Differential effects of alkaloids on sodium currents of isolated single skeletal muscle fibers

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Abstract The effects of the alkaloids ajmaline, lupanine, sparteine, serpentine, strychnine, and yohimbine were studied with the loose patch clamp technique on sodium currents of isolated single skeletal muscle fibers. The IC_{50} values for half-maximal blocking of the sodium currents were 6.6 μM for ajmaline, 55.7 μM for quinidine, 168.8 μM for sparteine, and 1.2 mM for lupanine. The observed Na^+ channel inhibition is in accordance with the use of ajmaline, quinidine and sparteine as antiarrhythmic drugs. The interference of alkaloids with Na^+ channels can also be interpreted as a means to strongly interfere with neuronal transmission in herbivores. Alkaloids thus serve as chemical defense compounds for the plants producing them.

Key words: Ajmaline; Lupanine; Sparteine; Yohimbine; Strychnine; Na^+ channel; Inhibition

1. Introduction

With more than 12 000 known structures alkaloids represent one of the largest groups of secondary metabolites of plants. There is good evidence that many of them serve as chemical defense compounds for the plants producing them against herbivorous animals or pathogens (for reviews see [1–4]). Since these natural products have apparently evolved as active metabolites some of them have useful medicinal properties and can be used therapeutically [5].

A few alkaloids, such as quinidine, ajmaline and sparteine (Fig. 1), are being used as antiarrhythmic agents to treat cardiac disorders. It has been deduced from pharmacological and electrophysiological studies that the mode of action is via a reduction of the Na^+ inward current, e.g. during the upstroke of cardiac action potentials. Though alkaloids have been comparatively tested on neuroreceptors, DNA intercalation, inhibition of DNA and RNA enzymes and inhibition of protein biosynthesis (see Table 1 and Section 4), there is still very little known about the differential action of alkaloids on voltage dependent ion channels which are responsible for the excitability of, for instance, nerve, heart smooth muscle and skeletal muscle cells. Therefore, to elucidate the mode of action of the alkaloids quinidine, ajmaline and sparteine on membrane excitability, we determined sodium currents in isolated muscle fibers by 'loose' patch clamp measurements [6–9]. The 'loose' patch clamp method allows to faithfully and reversibly measure voltage dependent Na^+ (and also K^+) currents from small patches (several μm diameter) of sarcolemma with a patch electrode in the 'cell attached' mode on (enzymatically) isolated single muscle fibers [9]. Thus, the method is ideally suited for detailed studies of the differential effects of drugs on voltage dependent ion channels. It should also be noted that to our knowledge none of these alkaloids, which included serpentine, lupanine, strychnine and yohimbine, were quantitatively tested on voltage dependent currents of skeletal muscle at all. Since the quinolizidine alkaloid lupanine is chemically related to sparteine it was of interest to us whether this alkaloid also inhibits Na^+ channels in skeletal muscle. The monoterpene indole alkaloids serpentine, strychnine and yohimbine were assayed as internal controls since they share structure similarities with ajmaline.

2. Materials and methods

2.1. Preparation of enzymatically isolated muscle fibers

Single muscle fibers (Mm. lumbricales) were obtained from the frog *Xenopus laevis*. The frogs were killed by decapitation. After dissection the muscles were incubated for 1 h at 29°C in normal Ringer solution (composition see below) plus 1 mg/ml collagenase, type IA (Sigma, Germany). Then the muscles were placed in another beaker for 2 min, which contained normal Ringer solution to wash off the enzyme. Following the collagenase treatment the muscles were incubated for 1 h in the presence of protease, type XXIV (0.2 mg/ml; Sigma, Germany) at room temperature (21–22°C) and rinsed 10 times with normal Ringer solution for removal of the enzyme. The muscles were then mechanically dissected by sucking them gently in a fire polished Pasteur pipette.

