Novel salmon cardiac peptide hormone is released from the ventricle by regulated secretory pathway

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NATRIURETIC PEPTIDES have been identified in various animal species of different phyla (3, 5, 27). They have been shown to have potent natriuretic and diuretic effects, as well as the ability to decrease blood pressure by relaxing vascular smooth muscle and by facilitating the transudation of plasma to the interstitium (27). In mammals the A- and B-type natriuretic peptides (ANP and BNP) have been found to be secreted by atrial and ventricular myocytes in response to mechanical stretch (4, 11, 15, 20). The peptide release may be a result of direct myocyte stretch or it may be due to paracrine factors such as endothelin-1, which has been shown to be able to stimulate natriuretic peptide secretion (8, 17). In the teleost eel ANP has been reported to respond to both volemic and osmotic stimuli, the latter being the more dominant (9).

ANP, as the prohormone, is stored in large quantities in the atria of all mammalian species studied (27), and a correspondingly higher number of secretory granules can be detected in the atrial myocytes (4). On the other hand, very little ANP with only an occasional secretory granule can be found in the ventricles in basal conditions (33). The secretion of ANP from the atria takes place via the regulated pathway, whereas the ventricles have been reported to utilize the constitutive pathway (1). The amount of BNP stored in the heart is at least an order of magnitude lower than that of ANP. Despite the fact that BNP is often thought of as a ventricular hormone, its basal concentration in mammalian atria is ~100 times higher than that in the ventricles (14). Its production in the ventricle per tissue weight increases to the atrial level only after induction by, e.g., abnormally high mechanical load. Because BNP has been reported to be stored in the same secretory granules as ANP (33), the mechanisms of secretion of the two hormones may be similar.

We have recently cloned and characterized from salmon a novel cardiac peptide hormone (sCP), whose peptide, mRNA, and gene structures represent an interesting mixture of mammalian A-, B-, and C-type (CNP) natriuretic peptides (32). sCP relaxes vascular smooth muscle, but its natriuretic and diuretic activity is not known. In addition to structure, another feature of sCP distinguishing it from the previously discovered cardiac hormones is its distribution. Its mRNA can be found in high concentrations in both chambers of the salmon heart, with only a three- to fourfold difference in favor of the atrium (32), and hence the majority of the total sCP in salmon heart appears to be produced in the ventricle. Therefore salmon ventricle represents a unique model for studying the general mechanisms of ventricular hormone secretion. In the present study we have devised an isolated perfused salmon ventricle preparation and used it to test the hypothesis that ventricular cardiomyocytes utilize the constitutive secretory pathway for peptide hormone release, suggested previously on the basis of results from a mammalian model (1).

MATERIALS AND METHODS

Experimental animals. Mature salmon (Salmo salar) of both sexes, weighing 728 ± 42 (SE) g, were purchased from...
the Finnish Game and Fisheries Research Institute at Taivalkoski. They were held in 350-liter tanks with circulated and aerated water at 3°C during the winter and 8°C during the autumn. The fish were kept in the tanks for 0–7 days, during which time they were not fed. The photoperiod mimicked the natural conditions at the time of the experiments, with a 12:12-h light-dark cycle during the autumn, and a 9:15-h light-dark cycle during the winter. The experiments were approved by the Animal Experimentation Review Board of the University of Oulu.

Perfusion of salmon ventricle in vitro. Salmon were stunned and placed on an operation board. The pericardium and ventral aorta were exposed by incising the ventral skin and retracting the muscle and connective tissues around the pericardium and ventral aorta. The pericardium was cut to expose the beating heart, which was removed. The atrium was cut off, and the ventricle (weight 856 ± 46 mg) was mounted in an organ bath formed by a glass vessel, which was constructed on the basis of the rat atrial perfusion system described previously (13). The simplicity of the setup is based on the fact that in the teleost heart there is no absolute requirement for a functional coronary circulation (6). The perfusion buffer was cooled to 6°C, using a K15 refrigerated osmometer (Haake, Karlsruhe, Germany). The osmolality was measured with aid of specific radioimmunoassay.

Radioimmunoassay of sCP. The sCP radioimmunoassays were performed using procedures described previously (32, 38). In brief, duplicate samples (100 µl) of each 1-min perfusate fraction were incubated overnight with the specific goat anti-sCP antiserum (100 µl) used at the final dilution of 1/8,000, followed by overnight incubation with 5,000–10,000 counts/min radioiodinated sCP (100 µl). Tyro-sCP was radioiodinated with the chloramine-T technique and purified by gel filtration using a 2-ml Sephadex G-25 M (Pharmacia, Uppsala, Sweden) column, followed by reverse-phase HPLC with a 4.6 × 300 mm Vydac C18 column (Separations Group, Hesperia, CA) and a 30-min 10 to 40% acetonitrile gradient in aqueous trifluoroacetic acid (TFA). Synthetic sCP-29, ranging from 8 to 2,000 pg/tube, was used as the standard. The bound and free fractions were separated by precipitation with 1 ml of 20% polyethylene glycol 6000 followed by centrifugation. The radioactivity in the precipitates was counted in a Wallac Clinigamma 1272 gamma-counter. Sensitivity of an assay was 10 pg/tube. Inter- and intra-assay coefficients of variation were <10% and <15%, respectively. Rat ANP, BNP, CNP, and endothelin-1 cross-reacted less than 0.1% in the assay.

