Effect of Isoproterenol on Protein Phosphorylation in Myocardial Ischaemia

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Abstract. Perfused rat hearts prelabelled with ^{32}P were made ischaemic by reducing the medium flow from 12 ml/min to 0.5 ml/min. There was a rapid decrease in the contractile performance, but no significant changes in the phosphorylation state of troponin I, myosin P-light chain, an 11 K protein or in the proportion of phosphorylase in the *a* form occurring up to 5 min of ischaemia. Control hearts stimulated with a bolus of isoproterenol showed a large increase in the contractile force and in the phosphorylation of troponin I, 11 K protein, and phosphorylase, respectively. These responses were progressively reduced by increasing periods of ischaemia. The reduction and loss of increased phosphorylation of these proteins on exposure to isoproterenol was parallelled with an inhibition of cyclic AMP accumulation in the ischaemic heart. Phosphorylation of the myosin P-light chain remained unchanged under all the conditions studied.

Key words: Myocardial ischaemia — Protein phosphorylation — Troponin I — Myosin P-light chain — Phosphorylase

Introduction

In normally perfused hearts catecholamines induce increased Ca^{2+} fluxes resulting in an increased contractility (Katz 1977). This is associated, and may indeed be brought about by increased phosphorylation of membrane and contractile proteins (England 1980; Katz 1981; Stull et al. 1981; Wollenberger and Will 1978). The contractile protein troponin I (TN—I) is phosphorylated both *in vitro* and *in vivo* by cyclic AMP-dependent protein kinase, while the myosin P-light chain is phosphorylated by a specific Ca^{2+}-calmodulin-dependent protein kinase (England 1976; Pires et al. 1974). Phospholamban, a 22,000 mol weight protein of the sarcoplasmic reticulum, is phosphorylated by a cyclic AMP-dependent as well as

* This research was supported by a grant from the Wellcome Foundation of Great Britain.

Ca^{2+}-calmodulin-dependent protein kinase *in vitro* (Katz 1981; Le Peuch et al. 1979) and possibly *in vivo* as well (Krainias and Solaro 1982; Le Peuch et al. 1980; Lindemann et al. 1983).

Heart ischaemia and anoxia is characterized by a rapid reduction in the contractile response (Theroux et al. 1974), although intracellular concentrations of ATP and H^+ ions are initially unchanged as compared to the aerobic state (Jacobus et al. 1982), and the level of cyclic AMP is elevated (Wollenberger et al. 1969). It has been suggested that a reduction in the Ca^{2+} influx across the sarcolemma may occur during ischaemia and anoxia, occasionally parallelled by a decrease in the phosphorylation of membrane proteins (Sperelakis and Schneider 1976).

The purpose of the present study was to investigate whether the reduced contractile response to ischaemia was reflected in alterations in protein phosphorylation. Moreover, the catecholamine responsiveness of the acute ischaemic tissue was studied, as a reduction or loss of cyclic AMP accumulation induced by isoproterenol during acute ischaemia have recently been described (Krause and England 1982).

Materials and Methods

Hearts from Wistar rats (220—260 g) were perfused by the Langendorff technique using modified Krebs-Henseleit bicarbonate-buffered medium containing 11 mmol/l glucose (England 1976) and gassed with O_2:CO_2 (19:1) at a flow rate (maintained by roller a pump) of 12 ml/min. Following a 5 min preperfusion the hearts were perfused with a medium containing 0.5 MBq $^{32}P_i$ per ml for 15 min in a recycling system. The hearts were then switched back for 1 min to non-radioactive medium to measure contractile response. Ischaemia was induced by reducing the flow to 0.5 ml/min. D, L-isoproterenol (70 pmole in 0.15 mol/l NaCl) was injected as a 5 μl bolus into the aortic cannula in various intervals following the onset of ischaemia, as indicated in Fig. 1, and 20 seconds later the hearts were freeze-clamped. Hearts not injected with isoproterenol were also freeze-clamped at the end of the perfusion. Changes in contractile force were monitored with a force displacement transducer (Ormed Engineering, Welwyn Garden City, Herts, U.K.) attached to the apex of the heart.

