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Electrophysiological expression of endothelin and angiotensin receptors in *Xenopus* oocytes injected with rat heart mRNA

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Functional endothelin and angiotensin receptors have been expressed in *Xenopus* oocyte following the microinjection of rat heart mRNA Under voltage clamp conditions, application of these peptides clearly induced oscillatory Ca^{2+}-activated chloride currents in a dose-dependent manner In addition, no direct modulation of expressed or native cardiac Ca channels was observed

Endothelin receptor, Angiotensin receptor, mRNA microinjected oocytes, Ca^{2+}-activated Cl current, Cardiac Ca channels, (Rat heart)

1. INTRODUCTION

In mammalian heart, elevation of intracellular Ca^{2+} is a key step for positive inotropism and two major physiological pathways are involved. The best known is the β-adrenergic stimulation that increases Ca^{2+} entry following cyclic AMP dependent phosphorylation of the high voltage-activated Ca channels [1]. On the other hand, α_1-adrenergic [2], muscarinic [3], P_2-purinergic [4] or angiotensin (AII) [5] receptors mediate inositol trisphosphate (IP_3) formation. This may trigger, in turn, an intracellular elevation of Ca^{2+} concentration as evidenced in many cell types, especially in *Xenopus laevis* oocyte [6].

Recently, a new potent vascular endothelium-derived vasoconstrictor peptide, endothelin (ET) has been identified [7] as a possible endogenous agonist for voltage-dependent Ca-channels (VDCC), especially in arterial smooth muscle [7–9]. Such a direct modulation of Ca channels is attractive for explaining the positive inotropism of ET [10] in heart muscle. However, high affinity sites for ^{125}I-ET [11,12] are distinct from known regulatory sites for L-type VDCC on rat cardiac membranes.

We have obtained expression of ET and AII receptors and characterized their functional responses in *Xenopus* oocytes injected with rat heart mRNA. We report evidence that both ET and AII, with regard to the induced activity of endogenous Ca^{2+}-activated Cl-channels, activate a complex intracellular pathway [13]. In addition, no direct modulation of either expressed or native Ca channel currents by ET, as for AII, could be observed. We conclude that inotropic effect of ET on heart involves an increase in phosphatidyl inositol turnover as demonstrated in aortic cells [14].

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2. MATERIALS AND METHODS

2.1 *Heart mRNA isolation and oocyte injection*

Total RNA was extracted from 10-day-old rat heart with a guanidine thiocyanate/phenol/chloroform procedure [15] Poly-A^+ messenger RNA (mRNA) was purified by affinity chromatography on oligo(dT)-cellulose and finally stored at a concentration of 2 mg/ml in sterile water in 2-μl aliquots. Stage V *Xenopus laevis* oocytes were enzymatically isolated and microinjected with 50 to 70 nl of the mRNA solution using classical protocols [16]

2.2 *Voltage clamp experiments on oocytes*

Whole-cell currents were measured with a 2 microelectrode voltage clamp technique The Ca^{2+}-activated Cl channel activity was studied in ND96 solution (in mM NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1 8, Hepes 5, pH 7 45 with NaOH) The Ca channel currents were recorded using Ba^{2+} as charge carrier [17] in Ba40 (in mM· NaOH 50, KOH 2, $Ba(OH)_2$ 50, Hepes 5, pH 7 45 with CH_3SO_3H) Data acquisition and analyses were monitored with an IBM PC using the pCLAMP software (Axon instruments)

2 3 *Voltage clamp on rat single ventricular cells*

Isolated ventricular myocytes were obtained by a collagenase treatment (1000 U/ml, 20 min) using 6-week-old rats. Ca channel currents were recorded using the whole-cell configuration of the patch clamp technique Bath solution contained (in mM) NaCl 140, $BaCl_2$ 1, $MgCl_2$ 2, Hepes 10, glucose 10, pH 7.2 with NaOH Tetrodotoxin (20 μM) was added to ensure nearly complete block of Na currents Recording pipettes contained (in mM)· CsCl 140, EGTA 10, Hepes 10, Mg- ATP 3, Mg-GTP 0 4, glucose 10, pH 7.2 (CsOH) Cs^+ (and also extracellular Ba^{2+}) completely block all K currents There was no evidence for T-type currents [18]

2 4 *Drug application*

Human endothelin-1 (ET) and angiotensin II (AII) were respectively purchased from Peptide Institute (Japan) and Sigma. Bay K 8644 was obtained from Bayer. The drugs were instantaneously bath-

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applied on ventricular cells and on oocytes during a stop in the perfusion of the experimental chamber