Gel filtration HPLC. Aliquots of the guanidine isothiocyanate supernatants obtained from the RNA extraction diluted in 400 µl of 40% acetonitrile in aqueous 0.1% TFA were directly used for the gel filtration HPLC analysis. Samples (3 ml) of the ventricular per fusates were first extracted with Sep-Pak C18 cartridges (Waters, Milford, MA) as follows. The perfusate samples were acidified with 600 µl 1 mol/l HCl and 1.6% glycine and applied into the cartridges preactivated with isopropanol and aqueous 0.1% TFA. After a wash with 10 ml 0.1% TFA, the peptide fraction was eluted with 2 ml 80%
acetonitrile in aqueous 0.1% TFA. The extracts were dried in a SpeedVac concentrator (Savant Instruments, Hicksville, NY) and reconstituted in 400 µl 40% acetonitrile in aqueous 0.1% TFA. The ProteinPak-125 column (Waters) was eluted with 40% acetonitrile in aqueous 0.1% TFA at the flow rate of 1 ml/min. Fractions of 0.5 ml were collected, dried in SpeedVac, and subjected in duplicate to the sCP radioimmunoassay. The column was calibrated with bovine serum albumin (68 kDa, void volume), rat proANP (14 kDa), synthetic sCP-29 (3 kDa), and 125I (total volume).

Isolation of mRNA and Northern blot analysis. The ventricles subjected to 20 min or 2 h of mechanical load of 13 cmH2O and their respective controls, perfused identically but without added load, were frozen and stored at −70°C. For RNA isolation, the ventricular samples were ground to powder in liquid N2 and homogenized in 4 mol/l guanidine isothiocyanate, 0.025 mol/l sodium citrate, pH 7, 0.1 mol/l 2-mercaptoethanol, 0.5% N-lauroylsarcosine. An aliquot of each homogenate was stored at −70°C for use in sCP radioimmunoassay. Total RNA was isolated from the remaining homogenates with the acidic phenol method (2). The RNA was sized-fractionated on 1% formaldehyde agarose gels, blotted to nylon membrane, and fixed by baking at 80°C for 2 h. The membrane was hybridized overnight with a [32P]dCTP-sCP cDNA probe (32) labeled to a specific activity of >108 dpm/µg by random-primed labeling and purified by Sephadex G-50 gel filtration. The hybridization membranes were washed with 0.2× saline-sodium citrate and 0.2% SDS for 60 min at 65°C and used to expose X-O-Mat films (Kodak, Rochester, NY) with an intensifying screen at −70°C for 3.5 h. To control potential differences in the RNA load, a 482 bp CDNA probe, corresponding to the sequence 922–1403 of the rat gene for 18S ribosomal RNA (35), was prepared by RT-PCR, cloned to the EcoR I site of pBlueScript SKII+, and the sequence was confirmed using the dideoxy method. The insert was labeled and used in the hybridizations as described for sCP.

Immunoelectron microscopy. Standard methods described previously for the localization rat cardiac ANP (25) were utilized, with the exception that the antiserum was the goat anti-sCP used in the radioimmunoassays, and the antigen used in the specificity studies to displace the staining was synthetic sCP-29.

Statistical analyses. Results are expressed as means ± SE. The data were analyzed using one-way ANOVA followed by the Newman-Keuls test. Differences at the 95% level were considered statistically significant.

RESULTS

To characterize sCP release, a perfusion system of isolated salmon ventricle was set up, which enabled easy manipulation of the mechanical load imposed on the tissue, as well as the collection of samples of the perfusate for radioimmunoassay (Fig. 1). In this in vitro system the level of immunoreactive sCP released from the perfused ventricle remained relatively stable at 80.0 ± 9.2 (SE) pmol/l (n = 4) during the 30-min study period (Fig. 2A), when no mechanical load was applied. However, when the ventricle was loaded me-
chanically by raising the perfusate outflow height, an almost instantaneous load-dependent elevation of the perfusate immunoreactive sCP levels was observed. The 4 cmH$_2$O load caused a 2.0 ± 0.3-fold increase in the perfusate immunoreactive sCP level (Fig. 2B, P < 0.01, n = 6) and the 8 cmH$_2$O load a 3.0 ± 0.6-fold increase (Fig. 2C, P < 0.01, n = 7). Further increase of load to 13 cmH$_2$O resulted in a 3.3 ± 0.8-fold increase (Fig. 2D, P < 0.01, n = 8). Figure 3 summarizes the dose-response relationship of the ventricular load and the perfusate immunoreactive sCP level.

