Sensitivity of Cardiac Background Inward Rectifying K^+ Outward Current (I_{K1}) to the Alkaloids Lepadiformines A, B, and C

Martin-Pierre Sauviat,*,† Joseph Vercauteren,‡ Nicole Grimaud,§ Marcel Jugé,§ Mohamed Nabil,⊥ Jean-Yves Petit,§ and Jean-François Biard||

Laboratoire d'Optique et Biosciences, INSERM U 696, CNRS UMR 7645, X, Ecole Polytechnique, 91128 Palaiseau Cedex, France, Laboratoire de Pharmacognosie, Faculté de Pharmacie, BP 14491, 34083 Montpellier Cedex 5, France, Laboratoire de Pharmacologie, Faculté de Pharmacie, BP 53508, 44035 Nantes Cedex 1, France, CERD, Djibouti, République de Djibouti, and SMAB, Faculté de Pharmacie, BP 53508, 44035 Nantes Cedex 1, France

Received June 15, 2005

Three marine alkaloids, purified from *Clavelina moluccensis*, were structurally identified as lepadiformines A, B, and C and studied on frog atrial myocytes I_{K1}, using the patch-clamp technique. Lepadiformine A (0.4 to 3.3 μM) blocked I_{K1} dose-dependently with an apparent dissociation constant (K_D) equal to 1.42 μM and a stoichiometry of 0.77. The block is voltage-dependent, suggesting that lepadiformine A occupies a receptor site located at about two-thirds of the membrane depth. The shortening of the aliphatic chain at position C13 of lepadiformine B decreased the potency of the molecule to block I_{K1} but not the affinity ($K_D = 1.56$ μM) and stoichiometry (0.72). Additional deletion of the oxygenated side chain at C2 in lepadiformine C markedly decreased the inhibitory effect of the molecule. In conclusion, lepadiformine modulates I_{K1} response in cardiac muscle. The oxygenated side chain in C2 is implicated in the affinity of lepadiformine, which behaved as an amine, for a receptor located near or inside the I_{K1} pore, and the aliphatic chain length at position C13 is involved in the degree of I_{K1} blockage.

In our continuing search for novel bioactive metabolites from marine natural organisms, *Clavelina lepadiformis* Müller from Tunisia has provided the alkaloid lepadiformine. This alkaloid has a moderate cytotoxic activity on KB cells and non-small-cell lung carcinoma (NSCLC-N6).[1] Among tunicates, *Clavelina* are prolific alkaloid-producing organisms, e.g., clavepictines,[2] wakayin,[3] pictamine,[4] piclavines,[5] cylindricines,[6] villatamide,[7] and lepadines.[7,8] More recently, we have isolated lepadiformine and two other derivatives from *C. moluccensis* Sluiter collected in Djibouti waters, and we proposed the names lepadiformines A, B, and C, respectively. At the same time, success in total synthesis of lepadiformine A indicates that the configuration proposed by Biard et al.[1] needed revision.

Biological studies of lepadiformines showed that intravenous injection of lepadiformine A isolated from *C. moluccensis* had a marked effect on the cardiovascular system. The substance (6 mg kg^{-1}) produced a transient decrease of arterial blood pressure in the rat and exerted a vasoconstrictor effect in the perfused rabbit ear.[9] Lepadiformine A also caused bradycardia and modified the electrocardiogram by prolonging the Q−T interval.[9] Our microelectrode measurements indicated that, in both rat left ventricular papillary muscle and frog auricle, micromolar concentrations of lepadiformine A prolonged the action potential (AP) duration (APD) and mimicked the effect of Ba^{2+}, a blocker of this background inward rectifying K^+ current (I_{K1}), indicating that the substance inhibited the current.[9] The cardiac inward rectifying K^+ channel (K_{ir}) is important in maintaining the maximum diastolic potential. $K_{ir}2.1$, $K_{ir}2.2$, and $K_{ir}2.3$ are coexpressed[10] and probably coassembled in cardiac muscle cells. Until now only a few K_{ir} channel inhibitors have been discovered. The channel is sensitive to the barbiturate thiopental[11] and blocked by tertiapin[12] and rLq2[13] isolated from bee and scorpion venoms, respectively. Therefore, the study of the mode of action of lepadiformines on cardiac muscle I_{K1} is of physiological interest. In the present work, we sought further insight into the structures of lepadiformines A, B, and C, and we analyzed the mechanism by which these molecules block cardiac muscle K_{ir} channel.

Results and Discussion

The EtOH extract of wet ascidians was partitioned between CH_2Cl_2 and H_2O. After evaporation, the organic residue was suspended in aqueous HCl. This suspension was filtered, washed with Et_2O, and then extracted with CH_2Cl_2 without any change of pH. Low-pressure chromatography (silica, CH_2Cl_2−MeOH from 100:0 to 70:30) gave product C and a mixture of compounds A and B, which were finally separated by HPLC (Et_2O−dioxane, 50:50). Chromatographic, mass, and NMR data indicated obvious similarities between these products and lepadiformine previously purified from *C. lepadiformis*.[1]

For compound A (lepadiformine A) (Figure 1a), complete matching of ^1H and ^{13}C NMR spectra (Table 1) and a protonated molecular peak $m/z = 294$ $[M + H]^+$ by LSI-MS corresponding to a molecular weight of $M = 293$ ($C_{19}H_{35}NO$) indicated that both *C. moluccensis* and *C. lepadiformis* synthesize lepadiformine A.

