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Short communication

Effects of (±)-kavain on voltage-activated inward currents of dorsal root ganglion cells from neonatal rats

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Abstract

Kava pyrones extracted from pepper *Piper methysticum* are pharmacologically active compounds. Since kava pyrones exhibit anticonvulsive, analgesic and centrally muscle relaxing properties, the influence of a synthetic kava pyrone, (±)-kavain, on voltage-dependent ion channel currents was studied. Effects of (±)-kavain on voltage-activated inward currents were analysed in cultured dorsal root ganglion cells derived from neonatal rats. Voltage-activated Ca^{2+} and Na^{+} currents were elicited in the whole-cell configuration of the patch clamp technique. Extracellularly applied (±)-kavain dissolved in hydrous salt solutions reduced voltage-activated Ca^{2+} and Na^{+} channel currents within 3–5 min. As the solubility of (±)-kavain in hydrous solutions is low, dimethyl sulfoxide (DMSO) was added to the saline as a solvent for the drug in most experiments. When (±)-kavain was dissolved in DMSO, the drug induced a fast and pronounced reduction of both Ca^{2+} and Na^{+} currents, which partly recovered within 2–5 min even in the presence of the drug. The present study indicates that (±)-kavain reduces currents through voltage-activated Na^{+} and Ca^{2+} channels.

Keywords: Sensory spinal ganglion cells; (±)-Kavain; Na channels; Calcium channels

1. Introduction

Kava pyrones are extracted from the root of the pepper plant *Piper methysticum* forster and used as an intoxicating beverage during traditional ceremonies by the inhabitants of the South Pacific Islands. The kava beverage induces relaxation, enhances a sense of sociability and promotes sleep. In Western nations kava is commercially distributed and clinically used as a pharmacologically active compound. Pharmacological investigations revealed that kava pyrones are acting on the central and peripheral nervous system, especially potentiating the barbiturate sleeping time (Klohs, 1979), anticonvulsive action (Kretschmar et al., 1970), analgesic properties (Jamieson and Duffield, 1990) and centrally muscle relaxant effects (Meyer, 1979; Meyer and Kretschmar, 1966). Currently, various sites or mechanisms of action of kava pyrones are discussed. Among these sites are voltage-dependent Na^{+} channels (Gleitz et al., 1995, 1996), γ-aminobutyric acid (GABA) receptors (Davis et al., 1992; Jussofie et al., 1994; Langosch et al., 1997), N-methyl-D-aspartic-acid (NMDA) glutamate receptors (Langosch et al., 1997; Walden et al., 1997). Because of their anticonvulsive action, kava pyrones may also affect voltage-activated Ca^{2+} channels as has been described for carbamazepine (Winkel and Lux, 1987; Walden et al., 1992; Schirrmacher et al., 1993; Walden et al., 1993). This would explain the similarities in the depressant action of kava pyrones and of organic Ca^{2+} antagonists on epileptic paroxysmal depolarizations (Witte et al., 1986; Bingmann and Speckmann, 1988; Walden and Speckmann, 1988). These findings led to the idea that the

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psychopharmacological effects of kava pyrones are also due to changes of voltage-dependent ion channel currents.

To elucidate the mechanisms as to how kava pyrones contribute to antiepileptic, analgesic and psychotropic actions, we analysed the effects of (±)-kavain on voltage-activated Ca^{2+} and Na^{+} channel currents of cultured rat dorsal root ganglion (DRG) cells (cf. Heyer and Macdonald, 1982; Tsien et al., 1988). High-threshold activated Ca^{2+} currents (L-type) were analysed predominantly, as they are involved in epileptogenesis (Meyer and Kretschmar, 1966). In addition, effects of (±)-kavain on voltage-dependent Na^{+} currents were analysed (cf. Gleitz et al., 1995, 1996).

