Journal of Physiology (1993), **460**, *pp.* 427–441
With 8 figures
Printed in Great Britain

MECHANISMS UNDERLYING CHEMORECEPTOR INHIBITION INDUCED BY ATRIAL NATRIURETIC PEPTIDE IN RABBIT CAROTID BODY

BY W.-J. WANG, L. HE, J. CHEN, B. DINGER AND S. FIDONE

From the Department of Physiology, University of Utah School of Medicine, 410 Chipeta Way, Research Park, Salt Lake City, UT 84108, USA

(*Received 10 December 1991*)

SUMMARY

1. Previous studies in our laboratory revealed the presence of atrial natriuretic peptide (ANP) in preneural chemosensory type I cells of the cat carotid body, and demonstrated that submicromolar concentrations of the peptide inhibited carotid sinus nerve (CSN) activity evoked by hypoxia. In the present study, we have evaluated the role of the cyclic nucleotide second messenger, cyclic GMP (cGMP), and the involvement of type I cells in rabbit chemosensory inhibition.

2. Submicromolar concentrations of the potent ANP analogue, APIII, greatly elevated both the content and release of cGMP from the carotid body. Denervation experiments confirmed earlier immunocytochemical studies which suggested that APIII-induced cGMP production occurs almost exclusively in type I cells; these experiments also indicate that both the sympathetic and sensory innervation to the carotid body exert a trophic influence on the metabolism of this second messenger.

3. Submicromolar concentrations of APIII inhibited the CSN activity evoked by hypoxia (79·8 ± 3·2% (mean ± S.E.M.) inhibition with 100 nM APIII) and nicotine (74·5 ± 3·6% inhibition with 100 nM APIII), but did not affect basal CSN activity established in 100% O_2-equilibrated superfusion solutions.

4. The biologically inactive analogue of ANP, C-ANP, failed to produce CSN inhibition; however, the inhibitory effects of APIII were mimicked by cell-permeant analogues of cGMP (dibutyryl-cGMP and 8-bromo-cGMP, 2 mM), which likewise did not alter basal CSN activity. Because we found that unmodified cGMP was an ineffective inhibitor of CSN activity, our data suggest that APIII inhibition is mediated intracellularly by cGMP produced within the type I cells.

5. APIII does not inhibit the CSN activity produced by 20 mM K^+ (in zero Ca^{2+} media), which very probably results from direct depolarization of the sensory nerve terminals.

6. Catecholamine release from the carotid body evoked by hypoxia is likewise not altered by APIII (100 nM).

7. The data are consistent with the notion that APIII and analogues of cGMP alter the release of excitatory and/or inhibitory transmitters from chemosensory type I cells in the carotid body.

MS 9953

INTRODUCTION

Studies in recent years have established that the heart contains a diuretic and natriuretic factor, atrial natriuretic peptide (ANP), which is released from cardiac myocytes in response to hypervolaemia and acute sodium load (de Bold, 1979; Genest, Cantin, Anand-Srivastava, Cusson, de Lean, Garcia, Cutkowska, Hamet, Kuchel, Larochelle, Nemer, Schiffrin, Schiller, Thibault & Tremblay, 1988). Circulating ANP acts directly via specific receptors in the kidney to promote sodium and water excretion (Genest *et al.* 1988), and the peptide also suppresses aldosterone secretion and causes vasodilatation in selected vascular beds (Murad, 1986). While the cellular mechanisms involved in these responses are not fully understood, recent demonstrations that the ANP receptor exhibits guanylate cyclase activity (Chinkers, Garbers, Chang, Lowe, Chin, Goeddel & Schulz, 1989) and that ANP stimulates cGMP formation (Hamet, Tremblay, Pang, Garcia, Thibault, Gutkowska, Cantin & Genest, 1984) suggest that this cyclic nucleotide may be a key mediator of the peptide's actions. The finding that cGMP mimics the effects of ANP further substantiates this hypothesis (Sen & Roy, 1986).

Studies in the central nervous system (CNS) have revealed effects mediated by ANP which complement its peripheral actions. ANP and ANP receptors have been localized in specific areas of the CNS, such as the subfornical organ, known to be involved in water and solute balance (see Samson, 1987, for review). Furthermore, central administration of ANP inhibits the release of the vasoconstrictor, arginine vasopressin (AVP; Samson, 1985; Samson, Aguila, Martinovic, Antunes-Rodrigues & Norris, 1987), and causes decreased salt-water intake in salt-depleted rats (Antunes-Rodrigues, McCann & Samson, 1986). Thus, the release of ANP from both peripheral and central sites appears to initiate a systemic response which mitigates the adverse hypertensive effects of acute hypervolaemia and increased salt intake.

Although reflexes initiated from the carotid body are involved in the regulation of blood pressure, classical views do not include a direct role for this arterial chemoreceptor organ in water and solute balance. However, an early study by Eyzaguirre and his colleagues (Gallego, Eyzaguirre & Monti-Bloch, 1979) demonstrated that carotid sinus nerve (CSN) fibres sensitive to hypoxia were also excited by increased osmolarity. Furthermore, in a recent review, Honig (1989) documented the relationship between arterial chemoreceptor activity and solute metabolism. The data suggest that reflexes initiated from the carotid body by hypoxic stimulation produce an increase in the renal excretion of sodium. Interestingly, this response appears to be mediated by a hormonal mechanism, although the possible involvement of ANP in these effects is uncertain (Honig, 1989).

In a preliminary investigation from our laboratory (Wang, He, Stensaas, Dinger & Fidone, 1991*b*), we reported that ANP-like immunoreactivity could be localized to the preneural, chemosensitive type I cells of the cat carotid body, and that submicromolar concentrations of the synthetic ANP analogue, atriopeptin III (APIII), profoundly inhibited chemoreceptor activity evoked by low O_2 stimuli. In the present study, we have attempted to elucidate the role of cGMP in this chemoreceptor inhibition. Our findings demonstrate that APIII activates guanylate cyclase in the rabbit carotid body, and that cGMP acts at an intracellular site in type I cells to mediate chemoreceptor inhibition.