Analysis of protein phosphorylation: Samples of frozen heart were prepared for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) essentially as described by Jeacocke and England (1977). Frozen tissue (0.1—0.2 g) was homogenised in 2 ml 5 mmol/l guanidine HCl, 0.1 mol/l glycine, 5 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, pH 1.5, insoluble protein was removed by centrifugation, and protein was precipitated by the addition of 6 ml 0.5 mol/l trichloroacetic acid. The precipitated protein was washed with 25 mmol/l sodium phosphate, pH 7.5, and finally redissolved in 10 % SDS, 20 % sucrose, 125 mmol/l TRIS, pH 6.8, by heating at 100 °C for 15 min. Protein was estimated in the final samples using the method of Zaman and Verwilghen (1979); 200 μg protein was run on each track. Electrophoresis was carried out by the method of Laemmli (1970) using either a 12.5 % gel or a 5 %—15 % gradient gel of polyacrylamide. Following staining with Coomassie Brilliant Blue the gels were dried, and ^{32}P in the protein band was determined by densitometric scanning after autoradiography of the gels. The autoradiographs were within the range of proportionality of absorbance and radioactivity. The specific radioactivity of ^{32}P-γ-ATP in the frozen

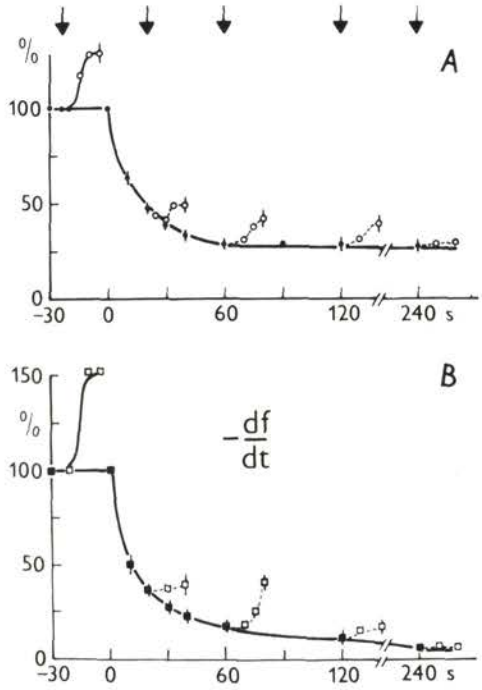

Fig. 1. Effect of ischaemia on the contractile force (*A*)and relaxation rate (*B*) of perfused heart, and the response to isoproterenol. (●, ■) untreated hearts (*n*: 6—9); (○, □) changes following a bolus injection of isoproterenol (arrows *n*: 3—5).

hearts was measured as described by England and Walsh (1975), and used to correct the densitometric values for each heart.

Other assays: ATP was assayed according to Lamprecht et al. (1970). The proportion of phosphorylase in the *a* form was assayed according to England (1976). Protein was determined by the Biuret method. All data are expressed as mean ±S.E.M (no. of observations).

Results and Discussion

Contractile and metabolic responses to ischaemia: The contractile performance of the heart, measured as the contractile force (defined as the difference between the tension developed at the systole and the diastole), relaxation rate and beating frequency, all showed a rapid deterioration from the onset of ischaemia. As shown in Fig. 1, contractile force and relaxation rate fell to less than 50 per cent of the control values after 15 s, and decreased thereafter to 25 and less than 10 per cent, respectively, after 4 min. The response of these parameters to isoproterenol varied depending on the ischemic interval. When isoproterenol was given in the control

