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Steroid-induced cardiac contractility requires exogenous glucose, glycolysis and the sarcoplasmic reticulum in rainbow trout

Richard S. Farrar*, Pavan K. Battiprolu*, Nicholas S. Pierson and Kenneth J. Rodnick†

Department of Biological Sciences, Idaho State University, Pocatello, ID 83209-8007, USA

*These authors contributed equally to this work

†Author for correspondence (e-mail: rodnkenn@isu.edu)

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Summary

Recent data from our laboratory suggest that sex steroids promote contractile function in cardiac muscle of rainbow trout (*Oncorhynchus mykiss* Walbaum), and there are sex differences in hormone signaling and cardiac function. The current study investigated whether steroid-induced inotropism in electrically paced (0.5 Hz, 14°C) ventricle strips at 90% L_{max} (1) has a metabolic requirement for exogenous glucose and (2) is associated with enhanced intracellular Ca^{2+} storage and release from the sarcoplasmic reticulum (SR). We also explored whether sex differences exist in extracellular Ca^{2+} (Ca^{2+}_o) or cardiac sensitivity to Ca^{2+}_o. In the absence or at low concentrations (1 or 2 mmol l^{-1}) of exogenous glucose, resting tension and relaxation time were increased selectively in cardiac tissue from females. Increasing glucose promoted twitch force in a bell-shaped manner, with 5 mmol l^{-1} representing the optimal concentration for both sexes. The positive inotropic effects of physiological concentrations of testosterone (T) and 17β-estradiol (E2) in male and female trout ventricle strips, respectively, developed slowly (10–45 min) and were not apparent in glucose-free medium, in medium containing iodoacetate (IAA), an inhibitor of glycolysis, or medium containing 5 mmol l^{-1} lactate or pyruvate. Male ventricle strips had increased inotropic responses to glucose and T compared with female strips exposed to glucose and E2. Furthermore, sexually maturing males showed a greater inotropic response than immature males or females. Pretreatment with ryanodine (a specific blocker of SR Ca^{2+} release) also eliminated the inotropic effects of sex steroids and exogenous glucose and reduced the post-rest potentiation of contractile force (a marker of SR Ca^{2+} storage). By contrast, the inotropic effects of epinephrine (Epi) or elevated Ca^{2+}_o were faster (developing within 1–3 min) and were not diminished by the presence or absence of glucose or by pretreatment with IAA or ryanodine. Sex differences were also found in responsiveness to caffeine (males > females) and the relationship between Ca^{2+} concentration and force development above baseline. The Ca^{2+}_{50} was lower in female cardiac tissue than males, suggesting greater Ca^{2+} sensitivity, and although plasma albumin was higher in females, total and ionized plasma Ca^{2+} did not differ between the sexes. For the first time, our study highlights the importance of extracellular glucose, glycolytic activity and SR Ca^{2+} storage and release for sex steroid-induced inotropism in the trout ventricle. Conversely, the inotropes Epi and elevated [Ca^{2+}_o] do not require the presence or metabolism of exogenous glucose or the SR for signaling their positive effects on contractility. These results also demonstrate novel sex-related differences in cardiac reliance on exogenous glucose, Ca^{2+} sensitivity and SR function and thus should be considered in future studies.

Key words: glucose, glycolysis, steroid hormone, cardiac function, inotropism, calcium, sarcoplasmic reticulum, rainbow trout, *Oncorhynchus mykiss.*

Introduction

For all vertebrates, the maintenance and regulation of cardiac contractility are crucial considerations, inasmuch as they help determine stroke volume of the heart, cardiac output and therefore the vascular delivery of oxygen and nutrients to tissues. We recently demonstrated that sex steroids and cortisol at physiological concentrations promote ventricular contractility in rainbow trout (*Oncorhynchus mykiss* Walbaum) in a sex-dependent manner (Farrar and Rodnick, 2004). Specifically, testosterone (T) and 17β-estradiol (E2) modulated cardiac function in males and females, respectively. These positive inotropic effects required hormone binding to specific receptors, synthesis of nitric oxide and polyamines and varied with hormone concentration and contraction frequency. Stimulation of the cardiac contractile state, or positive inotropy, can occur through several diverse mechanisms

(Siegl, 1986), many of which elevate intracellular Ca^{2+} (Ca^{2+}_i). Although we identified potential mechanisms and intracellular signals for steroid-induced inotropism and sex differences in hormone responsiveness, key issues remain to be resolved. For example, given an increased requirement for ATP production during elevated myocardial performance, the importance of metabolic mechanisms for steroid-induced inotropism should be considered. Moreover, to the best of our knowledge, a linkage between cardiac energetics, steroid-induced inotropism and Ca^{2+} storage and homeostasis in cardiomyocytes has not been examined previously in a non-mammalian species.

Most cells are dependent upon glucose uptake and metabolism as a source of ATP. Previous studies of the mammalian heart highlight a dependence of cardiac steroidal glycosides on extracellular glucose and glycolysis for their positive inotropic effects (Bhattacharyya and Vassalle, 1981; Ogbaghebriel and Dresel, 1988; Ogbaghebriel and Dresel, 1989). Glycolytically produced ATP appears to be uniquely suited for the control of myocardial Ca^{2+}_i levels in mammals (Kusuoka and Marban, 1994; Xu et al., 1995) and allows for effective relaxation of the teleost heart under hypoxic conditions (Bailey et al., 2000; Gesser, 2002). Our earlier studies demonstrated that contractile activity increases glucose uptake in the eel (*Anguilla rostrata* LeSueur) heart (Rodnick et al., 1997); however, it is not clear whether extracellular glucose and glycolysis influence teleost cardiac contraction and relaxation under normoxic conditions or in response to cardiotonic compounds. While the normal mammalian heart can use either carbohydrates (glucose, glycogen and lactate) or fatty acids for oxidative metabolism and the provision of ATP during continuous contraction, the contribution of the glycolytic pathway to ATP production is low under aerobic conditions (Neeley and Morgan, 1974). The fish heart, which receives a lower oxygen supply and experiences more variable extracellular conditions than the mammalian heart, may be more dependent upon exogenous glucose as a fuel source, and anaerobic glycolysis for ATP production (Driedzic, 1992). Theoretically, sex steroids could promote 'metabolic inotropism' in the heart of rainbow trout, whereby glucose uptake, glycolytic activity and metabolic production of ATP are enhanced.

Mechanisms that underlie the steroid-induced inotropism should increase systolic Ca^{2+}_i and there are several possible sources for the Ca^{2+} involved in cardiac muscle activation. In teleost fish, extracellular Ca^{2+} (Ca^{2+}_o) is considered to be the primary source of Ca^{2+} for cardiac contraction (Tibbits et al., 1991), and controversy still exists regarding the role of the sarcoplasmic reticulum (SR) in Ca^{2+} cycling. Although previous studies suggest that the SR is extremely limited in most fishes (Santer, 1985), and the release of Ca^{2+} from the SR is not necessary to activate contraction in trout (Tibbits et al., 1991; Keen et al., 1994), the SR is apparently capable of modifying Ca^{2+}_i homeostasis and excitation–contraction coupling (Aho and Vornanen, 1998; Hove-Madsen et al., 1998). Research on the rainbow trout suggests that body temperature and contraction frequency affect the contribution of SR Ca^{2+} to the activator Ca^{2+} concentration (Keen et al., 1994; Shiels and Farrell, 1997). Interestingly, there appears to be an optimum frequency (~0.5 Hz) for steroid-induced inotropism in the isolated ventricle strips at 14°C, and the onset of steroid actions is gradual (starting at 10–15 min, peaking after 30–40 min) compared with epinephrine (Epi; 1–3 min) (Farrar and Rodnick, 2004). We therefore hypothesized that a mechanism other than the rapid activation of voltage-gated Ca^{2+} channels and sarcolemmal Ca^{2+} flux is responsible for elevating systolic Ca^{2+}_i and promoting steroid-induced inotropism in the heart of rainbow trout. Moreover, because glycolytically produced ATP is thought to selectively modulate the activity of the SR Ca^{2+} pump in the mammalian heart (Xu et al., 1995) and endoplasmic reticulum of eukaryotic cells (Martinez Zaguilan and Wesson, 1996), preferential storage and mobilization of SR Ca^{2+} could be the central mechanism for steroid-induced inotropy in the trout heart.

There is a growing appreciation of sex differences in myocardial function and Ca^{2+} homeostasis in mammals. Evidence suggests that there are sex differences in (1) intrinsic contractile properties (Capasso et al., 1983); (2) the positive inotropic response to Ca^{2+}_o (Wang et al., 1998); (3) Ca^{2+} channels in the heart (Ishii et al., 1988) and (4) hormone responsiveness (Capasso et al., 1983; Schwertz et al., 1999). Whether there are sex differences in $[Ca^{2+}_o]$ or cardiac sensitivity to Ca^{2+} in fishes is not known. In addition, the possibility that sex differences exist in cardiac energy metabolism, Ca^{2+} homeostasis and contractility warrants investigation. Thus, the purpose of the present study was to investigate the role of exogenous glucose, glycolysis and the SR for steroid-enhanced cardiac contractility in male and female rainbow trout. We also examined whether there are sex differences in plasma Ca^{2+}, Ca^{2+} sensitivity and contractile properties of cardiac tissue in the presence of inotropic agents. Portions of this work have been presented previously in abstract form (Pierson et al., 2003; Battiprolu and Rodnick, 2004). The major finding is that glucose, by itself, can be regarded as a metabolic inotrope, and steroid-induced inotropism requires exogenous glucose and glycolytic activity and is closely related to SR function. For the first time, we also show sex differences in the effectiveness of glucose, Ca^{2+} sensitivity and contractile properties of ventricle strips *in vitro*.

