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ADRENERGIC STIMULATION OF SUBSTRATE UTILIZATION BY CARDIAC MYOCYTES ISOLATED FROM RAINBOW TROUT

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Summary

A method is described for the isolation of calcium-tolerant myocytes from adult rainbow trout. Isolated myocytes remain viable for at least 4 h in suspension as indicated by (1) maintenance of ATP, phosphocreatine (PCr) and glycogen levels; (2) maintenance of the integrity of cell membranes, shown by low rates of leakage of lactate dehydrogenase (LDH) to the medium and exclusion of Trypan Blue; (3) the ability to metabolize substrates; and (4) sensitivity to adrenergic agonists. CO_2 production from both glucose and lactate was sensitive to adrenergic stimulation, with the following order of potency: isoproterenol>noradrenaline≫adrenaline>phenylephrine, which indicates the presence of β_1-adrenoceptors. Myocytes isolated from trout acclimated to 20°C in the summer were more sensitive to β-adrenergic stimulation than myocytes isolated from trout acclimated to 9°C in either summer or winter. In the absence of exogenous fuel, there was a net reduction in myocyte glycogen content and glycogenolysis was further stimulated by 10^{-7} mmol l^{-1} noradrenaline. However, in the presence of exogenous fuel (either 5 mmol l^{-1} lactate or 5 mmol l^{-1} glucose), glycogen was 'spared' and noradrenaline-stimulated glycogenolysis was apparently inhibited.

Introduction

Cardiac contractility and its relationship to metabolic phenomena is of fundamental significance. While each has been the subject of much research in fish (Driedzic *et al.* 1983; Farrell, 1984; Sidell *et al.* 1984) the relationship between performance and metabolism has received scant attention (Driedzic and Hart, 1984; Sidell and Driedzic, 1985; Milligan and Farrell, 1991). *In vivo*, cardiac performance of trout hearts is stimulated by catecholamines (see Farrell, 1984). The adrenergic effect is manifested as increases in both the force and the frequency of contraction (Farrell, 1984; Farrell *et al.* 1986). As cardiac workload is increased *via* adrenergic stimulation, metabolism must increase in concert to meet these changing metabolic demands. Most studies on working trout hearts have

Key words: cardiac myocytes, metabolism, noradrenaline, adrenaline, glycogen, lactate, rainbow trout, *Oncorhynchus mykiss*.

been limited to examining the inotropic and chronotropic effects of catecholamines (e.g. Farrell *et al.* 1986; Graham and Farrell, 1989) and only recently have attempts been made to examine the relationship between cardiac performance and metabolism. Milligan and Farrell (1991) demonstrated that, in the *in situ* perfused trout heart, lactate utilization increased with increasing workload, created by changes in preload and afterload, in the absence of increased adrenergic stimulation. This observation indicates that changes in mechanical function alone alter metabolism. However, since cardiac performance *in vivo* is stimulated by catecholamines, of both neural and humoral origin, it is of interest to examine the role of catecholamines in regulating cardiac muscle metabolism.

In intact, working heart preparations, the distinction between the catecholamine-mediated increase in mechanical function and independent effects of catecholamines on metabolic pathways has not always been achieved. Isolated cells in suspension have provided useful insights into metabolic regulation in a number of other fish systems, most notably hepatocytes (see Moon *et al.* 1985, for a review). Isolated cardiac myocytes from rainbow trout offer a potential model system for the study of metabolic processes. Because Ca^{2+}-tolerant isolated myocytes (myocytes that are viable in the presence of physiological Ca^{2+} levels; Kammermeier and Rose, 1988) are mechanically at rest, the basal metabolic activity of the myocardium in the absence of contractile activity may be studied. This system offers the distinct advantage over the 'arrested heart' in that experiments can be done in physiological media (i.e. at physiological potassium levels).

In this study, the isolation, characterization and metabolic responses to catecholamines of Ca^{2+}-tolerant myocytes from rainbow trout are described. Given that the adrenergic responsiveness of trout cardiac performance varies with temperature and/or season (Graham and Farrell, 1989), the effects of temperature and seasonal acclimation on the adrenergic response of trout myocytes were also examined.

Materials and methods

Experimental animals

Rainbow trout *Oncorhynchus mykiss* (Walbaum), weighing 150–220 g, were obtained from Rainbow Springs Trout Hatchery, Thamesford, Ontario. Fish were held indoors in a large (500 l), cylindrical black plastic tank continuously supplied with flowing dechlorinated tapwater and fed a maintenance ration of commercial trout food three times weekly. Experiments on 'winter' fish were performed from December to February, when the ambient water temperature was constant at 9 °C, and those on 'summer' fish were performed in July and early August, when the ambient water temperature was constant at 20 °C. During the same period, a group of 'summer' fish was acclimated to 9±1 °C for at least 2 weeks (Graham and Farrell, 1989) prior to experimentation. These fish were used to assess the

influence of season and temperature on the adrenergic regulation of substrate utilization. Fish were kept under natural photoperiod conditions (40°N latitude).

