The Journal of Experimental Biology 207, 2083-2093
Published by The Company of Biologists 2004
doi:10.1242/jeb.00996

Sex-dependent effects of gonadal steroids and cortisol on cardiac contractility in rainbow trout

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Accepted 22 March 2004

Summary

The purpose of this study was to determine whether steroid hormones modulate cardiac function in rainbow trout (*Oncorhynchus mykiss* Walbaum). We assessed the effects of exogenously administered steroids on isolated ventricle strips and report that physiological concentrations of androgens, 17β-estradiol and cortisol rapidly (<10 min) enhance inotropism (30–40%) in a sex-specific manner. These effects were specific to the hormones studied, absent if animals were anesthetized chemically and dependent upon steroid concentration and contraction frequency. Based on the use of specific steroid receptor antagonists and key enzyme inhibitors, it appears that testosterone, 11-ketotestosterone and cortisol each act through specific intracellular receptors in males and that the positive inotropism requires the synthesis of polyamines and nitric oxide. Cortisol and 17β-estradiol, but not androgens, had similar effects in females and also involved similar signaling pathways. Androgen and cortisol effects were additive in males but cortisol and 17β-estradiol were not additive in females, suggesting sex differences in the pathways through which these hormones stimulate inotropism. In summary, gonadal steroids and cortisol promote ventricular contractility in a sex-dependent manner through mechanisms that appear multifaceted. Ultimately, steroid-mediated improvements in cardiac performance might involve non-genomic pathways and be physiologically important during migration, spawning or stressful periods.

Key words: steroid hormone, cardiac function, cortisol, 17β-estradiol, androgen, inotropism, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Steroid hormones regulate a large array of developmental and physiological functions in target tissues. Most of the time-honored effects of sex steroids (androgens and estrogens) are mediated by nuclear receptors and altered transcriptional activity (genomic mechanism); however, a growing number of studies describe rapid steroid effects through nongenomic mechanisms of action, several signal transduction pathways and even multiple cell types (Falkenstein et al., 2000). For example, mammalian cardiomyocytes contain specific steroid receptors and respond to androgenic stimulation with both prolonged and rapid effects. Endogenous androgens promote well-documented sex differences in ventricle size (male>female; Batterham et al., 1997). Testosterone (T) also rapidly increases Ca^{2+} availability and contractile force in rat myocytes through increased polyamine synthesis (Koenig et al., 1983) without changes in the expression of the ornithine decarboxylase (ODC) gene (Bordallo et al., 2001). Estrogen can also modulate [Ca^{2+}] in the mammalian heart, and this effect may involve direct effects on cardiomyocytes (Buitrago et al., 2000) or the synthesis and release of nitric oxide (NO) from nearby endothelial cells (Chambliss and Shaul, 2002).

Androgens promote dramatic sex differences in ventricle size and function during sexual maturation of salmonid fishes (Franklin and Davie, 1992). As rainbow trout reach sexual maturity, plasma levels of androgens and estrogens reach peak concentrations (Scott et al., 1980a,b; Lou et al., 1986). Elevated levels of 11-ketotestosterone (11KT), both during sexual maturation (Franklin and Davie, 1992) and as a result of chronic administration (Thorarensen et al., 1996), induce ventricular hypertrophy (Davie and Thorarensen, 1997), presumably through genomic mechanisms. Further, Slater et al. (1995) demonstrated binding of [^{3}H]testosterone to heart cytosolic fractions from rainbow trout, providing evidence of specific androgen receptors and the possibility for direct effects of androgens on cardiomyocytes. Despite the recognized chronic effect of androgens on ventricle size in salmonid fishes, the acute effects of gonadal steroids on myocardial contractility or cardiovascular function in fishes has not been studied.

Another steroid hormone of considerable interest in fishes is cortisol (C). This glucocorticoid is elevated by stress and reduces immune function, disease resistance and reproductive success in salmonid fishes (Carragher et al., 1989; Campbell et al., 1992). C levels are elevated in sexually maturing salmonids (Sower and Schreck, 1982). However, there is a

decrease in stress-induced C levels in both sexes during spawning compared with non-reproductive periods (Donaldson and Fagerlund, 1968; Pottinger et al., 1995). Similar to the sex steroids, positive inotropic actions of glucocorticoids have been demonstrated in cardiac tissue of mammals (Yano et al., 1994; Wehling, 1997; Falkenstein et al., 2000) and amphibians (Hajdu and Szent-Györgyi, 1952). Thus, we felt that it was also important to determine whether C serves as a potential modulator of cardiac function in fishes. To the best of our knowledge, only one previous study has examined the effects of exogenous C on acute cardiac function in fishes (Farrell et al., 1988). These authors saw no effect of C on isolated cardiac preparations.

Given this background, the objectives of the present study were to (1) examine whether gonadal steroids and/or C can rapidly promote cardiac contractility in male and female rainbow trout and (2) define potential mechanisms for steroid-induced positive inotropism in fishes. We hypothesized that, similar to mammals, sex steroids and C would increase cardiac contractility in fishes. Our results support this hypothesis and provide evidence for rapid inotropic mechanisms involving NO and polyamines.

Materials and methods

Experimental animals

10–12-month-old immature male (408±14 g; 31.8±0.4 cm, mean ± S.E.M., N=148) and female (380±13 g; 31.0±0.3 cm, N=123) and 24–28-month-old, sexually mature male (1098±58 g; 43.1±0.8 cm, N=26) rainbow trout were obtained from Clear Springs Foods, Inc. (Buhl, ID, USA). Fish were transported to Idaho State University and held in 1000-liter 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.

Ventricle strip preparation

Fish were netted rapidly and euthanized by a sharp blow to the head. The ventricle was excised, weighed and immediately placed in ice-cold modified teleost Ringer solution. This solution contained (in mmol l^{-1}): 111 NaCl; 5 KCl; 5 NaH_2PO_4; 10 $NaHCO_3$; 1.5 $CaCl_2$; 1.0 $MgSO_4$ and 5 glucose and was equilibrated with 0.5% CO_2:99.5% O_2 (pH of 7.6 at 14°C). To address potential effects of chemical anesthesia on the response of ventricular tissue to steroids, additional fish (N=10) were anesthetized with a buffered (0.2 g l^{-1} $NaHCO_3$) solution of MS-222 (tricaine methanesulfonate; 0.2 g l^{-1}) or benzocaine (ethyl p-aminobenzoate; 0.2 g l^{-1}) prior to excision of the ventricle. The sex of each fish was determined by visual or microscopic examination of the gonads, and four uniform strips (approximate dry mass, 1.5 mg) were cut from each ventricle. Each strip (approximate dimensions, 4.5 mm long × 0.7 mm wide × 0.5 mm thick) was 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. The temperature of the muscle baths was maintained at 14°C with a refrigerated recirculating bath. Strips were stimulated with a voltage that elicited full contraction (60 V) at 0.5 Hz with 5 ms square wave pulses (Grass S88 Stimulator; Grass Medical Instruments, Quincy, MA, USA), and the length of each strip was adjusted to produce maximal twitch force. After a 1 h equilibration period, we measured twitch force (F), time to peak force (t_p), time to 80% relaxation ($t_{0.8r}$), resting tension and ±dF/dT for 30 min using a data acquisition system (BioPac MP100; Goleta, CA, USA) and software (Acqknowledge v.3.5.5; BioPac). As pointed out by Hartmund and Gesser (1996), this preparation cannot be regarded as truly isometric because of its nonhomogenous orientation of contracting myocytes. Thus, changes in twitch force development and resting tension were normalized (%) to the measurements taken at the end of the initial equilibration period. Different fish and separate strips were used for each experimental condition and appropriate controls. All four strips from a given heart were prepared at the same time and incubated for the same duration. The same strip was used only once. Given the large number of incubation conditions (n=15 for males and n=14 for females), this ultimately resulted in unequal sample sizes for statistical comparisons.

