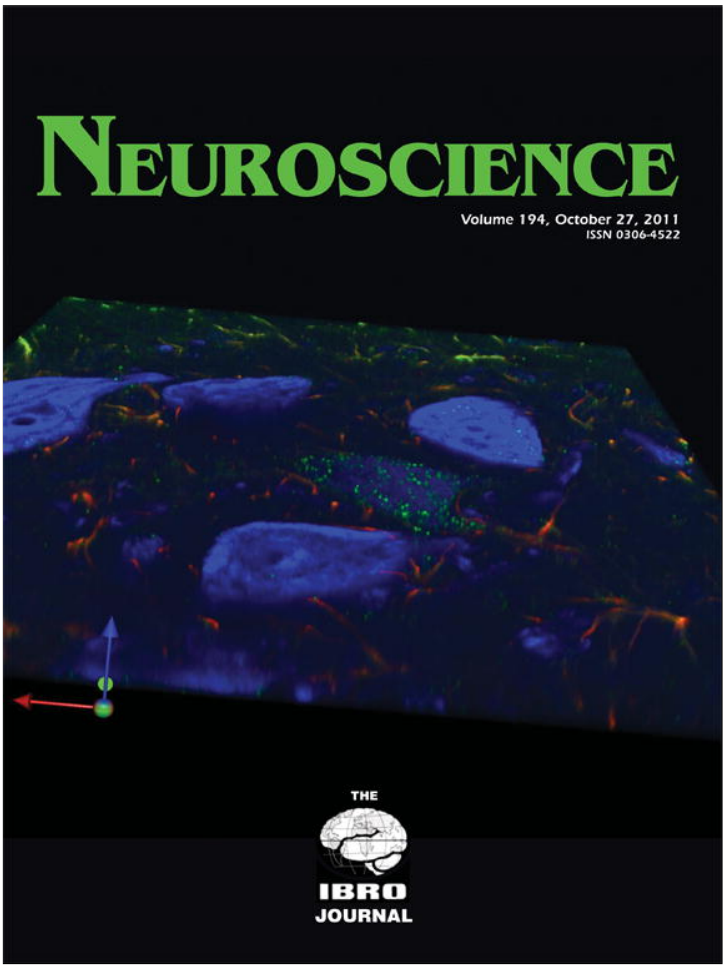

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THE EFFECT OF A GAP-JUNCTION BLOCKER, CARBENOXOLONE, ON ISCHEMIC BRAIN INJURY AND CORTICAL SPREADING DEPRESSION

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Abstract—Cortical spreading depression (CSD) has been shown to cause secondary cell loss in experimental models of brain injury and in patients, and blocking of CSD is a potential neuroprotective strategy. Here we tested the hypothesis that gap junctions affect CSD under physiological conditions as well as infarct development in a rat two-vein occlusion model suited to study pathophysiology of the penumbra (n=71). We applied the gap junction blocker carbenoxolone (CBX) or saline intra-ventricularly. Interestingly, CBX temporarily increased systemic blood pressure and cortical blood flow (41% and 53%, 15 min after 250 μg CBX). We induced CSD with cortical microinjection of potassium chloride (KCl), counted how many spontaneous CSDs after CSD induction were elicited and measured the propagation velocity. After 250 μg CBX administration, significant 37.5±6.5 additional CSDs were seen. CSD velocity increased significantly after 50 μg and 250 μg CBX. Occlusion of two adjacent cortical veins using Rose Bengal dye and fiberoptic illumination followed by 250 μg CBX or saline showed a significant more than doubling of infarct volumes 7 days after CBX. The current experiments provide evidence that CBX can accelerate the initiation and propagation of CSD suggesting opening of gap junctions is not required for CSD propagation. Blocking gap junctions worsens outcome from focal cerebral ischemia. Hence, measures intended to improve spatial buffering via astroglial gap junctions could have therapeutic potential in disease processes involving CSD.

Key words: gap junctions, carbenoxolone, cortical spreading depression, cerebral blood flow, focal cerebral ischemia.

Cortical spreading depression (CSD) is an electrophysiological phenomenon first described experimentally by Leão (Leao, 1944). CSD is a state of depressed cortical activity following electrical or mechanical stimulation spreading from its origin. Under physiological conditions CSD is transient and not associated with neuronal injury (Nedergaard and Hansen, 1988). Later, Leão also reported CSD following basilar and bilateral carotid artery occlusion in rabbits, that is, global cerebral ischemia (Leao, 1947). If ischemia is severe or anoxia occurs, a persistent electrical depression is induced that is referred to as anoxic depolarization (AD). CSD is characterized by rapid and almost complete depolarization of neurons and glia with a wave like redistribution of ions between intracellular and extracellular space travelling as a wave across the cerebral cortex. CSD is a process with spontaneous recovery propagating at a speed of 2–3 mm/min (Somjen, 2001). These ion shifts lead to cell swelling and shrinkage of the extracellular space and can be detected by electrical impedance measurement which assess changes of the extracellular space (Ochs and Van Harreveld, 1956). In order to normalize the disturbed extra- and intracellular ion balance, brain tissue requires significant energy reserves (Wolf et al., 1996) and the increased metabolism goes along with vasodilation (Leao, 1944). Under pathophysiological conditions (e.g. focal ischemia), however, an inverse hemodynamic reaction has been observed in regions with compromised blood flow (Sonn and Mayevsky, 2000). In this case gradients of cerebral blood flow (CBF), oxygen, and glucose exist that allow an initial persistent, anoxic depolarization in the ischemic core to become a transient depolarization in surrounding tissue with reduced CBF and energy supply and a CSD in healthy tissue (Nallet et al., 1999, 2000; Nedergaard et al., 1995; Shin et al., 2006). Consequently, vasoconstriction and increased energy demand leads to cell death in the peri-ischemic area around the core, thereby expanding the injury (Busch et al., 1996). CSD has been shown to cause secondary cell loss in various experimental models of brain injury and recently also in patients (Dreier et al., 2006; Oliveira-Ferreira et al., 2010; Von Baumgarten et al., 2008). Thus, blocking of initiation and propagation of CSD seems to be a neuroprotective strategy.

