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Determination of K_A values by controlled receptor expression in *Xenopus* oocytes

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1 In the present study we estimated the K_A value of endothelin-1 (ET-1) for ET_A-receptors by a new method in which the level of expression of ET_A-receptors in *Xenopus* oocytes was altered in a controlled way.

2 Kv1.2 (a delayed rectifier type K channel) cRNA at the fixed concentration of 0.2 $\mu g\ \mu l^{-1}$ was mixed with ET_A-receptor cRNA at various concentration ratios (10^{-3}–3). Oocytes were examined 2–4 days after the injection of the cRNA mixtures.

3 In these oocytes, ET-1 suppressed the amplitude of Kv1.2 current in a dose-dependent manner in the range of 0.1–100 nM; the maximum inhibition produced by ET-1 was larger and the EC_{50} value for the inhibition by ET-1 was smaller as the mixture ratio was increased. Double-reciprocal plots of equiactive concentrations of ET-1 in 1/1- and 1/30-injected oocytes yielded a K_A for ET-1 of 7.4 nM. The number of ET_A-receptors in 1/30-injected oocytes was 13% of that in 1/1-injected oocytes, whereas the inhibition of the current in 1/30-injected oocytes was about 60% of that in 1/1-injected oocytes. This suggests the presence of spare receptors of ET_A in the latter.

4 A saturation binding experiment estaimated a K_D value of 0.1 nM for ET-1 at ET_A-receptors and the number of ET_A-receptors in 1/30-injected oocytes was 23% of that in 1/1-injected ones. This value was not significantly different from that estimated by the above new method. However, there was a discrepancy between K_A and K_D, which could be due to factors unique to the expression system employed in the present study.

Keywords: Endothelin receptor; K_A; Kv1.2; spare receptor; *Xenopus* oocyte

Introduction

The dissociation equilibrium constant, K_A, of a full agonist has traditionally been estimated by comparison of dose-response curves in the presence and the absence (control) of an irreversible antagonist (Furchgott, 1966). The presence of an irreversible antagonist results in a parallel shift of the dose-response curve to the right until spare receptors are exhausted, and then the suppression of the maximal response occurs (Nickerson, 1956; Stephenson, 1956). The K_A value is calculated from comparison between the control dose-response curve and the curve in which the maximal response is suppressed. In this method, irreversible antagonists are used to eliminate a certain proportion of available receptors. However, attempts have also been made using functional antagonism (Amidon & Buckner, 1982; Leff *et al.*, 1985), post-receptor interventions (Gião & Rico, 1971) and receptor desensitization (Morgenstern, 1974) to estimate agonist affinities without irreversible antagonists.

Xenopus laevis oocytes have been used as powerful tools because they can translate faithfully foreign genetic information about receptors and channels and allow the expressed receptors to be arranged at the electrophysiological level (Soreq & Seidman, 1992). Since the amounts of expressed receptors or channels can be controlled by changing the amounts of foreign genetic information, this expression system could in principle be applied to investigate the relationship between receptor density and response. We have previously reported the cloning of a delayed rectifier type K channel, which corresponds to Kv1.2 according to the nomenclature by Chandy *et al.* (1991), and shown that stimulation of ET_A endothelin receptors by endothelin (ET)-1 suppressed the amplitude of Kv1.2 current in coexpressed oocytes (Ishii *et al.*, 1992). In the coexpressed oocytes, it is expected that the degree of suppression of Kv1.2 current by ET-1 would be different if the ratio of the number of ET_A-receptors to that of Kv1.2 channel is varied. The difference in the suppression, in turn, should make it possible to construct the concentration-inhibition curves which are usually obtained in the presence and the absence of irreversible antagonists.

Based on these assumptions, we conducted the present experiments to estimate the K_A of ET-1 for the ET_A-receptor in which expression of the ET_A-receptor in *Xenopus* oocytes was varied using the *Xenopus* oocyte expression system. The dissociation equilibrium constant, K_D, for ET-1 at ET_A-receptors was also estimated by saturation binding for comparison.