Experimental protocols

Chemicals

Cyclodextrin (2-hydroxypropyl-β-cyclodextrin) was obtained from Research Biochemicals International (Natick, MA, USA). Testosterone (4-androsten-17β-ol-3-one) and 11-ketotestosterone (4-androsten-17β-ol-3, 11-dione) were purchased from Steraloids (Newport, RI, USA). Aldosterone (4-pregnen-18, 20-diol-11β, 18-epoxy-3,20-dione), cholesterol (5-cholesten-3β-ol), 17β-estradiol [1,3,5(10)-estratriene-3, 17β-diol], hydrocortisone (11β–17α, 21-trihydroxypregn-4-ene-3, 20-dione), flutamide {2-methyl-N-[4-nitro-3-(triflouromethyl)-phenyl]propanamide}, mifepristone [11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one], tamoxifen [(Z)-1-(p-dimethylamino-ethoxyphenyl)-1,2-diphenyl-1-butene], DFMO [2-(diflouromethyl)-ornithine], GDP [guanosine 5′-O-(2-thiodiphosphate)], L-NAME (Nω–nitro-l-arginine methyl ester) and bovine serum albumin (BSA)-conjugated steroids were purchased from Sigma-Aldrich (St Louis, MO, USA). Unless noted otherwise, additional chemicals were also purchased from Sigma-Aldrich.

Effects of steroid concentration, sex and contraction frequency

In the first series of experiments, ventricle strips from male

and female rainbow trout were exposed to increasing concentrations (from physiological to pharmacological) of individual steroids for 40 min. Steroids were solubilized in either absolute ethanol or 10% (w/v) cyclodextrin. Other experimental compounds were solubilized in either Ringer solution [epinephrine, GDP, α-diflouromethylornithine (DFMO) and L-NAME] or absolute ethanol (aldosterone, BSA-conjugated steroids, cholesterol, flutamide, mifepristone and tamoxifen). Concentrations reported for all steroids and other chemicals are final concentrations in the tissue baths. Concentration–response curves were constructed for T and 11KT (0.3 nmol l^{-1}–30 μmol l^{-1}), C (0.1 nmol l^{-1}–100 μmol l^{-1}) and 17β-estradiol (E2; 0.01 nmol l^{-1}–100 μmol l^{-1}). Based on these studies, optimal concentrations (those that elicited the greatest increase in contractile force) of T, 11KT, C or E2 were administered to strips, with control strips receiving an equal volume of ethanol or cyclodextrin. The final concentrations of ethanol and cyclodextrin were 0.7 mmol l^{-1} and 2.4 μmol l^{-1}, respectively. Ventricle strips were exposed to cholesterol (1 nmol l^{-1}–100 μmol l^{-1}) or aldosterone (1 nmol l^{-1}–1 μmol l^{-1}) to evaluate whether a generic or non-physiological steroid can alter cardiac performance, respectively. We also determined whether steroid-induced changes in ventricle performance are frequency dependent by electrically stimulating strips over a physiological range of contraction frequencies (0.2–1.0 Hz), at optimal steroid concentrations.

Mechanisms responsible for steroid actions

To explore whether steroid actions were additive or involved a common mechanism of action, we treated strips with an optimal concentration of one steroid and then another after maximal effects of the first steroid were demonstrated. We also determined whether steroids' effects are mediated through their respective receptors by pre-treating ventricle strips with flutamide (0.1 mmol l^{-1}), mifepristone (0.1 mmol l^{-1}) or tamoxifen (0.25 mmol l^{-1}) to inhibit androgen, C and E2 receptors, respectively. We chose to pre-treat trout ventricle strips with these compounds 10 min prior to administration of steroids for several reasons. First, this time interval corresponds closely to the extracellular equilibration time of mannitol in eel ventricle strips (Rodnick et al., 1997) and the preincubation times in mammalian hearts (flutamide; Remmers et al., 1997) and isolated tissues (DFMO; Koenig et al., 1983). We also wanted to avoid the cytotoxic effects of these drugs, which can occur after extended exposure (Wang et al., 2002). To identify the possibility of membrane receptors for sex steroids and C, we used BSA-coupled conjugates of T (0.3 μmol l^{-1}), C (0.1 μmol l^{-1}) and E2 (1 nmol l^{-1}). BSA is a large protein (M_r=68 000) and, when conjugated to steroids, renders the molecule membrane impermeable (Stevis et al., 1999). BSA-conjugated steroids are commonly used to identify steroid actions that do not involve classical nuclear receptors (Falkenstein et al., 2000).

Specific intracellular pathways for possible steroid action on the heart were investigated using a single dose of inhibitory compounds prior to steroid administration. Inhibitory compounds included guanosine diphosphate (GDP; 500 μmol l^{-1}; an inhibitor of G-protein activation), (DFMO; 10 mmol l^{-1}; an inhibitor of ornithine decarboxylase and polyamine synthesis) and L-NAME (1 mmol l^{-1}; an inhibitor of NO synthase). In addition, the responsiveness of trout cardiac tissue to epinephrine (1 μmol l^{-1}) was tested, both in the presence and absence of steroids to address hormone additive effects and the effectiveness of GDP. Ventricle strips were always pre-treated with inhibitory and stimulatory agents 10 min prior to administration of steroids.

Data analysis

For each ventricle strip, force and other variables were calculated from data acquired at 0, 1, 2, 3, 5, 10, 20 and 30 min after a 1 h equilibration period. Data are expressed as means ± S.E.M. of the percent change of basal inotropism. Cardiac performance between control (strips exposed only to Ringer, steroid vehicle or inhibitor) and steroid-treated strips for both sexes was assessed by a two-way analysis of variance (ANOVA) with Bonferroni *post-hoc* corrections using SPSS software. A one-way ANOVA was used to examine the effects of contraction frequency on steroid-induced inotropism in males and females. Statistical significance was set at $P<0.05$.