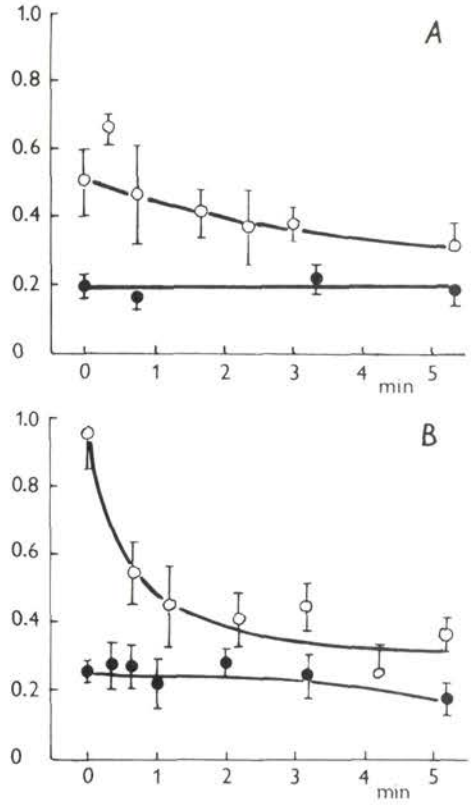

Fig. 2. Effect of isoproterenol on phosphorylation of 11 K protein (relative to that of the myosin P-light chain) (*A*) and troponin I (mole P/mole) (*B*) in perfused control and ischaemic rat hearts in relation to the duration of the ischaemic interval. (●) untreated hearts (n: 3—5); (○) following a bolus injection of isoproterenol and freeze clamped 20 sec later (n: 3—5). P—LC: myosin P-light chain.

period, the contractile force and relaxation rate increased by of approximately 30 and 50 per cent, respectively. These responses progressively diminished during ischaemia, and they were almost completely lost after 4 min. Similar changes were also observed in the frequency of contraction (data not shown). The intracellular concentration of ATP remained constant throughout the period of ischaemia, at a value of 21.2 ± 0.5 μmole/g protein ($n = 95$), and it remained unchanged upon isoproterenol administration.

Phosphorylation of proteins during ischaemia: Three proteins labeled with ^{32}P were selected for measurements in this study: TN—I, myosinP-light chain, and a protein of a relative molecular weight of 11,000 (11 K protein). The 11 K protein

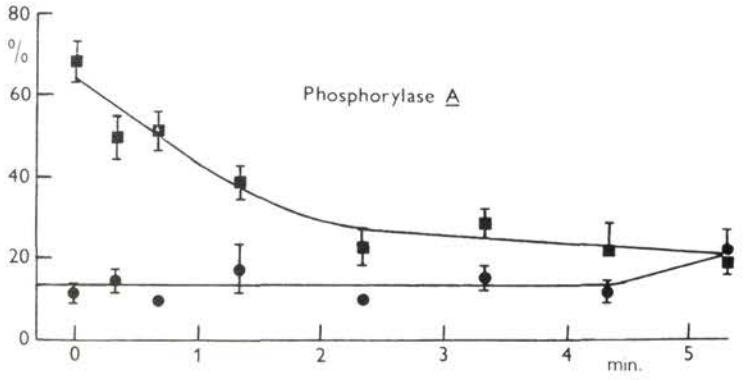

Fig. 3. The effect of ischaemia and isoproterenol on the proportion of phosphorylase *a*. (●) untreated hearts (*n*: 3—8; without bars *n*: 2); (■) hearts injected with a bolus of isoproterenol and freeze clamped 20 s later (*n*: 4—7).

may be a monomer of phospholamban or a protein of sarcolemmal origin, which can be phosphorylated *in vivo* (Lindemann et al. 1983). Figs. 2 and 3 show the effect of ischaemia as well as that of isoproterenol on the phosphorylation of 11 K protein, TN—I, and phosphorylase, respectively. In control perfusion the phosphorylation levels of TN—I, phosphorylase and the 11 K protein (the latter being often undetectable) were low. Myosin P-light chain showed a substantial phosphorylation of 0.5—0.6 mol of phosphate/mol. Ischaemia itself induced no changes in the phosphorylation of any of the proteins studied. Thus the phosphorylation states of the proteins examined are probably not causally related to the rapid deterioration in the heart contractile performance following ischaemia.

