

## Catecholamine secretion in trout chromaffin cells experiencing nicotinic receptor desensitization is maintained by non-cholinergic neurotransmission

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### Summary

The goal of the present study was to assess the catecholamine secretory capabilities of rainbow trout *Oncorhynchus mykiss* chromaffin cells experiencing desensitization of the nicotinic receptor. It was hypothesized that the potential to secrete catecholamines could be maintained under conditions of nicotinic receptor desensitization owing to activation of non-cholinergic release pathways. An *in situ* model for chromaffin cell nicotinic receptor desensitization was developed by perfusing a posterior cardinal vein preparation with saline containing  $10^{-5}$  mol l<sup>-1</sup> nicotine. Under such conditions of desensitization, the chromaffin cells were largely unresponsive to high-frequency (20 Hz) electrical stimulation; the minimal remaining secretory response was abolished by addition of the nicotinic receptor antagonist hexamethonium ( $10^{-3}$  mol l<sup>-1</sup>). In marked contrast, however, the capacity to secrete catecholamines in response to low-frequency (1 Hz) electrical stimulation was unaffected by nicotinic receptor desensitization or by cholinergic receptor blockade (hexamethonium plus atropine). In preparations experiencing nicotinic receptor desensitization, the stimulatory effect of low-frequency

(1 Hz) stimulation on catecholamine secretion was reduced by 43% in the presence of the VPAC receptor antagonist, VIP<sub>6-28</sub>. The stimulatory effect of high-frequency (20 Hz) stimulation was unaffected by VIP<sub>6-28</sub>. Catecholamine secretion evoked by cod VIP ( $10^{-11}$  mol kg<sup>-1</sup>) and homologous angiotensin II ([Asn<sup>1</sup>, Val<sup>5</sup>] Ang II;  $5 \times 10^{-7}$  mol kg<sup>-1</sup>) was markedly enhanced (107 and 97%, respectively) in desensitized preparations. However, the secretory response to the muscarinic receptor agonist methylcholine ( $1 \times 10^{-3}$  mol kg<sup>-1</sup>) was unchanged by desensitization. The results of this study demonstrate that exploitation of non-cholinergic mechanisms, including peptidergic pathways activated during low-frequency neuronal stimulation, is a potential strategy whereby catecholamine secretion from trout chromaffin cells can be maintained during periods of nicotinic receptor desensitization.

Key words: catecholamine, adrenaline, noradrenaline, VIP, PACAP, stress, nicotinic receptor, angiotensin II, muscarinic receptor, rainbow trout, *Oncorhynchus mykiss*.

### Introduction

In teleosts, the catecholamine hormones, adrenaline and noradrenaline, are synthesized, stored and released by chromaffin cells lining the posterior cardinal vein (Nandi, 1967; Nilsson et al., 1976; Perry and Bernier, 1999). They are released in response to severe acute stressors (e.g. hypoxia, hypercapnia, chasing) and function to reduce the detrimental effects that are often associated with stress (Wendelaar Bonga, 1997; Reid et al., 1998). The beneficial effects of catecholamines are achieved, in part, by modulation of the cardiovascular and respiratory systems (Perry and Gilmour, 1999) as well as by the mobilization of energy stores (Fabbri et al., 1998). The current model for catecholamine release in fish contends that a number of cholinergic and non-cholinergic neurotransmitters and/or neuromodulators interact either directly or indirectly with the chromaffin cells to influence secretion (Reid et al., 1998). The primary mechanism of catecholamine secretion, as in other vertebrates, is believed to

be cholinergic and involves the interaction of acetylcholine (ACh) with nicotinic or muscarinic receptors (Nilsson et al., 1976; Guo and Wakade, 1994). Non-cholinergic mechanisms of catecholamine secretion in fish include activation of the renin-angiotensin system (RAS; Bernier and Perry, 1997, 1999), direct actions of elevated levels of adrenocorticotrophic hormone (ACTH; Reid et al., 1996) or serotonin (Fritsche et al., 1993), and neuronal release of vasoactive intestinal polypeptide (VIP) and/or pituitary adenylyl cyclase activating polypeptide (PACAP; Montpetit and Perry, 2000).

For cholinergic-evoked catecholamine secretion, the relative involvement of nicotinic *versus* muscarinic receptor stimulation is species dependent. In rainbow trout (*Oncorhynchus mykiss*), activation of the nicotinic receptor is believed to be the principal direct pathway for secretion (Montpetit and Perry, 1999). Activation of the muscarinic receptor, while able to cause secretion at high agonist

concentrations (Julio et al., 1998), is mainly thought to enhance nicotinic-evoked catecholamine secretion (Montpetit and Perry, 1999).