3. RESULTS AND DISCUSSION

Fig.1 presents the typical membrane current responses observed after AII (fig.1A) or ET (fig.1B) application on oocytes injected with rat heart mRNA ($n=28$). No responses were observed on uninjected oocytes ($n=19$) from the same donors ($n=3$). We report here, for the first time, expression of cardiac metabotropic receptors, i.e. ET and AII receptors, in mRNA-injected oocytes. The current waveform indicates that these two receptors are both coupled to a common second messenger pathway that acts on open endogenous Ca^{2+}-activated Cl channels [16,19]. This is supported by the variable delay between drug application and current activation which indicates the involvement of intra-cellular mechanisms. The reversal potential of this current (−29 mV; fig.1C) is typical of Cl current in *Xenopus* oocyte [16]. With respect to the previous studies concerning the 7-helix receptor family [13,19] (for review see [20]), one can expect that these two receptors are both coupled to GTP binding proteins (G proteins) which mediate the phospholipase C activation; this hypothesis has been supported concerning brain AII receptors [21]. Thus, ET and AII stimulation directly activate phosphatidyl inositol (PI) metabolism and reconstitution of these receptors in the oocyte system opens new fields for studies of excitation-contraction coupling in cardiac tissues, by following IP_3 formation.

Because ET has been shown to increase the IP_3 turnover in aortic cells [14], it was crucial to determine whether a possible contamination in our heart mRNA preparation could account for the observed effects. The lack of Arg-vasopressin- ($n=4$) and serotonin- ($n=3$) metabotropic responses turned off this possibility. Thus, it appears obvious, that ET and AII metabotropic responses were directed by heart messenger RNA myocytes.

ET and AII responses occur in a dose-dependent manner on the Ca^{2+}-dependent Cl current (fig.2). The drug-induced currents correspond to the maximal inward peak of the oscillatory current. Fig.2B shows that high affinity of ET-mediated responses is conserved in the mRNA injected oocyte system ($EC_{50}=0.1\ \mu M$) with regard to native tissues [8,9,11,14]. Higher concentrations are necessary for AII-induced responses ($EC_{50}=2\ \mu M$). Although ET and AII responses are completely desensitized for large doses of both drugs, there is no evidence, in our experiments, for a cross-desensitization between these two responses in contrast with previous reports using the oocyte system [13].

ET and AII responses were distinguishable by their duration, longer for ET (4 to up to 15 min) than for AII (less than 3 min), and also by their maximal current amplitude (350 nA upon 0.1 μM of ET and 1200 nA under 10 μM of AII). Observations concerning