To determine whether sCP release can be induced by other factors in addition to mechanical load, we examined the effect of a relatively large dose of human endothelin-1. In mammalian experiments endothelin-1 has been found to be the potential paracrine factor inducing atrial ANP and BNP secretion. Endothelin-1 (n = 6) used in the relatively high concentration of 5 nmol/l, known to be effective in mammalian experiments, caused an approximately twofold increase (P < 0.01) in the immunoreactive sCP-release (Fig. 4) from the salmon ventricle preparation.

Immunoelectron microscopic results demonstrated the presence in salmon ventricle of a large number of granules containing immunoreactive sCP (Fig. 5). To quantify immunoreactive sCP and sCP mRNA stored in the tissue, the salmon ventricles used for the perfusion experiments were homogenized in conditions inhibiting proteolysis and RNA degradation. The immunoreactive sCP levels in different dilutions of the homogenates were measured with a specific radioimmunoassay, which showed parallel displacement between synthetic sCP standard and the ventricular extracts. Large amounts of immunoreactive sCP were detectable in the ventricle in the basal conditions (5.03 ± 0.50 nmol/g, n = 6), as well as after having been mechanically loaded with 13 cmH$_2$O for 20 min (6.95 ± 0.62 nmol/g, n = 8), with no statistically significant difference between the two. The same homogenates were used for the extraction of total RNA. Northern blot analysis demonstrated a strong 1.3-kb band hybridizing with the sCP cDNA probe in stringent wash conditions (Fig. 6). The intensity of the signal, quantified with densitometry, was no higher after 20 min or 2 h of mechanical loading than in the basal conditions (Fig. 6). In fact, the 20-min load was associated with a small but significant (P < 0.05) decrease in the sCP mRNA signal, when normalized to the 18S ribosomal RNA in the same samples.
Gel filtration HPLC analyses were performed to find the molecular form of immunoreactive sCP stored in the ventricular tissue and secreted to the perfusion fluid. Aliquots of the tissue extracts were used directly for the HPLC analysis, whereas samples of the perfusate were concentrated with solid phase C18 extraction before HPLC. The immunoreactivity in the tissue extracts, both in basal conditions and after mechanical load, was almost exclusively due to material with a molecular mass of 10 to 15 kDa (Fig. 7A). On the other hand, the immunoreactivity present in the perfusates, regardless of the degree of mechanical load, was due to material with a significantly lower molecular mass, coeluting with the biologically active 29-amino acid sCP used for the calibration of the column (Fig. 7, B and C).

The difference in the molecular forms between the stored and secreted peptide was not an artifact caused by the extraction of the perfusate samples, because gel filtration of unextracted perfusate samples from mechanically loaded ventricles displayed the same chromatographic pattern as that of samples extracted with C18 cartridges (data not shown).

**DISCUSSION**

Our results show that 1) salmon ventricle can be maintained in an organ bath with simple external perfusion, such that the mechanical load of the ventricle can be readily altered, 2) salmon ventricle stores large amounts of the prohormone of the novel cardiac
peptide (sCP) and the ventricular levels of mRNA encoding the peptide are correspondingly high, 3) a large number of granules staining with anti-sCP antiserum can be detected in the salmon ventricle by immunoelectron microscopy, 4) mechanical load and endothelin-1 induce an acute increase in the secreted sCP without causing corresponding rapid changes in the tissue peptide or mRNA levels, 5) immunoreactive sCP stored in the ventricle in basal and mechanically loaded conditions has a high-molecular weight of at least 10,000, whereas the secreted immunoreactivity corresponds in size to the 29-amino acid biologically active form with the molecular weight of 3,000.

Our present results demonstrate that the salmon ventricle stores large amounts of immunoreactive sCP and that a matching high level of sCP mRNA can be found in the ventricular extracts. The concentration of immunoreactive sCP in salmon ventricle (5,030 ± 500 pmol/g) is much higher than that previously reported for ANP (6.5–30 pmol/g) or CNP (0.6 pmol/g) in rat ventricle (14, 21) and also significantly higher than that of BNP (3–4 pmol/g) in rat ventricle (12, 21) or ventricular natriuretic peptide (=1,600 pmol/g) in eel ventricle (31), hormones considered to be characteristically ventricular. In keeping with the high tissue concentrations, we detected in salmon ventricular myocytes, using immunoelectron microscopic methods, a large number of vesicles staining with anti-sCP antiserum and resembling secretory granules. Thus salmon ventricle appears to produce sCP in a manner resembling the production of ANP in mammalian atrium with high basal levels of mRNA and hormone stores. This differs significantly from the production of ANP and BNP in postnatal mammalian ventricle, which appear to be made on demand in nonpathological situations (reviewed in Ref. 22). The level of immunoreactive sCP in salmon ventricle approaches that of ANP in mammalian atria. There is ~10 nmol/g immunoreactive ANP in rat atrium (15), compared with the 5 nmol/g of immunoreactive sCP we detected in the salmon ventricle.

