Simultaneous response of myocardial contractility and a major proteolytic process to β -adrenergic-receptor occupancy in the Langendorff isolated perfused rat heart

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The Langendorff isolated rat heart was adapted to the study of minute-to-minute percentage changes in bulk protein degradation by using non-recirculating perfusion. Hearts were perfused at 8 ml/min at 35 °C with Krebs–Henseleit buffer containing 11 mM-glucose, and only hearts with regular ventricular rhythm were employed. Proteins were labelled by infusion of [3 H]leucine for 0.5 h *in vitro*. A complete amino acid mixture was then added at 3 times normal rat extracellular concentrations. After labelling, the re-incorporation of [3 H]leucine was competitively inhibited by addition of either 4 mM-leucine or 20 μ M-cycloheximide. The residual unincorporated radioactivity and the preferentially labelled rapid-turnover proteins were eliminated during a 3 h preliminary perfusion period. The basal rate of release of [3 H]leucine and percentage changes were then determined at 1 min intervals, by using each heart as its own control. Leucine metabolism was inconsequential to results. Exchange of intracellular leucine pools with extracellular leucine and subsequent release in effluent perfusate was 95% complete within approx. 2 min. The basal rate of protein degradation was unchanged by electrical stimulation of the heart rate to 360 beats/min or cessation of contractile activity by membrane depolarization under 25 mM-KCl. Infusion of the β -agonist isoprenaline at 5–500 nM caused a graded inhibition of myocardial protein degradation within 5–6 min, with a maximum inhibition of 30%. This inhibition was sustained for at least 1 h of drug administration and was reversed within 4–6 min of cessation of isoprenaline or simultaneous infusion of 1 μ M of the β -receptor antagonist propranolol. Minute-to-minute adrenergic proteolytic control was a simultaneous co-variable with β -receptor-mediated inotropic changes in right-intraventricular systolic pressure. Stoppage of the heart in asystole by the Ca^{2+} -channel blocker nifedipine (0.7 μ M) delayed the onset, but did not cause sustained reversal, of adrenergic-inhibited degradation, indicating the absence of a direct obligatory mechanistic linkage between the events of the contraction–relaxation cycle and protein degradation in this preparation.

INTRODUCTION

Many aspects of myocardial metabolism are directly or indirectly integrated with the specialized function of the heart; however, the relationship between protein degradation and mechanical function has remained unknown, owing to experimental limitations. Two experimentally distinguishable types of controls can alter cardiac mechanical function and also elicit diverse metabolic responses [Starling (1918); Sarnoff *et al.* (1960); reviewed by Will-Sahab & Krause (1980), Demaille & Pechere (1983) and Ramachandran *et al.* (1983)]. The intrinsic controls can change myocardial mechanical function within one or more contractions in association with such variables as the pre-contraction sarcomere length, load, or heart rate. The extrinsic controls can change the so-called contractility status within several minutes of adrenergic-receptor occupancy. However, the exact interrelationships *in vivo* between these two types of controls remain uncertain (see the Discussion section).

Mammalian hearts can be perfused *in vitro* as either working preparations, contracting against a load, or non-working preparations, contracting only against a small amount of internal tissue pressure [reviewed by Neely & Rovetto (1975) and Kira *et al.* (1984), and see below]. In working heart preparations, it has previously been suggested that myocardial protein degradation did not change when mechanical work was experimentally

varied by presumably intrinsic controls in the absence of adrenergic stimulation [Schreiber *et al.* (1973), Smith & Sugden (1983), and literature reviewed therein]. In non-working heart preparations it is described below that decreases in a major proteolytic process are indeed co-ordinated with simultaneous increases in the contractility status caused by β -adrenergic-receptor occupancy. However, adrenergic control of this proteolytic process is experimentally demonstrated to be independent of any direct linkage to mechanical function or the events of the contraction–relaxation cycle in the non-working myocardial preparation (reported preliminarily by Lockwood, 1984). The present procedures have not previously been applied; thus the relationship of these results to other studies is uncertain [Schreiber *et al.* (1973), Waterlow *et al.* (1978), Chua *et al.* (1979, 1982), Morgan *et al.* (1980), Morgan (1982), Smith & Sugden (1983) and literature reviewed therein].

EXPERIMENTAL

Perfusion of hearts

As modified from the procedure described by Neely & Rovetto (1975), the hearts of 200 g male Sprague–Dawley rats were isolated and perfused by the Langendorff method. Heparin (Robbins; 9×10^3 units per animal) was

injected intramuscularly 1 h before decapitation. A glass-fibre perfusate filter, in line, removed particles of the size of red blood cells. Hearts were perfused with 8.0 ml of non-recirculating Krebs–Henseleit (1932) salt solution previously equilibrated with CO_2/O_2 (1:19)/min. This perfusate initially contained 0.2% bovine serum albumin (Sigma) and 11 mM-glucose, except as described below. After the 0.5 h [^3H]leucine incorporation period with no amino acids in the perfusate, as described below, a complete amino acid mixture was added at 3 times the normal rat plasma concentrations determined by Morgan *et al.* (1971), to promote maximal protein synthesis of the preparation, as described by Morgan *et al.* (1971).

