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Imidazoline Receptor Contributes to Ion and Water Transport across the Intestine of the Eel Acclimated to Sea Water

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ABSTRACT—Guanabenz, an I_2-imidazoline-related compound with high affinity for intestinal membrane of the eel (Kim *et al*., 1998), enhanced the transepithelial potential difference (PD) and short-circuit current (*Isc*) from serosa to mucosa after pretreatment with isobutylmethylxanthine (IBMX), serotonin (5-HT) and methacholine (MCh). The mucosal effect of guanabenz was not mimicked by adrenaline, indicating that the mucosal guanabenz binding site is not adrenoceptors. The mucosal guanabenz enhanced the *Isc* in a concentration-dependent manner. Similar enhancement in the *Isc* was also obtained after addition of other imidazoline derivatives such as ST93, clonidine, ST91, naphazoline and UK14,304 into the mucosal fluid. On the other hand, the effect of guanabenz was completely blocked by mucosal RX821002 or efaroxan, another imidazoline derivatives. Since some imidazoline derivatives act as agonists and others as antagonist, there must exist imidazoline receptor on the mucosal side of the eel intestine. Accompanied by an increase in the PD, NaCl and water absorption across the intestine was also enhanced by mucosal guanabenz. To search for endogenous ligands for the imidazoline receptor, luminal fluid in the intestine of the seawater eels was collected. However, most luminal fluid was ineffective. Only one among 10 samples showed guanabenz-like activity, suggesting that the endogenous ligands is secreted into the lumen under restricted condition alone.

INTRODUCTION

The concept of the imidazoline receptor or imidazoline/guanidinium receptive site was proposed at first, on the basis of structure/function studies, to be a non-adrenergic receptive site, mediating the hypotensive actions of clonidine in brain (Bousquet *et al*., 1984). Ligand binding sites soon proved the correctness of the surmise (Ernsberger *et al*., 1987), and soon established that imidazoline receptors existed in multiple forms, were expressed in a variety of organs, cells and tissues (Coupry *et al*., 1990; Jackson *et al*., 1992; Lachaud *et al*., 1992; Ivkovic *et al*., 1994; Regunathan and Reis, 1996). The identification of agmatine as an endogenous ligand(s) for imidazoline receptors (Li *et al*., 1994) and the probability that other "clonidine-displacing substances" exist (Atlas and Burstein, 1984; Ernsberger *et al*., 1988; Pinthong *et al*., 1995) suggest that imidazoline receptors may be a receptive component of a novel neurotransmitter, hormonal and/or paracrine system with widespread influences in the body's economy. Immunocytochemical localization of the imidazoline receptor is recently demonstrated in the mammalian central nervous system (Ruggiero *et al*., 1998), and this receptor is implicated in the pathogenesis of neurological disorders, based on changes in imidazoline receptor proteins and binding sites in the human brain in suicide victims (Garcia-Sevilla *et al*., 1996), aging (Garcia-Sevilla *et al*., 1995; Regunathan *et al*., 1996), Alzheimer disease (Ruiz *et al*., 1993; Garcia-Sevilla *et al*., 1998), Huntington's and Parkinson's disease (Reynolds *et al*., 1996). However, the precise roles of imidazoline receptors are not clear yet. A good model for elucidating roles of imidazoline receptors is required.

Epithelial membrane of the eel intestine contains specific clonidine binding sites. The specific binding is inhibited competitively by various imidazoline derivatives: the rank order being guanabenz > cirazoline = naphazoline = UK14304 = ST587 ≥ clonidine ≥ idazoxan = RX821002 = tolazoline > ST93 = oxymetazoline = amiloride = ST91 > yohimbine = efaroxan = rauwolscine ≥ adrenaline = ST567 = histamine = agmatine (Kim *et al*., 1998). Although this rank order suggests existence of imidazoline receptor, no identical orders are reported in mammals, an implication of new subtype of imidazoline receptors in the eel intestine.

Using eel intestine as a model system, the present study was aimed to elucidate the role of imidazoline receptor in tissue or cell level. Guanabenz was selected as a ligand to stimulate the receptor, because it exhibited highest affinity in the eel receptor (Kim *et al*., 1998). Mucosal application of guanabenz enhanced NaCl and water absorption across the intestine of the eel acclimated to sea water.