Cardiac myocyte isolation and viability criteria

Fish were anaesthetized with MS 222 (1:20 000), placed on an operating sling, injected with 0.5 ml of 1500 i.u. ml^{-1} sodium heparin in 0.9 % NaCl *via* the caudal complex, and returned to the anaesthetic solution. The heparin was allowed to circulate for at least 5 min, at which point fish were killed with an anaesthetic overdose. A mid-ventral incision was made to expose the heart, and the ventral aorta was cannulated with a blunted 16 gauge needle attached to PE 250 tubing. The atrium was cut to prevent a build-up of fluid in the ventricle and the heart was retrogradely perfused at 1.5 ml min^{-1} with a Gilson peristaltic pump for 10–15 min with perfusion saline (Table 1) to wash out the blood. Once the blood had been cleared, the heart was excised and transferred to a watch glass and the perfusion was continued for another 30 min with stage 1 enzyme medium (Table 1). Perfusion was then terminated, and the cannula, bulbus and atrium were removed. At this stage of digestion, the outermost layer of ventricular cells, the epicardium, was rarely digested, whereas the inner layer, the endocardium, was visibly 'softened'. Initial experiments showed that the epicardium was not readily

Table 1. *Composition of media used to isolate cardiac myocytes from rainbow trout*

Perfusion medium (for clearing blood)
- 125 mmol l^{-1} NaCl
- 5 mmol l^{-1} KCl
- 10 mmol l^{-1} Hepes
- 2.5 mmol l^{-1} NaH_2PO_4
- 5 mmol l^{-1} glucose
- 0.05 mmol l^{-1} $CaCl_2 . 2H_2O$
- 500 i.u. ml^{-1} sodium heparin

Gassed with 0.5 % CO_2:99.5 % air for 30 min, pH adjusted to 7.6 at 25 °C
- 10 mmol l^{-1} $NaHCO_3$

Enzyme medium (for cell dispersion)

Stage 1: perfusion medium, without heparin, but with
- 0.1 mmol l^{-1} $CaCl_2 . 2H_2O$
- 0.1 % collagenase (Type 1, Boehringer-Mannheim)
- 0.06 % hyaluronidase

Stage 2: stage 1 medium with
- 1 % bovine serum albumin, fatty-acid free

Suspension medium (for washing and final suspension)

Perfusion medium, without heparin and glucose, but with
- 0.5 mmol l^{-1} $CaCl_2 . 2H_2O$
- 4 % bovine serum albumin, fatty-acid free

Note: add substrate of preference (e.g. lactate, glucose, palmitate)

Modified from French *et al.* (1981).

dispersed by further digestion without substantial cell damage, so in all subsequent digestions the 'softened' endocardial layer was teased away from the epicardial layer, and minced with a sharp razor blade. To disperse the endocardial cells further, the minced tissue was incubated with stage 2 enzyme medium (Table 1) for an additional 15–20 min. Release of myocytes was greatly enhanced by gentle agitation of the tissue suspension. The dispersed tissue was filtered through a 250 μm nylon filter, then centrifuged for 5 min at 50 g. The cells were resuspended and washed twice in suspension medium (Table 1) and suspended to yield a final concentration of cells of approximately 50 mg wet mass cells ml^{-1}. Cell wet mass was obtained by centrifuging duplicate 500 μl samples in preweighed Eppendorf microcentrifuge tubes at 12 500 g for 5 min, removal of the supernatant (final traces removed with the aid of a paper tissue) and then reweighing the vial. Perfusion and incubation were all performed at experimental temperature.

The yield of viable myocytes was determined by mixing equal volumes of cell suspension and 0.4% Trypan Blue in 0.9% NaCl and counting cells on a haemocytometer. Viable cells were those that excluded the dye. This technique typically yields 85–90% viable cells. Preparations with less than 85% viable myocytes were discarded. Red cell contamination of the preparations varied, but rarely exceeded 3% of the total viable cell population. Yields of up to 40% of ventricular wet mass were obtained using this method.

Initial experiments were carried out to determine the duration of myocyte viability in suspension. Cell viability was assessed with the Trypan Blue exclusion method, maintenance of adenosine triphosphate (ATP) and phosphocreatine (PCr) levels and leakage of lactate dehydrogenase (LDH) to the suspension medium. After either 15 min, 1, 2 or 4 h in suspension, a sample of cells was taken to assess cell viability using Trypan Blue exclusion and LDH leakage to the medium. To the remaining cells, 100 μl of 70% $HClO_4$ was added and the cells were analyzed for ATP and PCr content. For comparison, ATP and PCr were also measured in freeze-clamped whole hearts. Heart samples were homogenized in 6% $HClO_4$, centrifuged and the supernatant analyzed for ATP and PCr. These initial experiments were performed on fish acclimated to 9°C in the winter.

Measurement of metabolism

To obtain enough cells for multifactorial experiments, cells from four hearts were routinely pooled. Incubations were carried out in 20 ml darkened glass vials containing 500 μl of cell suspension. All incubations were carried out in a shaking water bath (600 revs min^{-1}) at either 9 or 20°C. The incubation vials were sealed with a rubber septum fitted with a plastic centre well containing glass fibre filter paper. The vials were shaken for 15 min prior to beginning an experiment to allow the cells to equilibrate.

Substrate utilization was determined by measuring the production of $^{14}CO_2$ after incubating the cell suspensions with ^{14}C-labelled substrates, as described by French *et al.* (1981), assuming CO_2 production was linear over the 1 h incubation period. In the experiments with adrenergic agonists [(−)epinephrine (+)bitar-

trate; (−)arterenol; (−)isoproterenol hydrochloride; L-phenylephrine hydrochloride] and antagonists (DL-propranolol; phentolamine hydrochloride), drugs were prepared immediately before use in 0.9 % NaCl and added at the beginning of the 15 min equilibration period. Experiments were begun by adding uniformly labelled [^{14}C]lactate (7 mCi mmol^{-1}; ICN Biomedical), [^{14}C]glucose (90 mCi mmol^{-1}; ICN Biomedical) or [^{14}C]palmitate (819 mCi mmol^{-1}; Amersham) to yield a final specific activity of 0.2 μCi mmol^{-1}. [^{14}C]Palmitate was complexed with 4 % bovine serum albumin (BSA) before use (French *et al.* 1983). Cells were incubated for 1 h with a single labelled substrate. The experiment was terminated by the addition of 100 μl of 70 % $HClO_4$ through the cap.

To correct for breakdown and contamination of radioactive substrates, blanks (vials containing everything except cells) were run in triplicate with each experiment. These blank values, which differed for different substrates, were subtracted from the experimental value. Typically, blank vials yielded counts ranging from 80 to 200 cts min^{-1} (background=30 cts min^{-1}) *versus* 1200–2800 cts min^{-1} collected from experimental vials.