Right-ventricular pressure was monitored by catheterization through the pulmonary artery. The artery was tied around the polyethylene catheter (PE 90), thereby closing the chamber. A Grass pressure transducer located at the level of the heart was connected to a Gould amplifier-recorder, and calibrated with a manometer. A peristaltic pump maintained the flow rate constant at 8.0 ml/min. A large 1 mm-diameter aortic cannula provided negligible resistance to flow in comparison with the vasculature of the organ, as evidenced by nearly zero perfusion pressure in the absence of the heart. Various experimental solutes were infused into the flow of perfusate immediately above the heart in 10^2 – 10^3 -fold concentrated solutions, by using separate syringe pumps. All drugs were from Sigma and were dissolved 1 h before use in 0.9% NaCl, except as indicated. Catecholamine solutions contained 0.1% ascorbic acid, as antioxidant. The infused ascorbic acid was diluted more than 200-fold and had no effect on results as determined in vehicle control experiments. Approximately one in nine heart preparations exhibited alternating pressure pulses or gross arrhythmia or fibrillations after 1–2 h; such experiments were discontinued.

Electrical pacing of heart rate

Wire electrodes separated by approx. 2 mm were placed against the surface of the atrium. Use of a Grass square-wave generator, giving supramaximal pulses of 20 V, 4 ms duration, sufficed to pace the heart at the indicated frequencies. Visual observation indicated that at this voltage rhythmic ventricular contraction followed atrial contraction; therefore conduction did not take place by spurious direct surface current.

Labelling *in vitro* of myocardial proteins and measurements of degradative alterations

To label myocardial proteins, 400 μCi of [4,5- ^3H]leucine (60 mCi/mmol, 1.7 $\mu\text{Ci}/\text{ml}$; New England Nuclear) was infused at 35 °C over 0.5 h before addition of the amino acids described above. Non-radioactive leucine (4 mM) was then added in a concentrated solution, pH 7.4, as a competitive inhibitor of re-incorporation of labelled leucine. The effluent perfusate from the organ was directed into a fraction collector. A 3 h preliminary washout period preceded measurement of the percentage changes in leucine release [Goldberg *et al.* (1982) and see below]. The heart was washed at 0.1, 1.5 and 2.5 h after labelling by submersion in buffer for 5 min. To increase longevity of the preparation, hearts were routinely perfused at 32 °C for 2.5 h, and the temperature was then increased to 35 °C 0.5 h before the experimental measurement period (see below). Repeated alteration of the perfusion temperature between 37.5 and 25 °C, within

10 min, resulted in sustained degradative change within 1 min of sustained temperature change in two separate experiments (results not shown). When 20 μM -cycloheximide was used to inhibit [^3H]leucine re-incorporation (reviewed by Morgan *et al.*, 1980), a 3 h perfusion period preceded the addition of cycloheximide, and measurements began at 4 h. Radioactivity in 4 ml of perfusate was counted in 16 ml of Aquasure (New England Nuclear), to provide a radioactivity more than 30 times background. Quench of radioactivity by drugs was monitored in each fraction and rigorously corrected by internal standardization where necessary. Under all of the present conditions the amount of trichloroacetic acid-precipitable macromolecular radioactivity released was less than 1% of the acid-soluble radioactivity. As determined by methods previously described (Lockwood *et al.*, 1982b), the fractional rate of release of incorporated leucine from the total organ declined from approx. 6–7%/h at 3 h after labelling to 2–3%/h at 5 h after labelling, owing to progressive elimination of more rapidly degraded proteins; however, such values theoretically depend on the exact labelling and unlabelling protocol.

