ELSEVIER

European Journal of Pharmacology 282 (1995) 199–206

ejp

Functional analyses of α_1-adrenoceptor subtypes in rat hypothalamic ventromedial nucleus neurons

Lee-Ming Kow *, D.W. Pfaff

The Rockefeller University, 1230 York Avenue, Box 275, New York, NY 10021-6399, USA

Received 16 March 1995; revised 19 May 1995; accepted 29 May 1995

Abstract

Activation of α_1-adrenoceptors in rat hypothalamic ventromedial nucleus can excite neurons and facilitate female sexual behavior. To identify the α_1-adrenoceptor subtype(s) involved, the α_{1B}-adrenoceptor-specific antagonist chloroethylclonidine (100 μM) and/or the α_{1A}-adrenoceptor-selective antagonist 5-methyl urapidil (1 or 2.5 μM) or WB-4101 (0.1–10 μM) were applied to a recording chamber bathing the hypothalamic slice containing the ventromedial nucleus. In all the neurons tested, both types of antagonists blocked, often completely, excitatory responses to nonselective α_1-adrenoceptor agonists. Since the doses used were unlikely to make these antagonists nonselective, the results suggest that activation of both α_{1A}- and α_{1B}-adrenoceptor subtypes was necessary for α_1-adrenoceptor agonists to evoke an excitation, or that with the present application method – injection into the continuously perfused chamber – chloroethylclonidine did not act specifically. In preincubation (at 37°C for 90 min) where it was reported to act by specific alkylation, chloroethylclonidine (100 μM) but not the vehicle abolished the excitation evoked by an α_1-adrenoceptor agonist, but not that by carbachol or other excitants. Also, either in bath application or incubation, chloroethylclonidine worked equally efficiently on slices from ovariectomized rats, that reportedly contain few α_{1B}-adrenoceptors, and from those treated with estrogen which induces α_{1B}-adrenoceptors selectively, suggesting that α_{1B}-adrenoceptor was necessary even when in low abundance. Thus, it is likely that the activation of both α_{1A}- and α_{1B}-adrenoceptor subtypes and also thereby, their respective couplings to second messengers are necessary to mediate the actions of α_1-adrenoceptor agonists in exciting hypothalamic neurons.

Keywords: Brain slice; Chloroethylclonidine; Estrogen; 5-Methyl urapidil; WB-4101

1. Introduction

In an earlier study, we found that norepinephrine excited neurons in the hypothalamic ventromedial nucleus in brain slices through α_1-adrenoceptors and inhibited them through α_2-adrenoceptors (Kow and Pfaff, 1987). More recently we found that microinfusions of α_1-adrenoceptor, but not α_2-adrenoceptor, agonists into the ventromedial nucleus facilitated lordosis behavior in estrogen-primed female rats (Kow et al., 1992). These findings indicate that activation of α_1-adrenoceptors in the ventromedial nucleus can evoke neuronal excitation and facilitate lordosis. Since α_1-adrenoceptor consists of at least two subtypes, α_{1A} and α_{1B}, which have different genetic and pharmacological characteristics and couple differently to intracellular messengers (Minneman and Esbenshade, 1994), we further investigated the involvement of α_1-adrenoceptor subtypes (and, by implication, the corresponding intracellular signal pathways) in neuronal excitation and lordosis facilitation with chloroethylclonidine, which has been reported to be an α_{1B}-adrenoceptor-specific antagonist (Han et al., 1987; Minneman et al., 1988). When administered prior to the applications of α_1-adrenoceptor agonists, chloroethylclonidine blocked both the excitatory electrophysiologic action and the facilitatory behavioral effect of the agonists (Kow et al., 1992), suggesting that the mediation of these neuronal and behavioral functions involves α_{1B}-adrenoceptors and the intracellular messenger(s) coupled to them. However, in the electrophysiological experiments of that study we also found that the excitation evoked by

* Corresponding author. Tel. (212) 327-8669, fax (212) 327-8664, e-mail kowl@rockvac.rockefeller.edu.

SSDI 0014-2999(95)00326-6

α_1-adrenoceptor agonists from ventromedial nucleus neurons was virtually completely abolished by brief bath applications of chloroethylclonidine (Kow et al., 1992). This observation raised the possibilities that: (1) the α_1-adrenoceptor agonist-evoked excitation in the ventromedial nucleus is fully mediated by α_{1B}-adrenoceptors and, conversely, α_{1A}-adrenoceptors are either absent or not sufficient to mediate the excitation, (2) contributions by receptors from both α_1 subtypes are required to mediate α_1-adrenoceptor agonist-evoked excitation, or (3) under our experimental conditions, chloroethylclonidine behaved as a non-specific α_1-adrenoceptor antagonist blocking not only α_{1B}- but also α_{1A}-adrenoceptors. These possibilities were examined in a series of experiments, reported here.

Portions of the results have been reported in a preliminary form (Kow and Pfaff, 1992).

2. Materials and methods

2.1. Animals

Adult, female Sprague-Dawley rats were housed in an air-conditioned room under a reversed light/dark cycle with light off from 10:00 through 22:00 h, and were fed with ad libitum food and water supplies. They were ovariectomized or ovariectomized and treated with estrogen at least one week before they were killed for preparing hypothalamic slices. The estrogen treatment consists of a subcutaneous implantation of a silastic tubing containing 100% estradiol. The effect of the estrogen treatment was verified by examining the width of the uterus.