Following addition of $HClO_4$, 200 μl of 1 mol l^{-1} hyamine hydroxide was injected onto the glass filter through the septum to collect $^{14}CO_2$. The vials were shaken at 1000 revs min^{-1} for 1.5 h to ensure complete collection of the $^{14}CO_2$. The filter papers were removed and counted in 10 ml of scintillation fluor (ReadySafe; Beckman) in a Beckman LS9000 liquid scintillation counter. Preliminary trials with ^{14}C-labelled $NaHCO_3$ yielded a recovery of more than 95 % of $^{14}CO_2$ with this method.

Gluconeogenesis from labelled lactate was determined by monitoring the rate of [^{14}C]glucose production. Radiolabelled glucose was isolated by a method based on that described by Walton and Cowey (1979). After the glass filter had been removed, the $HClO_4$ precipitate was centrifuged (to remove protein) and the supernatant neutralized with 1.5 mol l^{-1} K_2CO_3 and recentrifuged. A sample (250 μl) of the final supernatant was added to 2.25 ml of 1 mol l^{-1} glucose plus 0.75 g of Amberlite MB3 mixed-bed resin (BDH Chemicals) and the sealed vials were vigorously shaken for 2 h. A 2 ml sample was removed, centrifuged to remove any resin and two 500 μl samples were counted in 10 ml of scintillation fluor.

Experimental series

Substrate utilization

In this series of experiments, the dependence of lactate and glucose utilization on substrate concentration was examined. Cells isolated from four hearts were pooled and about 25 mg of packed cells was suspended in 500 μl of suspension medium in the incubation flasks. Half the cell suspensions were incubated with various concentrations of lactate and the other half were incubated with various concentrations of glucose. ^{14}C-labelled lactate or glucose was added to a final specific activity of 0.2 μCi mmol^{-1}. Radiolabel incorporation into glucose was

investigated in those cells incubated in the presence of [^{14}C]lactate. Blanks were run in triplicate for each isotope. These experiments were performed on myocytes isolated from fish acclimated to 9°C in winter.

Adrenergic effects on substrate utilization

In this series of experiments, the effect of adrenergic agonists on substrate utilization was examined. Again, cells isolated from four hearts were pooled and subdivided into incubation vials containing about 25 mg of packed cells in 500 μl of suspension medium. The adrenergic agonists were added 15 min prior to the addition of labelled substrate. Each experimental run consisted of (1) three blanks; (2) two control vials, which contained cells in suspension, substrate and isotope but no drugs; and (3) four dose–response series (adrenaline, noradrenaline, isoproterenol and phenylephrine). The drugs were added to the final concentrations shown in Fig. 2. For each substrate examined (5 mmol l^{-1} lactate, 5 mmol l^{-1} glucose) five independent experimental runs (i.e. N=5) were performed. ^{14}C-labelled lactate or glucose was added to a final specific activity of 0.2 μCi $mmol^{-1}$. Because of the variation between experimental runs in terms of absolute amount of $^{14}CO_2$ produced, results are expressed as a percentage of the control value.

To delineate the receptor type mediating the response, a series of experiments was performed that attempted to block the adrenergic stimulation with known adrenergic antagonists. The concentration of adrenergic agonist that gave an approximately 50% response (approx. 10^{-7} mmol l^{-1}) was used. Phentolamine, an α-adrenergic antagonist, and propranolol, a general β-adrenergic antagonist, were added in excess (10^{-6} mmol l^{-1}) 15 min prior to the addition of agonist. Again, each experimental run consisted of three blanks, two controls and the appropriate combination of agonist and antagonist, as shown in Table 4. Four independent experimental runs (i.e. N=4) were performed for each substrate: 5 mmol l^{-1} lactate, 5 mmol l^{-1} glucose and 0.25 mmol l^{-1} palmitate. These experiments were performed on myocytes isolated from trout acclimated to 9°C in winter.

Effect of exogenous fuel on glycogen utilization

This series of experiments examined the interactive effects of exogenous fuel availability and adrenergic stimulation on glycogen utilization. Immediately after isolation, a subsample of the pooled myocytes was taken for measurement of glycogen content in these freshly isolated cells. Cell suspensions (approx. 25 mg in 500 μl of suspension medium) contained either no exogenous substrate, 5 mmol l^{-1} glucose or 5 mmol l^{-1} lactate. To one set of incubation vials containing glucose, lactate or no exogenous substrate, noradrenaline was added to a final concentration of 10^{-7} mol l^{-1}, 15 min prior to the addition of the radiolabel. At the end of the 1 h incubation period, myocyte glycogen content was measured. These experiments were performed on myocytes isolated from trout acclimated to 9°C in winter.

Seasonal and temperature effects

In this series of experiments, the effects of temperature and seasonal acclimation on the adrenergic response were assessed. The experimental protocol was essentially that described for investigating adrenergic effects on substrate utilization, except that the dose–response curves were run for isoproterenol and noradrenaline only since, of the agonists tested, they elicited the greatest response (see Fig. 2). Cell isolation and incubations were performed at the temperature to which the fish were acclimated, either 9°C or 20°C. Four independent experimental runs (i.e. $N=4$) were performed for each substrate and each acclimation condition, except acclimation to 9°C in winter, where the data were obtained in the series investigating adrenergic effects on substrate utilization, so $N=5$.

Metabolite and enzyme assays

ATP and PCr were assayed in cells (approx. 25 mg) or tissue (approx. 100 mg) homogenized in $HClO_4$. The homogenate was centrifuged at 12 500 ***g*** for 5 min, the supernatant was withdrawn and neutralized with 1.5 mol l^{-1} K_2CO_3, recentrifuged and the resultant supernatant analyzed according to the method described by Bergmeyer (1965) using a Shimadzu spectrophotometer. Myocyte glycogen was measured in cell homogenates digested with amyloglucosidase, assayed as glucose according to the methods described by Keppler and Decker (1974) and expressed as μmol glucose g^{-1} packed cell wet mass.