Materials and methods

Experimental animals

Ten to 12 month-old male and female rainbow trout were obtained from Clear Springs Foods, Inc. (Buhl, ID, USA). Male fish were either approaching sexual maturity [gonadosomatic index (GSI) >1.0%] with increased ventricle size [relative ventricle mass (RVM) >0.10%] or were immature, and all females were immature. Our initial studies of glucose concentration–response on cardiac performance (July–October 2004) included sexually maturing males (N=26), immature males (N=24) and immature females (N=21); however, all other experiments used immature males

and females. Fish were transported to the Aquatics Research Facility at Idaho State University and held in 1000-l circular tanks containing filtered, dechlorinated water at 14±1°C. Fish were fed commercial trout pellets (1% of body mass every other day), exposed to a constant 12 h:12 h light:dark photoperiod and held for at least one week before experiments. All experiments were conducted in accordance with the National Institutes of Health Guidelines in the USA, Department of Health Education and Welfare Publication No. NIH 78-23 (1978), and were approved by the Animal Welfare Committee at Idaho State University. Unless noted otherwise, chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and were of analytical grade.

Ventricle strip preparation

Fish were netted rapidly and killed by a sharp blow to the head. Blood for measurement of biochemical variables was drawn from the caudal vessels using sterile 22 g needles and 3 ml Vacutainers® that contained lithium heparin (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged (3000 ***g*** for 10 min at 4°C) to isolate plasma (see below). The ventricle was quickly excised, weighed and immediately placed in ice-cold, modified teleost Ringer. This basic medium contained (in mmol l^{-1}) 111 NaCl, 5 KCl, 0.5 NaH_2PO_4, 10 $NaHCO_3$, 1.5 $CaCl_2$ and 1.0 $MgSO_4$, was equilibrated with 0.5% CO_2:99.5% O_2 and had a pH of 7.6 at 14°C. Concentrations reported for all chemicals are final values in the tissue baths. The sex of each fish was determined by visual or microscopic examination of the gonads, and gonad mass was measured. Uniform strips (weighing 15–25 mg, approximate dimensions 4–5 mm long × 0.7 mm wide) were cut from each ventricle using a single-edge razor blade. Each strip was vertically mounted, clamped at its base, tied at the other end with surgical silk (3-0) and attached to a Kent isometric transducer (Model TRN002; Litchfield, CT, USA). Strips were suspended in 30 ml tissue baths containing Ringer solution, between platinum wires, and oxygenated throughout the experiment. Each ventricle strip was used for only one experiment. The temperature of the tissue baths was maintained at 14±1°C with a refrigerated recirculating bath. Strips were stimulated with a voltage that elicited full contraction (60 V) at a physiological frequency (0.5 Hz) with 5 ms square wave pulses (Grass S88 Stimulator; Grass Medical Instruments, Quincy, MA, USA). The length of each strip was increased gradually until maximum isometric force production (L_{max}) was achieved and then muscle length was reduced to 90% L_{max} to avoid damage to the preparation. In preliminary studies, we determined that the inotropic effects of both extracellular glucose and sex steroids are not realized when this preparation is maintained at 100% L_{max} (data not shown), suggesting possible tissue damage or common pathways for length-dependent, substrate- and hormone-induced inotropism.

After a 60 min equilibration period to allow for recovery from tissue cutting and stabilization of twitch force (F) at 90% L_{max}, we measured F, time to peak force (t_p), time to 80% relaxation ($t_{0.8r}$) and resting tension for another 60 min using a data acquisition system (BioPac MP100; Santa Barbara, CA, USA) and software (Acqknowledge v. 3.5.5; BioPac). As pointed out by Hartmund and Gesser (Hartmund and Gesser, 1996), this preparation cannot be regarded as truly isometric because of its nonhomogenous orientation of contracting myocytes. Thus, changes in F and resting tension were normalized (%) to the measurements taken at the end of the equilibration period. The 100% value for each strip (control and experimental) was established at the end of the equilibration period. Thus, depending on the degree of positive or negative inotropy, final performance measurements for control and experimental ventricle strips were above or below 100%, respectively.

Measurement of plasma characteristics

Osmolality was measured using a vapor pressure osmometer (Model 5520; Wescor, Ogden, UT, USA). Albumin concentrations were determined using the bromocresol green reagent (Eagle Diagnostics, Desoto, TX, USA) and bovine serum albumin standards according to manufacturer's directions. Glucose was measured using the Infinity® Glucose Hexokinase Liquid Stable Reagent (No. TR15498; ThermoTrace, Noble Park, Victoria, Australia).

Ionized and total calcium determinations

Blood was drawn anaerobically from the caudal vessels and centrifuged to isolate plasma. One ml of the plasma was immediately used for ionized Ca^{2+} measurements while the remaining plasma was frozen under liquid N_2 until assayed for total Ca^{2+}. Ionized Ca^{2+} was measured using a Ca specific electrode (Thermo Orion, Beverly, MA, USA). Plasma (1 ml) was added to 5 ml of a thermostatically controlled (14±1°C) buffer solution (Hepes, 20 mmol l^{-1}, pH 7.7), brought up in filtered (0.22 μm) 0.9% (w/v) NaCl. Millivolt readings were recorded using the Beckman pH meter (Model 11; Fullerton, CA, USA) and compared to values for $CaCl_2$ standards according to Beer's Law equation. Total plasma Ca was measured using a spectrophotometric assay (Arsenazo, Eagle Diagnostics, De Soto, TX, USA) according to the manufacturer's instructions.

Experimental protocols

Requirements of exogenous glucose and glycolysis for contractile performance

Preliminary experiments showed that extracellular glucose, by itself, exerted positive inotropic effects in cardiac tissue from male and female rainbow trout. To complete a dose response for glucose, ventricle strips from sexually maturing males, immature males and immature females were incubated initially in glucose-free media and then either remained glucose-free or received 1, 2, 5 or 10 mmol l^{-1} D-glucose or an isomolar concentration of mannitol at the end of the equilibration period (Fig. 1). For all subsequent experiments, only immature males and females were used. We then evaluated the importance of exogenous glucose at a physiological concentration (5 mmol l^{-1}) and glycolysis on