In the present study, using the isolated perfused salmon ventricle model, we found that the molecular weight of immunoreactive sCP stored in salmon ventricle (~10,000), as assessed by gel filtration HPLC, is considerably higher than that of the secreted peptide (~3,000, see below). The smaller molecular weight form is probably due to the biologically active 29-amino acid form of sCP (32). It therefore appears, analogously with mammalian proANP (37), that the final proteolytic processing of pro-sCP is closely connected to exocytosis of the secretory granules. In the mammalian atrium ANP is stored exclusively as proANP1–126 and the processing to ANP99–126 takes place in conjunction with exocytosis of the secretory granules (22, 34, 37). On the other hand, at least in humans and rats, proBNP appears to be processed while stored in the heart (23, 30). The ventricular secretion of mammalian natriuretic peptides is considered to take place via the constitutive pathway (1). The subtilisin-like proprotein convertase furin is one of the major processing enzymes of the constitutive secretory pathway (28). With regard to prepro-sCP, however, the lack of an arginine residue at position −4 from the cleavage site of prepro-sCP (32) makes it an unlikely substrate for furin.

Salmon ventricle offers a highly useful model for studying the effects of mechanical stimuli on cardiac secretion. Unlike mammalian ventricles, teleost ven-
The acute sensitivity of the secretion of sCP mechanical load resembles that of mammalian atrial ANP (15). Numerous studies have shown that stretching of atrial myocytes in vitro or by volume load or pressure in vivo stimulates the secretion of ANP within minutes, whereas the activation of ANP gene expression takes hours or days to develop (4, 26, 27, 29). In the case of BNP, on the other hand, mechanical load causes a rapid induction of gene expression, whereas the secretory response is slower to develop (4, 27). We did not observe any acute increase in sCP mRNA in response to loading for up to 2 h. On the contrary, the 20-min load was associated with a small but significant decrease of sCP mRNA levels, the cause of which is not yet known. It may be argued that the period of loading was too short for any changes in natriuretic peptide mRNA levels to be detected. However, a clear induction of the atrial BNP gene can be observed after an hour of mechanical loading in mammalian models (20).

Several previous studies have shown that endothelin-1 is a potent stimulator of ANP release in mammals (reviewed in Ref. 39). Endothelin has been shown to stimulate ANP release in a perfused rat heart preparation (19), as well as to elevate circulating ANP levels in anesthetized rats (24). Immunoreactive endothelin has been found in nonmammalian vertebrates such as fish (10, 36). In our study endothelin-1 had the ability to increase modestly the secretion of immunoreactive sCP from the isolated perfused salmon ventricle preparation. Our results therefore show that the secretion of sCP can be induced rapidly not only by mechanical load but by humoral factors, such as endothelin-1, as well. Thus the secretion of sCP from salmon ventricle has a resemblance to the natriuretic peptide secretion from mammalian atrium. In rats, for example, endothelin-1 has been found to be a strong stimulator of natriuretic peptides in the atria but not in the ventricle (17–19).

Our recent results with the ventricular secretion of the novel cardiac hormone sCP led to several interesting conclusions. On the basis of previous studies with mammalian cardiomyocytes, the ventricular secretion of the natriuretic peptides is believed to take place via the constitutive pathway, whereas the atrial secretion follows the regulated pathway (1). Our results show that this is not the case with sCP production in the salmon ventricle and therefore that the constitutive pathway is not an inherent property of ventricular hormone release. On the contrary, the secretion of sCP from salmon ventricle clearly utilizes the regulated pathway. It can be induced rapidly by mechanical load and modulated by paracrine factors such as endothelin-1. The ventricle appears to use secretory granules to store sCP in sufficient amounts for short-term sCP need, because no significant change can be detected in the ventricular sCP or its mRNA levels after 20-min or even 2-h stimulation of secretion by mechanical load, despite continuously elevated sCP release. The post-translational processing to mature sCP appears to take place in conjunction with the exocytosis of the secretory granules, as is the case with the processing of mammalian proANP. Considering the ease of use of salmon ventricle for in vitro perfusion experiments sCP is an excellent model for investigating cardiac secretion mechanisms, as well as the general cellular mechanisms and transduction pathways coupling mechanical stimuli to exocytosis of secretory products.

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REFERENCES