the hippocampus. In contrast, in another study, an A_1 adenosine receptor

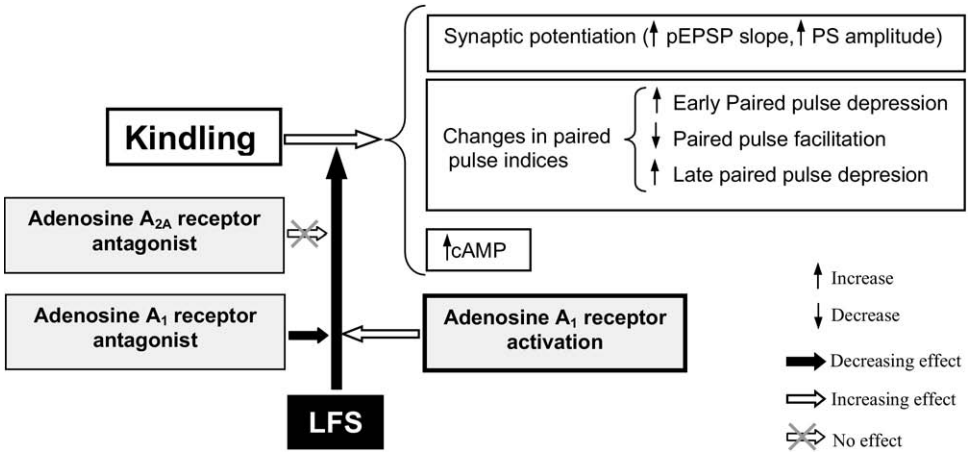

Fig. 6. A block diagram drawing of the anticonvulsant effect of LFS on kindling-induced changes in synaptic activity and cAMP concentration in the dentate gyrus during rapid perforant path kindling. The figure has been planned according to the results of the present study. LFS application prevents a) the kindling-induced-potentiation in synaptic activity, b) kindling-induced changes in paired-pulse indices and c) kindling-induced increase in cAMP concentration. Adenosine A_1, but not A_{2A}, receptor activity has a role in mediating these effects of LFS. See text for more details.

antagonist, 8-cyclopentyltheophylline, has been shown to inhibit the depotentiation in hippocampal CA1 neurons (Fujii et al., 1997). Our results are similar to studies reported that A_1 adenosine receptor activation is necessary for depotentiation (Fujii et al., 1997; Huang et al., 1999).

Similar to our previous report (Mohammad-Zadeh et al., 2007), application of LFS in this study retarded the kindled seizures acquisition. It increased the number of stimulations to reach different seizure stages. In addition, LFS prevented kindling-induced increase in the pEPSP slope and PS amplitude in the dentate gyrus. It has to be noted that while LFS had an antiepileptogenic effect on kindling rate and increased the number of stimulations required to reach stage 5 seizure, the animals were eventually kindled. It shows that LFS application failed to prevent kindling-induced changes in neural activity completely. Thus these data can be interpreted as the probable mechanism of this partial anticonvulsant effect of LFS.

Microinfusion of an adenosine A_1 receptor antagonist (CPT) suppressed these inhibitory effects of LFS on perforant path kindling, so that in the KLFS+CPT group the seizure parameters were shifted from the KLFS+vehicle to the kindled+vehicle group. It means that adenosine A_1 receptors may be involved in the inhibitory effect of LFS during kindling (Fig. 6).

In addition, LFS application suppressed potentiation of early and late paired pulse depression in the dentate gyrus. A_1 adenosine receptor blockade (by CPT) significantly reduced theses effects of LFS (Figs. 5 and 6). Early paired pulse depression involves a recurrent feedback inhibition of granule cells through $GABA_A$ receptors (Adamec et al., 1981; Tuff et al., 1983) and late paired pulse depression reflects a feedforward inhibition (Alger, 1984; Burdette and Masukawa, 1995) through activation of $GABA_B$ receptors and/or calcium dependent afterhyperpolarization (Thalmann and Ayala, 1982; Alger, 1984). Thus, LFS needs A_1 adenosine receptor activation to apply its inhibitory effects on kindling-induced potentiation of GABAergic transmission in the dentate gyrus. Of course, potentiation of GABAergic system during kindling, which has been also reported previously (de Jonge and Racine, 1987; Maru and Goddard, 1987), may be a compensatory mechanism against synaptic potentiation during kindling (Gutierrez, 2000; Maru et al., 2002; Mohammad-Zadeh et al., 2007) or may be involved in synchronization of neural cells for generating of ADs during seizure (Velazquez and Carlen, 1999; Higashima et al., 2000).