2.2. Pharmacological agents

The agents include norepinephrine; α_1-adrenoceptor agonists methoxamine and phenylephrine; α_{1A}-adrenoceptor-selective antagonists WB-4101 and 5-methyl urapidil; α_{1B}-adrenoceptor-specific antagonist chloroethylclonidine; cholinergic agonist carbachol and muscarinic agonist McN-A-343; and occasionally, serotonin and oxytocin. The agonists were obtained from Sigma Chemical Co., and the antagonists from Research Biochemicals International. All agents were dissolved in saline initially and then diluted with artificial cerebrospinal fluid (ACSF, see below) to the desired concentration just before use.

2.3. Preparation of hypothalamic slices

Individual rats were decapitated while deeply anesthetized with Metofane, and the brain was quickly removed and immersed in ice-cold sucrose-ACSF (S-ACSF), a modified ACSF. The hypothalamus was then blocked out, mounted rostral side down, and sliced coronally in the ventrodorsal direction with a Vibratome. Thin (300–400 μm) slices containing the ventromedial nucleus were collected relying on anatomical landmarks, especially the morphology of the third ventricle. In most experiments the slices were split at midline. All the slicing and splitting were also carried out in ice-cold S-ACSF. Afterward, the slices were incubated in S-ACSF at room temperature for 1 h and then rinsed and transferred to ACSF. They were incubated in ACSF at room temperature for at least 30 min to get equilibrated before being used for recording. The ACSF consisted of the following in mM: NaCl, 124; $NaHCO_3$, 26; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.4; and dextrose, 10. The S-ACSF was the ACSF with all of its NaCl replaced by equi-osmolar sucrose to prevent neurons from over-excitation by mechanical stimulation during slicing. All solutions were saturated with 95% O_2 and 5% CO_2 during use.

2.4. Single-unit recording and testing

One slice at a time was placed securely on a net in a chamber, which was perfused continuously with ACSF at 2 ml/min. The ACSF was pre-warmed to maintain the temperature in the chamber at 33–34°C. To facilitate the access of pharmacological agents, the slice was completely submerged in ACSF. Electric activity of single neurons was recorded extracellularly with glass micropipettes filled with ACSF (5–15 MΩ). All recordings were made from the ventrolateral half of the ventromedial nucleus, using the anatomical landmarks in the slice as the guide. The action potential waveform was monitored on an oscilloscope. The firing pattern and firing rate histogram were displayed on a chart recorder and also recorded on video cassettes for later analyses. Only the neurons that showed a stable firing rate for at least 5 min were tested with α_1-adrenoceptor agonists and/or antagonists. All test agents, except for the chloroethylclonidine used in incubation, were applied by a bolus injection into the perfusing tubing near the entrance to the chamber. In experiments where an agonist was applied repeatedly it was administered at intervals known (from previous studies) to be long enough to avoid desensitization. A response to an agonist was defined as a change of the firing rate following the agonist application of greater than two standard deviations of the resting rate, or when a silent unit became active or vice versa. The concentration of the agents was presented as the peak concentration in the bath. This transient peak was calculated using a dilution factor determined from calibration experiments. In experiments involving bath application of antagonists, each slice was used for studying only one unit to avoid possible cumulation of antagonist effects. Also, to assess the specificity of an antagonist in a

blockade, a neuron was often also tested with carbachol and/or other excitants following the abolition of the α_1-adrenoceptor agonist-evoked excitation by the antagonist. A reduction of a response amplitude by $\geq 50\%$ following an antagonist administration was considered as an blockade.

2.5. Statistical analyses

The Fisher exact probability test was used. All P values presented are two-tailed with $P < 0.05$ considered as significant.

3. Experiments

3.1. Experiment 1: effects of chloroethylclonidine and α_{1A}-selective antagonists, 5-methyl urapidil and WB-4101

With a preponderance of evidence about α_{1B}-adrenoceptors in ventromedial nucleus, this experiment was conducted to test whether α_1-adrenoceptor agonist-evoked excitation could also be mediated by α_{1A}-adrenoceptors, as well as to compare the effectiveness of chloroethylclonidine and α_{1A}-adrenoceptor-selective antagonists. The testing procedure was essentially the same as in a previous study (Kow et al., 1992). Briefly, a nonselective agonist was first applied to evoke an excitation (or an inhibition in some occasions for control). After an interval ($\geq$ 15 min) long enough to avoid sensitization or desensitization, the excitable neurons were then, in attempts to block the response, treated with an antagonist followed 2–3 min later by a second agonist application.

In experiments with α_{1A}-adrenoceptor-selective antagonists, all slices were prepared from ovariectomized rats treated with estrogen and the agonists employed were mainly phenylephrine (10 μM) and, occasionally, norepinephrine (5 μM) or methoxamine (10–20 μM). Fourteen units excited by α_1-adrenoceptor agonists were tested with 5-methyl urapidil (1 or 2.5 μM) and, as with chloroethylclonidine, the excitations from all units were either abolished or attenuated (Fig. 1 and Table 1). The 5-methyl urapidil effect, which often was long-lasting (Fig. 1A), was dose-related (Fig. 1B) and specific for an α_1-adrenoceptor agonist over a muscarinic agonist (Fig. 1C). Similarly, in 11 units tested with WB-4101 (0.1–10 μM), the α_1-adrenoceptor agonist-evoked excitation was abolished in all of the 11 units by this antagonist (Fig. 1 and Table 1). The effect of WB-4101 was also often long-lasting (Fig. 2A). It was also specific; it did not block the excitation evoked by carbachol (Fig. 2B) or the inhibitory response evoked by norepinephrine (Fig. 2C) known to be mediated by α_2-adrenoceptors (Kow and Pfaff, 1987). Therefore, even in preparations where α_{1B}-adrenoceptors were

