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Effects of Ca^{2+} channel blockers on cortical hypoperfusion and expression of c-Fos-like immunoreactivity after cortical spreading depression in rats

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1 We examined the effects of two Ca^{2+} channel blockers, lomerizine (KB-2796) and flunarizine, on the cortical hypoperfusion (measured by hydrogen clearance and laser Doppler flowmetry methods) and cortical c-Fos-like immunoreactivity that follow KCl-induced cortical spreading depression in anaesthetized rats. Cortical spreading depression was induced by application of 1 M KCl for 30 s to the cortical surface, 3.0 mm posterior to the area of cerebral blood flow measurement.

2 In control rats, KB-2796 (0.3 and 1 mg kg^{-1}, i.v.) dose-dependently increased cerebral blood flow significantly at 30 min and 15 min, respectively, after its administration. Flunarizine (1 mg kg^{-1}, i.v.) significantly increased cerebral blood flow 15 min after its administration. In contrast, dimetotiazine (3 mg kg^{-1}, i.v.), a 5-HT_2 and histamine H_1 antagonist, failed to affect cerebral blood flow significantly.

3 After KCl application to the cortex, cerebral blood flow monitored by the laser Doppler flowmetry method increased transiently, for a few minutes, then fell and remained approximately 20 to 30% below control for at least 60 min. Cerebral blood flow monitored by the hydrogen clearance method was also approximately 20 to 30% below baseline for at least 60 min after KCl application. KB-2796 (0.3 and 1 mg kg^{-1}, i.v.) and flunarizine (1 and 3 mg kg^{-1}, i.v.) administered 5 min before KCl application inhibited the cortical hypoperfusion that followed KCl application, but dimetotiazine (1 and 3 mg kg^{-1}, i.v.) did not.

4 An indicator of neuronal activation, c-Fos-like immunoreactivity, was detected in the ipsilateral, but not in the contralateral frontoparietal cortex 2 h after KCl application. No c-Fos-like immunoreactivity was seen on either side of the brain in the hippocampus, thalamus, striatum or cerebellum.

5 KB-2796 (1 mg kg^{-1}, i.v.) and flunarizine (3 mg kg^{-1}, i.v.), but not dimetotiazine (3 mg kg^{-1}, i.v.), significantly attenuated the expression of c-Fos-like immunoreactivity in the ipsilateral frontoparietal cortex.

6 These findings suggest that the inhibitory effects of KB-2796 and flunarizine on the cortical hypoperfusion and expression of c-Fos-like immunoreactivity induced by spreading depression are mediated via the effects of Ca^{2+}-entry blockade, which may include an increase in cerebral blood flow and the prevention of excessive Ca^{2+} influx into brain cells. KB-2796 and flunarizine may prove useful as inhibitors of cortical spreading depression in migraine.

Keywords: Ca^{2+} channel blocker; cerebral blood flow; c-Fos expression; cerebral hypoperfusion; cortical spreading depression; dimetotiazine; flunarizine; KB-2796 (lomerizine); migraine

Introduction

In the pathophysiology of migraine, cortical spreading depression (CSD) has been considered to be associated with aura and cerebral ischaemia (Lauritzen, 1987a: Siesjö & Bengtsson, 1989). Experimentally, CSD can be induced when KCl is applied to the surface of the rat cerebral cortex. CSD is characterized by a transient depolarization of nerve cells and followed by depression of evoked and spontaneous EEG activity which spreads at a rate of 2 to 5 mm min^{-1} across the cortical surface (Leão, 1944). After CSD, cerebral cortical blood flow (CBF) declines by 20 to 30% and remains low for at least 1 h in rats (Lauritzen *et al.*, 1982; Lauritzen, 1984). In migraine with aura, CBF is reduced by at least 20% during the aura and remains low for 4 to 8 h into the headache period (Olesen *et al.*, 1981; Lauritzen & Olesen, 1984). This closely resembles the reductions in CBF observed in experimental CSD and, for this reason, experimental CSD is considered by some investigators to be an animal model of migraine (Lauritzen, 1987b).

Various Ca^{2+} channel blockers such as flunarizine, nimodipine, verapamil, nifedipine and diltiazem have been shown to be effective in the prophylaxis of migraine (Louis, 1981; Solomon, 1985). Although these drugs are thought to prevent migraine by inhibiting arterial vasospasm, producing vasodilatation, inhibiting platelet aggregation and blocking platelet 5-hydroxytryptamine (5-HT) release and uptake, details of the mechanisms underlying their effects are unknown (Solomon *et al.*, 1983). In the rat experimental CSD model, flunarizine has been reported to block the depression of EEG activity and the negative shift in direct current potential that follow KCl application (Wauquier *et al.*, 1982). This result suggests that Ca^{2+} channel blockers may inhibit the initiation and/or propagation of CSD.

Recently, application of KCl to the brain surface of the rat has been reported to increase expression of the c-Fos protooncogene (Herrera & Robertson, 1990; Herrera *et al.*, 1993). Although the role of c-Fos in the central nervous system is not entirely clear, c-Fos-like immunoreactivity is useful since it can be used to identify areas of neuronal activity and to map functionally related neuronal pathways (Ehret & Fischer, 1991).

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1-[*Bis*(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride (lomeridine: KB-2796) is a newly synthesized Ca^{2+} channel blocker which is under development as a potential anti-migraine drug. KB-2796 inhibits specific [^{3}H]-nitrendipine binding to cerebral cortex membranes in the guinea-pig (Iwamoto *et al.*, 1988), inhibits selectively the constriction of cerebral arteries induced by various stimulants *in vitro* (Kanazawa & Toda, 1987) and increases cerebral blood flow in the cat (Kanazawa *et al.*, 1986). Furthermore, KB-2796 has been reported to inhibit both T-type and L-type Ca^{2+} currents in rat hippocampal CA1 pyramidal single neurones (Akaike *et al.*, 1993), to prevent glutamate-induced neurotoxicity in rat hippocampal primary cell cultures (Hara *et al.*, 1993b) and to exhibit protective effects in models of ischaemia and hypoxia (Yoshidomi *et al.*, 1989; Hara *et al.*, 1990; 1993a). However, little is known about the effects of KB-2796 on CSD, a putative animal model of migraine. Therefore, we examined the effects of KB-2796 on the cortical hypoperfusion and expression of c-Fos-like immunoreactivity that follow after CSD in anaesthetized rats and compared them with those of flunarizine, another Ca^{2+} channel blocker, and dimetotiazine, a 5-HT_2 and H_1 antagonist which possesses a prophylaxic effect during migraine (Hélène, 1992).

Methods

Animals

Male Wistar rats weighing 250 to 350 g (Japan SLC, Shizuoka, Japan) were housed in an air-conditioned room at 25 ± 1°C with 55 ± 5% humidity and given food and water *ad libitum*.

Cortical spreading depression (CSD)

Rats were anaesthetized with a single intraperitoneal dose of 1.2 g kg^{-1} urethane (Kishida Chemical, Osaka, Japan). The right femoral vein was cannulated with a polyethylene tube for the administration of drugs and/or vehicle and the animal placed in a stereotaxic frame (Narishige, Tokyo, Japan). Rectal temperature was maintained at about 37°C with the aid of a heating lamp. CSD was induced by application with a microsyringe of 5 μl of 1 M KCl to the cortex via an aperture 1.7 mm in diameter made in the dura, 6.5 mm posterior to Bregma and 1.5 mm left of the midline. After 30 s, KCl was carefully removed using cotton wool.

