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European Journal of Pharmacology 337 (1997) 227–233

ejp

Identification of pressor regions activated by central cholinergic stimulation in rat brain

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Received 15 July 1997; revised 11 August 1997; accepted 15 August 1997

Abstract

The acetylcholinesterase inhibitor neostigmine (2 μg) was microinjected into the lateral cerebral ventricle (i.c.v.) of unanesthetized rats to activate central cholinergic receptors. Changes in arterial blood pressure were correlated with changes in Fos-like immunoreactivity in the hypothalamus and forebrain following cholinergic stimulation. Neostigmine increased mean arterial pressure by 39 ± 3 mmHg at peak ($P < 0.05$) from a pretreatment level of 104 ± 4 mmHg. Blood pressure remained elevated for more than 30 min. Distinct Fos-like immunoreactivity was found in the posterior hypothalamic nucleus, the paraventricular nucleus and the supraoptic nucleus of the hypothalamus, the ventral premamillary nucleus, the central nucleus of amygdala, the lateral septum and the medial preoptic area. In contrast, only a very small amount of Fos-like immunoreactivity was scattered in those regions in a control group injected i.c.v. with saline. Pretreatment with the muscarinic receptor antagonist methylatropine (i.c.v., 0.5 μg) prevented the pressor response to neostigmine and evoked a reduced Fos-like immunoreactivity compared to animals given neostigmine without methylatropine. The pressor response to neostigmine was blocked after pretreatment with phenoxybenzamine, however, this did not prevent the development of Fos-like immunoreactivity. These results indicate that the pressor response induced by central cholinergic stimulation may result from muscarinic receptor activation in specific regions of the hypothalamus and the forebrain that are implicated in regulating cardiovascular activity.

Keywords: Arterial blood pressure; c-Fos; Cholinergic system, central; Neostigmine

1. Introduction

Intracerebroventricular (i.c.v.) injection of acetylcholinesterase inhibitors or muscarinic agonists increase blood pressure (Brezenoff, 1973; Lang and Rush, 1973; Brezenoff and Rusin, 1974; Day and Roach, 1977). The sites mediating this effect, however, are not completely known. Several regions in the hypothalamus and the forebrain have been implicated in the central cholinergic pressor response by local cholinergic activation following microinjection of acetylcholinesterase or muscarinic receptor agonists into those regions (Buccafusco and Brezenoff, 1979; Willette et al., 1984; Ohta et al., 1991; Scheucher et al., 1991; Ota et al., 1994). Although useful, microinjection and cardiovascular monitoring permits only a limited number of brain regions to be studied at a time. Thus, this technique does not lend itself to studies of the overall integrative sites that operate during central cholinergic activation.

Expression of c-*fos* has been used widely to label activated neurons under a variety of stimulated conditions (Morgan et al., 1987; Erickson and Millhorn, 1991; Sharp et al., 1991; Hughes and Dragunow, 1993). Mapping Fos protein allows for the investigation of large numbers of activated cells in the central nervous system in a single animal (Sagar et al., 1988; Dragunow and Faull, 1989). In the present study, we microinjected neostigmine i.c.v. and used expression of c-*fos* to detect regions in the hypothalamus and the forebrain that became active during the centrally-mediated cholinergic pressor response.

2. Material and methods

Male Sprague–Dawley rats obtained from Charles River Breeding Farm and weighing 200–250 g were used in all