Determination of one-pass extraction of leucine and conversion into CO_2

Buse *et al.* (1973) have described some amount of conversion of [^{14}C]leucine into $^{14}\text{CO}_2$ in a recirculated perfusate system under other conditions; therefore this compound was studied under the present conditions. [$\text{U-}^{14}\text{C}$]Leucine (10 $\mu\text{Ci}/\text{ml}$; New England Nuclear) was added to the perfusate reservoir bottle at leucine concentrations of 200 μM or 4.0 mM. The total percentage extraction of [^{14}C]leucine by the heart was determined by simultaneously sampling the perfusate before and after it traversed the organ. In order to trap CO_2 in the effluent perfusate, the fraction collector tubes contained a small volume of NaOH, providing a final pH of 11.0. In view of negative results on extraction and metabolism (see below), it was unnecessary to determine gaseous loss of $^{14}\text{CO}_2$ from the surface of the organ or fraction-collector tubes. To remove $^{14}\text{CO}_2$, a 3 ml sample from each tube was acidified to pH 1.5 with 1 ml of HCl, and shaken at 30 °C for 3 h in parallel with non-acidified samples containing 1 ml of water. Equal volumes of alkaline samples containing both [^{14}C]leucine and $^{14}\text{CO}_2$, and acidified samples containing only [^{14}C]leucine, were then added to separate scintillation vials containing excess acetate buffer, pH 4.0, and scintillation 'cocktail'. Vials were rapidly capped and counted for radioactivity after decline of chemiluminescence at 24 h. The percentage conversion of [^{14}C]leucine into volatile $^{14}\text{CO}_2$, and percentage extraction of [^{14}C]leucine by the heart, were determined by dividing the radioactivity of the acidified or alkalized effluent samples by the initial amount of perfusate radioactivity before passage through the organ. All samples were counted for radioactivity under identical conditions, with internal standardization.

T.l.c. studies of metabolism of label

After biosynthetic labelling, an internal chromatographic standard of [^{14}C]leucine was added to the [^3H]leucine radioactivity in the effluent perfusate from the heart at a $^3\text{H}/^{14}\text{C}$ ratio of 3:1. The bovine serum albumin of the perfusate sample was precipitated from 10% trichloroacetic acid. Less than 0.1% of the radioactivity was precipitable. To attain sufficient radioactivity, ten

Fig. 1. Rate of release of previously incorporated leucine from the perfused heart versus time after an 0.5 h incorporation period *in vitro*

Rat heart proteins were biosynthetically labelled by the routine labelling procedure described in the text. A 15 min period of perfusion with 4 mM non-radioactive leucine (at 32 °C) preceded the beginning of the determination, during which time the organ was rinsed repeatedly by submersion in buffer. Unlike other routine experiments, the temperature was not increased above 32 °C after 2.5 h (see the text). Perfusion at the lower temperature of 32 °C for the required 2.5 h preliminary period enhanced longevity (see the text).

separate spots were placed in parallel on a 16 cm × 16 cm Gelman silica-impregnated fibre pad, run in parallel, and counted for radioactivity together. The solvent was propan-2-ol/formic acid (20:1, v/v), Isotopic spill-over of ^{14}C standard was rigorously corrected by internal standardization.

RESULTS

Conditions of perfusion

In place of a constant perfusate pressure, with variable flow rate, a constant flow rate was required in order to maintain a minute-to-minute steady-state externalization of leucine (see below). At 37.5 °C, insufficient perfusate flow rates can injure the organ by hypoxia and/or ischaemia; and at any temperature excessive flow rates can cause injuriously excessive intravascular and tissue pressure, gross oedema, large increase in organ resistance to flow, and arrhythmia. Owing to these considerations, a constant perfusate flow rate of 8 ml/min and temperature of 35 °C or less were selected in order to optimize rhythmic functional longevity during extended perfusions. Under these conditions the aortic perfusion pressure remained constant at approx. 40–50 mmHg, and the right-ventricular diastolic pressure returned to zero throughout the 5 h perfusion period. At flow rates greater than 9.0 ml/min, a decrease in rhythmic longevity was preceded by a gradual increase in perfusion pressure, clearly visible oedematous swelling of the organ, and increases of the right-ventricular diastolic and systolic

Fig. 2. Effect of infusion of 4 mM extracellular non-radioactive leucine or 20 μM -cycloheximide on release of previously incorporated ^3H leucine from the perfused heart

After labelling, the hearts of both groups were perfused for 3.5 h with routine amino acid concentrations described in the text, except without additional 4 mM-leucine. From 3.5 h to the experimental zero time at 4.5 h, the hearts were perfused with no amino acids. The declining baseline of ^3H leucine release appeared similar to Fig. 3 below; however, this graphically determined baseline was used as the 100% control. After zero time each point is shown as a percentage of the extrapolated value of the previous baseline before zero time. At the indicated time, leucine (○) was infused at a final concentration of 4 mM (0.6 min measurements), or cycloheximide (●) was infused at 20 μM (1.0 min measurements). The means $\pm$ S.E.M. of the percentage increases in three experiments are shown at 10 min after additions. In each of three separate identical experiments the time to 95% of the final increased steady state was approx. 6 min with cycloheximide and 2 min with leucine.

pressures (results not shown). These conditions indicate oedema, increased vascular resistance and right-ventricular fluid accumulation respectively.

