Membrane proteins of the myocytes in cardiac overload

PASCAL MANSIER, BRIGITTE CHEVALIER, ERIC MAYOUX, DANIELLE CHARLEMAGNE, LAURENCE OLLIVIER, FRANCINE CALLENS-EL AMRANI & BERNARD SWYNGHEDAUW

Unité Inserm 127, Hôpital Lariboisière, 41 Boulevard de la Chapelle, 75010 Paris, France

1 Hypertrophy of the cardiac myocytes resulting from a mechanical overload may be responsible for major membraneous modifications, either at the sarcolemmal or at the sarcoplasmic level. In this study several sarcolemmal markers such as β -adrenoceptors, muscarinic receptors or $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ were investigated in an experimental model of cardiac hypertrophy, the chronic aortic stenosis in adult rats.

2 Left ventricular β -adrenoceptor density (expressed in fmol mg^{-1} protein) was decreased in the aortic stenosis group by about 30%; however, when expressed in number of receptors per cardiac cell β -adrenoceptor number in the hypertrophied myocytes was unchanged.

3 Similarly, the number of muscarinic receptors in the hypertrophied cells, expressed as number of receptors per cardiac cell, was unchanged.

4 The number of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ molecules with high affinity for ouabain was markedly increased in the hypertrophied myocytes, while those with low affinity for ouabain were not.

5 These results indicate the necessity in chronic hypertrophy to calculate receptors not only in density (fmol mg^{-1} protein) but also in number per cardiac cell. The unchanged number of β -adrenergic and muscarinic receptors present on the hypertrophied myocytes suggests a non-regulation for the genes coding for these receptors.

Keywords β -adrenoceptors muscarinic receptors $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ sarcoplasmic reticulum cardiac hypertrophy cardiac insufficiency

Introduction

In the adult heart, mechanical overload results in the hypertrophy of the myocytes in the absence of cell division and in the hypertrophy with cellular division of the non-myocyte cells (Korecky & Rakusan, 1978). The average cellular volume of the myocytes and hence their average cell surface increase in parallel with the degree of hypertrophy and the surface/volume ratio remains constant (Anversa *et al.*, 1978). The membranes such as the sarcolemma or the sarcoplasmic reticulum must adapt to the overload but the membrane hypertrophy is known to be non homogeneous; for example, for a hyper-

trophy of 60%, the actual surface of the sarcolemma increases by 35%, that of the intercalated discs by 55% and that of the T tubules by 107%. In hypertrophy, there may be a new distribution of the proteins which are unequally distributed on the membrane surface. One can schematically distinguish three groups of membrane proteins in the normal myocytes of the adult rat ventricle: — the very abundant membrane proteins, the $(\text{Ca}^{2+})\text{-ATPase}$ of the sarcoplasmic reticulum (SR) of which there are about 100 million molecules per myocyte; — those which are simply abundant, the iso-

Correspondence: Dr P. Mansier, Unité Inserm 127, Hôpital Lariboisière, 41 Boulevard de la Chapelle, 75010 Paris, France

forms of the (Na^+ , K^+)-ATPase and the calcium channels (100–500,000 molecules per cell) — and finally the rarer proteins, for example the muscarinic receptors (M2), β_1 - and α_1 -adrenergic receptors (10,000–80,000 molecules per cell).

In the present study, modulations of the (Na^+ , K^+)-ATPase, the β_1 and M2 receptors in chronic hypertrophy are reported, and changes in receptor density (expressed in fmol mg^{-1} protein) are compared with receptor number (expressed as number of receptors per cardiac cell) in the hypertrophied myocytes.

Methods

Aortic stenosis

Adult male Wistar rats (180–220 g) were operated and paired to sham-operated animals. Abdominal aortic stenosis was induced using a partially occluded haemoclip placed above the renal artery with Weck forceps. The method was modified as described previously (Chevalier *et al.*, 1989a). Animals were killed 4–6 weeks after the operation. Routine autopsy included search for congestive lungs or liver, and ascites.

Heart membrane preparation

For β -adrenoceptor and muscarinic receptor studies, homogenates of left ventricles have been prepared as previously described (Chevalier *et al.*, 1989b). For studies of ouabain binding sites, sarcolemmal vesicles were isolated according to our previously published procedure (Mansier & Lelièvre, 1982).

