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EPINEPHRINE STIMULATION OF GLUCOSE RELEASE FROM PERFUSED TROUT LIVER: EFFECTS OF ASSAY AND ACCLIMATION TEMPERATURE

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Summary

Since fish are poikilothermic, changes in temperature may perturb hormonal activation of cell function. To test this hypothesis, and determine the extent to which hormonal responses are thermally compensated, the effect of temperature on epinephrine-stimulated glucose release in perfused trout liver was studied. Thermally acclimated (5 and 20˚C) rainbow trout (*Oncorhynchus mykiss*) were responsive to epinephrine (0.5×10^{-6} mol l^{-1}) at both 5 and 20˚C. Metoprolol (β_2 antagonist) and propranolol (β_{1and2}) decreased the response significantly (to 1.4% and 8.4% of stimulated values, respectively) while phentolamine (α_{1and2}) was without effect, implying the response is β_2-mediated. Both basal (86 and 19 μmol g^{-1} liver h^{-1} in 5 and 20˚C trout, respectively) and epinephrine-stimulated (210 and 168 μmol g^{-1} h^{-1}) rates of glucose release were higher (2.4-fold higher for epinephrine-stimulated and 8.8-fold for basal) in 5˚C- than in 20˚C-acclimated fish, regardless of perfusion temperature. Although the dose–response curve for epinephrine was markedly temperature-dependent, cold- and warm-acclimated fish were affected in different ways. Cold-acclimated fish (5˚C) were less responsive to epinephrine when perfused at 5˚C (ED_{50} 6.8×10^{-9} mol l^{-1}) than when perfused at 20˚C (ED_{50} 8.2×10^{-10} mol l^{-1}); in contrast, warm-acclimated fish (20˚C) were less responsive to epinephrine when perfused at 20˚C (ED_{50} 4.6×10^{-7} mol l^{-1}) than at 5˚C (ED_{50} 6.6×10^{-9} mol l^{-1}). These results are interpreted as being indicative of adaptations to maintain the capacity for hepatic glucose mobilization at low temperature.

Introduction

Temperature exerts a fundamental constraint on poikilothermic organisms, determining both reaction rates and the conformation of biological structures stabilized by weak bonding forces (e.g. tertiary structure of proteins) (Hochachka and Somero, 1984). Thermal perturbations of structure and function are particularly acute for aquatic poikilotherms such as teleost fish, for which muscle temperatures have been measured to be within 1˚C of environmental temperature (Linthicum *et al.* 1972). One poorly studied aspect of thermal biology in poikilotherms is the impact of temperature change on endocrine function. The maintenance of integrated physiological function at different

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body temperatures would appear to require minimal perturbation of endocrine regulatory mechanisms. Yet, various aspects of endocrine function are markedly temperature-dependent. For example, in trout red blood cells, epinephrine-stimulated Na^+/H^+ exchange is markedly depressed (Q_{10}=7.9) at low (4°C) temperature (Cossins and Kilbey, 1990). Conversely, the binding of [^{3}H]cyclohexyladenosine to the A_1-adrenergic receptor isolated from rat and chicken brain increases two- to fourfold as temperature is decreased (Siebenaller and Murry, 1988). Furthermore, reductions in membrane fluidity, similar to those induced by cold temperature (Hazel, 1989), inhibit collision coupling between components of the β-adrenergic signal transduction pathway and thus depress rates of 3′,5′-cyclic adenosine monophosphate (cyclic AMP) production in plasma membranes of turkey erythrocytes (Atlas *et al.* 1980). The thermal sensitivity of endocrine processes, as delineated above, suggests that changes in temperature may significantly perturb endocrine regulation in poikilotherms and that acclimatory mechanisms may be required to offset the direct effects of temperature perturbation on endocrine control systems.

