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Aconitum sp. alkaloids: the modulation of voltage-dependent Na^+ channels, toxicity and antinociceptive properties

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Abstract

Alkaloids from *Aconitum* sp., used as analgesics in traditional Chinese medicine, were investigated to elucidate their antinociceptive and toxic properties considering: (1) binding to Na^+ channel epitope site 2, (2) alterations in synaptosomal Na^+ and Ca^{2+} concentration ($[Na^+]_i$, $[Ca^{2+}]_i$), (3) arrhythmogenic action of isolated atria, (4) antinociceptive and (5) acute toxic action in mice. The study revealed a high affinity group (K_i 1 μM) and a low affinity group (K_i 10 μM) of alkaloids binding to site 2. The compounds of the high affinity group induce an increase in synaptosomal $[Na^+]_i$ and $[Ca^{2+}]_i$ (EC_{50} 3 μM), are antinociceptive (ED_{50}, 25 μg/kg), provoke tachyarrhythmia and are highly toxic (LD_{50} 70 μg/kg), whereas low affinity alkaloids reduce $[Ca^{2+}]_i$, induce bradycardia and are less antinociceptive (ED_{50} 20 mg/kg) and less toxic (LD_{50} 30 mg/kg). These results suggest that the alkaloids can be grouped in Na^+ channel activating and blocking compounds, but none of the alkaloids seem to be suitable as analgesics because of the low LD_{50}/ED_{50} values.

Keywords: *Aconitum* alkaloid; Na^+ channel; Toxicity; Antinociception

1. Introduction

In traditional Chinese and Japanese medicine the crude drug 'bushi', the extract of *Aconitum* sp. tubers (mainly *A. japonicum* Thunberg, *A. carmichaeli* Debeaux) has been used for centuries as an important remedy with analgesic action. It was demonstrated by the tail pressure test and acetic acid-induced writhing test that aconitine and mesaconitine, the main *Aconitum* alkaloids, produced antinociception in rats and mice (Hikino et al., 1979). The structure of mesaconitine is closely related to aconitine and differs only from aconitine by a methyl group instead of an ethyl group at the nitrogen atom. Oyama et al. (1994) found that mesaconitine mainly contributes to the analgesic effect of the drug. Further studies on mesaconitine revealed that it is a centrally-acting drug which does not interact with opiate receptors (Murayama et al., 1984; Hikino and Murayama, 1985; Murayama and Hikino, 1985; Suzuki et al., 1994).

The second main alkaloid of the tubers, aconitine, is known to bind with high affinity to the open state of Na^+ channels at epitope 2, thus causing a persistent activation of Na^+ channels by blocking its inactivation. As a consequence of prolonged Na^+ channel activation, cells are depolarized by a sustained Na^+ influx finally leading to inexcitability (Catterall, 1980).

Apart from the main alkaloids mentioned above, other alkaloids, namely 6-benzoylheteratisine, heteratisine, 14-benzoyltalatisamine, talatisamine, 1-benzoylnapelline, napelline and songorine, are prepared from *Aconitum* species which are used as analgesics in traditional Chinese medicine (Bisset, 1981). These substances have a skeleton structure similar to aconitine, but differ by carrying various substituents. Except for 6-benzoylheteratisine, which has recently been shown to elicit antiarrhythmic and anticonvulsive effects (Heubach et al., 1997; Ameri et al., 1996a), there is only little information about the pharmacological properties of these compounds.

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The aim of the present study was to elucidate the mode of action, the antinociceptive efficacy and the acute toxicity of these alkaloids in comparison to aconitine by using different pharmacological in vitro and in vivo models. Because of their structural relationship to aconitine an interaction with Na^+ channels was suggested, which are targets of local anaesthetics, antiarrhythmics and anticonvulsants (Catterall, 1987). The affinity of these alkaloids to receptor site 2 of Na^+ channels was studied by the [^{3}H]batrachotoxinin A 20-α-benzoate binding assay (Creveling and Daly, 1992). Alterations in intracellular ion concentrations due to an interaction with Na^+ channels were detected by fluorometric measurements of free cytosolic Na^+ and Ca^{2+} concentration, ($[Na^+]_i$, $[Ca^{2+}]_i$). The antinociceptive potential of the alkaloids was investigated in the mouse formalin test by which peripheral and central activity of a drug can be detected. Referring to the toxicity, arrhythmogenic action of the alkaloids was studied on guinea-pig isolated atria and acute toxicity was evaluated in mice.

2. Materials and methods

2.1. Preparation of synaptosomes

Synaptosomes of rat cerebral cortex (male Wistar rats 180–200 g; Charles River, Sulzfeld, Germany) were prepared as recently described (Gleitz et al., 1996). For the [^{3}H]-batrachotoxinin-binding assay aliquots of the crude synaptosomal suspension were cryopreserved according to Gleitz et al. (1993). The fluorescent measurements of $[Na^+]_i$ and $[Ca^{2+}]_i$ were performed with freshly isolated crude synaptosomes. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

2.2. [^{3}H]batrachotoxinin A 20-α-benzoate binding assay

The assay was carried out as previously described with minor modifications (Gleitz et al., 1996). In competition experiments synaptosomal protein (final concentration 1 mg protein/ml) was incubated for 120 min at 37°C with tetrodotoxin (1 μM), scorpion venom (5 μg/ml), [^{3}H]batrachotoxinin A 20-α-benzoate (4 nM) and different concentrations of the alkaloids. Non-specific binding was determined in the presence of 100 μM veratridine.

2.3. Measurement of synaptosomal $[Na^+]_i$ and $[Ca^{2+}]_i$

$[Na^+]_i$ and $[Ca^{2+}]_i$ were determined by the ratio-fluorescence method with sodium-binding benzofuranisophthalate (Minta and Tsien, 1989) and calcium-binding Fura-2 (Grynkiewicz et al., 1985). The synaptosomes were suspended in incubation buffer (125 mM NaCl, 3.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5 mM $NaHCO_3$, 25 mM HEPES, 10 mM glucose, pH 7.4 at 37°C) and incubated at room temperature for 45 min with a final concentration of either 10 μM fura-2-acetoxymethylester (AM) or 15 μM sodium-binding benzofuranisophthalateacetoxymethylester and 0.071% Pluronic F127. Afterwards the suspension was washed twice with chilled washing buffer (320 mM sucrose, 1 mg/ml bovine serum albumin, 5 mM *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES)) and centrifuged at $28\,000 \times g$ for 10 min. The pellet was resuspended in incubation buffer at 37°C, recentrifuged at $10\,000 \times g$ for 5 min and stored on ice until measurement. After resuspension of the pellet 2 ml of incubation buffer (37°C) the suspension was transferred to a temperature-controlled stirred cuvette (37°C). Fluorescence measurements were carried out with a fluorescence spectrophotometer (Delta Scan, PhotoMed, Wedel, Germany) as described by Gleitz et al. (1996).