METHODS

Cyclic GMP content and release. Under pentobarbitone anaesthesia (35 mg kg^{-1}, I.P.), the CSN was restricted bilaterally in five New Zealand White rabbits. In a second group of three rabbits, the superior cervical ganglia were bilaterally removed. Ten to fifteen days after surgery the carotid bodies were removed from these animals and also from forty-one normal, previously unoperated rabbits. With the aid of a dissecting microscope, the carotid bodies were cleaned of surrounding connective tissue in a lucite chamber containing 100% O_2-equilibrated modified Tyrode solution at 0–4 °C (mM: NaCl, 112; KCl, 4·7; $CaCl_2$, 2·2; $MgCl_2$, 1·1; sodium glutamate, 42; HEPES buffer, 5; glucose, 5·6; pH = 7·4). The tissues were weighed on a Cahn electrobalance equipped with a humidified weighing chamber (mean weight of rabbit carotid bodies approximately 390 μg), and then transferred to glass scintillation vials in a water-bath shaker for a 30 min preincubation in 1 ml of 100% O_2–Tyrode solution at 37 °C. The preincubation medium was replaced with 1 ml of 100 % O_2–Tyrode solution with or without a selected concentration of atriopeptin III (APIII, Sigma; rat, 24 amino acid form). In some experiments (Table 1), utilization of the full 28 amino acid peptide, ANP (from rat) demonstrated similar potencies of the two forms with respect to cGMP production. After a 10 min incubation (37 °C), the carotid bodies were immersed in 600 μl of cold (4 °C) 6% trichloroacetic acid (TCA). The tissues were homogenized in a glass–glass homogenizer and the homogenates centrifuged at 13000 *g* for 10 min (4 °C). The supernatant was extracted three times in 3 ml of water-saturated ethyl ether, the remaining aqueous phase was dried under vacuum (Savant), and the sealed samples stored at 4 °C for up to one month prior to radioimmunoassay (RIA). The assays were performed using commercially available cGMP RIA kits (Dupont, NEN; acetylated assay; minimal detectable limit = 2·5 fmol) and the data are expressed as picomoles cGMP per milligram tissue. Replicates of cGMP standards varied by $< 10\%$; furthermore, each assay contained an appropriate control group and data for control (unstimulated) carotid bodies varied between different kits by $\leqslant 20\%$.

Cyclic GMP release was assessed from groups of six carotid bodies superfused with 1 ml of Tyrode solution in glass scintillation vials. The superfusates from sequential control (100% O_2 media; 10 min), stimulus (APIII, 0·01–10 μM; 10 min) and post-stimulus periods (100% O_2 media; 10 min) were collected in vials containing 100 μl of 6% TCA. The samples were extracted in water-saturated ethyl ether, dried and assayed by RIA for cGMP as described above. The data are expressed as picomoles cGMP released per ten minutes per milligram of tissue.

Lyophilized APIII (Sigma) was dissolved in modified Tyrode solution to a concentration of 100 μM, aliquoted and stored at −80 °C; under these conditions, the potency of the peptide was stable for up to 30 days. Significant batch variation was observed for APIII potency with respect to both generation of cGMP and inhibition of CSN activity. Dose–response relationships were therefore obtained from single batches.

Catecholamine release from carotid bodies. Carotid bodies were surgically removed from adult New Zealand White rabbits and cleaned of adjoining connective tissue in a bath of ice-cold modified Tyrode solution equilibrated with 100% O_2. The tissues were pooled and incubated for 2 h, as previously described (Fidone & Gonzalez, 1982), in media containing [^{3}H]tyrosine (20–40 Ci $mmol^{-1}$; 20–25 μM; Amersham) plus ascorbic acid (100 μM) and tyrosine hydroxylase co-factor (6-MPH_4; 50 μM). Following incubation, carotid bodies were separated and washed for 90 min (fresh media every 30 min) in standard scintillation vials containing modified Tyrode solution equilibrated with 100% O_2. Catecholamine (CA) release was assessed during 'stimulus cycles', each of which consisted of sequential control (100% O_2 media), stimulus (low O_2 media) and post-stimulus (100% O_2 media) periods. Superfusates were collected in vials containing ascorbic acid (10 mM), plus acetic acid (4 M), and stored overnight (4 °C) prior to [^{3}H]CA processing on alumina columns. [^{3}H]CAs were eluted with 1 N perchloric acid. [^{3}H]CAs extracted from carotid bodies homogenized in 0·4 N perchloric acid were similarly treated on alumina columns. Radioactivity eluted from the columns was measured in a Packard Model 1500 scintillation spectrometer. CAs were separated using conventional high-performance liquid chromatography (HPLC) with electrochemical (EC) detection as previously described (Gomez-Niño, Cheng, Yoshizaki, Gonzalez, Dinger & Fidone, 1990).

Carotid sinus nerve (CSN) activity. The carotid bodies along with their attached CSNs were removed from pentobarbitone-anaesthetized rabbits and placed in a lucite chamber containing 100% O_2-equilibrated modified Tyrode solution at 0–4 °C. With the aid of a dissecting microscope, each carotid body and CSN was carefully cleaned of surrounding connective tissue. This procedure

routinely requires less than 30 min. The preparation was then placed in a conventional superfusion chamber where the carotid body was continuously superfused (up to 4 h) with modified Tyrode solution maintained at 37 °C and equilibrated with a selected gas mixture. The CSN was drawn through a tiny hole in a glass coverslip into an adjoining chamber containing mineral oil, where it was positioned on two platinum wire electrodes for differential recording of chemoreceptor activity. The action potentials were led to an AC-coupled preamplifier, an oscilloscope and a magnetic tape-recorder. The amplified signals were also led through a window discriminator to (1) a frequency-to-voltage converter for the final display of the spontaneous neural activity on a chart recorder and (2) a digital counter-printer which read the total number of nerve impulses recorded during selected periods.

All statistical comparisons are based upon Student's t test for unpaired observations.

RESULTS

Effects of APIII on carotid body cGMP content

The cGMP level in normal rabbit carotid bodies incubated for 40 min in 100% O_2-equilibrated media was $0{\cdot}035 \pm {\cdot}002$ pmol (mg tissue)$^{-1}$. This concentration of cGMP, measured under non-stimulated, basal conditions, is similar to previously published values obtained from a variety of neural and non-neural tissues (Kuo & Greengard, 1972; basal levels of cAMP are generally 100-fold greater in most tissues, including the carotid body; Wang, Cheng, Dinger & Fidone, 1989). Figure 1*A* shows that incubation of carotid bodies in increasing concentrations of the synthetic ANP analogue, atriopeptin III (APIII), elevated the cGMP content of the tissue in a dose-related manner. Incubations for 10 min in 10, 100 and 1000 nM APIII produced increases in cGMP of 2·6-, 42·3- and 134·0-fold, respectively. No significant change in the cGMP content was observed with 1 nM APIII.

In related experiments (Fig. 1*B*), we assessed the effects on cGMP of chronic (10–14 days) transection of the CSN (CSN denervation) and chronic removal of the superior cervical ganglion (sympathectomized). Following sympathectomy, the basal levels of cGMP remain unaltered; however, the response to 0·1 μM APIII was reduced by 75% in the absence of the sympathetic innervation to the organ. In contrast, after CSN denervation the basal levels of cGMP in the carotid body were significantly elevated (45% increase; $P < 0{\cdot}025$), and the response to 0·1 μM APIII was greatly potentiated (181% increase; $P < 0{\cdot}01$). These results with denervated carotid bodies suggest that the cGMP in the organ is primarily of non-neural origin, which agrees with our recent immunocytochemical studies identifying type I cells in cat and rat carotid bodies as the primary locus of cGMP (Wang, Stensaas, deVente, Dinger & Fidone, 1991*c*). Another important observation is that the afferent (CSN) and sympathetic innervation to the origin is able to reciprocally modulate the metabolism of this second messenger, a phenomenon which closely parallels earlier findings from our laboratory demonstrating a similar influence of sensory and autonomic innervation on the activity of the catecholamine synthetic enzyme, tyrosine hydroxylase (Gonzalez, Kwok, Gibb & Fidone, 1979).