Extraction and metabolism of leucine

In the presence of 11 mM-glucose, the fraction of $[\text{U-}^{14}\text{C}]$ leucine that was converted into $^{14}\text{CO}_2$, or otherwise depleted from perfusate containing 200 μM - or 4 mM-leucine, during one passage through the heart was less than the experimental precision of 0.1% (results not shown). Infusion of the maximal amounts of isoprenaline used herein did not cause detectable conversion of $[\text{U-}^{14}\text{C}]$ leucine into $^{14}\text{CO}_2$ or extraction of leucine greater than the experimental uncertainty of 0.1% (results not shown). On silica-gel t.l.c., the migration of the ^3H radioactivity released from the heart was indistinguishable from the simultaneous co-migration of the $[\text{U-}^{14}\text{C}]$ leucine internal standard under basal conditions or with

Fig. 3. Protein degradation before and after cessation of contractile activity owing to increased K⁺ concentration

A control rhythm of 200 beats/min, 35 mmHg peak systolic pressure, with 4.7 mM-K⁺ is illustrated in expanded horizontal dimensions by the right intraventricular pressure pulse at 1 min (chart speed was 5 mm/s, leftward, and a 3 s interval is indicated). Elevation of perfusate K⁺ concentration to 25 mM by infusion of KCl, as indicated by the dashed trace at 9 min, promptly stopped the heart in less than 10 s. Perfusion continued at a rate of 8.0 ml/min by constant action of the peristaltic pump. In some preparations diastolic pressure increased slightly. A 4.0 h degradation period preceded the measurements. The actual curvature of the linearly approximated trace is empirically negligible over the short time interval shown. The continuously declining rate of leucine release before and after cessation of contractile activity represents an unchanged control of bulk protein degradation (see the text and below).

0.1 μ M-isoprenaline, i.e. the maximal amounts of the non-superimposed areas under the two curves describing perfusate ³H radioactivity and [¹⁴C]leucine internal-standard radioactivity versus distance co-migrated was less than 2–3% of the total areas, which is the limit of the experimental precision (results not shown). Thus the maximal possible amount of extraction and/or metabolism of [U-¹⁴C]leucine or [4,5-³H]leucine by the heart under the present conditions is negligible in comparison with the magnitude of isoprenaline-induced changes in release described below.

Preliminary elimination of unincorporated leucine and rapid-turnover proteins

At 15 min after the 0.5 h labelling period, the rapid initial release of [³H]leucine presumably consists of the elimination of unincorporated intracellular label combined in unknown proportion with the degradation of the distinct rapid-turnover subclass of cell proteins (Fig. 1) (Goldberg *et al.*, 1982). At 2.5 h after labelling, the semilogarithmic trace describing release of radioactivity versus time, at 32 °C, approached a second component of lesser slope. The initial, rapidly declining, component was also eliminated before 2.5 h at the routine experimental temperature of 35 °C; however, the longevity of the preparation was increased by preliminary perfusion at the lower temperature of 32 °C (see the Experimental section). The seemingly log-linear relationship shown in the semilogarithmic plot of Fig. 1 at 32 °C beginning at 2.0 h is only an approximation; and at 35 °C

the second component did not strictly approach log-linearity until later times (see the Experimental section, and below). Thus it is not possible to characterize protein degradation 3–5 h after labelling with a constant fractional rate of degradation in units of per cent per hour. For comparative illustration, the control degradative rates beginning 3 h after labelling are presented below as either the actual declining baseline of leucine release, or the 100% value of this baseline at each point.

Exchange of intracellular and extracellular leucine pools and externalization of label

Addition of large concentrations of non-radioactive leucine to the perfusate rapidly floods the intracellular leucine pools, competitively prevents re-incorporation of labelled amino acid, and promotes extracellular release (Poole & Wibo, 1973; Jefferson *et al.*, 1977; McKee *et al.*, 1978; Tischler *et al.*, 1982). When the perfusate concentration of non-radioactive leucine was increased from 0 to 4 mM, 4.5 h after labelling, the release of [³H]leucine from the organ increased by approx. 30% (Fig. 2), suggesting that approx. 30% of leucine degraded from cell protein had previously been recycled back into protein synthesis without extracellular release. The percentage increase in [³H]leucine release after infusion of 4 mM-leucine theoretically varies with the exact synthesis rate at the time of observation, although this was not rigorously investigated; 4 mM-leucine had no effect on contractile activity or longevity of this preparation (also reviewed by Lefer & Curtis, 1982). In two separate

Table 1. Effect of various experimental treatments on myocardial protein degradation

All procedures employed the routine perfusion conditions described in the text, except treatments (f) and (g) (see the text). In each individual experiment the preceeding 100% control basal degradation rate was determined with at least ten successive 1 min measurements of leucine release, as illustrated in Figs. 1–7. Each individual experimental condition represents at least eight successive 1 min determinations of a steady-state degradative rate after a transition period of 7 min or more under the indicated condition. In individual experiments the s.d. of measured control and experimental points above and below the fitted trace was less than $\pm 1\%$, as illustrated throughout Figs. 1–7. Thus each individual experimentally altered degradative rate represents a comparison of the means of at least ten normalized 100% control and eight experimental determinations in a single internally controlled experiment. The means of separate internally controlled experiments are shown $\pm$ the s.d. of these means. The time of measurement in (g) corresponds to the preliminary washout period at 20 min illustrated in Fig. 1. With Student's *t* test, the significance of the differences between the population of normalized 100% control means (s.d. = 0) and the experimental means are: **P* < 0.1; ***P* < 0.05; ****P* < 0.01; *****P* < 0.005.

