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Chemical and Pharmacological Characterization of *Erythrophleum lasianthum* Alkaloids

Luisella Verotta[1,4], *Talal Aburjai*[1], *Colin B. Rogers*[2], *Paola Dorigo*[3,4], *Ildebrando Maragno*[3], *Daniela Fraccarollo*[3], *Giovanni Santostasi*[3], *Rosa Maria Gaion*[3], *Maura Floreani*[3], *and Francesca Carpenedo*[3]

[1] Dipartimento di Chimica Organica e Industriale, via Venezian 21, I-20133 Milano, Italy
[2] Department of Chemistry, University of Durban-Westville, Private Bag X54001, Durban 4000, South Africa
[3] Dipartimento di Farmacologia, L.go E. Meneghetti 2, I-35131 Padova, Italy
[4] Address for correspondence

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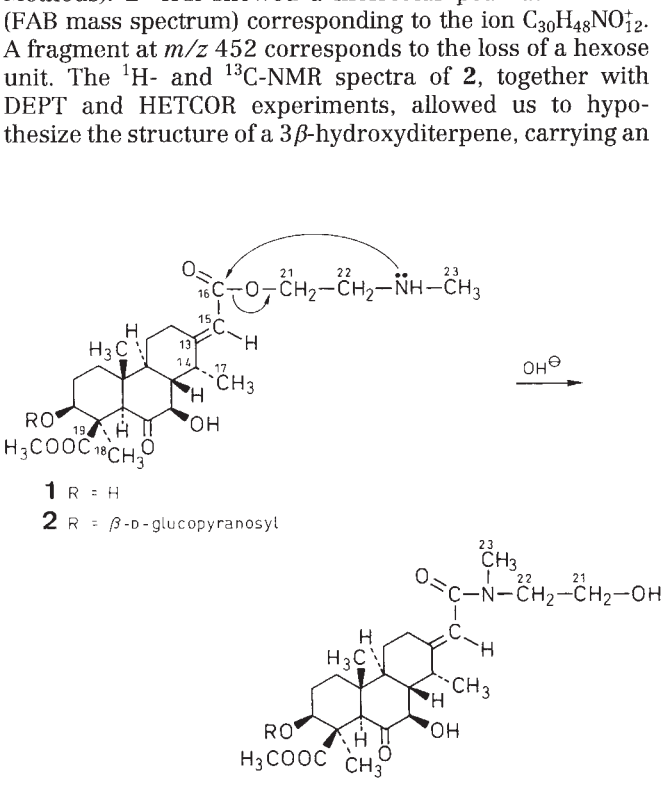
Abstract

Two alkaloids **1** and **2** were isolated from the seeds of *Erythrophleum lasianthum*. Their structures were assigned by spectroscopic and chemical means as 3β-hydroxynorerythrosuamine (**1**) and its 3-*O*-β-D-glucopyranoside (**2**). In spontaneously beating atria, both compounds **1** and **2** showed a marked and concentration-dependent positive inotropic activity and a weak negative chronotropic activity. The positive inotropic effect induced by **1** and **2** was not modified by propranolol, prazosin, carbachol, and ranitidine plus pyrilamine. Both **1** and **2** were very active in inhibiting the Na^+/K^+-ATPase isolated from bovine cardiac sarcolemmal vesicles.

Erythrophleum lasianthum Corbishley (Caesalpinoidae, Leguminosae) is a tree growing in dry sand forests of Natal (Republic of South Africa) and widely used by Zulu peoples in medicines and magic to heal as well as to kill. Since 1875 it has been known that all alkaloids isolated from *Erythrophleum* species exert remarkable digitalis-like activity on the heart (1). Many data are reported about the biological effects of cassaine and its analogues, the alkaloids occurring in the trees of the genus *Erythrophleum* (2, 3), but few data are reported about the chemical composition of *Erythrophleum lasianthum*; its major alkaloid was named erythrophleine but its structure and biological activity was never completely elucidated (4). Since small differences in the chemical structures could cause marked differences in the biological activities, we investigated also this species.