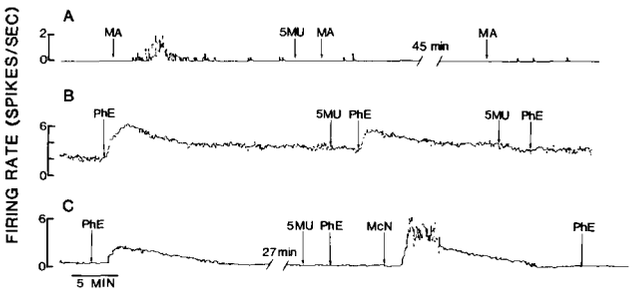

Fig. 1. Effects of the α_{1A}-adrenoceptor-selective antagonist 5-methyl urapidil on α_1-adrenoceptor agonist-evoked excitation. In this and the following figures, each trace (unless otherwise indicated) is the firing rate histogram recorded from an individual neuron. The arrows indicate the time points at which test agents shown by their abbreviations above the arrows were applied. The dose for all the MA (methoxamine) and the PhE (phenylephrine) applications shown in this and all the following figures was 10 μM. A: The excitation evoked by methoxamine from a ventromedial nucleus neuron was abolished by 5MU (5-methyl urapidil, 2.5 μM) for more than an hour. B: The excitation by phenylephrine was attenuated by 5-methyl urapidil at a lower dose (1.0 μM) and then abolished by a larger dose (2.5 μM). C: 5-Methyl urapidil abolished the excitation by phenylephrine but not that by a weak muscarinic agonist, McN-A-343 (McN, 50 μM).

induced by estrogen treatment (see below), α_{1A}-adrenoceptors still seemed to be required for the α_1-adrenoceptor agonist-evoked excitation.

In chloroethylclonidine experiments, only phenylephrine (10 μM), which can stimulate both α_{1A}- and α_{1B}-adrenoceptors (Piascik et al., 1990a,b; Takayanagi et al., 1991,1992), was used to evoke neuronal excitation. Some neurons were treated with chloroethylclonidine twice. As in the previous study (Kow et al., 1992), all the excitations evoked by phenylephrine were blocked by chloroethylclonidine, and there was no difference between preparations from ovariectomized rats and ovariectomized rats treated with estrogen (Table 1). Overall, these results show that both α_{1A}-adrenoceptor antagonists and chloroethylclonidine can block the excitation in all ventromedial nucleus neurons excitable by α_1-adrenoceptor agonists, and, thus, suggest

Table 1
Effects of 5-methyl urapidil, WB-4101 (0.1–10 μM), or chloroethylclonidine (100 μM) on the excitatory action of α_1-adrenoceptor agonists

Effects	5-Methyl urapidil		WB-4101	Chloroethylclonidine	
	1 μM	2.5 μM		OVX + E [a]	OVX [b]
Abolition of response	2	4	11	6	4
Attenuation	5	3	0	2	0
No effect	0	0	0	0	0

Numbers are the number of trials with the stated result (total $n = 37$). [a] On preparations from ovariectomized rats treated with estrogen. [b] On preparations from ovariectomized rats. Results are not statistically different from OVX + E.

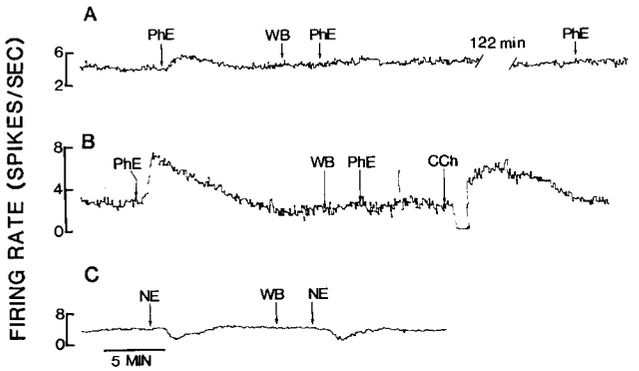

Fig. 2. Effects of the α_{1A}-adrenoceptor-selective antagonist WB-4101 on α_1-adrenoceptor agonist-evoked excitation. A: WB (WB-4101, 2.5 μM) abolished the excitation evoked by PhE (phenylephrine, all applications 10 μM, here and in B) for more than 2 h. B: WB-4101 (0.1 μM) abolished phenylephrine-evoked excitation but neither the inhibitory nor the excitatory component of the biphasic response evoked by CCh (carbachol, 10 μM). C: The inhibitory response by NE (norepinephrine, 5 μM) was not affected by the largest dose (10 μM) of WB-4101 ever used.

that each responsive ventromedial nucleus neuron contains both α_{1A}- and α_{1B}-adrenoceptors or that chloroethylclonidine under the conditions of these experiments may occlude α_{1A}-adrenoceptors. This suggestion prompted us to conduct the experiment 2 below.

