Volume 281, number 1,2, 145–148 FEBS 09574 April 1991

ADONIS 001457939100285B

An organophosphorus compound, Vx, selectively inhibits the rat cardiac Na^+,K^+-ATPase α_1 isoform

Biochemical basis of the cardiotoxicity of Vx

Pascale Robineau[1,*], Yves Leclercq[2], Alain Gerbi[2], Isabelle Berrebi-Bertrand[2] and Lionel G. Lelievre[2]

[1]*Centre d'Etudes du Bouchet, Division of Pharmacology, BP no. 3, 91710 Vert-le-Petit, France* and [2]*Laboratoire de Pharmacologie des Transports Ioniques Membranaires, Hall de Biotechnologies, Tour 54/5e, Université Paris 7, 2 Place Jussieu, 75251 Paris Cedex 05, France*

Received 9 February 1991

Serine-specific reagents, anticholinesterase organophosphorus compounds like Vx provoke, in the micromolar range, digitalis-like ventricular arrythmias of non-cholinergic origin in rodent hearts. The sensitivities of the two rat cardiac Na^+,K^+-ATPase isoforms (α_1 and α_2) to Vx (0.1–100 μM) were measured in sarcolemma vesicles. At 1 μM Vx, the inhibition of the total activity averaged 18% but never exceeded 75% with 100 μM. When the α_2 isoform activity was inhibited by 0.1 μM ouabain, α_1 was 35% inhibited by 1 μM Vx, i.e. a 16±4% inhibition of the total activity. The cardiac α_1 being related to the digitalis-induced toxicity, its selective inhibition by a micromolar dose of Vx fully accounts for the cardiotoxicity of Vx. Inasmuch as Vx had no effect on the rat kidney α_1, differentially inactivated the cardiac isozymes and specifically reacted with serine residues, the putative binding-site(s) of the organophosphorus compound on the Na^+,K^+-ATPase molecules has been considered.

α_1-Isoenzyme; Na^+,K^+-ATPase; Organophosphorus; Heart

1. INTRODUCTION

Organophosphorus compounds are known to irreversibly inhibit cholinesterases by reacting with serine residues [1]. They induce central and peripheral disorders resulting from their anticholinergic action. Cardiotoxicity was reported to involve a brief and intense sympathetic discharge, followed by an extreme parasympathetic tone [2]. A third phase, in which ventricular premature complexes and ventricular tachycardia occurred, was described in clinical reports [2,3]. Similar ventricular arrhythmias have been observed following administration of the organosphosphorus compound *S*-(2-diisopropylaminoethyl)-*O*-ethylmethylphospho-nothiolate (Vx) in either conscious or anaesthetized rats (12 μg/kg body weight s.c.) and dogs (3–6 μg/kg body weight s.c.) [4,6].

A recent electrophysiological work [7] in guinea-pig papillary muscles showed that Vx (5 μM) modified the course of the action potential. Depolarizing oscillations in resting membrane potential, so-called delayed afterdepolarizations, were recorded and, in some cases, led to the development of triggered activity in this non-automatic preparation. Moreover, these Vx-induced effects were found to increase when the electrical stimulation frequency applied to the preparation was increased. Such actions are classically reported [8] to characterize a digitalis-induced ventricular toxicity.

Inasmuch as a micromolar dose of Vx induced a digitalis-like toxicity in animals, the initial goal of this study was to determine whether or not this compound could inhibit the cardiac Na^+,K^+-ATPase activity, i.e. the pharmacological receptor for digitalis [9]. In a second aproach, we have looked for an either general or selective action of this compound on the cardiac and renal Na^+,K^+-ATPase isozymes. Then, in order to approach what could be the molecular basis of the Vx effects, the distributions of serine residues in the isoform-specific zones of the two enzyme molecules have been compared.

Molecular cloning and sequence of the α subunit encoding DNAs revealed the existence of three major α subunit isoforms in rat: α_1, α_2 and α_3 [10]. In adult rat heart, the first two isoforms are expressed [11,12].

