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DESENSITISATION OF CHROMAFFIN CELL NICOTINIC RECEPTORS DOES NOT IMPEDE CATECHOLAMINE SECRETION DURING ACUTE HYPOXIA IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

Experiments were performed on adult rainbow trout (*Oncorhynchus mykiss*) *in vivo* using chronically cannulated fish and *in situ* using a perfused posterior cardinal vein preparation (i) to characterise the desensitisation of chromaffin cell nicotinic receptors and (ii) to assess the ability of fish to secrete catecholamines during acute hypoxia with or without functional nicotinic receptors. Intra-arterial injection of nicotine (6.0×10^{-7} mol kg^{-1}) caused a rapid increase in plasma adrenaline and noradrenaline levels; the magnitude of this response was unaffected by an injection of nicotine given 60 min earlier. Evidence for nicotinic receptor desensitisation, however, was provided during continuous intravenous infusion of nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) in which plasma catecholamine levels increased initially but then returned to baseline levels. To ensure that the decline in circulating catecholamine concentrations during continuous nicotine infusion was not related to changes in storage levels or altered rates of degradation/clearance, *in situ* posterior cardinal vein preparations were derived from fish previously experiencing 60 min of saline or nicotine infusion. Confirmation of nicotinic receptor desensitisation was provided by demonstrating that the preparations derived from nicotine-infused fish were unresponsive to nicotine (10^{-5} mol l^{-1}), yet remained responsive to angiotensin II (500 pmol kg^{-1}). The *in situ* experiments demonstrated that desensitisation of the nicotinic receptor occurred within 5 min of receptor stimulation and that resensitisation was established 40 min later.

The ability to elevate plasma catecholamine levels during acute hypoxia (40–45 mmHg; 5.3–6.0 kPa) was not impaired in fish experiencing nicotinic receptor desensitisation. Indeed, peak plasma adrenaline levels were significantly higher in the desensitised fish during hypoxia than in controls (263±86 *versus* 69±26 nmol l^{-1}; means ± S.E.M., N=6–9). Thus, the results of the present study demonstrate that activation of preganglionic sympathetic cholinergic nerve fibres and the resultant stimulation of nicotinic receptors is not the sole mechanism for eliciting catecholamine secretion during hypoxia.

Key words: desensitisation, rainbow trout, *Oncorhynchus mykiss*, chromaffin cell, cholinergic receptor, adrenaline, noradrenaline, hypoxia, catecholamine, nicotinic receptor.

Introduction

In teleost fish, the catecholamine hormones (noradrenaline and adrenaline) are secreted into the bloodstream from the chromaffin tissue upon acute severe stress (for reviews, see Randall and Perry, 1992; Reid et al., 1998). The subsequent increase in circulating catecholamine levels ultimately serves to maintain or enhance oxygen levels in the blood and its delivery to the tissues. This is accomplished by several means, including contraction of the spleen to release stored red blood cells (Nilsson and Grove; 1974; Perry and Kinkead, 1989), enhancing the gill oxygen-diffusing capacity (Pettersson, 1983; Perry et al., 1985) and, in some species, by activating a β-adrenergic Na^+/H^+ exchanger (βNHE; Borgese et al., 1992) on the red blood cell membrane. The latter response is believed to increase red blood cell intracellular pH (pHi) and thereby enhance haemoglobin oxygen-binding affinity/capacity (for reviews, see Thomas and Perry, 1992; Motais et al., 1992; Nikinmaa and Boutilier, 1995). Stressors capable of causing catecholamine secretion include hypoxia (Ristori and Laurent, 1989), hypercapnia (Perry and Gilmour, 1996), intensive exercise (Primmett et al., 1986) and air exposure (Walhqvist and Nilsson, 1980).

In teleosts, the chromaffin cells line the walls of the posterior cardinal vein in the region of the head kidney (Nandi, 1961; Nakano and Tomlinson, 1967). In trout (*Oncorhynchus mykiss*), the chromaffin cells possess cholinergic and non-cholinergic receptors (for a review, see Reid et al., 1998). The primary mechanism initiating catecholamine release in trout is thought to involve increased neuronal stimulation by sympathetic preganglionic cholinergic nerve fibres that innervate the chromaffin cells (Nilsson et al., 1976; Montpetit and Perry, 1999). The subsequent release of the neurotransmitter acetylcholine and its interaction with

nicotinic and muscarinic cholinergic receptors elicits a series of Ca^{2+}-dependent events leading to catecholamine secretion (Nilsson et al., 1976; Montpetit and Perry, 1999; Furimsky et al., 1996). In trout, nicotinic receptor stimulation is considered to be the primary mediator of cholinergic-induced secretion of catecholamines (Reid et al., 1998). However, muscarinic receptor stimulation may enhance the nicotinic-evoked response or directly elicit secretion under conditions of intense stimulation (Montpetit and Perry, 1999).

The successful adaptation of many fish species to stressors depends, in part, upon the proper control of catecholamine secretion into the circulation. In certain tissues, excessive stimulation by catecholamines can lead to reduced sensitivity and attenuate responses to subsequent adrenergic stimulation (Thomas et al., 1991). In mammals, excessive secretion of catecholamines may be prevented by rapid desensitisation of nicotinic receptors (Boska and Livett, 1984; Malhotra et al., 1988), a process whereby receptors are inactivated after prolonged or repeated application of agonist. Although the control of catecholamine release from piscine chromaffin cells has been studied extensively, the possibility and physiological consequences of secretory desensitisation have received little attention.