The mechanisms of CSD initiation and propagation are still not completely understood. CSD is thought to be a cascade of interlocking mechanisms that involve the coupling of both intracellular and extracellular spaces, and very likely the coupling of populations of cells via gap junctions (Martins-Ferreira et al., 2000). Astrocytic gap junctions could play a pivotal role to prevent CSD and lesion expansion since they are crucial for spatial buffering of ions and small molecules and their function is reduced following cerebral ischemia (Leis et al., 2005).

Gap junction blockers such as octanol, halothane, or heptanol have been shown to reduce CSD propagation velocity and to block initiation of CSD (Nedergaard et al., 1995). In contrast, it was reported that astrocyte-directed inactivation of connexin 43, the most prominent compo-

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Abbreviations: CBF, cerebral blood flow; CSD, cortical spreading depression; IMP, impedance; 2-VO, two-vein occlusion.

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nent of astrocytic gap junctions, accelerated spreading depression (Theis et al., 2003) and carbenoxolone (CBX), which is considered to be the most selective gap junction blocker, did not prevent cortical spreading depression in hippocampal slices (Peters et al., 2003).

On the other hand, regardless of CSD there are some evidences that gap junction opening is necessary for spatial buffering of apoptotic agents, consequently opening of gap junction is neuroprotective against ischemia *in vitro* (Siushansian et al., 2001; Nakase et al., 2003b). However there have been reported to be opposite results in a global ischemia model, CBX could block the free radicals spread through astroglial gap junctional communication, and hippocampal cell death was reduced *in vivo* (Nodin et al., 2005) and cell death caused by oxygen-glucose deprivation was decreased with CBX in perinatal rats (de Pina-Benabou et al., 2005).

We have previously developed a venous infarct model with occlusion of two adjacent cortical veins (Nakase et al., 1995, 1996, 1997, 1998). At this location, CBF in the vicinity of occluded veins is reduced rather homogeneously, with a large penumbra, a slowly growing infarct, and delayed cell death (Heimann et al., 1999; Murata et al., 2001). This two-vein occlusion (2-VO) model therefore seems suited to the study of pathophysiological mechanisms, including infarct development in the penumbra.

In the present study we tested the hypothesis that gap junction blockade influences CSD and CBF under physiological conditions and infarct development in a two-vein occlusion model *in vivo*. We applied the gap junction blocker CBX into the rat lateral ventricle to elucidate its effects on initiation and propagation of CSD which was induced by cortical microinjection of potassium chloride. The initiation and propagation of CSD was evaluated by registering the frequency of elicited CSDs and their velocity. In addition, we measured the venous infarct volume to clarify the role of gap junctions in infarct development.

EXPERIMENTAL PROCEDURES

This study was conducted according to German animal protection legislation and has been reviewed by the regional ethics committee (Approval number AZ 177-07/011-1).

Animal preparation

The experiments were performed using 71 male Wistar rats (weight 360.8±4.53 g Charles River Laboratories, Sulzfeld, Germany). The animals were housed in individual cages and had free access to food and water. Anesthesia was induced by an i.p. injection of chloral hydrate (36 mg/100 g body weight), and the animals were premedicated with 1 mg of s.c. administered atropine. Anesthesia was maintained with chloral hydrate (12 mg/100 g body weight/h) administered hourly through a peritoneal catheter. All animals were intubated with silicon tubing (outer diameter, 2.5 mm) and mechanically ventilated using a rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA, USA) with 30% inspired oxygen and controlled end expiratory PCO_2 (Artema MM206C; Heyer, Sundbyberg, Sweden). Rectal temperature was kept at 37.0 °C by using a feedback-controlled heating pad (Harvard Apparatus), and the left temporal muscle temperature was monitored throughout the experiment. A polyethylene catheter (outer diameter, 0.96 mm; Portex; Smiths Industries Medical Systems Co., London, England) was inserted into the tail artery to monitor mean arterial blood pressure and arterial blood gases, pH, electrolytes, and glucose levels. Another polyethylene catheter was inserted into the left femoral vein. After rats were mounted in a stereotactic frame (Stoelting, Wood Dale, IL, USA), a midline skin incision was prepared and a left parietal cranial window was made to access the brain surface by using a high-speed drill under an operating microscope (OP-Microscope; Zeiss, Wetzlar, Germany). During the craniectomy, the drill tip was cooled continuously with physiological saline to avoid thermal injury to the cortex. The dura was left intact.

Measurement of tissue impedance

To measure cell swelling occurring together with DC-potential changes during cortical spreading depression and during ischemia, two impedance electrodes were introduced into the cortex (depth, 0.4–0.5 mm; distance, 3 mm) (Otsuka et al., 2000; Kempski et al., 2001). The impedance electrodes were made from two stainless steel wires (outer diameter, 0.5 mm) covered by polyvinyl chloride for electrical insulation except for the 0.3 mm sharp-pointed tip. Impedance (IMP) was measured at 1 kHz (10 mV, bias-free) throughout the experiment using a precision LCR monitor (4284A; Hewlett–Packard, Avondale, PA, USA). At that frequency, the alternating current travels through the extracellular space and impedance increases if extracellular space shrinks, that is, cells swell. DC-potential and impedance change together (Otsuka et al., 2000). We defined the stabilized value at 90 min after insertion of electrodes as baseline value (=1.0) and all data of impedance were calculated as ratio to the baseline.

Measurement of CBF

Local cerebral blood flow (lCBF) was assessed by laser Doppler (Model BPM 403a; Vasomedics, St Paul, MN, USA) with 0.8 mm needle probes. Flow is expressed in LD units, which are not arbitrary but have a low biological zero (0–1 LD units) and are one-point calibrated with latex beads at 25 °C in a Teflon vial.