2. Experimental procedures

Spinal ganglia from 14 neonatal rats (four different litters, 2- to 4-day-old) were excised after decapitation. Dissected ganglia were stored in ice-cold 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; 20 mM)-buffered minimum essential medium (MEM; 88 vol%) which contained fetal calf serum (FCS; 10 vol%), glutamine (200 mM; 1 vol%) and penicillin (5000 I.U./ml; 0.5 vol%)/streptomycin (5000 μg/ml; 0.5 vol%) in a Petri dish (Falcon). For enzymatic preparation of the ganglia, the media had been warmed to room temperature (21±1°C). Ganglia were washed three times with serum-free MEM. This medium was exchanged for 0.9 ml of MEM plus FCS and 0.1 ml collagenase [Sigma Type II; stock solution: 12.5 mg in 1 ml HEPES-buffered solution (HBS) containing (in mmol/l) NaCl 140, KCl 3, $CaCl_2$ 1.8, $MgSO_4$ 1.3, KH_2PO_4 1.25, glucose 11, HEPES 10; pH 7.4] was added for 16 min and stored in an incubator at 35°C. Collagenase containing medium was removed and the ganglia were washed three times with serum-free MEM. Then 0.9 ml serum-free MEM plus 0.1 ml trypsin (Sigma, Type IX; stock solution: 25 mg in 1 ml HBS) were added for another 6 min at 35°C. Trypsin containing medium was removed and the ganglia were washed three times in MEM plus FCS. Ganglia were transferred to a plastic tube and 1.5–2 ml MEM plus FCS with DNase (Sigma Type II; stock solution: 1 mg in 10 ml) were added. Solution containing the ganglia were triturated with a fire-polished glass pipette and 8 ml of MEM plus FCS was added. The cell suspension was passed through a nylon mesh (ø 200 μm) and placed on Poly-L-Lysine (Sigma, 0.1% w/v in water) covered glass coverslips in 50–100 μl aliquots in Petri dishes (Falcon) and maintained in a humidified atmosphere at 35°C. After 2 h, when the neurones had settled, the Petri dishes were filled with 1 ml MEM plus FCS. All media components were purchased from Flow Laboratories Meckenheim (Germany).

The neurones were used for electrophysiological recordings 12 h after plating for another 2 or 3 days in culture. Coverslips were transferred to a recording chamber mounted on an inverted microscope. The chamber (1.5×5 cm^2; volume 2 ml) was superfused at a rate of 4–6 ml/min with a physiological HBS (see below) at room temperature (21±1°C).

For whole-cell recordings glass pipettes were made from soft glass (ø 1.5 mm, Hilgenberg, Germany) in a two-step pulling procedure (P-87, Flaming/Brown, Sutter Instruments, Germany). When filled with the appropriate solution these electrodes had resistances of 2–4 MΩ. Pipettes were placed on the head-stage of an EPC 9 (HEKA, Germany) patch clamp probe. When the electrode made contact with the cell, gentle suction was applied to achieve a high resistance seal of several GΩ (Hamill et al., 1981). To gain access to the interior of the cell and to record ion currents, the cell membrane was ruptured by further suction. Cells were clamped at −80 mV. Voltage clamp command pulses were delivered by the EPC 9 unit which was controlled by an ATARI STE computer using the patch clamp software EPC 9 Screen. Series resistance compensation was automatically performed by the patch clamp software. Raw data were filtered at 2.3 kHz. All data were corrected for leak currents by a p/4 protocol as described by Chad and Eckert (1986). Raw and corrected data were stored on hard disc and analysed using M2-lab software on the computer. Ca^{2+} and Na^{+} currents were evoked at a rate of one per 5 s. Ca^{2+} currents were elicited by stepping from the holding potential of −80 mV to 0 mV for 70 ms and then back to the holding potential. Na^{+} currents were evoked by step depolarizations from the holding potential of −80 mV to 0 mV which lasted 25 ms.

