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Laboratory Research

Effects of Antiepileptic Drugs on Refractory Seizures in the Intact Immature Corticohippocampal Formation In Vitro

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Summary: *Purpose:* We developed a new in vitro preparation of immature rats, in which intact corticohippocampal formations (CHFs) depleted in magnesium ions become progressively epileptic. The better to characterize this model, we examined the effects of 14 antiepileptic drugs (AEDs) currently used in clinical practice.

Methods: Recurrent ictal-like seizures (ILEs, four per hour) were generated in intact CHFs of P7–8 rats, and extracellular recordings were performed in the hippocampus and neocortex. AEDs were applied at clinically relevant concentrations (at least two), during 30 min after the third ILE. Their ability to prevent or to delay the next ILE was examined.

Results: Valproic acid and benzodiazepines (clobazam and midazolam) but also phenobarbital and levetiracetam prevent the occurrence of seizures. In contrast, usual concentrations of carbamazepine (CBZ), phenytoin, vigabatrin, tiagabine, gabapentin, lamotrigine (LTG), topiramate, felbamate, and ethosuximide did not suppress ILEs. In addition, LTG and CBZ aggravate seizures in one third of the cases.

Conclusions: This intact in vitro preparation in immature animals appears to be quite resistant to most AEDs. Blockade of seizures was achieved with drugs acting mainly at the γ-aminobutyric acid $(GABA)_A$-receptor site but not with those that increase the amount of GABA. Drugs with a broad spectrum of activity are efficient but not those preferentially used in partial seizures or absences. We suggest that this preparation may correspond to a model of epilepsy with generalized convulsive seizures and could be helpful to develop new AEDs for refractory infantile epilepsies. **Key Words:** Models—Animal diseases—In vitro—Low magnesium-aCSF—Immature rats—Intact tissues—Corticohippocampal formation—Anticonvulsant drugs—Carbamazepine—Clobazam—Ethosuximide—Felbamate—Gabapentin—Lamotrigine—Levetiracetam—Midazolam—Phenobarbital—Phenytoin—Tiagabine—Topiramate—Vigabatrin—Valproic acid.

The development of new antiepileptic drugs (AEDs) devoted to adult patients requires initial studies on adult animal models of epilepsy to select the efficient compounds and to eliminate the toxic ones (1–3). A similar strategy cannot be applied to the age-dependent infantile epilepsies because models adapted for each critical developmental stage are not available. As a consequence, most currently available AEDs to treat infantile epilepsy have been developed initially for adults. However, adult and infantile epilepsies are largely distinct, and drugs that are effective for adults are not necessarily indicated for children that display a large variety of epilepsy syndromes. AEDs developed this way may even induce adverse effects (4) or worsen infantile epilepsy syndromes (5,6). Therefore a great need exists to develop relevant animal models that could discriminate the effects of currently available AEDs and contribute to the evaluation of new drugs.

We describe the effects of a large number of AEDs in a novel rat preparation: the intact corticohippocampal formation (CHF) in vitro, a preparation that allows preservation of most of the intrinsic and extrinsic connections within and between cortical and hippocampal regions. We have previously reported that during the first postnatal week, the isolated neonatal intact preparation depleted in magnesium ions becomes progressively epileptic with recurrent ictal-like events (ILEs) recorded as soon as P1 in both cortical and hippocampal regions (7). The effects of AEDs were therefore studied at the end of the first postnatal week. This critical period of development is characterized by an intense activity-dependent construction of the network, and the depolarizing effects of γ-aminobutyric acid (GABA), allowing us to test and to compare the effects of AEDs used in clinical practice in conditions

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relevant to infantile epilepsy within a developmental context. We now report that recurrent seizures are suppressed by few AEDs, resistant to most of them, and particularly to those that are efficient in partial epilepsies or in absences and aggravated by some others. Therefore this model that allows the discrimination of the commonly used AEDs may be suitable to determine the effects of novel AEDs in the developing brain.

METHODS

All protocols were designed according to INSERM guidelines for the care and use of animals. Experiments were performed on intact CHFs taken from Wistar rats at postnatal (P) days 7 and 8 as previously described (7). This preparation comprised limbic (hippocampus, septum, and entorhinal cortex) and nonlimbic areas (large parts of the neocortex). This intact preparation, in which the integrity of the network is preserved within and between brain regions, offers an excellent compromise between in vivo and in vitro preparations and allows the study of epileptic processes during the first week of life (7). Rats were killed and brains were extracted from the skull at 4°C. The hemispheres were separated and dissected to obtain two CHFs containing interconnected septum, hippocampus, entorhinal cortex, and a large part of neocortex. Each CHF was placed in oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (aCSF) with the following composition (in m*M*): NaCl, 126; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 1.3; $NaHCO_3$, 25; NaH_2PO_4, 1.2; glucose, 10 (pH 7.3). After $\geq$1 h rest at room temperature, the CHF was transferred to the recording chamber where it was fully submerged and superfused with oxygenated aCSF at 31 $\pm$ 1°C at a flow rate of 5.0 $\pm$ 0.2 ml/min. After 15–30 min, the CHF was then superfused with an aCSF without magnesium ions (low-Mg^{2+} aCSF). In these conditions, the extracellular concentration of Mg^{2+} ions does not completely decrease to zero because of the contamination by Mg^{2+} of the other constituents of the aCSF (8).

Extracellular recordings

In a previous study we showed that spontaneous seizures were synchronized in hippocampal and cortical regions (7). Recordings were performed with extracellular electrodes filled with aCSF, placed in the neocortex and the hippocampus. Bipolar twisted nichrome electrodes were used to stimulate Schaffer collaterals (15–30 V, 30-μs duration at 0.033 Hz), and recording electrodes were placed at a location of the highest evoked field potential response. Data were acquired by using a Digidata 1200B card (Axon Instruments, Foster City, CA, U.S.A.) and were analyzed by using Clampfit (Axon Laboratories, Foster City, CA, U.S.A.) software.

