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KAVA EXTRACT INGREDIENTS, (+)-METHYSTICIN AND (±)-KAVAIN INHIBIT VOLTAGE-OPERATED Na^+-CHANNELS IN RAT CA1 HIPPOCAMPAL NEURONS

E. I. MAGURA,*‡ M. V. KOPANITSA,* J. GLEITZ,† T. PETERS† and O. A. KRISHTAL*

*Department of Cellular Membranology, Bogomoletz Institute of Physiology, 4 Bogomoletz Strasse, Kyiv 252024, Ukraine

†Institut für Naturheilkunde, University Clinics Ulm, 20 Helmholtz Strasse, Ulm 89070, Germany

Abstract—The action of synthetic kava pyrones, (+)-methysticin and (±)-kavain, on voltage-operated Na^+-channels was studied in whole-cell patch-clamped CA1 hippocampal neurons. In doses of 1–400 μM, both compounds exerted a rapid and reversible inhibition of the peak amplitude of Na^+-currents. Shifting holding membrane potential (V_{hold}) to more positive values enhanced their blocking effect. The drugs studied did not demonstrate use-dependent properties at 10 Hz stimulation but shifted h_∞ curve toward more negative potentials, accelerated time-course of inactivation and slowed down the recovery from inactivation.

Voltage-dependence of Na^+-channel inhibition can be explained by interaction of (+)-methysticin and (±)-kavain with resting closed and inactivated states of Na^+-channel. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: *Piper methysticum*, kava pyrone, (+)-methysticin, (±)-kavain, sodium channels, hippocampal neurons.

Kava pyrones are pharmacologically active constituents prepared from the root of the intoxicating pepper (*Piper methysticum Forst*.), a remedy of the South Pacific islands.[16,23] The main kava pyrones (+)-kavain, (+)-dihydrokavain, (+)-methysticin and (+)-dihydromethysticin are characterized by their anticonvulsive, analgesic and centrally muscle relaxing properties.[6,17] Although the intoxicating pepper is not reported in folk medicine for the treatment of epilepsy, anticonvulsive action against electroshock- and pentylenetetrazole-induced seizures was demonstrated in mice.[13] Despite the similarity with benzodiazepines, kava pyrones fail to interfere with $GABA_A$ receptors as shown by radioligand binding assays[5] and pharmaco-electroencephalogram brain mapping analysis.[21]

Recently, Schmitz *et al.*[22] confirmed the anticonvulsive action of (+)-methysticin, which suppressed epileptiform activity of hippocampal and entorhinal tissue slices independent of different stimuli, suggesting that neuronal excitability is affected. Consistent with this idea, Gleitz *et al.*[7,8] showed that synthetic (±)-kavain blocks veratridine-sensitive Na^+-channels. This mode of action may indeed account for the anticonvulsive properties of kava pyrones since such antiepileptic drugs as carbamazepin and phenytoin exert their action via an inhibition of voltage-dependent Na^+-channels.[4,24] Also, neuroprotective effects of (+)-methysticin and dihydromethysticin but not (±)-kavain have been demonstrated in models of focal ischaemia.[1] Therefore, kava pyrones may be of interest as a new class of neuroprotective drugs. It should be noted that (+)-methysticin and (±)-kavain do not possess any potentially ionizable group (Fig. 1) and differ, in this respect, from local anaesthetics. To elucidate the mechanisms of Na^+-channel inhibition by (+)-methysticin and (±)-kavain, we studied their action on voltage-dependent Na^+-currents in freshly isolated rat hippocampal neurons.

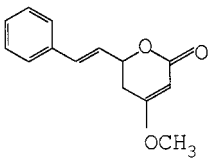
EXPERIMENTAL PROCEDURES

Cell preparation

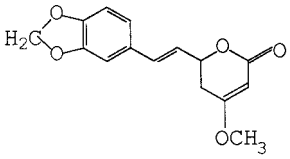
This study was carried out on protease-treated and acutely dispersed CA1 hippocampal neurons from 12–14-day-old Wistar rats (WAG/GSto, Moscow, Russia) as described in detail previously.[18] Briefly, the hippocampi were cut into 200–400 μm-thick slices and incubated with 2 mg/ml protease (type XXIII). After rinsing off the enzyme, single cells could be isolated by trituration of tissue pieces through several fire-polished pipettes with opening diameters from 0.5 to 0.1 mm. The pyramidal neurons were identified by their characteristic form and partially preserved dendritic arborization.

