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Inhibition of cardiac (Na^+, K^+)-ATPase isozymes by LND 623

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LND 623 [3β-rhamnosyl-14β-amino-20(*R*)-β-ol, 5β-pregnan*] is a novel positive inotropic drug with greater potency and lower arythmogenicity than digoxin [1–3]. On guinea pig papillary muscles, LND 623 exerts a significant positive inotropic effect at a three-fold lower dose than digoxin. The two compounds are equi-effective at inducing the same signs of toxicity [3]. The structure of LND 623 differs from the general structure of cardiac glycosides by two substitutions on the steroid skeleton: replacement of the lactone ring by an alcohol function (C20β) and the 14β-hydroxyl group by an amine function. LND 623 has been found to inhibit (Na^+, K^+)-ATPase (EC 3.6.1.37) activities in brain and cardiac preparations from human and guinea pig [4, 5]. In heart, the (Na^+, K^+)-ATPase isozymes of high and low affinity for ouabain [6–10] are involved in the inotropic and arythmogenic effects of cardiac glycosides, respectively [7, 9–14]. The apparent inconsistency between the inotropic efficacy of LND 623 and its inhibitory effect on both cardiac and non-cardiac (Na^+, K^+)-ATPases led us to investigate the activity of this compound on dog and rat cardiac (Na^+, K^+)-ATPase isozymes [9, 13]. Our results indicate that LND 623 might exert its large and potent inotropic activity via a direct and selective inhibition of the (Na^+, K^+)-ATPase isoforms of high affinity for cardiac glycosides.

Materials and Methods

Membrane preparations. Rat and dog (mongrel) hearts were perfused via coronary arteries with either an ice-cold medium containing a physiological Ca^{2+} level (2 mM) or a Ca^{2+}-free buffer according to the procedure described previously [13]. (Na^+, K^+)-ATPase-enriched preparations were isolated from canine [9] and rat cardiac ventricles [13]. Protein content was determined by the method of Lowry *et al.* [15]. Sarcolemmal vesicles resuspended in 100 mM NaCl, 250 mM sucrose and 30 mM imidazole-HCl pH 7.4, were kept frozen at −80° and used for enzymatic assays within 1 week. To make membranes permeable to substrates and ligands before (Na^+, K^+)-ATPase assays, all vesicles were subjected to repeated freezings and thawings or to sodium dodecyl sulfate treatments (0.2 mg/mg of protein, 30 min at 20°) [13].

(Na^+, K^+)-*ATPase assay and drug sensitivity.* Enzymatic activity, in the absence or presence of ouabain (from 0.3 nM to 2 mM) or LND 623 (from 0.3 nM to 30 μM), was determined at 37° using the coupled assay method [14] in a medium containing 100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 2 mM phosphoenol pyruvic acid, 1.4 mM NADH, 3.5 units of pyruvate kinase, 5 units of lactate dehydrogenase and 40 mM imidazole-HCl pH 7.4. (Na^+, K^+)-ATPase assays were carried out after a 30-min preincubation at 37° in the assay medium (without NADH) containing purified membranes and well defined drug concentrations. Control experiments have shown that, under these conditions, a stable level of enzyme inhibition is reached. The enzymatic reaction was initiated by placing an aliquot of this medium into a cuvet containing the complete assay mixture with the same drug concentration as used in the preincubation. The rates of NAD formation were then monitored continuously for 30 min. Enzyme activity in the presence of drug was measured at equilibrium, i.e. when the decrease in absorbance was a linear function of time. Control experiments demonstrated that LND 623 did not oxidize NADH if added to the incubation mixture or interfere with pyruvate kinase and lactate dehydrogenase activities.

Mathematical analysis. The dose–response curves were analysed assuming two separate and saturable inhibitory processes. We used a computerized non-linear regression program (Biosoft, Cambridge, U.K.) which adjusts the parameters to minimize the sum of relative squared errors.

Results and Discussion

LND 623 induced a biphasic dose-dependent inhibition of both canine and rat cardiac (Na^+, K^+)-ATPases (Fig. 1a and b). Up to 70% of the total enzymatic activity was inhibited by concentrations between 3 and 10 nM. Complete inhibition occurred with 1 and 300 μM in dog and rat, respectively. LND 623 inhibited both dog and rat (Na^+, K^+)-ATPase isoforms and discriminated between them.

