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Long-term Effect of Convulsive Behavior on the Density of Adenosine A_1 and A_{2A} Receptors in the Rat Cerebral Cortex

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Summary: *Purpose:* Adenosine is a neuromodulator that has been proposed to act as an anticonvulsant mainly via inhibitory A_1 receptors, but recent data show that genetic deletion of facilitatory A_{2A} receptors might also attenuate convulsions. Since both A_1 and A_{2A} receptors are prone to down- and upregulation in different stressful situations, we investigated if convulsive behavior leads to a long-term change in A_1 and A_{2A} receptor density in the rat cerebral cortex.

Methods: Stage 4-5 convulsions (Racine's scale) were induced in adult Wistar rats either through amygdala stimulation (kindling) or by intraperitoneal injection of kainate (10 mg/ml). Rats were killed after 4 weeks to evaluate adenosine A_1 and A_{2A} receptor density in the cerebral cortex using both Western blot and membrane binding assays.

Results: The binding density of the A_1 antagonist, ^{3}H-DPCPX, decreased by 40. ± 4.4% and by 20.7 ± 0.5% after kindling or kainate injection. Likewise, A_1 receptor immunoreactivity in cortical membranes from kindled or kainate-injected rats decreased by 19.1 ± 3.3% and 12.7 ± 5.7%, respectively. In contrast, the binding density of the A_{2A} receptor antagonist ^{3}H-SCH 58261 increased by 293 ± 34% and by 159 ± 32% in cortical membranes from kindled or kainate-injected rats, and A_{2A} receptor immunoreactivity also increased by 151 ± 12% and 79.6 ± 7.0%.

Conclusions: This indicates that after convulsive behavior there is a long-term decrease of A_1 receptors accompanied by an increased density of A_{2A} receptors, suggesting that A_{2A} antagonists rather than A_1 agonists may be more promising anticonvulsive drugs. **Key Words:** Adenosine—Cortex—Kindling—Kainate—Epilepsy—A_1 receptor—A_{2A} receptor.

Adenosine is a ubiquitous neuromodulator that mainly inhibits synaptic transmission and neuronal excitability through activation of the predominant adenosine A_1 receptors (1). The ability of adenosine to selectively depress glutamatergic excitatory pathways makes A_1 receptors interesting anticonvulsant targets [reviewed in (2)]. Accordingly, exogenous administration of A_1 receptor agonists attenuates seizures, and the use of A_1 receptor antagonists has proconvulsant effects [reviewed in (2)]. It has even been proposed that the loss of the A_1 receptor-mediated control of glutamatergic function could contribute to the implementation of epileptic status (3). However, conflicting results have been reported in relation to the impact of convulsive behavior on cortical A_1 receptors [reviewed in (4)]. In other brain regions, chronic stressful situations, such as amygdala kindling (5) or Alzheimer's disease (6), decreased the density of A_1 receptors.

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Adenosine can also activate another less abundant adenosine receptor subtype, i.e., A_{2A} receptors, with effects generally opposite to these mediated by A_1 receptors (7). These facilitatory A_{2A} receptors are most abundant in the basal ganglia and have a density 20 times lower in the cerebral cortex (8). However, despite their low abundance, and by mechanisms still to be resolved, pharmacological blockade or genetic inactivation of these A_{2A} receptors confer robust neuroprotection in the limbic and neocortex in different noxious brain situations [reviewed in (9)]. In particular, genetic inactivation of A_{2A} receptors decreases ethanol withdrawal-induced seizures (10), suggesting a role for A_{2A} receptors also in the control of convulsive behavior. Interestingly, studies in nonbrain preparations have documented that prolonged stressful situations, such as hypoxia (11) or exposure to cytokines (12), enhance the expression and density of these A_{2A} receptors, but this has not yet been documented in brain tissue.

Therefore, there is ground to consider both A_1 and A_{2A} receptors as possible targets for the development of anticonvulsants. However, since noxious situations lead to

changes in the density of both A_1 and A_{2A} receptors, it appears necessary to determine if the occurrence of seizures might also lead to changes in A_1 and A_{2A} receptor densities. Thus, this study was designed to investigate the long-term changes of the densities of A_1 and A_{2A} receptors in the cerebral cortex using two different strategies to induce episodic convulsive behavior: amygdala kindling (13) and intraperitoneal injection of kainate (14).

