Experimental Physiology (1998), **83**, 809–820
Printed in Great Britain

EFFECTS OF ARGININE VASOTOCIN, ISOTOCIN AND MELATONIN ON BLOOD PRESSURE IN THE CONSCIOUS ATLANTIC COD (*GADUS MORHUA*): HORMONAL INTERACTIONS?

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(MANUSCRIPT RECEIVED 10 JUNE 1998, ACCEPTED 25 AUGUST 1998)

SUMMARY

The effects of exogenous arginine vasotocin (AVT), isotocin (IT) and melatonin on the blood pressure of conscious Atlantic cod (*Gadus morhua*) were examined. Ventral aortic blood pressure (P_{VA}) was recorded from a cannula inserted into the afferent branchial artery of the third gill arch. Dorsal aortic blood pressure (P_{DA}) was recorded via a cannula implanted into the efferent branchial artery in the same gill arch. Each fish received two doses (10 and 50 ng kg^{-1}) of AVT, IT and melatonin. AVT was also administered in combination with melatonin. Injection of 10 ng kg^{-1} AVT produced significant hypertension, especially in animals injected during the daytime. In contrast, the same dose of IT induced no significant change in either P_{VA} or P_{DA}. Administration of 10 ng kg^{-1} melatonin at night caused a long-lasting decrease in both parameters. Melatonin also inhibited the increase in blood pressure elicited by AVT. These results indicate that AVT, but not IT, is vasopressor in the cod. The effects of combined administration of melatonin and AVT show the antihypertensive action of melatonin in AVT-induced hypertension.

INTRODUCTION

Arginine vasotocin (AVT) and isotocin (IT) are the teleost fish neurohypophysial hormones. Both AVT and IT are closely related to the mammalian hormone arginine vasopressin (AVP). Vasotocin is present in all vertebrate classes and is regarded as the ancestral neurohypophysial nonapeptide (Acher, 1995). Although the physiological role of AVT is poorly understood the literature strongly suggests the contribution of the hormone to the maintenance of salt and fluid balance and to regulation of some cardiovascular parameters and endocrine secretion in teleosts (Babiker & Rankin, 1979; Groves & Batten, 1986; Balment, Warne, Tierney & Hazon, 1993; Uchiyama & Murakami, 1994; Amer & Brown, 1995; Warne & Balment, 1995; Pierson, Guibbolini & Lahlou, 1996). Systemic administration of AVT induces elevation of blood pressure in various non-mammalian vertebrates (Babiker & Rankin, 1979; Le Mevel, Mabin & Vaudry, 1991; Chiu, Lee & Pang, 1993; Le Mevel, Pomantung, Mabin & Vaudry, 1993; Robinzon, Koike & Marks, 1993; Oudit & Butler, 1995) and the hormone may play a role in regional blood flow distribution in fish (Warne & Balment, 1997). It has been demonstrated that the neurohypophysial peptides constrict the branchial vessels in fish (Chan & Chester-Jones, 1969; Rankin & Maetz, 1971; Bennett & Rankin, 1986).

Melatonin (*N*-acetyl-5-methoxytryptamine), the principal hormone of the pineal gland and retina in vertebrates, is implicated in those physiological processes that are controlled by light: circadian and seasonal rhythms, reproduction and behaviour (Binkley, 1988; Reiter, 1991;

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Filadelfi & Castrucci, 1996). In all species examined, the circulating melatonin level rises in response to an increase in melatonin synthesis during darkness. Light suppresses or inhibits melatonin synthesis. In teleosts, the pineal organ is the major source of melatonin (Kezuka, Iigo, Furukawa, Aida & Hanyu, 1992; Zachmann, Knijff, Ali & Anctil, 1992). Initially, the central nervous system was presumed to be the main target for melatonin action. Now, it is known that other organs and systems respond to melatonin signals. Melatonin seems to be involved in blood pressure control in mammals (Zanoboni & Zanoboni-Muciaccia, 1967; Birau, Peterssen, Meyer, Gottschalk, 1981; Kawashima, Miwa, Fujimoto, Oohata, Nishino & Koike, 1987) but no data are available for fish.

Participation of both AVT and melatonin in systems controlling the physiological adaptation of fish to daily and seasonal environmental changes (light, temperature, salinity) and the AVT/AVP–melatonin interrelationship described in mammals (Yasin, Costa, Besser, Hucks, Grossman & Forsling, 1993; Juszczak, Debeljuk, Bartke & Stempniak, 1995; Kulczykowska, 1995*a*), provided the background for the present study of AVT–melatonin interaction in fish.

