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Influence of carbenoxolone on the anticonvulsant efficacy of conventional antiepileptic drugs against audiogenic seizures in DBA/2 mice

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Abstract

Carbenoxolone, the succinyl ester of glycyrrhetinic acid, is an inhibitor of 11β-hydroxy steroid dehydrogenase and gap junctional intercellular communication. It is currently used in clinical treatment of ulcer diseases. Systemic administration of carbenoxolone (1–40 mg/kg, intraperitoneally (i.p.)) was able to produce a dose-dependent decrease in DBA/2 audiogenic seizure severity score. Glycyrrhizin, an analogue of carbenoxolone inactive at the gap-junction level, was unable to affect audiogenic seizures at doses up to 30 mg/kg. In combination with conventional antiepileptic drugs, carbenoxolone, 0.5 mg/kg, i.p., which per se did not significantly affect the occurrence of audiogenic seizures in DBA/2 mice, potentiated the anticonvulsant activity of carbamazepine, diazepam, felbamate, gabapentin, lamotrigine, phenytoin, phenobarbital and valproate against sound-induced seizures in DBA/2 mice. This effect was not observed after the combination of glycyrrhizin (10 mg/kg, i.p.) with some conventional antiepileptic drugs. The degree of potentiation induced by carbenoxolone was greater for diazepam, felbamate, gabapentin, phenobarbital and valproate, less for lamotrigine, phenytoin and carbamazepine. This increase was associated with a comparable impairment in motor activity; however, the therapeutic index of combined treatment of antiepileptic drugs with carbenoxolone was more favourable than the combination with glycyrrhizin or saline. Since carbenoxolone did not significantly influence the total and free plasma levels of diazepam, felbamate, gabapentin, lamotrigine, phenytoin, phenobarbital, valproate and carbamazepine, pharmacokinetic interactions are not likely. However, the possibility that carbenoxolone can modify the brain clearance of the anticonvulsant drugs studied may not be excluded. In addition, carbenoxolone did not significantly affect the hypothermic effects of the anticonvulsants tested. In conclusion, carbenoxolone showed an additive anticonvulsant effect when administered in combination with some classical anticonvulsants, most notably diazepam, felbamate, gabapentin, phenobarbital, and valproate, implicating a possible therapeutic relevance of such drug combinations.

Keywords: Epilepsy; Carbenoxolone; Gap junction; Antiepileptic drug; Carbamazepine; Phenytoin; Valproate; Felbamate; Anticonvulsant potency; Audiogenic seizure; DBA/2, mouse

1. Introduction

Monotherapy is actually considered as the optimal treatment of epilepsy. However, around 30% of patients may experience seizures resistant to the currently available anticonvulsant drugs (Rogawski, 1998). Therefore, identification of novel pharmacological targets and analysis of drugs acting on them is still a priority in epilepsy research. It has been recently proposed that neuronal gap junctions, the molecular substrate of electrical synapses (Bennet, 1997; Belluardo et al., 1999; Condorelli et al., 2000; Rouach et al., 2000; Szeute et al., 2002), can represent a potential target for anticonvulsant therapy (Traub et al., 2001; 2002).

There is much in vitro experimental data concerning the involvement of gap junctions in epileptiform activity

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induction, in seizure propagation and epileptogenesis (Carlen et al., 2000; Perez-Velazquez and Carlen, 2000; Ross et al., 2000; Traub et al., 2001, 2002). The hippocampus plays a dominant role in the propagation of seizures (McNamara, 1994); recently, upregulation of gap junction connexins 30 and 32 has been described for hippocampal slices following bicuculline- or kainate-induced epileptiform activity (Li et al., 2001; Condorelli et al, 2002).

Carbenoxolone, a gap junction blocker and mineral corticoid agonist (Davidson et al., 1986; Ross et al., 2000; Jahromi et al., 2002), is able to reduce the frequency of spontaneous burst activity and to delay the induction of epileptiform activity (Ross et al., 2000; Perez-Velazquez and Carlen, 2000; Traub et al., 2001). Carbenoxolone (100 μmol/l) inhibits in vitro spontaneous 200 Hz oscillations in hippocampal slices and higher concentrations can suppress both very fast oscillations and seizure-like events induced by trimethylammonium combined with tetanic stimulation (Draguhn et al., 1998).

Until today little attention has been paid to changes in direct intracellular transmission via gap junctions (Carlen et al., 2000; Perez-Velazquez and Carlen, 2000) and no data are available about the interactions between compounds acting on gap junctions and anticonvulsant drugs.

The aim of the present study was to investigate the efficacy of conventional antiepileptic drugs such as carbamazepine, diazepam, felbamate, phenytoin, gabapentin, lamotrigine, phenobarbital and valproate administered together with carbenoxolone or glycyrrhizin (an analogue of carbenoxolone that does not block gap junctions) against audiogenic seizures in DBA/2 mice. Carbenoxolone and glycyrrhizin display different pharmacological profiles (Davidson and Baumgarten, 1988; Jellinck et al., 1993; Dodic et al., 1998; Dobbins and Saul, 2000) including their gap-junction activity (Szeute et al., 2002).

2. Materials and methods

2.1. Animals

The experiments were carried out on DBA/2 mice, weighing 6–12 g (22 to 26 days old) or 20 to 28 g (48 to 56 days old). Animals were purchased from Harlan Italy (Correzzana, Milan, Italy) and housed in groups of 8–10 in colony cages at room temperature, under a 12-h light/dark cycle (lights on at 7:00 a.m.). The animals were housed with free access to food pellets and tap water with food and water available ad libitum. Experimental groups, consisting of 10 animals, were assigned according to a randomised schedule, and each mouse was used only once. Control animals were always tested on the same day as the respective experimental groups. Procedures involving animals and their care were conducted in conformity with international and national law and policies (European Communities Council Directive of 24th November 1986, 86/609EEC).