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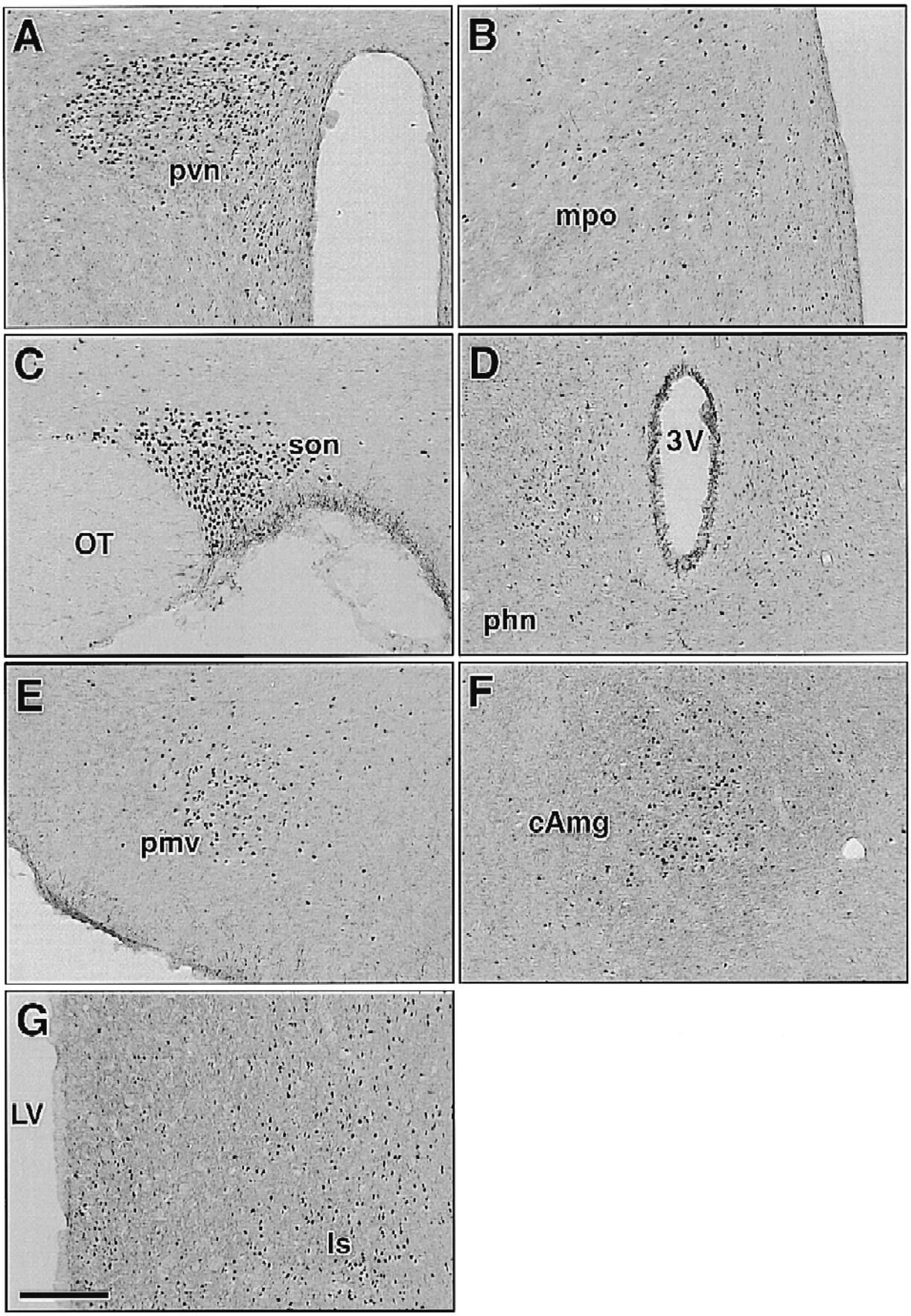
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experiments. Animals were kept on a 12 h light/dark cycle with food and water available.

2.1. Surgical procedures

Rats were anesthetized with tribromoethanol (200 mg/kg, i.p.) and mounted in a David Kopf stereotaxic instrument. For insertion of the i.c.v. injection guide, a burr hole was drilled 1.3 mm lateral to bregma and a 23 gauge stainless steel guide cannula was inserted through the hole to a depth 3.5 mm below the skull surface. The guide cannula was cemented with dental acrylic to stainless steel support screws placed in the skull. A 30 gauge stylet was inserted into the guide to maintain patency. After surgery, animals received 60 000 units of penicillin G, i.m. Animals were housed individually for at least 5 days to recover from surgery.

The day before the experiment, i.c.v.-cannulated rats were anesthetized again with tribromoethanol. A 2 cm longitudinal incision was made in the neck directly over the trachea. The underlying muscles were separated using blunt dissection and the left common carotid artery was catheterized with PE-50 tubing filled with heparinized saline. The catheter was threaded through the artery for a distance of 2.5 cm, which allowed the tip of the cannula to rest in or near the arch of the aorta. The left jugular vein was catheterized for i.v. administration of phenoxybenzamine (5 μg/kg, Sigma). The catheters were exteriorized and secured at the back of the neck. The free end of the catheters were plugged with a stylet and the rats were kept in individual cages with free access to water and food.