The impact of acute and acclimatory changes in temperature on hormonal activation of cell function was studied by measuring rates of basal and epinephrine-stimulated glucose release in perfused livers of thermally acclimated (5 and 20°C) rainbow trout (*Oncorhynchus mykiss*). The binding of epinephrine to specific receptors located in the plasma membrane stimulates (*via* G-proteins) the activity of adenylate cyclase, the production of cyclic AMP (Levitzki, 1988), the activation of glycogenolysis and, ultimately, the release of glucose (Ross, 1989). Two questions are explicitly addressed in this study. (1) Is epinephrine-stimulated glucose release from trout liver significantly perturbed by temperature? (2) Does thermal acclimation result in temperature compensation of endocrine function?

Materials and methods

Animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)], obtained from the Alchesay National Fish Hatchery, Whiteriver, AZ, were acclimated to either 5 or 20°C for a minimum period of 2 weeks and fed Glencoe Mills trout chow to satiation once per day.

Liver perfusion

Fish were anesthetized with neutralized (pH7.0), aerated MS-222 (1:2000 w/v) at the respective acclimation temperature. The abdominal cavity was opened and the hepatic portal vein cannulated 2.0cm from the liver with polyethylene tubing. Cannulae were securely sutured in place, all severed vessels were tied off, and the liver was perfused with Hepes-buffered, heparinized (3620units100ml^{-1}) trout Ringer's solution (NaCl, 1.76mmol l^{-1}; KCl, 0.054mol l^{-1}; $MgSO_4$, 8.1mmol l^{-1}; KH_2PO_4, 4.4mmol l^{-1}; Na_2HPO_4, 3.5mmol l^{-1}; $NaHCO_3$, 0.08mol l^{-1}; $CaCl_2$, 0.01mol l^{-1}; Hepes, 0.20mol l^{-1}, aerated with 98%O_2/2%CO_2), until cleared of blood (Moon *et al.* 1985). The cannulated

liver was carefully removed and transferred to a rigid, porous nylon-net platform positioned in a small glass funnel suspended over a fraction collector. When in position, the liver was covered with Parafilm to prevent drying. Perfusions were performed using a Harvard Apparatus variable-speed pump ($3.1 ml min^{-1}$) with trout Ringer's solution at 5 or 20°C. Perfusion was continued for 15–20min to allow the preparation to stabilize before experimental manipulation. In experiments involving changes in temperature, the liver was cooled or warmed as rapidly as possible by altering the temperature of the perfusate and supporting device. Temperature was measured with a Yellow-Springs Instrument Co. tele-thermometer employing a stainless-steel pancake probe placed in direct contact with the external surface of the liver. For dose–response curves, each concentration of epinephrine (1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 1×10^{-4} or $1\times10^{-3} mol l^{-1}$) was administered as a bolus (constant infusion of epinephrine in Ringer for 1min, at a rate of $3.1 ml min^{-1}$), in ascending order of concentration. Epinephrine was prepared fresh each day and shielded from direct light until used to minimize oxidation. Samples of perfusate were collected with a fraction collector at 1min intervals. Rates of glucose release were computed from the product of the glucose concentration in the perfusate and the perfusion flow rate (e.g. for a basal output of $0.1 \mu mol ml^{-1} g^{-1} \times 3.1 ml min^{-1} \times 60 min\, h^{-1} = 19 \mu mol\, h^{-1} g^{-1}$). Concentrations of phentolamine, propranolol and metoprolol were $0.5\times10^{-5} mol\, l^{-1}$; they were administered as a bolus in conjunction with epinephrine ($0.5\times10^{-6} mol\, l^{-1}$).

Analytical procedures

Glucose in the perfusate was assayed *via* the colorimetric glucose oxidase method of Kunst *et al.* (1974). Statistical analyses were performed using Student's *t*-test, with the level of significance at $P<0.05$.

Extent of perfusion for each preparation was determined at the end of each experiment by infusion of Direct Blue ($0.1 g\, 100 ml^{-1}$ Ringer) for 3min. Each liver was then homogenized (using an all-glass tissue homogenizer) in the perfusion medium (four times w/v), and the resulting homogenate was centrifuged for 2h at $25000 revs min^{-1}$ in a Beckman 50.1 Ti fixed-angle rotor. The absorbance at 505nm was measured spectrophotometrically and the absorbance per gram liver was used to correct the data within experimental groups relative to the best perfusion (the correction factor = absorbance $g^{-1}{}_x$/absorbance g^{-1} liver$_y$, where *y* is the most extensively perfused liver and *x* is a liver not as extensively perfused as *y*. The extent of perfusion by this criterion varied by no more than 10% among all experiments).