2.2. Solutions and alkaloids

Normal Ringer solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl and 5 mM HEPES, pH 7.2. High Ca^{2+}-Ringer contained 115 mM NaCl, 2.5 mM KCl, 4.9 mM CaCl and 5 mM HEPES, pH 7.2. Ringer with K^+-channel blockers contained 20 mM tetraethylammonium chloride (TEA-Cl), 10 mM CsCl and 2 mM 4-aminopyridine (4-AP). Ajmaline, quinidine, sparteine, serpentine, strychnine and yohimbine were from Sigma, Germany. Lupanine was isolated from *Lupinus albus* in our laboratory [10]. All compounds were of highest purity, as determined by HPLC and GLC. Sparteine and quinidine were dissolved in 5% DMSO. Ajmaline was directly added to the Ringer solution in the required concentration. For the other alkaloids Ringer stock solutions were prepared which either contained alkaloids in 10 mM (lupanine and sparteine) or 1 mM concentration (quinidine, strychnine and yohimbine). The pH was adjusted with HCl or NaOH.

2.3. Loose patch-clamp technique

The 'loose patch' clamp technique used to measure membrane currents has been described in detail elsewhere [6,9]. Micropipettes were pulled from borosilicate glass (Science Products, Germany) with a outer diameter of 1.5 mm, using a Flamming-Brown micropipette puller P-87, and were fire polished with a custom made microforge. Patch pipettes had a tip resistance between 200 and 900 kΩ and an inner tip diameter between 6 and 20 μm. Membrane currents were recorded with a Bio-Logic RK300 patch clamp amplifier. No filters were used. Data were acquired by a Tl-1 or Digidata 2000 interface (Axon Instruments, USA) and were analyzed on a 486 PC using pClamp version 6.03 software (Axon Instruments). The sampling frequency was set to 100 kHz. Leak currents and capacitive currents were on-line corrected using the P/N-method. In some cases the

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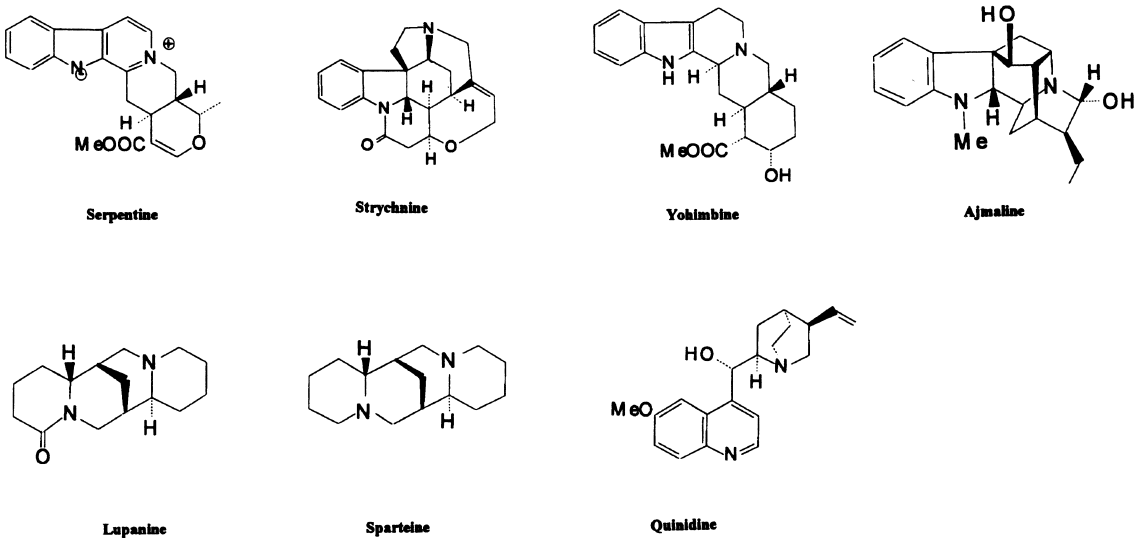

Fig. 1. Chemical structures of the alkaloids serpentine, strychnine, yohimbine, ajmaline, lupanine, sparteine and quinidine.

