Effect of Muscarinic Receptor Modulators in the Hypothalamic Supraoptic Nucleus of the Rat

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ABSTRACT—Muscarinic antagonists were injected into the hypothalamic supraoptic nucleus (SON) and their effects on the acetylcholine (ACh) release of this nucleus were studied by in vivo microdialysis techniques. Atropine, AF-DX116 (a M2-receptor antagonist), 4-DAMP (a M3-receptor antagonist) and pirenzepine (a M1-receptor antagonist) concentration-dependently increased the ACh release. The EC50 values for these antagonists were 15 nM for atropine, 7.8 μM for pirenzepine, 0.39 pM for AF-DX116 and 59 nM for 4-DAMP, suggesting the autoregulation of the ACh release through an activation of M2 and M3 subtypes of muscarinic receptors in the SON. The postsynaptic effect of muscarinic receptors on urine outflow was studied by microinjection of selective muscarinic receptor agonists and antagonists into the SON. McN-A-343 (a M1-receptor agonist) had no significant effect on urine outflow. Pre-microinjection of atropine, 4-DAMP, p-F-HHSiD (a M3-receptor antagonist) or pirenzepine into the SON concentration dependently attenuated the oxotremorine-induced antidiuresis. In contrast, AF-DX116 and methoctramine had no effect on this oxotremorine-induced action. These results suggest that the M3-subtype may contribute to the antidiuretic actions. Nicotine produced an increase in ACh release in the SON and also induced potent antidiuretic effects, both of which were inhibited by hexamethonium. Thus, in the SON, the ACh release may be autoregulated by M2- and M3-subtypes of muscarinic receptors and the antidiuretic effects of ACh produced through an activation of the M2-subtype.

Keywords: Muscarinic receptor subtype, Acetylcholine release, Antidiuresis, Supraoptic nucleus

The hypothalamic supraoptic nucleus (SON) contains magnocellular cell bodies that synthesize and release neurohypophyseal hormones such as vasopressin and oxytocin. Immunohistochemical and electrophysiological studies have demonstrated that cholinergic neurons were densely distributed on the magnocellular neurons in the SON (1, 2) and that iontophoretic application of acetylcholine (ACh) excited these neurons (3, 4), respectively.

We previously demonstrated that cholinergic agonists microinjected into the SON induce antidiuretic effects due to a stimulation of vasopressin release through an activation of muscarinic receptors in the nucleus (5, 6). Recently, we detected a spontaneous release of ACh from the SON by using a brain microdialysis method (7). The ACh-release was enhanced by infusion of atropine and pirenzepine; the former was more potent than the latter. These results could be interpreted to indicate that a non-M1-subtype autoreceptor is involved in the regulation of the ACh-release in the SON, and thus affects the urine outflows (7).

Muscarinic receptors are pharmacologically characterized into three subtypes, M1, M2 and M3 (8–10). Recent molecular biological studies indicate the existence of five genes (m1–m5) encoding muscarinic receptors (11, 12). The M2- or M3-subtype works as the presynaptic autoreceptors to modulate the ACh release from cholinergic neurons in the hippocampus and striatum (13–15). Our previous study has indicated that a non-M1-subtype of muscarinic receptor was related to the regulation of ACh release in the SON. However, it remains to be determined which subtype (M2 or M3) of muscarinic receptor functions as the presynaptic autoreceptor or mediates the decrease in urine outflow due to vasopressin release.

Using in vivo microdialysis and microinjection techniques, we studied the effects of selective muscarinic agonists and antagonists on the ACh release in the SON and also the urine outflow.
MATERIALS AND METHODS

Animals and drugs

Male Wistar rats (weighing 280–320 g) were used. The rats were maintained in a light-controlled environment with food and water given ad libitum.

The following drugs were used: atropine sulfate, pirenzepine dihydrochloride, physostigmine salicylate (Sigma Chemical Co., St. Louis, MO, USA); oxotremorine (Aldrich Chemical Co., Inc., Milwaukee, WI, USA); McN-A-343, methoctramine chloride, hexahydro-siladifenidol, \( p \)-fluoro analog (\( p \)-F-HHSD) and 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP) (Research Biochemicals, Inc., Natick, MA, USA); nicotine tartrate (Wako Pure Chemical Ind., Osaka); hexamethonium bromide (C6) (Nacalai Tesque, Kyoto). 11-[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX116) was kindly provided by Boehringer Ingelheim (Germany).