Radioligand binding experiments

Quantification of total β -adrenoceptors was performed using ^{125}I -pindolol (I-PIN) with a specific activity of $2,200 \text{ Ci mmol}^{-1}$ (New England Nuclear). An aliquot of the homogenate was incubated with I-PIN (8–400 pM) in a final volume of 500 μl either at room temperature during 40–60 min or overnight at 4°C . Non-specific binding was defined as binding in the presence of $1 \mu\text{M}$ (–)-propranolol. To evaluate the relative number of each subtype receptor, displacement curves were established for both groups employing a β_2 -adrenoceptor specific antagonist, ICI 118,551. Determination of muscarinic receptors density was performed using [^3H]-quinuclidinyl benzilate (^3H -QNB, specific activity = $42\text{--}44 \text{ Ci mmol}^{-1}$). An aliquot of the homogenate was incubated at room temperature for 150 min or overnight at

4°C in a final volume of 2 ml in presence of [^3H]-QNB (6–250 pM). Non-specific binding was defined as binding in the presence of $1 \mu\text{M}$ atropine. For both types of receptors, competition curves in presence of the relevant agonist allowed the determination of classes of receptors of high and low affinity. Equilibrium binding of [^3H]-ouabain was measured after 60 min at 37°C as described in a previous publication (Charlemagne *et al.*, 1986).

Expression of the results

The density of these different membrane proteins has been estimated by dividing the number of receptors per cell by the total membrane surface (7,868 μm^2 for the normal myocyte and 12,495 μm^2 for the hypertrophied cell) (Mayoux, personal communication) and by acknowledging that the number of myocytes per left ventricle remains constant during the hypertrophic process, which in this model is surely true.

Calculations and statistical analysis

Equilibrium binding data (saturation or displacement curves) were analyzed using LIGAND program. This program fits the binding data to equations describing the laws of mass action for one or two classes of binding sites and determines whether the one-site model is statistically better than the two-site fit. The affinities (K_D) of the radioligands were determined from Scatchard analysis. For agonist competition curves, the computer-aided analysis yielded the affinity and the density of each class of binding sites. The likelihood that more than one receptor subtype was present was statistically (F test) decided by comparison of the residual variances obtained. Statistical analysis comparing data from the hypertrophied left ventricle vs sham ones has been performed using the Student's t -test.

Results and Discussion

In this model, the hypertrophy was detectable at the level of the whole organ with 50–60% increase of the left ventricle weight (LV) in the aortic stenosis group (Table 1). The LV weight/body weight (LVW/BW) ratio which is a classical index of hypertrophy was also significantly increased by the same percentage.

The β -adrenergic system has recently been extensively explored following cardiac failure in man, as in experimental animal models. In the failing heart, the elevated circulating catecholamines levels down-regulate the β_1 -receptor

Table 1 Anatomical data

	Sham n = 72	Aortic stenosis n = 67
Body weight (BW) (g)	345 ± 5	334 ± 6
Left ventricle weight (LVW) (mg)	743 ± 14	1098 ± 24***
LVW/BW (mg g ⁻¹)	2.14 ± 0.05	3.24 ± 0.05***
LV wall thickness (mm)	3.2 ± 0.1	4.2 ± 0.1***

*** $P < 0.001$.

density (see for example Bristow *et al.*, 1986; Brodde, 1987). We have previously shown that in the homogenates from the aortic stenosis group, the β -receptors were decreased by 30% (Chevalier *et al.*, 1989b). In the present report, distinction was made between the receptors of β_1 - or β_2 -subtype since the receptors present on the myocytes have been shown to belong only to the β_1 class (Buxton & Brunton, 1985). The 30% decrease previously found concerned actually only the β_1 subtype. The observed decrease in β -receptors was also the case for most of the models of compensatory hypertrophy, in spite of a normal plasma content of catecholamines (Ayobe & Tarazi, 1984) (see Table 2 for

the rat heart). Furthermore, recent unpublished data from this laboratory have shown that, in addition, a new population of β_1 -receptors which has a low affinity for the agonist appeared. This finding suggested that the pathogenesis of the down-regulation was more complex than was initially expected and may be related to a change in the family of G proteins or in the cyclase itself. As a logical consequence of the receptor changes, a reduced inotropic response to isoprenaline has been reported at both stages of the cardiac overloading (Brodde, 1987; Chevalier *et al.*, 1989b). In Table 3, results were expressed in number of receptors per cardiac cell and revealed an unchanged number of β_1 -receptors in the hypertrophied myocytes.