2.2. Experimental protocols

DBA/2 mice were exposed to auditory stimulation, 60, 90, 120 or 180 min following intraperitoneal (i.p.) administration of carbenoxolone (1–40 mg/kg), glycyrrhizin (10–120 mg/kg) or saline and 30, 45, 60 or 120 min following i.p. injection of some antiepileptics. Each mouse was placed under a hemispheric perspex dome (diameter 58 cm) and 1 min was allowed for habituation and assessment of locomotor activity. Auditory stimulation (12–16 kHz, 109 dB) was applied for 1 min or until tonic extension occurred. The seizure response, as previously reported (De Sarro et al., 1984), was assessed using the following scale: 0 = no response, 1 = wild running, 2 = clonus, 3 = tonus, 4 = respiratory arrest. The maximum response was recorded for each animal. Rectal temperature was recorded immediately prior to auditory testing using an Elektrolaboratoriet thermometer type T.E.3. Behavioural changes were observed during the period between drug administration and auditory testing.

2.3. Determination of the plasma levels of the antiepileptic compounds

DBA/2 mice (20–28 g) were given i.p. either saline and one antiepileptic drug, carbenoxolone and one antiepileptic drug or glycyrrhizin and one antiepileptic drug. The same protocol was used for behavioural and pharmacokinetic studies. Older DBA/2 mice were used for pharmacokinetic studies because it is very difficult to collect blood from younger DBA/2 mice. No differences in pharmacokinetics were reported between 21–26 and 48–56 days old mice (De Sarro et al., 1996, 1998, 2000a, 2000b, 2002). The animals were lightly anaesthetized with ethyl ether and killed by decapitation at appropriate times and blood samples of approximately 1 ml were collected into Eppendorf tubes. The felbamate and lamotrigine assay was carried out using high-performance liquid chromatography (HPLC) analysis (Rizzo et al., 1997). The gabapentin assay was carried out using a HPLC method previously described by Tang et al. (1999).

Blood samples were centrifuged at 2000 rpm for 15 min for carbamazepine, diazepam, phenytoin and phenobarbital determination. The plasma was put into a MPS-1 system (Amicon, Danvers, MA, USA) for the separation of free from protein-bound microsolutes. Plasma samples, 60 μl, were transferred to special sample cups and inserted into an Automatic Clinical Analyser (ACA II, du Pont, Wilmington, DE, USA) which uses a method based on the homogenous enzyme immunoassay technique. For the valproate assay a serum sample, 50 μl, was diluted twice with Tris buffer and analysed using the same method. Control drug solutions were put before and after the respective antiepileptic experimental samples.

2.4. Effects on motor movements

Behavioural changes and their onset and duration were recorded after drug injection until the time of the rotarod test. In particular, two independent observers followed gross behavioural changes consisting of locomotor activity, ataxia, squatting posture and possible piloerection. These behavioural changes were noted but not statistically analysed. Groups of 10 DBA/2 mice, 8–12 g and 22–26 days old, were trained to do coordinated motor movements continuously for 2 min on a 3-cm diameter rotarod turning at 8 rev min^{-1} (U. Basile, Comerio, Varese, Italy). Impairment of coordinated motor movements was defined as inability of the mice to remain on the rotarod for a 2-min test period (Dunham and Miya, 1957). The ability of the mice to remain on the rotarod was tested 45 min after the i.p. administration of saline and one of the conventional antiepileptics, after the combined treatment with carbenoxolone and one of the antiepileptics or after the combined treatment with glycyrrhizin and one of the antiepileptic drugs.

2.5. Statistical analysis

Statistical comparisons of groups of control and drug-treated animals were made using Fisher's exact probability test (incidence of the seizure phases and influence on motor performance) or analysis of variance (ANOVA) and Dunnett's test (rectal temperature). The percent incidence of each phase per dose of the administered compound and the dose–response curves were fitted using linear regression analysis. ED_{50} values ($\pm$ 95% confidence limits) for each compound and each phase of seizure response were estimated using a computer program (SPSS for windows, SPSS, Chicago, USA) for the method of Litchfield and Wilcoxon (1949); the relative anticonvulsant activities were determined by comparison of respective ED_{50} values. The lines of best fit for conventional antiepileptic drug plus saline or in association with carbenoxolone or glycyrrhizin were compared using a χ^2-test, with results expressed for position, parallelism and heterogeneity. TD_{50} values ($\pm$ 95% confidence limits) for each compound were estimated using the method of Litchfield and Wilcoxon (1949). The plasma levels of the drugs are expressed as means $\pm$ S.E.M. of at least eight determinations and Student's *t*-test was used for statistical comparisons.

2.6. Drugs

The drugs and their sources were: carbamazepine (Novartis, Basel, Switzerland), diazepam (Hoffman La Roche, Basel, Switzerland), felbamate (Schering Plough, Milano, Italy), which were suspended in a 1% solution of Tween 80. Valproate (Mg^{2+} salt) (Sigma Tau, Pomezia, Italy), gabapentin (Parke Davies, Milano, Italy), phenobarbital (Na^+ salt, Bracco, Milano, Italy), phenytoin (Na^+ salt, Recordati, Milano, Italy), lamotrigine (Glaxo-Wellcome, Verona, Italy), carbenoxolone (water soluble 18-α-glycyrrethinic acid derivative or 18-α-glycyrrethinic acid 3-β-*O*-hemisuccinate), and glycyrrhizin (Sigma, St. Louis, MO, USA) were dissolved in sterile saline. All drugs were administered intraperitoneally (i.p.), the injection volume was always 0.1 ml/10 g of body weight. Control animals received equivalent volumes of the solvent at the respective times before the test.