Materials

All solutions were prepared in glass-distilled water. Na_2HPO_4 was purchased from EM Science. KCl, $MgSO_4$ and $CaCl_2$ were purchased from Baker. NaCl and $NaHCO_3$ were purchased from Fischer Scientific. Hepes, bovine serum albumin (BSA), Direct Blue, epinephrine bitartrate, heparin and propranolol were purchased from Sigma. Phentolamine and metoprolol were donated by Ciba-Geigy.

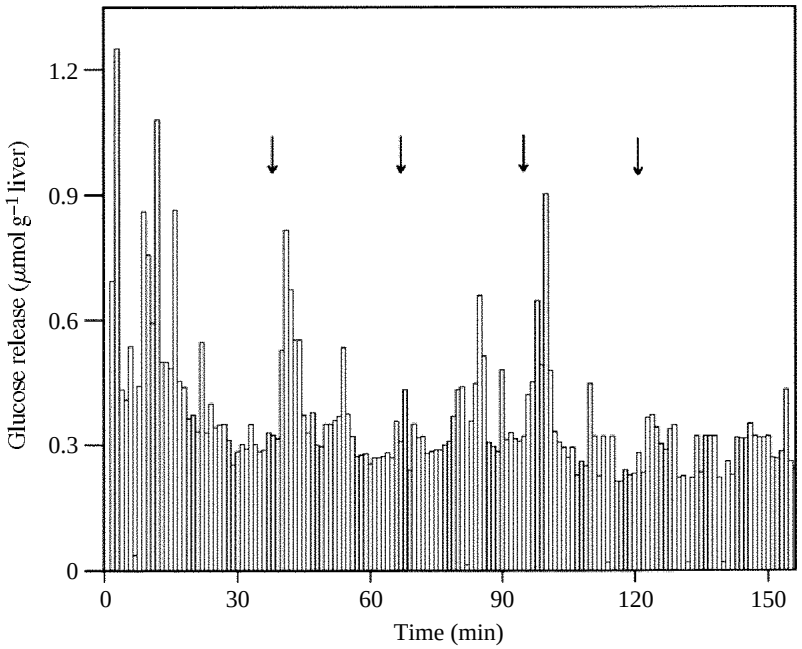

Fig. 1. Responsiveness of the perfused liver preparation to epinephrine over time. Representative example of the response of a perfused liver from a 20°C-acclimated trout to 0.5×10^{-6} mol l^{-1} epinephrine over a 2.5-h period. Arrows indicate the times at which epinephrine was administered in a 3.1ml bolus.

Results

Initial attempts to characterize epinephrine-stimulated glucose release utilized isolated hepatocytes prepared from trout liver by the technique of Moon *et al.* (1985). Although hepatocytes were responsive to epinephrine over the administered range, glucose release could not be inhibited by β-adrenergic antagonists (e.g. propranolol) as predicted from previous studies, showing this response to be β-mediated (Mommsen *et al.* 1988). We speculate that the failure of β-antagonists to block glucose release in our experiments may reflect an alteration in the adrenergic receptor caused by the collagenase used in harvesting the hepatocytes. Since we wished to study the properties of intact receptors, whole-organ perfusion was employed to circumvent potential problems that may arise as a result of receptor modification during hepatocyte isolation.

Perfusion conditions

After an initial washout period (lasting 15–20min), glucose release stabilized at what was considered to be basal levels (Fig. 1): stress from anesthesia, surgery and manipulation of the liver possibly contribute to the elevated levels of glucose release observed during the first 10min of collection. Perfused livers remained responsive to epinephrine (0.5×10^{-6} mol l^{-1}, when delivered as a 3.1ml bolus) for up to 90min (Fig. 1). Consequently, in all subsequent experiments, epinephrine was administered only in the interval between 15–20 and 90min following initiation of perfusion.

