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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