ELSEVIER

European Journal of Pharmacology 315 (1996) 89–97

ejp

Anticonvulsive action of (±)-kavain estimated from its properties on stimulated synaptosomes and Na^+ channel receptor sites

Johannes Gleitz *, Jutta Friese, Anne Beile, Angela Ameri, Thies Peters

University Clinics Ulm, Institute of Naturheilkunde, Helmholtzstraße 20, 89081 Ulm, Germany

Received 25 March 1996; revised 5 July 1996; accepted 9 July 1996

Abstract

Kava pyrones are constituents of the intoxicating pepper (*Piper methysticum* Forst.), which has been shown to be anticonvulsive. The question of how the excitability of neurons is affected was investigated by determining the interaction of (±)-kavain with epitopes (site 1, site 2) of voltage-dependent Na^+ channels and the action of (±)-kavain on 4-aminopyridine-stimulated synaptosomes as model of repetitive firing neurons. [^{3}H]Saxitoxin and [^{3}H]batrachotoxin were used for radioligand-binding assays performed with synaptosomal membranes. Glutamate released from 4-aminopyridine-stimulated cerebrocortical synaptosomes and the cytosolic concentrations of Na^+ and Ca^{2+} ($[Na^+]_i$, $[Ca^{2+}]_i$) were detected fluorometrically by using an enzyme-linked assay, sodium-binding benzofuranisophthalate (SBFI) and Fura-2, respectively. (±)-Kavain failed to compete with [^{3}H]saxitoxin up to 400 μmol/l but dose-dependently suppressed binding of [^{3}H]batrachotoxin with an IC_{50} value of 88 μmol/l ($K_i = 72$ μmol/l) although displacement of [^{3}H]batrachotoxin was restricted to 33% of control at 400 μmol/l (±)-kavain. In stimulated synaptosomes, 5 mmol/l 4-aminopyridine provoked an increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ by 9 mmol/l Na^+ and 235 nmol/l Ca^{2+}. Comparable to the reduction in [^{3}H]batrachotoxin binding, 400 μmol/l (±)-kavain suppressed the increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ to 38 and 29% of control, respectively. Consistent with the increase in $[Na^+]_i$ and $[Ca^{2+}]_i$, 5 mmol/l 4-aminopyridine provoked glutamate release (rate: 38 pmol/s*mg protein) which was dose-dependently diminished to 60% of control by 400 μmol/l (±)-kavain. KCl depolarization (40 mmol/l) provoked an increase in $[Ca^{2+}]_i$ and glutamate release almost identical to the responses elicited by 4-aminopyridine but 400 μmol/l (±)-kavain suppressed only the rate of glutamate release by 9% of control. The data suggest an interaction of (±)-kavain with voltage-dependent Na^+ and Ca^{2+} channels, thereby suppressing the 4-aminopyridine-induced increase in $[Na^+]_i$, $[Ca^{2+}]_i$ and the release of endogenous glutamate.

Keywords: Epilepsy; Kava pyrone; *Piper methysticum*; Na^+ channel; Synaptosome

1. Introduction

Kava pyrones are 4-methoxy-α-pyrone derivatives isolated from the root-stock of the intoxicating pepper *Piper methysticum* Forst. (Duve, 1981; Smith, 1983), a remedy indigenous to the islands of the South Pacific. Pharmacological studies revealed the main kava pyrones, (+)-kavain, (+)-dihydrokavain, (+)-methysticin, (+)-dihydromethysticin and yangonin, to be responsible for the analgesic (Jamieson and Duffield, 1990), sedative (Meyer, 1979; Capasso and Calignano, 1988) and spasmolytic action (Meyer, 1965) known from traditional medicine (Lebot et al., 1992; Singh, 1992). Although treatment of epilepsy by preparations of the intoxicating pepper is not reported in folk medicine (Lebot et al., 1992; Singh, 1992), the anticonvulsive action of the main kava pyrones against maximal electroshock-, strychnine- and pentylenetetrazole-induced convulsions have been demonstrated in mice (Kretzschmar and Meyer, 1969; Kretzschmar et al., 1969, 1970; Meyer, 1979). Recently, Schmitz et al. (1995) confirmed (+)-methysticin to have anticonvulsive properties, using hippocampal and entorhinal cortex slices as models of seizure-like events. Since (+)-methysticine, in the range of 10–100 μmol/l, suppressed epileptiform activity independent of the stimulus (low Ca^{2+}, low Mg^{2+} and high K^+ perfusion medium), the authors suggested a direct membrane action of (+)-methysticin on the excitability of neurons.

The aim of the current paper was to elucidate the mechanism of action of the synthetic kava pyrone, (±)-kavain, on the excitability of neurons, especially its properties on voltage-dependent Na^+ channels because they are a common target of diverse antiepileptic drugs. An interaction of (±)-kavain with receptor site 1 of Na^+ channels,

* Corresponding author. Tel.: (49-731) 502-4281; Fax: (49-731) 502-4299; e-mail: johannes.gleitz@medizin.uni-ulm.de

PII S0014-2999(96)00550-X

which is the epitope of antagonists, like saxitoxin and tetrodotoxin, and its influence on site 2, the binding site of agonists, including batrachotoxin, aconitine and veratridine (Catterall et al., 1979, 1981), were investigated by radioligand-binding assays. To mimic repetitive firing neurons, as observed in vivo during epileptic seizures, 4-aminopyridine-stimulated cerebrocortical synaptosomes were used as a model (Buckle and Haas, 1982; Tibbs et al., 1989; McMahon and Nicholls, 1991) to elucidate the anticonvulsive properties of (±)-kavain. 4-Aminopyridine is known to block K^+ channels (Thesleff, 1980; Cook, 1988; Castle et al., 1989), reducing the membrane potential sufficiently to activate voltage-dependent Na^+ channels (Tibbs et al., 1989), an effect correlated with an increase in cytosolic free Na^+, Ca^{2+} ($[Na^+]_i$, $[Ca^{2+}]_i$) and the release of endogenous glutamate. The anticonvulsive action of (±)-kavain was estimated by its interaction with receptor sites of Na^+ channels and the ability to suppress the 4-aminopyridine-induced increase in $[Na^+]_i$, $[Ca^{2+}]_i$ and release of glutamate.

2. Materials and methods

2.1. Preparation of synaptosomes

The experiments were conducted with 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 bovine serum albumin, 5 mmol/l *N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Tes), pH 7.4) with a Braun potter (Melsungen, Germany). The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant was recentrifuged at $28\,000 \times g$ for 20 min. The pellet was resuspended in homogenization buffer and for binding assays aliquots of the crude synaptosomal suspension (14 mg protein/ml) were cryopreserved according to Gleitz et al. (1993b). For measurements of $[Na^+]_i$, $[Ca^{2+}]_i$ and glutamate release, the crude synaptosomal suspension was purified by a discontinuous Ficoll gradient (Gleitz et al., 1993a). Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as standard.

