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(±)-Kavain Inhibits the Veratridine- and KCl-induced Increase in Intracellular Ca^{2+} and Glutamate-release of Rat Cerebrocortical Synaptosomes

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Summary—The action of (±)-kavain on the veratridine, monensin and KCl-depolarization evoked increase in free cytosolic Ca^{2-} concentration ($[Ca^{2+}]_i$), and its influence on the release of endogenous glutamate from rat cerebrocortical synaptosomes were investigated. $[Ca^{2+}]_i$ was fluorimetrically determined employing FURA as the Ca^{2+} sensitive fluorophore, and glutamate was detected by a continuous enzyme-linked fluorimetric assay. The incubation of synaptosomes in the presence of (±)-kavain up to a concentration of 500 μmol/l affected neither basal $[Ca^{2+}]_i$ nor spontaneous release of glutamate, but dose-dependently reduced both veratridine-elevated $[Ca^{2+}]_i$ (IC_{50} = 63.2 μmol/l) and glutamate-release (IC_{50} = 116.4 μmol/l). The inhibition of these parameters, attained with 500 μmol/l (±)-kavain, could be overcome by inducing an artificial Na^+ influx, using monensin as a Na^+ ionophore. An application of (±)-kavain after veratridine caused a decrease in veratridine-elevated $[Ca^{2+}]_i$, which was similar to the action of tetrodotoxin (TTX) with regard to time course, half-life of $[Ca^{2+}]_i$ decline and the final steady state level of $[Ca^{2+}]_i$. Concomitantly, veratridine-induced glutamate-release was blocked. The results indicate that specific inhibition of voltage-dependent Na^+ channels is a primary target of (±)-kavain, thus preventing a $[Na^+]_i$ provoked increase in $[Ca^{2+}]_i$ and glutamate-release. However, pathways related to the elevation of $[Ca^{2+}]_i$ by $[Na^+]_i$ itself, and the processes involved in normalization of elevated $[Ca^{2+}]_i$ and glutamate-release downstream to enhanced $[Ca^{2+}]_i$, seems to be unaffected by (±)-kavain. Using KCl-depolarized synaptosomes, 400 μmol/l (±)-kavain reduced, in analogy to Aga-GI toxin, KCl-evoked $[Ca^{2+}]_i$ and diminished the part of glutamate-exocytosis which is related to external Ca^{2+} to about 75% of control. At a concentration of 150 μmol/l, which is above the IC_{50} value necessary to block voltage-dependent Na^+ channels, (±)-kavain affected neither basal nor the KCl-induced increase in $[Ca^{2+}]_i$. These results might suggest that (±)-kavain at concentrations sufficient to block Na^+ channels completely, moderately inhibits the non-inactivating Ca^{2+} channels located on mammalian presynaptic nerve endings.

Keywords—Calcium channel, kavain, piper methysticum, sodium channel, spider toxin, synaptosomes.

Kava pyrones prepared from the rhizome of the intoxicating pepper, *Piper methysticum* Forst., have been shown to be responsible for the analgesic and anti-convulsive actions known from traditional medicine (Lebot *et al.*, 1992; Singh, 1992). The antinociceptive properties of (+)-kavain, (+)-dihydrokavain, (+)-methysticin and (+)-dihydromethysticin, which are major pyrone constituents of kava roots (Duve, 1981; Smith, 1983), has been confirmed by the tail immersion method and the abdominal constriction procedure (Jamieson and Duffield, 1990). Because naloxone, in doses that inhibit morphine-induced analgesia, was ineffective in reversing antinociceptive activities of kava pyrones, an interaction of these compounds with opioid receptors was excluded, and they were assumed to have a local anaesthetic action (Meyer, 1979; Singh, 1983). An anticonvulsive action against pentylenetetrazol-induced convulsions (Kretzschmar and Meyer, 1969), and inhibition of epileptiform discharges induced in different *in vitro* seizure models (Schmitz *et al.*, 1995) could be demonstrated with the natural kava pyrone (+)-methysticin. With respect to the dose response relationship and the duration of action and influence on the seizure pattern, the anticonvulsive activity of this kava pyrone resembles that of the local anaesthetic procaine (Kretzschmar and Meyer, 1969). Recently, Gleitz *et al.* (1995) showed that (±)-kavain, a synthetic kava pyrone, blocked veratridine-sensitive Na^+ channels of cerebrocortical synaptosomes. Since voltage-dependent Na^+ channels are known to be a common target of action for diverse antiepileptic and analgesic drugs (Loscher and Schmidt, 1994; Upton,

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1994), inhibition of Na^+ channels by kava pyrones may explain their pharmacological actions described above. However, no information exists about a possible action of kava pyrones on intracellular Ca^{2+} homeostasis and the release of neurotransmitters. Alterations of these parameters, induced by kava pyrones, may additionally contribute to their analgesic and anticonvulsive properties. Therefore, in the present paper, the action of (±)-kavain on the intrasynaptosomal free Ca^{2+} concentration ($[Ca^{2+}]_i$) and its influence on the release of endogenous glutamate from cerebrocortical synaptosomes induced by veratridine, KCl-depolarization, and the Na^+ ionophore monensin, was investigated.