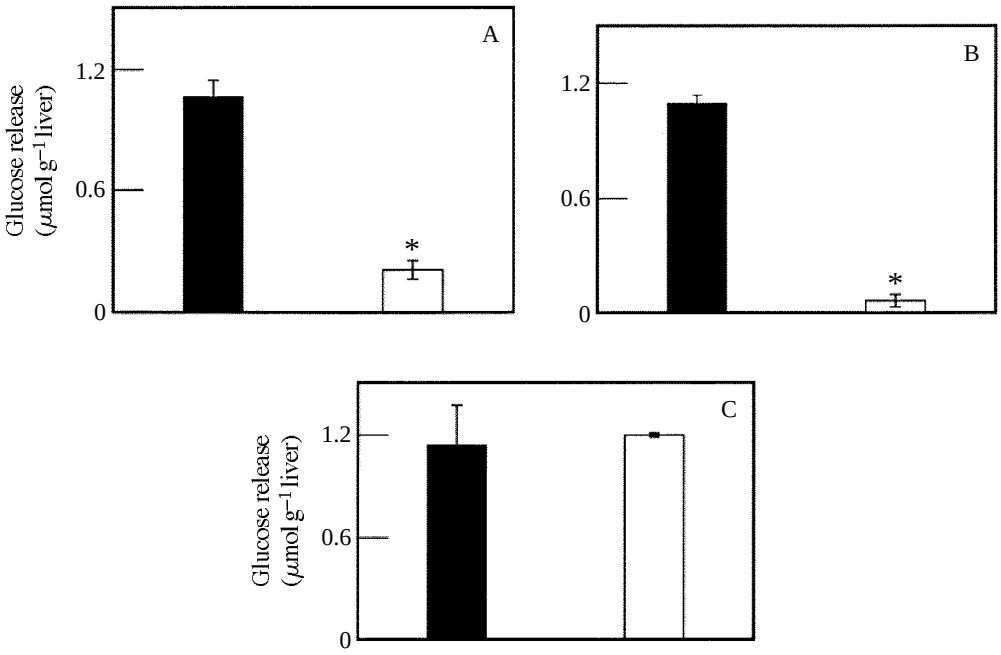

Fig. 2. Effects of α- and β-specific antagonists on epinephrine-induced glucose release from perfused livers. (A) The general (β_{1and2}) β-antagonist propranolol; (B) the β_2-specific antagonist metoprolol; (C) the general (α_{1and2}) α-antagonist phentolamine. Filled bars represent the response to 0.5×10^{-6} mol l^{-1} epinephrine alone; open bars represent the response to epinephrine in the presence of 0.5×10^{-5} mol l^{-1} antagonist. Plotted as mean ± S.E.M., N=3, where * indicates a significant (P<0.05) difference.

Pharmacology of the response

Propranolol, a general β-antagonist, inhibited glucose release by 3.5-fold (to 8.4% of control), indicating that the response is β-mediated (Fig. 2A). In addition, administration of metoprolol, a β_2-specific antagonist, decreased glucose release 5.8-fold (to 1.4% of control), demonstrating that the release of glucose in response to epinephrine is mediated by the β_2-adrenergic receptor (Fig. 2B). Furthermore, there was no significant reduction in glucose release when phentolamine, a general α-receptor antagonist, was administered in combination with epinephrine (Fig. 2C).

Rates of epinephrine-stimulated glucose release

The rates of neither basal (lowest non-stimulated activity) nor epinephrine-stimulated (highest value) glucose release were significantly affected by an acute change in temperature (achieved in 30min) in preparations from either acclimation group (Figs 3 and 4). However, both basal (86 and 19 μmol g^{-1} liver h^{-1} in 5°C- and 20°C-acclimated trout, respectively) and epinephrine-stimulated (210 and 168 μmol g^{-1} h^{-1}) rates of glucose release were respectively 2.4- and 8.8-fold higher in 5°C- than 20°C-acclimated fish, regardless of perfusion temperature (Fig. 5). Although rates of basal and stimulated