For the muscarinic receptors, information available at the present time suggests that they belong only to the M2 class in the cardiac muscle. In the hypertrophied cell, results shown in Table 3 suggest that the expression of the gene responsible for the M2 receptors was not enhanced during the process of hypertrophy since the number of M2 receptors per cell was unchanged.

The (Na⁺, K⁺)-ATPase is constituted of two different monomers, α and β , one of which, α , exists as several isoforms: α_1 which has a low affinity site for ouabain and α_2 which has a high affinity for the drug. These sites correspond to two different proteins coded by two different genes. In our laboratory, the number of molecules

Table 2 The myocardial β -adrenergic system in hypertrophied rat hearts

Models and references	β -adrenoceptors		Isoprenaline inotropic effect	Coupling with G _s
	Density (fmol mg ⁻¹) (*)	Quantity (fmol/LV) (*)		
<i>Renal arterial hypertension (Goldblatt)</i>				
Ayobe & Tarazi (1983)	↓ (36 – 28)	NC (2,140 – 1,970)	↓	
Ayobe & Tarazi (1984)	↓ (37 – 22)	NC (3,440 – 2,610)	↓	
Gende <i>et al.</i> (1985)	NC (43 – 42)		↓	
Kuchii <i>et al.</i> (1981)	NC			
Kumano <i>et al.</i> (1983)	↑ (38 – 51)	↑ (3,181 – 6,241)		
Woodcock <i>et al.</i> (1979)	(110 – 75)			
<i>Genetic hypertension (SHR)</i>				
Kumano <i>et al.</i> (1983)	↓ (48 – 34)	NC (2,440 – 2,181)		
Limas & Limas (1984)	↓ (106 – 78)			NC
Michel <i>et al.</i> (1987)	NC (27 – 25)			
Upsher & Khairallah (1985)	↓ (48 – 34)			
Will-Shahab <i>et al.</i> (1986)	↓ (480 – 188)	↓		
<i>Pressure overload (thoracic and abdominal aortic stenosis)</i>				
Chevalier <i>et al.</i> (1989a)	↓ (31 – 25)	NC (1,802 – 1,758)	↓	
Limas (1979)	↑ (98 – 138)			
Mansier (this report)		NC (1,578 – 1,602)		
Will-Schahab <i>et al.</i> (1986)	↓ (480 – 160)	↓		↓

NC = no change. * = 1st number: control value. 2nd number: hypertrophy value.

Table 3 Densities of some membrane proteins in rat cardiac hypertrophy

	Normal (sites/cell)	Hypertrophied (sites/cell)
<i>Sarcolemma</i>		
Total calcium channel ^a	120,000	200,000
Functional calcium channels ^b	10,000	17,000
(Na ⁺ ,K ⁺)-ATPase ^c		
High affinity	130,000	340,000
Low affinity	480,000	390,000
Muscarinic receptors ^d	70,000	80,000
β ₁ -adrenoceptors ^e	21,000	22,000
α ₁ -adrenoceptors ^f	80,000	
<i>Sarcoplasmic reticulum</i> ^g		
(Ca ²⁺)-ATPase	10,600 × 10 ⁴	11,400 × 10 ⁴

^a from Mayoux *et al.* (1988). ^b from Reuter (1983). ^c from Charlemagne *et al.* (1986). ^d and ^e from this report. ^f from Buxton & Brunton (1985). ^g from Lompré (personal communication).

of (Na⁺,K⁺)-ATPase present in the cardiac myocyte has been determined from the capacity of fixation of labelled ouabain onto membranes and onto isolated myocytes (Charlemagne *et al.*, 1986). In compensatory hypertrophy, the results are the following: (i) a diminution in the number of low affinity sites for ouabain, this site is responsible for the toxic effects of the drug; (ii) a 61% increase of the density of high affinity sites, these sites are only present in some tissues and are responsible in the heart for the therapeutic effects of the drug; (iii) a normal K_D ; (iv) a slowing of the dissociation rate constant for ouabain, k_1 . Physiological studies of the inotropic effects of ouabain on an isolated heart are in full agreement with these findings since they showed a slowing of the recovery of a normal contraction after a short infusion of the drug (Lelièvre *et al.*, 1986) and a diminished toxicity of the glycoside at high calcium (Chevalier *et al.*, 1989a). Again, when the results were expressed in number of sites per myocytes as in Table 3, they revealed a non significant change in the number of low affinity sites but a marked increase in that of high affinity sites.