Results

Concentration and sex-dependent effects of steroids

Concentration–response curves for T and 11KT (0.3 nmol l^{-1}–30 μmol l^{-1}), C (0.1 nmol l^{-1}–100 μmol l^{-1}) and E2 (0.01 nmol l^{-1}–100 μmol l^{-1}) can best be described as contractile force reaching a maximum, then declining, with maximum force occurring at physiological values [0.3 μmol l^{-1} for T and 11KT, 0.1 μmol l^{-1} and 0.01 μmol l^{-1} for C (in males and females, respectively) and 1.0 nmol l^{-1} E2; Fig. 1]. For all steroids tested, significant increases in inotropy were observed within 3 min of exposure, and maximal values were observed after approximately 10 min (Fig. 2). By contrast, epinephrine-induced inotropism took just 2–3 min to reach peak values (Fig. 2). Compared with ethanol and cyclodextrin controls, physiological concentrations of T and 11KT induced positive inotropism (35–40%) in strips from male rainbow trout (ANOVA, $F_{5,27}$=4.89, P=0.021 and $F_{5,27}$=5.24, P=0.018, respectively; Fig. 3A). We did not observe a difference between mature and immature males (ANOVA, $F_{4,28}$=2.15, P=0.216) and therefore pooled the data. This effect of androgens (T and 11-KT) was sex-dependent and not seen in immature female rainbow trout (ANOVA, $F_{6,23}$=1.89, P=0.249 and $F_{6,22}$=1.76, P=0.228, respectively). Although C increased contractile force in both males (30%) and females (35%) (ANOVA, $F_{6,21}$=6.96, P=0.027 and $F_{5,27}$=5.17, P=0.028), maximal contractile force in females was observed at a 10-fold lower concentration than in males (Fig. 1A,B). E2 increased contractile force by 30% in cardiac muscle from female trout (ANOVA, $F_{6,21}$=6.22, P=0.034;

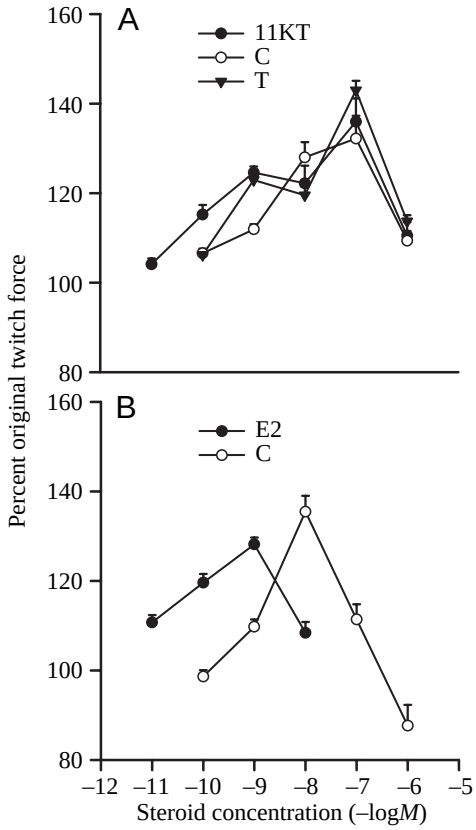

Fig. 1. Dose-dependent effects of 11-ketotestosterone (11KT), testosterone (T), cortisol (C) and estradiol (E2) on contractile force of ventricle strips from male (A) and female (B) rainbow trout. At the concentrations tested, contractile force in E2-treated ventricle strips from males did not differ from that in control strips. Likewise, 11KT- or T-treated strips from females did not differ from control strips. Values are means + S.E.M. (*N*=7–10 strips) for maximal response at each concentration.

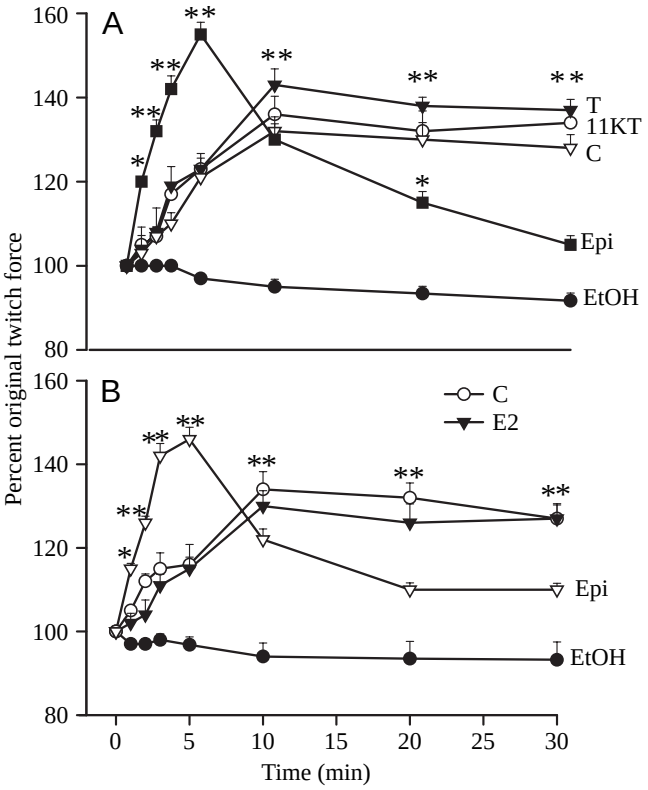

Fig. 2. Time course of contractile force in ventricle strips from male (A) and female (B) rainbow trout after exposure to ethanol (EtOH), epinephrine (Epi; 10 μmol l^{-1}) 11-ketotestosterone (11KT; 0.3 μmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), cortisol (C; 0.1 μmol l^{-1} in males and 0.01 μmol l^{-1} in females) and estradiol (E2; 1.0 nmol l^{-1}). Contractile force in E2-treated strips from males did not differ significantly from that in EtOH control strips. Similarly, force in 11KT- or T-treated strips from females did not differ significantly from EtOH control strips. All steroid values are means + S.E.M. (*N*=8–12). **P*<0.05 *versus* EtOH control strips (epinephrine only), ***P*<0.05 *versus* EtOH control strips for all steroids.

Fig. 3B). However, E2 had no effect on contractile performance of strips from male rainbow trout (ANOVA, $F_{5,24}$=2.07, *P*=0.312). When ventricle strips were incubated with maximally stimulating concentrations of 11KT+C, or T+C, in males, we observed greater contractility than that brought about by either hormone alone (Fig. 4). The effect was completely additive between androgens and C, suggesting that separate mechanisms of actions were responsible for androgen + C-enhanced inotropism. By contrast, we did not observe additive effects of E2 and C in ventricle strips from females (Fig. 4), signifying that these two steroids may be acting through a common mechanism.

