Novel [3H]Clonidine Binding Sites in the Intestine of the Eel Acclimated to Sea Water

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ABSTRACT—Novel clonidine binding sites were characterized in the intestinal membrane isolated from seawater eels. The specific clonidine binding sites consisted of at least two classes, high affinity ($K_d = 1.4 \pm 0.3$ nM, n = 5) and low affinity ($K_d = 175 \pm 34$ nM, n = 5) sites. The specific binding of 2 nM [3H]clonidine was most enhanced at 20°C and at pH 7.5, and reversed by unlabelled clonidine. Such binding was hardly inhibited by adrenaline, yohimbine or rauwolscine, indicating that most binding sites are distinct from α_2-adrenoceptor. The specific clonidine binding was inhibited by various imidazoline/guanidinium drugs, indicating existence of imidazoline/guanidinium receptive sites (IGRS) or imidazoline receptors in the eel intestine. Competition experiments revealed that rank order to displace 2 nM [3H]clonidine from their binding sites was as follows: guanabenz > cirazoline = naphazoline = UK14304 = ST587 $\geq$ clonidine $\geq$ idazoxan = RX821002 = tolazoline > ST93 = oxymetazoline = amiloride = ST91 > yohimbine = efaroxan = rauwolscine $\geq$ adrenaline = ST567 = histamine = agmatine. The rank order was different from those in I_1 or I_2 sites of IGRS reported in various mammalian tissues, suggesting existence of new IGRS, non I_1 and non I_2 sites, in the eel intestine. In addition, structure-affinity relationships are discussed from the results of competition experiments. Although physiological role of IGRS is not clear yet even in mammalian cells/tissues, eel intestine may be a good model to elucidate how the IGRS act in the cell and to decide what is the endogenous ligand for the IGRS, since eel intestine contains great amount of IGRS and it responds to guanabenz, an exogenous clonidine derivative.

INTRODUCTION

Intestinal NaCl and water absorption is higher in seawater than in freshwater eels (Oide and Utida, 1967; Skadhauge, 1969; Ando, 1975; Ando *et al.*, 1992). Following acclimation of eels to sea water, the intestine enhances sensitivity to adrenaline (Ando and Hara, 1994). Since adrenaline is known to enhance NaCl and water absorption in the eel intestine (Ando and Kondo, 1993; Ando and Omura, 1993), the higher sensitivity to adrenaline may contribute to the higher NaCl and water absorption in the seawater eel intestine, even if adrenaline levels are identical in both seawater and freshwater eels. The enhancement in adrenaline sensitivity in the seawater eel intestine may be due to an increase in receptor number, or an increase in affinity to adrenaline, perhaps by induction of new receptors with higher affinity to adrenaline.

In order to evaluate these two possibilities, the present study was planned to characterize adrenaline receptors in the eel intestine, by using [3H]clonidine as a radioligand, since our previous study had demonstrated that clonidine acts on the eel intestine as an adrenoceptor agonist (Ando and Kondo, 1993;

Ando and Omura, 1993). However, most [3H]clonidine binding in the seawater eel intestine was hardly displaced by adrenaline. Therefore, characterization of such novel [3H]clonidine binding sites was performed. Some characteristics of these binding sites were similar to the imidazoline/guanidinium receptive sites (IGRS) or imidazoline receptors reported in mammals (Coupry *et al.*, 1990; Jackson *et al.*, 1992; Lachaud *et al.*, 1992; Ivkovic *et al.*, 1994; Regunathan and Reis, 1996).

MATERIALS AND METHODS

Animals

Cultured Japanese eels (*Anguilla japonica*), weighing about 200 g, were kept in seawater (20°C) for more than one week without food. After decapitation, the intestine was excised and the lumen flushed with ice-cold Krebs bicarbonate Ringer's solution consisting of (mM); 118.5 NaCl, 4.7 KCl, 3.0 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 24.9 NaHCO₃. The intestine was then opened, and the mucosal epithelial layer was scraped and frozen at –80°C.

Membrane preparations

Frozen mucosal epithelia were homogenized in 10 vol. of ice-cold buffer (pH 7.5), containing 40 mM Tris/HCl, 40 mM NaCl and 100 mM sucrose, using a polytron (T8, setting 6, × 10s bursts). The homogenates were centrifuged at $900 \times g$ for 10 min at 4°C. The supernatant was collected and centrifuged at $11,000 \times g$ for 20 min at 4°C. The pellets were suspended in 10 vol. of the same buffer cited

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above and incubated at 25°C for 30 min to dissociate endogenous ligands. After centrifugation at 11,000 × g for 20 min, the pellets were resuspended with an assay buffer (pH 7.5) consisting of 40 mM Tris/HCl and 5 mM MgCl$_2$. Proteins were determined with micro BCA protein assay reagent kit (Pierce Chemical Company) in an aliquot of the suspension. Bovine serum albumin was used as a standard. Na$^+$/K$^+$ ATPase (ouabain-sensitive ATPase) activity of the membrane suspension was about 1.6 μmol Pi/mg protein for 20 min.