2.4. Guinea-pig isolated atria

Male Dunkin–Hartley guinea pigs (200–300 g) were killed by cervical dislocation and the heart was quickly removed. Left and right atria were cut off and fixed vertically in a 7.5 ml muscle chamber (FMI, Seeheim, Germany) containing Tyrode's solution (130 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 10 mM glucose) that was bubbled with 95% O_2 and 5% CO_2 to maintain a pH of 7.35–7.45. The temperature was maintained at 32 ± 0.5°C. The preparations were pre-stretched by about 10 mN to yield maximal contractile force. Left atria were driven by field stimulation (2 ms impulse duration, 1.5-fold threshold-current) with two platinum electrodes at 2.5 Hz, which corresponded to the spontaneous frequency of right atria (2.5 ± 0.4 Hz; $n = 23$).

Contractions were registered by an isometric transducer and recorded both by a digitizing device. After 90 min of equilibration spontaneous frequency of right atria and excitation threshold of left atria became constant. The atria were incubated with the test substances for 30–90 min and concentrations were increased cumulatively. In all experiments the final dimethylsulfoxide (DMSO) concentration was limited to 0.1% (v/v), a concentration where parameters to be measured were not affected in control experiments.

2.5. Animals used for the in vivo tests

Male NMRI mice (Iffa Credo, Brussels, Belgium) with a weight of 25–30 g were used in the formalin test and for evaluation of acute toxicity. The mice were maintained on a 12 h light–dark cycle with free access to water and food at a temperature of 23–25°C and humidity of 60 ± 10%. All experiments were conducted in a randomised manner during the light period between 7.30 and 18.00 h and were carried out in accordance with the German legislation for

the use of experimental animals and with the European Communities Council Directive of 24th November 1986 (86/609/EEC).

2.6. Formalin test

The formalin test was performed according to Dubuisson and Dennis (1977) and Coderre et al. (1993). Before drug or vehicle administration each mouse was moved to a single cage and left there for 30 min to habituate to the test environment. Afterwards the animal received i.v. (10 ml/kg) drug or vehicle administration. 5 min later the mouse was briefly restrained in a plexiglas tube allowing free access to its right hindlimb and 20 μl of formalin 5% was injected s.c. into the dorsal surface of the hindpaw. The mouse was then placed into an open cage with a large mirror mounted behind, which permitted unhindered observation of the animal. Mice were monitored individually for a period of 30 min following formalin injection using 4 behavioural categories, listed here with their category weight: 0 = normal behaviour, 1 = lifting/holding of the injected paw, 2 = flinching/shaking of the injected paw and 3 = licking/biting of the injected paw. The time during which the animal displayed a defined behaviour was recorded on line by means of a 4-channel data input to a personal computer excel programme (version 5.0). The total time spent in one of the defined behavioural states was summed up at 3 min intervals, i.e., 10 times per observation period. In addition, an average pain intensity score (pain rate, PR) was calculated according to the formula: $[T_0^* 0 + T_1^* 1 + T_2^* 2 + T_3^* 3]$:180; where T_0, T_1, T_2 and T_3, respectively, are the time in seconds the mouse spent in one of the 4 defined behavioural states and numerical values 0–3 represent the corresponding category weight. The area under the data (AUD) of the pain rate to time curves was calculated by use of the trapezoid rule.

2.7. Evaluation of acute toxicity in mice

Type, intensity and frequency of occurrence of different symptoms of drug-induced physiological and behavioural changes were monitored using a standardised protocol following a modified catalogue described by Irwin (1968). Behaviour and general condition of the animals were observed continuously up to 6 h following drug administration and were compared to normal behaviour and condition using 31 parameters. This included characterisation of the behavioural profile (awareness, mood, motor activity), neurological symptoms (central excitation, motor dis-/co-ordination, muscle tone, reflexes), autonomic peculiarities (optical signs, secretory signs, general signs), in general describing the toxic potential of a substance given to the animals. Symptoms were registered up to 1 week after drug administration. Each alkaloid was tested in 3–4 different doses with $n = 4$ per dose and the full protocol was determined in each group. An individual and qualitative description of the individual type of toxicity and cause of death for each alkaloid was obtained. Quantification of dose–response effects included definition of the highest dose tolerated without any symptoms of toxicity, definition of the lowest dose with relevant signs of toxicity, definition of the highest dose without lethality and an approximative calculation of a LD_{50} value.

2.8. Statistics and calculations

If not stated otherwise, results are expressed as mean values ± S.D. Statistical evaluation was performed by use of Student's *t*-test (unpaired, two sided). Significance was assumed when $P \leq 0.05$ (*), $P \leq 0.01$ (**) or $P \leq 0.001$ (***).

Fitting of the [^{3}H]batrachotoxinin A 20-α-benzoate binding data and calculation of the IC_{50} values were performed by employing the following equation: $y = B_0 - (B_0^* x)/(IC_{50} + x)$, where x is the concentration of the tested alkaloid, B_0 represents the specific binding of the control and y the measured value. Specific binding is defined as the difference between total binding and non-specific binding. The inhibition constant K_i was calculated according to the equation of Cheng and Prusoff (1973): $K_i = IC_{50}/(1 + [L]/K_d)$, where K_d represents the equilibrium dissociation constant of [^{3}H]batrachotoxinin A 20-α-benzoate binding ($K_d = 22.6$ nM) and [L] is the concentration of the radioligand.

Aconitine- and mesaconitine-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 alkaloid-stimulated cation concentrations, measured 15 s before and 400 s after application to synaptosomes. This interval was chosen because the cation concentrations tend to reach a steady state level and alterations in $[Na^+]_i$ and $[Ca^{2+}]_i$ about 400 s after alkaloid treatment were relatively small compared with the initial increase in $[Na^+]_i$ and $[Ca^{2+}]_i$. The $\Delta[Na^+]_i$ and $\Delta[Ca^{2+}]_i$ values were fitted employing the logic dose–response function, and EC_{50} values were calculated according to the fitted curves. 6-Benzoylheteratisine, heteratisine, 1-benzoylnapelline, napelline, songorine, 14-benzoyltalatisamine and talatisamine-induced action on the aconitine-stimulated increase in $[Ca^{2+}]_i$ was calculated as the difference between alkaloid-treated and aconitine-stimulated cation concentrations, measured 15 s before and 500 s after application of aconitine (5 μM) to synaptosomes.