3. Results

3.1. Anticonvulsant properties of carbenoxolone and glycyrrhizin in DBA/2 mice

To allow better evaluation of anticonvulsant activity, we exposed the animals to auditory testing at different times after carbenoxolone or glycyrrhizin administration. Carbenoxolone or (10, 15, 20 and 30 mg/kg, i.p.), produced dose-dependent significant protection ($P < 0.01$) against the clonic or tonic phase of the audiogenic seizure response in DBA/2 mice 60, 90, 120 and 180 min after administration (Table 1). Significant protection against the wild running phase was observed 90 and 120 min after carbenoxolone 30 mg/kg, i.p.; a larger dose of carbenoxolone (40 mg/kg, i.p.) was necessary to significantly antagonize the wild running phase when carbenoxolone was administered 60 or 180 min before auditory testing. After carbenoxolone 0.5 and 1 mg/kg, i.p., no significant anticonvulsant activity or behavioural changes were observed, whereas carbenoxolone 5 mg/kg, i.p., was able to significantly antagonize the tonic phase only. ED_{50} values ($\pm$ 95% confidence limits) of carbenoxolone against the different phases of audiogenic seizures are

Table 1
MED_{50} values ($\pm$ 95% confidence limits) of carbenoxolone and glycyrrhizin on audiogenic seizures in DBA/2 mice following various pretreatment times

Pretreatment time (min)	Seizure phase		
	Wild running	Clonus	Tonus
Carbenoxolone (60)	36.2 (25.9–50.6)	7.62 (5.3–10.9)	2.9 (1.9–4.43)
Carbenoxolone (90)	23.9 (16.8–34)	4.69 (2.33–9.31)	0.87 (027–2.85)
Carbenoxolone (120)	26.2 (18.9–36.4)	5.00 (2.36–10.6)	1.39 (0.92–2.1)
Carbenoxolone (180)	34.4 (26.2–45.2)	8.3 (6.1–11.3)	2.75 (1.74–4.35)
Glycyrrhizin (60)	>100	84.2 (61.3–115.6)	59.5 (46.7–75.8)
Glycyrrhizin (90)	92.8 (75.8–113.6)	47.68 (39.17–58.03)	33.2 (23.5–46.9)
Glycyrrhizin (120)	>100	54.4 (42.6–69.5)	43.06 (35.06–52.89)
Glycyrrhizin (180)	>100	73.3 (57.6–93.3)	57.3 (42.4–77.44)

All data above are expressed in mg/kg and were calculated according to the method of Litchfield and Wilcoxon (1949).

reported in Table 1. Parallel investigations with glycyrrhizin showed significant protection against the clonic and tonic phases of seizures following 80, 100 and 120 mg/kg, i.p., at all investigation times. Only at the doses of 100 or 120 mg/kg was glycyrrhizin able to significantly protect against the wild running phase of the audiogenic seizures 90 min before auditory testing. When the auditory test was carried out 90 or 120 min after the i.p. administration of glycyrrhizin (60, 80, 100 and 120 mg/kg) the clonic and tonic phases of the audiogenic seizure response were significantly antagonized in DBA/2 mice. ED_{50} values (± 95% confidence limits) of glycyrrhizin against the different phases of audiogenic seizures are reported in Table 1. The doses of carbenoxolone and glycyrrhizin used in the present study carried neither ataxia nor a fall in rectal temperatures but following the two highest doses of both compounds, a reduction in locomotor activity was evident. Since both compounds appeared to exert their maximal anticonvulsant activity at 90 min (Table 1), we decided to use this pretreatment time for the subsequent studies. The doses of 0.5 mg/kg of carbenoxolone and 10 mg/kg of glycyrrhizin were chosen, being the maximum ineffective dose in our animal model. In addition, as in previous studies, all the conventional anticonvulsants were administered 45 min before auditory testing (De Sarro et al., 1992, 1996, 1998, 2000a,b, 2002).

3.2. Influence of carbenoxolone on the anticonvulsant activity of conventional antiepileptic drugs against audiogenic seizures

The influence of carbenoxolone on the activity of the conventional antiepileptic drugs on the audiogenic seizure response varied according to the different classes. As shown in Table 2, diazepam, carbamazepine, felbamate, gabapentin, lamotrigine, phenobarbital, phenytoin and valproate exhibited anticonvulsant activity in the audiogenic seizure model of DBA/2 mice. Pretreatment (45 min before anticonvulsant administration) with carbenoxolone (0.5 mg/kg, i.p.) was able to produce a consistent shift to the left of the dose–response curves and a significant reduction of ED_{50} values of antiepileptics, suggesting an increase in anticonvulsant activity. All dose–response curves were parallel. The degree of potentiation by carbenoxolone varied among the anticonvulsant drugs, being greatest for diazepam, phenobarbital, gabapentin, valproate and felbamate, less for lamotrigine, phenytoin, and carbamazepine.