In an attempt to test the specificity of chloroethylclonidine action, slices from both ovariectomized rats and ovariectomized rats treated with estrogen were used in chloroethylclonidine experiments. It has been reported that the α_1-adrenoceptor population in the medial hypothalamus, which includes the ventromedial nucleus, of ovariectomized rats consists almost entirely of the α_{1A} subtype unless the rats were primed with estrogen, which increases α_{1B}-adrenoceptors selectively (Petitti et al., 1992). Accordingly, an α_{1B}-adrenoceptor-selective antagonist should have little or no effect on slice preparations from ovariectomized rats but should be effective on those from ovariectomized rats treated with estrogen. Based on this inference and the assumption that chloroethylclonidine was α_{1B}-adrenoceptor-specific, we expected that chloroethylclonidine would be much more effective on preparations from ovariectomized rats treated with estrogen than on those from ovariectomized rats. Contrary to this, the results show chloroethylclonidine worked about equally well on both kinds of preparations, suggesting either one or both of the above assumptions were not correct. This suggestion was further evaluated in experiments 2 and 3 more extensively under more stringent conditions.

3.2. Experiment 2: comparison of the effects of chloroethylclonidine and α_{1A}-adrenoceptor-selective antagonists exerted on individual neurons

In this experiment, the excitation evoked by phenylephrine (10 μM) from individual neurons was tested with both α_{1B}-and α_{1A}-adrenoceptor antagonists to see if both subtypes of receptors coexisted in the same neuron. Since neurons recover faster from chloroethylclonidine than from α_{1A}-adrenoceptor antagonists, the units were usually tested with chloroethylclonidine first. The second antagonist was applied only after a clear, though not always complete, recovery from the first had been observed.

A total of 27 phenylephrine-excitable units from estrogen-primed preparations and those not primed with estrogen were tested either with chloroethylclonidine and 5-methyl urapidil or with chloroethylclonidine and WB-4101. As summarized in Table 2, all the units tested were affected by both types of antagonists. In the majority (17/27) of cases, α_1-adrenoceptor agonist-evoked excitations were abolished by *both* antagonists (Fig. 3). The remaining responses were abolished by one and attenuated by the other antagonist (Table 2). There was no difference between preparations primed or not primed with estrogen. The effects of the antagonists were, again, specific for phenylephrine-evoked excitation, because application of other excitatory agents, such as carbachol, after the phenylephrine excitation was abolished, excited all of the 15 units examined (Fig. 3A′, B′ and C′). Thus, α_{1A}-adrenoceptor antagonists are as effective as chloroethylclonidine not only between two populations of ventromedial nucleus neurons sampled in experiment 1, but also on individual neurons.

Table 2
Comparisons of the effects of two types of α_1-adrenoceptor antagonists, the α_{1A}-adrenoceptor-selective WB-4101 (0.1–2.5 μM) or 5-methyl urapidil (1 or 2.5 μM) and the α_{1B}-adrenoceptor-selective chloroethylclonidine (50 or 100 μM), on the excitatory response evoked by α_1-adrenoceptor agonist phenylephrine (10 μM) from individual neurons

Preparations	Abolished by both types	Abolished by one and attenuated by the other type	Not affected by either type	P (Fisher exact probability)
OVX + E [a]	8	5	0	> 0.05
OVX [b]	9	5	0	

Total n = 27 units. [a] Ovariectomized and treated with estrogen. [b] Ovariectomized.

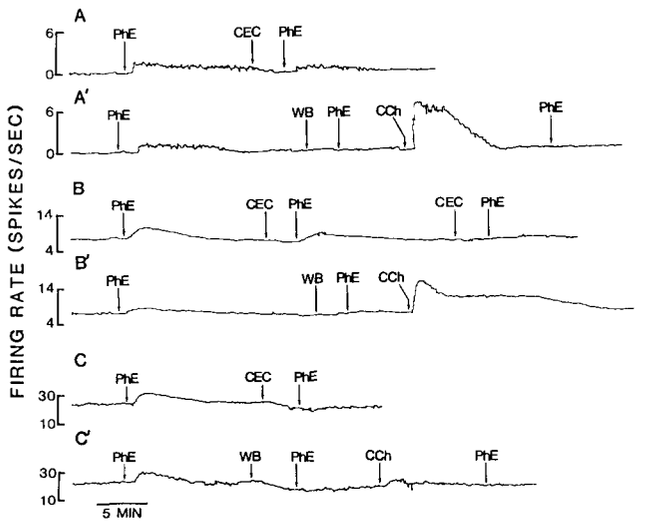

Fig. 3. Similarity in effects by chloroethylclonidine (CEC, 100 μM) and WB-4101 (WB, 2.5 μM) on neuronal excitations evoked by phenylephrine (PhE, 10 μM). Traces A and A' were taken from the continuous recording from a neuron with a gap of 52 min. The long gap was necessary for allowing the neuron to recover from chloroethylclonidine. Similarly, B and B' as well as C and C' were recordings from unit B with a 28-min gap and unit C a 51-min gap, respectively. Note, CCh (carbachol, 2 μM) was able to excite every neuron after the phenylephrine-evoked excitation was abolished (A', B' and C'). Note also the scales of the firing rate are compressed in this figure, making the responses to phenylephrine appear to be smaller than those in previous figures.