2.2. Recording of blood pressure

On the day of the experiment, the rats were placed in individual plastic cages ($28 \times 28 \times 34$ cm) that allowed the animals to move freely. Following 20–30 min to adapt to the new surroundings, the arterial catheter was connected to a Statham pressure transducer coupled to a Watanabe recorder. Before recording blood pressure, the arterial catheter was flushed with 0.1 ml heparinized saline via a three-way stopcock. Mean arterial pressure was calculated as diastolic pressure plus 1/3 pulse pressure.

2.3. Drug administration

Stainless steel 30 gauge injection cannulas were prepared in lengths appropriate to reach the selected cerebral site upon insertion through the guide cannulas. For i.c.v. injection, the injection cannula was connected via PE-20 tubing to a 5 μl microsyringe and lowered to a depth of 4.5 cm below the skull surface, according to the atlas (Paxinos and Watson, 1986). 0.5, 2 and 3 μg neostigmine and 0.5 μg methylatropine were prepared for i.c.v. injection (Sigma). Drugs were dissolved in 0.9% saline. The drugs were injected i.c.v. in a volume of 5 μl over a period of 30 s and the needles were allowed to remain in the ventricles for 2–3 min after injection.

In a preliminary study, 0.5 μg of neostigmine produced only a slight increase of arterial blood pressure, while 2 μg neostigmine evoked a prominent pressor response accompanied by stereotypic facial grooming and chewing movements. A 3 μg dose of neostigmine caused salivation, nasal secretion and tremors in addition to the pressor response. Therefore, the 2 μg dose of neostigmine was used in this study.

2.4. Immunocytochemical procedure

At 30, 60, 90 and 120 min ($n = 4$ for each group) and 24 h ($n = 2$) after neostigmine administration, the animals were anesthetized and perfused transcardially with 250 ml heparinized saline (100 unit/ml) followed by 450 ml fresh 4% paraformaldehyde. The brain was removed and post-fixed at 4°C in the same fixative for 2 h, then rinsed in PBS (phosphate-buffered saline, 0.1 M, pH: 7.4) for 15 min and stored overnight in 30% sucrose at 4°C.

Serial 40 μm tissue sections were cut on a freezing microtome and rinsed 30 min in PBS. The sections then were placed for 10 min in 0.5% hydrogen peroxide, 3×10 min in PBS, 15 min in PBS containing 1% normal goat serum and 0.1% Triton X-100 and then incubated with a rabbit antibody to Fos (Oncogene Science) diluted 1:200 in a PBS solution containing 1% normal goat serum and 0.1% Triton X-100 at room temperature on stirrer for 24 h.

The following day, the sections were rinsed twice in PBS for 10 min (2×10 min) and then once for 15 min in PBS containing 1% normal goat serum and 0.1% Triton X-100. The sections then were incubated with biotinylated goat anti-rabbit IgG (Immunoglobulin G, 1:200, 30 min) followed by ABC reagent (1:50, 30 min) (Vector Labs). The reaction product was made visible by incubating sections with hydrogen peroxide and diaminobenzidine (5 min) following rinse in Tris buffer (15 min). Finally, sections were rinsed in PBS, mounted onto gelatin-coated slides, and dried. The sections were cleared in ascending alcohols (50, 75, 90 and 100%) and in xylene, then coverslipped with permount medium.

2.5. Cell counts and statistical analysis

Tissue sections were examined under standard light microscope. The cell nuclei of activated cells showed the characteristic dark brown staining of oxidized diaminobenzidine as c-Fos labeling. Two to three sections that most closely matched the standard stereotaxic planes of Paxinos

Fig. 1. Photomicrographs of histologic sections stained immunohistochemically for Fos-like immunoreactivity in some regions of hypothalamus and forebrain are shown as follows: phn, posterior nucleus of hypothalamus; pvn, paraventricular nucleus of hypothalamus; son, supraoptic nucleus of hypothalamus; pmv, ventral premamillary nucleus; ls, lateral septum; mpo, medial preoptic area; cAmg, central nucleus of amygdala. Scale bar: 250 μm.

and Watson's atlas were selected for each of the brain structures of each animal. The total number of labeled cells were counted in each region for each animal. This number was then divided by the total number of sections counted to provide a mean cell count per slice for each region.

