Biochemical Pharmacology, Vol. 35, No. 20, pp. 3449–3455, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

RESPECTIVE INVOLVEMENTS OF HIGH- AND LOW-AFFINITY DIGITALIS RECEPTORS IN THE INOTROPIC RESPONSE OF ISOLATED RAT HEART TO OUABAIN

LIONEL G. LELIEVRE,* DANIELE CHARLEMAGNE, CHRISTIAN MOUAS and BERNARD SWYNGHEDAUW

INSERM U 127, Hôpital Lariboisière, Université Paris 7, 41 Bd de la Chapelle, 75010 Paris, France

(*Received* 28 *November* 1985; *accepted* 21 *March* 1986)

Abstract—High- and low-affinity digitalis receptors coexist in rat cardiac sarcolemma. In this study, their relative involvement in the inotropic effect of ouabain was evaluated on an isolated Langendorff rat heart preparation working under isovolumic conditions at a low external calcium concentration (0.25 mM). This involvement was estimated according to both the development of the inotropic response to ouabain (10^{-8}–10^{-4} M) and the time course of the washing out of the biological effect. In each phenomenon considered, and whatever the index of inotropy chosen, the high-affinity digitalis receptor (EC_{50}: 1–2 × 10^{-8} M) contributed to 25–40% of the maximal inotropy (evoked by 10^{-4} M ouabain). Low-affinity receptors (EC_{50}: 1–2 × 10^{-5} M) acounted for 60–75%. These apparent affinities were identical to those previously determined in sarcolemma isolated from rat heart perfused with 0.25 mM Ca^{2+}. The biphasic effect of ouabain was related to both the inhibition of high- and low-sensitivity Na^+, K^+-ATPase forms and the corresponding number of ouabain-binding sites occupied. These results support the concept that the Na^+, K^+-ATPase highly sensitive to ouabain as revealed by lowering calcium is the *in vivo* manifestation of the high-sensitivity inotropic component.

It is generally accepted that the inotropic response of ouabain is directly correlated with the degree of occupation of the pharmacological receptor, i.e. the Na^+, K^+-ATPase [1–4]. The inotropic response of rat heart to digitalis was initially attributed to the existence of low-affinity receptors [5]. Erdmann *et al.* [6] demonstrated the presence of both high- and low-affinity ouabain-binding sites and showed that inotropy was related to occupation of the high-affinity receptors. This heterogeneity has been recently confirmed as well in cell membranes [7–10] as in isolated myocytes [7]. Occupation of the low-affinity receptors led to an inhibition of at least 90% of the Na^+,K^+-ATPase activity. The high-affinity site would not be associated with enzyme inhibition. However, we demonstrated that both high- and low-affinity ouabain binding sites in sarcolemma were associated with enzyme inhibition provided the external calcium concentration in the source heart was lowered from 2 to 0.25 mM or less [10–14].

The physiological relevance of these two receptor forms has been demonstrated by Adams *et al.* [7]. The biphasic dose-effect of ouabain in right ventricular strips implied two site types. Different studies [1, 7, 9, 15, 16] quantified the relative contribution of the two sites: one-third of the maximum inotropic effect would be due to the high-affinity digitalis receptor.

However, all these *in vivo* results have been obtained with ventricular strips at low frequency [6, 7, 9, 15, 16]. This experimental model did not reflect all the constraints of the whole heart. The contributions of the two sites were estimated only from the pattern of development of inotropic response [1, 6, 7, 9, 15, 16] without considering the overall phenomenon: both development and reversal. From a theoretical point of view, the amplitude of high-sensitivity inotropic component (30%) [9, 15, 16] largely exceeded both the degree of Na^+, K^+-ATPase inhibition (less than 10%) and the fractional occupancy of the ouabain binding sites (13%) [1, 9].

The goal of the present paper is to determine the contribution of the high-affinity sites in the inotropic response of isolated rat heart working at low calcium under isovolumic conditions. From the biphasic patterns of both the development and the reversal of the inotropic effects of ouabain, it was established that 25–40% of the maximum response was due to the pharmacological receptor of high-affinity. This contribution is related to the degree of Na^+,K-ATPase inhibition and the percentage of ouabain-binding sites occupied.

MATERIALS AND METHODS

Biochemical study

1. Sarcolemma preparations. In order to isolate the sarcolemma vesicles from hearts maintained under the experimental conditions used to measure inotropism (see below), normal hearts were submitted to a coronary perfusion with the Krebs–Henseleit solution containing 0.25 mM Ca^{2+} and 5.85 mM K^+. The ventricles were used to prepare microsomes highly enriched in sarcolemma vesicles [11, 12, 14, 21]. The Na^+,K^+-ATPase activities were assayed in the final 31,000 *g*–30 min pellet. The yield of sarcolemmal proteins [22] was 0.3 mg/g of heart. In order to reveal the latent enzyme activity, the fraction was submitted to two freeze-thaw cycles separated by a 24 hr-period [11, 14, 21].