3.3. Influence of glycyrrhizin on the anticonvulsant activity of conventional antiepileptic drugs against audiogenic seizures

The anticonvulsant activity of diazepam, phenytoin and valproate against audiogenic seizures was slightly increased by glycyrrhizin (10 mg/kg, i.p.). In particular, glycyrrhizin lowered the ED_{50} value for clonus of diazepam from 0.28 to 0.20 mg/kg, that of phenytoin from 2.5 to 1.91 mg/kg and

Table 2
ED_{50} values (± 95% confidence limits) of saline + some antiepileptic drugs or in association with carbenoxolone (0.5 mg/kg, i.p.) or glycyrrhizin (10 mg/kg, i.p.) against audiogenic seizures in DBA/2 mice

Seizure phase	Drug + saline	Drug + carbenoxolone	Drug + glycyrrhizin
Wild running			
Carbamazepine	10.6 (8.1–13.8)	6.9 (5.2–9.2)[b]	9.6 (7.4–12.4)
Diazepam	0.49 (0.34–0.71)	0.21 (0.14–0.31)[a]	0.45 (0.31–0.65)
Felbamate	114.6 (92–142.7)	48.4 (32.2–72.7)[a]	118.2 (94.2–148.3)
Gabapentin	38 (16–51)	21.2 (17.1–26.2)[a]	38 (16–51)
Lamotrigine	6.1 (4.6–8.1)	3.9 (2.8–5.4)[b]	5.9 (4.4–7.9)
Phenobarbital	7.1 (5.6–9)	3.4 (2.5–4.6)[a]	6.9 (5.1–9.3)
Phenytoin	4.3 (3.1–6)	2.9 (2.1–4.0)[b]	3.8 (2.6–5.5)
Valproate	84 (63–114)	40 (25–64)[a]	68 (47–98.4)
Clonus			
Carbamazepine	4.4 (3.6–5.4)	2.6 (1.8–3.7)[b]	4.0 (3.1–5.2)
Diazepam	0.28 (0.2–0.39)	0.12 (0.09–0.16)[a]	0.2 (0.14–0.29)
Felbamate	48.8 (35.4–67.2)	26.6 (17.9–39.5)[a]	44.2 (31.1–62.8)
Gabapentin	20.3 (13.7–30.2)	9.6 (8.3–11.1)[a]	20.3 (13.7–30.2)
Lamotrigine	3.5 (2.4–5.1)	2.0 (1.4–2.9)[b]	3.3 (2.1–5.2)
Phenobarbital	3.4 (2.3–5)	1.5 (0.9–2.5)[a]	3.2 (2.1–4.9)
Phenytoin	2.5 (1.8–3.5)	1.4 (0.9–2.2)[b]	1.91 (1.22–3.0)
Valproate	43 (33–56)	19.5 (15.1–25.2)[a]	31.2 (22.1–44)
Tonus			
Carbamazepine	3.0 (2.6–3.8)	1.8 (1.2–2.7)[b]	2.8 (2.1–3.73)
Diazepam	0.24 (0.15–0.39)	0.11 (0.07–0.17)[a]	0.18 (0.12–0.27)
Felbamate	23.1 (12.1–44)	12.8 (9.9–16.5)[a]	22.6 (14.7–34.7)
Gabapentin	13.9 (8.7–22.3)	6.5 (4.9–8.6)[a]	13.9 (8.7–22.3)
Lamotrigine	1.1 (0.7–1.8)	0.7 (0.5–1.0)[b]	1.2 (0.8–1.8)
Phenobarbital	2.4 (1.7–3.4)	1.1 (0.7–1.73)[a]	2.2 (1.5–3.23)
Phenytoin	2.0 (1.6–2.5)	0.9 (0.6–1.35)[a]	0.19 (0.12–0.30)
Valproate	31 (22–43)	13.4 (9.5–18.9)[a]	26.2 (17.4–39.6)

All data above are expressed in mg/kg and were calculated according to the method of Litchfield and Wilcoxon (1949). Significant differences in the ED_{50} values between concurrent groups of saline plus antiepileptic drug, carbenoxolone plus antiepileptic drug or glycyrrhizin plus antiepileptic drug-treated groups are denoted by $^{b}P<0.05$ and $^{a}P<0.01$.

that of valproate from 43 to 31.2 mg/kg, respectively (Table 2). Glycyrrhizin was unable to significantly affect the antiseizure properties of any of the antiepileptics studied (Table 2).

3.4. Influence of carbenoxolone or glycyrrhizin on the motor impairment induced by antiepileptic drugs

No gross behavioural changes and neurological deficits were detected at any doses of carbenoxolone and glycyrrhizin. Carbenoxolone administered at dose levels up to 50 mg/kg, i.p., and glycyrrhizin administered at dose levels up to 120 mg/kg, i.p., did not significantly affect locomotor performance. When applied at doses equal to their ED_{50} values against the clonic phase of the audiogenic seizures, carbamazepine (4.4 mg/kg), diazepam (0.28 mg/kg), gabapentin (20.3 mg/kg), felbamate (48.8 mg/kg), lamotrigine (3.5 mg/kg), phenytoin (2.5 mg/kg), phenobarbital (3.4 mg/kg), and valproate (43 mg/kg) did not influence the motor performance of DBA/2 mice. Higher doses were necessary to produce motor impairment (Table 3). Concomitant treatment with one antiepileptic and carbenoxolone (0.5 mg/kg, i.p.) resulted in a weak increase of motor impairment (Table 3). In fact, the therapeutic index of combined treatment with carbamazepine + carbenoxolone, diazepam + carbenoxolone, felbamate + carbenoxolone, gabapentin + carbenoxolone, phenobarbital + carbenoxolone, lamotrigine + carbenoxolone, phenytoin + carbenoxolone or valproate + carbenoxolone was more favourable than that for carbamazepine + saline, diazepam + saline, felbamate + saline, gabapentin + saline, phenobarbital + saline, lamotrigine + saline, phenytoin + saline or valproate + saline (Fig. 1 and Table 3). Glycyrrhizin (10 mg/kg, i.p.) administered concomitantly with each antiepileptic did not significantly affect locomotor performance (data not shown).

