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Callipeltin A: sodium ionophore effect and tension development in vascular smooth muscle

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Abstract

Callipeltin A is a cyclic depsidecapeptide isolated from the marine sponges *Callipelta* sp. and *Latrunculia* sp. that has been previously shown to increase the force of contraction of guinea-pig atria through the inhibition of Na^+/Ca^{2+} exchanger (NCX). We investigated the effect of callipeltin A on guinea-pig aortic rings contracted by procedures that activate NCX in ''calcium entry mode''. Callipeltin A did not inhibit these contractions. Resting aorta responded to callipeltin A with a remarkable contraction that was concentration-dependent (EC50 0.44 μM). This contraction was not inhibited by the calcium channel blocker verapamil and was not mediated by the activation of alpha-adrenergic or endothelin-1 receptors. Pre-incubation of aortic rings with 0.5 mM amiloride, an inhibitor of NCX, completely prevented callipeltin A-induced contraction. Furthermore, callipeltin A (EC50 0.51 μM) increased Na^+ efflux of Na-loaded erythrocytes. ^1H and ^{13}C NMR resonances of callipeltin A revealed small but significant changes in the titration with K^+ and Na^+ salts. It is suggested that the effect of callipeltin A on cardiac and vascular preparations is linked to a Na-ionophore action.

Keywords: Callipeltin A; Natural depsipeptides; Na/Ca exchanger; Na-ionophore; Vascular smooth muscle contractility

Callipeltin A (Fig. 1) is a cyclic depsidecapeptide isolated from the marine sponges *Callipelta* sp. and *Latrunculia* sp., whose structure consists of a macrocyclic lactone containing three unusual amino acid residues: β-methoxytyrosine (βOMeTyr), (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (AGDHE), and (3*S*,4*R*)-3,4-dimethyl-L-glutamine (diMeGln) [1,2]. Natural depsipeptides have been intensively studied in recent years for their relevant biological activities, including anticancer, antiviral, antibacterial, antifungal, anti-inflammatory, and anti-clotting or anti-atherogenic properties (reviewed by Ballard et al. [3]). Since its purification and characterization, callipeltin A showed some interesting pharmacological properties. In fact, callipeltin A has antifungal activity and is one of the first natural products to show activity against HIV [1]. Furthermore, this compound exerts cytotoxic activity against various carcinoma cell lines, including NSCLC-N6 non-small-cell-lung carcinoma, E39 renal carcinoma, P388 murine leukemia and M96 melanoma [3]. It has been also reported that callipeltin A is highly cytotoxic to porcine PS cells [4]. The molecular mechanisms of these effects of callipeltin A are still unknown.

Starting from the observation of the positive inotropic action on rat heart of some natural cyclodepsipeptides (roseotoxin B, roseocardin) [5], and from the work of Khananshvili et al. [6] on a series of conformationally constrained cyclic peptides that inhibit Na^+/Ca^{2+} exchanger (NCX) activity, in a previous work we tested the effect of callipeltin A on cardiac preparations [7]. We found that callipeltin A inhibited NCX of cardiac sarcolemmal vesicles. NCX is a major mechanism responsible for Ca^{2+} extrusion, therefore, an inhibition of NCX in cardiac muscle results in a positive inotropic effect, since the amount of Ca^{2+} available for the contractile machinery is increased. Accordingly, on guinea-pig atria

Abbreviations: NCX, Na^+/Ca^{2+} exchanger; $[Na^+]_0$, extracellular Na^+ concentration; $[Na^+]_i$, intracellular Na^+ concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration

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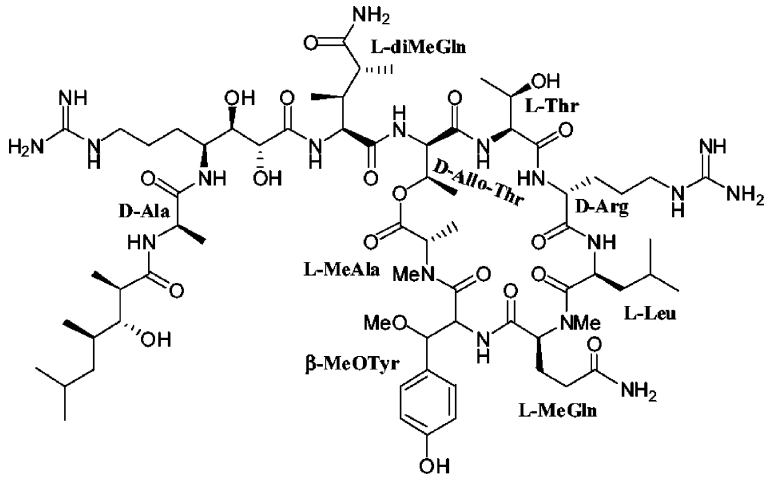

Fig. 1. Structure of callipeltin A.

callipeltin A exerted a positive inotropic effect [7]. Besides the heart, the presence of the NCX has been demonstrated also in vascular smooth muscle by analysis of RNA and protein expression [8]. Due to its localization adjacent to junctional sarcoplasmic reticulum (SR), it has been proposed that NCX could indirectly modulate the Ca^{2+} content of SR stores and thereby influence Ca^{2+} signalling and tension development [9,10]. In vascular smooth muscle preparations, experimental conditions inducing a decrease of the ratio $[Na^+]_0/[Na^+]_i$ promote Ca^{2+} influx through NCX working in "Ca^{2+} entry mode", thus triggering contraction [9–12]. Indeed, NCX countertransports Ca^{2+} for Na^+ in either direction across the cell membrane, the direction and the efficiency being determined by the membrane potential and the $[Na^+]_0/[Na^+]_i$ ratio values [8].