METHODS

Preparation of synaptosomes

The experiments were conducted using adult, male Wistar rats (180–220 g, Charles River, Sulzfeld, Germany). Synaptosomes of rat cerebral cortex were prepared according to Gleitz *et al.* (1993a). In brief, each cortex was homogenized in 15 ml of homogenization buffer (320 mmol/l sucrose, 0.5 mmol/l EDTA, 1 mg/ml BSA, 5 mmol/l TES, pH 7.4) with a Braun potter (Melsungen, Germany) rotating at 800 rev/min by 5 strokes. The homogenate was centrifuged at 1000 *g* for 10 min and the resultant supernatant was recentrifuged at 28,000 *g* for 20 min. The pellet was resuspended in homogenization buffer and layered on a discontinuous ficoll gradient [6%, 9%, 12% (w/v)]. After centrifugation at 90,000 *g* for 50 min, both synaptosomal interfaces, located within the 9% layer, were combined and filled up with a 10-fold volume of homogenization buffer without EDTA. Finally, the synaptosomal pellet was obtained by centrifugation at 20,000 *g* for 15 min. Freshly isolated synaptosomes were cryopreserved according to the method developed by Gleitz *et al.* (1993b). Briefly, the synaptosomal pellet was resuspended in homogenization buffer without EDTA to obtain a final protein concentration of about 14 mg/ml. Controlled freezing was performed with a programmable temperature controller (kryo 10-3.3/II, Messer Griesheim, Germany) according to the following cooling schedule: hold 5 min at 4°C, −1°C/min from 4°C to −30°C, −5°C/min from −30°C to −70°C, −10°C/min from −70°C to −140°C. Immediately after freezing synaptosomes were stored in liquid nitrogen.

FURA 2-AM loading

$[Ca^{2+}]_i$ was determined by the ratio fluorescence method according to Grynkiewicz *et al.* (1985) employing FURA as the Ca^{2+} sensitive fluorophore. For FURA loading, 495 µl of cryopreserved synaptosomes with a protein content of about 14 mg/ml protein were warmed in a water bath at 37°C until the suspension was just thawed. Five µl of a 1 mmol/l FURA 2-AM stock solution, dissolved in dimethylsulphoxide (DMSO), was added to obtain final concentrations of 10 µmol/l FURA 2-AM and 1% (v/v) DMSO. The suspension was incubated for 45 min on ice, subsequently diluted within 110 ml of incubation buffer (125 mmol/l NaCl, 3.5 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 1.2 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3$, 25 mmol/l HEPES, pH 7.4) and centrifuged at 12,000 *g* for 10 min. After washing the pellet with 100 ml incubation buffer, the pellet was resuspended in 12 ml incubation buffer, divided into 2 ml portions and centrifuged at 12,000 *g* for 5 min. The pellets were stored on ice until the measurement of fluorescence was performed.

$[Ca^{2+}]_i$ measurement

For $[Ca^{2+}]_i$ measurement, each FURA loaded synaptosomal pellet was resuspended in 2 ml incubation buffer to obtain protein concentrations of 0.5–0.7 mg/ml. Fluorescence measurements were made with a spectrofluorometer (Delta scan, PhotoMed GmbH, Wedel, Germany) in a temperature-controlled stirred cuvette at 37°C. At the end of the fluorescence measurement, calibration of each individual probe was carried out to calculate R_{max}, R_{min}, S_b and S_f (see below). Calculation of $[Ca^{2+}]_i$ and calibration was performed with modifications as described by Grynkiewicz *et al.* (1985). In brief, FURA fluorescence was measured alternately at excitation wavelengths of 340 and 380 nm with emission at 508 nm employing 8 nm bandpass slits for the excitation and emission monochromators. $[Ca^{2+}]_i$ was calculated as: $[Ca^{2+}]_i = (K_D * S_f/S_b) * (R - R_{min}) / (R_{max} - R)$, where R represents the ratio fluorescence intensities at 340 nm and 380 nm excitation, R_{min} is the ratio at 0.0 mol/l Ca^{2+}, R_{max} is the ratio at 1.2 mmol/l Ca^{2+}, S_f/S_b is the ratio of the excitation efficiencies of free FURA to Ca^{2+} bound FURA at 380 nm, and K_D is the dissociation constant of the FURA–Ca^{2+} complex. K_D was taken as 224 nM according to Grynkiewicz *et al.* (1985). For calculation of R_{max} and S_b, digitonin (30 µmol/l) and ionomycin (10 µmol/l) were added to synaptosomes at the end of the measurement to equilibrate $[Ca^{2+}]_i$ with the Ca^{2+} concentration (1.2 mmol/l) of the incubation buffer. Subsequently, EGTA (10 mmol/l) was applied to bind free Ca^{2+}, and R_{min} and S_f were calculated as described above. The half-life ($\tau_{1/2}$) of $[Ca^{2+}]_i$ decrease was calculated in analogy with the decay of radioactive isotopes by fitting the $[Ca^{2+}]_i$ values according to the exponential function: $[Ca^{2+}]_i = [Ca_0{}^{2+}]_i * e^{-k*t}$, where $[Ca_0{}^{2+}]_i$ represents maximal Ca^{2+} concentration, t is time and k is the coefficient of $[Ca^{2+}]_i$ decrease. The half-life was calculated as $\tau_{1/2} = \ln 0.5 / -k$.