2.2. [^{3}H]Saxitoxin- and [^{3}H]batrachotoxin-binding assays

[^{3}H]Batrachotoxin binding to synaptosomes was performed according to Postma and Catterall (1984) by a rapid filtration assay. Synaptosomes were suspended in Hepes-Tris buffer (5.4 mmol/l KCl, 0.8 mmol/l $MgSO_4$, 10 mmol/l glucose, 10 mmol/l pyruvate, 130 mmol/l choline chloride and 50 mmol/l Hepes-Tris, pH 7.4). The binding reaction was initiated by addition of 100 μl synaptosomal suspension to 150 μl Hepes-Tris buffer supplemented with tetrodotoxin (1 μmol/l), scorpion venom (5 μg/ml) and [^{3}H]batrachotoxin (1–300 nmol/l) to obtain a final concentration of 1.4–1.5 mg/ml protein. After an incubation at 37°C for 120 min, the reaction was stopped by addition of 2 ml ice-cold wash medium (1.8 mmol/l $CaCl_2$, 0.8 mmol/l $MgSO_4$, 163 mmol/l choline chloride, 5 mmol/l Hepes-Tris, pH 7.4) and synaptosomes were immediately collected on a glass-fiber filter (Whatman GF/C), washed with 2 ml wash medium for 3 times and counted (Beckman Instruments, LS6000 TA, USA). In competition experiments, synaptosomes were incubated with different concentrations of (±)-kavain and 10 nmol/l [^{3}H]batrachotoxin under the same conditions as described above. Non-specific binding was determined in the presence of 30 μmol/l veratridine.

Binding of [^{3}H]saxitoxin was determined analogously to [^{3}H]batrachotoxin binding as described above, using a final protein concentration of 0.5 mg/ml. The reaction was started by the addition of 100 μl of synaptosomal suspension to 150 μl Hepes-Tris buffer containing 1–40 nmol/l [^{3}H]saxitoxin. After an incubation at 37°C for 30 min, termination of reaction, washing procedure and measurement of radioactivity were performed as described for [^{3}H]batrachotoxin binding. For competition experiments, synaptosomes were incubated in the presence of 400 μmol/l (±)-kavain and 1.8 nmol/l [^{3}H]saxitoxin. Non-specific binding was determined in the presence of 1 μmol/l tetrodotoxin.

2.3. $[Na^+]_i$ and $[Ca^{2+}]_i$ measurement

$[Na^+]_i$ and $[Ca^{2+}]_i$ were determined by the ratiofluorescence method with sodium-binding benzofuranisophthalate (SBFI; Minta and Tsien, 1989) and Fura-2 (Grynkiewicz et al., 1985). For dye loading of synaptosomes suspended in 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 at 37°C), 495 μl suspension (14 mg/ml protein) was incubated at 25°C for 45 min with either 5 μl of 1 mmol/l of the acetoxymethyl ester (AM) of Fura-2 or 4.2 μl of 2 mmol/l SBFI-AM and 1.4 μl 20% (w/v) pluronic F127. Afterwards, the suspension was diluted with 110 ml incubation buffer, incubated at 25°C for additional 15 min and washed 3 times with incubation buffer by centrifugation at $12\,000 \times g$ for 10 min. The final synaptosomal pellets were stored on ice until measured.

Each pellet of dye-loaded synaptosomes was resuspended in 2 ml incubation buffer (37°C) to obtain a protein concentration of 0.5–0.7 mg/ml. Fluorescence measurements were made with a spectrofluorometer (Delta Scan, PhotoMed, Wedel, Germany) in a temperature-controlled stirred cuvette at 37°C. SBFI fluorescence was measured alternately at excitation wavelengths of 340 nm and 385 nm with emission at 500 nm and a 12-nm bandpass slit.

$[Na^+]_i$ was calculated as $[Na^+]_i = (K_d * S_f/S_b) * (R - R_{min})/(R_{max} - R)$ according to Minta and Tsien (1989). R_{min}, R_{max} and $K_d * S_f/S_b$ were calculated as described elsewhere (Sage et al., 1991) using 3 calibration buffers of the same composition as the incubation buffer, except for the Na^+ concentration, which amounted to 5, 50 and 130 mmol/l Na^+. Choline chloride was used as substitute for NaCl to obtain a final concentration of 130 mmol/l. Equilibration of the intrasynaptosomal Na^+ concentration with that of the calibration buffers was carried out by addition of the Na^+ ionophore monensin at a final concentration of 50 μmol/l.

$[Ca^{2+}]_i$ was determined by the method of Grynkiewicz et al. (1985) according to $[Ca^{2+}]_i = (K_d * S_f/S_b) * (R - R_{min})/(R_{max} - R)$, using a K_d of 224 nmol/l. In brief, Fura-2 fluorescence was measured at 508 nm at excitation wavelengths of 340 nm and 380 nm (8 nm bandpass slits). Calibration of each individual probe was carried out by addition of digitonin (30 μmol/l) and ionomycin (10 μmol/l) to induce a maximal Ca^{2+} influx and subsequent addition of 10 mmol/l EGTA to bind free Ca^{2+}.

2.4. Release of glutamate

Release of endogenous glutamate from synaptosomes was determined by continuous fluorometry according to 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 0.5–1.0 mg protein/ml. 2 ml of suspension was transferred to a stirred cuvette in a spectrofluorometer (Delta Scan, Photo Med, Wedel, Germany). Stock solutions of $NADP^+$, $CaCl_2$ and glutamate dehydrogenase (EC 1.4.1.3) were added to obtain final concentrations of 1 mmol/l $NADP^+$, 1.2 mmol/l $CaCl_2$ and 25 units of glutamate dehydrogenase. Generation of NADPH was monitored at 37°C by excitation at 340 nm and emission at 460 nm. Calibration was performed at the end of each individual measurement by addition of 10 nmol L-glutamate as internal standard. The amount of released L-glutamate was calculated according to the equation: glutamate [nmol/l] = $(F_{sample}/F_{standard}) * 10$ nmol/mg protein, where F represents fluorescence intensities of sample and standard at 460 nm.

2.5. Statistics and calculations

Results are expressed as means ± S.D. Statistical analysis was performed using Student's test (unpaired, two sided). Differences of means were considered significant if $P < 0.05$ (a), $P < 0.01$ (b) or $P < 0.001$ (c).

The calculation of non-specific binding (nsb), equilibrium dissociation constants (K_d) and maximum number of binding sites (B_{max}) was performed by fitting the binding curves of radio ligands according to the one binding site model (Mauz and Pelzer, 1990), using the equation $y = B_{max} * x/(K_d + x) + nsb * x$, where x represents the concentration of radioligand and y is total binding of radioligand which was experimentally determined.