Antiepileptic drugs

Carbamazepine (CBZ) and ethosuximide (ESM) were purchased from Sigma-Aldrich (France). Other AEDs were kindly supplied by the corresponding laboratories: clobazam (CLB), phenytoin (PHT), tiagabine hydrochloride (TGB), and sodium valproate (VPA) by Sanofi-Synthelabo, vigabatrin (VGB) and phenobarbital (PB) by Aventis, gabapentin (GBP) by Pfizer, felbamate (FBM) by Schering-Plough, midazolam maleate (MDL) by Roche, lamotrigine (LTG) by Glaxo-Smith-Kline, levetiracetam (LEV) by UCB-Pharma, and topiramate (TPM) by Janssen-Cilag. CLB and TPM were directly dissolved in aCSF. PHT was dissolved in NaOH (1N) adjusted to pH 7.3 with HCl (3N) and then diluted to the final concentration in aCSF. The other AEDs were dissolved in dimethylsulfoxide (DMSO) and diluted in aCSF to a final concentration of 0.1% DMSO. Previous studies have shown that such a concentration of DMSO has no effect on seizures. All drugs were applied by superfusion (5 ml/min).

Concentrations of AEDs

The concentrations used in this in vitro model were chosen according to the therapeutic range of each AED, when available (9–11). The therapeutic range represents the plasma concentrations at which most drugs are effective and well tolerated for most people. However, therapeutic ranges for GBP, LEV, MDL, TGB, and VGB are either unknown or not firmly established. For instance, a value >2 μg/ml of GBP (10) was thought to be effective, and 20 μg/ml appeared not to be toxic (12). For LEV, we took into account the value of 25 μg/ml reported recently in a study in which children (6–12 years old) received a single dose of LEV (20 mg/kg) as an adjunct to their stable regimen of a single concomitant AED (13). MDL is used in status epilepticus (14,15) and has been shown to be efficient in a similar in vitro model at concentrations of 50–100 μM (16,17). However, lower concentrations were also evaluated because they are more related to those required for activation of BZD receptors. Plasma concentrations for TGB were not defined in patients. We therefore selected the concentrations used by Sabau et al. (1999) on rat pup slices exposed to low-Mg^{2+}. The situation of VGB is different because it is a noncompetitive drug, and its plasma half-life is less critically related to its duration of action than is that of other AEDs (12). We therefore used VGB concentrations that have been evaluated in a similar in vitro model but in adult animal (18) or human slices (19). Table 1 summarizes the concentrations of AEDs used in this study and their therapeutic ranges in humans.

Statistical analysis

Results were expressed as mean values $\pm$ SEM of (n) independent experiments. A paired two-tailed *t* test between values obtained before and after drug application was performed for statistical evaluations: a p value <0.05 was considered a significant difference between mean values.

TABLE 1. *Therapeutic ranges of AEDs and their corresponding concentrations used in vitro*

AEDs	Therapeutic range (μg/ml)	Highest concentration (μM)	In vitro concentrations (μM)
CBZ	4 to 10 (a)	42	30, 100
CLB	0.3 to 0.8 (b)	3	0.3, 1, 3, 10
ESM	40 to 100 (a)	708	300, 1,000, 3,000
FBM	30 to 60 (a)	252	100, 300
GBP	>2 (c, d)[f]	58[f]	30, 100
LTG	1 to 5 (a)	20	10, 30
LEV	25 (e)[f]	253	30, 100, 300
MDL	Not known	–	1, 3, 30,100
PB	15 to 40 (a)	172	30, 100, 300
PHT	10 to 20 (a)	79	30, 100
TGB	Not known	–	10, 30
TPM	1 to 3	15	10, 30, 100
VGB	Not known	–	100, 300, 1,000
VPA	40 to 100 (c)	700	300, 1,000, 3,000

Values listed in the second column are from (a): Levy et al., 1995 (9), (b): Dulac, 1998 (11), (c): Froscher et al., 1999 (11), (d): Dichter et al., 1996 (12) and (e): Pellock et al., 2001 (13).

[f] The therapeutic range for these AEDs has not been determined, but plasma values are estimated (12) or available (13). The concentrations used in this study are reported in the last column. For each AED, several concentrations were tested in the low-Mg^{2+} model. They enclosed the highest concentration of AEDs evaluated in the plasma of epilepsy patients.

RESULTS

Low-Mg^{2+} aCSF-induced epileptiform activities in the intact CHF at P7–8

As previously shown, removing magnesium ions from the bathing medium (low-Mg^{2+}) induced spontaneous recurrent ILEs that are synchronized in the hippocampus and different cortical areas of intact CHFs (7). In the two brain regions, this activity is characterized by two consecutive components and starts (onset, 19.7 ± 0.8 min; n = 94) with a series (four to eight) of strong ILEs induced at a frequency of approximately four per hour (0.24 ± 0.02 per h; n = 94; Fig. 1A). Each ILE lasted 80–120 s and consists of an initial interictal-like burst, a tonic phase followed by a long-lasting tonic–clonic phase, and finally by the gradual development of recurring clustered bursts separated by short silent intervals of increasing duration (clonic phase; Fig. 1B). They are followed by late recurrent discharges (LRDs; onset, 89 ± 8 min; n = 48) that persist for ≥24 h even if the physiologic concentration of Mg^{2+} is restored (7). The mechanism of this transition is not known, but in adult tissues it is thought to be associated with a relative energy failure (20).

Therapeutic profile of low-Mg^{2+} aCSF-induced ILEs

We first examined the effects of AEDS on ILEs that represent seizures generated during the first postnatal week. A second study will be dedicated later to LRDs that are the consequences of the repetitive expression of ILEs. We found that AEDs display the same efficacy on ILEs induced either in P4–5 or in P7–8 CHFs. However, studying P7–8 rats allowed us to evaluate not only the positive effects of AEDs but also their adverse effects that cannot be evidenced at an earlier developmental stage.

In this study, the effects of 14 AEDs were evaluated by using concentrations around their therapeutic ranges (Table 1). Recordings were performed either simultaneously in the neocortex and in the CA1 area of the hippocampus, or only in the hippocampus. Because in the absence of drugs, the mean interval between two ILEs slightly decreased with time and remained <15 min (Fig. 1C), drugs were superfused for a period of 30 min that largely covers the interval between two ILEs. In the absence of drugs, the fourth ILE was always observed, so AEDs were applied usually after the third ILE, to have an internal control of the interval between ILEs 2 and 3 before application of the drug (Fig. 1). Therefore, AEDs were considered to be effective when no ILE was generated during this 30-min period of application of the drug. On the contrary, occurrences of long-lasting ILEs or LRDs, either during drug application or during the washout procedure, were taken as an indication of an "aggravation" of the epileptiform pattern. This assumption was made because long-lasting ILEs are the hallmark of the irreversible transition to LRDs that represent a persistent epileptic pattern in this immature model (7). In addition, with the same model in adults, LRDs were reported to be pharmacoresistant (16,17).