Thus, the first goal of the present investigation was to test the hypothesis that chromaffin cell nicotinic receptors of rainbow trout undergo desensitisation during repetitive or chronic cholinergic stimulation. A second goal was to test the hypothesis that trout exhibit a diminished capacity to secrete catecholamines during acute hypoxia when the nicotinic receptors are desensitised. Experiments were performed using a combination of *in vivo* and *in situ* techniques.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing between 200 and 500 g (mean mass 332.9±10.0 g; mean ± S.E.M., *N*=151) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and were held indoors in large fibreglass tanks supplied with dechlorinated City of Ottawa tap water that was maintained at 13 °C. Fish were allowed to acclimate to the aquarium for at least 3 weeks before experimentation. Fish were maintained on a 12 h:12 h L:D photoperiod and fed daily to satiation with a commercial salmonid diet.

Animal preparation

In vivo *experiments*

Rainbow trout were anaesthetised in a solution of ethyl-*P*-amino-benzoate (benzocaine; final concentration 2.4×10^{-4} mol l^{-1}) and placed on an operating table where the gills were continuously irrigated with anaesthetic solution. An indwelling polyethylene cannula (Clay-Adams PE50; internal diameter 0.580 mm, outer diameter 0.965 mm) was implanted into the dorsal aorta (Soivio et al., 1975) to permit injections and periodic blood sampling. For experiments requiring infusion of nicotine or saline, a second cannula was inserted into the caudal vein at the level of the caudal peduncle using standard surgical procedures (Axelsson and Fritsche, 1994). After surgery, trout were placed into individual opaque Perspex boxes supplied with aerated flowing water and allowed to recover for 24 h prior to experimentation.

In situ *posterior cardinal vein preparation*

Fish were killed by a sharp blow to the head, weighed and placed ventral side up on ice. A ventral incision was made along the length of the animal beginning at the anus and ending just anterior to the pectoral girdle. The tissue overlying the heart was removed by blunt dissection to expose the ventricle and the bulbus arteriosus. The ventricle was cannulated (Clay-Adams PE160 polyethylene tubing; internal diameter 1.14 mm, outer diameter 1.57 mm) by making an incision in the bulbus and inserting the tube through the bulbus into the ventricle. The cannula was secured with a ligature between the two chambers. This cannula served as the outflow in the perfusion system. The posterior cardinal vein (PCV) was also cannulated (Clay-Adams PE160 tubing) and served as the inflow cannula (Fritsche et al., 1993). The vein was perfused with aerated Cortland saline (Wolf, 1963), final pH 7.8, at a flow rate of approximately 1.5 ml min^{-1}. Perfusion was accomplished by siphon resulting from a positive pressure difference between the surface of the saline and the outflow cannula.

Experimental protocol

Demonstration of chromaffin cell nicotinic receptor desensitisation

Series 1. Nicotine injection/infusion in vivo. Fish were injected (1 ml kg^{-1}) twice *via* the dorsal aortic cannula with 6.0×10^{-7} mol kg^{-1} nicotine [(–)-nicotine di-D-tartrate] (Julio et al., 1998) or Cortland saline (control). Injections were separated by 60 min; initial experiments demonstrated that plasma catecholamine levels were essentially restored to baseline values within 60 min of nicotine injection. Blood samples (0.4 ml) were taken prior to nicotine or saline injection and at 2, 10, 15, 20, 30 and 60 min post-injection.

Fish were infused (0.2 ml min^{-1}) *via* the caudal vein with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) or saline (control) using a syringe infusion pump (Sage Instruments) for 60 min. Blood samples (0.4 ml) were withdrawn prior to infusion and at 5, 10, 15, 20, 30, 45 and 60 min after commencing infusion. An injection (0.3 ml kg^{-1}) of rainbow trout angiotensin II [10^{-6} mol kg^{-1} in 0.9 % (w/v) NaCl] was administered (Bernier and Perry, 1997) just prior to ending the infusion of nicotine; a blood sample was withdrawn 2 min post-injection. This final experiment was performed to determine whether the chromaffin cells in the nicotine-infused fish retained the capacity to secrete catecholamines in response to non-cholinergic stimulation.

Series 2. Nicotine infusion in vivo *followed by nicotine perfusion* in situ. Fish were infused for 60 min with nicotine or saline (control) as described above. Blood samples were withdrawn at 0 (pre), 5, 10 and 60 min of infusion. Fish were

killed by a sharp blow to the head and *in situ* posterior cardinal vein preparations were prepared as described above. Preparations were perfused with saline for 10 min to allow stabilisation of catecholamine secretion, after which a sample of outflowing perfusate was taken to determine the *in situ* basal secretion rates of catecholamines. This procedure was followed by a 10 min perfusion of nicotine (10^{-5} nmol l^{-1}), at the end of which chromaffin cell viability was assessed by injecting (1 ml kg^{-1}) of rainbow trout angiotensin II (500 pmol kg^{-1}). Perfusate samples were collected at 1, 2, 3, 4, 5 and 10 min of nicotine infusion and at 2 min post-angiotensin-II-injection.