The aim of this work was to assess the influence of exogenous arginine vasotocin, isotocin and melatonin on the blood pressure in the conscious Atlantic cod. Moreover, the potential interaction of these vasoactive hormones was studied by examining the responses to combined administration of AVT and melatonin.

METHODS

Experimental animals

Atlantic cod (*Gadus morhua*) of both sexes, weighing 550–850 g were caught off the Swedish coast and held at the Department of Zoophysiology (University of Göteborg, Sweden), where the experiments were performed. The animals were kept in seawater holding tanks at 9–10 °C for at least 2 weeks before experimentation. The fish were not fed while in captivity. The study was performed in November.

Cannulation of gill arteries

The fish were anaesthetized in a seawater solution of MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate, 100 mg l^{-1}; Sigma) until respiratory movement ceased. Then they were transferred to an operating table, where seawater containing 50 mg l^{-1} MS-222 was continuously passed over the gills during surgery. Two polyethylene cannulae (PE50) filled with heparinized (50–100 i.u. ml^{-1}) 0·9 % NaCl were placed in the afferent and efferent gill circulatory system, as described previously (Axelsson & Nilsson, 1986; Axelsson & Fritsche, 1994).

Ventral aortic blood pressure (P_{VA}) was recorded from a cannula inserted into the afferent branchial artery of the third gill arch. Heart rate was measured on the basis of the pulsatile P_{VA} signal. Dorsal aortic blood pressure (P_{DA}) was recorded via a cannula implanted into the efferent branchial artery in the same gill arch. During the experiments, the gill vessel cannulae filled with heparinized (50 i.u. ml^{-1}) 0·9 % NaCl were connected to a Statham P23 pressure transducer and Grass polygraph recorder system (model 79C). Calibration of pressure was made against a static water column. Mean P_{VA} and P_{DA} were determined as diastolic pressure + $\frac{1}{2}$ pulse pressure.

Blood samples (1·5 ml) were collected from the ventral aortic catheter twice a day: at 11.00 and 23.00 h. The volume was replaced by red blood cells resuspensed in 0·9 % NaCl. Hormones and vehicle were injected through the dorsal aortic cannula.

After the experiments, the fish were killed by overdose of MS-222.

Hormones

[Arg^8]vasotocin (molecular weight (MW), 1050·2; purity, 97 %), isotocin (MW, 966·1; purity, 97 %) and melatonin (MW, 232·3) were obtained from Sigma. AVT (1 mg ml^{-1}) and IT (1 mg ml^{-1}) were dissolved in HPLC-grade water and stored as stock solutions at –70 °C. Working standards and peptide solutions for injection were prepared directly before use in HPLC-grade water and 0·9 % NaCl, respectively.

The stock solution of melatonin (1 mg ml^{-1}) was prepared by dissolving in HPLC-grade methanol (J. T. Baker, Deventer, The Netherlands). Working standards and solutions for injection were prepared in HPLC-grade water and 0·9 % NaCl, respectively, immediately before use. The melatonin solutions were kept in the dark at −20 °C until use (but for no longer than 2 h) to avoid melatonin decomposition during storage.

Experimental protocol

The cod ($n = 6$) were transferred to experimental tanks and allowed to recover from surgery for 24 h. They were kept under controlled illumination (light on: 08.00–20.00 h). The stability of blood pressure and heart rate were checked for at least 1 h prior to the injections. Each fish received a control injection of 0·2 ml of 0·9 % NaCl, which produced no change in the measured cardiovascular parameters. The hormones were delivered in a volume of 0·1 ml and a 0·1 ml saline flush was given immediately after injection. Each fish received two doses (10 and 50 ng kg^{-1}) of AVT, IT and melatonin. AVT was also administered in combination with melatonin: the fish was first injected with 10 ng kg^{-1} melatonin, and 10 min later with 10 ng kg^{-1} AVT, or the AVT injection was given first followed 5 min later by the melatonin injection. Daytime experiments were started at 11.00 h, and night-time experiments were started at 23.00 h. Cardiovascular parameters were always allowed to return to pre-injection levels between injections and were required to remain constant for 1 h before subsequent injections.

P_{VA} and P_{DA} were recorded continuously during the 10 min period preceding intra-arterial injection of vehicle or hormones and 60 min post injection.

Plasma analysis

Plasma was separated from blood by centrifugation at 7000 *g* (microcentrifuge Wifug) and stored at −70 °C. Plasma was assayed for AVT, IT and melatonin using the HPLC methods described in detail elsewhere (Kulczykowska, 1995*b*; Kulczykowska & Iuvone, 1998). Osmolality was determined by vapour pressure measurement using a Wescor Vapor Pressure Osmometer (Wescor Inc., Logan, UT, USA). Plasma sodium was measured using an EasyLyte ions analyser (Medica Co., Bedford, MA, USA).