Although increases in contractile force (*F*) were observed, no significant changes were observed in t_p, $t_{0.8r}$ or ±d*F*/dT after

Table 1. *Contractile properties of rainbow trout ventricle preparations at 0.5 Hz, before (basal) and 20 min after exposure to optimal concentrations of testosterone (T; 0.3 μmol l^{-1}) in males and 17β-estradiol (E2; 1.0 nmol l^{-1}) in females*

Contractile variable	Males (basal)	Males (steroid-treated)	Females (basal)	Females (steroid-treated)
F (mN)	8.9±1.3	13.2±1.7	8.1±1.4	12.6±1.5
t_p (ms)	416±4.6	398±1.9	421±3.7	410±1.2
$t_{0.8r}$ (ms)	402±3.8	421±1.9	394±3.3	412±1.5
+d*F*/dT (mN ms^{-1})	1.51±0.7	1.66±0.2	1.50±0.5	1.56±0.1
–d*F*/dT (mN ms^{-1})	1.48±0.2	1.53±0.3	1.50±0.2	1.54±0.1

Values are means ± S.E.M. (*N*=8). *F*, maximum twitch force; t_p, time from stimulus to peak twitch force; $t_{0.8r}$, time from peak twitch force to 80% relaxation.

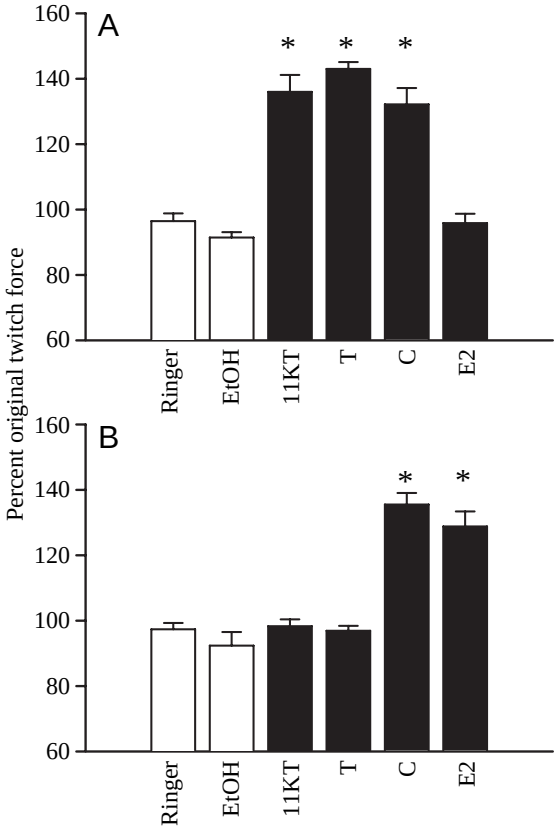

Fig. 3. Contractile force of ventricle strips from male (A) and female (B) rainbow trout with no chemical additions (Ringer only) and after exposure to ethanol (EtOH), 11-ketotestosterone (11KT; 0.3 μmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), cortisol (C; 0.1 μmol l^{-1} in males and 0.01 μmol l^{-1} in females) and estradiol (E2; 1.0 nmol l^{-1}). Contractile force in E2-treated strips from males did not differ significantly from that in EtOH control strips. Similarly, force in 11KT- or T-treated strips from females did not differ significantly from EtOH control strips. All steroid values are means + S.E.M. (N=8–12). *P<0.05 *versus* EtOH control strips.

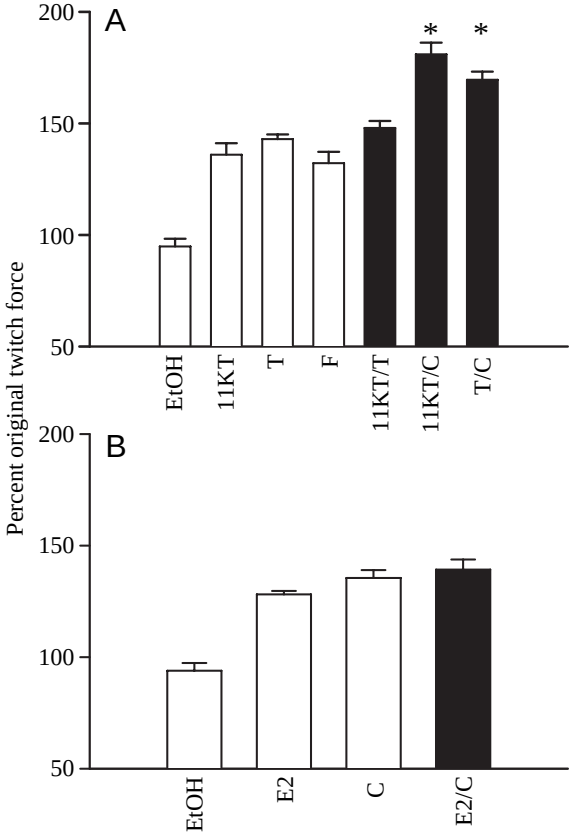

Fig. 4. Effects of multiple steroids on maximum contractile force developed by ventricle strips from rainbow trout at 0.5 Hz. Ventricle strips from both male (A) and female (B) rainbow trout were treated with 11-ketotestosterone (11KT; 0.3 μmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), cortisol (C; 0.1 μmol l^{-1} in males and 0.01 μmol l^{-1} in females), estradiol (E2; 1.0 nmol l^{-1}) or combinations of two different steroids at the same concentrations. In males, the maximal effects of 11KT+C (178±4%) and T+C (163±3%) were completely additive when compared with their independent effects (11KT, 143±2%; T, 136±5%; C, 132±5%). By contrast, maximal effects of E2 (129±4%) and C (136±4%) were not additive (143±3%). All values are means + S.E.M. (N=9). *P<0.01 compared with strips treated with one steroid hormone.

exposure to sex steroids or C (Table 1). Thus, although a sex-specific, positive inotropic effect was produced by steroid hormones on trout cardiac tissue, the timing of contraction and relaxation was conserved.

Contraction frequency and steroid-induced positive inotropism

We observed a negative force–frequency relationship over the range of contraction frequencies examined. Compared with 0.2 Hz, relative force production by the same strip was reduced by approximately 11, 31 and 46% at 0.5, 0.8 and 1.0 Hz, respectively (N=4). For males and females, the maximum inotropic effects of sex steroids and C were observed at 0.5 Hz (males ANOVA, $F_{3,28}$=5.19, P=0.017; females $F_{2,20}$=6.32, P=0.024), with reduced, yet significant, hormone effects occurring at 0.8 Hz (males ANOVA $F_{3,28}$=4.92, P=0.045; females $F_{2,20}$=5.03, P=0.039) (Fig. 5). The inotropic effects of steroids were not observed at 0.2 Hz and 1.0 Hz.