‡To whom correspondence should be addressed.

Abbreviations: DMSO, dimethylsulphoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; V_{hold}, holding membrane potential.



Kavain



Methysticin

Fig. 1. Chemical structures of kavain and methysticin.

Recording conditions

Na^+-currents were recorded by whole-cell patch-clamp technique[9] in the external solution of the following composition: NaCl 150 (in mM), KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 20, pH adjusted to 7.4 with NaOH. Test solutions were applied to the cell in a step-like manner by the fast concentration-clamp method.[14] Patch pipettes were filled with intracellular solution containing CsF 100, tris(hydroxymethyl)aminomethane chloride (Tris–Cl) 40 and had resistance values of 0.5–3 MΩ. Experiments were performed at room temperature (22–24°C).

Data analysis

Tetrodotoxin-sensitive Na^+-currents were digitally sampled at 50 μs intervals by 10-digit ADC-board and measured from baseline to peak value. Results are expressed as mean ± S.E.M. Curve fitting was performed by the least-squares procedure with the help of Microcal Origin v. 3.5 (Microcal Software, Inc., Northampton, MA, U.S.A.).

Solutions and chemicals

(+)-Methysticin and (±)-kavain were purchased from Extrasynthese (Lyon, France). All other drugs were obtained from Sigma. Stock solutions of (+)-methysticin and (±)-kavain (40 mM) were prepared in dimethylsulfoxide (DMSO) and diluted in external solution to 1–400 μM just before the experiment. In control experiments, a corresponding volume of DMSO was added to the extracellular solution (maximal concentration 1% v/v).

RESULTS

Extracellular applications of (+)-methysticin and (±)-kavain elicited rapid and reversible inhibition of the peak amplitude of Na^+-currents in the concentration range of 1–400 μM (Fig. 2A–D). Unfortunately, due to highly lipophylic nature of these kava ingredients and their limited solubility in external solution, complete block could not be obtained.

We have found that inhibitory action of (+)-methysticin and (±)-kavain increased with membrane depolarization. Change of holding membrane potential (V_{hold}) from −100 to −80 mV greatly increased the amount of block for both compounds tested. (Fig. 3A,B). Since voltage-dependence of Na^+-channel inhibition is characteristic of local anaesthetics and some anticonvulsant drugs,[20,24] we examined whether (+)-methysticin and (±)-kavain share other distinctive property of these substances, such as the frequency-dependent block. We found that the extent of the inhibition of Na^+-channel by (+)-methysticin and (±)-kavain did not significantly increase at 10 Hz frequency of stimulation (Fig. 4A,B), when local anaesthetics should already demonstrate significant frequency dependence.[2,19]

Therefore, in an attempt to explain the voltage-dependence of (±)-kavain block, we have investigated its influence on the inactivation processes. As seen from Fig. 5A and B, both (+)-methysticin and, slightly less, (±)-kavain shifted h_∞ curve toward more negative potentials. Thus, at membrane potentials positive to −100 mV, the drugs increase the degree of steady-state inactivation of Na^+-channels, whereas shifting V_{hold} to very negative values overcomes their inhibitory action.

To further evaluate the effects of kava ingredients on Na^+-channel inactivation, we investigated their action on the time course of inactivation and recovery from inactivation of Na^+-channels.[11] It was found that (+)-methysticin and (±)-kavain significantly accelerated the development of inactivation of Na^+-channels (Fig. 6A,B) and markedly slowed down the recovery from inactivation (Fig. 7A,B).