AVT and IT were extracted from plasma by solid phase extraction (SPE) using C_{18} Bakerbond cartridges (J. T. Baker, Phillipsburg, NJ, USA; pore size, 60 Å; particle diameter, 40 μm). HPLC was performed using a Beckman modular system (Beckman Instruments, San Ramon, CA, USA) with UV detector. Data were digitized using a Beckman 406 analog interface and processed using Beckman analytical series System Gold data acquisition software on an IBM compatible computer. Chromatographic separations were carried out on an Ultrasphere C_{18} column (i.d., 250 × 4·6 mm; particle diameter, 5 μm; pore size, 80 Å) connected to a precolumn (i.d., 45 × 4·6 mm) filled with the same material, both obtained from Beckman Instruments. Linear gradient elution from 20 to 40 % in 20 min was carried out with 0·1 % trifluoroacetic acid (TFA) in water and 0·1 % TFA in acetonitrile–water (3:1). The eluate was monitored at 215 nm. Plasma AVT and IT were identified by their retention times compared with those of standards. Quantitative determination of AVT and IT was performed on the basis of a standard curve. The inter- and intra-assay coefficients of variation were 17 % ($n = 15$) and 10 % ($n = 15$) for AVT and 15 % ($n = 15$) and 12 % ($n = 15$) for IT, respectively.

Melatonin was extracted from plasma by SPE using the same cartridge as described above. HPLC was performed with the Beckman modular system, mentioned above, connected to a Shimadzu spectrofluorometric detector (model RF-551). Chromatographic separations were carried out on the same Ultrasphere C_{18} column. Excitation and emission wavelengths were set at 286 and 352 nm, respectively. An isocratic elution system was prepared. The mobile phase was 60 % HPLC-grade methanol. The inter- and intra-assay coefficients of variation were 14 % ($n = 9$) and 10 % ($n = 9$), respectively.

Chemicals

HPLC-grade acetonitrile, water, methanol and TFA were purchased from J. T. Baker (Deventer, The Netherlands). Glacial acetic acid and hydrochloric acid were supplied by Merck.

Statistical analysis

For multiple comparisons, analysis of variance (ANOVA) and Dunnett's test were used. Significant differences between two means were identified using Student's paired *t* test. Significance was taken as $P < 0·05$. Data are given as means ± S.E.M.