Signaling pathways and possible mechanisms responsible for steroid actions

Flutamide, mifepristone and tamoxifen had no independent effects on ventricle strip performance (ANOVA, $F_{6,34}$=1.96, P=0.382). In males, pretreatment of ventricle strips with flutamide blocked the increased inotropism induced by T and 11KT (ANOVA, $F_{6,34}$=6.96, P=0.018). Pretreatment of female strips with tamoxifen inhibited the increase of contractile force induced by E2 (ANOVA, $F_{6,30}$=4.74, P=0.028). Pretreatment with mifepristone completely blocked effects of C in both males and females (ANOVA, $F_{6,37}$=5.32, P=0.020). By contrast, flutamide did not inhibit the positive inotropism in C-treated strips, and mifepristone did not affect inotropism in T- or 11KT-treated strips (Fig. 6). This suggests that each steroid is acting through separate receptor types in males. In females,

however, tamoxifen also inhibited the positive inotropism elicited by C. It is therefore possible that tamoxifen may block both E2 and C receptors in the heart of female rainbow trout, or act *via* a common intracellular mechanism.

Potential pathways by which steroid actions can be mediated include: (1) intracellular or membrane-bound receptors; (2) polyamine synthesis; (3) NO production and (4) G-protein activation. Based on negative results with physiological concentrations of BSA-conjugated T, C or E2 and studies using receptor antagonists, it appears that the improved inotropism induced by 'free' steroids involves intracellular binding to specific receptors. Pre-treatment of ventricle strips with DFMO had no independent effects on basal contractile performance, and the addition of steroids to DFMO-treated strips from both males and females showed no change in inotropism (Fig. 7). This suggests that polyamine synthesis plays an important role in promoting steroid-induced stimulation of myocardial contractility in rainbow trout. Pretreatment with L-NAME had no independent effect on ventricle strip performance. Strips receiving T, 11KT, C or E2 after treatment with L-NAME showed no improved inotropism (Fig. 8). This finding provides evidence that steroid-induced positive inotropism may also involve production of NO, either by myocytes or nearby endothelial cells, and subsequent intra- or intercellular signaling.

Unlike polyamines and NO, it doesn't appear that G proteins are mediators of the steroid-induced positive inotropism in trout cardiac muscle. Although GDP had no independent effects on ventricle strip performance, T, 11KT, C and E2

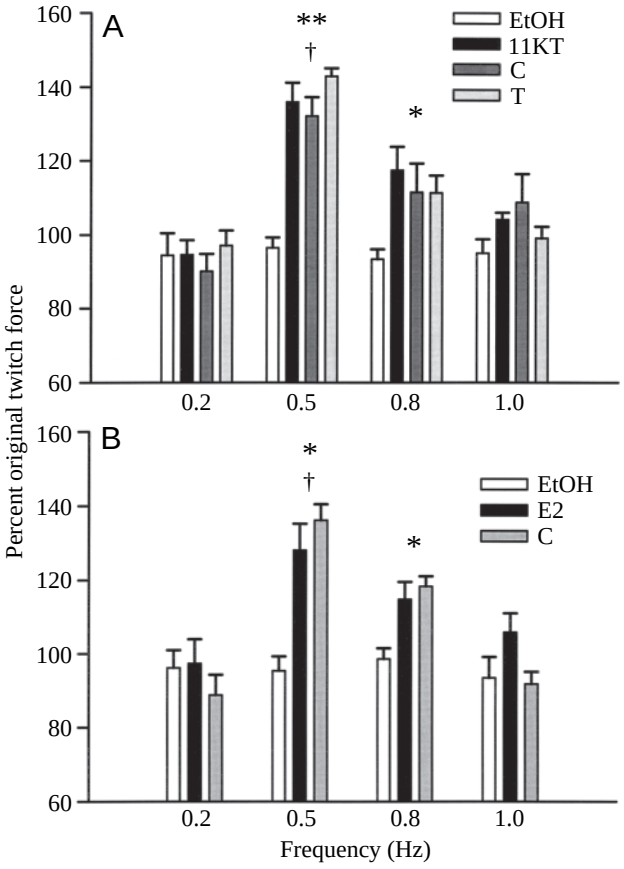

Fig. 5. Frequency-dependent effects of steroids on contractile force by ventricle strips from both male (A) and female (B) rainbow trout. Effects of 11-ketotestosterone (11KT), testosterone (T), cortisol (C) and estradiol (E2) are shown over physiological frequencies (0.2–1.0 Hz). Only strips contracting at 0.5 and 0.8 Hz responded significantly to steroids. All values are means + S.E.M. (N=6–8). *P<0.05, all steroid-treated strips *versus* EtOH control strips; **P<0.01, all steroid-treated strips *versus* EtOH control strips; †P<0.05, steroid-treated strips at 0.5 Hz *versus* steroid-treated strips at 0.2, 0.8 and 1.0 Hz.

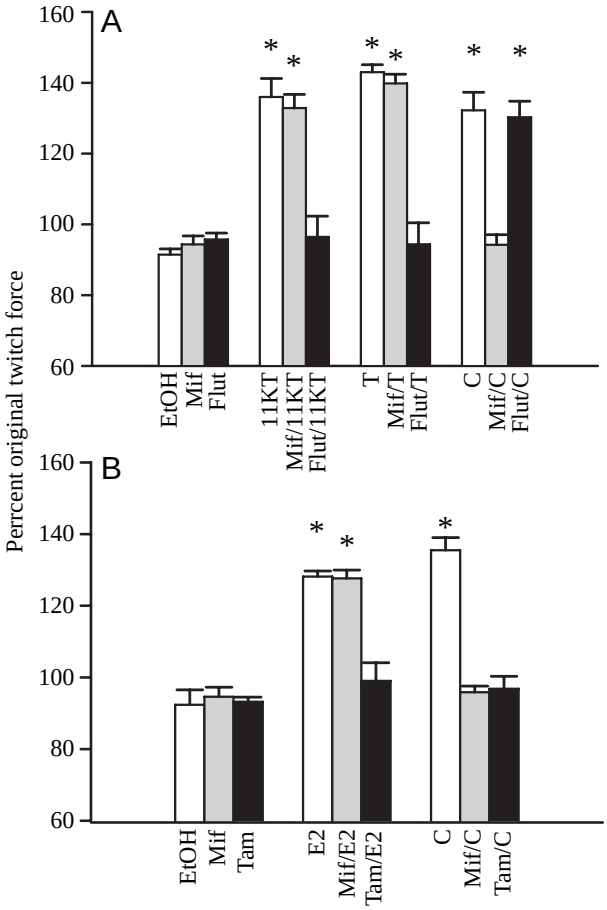

Fig. 6. Effects of specific receptor antagonists on maximum contractile force developed by steroid-treated ventricle strips from both male (A) and female (B) rainbow trout at 0.5 Hz. Strips were pretreated for 10 min with flutamide (Flut; 0.1 mmol l^{-1}), mifepristone (Mif; 0.1 mmol l^{-1}) or tamoxifen (Tam; 0.25 mmol l^{-1}) to inhibit androgen, cortisol and estradiol receptors, respectively. Flut, Mif and Tam had no independent effects on contractile force in males or females. Steroid concentrations used were: 0.3 μmol l^{-1} 11-ketotestosterone (11KT), 0.3 μmol l^{-1} testosterone (T), 0.1 μmol l^{-1} cortisol (C) in males, 0.01 μmol l^{-1} C in females, and 1 nmol l^{-1} estradiol (E2) in females. In males, Flut blocked the inotropic effects of androgens (11KT and T) but not C. Mif blocked the effects of C in males and females but not 11KT and T in males and E2 in females. In females, Tam prevented the positive effects of E2 and C. All values are means + S.E.M. (N=7–10). *P<0.05 *versus* EtOH control strips.