Investigations were carried out in a bath saline containing (concentrations in mM): NaCl 145, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10, and glucose 10. The pH value was 7.4 (with NaOH). To identify single ionic currents the normal saline was modified by ion substitution and/or by using specific channel blocking agents. To reduce K^{+} conductance, tetraethylammonium chloride (TEA-Cl) was added to the saline in exchange for Na^{+}. To isolate Ca^{2+} currents, $CaCl_2$ was replaced by $BaCl_2$. The modified solution contained (concentrations in mM): TEA-Cl 130, HEPES 10, MgCl 1.0, $BaCl_2$ 10, tetrodotoxin (TTX) 0.0004, and glucose 10, pH 7.2 (TEA-OH). Na^{+} currents were recorded in the normal bath solution. The pipette solution used for measuring Ca^{2+} and Na^{+} currents contained CsCl, which completely blocked K^{+} channels from the inside (concentrations in mM): CsCl 140, $MgCl_2$ 4, EGTA 10, HEPES–KOH 10, Na_2–ATP 2, adjusted to pH 7.2 with KOH. Patch clamp experiments were performed at room temperature (21±1°C). Experiments were excluded in which the currents showed a run-down of more than 10% of the total current within 3–5 min after whole-cell mode was attained.

The drug (±)-kavain (Krewel Meuselbach, Eitorf, Germany) was dissolved in dimethyl sulfoxide (DMSO, Sigma) to give a stock solution of 250 mM. Solutions were made freshly before each experiment. Addition of DMSO

or 250 mM (±)-kavain dissolved in DMSO, respectively, did not change the pH value of the bath solutions. The final DMSO concentration was 0.01%. Alternatively, (±)-kavain was dissolved directly in the hydrous salt solution and stirred for several hours.

3. Results

Effects of (±)-kavain on voltage-operated Ca^{2+} and Na^{+} channel currents were examined in 25 cultured DRG cells derived from different neonatal rats. We analyzed the influence of (±)-kavain on the amplitude of whole-cell currents measured under voltage clamp conditions. The drug was either applied via the bath superfusion system or added directly to the bath solution. The different treatments did not alter impedance of the cells. Due to difficulties of dissolving (±)-kavain in hydrous solutions in other experiments, the drug was either dissolved in hydrous salt solution or DMSO.

3.1. Effects of (±)-kavain dissolved in hydrous salt solution

In Ba^{2+}/TTX-containing solution, depolarisation of the membrane potential from −80 mV to 0 mV evoked inward currents which reached a peak within 5–10 ms and decayed slowly and, therefore, are Ca^{2+} channel currents (Fig. 1A). When the currents were stable 3 min after establishing whole-cell conditions (trace 1), DRG cells were superfused with a solution containing Ba^{2+} and TTX, in which (±)-kavain had been dissolved for 1–2 h (n=6; n=number of independent experiments in different cells). Under this condition, a decrease of the peak and steady-state channel currents shortly after application was observed (trace 2). The decrease of the peak amplitude of the Ca^{2+} channel currents during (±)-kavain exposure was not reversible after wash (trace 3).

Effects of (±)-kavain on voltage-activated Na^{+} currents were studied in normal HEPES-buffered solution using a similar pulse protocol as for the Ca^{2+} currents (Fig. 1B, trace 1). Exposure to (±)-kavain—diluted before in the bath solution for several hours—led to a decrease of the voltage-dependent Na^{+} current amplitude to 81±6% (n=5) of the original value (trace 2). This effect was only partly reversible in one out of five cells.

3.2. Effects of (±)-kavain dissolved in DMSO

As (±)-kavain showed effects on both Ca^{2+} and Na^{+} channel currents when directly dissolved in the bath solutions with unknown drug concentrations, we prepared stem solutions containing (±)-kavain dissolved in DMSO. When Ca^{2+} channel currents were stable during whole-cell recording (Fig. 2, trace 1), 2 μl DMSO (n=4) was acutely added to the bath solution, which reversibly reduced peak and steady-state Ca^{2+} channel currents within 1–2 min (trace 2) indicating a small, nonspecific effect of the solvent on this type of Ca^{2+} channel current. Subsequent addition of 2 μl (±)-kavain (250 mM in DMSO) to the superfusate induced a pronounced transient decrease of the