At the membrane level of cardiac muscles of rat [13,14], guinea-pig [15] and dog [16], two functional Na^+,K^+-ATPase isoforms (so-called α_1 and α_2) have been described. The α_2 isoform shows a high affinity for cardiac glycosides (apparent K_d values from 1 to 20 nM) and is responsible for their positive inotropic properties. The α_1 isoform is of low affinity for digitalis (apparent K_d values from 0.3 to 7 μM). In rat and

Correspondence address: L.G. Lelievre, Laboratoire de Pharmacologie des Transports Ioniques Membranaires, Hall de Biotechnologies, Tour 54/5e, Université Paris 7, 2 Place Jussieu, 75251 Paris Cedex 05, France. Fax: (33) 1) 44 27 69 66

Present address: Servier Institute, 11 Rue des Moulineaux, 92150 Suresnes, France.

guinea-pig hearts, α_1 inhibition by high doses of digitalis leads to both inotropic and toxic effects.

Our study show that Vx induced a selective inhibition of the cardiac α_1 isoform at a micromolar range, whereas the cardiac α_2 isoform was affected at higher Vx doses.

2. MATERIAL AND METHODS

Cardiac sarcolemmal vesicles were isolated according to our previously published procedure [17] from normal Wistar adult rat hearts perfused by a Ca^{2+}-free solution [13]. Rat kidney Na^+,K^+-ATPase was partially purified as described by Sweadner [18]. Enzyme activities were measured at 37°C as a function of time and amounts of proteins (from 0.1 to 2 μg). The relationships were linear. The enzymatic assays were carried out with vesicles permeabilized by LDS or SDS (lithium or sodium dodecyl sulfate) treatments (0.20 mg detergent/mg of proteins for 30 min at 20°C). The Na^+,K^+-ATPase activity was determined using the coupled assay method as previously described [19]. In the microsomal fractions, the specific activities of the Na^+,K^+-ATPase varied from 60 to 90 μmol of inorganic phosphate liberated per mg of protein per hour. The ouabain-insensitive activity, measured in the presence of 2 mM ouabain, accounted for less than 30% of the total ATPase activities.

In order to restrict the amplitude of the temperature and time-dependent denaturations of Vx during the assays, all the enzymatic measurements have been carried out at 30°C and did not exceed 20–22 min. After a 5-min preincubation of the reaction medium at 30°C, the enzymatic reaction was initiated by successive and rapid additions of Vx and LDS- (or SDS-) treated membranes and continuously monitored for up to 22 min. The inhibition level induced by a single dose of Vx remained stable during 22 min. In the assays, the maximum final concentrations of digitalis receptors (i.e. Na^+,K^+-ATPases) varied from 0.01 to 1 nM, with respective final concentrations of drug varying from 0.1 to 100 μM. Inhibition percentages of the total activity were calculated by comparing the activities in the presence or absence of drug after correcting for the ouabain-insensitive ATPase activity.

3. RESULTS AND DISCUSSION

The organophosphorus Vx, was solubilized into methylethylketone. This solvent interacted with the enzyme system at concentrations higher than 0.3% (v/v). In all the assays the vehicle concentrations never exceeded 0.1% (v/v), and the maximum Vx concentration was 100 μM.

The organophosphorus compound did not affect the ouabain-insensitive ATPase activity or the coupling enzymes (pyruvate kinase and lactodehydrogenase) (unpublished). When tested on the total Na^+,K^+-ATPase activity, Vx was found to achieve a stable inhibition within 90 s. Indeed, this effect did not vary from 90 s to 22 min (the longest kinetic period used). Thus, once bound, Vx would not be released.

As shown in Table I, no inhibition of the cardiac Na^+,K^+-ATPase activity was found with 0.1 μM Vx. The percentage of inhibition increased from 18 ± 6% up to 75% between 1 and 100 μM Vx in the assay. This inhibitory effect is consistent with previous reports showing an action of this type of compound on brain [20] and rat cardiac [21] Na^+,K^+-ATPase activities.