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MATERIALS AND METHODS

Japanese cultured eels, *Anguilla japonica*, weighing about 210 g were kept in seawater aquaria (20°C) for more than 1 week without food. After decapitation the intestine was removed and external muscle layers were carefully stripped off using tweezers according to Ando and Kobayashi (1978). The stripped intestine was opened and mounted as a flat sheet in an Ussing chamber with an exposed area of 0.5 cm^2. Both sides of the intestine were bathed with Krebs' bicarbonate Ringer solution consisting of (mmol l^{-1}): 118.5 NaCl, 4.7 KCl, 3.0 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$, and containing 5 mmol l^{-1} glucose and 5 mmol l^{-1} alanine. The bathing solutions (3.5 ml each) were kept at 20°C and circulated continuously by lifting with 95% O_2/5% CO_2 gas mixture (pH 7.4).

The transepithelial potential difference (PD) was recorded through a pair of calomel electrodes with a polyrecorder (EPR-151A, Toa Electronics, Tokyo, Japan) as the serosal potential with respect to the mucosa. To determine the tissue resistance (*Rt*), rectangular pulses, 30 μA for 500 ms were applied across the intestinal sheet every 5 min. Rt was calculated from the deflection of the PD. The short-circuit current (*Isc*) was obtained from the ratio of PD to *Rt*.

Net water flux was calculated directly from the difference between the rates of effluent and perfusate flow in the perfusion system. Details of simultaneous measurements of net water flux and PD have been described elsewhere (Ando *et al.*, 1986). Briefly, the serosal fluid was perfused through the everted intestine, whose muscle layers being stripped off, at a constant rate (around 150 μl min^{-1}) and the effluent was collected every 10 min. Therefore, the difference between the two rates (net water flux) was measured every 10 min. Net Na^+, K^+ and Cl^- fluxes were also calculated simultaneously from the collected fluid volume and ionic concentrations as described previously (Ando, 1983). Na^+ and K^+ concentrations were measured by flame photometry (FPF-2A, Hiranuma, Mito, Japan) and Cl^- concentration was determined with a chloride counter (CL-5M, Hiranuma).

After pretreatment with 10^{-5} mol l^{-1} isobutylmethylxanthine (IBMX), 10^{-6} mol l^{-1} serotonin (5-HT) and 10^{-6} mol l^{-1} methacholine (MCh), an experimental condition used to see the serosal effects of adrenaline, somatostatin and neuropeptide Y (Ando and Omura, 1993; Uesaka *et al.*, 1994; 1996), guanabenz was applied to the mucosal fluid.

Acetyl-β-methylcholine bromide (MCh), 3-isobutyl-1-methyxanthine (IBMX), 5-hydroxytryptamine creatine sulfate (5-HT), adrenaline HCl, clonidine HCl, yohimbine HCl, efaroxan HCl, torazoline HCl, naphazoline HCl and amiloride HCl were purchased from Sigma Chemical (St Louis, MO). RX821002 HCl, agmatine sulfate, idazoxan HCl, cirazoline HCl, oxymetazoline HCl, rauwolscine HCl, guanabenz acetate and UK14,304 were from Research Biochemicals Inc. (Natick, MA). ST91 HCl, ST93 HCl, ST567 HBr and ST587 nitrate were gift from Boeringer Ingelheim (KG, Germany). Eel somatostatin (eSS-25II) was isolated from eel intestine in our laboratory (Uesaka *et al.*, 1994).