It is well documented that in rats (Khiroug et al., 2002) and in humans (Ke et al., 1998), the nicotinic receptor undergoes desensitization after brief exposure to nicotinic receptor agonists. Desensitization results in an inactive receptor that does not allow for the passage of  $\text{Na}^+$ . Thus, further release of catecholamines *via* this pathway is diminished or prevented until the nicotinic receptor is resensitized (Reitstetter et al., 1999; Mahata et al., 1999). Recently, Lapner et al. (2000) developed an *in vivo* protocol to desensitize chromaffin cell nicotinic receptors in rainbow trout. Interestingly, the ability to secrete catecholamines during acute hypoxia was not impaired in those fish experiencing nicotinic receptor desensitization (Lapner et al., 2000). The continued ability to secrete catecholamines despite nicotinic receptor desensitization was later attributed to hypoxic activation of the RAS (Lapner and Perry, 2001). The results of these studies suggested that during periods of nicotinic receptor desensitization, the importance of normally minor pathways evoking catecholamine secretion might be substantially increased.

The neurotransmitters PACAP and VIP are potent secretagogues of adrenal catecholamine secretion in mammals (Lamouche and Yamaguchi, 2001; Fukushima et al., 2001), and in rainbow trout (Montpetit and Perry, 2000). It was recently demonstrated that in trout, these neuropeptides are preferentially released (i.e. in comparison to Ach) from the preganglionic fibres innervating the chromaffin cells under conditions of low-frequency (1 Hz) nerve stimulation (Montpetit and Perry, 2000). Because these neuropeptides (VIP and PACAP) and Ach are released from the same nerve fibers, but at different frequencies, it is possible that the neuropeptides might play an important role in maintaining catecholamine secretion during periods of nicotinic receptor desensitization. Thus, the goals of the present study were (i) to develop an *in situ* nicotinic receptor desensitization model that could be used in conjunction with electrical stimulation of the nerve fibers controlling catecholamine secretion and (ii) to test the hypothesis that catecholamine secretion could be sustained at such times owing to increased contribution of non-cholinergic and muscarinic pathways.

## Materials and methods

### *Experimental animals*

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of both sexes were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). The fish were held at University of Ottawa in large fiberglass tanks supplied with flowing, aerated, and dechlorinated city water. They (mean mass  $223 \pm 35$  g) were maintained at a temperature of  $13^\circ\text{C}$ , on a 12 h:12 h light:dark photoperiod and were fed daily with a commercial trout diet.

### *In situ saline-perfused posterior cardinal vein preparation*

The fish were killed by a sharp blow to the head, weighed and placed on ice. If electrical stimulation was to be administered, electrodes were sutured to the skin on each side of the fish in the anterior region of the body (Montpetit and Perry, 1999). A ventral incision was made from the anus to the pectoral girdle, and the tissue overlying the heart was removed by blunt dissection to expose the ventricle and the bulbus arteriosus. The fish were then cannulated (PE 160 polyethylene tubing, Clay-Adams, Maryland, USA), with the inflow cannula inserted into the posterior cardinal vein (PCV) and the outflow cannula inserted into the ventricle through the bulbus arteriosus. Each preparation was perfused for 20 min with modified aerated Cortland saline (Wolf, 1963; Montpetit and Perry, 1999) using positive pressure differences to maintain constant flow (approximately  $1.5 \text{ ml min}^{-1}$ ) through the PCV. The 20 min perfusion period allowed for the stabilization of the catecholamine secretion rates prior to the beginning of the experiment. Two samples were collected in pre-weighed microcentrifuge tubes following the 20 min period of perfusion to assess the catecholamine secretion rates prior to any experimental procedure. In the control group, perfusion with saline was continued whereas in the experimental groups, perfusion media were rapidly switched using a three-way valve. Perfusion media were identical except for the addition of specific agonists and/or antagonists. In both groups, perfusate samples were collected every 20 s within the first 2 min of the experiment, then at 3, 4, 5 and 10 min. Following the 10 min sample collection period, preparations were either given a bolus injection of an agonist or electrically stimulated using a previously validated field stimulation technique (Montpetit and Perry, 1999). Preparations were stimulated at 60 V at either high frequency (20 Hz), or low frequency (1 Hz) for 2 min; perfusate samples were collected at 20, 60 and 100 s to determine the maximum value. Catecholamine secretion rates post-stimulation were subsequently presented as maximal secretion rates. The high voltage used to electrically stimulate the nerves is required owing to the resistance imparted by the skeletal muscle; extensive prior validation (Montpetit and Perry, 1999) demonstrated that this procedure results in specific activation of nerves innervating the chromaffin tissue without non-selectively depolarizing the chromaffin cells.