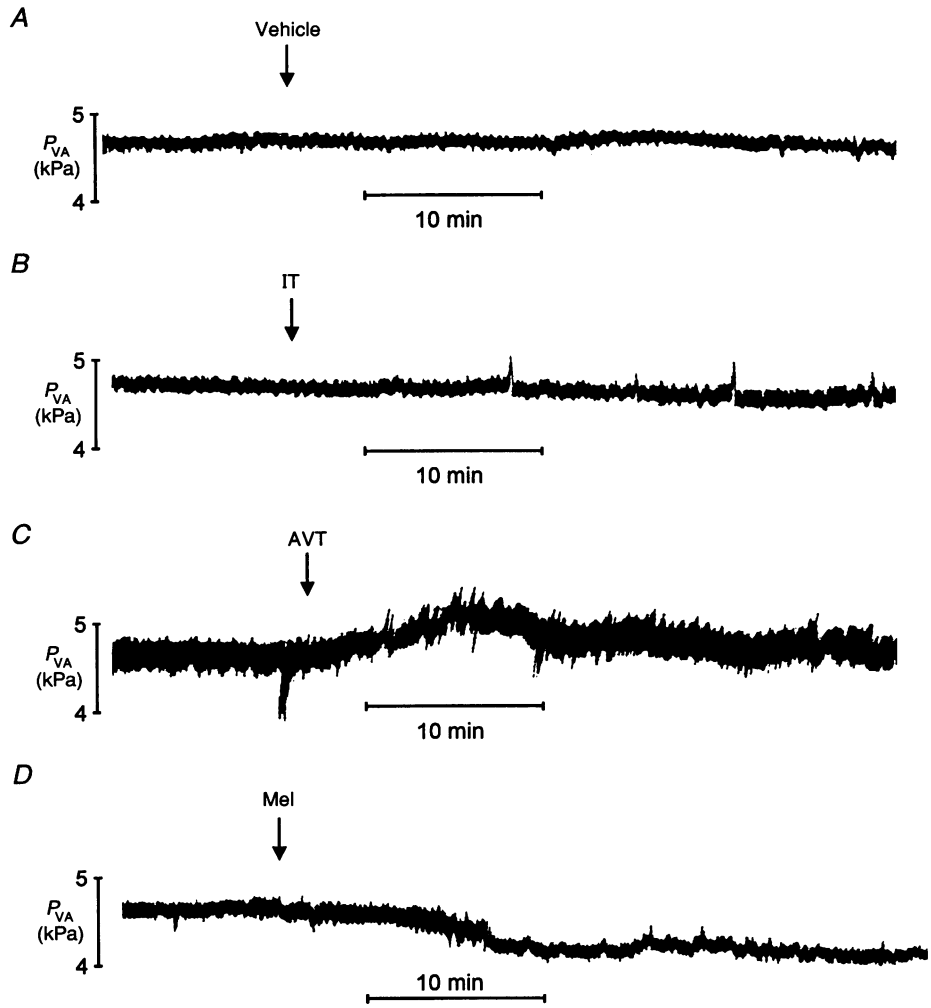

Fig. 1. Representative recordings of P_{VA} from the same fish after I.A. injections of vehicle (0·2 ml of 0·9 % NaCl; *A*), and 10 ng kg^{-1} doses of isotocin (IT; *B*), arginine vasotocin (AVT; *C*), and melatonin (Mel, *D*).

RESULTS

Vehicle (0·9 % NaCl) or hormones were injected (I.A.) into conscious cod in which blood pressures (P_{VA} and P_{DA}) and heart rate were stable. The control mean blood pressures measured during the night and day did not differ significantly. The patterns of change in P_{VA} and P_{DA} were similar in all experiments except one: the daytime administration of 50 ng kg^{-1} AVT.

Individual recordings illustrating changes in P_{VA} in the same fish during experimental procedures are presented in Figs 1, 2 and 3.

The mean percentage changes in P_{VA} following injection of vehicle or hormones are presented in Fig. 4. P_{DA} changes, not presented independently, were identical to those in P_{VA}.

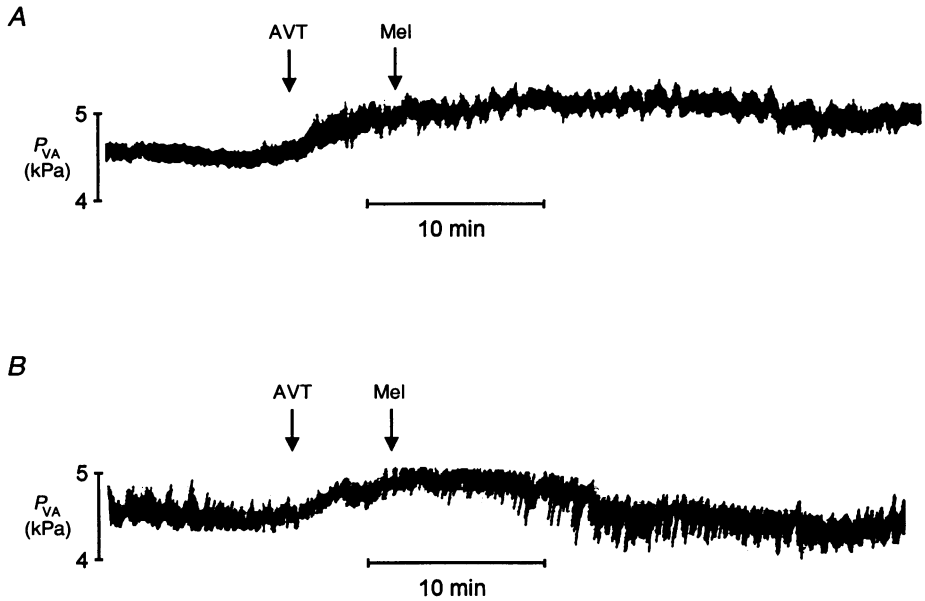

Fig. 2. Representative recordings of P_{VA} after combined treatment, AVT followed by melatonin (Mel) I.A. injections (10 ng kg^{-1} each) during the day (*A*) and at night (*B*).