increased contractile force (to a similar degree as strips receiving steroid only) in strips pretreated with GDP. Evidence for GDP effectiveness and G-protein importance to positive inotropism was provided when ventricle strips from male and female rainbow trout were pretreated with GDP and exposed to epinephrine. As expected, and yet in contrast to the steroids, GDP completely blocked the stimulatory effects of epinephrine on contractility (data not shown). In addition, adrenergic stimulation of inotropism with epinephrine was completely additive to the actions of sex steroids and C.

Cholesterol and aldosterone did not affect contractile performance of ventricle strips from male or female rainbow trout (ANOVA, $F_{6,37}$=1.65, P=0.347). Control strips receiving just ethanol showed an 8% decrease in contractile force independent of any other treatment (ANOVA, $F_{6,32}$=3.17, P=0.071), whereas cyclodextrin had no independent effects on contractile performance (ANOVA, $F_{6,30}$=1.27, P=0.431). It is noteworthy that ventricle strips from male and female fish anesthetized with buffered tricaine or benzocaine showed no response to concentrations of steroids that produced maximal positive inotropism in strips from animals that were euthanized by physical trauma to the head (Table 2; ANOVA, $F_{6,12}$=1.68, P=0.378).

Discussion

It is well known that androgens, E2 and C directly influence cardiac inotropism in mammals. The findings of the present study demonstrate for the first time that exposure to physiological concentrations of gonadal steroids (Scott et al., 1980a,b; Lou et al., 1986) and C (Sower and Schreck, 1982) can promote myocardial contractility in rainbow trout. From studies on mammals, it is also becoming increasingly clear that sex-specific differences exist in hormone signaling and the cardiovascular system (Lin et al., 1990; Beyer et al., 2001). Our data indicate that T, 11KT and C promote positive inotropism in hearts from sexually immature or mature male trout, whereas hearts from immature females only respond to E2 and C. The observation of sex-dependent responsiveness to steroids in the fish heart is consistent in most cases with the presence of particular hormones in the blood, with T being an exception. Blood levels of T and 11KT reach peak concentrations in male salmonids during sexual maturation, whereas E2 peaks in females at maturity (Campbell et al., 1980; Scott et al., 1980a). Female rainbow trout also have

Table 2. *Effects of chemical anaesthesia with buffered MS-222 or benzocaine prior to heart excision, or direct treatment of ventricle strips with epinephrine on the inotropic effects of 11-ketotestosterone (11KT; 0.3 μmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), cortisol (C; 0.1 μmol l^{-1} in males and 0.01 μmol l^{-1} in females) or 17β-estradiol (E2; 1.0 nmol l^{-1}) on contractile force of ventricle strips from male and female rainbow trout*

Treatments	MS-222 0.2 g l^{-1}	Benzocaine 0.2 g l^{-1}	Epinephrine 1 mmol l^{-1}
Males			
EtOH	93.4±3.0 (8)	94.5±1.8 (8)	155.6±3.8 (14)
T	94.6±2.2 (8)	95.1±2.7 (8)	182.8±5.6 (14)
11KT	96.7±3.8 (8)	93.5±2.2 (8)	173.4±4.7 (14)
C	93.7±4.0 (8)	92.4±1.9 (8)	179.6±5.4 (14)
Females			
EtOH	95.8±2.9 (9)	96.2±3.8 (9)	146.3±2.8 (12)
E2	93.7±3.1 (9)	94.9±2.8 (9)	168.1±4.6 (12)
C	93.1±2.4 (9)	93.6±2.7 (9)	178.7±5.2 (12)

Values are mean percentages ± S.E.M. (*N*) force achieved after equilibration (100% value).

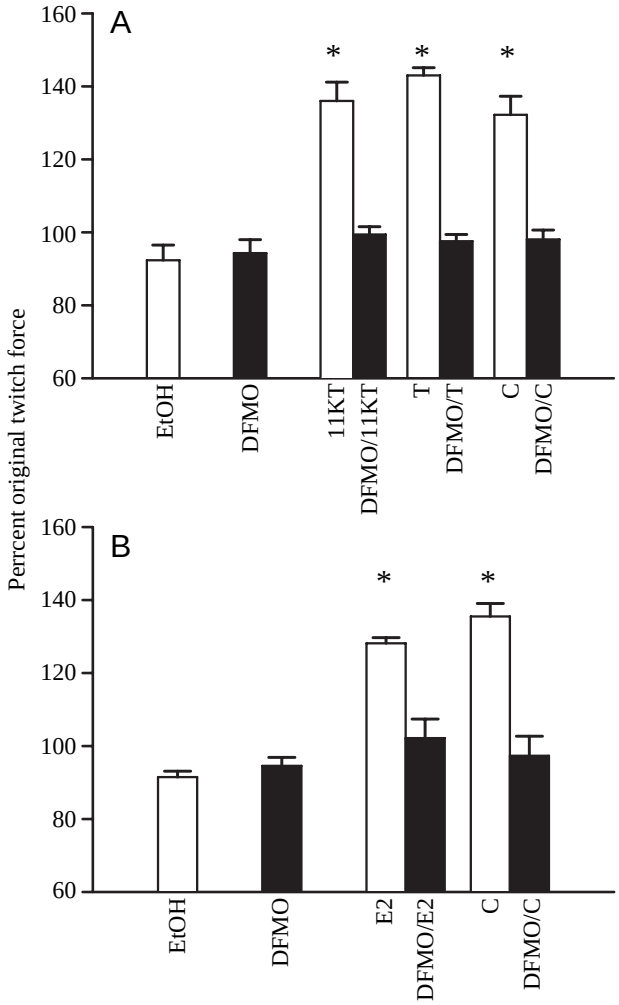

Fig. 7. Effect of diflouromethylornithine (DFMO) on contractile force in steroid-treated ventricle strips from both male (A) and female (B) rainbow trout. Strips were treated with DFMO (10 mmol l^{-1}) for 10 min prior to exposure to 11-ketotestosterone (11KT; 0.3 μmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), cortisol (C; 0.1 μmol l^{-1} in males and 0.01 μmol l^{-1} in females) or estradiol (E2; 1.0 nmol l^{-1}). DFMO had no independent effect on contractile force; however, DFMO did block the inotropic effects of androgens, C and E2. All values are means + S.E.M. (N=6–9). *P<0.05 *versus* EtOH control strips.

circulating T, and levels peak at ovulation (Scott et al., 1980b). However, we found that female heart tissue does not respond to T. As a result, an obvious question is why doesn't the female heart respond to T and exhibit positive inotropism? It is possible that the heart of female trout does not possess intracellular receptors for T or the required elements for a T-signaling system that affects cardiac contractility. Future studies will be necessary to resolve this issue.