Student's *t*-test was used for statistical comparison of changes in mean arterial pressure. A one way repeated measure analysis of variance (ANOVA) was used for statistical comparison the number of cells labeled with Fos-like immunoreactivity across time and a Student–Newman–Keuls post-hoc analysis was used to determine differences between groups. All values are presented as mean ± S.E.M. For all analysis, differences of $P < 0.05$ was considered significant.

3. Results

3.1. Blood pressure responses

Injection of 5 μl saline, i.c.v., had no significant effect on blood pressure. Control mean arterial pressure in this group of rats averaged 99 ± 4 mmHg and rose to only 102 ± 3 during 30 min after saline injection ($P > 0.05$). In contrast, mean arterial pressure following 2 μg neostigmine, i.c.v., increased from a basal level of 104 ± 4 to 143 ± 3 mmHg ($P < 0.05$). The pressor response began at 2–3 min after injection, peaked at 5–10 min and lasted over 30 min.

In rats pretreated 10 min earlier i.c.v. with 0.5 μg methylatropine, injection of 2 μg neostigmine was without significant effect. Mean arterial pressure in this case in-

Table 1
Changes of mean arterial pressure (mmHg)

Groups	Preinjection	Changes
Saline	99 ± 4	3 ± 1
Neostigmine	104 ± 4	39 ± 3 [a]
Neostigmine + methylatropine	98 ± 6	5 ± 2
Neostigmine + phenoxybenzamine	87 ± 3	11 ± 2

Values are mean ± SEM.
[a] $P < 0.05$, peak difference of mean arterial pressure versus basal level before injection, which are not significantly different among the groups.