In control perfusion isoproterenol increased the phosphorylation of TN—I, phosphorylase, and the 11 K protein, respectively. However, phosphorylation of these proteins gradually decreased with prolonged periods of ischaemia (Fig. 2 and 3). The phosphorylation state of the myosin P-light chain remained unchanged in all the above experiments (data not shown).

Both TN—I and the sarcoplasmic reticulum and/or sarcolemma protein (11 K protein) are phosphorylated by cyclic AMP-dependent protein kinase (Katz 1981; Le Peuch et al. 1979; Stull et al. 1981; Will et al. 1978). This enzyme is also capable of phosphorylating phosphorylase kinase (Walsh et al. 1971) increasing its activity to phosphorylate phosphorylase *b*.Thus the observed reduction in the phosphorylation of the proteins studied is probably due to a decrease in or loss of the capacity of the acutely ischaemic heart to accumulate cyclic AMP as a response to isoproterenol, as has recently been shown by Krause and England (1982). A decreased cyclic AMP concentration and a decreased TN—I phosphorylation were also found in infarcted areas of canine heart muscle (Antipenko et al. 1981).

The myosin P-light chain would not be expected to be responsive to changes in cyclic AMP, since it is phosphorylated by a Ca^{2+}-calmodulin-dependent protein kinase (Pires et al. 1974). The changes in phosphorylation of this protein observed in heart tissue following a period of ischaemia lasting for 30 min (Cummins et al. 1981) may only reflect subsequent alterations of the contractile protein in the heart. The effect of acute ischaemia on protein phosphorylation cannot be explained by a decrease in ATP concentration, since its total content remained unchanged throughout the ischaemic period.

There is strong evidence that cyclic AMP is involved in serious pathological sequelae of catecholamine stimulation in the heart, namely in the induction of cardiac arrhythmias including ventricular fibrillation (Opie et al. 1980). The loss or attenuation of isoproterenol-induced cyclic AMP accumulation and protein phosphorylation during short-term ischaemia may therefore be of importance in delaying partially the onset of catecholamines induced arrythmias. The rapid reduction of the contractile response, seems however, not to be associated with any detectable alterations in phosphorylation of the proteins studied: troponin I, the myosin P-light chain, and the 11 K protein. According to recently published data on anoxic rat heart (Kammermeier et al. 1982) the low level of free energy change of ATP hydrolysis rather than ATP deficiency, can be considered to be one major causal factor of the contractile failure during acute myocardial ischaemia. This could also be confirmed in our experiments.

References

Antipenko A. E., Choncharov O. G., Orovin B. G., Pepinova V. R. (1981): Cyclic AMP-dependent phosphorylation of troponin of experimentally infarcted heart muscle. Voprosy Med. Khimii **27**, 492—495 (in Russian)

Cummins P., Crome R., Yellon D. M., Hearse D. J. (1981): Changes in myosin light chain phosphorylation during regional myocardial ischaemia. J. Mol. Cell. Cardiol. **13**, 18

England P. J. (1976): Studies on the phosphorylation of the inhibitory subunit of troponin during modification of contraction in perfused heart. Biochem. J. **160**, 295—304

England P. J. (1980): Protein phosphorylation in the regulation of muscle contraction. In: Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation (Ed. P. J. Cohen), pp. 153—170, Elsevier/North-Holland Biomedical Press

England P. J., Walsh D. A. (1975): A rapid method for the measurement of (γ—^{32}P) ATP specific radioactivity in tissue extracts and its application to the study of $^{32}P_i$ uptake in perfused rat heart. Anal. Biochem. **75**, 429—435

Jacobus W. E., Pores I. H., Lucas S. K., Weisfeldt M. L., Flaherty H. T. (1982): Intracellular acidosis and contractility in the normal and ischemic heart as examined by ^{31}P NMR. J. Mol. Cell. Cardiol. **14**, Suppl. 3, 13—20

Jeacocke S. A., England P. J. (1977): Phosphorylation of myosin light chains in the perfused rat heart. Biochem. J. **188**, 763—768