Ischemia by two-vein occlusion (2-VO)

2-VO was performed by occlusion of two adjacent cortical veins by Rose Bengal dye (25–50 mg/kg b.W.) and fiberoptic illumination mercury lamp (6500–7500 lux, 540 nm) that was connected to a 100-μm optical fiber. The two veins were illuminated sequentially for 10–15 min until occluded (Nakase et al., 1996). LD probe was placed adjacent to the two occluded veins to measure lCBF in the penumbra.

Experimental design and treatment groups

Effect of carbenoxolone on CBF (study 1). Rats were assigned to three groups: saline (n=6), CBX 50 μg (n=6), CBX 250 μg (n=8). The laser Doppler probe was placed over a cortical region with flow values at 40–60 LDU representing the microcirculation (Otsuka et al., 2000). After CBF had stabilized for 10 min, rats received an i.c.v. injection of saline or carbenoxolone (=time 0), and mean arterial blood pressure and CBF were monitored for 1 h.

Effect of carbenoxolone on spontaneous CSD occurring during induction of 10 CSDs (study 2). After impedance electrode insertion, a glass micropipette for KCl injection was placed into the lateral parietal cortex for 1 mm in depth which was filled with 150 mmol/L KCl solution. Each rat received 10 injections of 5.0 μl KCl at 7-min intervals using a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden). Reversible cortical spreading depression was detected as a sudden increase (swelling) and decrease (recovery) of tissue impedance. We counted how many CSDs were elicited. Rats were randomly assigned to one of the

following three groups: (1) a vehicle-treated group receiving saline (n=6); (2) a carbenoxolone 50 μg-treated group (n=6); (3) a carbenoxolone 250 μg-treated group (n=6). In all groups, drugs were injected i.c.v. into the right cerebral ventricle (anteroposterior, −0.8 mm; lateral, 1.5 mm; and dorsoventral, 4.0 mm) (Paxinos). CBX was purchased from Sigma Chemical Co. and dissolved in saline. A total of 10 μl saline or carbenoxolone was administered just before the first induction of CSD.

Effect of carbenoxolone on CSD velocity (study 3). After insertion of impedance electrode and a glass micropipette, the closest distance between them was measured with a micrometer caliper. The time interval was measured between injection of KCl and peak of the IMP curve. Velocity (mm/min: the distance divided by the time) was calculated with these two values. We obtained ten data of velocity for each rat (i.e. first to tenth CSD velocity value obtained), and compared these for all groups.

Rats were randomly assigned to one of following two groups: (1) a vehicle-treated group receiving saline (n=7); (2) a carbenoxolone 50 μg-treated group (n=8). In both groups, drugs were injected into the right cerebral ventricle as above and 10 KCl injections were given thereafter.

Effect of carbenoxolone on infarct volume after two-vein occlusion (study 4). As illustrated with Fig. 2b two adjacent cortical veins occlusion was induced using Rose Bengal dye and fiberoptic illumination (6500–7500 lux, wavelength 540 nm) connected to a 100-μm optical fiber in 23 rats. The diameter of the occluded vein was approximately 100 μm. Rose Bengal dye was injected slowly without effect on systemic arterial pressure (50 mg/kg body weight); care was taken to avoid illumination of tissue and other vessels near the targeted vein (Otsuka et al., 2000). To exclude recanalization of the occluded vein, we injected drug 30 min after confirming that the two veins were occluded. Three groups were studied: saline treatment (n=8), CBX 50 μg i.c.v. (n=7), and CBX 250 μg i.c.v. (n=8). During the experiments animals were monitored for blood gases, arterial blood pressure and local cerebral blood flow and after 1 h the wound was closed and animals were brought back to their cages. After 7 days animals were perfusion-fixed with paraformaldehyde and infarct size was determined in Hematoxilin-Eosin stained sections: the infarcted area was evaluated in serial sections in 90-μm steps. The infarction volume V_I was calculated from infarct areas An in individual sections and the distance between sections according to the formula $V_I=\Sigma An * d$ (Otsuka et al., 2000).

Statistical analysis

Data are expressed as means±standard error of the mean. A one-way analysis of variance test was used to compare multiple groups with normal distribution, alternatively the Kruskal–Wallis test was applied. The t-test was used for two-group comparison. Statistical analyses were performed by using SigmaStat software (Jandel Scientific, SPSS, Erkrath, Germany). Statistical significance was assumed at an error probability of $P<0.05$.

RESULTS

Experiments were carried out according to four study designs which are listed in Table 1. Fig. 1A, B are the illustration of experimental setups and timing of drug administration.

Physiological variables

Physiological parameters and body weight of rats remained stable within normal range (Tables 2 and 3). One exception was arterial blood pressure which dose-dependently increased after i.c.v. CBX administration (Fig. 2a): at a CBX dose of 50 μg the increase was only moderate and reached statistical significance after 25 min, whereas at 250 μg the increase was significant from 5 min till 50 min after injection and reached a maximum at 15–20 min after injection (41% increase). Blood pressure had normalized after 60 min.

Table 1. Study design and treatment groups

- Study 1
  - Effects of CBX on CBF
    1. Vehicle NaCl n=6
    2. CBX 50 μg n=6
    3. CBX 250 μg n=8
    - Drugs (10 μl) were injected into the right parietal ventricle immediately before the first of 10 KCl-induced CSD in healthy tissue.
    - Parameter: velocity of CSD wave (time from KCl-injection until peak of IMP change).
    - No venous ischemia.
- Study 2
  - Effects of CBX on CSD velocity
    1. Vehicle NaCl n=7
    2. CBX 50 μg n=8
    - Drugs (10 μl) were injected into the right parietal ventricle immediately before the first of 10 KCl-induced CSD in healthy tissue.
    - Parameter: velocity of CSD wave (time from KCl-injection until peak of IMP change).
    - No venous ischemia.
- Study 3
  - Effects of CBX on frequency of spontaneous CSD
    1. Vehicle NaCl n=6
    2. CBX 50 μg n=6
    3. CBX 250 μg n=6
    - Drugs (10 μl) were injected into the right parietal ventricle immediately before the first of 10 KCl-induced CSD.
    - Parameter: frequency of spontaneous CSD.
    - No venous ischemia.
- Study 4
  - Effects of CBX on infarct size after venous ischemia
    1. Vehicle NaCl n=6
    2. CBX 50 μg n=6
    3. CBX 250 μg n=6
    - Drugs (10 μl) were injected i.c.v. after vein occlusion was completed.
    - Parameter: infarct size at day 7 post-ischemia.