Series 3. Repetitive nicotine perfusion in situ. The posterior cardinal vein was perfused for a period of 20 min to allow stabilisation of catecholamine secretion. A sample (approximately 1 ml) of outflowing perfusate was taken to determine the *in situ* basal catecholamine secretion rates. This was followed by a 10 min period of perfusion with saline containing nicotine (10^{-5} mol l^{-1}), during which samples were taken at 1, 2, 3, 4, 5 and 10 min. Preparations were then perfused with saline for 5, 10, 20, 30, 40 or 60 min (N=8 different preparations for each time period), followed by a second 10 min perfusion with saline containing nicotine (10^{-5} mol l^{-1}), during which perfusate samples were taken at 0 (pre), 1, 2, 3, 4, 5 and 10 min. In control fish (N=6 different preparations for each time period), the initial perfusate was saline rather than saline containing nicotine. Upon completion of each experiment, rainbow trout angiotensin II was injected, and a perfusate sample was taken 2 min post-injection.

Effects of acute hypoxia on fish experiencing nicotinic receptor desensitisation

Fish were infused (0.2 ml min^{-1}) *via* the caudal vein with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) or saline (control) using a syringe infusion pump (Sage Instruments) for 60 min. They were then subjected to 10 min of hypoxia (inspired P_{O_2}=40–45 mmHg; 5.3–6.0 kPa), while the infusion continued.

Acute hypoxia was achieved by replacing the air supplying a water/gas equilibration column with N_2. The desired water P_{O_2} (Pw_{O_2}) (40–45 mmHg) was established by adjusting the rate of water and/or N_2 flow through the column. The level of Pw_{O_2} was chosen on the basis of the study of Perry and Reid (1992), who demonstrated significant catecholamine release using this protocol. The Pw_{O_2} within the box was monitored continuously by allowing the water to flow by siphon through a P_{O_2} electrode (Cameron Instruments). Generally, the desired Pw_{O_2} in the experimental box was reached within 10 min and thereafter never varied more than ±5 mmHg (0.67 kPa).

Determination of catecholamine levels

Blood samples collected for catecholamine analysis were placed into micro-centrifuge tubes (1.5 ml) and immediately centrifuged (12 000 ***g*** for 20 s). The plasma was then transferred to micro-centrifuge tubes containing 10 µl (25 units) of heparin (ammonium salt). Samples were quick-frozen in liquid N_2 and then stored at −86 °C until subsequent analysis. *In situ* perfusate samples were collected in pre-weighed micro-centrifuge tubes (1.5 ml), placed in liquid N_2 and stored at −86 °C until subsequent analysis. The tubes were reweighed before thawing to estimate catecholamine secretion rates.

All plasma and perfusate samples were subjected to alumina extraction and then analysed by high-pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). 3,4-Dihydroxybenzylamine hydrobromide (DHBA) was used as an internal standard in all analyses.

Statistical analyses

The data are presented as means ±1 standard error of the mean (S.E.M.). Where appropriate, data were analysed statistically using one-way analysis of variance (ANOVA) followed by Dunn's multiple-comparison test. If assumptions for parametric statistics were violated, an ANOVA on ranks was performed followed by Dunn's multiple-comparison test. In other instances, data were analysed using Student's *t*-tests, and if assumptions for parametric statistics were violated, a Mann–Whitney rank sum test was performed. All statistical analyses were performed using commercial software (SigmaStat Version 2.0; SPSS); the fiducial limits of significance were set at 5 %.

Results

Demonstration of chromaffin cell nicotinic receptor desensitisation

Series 1. Nicotine injection/infusion in vivo

The temporal effects of a single intra-arterial injection of nicotine are illustrated in Fig. 1. These experiments were performed to confirm the effectiveness of nicotine as a secretory stimulant and to establish the time course of changes in circulating catecholamine levels. Plasma catecholamine levels were elevated after 2 min as a result of a significant increase in adrenaline levels (Fig. 1A). Although plasma noradrenaline levels appeared to increase, the high degree of variability in the data set prevented statistical confirmation (Fig. 1B). Plasma catecholamine levels were unaffected by injections of saline. Because plasma catecholamine concentrations were essentially restored to baseline levels after 60 min, subsequent experiments employed a 60 min waiting period between repetitive injections of nicotine.

The effects of serial nicotine injections are depicted in Fig. 2. As expected (see Fig. 1), the first injection of nicotine evoked significant elevations in plasma catecholamine levels, which then returned to pre-injection levels within 60 min. Plasma catecholamine levels were statistically elevated only at 2 min post-injection, and the data obtained from blood samples taken between 2 and 60 min have not, therefore, been included in Fig. 2. The response to the second injection of nicotine was essentially identical to the first. Thus, after 60 min of recovery, the ability of the chromaffin tissue to respond to nicotine did not appear to be impaired by prior short-term stimulation of chromaffin cell nicotinic receptors. This first series of experiments, therefore, was unable to provide evidence for