Determination of cerebral blood flow

Hydrogen clearance and laser Doppler flowmetry were used to measure CBF. In the hydrogen clearance method (CBF_{HC}), it was measured with a hydrogen electrode (top diameter 0.2 mm; MHD-60, MT Technical Institute, Tokyo, Japan) and a hydrogen monitor (DHM-3100, MT Technical Institute). After the removal of the dura mater (to make an aperture 1.7 mm in diameter), the electrode tip was inserted 1 mm into the cortex at an angle of 70°, 3.5 mm posterior to Bregma and 1.5 mm left of the midline. A reference electrode (MH-10, MT Technical Institute) was placed in the posterior neck muscles. Hydrogen was generated electrochemically by passing a constant current of 20 mA between the hydrogen and reference electrodes for 4 s. The CBF_{HC} values were derived from the hydrogen clearance curves using Aukland's equation (Aukland *et al.*, 1964) with minor modifications: $CBF_{HC}=[0.693\times(t_{C1/2}^{-1}-t_{D1/2}^{-1})]\times100$, where CBF_{HC} is expressed in ml min^{-1} 100 g^{-1} of tissue; $t_{C1/2}$ is the time in min needed for the current to decay by half the peak value; $t_{D1/2}$ is the time in min needed for the current to decay by half the peak value 45 min after death induced by 15% KCl, i.v., and 0.693 is the natural log function constant. All measurements were confined to the clearance phase (1 to 6 min) of the curve, which was consistently monoexponential. Starting 30 min after electrode insertion, CBF_{HC} was measured every 15 min until 3 consecutive baseline values were similar to each other. Drugs were then injected into the femoral vein slowly (over 2 min) and, 5 min later KCl was applied to the cortical surface. CBF_{HC} was measured 30 min and 60 min after drug administration.

CBF was also determined by laser Doppler flowmetry (CBF_{LDF}) with a flowmeter (ALF2100 and ALF21, Advance, Tokyo, Japan) fitted with a fine probe (1 mm diameter, Advance). After the removal of the dura mater as described above, the probe was carefully placed on the surface of the cortex and the probe signal recorded continuously using a recorder (U-228, Nippon Denshi Kagaku, Kyoto, Japan).

Arterial blood pressure and heart rate

The right common carotid artery was cannulated with a polyethylene tube to enable the monitoring of changes in arterial blood pressure and heart rate following drug administration. The variables were continuously monitored with a thermal array recorder.

Immunohistochemistry

To enable evaluation of the increase in c-Fos-like immunoreactivity, animals were perfused via the ascending aorta 2 h after application of KCl with 150 ml of saline (0.9%) containing heparin (200 iu), followed by 200 ml of paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.3). The brain with the upper cervical spinal cord attached was removed and kept in the same fixative buffer (pH 7.3) overnight at 4°C prior to sectioning. Brains were cut into coronal sections (70 μm thick) on a vibratome (Lancer Series 1000, AHS Japan, Tokyo, Japan) and the sections immunohistochemically processed by the avidin-biotin procedure using commercially available kits (Vectastain Elite ABC, Vector Labs, Burlingame, CA, U.S.A.) basically as described by Herrera & Robertson (1990) though with minor modifications. Briefly, sections were mounted on gelatin-coated slides and then incubated with 10% normal goat serum and 0.03% hydrogen peroxide in 0.01 M phosphate-buffered saline (PBS, pH 7.3) for 30 min at room temperature. Then, sections were incubated overnight at room temperature with rabbit anti-c-Fos polyclonal antibody (1:200 dilution, Lot No. 21930201, Oncogene Science, Cambridge, MA, U.S.A.) in PBS with 0.3% Triton X-100 (Bio Rad Labs., Tokyo, Japan) and 0.9% normal goat serum.

After washing the sections with PBS (15 min × 3), biotinylated goat anti-rabbit IgG antiserum (1:200 dilution in PBS with 1.5% normal goat serum, Vector Labs) was placed onto them for 1 h at room temperature. After further washing with PBS (15 min × 3), avidin-biotin-peroxidase complex was placed on the sections for 30 min at room temperature. Again, sections were washed with PBS (15 min × 3), then a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.2) was placed on them for 30 min. After the diaminobenzidine reaction, sections were air-dried and coverslipped.

The expression of c-Fos was quantified bilaterally in serial sections under a microscope. Anatomical boundaries were determined using coordinates derived from a rat brain atlas (Paxinos & Watson, 1982). For quantification of c-Fos protein expression, cells showing c-Fos-like immunoreactivity were counted within 3 separate frontoparietal (FrPa) areas (each 0.4 mm^2) of cortex on each side. The areas were: FrPa A (2.0 mm lateral to the midline and at a depth of 1.0 mm from the surface of the skull), FrPa B (6.0 mm lateral to the midline and at a depth of 3.5 mm from the surface of the skull) and FrPa C (6.5 mm lateral to the midline and at a depth of 6.0 mm from the surface of the skull) All labelled cells were counted regardless of staining intensity.

Experimental protocol

A number of different control groups were necessary. First, 52 rats in total were surgically prepared so that CBF_{LDF} could be

monitored and then the effect was assessed of KB-2796, flunarizine or dimetotiazine on CBF_{LDF} (Figure 1). Secondly, 70 rats in total were surgically prepared so that CBF_{LDF} could be monitored and then the effect of each drug was assessed on CBF_{LDF} after CSD (Figure 2). Thirdly, 75 rats in total were surgically prepared so that CBF_{HC} could be monitored and then the effect of each drug was assessed on CBF_{HC} after CSD (Figure 3). Sham-operated controls were included for the purpose of excluding injury as a cause of the CBF reductions observed in KCl-treated animals. Fourthly, 40 rats in total were surgically prepared so that arterial blood pressure and heart rate could be monitored (Figure 4). Lastly, 45 rats in total were surgically prepared so that c-Fos-like immunoreactivity could be measured after CSD and again after the administration of the drugs (Figures 5 and 6, Table 3). Vehicle or drug was administered i.v. 5 min before KCl application, and then each animal was perfused 2 h after CSD. Each animal received only vehicle or one drug at one concentration.

Drugs

KB-2796 (Kanebo, Osaka, Japan), flunarizine dihydrochloride (Sigma, St. Louis., MO, U.S.A.) and dimetotiazine mesylate (Shionogi, Osaka, Japan) were dissolved in a 2% dimethylacetamide solution containing 0.2% tartaric acid and injected i.v. in a volume of 1 ml kg^{-1} body weight.

Data analysis

Results are shown as means ± s.e.mean. Statistical analysis was performed by one-way ANOVA followed by a Dunnett's test (Figures 1–4, Tables 1–3), one-way ANOVA with repeated measures followed by a Dunnett's test (Figures 1–3) or two-way ANOVA with repeated measures (Figures 1–3), as appropriate. A P value <0.05 was considered statistically significant.

Results

Effects of drugs on CBF_{LDF} (Figure 1, Table 1)

The effects of KB-2796 (0.3 and 1 mg kg^{-1}, i.v.), flunarizine (1 and 3 mg kg^{-1}, i.v.) and dimetotiazine (3 mg kg^{-1}, i.v.) on CBF_{LDF} in anaesthetized normal rats are shown in Figure 1. Pretreatment values of CBF_{LDF} in vehicle- and drug-treated groups are shown in Table 1. There were no significant differences between the groups in terms of their pretreatment values. In the two-way ANOVA with repeated measures, there was a significant difference ($P<0.006$) between vehicle- and KB-2796-treated groups. However, there was no significant difference between vehicle- and flunarizine-treated groups ($P<0.47$), or between vehicle- and dimetotiazine-treated groups ($P<0.07$).