Taking into account the positive inotropic effect of callipeltin A, we decided to investigate its effect on guinea-pig vascular smooth muscle contractility, in order to widen the knowledge about the effects of this cyclic depsipeptide on the cardiovascular system and to gain insights on its mechanism of action. The data presented indicate that callipeltin A induces the contraction of guinea-pig aorta by means of an increase of sodium influx as shown by the effect of this compound on sodium fluxes in red blood cells.

1. Materials and methods

1.1. Chemicals

Callipeltin A and callipeltin D were isolated as previously described [1,2] and were dissolved in DMSO. Amiloride, ouabain, phenylephrine, and verapamil were purchased from Sigma and were dissolved in twice distilled water. Furosemide was from Sigma and was dissolved in methanol. $LiClO_4$, NaSCN, KSCN (spectroscopic grade reagents) were purchased from Aldrich.

1.2. Contractility of isolated guinea-pig aortic rings

Thoracic aorta excised from guinea-pig (250–350 g) were cleaned of connective tissue and then cut into two or three rings of 2 mm length. Rings were deprived of endothelium by gently rubbing the lumen with the tip of round-nose pliers. Two steel hooks were inserted into the lumen of each ring, which was then vertically placed in an organ bath filled with 15 ml of physiologic salt solution (PSS) aerated with 95% O_2 and 5% CO_2, and maintained at 35 °C (pH 7.36–7.40). One of the hooks was connected to an isometric transducer coupled to a pen recorder (Battaglia-Rangoni) for monitoring the developed tension. The composition of the PSS was (mM): NaCl, 125; KC1, 5; $CaCl_2$ 2.7; $MgSO_4$, 1; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11. Rings were stretched passively to impose a resting tension of about 1.5 g and allowed to equilibrate for at least 60 min before the experiment started. Each ring was then repeatedly stimulated with 1 μM phenylephrine until a reproducible response was obtained. Washout of phenylephrine for 20 min was performed before the beginning of the experiments. When sodium was omitted, sucrose was added (232 mM) to maintain osmolarity and $KHCO_3$ was substituted for $NaHCO_3$. In potassium-free PSS, KC1 was omitted and NaH_2PO_4 was substituted for KH_2PO_4.

For the concentration–response curve, callipeltin A was cumulatively added, the final DMSO concentration was always below 1 μL/mL, a concentration that has no effect on vascular contractility. The developed tension was measured in milligrams and expressed as percent of the contractile response elicited by 1 μM phenylephrine.

1.3. Sodium efflux of red blood cells

Blood was drawn into heparinized tubes and centrifuged at 3000 rpm for 4 min at 4 °C. Plasma and buffy coat were removed by aspiration, and red blood cells washed three

times with ice-cold washing solution (CWS) that contained (mM): choline chloride 149, $MgCl_2$ 1, Tris–MOPS 10, pH 7.4 (4 °C). Washed erythrocytes were resuspended to 50% hematocrit with the same solution. To measure the effects of callipeltin A and calipeltin D (an open chain derivative of callipeltin A [2]) on Na^+ efflux, the cell suspension was incubated in the CWS in the presence of ouabain, inhibitor of Na^+/K^+ ATPase, and of furosemide, inhibitor of Na^+–K^+–$2C1^-$ cotransport. Therefore, the cells were divided into the efflux medium (CWS) containing 1 mM ouabain and 1 mM furosemide in the absence and in the presence of increasing concentrations of callipeltin A or callipeltin D, as previously described [13]. The cells suspensions were divided into various tubes and were maintained at 37 °C in a shaking water bath. After 1, 5, 10, 40 min, three tubes were spun down and the Na^+ concentrations of the supernatant was measured by atomic absorption spectrophotometry (model 2380, Perkin Elmer) using appropriate Na^+ solution standards. The slope of Na^+ concentration in the efflux medium was linear in all the experiments. Efflux rate (mmol/L cells × h) was obtained by dividing the regression slope of the Na^+ concentration in the efflux medium versus time by the hematocrit level of the cell suspension. The callipeltin-induced efflux of Na^+ was measured by subtracting the efflux in the presence and in the absence of callipeltin A or callipeltin D.