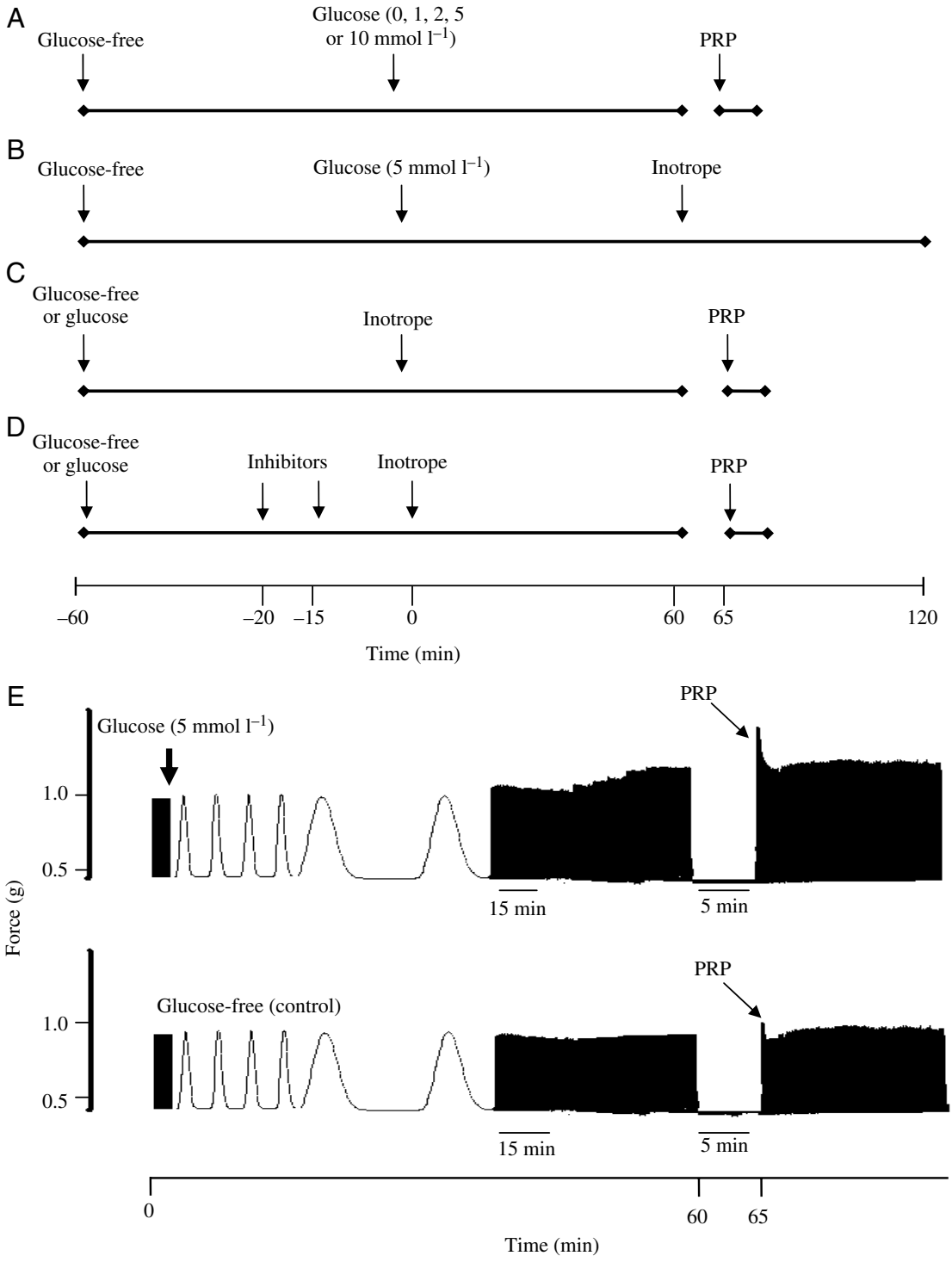

Fig. 1. Experimental design involving additions of D-glucose, inotropes and metabolic inhibitors to cardiac tissue *in vitro*. In all experiments, ventricle strips from rainbow trout were incubated for 60 min in either glucose or glucose-free media and electrically stimulated (0.5 Hz) at 14°C. (A) Glucose dose–response. Zero glucose reflects control ventricle strips remaining in glucose-free media for the entire experiment. (B) Combined effects of glucose (5 mmol l^{-1}) and inotropes: T (0.3 μmol l^{-1}) in males; E2 (1 nmol l^{-1}) in females; and Epi (1 μmol l^{-1}) or Ca^{2+}_o (5 mmol l^{-1}) in both sexes. (C) Effects of inotropes mentioned above and caffeine (8 mmol l^{-1}), with and without glucose. (D) Effects of inotropes in ventricle strips pretreated with inhibitors iodoacetate (IAA) (0.4 mmol l^{-1}) or ryanodine (10 μmol l^{-1}). (E) Original recording of isometric twitch force in ventricular muscle strips from a male rainbow trout. After ventricle strips were stretched to optimal length (90% L_{max}) and after mechanical stabilization for 60 min, glucose (5 mmol l^{-1}) was added to one strip and the other remained glucose-free (control) for 60 min. The extent of stored Ca^{2+} in the sarcoplasmic reticulum was determined by post rest potentiation (PRP). Stimulation of ventricle strips was discontinued for 5 min, prior to PRP measurements. PRP was higher in glucose-treated ventricle strips when compared with the corresponding glucose-free control. Values are means ± s.e.m. (N=6–11 strips per group).

contractile performance, both independently and after exposure of ventricle strips to one of the following inotropic agents: T (0.3 μmol l^{-1}), E2 (0.01 μmol l^{-1}), Epi (1 μmol l^{-1}; American Regent Laboratories, Inc., Shirley, NY, USA) or elevated Ca^{2+}_o (5.0 mmol l^{-1}). T and E2 were solubilized in absolute ethanol to produce stock solutions of 1.0 mmol l^{-1}, which were stored at –20°C. To determine whether glycolysis is necessary for the inotropic effects of glucose, ventricle strips receiving T, E2, Epi or Ca^{2+} were pretreated with iodoacetate (IAA) for 20 min prior to the addition of glucose, T, E2, Epi or additional Ca^{2+}. IAA affects a number of sulfhydryl group-containing enzymes; however, we used a concentration (0.4 mmol l^{-1}, made fresh and dissolved in Ringer) that is specific to glyceraldehyde-3-phosphate dehydrogenase and inhibits glycolysis in the trout heart by approximately 70% (Gesser, 2002).

Role of SR and Ca^{2+}_i stores in steroid-induced inotropism

Three complementary approaches were used to investigate the importance of the SR for cardiac contractility and steroid-induced inotropism in the rainbow trout heart. After the 60 min equilibration period, selected ventricle strips were pretreated with either ryanodine (10 μmol l^{-1}) or caffeine (8 mmol l^{-1}) for 15 min, a time that maximized the desired effects of both compounds without prolonged exposures. Both chemicals were dissolved in Ringer just prior to experiments. At these concentrations, ryanodine inhibits (El-Sayed and Gesser, 1989) and caffeine stimulates (Coyne et al., 2000) release of Ca^{2+} from the SR. To complement the ryanodine and caffeine experiments, we also used the measurement of post rest potentiation (PRP) of *F*. PRP is considered to be indicative of SR Ca^{2+} storage and subsequent release during the resumed contractions (El-Sayed and Gesser, 1989). After 15 min incubation with ryanodine or caffeine, T was added to the media for male ventricle strips and E2 for female strips. Strips from both sexes were also exposed to glucose (5 mmol l^{-1}), Epi or elevated Ca^{2+}, and contractile performance was monitored for an additional 45 min. Electrical stimulation of ventricle strips was discontinued at the end of the second hour of incubation for 5 min, just prior to PRP measurements. *F*, t_p and $t_{0.8r}$ were recorded for the first contraction following the resumption of stimulation. A summary of the experimental protocols is shown in Fig. 1.

Effects of extracellular Ca^{2+} on contractile force in ventricle strips

Ca^{2+} sensitivity was analyzed using the absolute and percent force changes by fitting the Ca^{2+}-twitch force relation to a modified Hill equation (van der Velden et al., 2003):

$$F(Ca^{2+}) / F_0 = [Ca^{2+}]^{nH} / (Ca_{50}{}^{nH} + [Ca^{2+}]^{nH}) ,$$

where *F* is the baseline force at 1.5 mmol l^{-1}, F_0 represents the force at a saturating level of extracellular Ca^{2+} (Ca^{2+}_o), nH is the steepness of the curve, and Ca^{2+}_{50} denotes the midpoint of the relation. Ca^{2+}_{50} defined the $[Ca^{2+}_o]$ at which half of the maximum change above baseline occurs and serves as a measurement of sensitivity to Ca^{2+}_o. We postulated that increasing $[Ca^{2+}_o]$ should increase trans-sarcolemmal Ca^{2+} influx in isolated cardiac tissue. Ventricle strips from male and female rainbow trout were exposed to increasing concentrations (0.5 mmol l^{-1} every 5 min) of Ca^{2+} for 60 min. Thus, the relationship between twitch force and Ca^{2+} was developed over a range of $[Ca^{2+}_o]$: 1.5–7.5 mmol l^{-1}. A 1.0 mol l^{-1} $CaCl_2$ stock solution was made with glucose-free Ringer or Ringer containing glucose (5 mmol l^{-1}). For each strip, the percent change of *F* was calculated at each time interval according to the baseline *F* value before Ca^{2+} additions.

Data analysis

Performance variables (*F*, t_p, $t_{0.8r}$ and resting tension) were averaged from continuous recordings of five waveforms at 5 min intervals after the 60 min equilibration period. Data are expressed as means ± s.e.m. of either absolute values (basal or active tension) or percent change of basal inotropism. Cardiac performance between control and experimental strips was assessed by a two-way (effect of sex and treatment) ANOVA with Bonferroni and LSD *post-hoc* corrections using SAS, Inc. software (Cary, NC, USA). Ca^{2+}_{50} values for male and female rainbow trout were also compared using a two-way (sex and glucose) ANOVA. A one-way ANOVA and Student's *t*-tests were conducted to analyze physical and plasma characteristics for both sexes, respectively. Significant statistical differences ($P<0.05$) are indicated in the text, tables and figures.

Table 1. *Physical characteristics of experimental rainbow trout*

Variables	Maturing males (*N*=26)	Immature males (*N*=64)	Immature females (*N*=60)
Body mass (g)	468±23	542±41	421±16
Fork length (cm)	33.4±0.9	34.8±1.0	31.3±1.3
Ventricle mass (mg)	630±3*	482±4	396±4
RVM (%)	0.24±0.04*	0.12±0.01	0.10±0.01
Gonad mass (g)	15.02±1.12*	0.58±2.01	0.82±0.87
GSI (%)	4.49±0.31*	0.12±0.37	0.19±0.17

Values are means ± s.e.m. RVM = (ventricle mass/body mass)×100; GSI = (gonad mass/body mass)×100. *$P<0.01$ *versus* immature males and females.

Table 2. *Plasma characteristics of male and female rainbow trout*

Variables	Males	Females
Osmolality (mosmol kg^{-1})	300±2 (49)	305±1 (49)
Albumin (g dl^{-1})	17.1±0.5 (49)	18.9±0.5* (49)
Glucose (mmol l^{-1})	5.12±0.24 (15)	5.09±0.23 (15)
Ionized Ca^{2+} (mmol l^{-1})	1.61±0.15 (25)	1.79±0.18 (25)
Total Ca^{2+} (mmol l^{-1})	2.22±0.20 (25)	2.37±0.24 (25)

Values are means ± s.e.m. (*N*). *$P<0.05$ *versus* males.

Table 3. *Effects of glucose at different concentrations on absolute basal tension (g)*

	Maturing males		Immature males		Immature females	
Glucose (mmol l^{-1})	Initial	Final	Initial	Final	Initial	Final
0	1.49±0.03	1.57±0.02	0.62±0.08	0.68±0.03	0.50±0.05	0.67±0.03[a,*]
1	1.12±0.04	1.16±0.06	0.52±0.05	0.56±0.06	0.84±0.07	1.03±0.05[b,*]
2	1.36±0.05	1.40±0.08	0.64±0.07	0.68±0.09	0.74±0.06	0.88±0.05[b,*]
5	1.11±0.04	1.13±0.07	0.59±0.03	0.61±0.08	0.63±0.04	0.68±0.07[c]
10	1.13±0.08	1.18±0.06	0.61±0.04	0.65±0.06	0.38±0.07	0.41±0.09[c]

Values are means ± s.e.m. N=8–26 strips per group. Absolute basal tension (g): initial values reflect the end of 60 min equilibration, and final values were taken at the end of experiments. Resting tension was selectively increased in female ventricle strips not receiving glucose (0), or at 1 and 2 mmol l^{-1} glucose. *P<0.05, differences between immature females and males (final minus initial values). Dissimilar letters denote significant differences between treatments (P<0.05). Percentage changes are shown in Fig. 2.