Experimental treatment	No. of observations	Protein degradation (% of previous basal rate, $\pm$ s.d.)
(a) Routine method, 4 mM-leucine		
(i) 5 nM-isoprenaline	3	95 $\pm$ 5*
(ii) 50 nM-isoprenaline	4	77 $\pm$ 3***
(iii) 500 nM-isoprenaline	3	71 $\pm$ 3****
(b) Electrical stimulation of heart rate to 350 beats/min	3	100 $\pm$ 1
(c) Cessation of contraction with 25 mM-K ⁺	3	99 $\pm$ 1
(d) Cessation of contraction with 0.7 μ M-nifedipine	3	102 $\pm$ 1**
(e) Cessation of contraction with 0.7 μ M-nifedipine and 0.5 μ M-isoprenaline	3	69 $\pm$ 3****
(f) 20 μ M-Cycloheximide and 50 nM-isoprenaline, no amino acids	3	74 $\pm$ 9****
(g) 0.1 μ M-Isoprenaline during preliminary washout period	3	97 $\pm$ 1**

experiments (results not shown) successive increases to 4 mM and 6 mM-leucine did not increase leucine release above that caused by 2 mM-leucine. Exchange of intracellular radiolabelled leucine pools with the extracellular unlabelled leucine of the perfusate and subsequent externalization attained approx. 90–95% of the new steady state within 2 min. Thus the results in Fig. 2 imply that changes in the rate of intracellular protein degradation are detectable by subsequent corresponding changes in the rate of leucine release in the effluent perfusate after an approx. 2 min time lag. None of the present results have been corrected for this 2 min period.

Leucine re-incorporation can also be prevented maximally by 20 μ M-cycloheximide (Morgan *et al.*, 1980). Although not rigorously quantified, the percentage increase in leucine release after cycloheximide infusion was similar to that after 4 mM-leucine; however, the observed time course of approx. 6 min appeared slightly longer (Fig. 2).

Absence of effect on protein degradation of conduction block and cessation of contraction

Increasing K⁺ to 25 mM stopped contraction of the heart in depolarized conduction block, but did not cause sustained change in protein degradation (Table 1, Fig. 3; Bandman & Strohm, 1982). However, irregular, transient 5–15% inhibitions of leucine release of several minutes' duration were observed in experiments in which the heart was stopped by gradually increasing the K⁺ concentration over 5 min (results not shown). Resumption of contraction by discontinuation of K⁺ infusion after 15 min caused no sustained change in protein degradation (results not shown).

Gradually increasing the concentration of tetrodotoxin to 6 μ g/ml caused transient cessation of contraction of approx. 1 min, followed by recovery of very slow rhythm

under continued tetrodotoxin infusion (Katzung, 1982; Morgan, 1982; Sperelakis, 1982). Leucine release was measured before, during and after three successive 10 min infusions of tetrodotoxin, in each of two separate experiments. Any possible effect of this procedure on leucine release was less than a slight transient inhibition of approx. 3%, despite the severe conduction impairment owing to blockage of Na⁺ activation channels (results not shown).

Absence of effect on protein degradation of electrical stimulation of heart rate

Under the present conditions, the basal heart rate of 220 beats/min was stimulated to approx. 350 beats/min by maximal concentrations of isoprenaline (see below). When the preparation used in Fig. 4 was electrically paced above the spontaneous rhythm of 180 beats/min to 360 beats/min, there was no deviation of the rate of leucine release from the basal value (Table 1). However, spontaneously arrhythmic or fibrillating hearts exhibited fluctuating basal degradative rates (results not shown), and the present results are exclusively from hearts with regular ventricular rhythm, except as indicated.