Study 1: effect of CBX on local cortical blood flow

Local CBF increased after 50 μg CBX i.c.v. administraion but reached statistical significance only at the higher dose (250 μg) beginning 10 min after injection and lasting for 30 min after injection (Fig. 2b) with a maximal increase of 53% after 15 min.

Study 2: effect of CBX on occurrence of spontaneous CSDs after CSD induction

We injected potassium chloride (KCl) 10 times at 7-min intervals and counted how many CSDs were elicited. Within 60 s after KCl injection into the cortex, one or more CSDs were always observed as a sudden tempo-

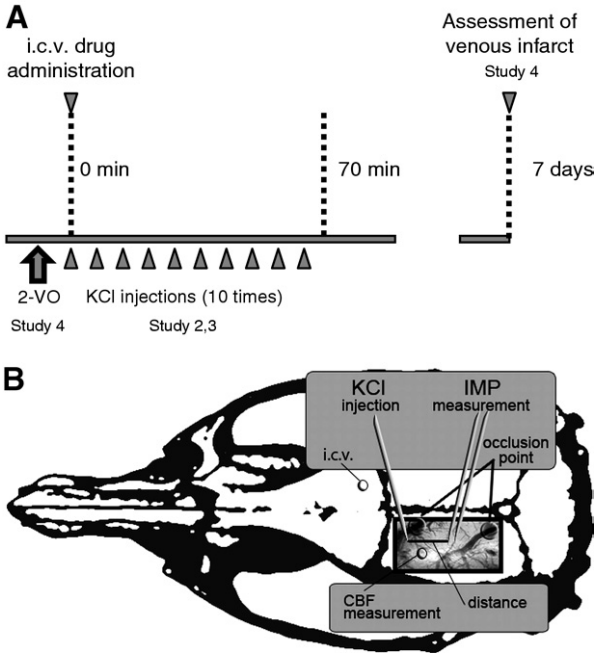

Fig. 1. Experimental setup and study design. (A) Schematic drawing of the experimental setup. The location of impedance electrodes, laser Doppler probe, glass micropipette for KCl injection and occlusion sites of two adjacent cortical veins of the parietal cortex are shown. (B) The study design was similar for all experiments with the exception that a focal ischemia by two-vein occlusion (2-VO) was only induced in study 4. Cortical spreading depression (CSD) was induced by 10 repetitive (7 min apart) KCl cortical microinjections in study 2 and 3. NaCl and CBX were injected after vein occlusion was completed and just before the first KCl injection.

ral increase of tissue impedance. Occasionally additional waves of CSD occurred spontaneously before impedance normalized after a single injection of KCl. Typical impedance changes for the three groups are shown in Fig. 3a–c. Anoxic depolarization-like alterations of impedance were never observed. The total number of CSDs which were induced by 10 cortical KCl injections was 24.83±4.03 in saline-treated animals during the 70-min observation time. This number increased significantly after treatment with 250 μg CBX (47.50±6.47, $P<0.05$), that is, 37.5 spontaneous depolarizations occurred in addition to the 10 induced ones.

Table 2. Physiological variables at baseline and at the end of study (studies 1–3) ($n=50$)

	pH	PO_2 (mmHg)	PCO_2 (mmHg)	MABP
Saline				
Baseline	7.43±0.01	158.4±5.4	41.5±1.3	81.2±2.4
Final	7.38±0.01*	139.1±4.1*	40.5±1.0	87.3±3.1
CBX50				
Baseline	7.43±0.01	153.4±5.8	39.8±0.7	80.4±4.3
Final	7.35±0.01*	145.1±4.9	43.4±1.2*	90.8±3.2
CBX250				
Baseline	7.41±0.01	149.6±7.8	42.0±3.4	86.3±2.6
Final	7.36±0.01*	144.2±7.6	41.2±1.0	94.7±1.8*

MABP, mean arterial blood pressure. Data are expressed as mean±standard error of the mean.
* $P<0.05$.

Table 3. Physiological variables at baseline and 100 min after two-vein occlusion in study 4 ($n=7$)

	pH	PO_2 (mmHg)	PCO_2 (mmHg)	MABP
Saline				
Baseline	7.42±0.03	168.4±4.9	40.1±1.0	89.3±2.0
Final	7.35±0.01*	135.8±9.5	42.1±1.0	91.4±2.8
CBX50				
Baseline	7.43±0.01	153.4±5.8	39.8±0.7	80.4±4.3
Final	7.34±0.01*	152.0±9.0	42.8±0.4	97.1±6.2
CBX250				
Baseline	7.43±0.02	191.1±14.1	39.6±1.9	95.0±2.2
Final	7.32±0.02*	134.9±9.2*	43.0±1.4	100.7±5.1

MABP, mean arterial blood pressure. Data are expressed as mean±standard error of the mean.
* $P<0.05$.