The two main alkaloids present in *Erythrophleum lasianthum*, compounds **1** and **2**, were isolated and characterized as stable hydrochlorides (Materials and Methods). **2** · HCl showed a molecular peak at *m/z* 614 (FAB mass spectrum) corresponding to the ion $C_{30}H_{48}NO_{12}^+$. A fragment at *m/z* 452 corresponds to the loss of a hexose unit. The ^{1}H- and ^{13}C-NMR spectra of **2**, together with DEPT and HETCOR experiments, allowed us to hypothesize the structure of a 3β-hydroxyditerpene, carrying an



unsatured carboxy exocyclic chain, esterified with a 2-methylamino residue and confirmed the presence of a β-D-glucopyranosyl mojety. A carbomethoxy group is present at C-4. Evidence for a 6α-hydroxy-7-keto grouping was provided by the H-7α signal which appeared as a doublet (J = 10.3 Hz) at δ 3.90 and the H-5 singlet at δ 2.66. From the spectroscopic data, biogenetic considerations and comparison with the literature (5–7), **2** was assigned the structure of 3β-hydroxynorerythrosuamine 3-*O*-β-D-glucopyranoside. Compound **1** · HCl ($C_{24}H_{37}NO_7$ · HCl) showed ^{1}H- and ^{13}C-NMR spectra superimposable with those of **2** except for the absence of the sugar part and the upper field shift of C3; **1** could be obtained by enzymatic hydrolysis from **2**, while the acid hydrolysis gave a dehydrated compound. From the same extract, the isomeric amides **1a** and **2a** were also isolated.

When tested on spontaneously beating atria, both compounds **1** and **2** were very active and induced positive inotropic effects in a concentration range corresponding to that of ouabain (Fig. 1). [EC_{50s} = 0.45 μM for **1**; 0.65 μM for **2** and 0.71 μM for ouabain]. Similarly to ouabain, the inotropic effect of **1** and **2** was associated with a slight reduction (−7.8% and −9.7% vs. the control) in the spontaneous beating rate of the atrial preparation. Concentrations of **1** larger than 2 μM and concentrations of **2** larger than 4 μM were toxic. The toxic effects induced by compounds **1** and **2** were similar to those induced by toxic concentrations of ouabain: a sustained increase in frequency rate, arrhythmias and, eventually, a block of the atria during systolic contraction. The positive inotropic effect induced by **1** and **2** was not related to an activation of adrenergic receptors inasmuch as it was modified neither by the β-blocker propranolol (0.1 μM) nor by the α-blocker prazosin (5 nM). The cardiac activity of **1** and **2** was not sustained by the endogenous release of catecholamines, since the experiments were performed on atria isolated from reserpine-treated animals, which did not respond to the tyramine test. Increases in intracellular cyclic adenosine monophosphate were not involved in the contractile effect of the alkaloids, inasmuch as this effect was not inhibited by carbachol (50 nM). Finally, the increase in contractile activity induced by the two compounds was not modified by the antihistaminic agents, pyrilamine (0.1 μM), and rantidine (10 μM) thus excluding an involvement of histamine receptors.

The concentration-effect curves of the inhibitory activity of **1** and **2** on Na^+/K^+-ATPase activity of bovine heart sarcolemmal vesicles (Fig. 2) were parallel to that of ouabain. The IC_{50s} calculated from these curves were 2 μM for **1**, 20 μM for **2**, and 10 μM for ouabain.

The Na^+/K^+-ATPase activity of sarcolemmal vesicles inhibited by 20 min preincubation with **1**, 2 μM, was completely restored when the vesicles were diluted 1 : 20 and incubated in a compound **1**-free medium, indicating that **1** combined reversibly with the enzyme. According to Lineweaver-Burk analysis (Fig. 3) **1** was a mixed inhibitor, as it increased the K_m of ATP from 0.45 to 0.92 mM, whereas it decreased the V_{max} of the enzyme from 24.3 to 14.7 μmoles/mg protein/h. The interaction of **1** with K^+ transport sites (8), was determined by testing its

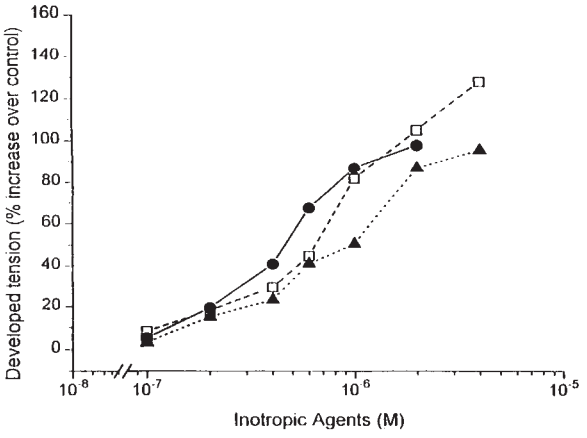


Fig. 1 Cumulative concentration-effect curves of the inotropic effect induced by **1** (●–●), **2** (□–□), and ouabain (▲–▲) in spontaneously beating atria. Each point is the mean ± SE of six to eight assays from eight different experiments.