Chemicals

[^{3}H]clonidine HCl (specific activity of 61.9 Ci/mmol) were obtained from New England Nuclear. The following reagents were purchased from Sigma; (–)adrenaline HCl, clonidine HCl, yohimbine HCl, efaroxan HCl, tolazoline HCl, naphazoline HCl, amiloride HCl. RX821002 HCl, agmatine sulfate, idazoxan HCl, cirazoline HCl, rauwolscine HCl, oxymetazoline HCl, guanabenz acetate and UK14,304, which was dissolved in DMSO, were from Research Biochemicals Inc.. ST91 HCl, ST93 HCl, ST567 HBr and ST587 nitrate were gifts from Boeringer Ingelheim KG, Germany.

[^{3}H]clonidine binding studies

Binding experiments were performed principally following a method by Azumaya and Tsutsui (1996). Saturation experiments were performed by incubating the membranes in 200 μl assay buffer (pH 7.5) for 60 min at 20°C with increasing concentrations of [^{3}H]clonidine. Non-specific binding was defined as a binding in the presence of 1 mM unlabelled clonidine. Incubation was terminated by the addition of 1 ml ice-cold assay buffer followed by centrifugation at 11,000 × g for 4 min at 4°C. The pellets were washed twice with cold buffer, and the radioactivity of resultant pellets was counted by means of crystal scintillation counting. The binding capacity was not different between the frozen and the unfrozen membrane preparations. The pH in the incubation media was adjusted with Tris/HCl between 7.0 and 10.0, and with phosphate buffer below pH 6.8. The binding was performed at 0°C on ice, 20 or 37°C in water bath.

Competition studies were carried out with 2 nM [^{3}H]clonidine and range of concentrations of various reagents under the same condition as the saturation experiments. At 2 nM [^{3}H]clonidine, the non-specific binding did not exceed 30% of total binding. Specific binding represented approximately 10% of the total radioligand concentration.

Data analysis

The results are expressed as the mean ± S.E.M. of n experiments performed in duplicate or tripricate. B$_{max}$ and K$_d$ were driven from a non-linear regression analysis using a program by Ishii *et al.* (1983). IC$_{50}$ and slope factors were deduced from pseudo-Hill plot of the competition data.

RESULTS

[^{3}H]Clonidine binding sites

Figure 1 shows saturation analysis of [^{3}H]clonidine binding to intestinal membrane homogenates. The specific binding (total binding – non-specific binding) increased with an increase in [^{3}H]clonidine concentrations (60 pM to 100 nM). The Scatchard plot of the specific binding was shown in Fig. 1B. Non-linear regression analysis shows that the clonidine binding sites consist of at least two different types, high affinity sites and low affinity sites. B$_{max}$ and K$_d$ of the high affinity sites were 16.9 ± 4.0 fmol/mg protein and 1.4 ± 0.3 nM, respectively (n = 5). B$_{max}$ = 984 ± 132 fmol/mg protein, K$_d$ = 175 ± 34 nM (n = 5) in the low affinity sites. Using 2 nM [^{3}H]clonidine, all of the following experiments were performed, concentrated only in high affinity sites.

The specific binding of 2 nM [^{3}H]clonidine was pH- and temperature-dependent. Maximal binding was obtained at 20°C (Fig. 2A), body temperature of the eel cultured at 20°C, and at pH of around 7.5 (Fig. 2B). This binding was reversed by addition of unlabelled clonidine (Fig. 3).

Competition experiments

In order to examine whether the clonidine binding sites in the eel intestine is identical with the adrenaline receptor, clonidine binding was examined in the presence of adrenaline. As shown in Fig. 4, however, adrenaline hardly displaced
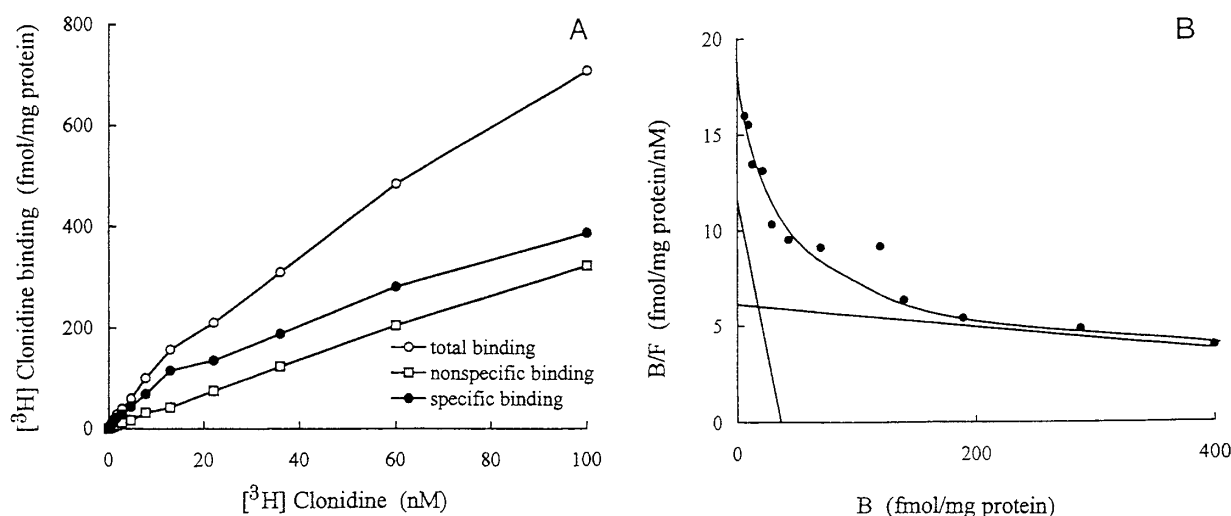