Cyclic GMP release evoked by APIII

Figure 2*A* shows the time course of cGMP release evoked from a group of six carotid bodies in response to a 10 min exposure to 100 nM APIII. Under basal conditions (100% O_2 media), cGMP release into the superfusate during the 10 min

period was 0·0073 pmol (mg tissue)$^{-1}$, or about 20% of the tissue cGMP content measured under these conditions. In the presence of 100 nM APIII, the superfusate concentration was elevated by 169-fold, to 1·23 pmol (mg tissue)$^{-1}$, which is approximately equivalent to the elevated tissue content of cGMP (1·47 pmol (mg

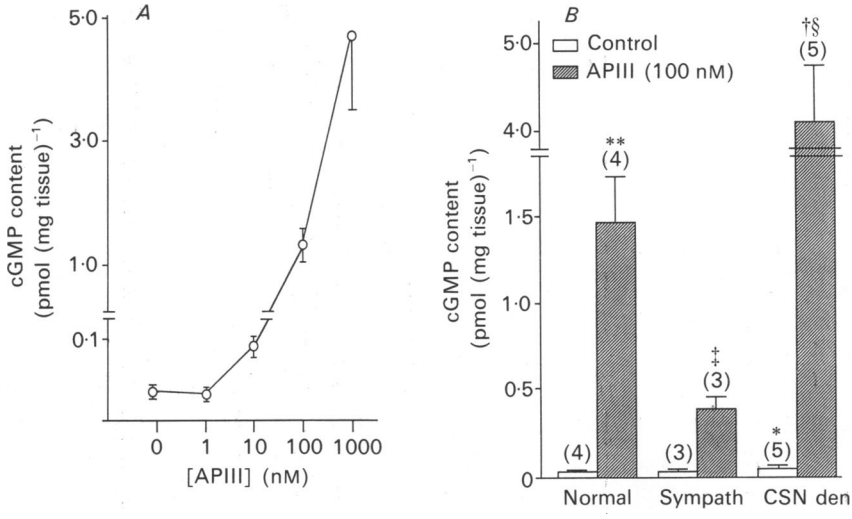

Fig. 1. *A*, changes in rabbit carotid body cGMP content in response to increasing concentrations of atriopeptin III (APIII). Values are means ± S.E.M. and represent measurements from five or six carotid bodies. *B*, effect of chronic (10–15 days) sympathectomy (removal of superior cervical ganglion; Sympath) and carotid sinus nerve denervation (CSN den) on cGMP content in carotid bodies incubated in the absence or presence of 100 nM APIII. Basal cGMP levels (in the absence of APIII) were unaffected by sympathectomy, but were elevated 45% following transection of the CSN. Sympathectomy diminished the response to APIII, whereas the response to the peptide was elevated following CSN denervation. Values are means ± S.E.M. * and ** indicate $P < 0·025$ and $P < 0·0025$, respectively, *vs.* normal carotid body control (minus APIII). † indicates $P < 0·01$ *vs.* normal carotid body with APIII. ‡ indicates $P < 0·005$ *vs.* sympathectomized control. § indicates $P < 0·0005$ *vs.* CSN denervated control. The number of carotid bodies in each group is given in parentheses.

tissue)$^{-1}$ achieved after a 10 min incubation in 100 nM APIII. During the first two 10 min post-stimulus periods, the concentration of cGMP in the superfusate rapidly declined to control values, and fully recovered to pre-stimulus conditions by the fourth post-stimulus period. Figure 2*B* shows the dose–response relationship for APIII-evoked release of cGMP (stimulus minus basal). Comparison of these data with the tissue content of cGMP (Fig. 1*A*) suggests that 50–90% of the cGMP generated in response to a 10 min exposure to APIII is rapidly released into the extracellular space.

Effect of APIII and cGMP on CSN activity

A typical effect of APIII on stimulus-evoked CSN activity in the rabbit is shown in Fig. 3. We examined the effects of the drug on the increased nerve activity evoked by media equilibrated with 20% O_2 (hypoxia; Fig. 3*A*), or containing 100 μM nicotine (Fig. 3*B*). Under control conditions, the mean basal nerve activity during

superfusion with 100% O_2 media was 242 ± 84 impulses $(10\ s)^{-1}$ (mean $\pm$ S.E.M.), as measured in thirteen preparations from eight rabbits. During the 5 min low O_2 stimulus period (20% O_2 medium), the discharge rate was elevated by an average of 2671 ± 210 impulses $(10\ s)^{-1}$ (Fig. 3*A*, Control); discharge rapidly subsided when 100% O_2 medium was reintroduced to the preparation. Administration of APIII at

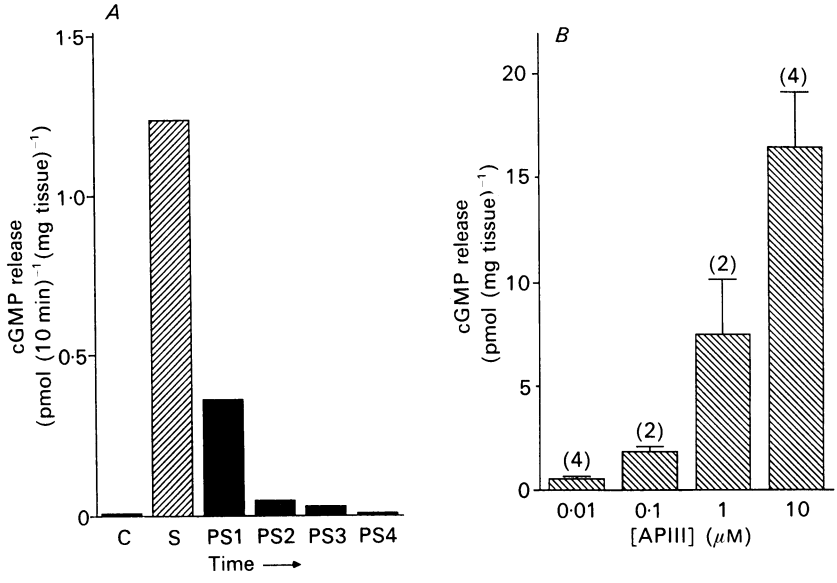

Fig. 2. *A*, time course of cGMP release evoked from a group of six carotid bodies (pooled) incubated in 100 nM APIII. Each bar represents the amount of cGMP collected during a 10 min superfusion period. C, control; S, stimulus (100 nM APIII); PS, post-stimulus . B, dose–response relationship for cGMP release evoked by APIII. The total amount released for each concentration of APIII was calculated as the combined excess (above basal release) observed in the presence of the peptide plus the excess released during the post-stimulus periods. Basal release was taken as the cGMP present in the 10 min control period immediately prior to stimulation. Values in parentheses equal the number of observations.

concentrations up to 1·0 μM did not alter basal CSN activity, but the drug significantly inhibited the response to hypoxia (Fig. 3*A*, Experimental response). APIII routinely reduced the peak and sustained discharge rates, and delayed the onset of the increase in CSN activity. The inhibition manifest in any given preparation consisted of various combinations of these effects. The response to hypoxia recovered completely following superfusion for 30–40 min in the absence of APIII (Fig. 3*A*, Recovery).