Methods

In vitro *transcription*

Rat Kv1.2 was inserted between ApaI and EcoRI sites of pBluescript vector. The plasmid containing rat Kv1.2 was linearized with EcoRI and capped run-off cRNA was synthesized *in vitro* with T7 RNA polymerase (Sambrook *et al.*, 1989). The plasmid containing ET_A was digested with XhoI and cRNA was transcribed with T3 RNA polymerase as described previously (Ishii *et al.*, 1992). The transcripts were dissolved in sterile water.

As regards relationships between the amount of injected cRNA and the expression level, at present two reports are available. In one work the amplitude of the current through HLK3 (or Kv1.3) increased biphasically over the range of 0.035–35 ng cRNA per oocyte in semilogarithmic plots (Honoré *et al.*, 1992). In another study whereas the 5-HT-induced current increased linearly in the range 2.5–10 ng rat brain mRNA per oocyte, injection of rat brain mRNA at amount of 50 ng per oocyte did not significantly increase the agonist-induced current, although voltage-dependent sodium currents were greatly increased after injection of 50 ng of rat brain mRNA (Lübbert *et al.*, 1987). Therefore, in view of these

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previous reports, the transcripts were prepared so that the mixtures consisted of 200 ng μl^{-1} Kv1.2 cRNA and 600, 200, 60, 20, 6, 2, and 0.02 ng μl^{-1} ET_A-receptor cRNA, respectively. A concentration ratio was given as $[ET_A]/[Kv1.2]$. When these mixtures were injected in a volume of 50 nl to each oocyte, 1/1-injected oocytes received 20 ng of total cRNA and those which were injected with a smaller ratio received less than 20 ng. 3/1-Injected oocytes received 40 ng of the mixture.

RNA injection into Xenopus *oocyte*

Pieces of ovarian lobes were removed surgically from *Xenopus laevis*, and dissected away in modified Barth's medium containing 10 $\mu g\ ml^{-1}$ of penicillin G and of streptomycin: composition (mM) NaCl 88, KCl 1, $NaHCO_3$ 2.4, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82 and HEPES 10, pH 7.4. Oocytes were defolliculated manually with forceps after 2 h incubation with 2 mg ml^{-1} collagenase in modified Barth's medium. Stage V and VI oocytes were selected and then injected with the mixture of transcripts in a volume of 50 nl per oocyte. Injected oocytes were incubated in modified Barth's medium for 2–5 days at 19°C.

Electrophysiological study

The standard two-microelectrode voltage-clamp method was used to measure whole cell currents of coexpressed oocytes. Oocytes were placed in the chamber and impaled by electrodes filled with 3 M KCl. After a 10 min equilibration period, membrane potential was held at –80 mV and then depolarized to +20 mV for 400 ms every 5 min. The current thus elicited immediately before exposure to ET-1 was defined as I_0. The oocytes which produced similar levels of I_0 were selected for equal levels of Kv1.2 expression. In such oocytes, the expression levels of ET_A-receptor were also expected to be similar. Only the oocytes in which the amplitude of the current did not decrease for at least 20 min were used. Electrophysiological experiments were carried out at 20–23°C with ND96 solution (composition, mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1 and HEPES 5, pH 7.5.

Radioligand binding

The binding reaction was performed at 22°C in 100 µl of the binding buffer (composition, mM): Tris-HCl 50, NaCl 154, EDTA 1, $MnCl_2$ 25, PMSF 0.5, BSA 2.5%, antipain 10 $\mu g\ ml^{-1}$ and leupeptin 10 $\mu g\ ml^{-1}$, pH 7.4. The binding reaction was started by adding [^{125}I]-ET-1 at a final concentration ranging from 7 pM to 1 nM for 1/1-injected or from 1 pM to 0.7 nM for 1/30-injected oocytes. After 3 h incubation, when equilibrium was reached, the reaction was terminated by rapid filtration of the incubation mixture through Whatman GF/C filter paper (that had been presoaked in 0.5% BSA) using a Brandel cell harvester (Gaithersburg, U.S.A.). Oocytes trapped on the filter were washed five times with 5 ml of washing buffer (composition, mM): Tris-HCl 50, $MnCl_2$ 5, BSA 0.1%, pH 7.4. Counts on the filter paper with oocytes were measured in a γ scintillation counter at 85.8% efficiency. Nonspecific binding was measured from the oocytes incubated in the presence of 1 µM unlabelled ET-1. Specific binding was calculated as the difference between total and nonspecific binding. The equilibrium saturation binding experiment was performed three times. Data from each experiment were subjected to Scatchard analysis, and the dissociation constant, K_D and the maximum receptor density, B_{max} were determined.