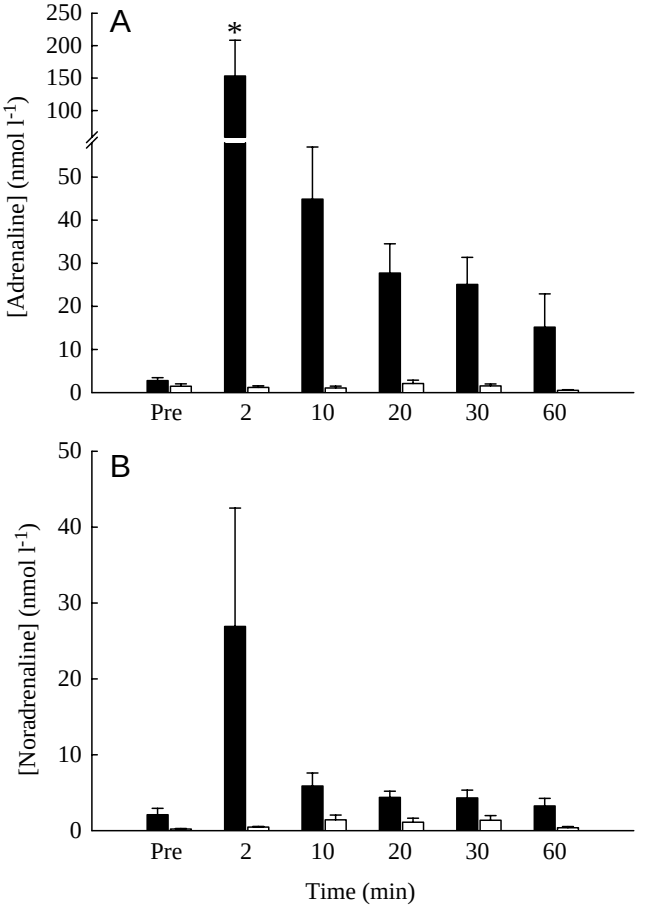

Fig. 1. The temporal effects of a single intra-arterial injection of 6.0×10^{-7} mol kg^{-1} nicotine (filled columns, N=9) or saline (open columns, N=7) on the plasma levels of (A) adrenaline or (B) noradrenaline in rainbow trout (*Oncorhynchus mykiss*). Pre-injection (Pre) samples were withdrawn immediately prior to the nicotine or saline injections. Values are shown as means + S.E.M. An asterisk denotes a significant difference (P<0.05) from the pre-injection value. The apparent increase in plasma [noradrenaline] at 2 min was not statistically significant (P=0.079).

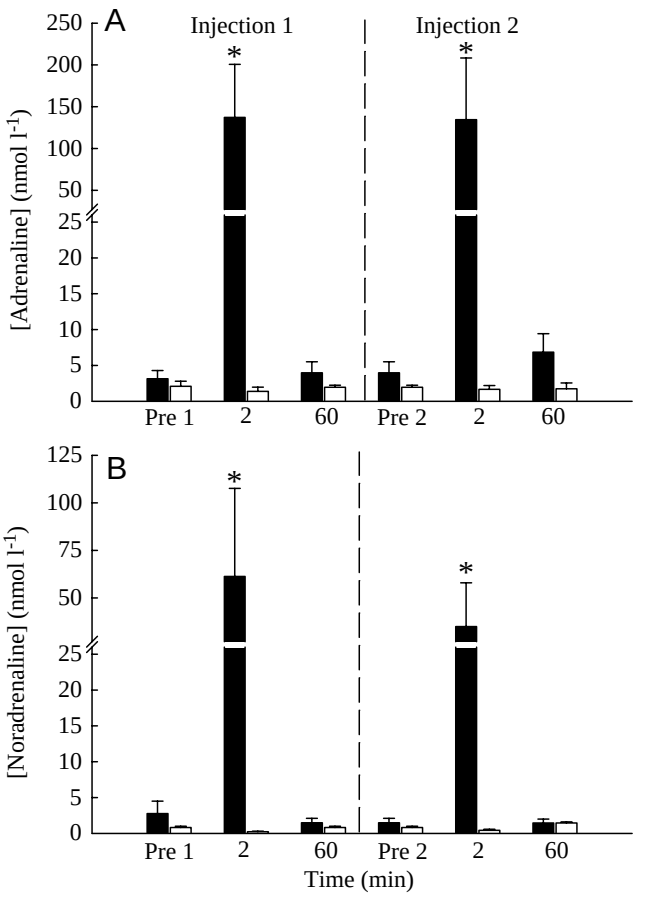

Fig. 2. The effects of serial intra-arterial injections of 6.0×10^{-7} mol kg^{-1} nicotine (filled columns; N=10) or saline (open columns; N=6) on plasma levels of (A) adrenaline or (B) noradrenaline in rainbow trout (*Oncorhynchus mykiss*). The second injection was administered 60 min after the first; note that the catecholamine values for 'Pre 2' were obtained from the 60 min sample of the first injection period. Values are shown as means + S.E.M. An asterisk denotes a significant difference (P<0.05) from the appropriate pre-injection value.

desensitisation of nicotinic receptors. Evidence for receptor desensitisation, however, was provided in a separate series of experiments in which fish were continuously infused with nicotine for 60 min (Fig. 3). Under these conditions, plasma catecholamine levels were elevated at 10 min of infusion but then declined gradually to baseline levels during the remaining 50 min period of infusion (Fig. 3B). Plasma catecholamine levels were unaffected by continuous intravenous infusion of saline (Fig. 3A). In response to angiotensin II injection, the saline- and nicotine-infused fish both exhibited increases in plasma catecholamine levels. However, the increase in plasma adrenaline concentration was significantly greater in the fish pre-infused with nicotine.