### *Series 1: Developing an in situ model for nicotinic receptor desensitization*

After the collection of pre-samples, the preparations were administered unmodified control saline or saline containing hexamethonium (nicotinic receptor antagonist;  $1 \times 10^{-3} \text{ mol l}^{-1}$ ; Sigma) or nicotine (nicotinic receptor agonist;  $1 \times 10^{-5} \text{ mol l}^{-1}$ ; Sigma). After 10 min, all preparations were electrically stimulated at 20 Hz.

### *Series 2: Catecholamine storage levels*

Catecholamine storage levels were measured to assess the potential contribution of non-chromaffin tissue to electrically evoked catecholamine secretion. Three tissues, heart, white

muscle (in the vicinity of the anterior PCV) and the anterior PCV were obtained by blunt dissection and frozen in liquid nitrogen. Tissues were cleaned using Cortland saline. Solution (1 ml) of 4% perchloric acid containing 2 mg ml<sup>-1</sup> EDTA and 0.5 mg ml<sup>-1</sup> sodium bisulphite was added to each tube (Woodward, 1982). Heart and white muscle were processed by mortar and pestle while the PCV was sonicated. The supernatant was diluted 100-fold with 4% perchloric acid and analysed by HPLC.

*Series 3: Assessing the mechanisms of catecholamine secretion in preparations experiencing nicotinic receptor desensitization*

This series of experiments was performed to determine if low-frequency electrical stimulation could elicit catecholamine secretion during nicotinic receptor desensitization, and if so, to determine if this secretion was non-cholinergic in origin. Preparations were perfused with either unmodified saline or saline containing nicotine (1×10<sup>-5</sup> mol l<sup>-1</sup>) or with hexamethonium (1×10<sup>-3</sup> mol l<sup>-1</sup>) plus atropine (1×10<sup>-5</sup> mol l<sup>-1</sup>). After 10 min, all preparations were electrically stimulated at low frequency (1 Hz).

In a separate group, preparations were either perfused with unmodified saline or saline containing nicotine (1×10<sup>-5</sup> mol l<sup>-1</sup>) plus VIP<sub>6-28</sub> (VIP receptor antagonist; Peninsula Laboratories, San Carlos, CA, USA; 1×10<sup>-6</sup> mol l<sup>-1</sup>). After 10 min, the preparations were divided into two groups; one group was stimulated at high frequency (20 Hz) and the other at low frequency (1 Hz).

*Series 4: Assessing the efficacy of cholinergic and non-cholinergic secretagogues in preparations experiencing nicotinic receptor desensitization*

Nicotinic receptors were desensitized during a 10 min infusion period with saline containing nicotine (see above). Following desensitization, separate preparations received a bolus injection of either cod VIP (1×10<sup>-11</sup> mol kg<sup>-1</sup> body mass), homologous angiotensin II ([Asn<sup>1</sup>, Val<sup>5</sup>] Ang II; 5×10<sup>-7</sup> mol kg<sup>-1</sup> body mass; Sigma) or the muscarinic receptor agonist methylcholine (1×10<sup>-3</sup> mol kg<sup>-1</sup> body mass; Sigma); control preparations received a bolus injection of saline.

*Analytical procedures*

Catecholamine levels in perfusate and tissue extracts were determined on alumina-extracted samples (200 µl) using high-pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). Concentrations were calculated relative to appropriate standards, using 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard. The secretion rates for adrenaline and noradrenaline were calculated then summed to yield total catecholamine secretion rates. Owing to a high degree of temporal variability, peak catecholamine secretion rates, generally obtained 2 or 3 min after stimulation/agonist addition, were calculated by taking the mean of the maximal secretion rates in response to stimulation for each fish within a given group.

*Statistical analyses*

The data are presented as means ± 1 standard error of the mean (S.E.M.). All data sets were analyzed using 'all pair-wise' two-way repeated measure analysis of variance (ANOVA) followed by Bonferroni's *t*-test.