Cortisol, the primary stress hormone in both male and female rainbow trout, fluctuates throughout a fish's life (Pottinger et al., 1995) and mediated a functional response in all trout hearts examined. However, cardiac muscle from females was more sensitive than males to C. This novel finding suggests that sex differences exist in receptor binding or intracellular signaling by C. It is noteworthy that Caldwell et al. (1991) demonstrated similar levels of total C in male and female rainbow trout, and yet mature females exhibit a lower free C compared with mature males and immature fish. This difference reflects a higher percent C bound to corticosteroid-binding protein in female fish. Ultimately, the concentration of unbound steroid hormones may determine whether steroid-induced inotropism has biological relevance and may explain sex differences in hormone sensitivity. Although it takes just 3–10 min for cardiac tissue to respond to steroid hormones *in vitro*, the fact that the increase in C during stressful periods takes much longer (~30 min; Gamperl et al., 1994) makes it unlikely that C-induced elevations in cardiac performance would occur following acute stressors.

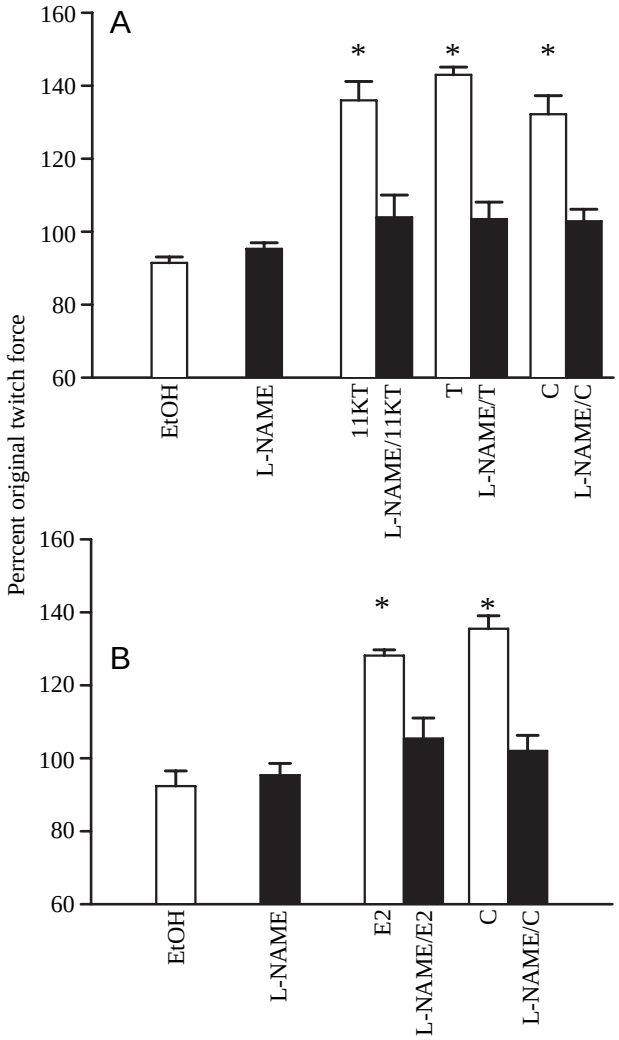

Fig. 8. Effect of L-NAME (Nω–nitro-l-arginine methyl ester) on contractile force by steroid-treated ventricle strips. Ventricle strips from both male (A) and female (B) rainbow trout were treated with L-NAME (1.0 mmol l^{-1}) for 10 min prior to exposure to 11-ketotestosterone (11KT; 0.3 μmol l^{-1}), testosterone (T; 0.3 μmol l^{-1}), cortisol (C; 0.1 μmol l^{-1} in males and 0.01 μmol l^{-1} in females) or estradiol (E2; 1.0 nmol l^{-1}). L-NAME had no independent effect on contractile force; however, L-NAME limited inotropic effects of androgens, C and E2. All values are means + S.E.M. (N=6–8). *P<0.05 *versus* EtOH control strips.

The finding of steroid-induced inotropism in the heart of male and female rainbow trout led us to study potential mechanisms of action *via* several complimentary approaches. First, we explored whether the type of anesthesia was important for realization of the inotropic effects of steroids. Only one previous study had examined the effects of C on cardiac function in fishes (Farrell et al., 1988), and these investigators concluded that C had a minimal effect on performance of perfused isolated hearts from rainbow trout. However, given our data, and the fact that Farrell et al. (1988) used chemically anesthetized rainbow trout, we were not surprised that they did not report any positive effects of C on isolated perfused hearts. In the current study, even short-term (<5 min) exposure of intact fish to common anesthetics prevented the subsequent response of isolated ventricle strips to C and sex steroids (Table 2). Anesthetics, including benzocaine, can compete with steroids for hydrophobic binding sites on the nicotinic acetylcholine receptor of *Torpedo marmorata* (Arias et al., 1990) and decrease the affinity of the canine Na^+/K^+-ATPase for ouabain (Kutchai et al., 2000). Thus, future studies should recognize the possibility that even brief chemical anesthesia of fishes can cause confounding effects of steroid hormone responsiveness in isolated cardiac tissue. We also studied the specificity of steroids for promoting inotropism by adding a generic precursor (cholesterol) and non-physiological steroid (aldosterone) to the experimental protocol. Based on the findings that these hormones were devoid of activity, it appears that there is selectivity for gonadal steroids and C to promote myocardial contractility in rainbow trout.

This study is also the first to investigate frequency-dependent effects of steroids on cardiac performance in fish. Under the defined experimental conditions, optimal steroid effects on increasing contractile force were observed at 0.5 Hz, were reduced at 0.8 Hz and absent at 0.2 Hz and 1.0 Hz. Physiological cardiac frequencies of rainbow trout at 14–15°C (Clark and Rodnick, 1999) fall within the range of frequencies tested. Generally speaking, the majority of teleost species show a negative force–frequency response (Shiels et al., 2002). In the current study, myocardial contractile force declined with

frequencies above 0.5 Hz, which is consistent with the findings of Hove-Madsen and Gesser (1989) in rainbow trout. Given this frequency dependency of steroid effectiveness *in vitro*, both body temperature and activity level may influence steroid action on the cardiovascular system *in vivo*.