DISCUSSION

In support of the results obtained previously with (±)-kavain on rat central synaptosomes[7] we report here that (±)-kavain and another kava pyrone, (+)-methysticin, inhibit voltage-dependent Na^+-channels in acutely dissociated rat CA1 hippocampal neurons. (+)-Methysticin was about four- to five-fold more potent blocker of peak current amplitude than (±)-kavain at different V_{hold} values (Fig. 3A,B). Also, (+)-methysticin caused a larger shift of h_∞ curve than (±)-kavain did (Fig. 5). These data may shed light on the mechanisms of anticonvulsive and analgesic actions of kava pyrones.[17,23]

The increase in the degree of Na^+-current inhibition with the depolarization of membrane potential may be a result of such processes as use-dependence of (±)-kavain block, voltage-dependence of drug binding or action on Na^+-channel inactivation.[3,12]

Use-dependence seems unlikely to play a significant role in the Na^+-channel block by kava pyrones for the following reasons: i) in all experiments the block was evident during the first pulse after

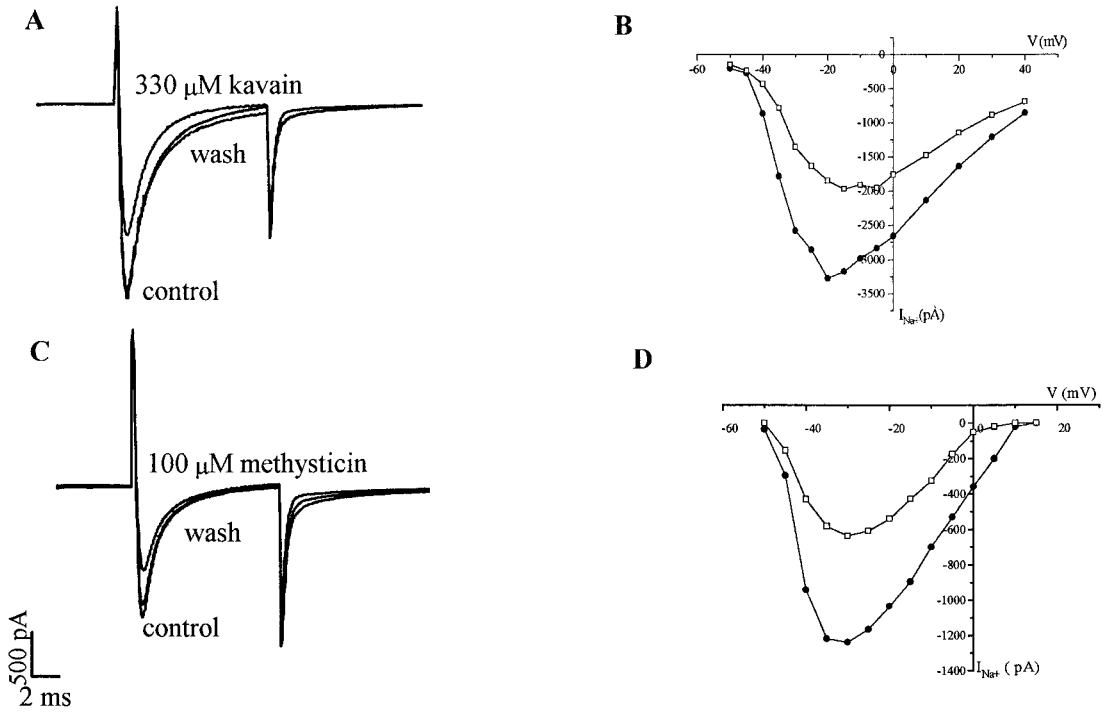


Fig. 2. (A) Inhibition of Na^+-current by 300 μM (±)-kavain. Inward Na^+-currents were elicited by 10 ms voltage pulses from holding potential −100 mV to −20 mV. Averaged records of five subsequent pulses at stimulation frequency 1 Hz are presented. (B) Effect of 300 μM (±)-kavain on I/V curve of Na^+-current. Na^+-currents were evoked by 10 ms voltage pulses from V_{hold} = −100 mV to the corresponding testing potentials at stimulation frequency 1 Hz. (C, D) same for 100 μM (+)-methysticin. Filled circles represent control experiments, open squares represent experiments with drug exposure.

exposure to either (+)-methysticin or (±)-kavain and the extent of inhibition did not change appreciably throughout the whole period of exposure to the drug; ii) experiments with higher frequency of stimulation failed to reveal any significant potentiation of the blocking effect (Fig. 4A,B).