The response of the rabbit carotid body to 100 μM nicotine is shown in Fig. 3*B* (Control). Upon exposure to this cholinergic agonist, CSN discharge rate rapidly peaked but then gradually subsided to near baseline levels, even during continued superfusion with the drug. The reason for this decline in the nicotine response is unknown, but may involve classical nicotinic receptor desensitization, which in other systems is regulated by receptor phosphorylation in the continued presence of

the agonist (Huganir, Delcour, Greengard & Hess, 1986). APIII significantly inhibited CSN activity evoked by 100 μM nicotine (Fig. 3*B*, Experimental response); the response to the drug recovered 30–40 min following removal of this synthetic peptide.

The specificity of the APIII inhibition was evaluated by comparing the effects of

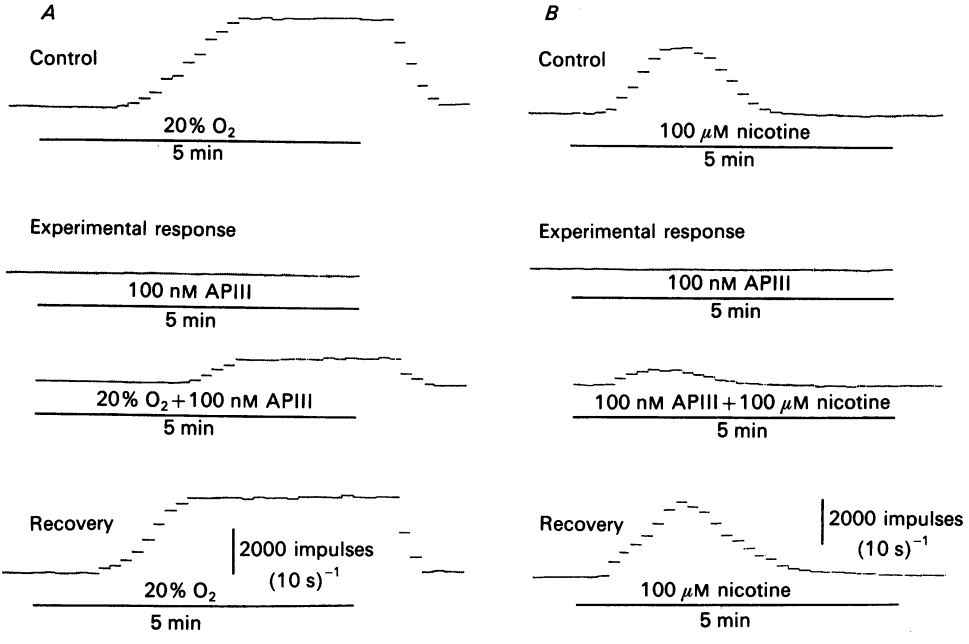

Fig. 3. *A*, effect of APIII on stimulus-evoked CSN activity. Control, basal CSN activity recorded in medium equilibrated with 100% O_2; hypoxic response evoked by 20% O_2 medium. Experimental response, two continuous traces demonstrate that APIII does not alter basal discharge rate, but response to 20% O_2 medium is greatly diminished. Recovery, response to hypoxia appears normal 30 min after wash-out of drug. *B*, same as in *A* except 100 μM nicotine is used to evoke CSN activity. Bar markers signal application of hypoxia, nicotine or APIII, as labelled. Calibration applies to all traces in *A* or *B*, respectively.

the peptide with those observed using 'clearance ANP' (C-ANP), a partial ring- and tail-deleted analogue of ANP which activates specific uptake mechanisms for the peptide in ANP-sensitive cells without evoking cGMP formation (Maack, Suzuki, Almeida, Nussenzveig, Scarborough, McEnroe & Lewicki, 1987). The data in Table 1 show that the cGMP content of rabbit carotid bodies was unchanged following 10 min incubations in 1, 10 and 100 nM C-ANP, while equivalent concentrations of ANP elevated cGMP levels in a dose-related manner. Figure 4*A* shows typical CSN responses obtained from a single preparation in which the effects of APIII and C-ANP were compared. In the presence of 100 nM APIII, there is a marked inhibition of the CSN activity evoked by hypoxia, in agreement with the data presented in Fig. 3*A*. In contrast, a 10-fold higher dose of C-ANP (1000 nM) failed to inhibit the hypoxic discharge. Results similar to these (summarized in Fig. 4*B*) were observed in six preparations using 100 and 1000 nM C-ANP.

TABLE 1. Effect of ANP *vs.* C-ANP on cGMP content of rabbit carotid bodies

	Peptide concentration (nM)		
	1	10	100
C-ANP	0·074 ± 0·009	0·067 ± 0·003	0·137 ± 0·041
ANP	0·123 ± 0·027*	0·279 ± 0·053**	1·92 ± 0·389***

Values for cGMP (means ± S.E.M) are expressed as pmol (mg tissue)$^{-1}$. Carotid bodies (n = 5–6) were incubated for 10 min in the presence of ANP or C-ANP. *, ** and *** indicate $P < 0·05$, $P < 0·001$ and $P < 0·0005$, respectively, compared to cGMP content measured with corresponding concentration of C-ANP.