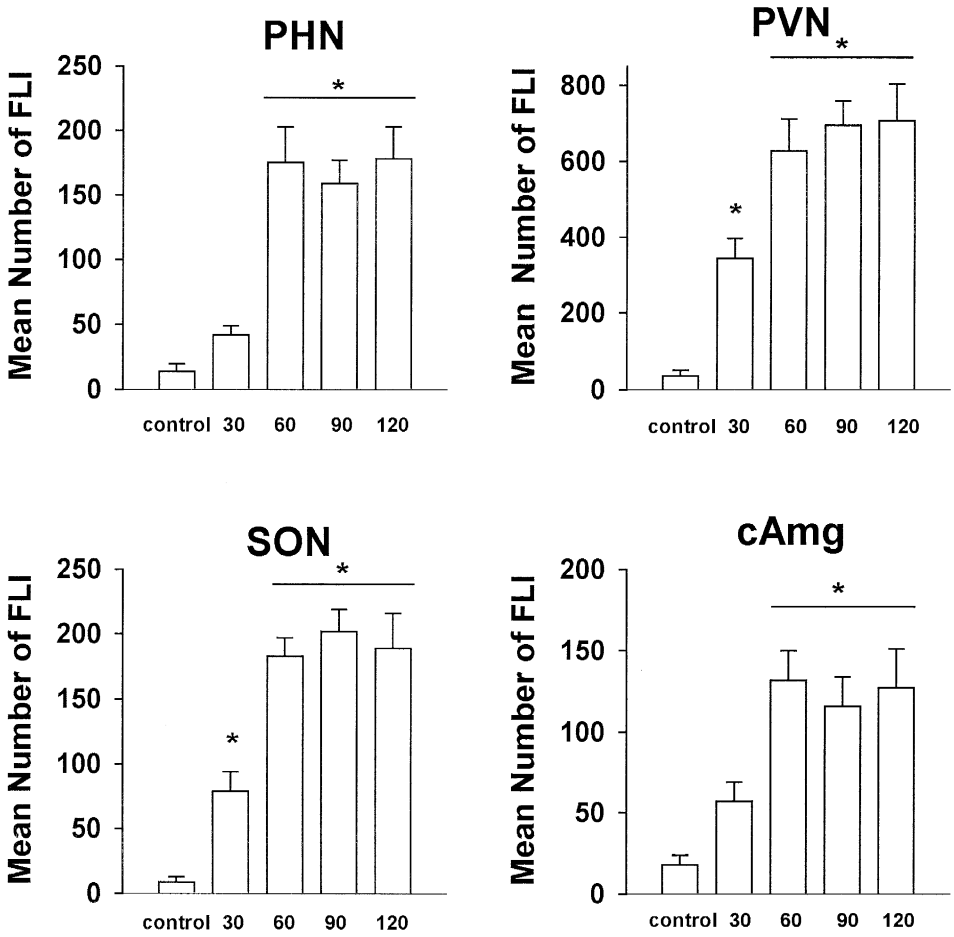

Fig. 2. Histogram showing the time course of mean number of Fos-like immunoreactivity cells in the posterior nucleus of hypothalamus (PHN), paraventricular nucleus of hypothalamus (PVN), supraoptic nucleus of hypothalamus (SON), and central nucleus of amygdala (cAmg) after central cholinergic stimulation. * $P < 0.05$ for 30, 60, 90 and 120 min after the end of i.c.v. 2 μg neostigmine injection versus control group of i.c.v. 5 μl saline. No significant differences among 60, 90 and 120 min after the end of neostigmine injection.

Table 2
Mean number of Fos-like immunoreactivity nuclei per section

	Saline	Neostigmine	Neostigmine + methylatropine
Posterior hypothalamic nucleus	14 ± 6	159 ± 18 [a,b]	51 ± 12
Paraventricular nucleus of the hypothalamus	37 ± 12	695 ± 64 [a,b]	282 ± 71 [a]
Supraoptic nucleus of the hypothalamus	9 ± 4	202 ± 17 [a,b]	63 ± 13 [a]
Central nucleus of amygdala	18 ± 10	116 ± 17 [a,b]	57 ± 12 [a]

Values are mean ± SEM.
[a] $P < 0.05$, neostigmine versus saline or neostigmine with methylatropine versus saline.
[b] $P < 0.05$, neostigmine versus neostigmine with methylatropine.

creased to only 103 ± 4 mmHg from a basal level of 98 ± 6 mmHg ($P > 0.05$). Likewise, after pretreatment with phenoxybenzamine (i.v., 5 μg/kg), the pressor response to neostigmine also was blocked, increasing to only 98 ± 3 mmHg from a level of 87 ± 3 mmHg ($P > 0.05$). The changes in mean arterial pressure are shown in Table 1.

3.2. Fos-like immunoreactivity

After i.c.v. injection of 2 μg neostigmine, distinct Fos-like immunoreactivity was found in the posterior nucleus of hypothalamus, the paraventricular nucleus and supraoptic nucleus of hypothalamus of hypothalamus, the ventral premamillary nucleus, the medial preoptic area, the lateral septum and the central amygdala.

Expression of c-*fos* was induced within 30 min after neural activation, while very little Fos-like immunoreactivity was found 24 h after injection of neostigmine. The level of Fos-like immunoreactivity was elevated significantly, but by about the same amount, at 60, 90 and 120 min. The time course is shown in Fig. 2 for representative regions. Based on these experiments, 90 min was permitted after neostigmine injection before terminating the experiment. Photomicrographs of histologic sections stained immunohistochemically for Fos-like immunoreactivity are shown in Fig. 1. Mapping of Fos-like immunoreactivity induced by i.c.v. neostigmine in the paraventricular nucleus of hypothalamus, the supraoptic nucleus of hypothalamus, the posterior nucleus of hypothalamus and the ventral premamillary nucleus are shown in Figs. 3 and 4.

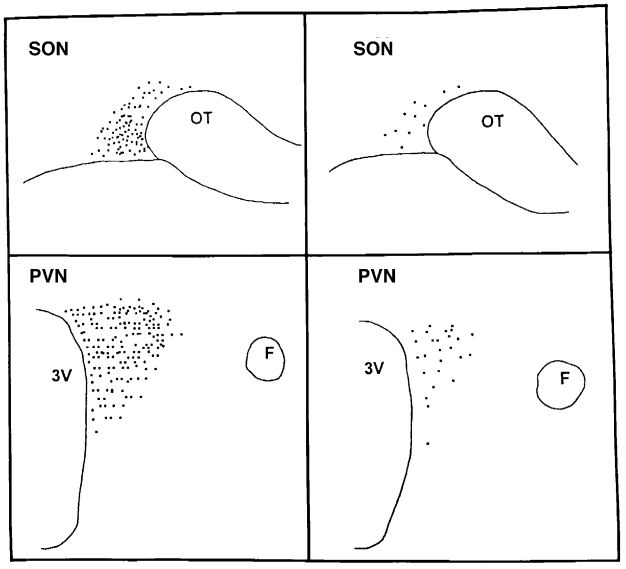

Fig. 3. Distribution of Fos-like immunoreactivity cells in representative coronal sections taken from the supraoptic nucleus of the hypothalamus (SQN) and the paraventricular nucleus of the hypothalamus (PVN) by camera-lucida mapping. Left panels are present with 2 μg neostigmine i.c.v. and right panels 2 μg neostigmine i.c.v. with 0.5 μg methylatropine i.c.v. Each dot represents two labeled cell nuclei. 3V, third ventricle; F, fornix.