Materials

Female *Xenopus laevis* aged more than 2 years were purchased from Seibu (Tokyo, Japan) and Jyofu (Chiba, Japan). Restriction enzymes were from Takara (Kyoto, Japan) and RNA polymerases were from Stratagene (La Jolla, U.S.A.). Heat-inactivated bovine serum albumin (BSA), PMSF (phenylmethylsulphonyl fluoride) and antipain were from Sigma Chemical (St. Louis, U.S.A.). Collagenase and leupeptin were from Wako (Osaka, Japan). [^{125}I]-ET-1 with a specific activity of 2,000 Ci $mmol^{-1}$ was from Amersham (Buckinghamshire, U.K.). Unlabelled ET-1 was from Peptide Institute, Inc. (Osaka, Japan).

Analysis of concentration-inhibition curves and statistics

Data are presented as mean ± s.e.mean. Statistical significance was evaluated by paired or non-paired t test between two groups and one-way analysis of variance (ANOVA) among more than three groups. If $P < 0.05$, the value was considered to be statistically significant.

The level of inhibition of $I_{Kv1.2}$ caused by ET-1 was expressed as a percentage of I_0. In constructing concentration-effect curves for ET-1 in inhibiting $I_{Kv1.2}$ (Figure 2a), each oocyte was exposed to a single concentration of ET-1 because oocytes were unable to tolerate multiple exposures to varied concentrations of ET-1 and washouts. Four or five oocytes were used to obtain the arithmetic mean of the effect of a single concentration of ET-1. The inhibitory response to a given dose of ET-1 in 1/1- and 1/30-injected oocytes was expressed as a percentage of the maximum inhibition of $I_{Kv1.2}$ produced by 100 nM ET-1 in 1/1-injected oocytes. The curves were computer-fitted using the following equation (Parker & Waud, 1971):

$$I = I_{max} \times \frac{A^n}{A^n + K^n} \quad (1)$$

where I is the inhibitory response of $I_{Kv1.2}$ to a given concentration of ET-1, I_{max} is the maximum inhibition of $I_{Kv1.2}$ (100%), A is the ET-1 concentration, K is the EC_{50} value, and n is the slope parameter. Since it seemed that there was neither cooperativity nor factors affecting the shape of the concentration-effect curve, the slope parameter, n, was assumed to be unity (Black & Leff, 1983). Equiactive concentrations were interpolated between these fitted lines using the upper part of the depressed concentration-response curve as recommended by Thron (1970). The interpolated values were fitted to the following equation (Furchgott, 1966):

$$\frac{1}{A} = \frac{1}{A'} \times \frac{1}{q} + \frac{1}{K_A} \times \frac{1-q}{q} \quad (2)$$

in which A and A′ are equiactive ET-1 concentrations of 1/1- and 1/30-injected oocytes, q is the ratio of receptor numbers under the two conditions, and K_A is the dissociation equilibrium constant.

Results

ET-1-induced suppression of $I_{Kv1.2}$ did not occur in the oocytes expressing only Kv1.2 (data not shown). Therefore it was considered that suppression was purely ET_A-receptor dependent. In order to examine the relationships between the amounts of receptor expressed in the oocytes and the level to which Kv1.2 was suppressed by receptor stimulation, oocytes which were injected with the two cRNAs at different concentration-ratios were prepared. The level of inhibition was expressed as a percentage of the amplitude of $I_{Kv1.2}$ at 30 min after the exposure to ET-1 to I_0 (Figure 1). The values of I_0 proved not to be different from each other by one-way ANOVA ($F(12,45) = 1.54$). When 100 nM or 10 nM ET-1 was applied, $I_{Kv1.2}$ was not inhibited at 1/1000-injected oocytes. As a cRNA ratio was increased, the level of inhibition became greater. In 1/1-injected oocytes $I_{Kv1.2}$ was suppressed to 30.6 ± 5.1% ($n = 5$) by 10 nM and 32.0 ± 10.0% ($n = 4$) by 100 nM ET-1. These values were not significantly different

from each other. Inhibition was not detected with application of 1 nM ET-1 in oocytes injected with the two cRNAs at a ratio lower than 1/100. $I_{Kv1.2}$ was suppressed by 1 nM ET-1 only to 65.6±11.2% (n=4) even in 1/1-injected oocytes.