Conclusions

In this report, results on three different membrane markers suggest that adaptation of the membrane to mechanical overload is not homogeneous. Furthermore, work on calcium channels and on (Ca²⁺)-ATPase of SR has been carried out and the findings are summarised in Table 3.

We have recently assayed the total number of

calcium channels by [³H]-dihydropyridine binding and found a normal density but an increased number of binding sites per left ventricle. In parallel the inotropic response to calcium, nifedipine, and Bay K 8644 was unmodified by the process of hypertrophy. These data suggest that in this model total and active calcium channels increase proportionally with the hypertrophy in a very harmonious way, as expected for an adaptational process (Mayoux *et al.*, 1988). Unpublished data using the patch-clamp technique have confirmed this finding. Data from both our laboratory (Lompré *et al.*, 1988) and from another (Nogai *et al.*, 1989) suggest that, both by immunoquantitation and by using a specific cDNA probe, the density of the (Ca²⁺)-ATPase of the sarcoplasmic reticulum is reduced, i.e. that the number of molecules of the enzyme per gram of tissue or per myocyte is diminished. This change may be the cause of the diminished calcium uptake which has been repeatedly reported (review in Swynghedauw & Delcayre, 1982) rather than an isoform shift. It may, in part, account for the slowing of the relaxation velocity which is observed in this model.

This analysis uncovers the necessity to calculate the receptors not only in density but also in number per cell. This is also the case for most of the studies for which calculation of the total quantity present in the ventricle was possible as shown in Table 2 which gives the number of β-receptors expressed in density or quantity in the total ventricle in rat heart with different models of cardiac hypertrophy. The unmodified number of receptors present on the myocyte suggests that the genes coding for the β₁ or M2 receptors

do not answer to the increase of the membrane surface. Studies are in progress in our laboratory to quantify mRNA to test this hypothesis. This apparent non response is in contrast to the increased level of mRNA obtained for Gi in a human heart failure (Feldman *et al.*, 1988, 1989).