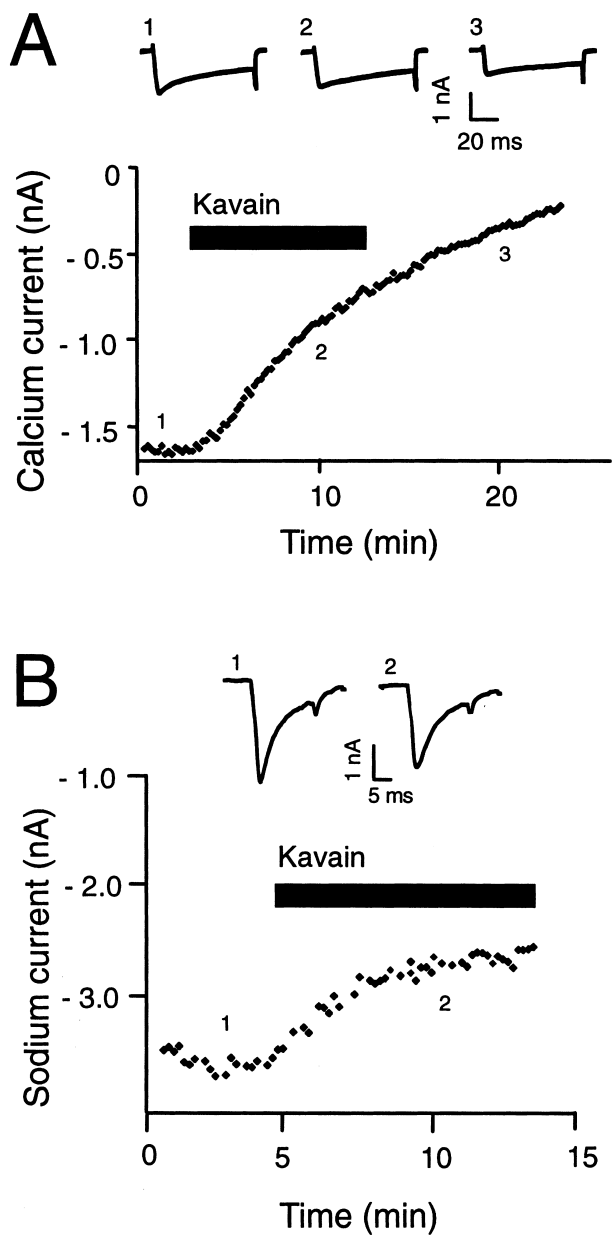

Fig. 1. Slow reduction of voltage-dependent Ca^{2+} and Na^{+} channel currents in rat dorsal root ganglion cells by (±)-kavain dissolved in the bath solution several hours before application. (A) Ca^{2+} channel currents elicited by a depolarising pulse (80 ms) from holding potential −80 mV to 0 mV in control solution containing 10 mM Ba^{2+} and 0.4 μM TTX (trace 1), during (±)-kavain exposure (trace 2), and after washing (trace 3). (B) Na^{+} currents elicited by a depolarising pulse (15 ms) from −80 mV to 0 mV in normal HEPES-buffered solution (trace 1) and during the application of (±)-kavain (trace 2). Effects on both, Ca^{2+} and Na^{+} channel currents were not reversible. (A, B) Upper parts show original current traces. Lower parts illustrate the changes of peak currents as a function of time during whole-cell recording.

