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Blockade of sodium channels by Bistramide A in voltage-clamped frog skeletal muscle fibres

Martin-Pierre Sauviat [1], Danièle Gouiffes-Barbin [2], Eudes Ecault [1] and Jean-Francois Verbist [2]

[1] *Laboratoire de Physiologie Comparée (URA CNRS 1121), Bât 443, Université de Paris XI, Centre d'Orsay, Orsay (France)* and [2] *Substances Marines à Activité Biologique, Faculté de Pharmacie, Nantes (France)*

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The effect of Bistramide A, a toxin isolated from *Bistratum lissoclinum Sluiter* (Urochordata), on the peak sodium current (I_{Na}) of frog skeletal muscle fibres was studied with the double sucrose gap voltage clamp technique. External or internal application of Bistramide A inhibited I_{Na} without alteration of the kinetic parameters of the current nor of the apparent reversal potential for Na. The steady-state activation curve of I_{Na} was unchanged while the steady-state inactivation curve of I_{Na} was shifted towards more negative membrane potentials. Dose-response curves indicated an apparent dissociation constant for Bistramide A of 3.3 μM and a Hill coefficient of 1.2 which suggested a one to one relation between the toxin and Na channel. The inhibition of I_{Na} occurred at rest, and was more important at more positive holding potentials. Bistramide A exhibited only a weak frequency-dependent effect. The toxin did not interact with the use-dependent effect of lidocaine. It mainly blocked Na channels at more depolarized holding potentials. The toxin blocked Na channels when it was internally applyed and when the inactivation gating system has been previously destroyed by internal diffusion of iodate. The data suggest that Bistramide A inhibited the Na channel both at rest and in the inactivated state and occupied a site which was not located on the inactivation gate.

Introduction

Bistramide A, an amided cyclic polyether toxin isolated from a New-Caledonian ascidian *Lissoclinum bistratum Sluiter* [1] is highly toxic with a rapid effect on the central nervous system leading to paresthesia and loss of muscle tone. In frog skeletal and heart muscle fibres Bistramide A does not alter the resting membrane potential. The amplitude and the duration of cardiac action potentials (AP) are decreased by the toxin and the interval between two consecutive APs was prolonged [2]. These observations suggested that the Na channel was one of the targets of the toxin. The aim of the present study was to analyze the mode of action of Bistramide A on the sodium membrane current of voltage-clamped frog skeletal muscle. Preliminary reports of part of these results have been published [3,4].

Correspondence: M.-P. Sauviat, Laboratoire de Physiologie Comparée (URA CNRS 1121), Bât 443, Université de Paris XI, Centre d'Orsay 91405, Orsay Cedex, France.

Methods and Materials

Voltage clamp experiments are performed at 5–8 °C on fine cut-end skeletal muscle fibres (100–200 μm in diameter, 5–6 mm in length) isolated from sartorius muscle of *Rana esculenta*. The double sucrose gap voltage clamp technique with vaseline seals was used [5]. Starting from a holding potential of −80 mV, the potential of the test gap was displaced in rectangular steps at a rate of 0.2 Hz; positive potentials corresponded to depolarizations. The sodium peak current (I_{Na}) was measured as net inward current if not otherwise specified. The apparent reversal potential for Na (V_{Na}) was obtained by subtracting the leak current (i.e. the current recorded after treatment with tetrodotoxin (TTX)) from the peak current recorded before TTX application. The time constant of the inactivation phase of I_{Na} (τ_h) was measured by semi-logarithmic plot of

the amplitude of the falling phase of the current versus time. The Na conductance (G_{Na}) was calculated according to the equation ($G_{Na} = I_{Na}/(V - V_{Na})$) in which V was the membrane potential. The steady-state parameter for activation (m_∞) was calculated from the peak Na conductance (G_{Na}) for each step potential and normalized to the maximum Na conductance ($\overline{G_{Na}}$) obtained in the whole family of pulses as $m_\infty = (G_{Na}/\overline{G_{Na}})^{1/3}$ [5]. The steady-state inactivation of the Na system (h_∞) was measured using a double pulse arrangement [6]. The potential of the test pulse (V_t) was kept constant at 0 mV applied from a holding potential of −120 mV for 60 ms, and the peak Na current associated with V_t was determined as a function of the initial conditionning step (V_c), 50 ms duration [5]. The dose-response curves were calculated by the modified Langmuir equation [7]