METHODS

Reagents

^{3}H-1,3-Dipropyl-8-cyclopentyladenosine (^{3}H-DPCPX, specific activity 109.0 Ci/mmol) was from DuPont NEN (Anagene, Portugal), and ^{3}H-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*] pyrimidine (^{3}H-SCH 58261, specific activity 77 Ci/mmol) was prepared by Amersham (Buckinghamshire, U.K.) and was a generous gift of Dr. Ennio Ongini (Shering-Plough, Milan, Italy). Kainate and adenosine deaminase (calf intestine suspension, 2000 U/ml, EC 3.5.4.4) were from Sigma (Reagente 5, Oporto, Portugal), 8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl}xanthine (XAC) was from Research Biochemical International (Reagente 5, Oporto, Portugal), goat purified IgG anti-adenosine A_{2A} receptor antibody (200 μg/ml) was from Santa Cruz Biotechnology-Europe (Freelab, Lisbon, Portugal), and rabbit purified IgG antiadenosine A_1 receptor antibody (1.8 mg/ml) was from Affinity Bioreagents (Golden City, CA, U.S.A.).

Animals and convulsive models

The experiments were performed using male Wistar rats from Harlan Ibérica (Barcelona, Spain) weighing 240–280 g for the amygdala-kindling experiments and 160–180 g for the kainate-injection experiments. The rats were handled according to the EU guidelines for use of experimental animals (86/609/EEC); they were anesthetized under halothane atmosphere before being killed by decapitation.

For the amygdala-kindling studies, the rats were divided into three groups: control rats, fully kindled rats, and sham-operated rats (electrode implemented but no stimulation delivered). Kindling was achieved by insertion of an electrode in the amygdala to allow chronic subthreshold stimulation to implement a fully kindled state, as previously described (5). Briefly, the rats were anesthetized with sodium pentobarbitone 60 mg/kg (3% wt/vol in saline) and coaxial bipolar electrodes (David Kopf) were implanted to the left amygdala. After a postoperative recovery of 1 week, animals were stimulated twice a day (10 a.m. and 4 p.m.) with a 50 Hz, 1 s, 500 μA, square wave with a positive pulse of 1 ms (Hugo Sachs stimulator, March-Hugstetten, Germany). After five consecutive stage 5 convulsions, fully kindled rats were allowed a 4-week recovery period before being killed for in vitro studies.

For the kainate injection studies, the rats were divided into two groups: control (saline-injected) and kainate-injected animals. Kainate (10 mg/ml) was injected intraperitoneally and the animal was placed in an observation box facing two experimenters who recorded the behavior changes displayed by the animals during a period of 4 hours according to the Racine's scale. Kainate-injected animals displayed a convulsive behavior that ranked at stage 4 (two rats) and stage 5 (one rat), whereas two other rats also reached stage 5 but died within the next hour, thus being excluded from the study. The rats were then allowed a 4-week recovery period before being killed for in vitro studies.

Adenosine receptor binding studies in cortical membranes

Saturation binding curves of the selective A_1 receptor antagonist, ^{3}H-DPCPX, or of the selective A_{2A} receptor antagonist, ^{3}H-SCH 58261, were performed as previously described using whole membranes from the cerebral cortex (8,15). Binding of ^{3}H-DPCPX (0–10 n*M*) or of ^{3}H-SCH 58261 (0–10 n*M*) was for 2 or 1 hours, respectively, at 37°C, with 32–159 μg of membrane protein in a final volume of 200 μl in an incubation solution containing 50 m*M* Tris-HCl and 10 m*M* $MgCl_2$, pH 7.4, with 2 U/ml adenosine deaminase. The binding reactions were stopped by vacuum filtration through Whatman GF/C glass fiber filters, followed by washing of the filters and reaction tubes with 8 ml of the incubation solution, kept at 4°C. Radioactivity retained in the filters was then determined after addition of 4 ml of scintillation liquid (Scintran Cocktail T, Wallac, Turku, Finland). Results are expressed as specific binding, determined by subtraction of the nonspecific binding, which was measured in the presence of 2 μM XAC, and normalized per amount of protein. All binding assays were performed in duplicate. To derive the binding parameters from saturation curves (K_D and B_{max} values), the data were fitted by a rectangular hyperbola using the GraphPad Prism software (San Diego, CA, U.S.A.).