Compound B (lepadiformine B) (Figure 1b) gave a molecular peak m/z 265.2399 in the EIMS corresponding to $C_{17}H_{31}NO$ (calcd value 265.24055). The fragmentation pattern showed peaks at m/z 234.2224 (calcd value 234.2221) for $M^+ - CH_2OH$, 208 for $M^+ - C_4H_9$, and 178 for $M^+ - CH_2OH - C_4H_9$. In comparison with product A, ^{13}C and ^1H NMR data (Table 1) showed the disappearance of two CH_2 groups (δ_C 26.11 and 31.36 in lepadiformine A) and the persistence of the NH (δ_H 10.10) and the oxygenated CH_2 group at C2 (δ_C 59.94, two diastereotopic H's at δ_H 3.61 and 4.15). Therefore, the core of lepadiformine A is conserved in lepadiformine B, but the aliphatic side chain at position C13 is shortened from C6 to C4. Such a shortening of the aliphatic side chain from hexyl to butyl has already been reported for the structurally related marine alkaloid cylindricines.[6]

Compound C (lepadiformine C) (Figure 1c) gave a peak $[M + H]^+$ at m/z 236.2371 in the HRESIMS, which corresponded to a molecular formula of $C_{16}H_{29}N$ (calcd for M + H 236.2378). The fragmentation pattern showed peaks at m/z 221.3, 207.3, 192.3, and 178.3 ($M^+ - C_4H_9$), indicating the progressive loss of a butyl side chain. Neither a peak at 204 ($M^+ - CH_2OH$) nor at 147 ($M^+ -$

* To whom correspondence should be addressed. Tel: (33) 01 69 33 47 34. Fax: (33) 01 69 33 30 17. E-mail: martin-pierre.sauviat@polytechnique.fr.
† Laboratoire d'Optique et Biosciences.
‡ Laboratoire de Pharmacognosie.
§ Laboratoire de Pharmacologie.
⊥ CERD.
|| SMAB.

10.1021/np050215s CCC: $33.50
Published on Web 02/23/2006

Table 1. ^{13}C and ^{1}H NMR Data for Lepadiformines A, B, and C (in $CdCl_3$)

	lepadiformine A		lepadiformine B		lepadiformine C	
$CdCl_3$ atoms	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	59.70	3.56	59.94	3.61		
		4.10		4.15		
2	63.23	3.61	63.50	3.63	46.86	2.85
						3.68
3	24.12	1.95	24.27	1.93	22.37	1.96
		2.32		2.35		2.10
4	26.20	1.75	26.42	1.75	27.64	1.71
		2.00		2.00		1.99
5	77.26		77.23		74.61	
6	33.60	2.10	33.75	2.13	35.46	2.03
						2.22
7	23.03	1.25−1.35	23.22	1.25−1.35	23.72	1.48
		1.75		1.75		1.85
8	24.71	1.25−1.35	24.88	1.25−1.35	25.14	1.25−1.35
		1.63		1.65		1.72
9	30.44	1.00	30.71	1.00	29.86	1.07
		1.63		1.65		1.61
10	36.00	2.00	36.19	2.00	37.82	1.95
11	22.27	1.30−1.40	22.52	1.30−1.40	20.96	1.52
		1.75		1.75		1.68
12	18.87	1.48	19.17	1.48	20.01	1.30−1.40
		1.89		1.90		1.87
13	58.47	3.61	58.67	3.63	55.29	3.52
14	29.64	1.20−1.30	29.52	1.20−1.30	30.80	1.22
		2.42		2.47		2.04
15	26.11	1.25−1.35	28.42	1.25−1.35	28.33	1.26
16	28.76	1.30−1.40	22.37	1.30−1.40	22.37	1.30−1.35
17	31.36	1.20−1.30	13.82	0.88	13.86	0.90
18	22.20	1.20−1.30				
19	13.73	0.82				
N*H*		10.10		10.10		n.o.[a]

[a] n.o.: not observed.

CH_2OH − C_4H_9) was observed, indicating the disappearance of the oxygenated side chain at position C2 (as in lepadiformine A or B). NMR data (Table 1) confirmed that the oxygenated CH_2 group at position C2 (δ_C 59.70, δ_H 3.56 and 4.10 in lepadiformine A) has disappeared and that the CH was converted to CH_2 at C2. No NH signal was observed in the proton NMR spectrum, while all the remaining signals were similar to those of lepadiformine B (Table 1). Thus, on the basis of the ^{1}H and ^{13}C NMR data mainly, lepadiformine C bears the same framework as lepadiformine B without the oxygenated side chain at C2. The absolute configuration of lepadiformine A was recently established by synthesis.[14−17] Lepadiformines B and C result most probably from the same biosynthetic pathway. Because of their matching NMR data, we propose the same absolute configuration for the new derivatives B and C. Such an assumption may be strengthened by referring to the observed $[\alpha]_D$ values for lepadiformines A, B, and C, which are very close. In addition, the NMR data and the use of HCl in the isolation process suggest that all three alkaloids were present as HCl salts.