The train of depolarizing pulses demonstrates that the steady-state level of blocking is achieved at the holding voltage. This suggests the interaction of the drug with the resting closed state of the Na^+-channel. This hypothesis is also in accordance with the observation that hydrophobic Na^+-channel blockers readily react with resting closed and inactivated states and are usually less dependent on the previous stimulation than more hydrophilic molecules.[10] We propose that (+)-methysticin and (±)-kavain also bind to the inactivated Na^+-channels and prolong the duration of the inactivation. This suggestion helps to explain the apparent voltage-dependence of their action, since at more positive V_{hold} values, larger number of channels are inactivated and therefore are more sensitive to the blocking effect. Interaction with the inactivated Na^+-channels also offers an explanation for the observed shift of h_∞ curve in more negative direction (Fig. 5) and acceleration of the time-course of inactivation (Fig. 6). Kinetics of recovery from inactivation are indicative of the ability of the neuron to fire repetitive action potentials. Many anaesthetic and anticonvulsant drugs have been shown to dramatically slow the rate of recovery from inactivation.[3,15] In our experiments, (+)-methysticin and (±)-kavain both slowed the recovery from inactivation (Fig. 7).

Voltage-dependent Na^+-channel blockers such as lidocaine, phenytoine, flunarizine, lifarizine, lamotrigine, riluzole are among the reference neuroprotective compounds.[4,24,25] They inhibit excessive Na^+ influx which accompanies many neurological disorders.

The results of the present study demonstrate that (+)-methysticin and (±)-kavain inhibit Na^+-channels in rat central neurons leading to a decrease of cellular excitability, which may be important for neuroprotection. This mechanism of action allows one to explain the well-known neuroprotective properties of kava constituents.[17] In addition, methysticin and ethysticin were recently demonstrated to have protective properties in two models of focal ischaemia.[1] Studying seizure-like events in hippocampal and entorhinal cortex slices, Schmitz *et al.* have shown anticonvulsive action of (+)-methysticin.[22]

CONCLUSION

We therefore conclude that the observed anticonvulsive and anti-ischaemic effects of kava ingredients

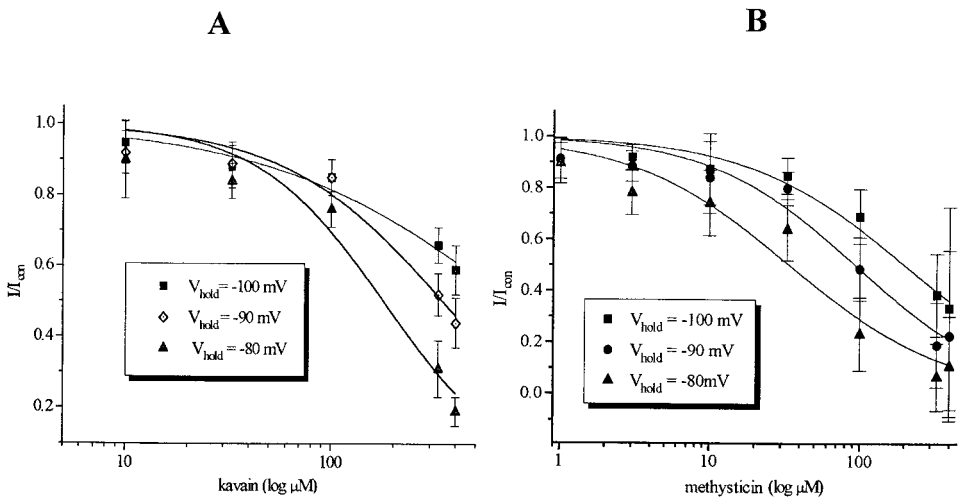


Fig. 3. (A) Dose-dependent block of Na^+-currents by (±)-kavain at different holding potentials. Testing voltage step to −20 mV, pulse duration 10 ms, stimulation frequency 1 Hz. Points represent mean ± S.E.M. (data from six cells for each data point are presented). Curves were drawn by least-square fitting of data by Hill's equation $I=I_{max}(IC_{50}{}^n/([C]^n+[IC_{50}]^n))$ where I_{max} and I current amplitudes measured in control and in the presence of (±)-kavain; [C] and IC_{50} are free concentration of (±)-kavain and concentration producing half-maximal inhibition respectively; *n* is a Hill's coefficient. Calculated theoretical IC_{50} values are 744.9; 347.6 and 178.8 for $V_{hold}=-100$ mV, $V_{hold}=-90$ mV and $V_{hold}=-80$ mV, respectively; n=0.7; n=1.1 and n=1.4 for $V_{hold}=-100$ mV, $V_{hold}=-90$ mV and $V_{hold}=-80$ mV respectively. (B) Same for (+)-methysticin. Calculated theoretical IC_{50} values are: 201.4; 94.2 and 34.3 for $V_{hold}=-100$ mV, $V_{hold}=-90$ mV and $V_{hold}=-80$ mV, respectively; n=0.8; n=0.9 and n=0.8 for $V_{hold}=-100$ mV, $V_{hold}=-90$ mV and $V_{hold}=-80$ mV, respectively.