4-Aminopyridine-induced increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ are expressed as $\Delta[Na^+]_i$ and $\Delta[Ca^{2+}]_i$, both calculated as the difference between basal and 4-aminopyridine-stimulated cation concentrations, measured 15 s before and 85 s after 4-aminopyridine was applied to synaptosomes.

The half-life ($\tau_{1/2}$) of the $[Ca^{2+}]_i$ decrease was calculated by fitting the $[Ca^{2+}]_i$ values according to the exponential function: $[Ca^{2+}]_i = [Ca_0^{2+}]_i * e^{-kt}$, 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 $\tau_{1/2} = \ln(0.5)/(-k)$.

The rate of glutamate release was calculated according to Nicholls et al. (1987) by linear regression analysis of glutamate released during the first 100 s after 4-aminopyridine was applied. The total amount of released glutamate (Glu_{max}) was calculated by non-linear fitting of the glutamate release kinetic according to $Glu = Glu_{max} * (1 - e^{-kt})$, where Glu is the amount of released glutamate, t is time and k is the coefficient of glutamate release. Glu_{max} was calculated by extrapolation of t to infinity.

2.6. Drugs and solvents

(±)-Kavain was prepared as 200-fold concentrated stock solution in DMSO. The final concentration of DMSO in the assays amounted to 0.5% (v/v) DMSO.

2.7. Materials

[^{3}H]Batrachotoxinin A 20-α-benzoate (specific activity: 1258 GBq/mmol) was purchased from Du Pont NEN Research Products (Bad Homburg, Germany). [^{3}H]Saxitoxin (specific activity: 814 GBq/mmol) was obtained from Amersham Life Science (Braunschweig, Germany). Bovine serum albumin, choline chloride, digitonin, EDTA, EGTA, ionomycin, Hepes, L-glutamate dehydrogenase (EC 1.4.1.3), monensin, ß-NADP, pyruvate (Na^+ salt), scorpion venom (*Leiurus quinquestriatus hebraeus*), tetrodotoxin, Tes and veratridine were purchased from Sigma (Deisenhofen, Germany). SBFI-AM and Fura-2-AM were obtained from MoBiTec (Göttingen, Germany). (±)-Kavain was purchased from Extrasynthese (Lyon, France). Tris was purchased from Fluka (Buchs, Switzerland). The other chemicals were supplied by Merck (Darmstadt, Germany).

3. Results

3.1. [^{3}H]Saxitoxin and [^{3}H]batrachotoxin binding

Fig. 1 demonstrates the saturable binding of [^{3}H]batrachotoxin and [^{3}H]saxitoxin to synaptosomes in

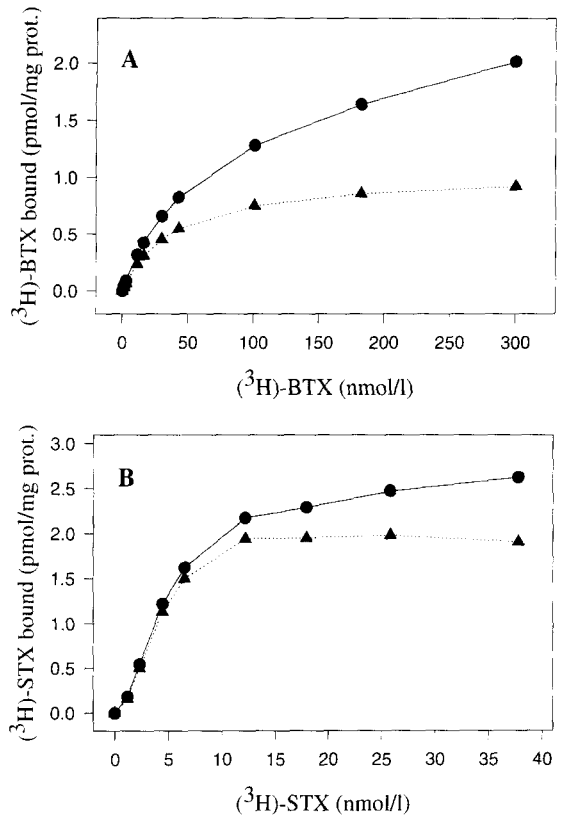

Fig. 1. Binding of [^{3}H]batrachotoxin and [^{3}H]saxitoxin to synaptosomes. The concentration-dependent binding of [^{3}H]batrachotoxin (A) and [^{3}H]saxitoxin (B) was determined by incubation of synaptosomes with different concentrations of radioligands. Total (●) and specific binding (▲) of [^{3}H]batrachotoxin ($n = 4$) and [^{3}H]saxitoxin ($n = 6$) are depicted as means. The K_d values determined for [^{3}H]batrachotoxin and [^{3}H]saxitoxin binding amounted to 52.6 and 8.5 nmol/l, respectively. Maximal binding of radioligands (B_{max}) was calculated to be 1.6 pmol [^{3}H]batrachotoxin/mg protein and 2.8 pmol [^{3}H]saxitoxin/mg protein.

the range of 1–300 and 1–40 nmol/l, respectively. According to the equation of the one binding site model (Mauz and Pelzer, 1990), the K_d and B_{max} of [^{3}H]batrachotoxin were calculated to be 52.6 nmol/l and 1.6 pmol/mg protein, respectively. The K_d of [^{3}H]saxitoxin binding amounted to 8.5 nmol/l and B_{max} was calculated to be 2.8 pmol/mg protein. As shown in Fig. 2, (±)-kavain dose-dependently displaced [^{3}H]batrachotoxin from site 2 of Na^+ channels with an IC_{50} value of 88 μmol/l ($K_i = 72$ μmol/l), although displacement was limited to 33% of control at 400 μmol/l (±)-kavain. Considering [^{3}H]saxitoxin binding, (±)-kavain failed to affect the binding of [^{3}H]saxitoxin to synaptosomes up to a concentration of 400 μmol/l as shown in the inset of Fig. 2.

3.2. Action of (±)-kavain on $[Na^+]_i$ and $[Ca^{2+}]_i$

As shown in the insets of Fig. 3, the addition of 4-aminopyridine to synaptosomes enhanced basal $[Na^+]_i$ and $[Ca^{2+}]_i$ within a few seconds. The 4-aminopyridine-induced increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ was calculated as described in Section 2 as the difference between basal and 4-aminopyridine-evoked cation concentrations, expressed as $\Delta[Na^+]_i$ and $\Delta[Ca^{2+}]_i$. Fig. 3 demonstrates that 4-aminopyridine dose-dependently enhanced $\Delta[Na^+]_i$ and $\Delta[Ca^{2+}]_i$, which reached their maximum values between 1 and 5 mmol/l 4-aminopyridine, despite the difficulty to detect small differences in $[Na^+]_i$ by the fluorescence method. In the following assays, 5 mmol/l 4-aminopyridine was applied to induce a maximal increase in $[Na^+]_i$ and $[Ca^{2+}]_i$.