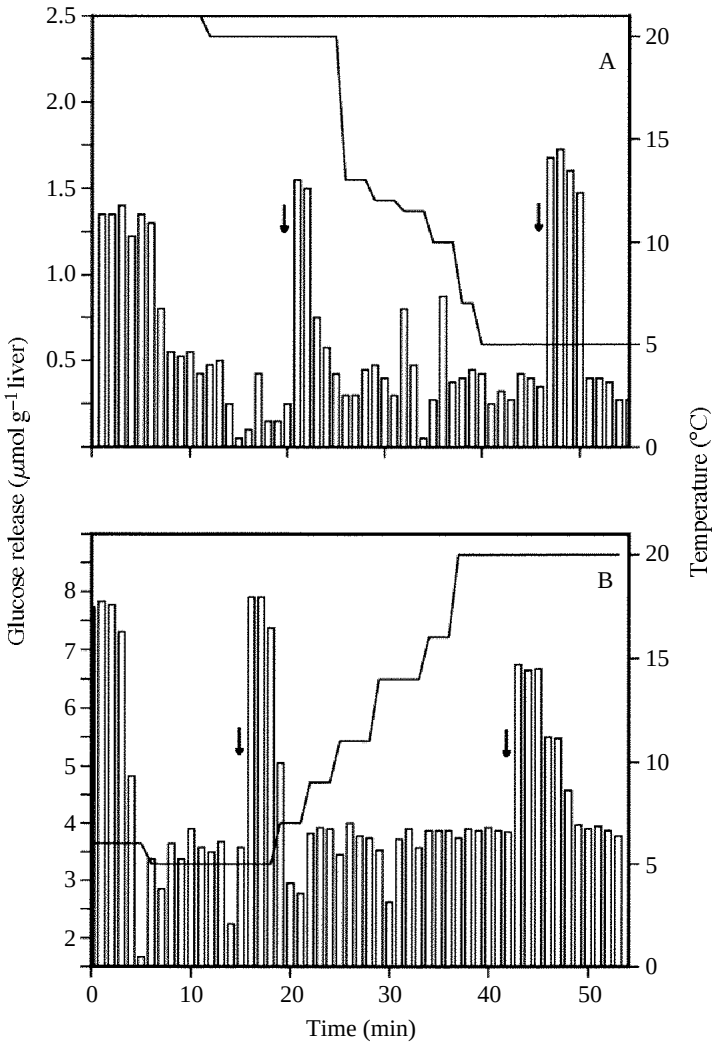

Fig. 3. Effect of assay temperature on hepatic release of glucose in response to epinephrine. (A) Liver from a 20˚C-acclimated fish initially perfused at 20˚C and rapidly cooled to 5˚C; (B) liver from a 5˚C-acclimated trout initially perfused at 5˚C and then warmed rapidly to 20˚C. Arrows indicate the points of epinephrine injection. The dashed line indicates the temperature of the liver as monitored by an external temperature probe.

glucose release are higher in cold- than in warm-acclimated fish, the ratio of stimulated to basal release is lower in cold-acclimated fish.

Dose–response curves

The dose–response curve for epinephrine was markedly temperature-dependent. Cold-acclimated fish were less responsive to epinephrine (by a factor of 10) when perfused at 5˚C (ED_{50} 6.8×10^{-9} mol l^{-1}) than when perfused at 20˚C (ED_{50} 8.2×10^{-10} mol l^{-1}), indicating an increased sensitivity to epinephrine with rising temperature. In contrast,

