Full Paper

Role of K^+ Channels in M_2 Muscarinic Receptor-Mediated Inhibition of Noradrenaline Release From the Rat Stomach

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Received July 29, 2004; Accepted September 9, 2004

Abstract. Previously we reported the cholinergic M_2 muscarinic receptor-mediated inhibition of noradrenaline release from the rat stomach (K. Yokotani, Y. Osumi. J Pharmacol Exp Ther. 1993;264:54–60). In the present study, we investigated the role of K^+ channels in oxotremorine (a muscarinic receptor agonist)-induced inhibition of noradrenaline release using isolated, vascularly perfused rat stomach. The gastric postganglionic sympathetic nerves were electrically stimulated twice at 2.5 Hz for 1 min and test reagents were added during the second stimulation. The electrically evoked release of noradrenaline was augmented by tetraethylammonium and 4-aminopyridine (non-selective K^+ channel blockers) and also by charybdotoxin (a blocker of big conductance Ca^{2+} -activated K^+ channel). On the other hand, apamin (a selective blocker of small conductance Ca^{2+} -activated K^+ channels) and glibenclamide (an ATP-activated K^+ channel blocker) had no effect on the evoked noradrenaline release. Oxotremorine-induced inhibition of noradrenaline release was attenuated by tetraethylammonium and 4-aminopyridine, while the inhibition was not influenced by charybdotoxin, apamin, and glibenclamide. These results suggest that tetraethylammonium- and 4-aminopyridine-sensitive K^+ channels (probably voltage-activated K^+ channels) are involved in the muscarinic receptor-mediated inhibition of noradrenaline release from the rat stomach.

Keywords: K^+ channel, muscarinic inhibition, noradrenaline release, sympathetic nerve, rat stomach

Introduction

K^+ channels play an important role in a number of different aspects of the electrical responses of the nervous system (1–3). For example, K^+ channel activity is involved in setting the membrane resting potential, determining the frequency of action-potentials, and the shape of the action-potential wave forms. Most of these channels permit the K^+ efflux from within the neurons, thereby tending to oppose depolarization or to cause repolarization or hyperpolarization, and resulting in a decrease of neurotransmitter release. Some K^+ channels, named inwardly rectifying K^+ channels, permit the K^+ influx from the outside the neurons to stabilize the resting membrane potential near the K^+ equilibrium

potential (4). These multiple tasks require a wide variety of K^+ channels.

K^+ channels are now classified into more than ten types based on their electrophysiological and pharmacological properties (5). Several types of K^+ channels including voltage-activated K^+ channels, calcium-activated K^+ channels, and adenosine 5'-triphosphate (ATP)-sensitive K^+ channels are known to be present on the central and peripheral nervous system to modulate neurotransmission (2, 6). However there is no direct evidence for the contribution of inwardly rectifying K^+ channels to modulate transmitter release (2, 4).

Previously, we reported that oxotremorine inhibits the release of noradrenaline by activation of cholinergic M_2 muscarinic receptors located on the sympathetic nerve terminals in the rat stomach (7). In the present study, therefore, we examined a role of K^+ channels in

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the M₂ muscarinic receptor-mediated inhibition of the gastric noradrenaline release.