5 mmol/l 4-aminopyridine provoked an increase in basal $[Na^+]_i$ from 16 ± 1 to 25 ± 1 mmol/l Na^+ ($n = 18$). To determine the effect of (±)-kavain on $\Delta[Na^+]_i$ and $\Delta[Ca^{2+}]_i$ at a concentration which caused maximal displacement of [^{3}H]batrachotoxin (Fig. 2), 400 μmol/l (±)-kavain was applied to synaptosomes 100 s before 4-aminopyridine. As shown in Fig. 4A, (±)-kavain itself failed to affect basal $[Na^+]_i$ but suppressed the 4-aminopyridine-induced increase in $[Na^+]_i$ to 38% of control, a value very similar to that seen in [^{3}H]batrachotoxin competition assay (Table 1). (±)-Kavain did not affect the $[Na^+]_i$ increase induced by 50 μmol/l of the Na^+

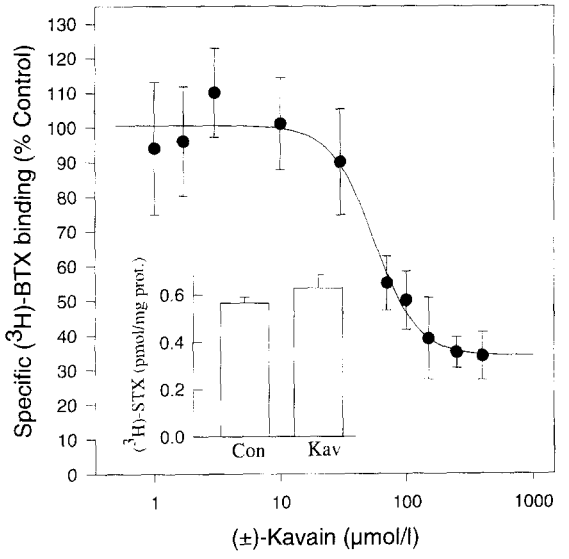

Fig. 2. The action of (±)-kavain on [^{3}H]saxitoxin and [^{3}H]batrachotoxin binding. Binding of [^{3}H]batrachotoxin was carried out by incubation of synaptosomes with 10 nmol/l [^{3}H]batrachotoxin and (±)-kavain at 37°C for 120 min. To obtain the dose-response curve, specific binding of [^{3}H]batrachotoxin in the presence of different (±)-kavain concentrations was calculated as the difference between total and non-specific [^{3}H]batrachotoxin binding, the latter being determined by incubating synaptosomes with [^{3}H]batrachotoxin and 30 μmol/l veratridine. The data represent mean ± S.D. ($n = 5$). For [^{3}H]saxitoxin-binding studies, synaptosomes were incubated with 1.8 nmol/l [^{3}H]saxitoxin and 400 μmol/l (±)-kavain at 37°C for 30 min. Non-specific binding of [^{3}H]saxitoxin was determined in the presence of 1 μmol/l tetrodotoxin. As shown in the inset, (±)-kavain (Kav) at a concentration of 400 μmol/l failed to affect specific [^{3}H]saxitoxin binding compared with control (Con). Results of [^{3}H]saxitoxin binding are depicted as mean ± S.D. ($n = 4$).

ionophore monensin, ruling out that (±)-kavain interferes with the method of fluorometric $[Na^+]_i$-determination (Fig. 4A).

As depicted in Fig. 4B, basal $[Ca^{2+}]_i$ increased after the addition of 4-aminopyridine and reached its maxium with a $\tau_{1/2}$ of 5 ± 2 s ($n = 5$). The steady-state level of $\Delta[Ca^{2+}]_i$, calculated as described above, amounted to 236 ± 8 nmol/l Ca^{2+} ($n = 15$). Comparable to the data obtained for $[Na^+]_i$ determination, (±)-kavain at a concentration of 400 μmol/l did not alter basal $[Ca^{2+}]_i$ but prevented the initial increase in $[Ca^{2+}]_i$, although it failed to block the increase in $[Ca^{2+}]_i$ completely (Fig. 4B). Despite the high concentration of (±)-kavain, a slight continuous increase in $[Ca^{2+}]_i$, which is difficult to detect in Fig. 4B, occurred upon the addition of 4-aminopyridine. However, as shown in Table 1, the 4-aminopyridine-induced $\Delta[Ca^{2+}]_i$ was only suppressed to 29% of control, a value which is in the same range as already observed for the (±)-kavain-dependent reduction of $\Delta[Na^+]_i$ and [^{3}H]batrachotoxin binding. Similar to the monensin-induced increase in $[Na^+]_i$, 400 μmol/l (±)-kavain failed to affect the enhancement of $[Ca^{2+}]_i$ induced by 50 μmol/l monensin, indicating that (±)-kavain did not influence the method of Ca^{2+} determination (Fig. 4B). When 400 μmol/l (±)-kavain was applied after 4-aminopyridine (Fig. 4B), the 4-aminopyridine-enhanced $\Delta[Ca^{2+}]_i$ declined with a $\tau_{1/2}$ of 9 ± 2 s ($n = 5$), but basal $[Ca^{2+}]_i$ was not reached. $\Delta[Ca^{2+}]_i$ was reduced by 73% of control ($n = 5$) upon the addition of 400 μmol/l (±)-kavain.

Similar to 4-aminopyridine, 40 mmol/l KCl induced an increase in $[Ca^{2+}]_i$ (Fig. 5) which peaked to a $\Delta[Ca^{2+}]_i$ value of 403 ± 38 nmol/l Ca^{2+} and declined to a steady-state level of $\Delta[Ca^{2+}]_i = 251 \pm 28$ nmol/l Ca^{2+} ($n = 6$). Application of 400 μmol/l (±)-kavain 100 s before KCl (Fig. 5) suppressed both values to the same extent (75–78% of control), viz. 315 ± 18 nmol/l Ca^{2+} (peak) and 189 ± 32 nmol/l Ca^{2+} (steady state, $n = 6$).