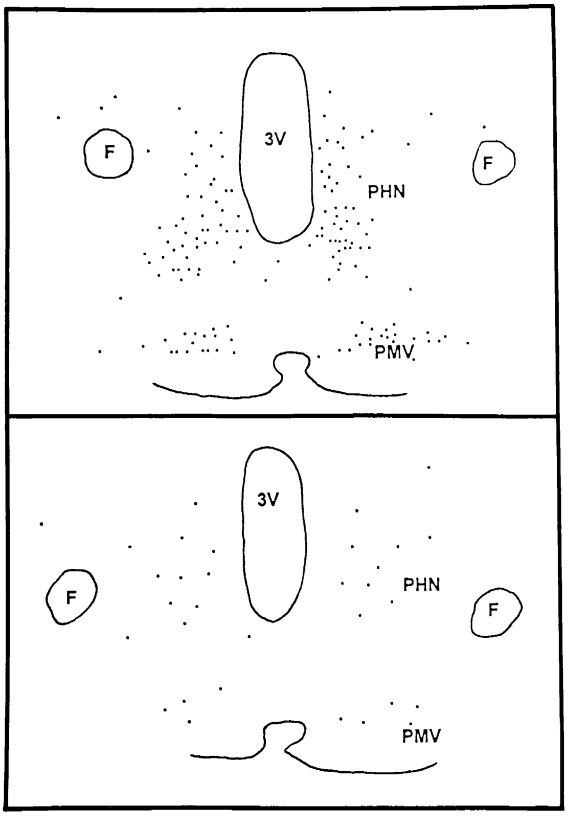

Fig. 4. Distribution of Fos-like immunoreactivity cells in representative coronal sections taken from the posterior nucleus of the hypothalamus (PHN) and the ventral premamillary nucleus (PMV) by camera-lucida mapping in animals following treatments, neostigmine i.c.v. injection (upper panel) and neostigmine i.c.v. injection pretreated with methylatropine injection i.c.v.(lower panel). Each dot represents two labeled cell nuclei.

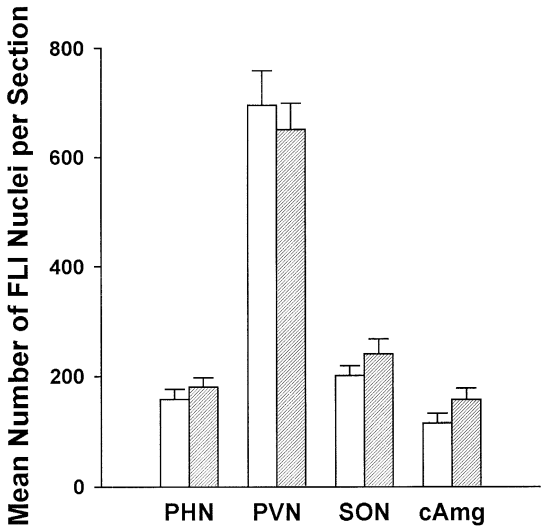

Fig. 5. Histogram showing the mean number of Fos-like immunoreactivity cells in the posterior nucleus of hypothalamus (PHN), paraventricular nucleus of hypothalamus (PVN), supraoptic nucleus of hypothalamus (SON), and central nucleus of amygdala (cAmg). Open bar, 2 μg neostigmine i.c.v. injection; shaded bar, 2 μg neostigmine i.c.v. injection with pretreatment of 5 μg/kg phenoxybenzamine, i.v. No significant differences between two groups.

In a control group, i.c.v. injection of 5 μl saline evoked only a very small amount of Fos-like immunoreactivity in the regions examined. The number of Fos-like immunoreactivity neurons was significantly less in the control group compared with the i.c.v. neostigmine injection group (Fig. 2).