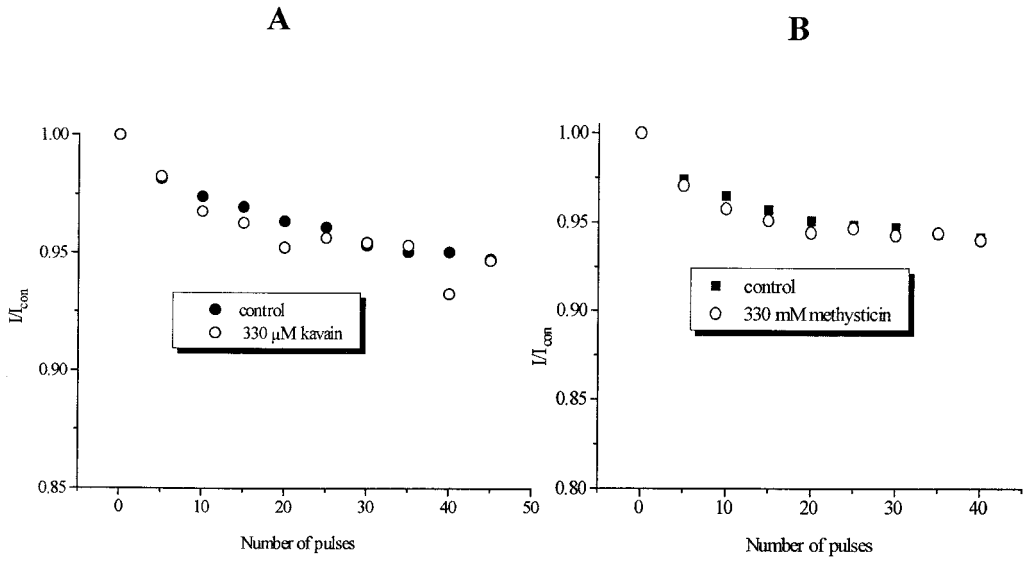


Fig. 4. (A) Time-course of Na^+-current inhibition at high stimulation frequency. Ten Hertz stimulation was applied in control and during incubation in 330 μM (±)-kavain. Test pulses of 10 ms duration from −100 to −20 mV were delivered. Data were normalized with respect to the peak amplitude of the first current trace and represent means of three cells. (B) Same for 330 μM (+)-methysticin.

may be in part mediated by the blockade of voltage-operated Na^+-channels. Considerable voltage-dependence of the observed inhibition of Na^+-currents by the studied substances is explained presumably by their interaction with resting closed and inactivated states of Na^+-channel.