$$Y = Y_{max}(X^m)/(K_d + (X)^m)$$

where Y is the percentage of inhibition of the ionic current, X the concentration of toxin, m the stoichiometric parameter and K_d the apparent dissociation constant, Y_{max} being taken as 100%. The temperature dependence of I_{Na} was determined by the temperature coefficient Q_{10} which was calculated from the following equation [8]:

$$Q_{10} = X_H / X_L^{\{10/(T_H - T_L)\}}$$

where X_L is the value of the experimental parameter at the lower absolute temperature (T_L) and X_H its value at the higher absolute temperature (T_H), the apparent activation energy (μ) was determined from equation:

$$\mu = (RT_L T_H/(T_H - T_L)) \ln(X_H/X_L)$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}).

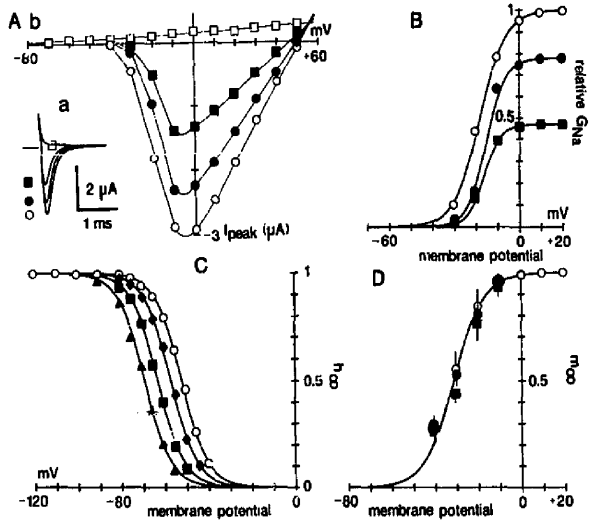

Fig. 1. Effect of Bistramide A on the peak sodium current. (Aa) Superimposed traces of Na current elicited by a 80 mV depolarizing pulse applied from a −80 mV holding potential. (○) Ringer solution containing TEA (10 mM) before and 5 min after successive applications of Bistramide A 1.4 µM (●), 5.6 µM (■), □, Bistramide A (5.6 µM) and tetrodotoxin (0.57 µM). (Ab) Current-voltage relationships of the peak Na current (I_{peak}). ○, Ringer solution containing TEA (10 mM), and 1.4 µM (●), 5.6 µM (■) Bistramide A; □, 5.6 µM Bistramide A and tetrodotoxin (0.57 µM). (B) Relative Na conductance (G_{Na})-membrane potential (V) relationships. G_{Na} was calculated from current-voltage curves of Fig. 1Ab according to the equation: $G_{Na} = I_{Na}/(V - V_{Na})$ with $E_{Na} = +56$ mV. I_{Na} was measured as the difference between the current recorded before and after tetrodotoxin application. ○, Ringer solution containing TEA (10 mM), 1.4 µM (●); 5.6 µM (■) Bistramide A. The line fitting the experimental data was drawn according to the equation $G_{Na} = \overline{G_{Na}}/(1 + \exp((V_{0.5} - V)/s))$ in which $\overline{G_{Na}}$ was the maximal conductance, $V_{0.5}$ was the membrane potential at which the conductance was half maximal, V the membrane potential. $V_{0.5}$ was −18 mV and −15 mV or −16 mV; s was 6 and 4.5 or 4 in the absence and in the presence of Bistramide A 1.4 µM or 5.6 µM in the Ringer solution, respectively. This experiment is the same as shown in Fig. 1Ab. (C) Curves for the steady-state inactivation (h_∞) of the Na system plotted on the same fibre before (○) and during 2.8 µM (◆); 5.6 µM (■), 11.2 µM (▲) Bistramide A application. Ordinate scale: relative magnitude of the Na current I_{Na}/I_{Namax} (h_∞) which developed under a 80 mV depolarizing test pulse applied from a −80 mV holding potential. The lines fitting the experimental data have been drawn according to Boltzmann equation ($h_\infty = 1/(1 - \exp(V_{h0.5} - V/s_h))$; $V_{h0.5}$ mid point and s_h slope factor were estimated using a least-square representation. $V_{h0.5}$ was −52 mV in the control solution and −57 mV, −63 mV, −69 mV in the presence of Bistramide A 2.8 µM, 5.6 µM, 11.2 µM in the control solution, respectively; s_h was 6 for the four curves. (D) Curve for the steady-state activation (m_∞) of the Na system plotted before (○) and during 1.4 µM (●), 5.6 µM (■) Bistramide A application. Ordinate scale relative G_{Na} ratio $(G_{Na}/\overline{G_{Na}})^{1/3}$ plotted as m_∞. The line fitting the experimental data has been drawn according to the Boltzmann equation ($m_\infty = 1/(1 + \exp(V - V_{m0.5}/s_m))$ with $V_{m0.5}$ the mid point equal to −32 mV and s_m the slope factor equal to 6.5. Vertical lines represented the S.E. values of means given in Table I for each solution