Series 2. Nicotine infusion in vivo *followed by nicotine perfusion* in situ

To confirm that the decrease in plasma catecholamine levels during continuous infusion of nicotine was a result of receptor desensitisation and not simply a consequence of increased catecholamine degradation/clearance, an additional series of experiments was performed that combined *in vivo* and *in situ* procedures (Fig. 4). Perfused posterior vein preparations derived from fish pre-infused for 60 min with saline demonstrated a pronounced secretion of adrenaline in response to 10^{-5} mol l^{-1} nicotine (Fig. 4A). The secretion of adrenaline was transient despite the continuing presence of nicotine in the perfusate. In contrast, perfused preparations derived from fish previously experiencing nicotine infusion did not secrete catecholamines during perfusion with 10^{-5} mol l^{-1} nicotine (Fig. 4C). Unlike the saline-infused group, in which plasma catecholamine levels were constant, the nicotine-infused fish displayed a transient elevation of circulating catecholamine levels (compare Figs 4B and D). To ensure that the lack of responsiveness of the preparations derived from nicotine-infused fish was not simply a consequence of non-viable

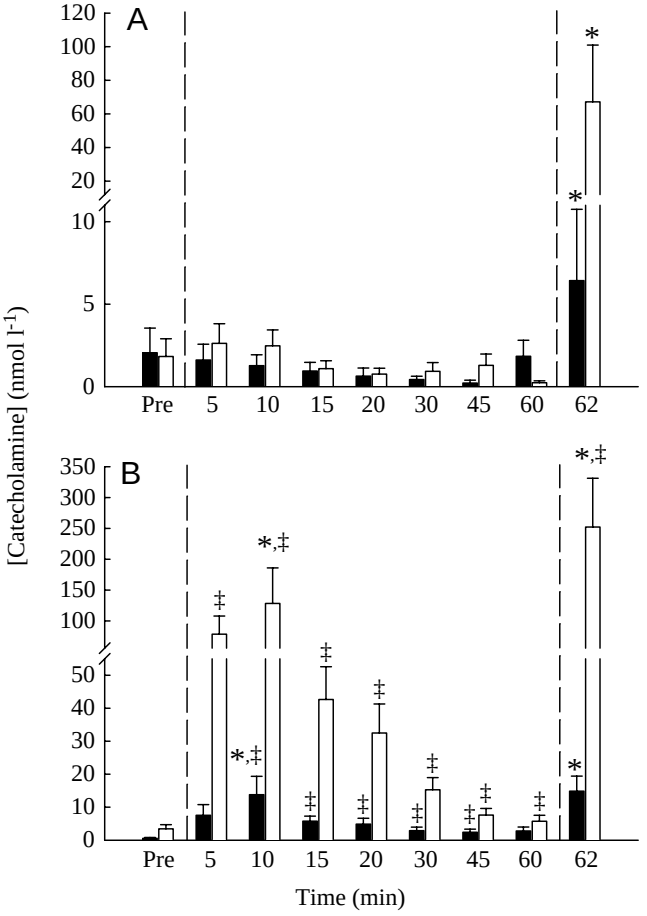

Fig. 3. The temporal effects of continuous intravenous infusion ($0.2\,ml\,min^{-1}$) of (A) saline (N=6) or (B) nicotine ($1.3\times10^{-5}\,mol\,kg^{-1}\,h^{-1}$; N=6) on the plasma levels of noradrenaline (filled columns) or adrenaline (open columns) in rainbow trout (*Oncorhynchus mykiss*). The first vertical dashed line indicates the commencement of infusion, and the second dashed line represents a bolus injection of trout angiotensin II ($10^{-6}\,mol\,kg^{-1}$). Values are shown as means + S.E.M. An asterisk denotes a significant difference (P<0.05) from the appropriate pre-injection value; a double dagger denotes a significant difference (P<0.05) from the corresponding value in the saline-infused group.