Table 3
TD_{50} values (± 95% confidence limits) of saline + various antiepiletics and carbenoxolone + antiepileptics obtained with the rotarod

Treatment	TD_{50} Locomotor deficit	TD_{50}/ED_{50}
Saline + carbamazepine	46.5 (37.9–57)	10.6
Carbenoxolone + carbamazepine	35.1 (26.4–46.7)	13.5
Saline + diazepam	3.8 (3.0–4.8)	13.6
Carbenoxolone + diazepam	2.9 (1.8–4.67)	24.2
Saline + felbamate	816 (590–1024)	16.7
Carbenoxolone + felbamate	746 (626–899)	28
Saline + gabapentin	290.3 (218.3–386)	14.3
Carbenoxolone + gabapentin	187.2 (140.7–249.1)	19.5
Saline + phenytoin	48.3 (50.9–68.4)	19.3
Carbenoxolone + phenytoin	29.6 (22.3–39.3)	21.1
Saline + lamotrigine	81 (55–118)	23.1
Carbenoxolone + lamotrigine	51.2 (43.1–60.8)	25.6
Saline + phenobarbital	139 (115–168)	40.9
Carbenoxolone + phenobarbital	84 (61–115.6)	56
Saline + valproate	290 (240–251)	6.7
Carbenoxolone + valproate	225 (181–279.7)	11.5

All data are expressed as mg/kg and were calculated according to the method of Litchfield and Wilcoxon (1949). TD_{50}/ED_{50} = therapeutic index which represents the ratio between TD_{50} and ED_{50} from the clonic phase of the audiogenic seizures. No significant differences were observed between concurrent groups.

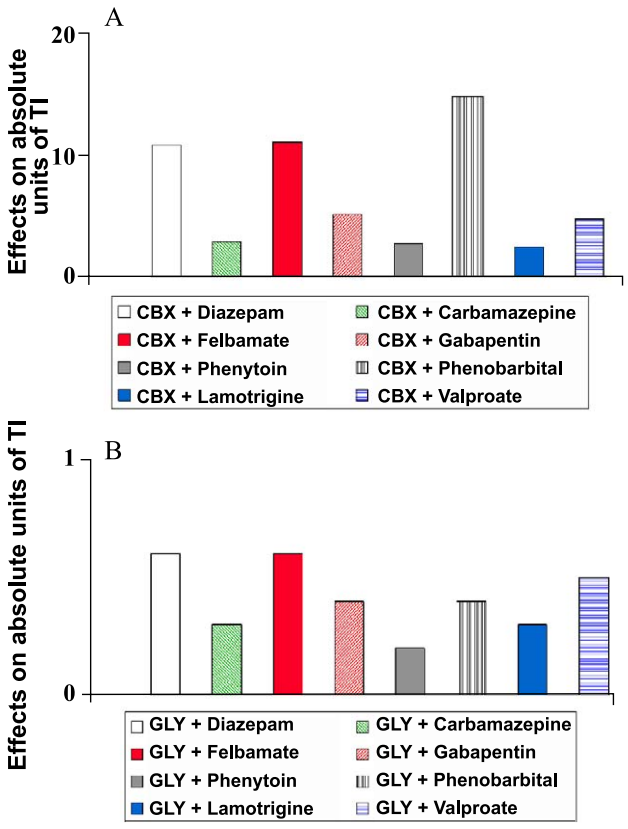

Fig. 1. (A) Effects of a single administration of carbenoxolone (0.5 mg/kg, i.p.) or glycyrrhizin (10 mg/kg, i.p.) in combination with some antiepileptics on absolute units of therapeutic index (TI). Note that the combined treatments with carbenoxolone (0.5 mg/kg, i.p.) combined with carbamazepine, diazepam, felbamate, gabapentin, lamotrigine, phenobarbital, phenytoin or valproate resulted in a favourable therapeutic index. On the contrary, a combination of glycyrrhizin (10 mg/kg, i.p.) with some antiepileptics (B) caused no marked changes in motor impairment.

3.5. Effects of combined treatment with carbenoxolone or glycyrrhizin with antiepileptic compounds on body temperature

The body temperature was recorded in animals given saline + anticonvulsant drugs or carbenoxolone or glycyrrhizin + anticonvulsant drugs. Hypothermic effects were observed only after administration of saline + the highest doses of carbamazepine (20, 30 and 50 mg/kg, i.p.), diazepam (3 and 5 mg/kg, i.p.) and valproate (100, 200 and 300 mg/kg, i.p.). In particular, we observed a dose-dependent reduction in body temperature of 2–4 °C only after these drugs. No significant differences among groups treated with saline + felbamate, gabapentin, lamotrigine, phenytoin, phenobarbital or low doses of carbamazepine, diazepam or valproate were evident (data not shown). A non-significant increase of hypothermic effects was observed in mice receiving carbamazepine, diazepam or val-

proate + carbenoxolone when compared to their concurrent saline + carbamazepine, diazepam or valproate group. On the contrary, mice treated with carbenoxolone (0.5 mg/kg, i.p.) + the other antiepileptic drugs showed no significant changes of hypothermic effects when compared with concurrent saline + antiepileptic drugs-treated animals. No significant changes in hypothermic effects were observed when antiepileptics were administered concomitantly with glycyrrhizin (data not shown).

3.6. Influence of carbenoxolone on the total and free plasma levels of antiepileptic drugs

Blood concentrations of carbamazepine, diazepam, felbamate, gabapentin, lamotrigine, phenytoin, phenobarbital and valproate are presented in Table 4. The dose of carbenoxolone studied did not significantly modify the plasma levels of carbamazepine (15 mg/kg, i.p.), felbamate (100 mg/kg, i.p.), lamotrigine (10 mg/kg, i.p.), phenytoin (10 mg/kg, i.p), phenobarbital (20 mg/kg, i.p), gabapentin (70 mg/kg, i.p.), valproate (200 mg/kg, i.p.) and diazepam (5 mg/kg, i.p.). A small increase of free plasma levels of carbamazepine, diazepam, phenytoin and valproate, was observed following concomitant treatment with carbenoxolone.