1.4. NMR analysis

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX 500 MHz spectrometer operating at 500 MHz (^1H) and 125 MHz (^{13}C), and are referenced to CD_3OD solvent signals at 3.30 and 49.0 ppm, respectively. Titration of callipeltin A (0.1 M in CD_3OD) with $LiClO_4$, NaSCN, KSCN (0.3 M in CD_3OD) were monitored by ^1H NMR and ^{13}C NMR spectroscopy. K^+ complex: ^1H NMR [500 MHz, CD_3OD, selected $\Delta\delta$ ($\delta_{\text{callipeltin A} + K^+} - \delta_{\text{callipeltin A}}$) values in ppm]: −0.07 (αH-D-Allo-Thr), −0.02 (αH-MeAla), +0.02 (αH-βMeOTyr), +0.01 (αH-MeGln), +0.01 (αH-Leu), −0.02 (αH-DiMeGln), +0.01 (αH-Arg), +0.02 (αH-Thr), +0.02 (NMe-MeAla), +0.02 (NMe-MeGln); ^{13}C NMR [125 MHz, CD_3OD, selected $\Delta\delta$ ($\delta_{\text{callipeltin A} + K^+} - \delta_{\text{callipeltin A}}$) values in ppm]: −0.2 (CO-D-Allo-Thr), −0.2 (CO-MeAla), +0.1 (CO-βMeOTyr), −0.3 (CO-MeGln), −0.1 (CO-Leu), +0.1 (CO-DiMeGln), −0.2 (CO-Thr). Na^+ complex: ^1H NMR [500 MHz, CD_3OD, selected $\Delta\delta$ ($\delta_{\text{callipeltin A} + Na^+} - \delta_{\text{callipeltin A}}$) values in ppm]: −0.02 (αH-D-Allo-Thr), −0.02 (αH-MeAla), +0.01 (αH-βMeOTyr), −0.01 (αH-MeGln), +0.01 (αH-Leu), −0.02 (αH-DiMeGln), −0.01 (αH-Arg), +0.02 (αH-Thr), +0.02 (NMe-MeAla); ^{13}C NMR [125 MHz, CD_3OD, selected $\Delta\delta$ ($\delta_{\text{callipeltin A} + Na^+} - \delta_{\text{callipeltin A}}$) values in ppm]: −0.2 (CO-D-Allo-Thr), −0.2 (CO-MeAla), +0.1 (CO-βMeOTyr), −0.3

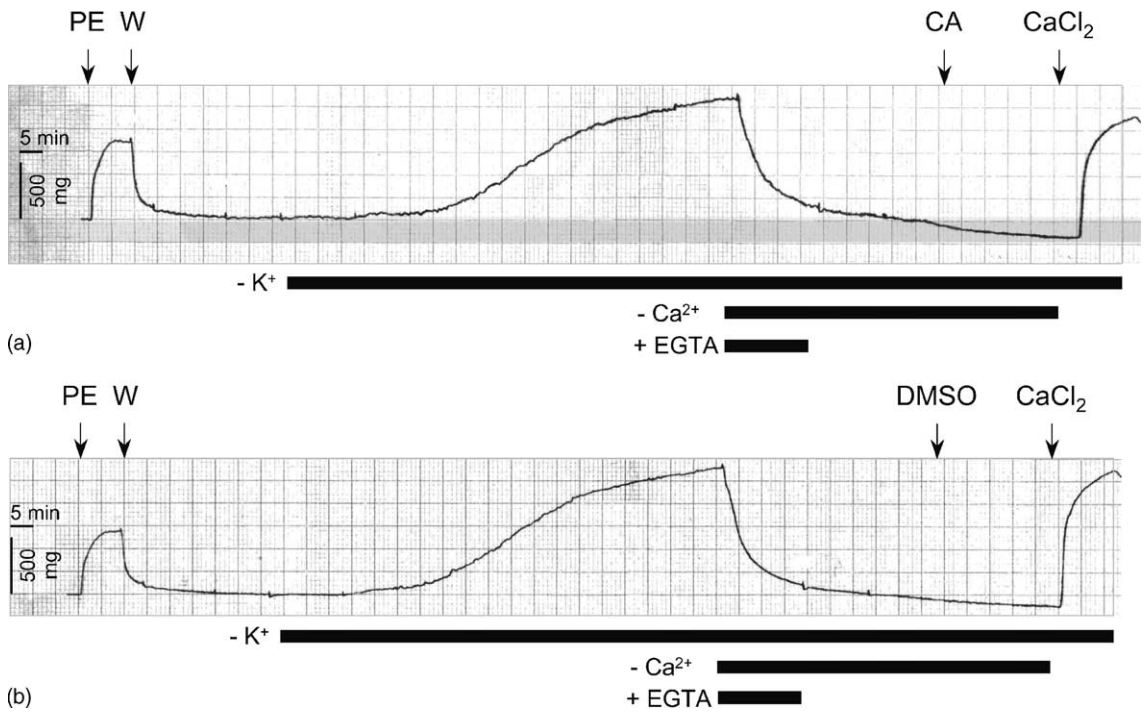

Fig. 2. Original traces of the contractile responses of two separate guinea-pig aortic rings showing the lack of effect of (a) 1 μM callipeltin A (CA) or (b) 3 μL DMSO on the contraction elicited by Ca^{2+} in K^+-free PSS. Following the stimulation with 1 μM phenylephrine (PE), the rings were washed (W) and contracted by exposure to K^+-free PSS. The rings completely relaxed upon washing with K^+- and Ca^{2+}-free PSS containing 1 mM EGTA to chelate all the extracellular calcium and re-contracted by the addition of 2.7 mM $CaCl_2$ in the absence of EGTA; CA and DMSO were added to the K^+- and Ca^{2+}-free PSS before the addition of 2.7 mM $CaCl_2$. Data obtained from three separated experiments showed that the developed tension was (in mg, mean ± S.E.): 1109 ± 23, 1087 ± 47 and 1180 ± 20, for control, callipeltin A and DMSO-treated rings, respectively.