Materials and Methods

Perfusion experiments

Male Wistar rats (Japan SLC, Inc., Hamamatsu) weighing about 300 g were fasted overnight before experiments. The isolated, vascularly perfused stomach preparations were made as described previously (8–10). Briefly, under urethane anesthesia, the abdomen was opened with a midline incision. After ligation of the abdominal aorta just above the branching of the celiac artery, the cannula was inserted into the celiac artery via an incision placed on the aorta and modified Krebs-Ringer solution (pH 7.4) bubbled with a mixture of 95% O₂ and 5% CO₂ was perfused with a constant flow rate of 2.5 ml per min. Modified Krebs-Ringer solution was composed of 117.5 mM NaCl, 4.7 mM KCl, 2.4 mM CaCl₂, 1.1 mM MgCl₂, 1.1 mM NaH₂PO₄, 25 mM NaHCO₃, 11.1 mM glucose, 0.05% of bovine serum albumin, 10 μ M pargyline, and 1 μ M phentolamine. A tube was inserted into the lumen of the stomach via a pylorus ring to drain the contents of the stomach throughout the experiment. The esophagus, duodenum, spleen, and pancreas were dissected after ligation of the vessels, and the vascularly perfused stomach was kept in a chamber prewarmed at 37°C. Each 2-min effluent from the portal vein was collected in chilled tubes containing 0.5 ml of 4 M perchloric acid, 1 ng of 3,4-dihydroxybenzylamine as an internal standard, and 50 μ l of 2% sodium pyrosulfite solution.

After an equilibration period of 60 min, the first electrical stimulation [consisting square-wave pulses at 2.5 Hz, 2-ms duration, 10 mA (supramaximal intensity) for 1 min] was applied to the periarterial nerves around the left gastric artery, which contain the postganglionic sympathetic nerves, with bipolar electrodes. The second electrical stimulation was carried out 26 min after the first stimulation. Perfusion medium containing test reagents such as K⁺ channel blockers and oxotremorine was changed 14 min before the second electrical stimulation. In some experiments, K⁺ channel blockers were added throughout experiments.

Noradrenaline assay in the medium and the stomach

At the end of each experiment, the stomach was homogenized in 20 ml of 0.4 M perchloric acid containing 18.6 mg of disodium EDTA dihydrate, 200 μ l of 2% sodium pyrosulfite solution, and 500 ng of 3,4-dihydroxybenzylamine as an internal standard. The homogenate was centrifuged for 10 min at 14,000 $\times$ g at 4°C. The supernatant was analyzed to determine the

tissue level of noradrenaline.

Catecholamines in the effluent and the supernatant of tissue homogenate were extracted by the method of Anton and Sayre (11), with a slight modification, and were assayed electrochemically with high-performance liquid chromatography (9, 10, 12). Specifically, 2 ml of effluent or an aliquot (0.1 ml) of supernatant was transferred to a centrifuge tube containing 30 mg of activated alumina and 3 ml of 1.5 M Tris Buffer (pH 8.6) containing 0.1 M disodium EDTA, after which the preparations were shaken for 10 min. The supernatant was discarded and the alumina was washed three times with double-deionized water. After centrifugation, the supernatant was discarded, and then the catecholamines adsorbed onto the alumina were eluted with 500 μ l of 4% of acetic acid containing 0.1 mM disodium EDTA. The recovery of catecholamines was about 85%.

The high-performance liquid chromatography-electrochemical detection system consisted of a pump (PU-980; JASCO, Tokyo), a sample injector (851-AS, JASCO), and an electrochemical detector (ECD-300; Eicom, Kyoto) equipped with a graphite electrode. Analytical conditions were as follows: detector, +450 mV potential against a Ag/AgCl reference electrode; column, Cosmosil μ C₁₈, 4.6 $\times$ 150 mm (Nakalai Tesque, Kyoto); mobile phase, 0.1 M NaH₂PO₄-Na₂HPO₄ buffer (pH 6.0) containing 50 mg/l disodium EDTA, 750 mg/l sodium 1-octanesulfonate (Nacalai Tesque), and 15% methanol at a flow rate of 0.5 ml/min. The amount of catecholamines in each sample was calculated using the peak height ratio relative to that of 3,4-dihydroxybenzylamine. This assay could determine 2 pg of noradrenaline accurately.

Evaluation and statistical analysis

Basal release of noradrenaline was calculated by averaging the amount of noradrenaline released in two subsequent samples before electrical stimulation. The release of noradrenaline is expressed as % of its tissue content per 2 min. In addition, the amounts of the evoked noradrenaline release above the basal level during 12 min after the first and second electrical stimulation are expressed as S₁ and S₂. The effects of test reagents are expressed as the ratios of S₂ to S₁. All values are expressed as the means $\pm$ S.E.M.