Fig. 1. (**A**) Saturation analysis of [^{3}H]clonidine binding (60 pM to 100 nM) to intestinal membrane homogenates at 20°C and pH 7.5: (○) total [^{3}H]clonidine binding, (●) specific binding and (□) non-specific binding (in the presence of 1 mM unlabelled clonidine). Each point depicts mean value of triplicate determinations. (**B**) The Scatchard plot of [^{3}H]clonidine specific binding indicating two classes of sites. The data from 5 experiments are B$_{max}$ = 16.9 ± 4.0 fmol/mg protein, K$_d$ = 1.4 ± 0.3 nM and B$_{max}$ = 984 ± 132 fmol/mg protein, K$_d$ = 175 ± 34 nM for the high- and low-affinity binding sites, respectively.
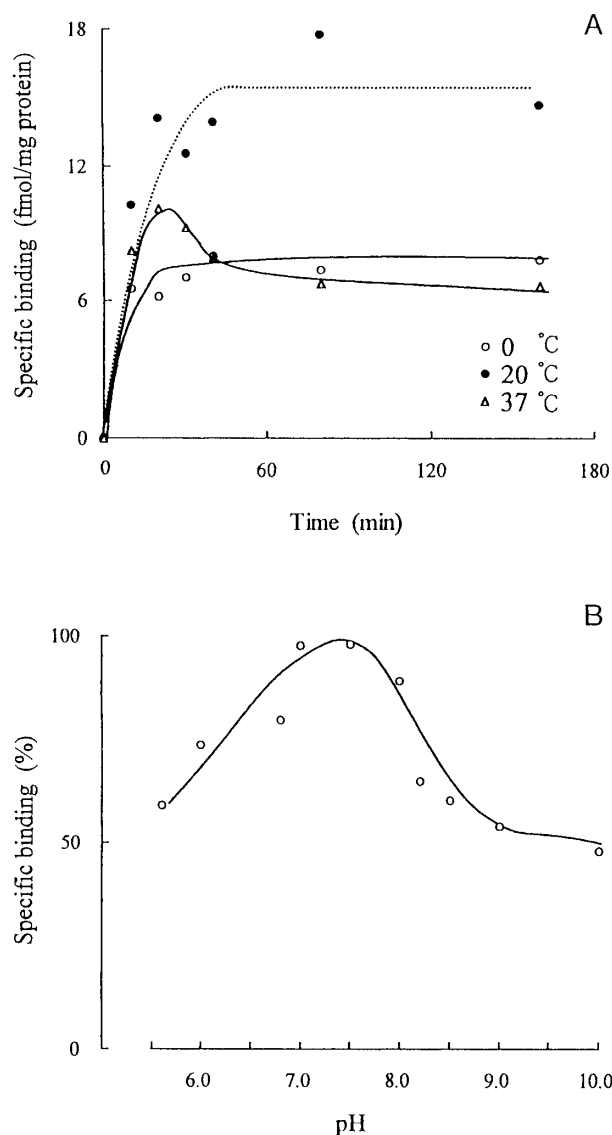
Fig. 3. Reversibility of 2 nM [³H]clonidine binding to intestinal membrane. After incubation of membrane fractions with 2 nM [³H]clonidine for 60 min, unlabelled clonidine (1 mM) was added (arrow). Each point represents mean value in triplicate at 20°C and pH 7.5.

Fig. 2. (**A**) Temperature dependency of 2 nM [³H]clonidine binding to intestinal membrane. The membrane fractions were incubated with [³H]clonidine at 0 (○), 20 (●) and 37 (△) °C, respectively (pH 7.5). Specific binding was determined after 10, 20, 30, 40, 80 and 160 min in triplicate. (**B**) pH dependency of 2 nM [³H]clonidine binding to the intestinal membrane fractions. The pH in the incubation media was adjusted with Tris/HCl between 7.0 and 10.0, and with phosphate buffer below pH 6.8. Specific binding is expressed as a ratio to the specific binding at pH 7.5 (100%). Each point depicts mean value of triplicate determinations at 20°C.