Under voltage clamp conditions, the current I_{K1} recorded in the control solution is inward directed in response to hyperpolarizing clamp steps and outward directed in response to depolarizing clamp steps applied from the HP (Figure 2Aa). Subsequent addition of lepadiformine A (1.6 μM) to the control solution decreases the magnitude of both (inward and outward) components of I_{K1} (Figure 2Ab). The blockade of the current induced by lepadiformine A is not time-dependent. The current remaining after lepadiformine A (1.6 μM) treatment is suppressed by subsequent addition of Ba^{2+} (2 mM) to the solution containing the alkaloid (Figure 2Ac). Total replacement of K^+ by Cs^+ in both control and pipet solutions prevents I_{K1} development, and subsequent addition of lepadiformine A (1.6 μM) to the K^+-free solution containing Cs^+ does not modify the amplitude of the current (Figure 2B). Current−voltage relationships plotted for I_{K1} show that lepadiformine A (1.6 μM) decreases the amplitude of the current and that the remaining current is suppressed by subsequent addition of Ba^{2+} (2 mM) to the solution containing lepadiformine A (Figure 2C). The blockade of I_{K1} by

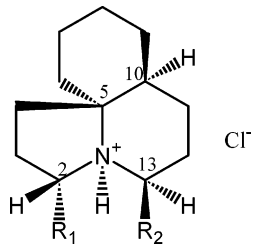

Figure 1. Structural formulas of lepadiformines (as hydrochloric salts): (1a) lepadiformine A (R_1: CH_2OH, R_2: C_6H_{13}); (1b) lepadiformine B (R_1: CH_2OH, R_2: C_4H_9); (1c) lepadiformine C (R_1: H, R_2: C_4H_9).

lepadiformine A was dose-dependent (Figure 3). The half-maximal response was reached at a drug concentration of 1.42 μM, and the Hill plot of the data gives a stoichiometric parameter value of 0.77.

The current−voltage curves of Figure 2C suggest that the lepadiformine A-induced I_{K1} blockade is potential-dependent, a situation that shares some similarities with the voltage-dependent block of Na^+ and K^+ channels produced by external acidification at the node of Ranvier.[18] Without making any specific assumption about the molecular events, the potential-dependent block of the current by lepadiformine A can formally be described by assuming that the equilibrium dissociation constant ($K_d = k_2/k_1$) of the reaction between the ion (X) and the channel receptor (R) changes with depolarization.

$$X + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X{\cdot}R$$

From the voltage-dependence of the inhibitory effect of lepadiformine A on I_{K1}, the voltage-dependence of the equilibrium dissociation constant (K_d) was calculated from the equation $K_d = k_2/k_1 = (a/(1 - a)) \times [X])$ (eq 2). In this equation a is the current ratio ($I_{\text{lepadiformine A}}/I_{\text{control}}$) between the net currents, which were measured by subtracting the current recorded at the end of the clamp steps before and after total blockade by Ba^{2+} (2 mM) in the presence

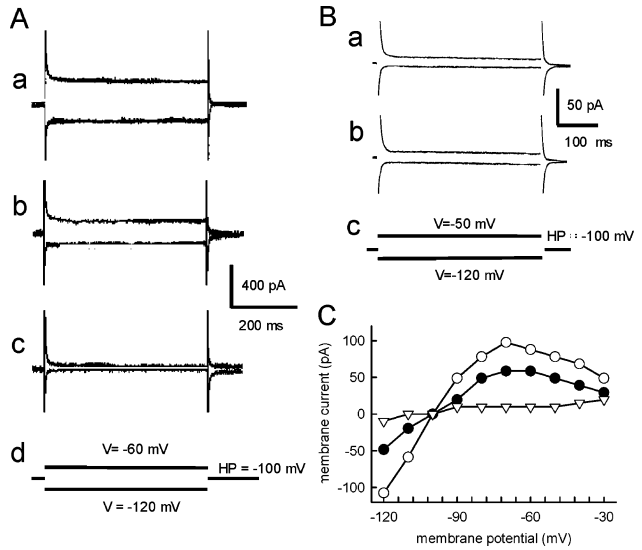

Figure 2. Effect of lepadiformine A on the background inward rectifying K^+ current. (A) Superimposed current traces recorded, in response to a −120 mV (lower traces) and −60 mV (upper traces) clamp step (V) applied from HP = −100 mV (d): (a) control Ringer solution, containing (b) lepadiformine A (1.6 μM) and (c) lepadiformine A and Ba^{2+} (2 mM). (B) Current traces recorded, in a in K^+-free control solution containing Cs^+, in response to a −120 mV (lower traces) and −60 mV (upper taces) clamp step (V) applied from HP = −100 mV (c), (a) before and (b) 5 min after lepadiformine A (1.6 μM) application. (C) Current−voltage relationships plotted for the current measured at the end (500 ms) of the clamp steps in the control Ringer solution before (open circles) and after (solid circles) 5 min lepadiformine A (1.6 μM) addition; (open triangles) subsequent addition of Ba^{2+} (2 mM) to the solution containing lepadiformine A; HP = −100 mV.