Lactate dehydrogenase (E.C. 1.1.1.27; reverse) activity was measured in suspension medium and lysed cells according to the methods previously described for fish tissues (Mommsen *et al.* 1980) using a Shimadzu spectrophotometer.

Chemicals, calculations and statistical analysis

All biochemicals were purchased from Sigma Chemical Co. (St Louis, MO) and Boehringer-Mannheim (Montreal, PQ) unless stated otherwise, and all chemicals were at least reagent grade. Total $^{14}CO_2$ and [^{14}C]glucose production were calculated from net cts min^{-1} collected (total minus blank), specific activities, packed myocyte wet mass and incubation time. It was assumed that all $^{14}CO_2$ produced was due to complete oxidation of the substrate. However, this may tend to overestimate substrate oxidation since uniformly labelled substrates were employed in this study; some $^{14}CO_2$ will be produced upon decarboxylation of pyruvate to acetyl CoA, and the fate of that acetyl CoA is uncertain. It may be used for fatty acid synthesis or enter the tricarboxylic acid cycle. All values are reported as means±1 S.E., with N referring to the number of independent experiments. Percentage values were transformed using the arc-sine transformation to normalize the data prior to statistical analysis (Zar, 1974). Significant differences between treatments were assessed with one-way analysis of variance (ANOVA) (Zar, 1974).

Results

Cell viability

When viewed with the phase-contrast microscope, cardiac myocytes isolated from rainbow trout were Ca^{2+}-tolerant; they showed the typical myocyte morphology in the presence of physiological Ca^{2+} levels. Myocytes were rod-shaped, branched, striated and approximately 65–80 nm in length by 8–10 nm in width. Only small quantities of cell debris, non-viable cells and erythrocytes were present. The degree of staining was variable, but, with practice, preparations were routinely isolated in which at least 85 % of the rod-shaped cells excluded the vital dye Trypan Blue (Table 2). Spontaneous contractile activity (indicative of Ca^{2+}-intolerant myocytes) was seen only in cells isolated from hearts initially perfused with Ca^{2+}-free saline (preliminary experiments). Non-viable myocytes appeared rounded with numerous 'blebs' at the cell boundaries and always stained blue.

Freshly isolated myocytes, that is cells fixed immediately after isolation, contained ATP and PCr levels comparable to those in freeze-clamped hearts (Table 2), except that ATP concentration was slightly, though significantly, reduced. Nonetheless, the isolated myocytes maintained constant ATP levels for up to 4 h in suspension (Table 2), indicating that the metabolic integrity of the cells was maintained. This maintenance of ATP and PCr levels was consistent with the low rate of leakage of LDH and the maintenance of cell viability estimated by Trypan Blue exclusion (Table 2).

Substrate utilization

Calcium-tolerant isolated cardiac myocytes are quiescent, so the values reported can be considered to be the basal metabolic activity of the myocytes.

Table 2. *Viability of isolated cardiac myocytes as a function of time in suspension*

Sample	[ATP] (μmol g^{-1} wet mass)	[PCr] (μmol g^{-1} wet mass)	LDH leakage (% activity in pellet)	% viable cells
Freeze-clamped heart	3.91±0.10 (N=5)	12.75±0.28 (N=5)		
15 min	2.83±0.35* (N=6)	12.84±0.22 (N=4)	ND	91.4±5.8 (N=6)
1 h	2.86±0.32* (N=6)	12.32±0.36 (N=4)	1.3±0.4 (N=4)	89.4±3.2 (N=6)
2 h	2.77±0.41* (N=6)	12.12±0.44 (N=4)	1.2±0.2 (N=5)	90.3±3.1 (N=6)
4 h	2.80±0.33* (N=6)	12.14±0.28 (N=4)	2.7±0.5 (N=5)	89.6±4.1 (N=6)

ND, not detectable.
* Significantly different ($P \leq 0.05$) from the corresponding value in freeze-clamped hearts.
Values are mean±S.E.
PCr, phosphocreatine; LDH, lactate dehydrogenase.

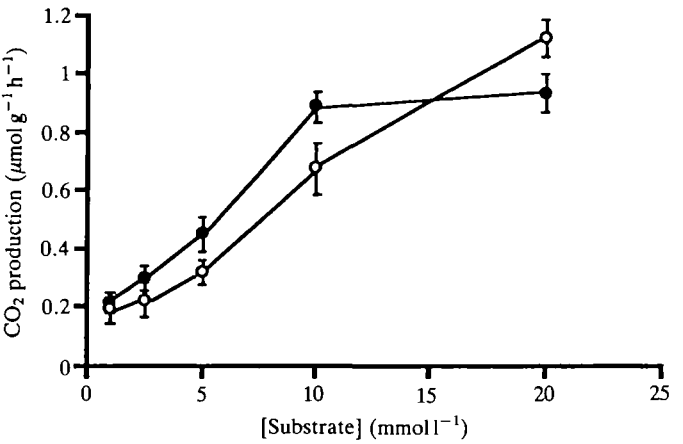

Fig. 1. Effect of substrate concentration on CO_2 production rates from glucose (○) and lactate (●) by cardiac myocytes isolated from adult rainbow trout. Values are means±1 S.E.M. (N=5 for each substrate).

Myocytes utilized both glucose and lactate in a concentration-dependent fashion (Fig. 1) and, except at the extremely high levels, $^{14}CO_2$ production from lactate exceeded that from glucose. Lactate utilization levelled off at 10–20 mmol l^{-1}, whereas glucose utilization continued to increase with increasing substrate concentration.