Fig. 4. Competition of specific binding of 2 nM [³H]clonidine by various adrenoceptor reagents or endogenous ligands. Values are mean ± S.E.M. of 5 to 17 independent experiments performed in duplicate. Intestinal membrane were incubated at 20°C for 60 min (pH 7.5) with 2 nM [³H]clonidine. Nonspecific binding was defined as the binding in the presence of 1 mM unlabelled clonidine. (●)clonidine; (○) adrenaline; (■) yohimbine; (□) rauwolscine; (▲) histamine; (△) agmatine. Number of experiments are shown in parentheses.

[³H]clonidine. Similar scarce competition were obtained in rauwolscine and yohimbine, α_2-adrenoceptor antagonists. Although both clonidine and histamine contain imidazole ring, histamine did not compete with [³H]clonidine, indicating that the clonidine binding sites do not recognize imidazole ring itself. Agmatine, an endogenous clonidine displacing substance in the bovine brain (Li *et al.*, 1994), did not compete with [³H]clonidine, either. Altogether, these results suggest existence of novel clonidine binding sites in the eel intestine, which is distinct from adrenaline-, histamine- and agmatine-
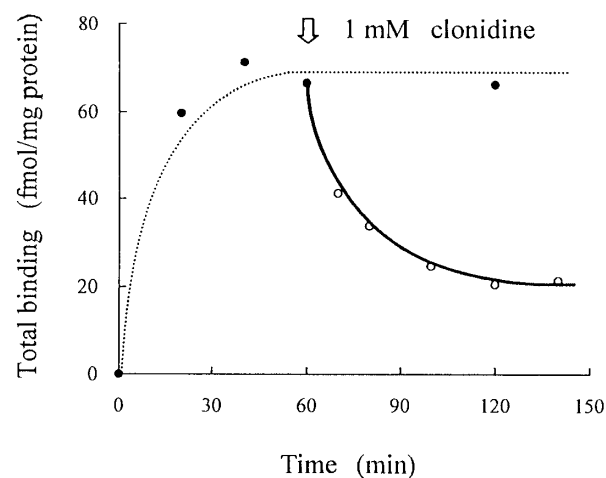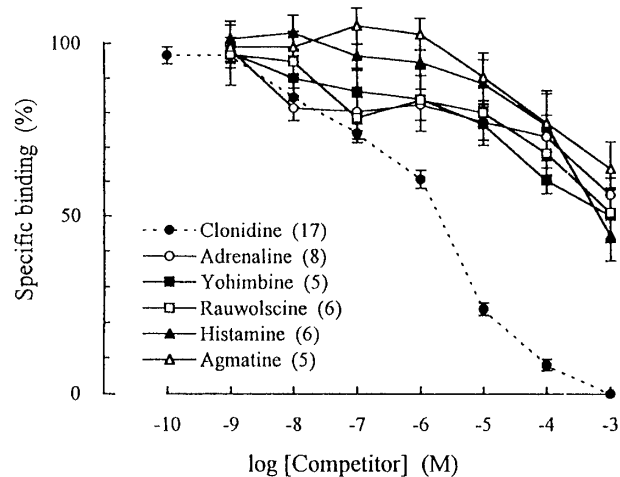
receptors.