**Results**

*Series 1: Developing an in situ model for nicotinic receptor desensitization*

Addition of nicotine to the perfusion fluid caused a marked and rapid increase in the rate of catecholamine secretion (Fig. 1A). Despite the continuing presence of nicotine in the perfusion fluid, catecholamine secretion rates were returned to basal levels within 3 min (Fig. 1A). Electrical stimulation at high frequency (20 Hz) caused marked catecholamine release in control preparations that was greatly reduced (though not abolished) in preparations receiving nicotine infusion (Fig. 1B). In the presence of the nicotinic receptor antagonist, hexamethonium, the preparations were unresponsive to nicotine and the ensuing stimulation at 20 Hz (Fig. 1B).

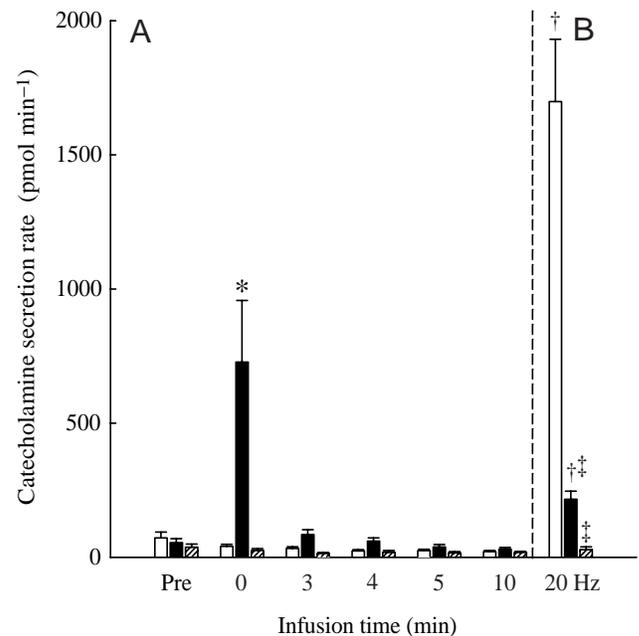


Fig. 1. (A) The effects of continuous infusion of control saline (unfilled bars; *N*=8) or rapidly switching to saline containing nicotine (10<sup>-5</sup> mol l<sup>-1</sup>; filled bars; *N*=8) or nicotine plus hexamethonium (10<sup>-3</sup> mol l<sup>-1</sup>; cross-hatched bars; *N*=8) on catecholamine secretion in an *in situ* perfused posterior cardinal vein preparation of rainbow trout *Oncorhynchus mykiss*. (B) After 10 min, the preparations were electrically stimulated using a frequency of 20 Hz and maximal catecholamine secretion rates were determined under the various conditions. An asterisk denotes a statistically significant difference from the 'pre-switch' (Pre) value; a dagger represents a significant difference from the secretion rate after 10 min; a double dagger represents a significant difference from the control (saline-perfused) group.

*Series 2: Catecholamine storage levels*

Fig. 2 illustrates the levels of stored catecholamines in several tissues. The catecholamine (adrenaline plus noradrenaline) concentration in either the heart or the white

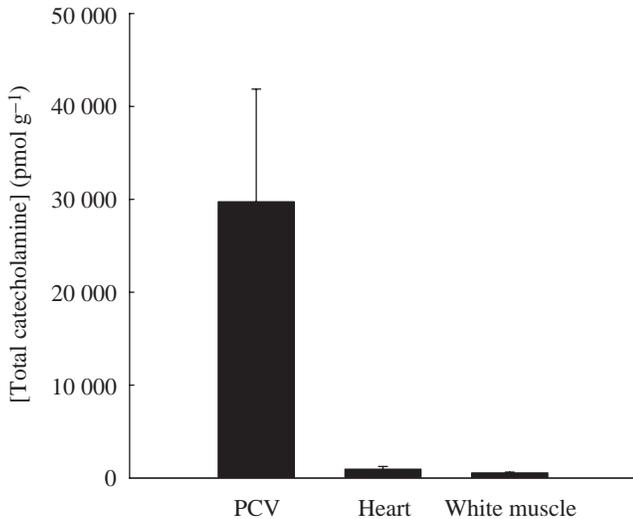


Fig. 2. Total catecholamine (adrenaline plus noradrenaline) levels in several tissues that could potentially contribute to catecholamine secretion in the perfused posterior cardinal vein (PCV) preparation;  $N=6$ .