RESULTS

Effects of guanabenz on PD, *Isc* and *Rt*

The seawater eel intestine in normal Ringer solution shows serosa-negative PD and *Isc*, which is due to active Cl^- transport from mucosa to serosa (Ando *et al.*, 1975). Under such condition, guanabenz (10^{-7}–10^{-6} mol l^{-1}) had no effect on PD, *Isc* and *Rt*. However, after pretreatment with IBMX, 5-HT and MCh, guanabenz enhanced the PD and *Isc* remarkably. Serosal effect of guanabenz was mostly inhibited by yohimbine (10^{-6} mol l^{-1}), an α_2 adrenoceptor antagonist, but mucosal effect was not. Figure 1 shows mucosal effect of guanabenz. Although mucosal adrenaline had no effects,

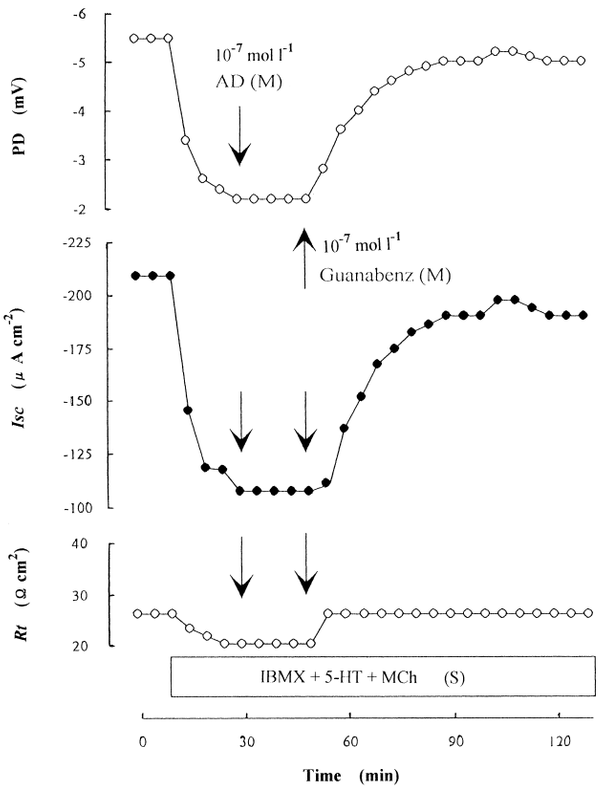

Fig.1. Effects of guanabenz on PD, *Isc* and *Rt*. Guanabenz (10^{-7} mol l^{-1}) was added to the mucosal fluid (M) after pretreatment with 10^{-5} mol l^{-1} isobutylmethylxanthine (IBMX), 10^{-6} mol l^{-1} serotonin (5-HT) and 10^{-6} mol l^{-1} methacholine (MCh) at the second arrows. Under the same condition, 10^{-7} mol l^{-1} adrenaline (AD) added to the mucosal fluid (M) had no effect on these 3 parameters (first arrows).

mucosal guanabenz enhanced the PD and *Isc* immediately, indicating that the mucosal effect of guanabenz is distinct from that of adrenaline. The effect of mucosal guanabenz was concentration-dependent; with a threshold of 10^{-9} mol l^{-1} and

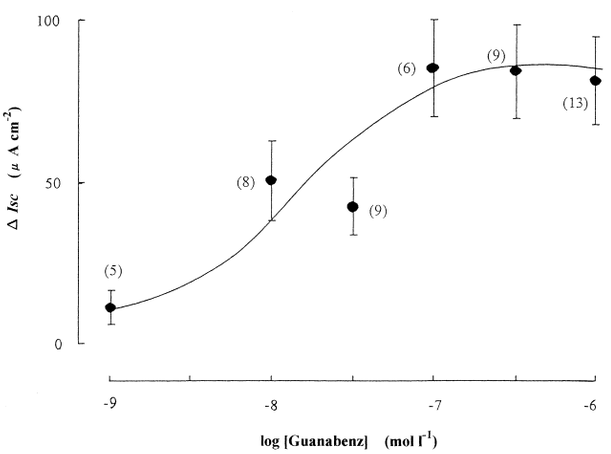

Fig. 2. Concentration-dependency of the effect of guanabenz. The change in the *Isc* (Δ*Isc*) after addition of guanabenz into the mucosal fluid was plotted against its concentration (on a logarithmic scale). Each point and vertical bar indicate the mean value and S.E.M.. Number of preparations are presented in parentheses.

maximal effect at 10^{-7} mol l^{-1} (Fig. 2). Similar enhancement in the PD and *Isc* was also observed after mucosal application of ST93 (10^{-7} mol l^{-1}). Although 10^{-7} mol l^{-1} clonidine, ST91, naphazoline and UK14,304 also enhanced the PD and *Isc*, their efficacy was low; 80% of guanabenz in clonidine, 78% in ST91, 63% in naphazoline and 28% in UK14,304 (data not shown). At the same concentration (10^{-7} mol l^{-1}), cirazoline, ST587, RX821002, tolazoline, oxymetazoline, amiloride, efaroxan, ST567 and agmatine did not enhance the PD and *Isc* at all (data not shown).