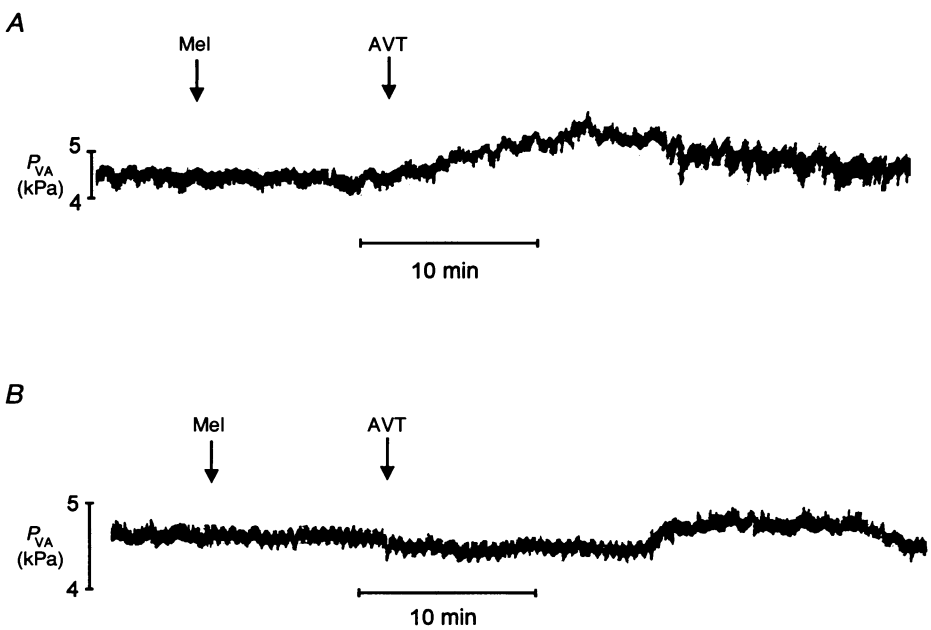

Fig. 3. Representative recordings of P_{VA} after combined treatment, melatonin (Mel) followed by AVT I.A. injections (10 ng kg^{-1} each) during the day (*A*) and at night (*B*).

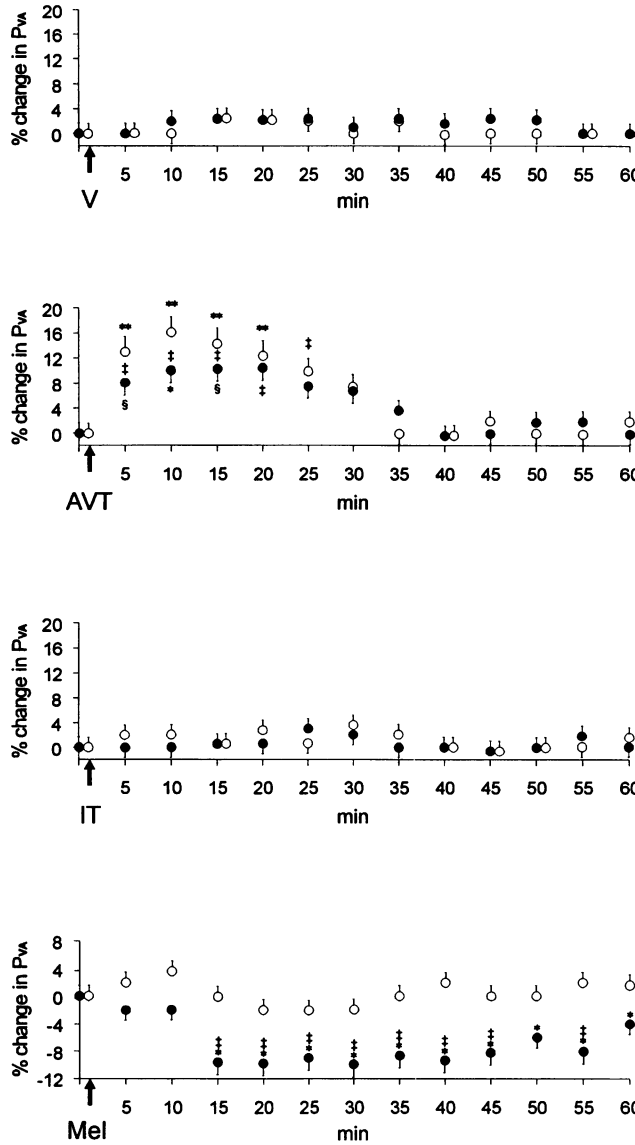

Fig. 4. Percentage changes in P_{VA} following vehicle (V, 0·2 ml of 0·9 % NaCl), AVT, IT and melatonin (Mel), given as single daytime (○; start 11.00 h) and night-time (●; start 23.00 h) I.A. injections (all at 10 ng kg^{-1}) in conscious cod. Symbols and error bars indicate means and S.E.M., respectively, $n = 6$. ** $P < 0·001$ compared with control; ‡ $P < 0·01$ compared with control; § $P < 0·05$ compared with daytime changes; * $P < 0·01$ compared with daytime changes.