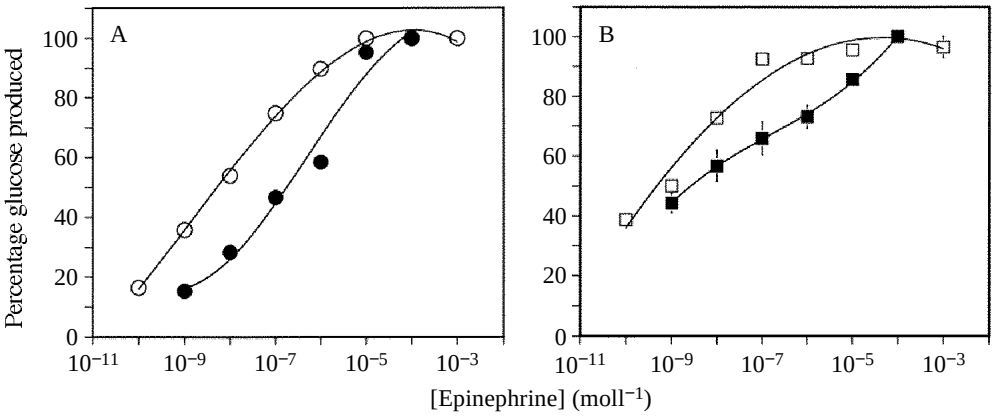

Fig. 4. Effects of assay and acclimation temperature on dose–response curves for epinephrine. (A) The livers of 20˚C-acclimated trout perfused at 20˚C (filled circles, $ED_{50}=4.6\times10^{-7}$ mol l^{-1}) and 5˚C (open circles, $ED_{50}=6.6\times10^{-9}$ mol l^{-1}). (B) Livers of 5˚C-acclimated trout assayed at 5˚C (filled squares, $ED_{50}=6.8\times10^{-9}$ mol l^{-1}) and 20˚C (open squares, $ED_{50}=8.2\times10^{-10}$ mol l^{-1}). Plotted as the mean ± S.E.M. (*N*=3).

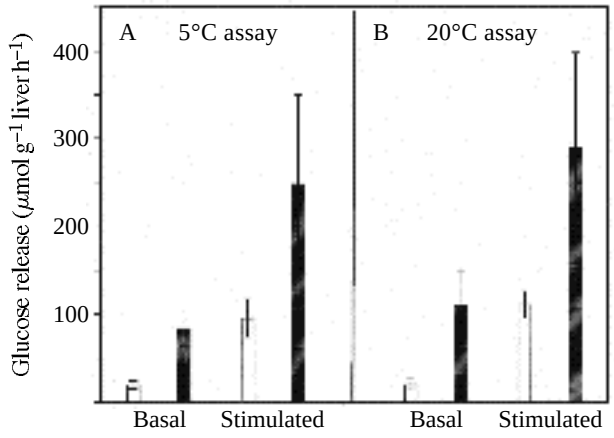

Fig. 5. Effects of assay and acclimation temperature on rates of hepatic glucose release from perfused liver. (A) 5˚C- (shaded bars) and 20˚C-acclimated (open bars) trout assayed at 5˚C. (B) 5˚C- and 20˚C-acclimated trout assayed at 20˚C. Open bars represents 20˚C, shaded bars 5˚C. For each panel, the basal response is represented by the first two bars on the left, and the epinephrine (0.5×10^{-6} mol l^{-1})-stimulated response by the two bars on the right. Plotted as the mean ± S.E.M. (*N*=3).

warm (20˚C)-acclimated fish were less responsive to epinephrine when perfused at 20˚C (ED_{50} 4.6×10^{-7} mol l^{-1}) than at 5˚C (ED_{50} 6.6×10^{-9} mol l^{-1}), indicating a heightened sensitivity (approximately 100-fold) at low temperature. Thus, dose–response curves of warm- and cold-acclimated fish are affected by acute changes in temperature in a fundamentally different manner, although in both cases sensitivity to epinephrine is enhanced when temperature is acutely varied towards the alternate acclimation

temperature (i.e. with warming in cold-acclimated fish and cooling in warm-acclimated fish).

Acclimation history also significantly influenced the sensitivity of trout liver to epinephrine as shown by differences in ED_{50} between acclimation groups. Although ED_{50} values were similar in both acclimation groups at 5°C (approximately 7×10^{-9} mol l^{-1}), they differed by three orders of magnitude (8.2×10^{-10} mol l^{-1} and 4.6×10^{-7} mol l^{-1}) at 20°C. When compared at their respective acclimation temperatures, the ED_{50} value for 20°C-acclimated fish (4.6×10^{-7} mol l^{-1}) was 100-fold higher than that of 5°C-acclimated (ED_{50} 6.8×10^{-9} mol l^{-1}) fish.