Each figure is representative of five to six experiments unless otherwise stated. Calculations are expressed as mean values ± S.E. mean; (n) corresponds to the number of preparations tested. The data were analyzed statistically using unpaired Student's t-test and a two-way analysis of variance with repeated measures. Differences were considered significant at $P < 0.05$. Transmembrane potentials and currents were displayed on a digital oscilloscope (Nicolet 310), recorded on the mass storage device of a desk-top computer (Hewlett Packard 9826), and plotted on an X-Y plotter (Ifelec or Hewlett Packard 7470).

The composition of the Ringer solution was (mM): NaCl, 110.5; KCl, 2.5; $CaCl_2$, 2. The pH of the solution was maintained at 7.3 with Hepes buffer (5 mM). Tetrodotoxin (TTX, 0.57 μM) and tetraethylammonium (TEA, 10 mM) were added to the Ringer solution to block the voltage-dependent Na and K currents, respectively. Lidocaine (xylocaine 1%) was from the Laboratoire Roger Bellon. The composition of the 'internal solution' which bathed the cut-ends of the fibres was (mM): K aspartate, 120; ATP, 5; phosphocreatine, 5; $MgCl_2$, 2; Hepes buffer, 5; pH 7.3 [5]. The toxin was dissolved in absolute ethanol at a concentration of 1.4 mM, kept at 4 °C and appropriately diluted just before use. Control solutions contained the same amount of ethanol as the test solution.

Results

Membrane currents

Under voltage clamp conditions, Bistramide A reduces the amplitude of the peak I_{Na} recorded in Ringer solution containing TEA in a dose-dependent manner (Fig. 1Aa). A further application of TTX to the toxin containing solution entirely inhibited the remaining current (Fig. 1Aa). Current-voltage relationships (I-V curves) of Fig. 1Ab show that Bistramide A decreased the amplitude of the peak I_{Na} without changing the position of the I-V curve on the voltage axis. The apparent reversal potential for Na was unchanged by the toxin. It was $+51 \pm 4$ mV ($n = 9$) in control and $+51 \pm 3$ mV ($n = 6$) and $+53 \pm 5$ mV ($n = 3$) in the presence of 1.4 μM and 5.6 μM Bistramide A, respectively. Fig. 1B shows that Bistramide A decreased the Na conductance whatever the membrane potential investigated. The toxin does not modify the time needed for I_{Na} to reach its peak value nor the time constant of the inactivation phase of the current (Fig. 1Aa). The steady-state inactivation of the Na system (h_∞)-membrane potential curve was significantly shifted in a dose-dependent manner towards more negative membrane potentials in the presence of Bistramide A (Fig. 1C). The membrane potential at which I_{Na} was half maximal and the slope factor of the curves are summarized in Table I. The steady-state activation of the Na system (m_∞)-membrane potential curve was not significantly modified by the toxin (Fig. 1D). The mid point of the activation voltage as well as the slope factor of the curve were not modified by Bistramide A (Table I). The inhibitory effect of the toxin on I_{Na} was insensitive to variation of the external pH in the range 5.8 to 8. Changing the temperature from 5 to 20 °C increased the amplitude of I_{Na} and accelerated the kinetic of the current. Q_{10} of I_{Na} decreased by 4% in the presence of toxin (control: 2.59 ± 0.04 ($n = 6$); Bis-

TABLE I

Effect of Bistramide A on the activation (m_∞) and inactivation (h_∞) steady-state parameters of the Na conductance

$V_{m0.5}$ and $V_{h0.5}$, s_m and s_h were the potential at which the half activation and inactivation occurred; the slope factors, respectively. ΔV was the difference between $V_{m0.5}$ or $V_{h0.5}$ recorded in the absence and in the presence of toxin. k/l represents the binding (k) and unbinding (l) ratio, D was the toxin concentration, n the number of fibres tested. Results are expressed as mean values ± S.E.