One possibility was that α_{1A}- and α_{1B}-adrenoceptor antagonists were equally effective because the antagonists were not selective due to overdose. The doses of chloroethylclonidine (100 μM), 5-methyl urapidil (1 or 2.5 μM) and WB-4101 (0.1–10 μM) employed in the present study were comparable to those used in other studies (e.g., Minneman and Atkinson, 1991; Piascik et al., 1991; Oriowo et al., 1992), where the antagonists worked in an appropriately selective manner. Furthermore, with the bolus injections in our experiments, the neurons were exposed to antagonists only briefly (2–3 min). Since such an application was much briefer than the incubation for 10 min or longer used by the other studies, overdose was unlikely to occur in our experiments. Therefore, the obliteration of selectivity due to an overdose is unlikely to be the explanation for the similarity in the effects between α_{1A}- and α_{1B}-adrenoceptor antagonists observed in experiments 1 and 2.

Nevertheless, in the previous (Kow et al., 1992) and the present studies, the blocking effect of bath-applied chloroethylclonidine faded or disappeared in 10–20 min, and hence, was not irreversible as it is supposed to be when chloroethylclonidine inactivates α_{1B}-adrenoceptors specifically by alkylation (Vargas et al., 1993). This observation raised the possibility that chloroethylclonidine in bath application may block α_1-adrenoceptors with a mechanism other than alkylation and, therefore, may not be α_{1B}-adrenoceptor-specific. Chloroethylclonidine has been reported to bind to both α_{1A}- and α_{1B}-adrenoceptors. However, it should be noted that this binding does not inactivate α_{1A}-adrenoceptors (Han et al., 1987; Johnson and Minneman, 1987; Michel et al., 1993; Minneman et al., 1988). To evaluate this alternative possibility raised by our observation, experiment 3 was conducted using a conventional method of chloroethylclonidine treatment – incubation.

3.3. Experiment 3: effect of chloroethylclonidine preincubation on the excitatory action of the α_1-adrenoceptor agonist phenylephrine

In this experiment, each hypothalamic slice was split. One half of a slice was preincubated in ACSF containing 100 μM chloroethylclonidine at 36–37°C for 30, 60 or 90 min in attempts to selectively block α_{1B}-adrenoceptors. The other, control, half was incubated in ACSF in parallel for 60 or 90 min. After these preincubations the slices were transferred back to ACSF and were further washed in the chamber with continuously perfusing ACSF for at least 20 min before a recording. No further antagonist was used beyond

Table 3
Effects of incubation of slices in chloroethylclonidine (CEC, 100 μM) solution or in artificial cerebrospinal fluid (ACSF) at 36–37°C for various durations on the excitatory action of the α_1-adrenoceptor agonist phenylephrine (10 μM)

Treatments	Responses to phenylephrine						*P*-values (for Pooled)	
	OVX + E [a]		OVX [b]		Pooled			
	↑	−/↓	↑	−/↓	↑	−/↓	vs. D	vs. E
A: CEC 30 min	2	1	1	1	3	2	n.s.	
B: CEC 60 min	5	12	6	2	11	14	< 0.05	
C: CEC 90 min	0	15	1	14	1	29	< 0.0001	< 0.0005
D: *ACSF 60 / 90 min*	*13*	*3*	*8*	*3*	*21*	*6*		
E: CEC 90 min plus recovery	3	1	4	3	7	4	n.s.	

The numbers in the table represent the numbers of units responding in the manner indicated (↑, excitation; −/↓, no response or inhibition). [a] Preparations from ovariectomized rats treated with estrogen. [b] Preparations from ovariectomized rats. Results are not statistically different from OVX + E for treatments B through E.

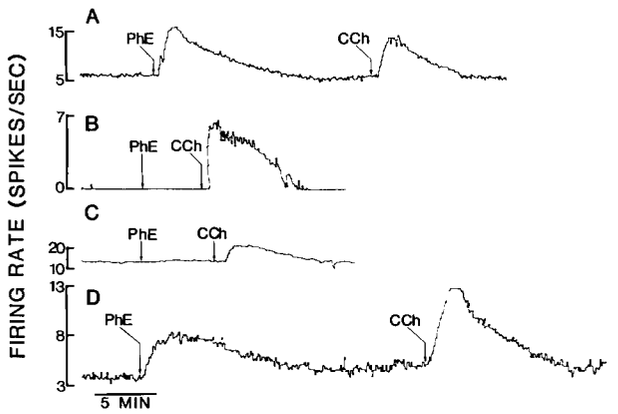

Fig. 4. Abolition of the excitatory action of the α_1-adrenoceptor agonist phenylephrine by a 90-min preincubation in chloroethylclonidine (100 μM). A: Recording of a control neuron from a slice preincubated in ACSF. This and many other control units were excited by both PhE (phenylephrine, 10 μM) and CCh (carbachol, 10 μM). B and C: Recordings from two neurons 1.25 and 6.5 h, respectively, after a 90-min chloroethylclonidine preincubation. These units no longer responded to phenylephrine but were still excited by carbachol. D: Responses of a neuron recorded 22 h after a 90-min chloroethylclonidine preincubation.

the preincubation. The α_1-adrenoceptor agonist phenylephrine, which can stimulate both subtypes, was then applied to see if excitation could still be evoked. Since our previous studies (Kow et al., 1992,1995) showed that a large majority of ventromedial nucleus neurons excited by α_1-adrenoceptor agonist were also excited by acetylcholine or carbachol, whenever possible carbachol was also administered following a phenylephrine application. Each slice was used for recording 1–3 neurons. In assessing the irreversibility of the blocking effect of chloroethylclonidine, neurons were tested from 27 min to 8 h after the preincubation. To see if the chloroethylclonidine blockade was permanent, some slices preincubated in chloroethylclonidine for 90 min and subjected to tests were saved overnight and tested again 21–25 h after the preincubation.