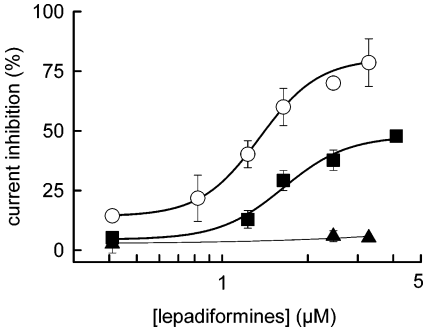

Figure 3. Dose−response curves for the inhibition of the background inward rectifying K^+ current by lepadiformines: (open circles) lepadiformine A (n = 4); (solid squares) lepadiformine B (n = 4); (solid triangles) lepadiformine C (n = 4). Curves were drawn according to eq 1: lepadiformine A (p = 0.77, K_D = 1.42 μM); lepadiformine B (p = 0.72, K_D = 1.56 μM); lepadiformine C (regression line drawn according to the equation $y = x + 2.54$; correlation coefficient = 0.98). Mean values ± SEM of n cells.

($I_{\text{lepadiformine A}}$) and in the absence ($I_{\text{control}}$) of lepadiformine A (1.6 μM) in the control solution, and [X] is the concentration of lepadiformine A. The lepadiformine A-induced voltage-dependence is described in more detail by plotting K_d against membrane potential values (Figure 4). Experimental data were best fit by a straight regression line, which indicated that K_d depends on membrane potential. In the Woodhull[18]-derived barrier model for voltage-dependent ionic channel block, voltage-dependent K_d's are used to determine the relative energy profile of ion channels at the site of blockage by determining the fraction (θ) of the applied transmembrane voltage field sensed by the ions. The voltage-dependent blockade of I_{K1} induced by lepadiformine A suggests

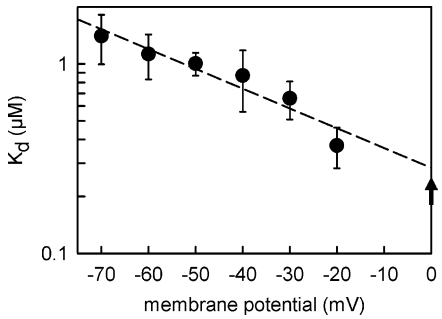

Figure 4. Voltage-dependence of the apparent dissociation constant K_d for the background inward rectifying K^+ current blockade induced by lepadiformine A (1.6 μM). K_d values obtained from eq 2 are plotted against membrane potential. The regression line was drawn according to the equation $y = -0.019x + 0.043$ (correlation coefficient = 0.98). (arrow) K_d = 0.3 μM at 0 mV. Mean values ± SEM of 6 cells.

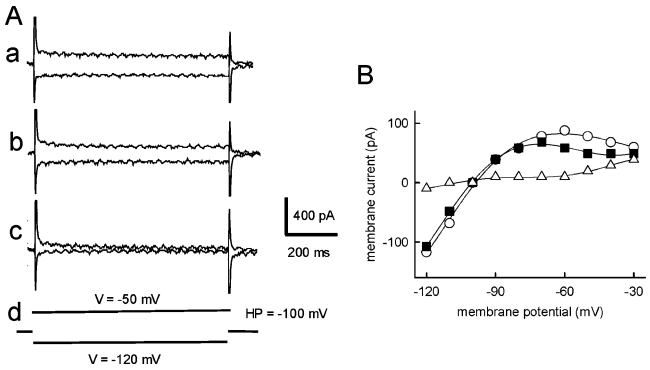

Figure 5. Effect of lepadiformine B on the background inward rectifying K^+ current. (A) Superimposed current traces elicited by −120 mV (lower traces) and −50 mV (upper traces) clamp steps applied from HP = −100 mV (d): (a) control Ringer solution containing (b) lepadiformine B (4.1 μM); (c) subsequent addition of Ba^{2+} (2 mM) to the solution containing lepadiformine B. (B) Current−voltage relationships plotted for the current measured at the end (400 ms) of the clamp steps in the control Ringer solution before (open circles) and after (solid squares) 5 min lepadiformine B (4.1 μM) application; (open triangles) subsequent addition of Ba^{2+} (2 mM) to the solution containing lepadiformine B; HP = −100 mV.

that the alkaloid may reach a site lying at an electrical distance θ from the inside of the membrane. Assuming that lepadiformine A bears at least one positive charge, as its chemical structure suggests, θ might be calculated according to the equation $K_d = k_2/k_1 \exp(z\theta FE/RT)$ (eq 3)[18] with z the valence of the molecule, F the Farad, R the gas constant, and T the absolute temperature. Since the slope of the regression line is 35 mV per e fold change in K_d, and zF/RT = 0.04159 mV^{-1} at 20 °C, θ is 0.69.