(CO-MeGln), −0.2 (CO-Leu), +0.1 (CO-DiMeGln), −0.4 (CO-Thr), +0.2 (Cα-Thr), +0.2 (Cβ-Thr).

1.5. Statistical analysis

Data on graphs are presented as mean ± standard error of the mean (S.E.M.) of at least three independent experiments. Graphs and EC50 determination were performed using GraphPad Prism version 3.03 for Windows, GraphPad Software.

2. Results

2.1. Effect of callipeltin A on guinea-pig aortic rings

In order to ascertain whether callipeltin A inhibits vascular smooth muscle NCX, guinea-pig aortic rings were exposed to experimental conditions which stimulated the NCX to work in "Ca^{2+} entry mode", thus inducing vascular smooth muscle contraction [11,12,14]. Ca^{2+} influx through NCX was obtained reducing the ratio $[Na^+]_0/[Na^+]_i$ by lowering the concentration of K^+ in the medium (Fig. 2), condition that induces the increase of $[Na^+]_i$ through the inhibition of Na^+/K^+ ATPase, or by decreasing $[Na^+]_0$ (Fig. 3). As shown in Figs. 2a and 3a, 1 μM callipeltin A did not inhibit the guinea-pig aortic ring contractions induced by activation of NCX forced to promote Ca^{2+} entry. In order to exclude any effect of the solvent, DMSO (0.02%, v/v) was tested in control rings (Figs. 2b and 3b).

As shown in Fig. 4, resting guinea-pig aortic rings exposed to callipeltin A (0.1–3 μM) developed concentration-dependent contractions. The maximal effect produced by callipeltin A was twofold the contraction induced by 1 μM phenylephrine, utilized as reference stimulus, the EC50 (0.44 μM) and the range of the effective concentrations was identical to that inducing a positive inotropic effect on guinea-pig atria [7]. Fig. 5 shows that verapamil (10 μM), a L-type calcium channel antagonist, neither prevented nor relaxed the contraction induced by 1.5 μM callipeltin A. Also the α-adrenergic antagonist phentolamine as well as the ET_A/ET_B endothelin-1 receptor antagonist bosentan failed to inhibit callipeltin A contraction (data not shown).

Considering that a decrease in $[Na^+]_0/[Na^+]_i$ produces Ca^{2+} influx through NCX, we explored the possibility that