Table 4
Influence of carbenoxolone on total and free plasma levels of some antiepileptic compounds (carbamazepine, diazepam, felbamate, gabapentin, lamotrigine, phenytoin, phenobarbital, and valproate) in DBA/2 mice

Treatment (time) (dose mg/kg)	Saline + compound		Carbenoxolone + compound	
	Total	Free	Total	Free
Carbamazepine (60 min) (15 mg/kg)	5.2 ± 0.7	0.62 ± 0.2	5.1 ± 0.5	0.66 ± 0.2
Diazepam (60 min) (5 mg/kg)	2.1 ± 0.2	0.15 ± 0.05	2.0 ± 0.3	0.21 ± 0.05
Felbamate (60 min) (100 mg/kg)	4.2 ± 0.3	3.1 ± 0.3	4.1 ± 0.3	3.0 ± 0.3
Gabapentin (45 min) (70 mg/kg)		10.2 ± 1.5		9.5 ± 1.5
Lamotrigine (45 min) (10 mg/kg)	1.8 ± 0.2	0.67 ± 0.07	1.9 ± 0.2	0.69 ± 0.1
Phenytoin (120 min) (10 mg/kg)	8.8 ± 1.8	0.9 ± 0.1	8.4 ± 2.0	1.2 ± 0.1
Phenobarbital (60 min) (20 mg/kg)	35.3 ± 3.1	4.4 ± 0.3	35.2 ± 3.2	4.5 ± 0.4
Phenobarbital (120 min) (20 mg/kg)	22.4 ± 2.5	3.1 ± 0.3	21.3 ± 2.4	3.0 ± 0.3
Valproate (30 min) (200 mg/kg)	251 ± 22	40.2 ± 3.9	245 ± 23	43 ± 3.9
Valproate (60 min) (200 mg/kg)	309 ± 29	49.4 ± 4.1	298 ± 31	60 ± 4.2

Drugs were administered i.p. Saline or carbenoxolone (0.5 mg/kg, i.p.) + lamotrigine and gabapentin 45 min, carbamazepine, diazepam, and felbamate 60 min, phenobarbital 60 and 120 min, phenytoin 120 min and valproate 30 and 60 min before drawing the sample. Values are means (μg/ml) of at least eight determinations ± S.E.M. Student's *t*-test was used for statistical analysis of the data.

3.7. Influence of glycyrrhizin on total and free plasma levels of antiepileptic drugs

Blood concentrations of carbamazepine, diazepam, felbamate, gabapentin, lamotrigine, phenytoin, phenobarbital and valproate were evaluated in mice receiving concomitantly one antiepileptic and glycyrrhizin (10 mg/kg, i.p.) or vehicle. The dose of glycyrrhizin studied did not significantly modify the plasma levels of carbamazepine (15 mg/kg, i.p.), felbamate (100 mg/kg, i.p.), lamotrigine (10 mg/kg, i.p.), phenytoin (10 mg/kg, i.p.), phenobarbital (20 mg/kg, i.p.), gabapentin (70 mg/kg, i.p.), valproate (200 mg/kg, i.p.) and diazepam (5 mg/kg, i.p.). A small increase of free plasma levels of carbamazepine, diazepam, phenytoin and valproate was observed following concomitant treatment with glycyrrhizin (data not shown).

4. Discussion

Our data demonstrated that carbenoxolone exerts anticonvulsant activity in audiogenic sensitive DBA/2 mice. Glycyrrhizin, a natural analogue of carbenoxolone, which is inactive as gap junction blocker (Davidson et al., 1986), is at least 20 times less potent than carbenoxolone to decrease seizures in DBA/2 mice.

Since glycyrrhizin is the glycosidic derivative of 18-β-glycyrrethinic acid, it is possible that the low activity observed with this molecule is due to the in vivo release of the aglycone portion of the molecule. Moreover, glycyrrhizin did not influence the activity of conventional anticonvulsants against audiogenic seizures in DBA/2 mice. The lack of efficacy of glycyrrhizin is consistent with the hypothesis that the block of gap junction communication is the main mechanism of action of carbenoxolone. The selective anticonvulsant effects of carbenoxolone in our mouse model agree with previous reports (Ross et al., 2000; Venance et al., 1998; Li et al., 2001). Indeed, carbenoxolone is lipophilic, easily penetrating to the brain in vivo (Jellinck et al., 1993; Dobbins and Saul, 2000).

The gap-junction blockade mediates intracellular coupling of carbenoxolone and was implicated as one of the major mechanisms behind its anticonvulsant action (Draguhn et al., 1998; Davidson et al., 1986; Carlen et al., 2000; Cross and Baldeweg, 2001; Kohling et al., 2001; Margineau and Klitgaard, 2001). As regards carbenoxolone, the majority of studies indicate that it is able to reduce the frequency of spontaneous bursts and to delay the induction of epileptiform activity (Ross et al., 2000; Traub

et al., 2001; Pais et al., 2003). The efficacy of the ketogenic diet for treating intractable seizures might be attributable to the block of gap-junctions via intracellular acidosis or by chemicals in the diet (Perez-Velazquez and Carlen, 2000). The present study is in agreement with a recent report by Hosseinzadeh and Nassiri Asl (2003), however, we have used lower doses which did not have any significant muscle relaxant effect. We first demonstrated that carbenoxolone might exert in vivo anticonvulsant actions in a genetic model of generalized clonic tonic seizures without displaying sedation and motor impairment. In addition, the administration of a low dose of this compound was able to enhance the action of some conventional antiepileptics.