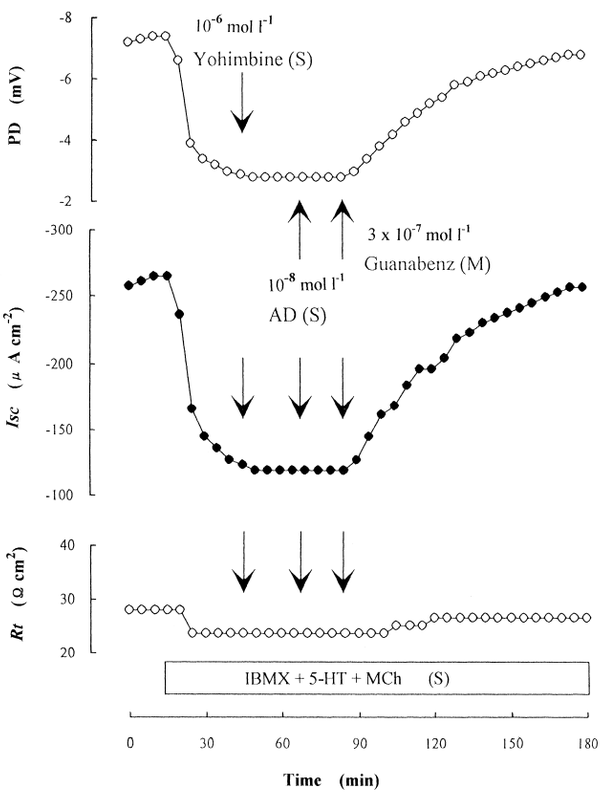

Fig. 3. Effects of guanabenz after blocking serosal adrenoceptor with yohimbine. After pretreatment with IBMX (10^{-5} mol l^{-1}), 5-HT (10^{-6} mol l^{-1}) and MCh (10^{-6} mol l^{-1}), 10^{-6} mol l^{-1} yohimbine was added to the serosal fluid (first arrows). Complete blockage of adrenoceptor was confirmed by no response of adrenaline (AD) added to the serosal fluid (second arrows). Then 3×10^{-7} mol l^{-1} guanabenz was added to the mucosal fluid (M) at the third arrows.

To deny a possibility that mucosal guanabenz stimulates catecholamine secretion from enterocytes such as enterochromaffin cells, the following experiment was performed. Since adrenoceptor exists in serosal side of the seawater eel intestine, and is blocked by yohimbine (Ando and Omura, 1993), effect of mucosal guanabenz was examined in the presence of serosal yohimbine. Even after blocking serosal adrenoceptor with yohimbine, mucosal guanabenz enhanced the PD and *Isc* immediately (Fig. 3).

Table 1 shows effect of mucosal guanabenz in various parts of the intestine. Eel intestine (ca. 15 cm) was divided into 6 parts; anterior, middle, posterior 3, posterior 2, posterior 1 and rectum, from rostral to caudal. Since the anterior part and rectum have lower PD and lower net water flux (Ando, 1980), these 2 parts were omitted in the present study. The enhancement in *Isc* by mucosal guanabenz was highest in the middle part of the intestine, and tended to decrease gradually to rectum. Although the *Rt* tended to increase after guanabenz, the effect was not significant statistically.

Effect on ion and water transport

After pretreatment with IBMX, 5-HT and MCh, mucosal guanabenz enhanced net Na^+, Cl^- and water fluxes 1.5 fold, accompanied by an increase in the PD (Table 2).