The effects of all AEDs tested were similar in neocortical and hippocampal regions. Drugs generally did not significantly affect the amplitude and duration of ILEs. Their main effect corresponded to a modification of the occurrence of the fourth ILEs. The results reported in Table 2 can be summarized as follows.

AEDs with complete efficacy

They corresponded to drugs that totally suppressed the epileptiform activity in hippocampal and cortical regions during their application period.

Phenobarbital. PB dose-dependently affected the occurrence of ILEs. Although a low concentration of PB (30 μM; n = 4) was not able to modify the discharge pattern, a concentration of 100 μM (n = 8) already significantly delayed the occurrence of the fourth ILE (n = 3) or completely suppressed its expression (n = 5; Fig. 2A). Finally, a higher concentration of PB (300 μM; n = 5), which is generally not used in clinical practice because of dose-related side effects, completely and reversibly prevented the occurrence of ILEs.

Valproic acid. At a low concentration, VPA (300 μM, n = 3) had no significant effect on the occurrence of ILEs. However, increasing the concentration to 1 mM (n = 8) significantly delayed the fourth ILE and, at 3 mM, ILEs were completely suppressed in eight experiments (Fig. 2B) and largely delayed in two others (Table 2).

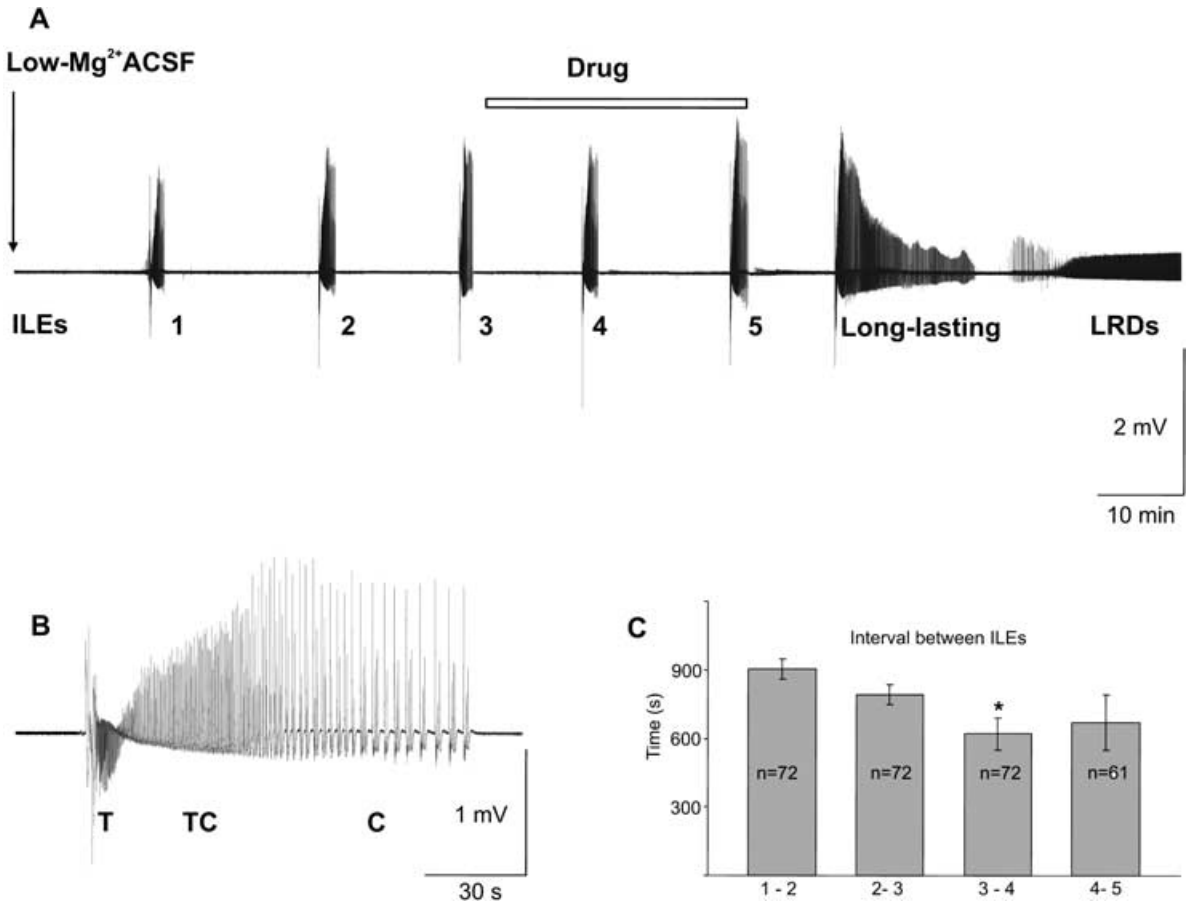

FIG. 1. Low-Mg^{2+} artificial cerebrospinal fluid (aCSF)-induced seizure-like pattern in immature rats. Intact corticohippocampal formations (CHFs) taken from P7–8 rats were continuously superfused with an aCSF from which Mg^{2+} ions have been omitted. **A:** Extracellular recordings were performed in the CA1 region. As previously reported (7), two consecutive patterns of activity were observed: ILEs, Ictal-like events; LRDs, late recurrent discharges. One long-lasting ILE usually constitutes a transition phase between these two patterns of activity**.** Note that in this example, no drug has been applied, but the bar (30 min) illustrates that ILEs are expected to occur during AEDs application. **B:** Enlargement of the first ILE showing the different phases of hyperactivity: T, tonic; TC, tonic–clonic; C, clonic. **C:** The mean time interval between two consecutive ILEs is represented. (*)The mean interval between ILEs three and four is significantly lower than that between ILEs two and three ($p < 10^{-3}$). Note that the interval between the third and the fourth ILEs is less than the duration of the AED application.

Levetiracetam. Initial studies were performed by using 30 μM (n = 4) and 100 μM (n = 5), concentrations that were indicated to be clinically relevant (21). In both cases, ILEs were not suppressed during application of LEV. However, there was already a significant increase in the interval between two ILEs at 100 μM (Table 2). At 300 μM (n = 4), LEV largely delayed the fourth ILEs in one experiment and suppressed it in the three others (Fig. 2C).