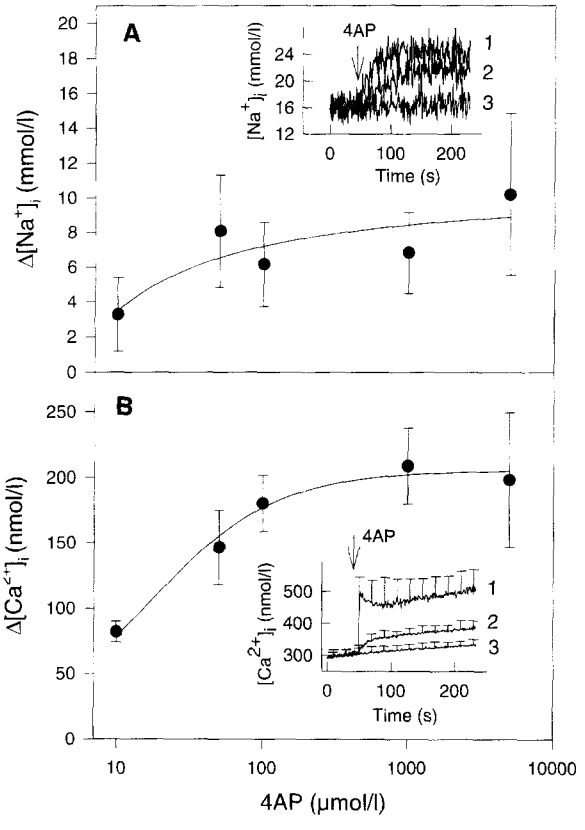

Fig. 3. Dose dependence of the 4-aminopyridine-induced increase in $[Na^+]_i$ and $[Ca^{2+}]_i$. As shown in the insets, synaptosomes were stimulated with different concentrations of 4-aminopyridine as indicated by arrows. $[Na^+]_i$ and $[Ca^{2+}]_i$ were continuously detected as shown for (1) 5 mmol/l 4-aminopyridine, (2) 0.01 mmol/l 4-aminopyridine and (3) control. Traces represent mean ± S.D. ($n = 6$). S.D. error bars of $[Na^+]_i$ traces are omitted for clarity. To obtain the dose-response curves, the 4-aminopyridine-dependent increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ was calculated as the difference between basal and 4-aminopyridine-evoked cation concentrations, expressed as $\Delta[Na^+]_i$ (A) and $\Delta[Ca^{2+}]_i$ (B). Results are shown as mean ± S.D. ($n = 6$).

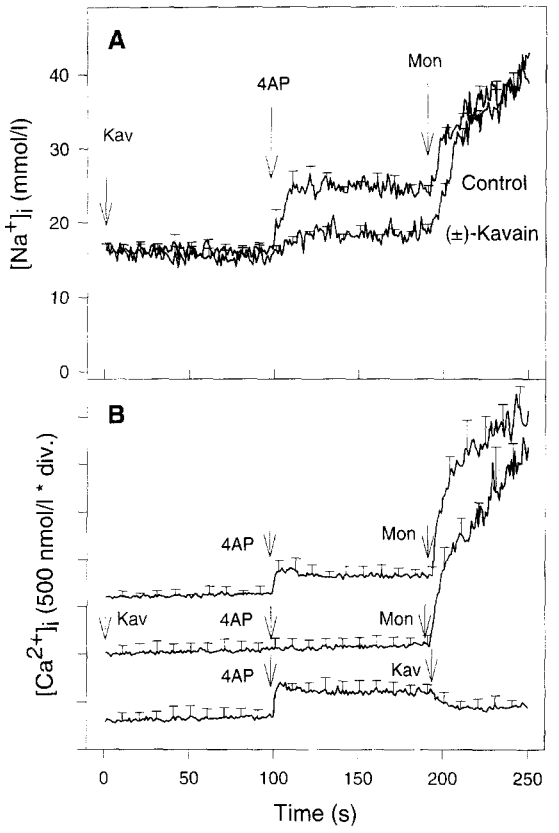

Fig. 4. Suppression of the 4-aminopyridine-induced increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ by (±)-kavain. To investigate the action of (±)-kavain on the 4-aminopyridine-induced increase in $[Na^+]_i$ (A) and $[Ca^{2+}]_i$ (B) at a concentration sufficient to attain maximal displacement of [^{3}H]batrachotoxin from synaptosomes (see Fig. 2), 400 μmol/l (±)-kavain (Kav) was applied to synaptosomes 100 s before 5 mmol/l 4-aminopyridine as shown by arrow. The Na^+ ionophore monensin (Mon, 50 μmol/l) was added as indicated to determine the effect of (±)-kavain on Na^+ channel-independent increase in $[Na^+]_i$ and $[Ca^{2+}]_i$.

Table 1
Action of (±)-kavain on [^{3}H]batrachotoxin binding and 4-aminopyridine-induced increase in $[Na^+]_i$, $[Ca^{2+}]_i$ and glutamate release

Parameter	Control	(±)-Kavain
[^{3}H]Batrachotoxin	224 ± 38.4	74.4 ± 16.9 [c]
(fmol/mg protein)	(n = 5)	(n = 5)
$\Delta[Na^+]_i$	8.7 ± 1.1	3.3 ± 1.7 [c]
(mmol/l)	(n = 6)	(n = 6)
$\Delta[Ca^{2+}]_i$	235.9 ± 20.3	67.7 ± 16.9 [c]
(nmol/l)	(n = 15)	(n = 5)
Glutamate release	38.1 ± 7.4	22.7 ± 2.0 [b]
(pmol/s per mg protein)	(n = 6)	(n = 6)
Total glutamate	15.0 ± 2.7	10.5 ± 1.3 [a]
(nmol/mg protein)	(n = 6)	(n = 6)

The amount of bound [^{3}H]batrachotoxin in the presence of 400 μmol/l (±)-kavain was calculated by a radioligand competition assay. To determine the action of (±)-kavain on the 4-aminopyridine-induced increase in $[Na^+]_i$, $[Ca^{2+}]_i$ and glutamate release, 400 μmol/l (±)-kavain was added to synaptosomes 100 s before the application of 4-aminopyridine (5 mmol/l). The 4-aminopyridine-dependent increase in $[Na^+]_i$ and $[Ca^{2+}]_i$, both calculated as the difference between basal and 4-aminopyridine-stimulated cation concentrations, is expressed as $\Delta[Na^+]_i$ and $\Delta[Ca^{2+}]_i$. The rate of glutamate release was calculated by linear fitting of the amount of glutamate released during the first 100 s after 4-aminopyridine was applied. Total amount of released glutamate was calculated as described in Section 2. Values are shown as means ± S.D. Differences between mean of control and (±)-kavain-treated synaptosomes were considered significant (t-test, unpaired, two-sided) if $P < 0.05$ (a), $P < 0.01$ (b) or $P < 0.001$ (c).

3.3. Glutamate release

As shown in the inset of Fig. 6, 4-aminopyridine (5 mmol/l) provoked a long-lasting release of glutamate under conditions of permanent depolarization. The rate and total amount of glutamate released, calculated as described in Section 2, amounted to 38.1 ± 7.4 pmol glutamate/s per mg protein and 15.0 ± 2.7 nmol glutamate/mg protein, respectively. Consistent with the earlier results, (±)-kavain itself failed to provoke glutamate release (Fig. 6, inset) but dose-dependently reduced both the rate of glutamate release and the total amount of glutamate released (Fig. 6), which were reduced by 400 μmol/l (±)-kavain to 60 and 70% of control, respectively (Table 1). The (±)-kavain-dependent suppression of glutamate release seemed to be biphasic, with a high-efficacy component up to about 70 μmol/l (±)-kavain and a low-efficacy component in the range of about 70–400 μmol/l (±)-kavain which accounted for about 10% inhibition.