All data were analyzed by repeated-measures analysis of variance, followed by post-hoc analysis with the Bonferroni method for comparing a control to all other means in Figs. 1–5. *P* values of less than 0.05 were taken to indicate statistic significance.

Drugs

The following drugs were used: oxotremorine

sesquifumarate, pargyline hydrochloride, phentolamine hydrochloride, tetraethylammonium chloride (Sigma Chemical Co., St. Louis, MO, USA); 4-aminopyridine (Research Biochemicals International, Natick, MA, USA); glibenclamide (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA); apamin, charybdotoxin (Peptide Institute Inc., Osaka). All other reagents were of the highest grade available (Nacalai Tesque). Glibenclamide was dissolved in 100% dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the perfusion medium was 0.1%.

Results

Effects of K^+ channel blockers on the electrically evoked release of noradrenaline from the rat stomach

The amount of noradrenaline remaining in the stomach was 673 ± 10 ng ($n = 216$). Spontaneous release of noradrenaline per 2 min was about 0.04% of its tissue content. Electrical stimulation of the gastric sympathetic nerves at 2.5 Hz for 1 min evoked an increase of noradrenaline release and this increase rapidly declined toward the basal level (Fig. 1). Repetitive stimulations evoked consistent and reproducible increases in noradrenaline release (Figs. 1 and 2).

After the first stimulation, perfusion medium was

changed to the next one containing K^+ channel blockers such as tetraethylammonium, 4-aminopyridine, charybdotoxin, apamin, or glibenclamide. The basal release of noradrenaline from the stomach was slightly increased by 4-aminopyridine [$0.08 \pm 0.01\%$ for 0.05 mM ($n = 4$); $0.11 \pm 0.01\%$ for 0.1 mM ($n = 6$); $0.15 \pm 0.01\%$ for 0.25 mM ($n = 5$)]. These values were significantly different from that of the control [$0.04 \pm 0.01\%$ of tissue content for the control ($n = 7$)] (Fig. 1). On the other hand, tetraethylammonium, charybdotoxin, apamin, and glibenclamide had no effect on the basal release (data not shown).

Tetraethylammonium (0.5, 1, and 2 mM) and 4-aminopyridine (0.1 and 0.25 mM) significantly and dose-dependently augmented the electrically evoked release of noradrenaline from the stomach (Figs. 1 and 2). Charybdotoxin ($0.03 \mu\text{M}$) also augmented the evoked noradrenaline release, while apamin (0.1 and $0.3 \mu\text{M}$) and glibenclamide (3 and $10 \mu\text{M}$) had no effect on the evoked release of noradrenaline from the stomach (Fig. 3).

Effects of K^+ channel blockers on the oxotremorine-induced inhibition of noradrenaline release from the rat stomach

Oxotremorine inhibited the evoked release of nor-

Fig. 1. Effects of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on the electrically evoked release of noradrenaline from the isolated rat stomach. Periaarterial nerves around the left gastric artery, which contain the gastric postganglionic sympathetic nerves, were electrically stimulated twice at a stimulus frequency of 2.5 Hz for 1 min. The first stimulation was carried out in the normal medium and the second stimulation was carried out in the medium containing 1 mM TEA or 0.1 mM 4-AP. The release of noradrenaline is expressed as % of its tissue content per 2 min. E.S., electrical stimulation of the gastric sympathetic nerves. Numbers in parentheses represent the number of experiments. Values are the means $\pm$ S.E.M. *Significant difference ($P < 0.05$) from the corresponding value of the control.