* Author to whom correspondence should be addressed.

2. (^{3}H)ouabain binding assays on sarcolemma vesicles were performed as previously described [10].

3. Na^+,K^+-ATPase inhibition. Two assays were used to study the inhibitory effect of ouabain on (Na^+,K^+)-ATPase activity. Anner and Moosmayer assay [23]: enzymatic activity was determined by measuring the release of inorganic phosphate from ATP at 37°. This method has been previously described in detail [10, 11]. Coupled optical assay [8, 14, 24]: the enzymatic activity was measured by continuously recording NADH oxidation in an ATP regenerating assay medium using a Gilford spectrophotometer. The final concentrations of ouabain in the assay medium varied from 10^{-9} to 10^{-4} M.

Inhibition percentage was calculated by comparing the activity in the presence of ouabain with that in the control after correcting for the ouabain-insensitive ATPase activity measured in the presence of 2×10^{-3} M ouabain. The Na^+,K^+-ATPase activities accounted for about 70% of the total ATPase activities of the preparations. The specific activity of Na^+,K^+-ATPase averaged 105 ± 16 μmoles of phosphate liberated/mg protein × hr.

Physiological study

Isolated hearts. An isolated rat heart preparation perfused at a constant coronary pressure was used [14]. In this preparation, a small cannulated fluid-filled balloon was placed in the left ventricle of the isolated heart and attached to a pressure transducer to monitor ventricular pressure. Since the balloon was non-compressible, contraction was isovolumic.

1. Perfusion technique. Rats were anesthetized. Hearts were rapidly removed. Retrograde coronary perfusion was performed at 37° at a pressure of 90 mmHg. The perfusate consisted of modified Krebs–Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.17 mM KH_2PO_4, 11 mM glucose and 0.25 mM $CaCl_2$ [13, 14]. By addition of either 2.68 or 4.68 mM KCl, the total K^+ content was adjusted to either 3.85 or 5.85 mM. After prolonged oxygenation in 5% CO_2, 95% O_2, the pH was 7.4 and the pO_2 around 600 mmHg. Hearts were paced at 360 beats/min (6 Hz) using two atrial electrodes attached to a PHILIPS TP 300 stimulator adjusted to 4 mA. A 1-msec rectangular unipolar impulse was used. A drain was placed in the apex of the left ventricle, the left atrium was opened and a collapsed latex balloon was inserted into the left ventricle.

A cannula was inserted into the right ventricle to collect and measure the coronary flow (around 14 ml/min). The perfusate efflux was not recirculated. The coronary flow was not maintained constant by a pump.

2. Measurement of mechanical function. It was performed as previously described [14]. The first pressure derivative was obtained from a Gould differentiator. Measurements were made every 5 min. Records were made in isovolumetry with balloon volume (110–120 μl of water) and diastolic pressures (10–15 mmHg) similar from heart to heart. To reflect the contractile state of the myocardium, it will be referred to the developed pressure, its first derivative (dP/dt), i.e. the rate of rise of intraventricular pressure [17] and $(dP/dt)\ max/P_{sys}$ [18–20]. The preparations in low external calcium were stable for 90 min [14].

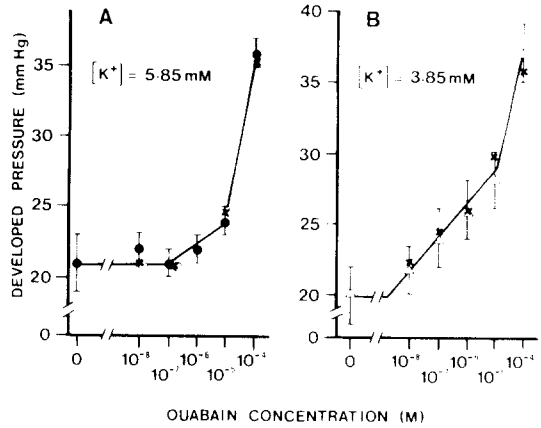

Fig. 1. Cumulative concentration–effect curves elicited by 10^{-8}–10^{-4} M ouabain (●). The isolated rat hearts were equilibrated for 20 min at 37° before addition of ouabain. Each point is the mean + S.E. of nine observations. Panel A: External potassium concentration, 5.85 mM. The theoretical values (×) are plotted assuming a unique low-sensitivity component with a K_D of 3×10^{-5} M. Note the discrepancy with experimental values in the presence of 10^{-6} M ouabain. Panel B: External potassium concentration, 3.85 mM (N = 5). Crosses (×) are the computed values derived from the model of two saturable components the parameters of which are reported in Table 2.