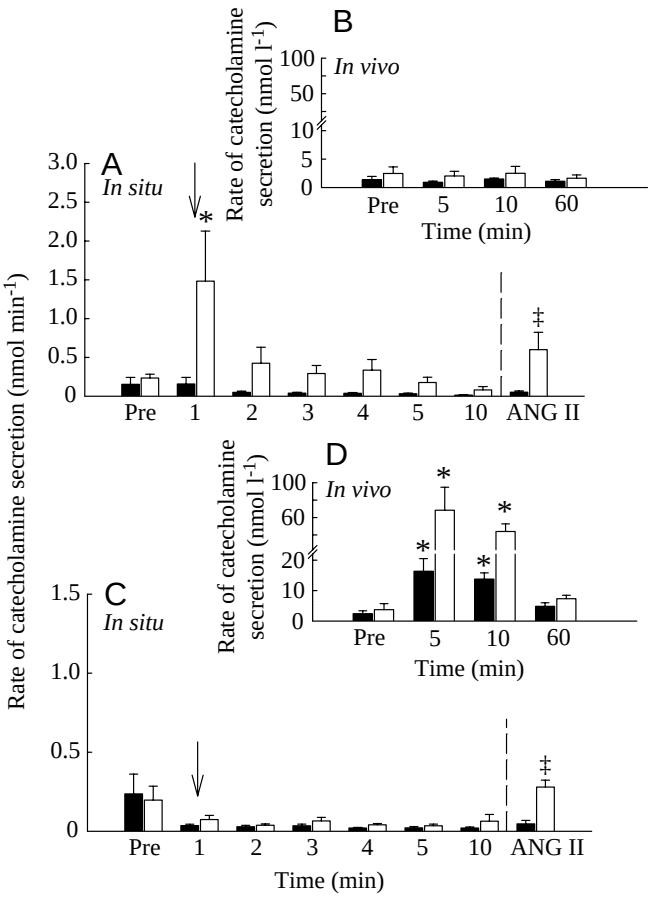

Fig. 4. The effects of nicotine ($10^{-5}\,mol\,l^{-1}$) injection (at the arrow) on noradrenaline (filled columns) or adrenaline (open columns) secretion in *in situ* perfused posterior vein preparations of rainbow trout (*Oncorhynchus mykiss*) obtained from fish pre-infused ($0.2\,ml\,min^{-1}$) *in vivo* for 60 min with (A) saline (N=8) or (C) nicotine ($1.3\times10^{-5}\,mol\,kg^{-1}\,h^{-1}$; N=7). The vertical dashed line indicates a bolus injection of trout angiotensin II (ANG II). B and D illustrate the effects of the prior saline and nicotine infusions, respectively, *in vivo* on plasma noradrenaline (filled columns) and adrenaline (open columns) levels. Values are shown as means + S.E.M. An asterisk denotes a significant difference (P<0.05) from the appropriate pre-injection (Pre) value *in vivo* or *in situ*; a double dagger denotes a significant difference (P<0.05) from the 10 min *in situ* values.

chromaffin tissue, the potent non-cholinergic secretagogue angiotensin II was delivered as a bolus injection prior to terminating each experiment. The control preparations (Fig. 4A) and the unresponsive (desensitised) preparations (Fig. 4C) exhibited similar increases in rates of adrenaline secretion after angiotensin II injection.

Series 3. Repetitive nicotine perfusion in situ

These experiments were performed to characterise further the nature of nicotinic receptor desensitisation and the time course of resensitisation; the results are illustrated in Fig. 5. After a single 10 min period of perfusion with nicotine, perfused posterior cardinal vein preparations were refractory for 30 min to further stimulation by nicotine. Responsiveness to nicotine was re-established after 40 min of recovery, and catecholamine secretion rates at 40 and 60 min were not statistically different from those of control preparations (Fig. 5).

Effects of acute hypoxia on fish experiencing nicotinic receptor desensitisation

Fig. 6 depicts the effects of acute hypoxia on peak plasma catecholamine levels in trout previously infused for 60 min under normoxic conditions with saline or nicotine. As previously shown, infusion with nicotine caused a pronounced increase in the circulating levels of adrenaline (Fig. 6A) and noradrenaline (Fig. 6B); saline infusion was without effect. Acute hypoxia caused significant increases in the levels of plasma catecholamines regardless of whether fish continued to

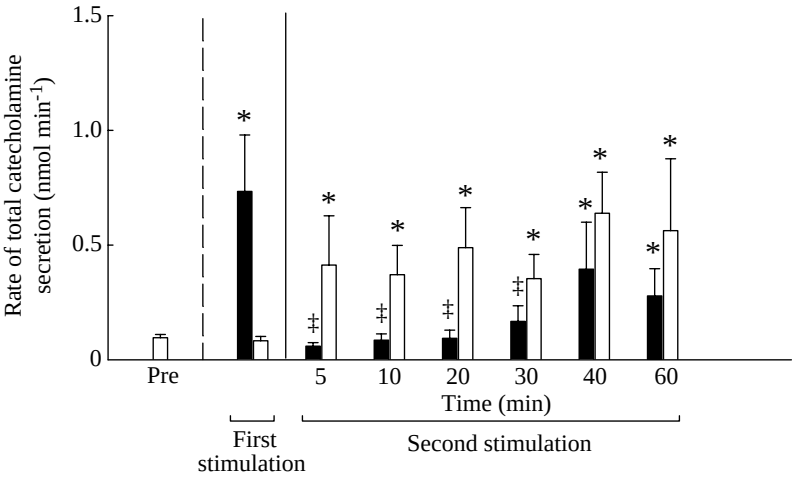

Fig. 5. The effects of nicotine (10^{-5} mol l^{-1}) on peak total catecholamine (noradrenaline plus adrenaline) secretion in *in situ* perfused posterior vein preparations of rainbow trout (*Oncorhynchus mykiss*). Experimental preparations (filled columns) were initially stimulated for 10 min with nicotine (first stimulation) and then perfused with saline for periods ranging from 5 to 60 min (N=8 different preparations for each time interval) before being re-stimulated with nicotine (second stimulation) for 10 min. Control preparations (open columns; N=6 for each time interval) were perfused with saline rather than nicotine during the first stimulation period. Values are shown as means + S.E.M. An asterisk denotes a significant difference (P<0.05) from the pre-stimulation value; a double dagger denotes a significant difference (P<0.05) between the control and experimental groups.

be infused with saline or nicotine. Indeed, the largest changes in plasma adrenaline levels were achieved in the fish that had been pre-infused with nicotine.