Table 4. *Effects of glucose (5 mmol l^{-1}) on the performance of rainbow trout ventricle strips at 0.5 Hz*

	Glucose-free			5 mmol l^{-1} glucose		
Variables	Maturing males	Immature males	Immature females	Maturing males	Immature males	Immature females
F (g)						
Initial	0.52±0.07	0.55±0.04	0.51±0.05	0.47±0.05	0.48±0.06	0.49±0.07
Final	0.62±0.05	0.64±0.07	0.58±0.05	0.72±0.03*	0.65±0.01*	0.61±0.03*
t_p (ms)	390±4	360±4	310±3*	410±3	380±3	360±4
$t_{0.8r}$ (ms)	350±3[†]	370±4[†]	420±4[†]	300±2	330±3	320±3

Values are means ± s.e.m. Maturing males (N=26), immature males (N=24), immature females (N=21). F, twitch force; initial values reflect the end of 60 min equilibration, and final values were taken at the end of experiments (percentage changes are shown in Fig. 3). t_p, time from stimulus to peak twitch force; $t_{0.8r}$, time from peak twitch force to 80% relaxation. *P<0.05, 5 mmol l^{-1} glucose *versus* glucose-free (final minus initial values). [†]P<0.05, glucose-free *versus* glucose for a given group.

Results

Physical characteristics of experimental animals and plasma measurements

Overall, body masses and fork lengths of male and female rainbow trout were not different (Table 1). However, ventricle mass, RVM, gonad mass and GSI were all significantly higher in sexually maturing male fish compared with immature males and females (ANOVA, $F_{2,147}$=164.27, P<0.01; Table 1). Plasma albumin was higher in female fish (t-test, P=0.040), although no sex differences were observed in osmolality, glucose and Ca^{2+}_o (total and ionized; Table 2).

Effects of glucose on cardiac performance: concentration and sex differences

Two traces of experiments involving ventricle strips are shown in Fig. 1E. To appreciate the magnitude of change for resting and developed tension (twitch force, F), both absolute (Tables 3, 4) and relative values (percentages; Figs 2, 3) are provided. For all experimental conditions, resting tension at 90% L_{max} ranged from ~0.4 g to 1.0 g at the end of equilibration (Table 3, initial values); however, sexually maturing males had slightly higher values than immature fish. When ventricle strips were incubated with glucose-free Ringer, resting tension increased selectively in female rainbow trout and was also elevated in the presence of 1 or 2 mmol l^{-1} glucose (Table 3; Fig. 2; ANOVA, $F_{7,63}$=7.78, P=0.032). However, resting tension remained stable in the presence of plasma levels of glucose (5 mmol l^{-1}). By contrast, resting tension in cardiac tissue from male fish (immature and sexually maturing) did not increase in the absence or presence of 1, 2, 5 or 10 mmol l^{-1} glucose (ANOVA, $F_{7,63}$=2.31, P=0.312). In the presence or absence of 5 mmol l^{-1} glucose, F did not decrease after the 60 min equilibration period for up to 2 h (data not shown). This finding provides evidence that strips did not fatigue under the experimental conditions employed (aerobic, 0.5 Hz, 14°C).

For both sexes, addition of glucose to the medium significantly improved contractile performance over glucose-free controls (Table 4; Fig. 3). The positive effect of glucose on F was concentration-dependent, reaching a maximum value at 5 mmol l^{-1}, and exhibited a bell-shaped relationship. At 5 mmol l^{-1} glucose, F increased to 149±3% in sexually maturing males after 45 min *versus* 135±2% in immature males and 123±2% in females after 25 min, compared with glucose-free conditions (ANOVA, $F_{7,63}$=11.53, P=0.021). The addition of glucose also increased t_p in females selectively (Table 4; ANOVA, $F_{7,63}$=5.14, P=0.047) and decreased $t_{0.8r}$ in both male (Table 4; ANOVA, $F_{7,63}$=5.42, P=0.043) and

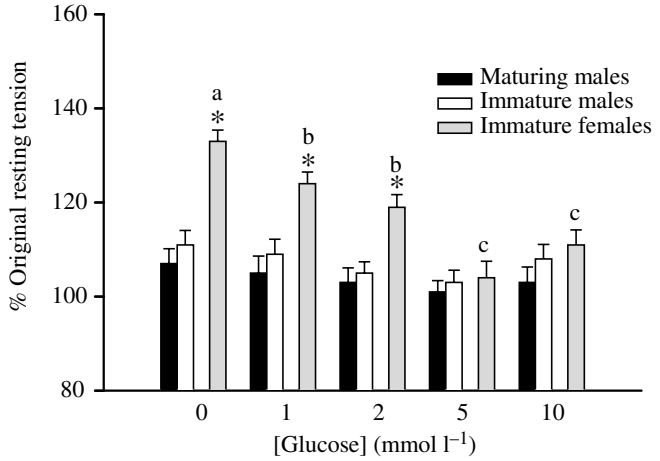

Fig. 2. Effects of exogenous glucose on resting tension. In females, resting tension was maintained in media containing 5 or 10 mmol l^{-1} glucose but increased in glucose-free (0), 1 and 2 mmol l^{-1} glucose conditions (dissimilar letters denote significant differences between treatments, *P<0.05). There were no significant differences in resting tension for males at all concentrations tested. Immature females also showed higher resting tension compared with immature and sexually maturing males at 0, 1 and 2 mmol l^{-1} glucose. Values are means ± s.e.m. (N=8–26 strips per group).

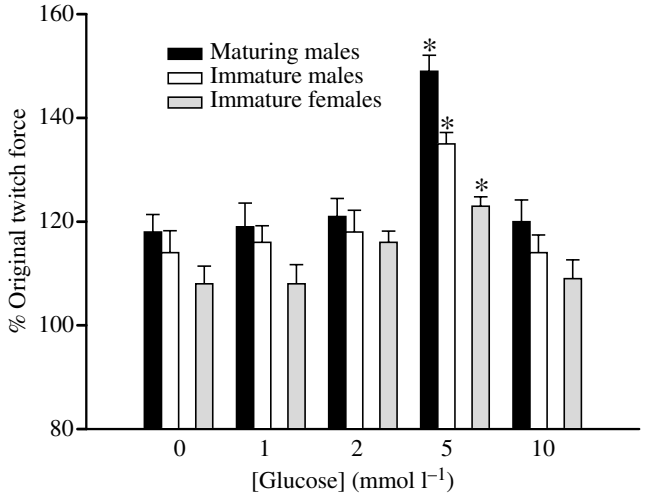

Fig. 3. Effects of exogenous glucose on twitch force (F). During the 60 min equilibration period, ventricle strips were incubated in the absence of any exogenous substrate. In sexually maturing males, F increased during the experimental period with 5 mmol l^{-1} glucose present, compared with 0, 1, 2 and 10 mmol l^{-1} glucose, and was higher than that in immature males and females at all concentrations tested. F was also higher in immature males at 5 mmol l^{-1} glucose compared with females and at all concentrations tested. In females, 5 mmol l^{-1} glucose increased F compared with 0, 1 and 10 mmol l^{-1} values. *P<0.05. Values are means ± s.e.m. (N=8–26 strips per group).

female fish (ANOVA, $F_{7,63}$=6.32, P=0.014), but to a much greater extent in females. PRP values at 5 mmol l^{-1} glucose were (1) elevated in sexually maturing males (178±4%, N=26) compared with immature males (163±3%, N=24) and females (160±4%, N=21, ANOVA, $F_{7,63}$=4.32, P=0.0473) and (2) increased relative to ventricle strips receiving 0, 1, 2 or 10 mmol l^{-1} glucose (ANOVA, $F_{7,63}$=5.38, P=0.029, N=8–10).

The importance of exogenous glucose and glycolysis for steroid-induced inotropism

Glucose-free medium failed to promote the inotropic effects of sex steroids on ventricle strips from sexually immature male and female rainbow trout (Fig. 4). However, similar to our previous studies (Farrar and Rodnick, 2004), F increased following the addition of T (males) or E2 (females) when ventricle strips were incubated in glucose-containing Ringer for 60 min (Fig. 4). Control ventricle strips (pooled in Figs 4–7) exhibited slightly, but not significantly, reduced F at the end of the experimental period. This observation reflects the presence of strips receiving ethanol (sex steroid controls) or lacking glucose (females only) in the incubation media. In related experiments (N=7–10 for males and females), we substituted glucose with an isomolar concentration (5 mmol l^{-1}) of lactate or pyruvate (oxidative carbohydrate substrates) and showed that (1) the inotropic effects of steroids were not realized and (2) PRP was not apparent with pyruvate or lactate present (data not shown).

Similar to incubation in the absence of exogenous glucose, exposure of ventricle strips to IAA (with or without 5 mmol l^{-1} glucose) completely blocked the inotropic effects of glucose and sex steroids (Fig. 5), which differed from comparable strips treated with these inotropes, but not IAA (ANOVA, $F_{5,40}$=4.93, P=0.019). Compared with controls (glucose and glucose-free conditions), the addition of IAA did not affect (1) F (Fig. 5; ANOVA, $F_{5,40}$=1.37, P=0.286), (2) resting tension (ANOVA, $F_{5,40}$=2.04, P=0.194) and (3) $t_{0.8r}$ in immature males (ANOVA, $F_{5,40}$=3.24, P=0.064). However, $t_{0.8r}$ was impaired in females in the presence (260±4 *vs* 320±3 ms, ANOVA $F_{5,40}$=5.26, P=0.041) or absence of glucose (270±2 *vs* 420±4 ms, ANOVA $F_{5,40}$=7.14, P=0.028). Together, these data indicate that exogenous glucose (not stored glycogen or other carbohydrate substrates) and glycolysis are essential for steroid signaling and increasing contractile performance of trout cardiac tissue. Epi and elevated Ca^{2+}_o also increased F (Fig. 4); however, in contrast to exogenous glucose and sex steroids, the effects of Epi or Ca^{2+}_o were rapid, reaching a maximum value within 2–3 min, after which contractility gradually decreased. Furthermore, pretreatment of ventricle strips from either sex with IAA, or withholding glucose from the media, did not diminish the inotropic effects of Epi or Ca^{2+}_o (Fig. 5; ANOVA, $F_{5,40}$=1.89, P=0.249).