In the one-way ANOVA followed by a Dunnett's test, the increase in CBF_{LDF} evoked by KB-2796 at 0.3 and 1 mg kg^{-1}, i.v., reached significance at 30 min and 15 min, respectively, after drug administration. Flunarizine significantly increased CBF_{LDF} 15 min after its administration (at 1 mg kg^{-1}, i.v.), but not at a high dose (3 mg kg^{-1}, i.v.). Dimetotiazine (3 mg kg^{-1}, i.v.) tended to decrease CBF_{LDF}, but not significantly. In the one-way ANOVA with repeated measures followed by a Dunnett's test, the CBF_{LDF} in the vehicle-treated group was not significantly different for 90 min after administration from pretreatment values in individual animals. KB-2796 (0.3 and 1 mg kg^{-1}, i.v.) significantly increased CBF_{LDF} over and above baseline at 30 min ($P<0.05$) after its administration. Flunarizine (1 and 3 mg kg^{-1}, i.v.) did not significantly change CBF_{LDF} for 90 min after its administration. On the other hand, dimetotiazine (3 mg kg^{-1}, i.v.) significantly decreased CBF_{LDF} at 5 min ($P<0.05$) after its administration.

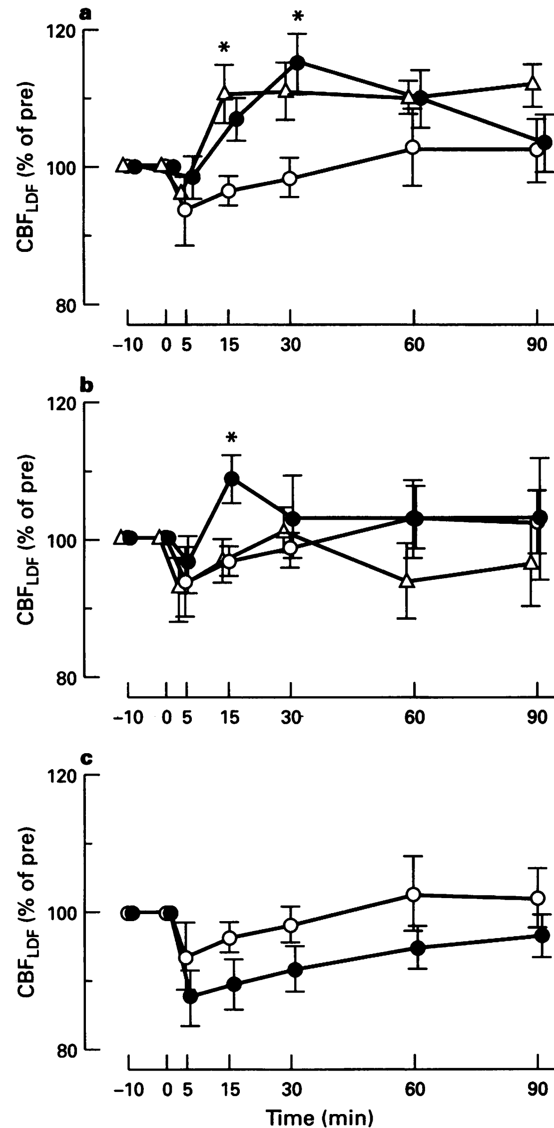

Figure 1 Effects of KB-2796, flunarizine and dimetotiazine on cerebral cortical blood flow measured by laser Doppler flowmetry (CBF_{LDF}) in anaesthetized rats. (a) KB-2796 (0.3 mg kg^{-1}, i.v., $n=7$, ●, or 1 mg kg^{-1}, i.v., $n=6$, △) or vehicle ($n=7$, ○); (b) flunarizine (1 mg kg^{-1}, i.v., $n=6$, ●, or 3 mg kg^{-1}, i.v., $n=6$, △) or vehicle ($n=7$, ○); (c) dimetotiazine (3 mg kg^{-1}, i.v., $n=6$, ●) or vehicle ($n=7$, ○). Data are expressed as percentage of baseline. Drugs were administered i.v. over 2 min or less. *$P<0.05$ vs. vehicle (Dunnett's test).

Effects of drugs on CBF_{LDF} after CSD in anaesthetized rats (Figure 2, Table 1)

The effects of KB-2796 (0.3 and 1 mg kg^{-1}, i.v.), flunarizine (1 and 3 mg kg^{-1}, i.v.) and dimetotiazine (1 and 3 mg kg^{-1}, i.v.) on CBF_{LDF} after CSD caused by KCl application are shown in Figure 2. Pretreatment values for CBF_{LDF} in the KCl + vehicle and KCl + drugs groups are shown in Table 1. There were no significant differences between the groups in terms of their pretreatment values. CBF_{LDF} transiently increased to approximately 160% of the pretreatment values (baseline) 1 to 3 min after KCl application and then decreased to approximately 20–30% below baseline for at least 60 min. In the two-way ANOVA with repeated measures, there was a significant difference ($P<0.02$) between KCl + vehicle- and KCl + KB-2796-treated groups. However, there were no significant differences between KCl + vehicle- and KCl + flunarizine-treated groups ($P<0.14$), or between KCl + vehicle- and KCl + dimetotiazine-treated groups ($P<0.73$).

In the one-way ANOVA followed by a Dunnett's test,

Table 1 Cerebral blood flow monitored by laser Doppler flowmetry (CBF_{LDF}) prior to drug administration

	Drugs (mg kg^{-1}, i.v.)		n	CBF_{LDF} (mV)
(1)	Vehicle		7	348.0±46.3
	KB-2796	0.3	7	450.9±37.7
		1	6	371.3±37.0
	Flunarizine	1	6	411.3±71.5
		3	6	359.3±38.9
	Dimetotiazine	3	6	328.7±33.2
(2)	KCl+vehicle		8	355.5±34.1
	KCl+KB-2796	0.3	8	322.0±31.6
		1	9	342.7±50.5
(3)	KCl+vehicle		11	414.2±44.6
	KCl+flunarizine	1	9	393.3±32.7
		3	9	392.4±34.4
	KCl+dimetotiazine	1	8	340.3±26.9
		3	8	380.5±40.0

CBF values are expressed as a mean±s.e.mean.