Release of glutamate

Release of endogenous glutamate from the cerebrocortical synaptosomes was determined by continuous fluorometry according to the method of Nicholls *et al.* (1987). Briefly, synaptosomal pellets were resuspended in incubation buffer without $CaCl_2$ for 5 min at 37°C to

obtain a suspension of 0.5–0.7 mg protein/ml. Two ml of suspension was transferred to a stirred cuvette located in a Delta Scan fluorimeter (Photo Med GmbH, Wedel), and stock solutions of $NADP^+$ (20 µl), $CaCl_2$ (20 µl), and glutamate dehydrogenase (GDH, E. C. 1. 4. 1. 3, 40 µl) were added to obtain final concentrations of 1 mmol/l $NADP^+$, 1.2 mmol/l $CaCl_2$ and 25 U GDH. Generation of NADPH was monitored at 37°C by excitation at 340 nm and emission at 460 nm employing an 8 nm bandpass for both monochromators. Calibration was performed at the end of each individual measurement by addition of 10 nmol L-glutamate as an internal standard. The amount of released glutamate was calculated according to the equation:

$$\text{Glutamate [nmol/mg protein]} = (F_{\text{Sample}}/F_{\text{Standard}}) * 10 \text{ nmol/mg protein},$$

where F represents fluorescence intensities of sample and standard at 460 nm. For the evaluation of drug-dependent alterations of glutamate-liberation, the rate of glutamate-release, expressed as pmol glutamate/sec * mg protein, was calculated according to Nicholls *et al.* (1987) by linear regression of glutamate-amounts released during the first 200 sec after the addition of veratridine, monensin or KCl. $\tau_{1/2}$ of glutamate-liberation was determined by fitting the glutamate values (GLU) according to the equation: $GLU = GLU_0 * (1 - e^{-k*t})$, were GLU_0 is the maximal amount of released glutamate, t represents time and k is the coefficient of glutamate-release. The half life was calculated as $\tau_{1/2} = \ln 0.5/ - k$.

Drugs and solvents

(±)-Kavain and monensin were prepared as 100-fold concentrated stock solutions employing DMSO and ethanol as solvents, respectively. The other drugs were dissolved in the incubation buffer. The final concentrations of solvents in the assays amounted to 0.1% (v/v) ethanol and 1% (v/v) DMSO. Drugs were added as indicated and measurements of $[Ca^{2+}]_i$ and glutamate were carried out in the presence of the applied drugs. Controls were treated with vehicle instead of drugs. If not otherwise mentioned in the text, final concentrations of veratridine and monensin amounted to 5 and 50 µmol/l, respectively.

Protein determination

Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

Statistics

Results are expressed as means ± SD. Statistical analysis was performed using Student's t-test (unpaired, double sided). Differences of means were considered significant if $P \leqslant 0.05$ (*), $P \leqslant 0.01$ (**) and $P \leqslant 0.001$ (***).

Materials

Bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), digitonin, ionomycin, L-glutamate dehydrogenase (E.C. 1.4.1.3), monensin, β-NADP, *N*-Tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), tetrodotoxin (TTX), and veratridine were purchased from Sigma Chemie GmbH (Deisenhofen, Germany). FURA 2-acetoxymethyl ester (FURA 2-AM) was obtained from MoBiTec GmbH (Göttingen, Germany). (±)-Kavain was purchased from Extrasynthese SA (Lyon, France). The other chemicals were supplied by Merck (Darmstadt, Germany).

RESULTS

Effect of (±)-kavain on veratridine-induced $[Ca^{2+}]_i$ and glutamate-release

Incubation of synaptosomes in the presence of veratridine, which is known to prolong the open state of voltage-dependent Na^+ channels thereby elevating $[Na^+]_i$ (Li and White, 1977; Ohta *et al.*, 1973), enhanced $[Ca^{2+}]_i$, and provoked a long lasting release of endogenous glutamate (Fig. 1). Five µmol/l veratridine induced an increase in $\triangle[Ca^{2+}]_i$ of 694 mmol/l, calculated as the

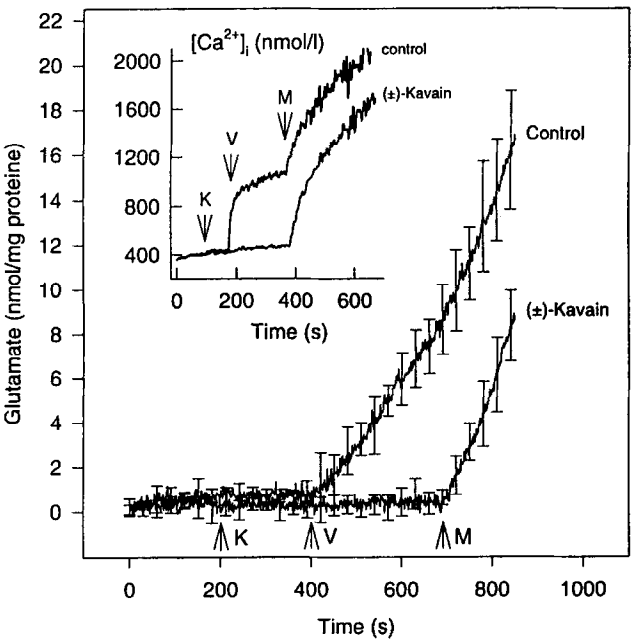

Fig. 1. Synaptosomal glutamate-release induced by veratridine and monensin in the presence [(±)-kavain] and absence (control) of (±)-kavain. (±)-kavain (K, 400 µmol/l), veratridine (V, 5 µmol/l) and monensin (M, 50 µmol/l) were added at the arrows. Traces represent means ± SD ($n = 6$) of individual determinations. *Inset:* action of (±)-kavain on veratridine and monensin evoked $[Ca^{2+}]_i$ (for legend see above). Values are means of 6 measurements (SD omitted for clarity).