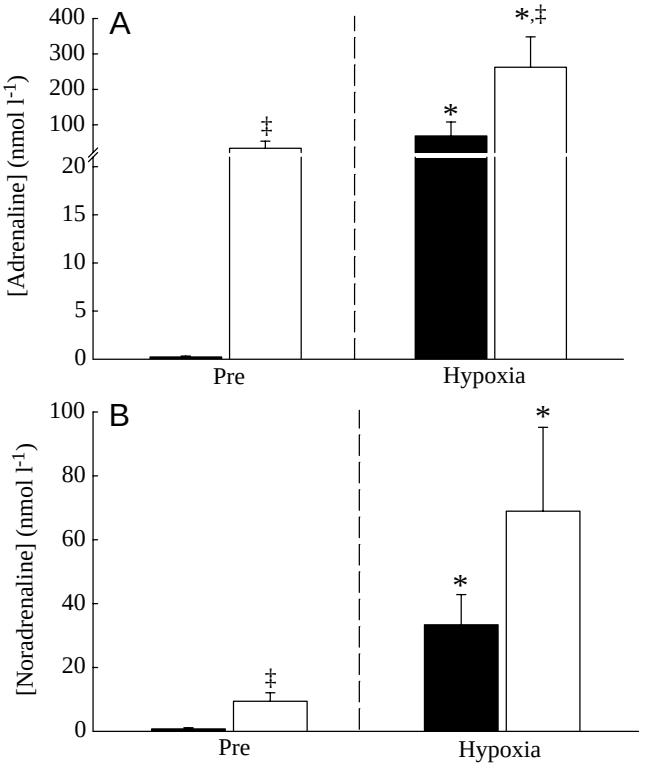

Fig. 6. The effects of acute hypoxia (40–45 mmHg; 5.3–6.0 kPa) on peak plasma levels (maximal concentration achieved during a 10 min hypoxic period) of (A) adrenaline or (B) noradrenaline in rainbow trout (*Oncorhynchus mykiss*) previously infused for 60 min with saline (filled columns; N=6) or 1.3×10^{-5} mol kg^{-1} nicotine (open columns; N=9) under conditions of normoxia. Values are shown as means + S.E.M. An asterisk denotes a significant increase (P<0.05) in peak plasma catecholamine levels during continuing infusion with saline or nicotine under hypoxic conditions; a double dagger denotes a significant difference (P<0.05) between the saline- and nicotine-infused fish.

Discussion

Desensitisation of chromaffin cell nicotinic receptors

This study is the first to report desensitisation of chromaffin cell nicotinic receptors in fish. Evidence for nicotinic receptor desensitisation was obtained from experiments conducted both *in vivo* and *in situ*. *In vivo*, continuous infusion of nicotine caused a transient rise in plasma catecholamine levels, suggesting that the secretion of catecholamines from the chromaffin cells was also transient. To demonstrate that the transient response to continuously infused nicotine was not related to depletion of catecholamine stores, fish were infused with angiotensin II, a potent secretagogue of catecholamines (specifically adrenaline) in trout (Bernier and Perry, 1999). In trout, angiotensin II mediates secretion solely *via* interaction with specific angiotensin II receptors (Bernier and Perry, 1997). Thus, the normal elevation of plasma adrenaline levels after injection of angiotensin II supports the idea of specific nicotinic receptor desensitisation rather than depletion of catecholamine stores or non-specific/secondary effects of nicotine infusion on chromaffin cell function. To exclude the possibility that the decreasing levels of plasma catecholamines during continuous infusion of nicotine were caused by increased metabolic degradation or clearance, chromaffin tissue from previously infused fish was perfused using an *in situ* posterior cardinal vein preparation. The results from that experimental series (see Fig. 4) confirmed that the chromaffin cell nicotinic receptors were, indeed, desensitised. Moreover, the rapid (within 5 min) decrease in catecholamine secretion *in situ* during sustained application of nicotine provided additional evidence of receptor desensitisation.

The mechanisms promoting desensitisation of the chromaffin cell nicotinic receptor were not evaluated in the present study. However, results from numerous previous studies utilising mammalian systems suggest that there are probably several processes that contribute simultaneously to desensitisation of the nicotinic receptor. Stimulation of a functional (i.e. sensitised) nicotinic receptor causes an increase in the conductance of Na^+ through a receptor-linked ion channel. The resultant influx of Na^+ elicits membrane

depolarisation which, in turn, causes an opening of voltage-dependent Ca^{2+} channels (Brandt et al., 1976; Wada et al., 1985; Liu and Kao, 1990). The inward flux of Ca^{2+} triggers a cascade of events leading to the secretion of catecholamines (Burgoyne et al., 1993). When desensitised, the nicotinic-receptor-linked ion channel is inactivated and membrane depolarisation is prevented (for reviews, see Marley, 1988; Ochoa et al., 1989). Inactivation of the ion channel is thought to involve phosphorylation of the nicotinic receptor following activation of several protein kinases (for a review, see Swope et al., 1999). In addition to nicotinic receptor desensitisation, the decreased responsiveness of chromaffin cells may be caused by post-receptor modifications of the intracellular reactions leading to catecholamine release.