Gluconeogenesis from labelled lactate was not detectable in isolated myocytes, even at very high (25 mmol l^{-1}) substrate concentrations.

Utilization of lactate and glucose was strongly stimulated by noradrenaline and only weakly stimulated by adrenaline (Fig. 2). The general β-adrenergic agonist isoproterenol elicited a stronger stimulation than noradrenaline, while the α-adrenergic agonist phenylephrine stimulated only at very high concentrations (10^{-5} mol l^{-1}) (Fig. 2). The agonist concentration yielding 50 % maximal response (apparent EC_{50}) was determined graphically from Fig. 2, assuming the stimulation of lactate and glucose utilization at an agonist concentration of 10^{-4} mol l^{-1} to be 100 %. For a given agonist, the apparent EC_{50} values for lactate and glucose stimulation were similar and the order of adrenergic potency was isoproterenol> noradrenaline≫adrenaline≫phenylephrine (Table 3).

Stimulation of lactate, glucose and palmitate utilization by noradrenaline and isoproterenol was inhibited by the non-specific β-antagonist propranolol (Table 4). Propranolol also inhibited the weak adrenaline stimulation (Table 4). The α-adrenergic antagonist phentolamine did not alter adrenaline, noradrenaline or isoproterenol stimulation of substrate utilization (Table 4). Neither propranolol nor phentolamine added alone had any significant effect on substrate utilization.

Glycogen content in freshly isolated myocytes did not vary significantly between preparations (Table 5). In the absence of exogenous substrate, the glycogen content of isolated myocytes was significantly lower than in those myocytes that

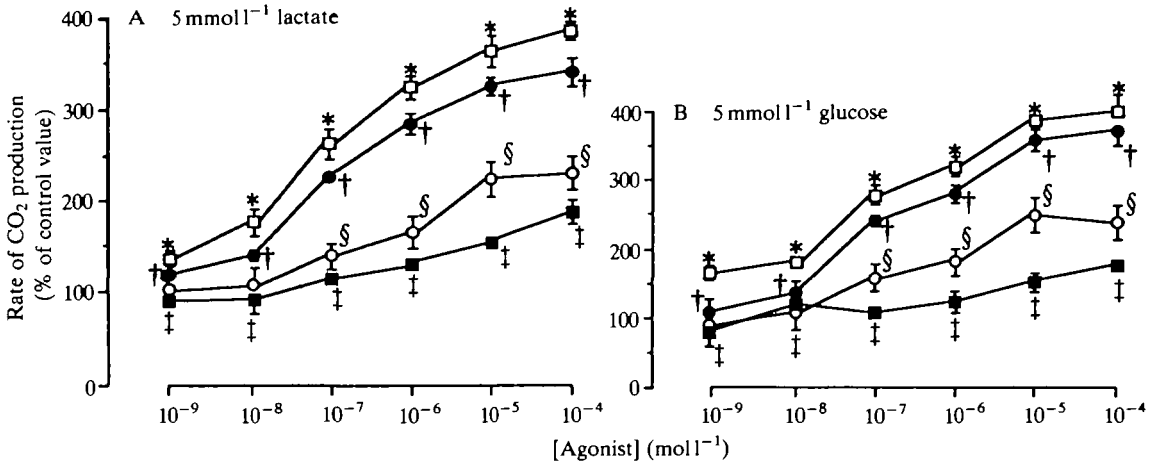

Fig. 2. Effects of various concentrations of adrenaline (AD, ○), noradrenaline (NAD, ●), isoproterenol (ISO, □) and phenylephrine (PHE, ■) on the rates of CO_2 production from 5 mmol l^{-1} lactate (A) and 5 mmol l^{-1} glucose (B). Values are means±1 S.E.M. (N=5 for each substrate). * indicates a significant difference ($P \leq 0.05$) from corresponding NAD, AD and PHE values; † indicates a significant difference ($P \leq 0.05$) from corresponding ISO, AD and PHE values; § indicates a significant difference ($P \leq 0.05$) from corresponding NAD, ISO, PHE values; ‡ indicates a significant difference ($P \leq 0.05$) from corresponding AD, ISO, NAD values.

Table 3. *Seasonal and temperature acclimation effects on apparent EC_{50} values for adrenergic stimulation of CO_2 production from ^{14}C-labelled lactate and glucose*

Season and temperature	Adrenergic agonist	Apparent EC_{50} (mol l^{-1}) Lactate	Glucose
Winter, 9°C	Adrenaline	4.7×10^{-7}	2.8×10^{-7}
	Noradrenaline	9.8×10^{-8}	9.7×10^{-8}
	Isoproterenol	6.9×10^{-8}	5.0×10^{-8}
	Phenylephrine	3.9×10^{-6}	2.6×10^{-6}
Summer, 20°C	Noradrenaline	4.2×10^{-8}	4.1×10^{-8}
	Isoproterenol	2.7×10^{-8}	1.6×10^{-8}
Summer, 9°C	Isoproterenol	6.6×10^{-8}	6.5×10^{-8}

Values determined graphically from Figs 2 and 3.

had been incubated in either 5 mmol l^{-1} glucose or 5 mmol l^{-1} lactate (Table 5). Addition of 10^{-7} mol l^{-1} noradrenaline stimulated glycogenolysis in the absence of exogenous substrate but in the presence of either 5 mmol l^{-1} glucose or 5 mmol l^{-1} lactate, glycogen utilization was substantially reduced (Table 5).