A possible interpretation to explain how A_1 adenosine receptor activation may be involved in the inhibitory effect of LFS on potentiation of GABAergic neurotransmission, is according to the inhibitory effect of A_1 adenosine receptors on GABA release (Yang et al., 2004; Jeong et al., 2003; Saransaari and Oja, 2005). Therefore, in the present study, LFS could not prevent the GABA release after CPT microinjection, i.e. blockade of A_1 adenosine receptors (note the similarity between the KLFS+CPT and kindled+vehicle groups in Fig. 5).

We also studied the effect of A_{2A} adenosine receptor antagonist on the anticonvulsant effect of LFS during kindling acquisition. A_{2A} adenosine receptor blockade by ZM microinjection could not prevent the inhibitory action of LFS on synaptic activity (pEPSP slope and PS amplitude) and paired pulse indices during kindling acquisition. The low density of A_{2A} adenosine receptors in the hippocampal formation (Haas and Selbach, 2000) compared with the high density of A_1 receptors (Fredholm et al., 2001; Ribeiro et al., 2003), may be considered as a possible interpretation to explain the effectiveness of CPT and ineffectiveness of ZM on LFS anticonvulsant action. We have recently shown that LFS application during perforant path kindling acquisition can decrease the A_{2A} receptor gene expression, in addition to preventing the decrease in A_1 receptor gene expression (unpublished observations).

On the other hand, microinjection of ZM (at the dose of 200 μM that had no significant effect on kindling rate) in the KLFS+ZM group increased the number of stimulations needed to achieve the seizure stages 2 and 3 compared with the KLFS+vehicle group. Considering the probable excitatory role of A_{2A} receptors on kindled seizures (Zeraati et al., 2006; Hosseinmardi et al., 2007), antagonizing of these receptors can decrease their excitatory effect and this can be added to the inhibitory effect of LFS. Thus, an increase in the anticonvulsant effect of LFS was observed in the KLFS+ZM group. It showed that activation of A_{2A} adenosine receptors may exert a weak inhibitory effect on anticonvulsant actions of LFS.

It has to be noted that some investigators have shown that activation of A_2 adenosine receptors exerts a facilitation effect on the synaptic components the depotentiation (Fujii et al., 1992, 2000a). It shows that despite similarities between kindling and LTP (Cain, 1989), the mechanisms of suppressing effect of LFS on kindling are not completely the same as mechanisms involved in depotentiation. Of course, there are also some reports showing that A_{2A} adenosine receptor activity has no role in depotentiation (Huang et al., 1999).

Our results showed that LFS application suppressed the kindling-induced increase in cAMP content in the dentate gyrus (Fig. 6). This effect was similar to previous studies in which application of forskolin, an adenylyl cyclase activator, during LFS application suppressed depotentiation in brain slices (Huang et al., 1999; Chen et al., 2001). In addition, application of LFS alone decreased cAMP content in the dentate gyrus.

cAMP has been shown to be epileptogenic following intracerebral injection in rats (Kuriyama and Kakita, 1980; Itagaki, 1983). In epileptic patients, the cAMP concentration in the cerebrospinal fluid is also elevated after an attack (Myllyla et al., 1975). However, it has remained unclear whether this cAMP elevation is a consequence or a cause of seizures.

The effect of CPT and ZM microinjection during LFS application on the cAMP was according to their effects on blocking the activity of proteins G_i and G_s, respectively. In KLFS+CPT, blocking the A_1 adenosine receptors prevented the decrease in cAMP. On the other hand, suppressing the A_{2A} adenosine receptors in KLFS+ZM group decreased the cAMP content in the dentate gyrus com-

pared with KLFS+vehicle. Thus, it may be postulated that an increase in the ratio of G_i/G_s protein activation has an important role in anticonvulsant action of LFS. Our previous study also showed that type 1 galanin receptor (GalR1), that activates G_i signaling pathway, has a role in mediating the anticonvulsant effects of LFS during perforant path kindling (Sadegh et al., 2007). More experiments are needed to determine the role of G proteins in LFS anticonvulsant actions. In addition, with regard to the evidence that galanin also plays a role in the antiepileptogenic effect of LFS, it should be emphasized that the effects of LFS are likely mediated through several systems.

It has also to be considered that there is an interaction between A_1 and A_{2A} adenosine receptors. It has been shown that a decrease in affinity of A_1 receptors induces A_{2A} receptor activation and the attenuation of A_1 responses causes A_{2A} receptor activation (Lopes et al., 1999).

Therefore, according to the present results it may be postulated that activation of adenosine A_1 but not A_{2A} receptors is involved in the inhibitory effect of LFS during kindling acquisition. The inhibitory effect of LFS on kindling-induced enhancement of cAMP levels in the dentate gyrus, which is consistent with the activation of adenosine A_1 receptors, may be considered as an important mechanism of LFS anticonvulsant effect. It has to be noted that only the antiepileptogenic effect of LFS during kindling acquisition was investigated in the present study, and it remains to be determined if the effect of LFS on fully kindled seizure is also mediated through the adenosine system.

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