The level of receptor-mediated inhibition produced by 100 nM ET-1 was not significantly different between 1/3-injected and 1/1-injected oocytes (39.4±1.8% *vs* 30.6±5.1%). To examine whether the receptors were expressed enough to saturate components of second messenger pathways in 1/1-injected oocytes, two groups of oocytes were prepared; one was 1/1-injected oocytes, the other was 1/30-injected ones. The values of I_0 were evaluated by one-way ANOVA ($F(7,27)=1.24$) and they were not significantly different from each other. ET-1 suppressed the amplitude of $I_{Kv1.2}$ in a dose-dependent manner at concentrations ranging from 0.1 nM to 100 nM. Maximum inhibition obtained with 100 nM ET-1 in 1/30-injected oocytes (40.9±6.5% of I_0; n=5) was 60.6% of that obtained with the same dose of ET-1 in 1/1-injected oocytes (67.5±3.3% of I_0; n=5) (Figure 2a). The pEC_{50} value for ET-1 in 1/30-injected oocytes (8.5±0.1; n=5) was significantly different from that in 1/1-injected oocytes (9.3±0.3; n=5). Double-reciprocal plots of equiactive concentrations of ET-1 in 1/1- and 1/30-injected oocytes yielded a straight line (Figure 2b). The q value was calculated to be 0.13 and the K_A 7.4 nM.

The ratio of the number of the expressed ET_A-receptor in 1/1-injected oocytes to 1/30-injected was measured directly using radioligand. Before the experiment, the expression level of the injected cRNAs was checked by measuring $I_{Kv1.2}$ from some of the oocytes to be used. $I_{Kv1.2}$ from 1/1-injected oocytes was not significantly different from that for 1/30-injected ones (2.69±0.80 μA *vs* 3.15±0.95 μA, respectively, n=8). The specific binding activity was detectable from one oocyte and increased linearly up to four oocytes (r=0.99; data not shown). In this assay, two (1/1-injected) or four (1/30-injected) oocytes per sample were used. [^{125}I]-ET-1 bound to the oocytes in a specific manner. Specific bindings of 1/1-injected and 1/30-injected oocytes were about 60% and 40% of total binding, respectively. The K_D and B_{max} values for 1/1-injected oocytes were 0.12±0.01 nM and 0.57±0.07 fmol/oocyte (n=3). Those for 1/30-injected were 0.11±0.01 nM and 0.13±0.02 fmol/oocyte (n=3) (Figure 3). The B_{max} was significantly different between the two groups. Its ratio for 1/30-injected to that of 1/1-injected oocytes was 0.23±0.02 (n=3). The K_D value was not different between the two groups.

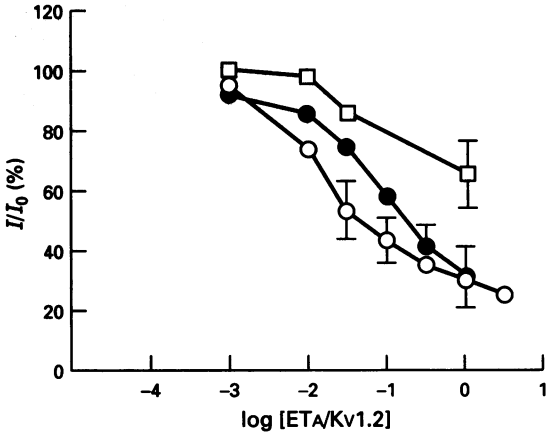

Figure 1 Relationships between the cRNA concentration ratio of ET_A to Kv1.2 and the level of inhibition of $I_{Kv1.2}$. Abscissa scale represents log of the cRNA concentration-ratio of ET_A to Kv1.2. ET-1; 100 nM (○), 10 nM (●) and 1 nM (□) was applied. Current was evoked by depolarizations from −80 mV to +20 mV at 30 min after the exposure to ET-1 and plotted as a percentage to I_0. Data are expressed as means ± s.e. mean of three to five experiments.