Microdialysis procedure

Brain microdialysis was carried out as described previously (7). Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and immobilized in a stereotaxic instrument (Takahashi Co., Tokyo) for implantation of a guide cannula for the microdialysis probe. The skull was then exposed, and a burr hole was drilled to insert the guide cannula (G-12; Eikom, Kyoto) into the SON (A, 6.28; L, 1.3; H, 7.8 in mm) according to the atlas of König and Klippel (16). The guide cannula was secured in place with dental cement. After a recovery period of one day, a microdialysis probe (BDP-I-12-02 or BDP-MI-12-02, Eikom) was inserted through the guide cannula into the SON. One end of the dialysis probe was connected to an HPLC system. At the end of each experiment, the position of the microdialysis probe was verified histologically by cutting the frozen brain at approx. 10 \( \mu \)m on microtome cryostat (Tissue-Tek II).

HPLC analysis of ACh

ACh in the dialysate was measured by HPLC combined with an enzyme-immobilized column and an electrochemical detector according to the method of Damsma et al. (17). For separation of ACh, an Eikompak AC-Gel column (6 \( \times \) 150 mm, Eikom) was used. An AC-Enzyme-Pak column (ACh esterase and choline oxidase-immobilized, Eikom) was used to convert ACh and choline to hydrogen peroxide, which was detected by an electrochemical detector with a platinum electrode (ECD-100, Eikom) at 450 mV. The mobile phase was delivered by a pump (EP-10, Eikom) at 1.0 ml/min and contained 0.1 M sodium phosphate buffer (pH 8.0), 300 mg/l sodium 1-decanesulfonate and 65 mg/l tetramethylammonium chloride. Quantification of collected ACh was performed by comparison with the peak height of the authentic standard (ethylhomocholine, EHC).

Measurement of urine outflow

Urine outflow was measured by the method of Dicker with some modifications (5, 18). The rats were starved overnight for approx. 17 hr, but had free access to water. The animals were loaded orally through a catheter with a volume of water equivalent to 5% of the body weight followed by the same volume of 12% ethanol. Cannulae were inserted into the trachea, bladder and external jugular vein. The rat was then immobilized in a stereotaxic instrument for rats (Takahashi Co., Tokyo). Drops of urine flowing from the urinary cannula were counted by a photo-electric drop counter (DCT 102; Unique Medical, Inc., Tokyo) and recorded as single pulses. Ethanol (3% in Locke solution) was infused at a constant rate of 0.10 ml/min through the cannula in the jugular vein in order to maintain a constant level of anesthesia and a constant rate of urine outflow. The following methods were used to stimulate vasopressin release: 1) Administration of hypertonic saline: Rats were given an i.p. injection of either 9.0% saline (25 ml/kg b.wt.) or an equal volume of 0.9% saline. 2) Hemorrhage: 3–4 ml of blood was withdrawn from catheters inserted into a femoral artery over 1–2 min.

Microinjection of drugs

A stainless steel cannula (outer diameter: 200 \( \mu \)m) was unilaterally inserted stereotaxically into the SON (A, 6.28; L, 1.3; H, 8.8 in mm) according to the atlas of König and Klippel (16). Drugs were dissolved in saline (approx. pH 7). A 1-\( \mu \)l aliquot of drug solution was micro-
injected through a microsyringe connected with the cannula when the urine outflow reached a constant rate. Then 2 μl of an artificial cerebrospinal fluid (CSF: 128 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 0.65 mM Na₂HPO₄, 4.8 mM NaHCO₃, pH 7.4) was inserted at a rate of approx. 0.3 μl/min for 10 min (total 3 μl). Effects of drugs on urine outflow were measured at 10-min intervals and expressed as a percent of the initial control urine outflow. To test the effects of pretreatment with muscarinic antagonists (atropine, pirenzepine, 4-DAMP, methoctramine, AF-DX116 and p-F-HHSiD), the first injection of oxotremorine was followed by a microinjection of the muscarinic antagonist into the SON and then a second injection of oxotremorine was performed. The position of the tip of the microinjection cannula was histochemically confirmed by the localization in a group of magnocellular cells in the SON positively stained by the method of Gomori (19).