Table 4. *Effects of various adrenergic agonists and antagonists on CO_2 production from ^{14}C-labelled substrates by isolated cardiac myocytes*

Adrenergic antagonist	Control	CO_2 production (expressed as % of control value) Adrenaline (10^{-7} mol l^{-1})	Noradrenaline (10^{-7} mol l^{-1})	Isoproterenol (10^{-7} mol l^{-1})
Lactate (5 mmol l^{-1})				
Control (N=4)	100†	115.2±9.9	189.9±12.1	202.1±8.5
Propranolol (10^{-6} mol l^{-1}) (N=4)	101.5±13.7	93.6±13.9*	106.8±6.8*	98.5±12.6*
Phentolamine (10^{-6} mol l^{-1}) (N=4)	98.8±5.9	114±14.2	185.9±12.4	196.7±14.7
Glucose (5 mmol l^{-1})				
Control (N=4)	100†	123.4±12.3	205.9±15.8	220.7±8.4
Propranolol (10^{-6} mol l^{-1}) (N=4)	99.8±9.3	104.1±12.7	84.3±12.7*	95.1±18.5*
Phentolamine (10^{-6} mol l^{-1}) (N=4)	114.2±10.6	133.1±11.9	221.4±18.2	206.5±14.7
Palmitate (0.25 mmol l^{-1})				
Control (N=5)	100†	147.5±12.3	284±15.8	310±14.7
Propranolol (10^{-6} mol l^{-1}) (N=5)	94.5±7.2	92.5±17.2*	107.9±15.3*	98.1±17.6*
Phentolamine (10^{-6} mol l^{-1}) (N=4)	105.8±6.9	140.3±11.9	272.4±10.9	326.4±18.9

* Significantly different (P<0.05) from corresponding value with agonist alone.

† Lactate, 100 % = 0.41±0.7 μmol g^{-1} h^{-1} (N=4); glucose, 100 % = 0.33±0.09 μmol g^{-1} h^{-1} (N=4); palmitate, 100 % = 0.15±0.04 μmol g^{-1} h^{-1} (N=5).

Antagonists were added 15 min before agonists.

Values are mean±S.E.

Effect of temperature acclimation

Temperature had a pronounced effect on the response of glucose and lactate utilization to noradrenaline and isoproterenol stimulation (Fig. 3). Myocytes isolated from fish acclimated to 9°C in either summer (July–August) or winter (December–February) were less sensitive and responsive to noradrenaline and isoproterenol stimulation than myocytes isolated from trout acclimated to 20°C in summer, as indicated by the rightward and downward shift in the dose–response curves (Fig. 3). The apparent EC_{50} values for noradrenaline and isoproterenol in myocytes isolated from summer fish at 20°C were 2–3 times those seen in myocytes isolated from fish acclimated to 9°C in either summer or winter (Table 3). Season alone was without effect, as the dose–response curves for myocytes isolated from fish acclimated to 9°C in either winter or summer were not significantly different (Fig. 3).

Table 5. *The effect of exogenous substrate availability and noradrenaline on glycogen utilization by myocytes isolated from adult rainbow trout*

Exogenous substrate	Glycogen content (μmol g^{-1} packed cell wet mass)		
	Freshly isolated myocytes‡	Control	Noradrenaline (10^{-7} mol l^{-1})
	12.8±0.7 (N=5)	9.8±2.1§ (N=5)	7.2±0.9* (N=5)
Lactate (5 mmol l^{-1})		12.7±1.8† (N=5)	11.2±0.8 (N=5)
Glucose (5 mmol l^{-1})		12.5±1.5† (N=5)	10.7±1.1 (N=5)

Control: no exogenous noradrenaline added.

* Indicates a significant difference (P<0.05) from the corresponding control value.

† Indicates a significant difference (P<0.05) from the control value in the absence of exogenous substrate.

‡ Freshly isolated myocytes are cells that were fixed for glycogen analysis immediately after isolation.

§ Indicates a significant difference (P<0.05) from the value for freshly isolated myocytes.

Values are mean±S.E.

Discussion

Methodology

The procedures described for isolating cardiac myocytes from mammalian cardiac muscle typically require a period of perfusion with Ca^{2+}-free medium (e.g. Piper *et al.* 1987), which aids in tissue disaggregation by facilitating separation of the intercalated disc regions between the cells (Yates and Dhalla, 1975). However, a common problem encountered with this technique is the 'calcium paradox': re-introduction of physiological calcium levels after incubation in calcium-free medium results in contraction and rounding of the cells; i.e. the cells are calcium-intolerant (Kammermeier and Rose, 1988). Myocytes from ectothermic vertebrates were thought to be less sensitive to changes in extracellular calcium concentration, thus less likely to be susceptible to the 'calcium paradox' (Lagerstrand *et al.* 1983). However, preliminary experiments indicated that myocytes isolated from trout are indeed sensitive to changes in extracellular calcium; upon re-introduction of physiological calcium levels, myocytes isolated in Ca^{2+}-free medium contracted spontaneously, rounded up and a high percentage of cells (>50 %) incorporated Trypan Blue, indicative of calcium-intolerance. In attempts to overcome this problem, various medium manipulations were tested and it was found that the presence of calcium at low levels (50 μmol l^{-1}) throughout the isolation procedure offered the best compromise between facility of tissue disaggregation and maintenance of cell integrity at physiological calcium levels; i.e. the isolated myocytes are calcium-tolerant.