3. Experimental protocols. Inotropic effects. After 20 min of equilibration, ouabain (from 10^{-9} to 10^{-4} M) was continuously infused for sequential 5 min periods. This protocol was chosen to rapidly get a dose response curve. Five-minute incubations at ouabain concentrations in the range of 10^{-8}–10^{-4} M were sufficient to achieve stable inotropic effects. Under our conditions, the plateau was reached within 90 sec.

Reversal. The recovery was initiated by a rapid change of the perfusate. The ouabain-free Krebs–Henseleit buffer (K^+ = 5.85 mM) was continuously infused for 30 min. The recovery was expressed in % below the last measurement made in the presence of 10^{-4} M ouabain.

4. Analysis of the results. As previously reported by Finet *et al.* [9], the experimental data from contractile studies were analyzed assuming that the relation between the ouabain concentration and the biological effect could be considered either as being due to one saturable component (Fig. 1a) or to the sum of two independent, non-interconvertible, saturable components. In the case of one saturable component (Fig. 1a):

$$I_{obs} = \frac{I_{max}}{\frac{K_{Dapp}}{(ouab)} + 1}.$$

In the case of two saturable components (Figs 1b, 2–4):

$$I_{obs} = I_{obs_1} + I_{obs_2} = \frac{I_{max_1}}{\frac{K_{Dapp_1}}{(ouab)} + 1} + \frac{I_{max_2}}{\frac{K_{Dapp_2}}{(ouab)} + 1}.$$

with $I_{max} = I_{max_1} + I_{max_2}$, where I_{obs} is the observed inotropic effect, I_{obs_1} the observed inotropic effect attributed to the high-sensitivity component, I_{obs_2} the observed inotropic effect attributed to the low-sensitivity component, I_{max} the maximum inotropic effect (obtained with 10^{-4} M ouabain), I_{max_1} the maximum inotropic effect attributed to the high-sensitivity component, I_{max_2} the maximum inotropic effect attributed to the low-sensitivity component, K_{Dapp}, the apparent concentration of ouabain producing a half maximal effect. K_{Dapp_1} and K_{Dapp_2} represent the value for the high- and low-sensitivity component, respectively, (ouab), the concentration of glycoside in the perfusate.

RESULTS

Our previous studies [10, 11, 14] indicate that, in rat heart, low external calcium concentrations (up to 0.25 mM) enable the detection of a Na^+,K^+-ATPase activity highly sensitive to ouabain in sarcolemma vesicles. In contrast, the existence and the K_D's values of high and low affinity ouabain-binding sites were not dependent upon the initial Ca^{2+} concentrations in the source heart [10]. These results are summarized in Table 1.

The possibility that the two receptor forms found in sarcolemma were involved in the pharmacological effect was examined by using isolated Langendorff rat heart preparation working under isovolumic conditions as a model [13, 14]. Ouabain only had a pronounced positive inotropic effect in the presence of 0.25 mM Ca^{2+} in the perfusing medium [14]. The glycoside toxicity (when it occurred) was observed with 10^{-4} M ouabain at 0.25 mM Ca^{2+}. It was characterized by irregular cardiac arrests and extrasystoles. It is for this reason that the inotropic effect of 10^{-4} M ouabain was considered to be maximal. At external calcium concentrations above 0.5 mM (up to 2 mM) ouabain had no effect or induced a negative inotropic action due to a three-fold increase in diastolic pressure [14].

Development of the inotropic response

In this model, a cumulative concentration–response curve of ouabain (10^{-8}–10^{-4} M) on developed pressure was obtained at two K^+ concentrations (Fig. 1). At a physiological K^+ (5.85 mM), the log concentration effect was not monophasic since a shoulder did exist at 10^{-6} M (Fig. 1a). An analysis with a unique glycoside receptor site assuming a K_D of 3×10^{-5} M did not fit the experimental values at 10^{-6} M. However, due to a wide degree of variation in the percentage increase in pressure produced at 10^{-6} M, the amplitude of the low concentration effect could not be determined accurately (Fig. 1a).

As potassium is known to antagonize ouabain action, potassium concentration was lowered to 3.85 mM. Figure 1b depicts a set of experiments following this procedure. The experimental data fitted a model assuming two saturable independent components. The high-affinity component (EC_{50} value 2×10^{-8} M) accounted for 25% of the maximal increase in developed pressure evoked by 10^{-4} M ouabain. The low-affinity component had an apparent affinity of 2×10^{-5} M for ouabain (Table 2).