leak and capacity compensation circuits of the amplifier were used. To establish the dose-dependence of alkaloids the following protocol was carried out: the high Ca^{2+}-Ringer solution was used to avoid a run down of the Na^+-current [9]. Seals were formed by pressing the pipette gently on the surface of the muscle fiber. A first test pulse train with five depolarizing pulses (generally from 99 to 120 mV micropipette potential: relative to that of the grounded bath solution) was applied to obtain a family of Na^+ inward currents (Fig. 1A). Thereafter the chamber was rinsed for 1 min with the alkaloid containing solution, washing the chamber volume at least 10 times. Then test pulses (generally 106–120 mV in 7-mV steps) were given 9–20 times every 20 or 30 s. The chamber was then rinsed for another minute to wash the chamber volume about 20 times assuring that at least 99% of the solution was changed. The electrical seal resistances were measured before the pulse train at 0 min and before the train after 1 min and after the last train. In some experiments we washed out the alkaloid with Ringer solution to test for the reversibility of the applied drugs. Then, pulse trains were applied during the wash-out-phases every minute or at longer times. In some experiments the K^+-channels were blocked by TEA-Cl, Cs-Cl and 4-AP (see Section 2.2). The correcting factor A for tip potential was always grater than 0.85 (see [6]).

2.4. Data analysis

Currents are expressed as the fraction of I_n/I_0, where I_0 is the current amplitude before drug application and I_n is the current measured with the nth applied pulse trains. The dose-dependence curves were fitted to the Hill equation

$$I/I_0 = 1/(1 + (x/K_i)^h \quad (1)$$

where x is the concentration of the inhibitor, h is the Hill coefficient and K_i gives the half-maximal inhibition.

3. Results

Fig. 2A shows a family of sodium inward currents (I_{Na}) recorded from the surface membrane of the enzymatically isolated lumbricalis muscle fibers at different test potentials. The activation and inactivation pattern of the sodium current is similar to that reported for other amphibian muscle fibers [11]. Also, the inactivation curves shown in Fig. 2B for the sodium currents closely resemble the h_∞-curves described by others under similar recording conditions [12,11]. A typical current-voltage relation (I-V-plot) for the inward sodium currents in our preparation is shown in Fig. 2C. The method allows a faithful recording of the peak sodium currents occurring at −10–0 mV. Some deviation from the expected current-

Table 1
Interaction of antiarrhythmic alkaloids with molecular targets (after [5,10,22–24])

Target	Ajmaline	Lupanine	Quinidine	Sparteine	Strychnine	Yohimbine
Neuroreceptors						
Adrenergic (α_1) IC_{50} μM	n.d.	> 500	29.7	> 500	25.1	n.d.
Adrenergic (α_2) IC_{50} μM	n.d.	> 500	1.3	127.7	172.3	n.d.
Serotonin (5-HT_2) IC_{50} μM	n.d.	> 500	14.4	> 500	51.6	n.d.
Acetylcholine (mACh) IC_{50} μM	n.d.	118.0	18.4	21.3	32.8	n.d.
Acetylcholine (nACh) IC_{50} μM	n.d.	5.3	> 500	330.8	10.2	n.d.
Butylcholine esterase (BChE) IC_{50} μM	n.d.	> 500	111.1	165.5	130.8	n.d.
DNA intercalation						
Increase in melting temp. (T_m °C)	2.3	0	8	0	n.d.	n.d.
Release of methyl green at 5 mM	33	0	88	3	3	2
Inhibition of DNA and RNA related enzymes						
Inhibition of DANN polymerase I (IC_{50} mM)	> 10	> 10	2.4	> 10	> 10	> 10
Inhibition of reverse transcriptase (IC_{50} mM)	7	> 10	1	> 10	7	> 10
Inhibition of protein biosynthesis						
In vitro translation at 1 mM (%)	40	0	63	0	20	59