Fig. 2. Effects of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on the electrically evoked release of noradrenaline from the isolated rat stomach. The gastric postganglionic sympathetic nerves were electrically stimulated twice at a stimulus frequency of 2.5 Hz for 1 min. TEA and 4-AP were added during the second electrical stimulation. The effects of these reagents are expressed as the S₂/S₁ ratio. Numbers in columns represent the number of experiments. *Significant difference ($P < 0.05$) from the control. Other conditions were the same as those in Fig. 1.

adrenaline from the stomach in a dose-dependent manner (0.01, 0.1, and 1 μ M) (Fig. 4). The inhibitory effects of oxotremorine (0.1 and 1 μ M) were attenuated by 1 mM tetraethylammonium or 0.1 mM 4-aminopyridine (Fig. 4), while oxotremorine-induced inhibitions of noradrenaline release were not influenced by 0.03 μ M charybdotoxin, 0.3 μ M apamin, and 10 μ M glibenclamide (Figs. 4 and 5).

Discussion

Transmitter release from noradrenergic neurons is modulated via presynaptic receptors for noradrenaline itself (adrenergic α_2 autoreceptors), in addition to presynaptic receptors for other neurotransmitters (heteroreceptors) (13). The α_2 adrenoceptor-mediated inhibi-

tion of sympathetic neurotransmission has been suggested to involve the activation of K⁺ channels in the rat vas deferens (14). Since the release of noradrenaline from the rat stomach is also regulated by presynaptic α_2 adrenoceptors (8), phentolamine (a non-selective α -adrenoceptor antagonist) was administered in the perfusion medium to block prejunctional α_2 -autoreceptors in the present experiments.

Tetraethylammonium has been regarded as a blocking agent for native voltage-activated K⁺ currents (especially delayed rectifier currents) (3, 15). About 2–3 mM of this compound is effective on the voltage-activated K⁺ currents in the sympathetic and parasympathetic ganglia (16). However, the selectivity of this compound is not really adequate. It also blocks the Ca²⁺-activated K⁺ currents (17). 4-Aminopyridine is a more selective blocker of the voltage-activated K⁺ currents than tetraethylammonium (18, 19). In neurons, 4-aminopyridine has been shown to block the voltage-activated K⁺ currents with IC₅₀ of approximately 2 mM (20). In the present experiment, the electrically evoked release of noradrenaline from the rat stomach was augmented by tetraethylammonium (0.5, 1, and 2 mM) and 4-aminopyridine (0.1 and 0.25 mM). Tetraethylammonium-induced activation of the sympathetic neurotransmission was reported in rat tail artery and mouse vas deferens (21) and rabbit facial vein (22). 4-Aminopyridine-sensitive activation of the sympathetic neurotransmission was also reported in the rabbit pulmonary artery (23), rabbit urethra (24), rat tail artery, and mouse vas deferens (21). Tetraethylammonium- and 4-aminopyridine-induced facilitation of ³H-noradrenaline release from the rat hippocampus has also been reported (25). From these evidence, it seems likely that tetraethylammonium- and 4-aminopyridine-sensitive K⁺ channels are involved in regulation of noradrenaline release from the rat stomach.

In the next experiment, we examined the effects of more selective blockers for each type of K⁺ channels in the following experiment. Recently, Tagaya et al. (26) reported that electrically evoked release of ³H-noradrenaline from canine pulmonary artery was augmented by apamin (0.01 μ M) [a selective blocker of small conductance Ca²⁺-activated K⁺ channels (27)] and iberiotoxin (0.01 μ M) [a selective blocker of big conductance Ca²⁺-activated K⁺ channels (28)]. In addition, glibenclamide, an ATP-activated K⁺ channel blocker (29, 30), has been shown to augment the electrically evoked release of ³H-noradrenaline from the isolated human right atrium (31). In the present experiment, the electrically evoked release of noradrenaline from the rat stomach was augmented by charybdotoxin (0.03 μ M) [a blocker of big conductance Ca²⁺-activated

Fig. 3. Effects of charybdotoxin, apamin, and glibenclamide on the electrically evoked release of noradrenaline from the isolated rat stomach. The gastric postganglionic sympathetic nerves were electrically stimulated twice at a stimulus frequency of 2.5 Hz for 1 min. Charybdotoxin, apamin, or glibenclamide was added during the second electrical stimulation. The control was cited from Fig. 2. *Significant difference ($P < 0.05$) from the control. Other conditions were the same as those in Figs. 1 and 2.