The addition of lepadiformine B (4.1 μM) to the control solution mainly inhibits the inward components of I_{K1} (Figure 5Aa,b). Subsequent addition of Ba^{2+} (2 mM) to the solution containing lepadiformine B suppressed the remaining current (Figure 5Ac). I_{K1} blockade is a function of lepadiformine B concentration (Figure 2). Compared to lepadiformine A, lepadiformine B (2.5 μM) significantly (P < 0.05) inhibits I_{K1} by only 54%. The half-maximal response was reached at a drug concentration of 1.56 μM. The Hill plot of the data gives a stoichiometric parameter value of 0.72. The current−voltage relationships of Figure 5B plotted for the blockade of I_{K1} show that lepadiformine B (4.1 μM) blocked I_{K1} in the membrane potential range −70 to −30 mV in a voltage-dependent manner (Figure 5B).

The addition of lepadiformine C (3.3 μM) to the control solution does not markedly alter any of the two components of the current

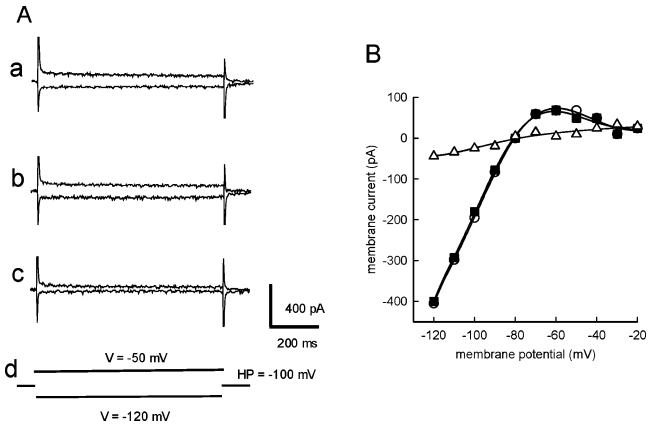

Figure 6. Effect of lepadiformine C on the background inward rectifying K^+ current. (A) Superimposed current traces recorded in response to a −120 mV (lower traces) and −50 mV (upper traces) clamp step (V) applied from HP = −100 mV (d): (a) control Ringer solution containing (b) lepadiformine C (3.3 μM) and (c) lepadiformine C and Ba^{2+} (2 mM). (C) Current voltage relationships plotted for the current measured at the end (400 ms) of the clamp steps. (open circles) Control Ringer solution containing (solid squares) lepadiformine C (3.3 μM) and (open triangles) lepadiformine C and Ba^{2+} (2 mM).

(Figure 6A). The current−voltage relationships of Figure 6B show that the amplitude of the current is not markedly modified in the presence of lepadiformine C (3.3 μM) in the control solution, whatever the membrane potential investigated. Increasing the lepadiformine C concentration in the control solution inhibits only weakly I_{K1} (Figure 2); compared to lepadiformine A, I_{K1} is inhibited only by 8.4% ($P < 0.05$) in the presence of lepadiformine C (2.5 μM) in the control Ringer solution.

Our present data show that the three marine alkaloids, lepadiformines A, B, and C, from *C. moluccencis* block the Ba^{2+}- and Cs^+-sensitive K_{ir} channels in frog auricle according to the sequence lepadiformine A > lepadiformine B > lepadiformine C.

I_{K1} plays an important role in the modulation of the duration of the repolarizing phase of cardiac AP.[19] The molecular structure of the K_{ir} channel consists of two transmembrane domains separated by a pore P loop that contains the signature K^+-selectivity sequence, as well as extended cytoplasmic N′ and C′ termini. The P loop as well as the C′ termini have been implicated in gating.[20] Our data show that I_{K1} is sensitive to micromolar concentrations of lepadiformine A. The stoichiometry of the block (0.77) suggests that at least one molecule is involved in the blockage of the channel. In addition, lepadiformine A I_{K1} channel blockage is not time-dependent, suggesting that the mode of inhibition of the substance differs from that of polyamine cations such as spermidine, which exert a time-dependent blockade on I_{K1}. Polyamine cations are supposed to occupy one or two sites located either in the core of the channel or on the carboxyl loop.[21] Polyamines are thought to block I_{K1} channels by entering deeply into a long, narrow pore, displacing K^+ to the outside of the membrane.[22]

Lepadiformine A causes a voltage-dependent pore block, indicating that I_{K1} inhibition is more pronounced at more positive membrane potentials and that at least one molecule or part of the molecule can enter the pore. The voltage-dependence of inward rectifier K^+ channels results primarily from the movement of K^+ ions across the transmembrane electric field, which accompanies the binding of a blocker.[23] From this voltage-dependence, we calculate that lepadiformine A may occupy a receptor site, lying at about two-thirds (0.69) of the apparent membrane depth, and may be located in close vicinity of or within the I_{K1} channel. This value is close to the one (0.64) reported for the binding of Ba^{2+} to a site located within the pore of $K_{ir}1.1b$ channels expressed in oocytes.[24]

Shortening the length of the lateral aliphatic chain at position C13 in lepadiformine B reduced the inhibition of I_{K1} by about one-half but does not modify the K_D value and the stoichiometry. This reveals that the affinity of lepadiformines A and B for the channel receptor is the same. The saturation of I_{K1} inhibition induced by lepadiformine B reveals that the length of the aliphatic chain plays an important role in the blockage of the pore, suggesting that it may penetrate into the channel. This mode of action shares some similarity with the effects of NMDA and AMPA receptor antagonists[25] or of the $K_v1.3$ channel blocker 5-alkoxypsoralens,[26] whose activity depends on the length of the chain linker. Therefore, our data reveal that the length of the aliphatic chain at position C13 is involved in K_{ir} channel blockage by lepadiformines A and B.