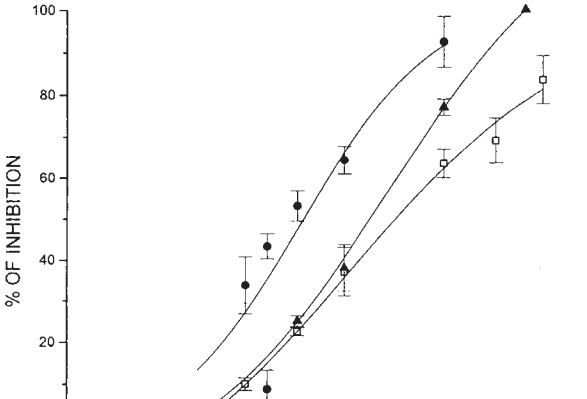


Fig. 2 Concentration-effect curves of the inhibition induced by **1** (●–●), **2** (□–□), and ouabain (▲–▲) on the Na^+/K^+-ATPase activity of cardiac sarcolemmal vesicles. Each point is the mean ± SE of four experiments in duplicate on different membrane preparations.

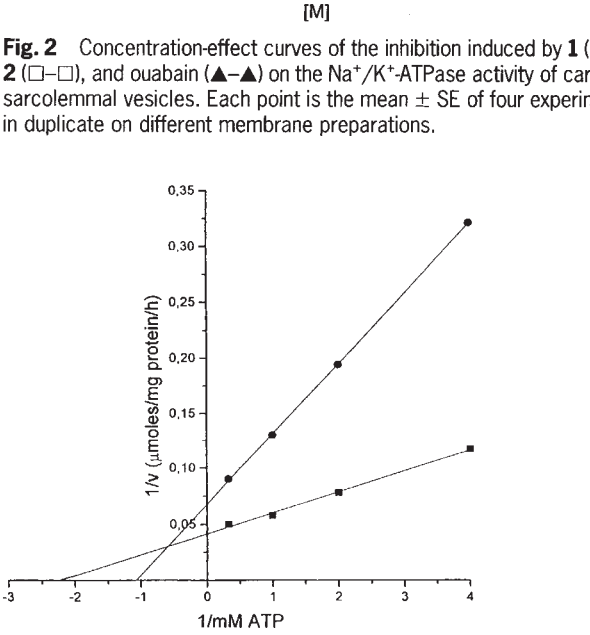


Fig. 3 Lineweaver-Burk plot for Na^+/K^+-ATPase activity of cardiac sarcolemmal vesicles in the absence (■–■) and in the presence (●–●) of **1**, 2 μM. The values represent the mean ± SE of four experiments in duplicate on different membrane preparations.