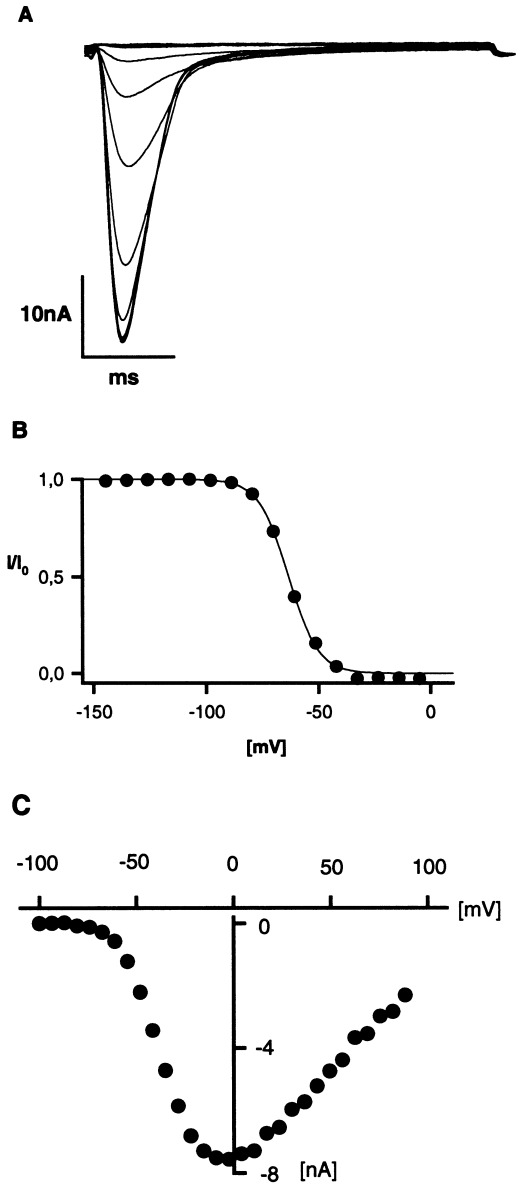

Fig. 2. Na+ inward currents from cell attached sarcolemma patches of skeletal muscle. A and B: h_∞-curve: prepulses of 90 ms duration from −144.5 up to −4.9 mV were given in 9.3-mV steps, then a second pulse to −9.6 mV (4.5 ms) was given. $R_S = 5.62$ kΩ, $R_P = 418$ MΩ and $A = 0.93$, membrane potential $E_m = -98$ mV. h_∞-curve was fitted after Bolzmann with 0.5 $h_\infty = -63.4$ mV and the slope is 6.55. C: *I-V*-plot for a Na^+-current. Seal resistance, $R_S = 5.21$ MΩ and pipette resistance $R_P = 400$ kΩ, correcting factor $A = 0.93$. Thirdly, pulses of 4.4 ms duration were given in 6.5-V steps,from −100 up to 88.5 mV, membrane potential $E_m = -100$ mV. Membrane potentials were measured after the pulse protocol with a microelectrode filled with 3 M KCl. K^+-channels were blocked (see Section 2).

voltage relation for I_{Na} is seen for depolarizations larger than +30 mV.

The inhibitory effects of the different alkaloids ajmaline, quinidine, sparteine, and lupanine on the peak sodium inward currents are summarized in Fig. 3A–D. All curves are normalized to the initial control current and were fitted with modified Hill equations. Most powerful inhibitory effects were observed with ajmaline (Fig. 3A), whereas quinidine was about 10-fold less effective (Fig. 3B). The Hill curve describing the sparteine effects was further shifted to higher concentrations than those of quinidine for similar inhibition (Fig. 3C). The least effective alkaloid to block sodium currents was lupanine, shown in Fig. 3D.

For a more detailed analysis the alkaloids serpentine, strychnine and yohimbine were tested. The comparative effects on the relative peak sodium currents at a given concentration of 10 μM for each substance are given in Fig. 4A. As DMSO was used to dissolve some of the substances it was also tested for a potential inhibitory effect. In comparison to the control values neither DMSO nor serpentine showed any significant effect on the peak sodium currents at 10 μM concentration. Whereas sparteine was only hardly inhibitory at 10 μM, quinidine, strychnine, yohimbine, and ajmaline exhibited a more pronounced Na^+-channel inhibition, with ajmaline as the most potent Na^+ channel blocker tested in our study.