Vehicle (0·2 ml of 0·9 % NaCl) injected during the day and at night induced no change in P_{VA} and P_{DA}. Administration of 10 ng kg^{-1} AVT produced a significant increase in both pressure values. Comparison of the effects of AVT (10 ng kg^{-1}) injected during the day and at night showed a significantly ($P < 0·05$) greater pressor response during the first 15 min in the daytime. In contrast, the same dose of IT induced no significant acute change in either P_{VA} or P_{DA}. Administration of 10 ng kg^{-1} melatonin caused no change in either P_{VA} or P_{DA} during

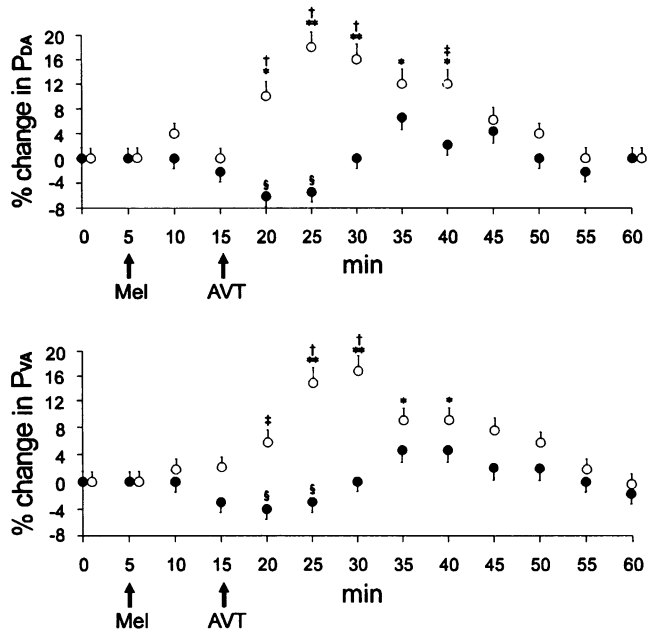

Fig. 5. Percentage changes in P_{VA} and P_{DA} after daytime (○) and night-time (●) I.A. injection of melatonin (Mel; 10 ng kg^{-1}) followed by AVT (10 ng kg^{-1}) in conscious cod. Symbols and error bars indicate means and S.E.M., respectively, $n = 6$. ** $P < 0{\cdot}001$ compared with control; * $P < 0{\cdot}05$ compared with control; § $P < 0{\cdot}05$ compared with 35 min value; † $P < 0{\cdot}001$ compared with night-time changes; ‡ $P < 0{\cdot}01$ compared with night-time changes.

the day, though there was a significant long-lasting (40 min) decrease in both parameters after melatonin injection during the night. Moreover, the night-time P_{VA} or P_{DA} values measured 15–60 min after melatonin injection were significantly lower than the corresponding daytime values ($P < 0{\cdot}01$).

Higher doses of IT and melatonin (50 ng kg^{-1}) injected during the day induced a slight but non-significant increase (maximum, 5 %) and a noticeable and significant decrease (maximum, 17 %; $P < 0{\cdot}05$), respectively, in both blood pressure values. The daytime administration of 50 ng kg^{-1} AVT caused increases in P_{VA} and P_{DA} (maximum, 30 %; $P < 0{\cdot}001$), preceded by a transient decrease in P_{DA} value (maximum, 15 %; $P < 0{\cdot}05$). These changes were associated with marked bradycardia (data not shown).

The results of combined hormone administrations are presented in Figs 5 and 6. Melatonin pretreatment did not alter the response to AVT in the daytime-treated animals but abolished the response in the night-time-treated fish, except for a transient increase at about 35–40 min after injection (Fig. 5). On reversal of the injection protocol, night-time injection of AVT followed by melatonin, AVT induced blood pressure elevation for a shorter period than following AVT alone at night. Moreover, melatonin appeared to be more effective in this protocol when administered at night (Fig. 6).

Plasma hormone levels were measured in samples taken before injection. Plasma AVT concentrations measured in fish at 11.00 and 23.00 h did not differ significantly (33 ± 18 and 47 ± 25 fmol ml^{-1}, respectively; $n = 3$). Plasma IT concentrations measured in the same fish did not vary significantly either (21 ± 10 and 28 ± 15 fmol ml^{-1}, respectively; $n = 3$). The night-time plasma melatonin concentration (259 ± 41 pg ml^{-1}; $n = 4$) was significantly higher