Search for imidazoline receptor antagonist

If mucosal guanabenz acts on the imidazoline receptor as an agonist, some imidazoline derivatives can be expected

Table 1. Effects of guanabenz on transepithelial potential difference (PD), short circuit current (*Isc*) and tissue resistance (*Rt*) in various parts of the intestine isolated from the seawater eels. Guanabenz (10^{-7} mol l^{-1}) was added to the mucosal fluid after pretreatment with isobutylmethyl-xanthine (IBMX, 10^{-5} mol l^{-1}), serotonin (5-HT, 10^{-6} mol l^{-1}) and methacholine (MCh, 10^{-6} mol l^{-1}). The latter 3 agents were added to the serosal fluid.

Condition	Middle (5)	Posterior 3 (5)	Posterior 2 (6)	Posterior 1 (6)
		PD (mV)		
Normal Ringer	−6.7±0.7	−7.9±0.6	−7.5±0.7	−6.9±1.2
IBMX+5-HT+MCh	−2.8±0.5	−3.5±0.5	−3.4±0.6	−2.1±0.8
Guanabenz	−4.9±0.5**	−5.8±0.5*	−6.5±0.6***	−5.4±0.8***
		Isc (μ A cm^{-2})		
Normal Ringer	−525.8±82.9	−567.1±106.7	−331.3±47.3	−194.1±37.2
IBMX+5-HT+MCh	−231.5±53.7	−271.8±70.9	−180.1±43.4	−68.9±19.4
Guanabenz	−393.9±87.7**	−414.1±79.4***	−279.4±32.3***	−123.9±23.4*
		Rt (Ω cm^2)		
Normal Ringer	13.4±1.0	15.3±2.0	24.5±3.5	37.8±3.6
IBMX+5-HT+MCh	12.8±1.1	14.7±1.9	21.0±3.4	26.5±5.4
Guanabenz	13.7±1.3	15.6±2.1	24.2±2.9	47.6±6.8

Values are means±S.E.M.. Number of preparations are shown in parentheses.
*P<0.01, **P<0.005, *** P<0.001 compared with those treated with IBMX, 5-HT and MCh (paired *t*-test).

Table 2. Effects of guanabenz on the PD, net ion fluxes (J^{Na}_{net}, J^{K}_{net}, J^{Cl}_{net}), and net water flux ($J^{H_2O}_{net}$) across the intestine of seawater eel. Guanabenz (3×10^{-7} mol l^{-1}) was added to the mucosal fluid after pretreatment with IBMX (5×10^{-5} mol l^{-1}), 5-HT (5×10^{-6} mol l^{-1}) and MCh (5×10^{-6} mol l^{-1}). The latter 3 agents were added to the serosal fluid.

	PD (mV)	J^{Na}_{net} (μ eq cm^{-2} 10 min^{-1})	J^{K}_{net} (μ l cm^{-2} 10 min^{-1})	J^{Cl}_{net}	$J^{H_2O}_{net}$
Normal Ringer	–8.0±0.5	2.3±0.3	0.0±0.0	2.3±0.2	14.7±1.8
IBMX+5-HT+MCh	–4.1±0.4	1.4±0.2	0.1±0.0	1.1±0.3	8.3±1.3
Guanabenz	–6.4±0.3*	2.2±0.2**	0.0±0.0	2.1±0.1**	13.2±1.0**

Values are means±S.E.M. (n=8)
* P<0.002, ** P<0.001, compared to those treated with IBMX, 5-HT and MCh (paired *t*-test).

to act as an antagonist. As shown in Fig. 4. mucosal RX821002 (10^{-6} mol l^{-1}) completely blocked the effect of guanabenz. Similar antagonistic effect was also observed in efaroxan (10^{-6} mol l^{-1}). However, cirazoline, naphazoline, UK14,304, ST587, clonidine, idazoxan, tolazoline, ST93, oxymetazoline, amiloride, ST91, yohimbine, rauwolscine, ST567 had no antagonistic effect (data not shown). Even after treatment with RX821002 or efaroxan, eel somatostatin, a potent stimulator of intestinal NaCl and water transport (Uesaka *et al*., 1994), enhanced the PD, *Isc* and Rt (Fig. 4), suggesting specific action of RX821002 and efaroxan on the imidazoline receptor.