The mechanisms causing the observed augmentation of effectiveness of the conventional antiepileptics mentioned are most probably not related to a pharmacokinetic interaction, as the free plasma levels of some compounds studied, with the exception of carbamazepine, diazepam, phenytoin and valproate remained unchanged in the presence of carbenoxolone. A possible increase of free plasma levels of the latter drugs might be due to an albumin-binding interaction. Under the conditions used, the antiepileptic potency of diazepam, phenobarbital, felbamate, gabapentin and valproate was affected by carbenoxolone much more than that of lamotrigine, phenytoin or carbamazepine.

What might account for such a selective action? Under our experimental conditions, the enhancing effect was less evident with drugs possessing membrane-stabilizing effects (i.e. carbamazepine, lamotrigine and phenytoin). An alternative explanation can also be suggested for the different mechanisms of action underlying the anticonvulsant activity of the antiepileptic drugs studied. Diazepam, phenobarbital, felbamate and valproate, in contrast to phenytoin, lamotrigine and carbamazepine, act mainly via other specific mechanisms (augmentation of γ-aminobutyric acid transmission, action on NMDA receptors and on voltage Ca^{2+} channels (White, 1997, 1999; Meldrum, 1997; Gareri et al., 1999). Assuming that the potentiation of antiepileptic activity observed here is related to the blockade of selective connexins exerted by carbenoxolone, it is possible to hypothesize that this compound should enhance the anticonvulsant action of some antiepileptics.

Both carbenoxolone and glycyrrhizin per se weakly influenced, at the doses studied, the motor activity of animals, as revealed by the rotarod test (data not shown). Carbenoxolone was able to increase the therapeutic index of the antiepileptics studied.

In summary, carbenoxolone significantly enhanced the antiepileptic activity of diazepam, gabapentin, phenobarbital, felbamate, and valproate against audiogenic seizures, whereas the action of lamotrigine, phenytoin and carbamazepine was less markedly changed by the co-administration of carbenoxolone. Although experimental data may be hard to extrapolate to clinical practice, it can be hypothesized that the use of drugs blocking the gap junctions might be potentially useful in the human therapy of some types of pharmacoresistant epilepsies. Future experiments should characterize whether or not the blockade of selective connexins has a similar anticonvulsant activity in other animal models.

References

Belluardo, N., Trovato-Salinaro, A., Mudò, G., Hurd, Y.L., Condorelli, D.F., 1999. Structure, chromosomal localization, and brain expression of human Cx36 gene. J. Neurosci. Res. 57, 740–752.

Bennet, M.V.L., 1997. Gap junctions as electrical synapses. J. Neurocytol. 26, 349–366.

Carlen, P.L., Skinner, F., Zhang, L., Naus, C., Kushnir, M., Perez-Velazquez, J., 2000. The role of gap junctions in seizures. Brains Res. Rev. 32, 235–241.

Condorelli, D.F., Belluardo, N., Trovato-Salinaro, A., Mudò, G., 2000. Expression of Cx36 in mammalian neurons. Brains Res. Rev. 32, 72–85.

Condorelli, D.F., Mudò, G., Trovato-Salinaro, A., Mirone, M.B., Amato, G., Belluardo, N., 2002. Connexin-30 mRNA is up-regulated in astrocytes and expressed in apoptotic neuronal cells of rat brain following kainate-induced seizures. Mol. Cell. Neurosci. 21, 94–113.

Cross, H., Baldeweg, T., 2001. A possible role for gap junctions in generation of very fast EEG oscillations preceding the onset of, and perhaps initiating, seizures. Epilepsia 42, 153–170.

Davidson, J.S., Baumgarten, I.M., 1988. Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure–activity relationships. J. Pharmacol. Exp. Ther. 246, 1104–1107.

Davidson, J.S., Baumgarten, I.M., Harley, E.H., 1986. Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. Biochem. Biophys. Res. Commun. 134, 29–36.

De Sarro, G.B., Croucher, M.J., Meldrum, B.S., 1984. Anticonvulsant actions of DS 103–282: pharmacological studies in rodents and the baboon *Papio papio*. Neuropharmacology 23, 526–530.

De Sarro, G.B., De Sarro, A., Trimarchi, G.R., Nisticò, G., 1992. Effects of some calcium antagonists upon the activity of common antiepileptic compounds on sound-induced seizures in DBA/2 mice. Gen. Pharmacol. 23, 75–82.

De Sarro, G.B., Nava, F., Aguglia, U., De Sarro, A., 1996. Lamotrigine potentiates the antiseizure activity of some anticonvulsants in DBA/2 mice. Neuropharmacology 35, 153–158.

De Sarro, G.B., Spagnolo, C., Gareri, P., Gallelli, L., De Sarro, A., 1998. Gabapentin potentiates the antiseizure activity of certain anticonvulsants in DBA/2 mice. Eur. J. Pharmacol. 349, 179–185.

De Sarro, G., Gareri, P., Falconi, U., De Sarro, G., 2000a. 7-Nitroindazole potentiates the antiseizure activity of some anticonvulsants in DBA/2 mice. Eur. J. Pharmacol. 394, 275–288.

De Sarro, G.B., Gratteri, S., Bonacci, F., Musumeci, S.A., Elia, M., De Sarro, A., 2000b. Topiramate potentiates the antiseizure activity of some anticonvulsants in DBA/2 mice. Eur. J. Pharmacol. 388, 163–170.

De Sarro, G., Donato Di Paola, E., Ferreri, G., De Sarro, A., Fischer, W., 2002. Influence of some β-adrenoceptor antagonists on the anticonvulsant potency of antiepileptic drugs against audiogenic seizures in DBA/2 mice. Eur. J. Pharmacol. 442, 205–213.

Dobbins, K.R., Saul, R.F., 2000. Transient visual loss after licorice ingestion. J. Neuro-ophthalmol. 20, 38–41.