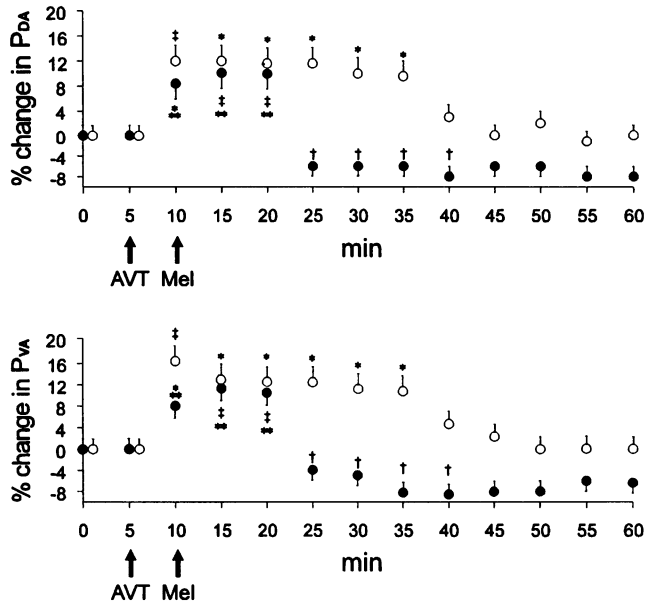

Fig. 6. Percentage changes in P_{VA} and P_{DA} after daytime (○) and night-time (●) I.A. injection of AVT (10 ng kg^{-1}) followed by melatonin (Mel; 10 ng kg^{-1}) in conscious cod. Symbols and error bars indicate means and S.E.M., respectively, $n = 6$. ‡ $P < 0{\cdot}01$ compared with control; * $P < 0{\cdot}05$ compared with control; ** $P < 0{\cdot}001$ compared with 25–60 min values; † $P < 0{\cdot}001$ compared with daytime values.

than that measured at 11.00 h (28 ± 11 pg ml^{-1}; $n = 4$) ($P < 0{\cdot}001$). Shortly after injections, plasma hormone concentration would be expected to increase by approximately 0·4 (dose of 10 ng kg^{-1}) and 2 ng ml^{-1} (dose of 50 ng kg^{-1}), assuming a blood volume of 3 ml blood (100 g body weight)$^{-1}$ and instantaneous distribution of the hormones within the intravascular compartment. The haematocrit value was approximately 20 % in all fish. Plasma osmolality and sodium concentration measured in the initial blood sample and the blood sample taken after the experiment showed no change.

DISCUSSION

Several studies have been performed on fish and other vertebrates to investigate the response of the cardiovascular system to exogenous arginine vasotocin (Babiker & Rankin, 1979; Le Mevel *et al.* 1991, 1993; Chiu *et al.* 1993; Robinzon *et al.* 1993; Oudit & Butler, 1995; Warne & Balment, 1997). However, this is the first attempt to compare the effects of melatonin, isotocin and a combination of melatonin and AVT in a single study performed in conscious teleost fish.

Arginine vasotocin has been found to increase the systemic blood pressure in conscious rainbow trout, flounder and eel (Le Mevel *et al.* 1993; Oudit & Butler, 1995; Warne & Balment, 1997). The mean plasma AVT levels reported in fish show great variation, probably due to differences in assay technique, species and physiological state (Perrott, Carrick & Balment, 1991; Balment *et al.* 1993; Warne, Hazon, Rankin & Balment, 1994; Kulczykowska & Stolarski, 1996; Kulczykowska, 1997). This makes it difficult to decide whether the dose of

hormone administered was physiological or pharmacological. In the present study, the doses used were based on measurement of circulating hormone levels and their pharmacokinetics. Directly after injection of the lower doses of hormones the theoretical plasma hormone concentration achieved would be above the physiological range. However, peptides and indolamines diffuse to the interstitial fluid and are rapidly cleared from the circulation, which results in a decrease in their effective plasma concentration. Assuming an AVT half-life of 7·5 min (Warne & Balment, 1997), the post-injection plasma AVT levels would decrease rapidly, to a level that could be considered physiological. On the other hand, both doses of IT used would be expected to produce plasma levels above the normal physiological range.

Administration of 10 ng kg^{-1} AVT induced a marked increase in P_{VA} and P_{DA} without affecting the heart rate. The significant increases in P_{VA} and P_{DA} were recorded during the first 20 min, when the predicted plasma AVT concentrations would still be 2- to 4-fold higher than the mean circulating levels in cod. The AVT levels measured in unstressed, hydrated fish under steady-state physiological conditions appear too low to contribute to the physiological regulation of blood pressure. However, during dehydration and haemorrhage, plasma AVT concentration might increase sufficiently to induce a significant pressor response in fish. The higher dose of AVT (50 ng kg^{-1}), which should be considered pharmacological, caused a pronounced increase of P_{VA} and P_{DA} associated with marked bradycardia. The initial fall in dorsal aortic blood pressure observed immediately after administration of 50 ng kg^{-1} AVT may have been due to AVT-dependent branchial vasoconstriction, as suggested by Warne & Balment (1997). The lower AVT dose did not cause any initial decrease in P_{DA}, which suggests that the biphasic response to AVT is of no physiological relevance. The pressor action of AVT observed in this study was similar to that reported in the conscious and anaesthetized rainbow trout (Le Mevel *et al.* 1991, 1993) and was probably mediated by a receptor similar to the mammalian V_1 vasopressinergic receptor, as shown by Warne & Balment (1997) in the flounder and Uchiyama & Murakami (1994) in the lamprey.

