PHOSPHOLAMBAN PHOSPHORYLATION IN THE PERFUSED RAT HEART IS NOT SOLELY DEPENDENT ON β-ADRENERGIC STIMULATION

Christian J. LE PEUCH*, Jean-Claude GUILLEUX and Jacques G. DEMAILLE
Faculté de Médecine de Montpellier and Centre de Recherches de Biochimie Macromoléculaire du CNRS, BP 5051, 34033 Montpellier, France

Received 23 February 1980

1. Introduction

Cardiac contraction is controlled by the cytosolic Ca^{2+} conc. Relaxation is induced by calcium removal from the regulatory site of troponin C, the calcium-binding protein that relieves the inhibition to actin–myosin interaction brought about by troponin I. The rate of relaxation is therefore dependent upon the calcium uptake by cardiac sarcoplasmic reticulum. Epinephrine is known to accelerate the rate of relaxation. This effect was shown in vitro to be paralleled by a cAMP-dependent phosphorylation of phospholamban, accompanied by an increase in the rate of Ca^{2+} uptake and in Ca^{2+}–Mg^{2+}-ATPase activity of the cardiac sarcoplasmic reticulum calcium pump [1–9].

Phospholamban was shown to be phosphorylated in vitro at two different sites by (i) the catalytic subunit of cAMP-dependent protein kinase and (ii) a calcium–calmodulin dependent, membrane-bound, protein kinase [9].

It was therefore important to assess the occurrence of the phospholamban phosphorylation in vivo, i.e., in perfused hearts, and to evaluate the extent of the β-adrenergic control of these covalent modifications under conditions of stimulation or blocking of the β-receptors. Conversely, phospholamban phosphorylation was also measured after perfusion in the presence and absence of fluphenazine, which inhibits the interaction of calmodulin with most of its target enzymes.

Abbreviations: cAMP, cyclic adenosine 3′:5′-monophosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; SDS, sodium dodecylsulphate; SR, sarcoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis

*Attaché de recherche INSERM; to whom correspondence and reprint requests should be addressed

2. Experimental

PMSF was from Sigma; carrier free [^{32}P]orthophosphoric acid was purchased from NEN. Polyacrylamide gel electrophoresis reagents were from Bio Rad. All other chemicals were from Merck or analytical grade. Fluphenazine dihydrochloride was kindly provided by Squibb.

2.1. *Heart perfusion*

Perfusion experiments were performed on rat hearts essentially as in [10,11]. Wistar rats (300–350 g) were heparinized and after 1 h anesthetized by intraperitoneal injection of 4.5 ml 10% urethane. After 5 min the heart was removed and washed in cold saline. The aorta was then canulated and tied. The heart was perfused under a constant hydrostatic pressure (80 cm H_2O) by the Langendorff technique with a bicarbonate medium (pH 7.3) containing 128.8 mM Cl^-, 25 mM CO_3H^-, 0.24 mM PO_4^{3-}, 1.2 mM SO_4^{2-}, 143 mM Na^+, 5.82 mM K^+, 2.54 mM Ca^{2+}, 1.19 mM Mg^{2+} and 11 mM glucose. The PO_4^{3-} concentration used was low in order to increase the specific radioactivity in the medium during perfusion with ^{32}P which was started 10 min after perfusion with the non-radioactive medium. After 30 min perfusion with medium containing $^{32}P_i$ (100 μCi/ml) the heart was perfused with the non-radioactive solution for 3 min and then for 60 s with the same solution containing 4 μM isoproterenol or 20 μM propranolol. The heart was immediately homogenized for 5 s at