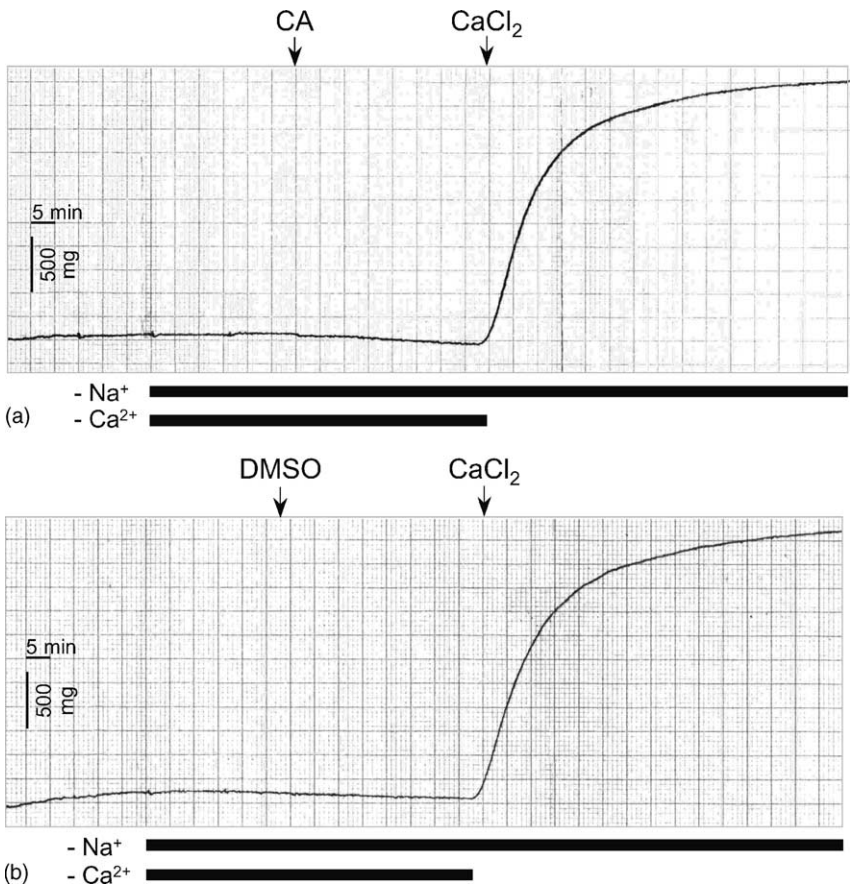

Fig. 3. Original traces of the contractile responses of two separate guinea-pig aorta rings showing the lack of effect of (a) 1 μM callipeltin A (CA) or (b) 3 μL DMSO on the contraction elicited by Ca^{2+} in Na^+-free PSS. Following the stimulation with 1 μM phenylephrine, aorta rings were washed with Na^+- and Ca^{2+}-free PSS containing 1 mM EGTA to chelate all the extracellular calcium (not shown in the traces). The rings were then incubated in Na^+- and Ca^{2+}-free PSS without EGTA; CA or DMSO were added before the addition of 2.7 mM $CaCl_2$ to the Na^+- and Ca^{2+}-free PSS. Data obtained from three separated experiments showed that the developed tension was (in mg, mean ± S.E.): 2104 ± 137 and 1891 ± 204, for control (with DMSO) and callipeltin A-treated rings, respectively.

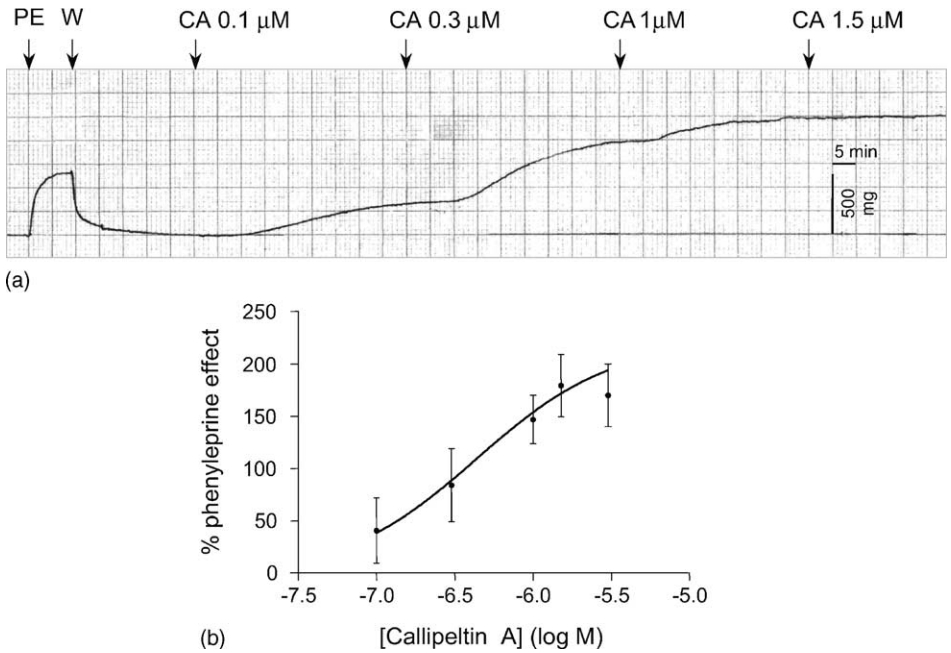

Fig. 4. (a) Original trace of the contractile response of a guinea-pig aorta ring exposed to increasing concentrations of callipeltin A (CA); stimulation with 1 μM phenylephrine (PE) is taken as reference stimulus; W, wash, (b) concentration–response curve of callipeltin A-induced contraction of guinea-pig aorta rings; developed tension induced by callipeltin A was expressed as percent of the contraction elicited by 1 μM phenylephrine; data are the mean ± S.E. of four independent experiments.

callipeltin A contracts aortic rings by promoting an increase of $[Na^+]_i$. To examine this hypothesis, we investigated if the inhibition of NCX could prevent the contraction induced by callipeltin A. Since we have previously shown that amiloride inhibits NCX-mediated contractions in guinea-pig aorta [11], we tested the effect of amiloride on the contraction elicited by callipeltin A. Amiloride (0.5 mM) completely prevented the contraction induced by maximal concentrations of callipeltin A (Fig. 6a). On the contrary, amiloride showed a negligible effect on the fully developed contractions, in agreement with our previous finding that this drug did not relax the rings contracted by Ca^{2+} entry through the NCX [11].