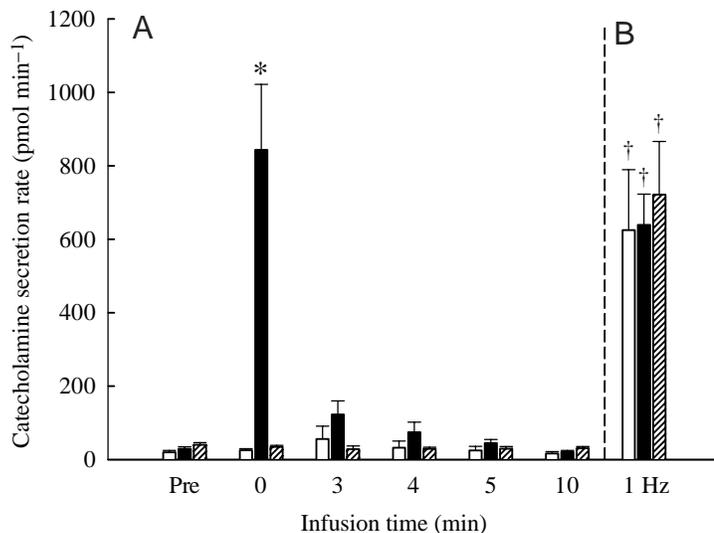


Fig. 3. (A) The effects of continuous infusion of control saline (unfilled bars;  $N=8$ ) or rapidly switching to saline containing nicotine ( $10^{-5}$  mol l<sup>-1</sup>; filled bars;  $N=8$ ) or nicotine plus a cocktail of  $10^{-3}$  mol l<sup>-1</sup> hexamethonium and  $10^{-5}$  mol l<sup>-1</sup> atropine (cross-hatched bars;  $N=8$ ) on catecholamine secretion in an *in situ* perfused posterior cardinal vein preparation of rainbow trout *Oncorhynchus mykiss*. (B) After 10 min, the preparations were electrically stimulated using a frequency of 1 Hz and maximal catecholamine secretion rates were determined under the various conditions. An asterisk denotes a statistically significant difference from the 'pre-switch' (Pre) value; a dagger represents a significant difference from the secretion rate after 10 min.

muscle was less than 1% of the concentration found in the anterior PCV.

*Series 3: Assessing the mechanisms of catecholamine secretion in preparations experiencing nicotinic receptor desensitization*

Fig. 3 illustrates the effect of low frequency (1 Hz) electrical stimulation on preparations treated with saline or nicotine. The nicotine treated group displayed the characteristic increase in catecholamine secretion in response to nicotine followed by a transient decrease to baseline levels (Fig. 3A). However, during electrical stimulation at 1 Hz, both the control and nicotine-treated (desensitized) groups showed an equivalent increase in maximum catecholamine secretion rate (Fig. 3B). The use of hexamethonium (nicotinic receptor antagonist) plus atropine (muscarinic receptor antagonist) to block all cholinergic receptors prevented the nicotine-evoked stimulation of catecholamine secretion (Fig. 3A) but was without effect on electrically evoked (1 Hz) secretion (Fig. 3B).

Regardless of the presence or absence of the VPAC receptor antagonist VIP<sub>6-28</sub> in the perfusate, all preparations responded similarly to nicotine addition by showing an increase in catecholamine secretion rates followed by a return to baseline levels (Fig. 4Ai,Bi). Both the control and the VIP<sub>6-28</sub> treated preparations exhibited similar increases in catecholamine secretion rates when stimulated at 20 Hz (Fig. 4Aii). However, when stimulated at lower frequency (1 Hz), the presence of VIP<sub>6-28</sub> significantly reduced the rate of evoked catecholamine secretion (Fig. 4Bii).

*Series 4: Assessing the efficacy of cholinergic and non-cholinergic secretagogues in preparations experiencing nicotinic receptor desensitization*