Western blot analysis of adenosine receptor immunoreactivity in cortical membranes

Adenosine A_1 and A_{2A} receptor immunoreactivity was evaluated by Western blot analysis, as previously described (5,15) in whole membranes from the rat cerebral cortex. Cortical membranes solubilized in 5% SDS were separated by SDS-PAGE (13%) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (0.45 μm from Amersham). After blocking, the membranes were incubated overnight at 4°C with either the antiadenosine A_1 receptor (1:1000 dilution) or anti-A_{2A} receptor antibodies (1:500 dilution), then with the alkaline phosphatase-conjugated anti-rabbit or anti-goat secondary antibody (1:10,000 dilution from Amersham Little Chalfont, Buckinghamshire, U.K.), and finally with Enhanced Chemi-Fluorescence (Amersham) for 5 min, then

analyzed densitometrically with Quantity one, Versadoc 3000 (BioRad).

Statistics

The values presented are mean ± SEM of *n* experiments. Comparison between the different animal groups was made using the two-tailed Mann–Whitney test. Statistical significance was considered at $p < 0.05$.

RESULTS

Modification of cortical A_1 and A_{2A} receptors in kindled rats

The selective A_1 receptor antagonist, ^{3}H-DPCPX, bound with a K_D of 0.77 n*M* (95% confidence interval: 0.58–0.96 n*M*, n = 4) and a B_{max} of 917 ± 60 fmol/mg protein (n = 4) to whole membranes from the cerebral cortex of control rats. As illustrated in Fig. 1A, there was a decrease of ^{3}H-DPCPX binding to cortical membranes obtained from kindled rats. In fact, in cortical membranes from kindled rats, the B_{max} of ^{3}H-DPCPX binding was decreased (40.4 ± 4.4%, $p < 0.05$) to 546 ± 52 fmol/mg protein (n = 4), whereas the K_D of ^{3}H-DPCPX binding was not significantly ($p > 0.05$) modified (K_D = 0.81 n*M*, 95% confidence interval of 0.45–1.37 n*M*, n = 4). This decreased density of A_1 receptors was not due to the surgical procedure since the K_D (0.77 nM, 95% confidence interval of 0.43–1.11 n*M*, n = 4) and the B_{max} of ^{3}H-DPCPX binding (866 ± 23 fmol/mg protein, n = 4) in cortical membranes from sham-operated rats were not different ($p > 0.05$) from control rats (Fig. 1A).

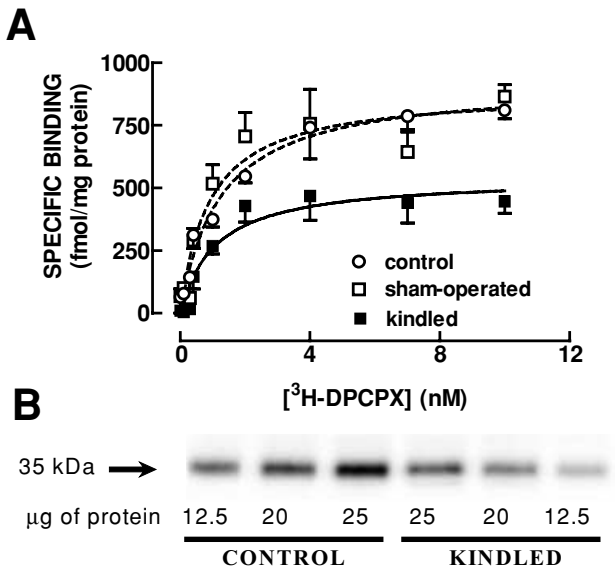

FIG. 1. Long-term effect of convulsions induced by amygdala kindling on the density of adenosine A_1 receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A_1 receptor antagonist ^{3}H-DPCPX in cerebral cortical membranes of control (open circles), sham-operated (open rectangles), and fully kindled rats (filled rectangles). The ordinates represent the specific binding of ^{3}H-DPCPX, obtained on subtraction of the nonspecific binding, determined in the presence of 2 μM XAC from total binding. The results are mean ± SEM of four experiments. **B:** A Western blot comparing the A_1 receptor immunoreactivity in membranes from the rat cerebral cortex of control (first three lanes from the left) and fully kindled rats (first three lanes from the right). The SDS-PAGE gel was loaded with three different amounts of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A_1 receptor protein is systematically lower in the membranes derived from kindled rats when compared to the same amount of loaded cortical membrane from control rats.