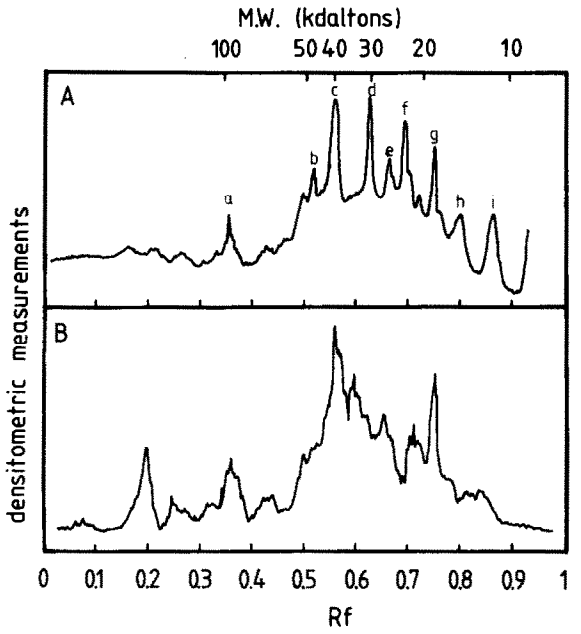

Fig.1. SDS (0.1%)–polyacrylamide (5–20% gradient) gel electrophoresis of isoproterenol-treated heart muscle homogenate (273 μg protein): (A) Autoradiogram; (B) Coomassie blue-stained gel.

full speed in an Omni-Sorvall mixer, equipped with a microhomogenizer accessory, in 8 vol. 70 mM NaF, 5 mM EDTA, 1 mM DTT, and 50 mM phosphate buffer (pH 7.0) [12] containing 0.3 M sucrose. In some experiments, 50 μM fluphenazine was added to the perfusion medium containing $^{32}P_i$. This 30 min perfusion time was chosen to allow at least partial entry of the drug within the cell, which recovered within 5–7 min after removal of fluphenazine, as monitored by electrocardiography.

2.2. *Sarcoplasmic reticulum preparation*

Sarcoplasmic reticulum was purified essentially as in [9]. The homogenate was centrifuged at 15 000 × *g* for 20 min, and the supernatant was centrifuged at 100 000 × *g* for 90 min. The 100 000 × *g* pellet was resuspended and incubated overnight in a minimal volume of 0.1 mM PMSF, 1 mM DTT, 0.3 M sucrose and 10 mM Hepes buffer (pH 7.5) containing 0.6 M KCl. The suspension was then centrifuged at 100 000 × *g* for 90 min and the final pellet resuspended and dissolved in the electrophoresis sample buffer containing 1% SDS. An aliquot of the muscle homogenate was also incubated with the same buffer for subsequent electrophoretic comparison. The samples were submitted to SDS–(0.1%) polyacrylamide (5–20% gradient) slab gel electrophoresis according to [13]. Molecular weight markers were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme, of mol. wt 97 400, 68 000, 43 000, 29 000, 17 500 and 14 300, respectively. The proteins were fixed and stained with Coomassie blue R 250. The gel was dried under vacuum and the radioactive bands detected by autoradiography after 24 h exposure of a Kodak X-Omat R film. The autoradiograms and the stained gels were scanned with a microdensitometer MK III CS from Joyce-Loebl. Individual protein bands were cut from the gel, dissolved in 30% hydrogen peroxide at 70°C, and counted in 10 ml dioxane–naphthalene scintillant.

3. Results and discussion

When homogenates from isoproterenol-stimulated hearts were submitted to SDS–PAGE, [^{32}P]phosphate appeared to be incorporated into 9 major polypeptide chains, as shown in fig.1. Their mol. wt was calculated to be 98 000, 48 000, 40 000, 30 000, 26 000, 23 000, 19 000, 15 000 and 11 500 for bands a–i, respectively. Bands a, d, f, g and i can tentatively be assigned to phosphorylase [14], troponin I [15], phospholamban dimer [9], myosin regulatory light chain [16,17] and phospholamban monomer [9,18], respectively. Cardiac troponin I does migrate upon SDS–PAGE with app. mol. wt 29 500 [17], which is significantly higher than the 23 550 mol. wt computed from the amino acid sequence [19].