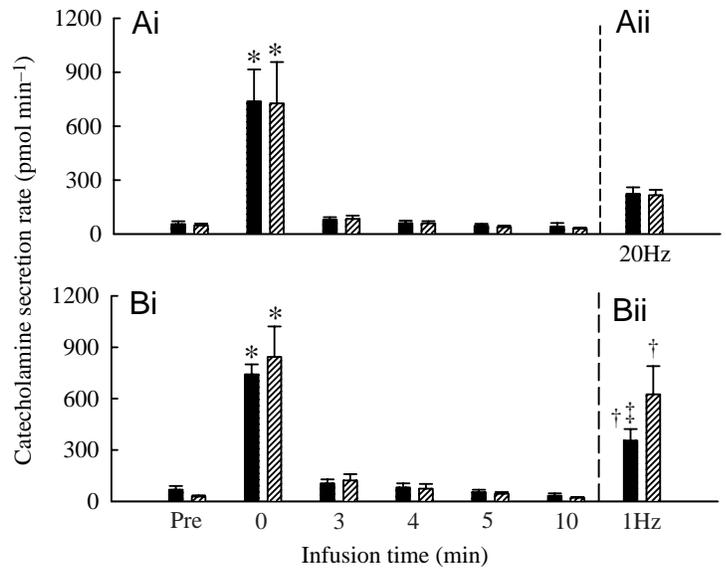
All preparations that received nicotine exhibited a transient elevation of catecholamine secretion rates (Fig. 5Ai-Ci), a response that is indicative of nicotinic receptor desensitization. However, the extent of catecholamine secretion in response to the subsequent addition of the non-cholinergic secretagogues, cVIP (Fig. 5Aii) and homologous Ang II (Fig. 5Bii), was markedly increased in preparations that had previously received nicotine. In contrast, addition of the muscarinic receptor agonist methylcholine caused identical increases in catecholamine secretion rates in the control and desensitized preparations (Fig. 5Cii).

**Discussion**

The predominant mechanism causing catecholamine release from vertebrate chromaffin cells is the activation of nicotinic receptors by acetylcholine (ACh) released from pre-ganglionic sympathetic nerve fibres. The chromaffin cell nicotinic receptor, however, undergoes rapid desensitization upon its binding to ACh (reviewed by Marley, 1988). In rainbow trout, the period required to

Fig. 4. (Ai,Bi) The effects of rapidly switching from control saline to saline containing nicotine ( $10^{-5}$  mol  $l^{-1}$ ; hatched bars;  $N=18$ ) or nicotine plus the VPAC receptor antagonist VIP<sub>6-28</sub> ( $10^{-6}$  mol  $l^{-1}$ ; filled bars;  $N=18$ ) on catecholamine secretion in an *in situ* perfused posterior cardinal vein preparation of rainbow trout *Oncorhynchus mykiss*. After 10 min, the preparations were electrically stimulated using a frequency of either (Aii) 20 Hz or (Bii) 1 Hz and maximal catecholamine secretion rates were determined under the various conditions. An asterisk denotes a statistically significant difference from the 'pre-switch' (Pre) value; a dagger represents a significant difference from the secretion rate after 10 min; a double dagger represents a significant difference from the control (saline-perfused) group.

resensitize nicotinic receptors in perfused PCV preparations may be as long as 30 min (Lapner et al., 2000). Because the ability of trout to secrete catecholamines *in vivo* during acute hypoxia is not impaired by nicotinic receptor desensitization, (Lapner et al., 2000), it would appear that alternative secretory mechanisms are activated, thereby ensuring that the ability to mount a humoral adrenergic stress response is not compromised. For example, Lapner and Perry (2001) demonstrated that the RAS, normally not an important



activator of catecholamine secretion during hypoxia, plays an essential role in this response when nicotinic receptors are desensitized. In the present study, we have extended these findings by developing an *in situ* model for chromaffin cell nicotinic receptor desensitization and demonstrating (i) that catecholamine secretion might be maintained during periods of receptor desensitization by peptidergic neurotransmission, and (ii) that nicotinic receptor desensitization is accompanied by an enhancement of catecholamine secretion evoked by the non-cholinergic secretagogues VIP and Ang II.

*The nature of nicotinic receptor desensitization*

In the present study, catecholamine secretion in a perfused PCV preparation was initially stimulated by nicotine, but then declined to baseline rates within 3 min despite the continuing presence of nicotine in the perfusion fluid. This biphasic response pattern