Benzodiazepines. Clobazam (CLB) was evaluated at clinically relevant concentrations (0.3 μM to 10 μM). ILEs were already suppressed in four experiments during the application of the lowest concentration of CLB (0.3 μM), whereas in four other experiments, the fourth ILE was significantly delayed. Increasing the concentration of CLB to 1 μM (n = 8; Fig. 3A) or 3 μM (n = 6) and 10 μM (n = 6) always suppressed the fourth ILEs (Table 2). At the highest concentrations, washing had to be performed for >2 h for ILEs to recover (Fig. 3B). Interestingly, an electrical stimulation of Schaffer collaterals (2–5 V, 30 μs) during the washing procedure of CLB (10 μM), immediately evoked an ictal-like response (Fig. 3C), suggesting that a long-lasting effect of CLB is responsible of the lack of spontaneously occurring ILEs.

We also tested another BZD that has a different chemical structure but that also acts on the modulatory site of the $GABA_A$ receptor. Midazolam (MDL) corresponds to a 1,4-BZD, whereas CLB is a 1,5-BZD. The efficacy of MDL was reported in the treatment of status epilepticus in adults and children (14,15), but its therapeutic range is not well established. We first used MDL concentrations (30 μM, n = 3, and 100 μM, n = 4) that were shown to block low-Mg^{2+}–induced ILEs in adult slices (16,17). At both concentrations, MDL was found to suppress ILEs completely during its application, but recovery of the fourth ILE usually required a long washout. Again,

TABLE 2. *Efficacy of AEDs on low-Mg^{2+}–induced ILEs*

Drugs	Conc. (μM)	n	Amplitude (%)	Duration (%)	Interval (%)
Drugs with complete efficacy					
PB	30	4	101 ± 2	110 ± 7	105 ± 11
PB	100	8	105 ± 5	104 ± 8	199 ± 34[b]
PB	300	5	108 ± 8	97 ± 13	493 ± 37[b]
VPA	300	3	99 ± 1	115 ± 6	100 ± 5
VPA	1,000	8	88 ± 14	123 ± 17	136 ± 7[b]
VPA	3,000	10	111 ± 8	92 ± 5	272 ± 24[c]
LEV	30	4	112 ± 4	91 ± 8	139 ± 15
LEV	100	5	99 ± 3	94 ± 4	191 ± 29[a]
LEV	300	4	105 ± 13	85 ± 8	243 ± 31[b]
CLB	0.3	8	104 ± 6	100 ± 8	189 ± 31[a]
CLB	1	8	89 ± 12	129 ± 17	281 ± 58[c]
CLB	3	6	110 ± 7	125 ± 9	394 ± 51[c]
CLB	10	6	102 ± 11	100 ± 15	422 ± 58[b]
MDL	1	3	88 ± 10	94 ± 18	268 ± 7[b]
MDL	3	4	98 ± 1	95 ± 5	336 ± 34[b]
Ineffective drugs					
ESM	300	4	101 ± 1	104 ± 6	100 ± 3
ESM	1,000	6	99 ± 1	91 ± 13	109 ± 8
ESM	3,000	7	95 ± 2	111 ± 2	119 ± 7
FBM	100	5	107 ± 7	137 ± 17	97 ± 20
FBM	300	5	109 ± 7	97 ± 6	112 ± 13
GBP	30	4	104 ± 3	113 ± 3	108 ± 7
GBP	100	6	102 ± 2	98 ± 8	86 ± 3[a]
PHT	30	4	97 ± 2	117 ± 4	96 ± 10
PHT	100	7	102 ± 1	86 ± 11	115 ± 12
TPM	10	5	99 ± 4	94 ± 8	85 ± 9
TPM	30	5	104 ± 3	113 ± 11	109 ± 30
TPM	100	5	96 ± 6	120 ± 16	95 ± 17
TGB	10	6	97 ± 3	114 ± 10	115 ± 9
TGB	30	6	97 ± 4	103 ± 12	92 ± 11
FMZ	10	4	103 ± 3	105 ± 5	103 ± 4
FMZ+CLB	10+10	6	99 ± 2	98 ± 7	122 ± 9
Ineffective drugs that modify or worsen ILEs					
CBZ	30	7	96 ± 8	115 ± 21	111 ± 18
CBZ	100	9	103 ± 1	146 ± 24	93 ± 10
LTG	10	10	106 ± 5	154 ± 30	94 ± 4
LTG	30	13	101 ± 3	144 ± 23	93 ± 7
VGB	100	3	99 ± 1	101 ± 4	100 ± 5
VGB	300	4	108 ± 6	86 ± 13	126 ± 16
VGB	1,000	8	98 ± 4	82 ± 14	87 ± 12

Intact CHFs were superfused with an artificial cerebrospinal fluid medium in which Mg^{2+} ions have been omitted. Spontaneous recurrent ILEs were induced approximately every 15 min. AEDs were superfused in the same medium for 30 min immediately after the third ILE. The amplitude and duration of the fourth ILE that occurs during AED application or during the washing procedure were compared with those measured in the absence of AED. Furthermore, the interval between the second and the third ILE was compared with that observed between the third and the fourth ILE. All values are then normalized, taking as 100% the values before AED application. AEDs differently affect the occurrence of the fourth ILE (last column). A value >100% indicated some kind of efficiency of the drug, whereas a lower value reflected a kind of worsening of the epileptiform pattern.

[a] $0.01 < p < 0.05$.
[b] $0.005 < p < 0.01$.
[c] $p < 0.005$.

electrical stimulation during the washing procedure elicited ILEs. Because the affinity of MDL for BZD binding sites was in the low micromolar range, we then tried lower concentrations and found that ILEs were blocked at 1 μM (n = 3; Fig. 3D) and 3 μM (n = 4).