2.2. Effect of callipeltin A on sodium fluxes

The data obtained with guinea-pig aortic rings indicate that the contraction induced by callipeltin A could be due

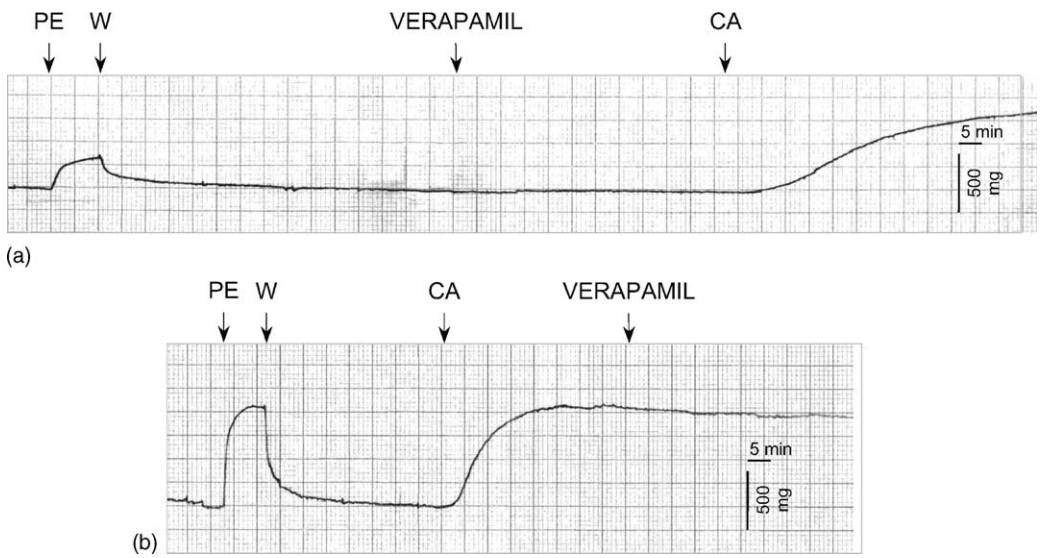

Fig. 5. Original traces showing the effect of verapamil on the contraction of guinea-pig aorta rings induced by callipeltin A. Verapamil (10 μM) was added either before (a) or after (b) the addition of 1.5 μM callipeltin A (CA). Data obtained from three separated experiments showed that the developed tension induced by callipeltin A was (in mg, mean ± S.E.): 813 ± 23 (control) and 757 ± 23 (verapamil pretreatment), whereas verapamil induced a decrease of 4 ± 1.47% (mean ± S.E., $n = 4$) when added after a fully developed contraction induced by callipeltin A.

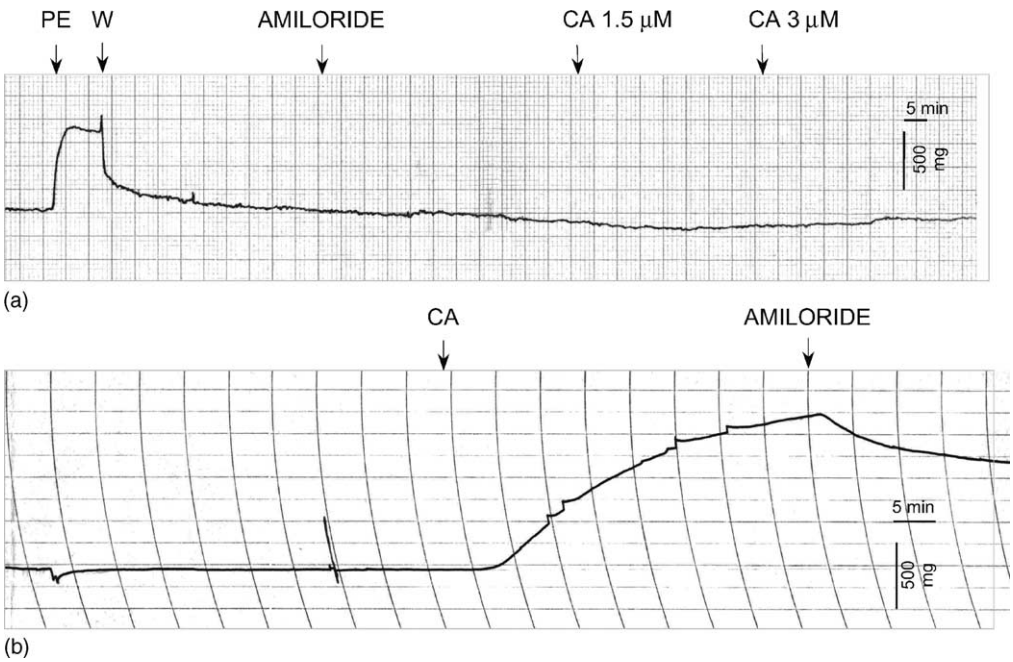

Fig. 6. Original traces showing the effect of amiloride on the contraction of guinea-pig aorta rings induced by callipeltin A. (a) Preincubation with 0.5 mM amiloride was performed before the addition of maximal concentrations (1.5 and 3 μM) of callipeltin A (CA). (b) Addition of 0.5 mM amiloride after a fully developed contraction elicited by 1.5 μM callipeltin A (CA). Data from five independent experiments showed that pretreatment with amiloride completely prevented callipeltin A-induced contraction, whereas amiloride induced a decrease of 14 ± 3% (mean ± S.E., $n = 3$) when added after a fully developed contraction induced by callipeltin A.

to an increase in Na^+ influx. Na^+ fluxes through the plasma membrane can be easily studied in red blood cells and therefore experiments to investigate the action of callipeltin A on those fluxes have been performed. In red blood cells, loaded with NaCl, callipeltin A increased Na^+ efflux in concentration-dependent manner with a EC50 of 0.51 μM (Fig. 7). At concentrations higher than 2 μM callipeltin A caused cell lysis. These data suggest that callipeltin A could mediate the transport of sodium ions through the plasma membrane.