To confirm that there was indeed a decreased density of A_1 receptors in cerebral cortical membranes of kindled compared to control rats, we compared by Western blot analysis the immunoreactivity against A_1 receptors in membranes from cortical membranes of control and fully kindled rats. As illustrated in Fig. 1B, there was a decrease in the densitometrically measured anti-A_1 receptor immunoreactivity in cortical membranes from kindled compared to control rats, with an average value of 19.1 ± 3.3% (n = 3).

The selective A_{2A} receptor antagonist, ^{3}H-SCH 58261, bound with a K_D of 0.98 n*M* (95% confidence interval: 0.47–1.49 n*M*, n = 4) and a B_{max} of 27.7 ± 3.1 fmol/mg protein (n = 4) to whole membranes from the cerebral cortex of control rats. As illustrated in Fig. 2A, there was a marked increase of ^{3}H-SCH 58261 binding to cortical membranes obtained from kindled rats. In fact, in cortical membranes from kindled rats, the B_{max} of ^{3}H-SCH 58261 binding was nearly tripled (293 ± 34% increase, $p < 0.05$) to 109.0 ± 3.5 fmol/mg protein (n = 4), with no change ($p > 0.05$) of K_D (1.07 n*M*, 95% confidence interval of 0.65–1.50 n*M*, n = 4). This increased density of A_{2A} receptors was not due to the surgical procedure since the K_D (0.88 n*M*, 95% confidence interval of 0.41–1.36 n*M*, n = 4) and the B_{max} of ^{3}H-SCH 58261 binding (30.6 ± 3.4 fmol/mg protein, n = 4) in cortical membranes from sham-operated rats were not different ($p > 0.05$) from control rats (Fig. 2A).

To confirm that there was indeed an increased density of A_{2A} receptors in cerebral cortical membranes of kindled compared to control rats, we compared by Western blot analysis the immunoreactivity against A_{2A} receptors in membranes from cortical membranes from control and fully kindled rats. As illustrated in Fig. 2B, there was an increase in the densitometrically measured anti-A_{2A} receptor immunoreactivity in cortical membranes on kindled compared to control rats, with an average value of 151 ± 12% (n = 3).

Modification of cortical A_1 and A_{2A} receptors in kainate-injected rats

To further strength the idea that a convulsive period caused a long-term effect on the density of adenosine receptors in the cerebral cortex, we decided to use a different experimental paradigm to trigger convulsions. Thus, rats were injected with kainate, which induced convulsions