To confirm that CLB and MDL interact with BZD binding sites, we used flumazenil (FMZ), a selective antagonist at $GABA_A$-receptor BZD sites. Because FMZ (10 μM, n = 4) did not significantly affect the interval (p = 0.4747) between ILEs three and four (Fig. 4A), we tried to antagonize the effects of BZD agonists in our model. Coapplication of FMZ (10 μM) and CLB (10 μM) had no effect on the occurrence of the fourth ILE or marginally delayed it (n = 6; p = 0.5247; Fig. 4B). Coapplication of FMZ (10 μM) and MDL (10 μM) gave similar results (n = 3). Furthermore, when ILEs were first prevented by a high concentration of CLB (10 μM, n = 4), the addition of FMZ (10 μM) in the washing medium allowed a recovery of ILEs within 4.3 ± 2.1 min (n = 4; Fig. 4C), compared with >2 h in the absence of FMZ. Similar results were obtained with MDL (10 μM, n = 3).

Ineffective AEDs

They corresponded to drugs that did not prevent the occurrence of ILE during their application at a therapeutic

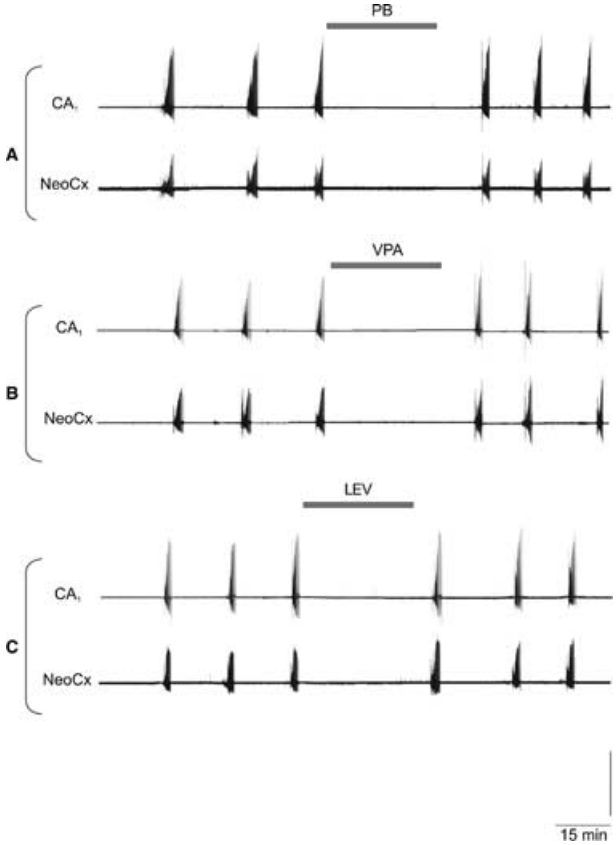

FIG. 2. Antiepileptic drugs (AEDs) that prevent ictal-like events (ILEs). Recordings were performed simultaneously in the CA1 region and in the neocortex (NeoCx) of P7–8 intact corticohippocampal formations (CHFs). Tissues were continuously superfused with low-Mg^{2+} artificial cerebrospinal fluid (aCSF). **A:** Phenobarbital (PB) (300 μM), **(B)** valproic acid (VPA) (3 m*M*), or **(C)** levetiracetam (LEV) (300 μM) were added after the third ILEs, for a period of 30 min (*bar*) and then washed out. No ILE occurs during the application of these drugs. The vertical scale bar represents 2 mV (CA1) and 0.75 mV (NeoCx).

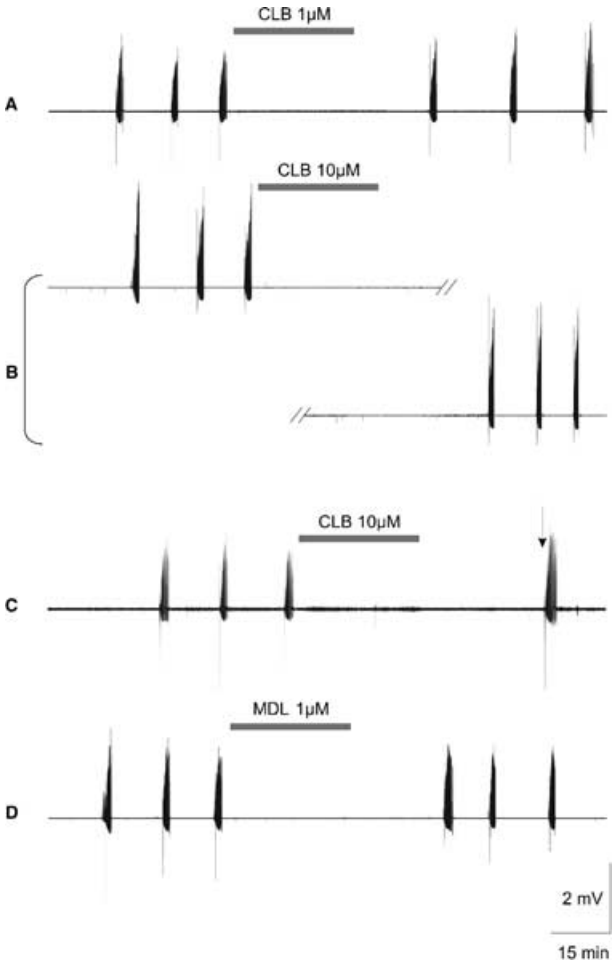

FIG. 3. Benzodiazepines prevent the occurrence of ictal-like events (ILEs). Extracellular recordings were performed in the CA1 region of P7–8 intact corticohippocampal formations (CHFs). Tissues were continuously superfused with low-Mg^{2+} artificial cerebrospinal fluid (aCSF). After the third ILEs, drugs were added to the superfusing medium for 30 min (*bar*) and then washed out. **A, B:** ILEs are suppressed during the application of clobazam (CLB; 1 μM) and CLB (10 μM, two traces). Note that after CLB (10 μM), the recovery of ILEs requires a longer washout time. **C:** Thirty minutes after the application (*bar*) of a high concentration of CLB (10 μM), an electrical stimulation of Schaffer collaterals (*arrow*) immediately evokes an ILE. **D:** Midazolam (1 μM) reversibly blocks the occurrence of ILEs during its application (*bar*).

concentration and that did not significantly affect the characteristics of the fourth ILE in cortical and hippocampal regions.

Ethosuximide. At clinically relevant concentrations (300 μM, n = 4 and 1000 μM, n = 6) ESM had no effect on the frequency of ILEs in the immature intact preparation. Because higher concentrations of ESM were reported to aggravate seizure-like events in adults slices (16), we also evaluated the effects of 3,000 μM ESM. At this concentration (n = 7), ESM was again unable to suppress ILEs but had no adverse effects (Table 2).