Statistical evaluation of the formalin test data included a test for differences between treatments by analyses of variance (ANOVA procedure), followed by posthoc comparisons using the non-parametric Dunnett test (one-sided). All statistical analysis were performed by means of a statistical programme package (SYSTAT for Windows, Version 5.0; Systat, Evanston, IL, USA). $P \leq 0.05$ was taken to be statistically significant. Arithmetic means ± S.E.M. are given throughout with $n = 10$ per group if not

stated otherwise, ED or LD values were calculated by linear regression analysis, confidence intervals were estimated according to Litchfield and Wilcoxon (1949).

2.9. Chemicals and materials

Mesaconitine was obtained from Latoxan (Rosans, France). The other alkaloids were kindly provided by Professor O.A. Krishtal (Bogomoletz Institute of Physiology, Kiev, Ukraine). Stock solutions were prepared either in DMSO or in hydrous solution.

[^{3}H]-batrachotoxinin A 20-α-benzoate (specific activity: 1258 Gbq/mM) was purchased from Du Pont NEN Research Products (Bad Homburg, Germany). Bovine serum albumin, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), choline chloride, ethylenediaminetetraacetic acid (EDTA), HEPES, pyruvate (Na^+ salt), digitonin, ionomycin, scorpion venom (*Leiurus quinquestriatus hebraeus*), tetrodotoxin, aconitine and veratridine were obtained from Sigma (Deisenhofen, Germany). Monensin was purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Sodium-binding benzofuranisophthalateacetoxymethylester and fura-2-am were obtained from MoBiTec (Göttingen, Germany). Tris was purchased from Fluka Chemie (Buchs, Switzerland). The other chemicals were obtained from Merck (Darmstadt, Germany).

3. Results

3.1. [^{3}H]batrachotoxinin A 20-α-benzoate binding

As depicted in Fig. 1 the tested alkaloids concentration-dependently displaced [^{3}H]batrachotoxinin A 20-α-benzoate from binding site 2 of Na^+ channels. A separation into two groups of inhibition curves becomes apparent: a high affinity group with K_i values of 0.86 ± 0.04 μM (1-benzoylnapelline), 1.17 ± 0.2 μM (aconitine), 2.17 ± 0.7 μM (mesaconitine), 7.6 ± 0.9 μM (14-benzoyltalatisamine), 12.32 ± 1.7 μM (6-benzoylheteratisine) and a low affinity group with K_i values of 145.5 ± 46.9 μM (napelline), 262.6 ± 50.9 μM (heteratisine), 323.5 ± 24.8 μM (songorine) and 1526 ± 118 μM (talatisamine).

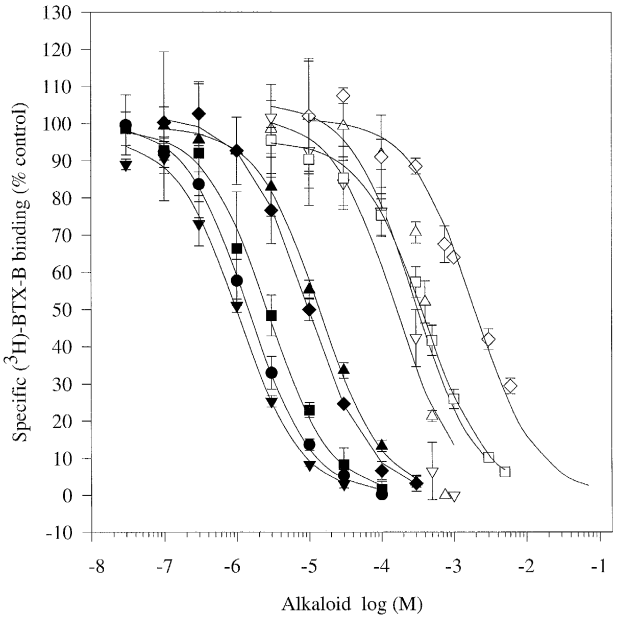

Fig. 1. The action of the alkaloids on [^{3}H]batrachotoxinin A 20-α-benzoate binding. [^{3}H]batrachotoxinin A 20-α-benzoate total binding was performed by incubating synaptosomal membranes with 4 nM [^{3}H]batrachotoxinin A 20-α-benzoate and different concentrations of alkaloids at 37°C for 120 min. The high affinity group (closed symbols): (▼) 1-benzoylnapelline, (●) aconitine, (■) mesaconitine, (◆) 14-benzoyltalatisamine, (▲) 6-benzoylheteratisine, and the low affinity group (open symbols): (▽) napelline, (△) heteratisine, (□) songorine and (◇) talatisamine. Non-specific binding of [^{3}H]batrachotoxinin A 20-α-benzoate was determined in the presence of 100 μM veratridine. The dose–response curves represent specific [^{3}H]batrachotoxinin A 20-α-benzoate binding (in % control), calculated as the difference between total binding and non-specific binding. The data represent means ± S.D. ($n = 3$).

3.2. Action of the alkaloids on $[Na^+]_i$ and $[Ca^{2+}]_i$

Aconitine and mesaconitine concentration-dependently enhanced $[Na^+]_i$ and $[Ca^{2+}]_i$ as shown in Fig. 2A and B, respectively. The ED_{50} values of aconitine- and mesaconitine-induced increases in synaptosomal $[Na^+]_i$ were calculated to be 2.31 ± 0.6 and 2.01 ± 0.5 μM, respectively. $[Ca^{2+}]_i$ was raised with ED_{50} values of 3.63 ± 0.7 μM (aconitine) and 5.19 ± 1.2 μM (mesaconitine). The other alkaloids failed to increase $[Ca^{2+}]_i$. To test whether these alkaloids may block Na^+ channels, synaptosomes were preincubated with each of them at 100 μM for 400 s before synaptosomes were stimulated with 5 μM aconitine. Fig. 3 demonstrates that 6-benzoylheteratisine, 1-benzoylnapelline and 14-benzoyltalatisamine suppress the aconitine-induced increase in $[Ca^{2+}]_i$ (in % control) to: 45.15 ± 9.4, 34.69 ± 7.5 and 33.35 ± 3.9, respectively, whereas the corresponding alkaloids, lacking the aromatic ester, did not affect the aconitine-stimulated increase of $[Ca^{2+}]_i$ (in % control): 113.03 ± 10.6 (heteratisine), 107.5 ± 5.6 (napelline), 101.53 ± 12.4 (talatisamine) and 77.32 ± 3.9 (songorine).