To characterize the novel clonidine binding sites further, competition with various clonidine derivatives were examined (Fig. 5). Displacing activity of ST567 was similar to those of adrenaline, histamine and agmatine. On the other hand, UK14304 displaced [³H]clonidine more effectively at lower concentrations (10⁻⁸–10⁻⁶ M) than unlabelled clonidine. Displacing activity of ST91 was lower than clonidine (Fig. 5A). As shown in Fig. 5B, ST93 and ST587 also interacted with [³H]clonidine, and the displacing activity was greater in ST587
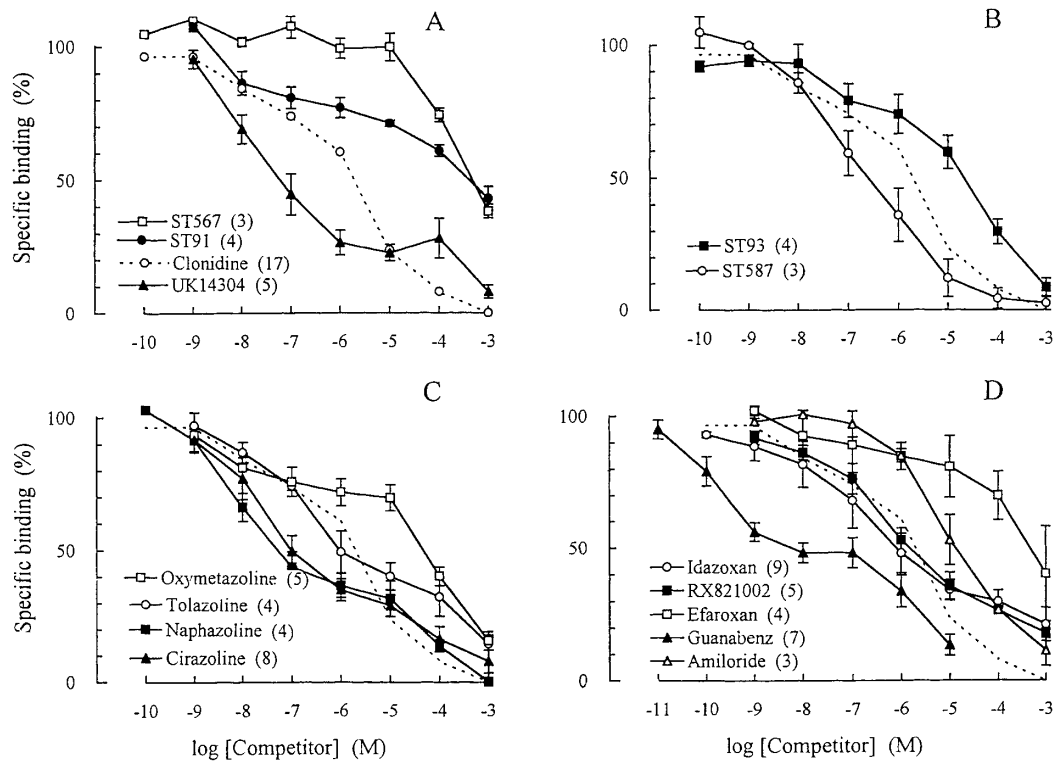
Fig. 5. Competition curves between [³H]clonidine and various clonidine derivatives. Procedures are the same as shown in Fig. 4 except for competitors. (**A**) (○) clonidine; (●) ST91; (□) ST567; (▲) UK14304. (**B**) (○) ST587; (■) ST93. (**C**) (○) tolazoline; (■) naphazoline; (□) oxymetazoline; (▲) cirazoline. (**D**) (○) idazoxan; (■) RX821002; (□) efaroxan; (▲) guanabenz; (△) amiloride. Number of experiments are shown in parentheses. Competition curve of clonidine is indicated as a dotted line for comparison in each figure.

than in ST93. Torazoline also displaced [³H]clonidine as well as clonidine (Fig. 5C). Naphazoline and cirazoline inhibited specific clonidine binding similarly, but their inhibitory effects were greater than that of tolazoline. On the other hand, the inhibition by oxymetazoline was smaller than that by tolazoline. Idazoxan also inhibited [³H]clonidine binding similarly as clonidine (Fig. 5D). Almost identical competition curve was obtained with RX821002. On the other hand, competition of [³H]clonidine binding by efaroxan was smaller than those by idazoxan or RX821002. Guanabenz displaced [³H]clonidine most effectively among all reagents tested in the present study (Fig. 5, Table 1). Amiloride also inhibited [³H]clonidine binding, but the effect was much smaller than that of guanabenz (Fig. 5D).

Table 1 summarizes IC_{50} and slope factors obtained from Figs. 4 and 5. Rank order of potencies to compete for [³H]clonidine binding was as follows: guanabenz > cirazoline = naphazoline = UK14304 = ST587 ≥ clonidine ≥ idazoxan = RX821002 = tolazoline ≥ ST93 = oxymetazoline = amiloride = ST91 > yohimbine = efaroxan = rauwolscine ≥ adrenaline = ST567 = histamine = agmatine.

In order to examine whether the binding sites of these clonidine derivatives are identical with that of clonidine, total binding was examined in the presence of idazoxan, cirazoline or guanabenz at their maximal concentrations, and in the mixture of these derivatives with 1 mM clonidine. As shown in Fig. 6, displacement of [³H]clonidine by cirazoline, idazoxan

Table 1. Inhibition of 2 nM [³H]clonidine binding to eel intestinal membranes by various clonidine derivatives tested in present study. Log [IC_{50}] and slope factor were calculated by pseudo-Hill plot of the data in Figs. 4 and 5.

Competitor	n	log [IC_{50}] (M)	Slope factor
Guanabenz	7	−7.7 ± 0.4**	0.4 ± 0.0
Cirazoline	5	−6.6 ± 0.4	0.4 ± 0.0
Naphazoline	4	−6.5 ± 0.2	0.4 ± 0.1
UK14304	5	−6.4 ± 0.3	0.4 ± 0.0
ST587	3	−6.4 ± 0.3	0.6 ± 0.1
Clonidine	17	−6.1 ± 0.1	0.5 ± 0.0
Idazoxan	9	−5.6 ± 0.5	0.4 ± 0.0
RX821002	5	−5.5 ± 0.2*	0.3 ± 0.0
Tolazoline	4	−5.4 ± 0.3*	0.4 ± 0.0
ST93	4	−5.2 ± 0.4*	0.4 ± 0.0
Oxymetazoline	5	−4.8 ± 0.2***	0.3 ± 0.0
Amiloride	3	−4.8 ± 0.2***	0.5 ± 0.1
ST91	4	−4.7 ± 0.2***	0.3 ± 0.0
Yohimbine	5	−3.1 ± 0.4**	0.3 ± 0.1
Efaroxan	4	−2.6 ± 1.3	0.3 ± 0.0
Rauwolscine	6	−2.4 ± 0.8**	0.2 ± 0.0
ST567	3	−1.4 ± 0.4***	0.3 ± 0.0
Adrenaline	8	−1.8 ± 1.0**	0.2 ± 0.0
Histamine	6	−1.2 ± 0.8**	0.3 ± 0.0
Agmatine	4	0.1 ± 1.6*	0.3 ± 0.0

Mean ± S.E.M.