After purification of SR vesicles from isoproterenol-treated hearts, the autoradiogram illustrated in fig.2A, upper trace, shows mainly the 40 000, 30 000, 23 000, 19 000 and 11 500 mol. wt [^{32}P]polypeptide chains. The Coomassie blue-stained electrophoretogram of SR vesicles obtained from either isoproterenol or propranolol-treated hearts (fig.2B) shows a slight contamination by contractile proteins, namely myosin heavy and light chains, actin and troponin. This explains the observed [^{32}P]phosphate incorporation into the 30 000 and 19 000 mol. wt bands which are troponin I and myosin regulatory light chain, respectively. Such a contamination was expected from the adopted purification procedure since it included

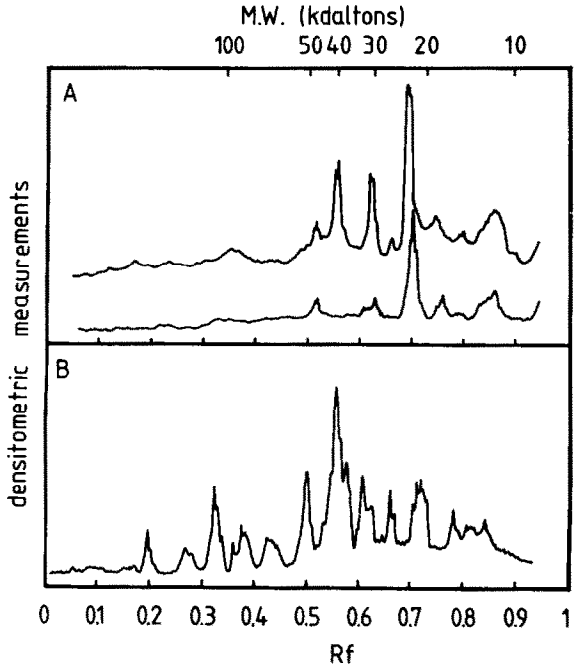

Fig.2. SDS (0.1%)–polyacrylamide (5–20% gradient) gel electrophoresis of rat heart sarcoplasmic reticulum (300 μg protein): (A) Autoradiogram of sarcoplasmic reticulum obtained from isoproterenol-stimulated heart (upper curve) and propranolol-treated heart (lower curve). Radioactivity under the 22 000 and 11 000 mol. wt peaks (upper curve) was 960 and 1040 cpm, respectively. (B) Coomassie blue-stained gel described in (A) (the same curve is obtained for both β-agonist and β-antagonist-treated hearts).

only a single high salt wash because of the scarcity of the starting material.

Major differences were observed when autoradiograms from isoproterenol-treated (fig.2A, upper trace) and propranolol-treated hearts (fig.2A, lower trace) were compared. The phosphorylation of a 48 000 mol. wt chain and of the 19 000 mol. wt regulatory light chain was unaffected by stimulation or blocking of the β receptors. In contrast, the phosphorylation of the 40 000 mol. wt protein and of troponin I (mol. wt 30 000) was essentially suppressed by propranolol, and appeared therefore to be catalyzed only by cAMP-dependent protein kinases, as already amply documented for troponin I [11,15,17]. The phosphorylation of phospholamban dimer and monomer was reduced after propranolol treatment to ~49% of the level in isoproterenol-stimulated hearts. Since troponin I phosphorylation was abolished after exposure to propranolol, the following two major conclusions can be drawn:

1. Phospholamban can be phosphorylated in vivo by a protein kinase that is different from cAMP-dependent protein kinase, presumably by the calcium–calmodulin-dependent protein kinase in [9];
2. Additional phosphorylation of phospholamban is brought about by cAMP-dependent protein kinase after stimulation by isoproterenol.

These conclusions are supported by the results of experiments in which fluphenazine was added to the $^{32}P_i$-containing perfusion medium. In the absence of either β-agonist or antagonist, the phosphorylation of phospholamban dimer + monomer was reduced by the fluphenazine treatment to ~37% of control. When the hearts were stimulated by isoproterenol, fluphenazine reduced phospholamban phosphorylation to ~32% of control. Comparison of propranolol-treated hearts with or without previous fluphenazine treatment was not performed because of cardiac arrest induced by the β-blocker on fluphenazine treated hearts.

Therefore, phospholamban phosphorylation appears to be inhibited independently either by β-antagonists or by fluphenazine, which is known to bind to calmodulin in the presence of Ca^{2+} and thereby to prevent its interaction with calmodulin-stimulated enzymes [20], assuming that the intracellular concentration of the drug was high enough. This independent effect of propranolol and fluphenazine seems to rule out the possibility that phospholamban is phosphorylated only by cAMP-dependent protein kinase, or that it is more slowly dephosphorylated than troponin I by phosphoprotein phosphatase.