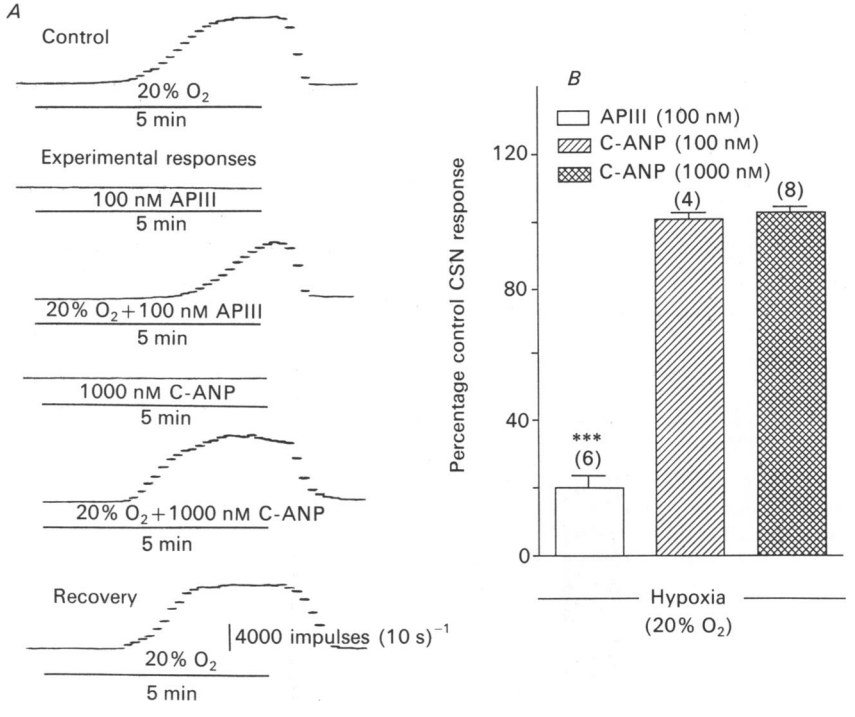

Fig. 4. *A*, CSN inhibition produced by APIII (100 nM) and ineffectiveness of C-ANP (1000 nM). Details as in Fig. 3. *B*, summary of CSN inhibition produced by APIII (100 nM) and C-ANP (100 and 1000 nM) in presence of superfusion media equilibrated with 20% O_2. *** indicates $P < 0·0005$ *vs.* control response. Values in parentheses indicate the number of observations.

Figure 5 shows the dose–response relationship for APIII inhibition of CSN activity evoked by hypoxia and nicotine. Clearly, the degree of inhibition produced by APIII appears to be independent of whether the nerve activity is evoked by hypoxia or nicotine. Moreover, a close correspondence exists between the effective dose–response ranges obtained for CSN inhibition and cGMP production (see Fig. 1*A*), further suggesting an important role for this cyclic nucleotide in the modulation of CSN activity.

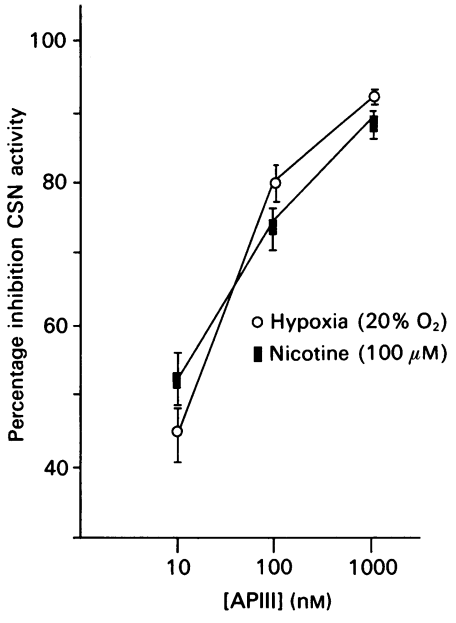

Fig. 5. Dose–response relationship for APIII inhibition of CSN activity evoked by hypoxia (20% O_2 media) or nicotine (100 μM). Each point represents data obtained from five to seven experimental trials as described in Fig. 3.

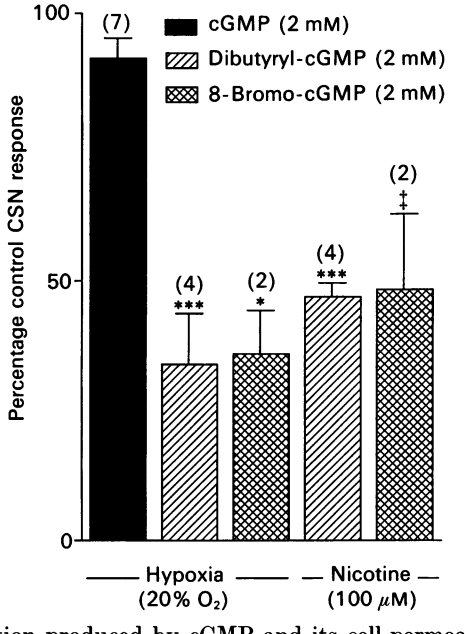

Fig. 6. CSN inhibition produced by cGMP and its cell-permeant analogues, dibutyryl-cGMP and 8-bromo-cGMP. CSN activity was evoked either by superfusion media equilibrated with 20% O_2 or containing 100 μM nicotine. Each bar represents data obtained from two to seven experimental trials. Significant inhibition is produced only by analogues of cGMP capable of achieving high intracellular concentration. ‡, * and ***, indicate $P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}005$, respectively.

In order to reveal the possible site of action of cGMP, we studied the effects of the cell-permeant analogues, dibutryryl-cGMP (db-cGMP) and 8-bromo-cGMP (8-b-cGMP), and compared their actions to those of unmodified cGMP, which is relatively impermeant to cell membranes. As shown in Fig. 6, moderate concentrations (2 mM) of db-cGMP or 8-b-cGMP produced greater than 50% inhibition of the CSN activity evoked by nicotine (100 μM) or hypoxia (20% O_2 media). Cyclic GMP itself (administered in the presence of the phosphodiesterase inhibitor isobutyl-methylxanthine; IBMX) produced only a small (< 10%) inhibition of the stimulus-evoked CSN activity, even at relatively high concentrations (10 mM). Basal CSN activity was unaffected by cGMP or its analogue (not shown).

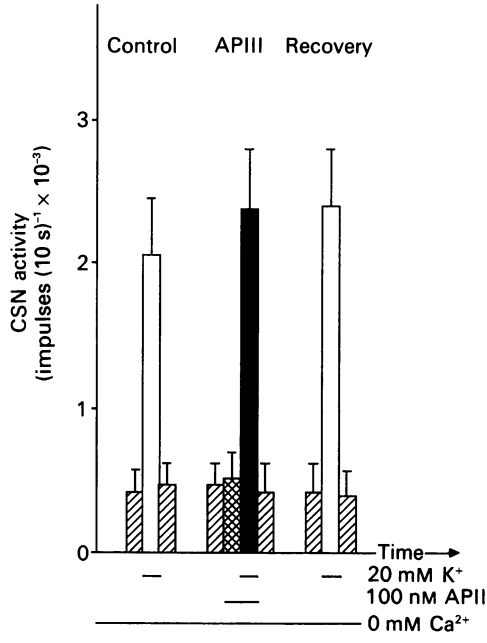

Fig. 7. APIII does not alter CSN activity evoked by elevated K^+ (20 mM). Each bar corresponds to a 5 min recording period. Data summarized from eight carotid body CSN preparations. The time axis indicates the application of 20 mM K^+ and 100 nM APIII in superfusate containing zero calcium. All solutions were equilibrated with 100% O_2.

Effect of APIII on CSN activity evoked by high [K^+]

Figure 7 shows that CSN activity evoked by media containing elevated K^+ and zero Ca^{2+} was not altered by APIII at a concentration (100 nM) which produced greater than 75% inhibition of the nicotine- and hypoxia-evoked activity. In these experiments, 20 mM KCl in the superfusates replaced an equimolar amount of NaCl; Mg^{2+} was increased from 1·1 to 2·1 mM. Because transmitter release from the carotid body is greatly reduced during superfusion with zero Ca^{2+} (Fidone, Gonzalez & Yoshizaki, 1982), CSN activity evoked under these conditions with high [K^+] can be largely attributed to direct depolarization of the nerve terminals. The inability of APIII to inhibit the potassium-evoked CSN discharge was consistently found in

eight preparations. In contrast, APIII (100 nM) always inhibited CSN activity evoked by hypoxia in these same preparations. These experiments provide further evidence that the APIII-induced inhibition involves the mediation of the type I cells.