Felbamate. At therapeutic concentrations (100 μM, n = 5, and 300 μM, n = 5), FBM was not able to suppress low-Mg^{2+}–induced ILEs (Table 2). Higher doses were not studied because of the well-known toxicity of FBM (22).

Gabapentin. The therapeutic range of GBP is not available. However, taking into account suggested plasma values between 2 and 20 μg/ml (12), we used concentrations of 30 (n = 4) and 100 μM (n = 6). At these concentrations, GBP did not prevent the occurrence of ILEs (Table 2), but at 100 μM, the duration of the fourth ILEs was significantly decreased (p = 0.0113), suggesting that this drug may precipitate ILEs (Table 2).

Phenytoin. At clinically relevant concentrations (30 μM, n = 4, and 100 μM, n = 6), PHT did not modify the occurrence of ILEs (Table 2). A modification of the discharge pattern was observed in one additional case at 100 μM (see below).

Topiramate. At therapeutic concentrations (10 μM, n = 5; 30 μM, n = 5), TPM was not able to suppress or delay the occurrence of ILEs. A higher concentration

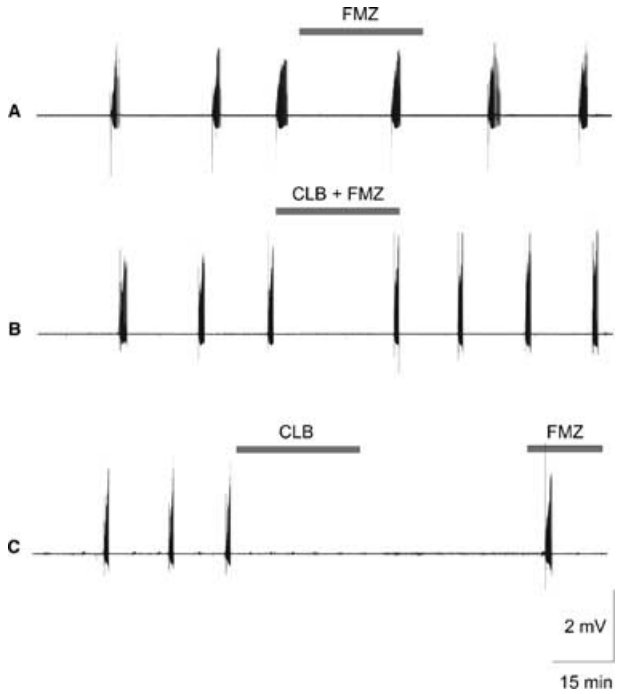

FIG. 4. Flumazenil (FMZ) antagonizes the effects of clobazam. Recordings were performed in the CA1 region of P7–8 intact corticohippocampal formations (CHFs). Tissues were continuously superfused with low-Mg^{2+} artificial cerebrospinal fluid (aCSF). After the third ILEs, drugs were added to the superfusing medium for 30 min (*bar*) and then washed out. **A:** Application of FMZ (10 μM), a specific benzodiazepine antagonist, has no significant effect on low-Mg^{2+} aCSF–induced seizures. **B:** Coapplication of FMZ (10 μM) with clobazam (CLB; 10 μM) during 30 min prevents the effects of CLB (see Fig. 3B). **C:** ILEs that are usually blocked for 2 h on the application of CLB (10 μM, see Fig. 3B) are immediately restored after the addition of FMZ (10 μM) in the washing medium (*bar*).

of TPM (100 μM, n = 5) also did not prevent the expression of the fourth ILEs (Table 2).

Tiagabine. There was no available therapeutic range for this drug. Therefore, we used concentrations of 10 μM and 30 μM that were reported to reduce or completely suppress ILEs induced by low-Mg^{2+} in cortical adult slices (23,24). In our conditions, TGB was ineffective to suppress or to significantly delay the occurrence of ILEs at these concentrations (10 μM, n = 6, and 30 μM, n = 6) (Table 2). Because TGB was reported to develop a proconvulsive effect at high concentrations (23), we did not test higher concentrations.

Ineffective AEDs that modify or worsen the seizure-like pattern

Carbamazepine. Application of CBZ concentrations in the therapeutic range (30 μM, n = 7, and 100 μM, n = 9) never suppressed the occurrence of ILEs (Table 2). However, the pattern of activity was frequently modified during the application of CBZ or during the washing procedure. Thus, in five of 13 experiments, long-duration ILEs or facilitated transitions to LRDs were observed at both concentrations, suggesting a kind of aggravation. On washing, a more complex pattern of discharge was observed with both interictal and several long-duration ILEs (Fig. 5A). In three additional cases at 100 μM, the pattern of discharge during CBZ application was modified, with a marked attenuation of the tonic phase and duration of the fourth ILE ($-44 \pm 12\%$). In addition, this modification was accompanied with a large amount of irregular interictal discharges (not shown).

Lamotrigine. Therapeutic concentrations of LTG (10 μM, n = 10, and 30 μM, n = 13) were unable to suppress the occurrence of ILEs (Table 2). Moreover, LTG induced repetitive long-lasting ILEs at 10 μM (two of eight experiments) and at 30 μM (four of 13 experiments, 236%; p = 0.0451), sometimes associated with the appearance of interictal events (Fig. 5B).

Phenytoin. A modification of the pattern of discharge similar to that reported above with CBZ was observed during the application of 100 μM PHT, but only in one of seven experiments (not shown).

Tiagabine. An attenuation of the tonic phase associated with reduction of the duration of the fourth ILE was observed in one case at 10 μM and at 30 μM. In contrast to CBZ and PHT, there were no interictal bursts during the application of the drug (not shown).

Vigabatrin. Application of VGB, 100 μM (n = 3), 300 μM (n = 4), or 1,000 μM (n = 8; Table 2) did not induce significant changes in amplitude, duration, and interval of ILEs. However, in three of eight experiments performed at 1,000 μM, VGB displayed a tendency to induce a transformation of the usual pattern of discharge.