The SR and inotropic effects of glucose and sex steroids

Ryanodine had no independent effects on F (Fig. 6; ANOVA, $F_{6,45}$=1.46, P=0.221), suggesting that SR Ca^{2+} release was not a requirement for myofilament activation under control conditions. Ryanodine also markedly reduced the

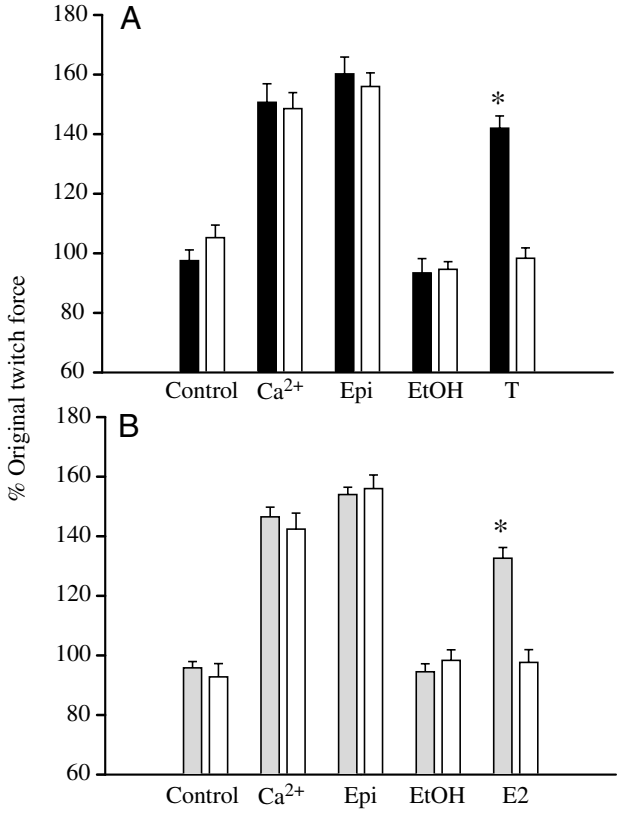

Fig. 4. Effects of Ca^{2+} (5 mmol l^{-1}), epinephrine (Epi; 1 μmol l^{-1}), ethanol (EtOH), testosterone (T; 0.3 μmol l^{-1}) or 17β-estradiol (E2; 1.0 nmol l^{-1}), in the presence (filled bars) or absence (open bars) of exogenous glucose (5 mmol l^{-1}), on performance of ventricle strips from males (A) and females (B). All ventricle strips received exogenous glucose during the 60 min equilibration period. There were only significant differences in ventricle strips exposed to sex steroids. *P<0.05. Values are means ± s.e.m. (N=7–9 strips per group).

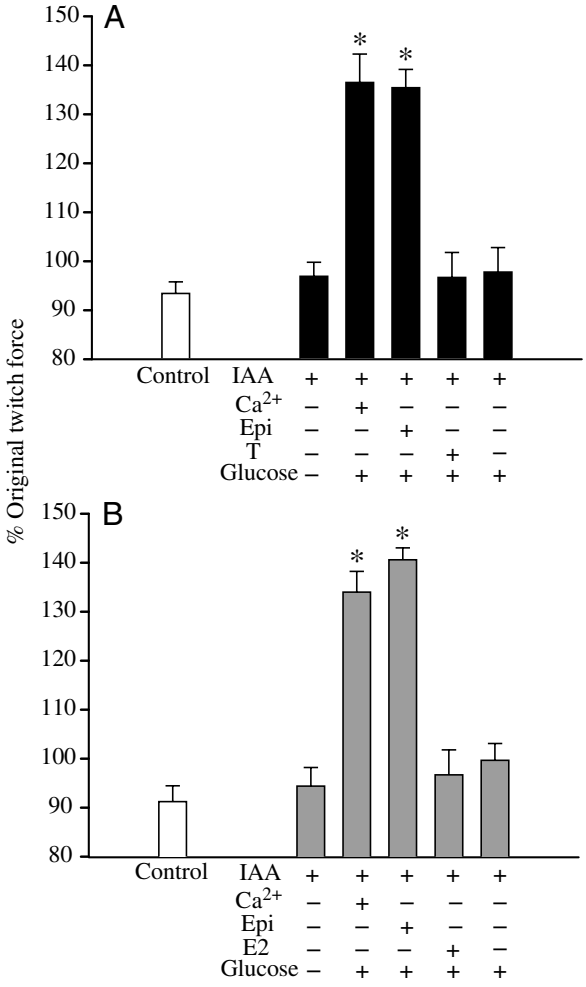

Fig. 5. Effects of glycolytic inhibitor iodoactetate (IAA; 0.4 mmol l^{-1}) on immature male (A) and female (B) cardiac tissue. After a 15 min incubation with IAA, ventricle strips were exposed to either increased Ca^{2+} (5 mmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), 17β-estradiol (E2; 1 nmol l^{-1}) or epinephrine (Epi; 1 μmol l^{-1}). Plus (+) and minus (–) denote presence and absence, respectively, of specific compounds in the incubation medium. For immature males and females, elevated Ca^{2+} or Epi, but not sex steroids, increased twitch force when glucose (5 mmol l^{-1}) was present. *P<0.05. Values are means ± s.e.m. (N=9–11 strips per group).

amplitude of the PRP (Fig. 7; ANOVA, $F_{6,45}$=6.22, P=0.011), providing evidence that Ca^{2+} released from the SR is responsible for the post-rest response. Pretreatment of ventricle strips from both males and females with ryanodine completely blocked the positive inotropism induced by exogenous glucose and T or E2 in the presence of glucose (Fig. 6; ANOVA, $F_{6,45}$=5.93, P=0.018). We also conducted supplementary studies with dantrolene (10 μmol l^{-1}, 15 min preincubation, N=6 for males and females), another inhibitor of Ca^{2+} release from the ryanodine receptor (Paul-Pletzer et al., 2005), and found identical results compared with ryanodine (data not shown). Together, these data suggest that the observed inotropism of glucose and sex steroids involves SR Ca^{2+} release. By contrast, the positive effects of Epi or Ca^{2+}_o on contractility were not diminished by ryanodine (Fig. 6; ANOVA, $F_{6,45}$=2.45, P=0.302) or dantrolene (data not shown). Similar to ryanodine, ventricle strips pretreated with IAA (ANOVA, $F_{5,40}$=4.45, P=0.022) or caffeine (ANOVA, $F_{5,40}$=5.67, P=0.018) diminished PRP (Fig. 7), providing evidence that glycolytic inhibition and caffeine reduced SR Ca^{2+} stores.

Caffeine significantly increased F in strips from both males and females, in the presence or absence of glucose (ANOVA, $F_{3,28}$=5.84, P=0.012 and ANOVA, $F_{3,28}$=4.82, P=0.023; Fig. 8), and the positive effect was more pronounced in males (236±12%) than females (177±7%) (ANOVA, $F_{1,14}$=6.10, P=0.013). Caffeine also completely blocked the inotropic effects of glucose, sex steroids, Epi and elevated Ca^{2+}_o (Fig. 8; ANOVA, $F_{7,56}$=1.84, P=0.392). However, we discovered that even after pretreatment with ryanodine, caffeine had significant inotropic effects in ventricle strips from male and female rainbow trout (data not shown). This finding suggests that caffeine's effects were not confined to the release of SR Ca^{2+}.

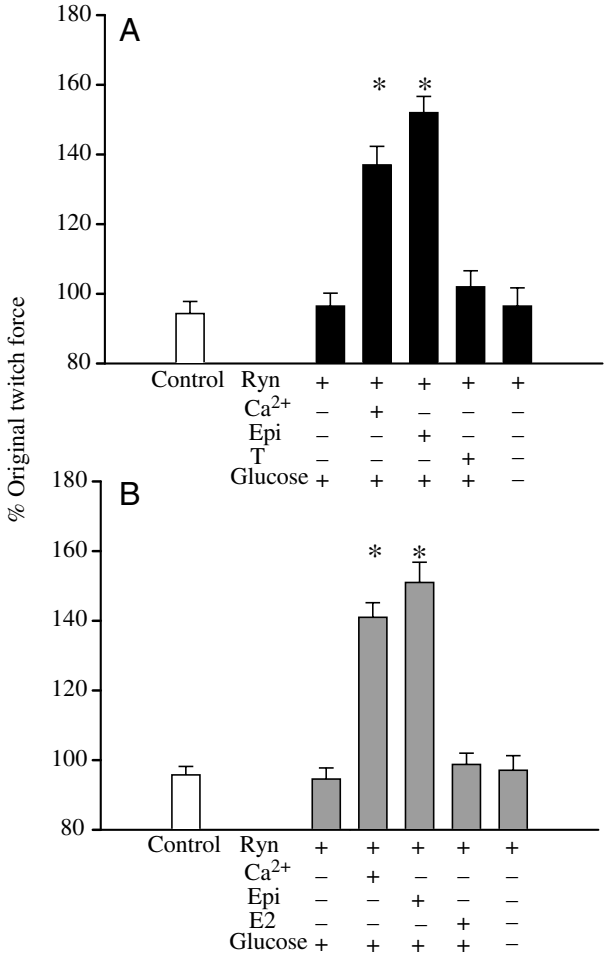

Fig. 6. Effects of ryanodine (Ryn; 10 μmol l^{-1}) in immature male (A) and female (B) cardiac tissue. Ventricle strips were incubated with Ryn 15 min prior to the addition of either increased Ca^{2+} (5 mmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), 17β-estradiol (E2; 1 nmol l^{-1}) or epinephrine (Epi; 1 μmol l^{-1}). Plus (+) and minus (–) denote presence and absence, respectively, of specific compounds in the incubation medium. For immature males and females, elevated Ca^{2+} or Epi, but not sex steroids, increased twitch force when glucose (5 mmol l^{-1}) was present. *P<0.05. Values are means ± s.e.m. (N=6–8 strips per group).