Table 1. Perfusions of rat heart with low or normal Ca^{2+} solutions. Effects on Na^+,K^+-ATPase activity and ouabain-binding sites in sarcolemma vesicles

Conditions of perfusion	Criteria	High-affinity sites (%)	Low-affinity sites (%)	K_D values 10^{-8} M	K_D values 10^{-5} M
Up to 0.25 mM Ca^{2+}	Ouabain-binding	10–20	80–90	3 ± 0.8	2.3 ± 0.8
	Na^+,K^+-ATPase	40–60	60–40	2 ± 1	6 ± 2
2 mM Ca^{2+}	Ouabain-binding	2–4	96–98	3 ± 1	3 ± 1
	Na^+,K^+-ATPase	N.D.	100	—	4 ± 2

N.D., not detectable.

Table 2. Contribution of high- and low-sensitivity components in the development of the inotropic effect of ouabain in isolated working rat heart. Computed parameters

Criteria	K^+ concentration (mM)	Computed parameters: High-sensitivity inotropic components (%)	Low-sensitivity inotropic components (%)	K_D values (M)
Developed pressure (P_{sys})	5.85	<10	90	N.D.–10^{-5}
	3.85	25	75	2×10^{-8}–2×10^{-5}
Rate of increasing pressure (dP/dt)	5.85	40	60	2×10^{-8}–10^{-5}
	3.85	30	70	10^{-8}–2×10^{-5}
$(dP/dt)/P_{sys}$	5.85	30	70	2×10^{-8}–2×10^{-5}
	3.85	30	70	2×10^{-8}–10^{-5}

N.D., not detectable.

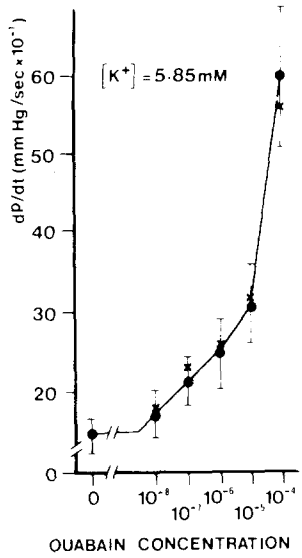

Fig. 2. Effect of cumulative addition of ouabain on the rate of rise of intraventricular pressure in the presence of a physiological K^+ concentration (5.85 mM) (●). The theoretical values (×) are calculated according to the parameters reported in Table 2.

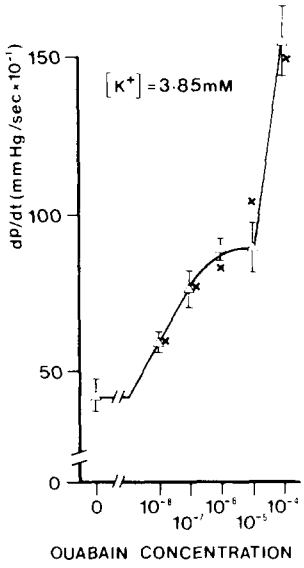

Fig. 3. Correlation between the rate of rise of intraventricular pressure (dP/dt) and ouabain concentrations at low external K^+ (3.85 mM) (△). Crosses (×) represent the theoretical values assuming two independent saturable components. For details, see Table 2.

If one refers to the rate of increasing pressure (dP/dt) as a true index of inotropy, the log concentration–effect curve was biphasic at both K^+ concentrations (3.85 and 5.85 mM) (Figs 2 and 3). The high-sensitivity inotropic component contributed to 35 ± 5% of the maximum increase in dP/dt with a computed affinity of $1.5 \pm 0.5 \times 10^{-8}$ M (Table 2). The calculated affinity of the low-sensitivity component was three orders of magnitude lower (2×10^{-5} M). When using $(dP//dt)_{max}/P_{sys}$ as an index of inotropy [18–

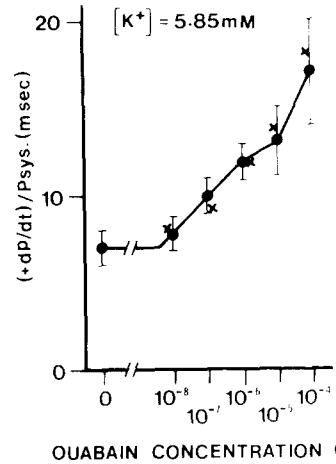

Fig. 4. Inotropic effects ($(dP/dt)/P_{sys}$ index) of ouabain in isolated rat heart. Cumulative addition of ouabain (10^{-8}–10^{-4} M) in the presence of a physiological potassium concentration (5.85 mM) (●); theoretical values (×).