	Control	Bistramide A (μM)			
		1.4	2.8	5.6	11.2
m_∞					
n	8	5	4	3	-
$V_{m0.5}$ (mV)	−31.5 ± 3.1	−31.2 ± 2.)	−33.7 ± 0.5	−29.3 ± 2.1	-
s_m	6.0 ± 0.8	6.0 ± 0.8	5.7 ± 0.5	6.1 ± 0.2	-
h_∞					
n	6	-	5	5	4
$V_{h0.5}$ (mV)	−55.5 ± 4.2	-	−59.4 ± 1.1	−63.2 ± 2.1 *	−68.7 ± 2.0 **
s_h	5.7 ± 0.7	-	5.5 ± 1.1	6.1 ± 0.7	6.2 ± 1.4
ΔV (mV)	-	-	3.9	7.7	13.2
k/l	-	-	0.18	0.13	0.08
$(k/l)D$	-	-	0.47	0.74	0.90

* $P < 0.02$; ** $P < 0.01$ when compared with control unpaired t-test.

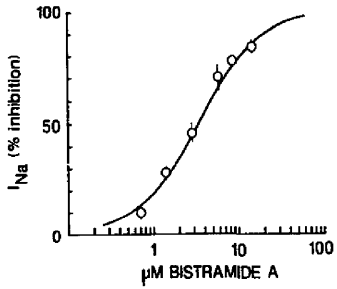

Fig. 2. Log-concentration-response relationship for the effect of Bistramide A on the peak I_{Na} amplitude (ordinate scale). Results are expressed as % of the current recorded in the absence of drug. The curve fitting the experimental data was drawn according to the modified Langmuir equation (see Methods) with $K_d = 3.3$ μM and $m = 1.2$. Vertical bars indicate S.E. values of means ($n = 6$).

tramide A: 2.49 ± 0.04 ($n = 9$)). The activation energy was unchanged (12.6 ± 1.2 kJ mol^{-1} ($n = 6$) before and 12.6 ± 2.5 kJ mol^{-1} ($n = 9$) and after toxin treatment, respectively). The inhibition of I_{Na} by Bistramide A was only partially reversible, in six experiments, the current recovered only by $14.3 \pm 5\%$ 6 min after washout of (2.8 μM) Bistramide A.

Dose-response curve

The dose-response curve for the inhibitory effect of Bistramide A on I_{Na} is shown in Fig. 2. The Hill plot of the data (log% inhibition/(100 − % inhibition) versus log Bistramide A concentration) gave a stoichiometric parameter of 1.2 suggesting a one to one relationship between the toxin molecule and the Na channel, the apparent dissociation constant was 3.3 μM.

Effect of the frequency of stimulation

The toxin (2.8 μM) inhibited I_{Na} in the absence of stimulation. After 5 min, the tonic block reached $18 \pm 4\%$ ($n = 6$) at −80 mV holding potential; the block was increased at less negative holding potential and reached $49 \pm 5\%$ ($n = 5$) at −70 mV. Fig. 3A shows that the inhibition of I_{Na} by Bistramide A exhibited a weak dependence on the frequency of stimulation. The peak I_{Na} was moderately reduced when the rate of stimulation increased. This additional frequency-dependent block of I_{Na} associated with the increase in stimulation rate never exceeded 20% of the control value. By contrast, lidocaine (0.4 mM) which induced a $26 \pm 5\%$ ($n = 9$) tonic block of I_{Na} (elicited by a 0 mV depolarizing step applied from a −80 mV holding potential) exhibited a clear use-dependence block of I_{Na} (Fig. 3A). The reduction of the peak I_{Na} with increased stimulation reached the same amplitude when the following solutions are applied to the fibre: (a) lidocaine alone; (b) a solution containing both Bistramide A and lidocaine; (c) Bistramide A for 5 min followed by a solution containing both Bistramide A and lidocaine (insert of Fig. 3A). The degree of channel occupancy by the toxin was estimated from the normalized value of I_{Na} taken as the ratio between the current recorded when the preparation was driven first at low (0.03 Hz) frequency and then at a higher frequency (2 Hz) for one minute each as a function of the holding membrane potential in the range −130 to −60 mV. After each run, the preparation was driven for two minutes at 0.03 Hz before changing the membrane potential. Fig. 3B shows that Na channels were occupied by Bistramide A at more depolarized membrane potentials than a solution containing both lidocaine and Bistramide A or lidocaine alone (not shown). Bistramide A had no effect upon the occupation curve evoked by lidocaine.