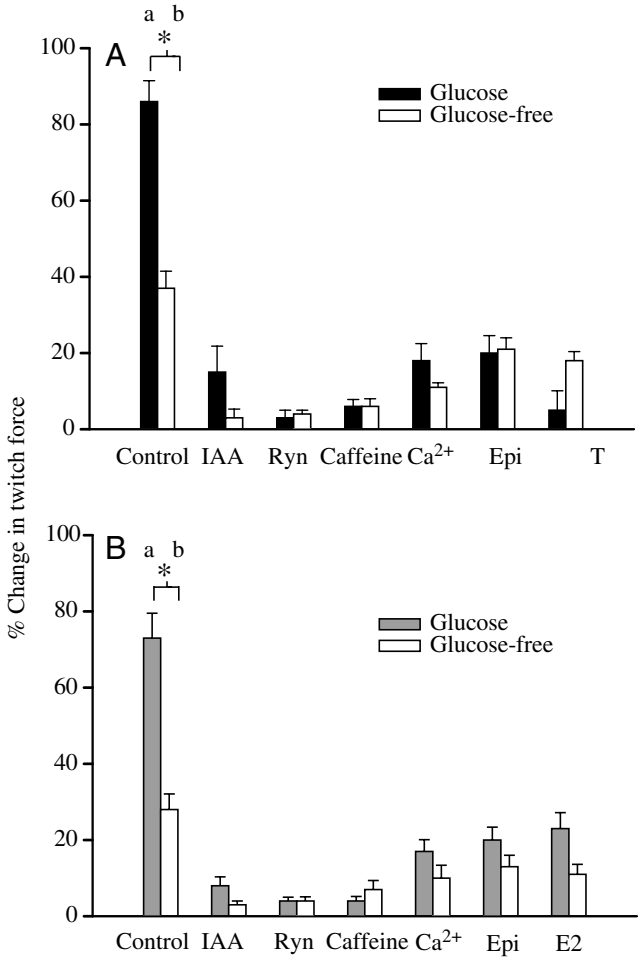

Fig. 7. Effects of various compounds (iodoacetate, (IAA; 0.4 mmol l^{-1}); ryanodine, (Ryn; 10 μmol l^{-1}); caffeine (8 mmol l^{-1}); Ca^{2+} (5 mmol l^{-1}); epinephrine (Epi; 1 μmol l^{-1}); testosterone (T; males; 0.3 μmol l^{-1}); or 17β-estradiol (E2; females; 1.0 nmol l^{-1}) on post-rest potentiation (PRP) in immature males (A) and females (B). In both sexes, ventricle strips receiving glucose had higher PRP than strips without glucose (*P<0.05). Control strips containing glucose also exhibited higher PRP than all other treatments when glucose was present (a denotes P<0.05). PRP for glucose-free, control strips were higher than all other treatments (b denotes P<0.05). However, other than control strips, no significance was observed between glucose *vs* glucose-free treatments (P=0.34). Values are means ± s.e.m. (N=6–11 strips per group).

Calcium sensitivity and calcium-induced contractile properties

Sex differences were found in the relationship between $[Ca^{2+}_o]$ and force development by ventricle strips (Fig. 9). As expected, increasing Ca^{2+}_o elevated F above baseline values ($[Ca^{2+}_o]$=1.5 mmol l^{-1}) in both sexes, with or without exogenous glucose (ANOVA, $F_{3,33}$=6.58, P=0.014), and there were no sex differences in Ca^{2+}-induced F (ANOVA, $F_{3,33}$=1.38, P=0.249). However, the plot of Ca^{2+}_o and percent change of F in females was left of the curve for males when glucose was present (Fig. 9A), and the corresponding Ca^{2+}_{50} was lower for females (2.52±0.09 mmol l^{-1}) than males (2.81±0.07 mmol l^{-1}, ANOVA, $F_{3,33}$=5.75, P=0.003), reflecting greater Ca^{2+} sensitivity in female cardiac tissue. In the absence of glucose, ventricle strips from both sexes were less sensitive to Ca^{2+}_o (male Ca^{2+}_{50}=3.07±0.10 mmol l^{-1}, female Ca^{2+}_{50}=2.93±0.09 mmol l^{-1}; ANOVA $F_{3,33}$=4.51, P=0.027) compared with tissue receiving glucose (Fig. 9B,C), but sex differences were not evident.

Discussion

The influence of sex and sex steroids on the energetics, performance and Ca^{2+} regulatory processes of fish cardiac muscle has been largely ignored. As a result, the present study

examined the role of exogenous glucose, glycolysis and the SR for steroid-enhanced cardiac contractility in ventricle strips from male and female rainbow trout. The data clearly demonstrate the importance of glucose and glucose concentration for contractile force production and the maintenance of resting tension. The current results also provide new evidence for metabolic inotropism in trout cardiac muscle *in vitro* and sex differences in Ca^{2+} homeostasis, Ca^{2+} sensitivity and mechanical function. The presence of exogenous glucose and an active glycolytic pathway are crucial prerequisites for increased contractility of trout ventricle strips following exposure to T and E2, but not Epi or elevated $[Ca^{2+}]_o$. Overall, there is a recurring theme that cardiac tissue from male and female rainbow trout is different, with exogenous glucose being more important for female cardiac function. Sexual maturity also appears to play an important role in cardiac energy metabolism and performance in male rainbow trout.

Sex-dependent effects of extracellular glucose on cardiac performance

Although the focus of this study was steroid-induced inotropism, it was important to measure substrate effects on myocardial function, independent of hormone actions. It's been known for over 55 years that D-glucose increases contractile force and the ability to relax in the substrate-depleted mammalian heart (Webb, 1950). In rat atria, 5.5 mmol l^{-1} glucose, but not similar concentrations of acetate, lactate, pyruvate or fructose, increased the force of contraction (Ko and Paradise, 1973). These authors promoted the early hypothesis that glucose was increasing $[Ca^{2+}]_i$ and therefore Ca^{2+} availability to the contractile apparatus. Under the current experimental conditions (well-oxygenated, contraction frequency 0.5 Hz, 14°C), exogenous glucose increased

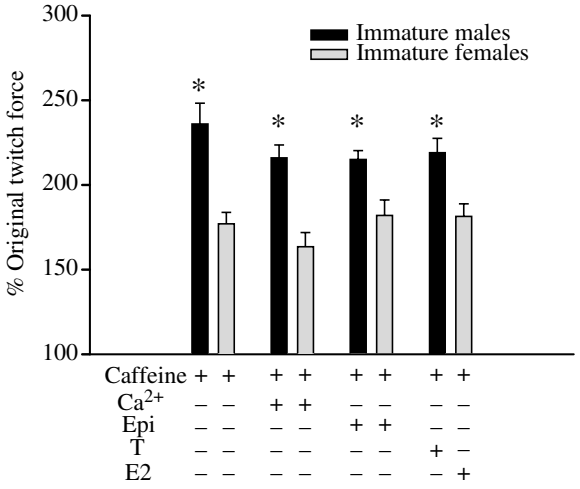

Fig. 8. Effects of caffeine (8 mmol l^{-1}) on contractile performance of cardiac tissue in the presence or absence of elevated Ca^{2+} (5 mmol l^{-1}), epinephrine (Epi; 1 μmol l^{-1}), testosterone (T; males; 0.3 μmol l^{-1}) or 17β-estradiol (E2; females; 1.0 nmol l^{-1}). All ventricle strips were exposed to 5 mmol l^{-1} glucose for 1 h prior to addition of caffeine. After a 15 min exposure to caffeine, each strip received one of the inotropes. Within a sex there were no differences between treatments; however, the increase in twitch force was greater in immature males than females (*P<0.05). Values are means ± s.e.m. (N=7 strips per group).

