Functional dopamine D₂ receptors on rat vagal afferent neurones

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1 In the present study *in vitro* electrophysiology and receptor autoradiography were used to determine whether rat vagal afferent neurones possess dopamine D₂ receptors.

2 Dopamine (10–300 µM) elicited a temperature- and concentration-dependent depolarization of the rat isolated nodose ganglion preparation. When applied to the tissue 15 min prior to agonist, raclopride (10 µM), clozapine (10 µM) or a mixture of raclopride and clozapine (10 µM each) all produced a threefold parallel shift to the right of the dopamine concentration-response curve. In contrast, SCH 23390 (100 nM), phentolamine and propranolol (1 µM each) failed to antagonize the dopamine-mediated depolarization.

3 [¹²⁵I]-NCQ 298 (0.5 nM), a D₂ selective radioligand, bound topographically to sections of rat brainstem. Densitometric quantification of autoradiograms revealed 93.8 ± 0.5% specific binding of this salicylamide radioligand, as determined by raclopride (10 µM, *n* = 10 animals). Binding was highest in the nucleus tractus solitarius (NTS), particularly the medial and gelatinous subnuclei. In addition, specific binding was also observed in the interpolar spinal trigeminal nucleus and the inferior olive.

4 Unilateral nodose ganglionectomy caused a 36.6 ± 3.0% reduction in specific binding in the denervated NTS compared to the contralateral NTS. Furthermore, the loss of binding was confined to the dorsal aspect of the medial subnucleus of the NTS. Sham surgery had no effect on the binding of [¹²⁵I]-NCQ 298 in rat brainstem.

5 The present data provide evidence for the presence of functionally relevant dopamine D₂ receptors on both the soma and central terminals of rat vagal afferent neurones. In addition, the majority of D₂ receptors in the rat NTS appear to be located postsynaptically with respect to vagal terminals, and are presumably located either on ascending glossopharyngeal terminals, descending terminals from higher brain regions or on neuronal cell bodies within the NTS.

Keywords: Dopamine; [¹²⁵I]-NCQ 298; nodose ganglion; vagal afferent nerves; nucleus tractus solitarius; D₂ receptors; electrophysiology; autoradiography

Introduction

It has been shown by molecular biological techniques that dopamine receptors form a heterogeneous population of protein molecules (receptor subtypes). Current nomenclature describes D₁ through to D₅ receptors, some of which exhibit alternate splice variants (Civelli *et al.*, 1993). With the exception of the D₃ receptors, all other dopamine receptor subtypes appear to be coupled to G-proteins (Sokoloff *et al.*, 1990).

Historically, much interest has centred on the involvement of dopaminergic systems and dopamine receptors in neurodegenerative disorders such as Parkinson's disease (Birkmayer & Hornykiewicz, 1962) and psychoses (Seeman *et al.*, 1976); however, both dopamine and its receptors have been implicated in cardiovascular control and chemoreflex pathways (Fidone *et al.*, 1988; Van Den Buuse & De Jong, 1991). For example, activation of dopamine D₂ receptors in the periphery mediates a hypotensive response via the inhibition of noradrenaline release from noradrenergic terminals (Nagahama *et al.*, 1986). In contrast, central D₂ receptor activation leads to a pressor effect, and the receptors mediating the central pressor response have been localized to a discrete region of the posterior nucleus tractus solitarius (NTS) (Yang *et al.*, 1990). Furthermore, microiontophoretic application of dopamine into the NTS has been shown to inhibit neuronal activity in this brainstem nucleus (Granata & Woodruff, 1982). The NTS, which receives inputs from arterial baroreceptor and chemoreceptor primary afferent fibres via the IXth and Xth cranial nerves, is established as

the pivotal relay nucleus in central cardiovascular control and chemoreflex pathways (Kalia, 1981).