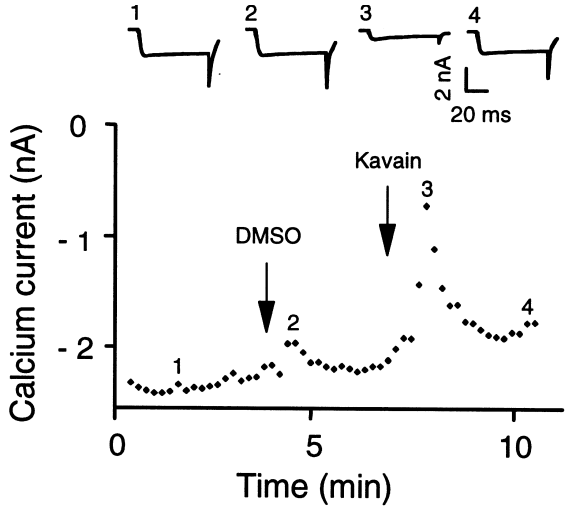

Fig. 2. Fast and transient reduction of voltage-dependent Ca^{2+} channel currents in rat dorsal root ganglion cells after acute addition of 2 μl dimethylsulfoxyde (DMSO) and 2 μl (±)-kavain (250 mM, dissolved in DMSO) to the bath solution containing 10 mM Ba^{2+} and 0.4 μM TTX. Upper part shows responses elicited by step depolarisations (60 ms) from holding potential −80 mV to 0 mV in control solution (trace 1), after exposure to DMSO (trace 2) as well as (±)-kavain (trace 3) and after washing (trace 4). Lower part demonstrates peak amplitudes of the Ca^{2+} channel currents as a function of time.

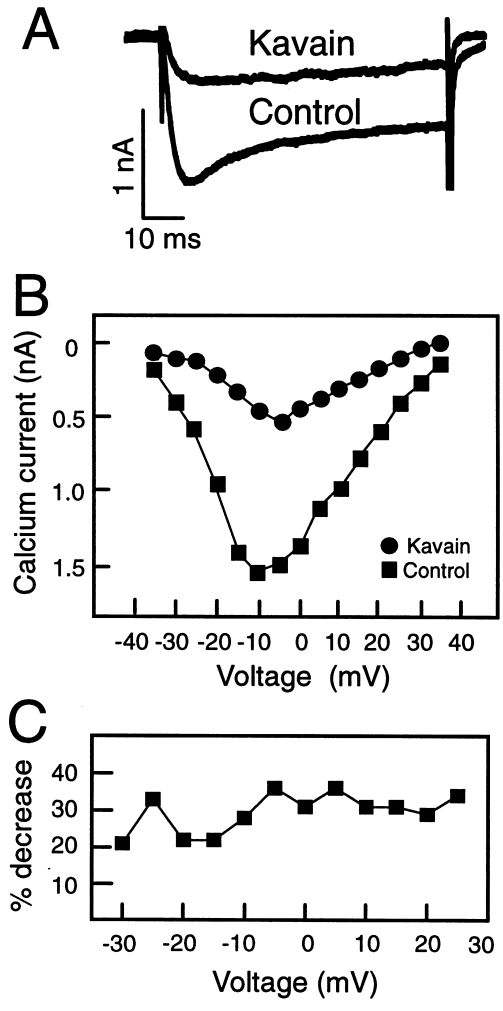

Fig. 3. Reduction of the voltage-activated Ca^{2+} channel currents elicited by a depolarisation from −80 mV to 0 mV for 70 ms by 100 μmol/l (±)-kavain (A). Current–voltage relation of the voltage-activated Ca^{2+} channel currents (peak values) before and after application of 100 μmol/l (±)-kavain (B). Percent decrease of the peak amplitude of the Ca^{2+} current in the voltage range between −30 and 30 mV (C).

peak amplitude within 1 min from 2.2 nA to 0.7 nA, which was 32% of the initial value (traces 3 and 4). Steady-state currents measured after 70 ms were affected in a similar way. In another cell, this way of (±)-kavain application caused a similar decrease of peak Ca^{2+} channel currents to 5%, which was reversible after washing. In two other DRG cells, the peak Ca^{2+} current was reversibly reduced by 2 μl (±)-kavain (250 mM in DMSO) added directly to the bath solution to 75% and 82% of the original value [63±27%, mean±standard error, $n=3$]. Fig. 3 illustrates reduction of the voltage-dependent Ca^{2+} currents by 100 μmol/l (±)-kavain. The peak amplitude of the Ca^{2+} currents were reduced to 35% of the control value after a step-depolarisation from −80 mV to 0 mV (Fig. 3A). The voltage-activated Ca^{2+} channel currents were reduced over the entire voltage range (Fig. 3B). In this experiment a shift in the peak of the current–voltage relationship of 5 mV was observed. Between −30 and 30 mV the decrease of the peak amplitude by 100 μmol/l (±)-kavain was similar indicating that high- and low-voltage activated Ca^{2+} channel currents are affected in a similar way (Fig. 3C).