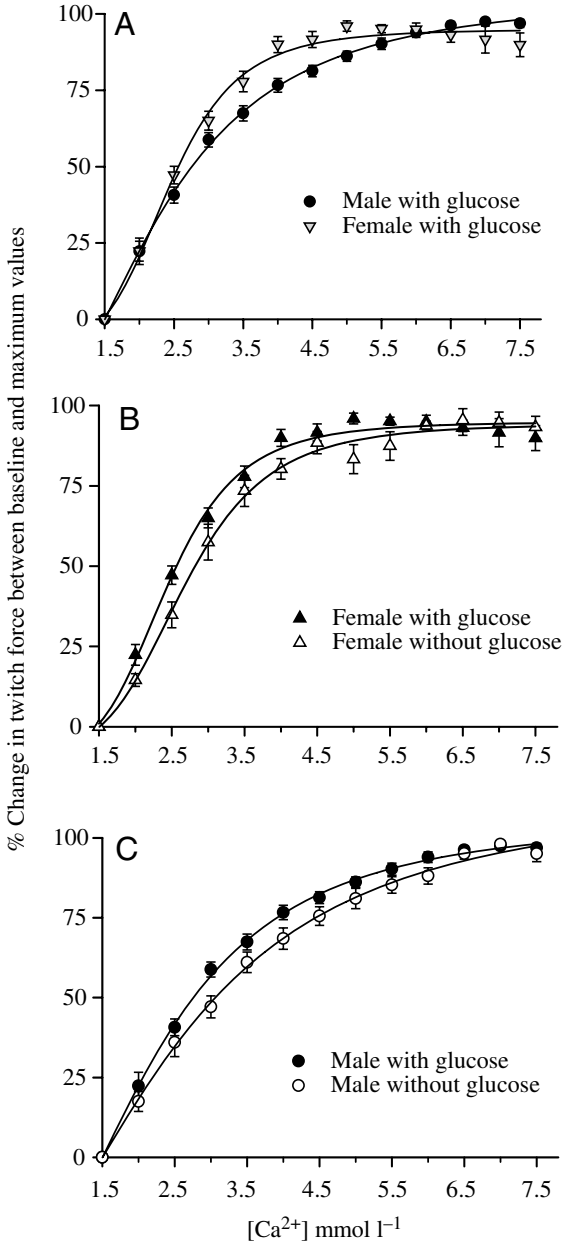

Fig. 9. The relationship between extracellular Ca^{2+} and contractile force. Twitch force is expressed as a percentage of the difference between maximum force development and baseline force at 1.5 mmol l^{-1} Ca^{2+} for (A) male and female ventricle strips receiving 5 mmol l^{-1} glucose; (B) female ventricle strips, glucose *vs* glucose-free and (C) male ventricle strips, glucose *vs* glucose-free. In the presence of glucose, the EC_{50} for Ca^{2+}-dependent force production was lower in females than males (P<0.01). Compared with tissue receiving glucose, glucose-free ventricle strips from both sexes were less sensitive to Ca^{2+} (P<0.05) but sex differences were not evident. Values are means ± s.e.m. (N=8–9 strips per curve).

contractile force of ventricle strips from sexually maturing male, immature male and female rainbow trout. Of particular interest were the findings that the positive effect of glucose on twitch force (F) was concentration dependent, showing a bell-shaped relationship, relatively slow to plateau (25–45 min) and more pronounced in sexually maturing males than immature males and females. In all groups, the maximally effective concentration of glucose for increasing F was 5 mmol l^{-1}, a value that mirrored plasma levels. On the other hand, a previous study by Gesser on rainbow trout (male and female, but not distinguished) ventricle strips using (1) extended exposure (120 min) to anoxia, (2) a higher concentration of glucose (10 mmol l^{-1}) and other aerobic substrates, (3) a higher stimulation frequency (0.2 Hz below the maximum rate) and (4) Epi (10 μmol l^{-1}) did not demonstrate a positive effect of glucose on F after 15 min (Gesser, 2002). Whether anoxia, the presence of higher extracellular glucose and other substrates, Epi or the timing of measurements explain the absence of glucose-induced inotropism cannot be addressed at this time. It is also noteworthy that increasing the concentrations of glucose from 5 to 10 mmol l^{-1} reduced cardiac contractility in the current study, possibly by feedback inhibition of glucose phosphorylation (hexokinase), and therefore glucose utilization, by glucose-6-phosphate accumulation as described in mammalian smooth muscle (Kusuoka and Marban, 1994) and endothelial cells (Vinals et al., 1999). The importance of 'early sexual maturity' for glucose-induced inotropism was a novel, yet incomplete, finding because precocious males, but not females, were common in the population of experimental animals during one of our sampling periods. This observation may reflect underlying, chronic effects of sex steroids on cardiac energy metabolism and Ca^{2+} homeostasis in salmonid fishes.

How does exogenous glucose enhance cardiac function in rainbow trout? The finding that pretreatment of ventricle strips from both males and females with IAA prevented the glucose-induced increase in contractility suggests that the glycolytic pathway plays an essential role. Our studies with ryanodine, dantrolene and PRP data also point to the SR as the major source of activator Ca^{2+} and a vital organelle for glucose and steroid-induced inotropism. Namely, pretreatment of ventricle strips with ryanodine completely blocked the positive inotropism induced by exogenous glucose (males and females), and T (males) or E2 (females) in the presence of glucose. In addition, both glucose- and steroid-induced effects were inhibited by dantrolene, an antagonist of the SR Ca^{2+} release channel. PRP, which reflects SR Ca^{2+} storage and release, was diminished when (1) exogenous glucose was absent, or present at low concentrations (1 or 2 mmol l^{-1}) and (2) ventricle strips were exposed to IAA. As a result, it is conceivable that stimulation of cardiac function is due to increased cardiomyocyte glucose uptake by simple or facilitated diffusion (Rodnick et al., 1997; Clow et al., 2004) and subsequent increases in ATP production, glycolytic intermediates and enhanced SR Ca^{2+} uptake, storage and release (see below).

The absence of exogenous glucose compromised resting tension and $t_{0.8r}$ selectively in ventricle strips from female trout, providing additional evidence for sex differences in cardiac energy metabolism and Ca^{2+} homeostasis. In contrast to females, ventricle strips from both immature and sexually maturing males maintained resting tension and probably diastolic Ca^{2+}_i under substrate-free conditions. A likely explanation for higher absolute resting tension (initial and final) in ventricle strips from sexually maturing males (Table 3) is a greater proportion of compact epicardium *versus* spongy endocardium than in immature fish (Clark and Rodnick, 1999).

Dysregulation of Ca^{2+} homeostasis may lead to an excess of myocardial Ca^{2+}_i and impaired relaxation. Relaxation still occurred, albeit at a slower rate in female cardiac tissue, and inclusion of plasma levels of glucose (5 mmol l^{-1}) preserved resting tension. These results are consistent with those of Gesser, who emphasized the importance of exogenous glucose and the glycolytic pathway to maintain resting tension and twitch force in trout ventricle strips under aerobic and elevated working conditions (Gesser, 2002). Similarly, Bailey and colleagues reported that extracellular glucose in the media was required to maintain resting tension ventricle strips from American eel (*Anguilla rostrata* LeSueur) under anoxic conditions or normoxic conditions during a Ca^{2+}_o challenge (Bailey et al., 2000).

The present work also demonstrated that IAA impaired diastolic relaxation to a greater extent in female ventricle tissue than in males. IAA alone, however, did not raise resting tension as did exogenous substrate deprivation. A possible explanation for this discrepancy is that 0.4 mmol l^{-1} IAA does not block glycolytic flux completely (Gesser, 2002) and there was adequate ATP production to maintain resting tension but not relaxation rate. Presumably, all cardiomyocytes will experience passive leakage of Ca^{2+} into the cytoplasm from both extracellular and SR sources. Evidence from the mammalian smooth muscle (Kusuoka and Marban, 1994) and heart (Aasum et al., 1998) indicates that glycolysis is especially important for the maintenance of cellular ion homeostasis and therefore normal or stable diastolic relaxation. Under aerobic conditions, exogenous glucose is essential for the ability of the SR in rat heart to accumulate Ca^{2+} (Muir et al., 1970). In addition, glycolytically derived ATP has been postulated to selectively fuel the SR Ca^{2+} pump (Xu et al., 1995) and sarcolemmal Na^+/K^+ pump (Dizon et al., 1998). Thus, an inadequate supply of glycolytically produced ATP for the Ca^{2+}-ATPase may increase diastolic $[Ca^{2+}]_i$ in female fish and endogenous glycogen cannot maintain high energy phosphate reserves localized to the SR. It appears that ventricle strips from female rainbow trout rely more on exogenous glucose and possibly glycolysis than males for maintenance of resting tension, relaxation rate and contractility. Conversely, males may utilize more endogenous glycogen for glycolytically produced ATP and maintain resting tension and $t_{0.8r}$ during exogenous fuel deprivation. The important question arises, therefore, as to why endogenous glycogen failed to prevent the development of elevated resting tension and presumably maintain Ca^{2+} homeostasis in female trout cardiac muscle? The

answer may involve selective compartmentation of endogenous *versus* exogenous carbohydrates and relate to the observation in rat hearts that exogenous glucose from glycogen is oxidized preferentially compared with exogenous glucose (Henning et al., 1996).

Glucose metabolism and SR Ca^{2+} support of steroid-induced inotropism

The results of this investigation are the first to demonstrate that exogenous glucose, but not endogenous glycogen, and glycolysis selectively facilitate steroid-induced contractile function in the trout heart. Preparations without glucose, pretreated with IAA, or even receiving other carbohydrate substrates (lactate or pyruvate) failed to respond to T or E2. Our studies also implicate the SR as a key downstream effector for steroid signaling in cardiac tissue from rainbow trout and provide indirect evidence for functional links between glycolysis, excitation–contraction coupling and the SR. Steroids, acting directly or indirectly, appear to potentiate glucose metabolism and increase loading (and subsequent release) of Ca^{2+} from the SR.