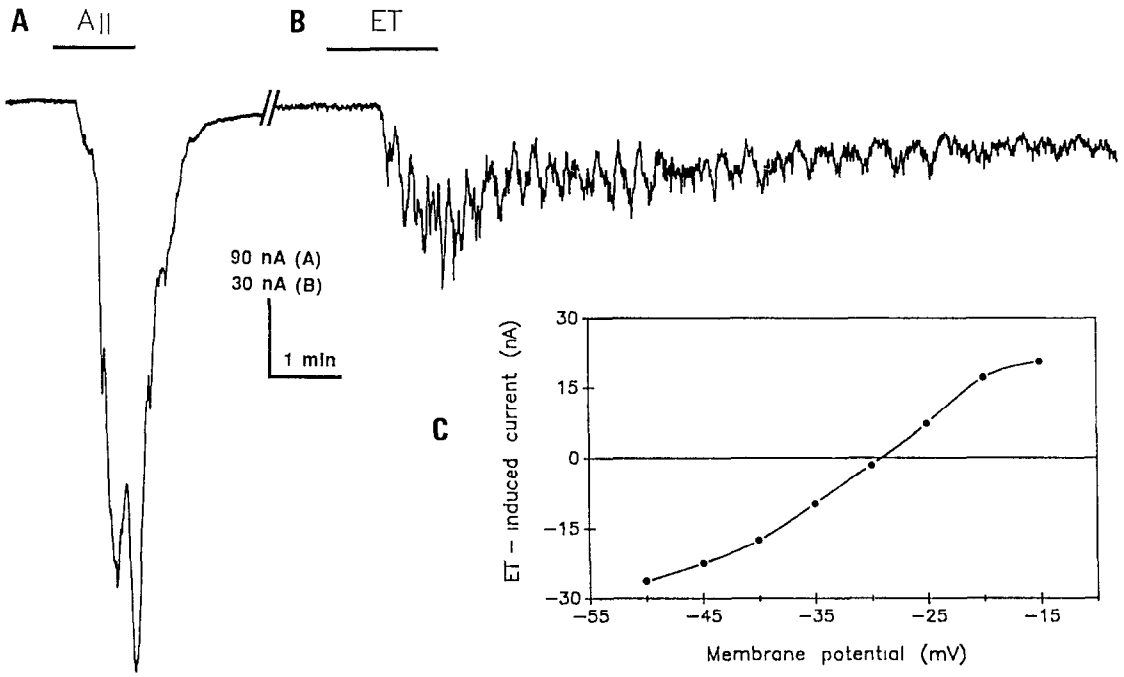

Fig 1 Typical current responses to AII and ET recorded in *Xenopus* oocytes injected with rat heart mRNA All experiments were performed on the same oocyte The holding potential was −80 mV (A) Current response to the application of AII (10 μM) (B) Current response to the application of ET (0 1 μM). A delay of 30 min was respected between the two drug exposures The current-potential relationship (C) was plotted for the current induced by ET, the reversal potential was −29 mV Note that current amplitude calibration is different for A and B

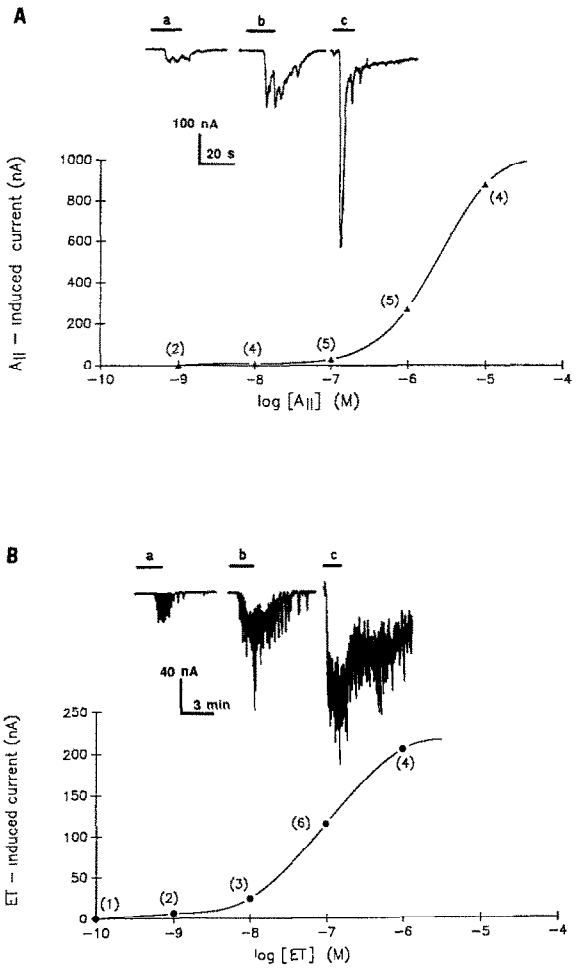

Fig 2 Dose-response curves of the inward Ca^{2+}-activated Cl currents induced by AII (A), and ET (B) in mRNA injected oocytes The holding potential was −80 mV Responses were expressed as the maximal inward peak current Two concentrations only were applied on each oocyte and a 30 min delay between drug exposures was respected The (*n*) values represent the number of tested oocytes for each data point. Insets show AII-induced current traces (A) and ET-induced current traces (B) In A, concentrations used were as follows· (a) 0 1 μM, (b) 1 μM, (c) 10 μM In B, concentrations used were· (a) 1 nM; (b) 0 1 μM; (c) 1 μM