References

- Anversa, P., Loud, A., Giacomelli, F. *et al.* (1978). Absolute morphometric study of myocardial hypertrophy in experimental hypertension. *Lab. Invest.*, **38**, 597–609.
- Ayobe, M. H. & Tarazi, R. C. (1983). Beta-receptors and contractile reserve in left ventricular hypertrophy. *Hypertension*, **5**, 192–197.
- Ayobe, M. H. & Tarazi, R. C. (1984). Reversal of changes in myocardial β -receptors and inotropic responsiveness with regression of cardiac hypertrophy in renal hypertensive rats (RHR). *Circulation Res.*, **54**, 125–134.
- Bristow, M. R., Ginsburg, R., Umans, V., Fowler, M., Minobe, W., Rasmussen, R., Zera, P., Menlove, R., Shah, P., Jamieson, S. & Stevison, E. B. (1986). β_1 and β_2 -adrenergic-receptor subpopulations in non failing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective β_1 -receptor down-regulation in heart failure. *Circulation Res.*, **59**, 297–309.
- Brodde, O. E. (1987). Cardiac beta-adrenergic receptors. *ISI Atlas of Sciences: Pharmacology*, **1**, 107–112.
- Buxton, I. L. O. & Brunton, L. L. (1985). Direct analysis of β -adrenergic receptor subtypes on intact adult ventricular myocytes of the rat. *Circulation Res.*, **56**, 126–132.
- Charlemagne, D., Maixent, J. M., Preteseille, M. *et al.* (1986). Ouabain binding and $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity in rat cardiac hypertrophy: expression of the neonatal forms. *J. biol. Chem.*, **261**, 185–189.
- Chevalier, B., Berribi-Bertrand, I., Mouas, C. *et al.* (1989a). Diminished toxicity of ouabain in the hypertrophied rat heart. *Pflügers Arch.*, **41**, 311–316.
- Chevalier, B., Mansier, P., Callens-El Amrani, F. *et al.* (1989b). The beta adrenergic system is modified in compensatory pressure cardiac overload in rats: physiological and biochemical evidence. *J. cardio-vasc. Pharmac.*, **13**, 412–420.
- Feldman, A. M., Cates, A. E., Bristow, M. R. *et al.* (1989). Altered expression of α -subunits for G proteins in failing human hearts. *J. Mol. Cell. Cardiol.*, **21**, 359–365.
- Feldman, A. M., Cates, A. E., Veazy, W. B., Herschberger, R. E., Bristow, M. R., Baughman, K. L., Baumgartner, W. A. & van Dop, C. (1988). Increase of 40,000 mol wt pertussis toxin substrate G protein in the failing human heart. *J. clin. Invest.*, **82**, 189–197.
- Gende, O. A., Mattiazzi, A., Camillion, M. C. *et al.* (1985). Renal hypertension impairs inotropic isoproterenol effect without β -receptor changes. *Am. J. Physiol.*, **249**, H814–H819.
- Korecky, B. & Rakusan, K. (1978). Normal and hypertrophic growth of the rat heart: changes in cell dimensions and number. *Am. J. Physiol.*, **234**, H123–H128.
- Kuchii, M., Fukuda, K., Hano, T. *et al.* (1981). Changes in cardiac beta-adrenoceptor concentrations in spontaneously hypertensive and experimental renal hypertensive rats. *Jap. Circulation J.*, **45**, 1104–1110.
- Kumano, K., Upsher, M. E. & Khairallah, P. A. (1983). Beta adrenergic receptor response coupling in hypertrophied hearts. *Hypertension*, **17**, 1–175–I-183.
- Lelièvre, L. G., Maixent, J. M., Lorente, P. *et al.* (1986). Prolonged responsiveness to ouabain in hypertrophied rat heart: physiological and biochemical evidence. *Am. J. Physiol.*, **250**, H923–H931.
- Limas, C. J. (1979). Increased number of β -adrenergic receptors in the hypertrophied myocardium. *Biochem. Biophys. Acta*, **588**, 174–178.
- Limas, C. & Limas, C. J. (1984). Decreased isoproterenol-induced 'down regulation' of beta-adrenergic receptors in myocardium of SHR. *Hypertension*, **6**, 131–139.
- Lompré, A. M., Levitsky, D., de la Bastie, D., Mercadier, J.-J., Rappaport, L. & Schwartz, K. (1988). Function of the sarcoplasmic reticulum and expression of its $\text{Ca}^{2+}\text{-ATPase}$ gene in pressure over-loaded rat myocardium. *Circulation*, **78**, Suppl. II, II-535 (Abstract).
- Mansier, P. & Lelièvre, L. (1982). Ca^{2+} free perfusion in rat heart reveals a $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ form highly sensitive to ouabain. *Nature*, **300**, 535–537.
- Mayoux, E., Callens, F., Swynghedauw, B. *et al.* (1988). Adaptational process of the cardiac Ca^{2+} channels to pressure overload: biochemical and physiological properties of the dihydropyridine receptors in normal and hypertrophied rat hearts. *J. cardiovasc. Pharmac.*, **12**, 390–396.
- Michel, R. C., Wang, X. L., Schliker, E. *et al.* (1987). Increased β_2 -adrenoceptor density in heart, kidney and lung of spontaneously hypertensive rats. *J. autonomic Pharmac.*, **7**, 41–51.
- Nogai, R., Zarain-Herzberg, Brandl, A. (1989). Regulation of myocardial $\text{Ca}^{2+}\text{-ATPase}$ and phospholamban mRNA expression in response to pressure overload and thyroid hormone. *Proc. Nat. Acad. Sci. USA*, **86**, 2966–2970.
- Reuter, H. (1983). Calcium channel modulation by

- neurotransmitters, enzymes and drugs. *Nature*, **301**, 569–572.
- Swynghedauw, B. & Delcayre, C. (1982). Biology of cardiac overload. *Pathobiol. Ann.*, **12**, 137–183.
- Upsher, M. E. & Khairallah, P. A. (1985). Beta-adrenergic receptors in rat myocardium during the development and reversal of hypertrophy and following chronic infusions of angiotensin II and epinephrine. *Arch. int. Pharmacodyn.*, **274**, 65–79.
- Will-Shahab, L., Küttner, I., Warbanow, W. *et al.* (1986). Signal transfer in cardiac muscle. Alteration of the β -adrenoceptor adenylate cyclase system in the hypertrophied myocardium. *Biomed. Biochim. Acta*, **45**, S199–S204.
- Woodcock, E. A., Funder, J. W. & Johnston, C. I. (1979). Decreased cardiac β -adrenergic receptors in deoxycorticosterone-salt and renal hypertensive rats. *Circulation Res.*, **45**, 560–565.