3.3. Antinociceptive efficacy of aconitine-like alkaloids in the mouse formalin test

S.c. injected formalin (5%) into the dorsal surface of one hindpaw induced nociception-related behaviour of three distinguishable categories: 1 = lifting/holding of the injected paw, 2 = flinching/shaking of the injected paw and 3 = licking/biting of the injected paw. Fig. 4 illustrates the time the animals behaved normal or exhibited one of these nociceptive reactions during the 30 min observation period. In mice receiving vehicle, the predominant re-

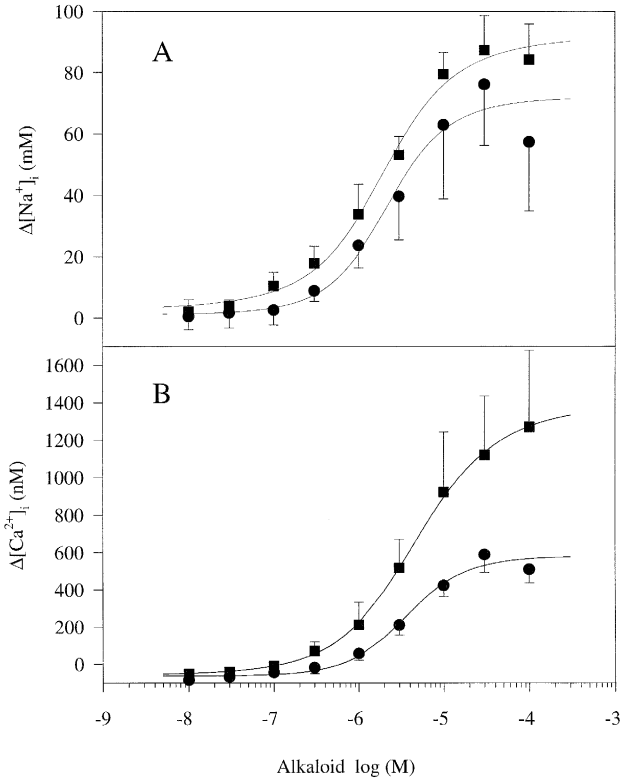

Fig. 2. Concentration-dependent action of (●) aconitine and (■) mesaconitine on $[Na^+]_i$ and $[Ca^{2+}]_i$. The concentration–response curves were obtained by calculating the alkaloid-stimulated increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ as difference between basal and alkaloid-stimulated ion concentration, expressed as $\Delta[Na^+]_i$ (A) and $\Delta[Ca^{2+}]_i$ (B). Results are shown as means ± S.D. ($n = 6$).

sponse to formalin was lifting/holding and licking/biting of the treated hindpaw, observed during 60% and 13%, respectively, of the monitored period. The remainder of time the animals either spent flinching/shaking the paw, or, 24% of the time, showed no nociceptive behaviour.

Aconitine dose-dependently reduced each of the 3 categories of nociceptive behaviour, accordingly mice treated with 30 μg/kg i.v. aconitine behaved normal during 75% of the observation period.

Based on the assumption that the different behaviours reflect different degrees of pain, the pain perception of the mice following formalin injection was assessed by calculating an average weighted intensity score (Dubuisson and Dennis, 1977; Coderre et al., 1993) for each of the ten 3 min intervals of the 30 min observation period (Fig. 5). In vehicle-treated animals the pain rate curve displayed a characteristic biphasic feature (see also Tjølsen et al., 1992). Animals treated with aconitine dose-dependently showed less nociceptive behaviour throughout the observation period, i.e., during the early, or first phase (0–15 min) and the late, or second phase (15–30 min) (Fig. 5); ED_{30} values of 17 (7–27) μg/kg for the first phase and 17 (10–24) μg/kg for the second phase were determined, the corresponding ED_{50} values were about 28 and 27 μg/kg. Further testing of higher doses was hampered by toxic effects.

The alkaloids mesaconitine, 6-benzoylheteratisine, 1-benzoylnapelline, songorine, heteratisine, 14-benzoyltalatisamine, talatisamine and napelline were tested according to the procedure already presented for aconitine. All alkaloids were dosed up to a maximum possible antinociceptive effect or up to the maximum tolerated dose at which nociception could be determined. Results are summarized in Tables 1 and 2.

Mesaconitine showed dose-dependent antinociceptive effects comparable and equipotent to those of aconitine (Table 1).

6-Benzoylheteratisine, 1-benzoylnapelline, songorine and heteratisine were tested in different doses. The lowest dose eliciting antinociception was 2.0, 21.5, 46.4 and 100 mg/kg, respectively, which in all cases was also the highest dose permitting testing for antinociception. In any case a reduction of the nociceptive behaviour of about 30–50% was observed; equipotent doses were 100- to 3000-fold higher compared to aconitine or mesaconitine.

14-Benzoyltalatisamine, talatisamine and napelline were investigated up to the highest dose permitting testing for antinociception: 10, 46.4, 68.1 mg/kg i.v., respectively, but did not show a pronounced antinociceptive effect (Table 2).

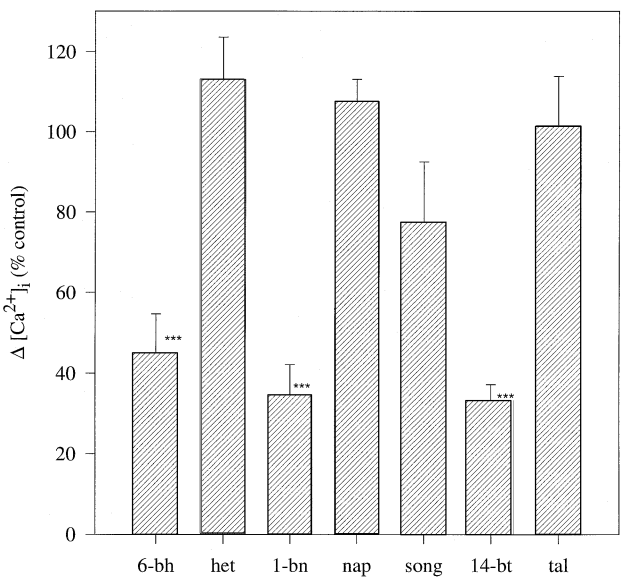

Fig. 3. Action of alkaloids on aconitine-induced increase in $[Ca^{2+}]_i$. To investigate a possible inhibiting action of alkaloids on the aconitine-stimulated increase in $[Ca^{2+}]_i$, 6-benzoylheteratisine (6-bh), 1-benzoylnapelline (1-bn), 14-benzoyltalatisamine (14-bt) and the corresponding non-aromatic alkaloids, heteratisine (het), napelline (nap), songorine (song) and talatisamine (tal), were applied to synaptosomes at a concentration of 100 μM 400 s before aconitine (5 μM) addition. The aconitine-stimulated increase in $[Ca^{2+}]_i$ was calculated as the difference between alkaloid-treated and aconitine-induced cation concentration, measured 15 s before and 500 s after aconitine application. Results are expressed as means ± S.D. ($n = 6$). Differences of means were assumed significant (Student's t-test) if $P \leq 0.001$ (***).