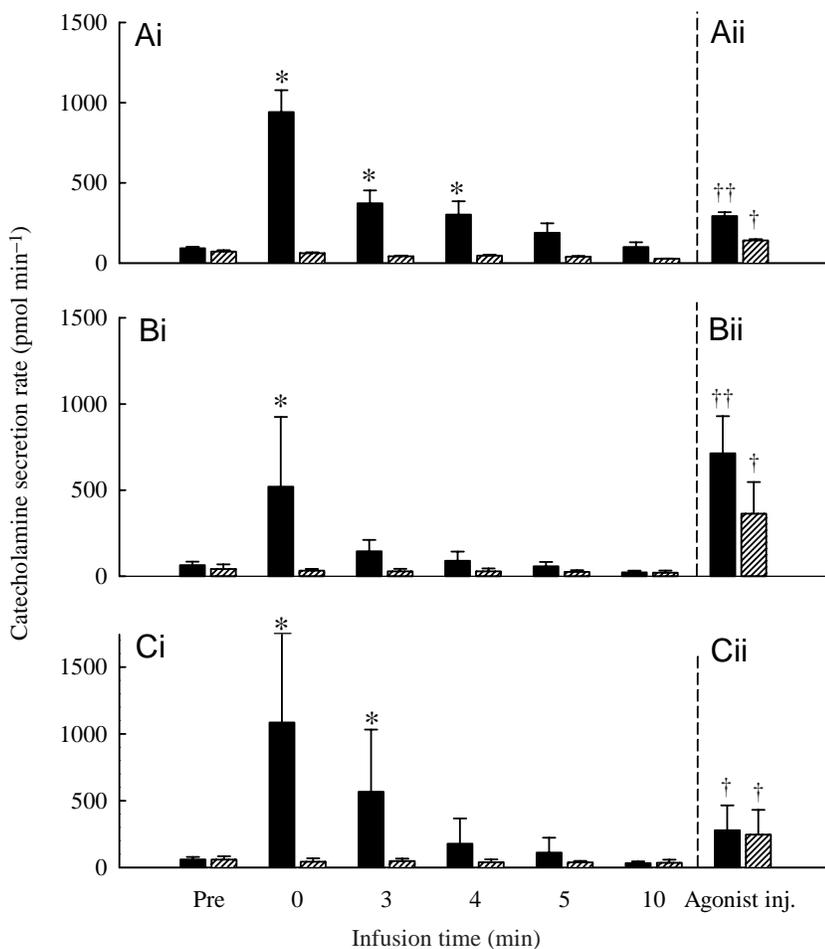


Fig. 5. The effects of (Aii) cVIP ( $10^{-6}$  mol  $l^{-1}$ ;  $N=15$ ), (Bii) Ang II ( $10^{-5}$  mol  $l^{-1}$ ;  $N=6$ ) and (Cii) methylcholine ( $10^{-3}$  mol  $l^{-1}$ ;  $N=6$ ) on catecholamine secretion rates (Ai–Ci) in posterior cardinal vein preparations previously perfused with control saline (hatched bars) or after rapidly switching to saline containing nicotine ( $10^{-5}$  mol  $l^{-1}$ ; filled bars). An asterisk denotes a statistically significant difference from the 'pre-switch' (Pre) value; a dagger represents a significant difference from the secretion rate after 10 min; a double dagger represents a significant difference from the control (saline-perfused) group. Agonist inj. indicates rate measured after agonist injection (see Materials and methods).

is indicative of nicotinic receptor desensitization. The decline in catecholamine secretion could not be explained by exhaustion of catecholamine stores or intracellular signaling molecules because stimulation of muscarinic (using methylcholine) or non-cholinergic receptors (using VIP or Ang II) was able to rapidly reactivate catecholamine secretion. It was previously demonstrated that high-frequency (20 Hz) electrical stimulation of the trout PCV preparation evokes catecholamine secretion *via* selective activation of the nicotinic receptor pathway (Montpetit and Perry, 1999). However, during low-frequency (1 Hz) stimulation, a significant component of catecholamine secretion is mediated by VIP and/or PACAP *via* activation of VPAC receptors (Montpetit and Perry, 2000). Thus, in the present study, further evidence for nicotinic receptor desensitization was provided by the fact that catecholamine secretion in preparations that had received nicotine was largely prevented during high-frequency electrical stimulation. Because the addition of the nicotinic receptor antagonist, hexamethonium, eliminated the small amount of residual catecholamine secretion during high-frequency stimulation, it would appear that the desensitization protocol used in this study, though effective, was unable to totally desensitize all nicotinic receptors.

Previous studies (e.g. Montpetit and Perry, 1999) that employed electrical stimulation to evoke secretion assumed that the catecholamines appearing in the perfusate originate from the chromaffin cells lining the PCV. However, owing to extensive neuronal innervation of the heart and the likely depolarization of skeletal muscle in the vicinity of the PCV, catecholamines could potentially also arise *via* spillover from cardiac adrenergic nerves or secretion from cardiac and/or muscle tissue. However, based on the low levels of catecholamines stored in the heart and skeletal muscle (less than 1% of anterior PCV), this is unlikely. The fact that catecholamine secretion during high-frequency electrical stimulation is prevented by nicotinic receptor blockade is further evidence that the chromaffin tissue is the predominant, if not exclusive, site of catecholamine release in the perfused PCV preparation.