As presented in Table 3, the majority of the neurons recorded from the control slices were excited by phenylephrine, often also by carbachol as well (Fig. 4A), regardless of the estrogen condition. Preincubation in the ACSF containing 100 μM chloroethylclonidine for 30 min did not seem to affect neuronal responsiveness to phenylephrine. After a 60-min preincubation in chloroethylclonidine, proportionally less neurons, as compared to the control, were phenylephrine-excitable in ovariectomized rats treated with estrogen though not in ovariectomized rat preparations (the difference between the two preparations was not significant, Table 3). However, when the incubation period was prolonged to 90 min, practically none of the ventromedial nucleus neurons was excited by phenylephrine, regardless of hormonal condition. The proportion of phenylephrine-excitable neurons after the 90-min chloroethylclonidine incubation is significantly different from that of the control in both ovariectomized rats treated with estrogen and ovariectomized rat groups (Table 3). Also, by applying both phenylephrine and carbachol on the same neurons we found that after the control preincubation 10 out of the 14 units that were excited by carbachol were also excited by phenylephrine. In contrast, of the 16 neurons excited by carbachol after the 90-min chloroethylclonidine preincubation only one was responsive to phenylephrine (Fig. 4B and C). The contrast is again statistically significant ($P < 0.0005$). Also, neurons unresponsive to phenylephrine after the chloroethylclonidine preincubation could still be excited by 'tickling' the neuron with micropipette ($n = 5$) or by application of oxytocin or serotonin ($n = 1$ each). The absence of response to phenylephrine after the chloroethylclonidine preincubation was due to a specific suppression of phenylephrine action.

One surprising finding from this experiment is that it took 60–90 min of chloroethylclonidine incubation to block phenylephrine action, whereas most reports showed that an incubation for 10–60 min was sufficient to inactivate α_{1B}-adrenoceptors (Hidenori and Honda, 1993; Piascik et al., 1991; Takayanagi et al., 1991). This discrepancy probably was due to the difference in sample preparations: membrane preparation or cell culture in most other reports, and brain slices in the present study. It may require more time for chloroethylclonidine to alkylate α_{1B}-adrenoceptors on the neurons in hypothalamic slices. It may also be due to the difference in the end-point observed: binding and biochemical effects in most others and electrical activity of neurons in the present study. More extensive receptor inactivation may be required to block the electrical response. Otherwise, the present findings are consistent with the notion that the suppression by the 90-min chloroethylclonidine incubation was due to alkylation. First of all, as observed by others and different from bath application, this suppressive effect of chloroethylclonidine was not reversed for at least several hours, since it was observed at various intervals from 33 min up to more than 6 h after the preincubation (Fig. 4B and C). However, the effect was not permanent, because after an overnight (20.5–26.5 h) recovery the responsiveness to phenylephrine was restored (Fig. 4D, Table 3). Whether this was due to the synthesis of new receptors or the reversal of the alkylation or both is not known, but it is obvious thereby that the suppression was not a result of a non-specific, permanent damage to neuronal membrane. This was further indicated by the fact that chloroethylclonidine preincubations abolished phenylephrine excitation but not the excitation evoked by carbachol, serotonin, oxytocin or electrode movement, nor the inhibition evoked by phenylephrine

or carbachol. Also, following a chloroethylclonidine preincubation the slices were washed for at least 30 min to several hours before the recording. Since this washing duration is longer than that for a neuron to recover from chloroethylclonidine blockade in a bath application, it is unlikely that any chloroethylclonidine from the preincubation was still present in the bath during the recording. Therefore, the blockade of phenylephrine action by chloroethylclonidine preincubation was not due to the competition for receptor binding between chloroethylclonidine and phenylephrine.

Although it is theoretically possible that chloroethylclonidine may inactivate α_1-adrenoceptors by a mechanism other than alkylation, we are not aware of any. This absence of a reasonable alternative and the discussion above strongly suggest that the chloroethylclonidine in our preincubation experiments acted by alkylation. It has additionally been reported that α_{1A}-adrenoceptors lack the substrate amino acid for chloroethylclonidine alkylation (Terman et al., 1990). Thus, altogether it is most likely that, in our hand, chloroethylclonidine acted as an α_{1B}-adrenoceptor-specific antagonist. This taken together with the finding that a 90-min chloroethylclonidine preincubation completely suppressed the excitation evoked by phenylephrine indicate, in turn, that α_{1B}-adrenoceptors are required for mediating phenylephrine excitation and that α_{1A}-adrenoceptors alone are not sufficient. Conversely, the results from experiments 1 and 2, that show α_{1A}-adrenoceptor antagonists could abolish excitation evoked by α_1-adrenoceptor agonists, including phenylephrine, would imply that α_{1A}-adrenoceptors are necessary and α_{1B}-adrenoceptors alone are insufficient. Thus, both α_{1A}- and α_{1B}-adrenoceptors appear to be necessary and neither alone is sufficient to mediate neuronal excitation evoked by α_1-adrenoceptor agonists.