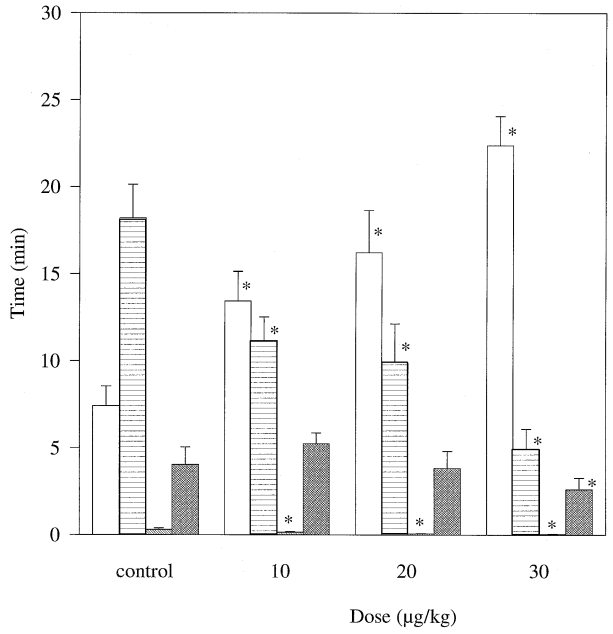

Fig. 4. Antinociceptive effect of aconitine in the formalin test in mice. Effect of aconitine on formalin-induced nociceptive behaviour in mice. Five min following i.v. administration of aconitine, 20 μl of formalin (5%) was injected s.c. into the dorsal surface of the right hindpaw. During subsequent 30 min each animal was monitored for nociception-related behaviour using 4 categories: 0 = normal behaviour (open columns), 1 = lifting/holding of the injected paw (horizontal striped columns), 2 = flinching/shaking of the injected paw (right-hatched columns) and 3 = licking/biting of the injected paw (cross-hatched columns). The overall time the animals behaved according to these categories was recorded in each group. Means ± S.E.M. of 10 animals per group, differences compared to vehicle group: $^{*}P \leq 0.05$.

Used in the doses indicated in Tables 1 and 2, none of the drugs changed the motor function as assessed in the rota rod standard procedure (according to Dunham and Miya, 1957; data not shown) or general behaviour of the mice.

3.4. Acute toxicity of aconitine-like alkaloids in mice

All substances showing antinociceptive efficacy and napelline were assessed for acute toxicity. The individual dose range tested for each substance and the results of quantification of dose response effects are given in Table 3.

The aconitine-induced toxicity can be characterized as follows: first symptoms (46.4 μg/kg i.v.) were enhanced and irregular respiratory activity, exophthalmia, Straub reaction, convulsions, disturbed locomotion activity, mydriasis and loss of body weight. At higher doses (68.1 μg/kg i.v.) gasping for breath, convulsions of higher intensity and frequency, lacrimation, salivation, reduced locomotion, loss of orientation, ventral or ventro-lateral recumbency and loss of different reflexes were observed additionally. At 100 μg/kg i.v. additionally muscle relaxation occurred and 2 out of 4 animals died shortly after administration; 147 μg/kg i.v. aconitine induced exophthalmia, convulsions, gasping for breath, ventral recumbency and cyanosis and the animals died within 1 min following drug administration.

Following administration of the alkaloids, mesaconitine, 6-benzoylheteratisine, 1-benzoylnapelline, songorine, heteratisine and napelline the qualitative findings were similar to those of aconitine. Quantitative differences between the different alkaloids are summarized in Table 2. The differences between doses leading to toxicologically relevant symptoms were remarkable: from as little as 0.005 mg/kg for aconitine up to 68.1–100 mg/kg for heteratisine, songorine or napelline, reflecting the individual dose range needed for eliciting antinociception (see Table 3 versus Table 1 or Table 2, respectively). For receptor affinity and pharmacological potencies of different aconitine-like alkaloids, see Table 4.

The approximative therapeutic index (LD_{50}/ED_{30AUD1}) was calculated for the alkaloids: 6.0 (aconitine), 3.7 (mesaconitine) and based on single dose antinociceptive effects: 1.1 (6-benzoylheteratisine), 1.5 (1-benzoylnapelline), 2.3 (songorine) and 1.6 (heteratisine).

3.5. Arrhythmogenic action of the alkaloids on guinea-pig isolated atria

In order to determine the threshold for the induction of arrhythmia and to characterize the type of arrhythmia left

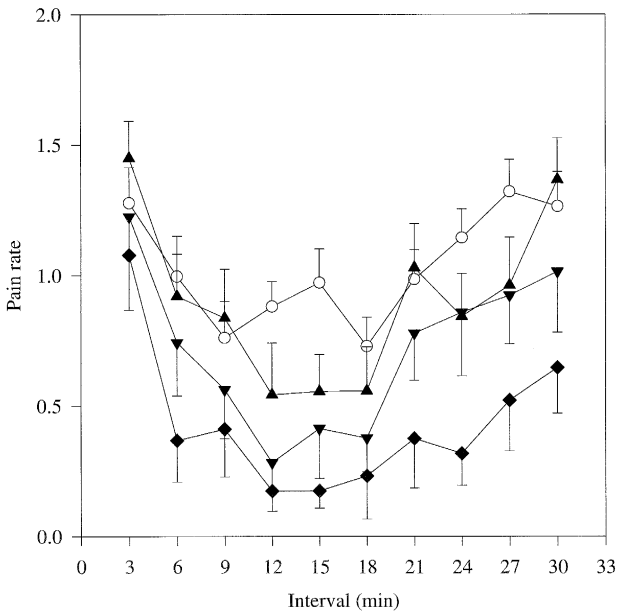

Fig. 5. The time-course of formalin-induced nociceptive behaviour in mice. Effect of aconitine on the time-course of formalin-induced nociceptive behaviour in mice. Five min following i.v. administration of vehicle (○) or aconitine; 10 μg/kg (▲), 20 μg/kg (▼), 30 μg/kg (◆), 20 μl of formalin (5%) was injected s.c. into the dorsal surface of the right hindpaw. During subsequent 30 min each animal was monitored for nociception-related behaviour (see Fig. 4), and an average pain intensity score (pain rate, PR) was calculated. Means ± S.E.M. of 10 animals per group, differences compared to vehicle group: $^{*}P \leq 0.05$.