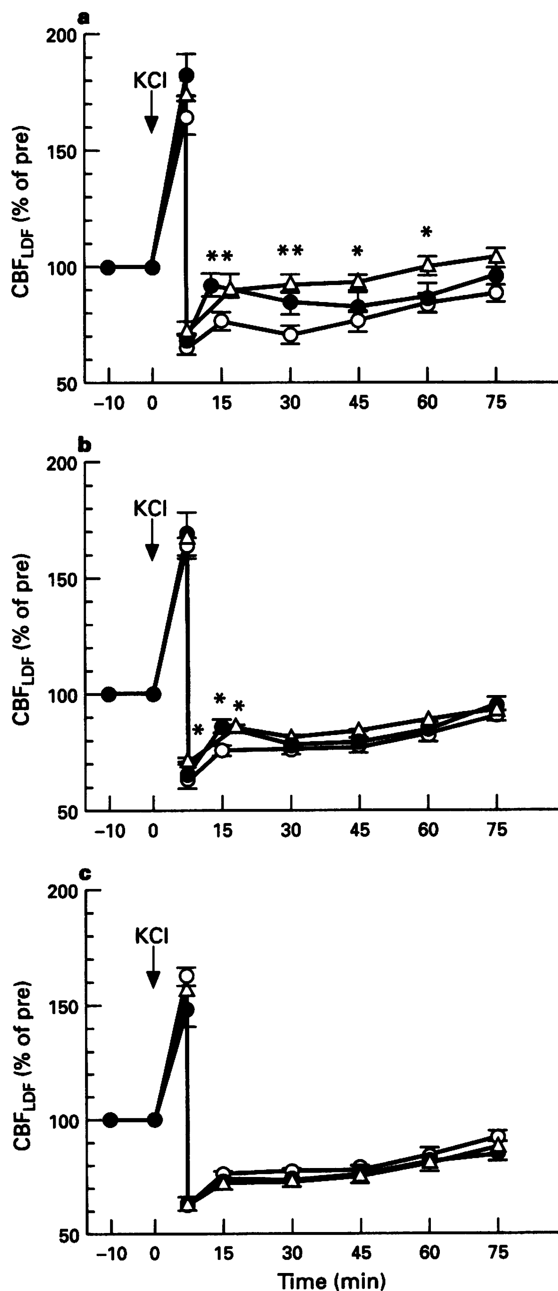

Figure 2 Effects of KB-2796, flunarizine and dimetotiazine on the cortical hypoperfusion following KCl-induced cortical spreading depression in rats (laser Doppler flowmetry). (a) KCl+vehicle (n=8, ○), KCl+0.3 mg kg^{-1} KB-2796, i.v. (n=8, ●), KCl+1 mg kg^{-1} KB-2796, i.v. (n=9, △); (b) KCl+vehicle (n=11, ○), KCl+1 mg kg^{-1} flunarizine, i.v. (n=9, ●), KCl+3 mg kg^{-1} flunarizine, i.v. (n=9, △); (c) KCl+vehicle (n=11, ○), KCl+1 mg kg^{-1} dimetotiazine, i.v. (n=8, ●), KCl+3 mg kg^{-1} dimetotiazine, i.v. (n=8, △). Data are expressed as percentage of baseline. Drugs were administered i.v. 5 min before KCl application. *$P<0.05$, **$P<0.01$ vs. KCl+vehicle (Dunnett's test).

KCl+KB-2796 (0.3 and 1 mg kg^{-1}, i.v.) significantly reduced the cortical hypoperfusion at 15–60 min after drug administration in a dose-dependent manner, but did not affect the transient cortical hyperperfusion (Figure 2a). Flunarizine (1 and 3 mg kg^{-1}, i.v.) significantly reduced the cortical hypoperfusion at 5 and 15 min after drug administration, but it too failed to affect the transient cortical hyperperfusion (Figure 2b). Dimetotiazine (1 and 3 mg kg^{-1}, i.v.) had no effect on either the transient cortical hyperperfusion or the cortical hypoperfusion following KCl application (Figure 2c).

In the one-way ANOVA with repeated measures followed by a Dunnett's test, KCl+vehicle significantly reduced CBF_{LDF} below pretreatment values in individual animals for 60 min ($P<0.01$) after its administration. The KCl+KB-2796 (0.3 mg kg^{-1}, i.v.) group showed no significant change in CBF_{LDF} at 15 min after drug administration but showed a fall at 30–75 min. KCl+KB-2796 (1 mg kg^{-1}, i.v.) caused no significant change in CBF_{LDF} from 15 to 75 min after drug administration. In this analysis, there were no significant differences between the KCl+vehicle-treated, KCl+flunarizine (1 and 3 mg kg^{-1}, i.v.)-treated and KCl+dimetotiazine (1 and 3 mg kg^{-1}, i.v.)-treated groups.

Effects of drugs on CBF_{HC} after CSD in anaesthetized rats (Figure 3, Table 2)

The effects of KB-2796 (0.3 and 1 mg kg^{-1}, i.v.), flunarizine (1 and 3 mg kg^{-1}, i.v.) and dimetotiazine (1 and 3 mg kg^{-1}, i.v.) on CBF_{HC} after CSD caused by KCl application are shown in Figure 3. Pretreatment values for CBF_{HC} in sham operated (No KCl application), KCl+vehicle- and KCl+drugs-treated groups are shown in Table 2. There were no significant differences between the groups in terms of their pretreatment values. The CBF_{HC} decreased to approximately 70 to 80% of values seen with vehicle alone (open columns in Figure 3) 30 and 60 min after KCl application. Pretreatment with KB-2796 (1 mg kg^{-1}, i.v.) inhibited significantly the cortical hypoperfusion at both 30 and 60 min after CSD (Figure 3a). Pretreatment with flunarizine (3 mg kg^{-1}, i.v.) inhibited the cortical hypoperfusion significantly only at 30 min after CSD (Figure 3b). In contrast, dimetotiazine (1 and 3 mg kg^{-1}, i.v.) did not affect the cortical hypoperfusion at all (Figure 3c).

In the one-way ANOVA with repeated measures followed by a Dunnett's test, the KCl+vehicle-treated group (Figure

Table 2 Cerebral blood flow monitored by hydrogen clearance (CBF_{HC}) prior to drug administration

	Drugs (mg kg^{-1}, i.v.)		n	CBF_{HC} (ml min^{-1} 100 g^{-1})
(1)	Sham		8	48.9±3.2
	KCl+vehicle		7	56.9±3.3
	KCl+KB-2796	0.3	6	47.2±2.3
		1	8	46.7±2.0
(2)	Sham		7	59.0±5.7
	KCl+vehicle		10	51.0±3.6
	KCl+flunarizine	1	6	53.1±3.5
		3	8	48.2±4.1
	KCl+dimetotiazine	1	7	50.2±4.5
		3	8	59.4±5.4

CBF values are expressed as a mean±s.e.mean.