20], the experimental values fitted the theoretical curves derived from the model of two saturable components, assuming 30% of the maximal increase to be due to the high-affinity receptors (EC_{50}: 2×10^{-8} M) and 70% to be due to the low-affinity receptors (EC_{50}: 2×10^{-5} M) (Fig. 4 and Table 2). These observations were valid at the two K^+ concentrations tested (data not shown).

Reversal of the inotropic response

The reversal of inotropic effect by washing out ouabain could also be considered to estimate the respective contribution of the low and high sensitivity inotropic components. An important assumption is made, namely that the loss of inotropic effect is directly related to the release of ouabain from the two receptor types. The resulting curve should fit an equation of the type:

$$I = I^0_{LA} e^{-k_{LA} \cdot t} + I^0_{HA} e^{-k_{HA} \cdot t}$$

where I is the amplitude of the inotropic response at time t, I^0 is the amplitude of the inotropic response at time zero, LA due to the low affinity component and HA due to the high-affinity component. k is the dissociation rate constant for low- and high-affinity components, LA and HA, respectively.

The loss of inotropism was analyzed in terms of two independent compartments. The data shown in Fig. 5 fulfilled these criteria. The decrease in developed pressure plotted on a logarithmic scale as a function of time was biphasic. The curvilinearity was considered as the sum of two first order kinetics: (1) a rapid phase corresponding to the low-affinity component (fast dissociation process); and (2) a slow phase representing the high-affinity component (slow dissociation process). The ordinate of the second slope corresponded to 20.9 ± 3.2% of the maximum developed pressure achieved at time zero.

The feasibility of this type of analysis was confirmed by a particular set of experiments. Isolated hearts were perfused for 30 min with a single dose of ouabain (10^{-5} M). This concentration was chosen

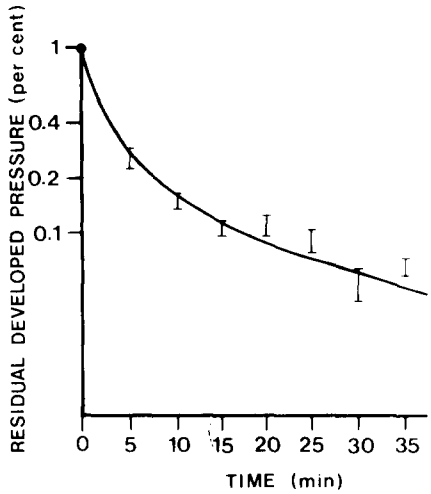

Fig. 5. Time course of reversal of developed pressure. The maximum inotropic effect was carried out with 10^{-4} M ouabain as outlined in Methods. At zero time a complete withdrawal of ouabain initiated the reversal. The external K^+ concentration was maintained at 5.85 mM throughout the complete experiment. Each point is the mean + S.E.M. of nine experiments.

in order to get both a clear-cut inotropic response and a substantial contribution of the high sensitivity component. The reversal of the inotropic response has been studied for 30 min. As shown in Fig. 6, the time course of the reversal ((dP/dt) P_{sys} index) was biphasic. The high sensitivity inotropic component (second slope) represented 62 ± 7% of the physiological response obtained with 10^{-5} M, i.e. 25 ± 4.5% of that with 10^{-4} M (see Fig. 4).

Relationship between inotropism and digitalis receptor forms in vitro

It was of interest to determine the possible relationship between the two inotropic components

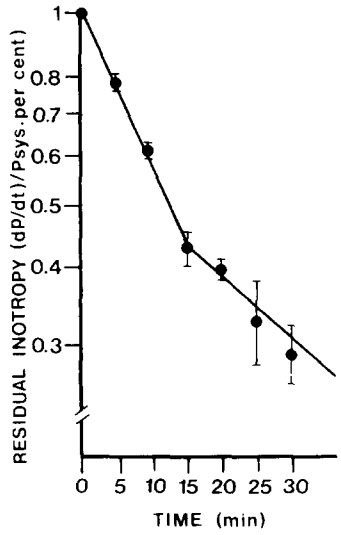

Fig. 6. Time course of reversal of inotropy elicited by a single addition of 10^{-5} M ouabain for 30 min. The external K^+ concentration was maintained at 5.85 mM during the equilibration, perfusion and recovery periods. Each point is the mean + S.E.M. of eight experiments.