Table 1. Effect of (±)-kavain on veratridine- and monensin-induced increase in $[Ca^{2+}]_i$ and glutamate-release

Drug	$\triangle[Ca^{2+}]_i$	Glutamate-release
5 μmol/l Veratridine (control)	694 ± 51 ($n = 4$)	25.6 ± 3.9 ($n = 11$)
500 μmol/l (±)-Kavain +5 μmol/l veratridine	58 ± 16*** ($n = 5$)	1.3 ± 0.7*** ($n = 5$)
50 μmol/l Monensin	1017 ± 137* ($n = 6$)	55.4 ± 13.7*** ($n = 6$)
500 μmol/l (±)-Kavain +50 μmol/l monensin	1010 ± 119* ($n = 6$)	59.3 ± 8.4*** ($n = 6$)

(±)-kavain was added to synaptosomes 100 sec ($\triangle[Ca^{2+}]_i$ determination) or 200 sec (glutamate determination) prior to application of veratridine or monensin. $\triangle[Ca^{2+}]_i$ (nmol/l) represents the difference between the basal and drug-induced increase in $[Ca^{2+}]_i$ measured 20 sec before and 180 sec after addition of veratridine or monensin. The rate of glutamate-release (pmol/sec*mg protein) during the first 200 sec, after the application of veratridine or monensin, was calculated by linear regression. Results are shown as means ± SD. Values different from control, were considered significant (Student's *t*-test, double sided, unpaired) if $P \leq 0.05$ (*) or $P \leq 0.001$ (***).

difference between basal and veratridine-stimulated $[Ca^{2+}]_i$, measured 20 sec before and 180 sec after addition of veratridine. This interval was chosen to calculate $\triangle[Ca^{2+}]_i$ because alterations in $[Ca^{2+}]_i$, determined about 180 sec after addition of veratridine, were relatively small compared to the initial increase in $[Ca^{2+}]_i$ (Fig. 1, inset). The rate of glutamate-release during the first 200 sec after application of veratridine amounted to 25.6 pmol/sec*mg protein (Table 1).

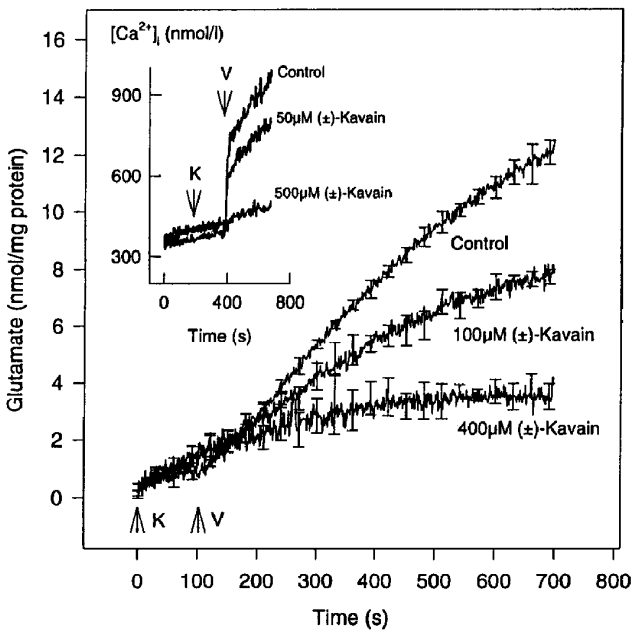

Fig. 2. Dose-dependent inhibition of veratridine-induced glutamate-release by (±)-kavain. Synaptosomes were incubated in the absence (control) and presence of different concentrations of (±)-kavain. (±)-kavain (K) and veratridine (V, 5 μmol/l) were applied as indicated. Values represent means ± SD ($n = 6$). *Inset:* dose-dependent inhibition of veratridine-evoked $[Ca^{2+}]_i$ by (±)-kavain. Drugs were added as indicated by arrow. Traces represent means ($n = 4$, SD omitted for clarity).

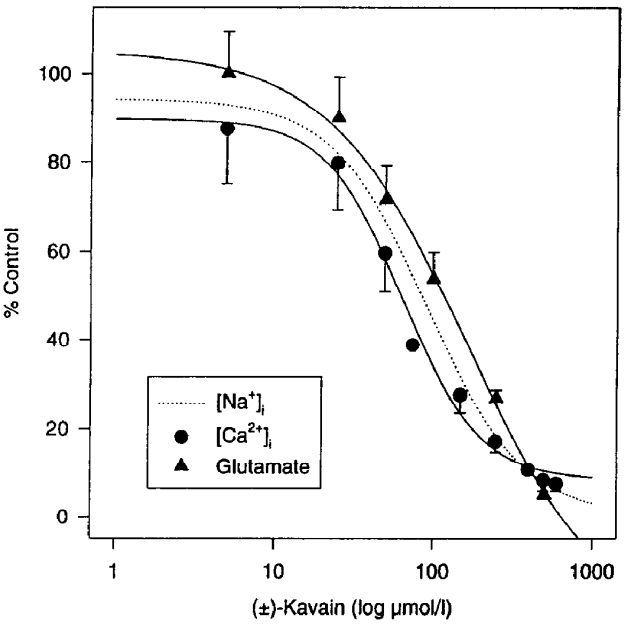

Fig. 3. Dose–response curves of (±)-kavain-dependent inhibition of veratridine evoked $[Ca^{2+}]_i$ ($n = 4$), glutamate-release ($n = 6$), and $[Na^+]_i$ as published by Gleitz *et al.* (1995). For calculation see text. Values represent means ± SD.