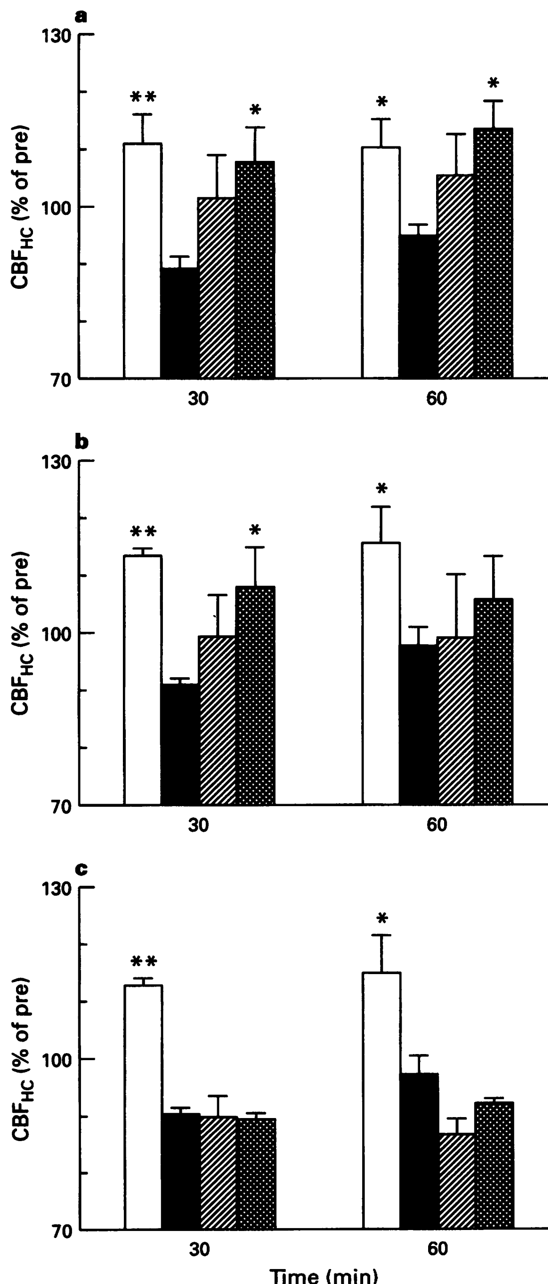

Figure 3 Effects of KB-2796, flunarizine and dimetotiazine on the cortical hypoperfusion following KCl-induced cortical spreading depression (hydrogen clearance). (a) Sham-operated group (no KCl; $n=8$, open columns), KCl+vehicle ($n=7$, solid columns), KCl+0.3 mg kg^{-1} KB-2796, i.v. ($n=6$, hatched columns), KCl+1 mg kg^{-1} KB-2796, i.v. ($n=8$, cross-hatched columns); (b) sham-operated group (no KCl; $n=7$, open columns), KCl+vehicle ($n=10$, solid columns), KCl+1 mg kg^{-1} flunarizine, i.v. ($n=6$, hatched columns), KCl+3 mg kg^{-1} flunarizine, i.v. ($n=8$, cross-hatched columns); (c) sham-operated group (no KCl; $n=7$, open columns), KCl+vehicle ($n=10$, solid columns), KCl+1 mg kg^{-1} dimetotiazine, i.v. ($n=7$, hatched columns), KCl+3 mg kg^{-1} dimetotiazine, i.v. ($n=8$, cross-hatched columns). Data are expressed as percentage of baseline. Drugs were administered i.v. 5 min before KCl application. $^{*}P<0.05$, $^{**}P<0.01$ vs. KCl+vehicle (Dunnett's test).

3a) showed a CBF_{HC} significantly reduced below pretreatment values in individual animals at 30 min ($P<0.05$) but not at 60 min after drug administration. The KCl+KB-2796 (1 mg kg^{-1}, i.v.) groups exhibited significantly increased CBF_{HC} at 60 min ($P<0.05$) after drug administration. The vehicle-treated group (sham-operated group) in Figure 3b showed significantly increased CBF_{HC} at both 30 and 60 min ($P<0.05$) after vehicle administration, whereas the KCl+vehicle-treated animals showed a significantly decreased CBF_{HC} only at 30 min ($P<0.05$). On the other hand, KCl+dimetotiazine (1 mg kg^{-1}, i.v.) significantly decreased CBF_{HC} at 30 min ($P<0.05$) and 60 min ($P<0.01$), whereas KCl+dimetotiazine (3 mg kg^{-1}, i.v.) significantly decreased CBF_{HC} only at 30 min ($P<0.05$).

Arterial blood pressure and heart rate (Figure 4)

The effects of KB-2796 (0.3 and 1 mg kg^{-1}, i.v.), flunarizine (1 and 3 mg kg^{-1}, i.v.) and dimetotiazine (1 and 3 mg kg^{-1}, i.v.) on mean arterial blood pressure (MABP) and heart rate (HR) are shown in Figure 4. Pretreatment-values for MABP with vehicle, KB-2796 (0.3 mg kg^{-1}, i.v.), KB-2796 (1 mg kg^{-1}, i.v.), flunarizine (1 mg kg^{-1}, i.v.), flunarizine (3 mg kg^{-1}, i.v.), dimetotiazine (1 mg kg^{-1}, i.v.) and dimetotiazine (3 mg kg^{-1}, i.v.) were, respectively, 76.3±3.9 ($n=9$), 98.0±6.9 ($n=5$), 75.8±3.2 ($n=5$), 67.8±4.4 ($n=5$), 87.0±9.6 ($n=5$), 77.8±2.4 ($n=5$) and 79.9±4.1 mmHg ($n=6$). Pretreatment-values for HR in the same groups were, respectively, 326.7±15.0 ($n=9$), 346.0±24.8 ($n=5$), 308.0±19.9 ($n=5$), 276.0±8.1 ($n=5$), 358.0±23.8 ($n=5$), 336.0±24.6 ($n=5$) and 331.7±9.5 beats min^{-1} ($n=6$). There were no significant differences between the groups in terms of their absolute values of MABP and HR before administration of the drugs.

In the one-way ANOVA followed by Dunnett's test, KB-2796 (0.3 and 1 mg kg^{-1}, i.v.) signficantly decreased MABP at 2 min after its injection compared with the vehicle-treated groups, but did not change HR. Flunarizine (1 mg kg^{-1}, i.v.) did not significantly change MABP or HR. Flunarizine (3 mg kg^{-1}, i.v.) significantly decreased MABP at 2 and 15 min and decreased HR at 15 and 30 min after its injection. Dimetotiazine (1 mg kg^{-1}, i.v.) significantly decreased MABP and HR at 15 min after its injection. Dimetotiazine (3 mg kg^{-1}, i.v.) significantly decreased MABP at 2, 15 and 30 min after its injection and decreased HR at 15, 30 and 45 min after its injection.