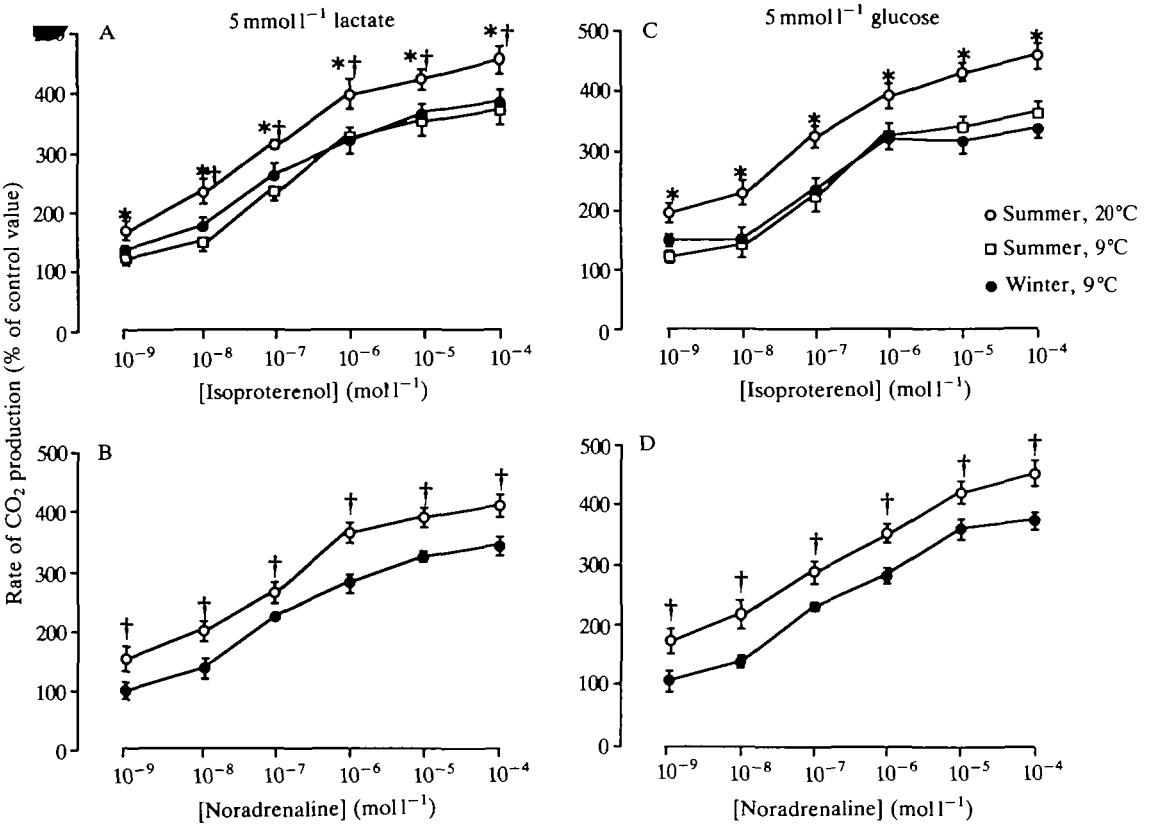

Fig. 3. Effects of season and temperature acclimation on the adrenergic responsiveness of the rates of CO_2 production from 5 mmol l^{-1} lactate (A, B) and 5 mmol l^{-1} glucose (C, D) by cardiac myocytes isolated from adult rainbow trout acclimated in the summer to 20°C (○, N=4); in the summer to 9°C (□, N=4), or in the winter to 9°C (●, N=5). Values are means±1 S.E.M. * indicates a significant difference ($P \leq 0.05$) from the corresponding values from summer 9°C- and winter 9°C-acclimated fish; † indicates a significant difference ($P \leq 0.05$) from the corresponding value from winter 9°C-acclimated fish.

Basal metabolism of isolated cardiac myocytes

Except at extremely high (20 mmol l^{-1}) concentrations, $^{14}CO_2$ production from lactate exceeded that from glucose, which is also true of intact fish hearts (Lanctin *et al.* 1980). The kinetic relationships demonstrated in this study (Fig. 1) indicate that, *in vivo*, in stressful situations (e.g. hypoxia, exercise), when plasma lactate levels are often greater than glucose concentrations, lactate utilization would exceed glucose utilization. This observation is consistent with those from intact, electrically paced brook trout (*Salvelinus fontinalis*) hearts or *in situ* perfused rainbow trout hearts, which indicate that, when plentiful, lactate is a preferred fuel (Lanctin *et al.* 1980; Milligan and Farrell, 1991).

The relationship between CO_2 production from lactate and substrate concentration is clearly hyperbolic, indicating saturation. A similar observation has been

made in electrically paced sea raven (*Hemitripterus americanus*) and ocean pout (*Macrozoarces americanus*) hearts, where it has been shown that lactate oxidation is limited by lactate oxidase activity (Driedzic *et al.* 1985). However, CO_2 production from glucose continued to increase, even at supra-physiological substrate levels. The substrate-dependence of glucose utilization has not been examined in other fish hearts; however, in the absence of insulin, ventricular myocytes isolated from rats show a similar relationship (Rovetto, 1981).

Adrenergic effects on substrate utilization

It is certainly well established that adrenergic stimulation results in enhanced cardiac performance, through both inotropic and chronotropic effects. Obviously, cardiac metabolism must increase to meet the increased energetic demands. However, in intact working hearts, it is difficult to differentiate between the direct effects of catecholamines on metabolism and the effects mediated by increased contractile force. The results from this study clearly demonstrate that catecholamines stimulate metabolism in trout cardiac muscle independently of their effects on contractility. Because uniformly labelled glucose and lactate were employed in this study, it cannot be assumed that all the $^{14}CO_2$ produced arises from the complete oxidation of either glucose or lactate. Some of the $^{14}CO_2$ produced results from the decarboxylation of pyruvate to acetyl CoA, which may then be channelled into fatty acid synthesis, not the tricarboxylic acid cycle (Driedzic *et al.* 1985). However, in the presence of adrenergic stimulation, at least in the mammalian heart (Crass *et al.* 1975), lipogenesis is inhibited and lipolysis favoured. Thus, if lipogenesis was occurring in the adrenergically stimulated cells, it was probably minimal.