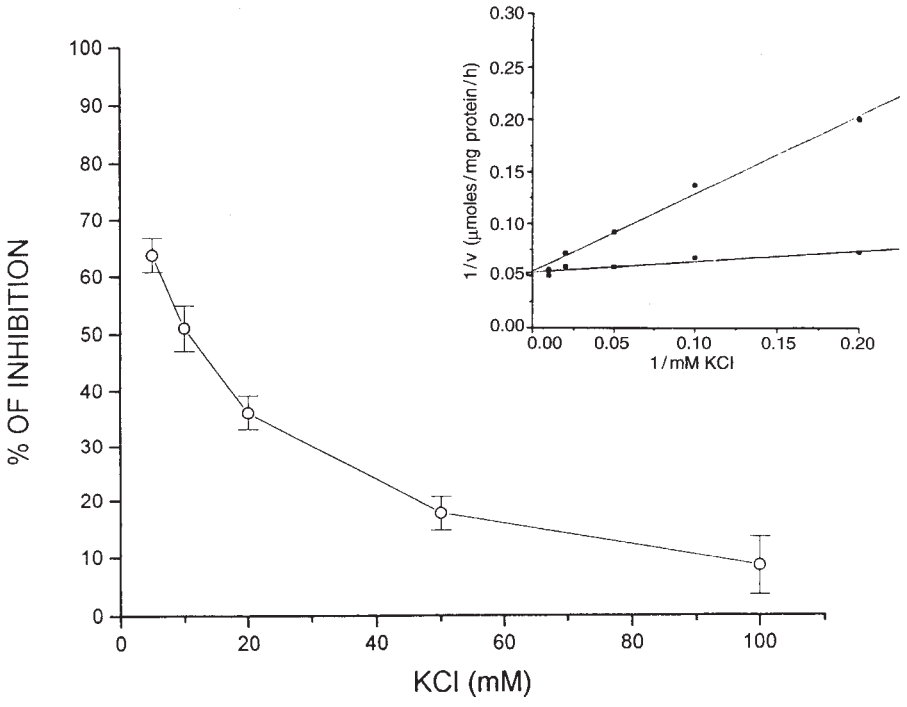


Fig. 4 Effect of increasing concentrations of KCl on the inhibition induced by **1**, 2 μM, on the Na^+/K^+-ATPase activity of cardiac sarcolemmal vesicles. The inset represents the Lineweaver-Burk plot for Na^+/K^+-ATPase activity in the absence (■) and in the presence (●) of **1**, 2 μM, at increasing concentrations of KCl. The values are the mean ± SE of four experiments.

inhibitory effect, at increasing concentrations of KCl. As reported in Fig. **4**, the inhibitory effect of **1**, 2 μM, was decreased by increasing concentrations of KCl in the incubation medium. The inset in Fig. **4** clearly shows that the antagonism was competitive.

When compound **1** was tested on other enzymatic systems known to control the cardiac contractility (sarcolemmal and sarcotubular Ca^{++}-ATPases, Na^+/Ca^{++} exchanger, and cAMP-dependent phosphodiesterases), it was found to be devoid of inhibitory influence (data not shown) suggesting the specificity of its interaction with Na^+/K^+-ATPase. Compound **2** behaved exactly like **1** even if at higher concentrations.

The range of concentrations at which **1** and **2** induced positive inotropic effect and inhibited Na^+/K^+-ATPase was similar to those of ouabain, cassaine (9, 10) and cassaine analogues (11), and the concentrations at which they produced toxic symptoms were only slightly larger than those at which they produced positive inotropic effect. For these reasons, we conclude that the alkaloids extracted from *Erythrophleum lasianthum* seeds behave like other known *Erythrophleum* alkaloids and probably do not represent a safer therapeutic approach for the treatment of cardiac heart failure.

Materials and Methods

M.p.s were measured on a Büchi 570 melting point apparatus and are uncorrected. Optical rotations were measured at 25 °C on a Perkin-Elmer 241 polarimeter. Precoated Kieselgel 60 F254 (Merck) was used for TLC, eluted with the organic phase of the mixture $CHCl_3$-MeOH-*n*-PrOH-H_2O, 5 : 6 : 1 : 4. Spots were detected by observing the UV absorption at 254 nm and spraying with H_2SO_4-MeOH (1 : 9) followed by heating. Kieselgel 60 (63 – 200 μm, Merck) was employed for CC. Sephadex LH 20 (Pharmacia), eluted with MeOH was used for gel filtration. Droplet counter current chromatography was performed on a DCC-A liquid/liquid chromatograph (300 tubes, 2 mm ID × 400 mm, Tokyo Rikakikai Co. Ltd, Tokyo, Japan) using the solvent system $CHCl_3$-MeOH-*n*-PrOH-H_2O (5 : 6 : 1 : 4) in the ascending mode (flow rate 0.4 ml/min). NMR spectra were recorded on a 200 MHz spectrometer (Bruker). TMS was used as internal standard. EI and FAB MS spectra (glycerol, positive mode) were obtained on a VG 7070 mass spectrometer. Noradrenaline hydrochloride, ouabain, propranolol hydrochloride, carbachol chloride, prazosin hydrochloride, ranitidin hydrochloride, pyrilamine maleate, Tris, ATP were from Sigma (St. Louis, Missouri).