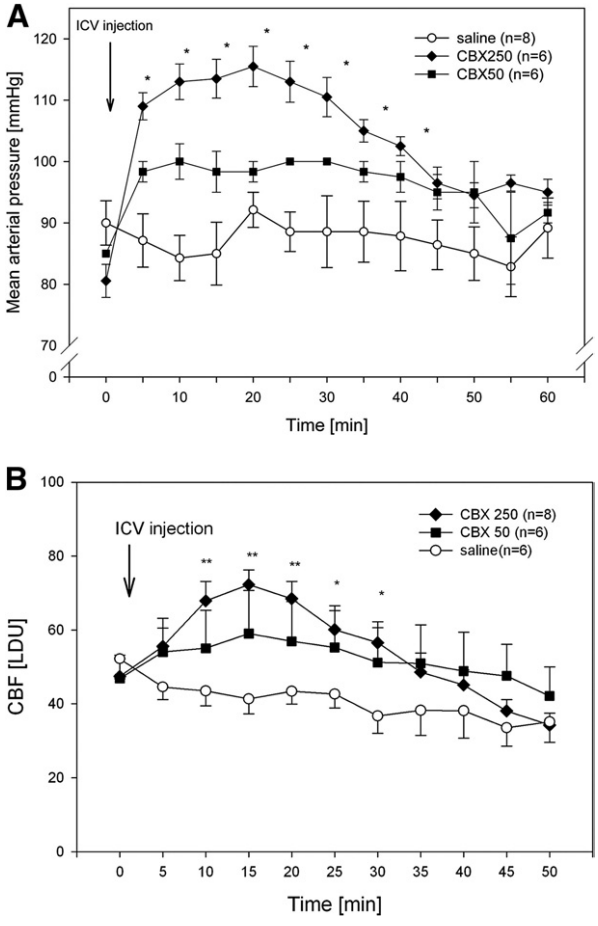

Fig. 2. Mean arterial pressure and CBF after intra-cerebroventricular injection of CBX. (a) Arterial blood pressure dose-dependently increased after CBX administration. At a dose of 250 μg the increase was significant from 5 till 50 min after injection and reached a maximum at 20 min after injection. Blood pressure had normalized after 60 min (* $P<0.05$). (b) Mean (±SEM) cortical blood flow (CBF) after NaCl ($n=6$), 50 μg ($n=6$) or 250 μg CBX ($n=8$) were injected-i.c.v. Both CBX concentrations elevated CBF for <10 min (LDU, Laser Doppler Unit) (** $P<0.01$, * $P<0.05$).

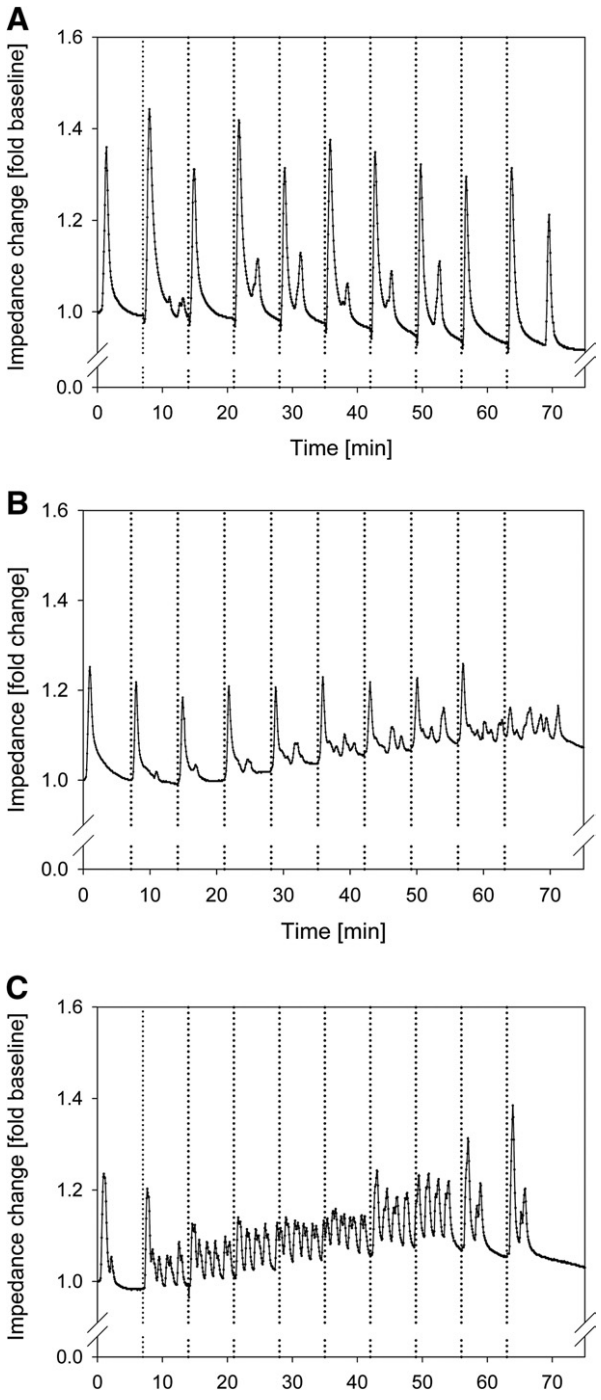

Fig. 3. Representative impedance changes. Baseline (=1.0) is the stable value 90 min after insertion of IMP probe. CBX was injected before first KCl-injection (first KCl injection at time=0, dotted lines indicate following KCl injections in 7-min intervals). (a) saline administration. (b) 50 μg CBX. (c) 250 μg CBX.

Treatment with 50 μg CBX increased the number of spontaneous CSDs slightly without reaching statistical significance (34.83±4.13). Fig. 4 summarizes these data. The number of additional spontaneous CSDs which were induced after each KCl injection gradually increased after individual KCl injections in the CBX- but not in the saline-treated group. A peak was reached after the fifth KCl-injection.