Our data show that lepadiformine C poorly blocked I_{K1}. The chemical structure of lepadiformine C is similar to that of lepadiformine B, except that the oxygenated side chain at position C2 is missing, which leads to a decrease of the ammonium charge. The fact that lepadiformine C poorly blocked I_{K1} indicates that the side chain at C2 plays a major role in the binding capacity to the channel receptor. Ammonium compounds move easily through the phospholipid bilayers to reach a receptor site located in the proximity of the inactivation gate of the Na^+ pore,[27] and quaternary amines compete with the inactivation gate to inhibit the K^+ current.[28] According to Zhou et al.,[29] the hydrophobic central cavity and inner pore of K^+ channels form the receptor site for both the inactivation gate and quaternary ammonium compounds. Lepadiformines may interact with one of the negatively charged amino acids located in the inner vicinity of the narrow K^+ selectivity filter. Obvious candidate residues would be D172, E224, or E229, which form a ring at an intracellular site and which are involved in channel blockade by natural polyamines and are critical for their high affinity.[23,30]

In conclusion, our data reveal that the blockage of I_{K1} by lepadiformines is of physiological interest in cardiac muscle since there are not many blockers specific for this current. The results reveal that the oxygenated side chain at C2 favors the binding of the amine part of the molecule to an intracellular site located inside the channel and the length of the aliphatic chain at position C13 is involved in the blockage degree.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Beckman DU-64 spectrophotometer. IR spectra were obtained (NaCl disks) on a Perkin-Elmer PC 1000 FT-IR spectrophotometer. NMR studies were performed on a Brüker AMX-500 operating at 500 MHz for ^1H and 125 MHz for ^{13}C and referenced to the residual nondeuterated solvent. HREIMS was recorded on a Varian MAT 311 for lepadiformine B. Positive ion, liquid secondary ionization mass spectra (LSIMS, Cs^+, 3-nitrobenzyl alcohol as matrix) for lepadiformine A and high-resolution electrospray ionization mass spectra (HRESIMS) for lepadiformine C were obtained on a ZabSpec TOF Micromass. HPLC was performed on a Gilson system with a model 115 UV/vis detector and a model 132 refractometer.

Animal Material. Samples of *C. moluccensis*, collected by one of the authors (J.-F.B.) at a depth of 5 m around the Musha Islands (Djibouti), were immediately frozen and maintained frozen until extraction. Systematic identification was performed by Dr. C. Monniot at the Muséum National d'Histoire Naturelle (Paris), where a voucher specimen is deposited.

Isolation and Identification of Lepadiformines A, B, and C. The frozen ascidians (2 kg) were extracted twice by 95% EtOH to yield a crude extract, which was partitioned between CH_2Cl_2 and H_2O to yield 6.7 g of organic extract. This extract was then dispersed in aqueous HCl (1 N, 2 L). The resulting suspension was filtered, washed by Et_2O (4 × 500 mL), then directly extracted by CH_2Cl_2 (4 × 500 mL) without change of pH to obtain purified extract (2.2 g). This extract was chromatographed on silica (glass column, silica 40−60 μm, 65 g, extract sample 1.1 g) with a step-gradient of CH_2Cl_2−MeOH to give raw

lepadiformine C (94:6, 250 mL, 75 mg) and a mixture of A and B (92:8, 750 mL, 1 g). Pure lepadiformine C was obtained by preparative silica TLC (CH_2Cl_2−MeOH, 90:10, visualization by sulfuric acid vanillin). A mixture of lepadiformines A and B was resolved by HPLC (Lichrosorb, 25 × 4 mm, 5 μm, isocratic Et_2O−dioxane, 50:50, 1 mL/min, sample 10 mg per cycle) to afford pure lepadiformine A (1.4 g) and lepadiformine B (150 mg).

Lepadiformine A (Figure 1a): pale yellow oil; $[\alpha]_D$ +4.0 (*c* 1.00, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 310 (1,56), 206 (2,74); IR (film, NaCl) ν_{max} 3316, 2931, 2854, 2690, 2597, 1723, 1630, 1469, 1415, 1379, 1266, 1123, 1086, 953, 856, 728, 589 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LSIMS (Cs^+, nMBA), *m/z* 294.3 $[M + H]^+$. See also ref 1.

Lepadiformine B (Figure 1b): pale yellow oil; $[\alpha]_D$ +3.0 (*c* 1.00, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 296 (2,55), 215 (2,98); IR (film, NaCl) ν_{max} 3318, 2933, 2854, 2679, 2587, 1633, 1469, 1333, 1256, 1085, 950, 856, 738, 590 cm^{-1}; 1H and 13C NMR data, see Table 1; HREIMS *m/z* 265.2399, calcd for $C_{17}H_{31}NO$, 265.24055 (Δ + 0.65 mμ).