Inhibition of myocardial protein degradation by a β -adrenergic agonist

In the experiment shown in Fig. 5, infusion of isoprenaline (0.1 μ M) caused a reversible 25% inhibition of the rate of leucine release which could be sustained for 1 h of drug infusion. Although the inhibition of protein degradation by isoprenaline could be induced and reversed repeatedly (Fig. 6), prolonged administration of 0.1 μ M-isoprenaline was eventually injurious and caused arrhythmias or fibrillation after approx. 1 h. The percentage β -adrenergic-induced decreases in protein degradation, and repeated reversals thereof, were at least

Fig. 4. Effect on protein degradation of electrical pacing of heart rate

Beginning at 22 min, the spontaneous heart rate was increased, then decreased by electrical stimulation of the atrium (see the text). The heart maintained a regular rhythm throughout. In contrast with Fig. 5, the baseline rate of leucine release was unchanged by the stimulation of heart rate; therefore the basal rate of protein degradation was unchanged.

similar over the time period used here from 3 to 5 h after labelling (Figs. 5 and 6). Therefore the adrenergic degradative control is independent of any progressive change in the labelled protein population caused by preferential elimination of those proteins with more rapid intrinsic turnover rates. Moreover, the intracellular factors controlling degradation remain functionally stable under the present conditions. During the preliminary washout period, the release of previously unincorporated leucine from cytoplasmic pools and/or the degradation of rapid turnover proteins was nearly unchanged by infusion of isoprenaline at times corresponding to the initial steep decline in Fig. 1 (Table 1).

When leucine re-incorporation was inhibited with 20 μM -cycloheximide in the absence of all exogenous amino acids, the effect of isoprenaline on degradation was at least qualitatively similar to the present method (Table 1). Cycloheximide caused arrhythmias after 2 h of perfusion, and could not be routinely used.

Simultaneity of degradative control and inotropic response to β -adrenergic agonism

When stretched to the optimum of the length-tension curve, fixed isometric cardiac-muscle preparations respond mechanically to isoprenaline with a graded concentration-dependence from approx. 5 to 500 nM [reviewed by Scholz (1980) and Will-Sahab & Krause (1980)]. Under the present conditions, the peak right-intraventricular systolic pressure of the preparation reaches a constant maximum value at approx. 10 nM-isoprenaline (Fig. 6), and does not directly indicate corresponding changes in wall tension. Moreover, the exact mechanical responses of the left and right sides of the heart to isoprenaline might also differ quantitatively, although not qualitatively. For these reasons, interpretation of the changes in mechanical function observed here

Fig. 5. Sustained inhibition of myocardial protein degradation by isoprenaline

Methods were as described in the text, except that the temperature was 34 °C, to enhance longevity. From 3 to 3.8 h 0.1 μM -isoprenaline (Iso) was infused. To prevent fibrillation, this concentration was attained gradually over the first 1 min. Discontinuation of lower concentrations of isoprenaline (10 nM) resulted in a more prompt return to the control degradative rate within 5 min, presumably owing to differences in the kinetics of drug elimination from the heart (see Fig. 6). At 4.25 h, a second infusion of 0.1 μM -isoprenaline was begun. The organ became arrhythmic at 4.6 h. The actual curvilinear basal control degradative rate has been approximated by the linear dashed trace, and the method does not permit quantitative comparison of basal and inhibited slopes during the maximal possible measurement period.

is limited to a qualitative indication of the time course of adrenergic agonism-antagonism; and no graded relationship between ventricular pressure and protein degradation was investigated.

Acute sustained infusion of isoprenaline in the present preparation causes a well-known, but unexplained, transient systolic-pressure increase, within 30 s, which is followed by a partial compensation within approx. 1–2 min (Fig. 6) (reviewed by Harden, 1983). Administration of the submaximal concentration of 10 nM-isoprenaline at 20 min in Fig. 6 caused a reversible decrease in leucine release in approximate temporal correlation with characteristic inotropic and chronotropic responses. Although mechanical parameters generally returned to previous control values after discontinuation of isoprenaline, Fig. 6 illustrates a preparation in which rate and peak systolic ventricular pressure declined below previous values. However, the basal degradative rate was not directly affected by such unexplained fluctuations in basal heart rate and pressure of the preparation *in vitro*. Indeed, at 112–116 min the rate and pressure returned abruptly to the indicated previous control values, with no accompanying change in basal protein degradation. In Fig. 6, beginning at 62 min, the isoprenaline concentration was gradually increased to 100 nM at 80 min, resulting in a lesser initial pressure peak. The second decrease in protein degradation at 86 min had not attained the maximum inhibited values shown in Fig. 5 before

Fig. 6. Changes in myocardial protein degradation, heart rate and peak right-ventricular pressure, in response to isoprenaline, and to propranolol simultaneous with isoprenaline

Unlike all other experiments described here, the protein degradation rate was determined at 2 min intervals. Additional measured points have been inserted to define inflections in heart rate. Continuously measured values describing pressure (not shown) deviated by less than 1% from the indicated trace. After the isoprenaline administration protocol described in the text, 1 μ M-propranolol was infused continuously at 95 min simultaneous with the continued infusion of isoprenaline (see the text).

discontinuation of isoprenaline at 20% inhibition. At 86 min the isoprenaline infusion in Fig. 6 was interrupted for 2 min, causing partial return of leucine release toward control values. The period from 64 to 90 min in Fig. 5 illustrates dissociation of the exact time course and concentration-response relationships of chronotropy,

inotropy and protein degradation in this preparation. When the identical isoprenaline concentration was resumed at 88 min, protein degradation declined for the third time (Fig. 6).