4. Discussion

In the present study three findings with regard to the blockade of the excitation evoked by α_1-adrenoceptor agonists from ventromedial nucleus neurons were that two different types of antagonists, the α_{1A}-adrenoceptor-selective 5-methyl urapidil and WB-4101 and the α_{1B}-adrenoceptor-specific chloroethylclonidine appeared to be equally effective. Second, either type of antagonist could suppress the excitatory neuronal response to phenylephrine (which activates both α_{1A}- and α_{1B}-adrenoceptors). Third, whether by bath application or in preincubation, chloroethylclonidine was equally effective in blocking the phenylephrine-evoked excitation from estrogen-primed preparations, which contain α_{1B}-adrenoceptors, as well as from preparations not primed with estrogen, that reportedly have very few receptor of this subtype (Petitti et al., 1992). These observations raised two major possibilities: (1) the antagonists used were not specific for the respective subtypes, or (2) there are no distinct and completely independent α_{1A}- and α_{1B}-adrenoceptors in the ventromedial nucleus. Interestingly, similar observations and suggestions have also been reported for other kinds of preparations using different methods (Esbenshade et al., 1993; Minneman and Atkinson, 1991; Oriowo et al., 1992; Piascik et al., 1991).

A specific antagonist may become non-specific as a result of an overdose or the manner of application. However, as discussed in experiments 2 and 3, neither was the case in the present study. Therefore, possibility 2, a lack of α_{1A}/α_{1B}-adrenoceptor independence, is more likely. This, in turn, may be due to several mechanisms. Firstly, it could be that, in the ventromedial nucleus, the numbers or the densities alone of either α_{1A}- or α_{1B}-adrenoceptor subtype was insufficient to mediate the excitation. Here, for α_1-adrenoceptor agonist-evoked excitation to occur, a summation of the effects mediated by receptors from both subtypes would be needed. Secondly, in the ventromedial nucleus, there may be a new α_1-adrenoceptor subtype that is neither pure α_{1A} nor pure α_{1B} but possesses some characteristic of both subtypes, as initially proposed by others (Minneman and Atkinson, 1991; Piascik et al., 1991). Such could result from the proven existence of an alternate transcript from an α_{1B}-adrenoceptor gene (McGehee and Cornett, 1991). Indeed, a receptor expressed from a clone from bovine brain cDNA library is similar to, but distinct from either α_{1A}- or α_{1B}-adrenoceptor (Schwinn et al., 1990). Thirdly, the responsive ventromedial nucleus neurons may have both α_{1A}- and α_{1B}-adrenoceptor binding sites, and both types of sites have to be occupied to excite the neuron. This type of possibility has been raised by the finding that activation of both δ- and μ-opioid receptors in an opioid-sensitive system is required for β-endorphin to exert a behavioral effect (Smith et al., 1983). Similarly, there is evidence that a receptor for nerve growth factor is composed of two binding sites and that binding to both sites is required for a response (Hempstead et al., 1991). Fourthly, in addition, some kinds of interaction between α_{1A}- and α_{1B}-adrenoceptor subtypes might be required to mediate the α_1-adrenoceptor agonist-evoked excitation. A precedent for this is the interaction between dopamine D_1 and D_2 receptors (Bertorello et al., 1990; Calabreski et al., 1992; Piomelli et al., 1991).

None of these four mechanisms discussed above can yet be definitely ruled out or proven. Therefore, the exact subtype(s) or form(s) of the receptor responsible for mediating the α_1-adrenoceptor agonist-evoked excitation of ventromedial nucleus neurons remain to be

determined. Nevertheless, for ventromedial nucleus neurons at least, activation of both α_{1A}- and α_{1B}-adrenoceptors is necessary for evoking α_1-adrenoceptor agonist-evoked excitation. This also appears to be true for the stimulation of the phosphoinositide pathway because in hypothalamus the stimulation of phosphoinositol hydrolysis by the activation of α_1-adrenoceptors could be blocked by either α_{1A}- or α_{1B}-adrenoceptor-specific antagonists (Karkanias et al., 1995).

Acknowledgements

This study was supported by NIH grants NS30824 (L.-M.K.) and HD05751 (D.W.P.).

References

Bertorello, A.M., J.F. Hopfield, A. Aperia and P. Greengard, 1990, Inhibition by dopamine of $(Na^+ + K^+)$ATPase activity in neostriatal neurons through D_1 and D_2 dopamine receptor synergism, Nature 347, 386.

Calabreski, P., R. Maj, N.B. Mercuri and G. Bernardi, 1992, Coactivation of D_1 and D_2 dopamine receptors is required for long-term synaptic depression in the striatum, Neurosci. Lett. 142, 95.

Esbenshade, T.A., C. Han, T.J. Murphy and K.P. Minneman, 1993, Comparison of α_1-adrenergic receptor subtypes and signal transduction in SK-N-MC and NB41A3 neuronal cell lines, Mol. Pharmacol. 44, 76.

Han, C., P.W. Abel and K.P. Minneman, 1987, Heterogeneity of α_1-adrenergic receptors revealed by chlorethylclonidine, Mol. Pharmacol. 32, 505.

Hempstead, B.L., D. Martin-Zanca, D.R. Kaplan, L.F. Parada and M.V. Chao, 1991, High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor, Nature 350, 678.

Hidenori, Y. and K. Honda, 1993, α_1-Adrenoceptor subtype in the rat prostate is preferentially the α_{1A} type, Jpn. J. Pharmacol. 62, 297.