Dodic, M., May, C.N., Coghlan, J.P., 1998. Carbenoxolone does not cause a syndrome of mineralcorticoid excess in sheep. Steroids 63, 99–104.

Draguhn, A., Traub, R.D., Schmitz, D., Jefferys, J.G., 1998. Electrical coupling underlies high-frequency oscillations in the hippocampus in vitro. Nature 394, 189–192.

Dunham, N.W., Miya, T.S., 1957. A note on a simple apparatus for detect-

ing neurological deficit in rats and mice. J. Am. Pharm. Assoc. 46, 208–209.

Gareri, P., Gravina, T., Ferreri, G., De Sarro, G., 1999. Treatment of epilepsy in the elderly. Prog. Neurobiol. 58, 389–407.

Hosseinzadeh, H., Nassiri Asl, M., 2003. Anticonvulsant, sedative and muscle relaxant effects of carbenoxolone in mice. BMC Pharmacol. 3, 3.

Jahromi, S.S., Wentlandt, K., Piran, S., Carlen, P.L., 2002. Anticonvulsant actions of gap junctional blockers in an in vitro seizure model. J. Neurophysiol. 88, 1893–1902.

Jellinck, P.H., Monder, C., McEwen, B.S., Sakai, R.R., 1993. Differential inhibition of 11beta-hydroxysteroid dehydrogenase by carbenoxolone in rat brain regions and peripheral tissues. J. Steroid Biochem. Mol. Biol. 46, 209–213.

Kohling, R., Gladwell, S.J., Bracci, E., Vreugdenhil, M., Jefferys, J.G., 2001. Prolonged epileptiform bursting induced by O–Mg (2+) in rat hippocampal slices depends on gap junctional coupling. Neuroscience 105, 579–587.

Li, J., Shen, H., Naus, C.C.G., Zhang, L., Carlen, P.L., 2001. Upregulation of GAP junction connexin 32 with epileptiform activity in the isolated mouse hippocampus. Neuroscience 105, 589–598.

Litchfield, J.T., Wilcoxon, F., 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96, 99–113.

Margineau, D.G., Klitgaard, H., 2001. Can gap-junction blockade preferentially inhibit neuronal hypersynchrony vs. excitability? Neuropharmacology 41, 377–383.

McNamara, J.O., 1994. Cellular and molecular basis of epilepsy. J. Neurosci. 14, 3413–3425.

Meldrum, B.S., 1997. Identification and preclinical testing of novel antiepileptic compounds. Epilepsia 38, S7–S15.

Pais, I., Hormuzdi, S.G., Monyer, H., Traub, R.D., Wood, I.C., Buhl, E.H., Whittington, M.A., LeBeau, F.E., 2003. Sharp wave-like activity in the hippocampus in vitro in mice lacking the gap junction protein connexin 36. J. Neurophysiol. 89, 2046–2054.

Perez-Velazquez, J.L., Carlen, P.L., 2000. Gap junctions, synchrony and seizure. Trends Neurosci. 23, 68–74.

Rizzo, M., Morrone, L., Longo, P., Sino poli, V.A., Spagnolo, C., Lo Pilato, R., Pelaggi, T., David, E., Rotiroti, D., De Sarro, G.B., 1997. Simultaneous determination of lamotrigine, felbamate and some conventional antiepileptic drugs using high performance liquid chromathography. Pharmacol. Res. 35, 105–109.

Rogawski, M.A., 1998. Mechanism-specific pathways for new antiepileptic drug discovery. In: French, J., Leppik, I., Dichter, M.A. (Eds.), Antiepileptic Drug Development. Lippincott-Raven Publishers, Philadelphia, pp. 11–27.

Ross, F.M., Gwyn, P., Spanswick, D., Davies, S.N., 2000. Carbenoxolone depresses spontaneous epileptiform activity in the CA1 region of rat hippocampal slices. Neuroscience 100, 789–796.

Rouach, N., Glowinski, J., Giaume, C., 2000. Activity-dependent neuronal control of gap-junctional communication in astrocytes. J. Cell Biol. 149, 1513–1526.

Szeute, M., Gajda, Z., Said Ali, K., Hermesz, E., 2002. Involvement of electrical coupling in the in vivo ictal epileptiform activity induced by 4-aminopyridine in the neocortex. Neuroscience 115, 1067–1078.

Tang, P.H., Miles, M.V., Glauser, T.A., DeGrauw, T., 1999. Automated microanalysis of gabapentin in human serum by HPLC with fluorometric detection. J. Chromatogr., B, Biomed. Sci. Appl. 727, 125–129.

Traub, R.D., Whittington, M.A., Buhl, E.H., Lebeau, F.E., Bibbig, A., Boyd, S., Cross, H., Baldeweg, T., 2001. Very fast EEG oscillations preceding the onset of, and perhaps initiating seizures. Epilepsia 42, 153–170.

Traub, R.D., Draguhn, A., Whittington, M.A., Baldewag, T., Bibbig, A., Buhl, E.H., Schmitz, D., 2002. Axonal gap junctions between principal neurons: a novel source of network oscillations and perhaps epileptogenesis. Rev. Neurosci. 13, 1–30.

Venance, L., Premont, J., Glowinski, J., Giaume, G., 1998. Gap-junctional communication and pharmacological heterogeneity in astrocytes cultured from the rat striatum. J. Physiol. 510, 429–440.

White, H.S., 1997. Clinical significance of animal seizure models and mechanisms of action: studies of potential antiepileptic drugs. Epilepsia 38, S9–S17.

White, H.S., 1999. Comparative anticonvulsant and mechanistic profile of the established and newer antiepileptic drugs. Epilepsia 40, S2–S10.