In mammals, steroid hormones affect cellular function by a variety of mechanisms, including binding to membrane or nuclear receptors and several complex signal-transduction pathways. To elucidate the underlying mechanism that steroids use to modulate myocardial contractile performance in trout, we exposed ventricle strips to (1) BSA-conjugated steroids and (2) chemical inhibitors of steroid receptors and signaling cascades involving synthesis of polyamines, NO and G-protein activation. Although it is possible that the observed steroid effects involved a membrane-bound receptor rather than a traditional nuclear receptor, the fact that free steroids, but not BSA-conjugated steroids, had effects on contractility implies that membrane-bound receptors were not responsible for the observed effects. The fact that the positive inotropic actions of T, 11KT, E2 and C were inhibited completely by pretreatment with receptor blockers also suggests that the signaling mechanism for each hormone involves binding to its intracellular receptor. One important limitation of our tissue preparation is that we cannot identify whether the binding of steroids and chemical signal(s) originated in myocytes or some other cell near the myocyte (e.g. endothelial cell). Future studies involving isolated myocytes or nonmyocyte cells will be necessary to identify the target of steroid binding and signaling in trout cardiac tissue.

Based on the rapid timing of enhanced cardiac contractility following exposure to steroid hormones (observed within 3 min and maximal after 10 min), it appears that the mechanism of action involves a nongenomic pathway. The observed time course of inotropic effects in the rainbow trout ventricle agrees with previous studies showing rapid effects of C on muscle glycogen metabolism (Milligan, 2003) and similar effects in rat cardiac tissue (Rubín et al., 1999). However, we cannot rule out the possibility that a genomic mechanism is responsible. For example, the synthesis of ornithine decarboxylase, which appears to play an important role in the myocardial response to steroid hormones, has a 10–30 min turnover time in mammalian cells and increases rapidly after hormone stimulation (Bachrach, 1984). Additional studies of mRNA transcription and protein expression will be required to define whether the steroid response in cardiac tissue is genomic or nongenomic.

Rapid effects of steroids are likely to be mediated through signaling cascades involving polyamines, NO and G-protein activation. The aliphatic amines putrescine, spermidine and spermine are ubiquitous cellular compounds that appear important in cell growth and differentiation (Marton and Pegg, 1995). Previous research in mammals has indicated that polyamines contribute to androgenic stimulation of calcium flux and membrane transport (Koenig et al., 1989). Based on the selective inhibition of ornithine decarboxylase, the rate-regulating enzyme of polyamine synthesis, our results demonstrate that polyamine synthesis is required for steroid-enhanced force in ventricle strips from rainbow trout. At this point, however, we do not know which polyamine is the active species or the link between polyamine synthesis and other signaling pathways that ultimately promote cardiac inotropism in fishes.

NO, produced autocrinally by cardiomyocytes or paracrinally by endothelial cells, has also been implicated as a key molecule in the regulation of contractile performance. In mammalian cardiac muscle, NO can exert positive and negative effects on myocardial contractility, depending on the concentration of NO, the status of the endocardial endothelium and the degree of cholinergic or adrenergic stimulation (Mohan et al., 1996; Balligand and Cannon, 1997). An NO-induced reduction in stroke volume and work has been described in the eel heart (Imbrogno et al., 2001), although NO signaling is involved in angiotensin II-mediated inotropism (Imbrogno et al., 2003). Recent research in mammals has suggested that E2 has rapid effects on endothelial cell function, including stimulation of NO synthase (Chambliss and Shaul, 2002). Furthermore, inhibitors of NO synthase, such as L-NAME, have been shown to increase the response of myocytes to adrenergic agonists (Balligand et al., 1993). Our studies demonstrate that L-NAME effectively blocks the steroid-induced positive inotropism in the trout ventricle and provide evidence that steroid effects on contractile performance require products of NO synthase. Whether we inhibited endothelial and/or cardiomyocyte NO synthase remains to be determined. Ultimately, elucidation of the signaling mechanism that NO and polyamines use will enhance understanding of the role of steroid hormones in modulating cardiac function in fishes. One possible target for both polyamines and NO is the enzyme guanylate cyclase, which, when activated, could raise intracellular cGMP and subsequently stimulate cGMP-dependent protein kinase activity (Tantini et al., 2001).

Although several, rapid, nongenomic effects of steroids appear to be mediated through G-protein activation (Cato et al., 2002), this was not the case for the positive inotropic effects of steroids on the trout heart. This disassociation was inferred from the observations that (1) GDP had no independent effects on ventricle strip performance, and all the steroids tested (T, 11KT, C and E2) increased contractile force in strips pre-treated with GDP, and (2) the positive inotropism induced with epinephrine was completely additive to the actions of sex steroids and C. Finally, it will be of interest in future studies to distinguish the separate pathways or mechanisms that sex steroids and C use to modulate cardiac inotropism in male and female rainbow trout. As mentioned previously, we observed additive effects for androgens (T and 11KT) and C on promoting contractility of ventricle strips from males. Additive effects, and therefore the involvement of separate signaling pathways, were not observed between E2 and C in females.

Ultimately, care must be taken when attempting to extrapolate the results of the current study to *in vivo* cardiac performance and draw conclusions about the overall

importance of steroid hormones. One limitation of this study is the use of a multicellular isometric muscle preparation. Future research should include single-cell experiments on cardiomyocytes and endothelial cells to better define the target of steroid hormones, possible intercellular/paracrine and autocoid signaling, and intracellular pathways/mechanisms of action. In addition, it will be important to conduct experiments on whole animals to validate the physiological relevance of steroid-induced cardiac inotropism in fishes. Our study also strengthens the need to document the sex of fishes when conducting studies of hormone responsiveness and, more specifically, to include both sexes when performing studies on the regulation of myocardial contractility in salmonid fishes.

Summary

This study demonstrates that steroid hormones promote myocardial contractile performance *in vitro* in male and female rainbow trout. The observed effects of gonadal steroids and C depend upon (1) hormone concentration, (2) contraction frequency, (3) sex of the animal and (4) involve specific receptor types. The positive inotropism of sex steroids and cortisol was additive in males, but not females, and these effects were abolished when fish were anesthetized with either benzocaine or MS-222. Maximal effects are realized within 10 min and it appears that the improved inotropism involves (1) intracellular binding of steroids, (2) production of polyamines and NO but (3) no activation of G proteins.

This work was supported in part by NIH Grant Number P20 RR16454 from the BRIN Program of the National Center for Research Resources, the MSTI/MSMRI Research Institute of the St Luke's Regional Medical Center, and the Graduate Student Research and Scholarship Committee at Idaho State University.

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