Neuronal cell bodies of centrally projecting glossopharyngeal and vagal afferent neurones, including baroreceptor and chemoreceptor afferents, are located in the petrosal and nodose ganglia respectively (Kalia, 1981; Kumada *et al.*, 1990). Immunocytochemical studies have demonstrated the presence of the necessary enzymes for the synthesis of dopamine in petrosal ganglia cell bodies (Finley *et al.*, 1992). Furthermore, hypoxic insult causes an increase in dopamine release in the rabbit NTS (Goigny *et al.*, 1991). Taken together, these data provide strong evidence for dopamine as a neurotransmitter or modulator of chemoreceptor primary afferent neurones. In addition, neuronal perikarya of the carotid body, petrosal ganglion and superior cervical ganglion express the genetic information encoding the D₂ but not the D₁ receptor (Czyzyk-Krzeska *et al.*, 1992).

In contrast to the depth of information regarding the presence of dopamine and dopamine receptors in chemoreceptor afferent neurones, relatively little is known about the involvement of dopaminergic systems and dopamine receptors in baroreceptor primary afferents. The present study has therefore addressed this question in rats, using functional and anatomical techniques. In light of the documented ability of dopamine to depolarize mammalian primary afferent neurones (Zapata, 1975; Gallagher *et al.*, 1980; Monti-Bloch *et al.*, 1993), the isolated nodose/vagal grease-gap technique was employed to determine whether activation of dopamine receptors on the soma membranes of nodose ganglia could evoke an electrophysiological response.

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This methodology also gives an indication of the presence and properties of receptors on central vagal afferent terminals (Round & Wallis, 1986). In addition, the salicylamide [¹²⁵I]-NCQ 298, a selective radioligand for dopamine D₂ receptors (Hall *et al.*, 1991), was utilized in autoradiographic experiments to assess the distribution of D₂ binding sites in the rat brain stem. Specifically, the binding of [¹²⁵I]-NCQ 298 to sections of rat brain stem was studied following unilateral vagal deafferentation (nodose ganglionectomy) to determine whether labelled sites were pre- or post-synaptic in location with respect to central vagal afferent terminals.

Methods

All the experiments described here were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the NH & MRC Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

In vitro electrophysiology

Male Sprague-Dawley rats (250–350 g) were killed by cervical dislocation and the nodose ganglia with attached distal vagal trunk were removed and desheathed. The tissue was then placed in a twin chambered perspex bath with the nodose ganglion placed in one compartment of the bath and isolated from the vagal nerve trunk in an adjacent compartment by a silicone grease seal, as previously described (Widdop *et al.*, 1990). The preparation was superfused with Krebs buffer (36°C, 2 ml min⁻¹) of the following composition (in mM): NaCl 118, NaHCO₃ 24.9, KH₂PO₄ 1.3, KCl 4.7, CaCl₂ 2.6, glucose 11, MgSO₄ 1.2, gassed with 95% O₂/5% CO₂, pH approximately 7.4. In experiments employing dopamine, the superfusate also contained ascorbic acid (0.11 mM) and EDTA (1 mM) which were present throughout the whole experimental period and at the concentration employed did not cause any depolarization. The d.c. potential between the two compartments following drug administration to the nodose ganglion was recorded by calomel electrodes connected to the preparation through agar-KCl bridges. The potential changes were amplified and displayed on a Grass Polygraph (Model 79D). Drugs were applied non-cumulatively and remained in contact with the tissue until apparent equilibrium was reached. This was followed by a washout and recovery period (10–20 min) to allow full repolarization prior to another drug addition. When used, antagonists were added to the superfusate 15 min before agonist application. This method allowed stable responses to be measured over a 5–6 h period. The magnitude of an observed depolarization was measured from a projection of the baseline preceding the response to account for any drift in the preparation. At the beginning and end of each experiment a positive control of a previously determined maximal concentration of 5-hydroxytryptamine (5-HT, 3 µM; Widdop *et al.*, 1992) was applied to the nodose ganglia in order to check the viability of the preparations.

Preparation of radioligand

[¹²⁵I]-NCQ 298 ((S)-3-iodo-N-[(1-ethyl-2-pyrrolidinyl)methyl]-5,6-dimethoxysalicylamide) was prepared from NCQ 634 (the corresponding des-iodo derivative) by the chloramine-T radioiodination technique and purified by paper chromatography, as described previously (Adams & Jarrott, 1982). The binding profile of this radioligand has been reported (Hall *et al.*, 1991).