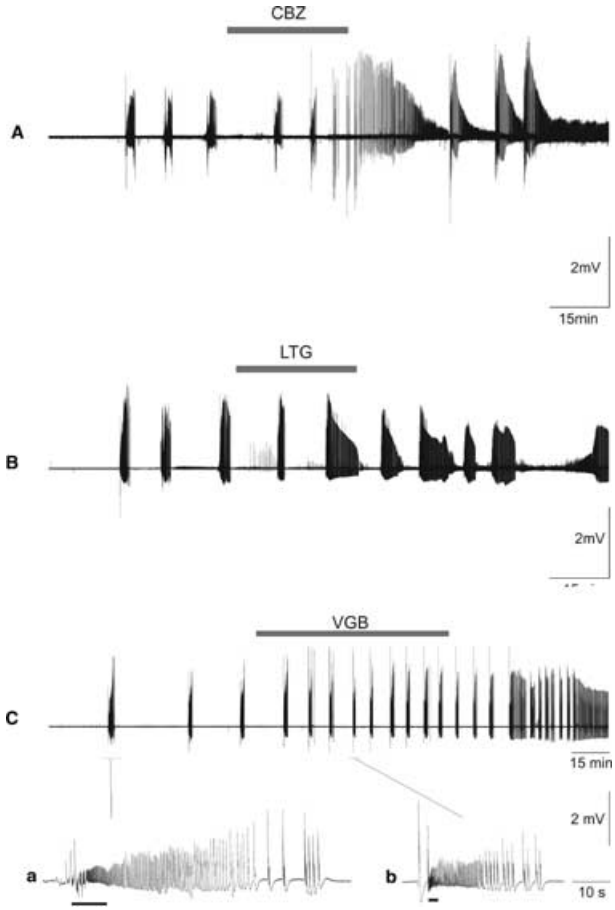

FIG. 5. Antiepileptic drugs (AEDs) that modify the seizure-like pattern. Recordings were performed in the CA1 region of P7–8 intact corticohippocampal formations (CHFs). Tissues were continuously superfused with low-Mg^{2+} artificial cerebrospinal fluid (aCSF). After the third ILEs, drugs were added to the superfusing medium for 30 min (*bar*) and then washed out. **A, B:** Carbamazepine (CBZ) and lamotrigine (LTG) aggravate seizure-like events. Application of CBZ (30 μM) or LTG (30 μM) does not suppress the occurrence of ILEs. In contrast, long-lasting ILEs were triggered on washing of CBZ or during the application of LTG. They persisted and precipitated the occurrence of late recurrent discharges (LRDs). **C:** Vigabatrin (VGB) modifies seizure-like events. ILEs are not suppressed during superfusion of VGB for 60 min (*bar*), but in contrast, their frequency dramatically increased. Note that during VGB application, the duration of the fourth ILE and that of its tonic phase (*bars*) were modified. On washing, long-duration ILEs and LRDs occurred.

Because the efficacy of VGB may require longer application time (18), additionnal experiments were performed with a 60-min application of VGB (1,000 μM, n = 4). VGB now induced a significant reduction of (a) the interval (-25%, p = 0.04569); (b) the duration of the fourth ILE (-36%, p = 0.0089); and (c) its tonic phase (-45%, p = 0.0154), and also increased the number of ILEs (10 ± 2 ILEs; p = 0.02530; Fig. 5C). These repetitive ILEs were followed by bursts of interictal discharges that progressively gave rise to LRDs.

DISCUSSION

The main observation of this study is that at the end of the first postnatal week, low-Mg^{2+}–induced ILEs in hippocampal and cortical regions from intact CHFs are resistant to a large number of presently available AEDs. Only two classes of AEDs are able to suppress completely the recurrence of seizures at therapeutic concentrations, BZDs and VPA, both being drugs of choice in the treatment of generalized convulsive seizures in humans. In contrast, most AEDs used in partial seizures and ESM used in absences are ineffective. Such a preparation may therefore correspond to a model of convulsive generalized seizures. Because this therapeutic profile is specific for immature tissues, it could be helpful for developing new drugs for infantile epilepsies.

Seizure-like events induced by low-Mg^{2+} are pharmacoresistant

The better to characterize the therapeutic profile of the low-Mg^{2+} model of ILEs induced in intact immature tissues, we studied the effects of a large panel of AEDs used in epilepsy patients. For this purpose, we tested concentrations that corresponded to those reached in plasma for the therapeutic range of each AED. Among the 14 AEDs representing 12 classes of chemical drugs, few of them are able to suppress ILEs. Thus only VPA and BZDs (CLB, MDL) are efficient classes of drugs in this model. PB and LEV also prevent ILEs but at concentrations that are at the limit or higher than those used in clinical studies. In contrast, CBZ, FBM, GBP, PHT, TGB, TPM, VGB, and ESM have generally no effect at their clinically relevant concentrations. However, CBZ and LTG may aggravate the epileptiform pattern because long-duration ILEs and LRDs were observed during their application or during the washing procedure. Furthermore, at higher concentrations, CBZ, PHT, TGB, and VGB were still not able to suppress the occurrence of ILEs, but they altered the pattern of discharges. Although such a modified pattern has not yet been thoroughly studied, it is unlikely that it could be associated with an improvement of seizure-like events.

The therapeutic profile is specific for immature tissues

The characteristics of low-Mg^{2+}–induced ILEs in immature and adult tissues present some similarities. Thus the morphology of seizure-like events is quite similar in both cases, with an initial tonic phase followed by a tonic–clonic and then by a clonic phase (25). In addition, VPA and PB are efficient in immature and adults tissues (16) at similar concentrations. One benzodiazepine, MDL, also blocks seizure-like events in mature tissues (16) but at a concentration that is 50 times higher than that required in the immature preparation. Because relevant therapeutic concentrations of MDL have not been yet tested, and because no other BDZ has been tested, the possibility that MDL acts through sodium channels (26) in the adult model cannot be excluded.

In spite of these similarities, the large range of AEDs studied here allowed us to conclude that low-Mg^{2+}–induced ILEs are more resistant to AEDs in the immature model than in the adult one. For instance, PHT (16) and TGB (23) are efficient in corticohippocampal slices from adult rats, but not in the immature model at the same concentration [see also (23)]. In addition, LTG, VGB, and FBM are efficient in adult hippocampal slices (18,27–29), but not in the immature model, where they occasionally aggravate seizure-like events at similar concentrations. Conversely, ESM facilitates the transition to LRDs in adult corticohippocampal slices (16), whereas it does not display this adverse effect in the immature preparation. These observations show that the therapeutic profiles of the two models are different and support the idea that AEDs that are developed for adult patients are not necessarily indicated for infants.