Statistical analyses

The significance of differences between mean values was determined by Student’s t-test. Differences were considered significant at P < 0.05.

The EC₅₀ and IC₅₀ values for muscarinic receptor agonists and antagonists were calculated from the dose-effect curves, drawn by the computer-assisted linear least squares method.

RESULTS

Effect of microinjection of muscarinic receptor antagonists on ACh release in the SON

The mean value of spontaneous ACh release in the presence of 10⁻⁴ M physostigmine was 1.438 ± 0.344 pmol/40 μl/20 min during the 20 min before the infusion of muscarinic receptor antagonist (±S.E.M., n = 27). The ACh release was constantly detected for at least 3 hr. Although the value of spontaneous ACh release before infusion of each antagonist was variable (Fig. 1), the high K⁺ (100 mM)-induced increase in the ACh release observed at the end of each experiment was almost equal (300–400%). There might be a great individual variation since the microdialysis was carried out under unanesthetized and unrestrained conditions. Following injection of four different muscarinic receptor antagonists (atropine, 4-DAMP, AF-DX116 and pirenzepine, each at the concentration of 10 μM) into the SON, the releases of ACh from the nucleus were gradually increased. The maximum values were reached at 40–80 min after the injection (Fig. 1). The potency for the ACh release was in the order of atropine (about 260% of the initial control) > 4-DAMP = AF-DX116 (about 160% of the control) > pirenzepine (about 130% of the control). The atropine increased the ACh release over 200% of the control within 20–40 min after the start of infusion, and then the release gradually decreased with time until 120 min. On the other hand, 4-DAMP or AF-DX116 produced a small but sustained increase in the ACh-release for 100 min after the injection. Simultaneous infusion of AF-DX116 with 4-DAMP increased the maximum values of the ACh release induced by an individual antagonist alone (about 270% of control).

The effect of atropine, pirenzepine, AF-DX116 or 4-DAMP injected into the SON on ACh release from the nucleus were shown to be concentration-dependent (Fig. 2). The maximum effect was obtained at 0.1 μM for atropine, 10 μM for AF-DX116, 10 μM for pirenzepine and 10 μM for 4-DAMP. The EC₅₀ values calculated from the curves were 15 nM for atropine, 59 nM for 4-DAMP, 0.39 nM for AF-DX116 and 7.8 μM for pirenzepine. The affinity profiles of these muscarinic receptor antagonists are shown in Fig. 3.

Effect of microinjection of muscarinic receptor stimulants into the SON on urine outflow

Figure 4 shows the effects of microinjected oxotremorine and McN-A-343 into the SON on the urine outflow. Oxotremorine, but not McN-A-343, attenuated the urine outflow, in a concentration-dependent manner (EC₅₀ = 0.3 mM, n = 15 (5)). The effects of microinjection of oxotremorine on urine outflow were reproducible; i.e., the antidiuretic effect of the second injection of oxotremorine (at 60–90 min after the first) was almost equal to that in-
The effects of various muscarinic receptor antagonists on urine outflow decreased by oxotremorine were studied. Infusion of atropine, 4-DAMP, pirenzepine or p-F-HHSiD into the SON did not modify the urine outflow by itself but attenuated the inhibitory actions of oxotremorine on urine outflow (Fig. 5). The IC50 values for the antagonists were 1.5 pM for atropine, 50 fM for 4-DAMP, 0.3 mM for pirenzepine and 0.5 mM for p-F-HHSiD (n=5). In contrast, AF-DX116 and methoctramine (an antagonist of M2-receptor subtype) had no effect on this oxotremorine-induced action.

Effects of stimulants that induce vasopressin release on the urine outflow and ACh release in the SON

Microinjection of nicotine (100 nmol/μl) into the SON transiently increased the release of ACh from the nucleus and decreased the urine outflow. These effects of nicotine were inhibited by a pre-application of hexamethonium (300 nmol/μl) (Fig. 6), suggesting that nicotine may induce the decrease in urine outflow through a mediation of ACh-action in the SON. The concentration changes in ACh in the SON following the decrease in blood volume and intraperitoneal injection of hypertonic saline (9%), procedures known to stimulate vasopressin release, were measured, the objective being to study the physiological significance of the increase in ACh release in the SON.