Unilateral vagal deafferentation

Male rats (250–320 g) were anaesthetized with sodium methohexitone (60 mg kg⁻¹, i.p.) and placed on their back. A

midline incision was made in the neck and the left nodose ganglion was exposed and excised (*n* = 5) including trunks of vagus, superior laryngeal and inferior pharyngeal nerves at the respective points of contact with the nodose ganglion. In a second group (*n* = 5), the left nodose ganglion was exposed but not removed (sham control). After a 14 day recovery period, the animals were re-anaesthetized with sodium pentobarbitone (60 mg kg⁻¹, i.p.) and the rats were then immediately killed by decapitation and their brainstems removed. All tissue was frozen over liquid nitrogen at the time of removal and stored at -80°C until further processed.

Autoradiography

Cryostat-cut sections of brainstem (10 µm) were taken between bregma -14.3 mm to bregma -12.8 mm (Paxinos & Watson, 1986) and thaw-mounted onto gelatin/chrome alum/bovine serum albumin coated microscope slides. Autoradiographic experiments were carried out as previously described (Hall *et al.*, 1991). In short, tissue sections were allowed to warm to room temperature and then pre-incubated (30 min, room temperature) in Tris HCl buffer (170 mM, pH 7.6) containing NaCl 120 mM, KCl 5 mM, CaCl₂ 2 mM, MgCl₂ 1 mM and ascorbic acid 0.001% w/v. The sections were then incubated with [¹²⁵I]-NCQ 298 (0.5 nM) for 60 min at room temperature in the same buffer used for the pre-incubation. Non-specific binding was defined as that remaining in the presence of raclopride (10 µM).

The slide-mounted sections were then washed in ice-cold Tris HCl buffer (170 mM, pH 7.6, 4 × 2 min) and finally rinsed in ice-cold distilled water (2 × 30 s). Following this, the sections were dried under a gentle stream of cool air and apposed to X-ray film (Kodak XAR-5) for 36 h at 4°C, in the presence of standard ¹²⁵I microscaler (Amersham). Developed autoradiograms were quantified with an MCID M4 image analysis system (Imaging Research Inc., Canada), by comparison of the optical densities of autoradiographic images with those of the standard microscaler. Brain regions were identified following microscopic examination of adjacent tissue sections stained with 0.1% thionin with reference to a stereotaxic atlas (Paxinos & Watson, 1986).

Materials

The salicylamide compounds NCQ 298, NCQ 634 and raclopride were gifts from Astra Pharmaceuticals, Sweden. Clozapine was a gift from Sandoz, Australia. Dopamine hydrochloride and 5-hydroxytryptamine creatinine sulphate were obtained from Sigma Chemicals, St Louis, MO, U.S.A. SCH 23390 was from Research Biochemicals Inc., U.S.A., phentolamine from Ciba-Geigy and propranolol from I.C.I. Other reagents were of either laboratory or analytical grade from various suppliers.

Statistics

A paired *t* test (two-tailed) was employed to analyse differences in [¹²⁵I]-NCQ 298 binding between the denervated and intact sides of the NTS. *P* < 0.05 was considered significant. Concentration-response curves on the isolated nodose ganglion preparation were analysed by potency ratios.

Results

In vitro electrophysiology

Addition of dopamine to the superfusate bathing the isolated nodose ganglia elicited a concentration-dependent depolarization (Figure 1). Furthermore, the action of dopamine on the isolated nodose ganglion was also temperature-dependent,

Figure 1 Polygraph trace showing a representative series of responses to different concentrations of dopamine applied to the rat isolated nodose ganglion preparation. The first depolarization represents an initial positive control of 5-hydroxytryptamine (5-HT, 3 μ M). Scales as shown.