The IC_{50} values of ajmaline ($IC_{50} = 6.6$ μM) in comparison to lupanine ($IC_{50} = 1.2$ mM), quinidine ($IC_{50} = 55.7$ μM) and sparteine ($IC_{50} = 168.8$ μM) were evaluated for the sodium channel inhibition and are summarized in Fig. 4B.

4. Discussion

Our patch clamp measurements clearly show that the alkaloids ajmaline, lupanine, quinidine, sparteine, strychnine, and yohimbine inhibit sodium channels of *Xenopus laevis* muscle fibers. Of the alkaloids studied ajmaline is the most effective blocking agent under experimental conditions, which is more than 100 times more effective than lupanine and more than 20 times more effective than sparteine and approximately 5-fold more inhibitory than quinidine. Interestingly, the sensitivity of sodium channels in skeletal muscle membrane to sparteine (see also [13]) and lupanine seems to be very similar to that of the squid giant nerve axon [14]. Also, our findings agree with the use of ajmaline, quinidine and sparteine on heart muscle cells as antiarrhythmic agents [15]. It should be noted that the loose patch-clamp method allowed a very effective quantitative examination of the different alkaloids used in our study on the sodium inward currents. This method had so far been used mainly for quantitative mapping of ion channel conductances in various muscle preparations, but proved to be extremely useful for our study of the differential effects of various alkaloids.

Our results with the different alkaloids may help to clarify the molecular structure-function relation of the essential binding sites of the alkaloids with those of the voltage dependent sodium channel. Thus, the study may provide a basis for further more detailed studies on the actual blocking characteristics of the different alkaloids at the level of the single sodium channel (for review see e.g. [16]). Sodium channels are the most important ion channels for excitability and very ubiquitous in the animal kingdom (e.g. tetrodotoxin-sensitive Na^+ channels are present in animals with organized nervous systems down to plathelminths, see [17]). Therefore, it is interesting that experimental and circumstantial evidence strongly suggests that sparteine and lupanine function as defense compounds against herbivores and are therefore important for the fitness of the plants producing them. Both alkaloids are either feeding deterrents or toxins for animals (reviews [18,19,3]). Although not determined in detail for the other alkaloids, it is likely that the same conclusion applies to them.