The application of 40 mmol/l KCl provoked a release of glutamate almost identical to that of 4-aminopyridine-stimulated synaptosomes. The rate of glutamate release was 35.2 ± 4.3 pmol/s per mg protein (n = 6) and was diminished by 9% (32.2 ± 3.5 pmol/s per mg protein, n = 6) when 400 μmol/l (±)-kavain was added 100 s before KCl.

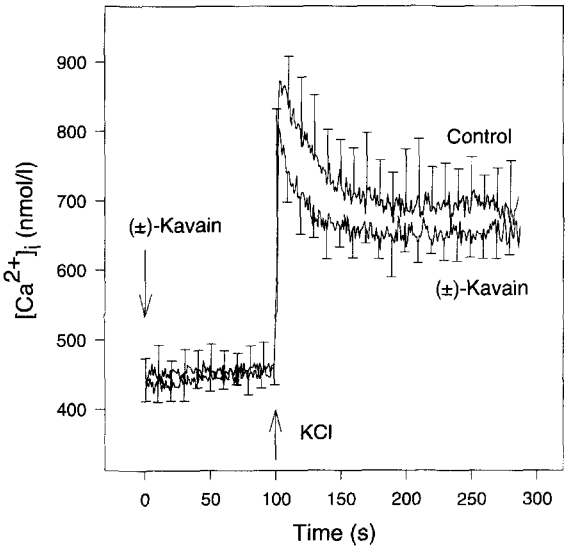

Fig. 5. Suppression of the KCl-induced $[Ca^{2+}]_i$ increase by (±)-kavain. Synaptosomal $[Ca^{2+}]_i$ was increased by the addition of 40 mmol/l KCl as indicated. (±)-Kavain (400 μmol/l) was applied at the onset of measurement. Traces are shown as mean ± S.D. (n = 6).

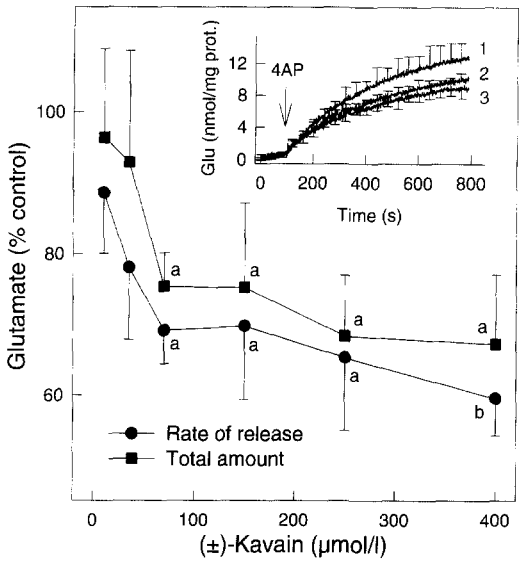

Fig. 6. Dose-dependent suppression of 4-aminopyridine-induced glutamate release by (±)-kavain. As shown in the inset, release of endogenous glutamate from synaptosomes was induced by addition of 5 mmol/l 4-aminopyridine as indicated. Application of (±)-kavain 100 s before 4-aminopyridine (5 mmol/l) suppressed glutamate release dose-dependently. Traces represent mean ± S.D. (n = 6) of control (1), 70 μmol/l (±)-kavain (2) and 400 μmol/l (±)-kavain (3). The (±)-kavain-dependent suppression of glutamate release, expressed as the reduction in total amount (■) and the rate (●) of glutamate released, was calculated as described in text. Data are depicted as mean ± S.D. (n = 6). Differences between means of control and (±)-kavain-treated synaptosomes were considered significant (t-test, unpaired, two-sided) if $P < 0.05$ (a) and $P < 0.01$ (b).

4. Discussion

4-Aminopyridine is known to block K^+ channels (Thesleff, 1980; Cook, 1988; Castle et al., 1989), thereby inducing a partial depolarization of the cell membrane

(Buckle and Haas, 1982; Pocock and Nicholls, 1992) to a level sufficient for activation of voltage-dependent Na^+ channels yet leaving the membrane sufficiently polarized for their subsequent reactivation (Tibbs et al., 1989), leading to Na^+ influx. Concomitantly, $[Ca^{2+}]_i$ increases, possibly by activation of voltage-sensitive Ca^{2+} channels (Tibbs et al., 1989; Barrie et al., 1991; McMahon and Nicholls, 1991) although a $[Ca^{2+}]_i$ increase, mediated by the Na^+/Ca^{2+} exchanger of the plasma membrane, can not be excluded (Tibbs et al., 1989). Finally, as demonstrated with 4-aminopyridine-stimulated synaptosomes, the enhanced $[Ca^{2+}]_i$ results in the release of several neurotransmitters, including glutamate (Tibbs et al., 1989; McMahon and Nicholls, 1991; Verhage et al., 1991; Pocock and Nicholls, 1992), dopamine (Arzate et al., 1986), acetylcholine (Tapia and Sitges, 1982) and γ-aminobutyric acid (Tapia et al., 1985).

In the current investigation, 4-aminopyridine-stimulated synaptosomes were used as a model of repetitively firing neurons (Tibbs et al., 1989; McMahon and Nicholls, 1991) to elucidate the anticonvulsive action of (±)-kavain. In accordance with the proposed mechanism of 4-aminopyridine-induced transmitter release described above, the release of glutamate and an increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ were induced by 4-aminopyridine, with the increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ being diminished to a comparable extent by (±)-kavain. This similar efficacy of (±)-kavain on both cation concentrations suggests an inhibition of voltage-dependent Na^+ channels, thus, suppressing the 4-aminopyridine-evoked Na^+ influx and the consequent increase in $[Ca^{2+}]_i$, finally leading to a suppression of glutamate release. (±)-Kavain (400 μmol/l) suppressed also the KCl-provoked increase in $[Ca^{2+}]_i$ but diminished the rate of glutamate release by only 9% of control. These results suggest an additional inhibition of voltage-dependent Ca^{2+} channels, but compared with the action of (±)-kavain on 4-aminopyridine-induced $[Ca^{2+}]_i$ and glutamate release, this inhibition was less pronounced.