Study 3: effect of CBX on CSD velocity

At 50 μg CBX-induced CSDs could be discriminated from spontaneous CSDs by the smaller changes of cell volume (i.e. impedance) seen in spontaneous CSDs following the induced ones. This permitted to measure velocity reproducibly which would not have been possible with 250 μg CBX (cf. Fig. 3b, c). The distance between impedance electrode and a glass micropipette (Fig. 1B) was not significantly different between the groups (2.7±0.2 mm in CBX group, 2.3±0.2 mm in the saline group). CSD velocity was acutely accelerated with 50 μg CBX reaching its fastest speed after the fifth KCl injection. Thereafter, velocity of the following KCl-induced CSD waves decreased gradually. The velocity of CSD waves was significantly increased by CBX following the 5th to 8th KCl-injection (2.97±0.21, 2.73±0.23, 2.92±0.18, 2.65±0.17 mm/min) when compared to saline treatment (1.75±0.17, 1.52±0.20, 1.33±0.11, 1.45±0.16 mm/min, $P<0.01$, $P<0.05$, $P<0.01$, $P<0.01$, respectively, Fig. 5).

Study 4: effect of CBX treatment after two-vein occlusion

Immediately after vein occlusion, local cortical blood flow dropped from 45–55 LDU to 20 LDU and below. After i.c.v. injection of CBX, 30 min after two-vein occlusion there was no significant effect on local flow which remained below 20 LDU in all animals (Fig. 6a), although blood pressure increased after 250 μg CBX (data not shown) just as seen in experiments of study 1. There was no significant difference of frequency of spontaneous CSDs elicited after CBX injection.

After 7 days there was a significant more than doubling of infarct volumes after 250 μg CBX. 50 μg also enlarged infarct size without reaching statistical significance, however (Fig. 6b).

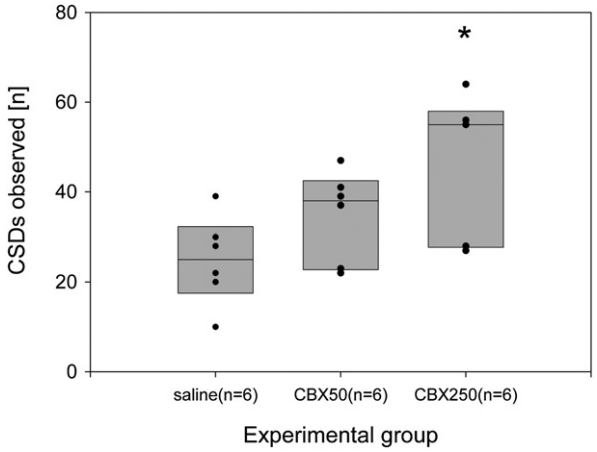

Fig. 4. Number of CSDs observed. Box plots of number of induced plus spontaneously occurring CSDs following saline or CBX (± 5th and 95th percentiles). Drugs were administered i.c.v. immediately before start of 10 cortical microinjections of KCl (* $P<0.05$ vs. NaCl).

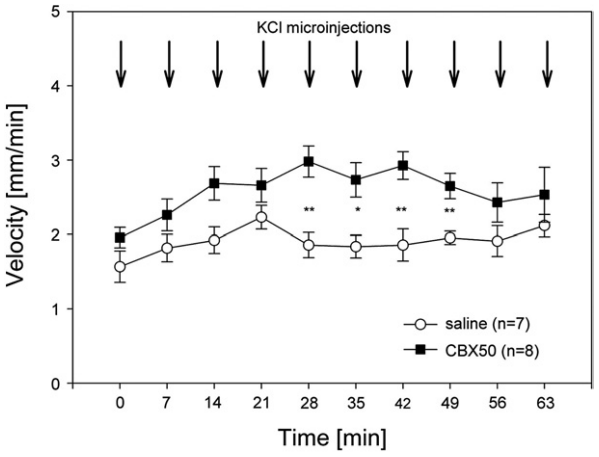

Fig. 5. CSD velocity. Velocity of KCl-induced CSD waves. Carbenoxolone (CBX) and NaCl were injected i.c.v. before the first KCl injection. The velocity of CSD waves was significantly increased by CBX following the 5th to 8th KCl-injection (means±SEM; ** $P<0.01$, * $P<0.05$).

DISCUSSION

Carbenoxolone as gap junction blocker

Carbenoxolone is related to glycyrrhetinic acid, and is thought to bind directly to connexins, inducing a conformational change and causing a closure of gap junctions (Rozental et al., 2001). Until now, CBX has been commonly used to block gap junction communication in the central nervous system, because it has been believed to have no direct effect on neuronal activities: CBX has been reported to have no effect on evoked synaptic responses (Ross et al., 2000; Köhling et al., 2001), postsynaptic responses to neurotransmitters (Yang and Michelson, 2001), intrinsic neuronal properties (Travagli et al., 1995; Köhling et al., 2001; Schmitz et al., 2001; Yang and Michelson, 2001), neuronal excitability (Köhling et al., 2001; Margineanu and Klitgaard, 2001; Schmitz et al., 2001) and cell conductances (Travagli et al., 1995). On the other hand there are some reports showing the opposite: CBX interferes with neuronal activities. Substantial effects of CBX on neuronal membrane properties have been described in respiratory neurons of the brain stem, where the input resistance and firing properties were reduced by CBX application (Rekling et al., 2000). The neuronal spontaneous synchrony in neuron–astrocyte co-cultures was reported to be reduced with CBX independently of gap junction blockade (Rouach et al., 2003). Recently, Tovar et al. (Tovar et al., 2009) have reported that CBX has non-gap junctional effects including reduction in excitatory and inhibitory synaptic currents, attenuation of membrane repolarization and spike rate, and decreases in input resistance. But there has been no report that CBX increases excitability of neurons themselves independently of gap junction communication. Direct electrical coupling between neurons with gap junctions is thought to contribute to ictogenesis by facilitating the spread of electrical activity between neurons and by enhancing synchronous activity (Carlen et al., 2000; Perez-Velazquez et al., 1994). Traub et al. (Traub et al., 2001) have suggested that gap junctions may also be important for seizure initiation and many groups have reported anticonvulsant effects of gap junction blockers including CBX (Carlen et al., 2000; Jahromi et al., 2002; Medina-Ceja et al., 2008). Some recent studies have shown that CBX causes paradoxical excitatory effects within the cerebral cortex (Yang and Ling, 2007) and at the cellular level in the hippocampus (Jahromi et al., 2002). But they concluded that the excitatory effects of CBX could depend on the blockade of gap junction between inhibitory interneurons.