Is it possible to explain the ecological functions on the base

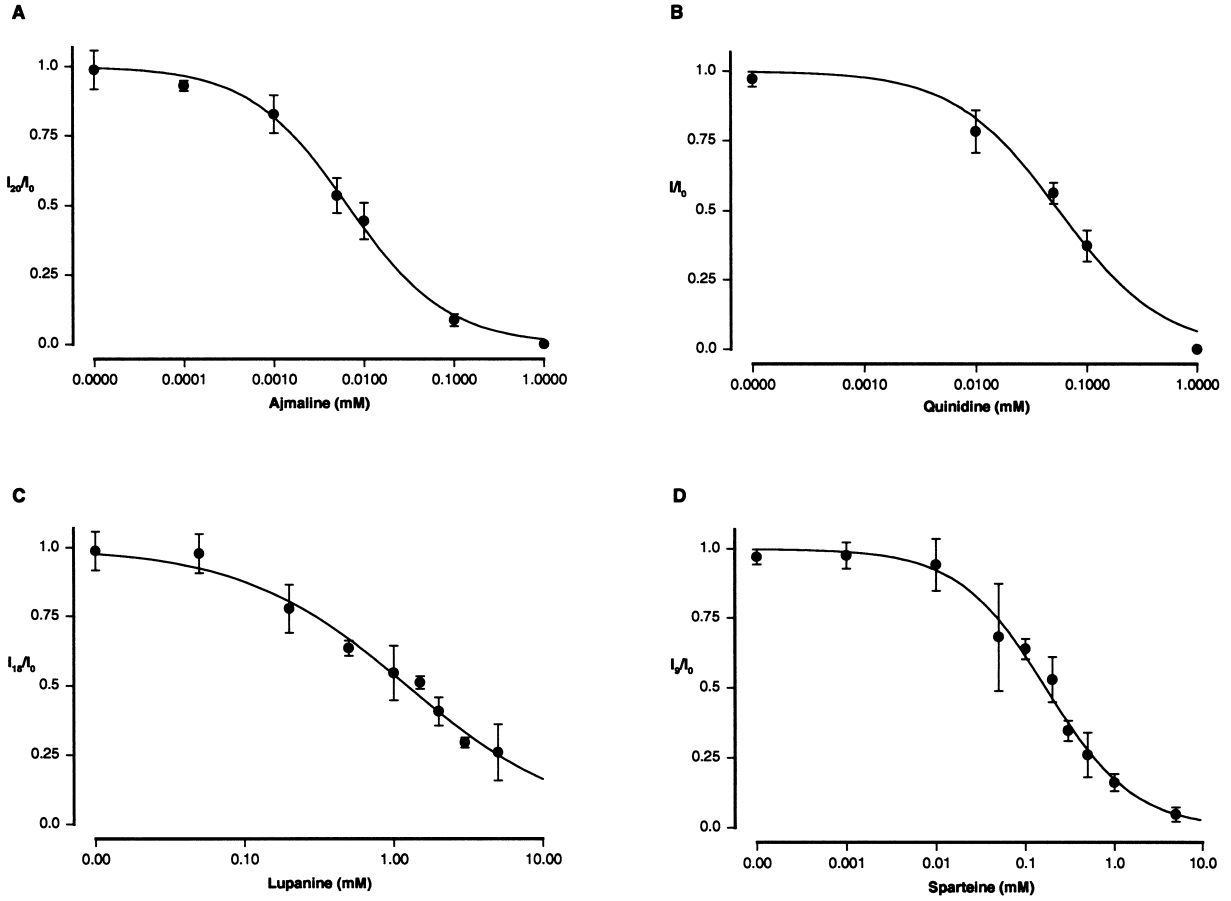

Fig. 3. Dose dependence of Na^+ channel inhibition by alkaloids. Dose-inhibition curves are fitted to Eq. 1. Values are given as a fraction of I_0. At the control values the bath was rinsed with Ringer solution, with the exception of sparteine (2.5% DMSO). A: Ajmaline. Hill parameters: $K_i = 6.6$ μM and $h = 0.79$. B: Quinidine. $K_i = 55.7$ μM, $h = 0.93$. C: Lupanine. $K_i = 1.22$ mM and $h = 0.78$. D: Sparteine. $K_i = 0.17$ mM and $h = 0.88$. The vertical lines indicate S.D. Note the different x-axes.

of the interaction of these alkaloids with molecular targets? In a comparative approach we have previously studied the interactions of ajmaline, lupanine, quinidine, sparteine, strychnine, and yohimbine with potential molecular targets, such as DNA/RNA and related processes, membrane stability, or binding to neuroreceptors [10,20,21,5]. A summary of the corresponding results is given in Table 1, and Figs. 3 and 4. It is apparent that the quinolizidine alkaloids affect at least 4 different molecular targets, i.e. nAChR (nicotinic acetylcholine receptor), mAChR (muscarinic acetylcholine receptor), Na^+, and K^+ channels. Although chemically very similar (Fig. 1), lupanine and sparteine differ in their activity spectrum. Whereas sparteine modulates Na^+, K^+ currents and mAChR, lupanine is very active at nAChR, but less so at the other targets. Since both sets of targets are crucial for neuronal signal transduction, it is plausible that any interference should lead to strong adverse effects. But are the concentrations of lupanine or sparteine, which occur in many members of the Genisteae (family Leguminosae), high enough in the herbivore to effectively interfere with the molecular targets?

