The Effect of Zaldaride Maleate, an Antidiarrheal Compound, on Acetylcholine-Induced Intestinal Electrolyte Secretion

Nobuo AIKAWA,* Nobuyuki KISHIBAYASHI, Akira KARASAWA, and Kenji OHMORI

The Drug Development Research Laboratories, Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan. Received February 21, 2000; accepted July 19, 2000

The effect of zaldaride on acetylcholine-induced colonic electrolyte secretion was examined. The short-circuit current response to acetylcholine was partially reduced by tetrodotoxin, a neuronal blocker, and was completely inhibited by atropine, an acetylcholine M receptor antagonist, in the rat colonic preparations. The tetrodotoxin sensitive effect was significantly inhibited by zaldaride, whereas the tetrodotoxin insensitive effect was not affected. Acetylcholine release from synaptosomes of submucosal nerves of guinea-pig colon was significantly reduced by zaldaride. Zaldaride may reduce colonic electrolyte secretion by acetylcholine due to the inhibition of acetylcholine release from synaptosomes of colonic submucosal nerves.

Key words zaldaride maleate; acetylcholine release; short-circuit current (Isc); calmodulin

Calmodulin is a Ca^{2+} -binding protein which plays important roles in the transport of electrolytes and water in the intestinal tract.¹⁾ Zaldaride maleate is a selective inhibitor of calmodulin, reported to ameliorate secretory diarrhea without reducing gastrointestinal propulsive motility.²⁻⁴⁾ Acetylcholine is a physiologically important substance, considered to increase cytosolic Ca^{2+} levels and regulate electrolyte transport in the intestinal tract.^{5,6)} In the present study, we examined the effect of zaldaride on acetylcholine-induced increases in short-circuit current (Isc), indicating the transport of electrolytes, in rat colonic preparations using Ussing chambers.

MATERIALS AND METHODS

All experiments were conducted in compliance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and according to experimental protocols approved by the Ethical Committee of the Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.

Ussing Chamber Experiment Male Sprague-Dawley rats (Charles River, Atsugi, Japan) weighing 250–450 g were killed, and the distal colon was removed. Colonic preparations were prepared by stripping away smooth muscle and mounted it in Ussing chambers (surface area 0.693 cm²). Each side of the preparation was bathed with 10 ml of Krebs-Henseleit solution containing (mM) NaCl 119.0, KCl 4.7, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, KH_2PO_4 1.2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.8, NaHCO_3 24.9, and glucose 11.1 (pH 7.4), warmed to 37 °C, oxygenated with carbogen (5% CO_2 in 95% O_2).

The change in Isc was measured continuously and recorded.⁷⁾ Each compound was added to perfused Krebs-Henseleit solution 10 min before the addition of secretagogues. All compounds were applied to the basolateral (serosal) side of the colonic preparation.

Acetylcholine Release Experiment This experiment was a slight modification of the method previously described by Shinozuka *et al.*⁸⁾ Heartley male guinea-pigs (SLC, Hamamatsu, Japan) weighing 250–400 g were killed, and the colon was removed. The colonic preparation was prepared by removing smooth muscle. After 0.32 M sucrose–

3 mM sodium phosphate buffer (pH 7.2) was added to the preparation at 5 ml/mg protein of the preparation, the preparation was crushed with plytron-homogenizer (PT10-35, Kinematica GmbH Littau, Lucern, Switzerland) followed with a Teflon-homogenizer (Wheaton, Millville, NJ, U.S.A.). After centrifugation (4 °C, 1000×g, 10 min), the supernatant was collected and supercentrifuged (4 °C, 17000×g, 20 min) and the pellet was collected. Three milliliters of normal buffer containing (mM) NaCl 119.5, KCl 17.5, CaCl_2 1.2, MgCl_2 1.3, NaHPO_4 1.2, glucose 10.0, Tris 20.0 (pH 7.4), gassed with carbogen, was added to the pellet and suspended. One micromole [³H]choline was incubated for 30 min at 37 °C. After being washed twice in normal buffer, the pellet was suspended in 1.5 ml of normal buffer containing 100 μM neostigmine. One hundred microliters of suspension was added to 400 μl of high potassium buffer containing 15 mM KCl with zaldaride or vehicle at 37 °C for 10 min, after which radioactivity was measured in a liquid scintillation spectrometer.

Materials The compounds used in the present study were zaldaride maleate (Novartis Consumer Health, Nyon, Switzerland); acetylcholine hydrochloride and neostigmine methyl sulfate (Sigma, St. Louis, MO, U.S.A.); tetrodotoxin and atropine sulfate (Wako, Osaka, Japan); [³H]choline (specific activity 3148.7 GBq/mmol) (New England Nuclear, Boston, MA, U.S.A.). Acetylcholine, atropine and tetrodotoxin were dissolved in distilled water. Neostigmine was dissolved and [³H]choline was diluted in a normal buffer. Zaldaride was dissolved in dimethylsulfoxide.