Internal diffusion experiments

The application of Bistramide A (0.3 mM) by internal diffusion through the cut-ends also reduced the peak I_{Na} by about 50% within 20 min (Fig. 4A). Fig. 4B shows that external application of Bistramide A reduced the peak I_{Na} whose inactivation phase has

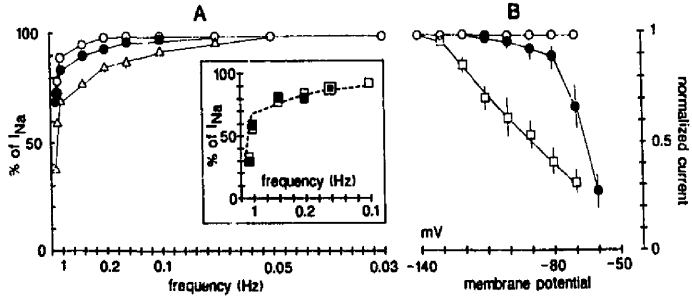

Fig. 3. Frequency-dependent effect of Bistramide A (5.6 μM). (A) Effect of stimulation frequency on the block of peak inward current (I_{Na}) by Bistramide A (●); ○, Ringer solution containing TEA (10 mM). The open triangles represent the effect of (0.4 mM) lidocaine. The insert represents the effect of lidocaine (dashed line) and a mixture of Bistramide A and lidocaine applied together (□) or after 5 min pretreatment with Bistramide A (■). The depolarizing test pulse was 80 mV amplitude (holding potential −80 mV). The preparation was driven for 1 min at the chosen rate before recording the current. (B) Voltage-dependent block of I_{Na} by Bistramide A (●) and a solution containing Bistramide A and lidocaine (0.4 mM) (□). ○, Ringer solution containing 10 mM TEA. Vertical bars represent the S.E. values of means of six experiments.

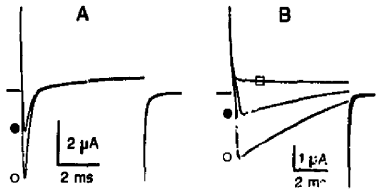

Fig. 4. Effect of Bistramide A on the internal surface of the fibre membrane. (A) Superimposed traces of the Na current recorded in the Ringer solution before (○) and after (●) 20 min diffusion of Bistramide A (0.3 mM) through the cut-ends of the fibre. (B) Superimposed traces of Na current recorded in the Ringer solution containing TEA (10 mM) with iodate (30 mM) applied internally for 30 min (○) before and (●) after external Bistramide A (5.6 μM) addition to the Ringer solution. □, Control solution containing Bistramide A and TTX (0.57 μM). In (A) and (B), the current was recorded under a 80 mV depolarizing pulse applied from a −80 mV holding potential.

been previously reduced by internal application of iodate through the cut-ends.

Discussion

The most prominent effect of Bistramide A on voltage clamped cut-end frog skeletal muscle fibre was a reduction of peak Na current (I_{Na}). It is generally accepted that the Na conductance (G_{Na}) is governed by the product of a constant value, the maximal Na conductance ($\overline{G_{Na}}$), and two kinetic factors m and h; m governs the activation process and h the inactivation process [6]. The reduction in I_{Na} brought about by the toxin does not seem to be related to an alteration of the apparent equilibrium potential for Na ions. I-V curves indicated that Bistramide A reduced the maximum I_{Na} without shift of the membrane potential at which it occurred. Moreover, the m_∞-membrane potential curve were unchanged in the presence of toxin suggested that Bistramide A did not affect the opening process of the Na channel. All these observations indicated that Bistramide A decreased $\overline{G_{Na}}$. However, Bistramide A shifted the steady-state inactivation curve of I_{Na} towards more negative membrane potentials although the time constant of the inactivation phase of the I_{Na} remained unaltered. A similar situation has already been reported for local anesthetics such as lidocaine. Most of the anesthetic reduce $\overline{G_{Na}}$ and shifted the steady-state inactivation curve of I_{Na} towards more negative membrane potentials while τ_h was unchanged [9]. Elliot and Haydon [10] raised the possibility that the reduction in $\overline{G_{Na}}$ by some neutral substances might be linked to an increase of membrane thickness, linked to the adsorption of molecules into the membrane, which would also shift the inactivation curve in the hyperpolarizing direction by reducing the electric field across the membrane. According to Hodgkin-Huxley theory [6], three states contribute to voltage-dependent Na channel excitation. Starting from a resting state (R), they activated (A), inactivated (I) and returned to the resting state according to Fig. 5 using Hodgkin-Huxley kinetic parameters.