As shown in Fig. 1, addition of 500 μmol/l (±)-kavain or vehicle (1% DMSO) prior to veratridine changed neither basal $[Ca^{2+}]_i$ nor basal release of glutamate but blocked both the veratridine-induced increase in $\triangle[Ca^{2+}]_i$ and the rate of released glutamate, which were reduced to 8.4% and 5.1% of control, respectively (Table 1). This inhibition could be overcome by inducing an artificial Na^+ influx with the Na^+ ionophore monensin excluding at least unspecific interactions, like fluorescence quenching by (±)-kavain or its intrasynaptosomally generated derivatives, with the methods of $[Ca^{2+}]_i$ and glutamate determination. As shown in Table 1, *monensin*-induced alterations in $\triangle[Ca^{2+}]_i$ and glutamate-release were unaffected by (±)-kavain at a concentration of 500 μmol/l.

Figure 2 demonstrates that (±)-kavain dose-dependently reduced both the veratridine-stimulated increase in $[Ca^{2+}]_i$ and the release of glutamate. With respect to the kinetics of the increase in $[Ca^{2+}]_i$, time courses were independent of the actual (±)-kavain concentration (Fig. 2, inset). Therefore, to calculate the dose–response curve, $\triangle[Ca^{2+}]_i$ values ($n = 4$) were determined as described above. $\triangle[Ca^{2+}]_i$ values and rates of glutamate release related to the (±)-kavain concentrations were fitted by the logistic dose response function, and IC values were calculated according the fitted curve. As shown in Fig. 3, incubation of synaptosomes in the presence of (±)-kavain reduced $\triangle[Ca^{2+}]_i$ and the release of glutamate at IC_{50} values of 63.2 and 116.4 μmol/l (±)-kavain, respectively. For comparison, the dose–response curve obtained for (±)-kavain-dependent inhibition of veratridine-elevated $[Na^+]_i$ ($IC_{50} = 86.0$ μmol/l), as recently

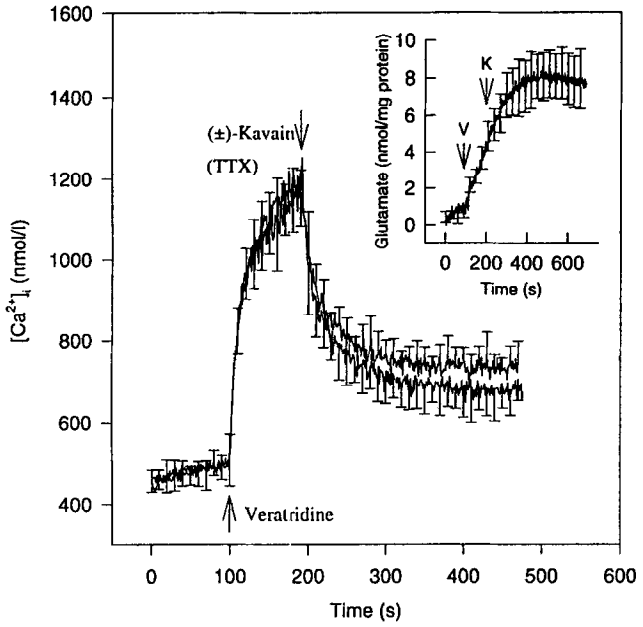

Fig. 4. Action of (±)-kavain (upper trace) and TTX (lower trace) on the recovery of veratridine-elevated $[Ca^{2+}]_i$. Veratridine (5 μmol/l), TTX (10 μmol/l), and (±)-kavain (400 μmol/l) were added as indicated. Traces are means ± SD ($n = 6$). *Inset:* inhibition of veratridine-induced glutamate-release by (±)-kavain. (±)-kavain (K, 400 μmol/l) was applied 100 sec after addition of veratridine (V, 5 μmol/l). Trace is depicted as mean ± SD ($n = 6$).

published by Gleitz *et al.* (1995), is depicted as a dotted line. With regard to the IC_{50} values and slopes, the curves were in the same range. The linear part of the $\triangle[Ca^{2+}]_i$ dose–response curve includes the interval between 27.1 (IC_{25}) and 140.8 (IC_{75}) μmol/l (±)-kavain, whereas the IC_{25} and IC_{75} values related to inhibition of glutamate-release, amounted to 47.4 and 253.0 μmol/l (±)-kavain, respectively.