Voltage-dependent Na^+ currents were elicited by depolarising pulses from a holding potential of −80 mV to 0 mV for 25 ms. The Na^+ current increased within 1 ms to a peak of 5.3 nA and then decayed to almost zero (Fig. 4, trace 1). The half time of this decay was 3 ms. A 22 min lasting exposure to (±)-kavain dissolved in normal saline induced a transient decrease of the amplitude of the Na^+ current of 500 pA, which was 10% of the initial value (trace 2). Subsequent addition of 100 μM (±)-kavain (in DMSO) hardly affected this peak current (trace 3). However, addition of 400 μM (±)-kavain transiently decreased the Na^+ current amplitude from 5 nA to 1.7 nA which was 34% of the initial value (traces 4 and 5).

Superfusion of the DRG cells with 100 μM or 400 μM (±)-kavain reduced the Na^+ currents to 65% ($n=2$) or 41±4% ($n=5$), respectively, which in most cases were transient or reversible after washing. However, 50 μM (±)-kavain did not affect Na^+ currents (96±9%, $n=6$). In one of six cells, the Na^+ current was reversibly reduced to 77% of the initial value by 50 μM (±)-kavain. Thus, low concentrations of (±)-kavain up to 50 μM hardly affected

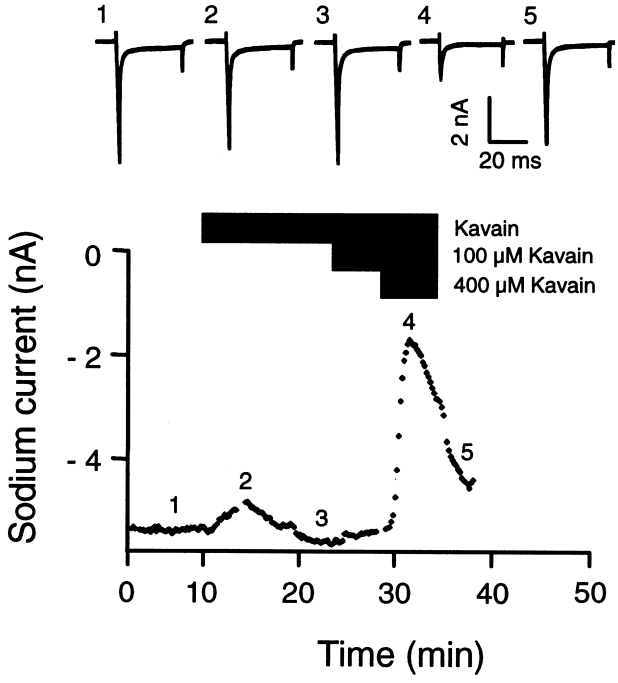

Fig. 4. Fast and transient reduction of voltage-dependent Na^+ currents in rat dorsal root ganglion cells after subsequent exposure to (±)-kavain dissolved in HEPES-buffered solution, 100 μM and 400 μM (±)-kavain (dissolved in dimethyl sulfoxide, DMSO). Upper part shows responses elicited by step depolarisations (30 ms) from holding potential −80 mV to 0 mV in control solution (trace 1), during exposure of (±)-kavain (trace 2), 100 μM (±)-kavain (trace 3), 400 μM (±)-kavain (trace 4) and after washing (trace 5). Lower part demonstrates peak amplitudes of the Na^+ currents as a function of time.

voltage-dependent Na^+ currents in DRG cells, whereas higher concentrations of up to 400 μM induced prominent reductions of this current.