*,**,*** P<0.05, P<0.01, P<0.001 compared to clonidine (Student *t*-test or Cochran-Cox test). IC_{50} in each group divided by dotted line were not significantly different.
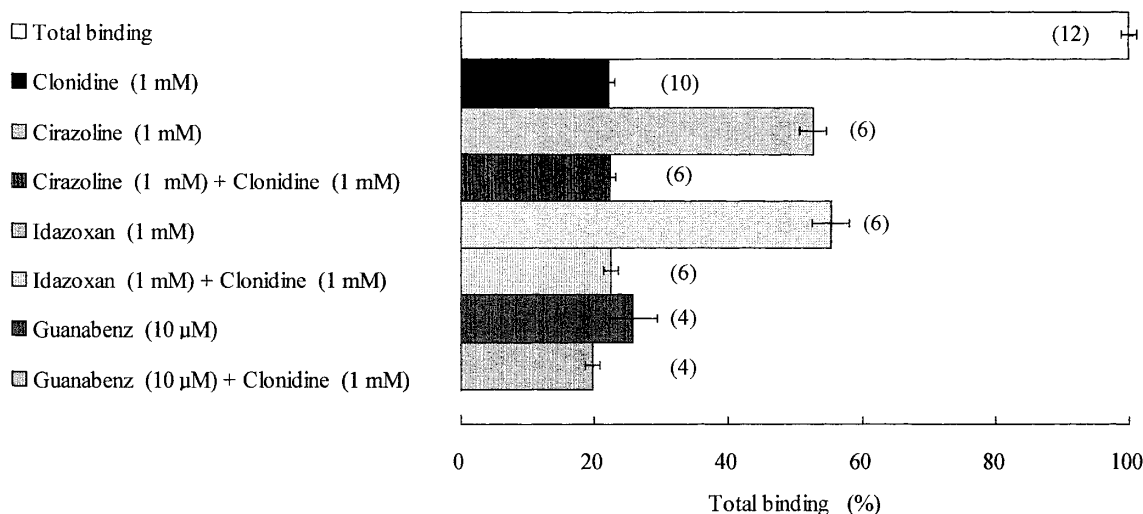
Fig. 6. Comparison of clonidine displacing activity of clonidine, cirazoline, idazoxan and guanabenz. Total binding of 2 nM [^{3}H]clonidine was examined in the presence of 1 mM unlabelled clonidine, cirazoline and idazoxan, and of 10 μM guanabenz, and expressed as a ratio to the count in the absence of competitor (100%). In the mixture of cirazoline, idazoxan or guanabenz with clonidine, the total count was almost equal to the nonspecific binding obtained in the presence of clonidine alone. Number of experiments are shown in parentheses.

and guanabenz was not additive to that by clonidine alone, suggesting that clonidine and these derivatives compete at the common binding sites.

DISCUSSION

The present study demonstrates there exist [^{3}H]clonidine binding sites in the eel intestine. Opposed to the initial object, however, most of the [^{3}H]clonidine binding sites were distinct from adrenoceptors, indicating two possibilities whether adrenoceptors in this tissue are very few or hardly recognized by [^{3}H]clonidine. But the latter case seems to be unlikely, because clonidine and adrenaline actually enhance the short-circuit current, an indicator of active Cl$^-$ absorption, across the eel intestine and both effects are completely blocked by yohimbine (Ando and Kondo, 1993; Ando and Omura, 1993).

Most [^{3}H]clonidine were displaced by various imidazoline/guanidinium drugs from their binding sites (Fig. 5). This indicates that these clonidine binding sites resemble to the imidazoline/guanidinium receptive sites (IGRS) or imidazoline receptors reported in a number of cells/tissues including kidney (Coupry et al., 1990; Lachaud et al., 1992; Ivkovic et al., 1994; Le Rouzic et al., 1995), liver (Zonnenschein et al., 1990; Atlas, 1991; Tesson et al., 1992; Ivkovic et al., 1994), brain (Tesson and Parini, 1991; Mallard et al., 1992; Reis et al., 1992; Regunathan et al., 1993; Bricca et al., 1994; De Vos et al., 1994; Ivkovic et al., 1994; Reis et al., 1994; Le Rouzic et al., 1995), cultured astrocytes (Regunathan et al., 1993), adipocytes (Lafontan et al., 1992), urethra (Yablonsky et al., 1991), platelets (Petrusewicz and Kaliszan, 1986), pancreatic β-cells (Brown et al., 1993; Lacombe et al., 1993; Reis et al., 1994), placenta (Diamant et al., 1992), adrenal chromaffin cells (Regunathan et al., 1991), and colon (Senard et al., 1990).