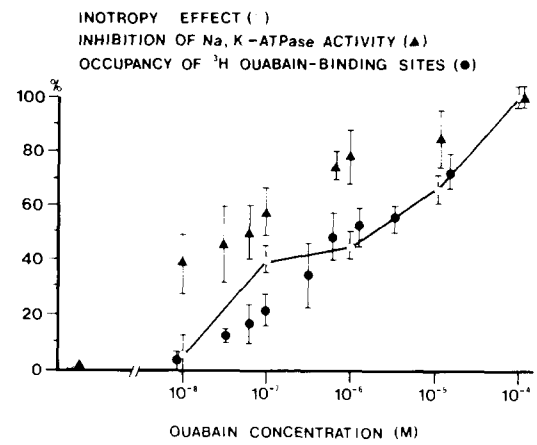

Fig. 7. Correlation between the increase in intraventricular pressure (dP/dt) (○), the fraction of receptors occupied by (^{3}H)ouabain (●) and the relative inhibition of Na^+,K^+-ATPase activity (▲). The latter two types of analyses have been performed on sarcolemma vesicles isolated from rat hearts perfused by the buffer used to measure inotropism (see Methods).

and the degree of occupancy *in vitro* of the putative pharmacological receptor, i.e. the Na^+,K^+-ATPase. This receptor was characterized on isolated sarcolemma vesicles from rat cardiac muscle by the sensitivity of Na^+,K^+-ATPase activity to ouabain and the (^{3}H)ouabain-binding measurements (Table 1 and Refs [10, 14]). The respective contribution of the two inotropic components correlated with the heterogeneous forms of the receptor as detected by both criteria. As shown in Fig. 7, the correlation was undoubtedly strict for the low-sensitivity component (doses of ouabain higher than 10^{-7} M). For the high-affinity component (doses of ouabain lower than 10^{-7} M), the initial parts of the dose–response curves slightly differed from each other. Indeed, the amplitude of the inotropic effect is intermediate between the other two parameters: it is lower than the degree of enzyme inhibition but higher than the number of ouabain-binding sites occupied.

Note that the high- and low-affinity digitalis receptors *in vitro* had apparent affinities for ouabain identical to those of high and low sensitivity inotropic components respectively (Tables 1 and 2).

DISCUSSION

The present study shows that, using isolated rat heart as a model, a high sensitivity inotropic component associated with inhibition of Na^+,K^+-ATPase accounted for 25–40% of the maximum inotropic effect of ouabain.

In spite of controversial data concerning the inotropic action of digitalis in rats [25–28], there are apparently two conditions under which ouabain increases force in rat heart: (1) by pacing at very low frequency, around 1 Hz [6, 7, 9, 15, 16], which in rats was only possible on papillary muscle [29] and ventricular strips [7, 15, 16, 30]; and (2) by reducing external calcium concentrations to 0.25 mM, and pacing at 6 Hz [13, 14]. This latter procedure renders the papillary muscle of young adults sensitive to 10^{-4} M ouabain [31, 32].

The involvement of an inotropic component highly sensitive to ouabain in the overall physiological response has been already reported [7, 9, 15, 16, 36]. This high sensitivity component would represent about 30% of the total inotropic response implying both high and low sensitivity components. It is noteworthy that these groups used right ventricular strips as a biological model (see above) and only analyzed the development of the isometric tension.

With isolated Langendorff rat heart preparation working under isovolumic conditions, one cannot refer to the developed systolic pressure to determine the involvement of the high-sensitivity component precisely. The problem with this index of inotropism was experimental variability at ouabain concentrations lower than 10^{-6} M. In order to circumvent this problem, the external K^+ concentration was lowered from 5.85 to 3.85 mM (Fig. 1). This lowering allowed an accurate detection (Table 2) of the high-sensitivity component.

If one considers both the rate of increasing pressure $(+dP/dt)$ and the $(+dP/dt)_{max}/P_{sys}$ values it appears that:

(a) the contribution of the high-sensitivity inotropic component accounted for 30% of the maximal physiological effect, and

(b) changes in potassium concentration (3.85 instead of 5.85 mM) did not affect this ratio significantly.

Obtained with a different biological system which takes into account the constraints of the whole heart, these results reinforce the previous observations [1, 7, 9, 15, 16]. The low-sensitivity inotropic component exhibited an apparent affinity of $1–2 \times 10^{-5}$ M whatever the index chosen (Table 2).

The respective contribution of the high- and low-sensitivity inotropic components should be identical regardless of the methodologies used to study it. Among these, reversibility of inotropism would yield results concurring with those stated above.

According to the biphasic time course of the reversal (Figs. 5 and 6), the high-sensitivity inotropic component accounted for 20–25% of the maximum inotropic response. It is noteworthy that the high sensitivity component undetectable in the developed pressure at normal potassium concentration (Fig. 1a) can be esimated accurately using the recovery of normal pressure (Fig. 5). This would imply that the association process (development of the response) by cumulative addition was a less sensitive methodology to study inotropism than the dissociation process (reversal of the response).