4. Discussion

Kava pyrones exert a variety of effects predominantly anticonvulsive, analgesic and centrally muscle relaxant (Kretschmar et al., 1970; Klohs, 1979; Meyer, 1979). In the present study, cultured DRG cells were selected although these cells are not likely to be a target of kava pyrones in most circumstances and are probably not involved in epileptogenesis. Nevertheless, these cells facilitate the analysis of postsynaptic drug effects, because they allow for a better control of local drug concentrations than, e.g., neurones in slice preparations. In line with the depressant effects of kava pyrones, (±)-kavain reduced voltage-dependent L-type Ca^{2+} channel currents and Na^+ currents in most of the DRG cells.

Due to difficulties of the solubility of (±)-kavain in hydrous solutions, we used DMSO as a solvent for the drug as well as different forms of application. Depending on the kind of solvent and application method, effects of (±)-kavain occurred with two different time courses. First, extracellularly applied (±)-kavain dissolved in the recording solution started to reduce Ca^{2+} and Na^+ channel currents within 2–3 min, which was not reversible after a 10–15 min lasting washing period. Effects of (±)-kavain on Ca^{2+} channel currents were similar to those of the antiepileptic drug carbamazepine in DRG cell cultures (Schirrmacher et al., 1995). The reason why effects of (±)-kavain were not reversible when dissolved directly in the hydrous salt solution is not known. It is likely, however, that precipitated crystals of (±)-kavain were still present in the bath during washing. Second, when (±)-kavain dissolved in DMSO was added to the bath either acutely or via the perfusion system, the drug induced a fast and pronounced reduction of both Ca^{2+} and Na^+ channel currents, which partly recovered within 2–5 min even in the presence of the drug. This effect resembles desensitisation characteristics known for other substances.

The present data on the inhibitory influence of (±)-kavain on Na^+ currents in DRG cells is consistent with those on veratridine-activated Na^+ channels in synaptosomes prepared from the rat cerebral cortex (IC_{50}=86 μM, Gleitz et al., 1995). As significant pharmacological effects of kava pyrones are predicted for plasma concentrations of about 50 to 150 μM (Kretschmar and Meyer, 1968), the concentrations of (±)-kavain used in our experiments (10 to 400 μM) were of physiological relevance (cf. Gleitz et al., 1995; Walden et al., 1997). In contrast to our study, where final concentrations of the solvent DMSO even of 0.01% affected ion channel currents, 1% DMSO seemed to have no effects on synaptosomes (Gleitz et al., 1995).

The spasmolytic, analgesic, and anticonvulsive actions of kava pyrones might be explained by inhibition of Na^+ currents. The question whether the reduction of Na^+ channel currents is also responsible for their psychotropic action cannot be answered easily, because of the large dosages used and of the observed fast elimination time (Keledjian et al., 1988; cf. Gleitz et al., 1995). Similarly, field potential changes induced by the removal of Mg^{2+} from NMDA receptors consecutively activating voltage-dependent Ca^{2+} channels in hippocampal neurones can be blocked by (±)-kavain (Walden et al., 1997). Thus, it may be assumed that the Ca^{2+} antagonistic properties of (±)-kavain may explain the effectiveness of the drug in the treatment of epilepsies (Speckmann et al., 1986) as well as affective disorders (Schirrmacher et al., 1993; Walden et al., 1995). In contrast to (±)-kavain, the anticonvulsive drug carbamazepine specifically reduced Ca^{2+} currents, but did not significantly modify voltage-activated Na^+ currents of DRG or hippocampal neurones (Hood et al., 1983; Zona et al., 1990; Schirrmacher et al., 1995). As there is evidence that also Na^+ channels are a common target of a variety of structurally diverse antiepileptic drugs

(Upton, 1994), the effects of (±)-kavain on both, voltage-activated Ca^{2+} and Na^{+} channel currents may intensify, e.g., anticonvulsive properties of this drug, which up to now were attributed primarily to GABAergic effects.

In conclusion, the anticonvulsive and analgesic effects of (±)-kavain could be partly explained by Na^{+} and Ca^{2+} antagonistic action.

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