The IGRS have been divided into two groups (I$_1$ and I$_2$ sites) in mammals, following their affinity for radioligands and drug-displacing profile (Regunathan and Reis, 1996). I$_1$ sites prefer clonidine to guanabenz, while I$_2$ sites prefer cirazoline or idazoxan rather than clonidine (see Table 2). The fact that the binding sites are recognized by [^{3}H]clonidine indicates similarity to I$_1$ sites. However, the rank order for displacing [^{3}H]clonidine is different from that in I$_1$ sites, especially guanabenz > clonidine (Table 2). In addition, the specific clonidine binding was not affected by GTPγS (data not shown), whereas I$_1$ sites are considered to be coupled to G-protein (Ernsberger et al., 1995; Regunathan and Reis, 1996).

Although higher affinity for guanabenz indicates similarity to I$_2$ sites in mammalian kidney, brain and liver, the relatively low affinity for idazoxan is different from these mammalian I$_2$ sites (Table 2). In mammals, I$_2$ sites are considered to be mainly localized in mitochondria (Tesson and Parini, 1991; Parini et al., 1992; Tesson et al., 1992; Bousquet, 1997; Ozaita et al., 1997). Recently, however, we have observed extracellular guanabenz immediately enhances the short-circuit current across the seawater eel intestine even in the presence of yohimbine (Kim et al., in preparation), suggesting existence of specific guanabenz binding sites which are distinct from α$_2$-adrenoceptor. The immediate response also indicates that the guanabenz binding sites are not in the mitochondria. Probably, the IGRS in the eel intestine may act as a receptor, since the binding is most enhanced under physiological condition (pH 7.5 at body temperature of 20°C), and since the binding is reversible. Altogether, these pharmacological profiles indicate that the eel intestinal IGRS are considerably different from mammalian I$_1$ and I$_2$ sites. Whether such discrepancy is due to species difference or due to existence of new IGRS, such as I$_3$ sites, is not clear yet. Recently, however, non-I$_1$ and non-I$_2$ imidazoline receptors are proposed in human heart

Table 2. Pharmacological profile of imidazoline/guanidinium receptive sites (IGRS)

Drug affinities	Distributions	References
guanabenz > cirazoline = naphazoline = UK14304 = ST587 ≥ clonidine ≥ idazoxan = RX821002 = tolazoline ≥ ST93 = oxymetazoline = amiloride = ST91 > yohimbine = efaroxan = rauwolscine > adrenaline =ST567 = histamine = agmatine	intestine	Present study
I_1-sites		
clonidine = phentolamine = idazoxan > rilmenidine = moxonidine > efaroxan ≫ guanabenz ≫ adrenaline = rauwolscine	kidney, PC12cells, chromaffin cells, platelets	(Regnathan and Reis, 1996)
clonidine, moxonidine > idazoxan, phentolamine > aganodine, guanabenz	adrenal medulla	(Molderings *et al.*, 1993)
I_2 - sites		
cirazoline > idazoxan = BFI > naphazoline = tolazoline > guanabenz ≫ clonidine ≫ adrenaline = rauwolscine	kidney, adipocytes, brain	(Regnathan and Reis, 1996)
idazoxan, phentolamine = aganodine, guanabenz > clonidine, moxonidine	brain	(Miralles *et al.*, 1993)
LSL61112 > idazoxan > BFI = cirazoline > guanabenz > oxymetazoline ≫ clonidine	liver (mitochondrial fractions)	(Ozaita *et al.*, 1997)
cirazoline > clonidine > UK14304 > guanabenz	prostate	(Regnathan *et al.*, 1993)

(Likungu *et al.*, 1996) and in rabbit sympathetic nerves (Gothert *et al.*, 1995) and aorta (Molderings and Gothert, 1995).

The membrane fractions used in the present study appears to contain heterogenous components, since Scatchard plots are hyperbolic and the slope factors are less than unity.