Lepadiformine C (Figure 1c): pale yellow oil; $[\alpha]_D$ +11.0 (*c* 1.00, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 275 (1.63), 205 (2.57); IR (film, NaCl) ν_{max} 3411, 2934, 2865, 2710, 2638, 1723, 1633, 1469, 1348, 1266, 1117, 1030, 989, 943, 825 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 236.2371 $[M + H]^+$, calcd for $C_{16}H_{29}N$, 236.2378 (Δ + 0.70 mμ).

Solutions. The composition of the standard Ringer solution was (mM) NaCl, 110.5; $CaCl_2$, 2; KCl, 2.5; $MgCl_2$, 1; Na^+ pyruvate, 5; glucose, 10; and HEPES (NaOH) buffer, 10; pH 7.3. The control Ringer solution used in patch-clamp experiments contained 0.6 μM tetrodotoxin (TTX) to block the peak Na^+ current; 2 mM 4-aminopyridine (4-AP) and 10 mM tetraethylammonium (TEA) to inhibit the transient and the delayed outward K^+ currents; 1 mM $CdCl_2$, 1 mM $CoCl_2$, and 0.5 mM $NiCl_2$ to respectively suppress L-type, T-type, and N-type Ca^{2+} currents; and 2 mM 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) to inhibit the chloride current. In some experiments KCl was replaced by CsCl in both the control solution and the patch-clamp pipet solution to suppress I_{K1}. $BaCl_2$ (2 mM) was added to the control solution to inhibit I_{K1}.[31] The Ca^{2+}-free solution used for cell isolation was obtained by simple Ca^{2+} removal. Lepadiformines were dissolved in ethanol.

Electrophysiological Experiments. Experiments were performed at 20−21 °C on myocytes enzymatically isolated from the sino-atrial region of adult frog (*Rana esculenta*) heart.[31,32] Briefly, the auricle was isolated from the heart and opened; then the external epithelial sheet surrounding the auricular tissue was carefully detached and removed, and the cleaned auricle was placed in the isolating chamber. The solutions used for the dissociation were filtered, oxygenated, maintained at 35 °C, and gently stirred with a small magnet. The auricle was successively bathed for 30 min in a Ca^{2+}-free Ringer solution, 20 min in a Ca^{2+}-free Ringer solution containing EGTA (0.1 mM), and 20 min in a Ca^{2+}-free Ringer solution. Then the auricle was bathed for 30 min in a Ca^{2+}-free solution containing 600 mU mL^{-1} type I collagenase (Sigma) and 1.5 mU mL^{-1} type XIV protease (Sigma). When the tissue was digested, the auricle was rinsed (20 min) with a Ca^{2+}-free Ringer solution, then bathed in standard Ringer solution and kept at 4 °C. Cells were dispersed in experimental dishes filled with Ringer solution by gentle agitation of the digested auricle. A giga seal was obtained, and the membrane was ruptured to allow a whole cell voltage clamp. The cell current was monitored using an Axopatch 200B amplifier feedback amplifier (Axon Instruments, Foster City, CA). Patch clamp pipets (Propper Manufacturing glass, i.d. 1.2 mm, wall 0.2 mm) with a resistance of 1.5 to 2.5 MΩ were filled with a solution containing (mM) KCl or CsCl, 150; Na_2-creatine phosphate, 5; K^+-adenosine phosphate, 5; ethyleneglycoltetracetic acid (EGTA) neutralized with KOH, 5; and HEPES (KOH) buffer, 10; pH 7.3. Starting from a holding potential (HP) of −100 mV, the membrane potential (V) was displaced in rectangular steps of 10 mV at a rate of 0.2 Hz. Positive potentials corresponded to depolarization; positive currents corresponded to outward current. Currents were recorded with a Labmaster acquisition card (DMA 100 OEM), driven by the software Acquis 1 linked to the mass storage of a desk computer (AT 80486 DX 33), displayed on an Nicolet 310 oscilloscope (Nicolet, Madison, WI). In our experiments, the series resistance and capacitance were not compensated for. The current magnitude was measured as the difference between the current obtained before and after complete inhibition by subsequent addition of Ba^{2+} to the test solution. Dose−response curves were fitted according to the modified Langmuir equation: $Y = Y_{max}X^p/(K_D{}^p/(K_D{}^p + X^p)$ (eq 1), where Y is the percentage of inhibition of the current, X the concentration of substance, p the stoichiometric parameter, K_D the apparent dissociation constant, and Y_{max} a constant value. Numerical data are expressed as mean values ± SEM; n corresponds to the number of preparations tested. The data were analyzed using the paired Student's *t*-test. Differences were considered significant at $P < 0.05$.

Acknowledgment. The authors are grateful to the European Community for financial support (MAST III contract); the Ardoukoba Association and the Daniel Jouvance Foundation for technical dive support (Mission Auracéa/Djibouti 1996); Dr. C. Monniot (Museum National d'Histoire Naturelle, Paris) for systematic identification of *C. moluccensis*; Prof. Guénot (Centre Régional de Mesures Physiques de l'Ouest, Université de Rennes, France) for mass spectra determination; and M. C. Boumard and F. Luce for technical support.