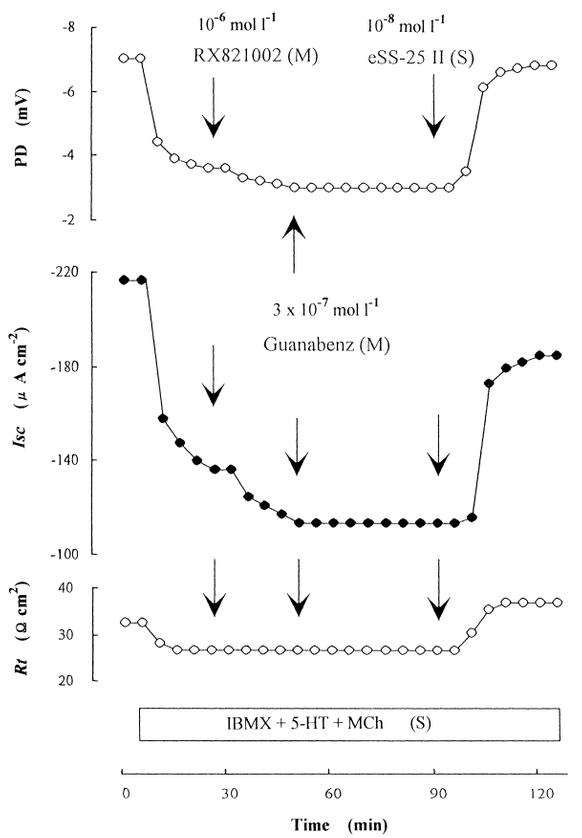

Fig. 4. Antagonistic action of RX821002, an imidazoline derivative. After pretreatment with IBMX (10^{-5} mol l^{-1}), 5-HT (10^{-6} mol l^{-1}) and MCh (10^{-6} mol l^{-1}), 10^{-6} mol l^{-1} RX821002 was added to the mucosal fluid (first arrows). In the presence of RX821002, mucosal guanabenz (3×10^{-7} mol l^{-1}) had no effects (second arrows). At the third arrows, 10^{-8} mol l^{-1} eel somatostatin (eSS-25II)was added to the serosal fluid (S).

Search for endogenous ligands for the imidazoline receptor

Since both agonists and antagonists act from mucosal side of the intestine, the endogenous ligands should be present in the luminal fluid. Therefore, after collecting luminal fluid in the intestine of the seawater eel, the collected fluid was added to the mucosal side in the Ussing chamber. However, most luminal fluids were ineffective. Only one among 10 samples showed guanabenz-like activity as shown in Fig. 5. Although much luminal fluid collected from 20 seawater eels was forced through C18 cartridge (Sep-Pak, Millipore, Milford, MA), active substance was not detected from the retained material.

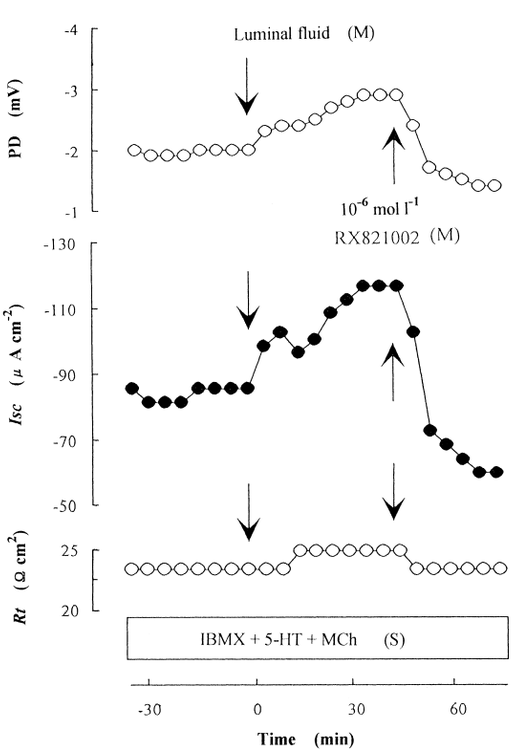

Fig. 5. Effects of luminal fluid obtained from the intestine of the seawater eel. After pretreatment with IBMX (10^{-5} mol l^{-1}), 5-HT (10^{-6} mol l^{-1}) and MCh (10^{-6} mol l^{-1}), 1/3 parts of the luminal fluid from one eel was added to the mucosal fluid (first arrows). At the second arrows, 10^{-6} mol l^{-1} RX821002 was added to the mucosal fluid (M). However, this response was exceptional; observed only in one sample and other 9 samples had no effect.