However, most abundant 2 nM [^{3}H]clonidine binding sites, high affinity sites, seem to be common among clonidine and various clonidine derivatives, since the non-specific binding are almost identical even in the presence of idazoxan, cirazoline and guanabenz and since these derivatives are structurally
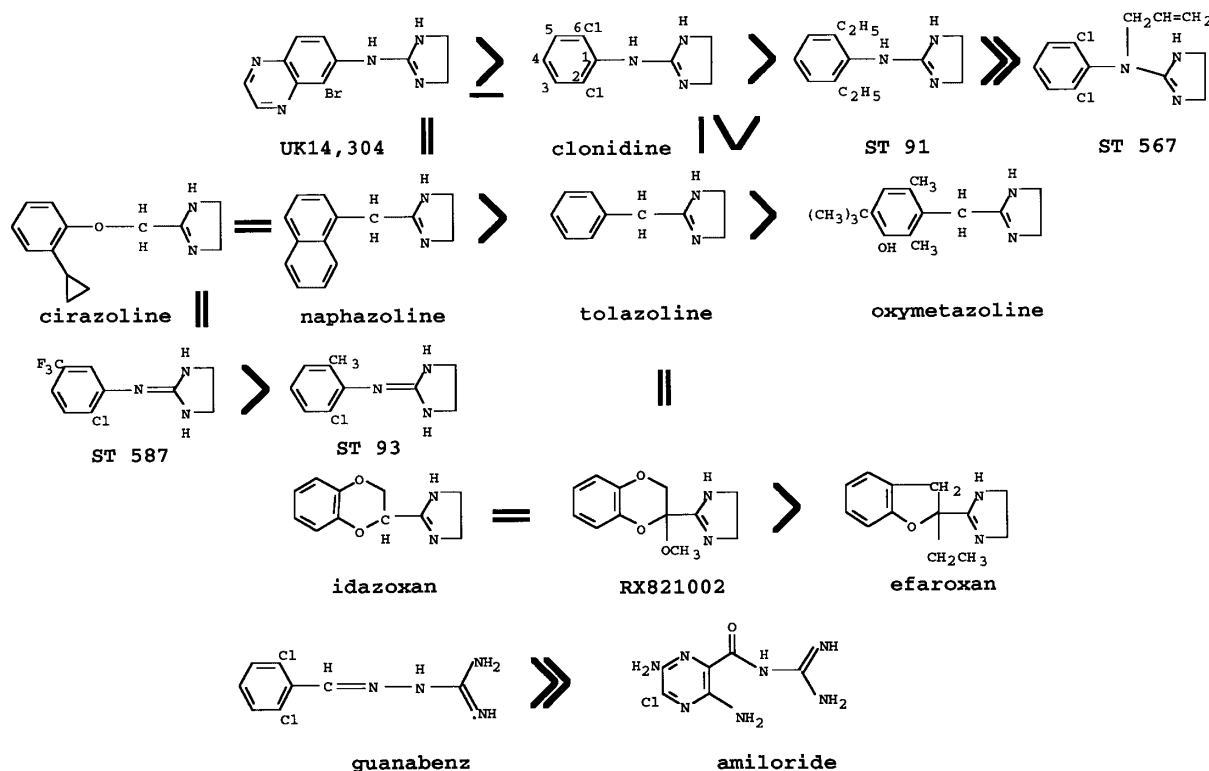
Fig. 7. Structure of imidazoline/guanidinium drugs used in the present study. The signs of equality and inequality indicate the rank order of potencies to displace 2 nM [^{3}H]clonidine from the novel binding sites in the seawater eel intestine.

similar (Figs. 6, 7). Assuming that 2 nM [³H]clonidine and various clonidine derivatives recognize the common IGRS, structure-affinity relationship can be discussed. Clonidine consists of phenyl and imidazole rings with inserted imino bridge between them (Fig. 7). The affinity seems to be lowered by replacement of H at position 6 of phenyl ring with Cl, CH_3 or C_2H_5, since ST587 > ST93, UK14304 ≥ clonidine > ST91 and tolazoline ≥ oxymetazoline. In contrast, benzene-, cyclopropyl-, Cl- or Br-substitution at position 2 of phenyl ring seems to enhance the affinity, since UK14304 = cirazoline = naphazoline > tolazoline. Imidazole ring does not seem to be essential, since guanabenz >> clonidine and amiloride also displace [³H]clonidine. Imino and methylene bridges between phenyl and imidazole rings seems to be equivalent, since UK14304 = naphazoline and ST91 = oxymetazoline. CH_2 and $O-CH_2$ bridges also seems to be equivalent, since cirazoline = naphazoline. Addition of lipophilic side chains, such as $CH_2-CH = CH_2$ or C_2H_5, to the bridge seems to decrease the affinity, since clonidine >> ST567 and idazoxan > efaroxan. In contrast, $O-CH_3$ addition to the bridge does not affect the affinity, because idazoxan = RX821002.

The concept of imidazoline receptor or IGRS evolves from efforts to understand the receptors through which clonidine, a centrally active antihypertensive agent, lowers arterial pressure in cat (Bousquet *et al.*, 1984). The presence of a nonadrenergic binding site for clonidine in the ventral medulla is directly demonstrated by Ernsberger *et al.* (1987), just like our present study. Recently, IGRS are reported in many cells/tissues as mentioned above. However, it is still not known how the IGRS work in cells and what are the endogenous ligands to these respective sites, except for agmatine (Li *et al.*, 1994). The eel intestine may be a good model to elucidate mechanisms how the IGRS work, and to identify the endogenous ligand(s), which may not be agmatine because agmatine does not effectively bind to the eel intestinal IGRS (Fig. 4).

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