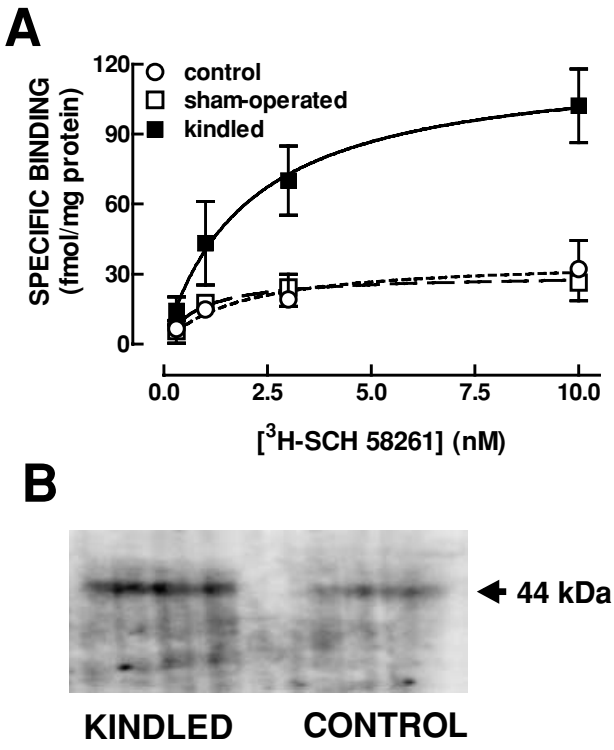

FIG. 2. Long-term effect of convulsions induced by amygdala kindling on the density of adenosine A_{2A} receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A_{2A} receptor antagonist ^{3}H-SCH 58261 in cerebral cortical membranes of control (open circles), sham-operated (open rectangles), and fully kindled rats (filled rectangles). The ordinates represent the specific binding of ^{3}H-SCH 58261, obtained on subtraction of the nonspecific binding, determined in the presence of 2 μM XAC from total binding. The results are mean ± SEM of four experiments. **B:** A Western blot comparing the A_{2A} receptor immunoreactivity in membranes from the rat cerebral cortex of control **(right lane)** and fully kindled rats **(left lane)**. The SDS-PAGE gel was loaded with 200 μg of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A_{2A} receptor protein is higher in the membranes derived from kindled compared to control rats.

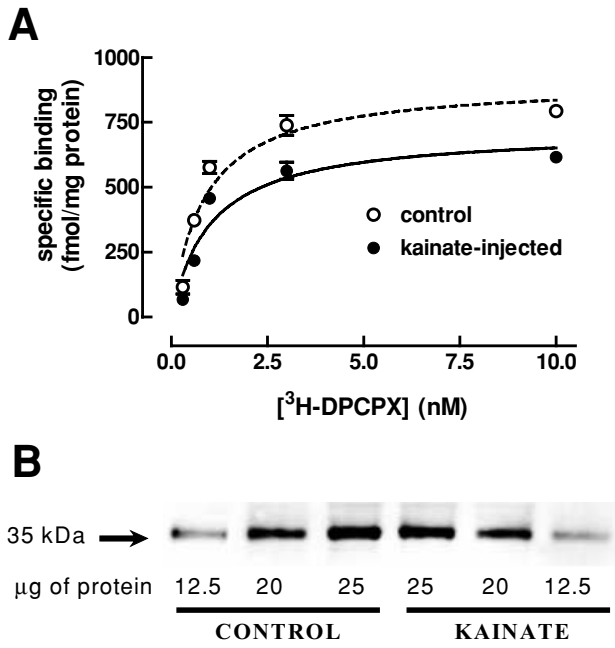

FIG. 3. Long-term effect of convulsions caused by the intraperitoneal injection of kainate on the density of adenosine A_1 receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A_1 receptor antagonist ^{3}H-DPCPX in cerebral cortical membranes of control (open circles) and kainate-induced kindled rats (filled rectangles). The ordinates represent the specific binding of ^{3}H-DPCPX, obtained on subtraction of the nonspecific binding, determined in the presence of 2 μM XAC from total binding. The results are mean ± SEM of three experiments. **B:** A Western blot comparing the A_1 receptor immunoreactivity in membranes from the rat cerebral cortex of control (first three lanes from the left) and fully kindled rats (first three lanes from the right). The SDS-PAGE gel was loaded with three different amounts of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A_1 receptor protein is systematically lower in the membranes derived from kainate-injected rats when compared to the same amount of loaded cortical membrane from control rats.

reaching a stage 4-5 between 30 and 240 min after kainate injection, and the density of cortical A_1 and A_{2A} receptors was evaluated after 30 days. As was observed in amygdala-kindled rats, there was a decrease in the binding density of the selective A_1 receptor antagonist, ^{3}H-DPCPX. Thus, ^{3}H-DPCPX bound with a K_D of 1.04 nM (95% confidence interval: 0.50–1.51 nM, n = 3) and a B_{max} of 907 ± 4.5 fmol/mg protein (n = 3) to whole membranes from the cerebral cortex of control (i.e., saline-injected rats). As illustrated in Fig. 3A, in cortical membranes from kindled rats, the B_{max} of ^{3}H-DPCPX binding was decreased to 719 ± 15 fmol/mg protein (20.7 ± 0.5% decrease, $p < 0.05$, n = 3) without change in K_D (K_D= 1.03 nM, 95% confidence interval of 0.82–1.24 nM, n = 3). This decreased density of A_1 receptors was also confirmed by Western blot analysis. In fact, as illustrated in Fig. 3B, there was a decrease in the densitometrically measured anti-A_1 receptor immunoreactivity in cortical membranes of kainate-injected compared to control rats, with an average value of 12.7 ± 5.7% (n = 3).