Table I

Comparative effects of Vx on the Na^+,K^+-ATPase activities associated with the α_1 isoform or with α_1 plus α_2 isoforms

Vx concentrations	Percent inhibition of	
	($\alpha_1 + \alpha_2$)	α_1
0.1 μM	L.D.	L.D.
1 μM	18 ± 6 (n = 12)	35 ± 4 (n = 5)
6.6 μM	29 ± 6 (n = 4)	40 ± 3 (n = 4)
10 μM	35 ± 8 (n = 10)	42 ± 5 (n = 7)
100 μM	70 ± 5 (n = 3)	55 ± 6 (n = 11)

L.D. = limit of detection.

In rat cardiac sarcolemma vesicles, the proportional contributions of the α_1 plus α_2 isoform activities in the total Na^+,K^+-ATPase activity were 45 ± 8% and 55 ± 8%, respectively [13,19].

In order to determine whether Vx had either a selective or a specific effect on one of the two cardiac Na^+,K^+-ATPase isoenzymes, α_1 and/or α_2, the responsiveness to Vx has been studied in the presence of 0.1 μM ouabain. At this ouabain concentration, 90% of the activity due to the α_2 isoform was inhibited (IC_{50} = 10 nM) whereas no detectable effect could be found on the activity associated with the α_1 isoform (IC_{50} = 7 μM). Consequently, in the presence of 0.1 μM ouabain, the activity due to the isoform α_1 represented more than 86% of the total activity measured.

In the presence of this submaximal ouabain concentration, the association process of Vx to its site(s), the stability of the complex and its apparent irreversibility were indistinguishable from those found when assaying the Na^+,K^+-ATPase activity in the absence of ouabain.

As shown in Table I (right panel), 0.1 μM Vx did not induce any inhibition whereas a stable inhibition (35 ± 4%) was found with 1 μM Vx. There was a sharp increase in the inhibition, from 0% to 35% between 0.1 and 1 μM Vx whereas at a 100-fold higher concentration (100 μM), Vx induced a 20% increase in the inhibition of the activity associated with α_1. There was no apparent relationship between the development rate of the inhibitory process, the Vx concentration and the isoform considered.

The inhibition of α_1 did not exceed 61% (Table I). It is very unlikely that the non-inhibited activity represented α_1 activity trapped into impermeable vesicles and inaccessible to Vx. Indeed, this isoform, to be assayed, should also be freely permeable to all the ligands of the enzyme. Furthermore, all Na^+,K^+-ATPase assays have been carried out with permeabilized (detergent-treated) vesicles. An alternative explanation would be that the Vx-resistant activity was of non-muscle origin. This possibility also has to be ruled out: only a few percent of our sarcolemmal vesicles isolated from normal rat heart were of non-muscle origin [17].

The percentage of inhibition of α_1 found with 1 μM Vx fully accounts for the 18 ± 6% inhibition of the total

activity (Table I and Fig. 1). Indeed, 35% inhibition of α_1 × 45% contribution represents a 16% inhibition of the total Na^+,K^+-ATPase activity. α_2 would not be inhibited at 1 μM Vx. At higher Vx levels, α_2 was more and more inhibited (Fig. 1). At 100 μM Vx, the maximum inhibition found, 75% of the total activity would represent the sum: 25% (55% × 45%) due to α_1 plus 50% (75–25%) due to α_2. The latter isoform was almost completely inhibited: 90% (50%/55%).

The present study clearly shows that the digitalis-like cardiotoxicity, of non-cholinergic origin, induced by a micromolecular dose of Vx was due to a selective inhibition of the cardiac α_1 isoform of the Na^+,K^+-ATPase. Inhibition by cardiac glycosides of this isoform α_1 of low affinity for ouabain also led to toxic effects in rat as well as in guinea-pig. Note that, at a Vx dose 5-fold lower than the toxic one [7], we already observed a significant (35%) inhibition of the α_1 isoform.

The relatively high sensitivity of the cardiac α_1 isoform to Vx was not observed with the rat kidney α_1 isoform; Vx (up to 30 μM) did not inhibit this activity (data not shown).

A comparison of the amino acid sequences of the two α subunits of the Na^+,K^+-ATPase as presented by [10] provides a basis to determine, at the protein level, which part(s) of the molecule might be involved in these different reactivities to Vx. The prediction is that regions of the molecule that vary in serine composition from α_1 isoform to α_2 are regions important in terms of sensitivity to Vx.