DISCUSSION

The present study demonstrates existence of guanabenz receptive site(s) and also demonstrates that this site(s) works actually in the seawater eel intestine. The guanabenz-specific receptive site(s) seems to be located on the mucosal side of the intestine, since guanabenz acts from mucosal side and starts to enhance the *Isc* within 2 min. The mucosal effect of guanabenz was not mimicked by mucosal adrenaline, indicating that the mucosal guanabenz-binding site(s) is distinct from the adrenoceptors. The mucosal effect of guanabenz was concentration-dependent. Other imidazoline derivatives, such as ST93, clonidine, ST91, naphazoline and UK14,304, also showed guanabenz-like action, though higher concentrations are needed in the latter four cases. On the other hand, the effect of mucosal guanabenz was completely blocked by RX821002 or efaroxan, another imidazoline derivatives. All together, these results indicate existence of imidazoline receptor(s) on the mucosal side of the intestine.

The enhancement in the serosa-negative PD and *Isc* can be explained by an increase in active Cl^- absorption, since the serosa-negative PD and *Isc* is due to active Cl^- transport from mucosa to serosa in the eel intestine (Ando *et al*., 1975). In fact, mucosal guanabenz enhances net Na^+, Cl^- and water fluxes (Table 2).

Similar enhancement in the PD, *Isc*, NaCl and water transport has been observed after serosal adrenaline (Ando and Kondo, 1993; Ando and Omura, 1993), eel somatostatin (eSS-25II, Uesaka *et al*., 1994) or eel neuropeptide Y (eNPY, Uesaka *et al*., 1996). Therefore, it is likely that mucosal guanabenz stimulates adrenaline, eSS-25II or eNPY secretion from paracrine cells, and one or some of these regulators stimulate(s) NaCl and water absorption across the intestinal epithelia. However, a possibility of adrenaline secretion can be denied, because the effect of guanabenz still remains even after blocking serosal adrenoceptor with yohimbine (Fig 3). Since suitable antagonists for eSS-25II or eNPY, both extracted from the eel intestine, are not found yet, participation of these peptides is not examined.

Since the imidazoline receptor(s) exists on the luminal side of the intestine, endogenous ligands should be present in the luminal fluid. However, most luminal fluid was ineffective. Only one among 10 samples showed guanabenz-like activity, suggesting that the endogenous ligands are secreted into the lumen under restricted condition alone. If such ligands are secreted into the lumen, the ligand-secreting organ(s) must be upper parts of the digestive tract. Therefore, we are now extracting endogenous ligands from esophagus, stomach and intestine. Recently, it is demonstrated that eel stomach contains such guanabenz-like substances (Takase *et al*., unpublished observation).

In mammals, only agmatine is authorized as an endogenous ligand for the imidazoline receptor in mammals (Li *et al*., 1994; Reis and Regunathan, 1998). However, agmatine does not seem to be an endogenous ligand in the eel intestine, because it has no effect on the PD and *Isc* and because it does not displace [^{3}H]clonidine from epithelial membranes isolated from the eel intestine (Kim *et al*., 1998).

The effect of guanabenz is observed only after inhibiting NaCl and water absorption with IBMX, 5-HT and MCh. Similar restoration of water absorption has been observed after serosal application of eSS-25II (Uesaka *et al*., 1994), adrenaline (Ando and Omura, 1993) and eNPY (Uesaka *et al*., 1996). Since water absorption across the intestine is vital in seawater teleosts, and its inhibition leads to death in sea water (Takei *et al*., 1998), such restoration mechanism may be advantageous in seawater teleosts. The present study demonstrates existence of imidazoline receptor on the lumen, and contribution of such receptor to the restoration of water absorption. Although characteristics of the endogenous ligands to act on the imidazoline receptor is not clarified yet, such ligands may be secreted into the lumen from the upper part of the digestive tract as a “luminocrine”. Such ligands may be novel, distinct from agmatine authorized in mammals.

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