Steroids have been shown previously to have rapid, metabolic effects in mammalian striated muscle and the fish gut. Specifically, Bihler and Sawh noted that inotropic concentrations of ouabain (a cardiac glycoside and steroid) in rat and guinea pig atria promoted sugar (3-*O*-methyl-D-glucose) transport *in vitro* (Bihler and Sawh, 1975). In rat cardiomyocytes, T ($10\,nmol\,l^{-1}$) rapidly enhanced 2-deoxyglucose uptake (Koenig et al., 1989). Tsai and Sapolsky also demonstrated that T ($1\,\mu mol\,l^{-1}$), but not corticosterone, rapidly (1–4 min) enhances 2-deoxyglucose uptake and energy metabolism in cultured mouse C_2C_{12} myotubes (Tsai and Sapolsky, 1996). In tilapia (*Oreochromis mossambicus*), 17α-methyltestosterone increased glucose uptake in the intestine within 20 min (Hazzard and Ahearn, 1992). Thus, a possible 'metabolic' explanation for the observed steroid-induced inotropism in rainbow trout cardiac tissue is a stimulatory effect of T (males) and E2 (females) on glucose uptake and glycolytic activity. Glycolytic enzymes in the mammalian heart are associated with the SR (Xu et al., 1995), and oscillations in SR Ca^{2+} release correlate with alterations in glucose metabolism *via* glycolysis (O'Rourke et al., 1994). In addition, certain sugar phosphates (glycolytic intermediates) can activate cardiac ryanodine receptors (Kermode et al., 1998). Whether elevated glycolytic activity and/or intermediates increase the open probability of ryanodine receptors in the heart of rainbow trout will require further study.

Ca^{2+} homeostasis and contractile function in cardiomyocytes are largely governed by the function of key proteins within the sarcolemma and SR (Bers, 2001). The potential effects of glucose and sex steroids on SR function in the fish heart are of particular interest because SR function is not universally viewed as important in ectotherms. In the current study, we used ryanodine, dantrolene, PRP and caffeine to indirectly examine the importance of the SR Ca^{2+} for steroid-induced inotropism. The effectiveness of ryanodine on the trout heart *in vitro* is dependent on fish acclimation temperature, test temperature, pacing frequency and the presence of Epi (Keen et al., 1994; Shiels and Farrell, 1997). Consistent with previous work (Driedzic and Gesser, 1988; Hove-Madsen and Gesser, 1989; Harwood et al., 2000), ryanodine did not have independent effects on contraction or relaxation of the trout cardiac tissue. This suggests that ventricular myocytes of both male and female rainbow trout can develop full, but not maximal, contraction force using only the Ca^{2+} entering across the sarcolemma. However, from our observations of contractility under aerobic conditions and fixed stimulation frequency (0.5 Hz) and temperature (14°C), the contribution of SR Ca^{2+} for steroid-induced inotropism in trout cardiac tissue is suggested by a reduction in contractile force in ryanodine- or dantrolene-treated ventricle strips. To the best of our knowledge, we are unaware of any previous studies using dantrolene on fish cardiac tissue or hearts to block SR function. Possible explanations for enhanced cardiac contractility involving the SR include (1) enhanced Ca^{2+} release mechanism by increasing the open probability of the ryanodine receptor in the SR membrane and (2) stimulation of the SR Ca^{2+} ATPase. Given our data, it seems likely that enhanced SR function is linked to glucose and steroid-induced inotropism. In agreement with our results, others have demonstrated a rapid (nongenomic) effect of steroids on intracellular Ca^{2+} mobilization (Morley et al., 1992; Buitrago et al., 2000).

Other inotropes (Epi and Ca^{2+}) do not require exogenous glucose, glycolysis or the SR

The observation that the inotropic responses to Epi and elevated Ca^{2+}_o occurred in the absence of exogenous glucose, and in the presence of IAA, ryanodine or dantrolene, suggests that the glycolytic pathway and SR are not involved, and more than one mechanism for augmented contractile force exists in fishes. Consistent with the current work, previous studies on mammalian hearts demonstrated that the inotropic effects of isoproterenol (Ogbaghebriel and Dresel, 1988) and Ca^{2+}_o (Ogbaghebriel and Dresel, 1988) were not blocked by cytochalasin B, an inhibitor of glucose transport, or IAA (Ogbaghebriel and Dresel, 1989). Likewise, an intact glycolytic pathway may not be necessary for an Epi-induced increase in contractile activity (MacLeod and Prasad, 1969) or preservation of myocardial Ca^{2+} transport during β-adrenergic stimulation (Bendjelid et al., 2003). By contrast, a study involving rat ventricular myocytes (Aasum et al., 1998) suggests that glycolysis is essential for the inotropic effects that accompany an elevation in Ca^{2+}_o. In rainbow trout, increases in Ca^{2+}_o result in increased cardiomyocyte Ca^{2+} influx through L-type Ca^{2+} channels (Coyne et al., 2000). Thus, in contrast to sex steroids, it appears that the positive inotropy observed with Epi and Ca^{2+}_o in the ventricle of rainbow trout is mediated predominantly through trans-sarcolemmal Ca^{2+} influx.

Sex differences in Ca^{2+} sensitivity and storage

Vertebrate cardiac muscle is absolutely dependent upon Ca^{2+}_o for contraction, and the strength of contraction is related to the

[Ca^{2+}_o]. Our data are the first to demonstrate sexual dimorphism in teleost cardiac sensitivity (females had greater sensitivity than males), but not responsiveness, to elevated Ca^{2+}_o, and effects of caffeine. Studies in mammals have previously documented sex differences in myocardial function (Capasso et al., 1983), including higher sensitivity to Ca^{2+} in atria from female rat hearts compared with male hearts (Wang et al., 1998; Schwertz et al., 1999). In addition, Vizgirda et al. hypothesized that SR Ca^{2+} uptake *via* Ca^{2+}-ATPase was less efficient in females, resulting in reduced Ca^{2+} uptake and storage (Vizgirda et al., 2002). Interestingly, this idea is consistent with the current data and may help explain sex and/or maturity differences in trout cardiac tissue. For example, given our findings that PRP was higher in sexually maturing males than immature males or females in the presence of 5 mmol l^{-1} glucose, and males had a larger inotropic response to caffeine than females, it is possible that males have a more extensive SR, higher activities of the SR Ca^{2+}-ATPase and therefore greater SR Ca^{2+} content and Ca^{2+} release. This unifying hypothesis is also consistent with the observations that glucose and T promote greater inotropism in males than glucose and E2 do in females. However, analysis of the actions of caffeine is complicated because of its multiplicity of actions in cardiac muscle. Although caffeine increases the rate of activator Ca^{2+} from the SR and inhibits post-rest stimulation in mammals (Siegl, 1986), studies have also indicated that caffeine can modulate Ca^{2+} sensitivity of contractile proteins (Wendt and Stephenson, 1983), increase activator Ca^{2+} through inhibition of phosphodiesterase and subsequent increase flux through the sarcolemma (Siegl, 1986), decrease Ca^{2+}-ATPase activity (Gupta et al., 1990) and even stimulate the reverse mode of the Na^+/Ca^{2+} exchanger (Léoty et al., 2001). While the present experiments of caffeine-induced contraction provide further evidence that sex differences exist in Ca^{2+} handling by trout cardiac tissue, the exact mechanism remains to be elucidated. More definitive studies are warranted to characterize sex differences in SR function and metabolic support by glucose-dependent mechanisms.

In contrast to our measurements of cardiac Ca^{2+} sensitivity, we did not observe any sex differences in ionized and total Ca^{2+} in plasma. Our numbers and results agree with previous studies by Andreasen (Andreasen, 1985) and Miguel et al. (Miguel et al., 1988) on rainbow trout, respectively; however, the sex of fish in the first study was not mentioned or known. In contrast to Ca^{2+}, albumin levels were slightly (11%) higher in female plasma. Miguel and colleagues reported lower albumin values than the current data and did not observe a sex difference (Miguel et al., 1988). Whether small differences in albumin concentration (or other plasma proteins) affect the binding and availability of circulating Ca^{2+} (Schjeide, 1985) for the contracting trout myocardium is uncertain.

Other possibilities, implications and limitations

Although the experimental data support enhanced glycolytic activity and SR function as explanations for both glucose- and steroid-induced inotropism, other possibilities exist. Recent evidence suggests that Na^+/Ca^{2+} exchange is an important mechanism for the regulation of SR Ca^{2+} content, Ca^{2+} release and contraction in trout cardiomyocytes (Hove-Madsen et al., 2003). We cannot exclude the possibility that exogenous glucose and or sex steroids elevate [Na^+_i], increase reverse-mode Na^+/Ca^{2+} exchange and therefore have direct effects on [Ca^{2+}_i], resting tension and trigger more Ca^{2+} release from the SR. This mechanism would still be consistent with our assertion that the SR plays a pivotal role for glucose-mediated intracellular Ca^{2+} buffering and enhanced contractility following exposure to sex steroids but disagrees with the observation that glycolytically derived ATP fuels the Na^+/K^+ pump (Dizon et al., 1998) and therefore helps to reduce Na^+_i.

There are also some important implications and limitations of our study. First, it is now evident that sex differences in cardiac function exist in fish and contribute to the complexities of steroid hormone actions. These differences should raise new questions about our current understanding of cardiac energetics and mechanical function and the appropriate design for future experiments. It remains to be determined whether the observed differences between male and female rainbow trout apply to other fish species. Second, contrary to the general view of a limited role of SR function in fish heart, these experiments propose an additional function of the SR: steroid-induced inotropism. However, as in our study, *in vitro* preparations do not use a full complement of exogenous substrates (carbohydrates, free fatty acids, amino acids) and hormones or reflect the normal physiological state. In addition, our conclusions are based mainly on indirect measurements using pharmacological agents, and these compounds have nonspecific effects. Complementary measurements of glucose uptake, glycolytic activity and SR Ca cycling are needed for a more integrated perspective of steroid-induced inotropism. Finally, the functional consequences of elevated sex steroids on cardiovascular function in the intact rainbow trout are unknown, and yet it is tempting to speculate that elevated circulating sex steroids during spawning periods would trigger a metabolic inotropic effect, enhancing intracellular Ca^{2+} storage and release from the SR in cardiomyocytes.

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