A marginal decrease in degradation of approx. 5% was observed at a minimal concentration of approx.

Fig. 7. Effect on basal and adrenergic-inhibited protein degradation of cessation of contractile activity under nifedipine

(a) The heart was perfused under routine conditions described in the text. Leucine release shown before zero time represents a 20 min control period. In (b) chart speed was 2 mm/s leftward throughout, and times (min) correspond to those indicated by arrows in (a). The control heart rate and right-ventricular pressure were 190 beats/min and 24 mmHg (b, 0 min). The gradually increasing nifedipine infusions beginning at 13 and 76 min are described in the text, as is the indicated corresponding control of protein degradation. The attainment of $0.5 \mu\text{M}$ -nifedipine was just sufficient to stop the atria and ventricles at 42.3 min; however, as indicated at 43 min, the heart exhibited occasional strong irregular contractions. The off-scale peak systolic pressure beginning at 42.4 min was approx. 20% greater than that shown as determined by change of amplifier scale immediately after the time shown. Nifedipine was discontinued at 46 min, and by 49 min the heart had regained a very slow regular rhythm of 24 beats/min after partial elimination of the drug. At 62 min, nifedipine had not been completely eliminated before the indicated infusion of $0.5 \mu\text{M}$ isoprenaline. The unexplained delayed and aberrant mechanical response and delayed inhibition of protein degradation are as described in the text. At 72 min a gradually increasing infusion of nifedipine was begun simultaneous with continued infusion of isoprenaline, causing the initial slight decrease in peak systolic pressure shown at 75 min (b). The isoprenaline-stimulated rate decreased from 260 beats/min at 66 min to 149 beats/min at 75 min. The nifedipine concentration was gradually increased to $0.7 \mu\text{M}$, at which concentration the ventricles ceased contraction at 105–115 min. The small apparent pressure pulse shown at 105 min is the mechanical disturbance of the catheter owing to the continued stimulated atrial contraction (see text). After discontinuation of nifedipine at 136 min, the ventricles regained contraction by 139 min, but protein degradation remained inhibited in the continued presence of isoprenaline.

5 nM-isoprenaline (Table 1). The maximal percentage inhibition of degradation was approx. 30% at isoprenaline concentrations greater than 300 nM (Table 1, Fig. 7 and other results not shown).

Reversal of proteolytic responsiveness to the β -agonist by β -receptor blockade

When an excess of the β -receptor-blocking drug propranolol was infused in the continued presence of isoprenaline, the inhibited rate of protein degradation and stimulated contractile parameters both exhibited a reversal beginning within 2 min, as shown at 95 min in Fig. 6. Protein degradation under sudden β -blockade increased transiently to a value above the unexposed control degradative rate from 98 to 108 min; however, such infusion of propranolol did not cause sustained increases in degradation above the baseline.

Absence of direct obligatory linkage between the adrenergic-responsive degradative process and the contraction-relaxation cycle

Nifedipine blocks function of the voltage-dependent Ca^{2+} slow channel, causing atrioventricular conduction block and cessation of ventricular contraction in asystole. Nifedipine might also cause other suppressive effects on membrane conduction (reviewed by Fleckenstein *et al.*, 1982). As shown in Fig. 7 and Table 1, a gradual increase in nifedipine concentration to 0.5 μM caused a negligible effect on basal protein degradation in association with cessation of contraction. After discontinuation of nifedipine, basal degradation was also unchanged as the ventricles gradually regained contractile activity during the slow elimination characteristic of this drug. After the drug action had been partially reversed and the heart had regained slow irregular contractions, isoprenaline was infused at the concentration producing maximal inotropic effects in isometric cardiac muscle preparations, 0.5 μM (Scholz, 1980). In contrast with typical isoprenaline stimulations shown in Fig. 6, the onset of mechanical stimulation was aberrant and delayed by 2–3 min under the action of residual concentrations of nifedipine in this preparation. The residual nifedipine was also sufficient to protect against the arrhythmias or fibrillations that generally resulted from this isoprenaline concentration, as was continuous infusion of 0.1 μM -nifedipine (Fig. 7, and other results not shown).