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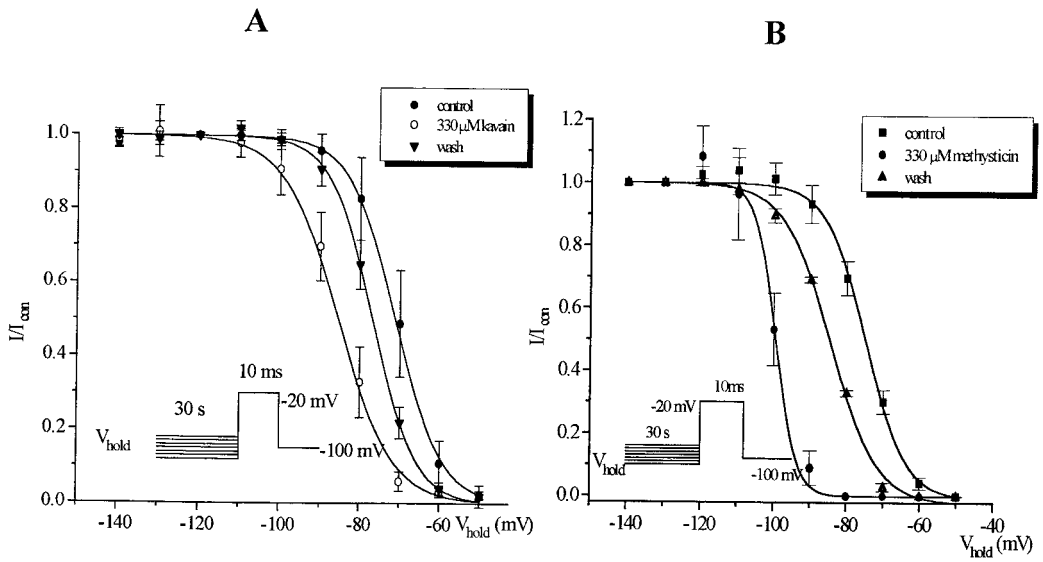

Fig. 5. (A) Effect of 330 μM (±)-kavain on the steady-state inactivation of Na^+-channels. The protocol of the experiments is shown in the inset. The cell was held at different membrane potentials for 30 s, and a 10 ms voltage step to −20 mV was applied thereafter. Na^+-current amplitudes were normalized in respect to values obtained at V_{hold} = −120 mV. Points represent mean ± S.D. (n=3). Smooth curves are the least square fits of the data by Boltzmann's equation $y=1/(1+\exp(V-V_{1/2})/k)$ where V is a prepulse holding potential, $V_{1/2}$ is a potential at which 50% of Na^+-channels were inactivated and k is a slope factor. Theoretical calculations were as follows: control: $V_{1/2}$ = −71.1; k=5.7; (±)-kavain: $V_{1/2}$ = −85.4; k=6.7; wash: $V_{1/2}$ = −76.8; k=5.3. (B) Same for 330 μM (+)-methysticin. Theoretical calculations were as follows. Control: $V_{1/2}$ = −74.9; k=5.5; (+)-methysticin: $V_{1/2}$ = −99.5; k=2.8; wash: $V_{1/2}$ = −84.5; k=6.4.