Effect of APIII on stimulus-evoked catecholamine release

Type I cells in the carotid body contain high concentrations of catecholamines, particularly dopamine, and current views of chemoreception suggest that these cells

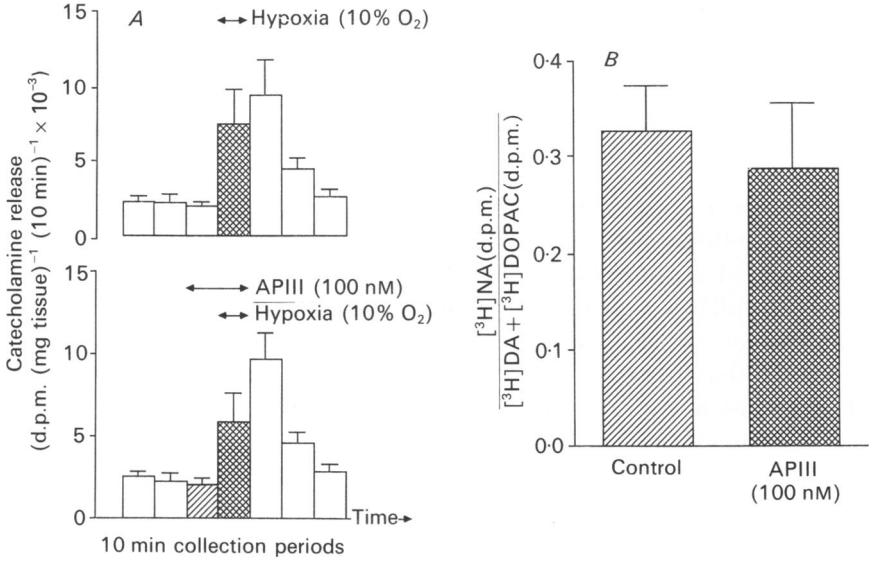

Fig. 8. *A*, effect of APIII on catecholamine release evoked by media equilibrated with 10% O_2. Upper panel shows typical profile including basal and stimulus-evoked release. Comparison with lower panel shows that overall basal and evoked release are unchanged in the presence of APIII. Each bar represents mean ± S.E.M. from four independent observations. *B*, results from HPLC–EC analysis of released radiolabelled catecholamines show that the ratio of noradrenaline (NA) to dopamine (DA) plus its metabolite dihydroxyphenyl-acetic acid (DOPAC) is unchanged in the presence of APIII. Values are means ± S.E.M. from four independent observations.

act as transducer elements in the organ, releasing neurotransmitters in response to natural and pharmacological stimuli (Fidone, Gonzalez, Obeso, Gomez-Niñ & Dinger, 1990). Because dopamine has been frequently proposed as an inhibitory transmitter in the carotid body, we have explored the possibility that APIII inhibition of CSN activity is mediated by alterations in catecholamine release from the type I cells. In these experiments, we monitored the basal and stimulus (hypoxia)-evoked release of [^{3}H] catecholamines ([^{3}H]CA) from the organ, in the presence or absence of 100 nM APIII. Figure 8*A* shows that with media equilibrated with 100% O_2 the drug did not alter basal [^{3}H]CA release during the 10 min collection period. Furthermore, the continued presence of APIII during the subsequent 10 min exposure to hypoxia likewise did not modify the evoked release of [^{3}H]CA (Fig. 8*A*). Three experiments, including nine carotid bodies in the control group and eleven which were exposed to 100 nM APIII, all produced similar results. HPLC analysis of

the released [^{3}H]CA showed further that APIII did not alter the ratio of [^{3}H]noradrenaline/[^{3}H]dopamine ([^{3}H]NA/[^{3}H]DA) released during hypoxia (Fig. 8*B*).

DISCUSSION

In the present study, we have shown that the biologically active ANP fragment, APIII, produces a dose-related inhibition of CSN activity evoked by either hypoxia or nicotine. The close correspondence between the effective dose range for this inhibition and the generation/release of cGMP, plus the fact that cell-permeant analogues of cGMP likewise inhibit CSN activity, suggest that the actions of APIII (ANP) are mediated by this cyclic nucleotide second messenger. Interestingly, previous studies in our laboratory demonstrated that hypoxia decreases the cGMP content in the carotid body (Wang *et al.* 1989), which is consistent with an inhibitory role for this putative second messenger in chemoreception. Cyclic GMP appears to act at an intracellular site because even high concentrations (10 mM) of the impermeant form of the nucleotide failed to alter the chemoreceptor response. These findings make it unlikely that the inhibition is caused by locally high concentrations of the cyclic nucleotide (e.g. consequent to release from type I cells; cf. Fig. 2), which in other systems have been shown to decrease spike amplitude and raise firing threshold in peripheral nerve (Vande Berg, 1974; see also Horn & McAfee, 1977). The release of large amounts of cGMP from the type I cells may instead constitute a mechanism for inactivation of the active substituent.

The location and characterization of ANP receptors in the carotid body has not yet been established, and therefore the precise locus of APIII actions with respect to type I cells *versus* chemoreceptive nerve terminals remains uncertain. In other tissues, two subtypes of ANP receptors have been identified; the receptor subtype thought to mediate natriuresis and diuresis in the kidney incorporates guanylate cyclase activity (B-ANP receptor), whereas the receptor subtype which occurs at much higher concentrations appears to be a transport protein involved in the metabolic clearance of ANP (C-ANP receptor; see Maack *et al.* 1987). The ring-deleted analogue of ANP, C-ANP, binds to the C-ANP receptor with high affinity but does not activate the guanylate cyclase activity of the B-ANP receptor (Fuller, Porter, Arfsten, Miller, Schilling, Scarborough, Lewicki & Schenk, 1988; Almeida, Suzuki, Scarborough, Lewicki & Maack, 1989). Our finding that C-ANP did not inhibit either basal or evoked CSN activity suggests that APIII actions in the carotid body are mediated by a specific B-ANP receptor and that the response requires the generation of cGMP.

The locus of cGMP generation in the carotid body appears to be the type I cells. Recent immunocytochemical studies in our laboratory have localized cGMP to the type I cells in the organ, and further have demonstrated that these cells constitute the principal source of increased immunostaining for the cyclic nucleotide following exposure to APIII (Wang *et al.* 1992*c*). Also, as we have shown in this study, the increases in cGMP in response to APIII persists in chronic CSN-denervated and sympathectomized carotid bodies. Changes in the cGMP response following these chronic denervations may be attributable to trophic influences from the neural innervation in the organ, a phenomenon which has previously been demonstrated for

neurotransmitter and cAMP metabolism in the carotid body (Gonzalez *et al.* 1979; Hanbauer, Karoum, Hellström & Lahiri, 1981; Gomez-Niño *et al.* 1990; Wang, Cheng, Yoshsizaki, Dinger & Fidone, 1991*a*).