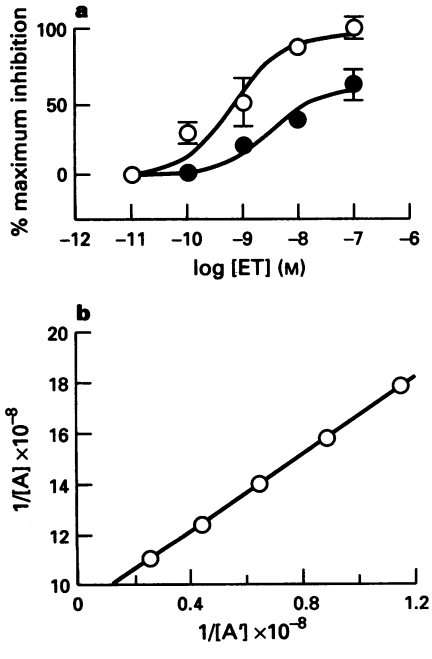

Figure 2 (a) Concentration-response relationships of ET-1-induced inhibition of $I_{Kv1.2}$ in 1/1-injected (○) and 1/30-injected oocytes (●). Current was evoked by depolarizations from −80 mV to +20 mV at 30 min after the exposure to ET-1 and plotted as a percentage of the maximum inhibition of $I_{Kv1.2}$ obtained with 100 nM ET-1 in 1/1-injected oocytes. Data are expressed as means of three to five experiments. Vertical bars are s.e.mean (b) Double-reciprocal plot for K_A estimation. [A] and [A'] are equiactive agonist concentrations in 1/1-injected and 1/30-injected oocytes. Intercept=9.0; slope=7.7; K_A=7.4 nM; q=0.13.

Discussion

The present study has shown that, under conditions where similar numbers of effector, Kv1.2, have been expressed, a maximal concentration of ET-1 (100 nM) produced a lower degree of maximum inhibition of $I_{Kv1.2}$ in oocytes expressing a smaller number of ET_A-receptors than in those expressing a larger number. Thus, the responses obtained from the two groups of the cell mimicked the effects of irreversible antagonist. Based on the same reasoning that allows estimation of the dissociation equilibrium constant K_A for a full agonist by using an irreversible antagonist (Furchgott, 1966), we made double-

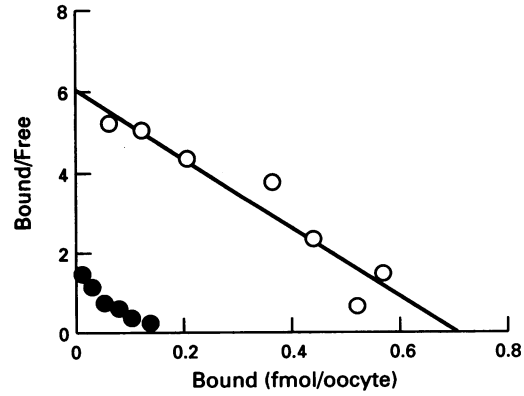

Figure 3 Scatchard plot of [^{125}I]-ET-1 binding to 1/1-injected (○) and 1/30-injected (●) oocytes. Each point is expressed as a mean of triplicate determinations. The data shown are representative of three experiments. The binding parameters for 1/1-injected and 1/30-injected oocytes yielded from this experiment are as follows: K_D=0.12 and 0.10 nM, B_{max}=0.71 and 0.15 fmol/oocyte, respectively.

reciprocal plots of equiactive concentrations of ET-1 in 1/1- and 1/30-injected oocytes. The q and K_A values obtained from these plots were 0.13 and 7.4 nM.