As described in detail previously for the reactivity of ouabain on the same preparations [9], the biphasic pattern could represent the sum of two inhibitory processes, each of them being characterized by an IC_{50} value and a proportional contribution. Computed IC_{50} values for LND 623 were lower than 0.1 nM for the process of high affinity and averaged 200 nM for the low affinity process. In both dog and rat cardiac preparations, the former process represented 65 ± 4% of the total inhibition induced by LND 623.

In striking contrast with ouabain, LND 623 had a constant and very high affinity for the inotropic sites (less than 0.1 nM). However, it exhibited the same affinities as ouabain for the low affinity isozymes. For LND 623, the ratio of the IC_{50} values of low to high affinity inhibitory sites was approximately 1500 whereas it was 150 for ouabain.

The Ca^{2+}-dependent expression of the rat cardiac (Na^+, K^+)-ATPase isoform with high sensitivity for ouabain [13] was not observed with LND 623. Indeed, whatever the Ca^{2+} concentration during the heart perfusion, LND 623 always discriminated 65 ± 4% of enzyme activity associated with high affinity sites (Fig. 1b).

The well known species difference in sensitivity to cardiac glycosides between dog and rat cardiac (Na^+, K^+)-ATPases was abolished with LND 623 but only for the high affinity (inotropic) sites. The fact that LND 623 exerted a significant positive inotropic effect at a lower dose than ouabain (Fig. 1) could be explained by the higher reactivity of LND 623 as compared to ouabain on the high affinity isoform. The similar potency of LND 623 and ouabain in producing the same signs of toxicity in dog (Fig. 1) reflects their similar reactivity on the low affinity sites.

Thus, LND 623 exhibits a similar and very high affinity (0.1 nM) for the rat and dog heart (Na^+, K^+)-ATPase isozymes responsible for the inotropic effects. This selectivity towards the "inotropic isozymes" not detected with ouabain could constitute the molecular basis of the strong cardiotonic action of LND 623.

* Abbreviation: LND 623, 3β-rhamnosyl-14β-amino-20(*R*)-β-ol,5β-pregnan.

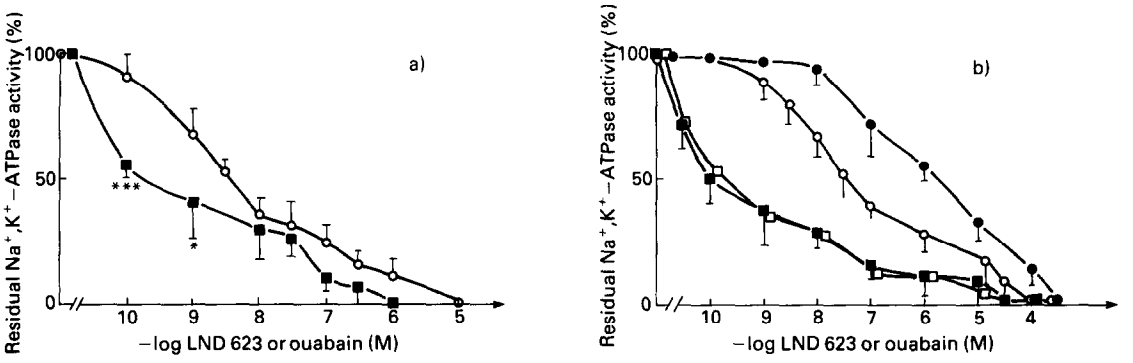

Fig. 1. (a) Dose–response curves of (Na^+, K^+)-ATPase activity to drug concentrations (logarithmic scale) in canine cardiac sarcolemmal vesicles. The drugs used are ouabain (○) and LND 623 (■). The final concentration of (Na^+, K^+)-ATPase molecules was 20 pM. The specific activities are: 110 ± 20 μmol/mg protein × hr. Ouabain insensitive ATPase activities represent less than 30% of the total ATP hydrolysis activity. Results obtained with the two drugs are means of three determinations on six membrane preparations from dog hearts. The standard error of the mean ranged from 6 to 10%. *** $P < 0.001$; * $P < 0.01$. (b) Dose–response curves of (Na^+, K^+)-ATPase activity to drug concentrations (logarithmic scale) in rat cardiac sarcolemmal vesicles. Ouabain (○), the source hearts were maintained at a physiological Ca level. Ouabain (●); the source hearts were perfused with a Ca-free buffer [13]. LND 623 (■); source hearts ± Ca. The sensitivity was tested in triplicate in three different preparations. The specific activities are: 160 ± 30 μmol/mg protein × hr. The standard error of the mean ranged from 8 to 15%.

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