A simple calculation can clarify this question: Alkaloid contents in *Lupinus* species range between 0.6 and 6% dry weight with comparatively high values in flowers and fruits which are important for reproduction and survival. The two major alkaloids usually are lupanine and sparteine. Assuming an alkaloid concentration of 2 g/100 g dry weight, which is equivalent to approximately 200 mg/100 g fresh weight and a small herbivore with a body weight of 1000 g; if this animal would ingest 100 g of an alkaloid-producing plant it would ingest 200 mg of alkaloids. Suggesting that the alkaloids are completely resorbed and equally distributed in the body, we would obtain a concentration of 200 mg alkaloids/kg body weight. Taking a molecular weight of 234 for sparteine, the alkaloid concentration in our herbivore would be 854 μM, which would be high enough to partially or completely block the binding of acetylcholine at their receptors or to block sodium and potassium currents in muscle cells (compare IC_{50} values in Table 1, Fig. 4B). In reality, alkaloids are never completely reabsorbed and usually quickly degraded in vertebrates; but even a 50% or 90% lower internal concentration would guarantee a substantial inhibition.

Quinidine has an even wider range of biochemical activities: it intercalates DNA, inhibits several enzymes, binds to adrenergic, serotonin and mACh receptors and inhibits Na^+ channels (Table 1, Fig. 3B), which could explain its cytotoxic, antiarrhythmic, antiparasitic, insecticidal, analgetic and muscle relaxant properties. Similar data can be tabulated for ajmaline, strychnine and yohimbine [5].

A main conclusion can be drawn from these findings: alkaloids often interact with more than one molecular target that

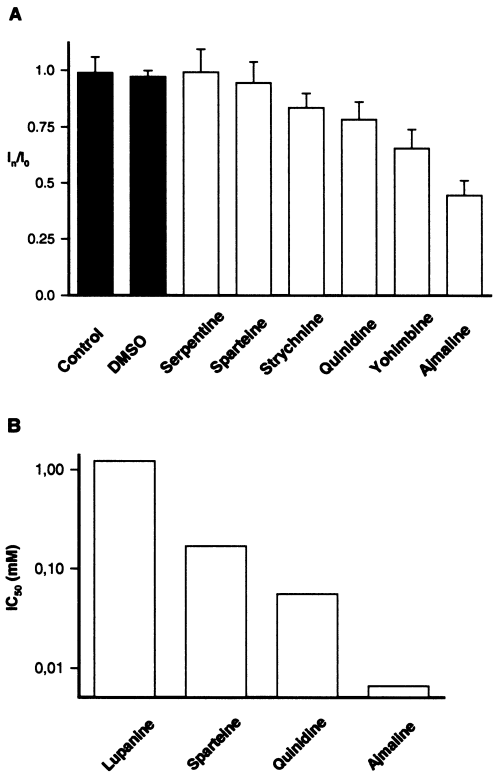

Fig. 4. A: Comparison of Na^+ channel inhibition by alkaloids (at 0.01 mM). Values are given as a fraction of I_0. Student's t-test values: ajmaline is different from yohimbine, $P < 0.005$; yohimbine is different from strychnine, $P < 0.02$; quinidine is different from strychnine, $P < 0.4$; yohimbine is different from quinidine, $P < 0.04$; strychnine is different from control, $P < 0.035$; quinidine is different from control, $P < 0.005$; strychnine is different from sparteine, $P < 0.15$. Vertical lines indicate ± S.D. B: Comparison of IC_{50} for ajmaline, quinidine, lupanine (1.22 mM) and sparteine.

is vital for a cell or organ. If these alkaloids are used as medicinal agents, i.e. as antiarrhythmic drugs, negative side effects are inherent properties of these molecules. However, the same properties are apparently favored from the point of view of ecology and evolution. If a compound can interfere with several targets, it is more likely that it can serve as defense substance against a wider variety of organisms. Furthermore, the simultaneous modulation of several, often unrelated molecular targets, might affect a herbivore more drastically than an interaction at a single target. Herbivores, especially insects, are genetically flexible in that they can develop resistance at a particular molecular target, so that the alkaloid can no longer interfere. If several targets are affected, concomitant mutations at several targets would be required to overcome the inhibitory effects. Since the statistical chances are very small for such an event, it appears not surprising that plants which accumulate sparteine, lupanine, quinidine, strychnine etc. are usually avoided by most herbivores. And adapted specialists which have overcome the evolutionary obstacles are indeed usually very rare in these plants.

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