Table 1
Antinociceptive effect of aconitine and mesaconitine in the formalin test

Group	Dose (mg/kg) i.v.	Early phase) (0–15 min) AUD (sec * score)	% Change to control	Late phase (15–30 min) AUD (sec * score)	% Change to control
Control 1		879.4 ± 60.9		979.1 ± 48.2	
Aconitine	0.010	774.9 ± 91.5	−11.9	856.5 ± 100.7	−12.5
	0.020	580.9 ± 122.5 [a]	−33.9	711.5 ± 117.0	−27.3
	0.030	397.4 ± 103.0 [a]	−64.8	377.5 ± 98.6 [a]	−61.4
ED_{30} (mg/kg)	0.017 (0.007–0.027)			0.017 (0.010–0.024)	
ED_{50} (mg/kg)	ca. 0.028			ca. 0.027	
Control 2		871.4 ± 58.5		1023.2 ± 54.9	
Mesaconitine	0.010	941.0 ± 104.2	+8.0	1087.9 ± 40.9	+6.3
	0.020	594.5 ± 89.7 [a]	−31.8	781.3 ± 103.4 [a]	−23.6
	0.030	305.5 ± 66.4 [a]	−64.9	324.2 ± 55.5 [a]	−68.3
ED_{30} (mg/kg)	0.018 (0.015–0.022)			0.019 (0.016–0.021)	
ED_{50} (mg/kg)	0.025 (0.021–0.034)			0.025 (0.022–0.031)	

Effect of different aconitine-type alkaloids on formalin-induced nociceptive behaviour in mice. 5 min following i.v. administration of the alkaloid, 20 μl of formalin (5%) was injected s.c. into the dorsal surface of the right hindpaw. During subsequent 30 min each animal was monitored for nociception-related behaviour and an average pain intensity score (pain rate) was calculated. The area under the data (AUD) of the pain rate to time curves was calculated by use of the trapezoid rule. Data are shown as means ± S.E.M. of 10 animals per group, differences compared to vehicle group: [a] $P \leq 0.05$.

Table 2
Antinociceptive effect of different aconitine-type alkaloids in the formalin test

Group	Dose (mg/kg) i.v.	Early phase (0–15 min) AUD (sec * score)	% Change to control	Late phase (15–30 min) AUD (sec * score)	% Change to control
Control 3		1026.0 ± 62.4		1068.9 ± 89.0	
6-Benzoylheteratisine	2.0	695.3 ± 68.4 [a]	−32.2	1158.1 ± 50.2	+8.3
Control 4		1112.8 ± 38.9		1158.5 ± 40.1	
1-Benzoylnapelline	21.5	734.7 ± 99.5 [a]	−34.0	960.2 ± 107.4 [a]	−17.1
Control 5		1117.8 ± 41.8		1154.9 ± 38.1	
Songorine	46.4	792.0 ± 51.7 [a]	−29.1	1065.0 ± 38.6	−7.8
Control 6		1086.2 ± 30.6		1190.4 ± 48.5	
Heteratisine	100.0	561.0 ± 87.6 [a]	−48.4	736.1 ± 110.3	−38.2
Control 7		1143.2 ± 42.4		1216.8 ± 29.0	
14-Benzoyltalatisamine	10.0	999.7 ± 18.8 [a]	−12.6	1187.6 ± 39.7	−2.4
Control 8		1156.4 ± 35.7		1217.1 ± 29.0	
Talatisamine	46.4	1110.5 ± 41.7	−4.0	1321.2 ± 47.7 [a]	+8.6
Control 9		1085.3 ± 33.0		1129.0 ± 37.3	
Napelline	68.1	1019.7 ± 34.4	−6.0	1148.1 ± 57.2	+1.7

For legends see Fig. 1. Data are shown as means ± S.E.M. of 10 animals per group, differences compared to vehicle group: [a] $P \leq 0.05$.

Table 3
Acute toxicity of different aconitine-like alkaloids

Drug	Dose range tested i.v. (mg/kg)	Lowest dose with relevant signs of toxicity (mg/kg)	Highest dose without lethality (mg/kg)	Approximative LD_{50} value (95% confidence limit) (mg/kg)
Aconitine	0.0464–0.147	0.0464	0.0681	0.100 (0.860–0.116)
Mesaconitine	0.0316–0.100	0.0316	0.0464	0.0681 (0.059–0.079)
6-Benzoylheteratisine	1.47–4.64	2.15	2.15	2.15–3.16
1-Benzoylnapelline	14.7–46.4	21.5	31.6	31.6–46.4
Songorine	46.4–147	68.1	68.1	106 (94–121)
Heteratisine	68.1–147	68.1	100	147 (127–171)
Napelline	68.1–147	100	147	> 147

Evaluation of acute toxicity of different aconitine-like alkaloids in mice. Behaviour and general condition of the animals were monitored continuously up to 6 h following administration of the alkaloid. Each substance was tested in 3–4 different doses with $n = 4$ per dose.

Table 4
Receptor affinity and pharmacological potencies of different aconitine-like alkaloids

Drug	Receptor affinity K_i value (μM ± S.D.)	Antinociceptive effect ED_{50} value (mg/kg), single dose (mg/kg), respectively	Acute toxicity LD_{50} value (mg/kg) (95 % confidence limit)
Mesaconitine	2.17 ± 0.7	0.025	0.0681 (0.059–0.079)
Aconitine	1.17 ± 0.2	0.028	0.1 (0.86–0.116)
6-Benzoylheteratisine	12.32 ± 1.7	2.0	2.15–3.16
1-Benzoylnapelline	0.86 ± 0.04	21.5	31.6–46.4
14-Benzoyltalatisamine	7.6 ± 0.9	10.0	n.d.
Songorine	323.5 ± 24.8	46.4	106 (94–121)
Heteratisine	262 ± 50.9	100	147 (127–171)
Napelline	145.5 ± 46.9	68.1	> 147
Talatisamine	1526 ± 118	46.4	n.d.

and right guinea-pig isolated atria were incubated at increasing alkaloid concentrations.

The arrhythmogenic potential of mesaconitine was slightly higher than that of aconitine (6–10 and 10 nM, respectively; $n = 12$) and there was a good correlation between left and right atria. The pattern of arrhythmia was also very similar: as the individual arrhythmogenic concentration was reached, a transient increase of contractile force could be observed in both atria. At 10 nM mesaconitine and 20 nM aconitine the force of contraction of still regularly beating left atria was increased by about 40%. After some minutes this positive inotropic effect shifted abruptly into an arrhythmia characterized by tachycardic and strongly irregular contractions.