Regarding the relationship between inotropism and heterogeneous digitalis receptors detected *in vitro* [10, 14], it has been clearly shown that the high-sensitivity inotropic component present in right ventricular strips was correlated with existence of the high-affinity ouabain-binding sites [7, 9, 33]. However, inhibition of Na^+,K^+-ATPase by very low doses of ouabain (10^{-7} M and less) would not have been demonstrated [6, 7, 33]. We have shown that low calcium concentrations (up to 0.25 mM) in rat hearts revealed a Na^+,K^+-ATPase activity highly sensitive to ouabain (K_D: 2×10^{-8} M). This enzyme form contributed $50 \pm 10\%$ of the total enzymatic activity (Fig. 7). It is important to note that the high-sensitivity inotropic component revealed here (Figs. 1–6) displayed the same apparent affinity (2×10^{-8} M, Table 2) as the high affinity Na^+,K^+-ATPase form (Table 1 and Refs [14, 33]), even though its contribution was lower (20–30%) than that of the enzyme form ($50 \pm 10\%$) (Fig. 7). Thus, the high-sensitivity inotropic component present in rat heart is linked to concomitant inhibition of Na^+,K^+-ATPase activity highly sensitive to ouabain. Under the same conditions, i.e. low Ca^{2+} concentrations in the source heart, the high-affinity ouabain binding sites (K_D: 3×10^{-8} M) represented $15 \pm 5\%$ of the total specific ouabain binding capacity found in sarcolemma [10]. This, again, is consistent with the participation of the high-sensitivity component in the maximal inotropy. The two ouabain-receptors described here are present on the sarcolemma of rat cardiac myocytes [11]. However, there is no evidence that these sites exist on the same Na^+,K^+-ATPase molecule or are separate isoenzymes. By immunoreaction with specific polyclonal antibodies raised against purified Na^+,K^+-ATPase, one could not detect two isoenzymes although these antibodies react with the two enzyme forms (α, α^+) present in rat brain [34] (data not shown).

The high degree of homology between the *in vivo* and *in vitro* experiments emphasized those reported by Finet *et al.* [9]. According to these authors, high-affinity ouabain-binding sites representing 10% of the total binding capacity accounted for 30% of the total inotropic effect. The very good correlation we found may be relevant to the partial Ca^{2+} depletion used as well *in vivo* as *in vitro*. When the extracellular Ca^{2-} concentration was lowered, the inotropic response to different agents such as ouabain [14], isoproterenol and phosphodiesterase inhibitors was potentiated (B. Swynghedauw, unpublished data, see also Ref. [31]). This could simply be attributed to a previous depletion of Ca^{2+} stores. Nevertheless, *in vitro* data [11, 12] showed that a pretreatment of the cardiac tissue by calcium-free (or low calcium) solutions reveals high-affinity sites for ouabain in the rat. Thus, the relationship between Ca^{2+} and the expression of ouabain receptors is complex. It may involve a calcium-binding protein such as calmodulin as demonstrated in the murine plasmocytoma model [35].

From the data presented here, three main conclusions can be drawn:

First, the high-sensitivity inotropic component detected in isolated working rat heart is linked to an inhibition of the Na^+,K^+-ATPase activity attributable to binding of cardiac glycosides to high-affinity pump sites. This has been very recently proposed by Grupp *et al.* [36] and Rasmussen *et al.* [37].

Second, there is a strict relationship between the fraction occupancy of high-affinity (^3H) ouabain-binding sites and the inotropic effect elicited by low doses of ouabain.

Third, the Ca^{2+}-dependent expression of the high-sensitivity inotropic component in rat presents a striking homology with the *in vitro* expression of the forms of the pharmacological receptor.

The differences in both Ca^{2+}-dependency and affinity for ouabain, *in vivo* as well as *in vitro*, could

mean a physiological role which is different for each receptor type. The high affinity digitalis receptor (Ca^{2+}-dependent) would be responsible for the inotropic effect only, whereas the occupation of the low-affinity receptors (Ca^{2+}-independent) would lead to inotropism and toxicity.

Acknowledgements—This work was supported by grants from the M.I.R. P.R.C. Medicaments. Convention No. 131030 (L.G.L.), the Université Paris 7. No. C. 32R01 (L.G.L.), the Fondation des Maladies Cardiovasculaires (B.S.) and the M.G.E.N. (B.S.).