References and Notes

(1) Biard, J. F.; Guyot, S.; Roussakis, C.; Verbist, J.-F.; Vercauteren, J.; Weber, J.-F.; Boukef, K. *Tetrahedron Lett.* **1994**, *35*, 2691−2694.
(2) Raub, M. F.; Cardellina, J. H.; Choudhary, M. I.; Ni, C. Z.; Clardy, J. *J. Am. Chem. Soc.* **1991**, *113*, 3178−3180.
(3) Copp, B. R.; Ireland, C. M.; Barrows, L. R. *J. Org. Chem.* **1991**, *56*, 4596−4597.
(4) Kong, F.; Faulkner, D. J. *Tetrahedron Lett.* **1991**, *32*, 3667−3668.
(5) Raub, M. F.; Cardellina, J. H. I.; Spande, T. F. *Tetrahedron Lett.* **1992**, *33*, 2257−2260.
(6) Li, C. P.; Blackman, A. J. *Aust. J. Chem.* **1995**, *48*, 955−965.
(7) Kubanek, J.; Williams, D. E.; Dilip de Silva, E.; Allen, T.; Andersen, R. J. *Tetrahedron Lett.* **1995**, *36*, 6189−6192.
(8) Wright, A. D.; Goclik, E.; Kšnig, G. M.; Kaminsky, R. *J. Med. Chem.* **2002**, *45*, 3067−3072.
(9) Jugé, M.; Grimaud, N.; Biard, J.-F.; Sauviat, M.-P.; Nabil, M.; Verbist, J.-F.; Petit, J. *Toxicon* **2001**, *39*, 1231−1237.
(10) Liu, G. X.; Derst, C.; Schlichthorl, G.; Heinen, S.; Seebohm, G.; Bruggemann, A.; Kummer, W.; Veh, R. W.; Daut, J.; Preisig-Muller, R. *J. Physiol.* **2001**, *532*, 115−126.
(11) Pancrazio, J. J.; Frazer, M. J.; Lynch, C., III. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 358−365.
(12) Jin, W.; Lu, Z. *Biochemistry* **1999**, *38*, 14286−14293.
(13) Lucchesi, K.; Ravindran, A.; Young, H.; Moczydlowski, E. *J. Membr. Biol.* **1989**, *109*, 269−281.
(14) Weinreb, S. M. *Acc. Chem. Res.* **2003**, *36*, 59−65.
(15) Sun, P.; Sun, C.; Weinreb, S. M. *Org. Lett.* **2001**, *3*, 3507−3510.
(16) Sun, P.; Sun, C.; Weinreb, S. M. *J. Org. Chem.* **2002**, *67*, 4337−4345.
(17) Abe, H.; Aoyagi, S.; Kibayashi, C. *Angew. Chem., Int. Ed.* **2002**, *41*, 3017−3020.
(18) Woodhull, A. *J. Gen. Physiol.* **1973**, *61*, 687−708.
(19) Kass, R. S.; Arena, J. P.; Walsh, K. B. *Drug. Dev. Res.* **1990**, *19*, 115−127.
(20) Loussouarn, G.; Rose, T.; Nichols, C. G. *Trends Cardiovasc. Med.* **2002**, *12*, 253−258.
(21) Isomoto, S.; Kondo, C.; Kurachi, Y. *Jpn. J. Physiol.* **1997**, *47*, 11−39.
(22) Pearson, W. L.; Nichols, C. C. *J. Gen. Physiol.* **1998**, *112*, 351−363.
(23) Guo, D.; Lu, Z. *J. Gen. Physiol.* **2003**, *122*, 485−500.
(24) Choe, H.; Sackin, H.; Palmer, L. G. *J. Gen. Physiol.* **1998**, *112*, 433−446.
(25) Bolshakov, K. V.; Kim, K. H.; Potapjeva, N. N.; Gmiro, V. E.; Tikhonov, D. B.; Usherwood, P. N.; Mellor, I. R.; Magazanik, L. G. *Neuropharmacology* **2005**, *49*, 144−155.
(26) Vennekamp, J.; Wulff, H.; Beeton, C.; Calabresi, P. A.; Grissmer, S.; Hansel, W.; Chandy, K. G. *Mol. Pharmacol.* **2004**, *65*, 1364−1374.
(27) Hille, B. *J. Gen. Physiol.* **1977**, *69*, 497−515.
(28) Choi, K. I.; Aldrich, R. W.; Yellen, G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5092−5095.
(29) Zhou, M.; Morais-Cabral, J. H.; Mann, S.; Mackinnon, R. *Nature* **2001**, *411*, 657−661.
(30) Kubo, Y.; Murata, Y. *J. Physiol.* **2001**, *513.3*, 645−660.
(31) Sauviat, M.-P.; Colas, A.; Pages, N. *B. M. C. Pharmacology* **2002**, *2*, 15.
(32) Sauviat, M.-P.; Benoit, A. G.; Debitus, C.; Pouny, I.; Laurent, D. *Photobiology* **2001**, *74*, 115−119.

NP050215S