In rats pretreated i.c.v. with 0.5 μg methylatropine, injection of the 2 μg dose of neostigmine ($n = 3$) induced significantly less Fos-like immunoreactivity, compared with injection of neostigmine alone (Table 2). Figs. 3 and 4 show mapping of Fos-like immunoreactivity induced by neostigmine and neostigmine with pretreatment of methylatropine in the paraventricular nucleus of hypothalamus, the supraoptic nucleus of hypothalamus, the posterior nucleus of hypothalamus and the ventral premamillary nucleus. In contrast, i.v. pretreatment with phenoxybenzamine (5 μg/kg; $n = 3$) did not prevent the development of Fos-like immunoreactivity (Fig. 5).

4. Discussion

In the present study, central cholinergic activation by i.c.v. injection of neostigmine produced distinct expression of c-*fos* in several brain nuclei, including the posterior nucleus of hypothalamus, paraventricular nucleus of hypothalamus, supraoptic nucleus of hypothalamus, ventral premamillary nucleus, central nucleus of amygdala, lateral septum, and medial preoptic area. These regions have been implicated in regulating cardiovascular activity (Buccafusco and Brezenoff, 1979; Ciriello and Calaresu, 1980; Willette et al., 1984; Ohta et al., 1991; Scheucher et al., 1991; Ota et al., 1994) and cholinergic stimulation in some of these sites causes a pronounced pressor response. For example, increases in blood pressure have been reported following injection of muscarinic receptor agonists or acetylcholinesterase inhibitors into the posterior nucleus of hypothalamus (Buccafusco and Brezenoff, 1979), central nucleus of amygdala (Ohta et al., 1991), supraoptic nucleus of hypothalamus and lateral septum (Scheucher et al., 1991; Ota et al., 1994). Likewise, injection of the cholinomimetic carbachol into the paraventricular nucleus of hypothalamus increased blood pressure and caused vasoconstriction of the renal, superior mesenteric and hindquarter vascular beds (Bachelard et al., 1994).

Expression of c-*fos* following i.c.v. carbachol (Rowland et al., 1994) or systemic injection of the muscarinic receptor agonist pilocarpine (Hughes and Dragunow, 1993) or the acetylcholinesterase inhibitor soman (Denoyer et al., 1992; Chollat-Namy et al., 1993) previously has been shown to induce Fos-like immunoreactivity in some of the regions in which we observed Fos-like immunoreactivity. Indeed, potent acetylcholinesterase inhibitor soman evoked a much more wide-spread expression of c-*fos* than observed in the present study, including the piriform, entorhinal, frontoparietal, retrosplenial and temporal cortices (Denoyer et al., 1992; Chollat-Namy et al., 1993). The more localized effect in our study probably is dose related, since the dose of soman was sufficient to cause seizure.

Since Fos-like immunoreactivity in the present study was localized primarily to brain regions involved in cardiovascular regulation, our results suggest that those sites may be involved in generation of the pressor response to central cholinergic stimulation by neostigmine. It is also possible, however, that neuronal activation in those brain regions could be a consequence of the increased blood pressure caused by other pathways. For example, activation of baroreflex pathway by increases in blood pressure has been reported to induce expression of c-*fos* in some brain regions (Erickson and Millhorn, 1991). This does not appear to be the case since, in the present study, i.v. injection of the α-adrenergic receptor blocking agent phenoxybenzamine prevented the pressor response to i.c.v. neostigmine but did not prevent the expression of c-*fos* in those brain regions. This indicates that the increase in Fos-like immunoreactivity is not the result of changes in blood pressure or cardiovascular reflexes. Instead, the increase in Fos-like immunoreactivity appears to be directly related to the actions of neostigmine on central cholinergic receptors. Furthermore, since i.c.v. injection of methylatropine prevented both the pressor response and the increase in Fos-like immunoreactivity following injection of neostigmine, it is likely that both responses are mediated by central muscarinic receptors on neurons involved in cardiovascular function.

Our results do not offer any conclusion concerning the relative contributions of the activated regions to the pres-

sor response, nor do they suggest any particular pathway. Previous experiments, however, have shown that the cholinergic activation in those regions are important to the generation of the pressor response. By combining i.c.v. and intranuclear injections with Fos-mapping it may be possible to extend our knowledge of these pathways.

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