REFERENCES

1. A. Schwartz, K. Whitmer, G. Grupp, I. Grupp, R. J. Adams and S. W. Lee, *Ann. N.Y. Acad. Sci.* **402**, 253 (1982).
2. T. Akera and T. M. Brody, *Ann. Rev. Physiol.* **44**, 375 (1982).
3. D. Noble, *Cardiovasc. Res.* **14**, 495 (1980).
4. L. H. Michael, A. Schwartz and E. T. Wallick, *Molec. Pharmac.* **16**, 135 (1979).
5. K. H. Repke, in (Ed. W. Wilbrandt) *Proc. 1st Int. Pharmacological Conference, Stockholm*, Vol. 3, p. 47. Pergamon Press, London (1963).
6. E. Erdmann, G. Philip and H. Scholz, *Biochem. Pharmac.* **29**, 3219 (1980).
7. R. J. Adams, A. Schwartz, I. Grupp, G. Grupp, S. W. Lee, E. T. Wallick, T. Powell, V. W. Twist and P. Gathiram, *Nature, London.* **296**, 167 (1982).
8. F. Noel and T. Godfraind, *Biochem. Pharmac.* **33**, 47 (1984).
9. M. Finet, T. Godfraind and F. Noel, *Br. J. Pharmac.* **80**, 751 (1983).
10. D. Charlemagne, J. M. Maixent, M. Preteseille and L. G. Lelievre, *J. biol. Chem.* **261**, 185 (1986).
11. P. Mansier and L. G. Lelievre, *Nature, Lond.* **300**, 535 (1982).
12. P. Mansier, P. S. Cassidy, D. Charlemagne, M. Preteseille and L. G. Lelievre, *FEBS lett.* **153**, 357 (1983).
13. L. G. Lelievre, P. Mansier, D. Charlemagne and B. Swynghedauw, *Bas. Res. Cardiol. (suppl)* **79**, 128 (1984).
14. L. G. Lelievre, J. M. Maixent, Ch. Mouas, D. Charlemagne and B. Swynghedauw, *Am. J. Physiol.* in press.
15. I. Grupp, G. Grupp and A. Schwartz, *Life Sci.* **29**, 2789 (1981).
16. G. Grupp, A. De Pover, I. L. Grupp and A. Schwartz, *Proc. Soc. exp. Biol. Med.* **175**, 39 (1984).
17. C. S. Apstein, R. M. Mueller and W. B. Hood Jr., *Circulation Res.* **41**, 206 (1985).
18. W. Grossman, F. Haynes, J. A. Paraskos, S. Saltz, J. F. Dan, and L. Dexter, *Circulation Res.* **31**, 83 (1972).
19. D. T. Mason, *Am. J. Cardiol.* **23**, 516 (1969).
20. D. T. Mason, in *Experimental Pharmacology* (Ed. K. Greff), p. 497. Springer, Berlin (1981).
21. P. Mansier, D. Charlemagne, B. Rossi, M. Preteseille, B. Swynghedauw and L. G. Lelievre, *J. biol. Chem.* **258**, 6628 (1983).
22. O. H. Lowry, J. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. B. Anner and M. Moosmayer, *Analyt. Biochem.* **65**, 305 (1974).
24. R. Thomas, J. Allen, B. J. R. Pitts and A. Schwartz, *Eur. J. Pharmac.* **53**, 227 (1974).
25. J. Dietrich and J. Diacono, *Life Sci.* **10**, 499 (1974).
26. P. M. Mathews, S. R. Williams, A. M. Seymour, A. Schwartz, G. Dube, D. G. Gadian and G. K. Radda, *Biochim. biophys. Acta* **720**, 163 (1983).
27. G. A. Langer, A. J. Brady, S. T. Tan, S. D. Serena, *Circulation Res.* **36**, 744 (1975).
28. F. S. Fein, R. S. Aronson, C. Nordin, B. Miller-Green, E. H. Sonnenblick, *J. molec. cell. Cardiol.* **15**, 769 (1983).
29. M. Reister, *Experimental Pharmacology*, **9**, 153. Springer–Verlag. Berlin (1981).
30. D. T. Masuoka, P. R. Saunders, *Proc. Soc. exp Biol. Med.* **74**, 879 (1950).
31. G. Gerstenblith, H. A. Spurgeon, J. P. Froelich, M. L. Weisfeld and E. G. Lakatta, *Circulation Res.* **44**, 517 (1979).
32. J. L. Sutko and J. T. Willerson, *Circulation Res.* **46**, 332 (1980).
33. S. Herzig and K. Mohr, *Br. J. Pharmac.* **82**, 135 (1984).
34. K. J. Sweadner, *J. biol. Chem.* **254**, 6060 (1979).
35. L. G. Lelièvre, J. D. Potter, M. Piacik, E. T. Wallick, A. Schwartz, D. Charlemagne and B. Geny, *Eur. J. Biochem.* **148**, 13 (1985).
36. I. Grupp, W. B. Im, C. O. Lee, S. W. Lee, M. S. Pecker and A. Schwartz, *J. Physiol.* **360**, 149 (1985).
37. H. H. Rasmussen, R. E. Ten Eick, G. T. Okita, R. S. Hartz, D. H. Singer, *J. Pharmac. exp. Ther.* **235**, 629 (1985).