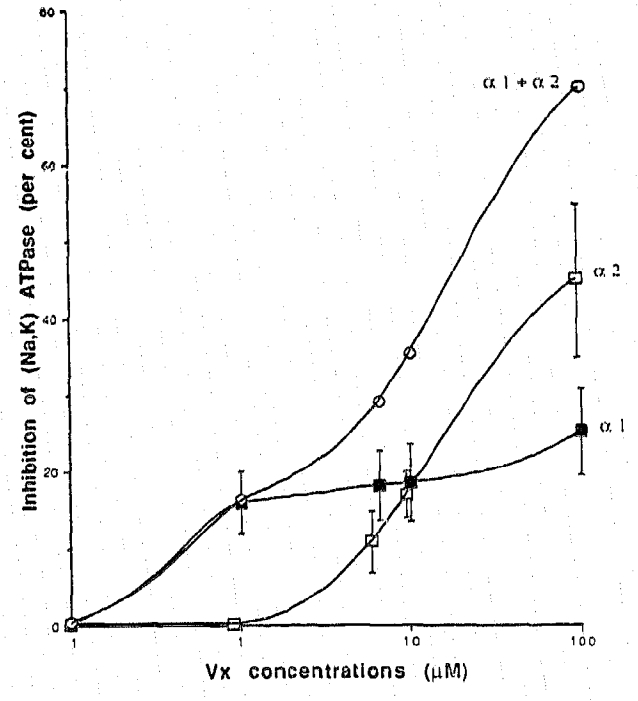

Fig. 1. Respective inhibitions by Vx of the two active rat cardiac Na^+,K^+-ATPase isoenzymes α_1 (■) and α_2. (□) The percentages of inhibition of α_1 were calculated from the values given in Table I (right panel) and assuming that the α_1 isoform activity represented 45% of the total enzymatic activity. The percentages of α_2 inhibition were the differences between the inhibitions of total activity and the α_1 inhibitions.

Out of the identical zones of α subunit isoform structures [10,22,23], the regions with the greatest sequence variations showing isoform-specific features, as defined by [23], occurred in clusters, in the NH_2-terminal half, in the extracellular loop H1H2 implicated in ouabain binding [24] and near the fluorescein isothiocyanate (FITC)-reactive site [23]. Seven positions of serine only occurred in the α_1 isoform and at least one of them should account for the higher apparent sensitivity to Vx. It is not yet possible to say whether all 7 serine residues contribute to the selectivity, although a predominant role of this that adjoins the nucleotide-binding site seems likely.

A particular reactivity of a single serine could fully account for the Vx/α_1 isoform interactions. Indeed, the magnitude of the α_1 inhibition did not significantly increase when [Vx] varied from 1 to 100 μM (Fig. 1) suggesting a limited number of serines involved in the chemical reaction with Vx. Furthermore, the kidney α_1 isoform, very similar to the cardiac one, was not inhibited by Vx.

So far, the simplest interpretation would be that the binding of Vx to α_1 occurred at a single site near, but not intrinsic to, the conserved sites essential for activity. According to this hypothesis, the serine-494 near the ATP-binding site (FITC-reactive site) would be a good candidate since there are significant sequence variations in α_1 and α_2 isoforms in the variable region to the left of the FITC-reactive lysine, mainly regarding the serine residues. However, it is noteworthy that these comparisons and suggestions suppose that rat brain and cardiac α_1 isoforms displayed the same amino acid sequences. This has not, as yet, been demonstrated.

The incomplete inhibition of the α_1 activity might be explained assuming that α_1 was heterogeneous and consisted of two forms, one responsible for the inotropic effect of high doses of ouabain [25] and insensitive to Vx and one responsible for the toxic effects of digitalis and sensitive to Vx. However, we have not been able yet to physically separate these putative two forms in hearts.