Mechanisms of action of AEDs in immature CHFs

We have previously shown that ILEs are highly sensitive to $GABA_A$-receptor agonists and to *N*-methyl-D-aspartate (NMDA), but not AMPA/kainic acid (KA)-receptor antagonists (7). Therefore, it was of interest to examine the effects of AEDs that affect glutamate or GABA transmission [for recent reviews on the mechanisms of action of AEDs, see (30,31)].

Among the currently used AEDs, only FBM is reported to affect NMDA receptors in vitro. Although FBM (100 μM) was shown already to reduce NMDA receptor–mediated responses (32), it was quite inactive in our model at this concentration. This suggests that the effect of FBM on NMDA receptors might not be sufficient to prevent ILEs in this model. LEV has been reported to decrease NMDA-induced bursting (33), but it is not yet known whether this property contributes to its antiepileptic properties. Nevertheless, LEV displays some efficiency in this model at high concentrations. TPM, which has been shown to modify AMPA/KA, but not NMDA-receptor responses (34), is quite inefficient in this model, in agreement with our observation that ILEs are not suppressed by AMPA/KA antagonists (7).

More interesting are the effects of AEDs known to modify GABA transmission [see (30,31)]. GABA concentrations may be increased by TGB via an inhibition of the GAT1 transporter, and by VGB via the inhibition of the metabolism enzyme GABA transaminase [see also (35)]. TPM was also found to increase GABA concentrations, but its mechanism remains to be clarified. Nevertheless, these three drugs are not efficient in our immature model, at least for concentrations evaluated here. In contrast, drugs that act at postsynaptic level, such as BZDs and PB, appear to suppress ILEs. These drugs interact

with specific sites located on the $GABA_A$-receptor complex and modulate GABA transmission either by increasing the open-time probability (PB) of $GABA_A$ receptor or by increasing its open-time frequency (BZDs). Their antiepileptic effects are mainly associated with this modulation. This suggests that in this model, modulation of $GABA_A$ response is more important than the availability of GABA itself. The possibility that in our conditions, $GABA_A$ receptors are already saturated may explain this discrepancy between pre- and postsynaptic-acting AEDs. However, at high concentrations, PB and BZDs may also interact with other sites. A direct action of PB on the agonist recognition site (31) cannot be excluded because the efficacy of PB in our model was observed at the limit of therapeutic concentrations. In contrast, the effects of BZDs on voltage-dependent sodium or calcium channels (31) could be excluded because low concentrations of CLB already prevent the occurrence of ILEs. In addition, the efficiency of two structurally distinct BZDs is reversed by FMZ, a specific antagonist of BZD modulatory sites. The fact that FMZ did not antagonize the effects of BZDs on sodium channels (26,36) also suggests that the efficacy of these AEDs is unlikely to be mediated by sodium channels. Furthermore, drugs that are well known to inhibit voltage-dependent sodium channels [CBZ, PHT, FBM, LTG, and TPM; see (30,31)] are unable to suppress the recurrence of ILEs. The only exception is VPA, which has a much broader mechanism of action. Finally, some tested AEDs are also thought to act through their blockade of voltage-dependent calcium channels (VDCCs). The efficacy of ESM to block low-threshold thalamocortical T-type VDCCs is frequently associated with its antiabsence property. However, in this model, ESM was devoid of effect on the recurrence of ILEs. In addition, FBM, CBZ, LTG, and GBP, which block L-type VDCCs, were not efficient blockers of ILEs in low-Mg^{2+}–induced ILEs in immature tissue.

Therefore the analysis of the mechanism of action of AEDs indicates that drugs modulating $GABA_A$-receptor responses are efficient to suppress or to delay ILEs induced by low-Mg^{2+} in immature tissues, whereas drugs acting either on GABA release or on AMPA/KA and on sodium or calcium channels rather exhibit a poor efficacy or aggravate ILEs.

A model of generalized epilepsy?

The therapeutic efficacy of the new AEDs is defined by controlled therapeutic trials, and that of classic AEDs is based on numerous years of clinical practice in enormous cohorts of patients. Relatively few drugs have a limited indication, such as ESM for absences or GBP and TGB for partial seizures. Most of them have a large spectrum of potential efficacy, both in partial and in generalized seizures. However, because of their different tolerability profiles or because some AEDs may aggravate specific seizure types or specific epilepsy syndromes, the following strategy of treatment is commonly used in children with epilepsy: first-line drug is VPA for generalized seizures and CBZ for partial seizures; PHT, VGB, GBP, and TGB are rather used as adjuncts in resistant partial seizures, and BZD, PB, and FBM in generalized seizures; LTG and TPM are used in adjunction in both situations (37). Although not yet approved in children, LEV could also be efficient in both types of epilepsies. Therefore, in addition to other criteria, the use of a large panel of AEDs would be helpful the better to characterize our model.

It should first be emphasized that seizure-like events that are synchronized in cortical and hippocampal areas (7) display the same therapeutic profile in the two regions. CBZ and PHT, which are recognized as the prototypes of the classic drugs indicated for partial epilepsies, have no effect on the recurrence of ILEs induced by low Mg^{2+} in immature tissues. Interestingly, in more than one third of the cases, CBZ was found to have adverse effects in this model because it increases the transition toward a permanent form of hyperactivity (LRDs). All the drugs indicated as adjunctive therapy in partial seizures (FBM, GBP, LTG, TPM, TGB, and VGB) also display poor efficacy in this model, suggesting that it is not adapted to study focal epilepsies. In contrast, the three efficient drugs were the classic AEDs currently used in generalized seizures (VPA, BZD, PB). Generalized seizures comprise convulsive and nonconvulsive seizures, the latter consisting of absences. The present model is not a model of absences because of the lack of efficacy of ESM, the prototype of the antiabsence drug. Furthermore, the morphology of synchronized discharges is not consistent with spikes and waves usually reported in absences, but rather with tonic–clonic discharges. Finally, such a therapeutic profile that is similar in cortical and hippocampal areas suggests that seizures induced by low Mg^{2+} in immature tissue may correspond to a model of generalized convulsive seizures. Other approaches must be performed to confirm this proposal, but this in vitro immature preparation, which exhibits a rather specific therapeutic profile, could already be useful for developing new drugs for severe infantile convulsive epilepsies.

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