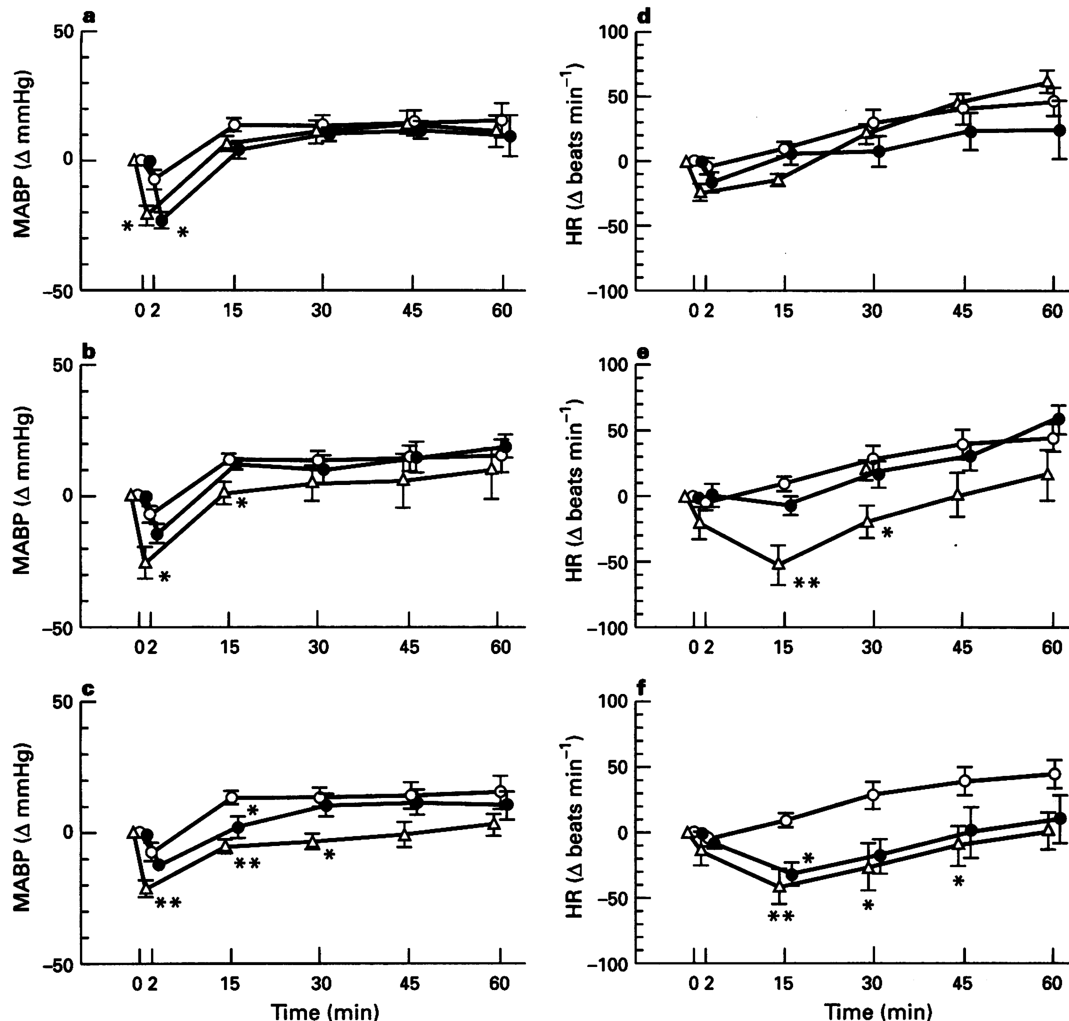

Figure 4 Effects of KB-2796, flunarizine and dimetotiazine on mean arterial blood pressure (MABP) and heart rate (HR) levels following drug administration. (a,d) KB-2796 (0.3 mg kg^{-1}, i.v., $n=5$, ● or 1 mg kg^{-1}, i.v., $n=5$, △) or vehicle ($n=9$, ○); (b,e) flunarizine (1 mg kg^{-1}, i.v., $n=5$, ● or 3 mg kg^{-1}, i.v., $n=5$, △) or vehicle ($n=9$, ○); (c,f) dimetotiazine (1 mg kg^{-1}, i.v., $n=5$, ● or 3 mg kg^{-1}, i.v., $n=6$, △) or vehicle ($n=9$, ○). Data are expressed as delta values with respect to baseline. Drugs were administered i.v. over 2 min or less. $^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle (Dunnett's test).

Effects of drugs on c-Fos-like immunoreactivity after CSD in anaesthetized rats (Table 3, Figures 5 and 6)

An increase in c-Fos-like immunoreactivity was observed in the cell nuclei of the ipsilateral cingulate, piriform and neocortex 2 h after KCl application, though much less immunoreactivity was found in the corresponding contralateral areas (Figure 5). The intensity of the immunoreactivity was always higher on the ipsilateral side than on the contralateral side 2 h after KCl application. No c-Fos-like immunoreactivity was seen in the cerebellum, hippocampus, thalamus or striatum. The effects of KCl + KB-2796 (1 mg kg^{-1}, i.v.), KCl + flunarizine (3 mg kg^{-1}, i.v.) and KCl + dimetotiazine (3 mg kg^{-1}, i.v.) in terms of the number of cells containing c-Fos-like immunoreactivity in 3 cortical areas of the coronal section are shown in Figure 6 and Table 3. Pretreatment with KB-2796 or flunarizine signficantly reduced the numbers on the c-Fos-like positive cells in the 3 areas of ipsilateral side. However, pretreatment with dimetotiazine did not affect the numbers of c-Fos positive cells in the 3 areas on either side of the cortex (Figure 6 and Table 3). There were no significant differences between the KCl + vehicle-treated group and any of the KCl + drugs-treated groups in terms of the numbers of c-Fos-like immunoreactive cells in the 3 cortical areas on the contralateral side (Table 3).

Discussion

CBF_{LDF} increased transiently to approximately 160% of the pre-CSD level immediately after KCl application and then decreased to 70–80% of the pretreatment-value for at least 60 min. CBF_{HC} was also changed to 70–80% of baseline at 30 and 60 min after KCl application. Thus, the change in CBF indicated by laser Doppler flowmetry was well correlated with the results of the hydrogen clearance method: this is consistent with previous reports which showed a good correlation between the two methods (Saeki *et al.*, 1990; Tamura *et al.*, 1992). Furthermore, these changes in CBF are consistent in magnitude with those reported by others (Lauritzen *et al.*, 1982; Lauritzen & Olesen 1984; Duckrow, 1991; Lacombe *et al.*, 1992). The reduction in CBF cannot be attributed to injury because the sham-operated group did not show a similar decline in CBF_{HC}. Moreover, Hall *et al.* (1991) have reported that arterial blood pressure does not change after KCl application to the rat cortex, so the changes induced in CBF after KCl treatment are unlikely to be due to induced changes in arterial blood pressure. These findings indicate that topical application of 1 M KCl to the exposed cortex for 30 s induces CSD, with cortical hyperperfusion for a few minutes, followed by cortical hypoperfusion for at least 60 min.

CSD has been reported to be propagated via diffusion and

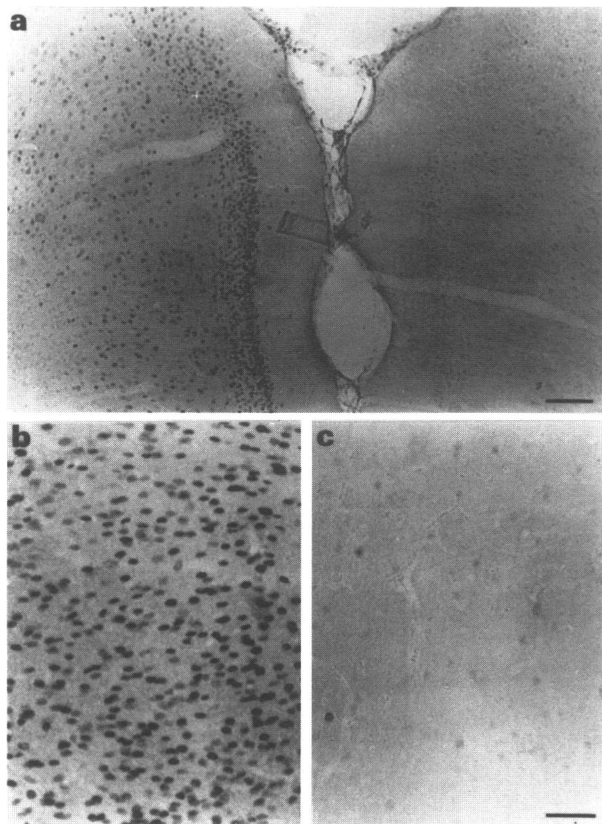

Figure 5 Photomicrographs showing c-Fos-like immunostaining in the cingulate cortex (from sections 70 μm thick, taken from 3.5 mm posterior to Bregma) 2 h after exposure of the left parietal cortex to 1 M KCl for 30 s. (a) Left (ipsilateral to side exposed to KCl) and right (contralateral) sides; (b) left (ipsilateral) side and (c) right (contralateral) side. Scale bar = 250 μm (a) and 100 μm (b and c).

release of K^+, glutamate and arachidonic acid in the extracellular space, resulting in an influx of Na^+ and Ca^{2+} into the intracellular space (Hansen & Zeuthen, 1981; Marranes *et al.*, 1988; Siesjö & Bengtsson, 1989; Lauritzen *et al.*, 1990; Fabricius *et al.*, 1993). Blockade of the *N*-methyl-D-aspartate (NMDA) receptor has been reported to increase the CSD threshold and also to decrease the propagation velocity and the duration of the accompanying extracellular direct current (Marrannes *et al.*, 1988). Several lines of evidence indicate that endogenous release of glutamate, activation of NMDA receptors and Ca^{2+} influx play an important role in the initiation, propagation and duration of CSD. However, the mechanisms underlying the CBF changes after CSD are not well understood. Interestingly, nitric oxide (NO) has been reported to participate in the transient hyperperfusion on the grounds that N^{ω}-nitro-L-arginine methylester (L-NAME), an NO synthase inhibitor, suppresses the transient increase, but not the subsequent decrease in CBF after CSD in rats (Duckrow, 1993) Furthermore, Pavlásek *et al.* (1993) have reported that CSD increases the catecholamine content of the extracellular space of rat cerebral cortex. These results indicate that neurotransmitters such as arachidonic acid and catecholamine may overflow during CSD and participate in the long-lasting hypoperfusion.