Fig. 4. Effects of tetraethylammonium (TEA), 4-aminopyridine (4-AP), and charybdotoxin on the oxotremorine-induced inhibition of noradrenaline release from the rat stomach. The gastric postganglionic sympathetic nerves were electrically stimulated twice at a stimulus frequency of 2.5 Hz for 1 min. TEA (1 mM), 4-AP (0.1 mM), or charybdotoxin (0.03 μM) was added throughout the experiment. Oxotremorine (0.01, 0.1, and 1 μM) was added during the second electrical stimulation. *Significant difference ($P < 0.05$) from each control. #Significant difference ($P < 0.05$) from the vehicle-treated group. Other conditions were the same as those in Figs. 1 – 3.

K⁺ channels (32)], while apamin (0.1 and 0.3 μM) and glibenclamide (3 and 10 μM) had no effect. These results suggest that charybdotoxin-sensitive K⁺ channels are also involved in regulation of the electrically evoked release of noradrenaline from the rat stomach.

In the next experiments, we examined the role of K⁺ channels in the cholinergic M₂ muscarinic receptor-mediated inhibition of noradrenaline release from the rat stomach. Previously we reported that oxotremorine, a muscarinic receptor agonist, inhibits the release of

Fig. 5. Effects of apamin and glibenclamide on the oxotremorine-induced inhibition of noradrenaline release from the rat stomach. The gastric postganglionic sympathetic nerves were electrically stimulated twice at a stimulus frequency of 2.5 Hz for 1 min. Apamin (0.3 mM) or glibenclamide (10 μ M) was added throughout the experiment. Oxotremorine (0.01, 0.1, and 1 μ M) was added during the second electrical stimulation. *Significant difference ($P < 0.05$) from each control. Other conditions were the same as those in Figs. 1 – 4.

noradrenaline from the stomach by M₂ muscarinic receptor-mediated and pertussis toxin-insensitive mechanisms, while the reagent exhibits negative chronotropic and inotropic effects on rat atria by pertussis toxin-sensitive mechanisms (7). The M₂ muscarinic receptor-mediated negative chronotropic effect has been shown to be mediated by activation of a certain type of rectifying K⁺ channels (33 – 35) and by pertussis toxin-sensitive mechanisms (36, 37). These results suggest that the inhibitory mechanisms of presynaptic M₂ muscarinic receptors on noradrenaline release from the rat stomach are different from those of postsynaptic M₂ muscarinic receptors located on rat atria. The muscarinic negative chronotropic effect has been shown to be attenuated by tertiapin, a blocker of a certain type of rectifying K⁺ channels (38, 39), while the oxotremorine-induced inhibition of noradrenaline release from the rat stomach was not influenced by tertiapin in the present experiment (data not shown).

The oxotremorine-induced inhibition of noradrenaline release from the rat stomach was attenuated by tetraethylammonium and 4-aminopyridine, while charybdotoxin, apamin, and glibenclamide had no effect on the oxotremorine-induced inhibition. These results suggest that the activation of the tetraethylammonium- and 4-aminopyridine-sensitive (probably voltage-activated) K⁺ channels located on the sympathetic nerve terminals are involved in the oxotremorine-induced inhibition of

noradrenaline release from the rat stomach.

In conclusion, the present study indicates that tetraethylammonium- and 4-aminopyridine-sensitive K⁺ channels (probably voltage-activated K⁺ channels) and charybdotoxin-sensitive K⁺ channels (big conductance Ca²⁺-activated K⁺ channels) play a modulatory role in noradrenaline release from the rat stomach. The cholinergic M₂ muscarinic receptor-mediated inhibition of noradrenaline release seems to be mediated by activation of tetraethylammonium- and 4-aminopyridine-sensitive K⁺ channels (probably voltage-activated K⁺ channels).

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