Statistical Analysis Changes in Isc in response to acetylcholine are shown as peak values. The amount of released acetylcholine was shown by tritiated activity. Each value indicated the mean ± S.E.M. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's-test or Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Ussing Chamber Experiment The Isc response to acetylcholine (30 μM) was increased Isc, which was partially reduced by 1 μM tetrodotoxin and was completely abolished

* To whom correspondence should be addressed. e-mail: AIKAWA@kyowa.co.jp

Fig. 1. Effect of Zaldaride on the Acetylcholine (30 μ M)-Induced Increase in Isc in Rat Colonic Preparations

Each column with a bar indicates the mean $\pm$ S.E.M. of 6 experiments. ** $p < 0.01$; statistically significant vs. the value of the vehicle group.

Fig. 2. Effect of Zaldaride on the Acetylcholine (30 μ M)-Induced Increase in Isc in Rat Colonic Preparations Treated with 1 μ M Tetrodotoxin

Each column with a bar indicates the mean $\pm$ S.E.M. of 6 experiments. ** $p < 0.01$; statistically significant vs. the value of the vehicle group.

Table 1. Effect of Zaldaride on [3 H]acetylcholine Release Stimulated by 15 mM KCl from the Synaptosomes of Submucosal Nerves of Guinea-Pig Colon

Zaldaride (μ M)	n	[3 H]Acetylcholine release ($\times 10^3$ dpm/mg protein)
Control	6	5.14 $\pm$ 0.10
Vehicle	6	6.48 $\pm$ 0.20 [#]
0.1	6	6.37 $\pm$ 0.16
0.3	6	5.97 $\pm$ 0.12*
1	6	5.95 $\pm$ 0.08*
3	6	5.78 $\pm$ 0.07**
10	6	5.67 $\pm$ 0.05**
30	6	5.55 $\pm$ 0.10**

Each value indicates the mean $\pm$ S.E.M. of 6 experiments. * $p < 0.05$, ** $p < 0.01$; statistically significant vs. the value of the vehicle group. [#] $p < 0.01$; statistically significant vs. the value of the control group. n: number of experiments.

by 3 μ M atropine (Figs. 1, 2). In the absence of tetrodotoxin, zaldaride at 10 μ M and higher significantly inhibited the acetylcholine-induced increase in Isc (Fig. 1). However, in the presence of tetrodotoxin, zaldaride at 3–30 μ M did not affect the acetylcholine-induced effect (Fig. 2).

Acetylcholine Release Experiment The application of 15 mM KCl increased the release of acetylcholine from synaptosomes of colonic submucosal nerves (Table 1). Zaldaride at 0.3 μ M and higher significantly decreased the release of acetylcholine from the synaptosomes of colonic submucosal nerves (Table 1).

DISCUSSION

Acetylcholine increased Isc in the rat colonic preparations. It has been reported that the Isc response to acetylcholine in the rat small intestine is mediated by acetylcholine M_3 receptors positioned on the enterocytes and by acetylcholine M_1 receptors located on the submucosal nerves.⁹ In our study, the Isc response to acetylcholine was completely inhibited by atropine, a non-selective acetylcholine M receptor antagonist, and was partially attenuated by tetrodotoxin, a neuronal blocker. These findings suggest that acetylcholine increases Isc in the rat colon due to the direct activation of acetylcholine M_3 receptors potentiated on the colonic epithelium and to acetylcholine release from submucosal nerves mediated by acetylcholine M_1 or N receptors located on the submucosal nerves.

Zaldaride significantly reduced the acetylcholine-induced increase in Isc, whereas in the presence of tetrodotoxin it had no effect. Zaldaride also inhibited acetylcholine release from the synaptosomes of colonic submucosal nerves. Acetylcholine release from enteric cholinergic nerves is regulated by calmodulin.^{10–13} Neurotransmitter release in the mammalian nervous system are modulated by the phosphorylation of synapsin I, and regulated by Ca^{2+} /calmodulin-dependent protein kinase I or II.^{14–17} From these findings, the authors presume that zaldaride reduced the release of acetylcholine from colonic submucosal nerves due to the inhibition of Ca^{2+} /calmodulin-dependent protein kinase I or II activity.

In conclusion, it is suggested that zaldaride inhibits acetylcholine-induced electrolyte secretion due to the reduction of acetylcholine release from enteric submucosal nerves. To determine the mechanisms of action of zaldaride on the acetylcholine release from enteric nerves, further studies are needed in different species.

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