KB-2796 (0.3 and 1 mg kg^{-1}, i.v.) increased normal CBF_{LDF} 15 and 30 min after its administration in anaesthetized rats. This is consistent with previous reports that KB-2796 increases regional CBF in pancuronium-immobilized cats (Harada *et al.*, 1987) and in anaesthetized dogs (Kanazawa *et al.*, 1990). KB-2796 significantly decreased MABP 2 min after its administration, but pressure had returned to the pretreatment level by 15 min. Flunarizine (1 mg kg^{-1}, i.v.) also increased normal CBF_{LDF} 15 min after its administration, but not when given at a high dose (3 mg kg^{-1}, i.v.). Since flunarizine did not significantly change MABP at the lower dose, but did significantly decrease MABP at the high dose, the failure of flunarizine at the high dose to affect CBF_{LDF} may be due to the compensatory effect on blood flow of the strong decrease in MABP. On the other hand, dimetotiazine (3 mg kg^{-1}, i.v.) tended to decrease normal CBF_{LDF} and to decrease MABP. Dimetotiazine non-selectively inhibits the contraction induced by K^+ and 5-HT in the basilar, coronary and mesenteric arteries of dogs (H. Hara *et al.*, data not shown). Accordingly, the tendency of dimetotiazine to decrease CBF may be due to an induced decrease in MABP.

KB-2796 and flunarizine each inhibited the hypoperfusion that followed KCl-induced CSD whichever flow-recording method was used, but did not affect the transient hyperperfusion. The potency of KB-2796 was 3 times higher than that of flunarizine on a mg basis. This ratio is similar to that between their potencies in inducing inhibitory effects on specific [^{3}H]-nitrendipine binding in dog aortic membranes (Iwamoto *et al.*, 1991) and guinea-pig cerebral cortex membranes (Iwamoto *et al.*, 1988), and in inducing effects on CBF in anaesthetized dogs (Kanazawa *et al.*, 1990). Dimetotiazine had little effect on the transient hyperperfusion or on the cortical hypoperfusion after CSD. As arachidonic acid levels in cortical tissue increase during the first 3 min after the shift in d.c. potential, release of arachidonic acid after CSD may also trigger events leading to vasoconstriction and the consequent reduction in blood flow (Lauritzen *et al.*, 1990). KB-2796 has been shown to inhibit the increase in free fatty acids in decapitated rat brain (Kanazawa *et al.*, 1986) and to inhibit the contractions induced by prostaglandin (PG) $F_{2\alpha}$, K^+ and 5-HT in dog cerebral arteries in a non-competitive manner (Kanazawa & Toda, 1987), Moreover, flunarizine has been reported to have the same actions (Van Nueten, 1984). Taken collectively, these findings suggest that the inhibitory effects of KB-2796 and flunarizine on the CSD-induced hypoperfusion may have resulted, at least in part, from the inhibitory effect of stimulants such as $PGF_{2\alpha}$ on the contraction of cerebral arteries and the subsequent decrease in CBF.

c-Fos protein, synthesized by an immediate early gene, is a nuclear phosphoprotein which regulates the transcription rate of target genes (Sambucetti & Curran, 1986; Sheng & Greenberg, 1990; Morgan & Curran, 1991). c-Fos protein is induced by a large number of stimuli including pharmacological agents and ischaemia (Kruijer *et al.*, 1984; Onodera *et al.*, 1989). The expression of c-Fos, as measured by c-Fos protein immunocytochemistry, can be used to identify areas of neuronal activity and to map functionally-related neuronal pathways (Ehret & Fischer, 1991). Topical application of 3 M KCl to the brain surface has been found to be accompanied by an ipsilateral increase in c-Fos immunolabelling in rats (Herrera & Robertson, 1990). In the present study, topical application of 1 M KCl to the brain surface was sufficient to produce an increase in c-Fos-like immunoreactivity in rat cerebral cortex. c-Fos immunostaining was seen in the ipsilateral frontoparietal cortex, but to a much lesser extent in the contralateral cortex. No c-Fos-like immunoreactivity was seen in the cerebellum, hippocampus, thalamus or striatum. The expression of c-Fos-like immunoreactivity was not markedly different between the two sides in the sham-operated group in which 0.9% NaCl was applied to the brain surface. Furthermore, there were no marked differences between the c-Fos expression in sham-operated cortices and those contralateral to 1 M KCl application (data not shown). Moreover, Moskowitz *et al.* (1993) have reported that the expression of c-Fos-like immunoreactivity was not markedly different between the ipsilateral and contralateral sides in a sham-operated group which had received 1 M NaCl by microinjection instead of 1 M KCl.

KB-2796 and flunarizine reduced the expression of c-Fos-like labelled cells in the ipsilaterial frontoparietal cortex after KCl-induced CSD, but induced no more effect on the contralateral side than vehicle alone. L-type voltage-sensitive Ca^{2+} channel blockers such as PN200-110, nifedipine and ni-