The rate of glutamate release from 4-aminopyridine-stimulated synaptosomes was only diminished to 60% of control at 400 μmol/l (±)-kavain. This apparently inconsistent efficacy between inhibition of 4-aminopyridine-induced $[Ca^{2+}]_i$ increase and glutamate release may be unravelled by the mechanism of glutamate release. Less than 50% of glutamate release is dependent on Ca^{2+}, reflecting vesicular bound glutamate, if glutamate release is induced by Na^+ influx (Nicholls et al., 1987), whereas the rest is thought to derive from cytosol via a reversal of the glutamate/Na^+ carrier (Sanchez-Prieto et al., 1987). Since 400 μmol/l (±)-kavain suppressed the 4-aminopyridine-induced increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ to only 38 and 29% of control, the additive action of both cation concentrations on glutamate release may account for the relatively high rate of glutamate release (60% of control) observed at this (±)-kavain concentration. Considering the biphasic inhibition of glutamate release, 4-aminopyridine may also activate voltage-dependent Ca^{2+} channels which seem to be less affected than Na^+ channels by (±)-kavain, as deduced from its action on KCl-evoked glutamate release. Therefore, in accordance with the results for [^{3}H]batrachotoxin displacement by (±)-kavain (see below), effective suppression of glutamate release by up to about 70 μmol/l (±)-kavain may reflect inhibition of Na^+ channels whereas the low efficacy of (±)-kavain in the range of 70–400 μmol/l may be predominantely attributed to inhibition of Ca^{2+} channels since the latter account for about 10% of the inhibition of glutamate release, a value closely related to the suppression (9%) of KCl-evoked glutamate release by 400 μmol/l (±)-kavain.

In order to determine whether (±)-kavain exerts its effect through an interaction with epitope 1 of Na^+ channels, a competition assay was performed with [^{3}H]saxitoxin. Like several local anaesthetics (Catterall, 1987), (±)-kavain failed to displace [^{3}H]saxitoxin, ruling out the possibility that (±)-kavain blocks Na^+ channels by an interaction with receptor site 1. However, (±)-kavain suppressed dose-dependently the binding of [^{3}H]batrachotoxin, a mode of action often observed for local anaesthetics (Catterall, 1987), suggesting an inhibition of Na^+ channels by an interaction of (±)-kavain with receptor site 2. But, in contrast to, e.g., tetracaine and lidocaine (Catterall, 1987), (±)-kavain failed to displace [^{3}H]batrachotoxin completely and maximal displacement of [^{3}H]batrachotoxin was restricted to 33% of control. It is tentative to speculate that (±)-kavain interacts specifically with Na^+ channel subtypes, e.g., type II which is predominantly located in axons, although no major differences in drug sensitivity have been described between type I and II channels so far (Taylor and Meldrum, 1995). However, the limited [^{3}H]batrachotoxin displacement (33% of control) by 400 μmol/l (±)-kavain is in line with the suppression of the 4-aminopyridine-induced increase in $[Na^+]_i$ and $[Ca^{2+}]_i$, which were diminished to 38 and 29% of control at the same (±)-kavain concentration.

Inhibition of voltage-dependent Na^+ channels as a mechanism of the anticonvulsive action of (±)-kavain is consistent with published results about the action of the local anaesthetic procaine and natural kava pyrones, including (+)-kavain and (+)-methysticin (Kretzschmar and Meyer, 1969). Regarding the dose-response relation, the influence on the seizure pattern and the duration of action against pentylenetetrazole-induced seizures in mice, the anticonvulsive properties of kava pyrones resemble those of procaine. Schmitz et al. (1995) showed (+)-methysticin to block the epileptiform activity of hippocampal and entorhinal cortex slices induced by either low Ca^{2+}, low Mg^{2+} or high K^+ perfusion medium. (+)-Methysticin was most effective against recurrent discharges in the hippocampal area CA1 induced by low Ca^{2+} medium. Since low Ca^{2+}-induced discharges were hardly affected by glutamate antagonists, the authors proposed a minor action of (+)-methysticin on glutamate receptors, and

suggested that (+)-methysticin affects the excitability of neurons, an assumption consistent with the inhibition of Na^+ channels as detected for (±)-kavain in the present paper.

The lowest effective dose used by Schmitz et al. (1995) to block discharges in CA1 was 10 μmol/l (+)-methysticin, a concentration lower than that of (±)-kavain (about 200 μmol/l) necessary to attain maximal displacement of [^{3}H]batrachotoxin. In contrast to these observations, a structure-activity study revealed equal efficacy against electroshock-induced convulsions for (+)-methysticin and (+)-kavain, when these drugs were i.v. injected in mice (Meyer and Kretzschmar, 1969). The latter data suggest that both compounds should be able to block Na^+ channels with similar efficacy, provided (+)-methysticin and (+)-kavain exert their action on Na^+ channels only. A stereo-specific action of the natural (+)-enantiomers as a cause for the different efficacies of (+)-methysticin and (±)-kavain seems to be unlikely because natural (+)-kavain and its synthetic racemate were equipotent in the maximal electroshock model (Meyer and Kretzschmar, 1969). Therefore, the question arises whether kava pyrones might accumulate in cell membranes. According to our data, 70 μmol/l (±)-kavain suppressed the rate of glutamate release to about 70% of control within less than 3 min. This observation might fit with the results of Schmitz et al. (1995), who reported a concentration- and time-dependent inhibition of epileptic activity by (+)-methysticin, which could be a hint that (+)-methysticin accumulates in the plasma membrane during prolonged perfusion. An incubation time of 55 min was necessary to block discharges in the CA1 with 10 μmol/l (+)-methysticin whereas the action of 50 μmol/l (+)-methysticin took place in 16 min.

In conclusion, the data indicate an inhibition of voltage-dependent Ca^{2+} and Na^+ channels by (±)-kavain. (±)-Kavain resembles local anaesthetics with respect to the results of [^{3}H]saxitoxin- and [^{3}H]batrachotoxin-binding studies, and suppresses the 4-aminopyridine-induced increase in $[Na^+]_i$, $[Ca^{2+}]_i$ and the release of endogenous glutamate.

References

Arzate, E.M., J. Morán and H. Pasantes-Morales, 1986, Inhibitory effects of taurine on 4-aminopyridine-stimulated release of labelled dopamine from striatal synaptosomes, Neuropharmacology 25, 689.

Barrie, A.P., D.G. Nicholls, J. Sanchez-Prieto and T.S. Sihra, 1991, An ion channel locus for the protein kinase C potentiation of transmitter glutamate-release from guinea pig cerebrocortical synaptosomes, J. Neurochem. 57, 1398.

Bradford, M.M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248.

Buckle, P.J. and H.L. Haas, 1982, Enhancement of synaptic transmission by 4-aminopyridine in hippocampal slices of the rat, J. Physiol. 326, 109.

Capasso, A. and A. Calignano, 1988, Synergism between the sedative action of kava extract and D,L-kawain, Acta Ther. 14, 249.

Castle, N.A., D.G. Haylett and D.H. Jenkinson, 1989, Toxins in the characterization of potassium channels, TINS 12, 59.