Plant material was collected in False Bay (KwaZulu, Natal, South Africa) in December 1991. A voucher specimen (no. 14122/1) is deposited in the Ward Herbarium in Durban (South Africa). Defatted seeds (100 g) were wetted with 10 % ammonia solution (20 ml) and rapidly extracted with MeOH (600 ml), under mechanical stirring, at room temperature (3 h). HCl was blown into the filtered solution (pH 1) which was later concentrated under vacuum and stripped at 20 °C. 12 g of crude extract were obtained, which were taken up with H_2O (200 ml) and extracted with EtOAc (2 × 100 ml). The aqueous residue (10.7 g) was purified on Sephadex LH 20 (5 cm ID × 100 cm, flow rate 12 ml/h, t_R between 680 and 800 ml). Combined fractions were submitted to DCCC obtaining **1** (t_R of **1** between 60 and 65 ml), **2** (t_R 72 – 81 ml), **1a** (t_R 96 – 117 ml), and **2a** (recovered with the stationary phase).

1 · HCl (170 mg) crystallized from $CHCl_3$-EtOAc (1 : 4); m.p. 238 °C; $[\alpha]_D^{25}$: −63.74° (*c* 0.7, MeOH); FAB MS: *m/z* 452 $[C_{24}H_{38}NO_7]^+$; ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 5.71 (1H, s, H-15), 4.24 (2H, m, H-21), 3.88 (1H, d, J = 10.3 Hz, H-7), 3.55 (3H, s, OCH_3), 3.33 (1H, dd, J = 11.9, 5.1 Hz, H-3), 3.13 (2H, m, H-22), 2.60 (1H, bs, H-5), 2.52 (3H, s, H-23), 1.15 (3H, s, H-18), 1.08 (3H, d, J = 7.0 Hz, H-17), 0.82 (3H, s, H-20). ^{13}C-NMR Table **1**.

2 · HCl (490 mg) crystallized from EtOAc-EtOH (9 : 1); m.p. 190 °C, $[\alpha]_D^{25}$: −119.4° (*c* 0.9, MeOH), FAB MS (positive): *m/z* 614 $[C_{30}H_{48}NO_{12}]^+$, 435 [614 − 179]$^+$. ^{1}H-NMR (200 MHz, DMSO-d_6) (after D_2O exchange) δ: 5.71 (1H, s, H-15), 4.25 (2H, m, H-21), 4.17 (1H, d, J = 7.8 Hz, H-1 Glc), 3.90 (1H, d, J = 10.3 Hz,

Table 1 ^{13}C-NMR resonances of compounds **1, 2, 1a, 2a** (50.32 MHz, DMSO-d_6).

C	2*	1*	2a§	1a§	m
1	36.23	36.30	36.22	36.35	t
2	23.61	23.56	24.27	24.24	t
3	86.77	76.28	86.80	76.31	d
4	41.63	41.84	41.71	41.97	s
5	63.11	62.98	63.16	63.06	d
6	208.51	208.71	208.70	208.92	s
7	75.34	75.33	75.48	75.60	d
8	50.49	50.48	50.32	50.41	d
9	45.00	45.12	45.24	45.41	d
10	46.54	47.23	46.52	47.23	s
11	26.16	27.02	25.98	27.07	t
12	25.62	26.15	25.65	26.02	t
13	165.49	165.69	154.41	154.43	s
14	40.01	39.97	38.62	38.80	d
15	112.12	112.21	115.82	115.87	d
			115.60	115.64	
16	167.76	167.43	167.53	167.40	s
			167.26	167.14	
17	13.56	13.56	13.63	13.67	q
18	14.18	14.25	14.19	14.32	q
19	173.19	173.41	173.25	173.43	s
20	25.21	25.43	25.20	25.45	q
21	58.94	60.32	58.61	58.76	t
22	46.92	48.69	51.98	52.03	t
			49.15	49.22	
23	32.68	31.34	36.89	36.87	q
			32.83	32.83	
OCH_3	51.13	50.91	51.15	50.90	q
Glc-1	105.09	–	105.04	–	d
2	73.96	–	73.78	–	d
3	77.02*	–	76.77	–	d
4	70.01	–	69.91	–	d
5	76.90*	–	76.77	–	d
6	61.12	–	61.03	–	t

* Interchangeable values.
\# As hydrochlorides.
§ The double resonances are due to the presence of the *syn* and *anti* configurations.

H-7), 3.53 (3H, s, OCH_3), 3.33 (1H, dd, J = 11.9, 5.1 Hz, H-3), 3.18 (2H, m, H-22), 2.66 (1H, bs, H-5), 2.57 (3H, s, H-23), 1.22 (3H, s, H-18), 1.10 (3H, d, J = 7.0 Hz, H-17), 0.82 (3H, s, H-20). ^{13}C-NMR Table **1**.