A possible direct role for CSN terminals in the APIII-mediated inhibition is not supported by our experimental results using elevated potassium ($[K^+] = 20$ mM) as a stimulant, without Ca^{2+} in the media. Elevated concentrations of K^+ have previously been shown to stimulate the carotid body *in vivo* (Band & Linton, 1986; Burger, Estavillo, Kumar, Nye & Patterson, 1988) and to evoke the Ca^{2+}-dependent release of catecholamines *in vitro* (Almarez, Gonzalez & Obeso, 1986); but in the absence of Ca^{2+}, the release of neurotransmitters from type I cells is depressed by over 90% (Fidone *et al.* 1982; Almarez *et al.* 1986). Thus, the failure of APIII to inhibit CSN activity elicited by elevated K^+ (in zero Ca^{2+}) suggests that depolarized chemoreceptive nerve terminals remain capable of generating propagated action potentials in the presence of the drug. Conversely, the absence of APIII effects on CSN axons and terminals provides additional support for the notion that the chemoreceptor inhibition is mediated by altered type I cell function.

The precise mechanisms by which the type I cells bring about the APIII-mediated inhibition of CSN activity cannot be determined from the present data. Many investigators have suggested an inhibitory role for dopamine in the rabbit carotid body (see Docherty & McQueen, 1979; Leitner & Roumy, 1986); however, the fact that APIII fails to alter the hypoxia-evoked release of catecholamines (which in the rabbit comprises primarily dopamine) would seem not to implicate this putative transmitter in the observed inhibition of CSN activity. Possible effects of the natriuretic peptide on the release of other potential inhibitory transmitters also needs to be explored, such as Met-enkephalin, which has been shown in pharmacological experiments to depress CSN excitation produced by hypoxia (W.-J. Wang, L. He, J. Chen, B. Dinger & S. Fidone, unpublished observations) and pharmacological agents (Monti-Bloch & Eyzaguirre, 1985).

It should be noted that the effects of ANP (APIII) in the carotid body are observed at submicromolar concentrations while peak plasma levels of ANP evoked by hypervolaemia or an acute sodium load are reported in the picomolar range (e.g. Katsube, Schwartz & Needleman, 1985; Ross, Ervin, Lam, Leake & Fisher, 1988). These circumstances suggest that the circulating peptide does not play a role in modulating chemosensory activity. Preliminary results in our laboratory, however, suggest that ANP contained in type I cells is released by micromolar concentrations of calcitonin gene-related peptide (CGRP) and that CGRP inhibits hypoxia-evoked CSN activity. Furthermore, Kondo & Yamamoto (1988) and Kummer & Fischer (1990) have described a subpopulation of CGRP containing afferent fibres which penetrate lobules of type I and type II cells in the carotid body. Thus, activation of these fibres and the release of CGRP may initiate a cascade of events involving the release of ANP, cGMP production and modulation of CSN activity.

In summary, the results of this study suggest that APIII acts upon type I cells in the rabbit carotid body to produce inhibition of stimulus-evoked CSN activity. The failure of C-ANP to inhibit nerve activity, and the ability of cell-permeant analogues of cGMP to mimic this inhibition, suggest that CSN activity is modulated by cGMP production in type I cells. The function of cGMP in the carotid body appears,

therefore, to contrast with that of cAMP, which has been shown in our earlier studies to enhance CSN activity and promote catecholamine release (Wang *et al.* 1991*a*). Finally, the present results suggest that ANP, which has been localized to the type I cells (Wang *et al.* 1991*b*), may play an important role in modulating the cardiovascular and ventilatory reflexes which originate from the carotid body.

This work was supported by US Public Health Service grants NS12636 and NS07938.

REFERENCES

ALMARAZ, L., GONZALEZ, C. & OBESO, A. (1986). Effects of high potassium on the release of [^{3}H]dopamine from the cat carotid body *in vitro*. *Journal of Physiology* **379**, 293–307.

ALMEIDA, F. A., SUZUKI, M., SCARBOROUGH, R. M., LEWICKI, J. A. & MAACK, T. (1989). Clearance function of type C receptors of atrial natriuretic factor in rats. *American Journal of Physiology* **256**, R469–475.

ANTUNES-RODRIGUES, J., MCCANN, S. M. & SAMSON, W. K. (1986). Central administration of atrial natriuretic factor inhibits saline preference in the rat. *Endocrinology* **118**, 1726–1728.

BAND, D. M. & LINTON, R. A. F. (1986). The effect of potassium on carotid body chemoreceptor discharge in the anaesthetized cat. *Journal of Physiology* **381**, 39–47.

BURGER, R. E., ESTAVILLO, J. A., KUMAR, P., NYE, P. C. G. & PATTERSON, D. J. (1988). Effects of potassium, oxygen and carbon dioxide on the steady-state discharge of cat carotid body chemoreceptors. *Journal of Physiology* **401**, 519–531.

CHINKERS, M., GARBERS, D. L., CHANG, M.-S., LOWE, D. G., CHIN, H., GOEDDEL, D. V. & SCHULZ, S. (1989). A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* **338**, 78–83.

DE BOLD, A. J. (1979). Heart atria granularity. Effects of changes in water and electrolyte balance. *Proceedings of the Society for Experimental Biology and Medicine* **161**, 508–511.

DOCHERTY, R. J. & MCQUEEN, D. S. (1979). The effects of acetylcholine and dopamine on carotid chemosensory activity in the rabbit. *Journal of Physiology* **288**, 411–423.

FIDONE, S. & GONZALEZ, C. (1982). Catecholamine synthesis in rabbit carotid body *in vitro*. *Journal of Physiology* **333**, 69–79.

FIDONE, S. J., GONZALEZ, C., OBESO, A., GOMEZ-NIÑO, A. & DINGER, B. (1990). Biogenic amine and neuropeptide transmitters in the carotid body chemotransmission: experimental findings and perspectives. In *Hypoxia: The Adaptations*, ed. SUTTON, J. R., COATES, G. & REEMERS, J. E., pp. 116–126. Decker, Toronto.

FIDONE, S. J., GONZALEZ, C. & YOSHIZAKI, K. (1982). Effects of low oxygen on the release of dopamine from the rabbit carotid body *in vitro*. *Journal of Physiology* **333**, 93–110.

FULLER, F., PORTER, J. G., ARFSTEN, A. E., MILLER, J., SCHILLING, J. W., SCARBOROUGH, R. M., LEWICKI, J. A. & SCHENK, D. B. (1988). Atrial natriuretic peptide clearance receptor. *Journal of Biological Chemistry* **263**, 9395–9401.