To estimate the action of (±)-kavain on veratridine-elevated $[Ca^{2+}]_i$ and glutamate-release, which had already been induced (±)-kavain was applied after veratridine. As shown in Fig. 4, post-application of 400 μmol/l (±)-kavain diminished the veratridine-enhanced $[Ca^{2+}]_i$. Immediately after the addition of (±)-kavain $[Ca^{2+}]_i$ decreased with a half-life of 22.9 ± 4.4 sec ($n = 6$). However, complete recovery to the basal $[Ca^{2+}]_i$ level, measured prior to the application of veratridine, was not reached. Similar results were obtained with the Na^+ channel antagonist tetrodotoxin (TTX, Fig. 4). Like (±)-kavain, TTX at a concentration of 10 μmol/l reduced $[Ca^{2+}]_i$ with a half time of 21.3 ± 3.6 sec ($n = 6$). Corresponding to the reduction in $[Ca^{2+}]_i$, evoked release of glutamate was blocked with $\tau_{1/2}$ of 58.4 ± 7.9 sec ($n = 6$), after 400 μmol/l (±)-kavain was applied (Fig. 4, inset). Comparing Fig. 2 (control trace) with the inset of Fig. 4, post glutamate release application of (±)-kavain reduced the maximum amount of released glutamate to about ⅔ of control. Additionally, as shown in Fig. 4, (inset), veratridine-stimulated glutamate-release was blocked completely about 200 sec after (±)-kavain was applied.

Effect of (±)-kavain on the KCl-induced increase in $[Ca^{2+}]_i$ and glutamate-release

To determine the effect of (±)-kavain on voltage dependent Ca^{2+} channels, synaptosomes were depolarized by 40 mmol/l KCl. Immediately after the addition of KCl, $[Ca^{2+}]_i$ peaked to $\triangle[Ca^{2+}]_i = 403$ nmol/l, calculated as the difference between basal and peak values (Fig. 5, inset). The initial Ca^{2+} peak declined with $\tau_{1/2}$ of 13.9 sec to a steady-state level of $\triangle[Ca^{2+}]_i = 251$ nmol/l, measured 250 sec after the addition of KCl (Table 2). A reduction of the KCl-induced increase in $[Ca^{2+}]_i$ could only be observed at high (±)-kavain concentrations (Fig. 5, inset). With respect to $\triangle[Ca^{2+}]_i$ of the peak and the steady-state level, 400 μmol/l (±)-kavain diminished both to the same degree to 78 and 75% of control, respectively (Table 2), demonstrating that the Ca^{2+} peak itself was unaffected by (±)-kavain (Fig. 5). Considering glutamate liberation, KCl-depolarization of synaptosomes provoked the release of glutamate at a rate of 35.2 pmol glutamate/sec * mg protein, which was diminished to 91% if the synaptosomes were incubated

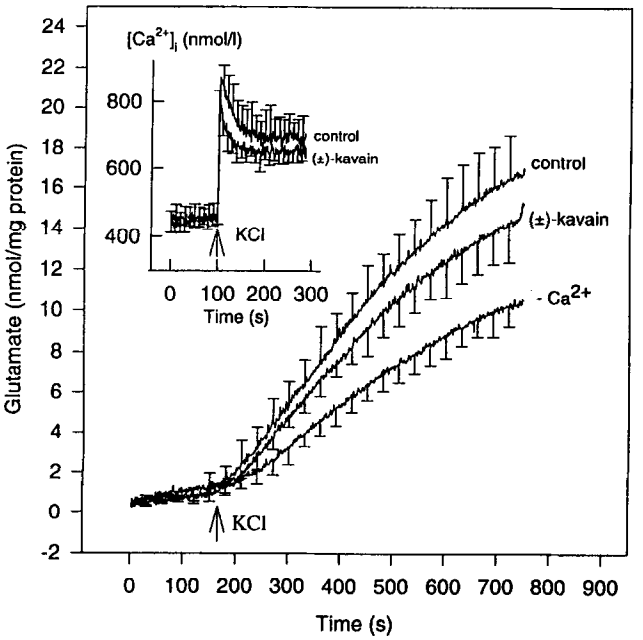

Fig. 5. Action of (±)-kavain on the KCl-depolarization dependent release of glutamate and $[Ca^{2+}]_i$. *Control* and (±)-*kavain* designed traces represent glutamate-release of synaptosomes incubated in Ca^{2+} containing incubation buffer. (±)-kavain (400 μmol/l) was applied at the onset of the measurement. — *Ca^{2+}* shows glutamate-release of synaptosomes resuspended in Ca^{2+} free incubation buffer supplemented with 0.5 μmol/l EGTA. KCl at a final concentration of 40 mmol/l was added as indicated. Traces are means ± SD ($n = 6$). *Inset:* reduction of KCl-induced increase in $[Ca^{2+}]_i$ by 400 μmol/l (±)-kavain that was applied at the onset of measurement. Values represent means ± SD ($n = 6$).

Table 2. Action of (±)-*kavain* on the KCl-depolarization induced increase in $[Ca^{2+}]_i$ and glutamate-release ($n = 6$)

Treatment	$\triangle[Ca^{2+}]_i$		$\tau_{1/2}$	Glutamate-release
	Peak	Steady-state		
Control	403 ± 38	251 ± 28	13.9 ± 3.0	35.2 ± 4.32
(±)-Kavain	315 ± 18*	189 ± 32	12.7 ± 2.3	32.2 ± 3.5
$-Ca^{2+}$	n.d.	n.d.	n.d.	22.5 ± 3.5***