Discussion

Epinephrine stimulates glucose release from teleost liver (Fig. 1 and Moon *et al.* 1985), presumably by binding to a specific receptor in the hepatic plasma membrane, activating (*via* a diffusion-coupled process involving a stimulatory G-protein as an intermediate) adenylate cyclase, and producing the intracellular second messenger cyclic AMP (Levitzki, 1988). Cyclic AMP in turn modulates the activity of protein kinases and phosphatases, which then influence (*via* phosphorylation) regulatory enzymes that determine the rate of carbon flux through the pathways of both glycolysis and gluconeogenesis (Krebs, 1989). In isolated trout hepatocytes, epinephrine has been reported to inhibit glycolysis (*via* inhibition of pyruvate kinase) and activate both glycogenolysis (glycogen phosphorylase) and gluconeogenesis, with glycogenolysis accounting for the vast majority (approximately 97%) of glucose released to the medium (Mommsen *et al.* 1988). We made no attempt in the present experiments to determine the proportion of glucose derived from glycogenolysis as opposed to gluconeogenesis, but based on the above results and the lack of a gluconeogenic precursor in the perfusion saline, believe that glycogenolysis is probably the pathway activated to a greater extent by epinephrine stimulation.

The isolated, perfused liver preparation used in these experiments remains viable and responsive to epinephrine for approximately 90min (Fig. 1), which is considerably shorter than the maintenance of viability (assessed by the stability of intracellular ATP concentrations) in isolated hepatocytes (up to 3h, Moon *et al.* 1985). Nevertheless, within this time frame, the perfused liver is a suitable *in vitro* system in which to study hormonal activation of cell function. In addition, basal rates of glucose release (19–86 μmol g^{-1} h^{-1}) are slightly higher than those obtained in isolated hepatocytes (6–10 μmol g^{-1} wetmass h^{-1}) (Reid *et al.* 1992). Furthermore, based on the sensitivity of the response to inhibition by propranolol (β_{1and2}), metoprolol (β_2) and phentolamine (α_{1and2}) (Mommsen *et al.* 1988; Exton *et al.* 1981), glucose release from trout liver in response to epinephrine is evidently mediated *via* a β_2-adrenergic receptor (Fig. 2), confirming and extending the previous observations of Birnbaum *et al.* (1976). In addition, the ED_{50} values reported in this study are mid-way between resting and stressed concentrations of circulating epinephrine in trout (10^{-9}–10^{-6} mol l^{-1}; Tetens and Lykkeboe, 1988) and within the range (10^{-7}–10^{-6} mol l^{-1}) found by Bourne and Cossins

(1982) to stimulate the red blood cell Na^+/H^+-exchanger half-maximally (Tetens and Lykkeboe, 1988).

Acute thermal effects

Surprisingly, neither basal nor hormone-stimulated rates of glucose release were significantly influenced by temperature (Q_{10}≈1) in either acclimation group (Fig. 3). Neither was the extent of activation (relative to the unstimulated control) altered by acute temperature change. Enhanced binding of hormone to receptor at cold temperatures, and the longer lifetime of the hormone–receptor complex that results, may compensate for thermal rate limitations (as suggested by the temperature-dependence of [^{3}H]cyclohexyladenosine binding to A_1-receptors in brain preparations from seven diverse species of fish) at low temperature, thus reducing the apparent temperature sensitivity of hormone action (Siebenaller and Murry, 1988). The data for warm-acclimated trout are consistent with this suggestion, but the higher ED_{50} at 5°C compared to 20°C for cold-acclimated trout is difficult to explain in these terms. Regardless of the mechanism, the temperature-independence of the response may be of major significance to an ectotherm by ensuring that thermal perturbations are prevented from adversely affecting endocrine regulation in this system.