The modulated receptor hypothesis (MRH) [9,11] postulated that the channel cycle can be altered by positioning one or many states by a drug or a toxin which can interact with the channel in each of its states (R', A', I'). The association (k) and dissociation (l) rate constants for interaction of the substance with channels in each of the three states are characteristic for the individual substances. Our observations indicated that the steady state inactivation curves can be fitted by Boltzmann equations and suggested that Bistramide A binds to inactivated Na channels. In the extended MRH, based on the minimal blockade model [12,13], the analysis of h_∞-V relations allows the determination of the affinity of a drug (D) to bind to available sites and block them. The variation of the mid point of the h_∞-membrane potential curve ($\Delta V_{h0.5}$) was used to estimate the affinity for binding to a sustained site. The binding (k) and unbinding (l) ratio (k/l) of the drug to available inactivated Na channels (I_a) to gave blocked channels (I_a^*) according to reaction:

$$I_a + \text{Drug} \underset{l}{\overset{k}{\rightleftharpoons}} I_a^*$$

can be obtained according to the equation $k/l = (e^{-V_{h0.5}/s_h} - 1)/D$ in which $\Delta V_{h0.5}$ was the difference between drug and control availability mid point and s_h the slope factor of the control inactivation curve. Table I shows that the ratio (k/l) decreased with increasing the toxin concentration and suggests that the number of occupied available sites increased with increasing toxin concentration. Moreover, the estimated ratio $(k/l)D$ reported in Table I which corresponded to an equilibrium block for the continuously available site [13] agree with the block of I_{Na} in the dose-response curve. This led to the conclusion that the shift in the $h_\infty - V$ curves account for the reduction of I_{Na} by

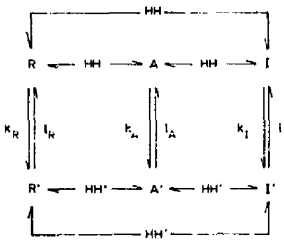

Fig. 5. Schematic representation of the modulated receptor hypothesis (see text for explanation).

Bistramide A. Diffusion experiments indicated that the toxin also reaches its site of action when it was applied from the internal side of the membrane. Bistramide A behaves like neutral or quaternary anesthetic molecules which are able to pass either through the membrane or the channel to block or exit from the channel [9]. The high lipophilicity of the molecule [1] might account for the toxin effect on G_{Na}. The absence of marked modification of the activation energy suggested that Bistramide A did not change the fluidity of membrane lipids surrounding the receptor or channel protein [14]. We also show that the receptor for Bistramide A was not located on the inactivation gate itself since the blockage of I_{Na} persisted when the inactivation subunit was destroyed by internal iodate application [5]. These observations also indicated that Bistamide A bypasses the inactivation gate to block the Na channel.

Bistramide A induced a resting block by inhibiting resting Na channels (i.e. in the absence of stimulation). This block develops with a greater affinity at more positive membrane potential levels, when more Na channels are in the inactivated state. It can therefore be suggested that this type of block results from an interaction of the toxin with closed Na channels. However, Bistramide A exhibited a weak frequency-dependent effect and did not antagonize the use-dependent effect of lidocaine. The voltage-dependence of the fraction of blocked/unblocked Na channels indicated that Na channels are occupied by Bistramide A at more positive membrane potentials than lidocaine which was also found to reach its site of action, in the absence or in the presence of Bistramide A, when the Na system is fully available or partially inactivated. This suggested that these molecules interacted with two independent receptors. The observation that the cummulative inhibition of I_{Na} increased with the polarization of the membrane indicated that the toxin binds to inactivated Na channel. For this point of view, Bistramide A shares some properties with anticonvulsant molecules which are cyclic amides [15].

In conclusion, Bistramide A is a new blocker of Na channels with potent anesthesic and anticonvulsant properties. The inhibition of the Na conductance, more marked at more positive membrane potentials, suggests the possibility that Bistramide A inhibited Na channels both at rest and in the inactivated state.

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