The above reasoning would be valid only if sufficient and equal quantities of Kv1.2 were expressed in the two groups and ET_A-receptors were expressed in the intended way. Kv1.2 was considered to be expressed in enough and even amounts in the oocytes examined, because we chose oocytes which produced a similar level of I_0 (about 3 μA). However, in most 3/1-injected oocytes I_0 was approximately 1 μA. 3/1-Injected oocytes received an injection of 40 ng of cRNA mixture. Thus, the low level of expression of Kv1.2 in these oocytes seemed to result from their limited translational capacity; competition in translating ET_A and Kv1.2 cRNAs. Likewise it matters whether the relationship between ET_A-receptor expression and injected ET_A cRNA was quantitatively reliable. In the present experiments, the degree of inhibition of $I_{Kv1.2}$ as a response to ET_A-receptor stimulation by ET-1 showed a monophasic increase on a semilogarithmic scale as the ET_A cRNA concentration was increased. Thus, it is reasonable to conclude that the ET_A-receptor was expressed in a quantified manner. There are reports indicating that *Xenopus* oocytes have a limited capacity for translating a membrane-associated mRNA; 20 ng or less (Richter & Smith, 1981) and 35 ng or more (Honoré *et al.*, 1992), and that membrane-associated mRNAs competed for the limited translational capacity if they were injected into the same oocyte (Richter & Smith, 1981). In the present experiments two cRNAs were injected in an amount less than 20 ng per oocyte except for 3/1-injected oocytes. This also supports the above reasoning that injected Kv1.2 was expressed sufficiently and equally, and ET_A-receptors were expressed depending on the amount of its RNA injected.

It appears reasonable, therefore, to assume that the concentration-response relationship between ET_A-receptor stimulation and inhibition of $I_{Kv1.2}$ obtained using two different groups of oocytes mimicked the effect of irreversible antagonist. The q and K_A values were obtained from double-reciprocal plots of equiactive concentrations of ET-1 in 1/1- and 1/30-injected oocytes. The q value was originally defined as a fraction of total receptor population remaining operative after irreversible blockade of fraction 1-q with an irreversible antagonist (Furchgott, 1966). The q value of 0.13 obtained from the double-reciprocal plots indicates that the number of ET_A-receptors operative (or expressed) in 1/30-injected oocytes was about 1/7.7 that of ET_A-receptor operative (or expressed) in 1/1-injected oocytes. Nevertheless, the inhibition of $I_{Kv1.2}$ produced by a maximum concentration (100 nM) of ET-1 in 1/30-injected oocytes was about 60% of that in 1/1-injected oocytes, indicating the existence of spare ET_A-receptor in the latter oocytes. The ratio of B_{max} determined by saturation binding of [^{125}I]-ET-1 to the coexpressed oocytes was 0.23 ± 0.02 and was indeed close to the q value of 0.13.

The K_A value obtained from the double-reciprocal plots was different from the K_D value obtained from the saturation binding experiment; 7.4 nM *vs* 0.11–0.12 nM. However, the K_D value obtained in the present experiments was consistent with the K_D value (0.11 nM) for ET_A-receptor determined from ET_A-transfected COS cells (Hori *et al.*, 1992). Why the two estimates of affinity are different is not known, although it is not uncommon for such differences to arise between binding and functional studies. It is possible to explain overestimation of agonist affinity on the basis that agonist-activated receptors are distributed into multiple states (Black & Shankley, 1990; Mackay, 1990; Leff *et al.*, 1990). Under these circumstances, the affinity estimate reflects distribution of the receptor into high affinity forms, perhaps a G-protein complex or a desensitized state, not simply agonist-receptor occupancy. In principle, such receptor distribution could affect the measured affinity in binding or functional studies. In the present case it would have to be argued that the binding experiments were more susceptible to receptor distribution than were expression studies. Whether or not this explanation is true does not detract, we feel, from the importance of the expression system as a method of obtaining a functional K_A estimate.

There have been many attempts to estimate the K_A value for a full agonist without using irreversible antagonists. The method presented in the present study, in which concentration-response relationships were compared in *Xenopus* oocytes with different levels of ET_A-receptor and a similar level of Kv1.2, does not require irreversible antagonists. This method could provide a new approach to the functional estimation of the K_A value for a cloned receptor. However, it should be borne in mind that there is a number of factors unique to a coexpression system presented above when the K_A value is estimated by the method presented.

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