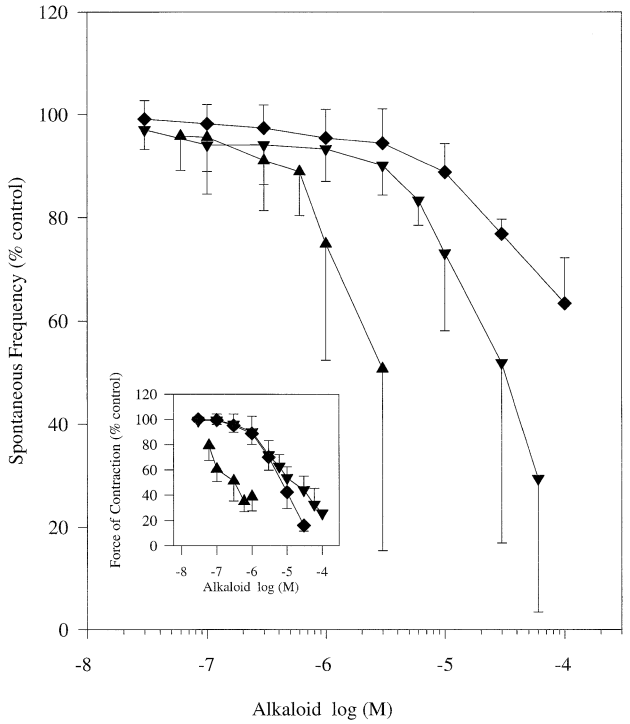

Fig. 6. Effect of the alkaloids on guinea-pig isolated atria. Effects of (▲) 6-benzoylheteratisine, (▼) 1-benzoylnapelline and (♦) 14-benzoyltalatisamine on spontaneous frequency were monitored on right atria. At control atria spontaneous frequency remained constant during experiments. Data are shown as mean values ± S.D. ($n = 4$–6). The influence of 6-benzoylheteratisine, 1-benzoylnapelline and 14-benzoyltalatisamine on force of contraction was investigated on left atria (inset). Data are shown as mean values ± S.D. ($n = 4$–6).

In contrast to the two former alkaloids, 1-benzoylnapelline and 14-benzoyltalatisamine showed a negative inotropic effect and caused bradycardic forms of arrhythmia (Fig. 6) as previously described for 6-benzoylheteratisine (Heubach et al., 1997). 1-Benzoylnapelline caused asystolia at a concentration of 30 μM in one out of four right atria, while 14-benzoyltalatisamine did not induce asystolia up to the highest concentration tested in our experiments (100 μM, $n = 4$).

The other alkaloids, namely heteratisine, talatisamine, napelline and songorine did not induce arrhythmia and had no major effects on force of contraction and spontaneous frequency up to a concentration of 100 μM.

4. Discussion

The data of the present study suggest a separation of the investigated alkaloids into two groups: (1) Na^+ channel activators and (2) Na^+ channel inhibitors. The first group is represented by aconitine and mesaconitine. The high affinity binding to site 2 of Na^+ channels is reflected by their small K_i values evaluated in the [^{3}H]batrachotoxinin A 20-α-benzoate binding assay. Consistent with a Na^+ channel activation by these compounds is the increase in $[Na^+]_i$ in synaptosomes, which subsequently raises $[Ca^{2+}]_i$ (Taylor and Meldrum, 1995). In line with this finding is the transient enhancement of contractile force of guinea-pig isolated atria by the two alkaloids. This effect is suggested to be due to a prolonged Na^+ influx, accompanied by an increase in $[Ca^{2+}]_i$, thereby enhancing atrial contractility. This mode of action agrees with the finding of Honerjäger and Meissner (1983) who demonstrated a positive in-

otropic action of aconitine on guinea-pig papillary muscle. Na^+ channel activation by aconitine and mesaconitine seems also to be responsible for their extremely high toxicity. Toxicological studies demonstrated that these alkaloids elicited a wide range of physiological responses as salivation, respiratory paralysis, muscular weakness and convulsions in different animal species (Benn and Jacyno, 1983). In the current study, administration of aconitine and mesaconitine in mice provoked similar symptoms of intoxication. The toxic potential of both alkaloids is reflected by their low LD_{50} values which are in good accordance with the LD_{50}s reported by others (Benn and Jacyno, 1983), the high affinity to site 2 of Na^+ channels and the high efficacy to induce an increase in synaptosomal $[Na^+]_i$ and tachyarrhythmia in atria, suggesting for both alkaloids that Na^+ channel activation is the primary target for their toxicity.

According to Tjølsen et al. (1992) the biphasic time-course of nociceptive behaviour in the formalin test is related to two distinctly different stimuli: a direct chemical stimulation of nociceptors during the first phase, and during the second phase a combination of peripheral inflammation and changes in spinal processes which are initiated in the first phase. The expression of the first phase and the local inflammatory changes are both necessary for the complete manifestation of the second phase of nociception. In the present study, aconitine and mesaconitine induced a suppression of both phases in the mouse formalin test. Based on the synaptosomal data which demonstrated a Na^+ influx induced by aconitine and mesaconitine, we suggest that both alkaloids might inhibit neuronal transmission due to a permanent depolarization of neurons, thus suppressing the formalin-induced behavioural changes. This hypothesis is supported by recent investigations on rat hippocampal slices which demonstrated an inhibitory action of aconitine on neuronal activity following an initial neuronal activation (Ameri et al., 1996b). Since inhibition was proportional to the frequency of neuronal activity (use-dependent) (Ameri et al., 1996b) the formalin-stimulated and therefore activated nociceptors may be regarded as a preferred target of aconitine. However, a specific antinociceptive action of aconitine and mesaconitine is questionable because of the low LD_{50}/ED_{50} values, suggesting that a severe intoxication may be responsible for their antinociceptive properties.

Concerning the structural relationship to aconitine the alkaloids, 6-benzoylheteratisine, heteratisine, 14-benzoyltalatisamine, talatisamine, 1-benzoylnapelline, napelline and songorine were supposed to act on Na^+ channels. In the current study, this was proved by using the [^{3}H]batrachotoxinin A 20-α-benzoate binding assay. The evaluated K_i values of these alkaloids revealed a separation into a high and low affinity group suggesting that binding to receptor site 2 of Na^+ channels is correlated with the aromatic ester moiety. This grouping is consistent with their pharmacological effects, thus indicating that the arylester group seems to be important for exerting pharmacological activity.

An exception are heteratisine and songorine which showed weak antinociceptive effects in the formalin test. These alkaloids reduced the nociceptive behaviour in the mouse formalin test to 30–50%, but at doses which were 2000- to 5000-times higher than those of aconitine.

An interesting finding is the different action of the two alkaloids on the two phases of the formalin test. Songorine suppressed only the first phase by about 30%, suggesting a central site of action. This effect is consistent with results reported by Benn and Jacyno (1983) showing songorine to have significant effects on central nervous system activity. In contrast to songorine, heteratisine induced a suppression of both phases like aconitine and mesaconitine. However, songorine and heteratisine failed to affect synaptosomal $[Na^+]_i$ and $[Ca^{2+}]_i$ and did not have any effect on isolated atria, suggesting that both alkaloids did not modulate Na^+ channels.