Although rates of glucose release were independent of assay temperature, dose–response curves for epinephrine were markedly, but differently, temperature-sensitive in both acclimation groups: dose–response curves shift to the left in warm-acclimated trout (100-fold change in ED_{50}), but to the right in cold-acclimated trout (10-fold change) with a 15°C drop in assay temperature (Fig. 4). Interestingly, there is a heightened sensitivity to epinephrine when either a warm-acclimated fish is acutely exposed to cold temperatures or a cold-acclimated fish is warmed abruptly. Enhanced binding of ligands to proteins at low temperature is a common observation (Somero, 1978) and may partially account for the shift in ED_{50} in warm-acclimated trout. However, the apparent reduction in the ED_{50} for epinephrine at low temperature in cold-acclimated trout cannot be explained in these terms and may reflect an effect of temperature on other processes. In this regard, the dose–response curves for perfused liver reported in Fig. 4 cover a much broader range of ligand concentrations than is typically seen in epinephrine binding studies (Tetens and Lykkeboe, 1988; Cossins and Kilbey, 1989). This is probably attributable to the complexity of the perfused liver system. For example, vasomotion, which controls the extent of perfusion and access of hepatocytes to hormones, may be temperature-dependent (Flavahan, 1992; Armah *et al.* 1990). Furthermore, glucose release is the final step in a multistep pathway and may be influenced at multiple control points (Hochachka and Somero, 1984; Cossins and Bowler, 1987).

Acclimatory thermal effects

Temperature acclimation significantly modifies the response of perfused trout liver to epinephrine. Rates of glucose release were higher (8.8-fold for basal and 2.4-fold for epinephrine-induced) in cold- than in warm-acclimated fish, possibly reflecting a greater reliance on glucose in cold or over-wintering trout. This hypothesis is consistent with a

possible obligatory dependence of the myocardium on glucose oxidation, based on enzyme data in cold-acclimated sea raven *Hemitripterus americanus* (Sephton *et al.* 1990). In addition, elevated levels of serum glucose may also serve a protective function in vertebrate poikilotherms at low temperature. For example, restriction of ice nuclei growth at freezing temperatures is of paramount importance if damaging ice crystal formation is to be prevented. Umminger has demonstrated a large increase (432% over 20˚C) in serum glucose levels in *Fundulus heteroclitus* maintained between 2 and 4˚C, which depresses the freezing point of serum to −0.8˚C (Umminger, 1971*a*,*b*). Thus, the heightened sensitivity of hepatic glycogenolysis to epinephrine observed in cold-acclimated fish in the present study may provide a mechanism for the regulated onset of hyperglycemia at low temperatures in vertebrate poikilotherms. Similarly, Storey and Storey (1992) have found that elevated concentrations (1000-fold) of glucose in the wood frog are rapidly induced with the onset of freezing.

Temperature acclimation also shifts the position of the dose–response curve and the sensitivity to epinephrine. Whereas the ED_{50} values for epinephrine are nearly comparable in both acclimation groups at 5˚C, at 20˚C, cold-acclimated fish are nearly two orders of magnitude more sensitive to epinephrine than are warm-acclimated fish. This is in contrast to the situation in erythrocytes, where the affinities of β-adrenoceptors (reported as ED_{50}) did not differ significantly between rainbow trout acclimated to winter (2˚C) and summer (20˚C) conditions (Tetens and Lykkeboe, 1988). When comparisons are made at the respective acclimation temperatures, cold-acclimated fish remain more sensitive to epinephrine then do warm-acclimated fish, reinforcing the previous suggestion that cold-acclimated trout have a greater capacity for glucose mobilization at low temperature (Umminger, 1971*c*,*d*).

In conclusion, the β-adrenergic regulation of hepatic glucose release responds to both acute and acclimatory changes in temperature in a manner that reinforces a common theme in the adaptation of vertebrate poikilotherms to low temperature, namely the maintenance of the capacity to elevate glycogenolysis (and possibly initiate hyperglycemia). The present study documents that the ability to maintain glucose production in, and release from, trout liver at low temperature is made possible by a variety of unique characteristics and adaptations that include: (1) insensitivity of rates of glucose release to acute changes in temperature, (2) a higher rate of glucose release in cold- than in warm-acclimated fish, and (3) a greater sensitivity of cold-acclimated fish to epinephrine as shown by the lower concentrations required to elicit a response.

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