#### *The potential role of non-cholinergic neurotransmitters during nicotinic receptor desensitization*

Vasoactive intestinal polypeptide (VIP) and pituitary adenylyl cyclase-activating polypeptide (PACAP) are potent secretagogues of catecholamine release from chromaffin cells in mammals (Fukushima et al., 2001; Geng et al., 1997; Mazzocchi et al., 2002) and fish (Montpetit and Perry, 2000). Furthermore, previous studies have demonstrated that VIP and PACAP are localized in pre-ganglionic sympathetic nerve fibers in the vicinity of chromaffin cells in rats (Holgert et al., 1996; Lamouche and Yamaguchi, 2001) and several fish species (Reid et al., 1995). In the perfused rat adrenal gland, it was shown that these endogenous neuropeptides are released into the perfusate during splanchnic nerve stimulation (Arimura and Shioda, 1995). On the basis of studies employing electrical stimulation of chromaffin tissue,

a model has emerged in which the non-cholinergic neurotransmitters, VIP and PACAP, are preferentially released in mammals (Wakade et al., 1991) and trout (Montpetit and Perry, 2000) during low-frequency neuronal stimulation. An important difference between mammals and fish, however, is that the catecholaminotropic response to VIP/PACAP is mediated by PACAP type receptors in mammals (Hamelink et al., 2002) and VPAC type receptors in trout (Montpetit and Perry, 2000). PACAP receptors exhibit a much greater efficacy for PACAP binding than for VIP binding, whereas VPAC receptors exhibit similar affinities for both PACAP and VIP. Thus, in mammalian chromaffin cells, PACAP is about 1000× more potent than VIP (Watanabe et al., 1995; Geng et al., 1997; Fukushima et al., 2001; Hamelink et al., 2002) whereas in fish, it would appear that VIP and PACAP are equally potent as catecholamine secretagogues (Montpetit and Perry, 2000).

In the present study, desensitization of the nicotinic receptor or blockade of the nicotinic receptor using hexamethonium did not impair the ability of chromaffin cells to secrete catecholamines in response to low-frequency electrical stimulation. Moreover, in the presence of the VPAC receptor blocker VIP<sub>6-28</sub>, catecholamine secretion evoked by low-frequency electrical stimulation was markedly reduced in the desensitized preparations. Conversely, VIP<sub>6-28</sub> was without effect during high-frequency stimulation. Because blockade of all cholinergic (nicotinic and muscarinic) receptors did not affect catecholamine secretion elicited by low-frequency stimulation, it is clear that neuronal release of VIP and/or PACAP and subsequent binding to chromaffin cell VPAC receptors is the principal mechanism underlying catecholamine secretion in desensitized preparations subjected to low-frequency excitation.

#### *Upregulation of non-cholinergic pathways during nicotinic receptor desensitization*

Not only are the non-cholinergic secretagogues VIP/PACAP (this study) and Ang II (Lapner and Perry, 2001) implicated in sustaining catecholamine secretion capabilities during periods of nicotinic receptor desensitization, their ability to evoke catecholamine secretion at such times is actually enhanced. Indeed, after only 10 min of nicotinic receptor desensitization, the rate of catecholamine secretion evoked by cVIP or homologous Ang II was approximately doubled in preparations experiencing desensitization. The mechanism(s) underlying this interesting phenomenon has not yet been established. However, owing to the rapidity of the response, it is likely to be a non-genomic mechanism that may involve modulation of existing membrane receptors or one or more intracellular signaling pathways.

#### *Conclusion*

The results of this study have demonstrated a potential novel mechanism for sustaining catecholamine secretion during periods of nicotinic receptor desensitization that involves low-frequency neuronal transmission in nerve fibres innervating the

chromaffin tissue. It remains to be seen, however, whether this strategy is actually exploited by fish to preserve the acute humoral adrenergic stress response at times when the nicotinic receptor is in a refractory state. In future research, it would be interesting to record from afferent nerve fibres during acute stress to ascertain whether the frequency of neuronal impulses is modulated according to the sensitivity state of the nicotinic receptor.

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