Despite their high affinity to binding site 2 of Na^+ channels, 6-benzoylheteratisine, 1-benzoylnapelline and 14-benzoyltalatisamine showed quite different pharmacological effects than aconitine and mesaconitine. These alkaloids significantly inhibited the aconitine-stimulated $[Ca^{2+}]_i$ increase which seems to be attributable to a Na^+ channel inhibition. Consistent with this finding is their bradycardic and negative inotropic action on guinea-pig isolated atria supporting the idea that Na^+ channels are blocked by these compounds. This hypothesis is in line with results of a recent electrophysiological investigation on guinea-pig isolated papillary muscles with 6-benzoylheteratisine (Heubach et al., 1997). It seems to be reasonable that the negative inotropic effects of these compounds were caused by a Na^+ channel inhibition, resulting in a decrease in $[Ca^{2+}]_i$. In the formalin test in mice 14-benzoyltalatisamine showed only a weak reduction of the nociceptive behaviour, whereas 6-benzoylheteratisine and 1-benzoylnapelline significantly reduced the first or both phases, respectively. However, like aconitine and mesaconitine, the Na^+ channel blocking alkaloids are characterized by low $LD_{50}/ED50$ values in the range of 1–6, suggesting that also in this case a severe intoxication may be due to suppressed behavioural changes in the formalin assay.

In summary, the in vitro data indicate that the investigated alkaloids can be grouped in Na^+ channel activators and blockers. Their affinities to Na^+ channels correlate with their effective doses determined for acute toxicity, suggesting a modulation of Na^+ channels responsible for intoxication. Because of the low LD_{50}/ED_{50} quotients in the range of 1 to 6, specific antinociceptive properties of the alkaloids are questionable and may be due to severe intoxication. Concerning the structure–activity relationship, it is concluded that the aromatic ester group is important for a binding to epitope 2 of Na^+ channels, responsible for the antinociceptive and toxic action.

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References

Ameri, A., Gleitz, J., Peters, T., 1996a. Inhibition of neuronal activity in rat hippocampal slices by *Aconitum* alkaloids. Brain Res. 738, 154–157.

Ameri, A., Shi, Q., Aschoff, J., Peters, T., 1996b. Electrophysiological effects of aconitine in rat hippocampal slices. Neuropharmacology 35, 13–22.

Benn, M.H., Jacyno, J.M., 1983. The toxicology and pharmacology of diterpenoid alkaloids. In: Pelletier, S.W. (Ed.), Alkaloids: Chemical and Biological Perspectives. John Wiley and Sons, New York, NY, pp. 153–210.

Bisset, N.G., 1981. Arrow poisons in China. Part II. *Aconitum*: Botany, chemistry and pharmacology. J. Ethnopharmacol. 4, 247–336.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgramm quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Catterall, W.A., 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu. Rev. Pharmacol. Toxicol. 20, 15–43.

Catterall, W.A., 1987. Common modes of drug action on Na^+ channels: Local anesthetics, antiarrhythmics and anticonvulsants. Trends Pharmacol. Sci. 8, 57–65.

Cheng, Y.-C., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Coderre, T.J., Fundytus, M.E., McKenna, J.E., Dalal, S., Melzack, R., 1993. The formalin test: A validation of the weighted-scores method of behavioural pain rating. Pain 54, 43–50.

Creveling, C.R., Daly, J.W., 1992. Batrachotoxinin A [^{3}H]benzoate binding to sodium channels. In: P.M. Conn (Ed.), Methods in Neurosciences. Academic Press, San Diego, CA, pp. 25–37.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: A quantitative study of the analgesic effects of morphine, meperidine and brain stem stimulation in rats and cats. Pain 4, 161–174.

Dunham, N.W., Miya, T.S., 1957. A note on a simple apparatus for detecting neurological deficit in rats and mice. J. Am. Pharm. Assoc. 46, 208–209.

Gleitz, J., Beile, A., Wilffert, B., Tegtmeier, F., 1993. Cryopreservation of freshly isolated synaptosomes prepared from the cerebral cortex of rats. J. Neurosci. Methods 47, 191–197.

Gleitz, J., Friese, J., Beile, A., Ameri, A., Peters, T., 1996. Anticonvulsive action of (±)-kavain estimated from its properties on stimulated synaptosomes and Na^+ channel receptor sites. Eur. J. Pharmacol. 315, 89–97.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Heubach, J.F., Heer, F., Wilhelm, D., Peters, T., 1997. 6-Benzoylheteratisine: A class-I antiarrhythmic substance from *Aconitum tanguticum* (Maxim.) Stapf. Phytomedicine, in press.

Hikino, H., Murayama, M., 1985. Mechanism of the antinociceptive action of mesaconitine: Participation of brain stem and lumbar enlargement. Br. J. Pharmacol. 85, 575–580.

Hikino, H., Ito, T., Yamada, C., Sato, H., Konno, C., Ohizumi, Y., 1979. Analgesic principles of *Aconitum* roots. J. Pharm. Dyn. 2, 78–83.

Honerjäger, P., Meissner, A., 1983. The positive inotropic effect of aconitine. Naunyn-Schmiedebergs Arch. Pharmacol. 322, 49–58.

Irwin, S., 1968. Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioural and physiologic state of the mouse. Psychopharmacologia 13, 222–257.

Litchfield, J.T., Wilcoxon, F. Jr., 1949. A simplified method of evaluating dose–effect experiments. J. Pharmacol. Exp. Ther. 95, 99–113.

Minta, A., Tsien, R.Y., 1989. Fluorescent indicators for cytosolic sodium. J. Biol. Chem. 264, 19449–19457.

Murayama, M., Hikino, H., 1985. Effect of cyclic AMP on mesaconitine-induced analgesia in mice. Eur. J. Pharmacol. 108, 19–23.

Murayama, M., Ito, T., Konno, C., Hikino, H., 1984. Mechanism of analgesic action of mesaconitine. I. Relationship between analgesic effect and central monoamines or opiate receptors. Eur. J. Pharmacol. 101, 29–36.

Oyama, T., Isono, T., Suzuki, Y., Hayakawa, Y., 1994. Anti-nociceptive effects of *Aconiti* tuber and its alkaloids. Am. J. Chin. Med. 22, 175–182.

Suzuki, Y., Oyama, T., Ishige, A., Isono, T., Asami, A., Ikeda, Y., Noguchi, M., Omiya, Y., 1994. Antinociceptive mechanism of the aconitine alkaloids mesaconitine and benzoylmesaconine. Planta Med. 60, 391–394.

Taylor, C.P., Meldrum, B.S., 1995. Na^+ channels as targets for neuroprotective drugs. Trends Pharmacol. Sci. 16, 309–316.

Tjølsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: An evaluation of the method. Pain 51, 5–17.