the catecholamine being ineffective at room temperature ($\sim 22^\circ\text{C}$). Over the concentration-range employed (10–300 μ M), dopamine did not consistently achieve an apparent maximum response; however, higher concentrations were not applied due to the possibility of actions at non-dopamine receptors. Therefore, data have been quantified in terms of potency ratios and plotted in a similar manner to *in vitro* electrophysiological studies of cholecystokinin receptors on 5-hydroxytryptaminergic neurones of the dorsal raphe nucleus (Boden *et al.*, 1991). The D₂ antagonist, raclopride (10 μ M, Köhler *et al.*, 1985) shifted the concentration-response curve for dopamine in a parallel fashion to the right by a factor of 2.96 ($n = 3$), yielding a potency ratio of 0.338 (Figure 2a). The D₄/D₂ antagonist clozapine (10 μ M, Lahti *et al.*, 1993) had a similar effect, causing a 3.00 fold rightward shift of the dopamine concentration-response curve (potency ratio = 0.333; $n = 6$), as shown in Figure 2b. A combination of both raclopride (10 μ M) and clozapine (10 μ M) shifted the concentration-response curve for dopamine evoked depolarizations to the right by a factor of 2.89 (potency ratio = 0.346, $n = 6$, Figure 2c). In contrast to raclopride and clozapine, the selective D₁ antagonist, SCH 23390 (100 nM, Hyttel, 1983) had no effect on the dopamine concentration-response curve (Figure 2d). The ability of dopamine to depolarize the rat isolated nodose ganglion was unaffected by the addition of phentolamine and propranolol (1 μ M each, $n = 3$; data not shown).

In vitro autoradiography

The binding of [¹²⁵I]-NCQ 298 has been fully characterized previously (Hall *et al.*, 1991) and in our hands this radioligand (0.5 nM) bound avidly to sections of rat brainstem. Raclopride (10 μ M) markedly inhibited the binding of [¹²⁵I]-NCQ 298. Thus, densitometric quantification estimated specific binding to represent $93.8 \pm 0.5\%$ of total binding ($n = 10$ animals; minimum of 18 sections/animal analysed). Specific binding of [¹²⁵I]-NCQ 298 was predominantly localized to the NTS/dorsal motor nucleus of vagus, central/medial inferior olive and the interpolar spinal trigeminal nucleus (Figure 3). At most anatomical levels through the brainstem analysed, unilateral deafferentation of the vagus failed to have an effect on the density of raclopride-sensitive binding sites in the NTS. However, between bregma -13.4 and bregma -13.2 , unilateral nodose ganglionectomy caused a $36.6 \pm 3.0\%$ reduction in the specific binding of [¹²⁵I]-NCQ 298 in the denervated (left) NTS (24 ± 3 d.p.m. mm^{-2}) compared to the contralateral (right) NTS (38 ± 4 d.p.m. mm^{-2} ; $n = 7$ sections from 5 animals; $P = 0.0001$ paired *t* test, intact versus denervated NTS), as shown in Figure 3b. At this anatomical level, the specific binding of [¹²⁵I]-NCQ 298 in the rat NTS described a crescent-like shape, being restricted to the medial, gelatinous and lateral subnuclei and absent in the solitary tract itself. The loss of binding was isolated to the dorsal aspect of the medial NTS (Figure 3b). In contrast,

Figure 2 Concentration-response curves of dopamine on the rat isolated nodose ganglion preparation, in the absence ($\square$) or presence ($\blacksquare$) of: (a) raclopride (10 μ M, $n = 3$); (b) clozapine (10 μ M, $n = 6$); (c) a combination of raclopride and clozapine (10 μ M each, $n = 6$); (d) SCH 23390 (100 nM, $n = 4$). Data points are mean $\pm$ s.e. of mean.

sham surgery did not affect raclopride-sensitive binding of [¹²⁵I]-NCQ 298 in the rats NTS ($n = 5$, Figure 3a). Figure 3c schematically demonstrates the anatomical subdivisions within the rat NTS. The medial NTS contains five subnuclei: medial, dorsal, intermediate, ventral and gelatinous whereas the lateral NTS is smaller and composed of four subnuclei: interstitial, lateral, ventrolateral and dorsolateral (Van Giersbergen *et al.*, 1992). A comparison between Figure 3b and Figure 3c demonstrates the loss of binding following unilateral nodose ganglionectomy is clearly restricted to the dorsal subdivision of the medial NTS.