From these findings, the accelerated velocity of CSD and the increase of spontaneous CSD in this study most likely is due to the effect of gap junction blockade by CBX, and not due to the effect of neuronal activity.

Carbenoxolone and the blood–brain barrier

CBX does not cross the blood–brain barrier (Leshchenko et al., 2006). So i.c.v. application was needed to study effects of CBX on brain. Although there are some studies

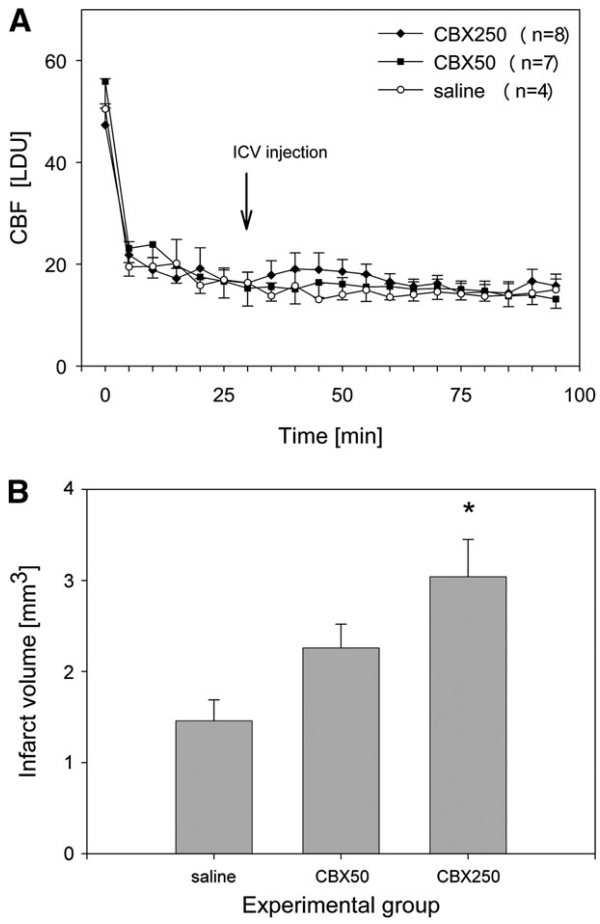

Fig. 6. CBF and infarct volume after two vein occlusion and CBX injection. (a) Occlusion of two bridging veins caused a 60% CBF drop from baseline (mean±SEM). There was no significant difference among groups at any time point. (b) Mean (±SEM) infarct size 7 d after venous ischemia: Carbenoxolone (CBX) or 0.9% NaCl were injected after 2-VO (two-vein occlusion) and caused a significant aggravation of ischemic damage (* $P<0.05$). CBX groups did not differ significantly.

with CBX *in vivo* application, the half-life of it has not been clarified especially in i.c.v. application. We saw that the effects of CBX on blood pressure and CSD lasted for about 60 min after single administration. It is not clear whether the attenuation of these effects depend on the inactivation of the drug itself or compensatory mechanisms after the blockade of gap junctions with CBX. When we injected CBX every 20 min into the lateral ventricle, the increased velocity and frequency of CSD did not decrease 60 min after the first injection (data not shown), which would suggest that the attenuation of the effects of CBX depend on the inactivation of the drug.

Cortical spreading depression

Cortical spreading depression is characterized by rapid depolarization of both neurons and glia (Gorji, 2001) accompanied by a breakdown of ion gradients and cell swelling. Despite of a vast numbers of studies, the mechanisms of CSD initiation are still not completely understood. CSD is thought to result from a cascade of interlocking mechanisms that involve the coupling of populations of cells via gap junctions (Martins-Ferreira et al., 2000). CSD represents a combined reaction of neurons and glial cells to elevations in extracellular potassium and glutamate (Somjen et al., 1992) and is associated with large increases in extracellular potassium whereas sodium, calcium, and chloride ions enter neurons and glia (Nicholson, 1980; Somjen et al., 1992). As a consequence, water is shifted from the extracellular space (ECS) into intracellular compartments, which leads to cell swelling and a reduction of the ECS and thereby causes further elevations in extracellular potassium and glutamate concentration (Theis et al., 2003).

Cerebral tissue impedance is determined by the intra/extracellular space ratio. Owing to the high electrical resistance of cell membranes a low-frequency alternating current flows mainly through the extracellular space (Van Harreveld et al., 1965) and impedance increases if the intracellular space expands. Therefore, impedance is a reliable parameter reflecting extracellular volume changes during CSD, and, hence provides additional information about volume regulatory phenomena during the recovery phase which is not available from DC-potential measurements. Actually the impedance signal is more reproducible, and during focal ischemia correlates far better with the resulting infarct size than the DC negativity (Otsuka et al., 2000).

Gap junction-coupled glial cells have a role in the regulation of extracellular potassium (Orkand et al., 1966; Ransom et al., 1996; Amzica et al., 2002). When extracellular potassium is elevated locally, glial cells become depolarized, and this depolarization spreads through gap junctions into neighboring astrocytes. In this way potassium diffusion is strongly accelerated in all directions, a mechanism termed spatial buffering (Orkand et al., 1966; Gardner-Medwin, 1981). Thus, gap junction communication among astrocytes has a pivotal role for spatial buffering of potassium. By means of spatial buffering astrocytic gap junctions might attenuate the propagation of CSD by facilitating the uptake of potassium ions (Ransom et al., 1996; Amzica et al., 2002) and glutamate (Blanc et al., 1998; Hansson et al., 2000) released during the depolarization phase of CSD (Martins-Ferreira et al., 2000).

During CSD high extracellular potassium can promote the overflow of glutamate. It is well known that astrocytes also modulate extracellular glutamate concentrations, thereby contributing to extracellular neurotransmitter homeostasis and astrocyte–neuron signaling (Anderson and Swanson, 2000). Cx43-containing astrocytic gap junctions might support astrocytic glutamate uptake (Hansson et al., 2000), and impaired glutamate transport in astrocytes has been observed on gap junction uncoupling (Blanc et al., 1998).