Kammermeier H., Schmidt P., Jüngling E. (1982): Free energy change of ATP hydrolysis: a causal factor of early hypoxic failure of the myocardium? J. Mol. Cell. Cardiol. **14**, 267—277

Katz A. M. (1977): The Physiology of the Heart. Raven Press, New York

Katz A. M. (1981): Regulation of calcium transport in cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase. Cold Spring Harbor Conf. Cell. Profil. **8**, 849—858

Krainias E. G., Solaro R. J. (1982): Phosphorylation of troponin I and phospholamban during catecholamine stimulation of rabbit heart. Nature **298**, 182—184

Krause E. G., England J. P. (1982): Loss of the cyclic AMP accumulation induced by isoproterenol during acute ischaemia in the isolated rat heart. J. Mol. Cell. Cardiol. **14**, 611—613

Laemmli U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277**, 680—685

Lamprecht W., Stein P., Heinz F., Weisser H. (1970): In: Methoden der enzymatischen Analyse (Ed. H. U. Bergmeyer), pp. 1734—1737, Akademie-Verlag, Berlin

Le Peuch C. J., Gaillex J.—C., Demaille J. G. (1980): Phospholamban phosphorylation in the perfused heart is not solely dependent on β-adrenergic stimulation. FEBS Lett. **114**, 165—168

Le Peuch C. J., Haiech J. Demaille J. G. (1979): Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate dependent and calcium-calmodulin-dependent phosphorylation. Biochemistry **18**, 5150—5157

Lindemann J. P., Jones L. R., Hathaway D. R., Henry B. G., Watanabe A. M. (1983): β-Adrenergic stimulation of phospholamban phosphorylation and Ca^{2+}-ATPase activity in guinea pig ventricles. J. Biol. Chem. **258**, 464—471

Opie L. H., Müller C., Nathan D., Daries P., Lubbe W. F. (1980): Evidence for role of cyclic AMP as second messenger of arrhythmogenic effects of beta-stimulation. Adv. Cyclic Nucleotide Res. **12**, 63—69

Pires E. N. V., Perry S. V., Thomas M. A. W. (1974): Myosin light-chain kinase, a new enzyme from striated muscle. FEBS Lett. **41**, 292—296

Sperelakis N., Schneider J. A. (1976): A metabolic control mechanism for calcium ion influx that may protect the ventricular myocardial cell. Amer. J. Cardiol. **37**, 1079—1085

Stull J. T., Sanford C. F., Manning D. R., Blumenthal D. K., High C. W. (1981): Phosphorylation of myofibrillar proteins in striated muscles. Cold Spring Harbor Conf. Cell. Prolif. **8**, 823—840

Theroux P., Franklin D., Ross J., Kemper W. S. (1974): Regional myocardial function during acute coronary occlusion and its modification by pharmacologic agent in the dog. Circ. Res. **35**, 896—908

Walsh D. A., Perkins J. P., Brostrom C. O., Ho E. S., Krebs E. G. (1971): Catalysis of the phosphorylase kinase activation reaction. J. Biol. Chem. **246**, 1968—1976

Will H., Levchenko T. S., Levitzky D. O., Smirnov V. N., Wollenberger A. (1978): Partial characterization of protein kinase catalyzed phosphorylation of low molecular weight proteins in purified preparations of pigeon heart sarcolemma and sarcoplasmic reticulum. Biochim. Biophys. Acta **543**, 175—193

Wollenberger A., Will H. (1978): Protein kinase catalyzed membrane phosphorylation and its possible relationship to the role of calcium in the adrenergic regulation of cardiac contraction. Life Sci. **22**, 1159—1178

Wollenberger A., Krause E.—G., Heier G. (1969): Stimulation of 3′, 5′-cyclic AMP formation in dog myocardium following arrest of blood flow. Biochem. Biophys. Res. Commun. **36**, 664—670

Zaman Z., Verwilghen R. L. (1979): Quantitation of proteins solubilized in sodium dodecylsulfate-mercaptoethanol-Tris electrophoresis buffer. Anal. Biochem. **100**, 64—69

Received October 12, 1983/Accepted November 2, 1983