The responsiveness of the cardiac α_2 isoform clearly differed from that of α_1 (Fig. 1). The α_2 inhibition sharply increased with Vx concentrations suggesting a successive recruitment of several α_2-specific serine residues. The high concentrations of Vx necessary to inhibit this isoform (up to 100 μM) reflect the low sensitivity of the reacting site(s) consistent with the general inhibitory effects of different organophosphorous compounds (diisopropylfluoridate, paraoxon and parathion) on various ATPases including the cardiac Ca^{2+}-ATPases [21].

Acknowledgements: This study was supported by grants from le Centre d'Etudes du Bouchet (Convention 25071/89/ETCA/CEB/CBS).

REFERENCES

[1] Schaffer, N.K., May, S.C. and Summerson, W.H. (1954) J. Biol. Chem. 206, 201–207.
[2] Ludomirsky, A., Klein, H.O., Sarelli, P., Becker, B., Taitelman, U., Barzilai, J., Lang, R., David, D., Disegni, E. and Kaplinsky, E. (1982) Am. J. Cardiol. 49, 1654–1658.
[3] Brill, D.M., Maisel, A.S. and Prabhu, R. (1984) J. Electrocardiol. 17, 97–102.
[4] Robineau, P. (1987) Toxicol. Appl. Pharmacol. 87, 206–211.
[5] Robineau, P. and Guittin, P. (1987) Toxicol. Lett. 37, 95–102.
[6] Robineau, P. (1988) J. Pharmacol. Methods 19, 127–133.
[7] Corbier, A. and Robineau, P. (1989) Arch Int. Pharmacodyn. Ther. 300, 218–230.
[8] Rosen, M.R. (1985) J. Am. Coll. Cardiol. 5, 22A–34A.
[9] Schwartz, A., Whitmer, K., Grupp, I., Adams, R.J. and Lee, S.W. (1982) Ann. NY Acad. Sci. 402, 253–271.
[10] Shull, G.E., Greeb, J. and Lingrel, J.B. (1986) Biochemistry 25, 8125–8132.
[11] Emanuel, J.R., Garetz, S., Stone, L. and Levenson, R. (1987) Proc. Natl. Acad. Sci. USA 84, 9030–9034.
[12] Orlowski, J. and Lingrel, J.B. (1988) J. Biol. Chem. 263, 10436–10442.
[13] Mansier, P. and Lillèvre, L.G. (1982) Nature 300, 535–537.
[14] Charlemagne, D., Mayoux, E., Peyard, M., Oliviero, P. and Geering, K. (1987) J. Biol. Chem. 262, 8941–8944.
[15] Berrebi-Bertrand, I., Maixent, J.M., Guede, F.G., Gerbi, A., Charlemagne, D. and Lelièvre, L.G. (1991) Eur. J. Biochem. (in press).
[16] Maixent, J.M., Charlemagne, D., de la Chapelle, D. and Lelièvre, L.G. (1987) J. Biol. Chem. 262, 6842–6848.
[17] Mansier, P., Charlemagne, D., Rossi, B., Preteseille, M., Swynghedauw, B. and Lelièvre, L.G. (1983) J. Biol. Chem. 258, 6628–6635.
[18] Sweadner, K.J. (1979) J. Biol. Chem. 254, 6060–6067.
[19] Lelièvre, L.G., Maixent, J.M., Lorente, P., Mouas, C., Charlemagne, D. and Swynghedauw, B. (1986) Am. J. Physiol. 250, H923–H931.
[20] Hokin, L.E., Yoda, A. and Sandhu, R. (1966) Biochim. Biophys. Acta 126, 100–116.
[21] Dierkes-Tizek, Von U., Glaser, Oldiges, H. and Hettwer, H. (1984) Arzneim. Forschung. 34, 671–678.
[22] Sweadner, K.J. (1989) Biochim. Biophys. Acta 988, 185–220.
[23] Takeyasu, K., Lemas, V. and Fambrough, D.M. (1990) Am. J. Physiol. 259, C619–C630.
[24] Price, E.M. and Lingrel, J.B. (1988) Biochemistry 27, 8400–8408.
[25] Lelièvre, L.G., Charlemagne, D., Mouas, Ch. and Swynghedauw, B. (1986) Biochem. Pharmacol. 35, 3449–3455.