CALLEGO, R., EYZAGUIRRE, C. & MONTI-BLOCH, L. (1979). Thermal and osmotic responses of arterial receptors. *Journal of Neurophysiology* **42**, 665–680.

GENEST, J., CANTIN, M., ANAND-SRIVASTAVA, M. B., CUSSON, J. R., DE LEAN, A., GARCIA, R., GUTKOWSKA, J., HAMET, P., KUCHEL, O., LAROCHELLE, P., NEMER, M., SCHIFFRIN, E. L., SCHILLER, W., THIBAULT, G. & TREMBLAY, J. (1988). The atrial natriuretic factor: its physiology and biochemistry. *Reviews in Physiology, Biochemistry and Pharmacology* **110**, 1–145.

GOMEZ-NIÑO, A., CHENG, G.-F., YOSHIZAKI, K., GONZALEZ, C., DINGER, B. & FIDONE, S. J. (1990). Regulation of the release of dopamine and norepinephrine from rabbit carotid body. In *Arterial Chemoreception*, ed. EYZAGUIRRE, C., FIDONE, S. J., FITZGERALD, R. S., LAHIRI, S. & MCDONALD, D. M., pp. 92–99. Springer-Verlag, New York.

GONZALEZ, C., KWOK, Y., GIBB, J. W. & FIDONE, S. J. (1979). Reciprocal modulation of tyrosine hydroxylase activity in rat carotid body. *Brain Research* **172**, 572–576.

HAMET, P., TREMBLAY, J., PANG, S. C., GARCIA, R., THIBAULT, G., GUTKOWSKA, J., CANTIN, M. & GENEST, J. (1984). Effect of native and synthetic atrial natriuretic factor on cyclic GMP. *Biochemical and Biophysical Research Communications* **123**, 515–527.

HANBAUER, I., KAROUM, F., HELLSTRÖM, S. & LAHIRI, S. (1981). Effects of hypoxia lasting up to one month on the catecholamine content in rat carotid body. *Neuroscience* **6**, 81–86.

HONIG, A. (1989). Peripheral arterial chemoreceptors and reflex control of sodium and water homeostasis. *American Journal of Physiology* **257**, R1282–1302.

HORN, J. P. & MCAFEE, D. A. (1977). Modulation of cyclic nucleotide levels in peripheral nerve without effect on resting or compound action potentials. *Journal of Physiology* **269**, 753–766.

HUGANIR, R. L., DELCOUR, A. H., GREENGARD, P. & HESS, G. P. (1986). Phosphorylation of the nicotine acetylcholine receptor regulates its rate of desensitization. *Nature* **32**, 774–776.

KATSUBE, N., SCHWARTZ, D. & NEEDLEMAN, P. (1985). Release of atriopeptin in the rat by vasoconstrictors or water immersion correlates with changes in right atrial pressure. *Biochemical and Biophysical Research Communications* **133**, 937–944.

KONDO, H. & YAMAMOTO, M. (1988). Occurrence, ontogeny, ultrastructure and some plasticity of CGRP (calcitonin gene-related peptide)-immunoreactive nerves in the carotid body of rats. *Brain Research* **473**, 283–293.

KUMMER, W. & FISCHER, A. (1990). Tachykininergic axons in the guinea pig carotid body: origin, ultrastructure and coexistence with other peptides. In *Arterial Chemoreception*, ed. EYZAGUIRRE, C., FIDONE, S. J., FITZGERALD, R. S., LAHIRI, S. & MCDONALD, D. M., pp. 229–234. Springer-Verlag, New York.

KUO, J.-F. & GREENGARD, P. (1972). An assay method for cyclic AMP and cyclic GMP based upon their abilities to activate cyclic AMP-dependent and cyclic GMP-dependent protein kinases. *Advances in Cyclic Nucleotide Research* **2**, 41–51.

LEITNER, L.-M. & ROUMY, M. (1986). Chemoreceptor response to hypoxia and hypercapnia in catecholamine depleted rabbit and cat carotid bodies in vitro. *Pflügers Archiv* **406**, 419–423.

MAACK, T., SUZUKI, M., ALMEIDA, F. A., NUSSENZVEIG, D., SCARBOROUGH, R. M., MCENROE, G. A. & LEWICKI, J. A. (1987). Physiological role of silent receptor of atrial natriuretic factor. *Science* **238**, 675–678.

MONTI-BLOCH, L. & EYZAGUIRRE, C. (1985). Effects of methionine-enkephalin and substance P on the chemosensory discharge of the cat carotid body. *Brain Research* **338**, 297–307.

MURAD, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. *Journal of Clinical Investigation* **78**, 1–5.

ROSS, M. G., ERVIN, M. G., LAM, R. W., LEAKE, R. D. & FISHER, D. A. (1988). Fetal atrial natriuretic factor and arginine vasopressin responses to hyperosmolarity and hypervolemia. *Pediatric Research* **24**, 318–321.

SAMSON, W. K. (1985). Atrial natriuretic factor inhibits dehydration and hemorrhage-induced vasopressin release. *Neuroendocrinology* **40**, 277–279.

SAMSON, W. K. (1987). Atrial natriuretic factor and the central nervous system. *Endocrinology and Metabolism Clinics of North America* **16**, 145–161.

SAMSON, W. K., AGUILA, M. C., MARTINOVIC, J., ANTUNES-RODRIGUES, J. & NORRIS, M. (1987). Hypothalamic action of atrial natriuretic factor to inhibit vasopressin secretion. *Peptides* **8**, 449–454.

SEN, I. & ROY, P. (1986). Atrial natriuretic factor induced phosphorylation of human placental membrane protein: an effect mimicked by guanosine 3′:5′-cyclic monophosphate. *Biochemical and Biophysical Research Communications* **139**, 431–438.

VANDE BERG, J. S. (1974). Inhibitory effects of dibutyryl and cyclic AMP on the compound action potential in the frog (Rana pipiens) sciatic nerve. *Experientia* **30**, 1025–1027.

WANG, W.-J., CHENG, G.-F., DINGER, B. G. & FIDONE, S. J. (1989). Effects of hypoxia on cyclic nucleotide formation in rabbit carotid body in vitro. *Neuroscience Letters* **105**, 164–168.

WANG, W.-J., CHENG, G.-F., YOSHIZAKI, K., DINGER, B. & FIDONE, S. (1991*a*). The role of cyclic AMP in chemoreception in the rabbit carotid body. *Brain Research* **540**, 96–104.

WANG, Z.-Z., HE, L., STENSAAS, L. J., DINGER, B. G. & FIDONE, S. J. (1991*b*). Localization and in vitro actions of atrial natriuretic peptide in the cat carotid body. *Journal of Applied Physiology* **70**, 942–946.

WANG, Z.-Z., STENSAAS, L. J., DEVENTE, J., DINGER, B. & FIDONE, S. J. (1991*c*). Immunocytochemical localization of cAMP and cGMP in cells of the rat carotid body following natural and pharmacological stimulation. *Histochemistry* **96**, 523–530.