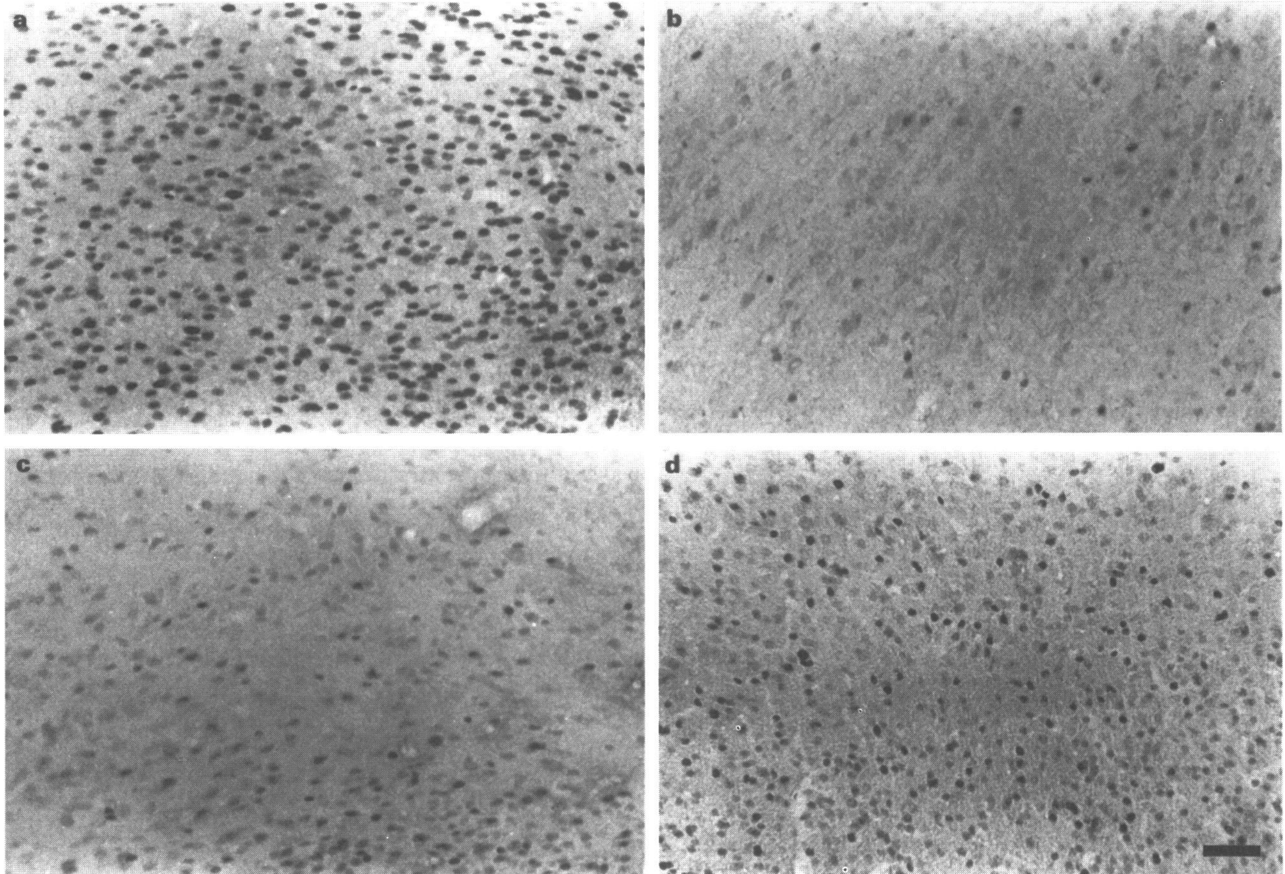

Figure 6 Photomicrographs showing c-Fos-like immunostaining in frontoparietal cortex (from sections 70 μm thick, taken from 3.5 mm posterior to Bregma) on ipsilateral side 2 h after exposure of left posterior parietal cortex to 1 M KCl for 30 s following pretreatment with KCl + vehicle (a), KCl + 1 mg kg^{-1} KB-2796, i.v. (b), KCl + 3 mg kg^{-1} flunarizine, i.v. (c) and KCl + 3 mg kg^{-1} dimetotiazine, i.v. (d). Scale bar = 100 μm.

Table 3 Effects of KB-2796, flunarizine and dimetotiazine on the expression of c-Fos-like protein after KCl-induced cortical spreading depression in the rat cortex

			Number of c-Fos-like positive cells (number/region)					
			Ipsilateral cortex			*Contralateral cortex*		
Drug (mg kg^{-1}, i.v.)		n	*FrPa A*	*FrPa B*	*FrPa C*	*FrPa A*	*FrPa B*	*FrPa C*
KCl + vehicle		9	409.6 ± 59.0	438.3 ± 61.8	458.6 ± 66.6	14.0 ± 3.9	12.6 ± 5.1	21.7 ± 11.4
KCl + KB-2796	1	9	183.1 ± 34.0**	171.2 ± 54.6**	247.2 ± 54.6*	10.9 ± 5.2	2.6 ± 1.2	3.7 ± 1.1
KCl + vehicle		9	542.9 ± 80.7	385.4 ± 55.0	529.2 ± 66.3	24.9 ± 7.7	13.1 ± 5.1	19.0 ± 6.8
KCl + flunarizine	3	9	319.2 ± 61.2*	222.0 ± 39.7**	223.3 ± 39.7**	18.2 ± 9.6	4.6 ± 2.7	22.2 ± 7.8
KCl + dimetotiazine	3	9	526.1 ± 46.5	401.6 ± 45.3	425.7 ± 39.9	15.9 ± 4.0	5.0 ± 1.2	16.8 ± 6.3

Drugs were administered i.v. 5 min before KCl application. Data are expressed as a mean ± s.e. In order to quantitate the expression of c-Fos-like protein, cells showing c-Fos-like immunoreactivity within 3 separate frontparietal (FrPa) area (0.4 mm^2) of bilateral cortex, coronary section (70 μm) of 3.5 mm posterior to Bregma; FrPa A [2.0 mm laterally of the midline and a depth of 1.0 mm from the surface of the skull], FrPa B [6.0 mm laterally of the midline and a depth of 3.5 mm from the surface of the skull] and FrPa C [6.5 mm laterally of the midline and a depth of 6.0 mm from the surface of the skull], were counted. $*P<0.05$, $**P<0.01$ vs. KCl + vehicle-treated animals (Dunnett's test).

trendipine have been reported to reduce basal c-Fos-like immunoreactivity in cultured cortical neurones and PN200-110 has been reported to block the expression of c-Fos protein induced by kinate (Murphy *et al.*, 1991). Furthermore, Bay K 8644, an L-type voltage-sensitive Ca^{2+} channel agonist, enhanced basal c-Fos expression (Murphy *et al.*, 1991). However, in contrast to this published work which seems to indicate that Ca^{2+} blockers inhibit basal c-Fos-like immunoreactivity, our results show that the drugs we examined have no effect on basal c-Fos-like immunoreactivity. Although the reason for this discrepancy is not entirely clear, it may result from a difference in the sensitivities of the antibodies used, the different reaction times of the different drugs or to a difference between *in vitro* and *in vivo* experiments. Taken together, the results indicate that the Ca^{2+} channel participates in the mechanism underlying the expression of c-Fos protein. KB-2796 and flunarizine have been shown to increase the latency to onset of hypoxia-induced SD and to inhibit hypoxia- or KCl-induced $^{45}Ca^{2+}$ uptake in rat hippocampal slices, independently of changes in the vascular system (Takagi *et al.*, 1994). In this regard, the potency of KB-2796 was 10 times higher than that of flunarizine. Flunarizine has also been reported to elevate the threshold for eliciting CSD in rats (Wauquier *et al.*,1985). In rat hippocampal slices, blockade of voltage-sensitive Ca^{2+}

channels with either Ni^{2+} or Co^{2+} blocked the propagation, but not the initiation of K^+-induced SD (Jing *et al.*, 1993). These reports, which show that inhibition of SD can be achieved by blockade of Ca^{2+} channels, suggest that Ca^{2+} channel blockers may prevent migraine by inhibiting SD.

Dimetotiazine had no effect on the cerebral hypoperfusion or the expression of c-Fos-like immunoreactivity that followed KCl-induced CSD. Thus, in this model, the profiles of KB-2796 and flunarizine differed from that of dimetotiazine and the cortical hypoperfusion and the c-Fos expression that followed KCl-induced CSD may not have involved interaction with $5\text{-}HT_2$ and H_1 receptors.

In conclusion, KB-2796 and flunarizine were each able to inhibit cortical hypoperfusion and the expression of c-Fos-like immunoreactivity in the cortex following KCl-induced CSD. These inhibitory effects may be mediated by the effects of Ca^{2+}-entry blockade, such as an increase in CBF and the prevention of excessive Ca^{2+} influx into brain cells Accordingly, KB-2796 and flunarizine may prove useful in inhibiting CSD in migraine.

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