Convulsions induced by kainate also caused a marked upregulation of A_{2A} receptors, as occurred in kindled rats. In fact, as illustrated in Fig. 4, there was a near doubling (159 ± 32% increase, $p < 0.05$) of the density of ^{3}H-SCH 58261 binding in kainate-injected rats (B_{max} of 70.2 ± 5.8 fmol/mg protein, n = 3) compared to control (B_{max} of 26 ± 2.9 fmol/mg protein, n = 3), with no change in K_D (0.89 nM, 95% confidence interval: 0.78–1.01 nM, n = 3, in control vs. 0.91 nM, 95% confidence interval: 0.59–1.21 nM, n = 3, in kainate-injected rats). This increased density of A_{2A} receptors was also confirmed by Western blot analysis. In fact, as illustrated in Fig. 4B, there was a marked increase in the densitometrically measured anti-A_{2A} receptor immunoreactivity in cortical membranes of

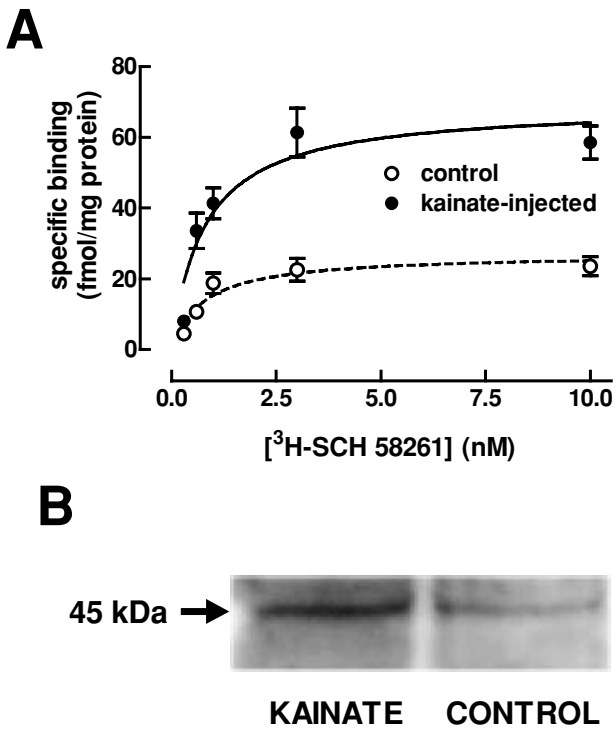

FIG. 4. Long-term effect of convulsions caused by the intraperitoneal injection of kainate on the density of adenosine A_{2A} receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A_{2A} receptor antagonist ^{3}H-SCH 58261 in cerebral cortical membranes of control (open circles) and kainate-induced kindled rats (filled rectangles). The ordinates represent the specific binding of ^{3}H-SCH 58261, obtained on subtraction of the nonspecific binding, determined in the presence of 2 μM XAC from total binding. The results are mean ± SEM of three experiments. **B:** A Western blot comparing the A_{2A} receptor immunoreactivity in membranes from the rat cerebral cortex of control **(right lane)** and kainate-injected rats **(left lane)**. The SDS-PAGE gel was loaded with 180 μg of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A_{2A} receptor protein is higher in the membranes derived from kindled compared to control rats.

kainate-injected compared to control rats, with an average value of 79.6 ± 7.0% (n = 3).

DISCUSSION

The neuromodulator adenosine has been associated with epileptic phenomena [reviewed in (2)] since there is a massive amount of adenosine released during seizures (16) and manipulation of the activity of either A_1 [reviewed in (2)] or A_{2A} receptors (10) affects convulsive behavior. We now found that convulsive behavior triggers a long-term decrease of A_1 receptor density and an increase of A_{2A} receptor density in the cerebral cortex. This conclusion was reached using two different and well-established paradigms to trigger convulsive behavior, amygdala kindling (13) and kainate injection (14), and two different experimental approaches to measure the density of adenosine A_1 and A_{2A} receptors, i.e., radioligand binding with selective antagonists and using selective antibodies against each adenosine receptor subtype. Thus, the observed remarkable qualitative similarity between the findings obtained when using two different ways to trigger convulsions and two different methods to evaluate changes in adenosine receptor density emphasizes the robustness of this major conclusion, i.e., that convulsive behavior causes a long-term increase in A_{2A} receptor density and a decrease of the density of A_1 receptors.