Although neither neuronal firing nor synaptic transmission is required for CSD generation, activated NMDA channels are thought to play a role (Sheardown, 1993). In a computer simulation study, when both, dendrites inwardly currents controlled by NMDA receptors, and slowly inactivating inwardly sodium currents were operated, the threshold for generation of CSD became lower and its latency shorter, and its duration longer (Somjen, 2001). Thus although glutamate is thought to mediate the initiation, the onset of CSD initiation depends mainly on the rate of increase in extracellular potassium. That is, the initiation of CSD would be facilitated by impeded glial buffering of extracellular potassium (Kager et al., 2002). From these findings, the frequency of CSD elicited by KCl injection in this model could depend on the disturbance of buffering capacity for extracellular potassium.

Gap junction communication is not necessary for propagation of CSD

Although the mechanisms of the propagation of CSD are not completely understood so far, there are mainly four hypotheses for the CSD propagation, two of which are based on the interstitial diffusion of either potassium or glutamate (Grafstein, 1956; Van Harreveld, 1959). The other two postulate mediation through gap junctions among either glial cells or neurons (Reid et al., 1988; Herreras et al., 1994). Glial and/or neuronal gap junctions have been discussed to play a pivotal role in the propagation of CSD (Nedergaard et al., 1995; Martins-Ferreira et al., 2000). Following their hypothesis selective gap junction blockers should block the propagation of CSD. In the present study, we observed that the velocity of cortical spreading depression induced with KCl was accelerated after i.c.v. application of CBX and was never blocked. Moreover these data show that CBX did not block the initiation and propagation of induced CSD.

The gap junction blockers used in studies with opposite results to ours were octanol, heptanol or halothane which are less specific and have suppressive effects on membrane functions (Largo et al., 1997). The suppressive effect on CSD, therefore, might not have been due to gap junctional blockade but could rather be a result of membrane stabilization. Interestingly, Martins-Ferreira and Ribeiro (Martins-Ferreira and Ribeiro, 1995) observed a biphasic, dose-dependent effect of heptanol and octanol on

CSD. Low concentrations led to an increase of CSD velocity, whereas higher doses blocked CSD completely. Perhaps, low concentration of these drugs acted as pure gap junction blockers whereas with high doses the membrane stabilizing effect prevailed.

Neuronal gap junctions mainly contain connexin36 whereas connexin43 is typical for astroglial gap junctions. CBX acts as a broad spectrum gap junction blocker and a contribution of neuronal gap junction blockade in our results is also possible. However, in most areas of the CNS, the number of neuron to neuron gap junctions was found to be very low, compared to the ones between astrocytes (Rash et al., 2001; Sotelo and Korn, 1978; Venance et al., 2000). Thus, it is suggested that the results that we obtained in this study depend on the blockade of astroglial gap junctions and the ensuing reduction of the potassium buffering capacity.

Effects of CBX on local cerebral blood flow and systemic blood pressure

Gap junctions are also found on cells of the vascular system, and their inhibition with CBX can reduce endothelium-dependent contractions (Tang and Vanhoutte, 2008). The observed CBF increase might be a result of such a vascular action of CBX. Another possible explanation would be a loss of autoregulation due to CBX and ensuing flow increase due to the blood pressure increase triggered by CBX. The experimental conditions, that is, chloral hydrate anesthesia, have been shown not to affect autoregulation (Heimann et al., 1994). We also observed that CBF did not increase due to CBX after two-vein occlusion (Fig. 6a). The data indicate that flow was actually measured in the penumbra: flow decreased to ~40% baseline flow whereas in the ischemic core 20% are typically found (Nakase et al., 1997). In the ischemic penumbra pial and penetrating arterioles are robustly dilated (Shih et al., 2009) and blocking of astroglial gap-junctions by CBX therefore will have no additional effect on CBF.

The observed increase of arterial blood pressure confirms earlier studies (Zhang et al., 2006) and is the result of the inhibition of 11 beta-hydroxysteroid dehydrogenase type 2 which is another known effect of CBX. 11 beta-hydroxysteroid dehydrogenase type 2 regulates responses in the paraventricular nucleus (PVN) of the hypothalamus implicated in sympathetic regulation (Zhang et al., 2006). Blood pressure responses to CBX can be prevented by adrenalectomy (Zhang et al., 2006).

Effects of CBX on outcome from focal venous ischemia

CBX increased infarct volumes after two-vein occlusion dose dependently. In the present two-vein occlusion model, widespread penumbra-like low flow areas were created and energy supply was restricted in these areas. By blocking spatial buffering of potassium with CBX, brain tissue could require more energy to normalize the disturbed extra- and intracellular ion balance under penumbra-like conditions and this could be the reason why infarct volume was enlarged with CBX.

In the periischemic lesion of cortex of Cx43 null mice, apoptosis did not cease even several days after focal ischemia (Nakase et al., 2003a). And it was reported that gap junctions were opened in ischemic conditions (Cotrina et al., 1998). These findings indicate the requirement of opening of astroglial gap junction to stop apoptosis in the penumbra. In our previous studies, a mitochondrial K–ATP channel blocker and a caspase inhibitor injected in the perioperative period reduced the infarct volume after 7 days (Nakagawa et al., 2005; Nishioka et al., 2006) in this model. Thus, apoptosis has a crucial role in exaggeration of infarct volume.

CONCLUSION

In conclusion, the current experiments provide evidence that CBX can accelerate the initiation and propagation of CSD suggesting opening of gap junctions is not required for CSD propagation but is rather necessary for extracellular homeostasis after CSD. Blocking gap junctions worsens outcome from focal cerebral ischemia suggesting that measures intended to improve spatial buffering via astroglial gap junctions could have therapeutic potential in disease processes involving CSD.

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