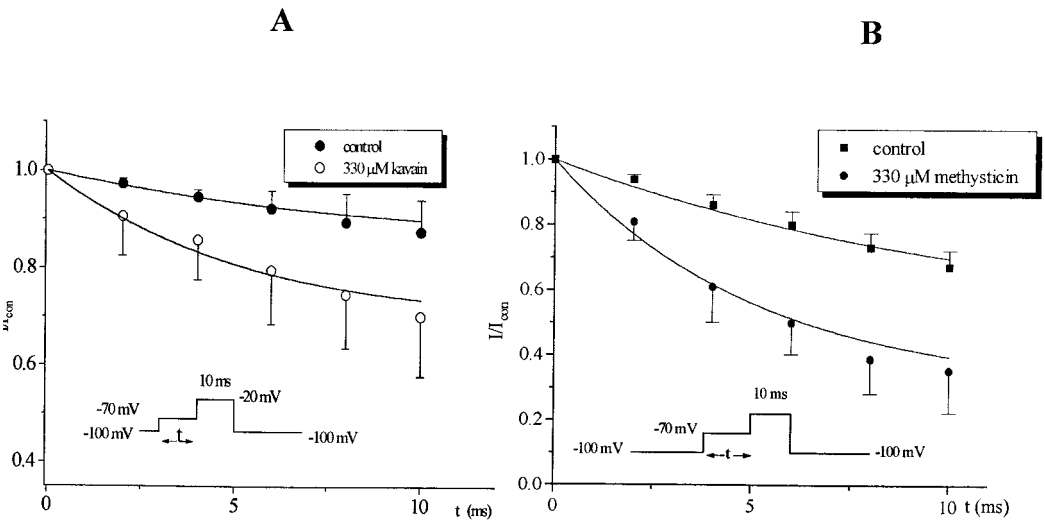

Fig. 6. (A) Time-course of inactivation in control and in the presence of 330 μM (±)-kavain. Experimental scheme is shown in the inset. Prepulses from the holding potential −100 mV to −70 mV were followed by the testing voltage step to −20 mV. Data from records with different prepulse duration were normalized in respect to the peak amplitude at t=0. Smooth curves were drawn according to the least square fits of data by the equation $y=y_\infty-[(y_\infty-1)\exp(-t/\tau_h)]$, where y_∞ is the ordinate at $t=\infty$ and τ_h is the time constant of inactivation. Calculated values of y_∞ are correspondingly 0.85 in control and 0.7 in 330 μM (±)-kavain; τ_h values are respectively 9.05 and 4.91. (B) Same for 330 μM (+)-methysticin. Theoretical calculations were as follows: calculated values of y_∞ are correspondingly 0.7 in control and 0.35 330 μM (+)-methysticin; τ_h values are, respectively, 10.9 and 5.1.

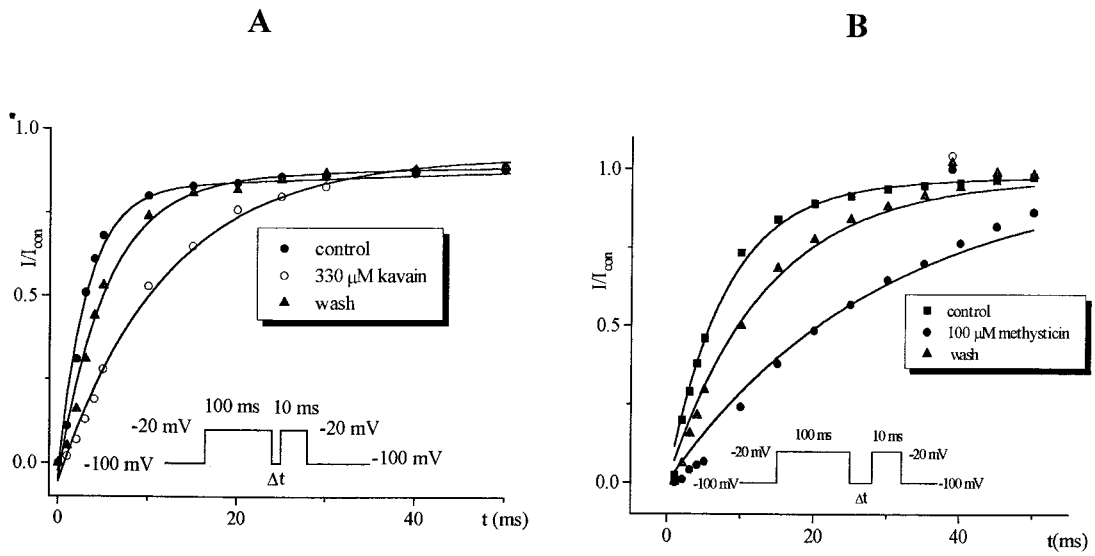

Fig. 7. (A) Action of (±)-kavain on the recovery of Na^+-channels from inactivation. The experimental protocol in shown in the inset. The cell was held at −20 mV for 100 ms, then V_{hold} was set to −100 mV for variable time intervals Δt. Thereafter, testing voltage pulse to −20 mV was applied to elicit Na^+-current. The ratio of this current amplitude to the control value was plotted against Δt values. Points represent means of three cells. Smoothing was performed by fitting data with the two exponential equation, describing the time-course of the recovery from inactivation: $I/I_{max}=1-[A\exp(-\Delta t/\tau_1)+B(-\Delta t/\tau_2)]$ where τ_1 and τ_2 are time constants for fast and slow components of the recovery respectively; A and B are fractions of Na^+-channel recovering correspondingly in a fast and slow way. Calculated parameter values in control: τ_1=3.17, τ_2=158.43, A=0.85, B=0.15; in (±)-kavain: τ_1=11.77, τ_2=500.01, A=0.97, B=0.03; in wash: τ_1=5.34, τ_2=191.47, A=0.89, B=0.11. (B) Same for 100 μM (+)-methysticin. Theoretical calculations were as follows: control: τ_1=8.16, τ_2=620, A=0.98, B=0.02; in (+)-methysticin: τ_1=28.8, τ_2=890, A=0.98, B=0.02; in wash: τ_1=13.53, τ_2=890, A=0.98, B=0.02.

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