Johnson, R.D. and K.P. Minneman, 1987, Differentiation of α_1-adrenergic receptors linked to phosphatidylinositol turnover and cyclic AMP accumulation in rat brain, Mol. Pharmacol. 31, 239.

Karkanias, G.B., N. Petitti and A.M. Etgen, 1995, Progesterone attenuation of α_1-adrenergic receptor stimulation of phosphoinositol hydrolysis in hypothalamus of estrogen-primed female rats, Endocrinology 136, 1993.

Kow, L.-M. and D.W. Pfaff, 1987, Responses of ventromedial hypothalamic neurons in vitro to norepinephrine: dependence on dose and receptor type, Brain Res. 413, 220.

Kow, L.-M. and D.W. Pfaff, 1992, Relations between α_{1a}- and α_{1b}-adrenoceptor subtypes: an electrophysiological assessment in hypothalamic neurons, Soc. Neurosci. Abstr. 18, 589.

Kow, L.-M., G.D. Weesner and D.W. Pfaff, 1992, α_1-Adrenergic agonists act on the ventromedial hypothalamus to cause neuronal excitation and lordosis facilitation: electrophysiological and behavioral evidence, Brain Res. 588, 237.

Kow, L.-M., Y.-F. Tsai, N.G. Weiland, B.S. McEwen and D.W. Pfaff, 1995, In vitro electro-pharmacological and autoradiographic analyses of muscarinic receptor subtypes in rat hypothalamic ventromedial nucleus: implications for cholinergic regulation of lordosis, Brain Res. (in press).

McGehee Jr., R.E. and L.E. Cornett, 1991, Alternative mRNAs encoding the α_{1b}-adrenergic receptor are expressed in a tissue-dependent manner in the Sprague-Dawley rat, J. Receptor Res. 11, 773.

Michel, M.C., J. Kerker, T.A. Branchek and C. Forray, 1993, Selective irreversible binding of chloroethylclonidine at α_1- and α_2-adrenoceptor subtypes, Mol. Pharmacol. 44, 1165.

Minneman, K.P. and B. Atkinson, 1991, Interaction of subtype-selective antagonists with α_1-adrenergic receptor-mediated second messenger responses in rat brain, Mol. Pharmacol. 40, 523.

Minneman, K.P. and T.A. Esbenshade, 1994, α_1-Adrenergic receptor subtypes, Annu. Rev. Pharmacol. Toxicol. 34, 117.

Minneman, K.P., C. Han and P.W. Abel, 1988, Comparison of α_1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB4101, Mol. Pharmacol. 33, 509.

Oriowo, M.A., R.D. Bevan and J.A. Bevan, 1992, Vascular α-adrenoceptor affinity variation is not due to varying populations of subtypes distinguished by WB 4101 and chlorethylclonidine, Eur. J. Pharmacol. 216, 415.

Petitti, N., G.B. Karkanias and A.M. Etgen, 1992, Estradiol selectively regulates α_{1B}-noradrenergic receptors in the hypothalamus and preoptic area, J. Neurosci. 12, 3869.

Piascik, M.T., B.T. Butler and T.A. Pruitt, 1990a, The role of α_1-adrenoceptor subtypes in the regulation of arterial blood pressure, Eur. J. Pharmacol. 199, 279.

Piascik, M.T., B.T. Butler, T.A. Pruitt and J.W. Kusiak, 1990b, Agonist interaction with alkylation sensitive and resistant α_1 receptor subtypes, J. Pharmacol. Exp. Ther. 254, 982.

Piascik, M.T., M.S. Sparks, T.A. Pruitt and E.E. Soltis, 1991, Evidence for a complex interaction between the subtypes of the α_1-adrenoceptor, Eur. J. Pharmacol. 199, 279.

Piomelli, D., C. Pilon, B. Giros, P. Sokoloff, M.-P. Martres and J.-C. Schwartz, 1991, Dopamine activation of the arachidonic acid cascade as a basis for D_1/D_2 receptor synergism, Nature 353, 164.

Schwinn, D.A., J.W. Lomasney, W. Lorenz, P.J. Szklut, R.T. Fremeau Jr., T.L. Yang-Feng, M.G. Caron, R.J. Lefkowitz and S. Cotecchia, 1990, Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype, J. Biol. Chem. 265, 8183.

Smith, A.P., N.M. Lee and H.H. Loh, 1983, The multiple site β-endorphin receptor, Trends Pharmacol. Sci. 4, 163.

Takayanagi, I., M. Harada, K. Koike and M. Satoh, 1991, Difference in α_1-adrenoceptor mechanisms for phenylephrine and tizanidine in rabbit thoracic aorta and common iliac artery, Can. J. Physiol. Pharmacol. 69, 1819.

Takayanagi, I., K. Shiraishi and N. Kokubu, 1992, α_{1B}-Adrenoceptor mechanisms in rabbit iris dilator, Jpn. J. Pharmacol. 59, 301.

Terman, B.I., R.P. Riek, A. Grodski, H.-J. Hess and R.M. Graham, 1990, Identification and structural characterization of α_1-adrenergic receptor subtypes, Mol. Pharmacol. 37, 526.

Vargas, H.M., D. Cunningham, L. Zhou, H.B. Hartman and A.J. Gorman, 1993, Cardiovascular effects of chloroethylclonidine, an irreversible α_{1B}-adrenoceptor antagonist, in the unanesthetized rat: a pharmacological analysis in vivo and in vitro, J. Pharmacol. Exp. Ther. 266, 864.