In association with the marked delayed stimulation of heart rate and peak systolic pressure, the rate of protein degradation declined by 33%, similar to the maximal inhibition observed with isoprenaline without nifedipine (Fig. 7). However, the time course of the inhibition of degradation to this value was markedly increased, from 4–6 min (Figs. 5 and 6) to approx. 11–12 min (Fig. 7), although this was not rigorously determined. Under continued isoprenaline infusion, nifedipine infusion was again initiated and the concentration was gradually increased to 0.7 μM . Rate and pressure gradually declined until the ventricles stopped. Under continued maximal concentration of isoprenaline the stimulated atria continued to beat; however, atrioventricular conduction was blocked, as is characteristic with high nifedipine concentration. The ventricular mass is more than 97% of the total rat heart mass. Cessation of ventricular contraction was associated with a small, transient, increase in degradation, but no reversal of the adrenergic-inhibited degradative rate was observed (Fig. 7). Upon

discontinuation of nifedipine under continued isoprenaline infusion, the heart regained ventricular contractile function; however, degradation remained inhibited. It was not determined whether Ca^{2+} -channel blockers might antagonize degradative changes caused by submaximal catecholamine concentration, or might exert a damping effect on brief exposures.

DISCUSSION

Metabolism, re-incorporation and transport of leucine

Under the present conditions, a non-recirculating perfusate volume equivalent to nearly 10 times the total fluid volume of the heart traverses the organ each minute. At 4 mM, the leucine concentration is approx. 40 times the normal intra- and extra-cellular leucine concentration in the rat (Morgan *et al.*, 1971). In diverse cell types, at extracellular concentrations of 0.5 mM or greater, the specific radioactivities of extracellular and intracellular pools of leucine and some other amino acids have been reported to equalize rapidly, with a time course consistent with that shown in Fig. 2 (Hod & Hershko, 1976; Jefferson *et al.*, 1977; McKee *et al.*, 1978; Tischler *et al.*, 1982). Therefore, a rapidly exchanging amount of leucine equivalent to several hundred times the normal total organ content of leucine traverses the organ each minute, causing an overwhelming saturation of any possible metabolism or re-incorporation of radiolabelled leucine degradative pools. However, a possible effect of amino acids on basal protein degradation was not studied, and the present results cannot be compared with other reports of an effect of leucine on protein degradation, in view of different methods (Morgan, 1982).

The validity of the present measurement of degradative change is not contingent on a constant control of transmembrane leucine efflux or active re-uptake before and after isoprenaline infusion in the presence of non-recirculating 4 mM-leucine. The accuracy of the measurement rather depends only on the establishment of a steady-state cascade of labelled degradation product, in the absence of appreciable re-incorporation or metabolism, before and during the observed degradative change. Several studies in addition to Fig. 2 indicate that the time course of exchange of intracellular and extracellular leucine pools is much more rapid than the 1 h duration of the sustained reversible decrease in leucine release rate shown in Fig. 5 (Poole & Wibo, 1973; Hod & Hershko, 1976; Tischler *et al.*, 1982). Therefore, if the initial decrease in leucine release (Figs. 5, 6 and 7) were attributable to an increase in the intracellular leucine pool content in the absence of degradative change, then the continued rapid replacement of the cytoplasmic leucine pool with excess of unlabelled non-recirculating exogenous leucine would restore the rate of release to the previous steady-state control value in several minutes, which was not observed. Moreover, if the decreased release shown in Figs. 5 and 6 were attributable to increased leucine-pool retention by changes in efflux or reuptake, then the rate of release would transiently exceed the baseline after discontinuation of isoprenaline, which did not appreciably occur. Finally, the absence of an effect of isoprenaline on release of leucine during the initial washout of unincorporated cytoplasmic label and/or label from rapidly degraded proteins provides a fortuitous additional internal control for overall changes

in transport and bulk regional perfusion under 4 mm-leucine (Table 1).

Inference of co-ordination *in vivo* of myocardial contractility status and the β -adrenergic-responsive degradative pathway

The results of Figs. 3, 4 and 7 suggest the absence of a direct obligatory link between protein degradation and the events of the contraction-relaxation cycle or any possible intrinsic control of the work of this preparation. However, diverse and complex intracellular factors can influence the contractility status at numerous regulatory points, and it remains unknown whether intrinsic controls might somehow influence the contractility status *in vivo* (Demaille & Pechere, 1983). Therefore, the present results do not eliminate the possibility that intrinsic controls or non-receptor-mediated changes in other intracellular factors could indirectly influence the degradative rate *in vivo* by unknown linkages that are not coupled in the non-working heart.

Under repeated isoprenaline injection, and some other adrenergic-related conditions associated with myocardial hypertrophy, the myocardial protein mass typically increases by 30–50% (Kaltenbach & Epstein, 1982). Regardless of its particular half-life, the eventual steady-state change in the amount of a protein is hypothetically proportional directly to the change in ratio of synthesis to degradation, assuming ideal zero-order synthesis and first-order degradation. Thus, if the presently observed fluctuation of 30% in the degradation of labelled proteins is representative of the majority of proteins (Morgan *et al.*, 1980), then a large fraction of the observed adrenergic control of bulk myocardial protein mass might be mediated by co-ordination of protein degradation and contractility.

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