The data in the present study do not provide any insights into the possible mechanisms of the stimulation of metabolism by adrenergic agonists. In guinea pig hearts, when the stimulatory effect of noradrenaline on myocardial contractility is inhibited, pyruvate dehydrogenase (PDH) activity is elevated (*via* a stimulation of mitochondrial PDH phosphate phosphatase activity; Hiraoka *et al.* 1980; Rovetto, 1981). This stimulation of PDH leads to increases in pyruvate oxidation, in the absence of increased metabolic demand (Bünger *et al.* 1983), which may account for the stimulation of glucose and lactate utilization. The stimulation of palmitate metabolism is more difficult to explain and suggests that the adrenergic stimulation of metabolism is perhaps *via* an indirect route; i.e. by stimulation of ATP demand owing, for example, to increased Na^+/K^+-ATPase activity (Wasserstrom *et al.* 1982; Lee and Vasalle, 1983; Danziger *et al.* 1990). It would appear that the mechanism of adrenergic stimulation of trout myocyte metabolism is complex.

In the absence of exogenous fuel, trout cardiac myocytes rely upon endogenous glycogen to meet energy demand and glycogenolysis is further stimulated by 10^{-7} mol l^{-1} noradrenaline. However, when exogenous substrate is present, not only is endogenous glycogen 'spared', but the effect of noradrenaline on glycogenolysis appears to be inhibited; there is no net reduction in glycogen levels,

A similar effect was seen in the perfused rat heart; adrenergic stimulation resulted in an almost complete conversion of glycogen phosphorylase b to glycogen phosphorylase a, yet, in the presence of adequate oxygen and exogenous substrate, there was no net decrease in cardiac glycogen stores (Williams and Mayer, 1966; Neely *et al.* 1970; Mayer, 1974). To explain this apparent contradiction in the rat heart, it was suggested that, under aerobic conditions, the concentration of inorganic phosphate in the heart is not high enough to support the transformation of glycogen to glucose 1-phosphate and that glycogen synthase is not inhibited by adrenergic stimulation (Neely *et al.* 1970; Mayer, 1974). Furthermore, increases in glucose 6-phosphate concentration, resulting from glucose utilization, would activate glycogen synthase and inhibit glycogen phosphorylase b, thus promoting glycogen synthesis (Rovetto, 1981). Whether the same mechanism is operating in trout heart is not clear. It is evident, however, that exogenous substrates are used by isolated trout myocytes in preference to endogenous stores, which is consistent with observations on the *in situ* perfused trout heart (Milligan and Farrell, 1991).

Over the physiological range of plasma catecholamine concentrations (maximum of approximately 10^{-7} mmol l^{-1}; Milligan and Wood, 1987), substrate utilization by resting myocytes was increased nearly twofold. In the *in situ* perfused trout heart, a fourfold increase in workload, achieved by increasing afterload only, regardless of changes in adrenergic stimulation, led to a twofold increase in lactate utilization (Milligan and Farrell, 1991). Taken together, these results suggest that, *in vivo*, catecholamines not only enhance cardiac performance directly but also facilitate the supply of available exogenous substrates to satisfy the commensurate increase in energy demand.

The order of sensitivity of both glucose and lactate utilization to adrenergic stimulation is isoproterenol$\geqslant$noradrenaline$\gg$adrenaline. Following the classification of adrenergic receptors established for mammals (Exton, 1985), this suggests that β_1-adrenoceptors are mediating the response. The adrenergic potency for myocyte substrate utilization differs markedly from that reported for contractility for a variety of teleost hearts where isoproterenol>adrenaline$\gg$noradrenaline, indicative of β_2-adrenoceptors (Laurent *et al.* 1983; Farrell, 1984; Farrell *et al.* 1986). However, it is similar to the order of potency reported for adrenergically stimulated metabolic effects in trout erythrocytes (Tetens *et al.* 1988), coho salmon (*Oncorhynchus kisutch*) liver (Sheridan, 1987) and copper rockfish (*Sebastes caurinus*) hepatocytes (Danulat and Mommsen, 1990). The results of the present study and those on contractility tend to suggest that both β_1- and β_2-adrenoceptors are present in the trout heart, but mediate different responses.

Phenylephrine, an α-adrenergic agonist, was without effect on substrate utilization, except at pharmacological doses. This observation is consistent with those from studies on the adrenergic regulation of contractility (Ask *et al.* 1980; Farrell *et al.* 1986), indicating that α-adrenoceptors do not play a significant role in the regulation of either cardiac metabolism or performance.

Temperature and seasonal effects of adrenergic stimulation

Substrate utilization by myocytes isolated from trout acclimated to 9°C in either winter or summer was not different and these myocytes were less sensitive and responsive to adrenergic stimulation than those isolated from trout acclimated to 20°C in the summer. This lack of seasonal effect suggests that temperature acclimation is the more important factor determining the sensitivity of cardiac myocyte metabolism to adrenergic stimulation. A similar temperature effect on adrenergic responsiveness has been reported for the *in situ* perfused trout heart. Hearts from trout acclimated to 15°C showed greater inotropic and chronotropic responses to adrenergic stimulation than did hearts from trout acclimated to 5°C (Graham and Farrell, 1989). Temperature and/or seasonal variations in adrenergic sensitivity have also been reported for trout erythrocytes (Cossins and Kilbey, 1989; Nikinmaa and Jensen, 1986), though others have reported no effect (Milligan *et al.* 1989; Tetens *et al.* 1988). It is not clear from the present studies, or those cited, why the adrenergic responsiveness changes with temperature; that is, whether there is a change in β-adrenoceptor density, affinity or, perhaps, intracellular signalling mechanism. This is an area that requires further investigation.

In conclusion, this study has demonstrated that cardiac myocytes isolated from rainbow trout offer a viable experimental model for studies on basal cardiac muscle function on the basis of several criteria: microscopic appearance; integrity of cell membranes, as indicated by the exclusion of Trypan Blue and low leakage of LDH to the medium; maintenance of ATP, PCr and glycogen levels; the ability to metabolize substrates; and sensitivity to adrenergic stimulation. Therefore, the isolated myocyte offers a good *in vitro* model system for further studies on the regulation of cardiac metabolism as well as sarcolemmal transport processes.

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