1a was crystallized from diisopropyl ether, m.p. 160 °C; $[\alpha]_D^{25}$: – 33.6° (*c* 0.6, MeOH); EI-MS *m/z* (%): 451 (100), 377 (38); ^{1}H-NMR (200 MHz, DMSO-d_6, 40 °C) δ: 5.96 and 5.83 (1H, bs, H-15), 3.87 (1H, d, J = 10.7 Hz, H-7), 3.55 (3H, s, OCH_3), 3.47 (2H, m), 3.34 (2H, m), 2.98 and 2.82 (1H, s, NCH_3), 2.57 (1H, s, H-5), 1.17 (3H, s, H-18), 1.09 (3H, d, J = 6.8 Hz, H-17), 0.85 (3H, s, H-20); (200 MHz, $CDCl_3$) δ: 6.02 and 5.92 (1H, bs, H-15), 3.94 (1H, d, J = 10.7 Hz, H-7), 3.80 (2H, m), 3.75 (3H, s, OCH_3), 3.35 (1H, dd, J = 11.9, 5.1 Hz, H-3), 3.10 and 3.00 (3H, s, NCH_3), 2.37 (1H, s, H-5), 1.37 (3H, s, H-18), 1.22 (3H, d, J = 6.8 Hz, H-17), 0.92 (3H, s, H-20). ^{13}C-NMR Table **1**.

2a (434 mg) crystallized from EtOAc; m.p. 179 °C; $[\alpha]_D^{25}$: – 53.98° (*c* 0.64, MeOH); FAB MS (positive) *m/z*: 636 [M + Na]$^+$, 458 [M – 179]$^+$; ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 5.95 and 5.85 (1H, bs, H-15), 5.01–4.43 (7H, exchangeable with D_2O), 4.20 (1H, d, J = 7.8 Hz, H-1 Glc), 3.87 (1H, d, J = 10 Hz, H-7), 3.53 (3H, s, OCH_3), 2.95 and 2.80 (3H, s, NCH_3), 2.63 (1H, bs, H-5), 1.22 (3H, s, H-18), 1.08 (3H, d, J = 6.7 Hz, H-17), 0.80 (3H, s, H-20). ^{13}C-NMR Table **1**.

Hydrolysis of **2a**

2a (100 mg), was dissolved in 3 ml of water and hydrolyzed with 1.2 ml of β-glucuronidase from *Helix pomatia* (4114, Merck) at 37 °C for 48 h. The mixture was extracted with $CHCl_3$ (3 × 3 ml). In the aqueous phase, glucose was identified by TLC and GLC by comparison with an authentic sample. The organic phase was dried then evaporated under vacuo, obtaining 56 mg of **1a**. It was purified by chromatography on silica gel (5 g) eluted with $CHCl_3$-*i*-PrOH (9 : 1) (80 ml) (t_R between 40 and 64 ml). Using the same procedure, **2** was hydrolyzed to give **1** which was not further purified.

Biological tests

All the biological experiments were performed on **1** and **2** as hydrochlorides. Spontaneously beating atria were isolated from reserpine treated (2 mg/kg *i.p.* daily for two days) guinea-pigs and suspended vertically in a physiological salt solution as already reported (12). The developed control tension ranged from 0.09 to 20 mN. Changes in developed tension were recorded by a writing oscillograph (Mod. 7050, Basile Unirecord System, Italy).

The inotropic agent was added cumulatively and the inotropic effect was recorded for 5 min after it had reached its maximum before adding a higher concentration. The basal and the stimulated contractile activity was determined by measuring the amplitude of developed tension. The data are expressed as percent variation from the control (i.e., atria incubated without drugs). Unless otherwise specified, the Na^+/K^+-ATPase activity was determined on sarcolemmal membrane vesicles prepared from bovine cardiac tissue according to Slaughter et al. (13). Typically 10 μg of sarcolemmal proteins were incubated for 20 min at 37 °C in a medium (final volume 1 ml) of the following mM composition Tris-HCl (pH 7.4) 50, NaCl 100, KCl 20, $MgCl_2$ 3 and ATP 3. The incubation was stopped by addition of ice-cold 12.5 % trichloroacetic acid. Pi formed from ATP was spectrophotometrically determined. Na^+/K^+-ATPase activity was evaluated as the difference between the samples incubated in the presence and in the absence of NaCl and KCl.

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