Decrease in body blood volume (3–4 ml) produced a decrease in the urine outflow and concomitantly caused a slight increase in the release of ACh in the SON (Fig. 6). In contrast, the injection of hypertonic solution reduced...
DISCUSSION

In the present experiment using in vivo microdialysis techniques, we found that infusion of muscarinic receptor antagonists into the SON concentration-dependently increased the release of ACh in the SON-dialysate. The results suggest that the muscarinic receptor antagonists may activate the release of ACh through an inhibition of presynaptic muscarinic autoreceptors in the SON (7, 20–22). The affinity constants for the muscarinic receptor antagonists have been determined by the functional studies that were generally carried out on the vas deferens or superior cervical ganglion (M₁), heart (M₂) or longitudinal smooth muscle of the ileum (M₃) (23). The -log values for atropine, pirenzepine, AF-DX116 and 4-DAMP used in this study at the M₂-subtype were 9.3, 6.5, 7.2 and 7.9, respectively. Figure 3 showed that the -log EC₅₀ for the four antagonists (atropine, 7.8; pirenzepine, 5.1; AF-DX116, 6.4; 4-DAMP, 7.2) correlated well with the affinity constants for these antagonists at the M₂-subtype, but not at the M₁- and M₃-subtype (M₁, r² = 0.3026; M₃, r² = 0.6875). The analysis suggests that the M₂-subtype muscarinic receptor may be responsible for this autoregulation. This hypothesis is consistent with the previous autoradiographic study in that the M₂-, but not M₁-, subtype of muscarinic receptors is localized in the SON (24). However, the maximal increase in the ACh
release induced by atropine (260% of the control) was significantly higher than that induced by the other muscarinic antagonists. Furthermore, simultaneous infusion AF-DX116 and 4-DAMP enhanced the maximum increase in the ACh release induced by each agent alone (about 270% of the control). Thus, these results suggest that not only the M2-type but also other types of muscarinic receptors (probably the M3-subtype) may be responsible for the autoregulation of ACh release in cholinergic nerve terminals in the SON.

We also found that microinjection of cholinergic receptor stimulants into the SON decreased the urine outflow, in which the M2-receptor agonist McN-A-343 is less stimulatory than oxotremorine. Furthermore, an antagonist for the M2-subtype receptor did not modify the antiuretic effect of microinjected oxotremorine. The potency for the inhibition of oxotremorine-induced antiuretic effects was in the order of atropine > 4-DAMP > pirenzepine = p-F-HHSiD. These results shown by receptor-subtype-selective agonist and antagonists suggest that in the SON, stimulation of postsynaptic cholinergic receptors produces the antidiureses through an activation of the M3-subtype of muscarinic receptors.

In the present experiments, microinjection of nicotine into the SON markedly decreased the urine outflow and caused a transient increase in the ACh release. Decrease in body blood volume (3 - 4 ml) also produced a decrease in the urine outflow concomitant with a slight increase in the release of ACh in the SON, but intraperitoneal application of hypertonic solution reduced the urine outflow without any change in the release of ACh in the SON. The results indicate that although these conditions are known to stimulate vasopressin release, injection of nicotine, but not hypertonic stimulation, produces an activation of cholinergic neurons in the SON; i.e., a specific stimulation may be required for ACh release in the SON. We have previously shown that the nicotine-induced antiuretic effect is inhibited by both muscarinic and nicotinic receptor antagonists (5). The finding suggested that nicotine may release ACh through the nicotinic receptor from presynaptic cholinergic terminals in the SON as demonstrated in a synaptosomal preparation of the myenteric plexus (25). The result in this study is agreement with the previous suggestion. On the other hand, the effects induced by a decrease in blood volume show discrepancies between the extent of decrease in urine outflow and the extent of increase in the ACh release when compared with the effects of nicotine. The suggestion that the decrease in blood volume stimulates vasopressin release by activating cholinergic neurons in the SON must await further substantiation.

Thus, it is considered that M1- and M2-subtypes of receptors may regulate ACh release as an autoinhibitory mechanism at cholinergic presynaptic nerve terminals, and the M2-subtype of receptor may be responsible for the postsynaptic events resulting in the decrease in urine outflow, possibly through an activation of vasopressin release. The localization and function of the muscarinic receptor subtypes in the SON may be different at the pre- and postsynaptic nerve terminals.

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