Catterall, W.A., 1987, Common modes of drug action on Na^+ channels: local anesthetics, antiarrhythmics and anticonvulsants, Trends Pharmacol. Sci. 8, 57.

Catterall, W.A., C.S. Morrow and R.P. Hartshorne, 1979, Neurotoxin binding to receptor sites associated with voltage-sensitive sodium channels in intact, lysed, and detergent-solubilized brain membranes, J. Biol. Chem. 254, 11379.

Catterall, W.A., C.S. Morrow, J.W. Daly and G.B. Brown, 1981, Binding of batrachotoxin A 20-α-benzoate to a receptor site associated with sodium channels in synaptic nerve ending particles, J. Biol. Chem. 256, 8922.

Cook, N.S., 1988, The pharmacology of potassium channels and their therapeutic potential, Trends Pharmacol. Sci. 9, 21.

Duve, R.N., 1981, Gas-liquid chromatographic determination of major constituents of *Piper methysticum*, Analyst 106, 160.

Gleitz, J., A. Beile, S. Khan, B. Wilffert and F. Tegtmeier, 1993a, Anaerobic glycolysis and postanoxic recovery of respiration of rat cortical synaptosomes are reduced by synaptosomal sodium load, Brain Res. 611, 286.

Gleitz, J., A. Beile, B. Wilffert and F. Tegtmeier, 1993b, Cryopreservation of freshly isolated synaptosomes prepared from the cerebral cortex of rats, J. Neurosci. Methods 47, 191.

Grynkiewicz, G., M. Poenie and R.Y. Tsien, 1985, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260, 3440.

Jamieson, D.D. and P.H. Duffield, 1990, The antinociceptive actions of kava components in mice, Clin. Exp. Pharmacol. Physiol. 17, 495.

Kretzschmar, R. and H.J. Meyer, 1969, Vergleichende Untersuchungen über die antikonvulsive Wirksamkeit der Pyronverbindungen aus *Piper methysticum* Forst., Arch. Int. Pharmacodyn. 177, 261.

Kretzschmar, R., H.J. Meyer, H.J. Teschendorf and B. Zöllner, 1969, Antagonistische Wirkung natürlicher 5,6-hydrierter Kawa-Pyrone auf die Strychninvergiftung und den experimentellen Tetanus, Arch. Int. Pharmacodyn. 182, 251.

Kretzschmar, R., H.J. Meyer and H.J. Teschendorf, 1970, Strychnine antagonistic potency of pyrone compounds of the kavaroot (*Piper methysticum* Forst.), Experientia 26, 283.

Lebot, V., M. Merlin and L. Lindstrom, 1992, Kava. The Pacific Drug (Harvard University Press, New Haven, CT) p. 119.

Mauz, A.B.M. and H. Pelzer, 1990, β-Adrenoceptor-binding studies of the cardioselective β-blockers bisoprolol, H-I 42 BS, and HX-CH 44 BS to heart membranes and intact ventricular myocytes of adult rats: two β_1-binding sites for bisoprolol, J. Cardiovasc. Pharmacol. 15, 421.

McMahon, H.T. and D.G. Nicholls, 1991, Transmitter glutamate-release from isolated nerve terminals: evidence for biphasic release and triggering by localized Ca^{2+}, J. Neurochem. 56, 86.

Meyer, H.J., 1965, Spasmolytische Effekte von Dihydromethysticin, einem Wirkstoff aus *Piper methysticum* Forst., Arch. Int. Pharmacodyn. 154, 449.

Meyer, H.J., 1979, Pharmacology of kava, in: Ethnopharmacologic Search for Psychoactivedrugs, eds. D.H. Efron, B. Holmsted and N.S. Kline (Raven Press, New York, NY) p. 133.

Meyer, H.J. and R. Kretzschmar, 1969, Untersuchungen über Beziehungen zwischen Molekularstruktur und pharmakologischer Wirkung C_6-arylsubstituierter 4-Methoxy-α-pyrone vom Typ der Kawa-Pyrone, Arzeim. Forsch. 19, 617.

Minta, A. and R.Y. Tsien, 1989, Fluorescent indicators for cytosolic sodium, J. Biol. Chem. 264, 19449.

Nicholls, D.G., T.S. Shira and J. Sanchez-Prieto, 1987, Calcium dependent and independent release of glutamate from synaptosomes monitored by continuous fluorometry, J. Neurochem. 49, 50.

Pocock, J.M. and D.G. Nicholls, 1992, A toxin (Aga-GI) from the venom

of the spider Agelenopsis aperta inhibits the mammalian presynaptic Ca^{2+} channel coupled to glutamate exocytosis. Eur. J. Pharmacol. 226, 343.

Postma, S.W. and W.A. Catterall, 1984, Inhibition of binding of ^{3}H-batrachotoxinin A 20-α-benzoate to sodium channels by local anesthetics, Mol. Pharmacol. 25, 219.

Sage, S.O., T.J. Rink and M.P. Mahaut-Smith, 1991, Resting and ATP-evoked changes in cytosolic free sodium concentration in human platelets loaded with the indicator SBFI, J. Physiol. 441, 559.

Sanchez-Prieto, J., T.S. Sihra and D.G. Nicholls, 1987, Characterization of exocytotic release of glutamate from guinea-pig cerebral cortical synaptosomes, J. Neurochem. 49, 58.

Schmitz, D., C.L. Zhang, S.S. Chatterjee and U. Heinemann, 1995, Effects of methysticin on three different models of seizure like events studied in rat hippocampal and entorhinal cortex slices, Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 348.

Singh, Y.N., 1992, Kava: an overview, J. Ethnopharmacol. 37, 13.

Smith, R.M., 1983, Kava lactones in *Piper methysticum* from Fiji, Phytochemistry 22, 1055.

Tapia, R. and M. Sitges, 1982, Effect of 4-aminopyridine on transmitter release in synaptosomes, Brain Res. 250, 291.

Tapia, R., M. Sitges and E. Morales, 1985, Mechanism of the calcium-dependent stimulation of transmitter release by 4-aminopyridine in synaptosomes, Brain Res. 361, 373.

Taylor, C.P. and B.S. Meldrum, 1995, Na^{+} channels as targets for neuroprotective drugs, Trends Pharmacol. Sci. 16, 309.

Thesleff, S., 1980, Aminopyridines and synaptic transmission, Neuroscience 5, 1413.

Tibbs, G.R., A.P. Barrie, F.J. van Mieghem, H.T. McMahon and D.G. Nicholls, 1989, Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: effect on cytosolic free Ca^{2+} and glutamate release, J. Neurochem. 53, 1693.

Verhage, M., H.T. McMahon, W.E.J.M. Ghijsen, F. Boomsma, G. Scholten, V.M. Wiegant and D.G. Nicholls, 1991, Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals, Neuron 6, 517.