Furthermore, we found that callipeltin D, a natural derivative of callipeltin A that has no effect either on cardiac sarcolemmal vesicles or guinea-pig atria [2], did not promote Na^+ efflux of NaCl-loaded erythrocytes (Fig. 7). This indicates that there is a correlation between the capacity of the compound in increasing Na^+ fluxes and the pharmacological effects observed in cardiac and vascular preparations.

2.3. NMR analysis

Titration of callipeltin A (0.1 M in CD_3OD) with $LiClO_4$, NaSCN, KSCN in CD_3OD were monitored by ^{1}HNMR. Whereas no changes in chemical shifts were observed in the Li^+ titration even after the addition of 6 equivalents of the cation, small but significant changes were observed in the titration with K^+ and Na^+ salts. After addition of 1.0 equivalent of the monovalent cation, no additional changes were observed by further addition of 2, 3 or 4 equivalents. The observed $\Delta\delta$ ($\delta_{\text{callipeltin A}+M^+} - \delta_{\text{callipeltin A}}$) for the ^{1}H and ^{13}C NMR resonances affected by the addition of the Na^+ or K^+ salts (1 equivalent) were reported in the experimental section. Significant changes in chemical shifts were observed especially for H-α protons and carbonyl signals of the aminoacidic units of the macrocyclic core. Figs. 8 and 9 show, respectively, the ^{1}H NMR and ^{13}C NMR spectra of callipeltin A and callipeltin A + 1 equivalent of NaSCN. Even if the small variations observed do not suggest the formation of a tight complex, NMR data support the hypothesis of an

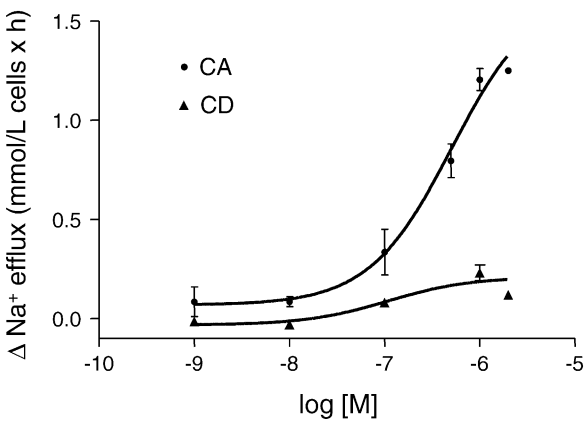

Fig. 7. Concentration–response curve of the effect of callipeltin A and callipeltin D on Na^+ efflux in red blood cells. Na^+-loaded cells were incubated in the absence or in the presence of either callipeltin A (CA) or callipeltin D (CD) as described in Section 1. Data are expressed as the difference between Na^+ efflux in treated vs. control cells and are the mean ± S.E. of three experiments.

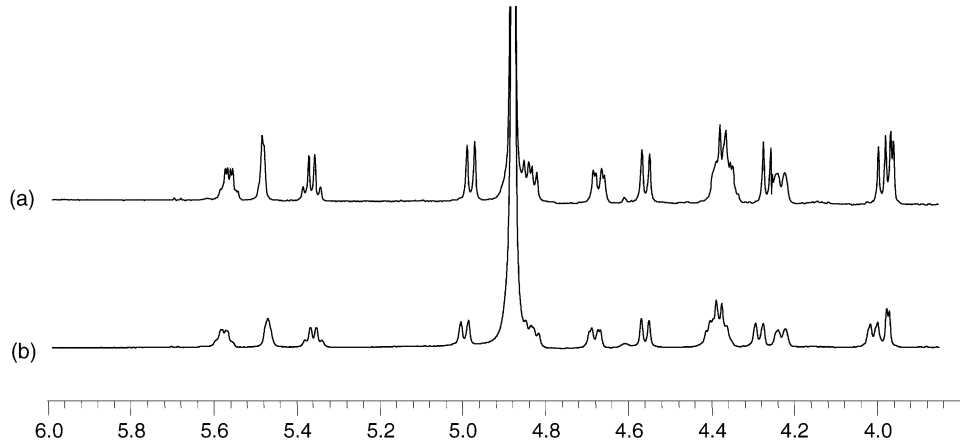

Fig. 8. (a) ^{1}H NMR (partial) of callipeltin A. (b) Callipeltin A + 1 equivalent of NaSCN.

interaction between callipeltin A and K^+ and Na^+ cation, that appears more efficient with potassium ion.