These data are fully consistent with the observations made on purified canine cardiac SR vesicles phosphorylated in vitro by either cAMP or Ca^{2+}-dependent protein kinases [9]. They also indicate that phospholamban does not seem to be in the dephospho form in vivo even under conditions where the cAMP concentration is at its basal level. It can be assumed that SR vesicle preparations are dephosphorylated in vitro by phosphoprotein phosphatase and cannot be rephosphorylated at the Ca^{2+}-dependent site in the absence of exogenous calmodulin, under the experimental conditions used to measure the rate of Ca^{2+} uptake. Therefore, figures previously reported for the maximal calcium uptake activity of cardiac SR vesicles may be significantly lower than the rate obtained in vivo, where the Ca^{2+}-dependent phos-

phorylation appears to occur independently of the cAMP-dependent one. This raises the interesting possibility that the cardiac SR has the capability of moving in and out of the cytosol the whole of the Ca^{2+} that are transported during systole and diastole, without the postulated help of the mitochondrial calcium pump [21].

Acknowledgements

This work was supported in part by grants from CNRS (ATP Modulation de l'action des hormones au niveau cellulaire), from INSERM (CRL 78.4.086.1 and 79.4.151.3 and ATP 63.78.95 Biologie et pharmacologie de la fibre musculaire cardiaque), from DGRST (ACC Biologie et fonction du myocarde), from Faculté de Médecine, NATO (grant no. 1688) and Fondation pour la Recherche Médicale Française.

References

[1] Wray, H. L., Gray, R. R. and Olsson, R. A. (1973) J. Biol. Chem. 248, 1496–1498.
[2] Kirchberger, M. A., Tada, M. and Katz, A. M. (1974) J. Biol. Chem. 249, 6166–6173.
[3] Tada, M., Kirchberger, M. A. and Katz, A. M. (1975) J. Biol. Chem. 250, 2640–2647.
[4] Kirchberger, M. A. and Tada, M. (1976) J. Biol. Chem. 251, 725–729.
[5] Schwartz, A., Entman, M. L., Kaniike, K., Lane, L. K., Van Winkle, W. B. and Bornet, E. P. (1976) Biochim. Biophys. Acta 426, 57–72.
[6] Will, H., Blanck, J., Smettan, G. and Wollenberger, A. (1976) Biochim. Biophys. Acta 449, 295–303.
[7] Wray, H. L. and Gray, R. R. (1977) Biochim. Biophys. Acta 461, 441–459.
[8] Tada, M., Ohmori, F., Yamada, M. and Abe, H. (1979) 254, 319–326.
[9] Le Peuch, C. J., Haiech, H. and Demaille, J. G. (1979) Biochemistry 18, 5150–5157.
[10] Krebs, H. A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–66.
[11] England, P. J. (1975) FEBS Lett. 50, 57–60.
[12] Holroyde, M. J., Small, D. A. P., Howe, E. and Solaro, R. J. (1979) Biochim. Biophys. Acta 587, 628–637.
[13] Laemmli, U. K. (1970) Nature 227, 680–685.
[14] McCullough, T. E. and Walsh, D. A. (1979) J. Biol. Chem. 254, 7345–7352.
[15] England, P. J. (1976) Biochem. J. 160, 295–304.
[16] Solaro, R. J., Moir, A. J. G. and Perry, S. V. (1976) Nature 262, 615–617.
[17] Kopp, S. J. and Barany, M. (1979) J. Biol. Chem. 254, 12007–12012.
[18] Le Peuch, C. J., Le Peuch, D. A. M. and Demaille, J. G. (1980) Biochemistry in press.
[19] Grand, R. J. A., Wilkinson, J. M. and Mole, L. E. (1976) Biochem. J. 159, 633–641.
[20] Levin, R. M. and Weiss, B. (1977) Mol. Pharmacol. 13, 690–697.
[21] Carafoli, E. and Crompton, M. (1978) Curr. Top. Membr. Trans. 10, 151–216.