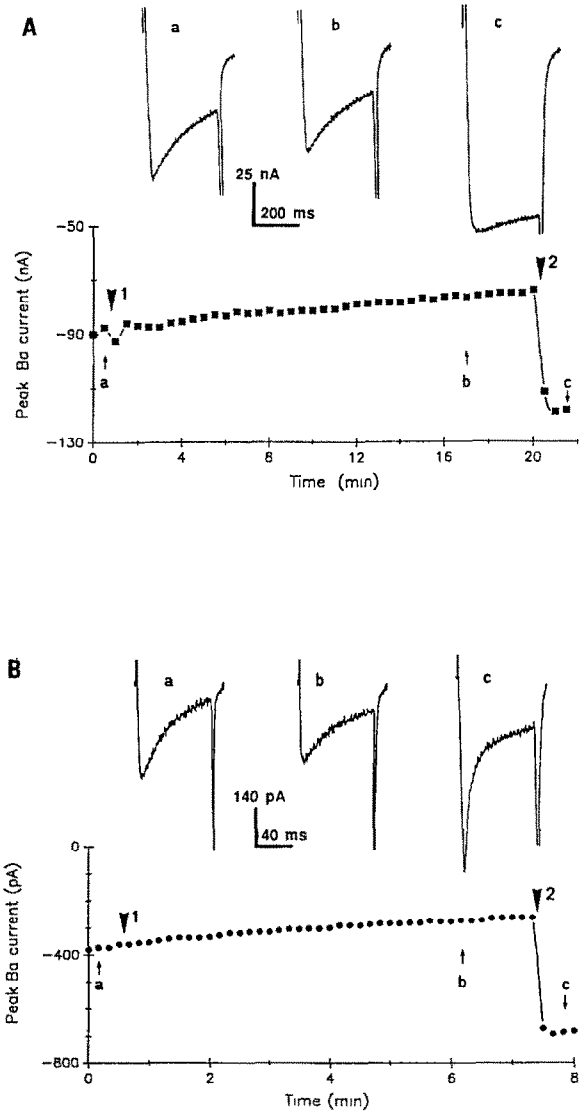

Fig.3 Application of ET on cardiac Ca channel currents. Ba^{2+} was used as charge carrier (see section 2) Expressed Ba current in mRNA injected oocytes (A) and native Ba current in ventricular myocytes (B) were recorded before and during exposure of ET (depolarizing pulse, 0 mV, holding potential, −80 mV) Arrows 1 and 2 indicate the application of ET (0.1 μM) and Bay K 8644 (1 μM) respectively in the bath Insets (a, b, c) show Ba current recordings in mRNA injected oocytes (A) and in ventricular cells (B) at 3 selected times

desensitization and time-course of ET- and AII-induced membrane currents suggest that partly distinct mechanisms for mediation of the effects are involved: AII induces a Ca^{2+} flash and is rapidly desensitized; in contrast, the ET response involves a sustained intracellular Ca^{2+} increase and show very slow desensitization. Thus, this ET-mediated response seems consistent with the extremely long-lasting contraction observed both in vivo and in vitro on various smooth muscles [7,14] and heart muscle [10] which is associated with a sustained increase in the intracellular Ca^{2+} [8,14]. In addition, cumulative effects on Cl current could be obtained by adding successively ET and AII (data not shown) indicating distinct receptors for the two agonists.

We tested whether ET could also act as a direct endogenous activator of L-type Ca channels to partly account for the rise in intracellular Ca^{2+} following exposure to ET. Voltage clamp experiments were performed both on cardiac Ca channels expressed in the oocyte system and, for comparison, on natural single ventricular rat myocyte using the whole-cell clamp technique. In the oocyte system, chloride conductance can be eliminated by replacing Ca^{2+} with barium (Ba^{2+}) and Cl^- with methane sulfonate (Ba40) in order to study Ca channel activity [17,22]. Nevertheless, application of ET in Ba40 induces a transient oscillation

in the holding membrane current due to the Cl channel activation (see fig.3A, following arrow 1). Fig.3A shows that ET (0.1 μM) is unable to enhance the Ba current recorded on a mRNA injected oocyte despite a long exposure (20 min; $n=4$), although the same currents were highly sensitive to the L-type specific dihydropyridine activator Bay K 8644 (arrow 2) which demonstrates the exogenous nature of these currents (endogenous currents are insensitive to DHPs [17]).

Expressed Ca channels present properties similar to native L-type Ca channels [17,22] but it was necessary to confirm the above results on ventricular myocytes. We did not find the typical Cl-conductance activated by Ca-mobilizing agonists (AII, ET) as observed in the oocyte system (data not shown). In addition, whole-cell Ca channel currents, as Ba currents, can be perfectly isolated from other contaminating conductances such as Na and K currents (see section 2). Under these experimental conditions, no potentiation of native Ba currents was detected upon application of 0.1 μM ET (fig.3B). In contrast, only a time-dependent decrease of Ba current amplitude occurred, which probably corresponds to the rundown of Ca channel currents in native cells [23]. On the other hand, the same currents were markedly increased following exposure to Bay K 8644 ($n=7$). Moreover, Bay K 8644 acts after ET exposure as in control conditions both on native and expressed Ca channels which exclude a common site for the two drugs [11]. AII also did not directly modulate Ca channel currents, both in mRNA injected oocytes ($n=5$) and in native cells ($n=2$; data not shown).

Although we found no evidence for a direct modulation of cardiac Ca channels, it is quite possible that the Ca channel activity could be mediated by intracellular messengers [8,9,14]. This role could be devoted to the subsequent elevation of intracellular Ca^{2+}, likely to trigger a variety of cellular responses [6], including a Ca-dependent phosphorylation of L-type VDCC which enhances Ca channel currents in mammalian heart [24].

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