The absence of any vasopressor response to the lower dose of IT suggests that this neurohypophysial peptide does not contribute to the physiological regulation of blood pressure in the cod. This further supports the idea that the synthesis and/or release of AVT and IT are controlled independently and the role of each hormone is different, as previously suggested (Kulczykowska & Stolarski, 1996; Kulczykowska, 1997).

The half-life of melatonin in the blood has been estimated to be about 10–40 min (Filadelfi & Castrucci, 1996). Thus, the melatonin dose of 10 ng kg^{-1} can be considered physiologically relevant; when added to the circulation during the day, this amount of the hormone may mimic the natural nocturnal level of the hormone. When administered to cod during the day, melatonin did not alter the blood pressure. Conversely, the same dose of melatonin given at night caused significant and prolonged decreases in both P_{VA} and P_{DA}. The diurnal variation in the response to melatonin could be due to a number of factors including a diurnal change in the number of receptors present and desensitization of the receptors on exposure to melatonin. On the other hand, the dose of melatonin injected could be insufficient to exert the effect during the day, when the level of endogenous melatonin is very low. There is a possibility that a threshold exists for the response to melatonin to bring about the blood pressure changes. The same dose added to the high endogenous melatonin content at night may elicit an effect which, however, can be considered pharmacological. The hypotensive effect of melatonin and its vascular receptors were described in mammals (Zanoboni & Zanoboni-Muciaccia, 1967; Birau *et al.* 1981; Kawashima *et al.* 1987; Viswanathan, Laitinen & Saavedra, 1993; Cagnacci, Arangino, Angiolucci, Mascio, Longu & Melis, 1997). The cardiovascular response to

melatonin may be exerted in part by the direct action of the hormone on blood vessels, in addition to a reduction in noradrenaline activity. Further work is required to confirm these data in the vascular system of the teleost fish.

The combined administration of melatonin and AVT revealed the antihypertensive action of melatonin in AVT-induced hypertension, especially in cod injected at night. This observation may suggest that in addition to direct action via hormone receptors in blood vessels, the hormone action can be mediated by the sympathetic nervous system. The indirect action of melatonin involving catecholamines and/or the renin–angiotensin system in the hypotensive effect observed within the first 15 min after melatonin injection is improbable. By contrast, during prolonged melatonin-induced blood pressure decrease catecholamines and angiotensin could be involved. In mammals melatonin can directly inhibit the release of AVP (Yasin *et al.* 1993; Juszczak *et al.* 1995); a similar inhibition of basal hypothalamic arginine vasotocin release in fish could also be considered. It is worth noting that the P_{VA} and P_{DA} changes within the first 15 min after AVT injection were significantly higher in the daytime-treated than in the night-time-treated fish. This may reflect an interdependence of AVT and the circadian rhythm of melatonin secretion, which is in agreement with the inhibitory effect of melatonin on AVT-induced hypertension shown in the present work.

In summary, AVT was shown to be a vasopressor in cod, but the pressor response was elicited with circulating AVT concentrations higher than the basal steady-state value. Isotocin did not alter blood pressure in cod. When injected into the fish during the night melatonin did decrease the blood pressure. The combined administration of melatonin and AVT revealed the antihypertensive action of melatonin in AVT-induced hypertension and provided the evidence for a potential physiological interaction between the two hormones. Further studies are needed to elucidate the mechanisms involved.

The excellent technical assistance of Gunilla Rydgren (Department of Zoophysiology, Göteborg University) is gratefully acknowledged. Author wishes to thank Professor Michael Axelsson (Department of Zoophysiology, Göteborg University) for making his expertise in the use of the aquatic facilities available to the author and all staff of the Department for a friendly atmosphere during experiments. Special thanks go to Professor Janusz Sadowski (Institute-Medical Research Centre of Polish Academy of Sciences, Warsaw, Poland) and Professor P. Michael Iuvone (Emory University School of Medicine, Atlanta, USA) for critically reading the manuscript and providing helpful advice.

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