400 μmol/l (±)-kavain was applied to synaptosomes 100 sec prior to the addition of KCl (40 mmol/l). $-Ca^{2+}$ represents synaptosomes incubated in Ca^{2+}-free incubation buffer supplemented with 0.5 μmol/l EGTA. $\triangle[Ca^{2+}]_i$ (nmol/l) values were calculated as the difference between the basal and peak concentration of $[Ca^{2+}]_i$ (peak), and as the difference between basal and steady state level of $[Ca^{2+}]_i$ (steady state). $\tau_{1/2}$ (sec) is the half-life of $[Ca^{2+}]_i$ declining from a peak to a steady-state level of $[Ca^{2+}]_i$. Glutamate-release (pmol/sec*mg protein) was determined as described in Table 1. Values not determined are designated as n.d. Results are represented as means ± SD. Means were considered different from control (Student's *t*-test, double sided, unpaired) if $P \leqslant 0.05$ (*) or $P \leqslant 0.001$ (***).

in the presence of 400 μmol/l (±)-kavain (Fig. 5, Table 2). The amount of released glutamate was reported (Nicholls *et al.*, 1987) to depend less than 50% on the external Ca^{2+} concentration ($[Ca^{2+}]_o$). In order to estimate the action of (±)-kavain on this $[Ca^{2+}]_o$ dependent part of glutamate-exocytosis, synaptosomes were resuspended in a Ca^{2+}-free incubation buffer supplemented with 0.5 μ mol/l EGTA prior to KCl-depolarization. As demonstrated in Fig. 5 and Table 2, omission of external Ca^{2+} reduced the rate of glutamate-exocytosis to 64% of control. Subtraction of this $[Ca^{2+}]_o$ independent rate from the control and (±)-kavain group revealed rates of $[Ca^{2+}]_o$ dependent glutamate-release of 12.7 and 9.7 pmol/s*mg protein, respectively. According to this calculation, 400 μmol/l (±)-kavain reduced the $[Ca^{2+}]_o$ dependent glutamate-exocytosis to 76% of control, a value closely related to the reduction (75% of control) of the steady state level of $\triangle[Ca^{2+}]_i$ (see above).

DISCUSSION

In the present investigation, the action of (±)-kavain on veratridine, monensin and KCl-depolarization induced alterations of $[Ca^{2+}]_i$ and glutamate-liberation were studied. Basal $[Ca^{2+}]_i$ and spontaneous release of glutamate were unaltered by (±)-kavain at concentrations of up to 500 μmol/l, suggesting that Ca^{2+} homeostasis of non stimulated synaptosomes was unaffected by (±)-kavain, and rules out the possibility that (±)-kavain directly evoking glutamate-release is responsible for its psychotropic action (Lebot *et al.*, 1992; Singh, 1992).

Referring to the action of (±)-kavain on the veratridine-induced increase in $[Ca^{2+}]_i$, veratridine is known to prolong the open state of Na^+ channels (Ohta *et al.*, 1973; Garber and Miller, 1987), inducing an increase in $[Na^+]_i$ (Li and White, 1977) accompanied by a decrease in $[K^+]_i$ (Blaustein, 1975), a reduction of membrane potential (Heinonen *et al.*, 1985; Dagani *et al.*, 1990; Deri and Adam-Vizi, 1992) and an elevation of $[Ca^{2+}]_i$ triggering the release of neurotransmitters. The $[Na^+]_i$ dependent increase in $[Ca^{2+}]_i$ has been attributed to several pathways, including Ca^{2+} entry through veratridine-modified Na^+ channels (Adam-Vizi and Ligeti, 1986), Ca^{2+} influx via voltage-dependent Ca^{2+} channels (Carafoli, 1987; Okada *et al.*, 1989; Adam-Vizi and Ligeti, 1986), a displacement of Ca^{2+} from intracellular binding sites (Carafoli, 1987), and a reversal of the Na^+/Ca^{2+} exchanger present in both plasma membrane (Allen, 1990; Canzoniero *et al.*, 1992) and mitochondria (Heinonen *et al.*, 1984; Gunter and Pfeiffer, 1990). In the present study, the presence of (±)-kavain blocked dose-dependently veratridine-evoked $[Ca^{2+}]_i$ and the release of glutamate. This inhibition could be overcome by inducing an artificial Na^+ influx using the Na^+ ionophore monensin, which is 350,000 times more effective in transporting Na^+ than Ca^{2+} (Pressmann, 1976; Antonenko and Yaguzhinsky, 1988; Ambroz *et al.*, 1990), thus excluding a substantial Ca^{2+} entry by monensin itself. (±)-Kavain, at a concentration of 500 μmol/l, sufficient to block the veratridine-induced increase in $[Ca^{2+}]_i$ and glutamate-release almost completely, affected neither the *monensin*-induced increase in $[Ca^{2+}]_i$ nor the diminished release of glutamate. These results may indicate a specific inhibition of Na^+ channels to be a primary target of (±)-kavain, thereby preventing Na^+ uptake and an $[Na^+]_i$ dependent increase in $[Ca^{2+}]_i$. As shown by monensin, $[Na^+]_i$ itself mediates alterations of $[Ca^{2+}]_i$ by the pathways mentioned above, and the cascade of glutamate-release downstream of elevated $[Ca^{2+}]_i$, e.g. mobilization of synaptic vesicles from their association with the cytonet and processes of exocytosis (Zimmermann, 1993), seems to be unaffected by (±)-kavain.