3. Discussion

In our previous work on the cardiac effect of callipeltin A, we found a powerful inhibition of the cardiac sarcolemmal vesicles NCX activity measured as Na-dependent $^{45}Ca^{2+}$ uptake in NaCl-loaded vesicles. On the basis of this finding, it would be expected that callipeltin A prevents aortic rings contraction induced by Ca^{2+} influx consequent to the activation of NCX working in "Ca^{2+} entry mode". The results of the present study show that this is not the case and, surprisingly, callipeltin A exerts a vasoconstrictor effect in guinea-pig aortic rings. The inhibition by callipeltin A of NCX can hardly explain vasoconstriction, since it has been shown that in resting vascular smooth muscle cells the inhibition of the NCX does not produce enough increase in intracellular calcium concentration to activate contraction, being the activity of the plasma membrane Ca^{2+} pump sufficient to maintain $[Ca^{2+}]_i$ within resting levels [15]. Also the activation of either L-type calcium channels or of α-adrenergic or endothelin-1 receptors is unlikely as a mechanism mediating this effect, being the calcium entry blocker verapamil, the α-adrenergic antagonist phentolamine, and the ET_A/ET_B endothelin-1 receptor antagonist bosentan ineffective in antagonizing the contraction induced by callipeltin A. Furthermore, it could be excluded that callipeltin A induces a release of calcium from internal stores since it elicits a contractile response only in the presence of extracellular calcium (Figs. 2a and 3a).

Our previous finding that callipeltin A inhibits the NCX activity comes from experiments in NaCl-loaded sarcolemmal vesicles, where the activity of NCX is measured as $^{45}Ca^{2+}$ transport in dependence on Na^+ gradient [7]. This does allow to ascertain an inhibitory effect on NCX activity, but does not elucidate the mechanism of action underlying this effect. We have therefore reconsidered the finding on sarcolemmal vesicles and hypothesized that callipeltin A could inhibit the $^{45}Ca^{2+}$ transport by dispersing the Na^+ gradient. The experiments performed with Na-loaded erythrocytes validate this idea, since callipeltin A produces a significant increase in Na^+ efflux, thus acting as a Na^+-ionophore that promotes the transmembrane transport of Na^+ ions according to the concentration gradient. The inhibition induced by callipeltin A of NCX activity shown in NaCl-loaded sarcolemmal vesicles, therefore, is an indirect effect and can now be explained by the dispersion of the Na-gradient, consequent to Na^+ efflux, that does not support NCX activity. This effect can also explain both the positive inotropic effect in guinea-pig atria [7] and the vasoconstrictor effect on aortic rings of this cyclodepsipeptide. Actually, in intact tissues, a Na-ionophore action promotes Na^+ influx, thus inducing a

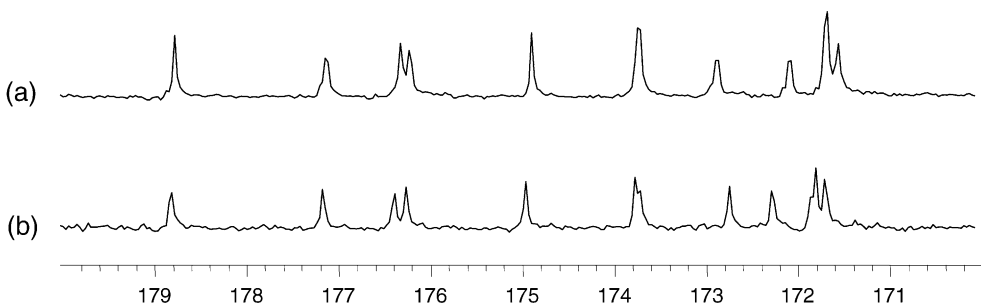

Fig. 9. (a) ^{13}C NMR (carbonyl region) of callipeltin A. (b) Callipeltin A + 1 equivalent of NaSCN.

decrease in $[Na^+]_0/[Na^+]_i$ ratio that in vascular smooth muscle activates contraction by stimulating Ca^{2+} entry through NCX [8,12]. Accordingly, amiloride was able to prevent and not to resolve the contraction of aortic rings elicited by callipeltin A, in agreement with the observations that amiloride prevents NCX-mediated contractions in guinea-pig aorta, as previously reported [11]. Furthermore, the lack of effect of callipeltin D shown in this study (Fig. 7) supports the conclusion that cardiovascular effects of callipeltin A are linked to its capacity of mediating Na^+ transport. In fact, callipeltin D, an open chain derivative of callipeltin A, did not affect Na^+ efflux from erythrocytes and, as previously shown, had no effect on guinea-pig atria contractility as well as on $^{45}Ca^{2+}$ uptake of cardiac sarcolemmal vesicles [2].

^1H NMR and ^{13}C NMR spectra indicate an interaction of callipeltin A with Na^+ and K^+. Although there is no formation of a tight complex between Na^+ and callipeltin A, the ionophore activity for this compound is evident. The Na^+-ionophore effect may also explain the antifungal and cytotoxic properties of callipeltin A. In fact, the mechanism of action of several antimicrobial agents including valinomycin, monensin, nystatin, amphotericin B is linked to the ability of these compounds of transporting ions across the membranes thus dissipating the transmembrane ion gradients [16–18].

In conclusion, the finding that callipeltin A acts as a ionophore compound gives an indication about the molecular mechanism responsible of some biological activities of this cyclodepsipeptide.

Acknowledgments

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