In mammalian systems, the time course of nicotinic receptor resensitisation is highly variable and related to the duration and strength of prior receptor stimulation (Rowell and Duggan, 1998). In the present study, perfusion of the posterior cardinal vein for 10 min with 10^{-5} mol l^{-1} nicotine caused the chromaffin cells to be unresponsive for 30 min to further application of nicotine; resensitisation to nicotine was established after 40 min. Extrapolation of these results to the intact animal is confounded by uncertainty about the concentrations of acetylcholine achieved in the vicinity of chromaffin cells during natural stimulation of the preganglionic sympathetic nerve fibres and the duration for which these nerves are active. Thus, it is premature to conclude that nicotinic receptor desensitisation in rainbow trout actually occurs during or immediately following an acute adrenergic stress response. However, if it does occur, it is likely that a significant recovery period would be required prior to the re-establishment of normal nicotinic receptor function. In the present study, catecholamine secretion *in vivo* was unaffected by a bolus injection of nicotine given 60 min earlier. Assuming that desensitisation had occurred, the restoration of normal function after 60 min is consistent with the results of the *in situ* experiments that demonstrated resensitisation within 40 min (see above).

Physiological consequences of nicotinic receptor desensitisation

During acute stress in fish, catecholamine release into the circulation is thought to be mediated predominantly by stimulation of chromaffin cell nicotinic receptors by acetylcholine released from preganglionic sympathetic nerve fibres (for a review, see Reid et al., 1998). Thus, it was hypothesised that, during exposure of trout to acute hypoxia, catecholamine release would be impaired in fish experiencing nicotinic receptor desensitisation. Thus, after developing and validating a protocol to elicit nicotinic receptor desensitisation *in vivo* (see above), peak plasma catecholamine levels were determined in fish experiencing an acute reduction of inspired P_{O_2} to 40–45 mmHg. The results clearly demonstrated that catecholamine release (as indicated by circulating catecholamine levels) was not impaired in fish experiencing nicotinic receptor desensitisation. Indeed, the fish experiencing receptor desensitisation actually exhibited significantly greater levels of adrenaline during hypoxia than the control fish. This finding is significant because it suggests that the role of the nicotinic receptor in promoting catecholamine release during hypoxia has been greatly overestimated. An alternative interpretation of the results is that, under normal conditions, activation of the nicotinic receptor is the principal (or a significant) mechanism eliciting catecholamine secretion but that, when these receptors are inactivated through desensitisation, alternative pathways leading to secretion are switched on. Indeed, there are numerous mechanisms of catecholamine secretion in both fish (see below) and mammals (Livett and Marley, 1993) that are not reliant on nicotinic receptor stimulation. For example, in rainbow trout (Julio et al., 1998; Montpetit and Perry, 1999) and other teleosts (Gfell et al., 1997; Abele et al., 1998), muscarinic receptors have been implicated in the secretory process. Other factors that may be involved include non-cholinergic neurotransmitters such as vasoactive intestinal polypeptide (VIP) and pituitary adenylate-cyclase-activating polypeptide (PACAP; Reid et al., 1995; C. Montpetit and S. Perry, unpublished data). These neurotransmitters are known to have stimulatory effects on catecholamine secretion in non-piscine vertebrates (Watanabe et al., 1995; Yamaguchi, 1993), and recent work has shown that they may be involved in catecholamine secretion in rainbow trout (C. Montpetit and S. Perry, unpublished data). Another potent non-cholinergic catecholamine secretagogue is angiotensin II (Bernier and Perry, 1997). In rainbow trout, angiotensin II is produced by the systemic renin–angiotensin system and has been shown to mediate catecholamine release during hypotensive conditions (Bernier and Perry, 1999). In mammals, there is evidence that acute hypoxia stimulates renin secretion (Ritthaler et al., 1997). If a similar situation exists in fish, activation of the renin–angiotensin system may be a significant mechanism leading to catecholamine secretion during hypoxia in trout. It is noteworthy that injections of angiotensin II into desensitised fish (Fig. 3) caused a greater elevation of plasma adrenaline levels than in the corresponding control fish. This suggests that desensitisation of the nicotinic receptor may increase the responsiveness of the chromaffin cells to non-nicotinic agonists, including angiotensin II. In mammalian systems, desensitisation of the nicotinic receptor causes an up-regulation of nicotinic receptor numbers (Schwartz and Kellar, 1983). It is conceivable that, in trout, desensitisation of the nicotinic receptor leads to an increase in the number of chromaffin cell angiotensin II receptors. Other factors that may be involved in evoking catecholamine secretion in the desensitised fish include serotonin (Fritsche et al., 1993), adrenocorticotropic hormone (ACTH; Reid et al., 1996) and a variety of autocrine/paracrine agents (Epple et al., 1993, 1994; Reid et al., 1996). Although in Atlantic cod (*Gadus morhua*), local hypoxia in the vicinity of the chromaffin cells can directly evoke catecholamine secretion (Perry et al., 1991), a similar mechanism does not appear to exist in trout (S. F. Perry, C. Montpetit and M. Borowska, in preparation).

The redundancy and complexity of the mechanisms promoting catecholamine secretion in fish and other vertebrates reflects the crucial importance of the adrenergic stress response under conditions of severe and acute stress. The multiple mechanisms eliciting catecholamine secretion ensure, among other things, that the adrenergic stress response can still occur even when the chromaffin cell nicotinic receptor is inactivated *via* desensitisation.

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