Several studies have shown that A_1 receptors undergo desensitization on prolonged activation (17–19), which is expected due to the massive and prolonged increase of extracellular adenosine occurring upon ictal activity (16,20,21). However, previous studies failed to reach an agreement on the effects of seizure activity on the density of A_1 receptors in the brain [reviewed in (4)]. In fact, most studies evaluating acute or short-term effects (i.e., after 24 h) of convulsions most commonly found an increase in the density of A_1 receptors in different brain regions, either upon induction of seizures with pentylenetetrazole (22,23), bicuculline (24,25), or 3-mercaptopropionate (26). In contrast, no acute or short-term changes in the density of brain A_1 receptors were found after electroconvulsive stimulation (27,28) or upon intraperitoneal injection of kainate (29). Changes in the density of A_1 receptors occurring 2–15 days after the observation of convulsive behavior also seem to depend on the strategy used to trigger convulsions. Thus, convulsions triggered by pentylenetetrazole (22) or repeated electroconvulsive stimulation (27,28) enhanced the density of A_1 receptors in most brain regions, whereas no measurable modification of A_1 receptor density was reported upon amygdala kindling (30)] or kainate injection (29,31). Interestingly, most studies seem to find a long-term decrease in the density of adenosine A_1 receptors, at least in the hippocampus (5,29,31). Thus, there is a general trend indicating that convulsive behavior leads to a long-term decrease in the density of A_1 receptors, as we now report has occurred in the cerebral cortex. This conclusion is in general agreement with the development of tolerance in relation to the anticonvulsive effects of A_1 receptor agonists (3,32).

However, the currently observed decrease of A_1 receptor density does not necessarily exclude A_1 receptors as potential targets for the development of new anticonvulsant drugs. In fact, while the efficiency of most anticonvulsants decreases markedly with increasing severity of seizures, A_1 receptor activation is able to suppress seizures in an animal model of pharmacoresistant epilepsy (33). This may be understood if one keeps in mind that seizures cause a long-term greater modification of the extracellular levels of adenosine able to activate A_1 receptors than a desensitization of responses mediated by A_1 receptors [(5), reviewed in (4)]. This suggests that strategies aimed at increasing the extracellular levels of adenosine may be more

effective than using A_1 receptor agonists (34,35), which also have the disadvantages of poor brain penetration and causing potent peripheral side effects (36).

In contrast to the well-defined potential of A_1 receptor activation to control seizures, the role of A_{2A} receptors is less well established. In fact, some reports with purportedly selective A_{2A} receptor agonists consistently observed anticonvulsant effects (32,37–39). However, despite the established presence of low amounts of A_{2A} receptors in cortical brain regions, it has recently been concluded that A_{2A} receptor agonists (like CGS 21680) mostly bind to A_1 rather than to A_{2A} receptors in cortical regions (8). This suggests that the activation of A_1 receptors might underlie the anticonvulsive effects of these purportedly selective A_{2A} receptor agonists (40). However, there are reasons to believe that A_{2A} receptors might play a role in the control of seizures and/or epileptogenesis. In fact, the group of Vaugeois showed that pharmacological blockade or genetic deletion of A_{2A} receptors decrease ethanol withdrawal-induced seizures in mice (10). Likewise, it has systematically been shown that blockade of A_{2A} receptors is neuroprotective in different noxious situations that involve limbic or cortical degeneration [reviewed in (9)], as is the case of temporal lobe epilepsy (41). This preliminary evidence, together with the presently observed robust increase in the density of adenosine A_{2A} receptors in the cerebral cortex of rats that had undergone a convulsive period, make A_{2A} receptor antagonists an attractive novel class of anticonvulsive drugs.

In conclusion, the presently observed long-term decrease in the density of A_1 receptors and the parallel increase of the density of A_{2A} receptors in the cerebral cortex provide a preliminary rationale for the development of novel anticonvulsive strategies targeting the adenosine neuromodulatory systems, which might be based on the combined use of strategies to burst the extracellular levels of adenosine to activate inhibitory A_1 receptors together with antagonists of facilitatory A_{2A} receptors.

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