Discussion

The present results demonstrate, for the first time, the presence of functional D₂ receptors on the soma membrane of rat vagal afferents. These findings are in agreement with the recent visualisation of D₂ binding sites on human inferior vagal ganglia (Lawrence & Jarrott, 1994). In addition, these data confirm the ability of dopamine to depolarize primary afferent neurones (Gallagher *et al.*, 1980; Monti-Bloch *et al.*, 1993). While the receptors mediating the dopamine-induced depolarization of the isolated rat nodose ganglia belong to the D₂ family, the current data cannot differentiate between the short and long forms of dopamine D₂ receptors (Monsma *et al.*, 1989). In addition to electrophysiological characterization, autoradiographic experiments in conjunction with unilateral vagal deafferentation confirm the presence of a population of dopamine D₂ receptors on central vagal afferent terminals within the rat NTS. Taken together, these observations suggest that D₂ receptors are synthesized in nodose perikarya and axoplasmically transported to the central nerve terminals in the medial subnucleus of the NTS.

Figure 3 Autoradiograms of [¹²⁵I]-NCQ 298 binding to sections of rat brain stem. Specific binding represented >90% of total binding. (a) Sham nodose ganglionectomy control. Anatomical level shown corresponds to bregma -13.3 mm. D₂ binding sites are predominantly restricted to the medial and gelatinous subnuclei of the rat NTS. Less dense binding is apparent in the interpolar spinal trigeminal nucleus and the inferior olive. Scale bar = 1.04 mm. (b) Unilateral nodose ganglionectomy. Anatomical level shown corresponds to bregma -13.3 mm. A $36.6 \pm 3.0\%$ ($n = 5$) reduction in D₂ binding can be observed in the denervated NTS (arrowhead) compared to the contralateral NTS. Loss of binding is confined to the dorsal aspect of the medial subnucleus (arrowhead, see (c) for definition of NTS subdivisions). Scale as for (a). (c) Schematic diagram showing subdivisions of the rat NTS at the anatomical level of the autoradiograms. A complete section of brain stem shows the relative position of the NTS on the dorsal surface of the brain stem. The boxed area is shown enlarged above the main diagram to allow definition of NTS subnuclei. Annotation as follows: (1) medial; (2) dorsal; (3) intermediate; (4) ventral; (5) gelatinous; (6) interstitial; (7) dorsolateral; (8) lateral; (9) ventrolateral; DVN, dorsal vagal nucleus; TS (shaded area), solitary tract; IV, fourth ventricle. Adapted from Paxinos & Watson (1986) and Van Giersbergen *et al.* (1992).

The ability of dopamine to depolarize the isolated nodose ganglion was shifted to the right in a parallel manner by the selective D₂ antagonist raclopride (Köhler *et al.*, 1985) and the D₄/D₂ antagonist clozapine (Lahti *et al.*, 1993). The fact that both antagonists caused a threefold rightward shift, which was unchanged by use of a combination of the two drugs, suggests that the receptor involved is indeed a D₂ receptor and not a D₄ receptor. Furthermore, clozapine is reported less than three fold selective for the D₄ over the D₂ receptor in membrane binding assays (Lahti *et al.*, 1993). In contrast, the dopamine-induced depolarizations were insensitive to D₁ receptor blockade by SCH 23390 (Hyttel, 1983). Additionally, phentolamine and propranolol were ineffective in preventing the dopamine-evoked response, discounting the possibility of an action at α - or β -adrenoceptors. The temperature-dependence of agonist-induced depolarization of rat vagal afferents has been previously described for angiotensin II (Widdop *et al.*, 1990; 1992), supporting the notion that dopamine is stimulating the synthesis of a second messenger through an enzymatic process. While these data support the presence of D₂ receptors on the cell bodies of centrally-projecting vagal primary afferent neurones, one must remember that the isolated nodose ganglion technique measures a population response, thus the neurone-types involved, such as baroreceptor, cannot be distinguished.