This suggestion is supported by the similarity of the dose–response curves. With regard to IC_{50} values, inhibition of Ca^{2+} entry ($IC_{50} = 63.2$ μmol/l) and glutamate-release ($IC_{50} = 116.4$ μmol/l) were in the same range as the published IC_{50} (86.0 μmol/l) necessary to reduce veratridine-induced Na^+ uptake (Gleitz *et al.*, 1995). Compared with the IC_{50} for inhibition of Na^+ influx, the somewhat smaller IC_{50} obtained for inhibition

of Ca^{2+} uptake, might reflect a slight but additional inhibition of Ca^{2+} entry via voltage-dependent Ca^{2+} channels (see below). However, the somewhat higher (±)-kavain concentration, necessary to block 50% of glutamate-release might depend on the additive action of $[Na^+]_i$ *and* $[Ca^{2+}]_i$ on glutamate liberation, since both cations were reported to stimulate the release of glutamate (Nicholls *et al.*, 1987).

Further evidence that Ca^{2+} homeostasis was unaffected by (±)-kavain is provided by the Na^+ channel antagonist TTX (Li and White, 1977). Like (±)-kavain, TTX, if added subsequently to veratridine, caused a decrease of veratridine-elevated $[Ca^{2+}]_i$ which was similar to that of (±)-kavain with regard to time course, $\tau_{1/2}$ of $[Ca^{2+}]_i$ decrease, and the final steady state level of $[Ca^{2+}]_i$. These results may indicate that the pathways responsible for normalization of elevated $[Ca^{2+}]_i$, e.g. Ca^{2+}-ATPase, Na^+/Ca^{2+} exchange and mitochondrial Ca^{2+} cycle, are unaffected by (±)-kavain. However, since $[Ca^{2+}]_i$ reflects the apparent output of several pathways acting in concert, each of them with an unknown contribution to $[Ca^{2+}]_i$ normalization, a slight interference of (±)-kavain with one single system involved in Ca^{2+} homeostasis cannot be excluded.

Concomitantly to normalization of $[Ca^{2+}]_i$, veratridine-evoked release of glutamate was blocked after (±)-kavain was added. The decline in glutamate-release seems to be only related to normalization of veratridine-elevated $[Na^+]_i$ *and* $[Ca^{2+}]_i$ by (±)-kavain, since $\tau_{1/2}$ of the reduction of glutamate-release (63 sec) represents an intermediate value between $\tau_{1/2}$ of $[Ca^{2+}]_i$ (22 sec) and $[Na^+]_i$ decrease (83 sec) as published by Gleitz *et al.* (1995). The rapid decrease in veratridine-elevated $[Ca^{2+}]_i$ and the inhibition of glutamate-release after the addition of (±)-kavain is in line with the fast action of kava pyrones, proceeding within 1–5 min as demonstrated by their spasmolytic activity (Meyer, 1965), the inhibition of the tonic stretch reflex (Meyer, 1979) and the anticonvulsive action (Kretzschmar and Meyer, 1969).

With respect to the results obtained with KCl-depolarization, it is known that KCl-induced Ca^{2+} uptake in synaptosomes proceeds through voltage-dependent Ca^{2+} channels (Adam-Vizi and Ligeti, 1986; Okada *et al.* 1989). The suggested presynaptic Ca^{2+} channel of mammals, proposed to be coupled to the release of glutamate (McMahon and Nicholls, 1991; Verhage *et al.*, 1991), neither undergoes voltage inactivation nor does it belong to the L, T and N type categories (Turner and Goldin, 1989; McMahon and Nicholls, 1991). To date, only the toxin Aga-GI, prepared from the venom of Agelenopsis aperta, has been shown to block this Ca^{2+} channel efficiently resulting in a 50% reduction of the KCl-induced sustained plateau of $[Ca^{2+}]_i$, finally leading to complete inhibition of glutamate-exocytosis (Pocock and Nicholls, 1992). In the present study, like Aga-GI, (±)-kavain reduced the steady state level of $[Ca^{2+}]_i$ (75% of control), whereas the initial Ca^{2+} peak, attributed to inactivating Ca^{2+} channels seems to be unaffected by (±)-kavain. Concomitantly to the reduction of the $[Ca^{2+}]_i$ plateau, the amount of glutamate release depending on external Ca^{2+} was reduced to the same degree (76% of control). These results might suggest that (±)-kavain blocks the non inactivating Ca^{2+} channel located on mammalian presynaptic nerve endings.

In conclusion, (±)-kavain blocked dose-dependently the veratridine-induced increase in $[Ca^{2+}]_i$ and glutamate-release, indicating voltage-dependent Na^+ channels to be the primary target, and thereby preventing a $[Na^+]_i$ mediated increase in $[Ca^{2+}]_i$ and the release of glutamate. Pathways involved in Ca^{2+} homeostasis seem to be unaffected by (±)-kavain because it did not affect basal $[Ca^{2+}]_i$, basal release of glutamate, normalization of elevated $[Ca^{2+}]_i$ or release of glutamate subsequent to enhanced $[Ca^{2+}]_i$. At (±)-kavain concentrations (150 μmol/l) around the IC_{50} value (86 μmol/l) necessary for the inhibition of voltage-dependent Na^+ channels, no alterations in the basal and KCl-induced increase in $[Ca^{2+}]_i$ was observed. However, at concentrations sufficient to block Na^+ channels almost completely, (±)-kavain inhibits, like Aga-GI toxin, KCl-depolarization elevated $[Ca^{2+}]_i$ and the release of glutamate moderately.

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