The autoradiographic pattern of [¹²⁵I]-NCQ 298 binding in the rat brainstem is largely similar to that recently found

with another D₂ selective radioligand [³H]-YM-09151-2 (Yokoyama *et al.*, 1994), with the exception that the latter radioligand visualized a population of sites in the hypoglossal nucleus which was not observed in the present study. The reason for this difference is unclear; however, Yokoyama and colleagues (1994) were 'surprised' to observe specific binding of [³H]-YM-09151-2 in a number of brainstem motor nuclei. Nonetheless, both studies describe high affinity D₂ binding in the rat NTS. The NTS is well established as the site of the first synapse for centrally-projecting vagal afferent neurones, including arterial baroreceptor afferents (Kumada *et al.*, 1990). While the majority of binding sites visualized appear to be postsynaptic to the vagal afferent terminals, the data presented herein demonstrate, for the first time, a population of presynaptic D₂ receptors on the central terminals of the vagus, particularly in the medial subnucleus of the NTS, the main location of primary arterial baroreceptor afferent terminals (Kumada *et al.*, 1990). Dopamine D₂ receptors in the rat NTS have been demonstrated to mediate a pressor response following microinjection of quinpirole (Yang *et al.*, 1990). This effect may, at least in part, be due to release-regulating D₂ receptors on baroreceptor afferent neurones. However, two thirds of the D₂ receptors in the NTS remain following nodose ganglionectomy, suggesting that postsynaptic receptors may also be involved in the central cardiovascular actions of dopamine. It is unlikely that the incomplete loss of D₂ binding sites in the NTS represents only a partial

denervation of the vagal projections. We have previously used this technique to demonstrate the presynaptic location of adenosine A_{2a} receptors (Castillo-Meléndez *et al.*, 1994); angiotensin II receptors (Lewis *et al.*, 1986) and vasopressin V₁ receptors (Gao *et al.*, 1992) in the rat NTS. In addition, nodose ganglionectomy has also been employed to study the cellular localization in the NTS of glutamate receptors (Lewis *et al.*, 1988), neurotensin receptors (Kessler & Beaudet, 1989), cholecystokinin receptors (Ladenheim *et al.*, 1988), muscarinic cholinergic and delta-opioid receptors (Leslie *et al.*, 1989), 5-HT₃ receptors (Merahi *et al.*, 1992) and substance P receptors (Manaker & Zucchi, 1993).

The dopamine D₂ receptors located postsynaptic to vagal afferent terminals in the NTS are likely to represent a combination of soma-membrane receptors and receptors on glossopharyngeal terminals. Dopamine-immunoreactive cell-bodies have been demonstrated in the rat NTS (Dahlström & Fuxe, 1964; Hökfelt *et al.*, 1984), while arterial baro- and chemoreceptor afferents also project through the glossopharyngeal nerve from the carotid sinus and petrosal ganglia (Kalia, 1981). Furthermore, 96% of carotid body afferent neurones in the petrosal ganglion are immunopositive for tyrosine hydroxylase while only 2% of such neurones stain for tyrosine hydroxylase in the nodose ganglion (Finley *et al.*,

1992). Indeed, dopamine is well-established as an important neuromodulator of chemoreceptor afferent neurones (Zapata, 1975; Fidone *et al.*, 1988; Goiny *et al.*, 1991; Czyzyk-Krzeska *et al.*, 1992; Finley *et al.*, 1992; Marchal *et al.*, 1992; Monti-Bloch *et al.*, 1993; Bairam *et al.*, 1993).

In conclusion, the present study has determined the ability of dopamine to depolarize rat vagal afferent neurones in a concentration- and temperature-dependent manner. Furthermore, the action of dopamine on the isolated nodose ganglion appears to be mediated by activation of D₂ receptors. In addition, the binding of [¹²⁵I]-NCQ 298 to slide-mounted sections of rat brainstem has provided anatomical confirmation of the presence of presynaptic D₂ receptors on central afferent terminals of the vagus nerve in the rat NTS. Whether these presynaptic D₂ receptors are involved in the central cardiovascular actions of dopamine remains to be elucidated.

This work was supported by a grant from the NH & MRC, Australia. A.J.L. is the recipient of a Wellcome Trust research travel grant. We are indebted to Astra Pharmaceuticals for the gifts of NCQ 298, NCQ 634 and raclopride; and to Sandoz for the gift of clozapine pure substance.

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(Received September 19, 1994

Revised December 6, 1994

Accepted December 9, 1994)