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The angiotensin AT_2 receptor modulates T-type calcium current in non-differentiated NG108-15 cells

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We report here that angiotensin II (AII) and the AT_2 receptor-selective ligand, CGP 42112, modulate the T-type calcium current in non-differentiated NG108-15 cells, which express only AT_2 receptors. Both peptides decrease the T-type calcium current at membrane potentials above −40 mV and shift the current–voltage curve at lower potentials with maximal effect between 5 and 10 min after application. These data describe a new cellular response to AII and suggest that the AT_2 receptor mediates certain neurophysiological actions of this hormone.

Angiotensin; Angiotensin receptor; Calcium current; CGP 42112; Neuronal cell; NG108-15

1. INTRODUCTION

Since 1989, use of peptidic and non-peptidic analogs have clearly identified two classes of angiotensin II (AII) receptors, which have been designated AT_1 and AT_2 [1–4]. The AT_1 receptor subtype is located in brain areas [5–10] and peripheral tissues [1–3] closely associated with the regulation of vascular tone and extracellular fluid volume. In these tissues, AII is implicated in the regulation of intracellular calcium concentration, acting both on calcium influx (via specific voltage-dependent channels) and on calcium release from intracellular stores [11–13]. The distribution of AT_2 binding sites in brain suggests rather a role in sensory and motor control [7–10]. Moreover, the abundance of AT_2 subtype in many fetal tissues (brain, skin, muscle) suggests a role for AT_2 during development [14,15]. Possible new roles for AII in the central nervous system have begun to emerge [16,17]. The AT_1 receptor has been recently cloned [18,19] and displays all of the well-known characteristics of a G-protein-linked receptor [20]. In contrast, no signal transduction mechanism has been clearly established for the AT_2 receptor subtype to date, but it does not interact with G-proteins in peripheral tissues [15,20–23]. It has been suggested that AII decreases the intracellular cGMP level through the AT_2 receptor in neurons [24]. In rat adrenal glomerulosa and PC12W cells, AT_2 receptors inhibit basal and atrial natriuretic peptide (ANP)-stimulated particulate guanylate cyclase activity, and the involvement of a phosphotyrosine phosphatase (PTPase) in this mechanism has been proposed [25]. In brain, potential interaction between G-proteins and a subclass of AT_2 receptors has been recently described [26], suggesting heterogeneity in the AT_2 receptor subtype. Many brain areas present high densities of either one or both types of AII receptors [6–10]. As mammalian neurons display many different types of ionic currents [27], it was of particular interest to look for interactions between AT_2 receptors and membrane conductances. Of particular value for this type of investigation is a neuronal cell-line expressing exclusively the AT_2 receptor.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

NG108-15 cells (passage 4 to 17) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Burlington, Canada), with HAT supplement (Gibco BRL, Burlington, Canada), 50 mg/l gentamycin and 10% fetal bovine serum (Gibco BRL, Burlington, Canada) at 37°C in a humidified atmosphere of 90% air and 10% CO_2.

2.2. *Competition binding experiments*

Non-differentiated NG108-15 cells were harvested in PBS/EDTA, and plasma membrane particulate was prepared as described previously [1]. 20 μg of particulate was incubated in 200 μl buffer (50 mM Tris, pH 7.4, 125 mM NaCl, 6.5 mM $MgCl_2$, 1 mM EDTA, 0.2% BSA, 1 mM benzamidine, 0.01% baeitracin and antipain, phosphoramidon, pepstatin A, bestatin and amastatin all at 1 μg/ml) for 90 min at 25°C in the presence of [^{125}I]Sar^{1}Ile^8 AII (0.25 mM) as a tracer and with the different competitors. Bound ligand was separated by filtration through glass-fiber filters. Non-specific binding determined in the presence of 1 μM AII (<10% of total binding) was subtracted from total binding. Degradation of [^{125}I]Sar^{1}Ile^8 AII as measured by thin-

layer chromatography was less than 5%. Data are the mean of three experiments analyzed with the EBDA-LIGAND program [41].

2.3. *Electrophysiological recordings*

For patch-clamp recordings, the cells were used between 24 and 72 h after subculture. All experiments were conducted at room temperature (22°C). A hollowed piece of plexiglass forming a 350 µl microcurve above the cells was held in position in the Petri dish with a thin film of silicone gel. The cells were then continuously superfused at 300 µl/min with an extracellular solution containing (in mM): 100 NaCl, 10 $CaCl_2$, 1 $MgCl_2$, 5 CsCl, 35 TEA, 5 HEPES and 0.5 µM tetrodotoxin (TTX), pH 7.35; the osmolarity was adjusted to 300 mOsm with glucose. Angiotensin II analogs and drugs were each dissolved in the extracellular solution for superfusion. It took about 10 s for the substances to reach the cells. Sylgard-coated Pyrex glass electrodes (resistance between 2 and 6 MΩ) were filled with the following intracellular solution (in mM): 20 NaCl, 120 CsCl, 1 $CaCl_2$, 11 EGTA, 2 $MgCl_2$, 5 HEPES, 3 ATP, 0.2 GTP, pH 7.35; the osmolarity was adjusted to 305 mOsm. All solutions were passed through 0.2 µm filters before use. The intracellular solution was kept on ice. Ionic current recordings were obtained using the whole-cell configuration of the patch-clamp method [30] and employing an Axopatch 1B amplifier (Axon Instruments) piloted by pClamp software (Axon Instruments) on an IBM-PC computer. The junction potential, as well as the capacitive transient, were compensated for. After membrane disruption, the series resistance and the capacity were compensated for. Leak subtraction was performed with the P/N protocol. Currents were low-pass filtered at 1 kHz, sampled at 4 kHz, stored on floppy disks and analyzed with the pClamp software.

2.4. *Steady-state inactivation curves*

Starting from a −80 mV holding potential, a 600 ms conditioning voltage step was applied at various levels and immediately followed by a 200 ms test potential at −30 mV. The currents were normalized with respect to the current of maximal amplitude (holding potential = −80 mV). V_h and k were calculated with the Boltzmann equation:

$$I_{max}/(1 + \exp((V - V_h)/k))$$

where V_h represents the potential of half-inactivation and k the slope factor. Results are all expressed as mean ± S.E.M.

3. RESULTS AND DISCUSSION

3.1. *Exclusive expression of angiotensin AT_2 receptors by non-differentiated NG108-15 cells*

Non-differential NG108-15 cells express the complete renin-angiotensin system [28], and preliminary studies indicate that these cells express only one class of AII receptors which are not coupled to phosphatidyl inositide hydrolysis [29]. As shown in Fig. 1A, competition binding experiments performed on plasma membrane particulate prepared from these cells reveal monophasic competition curves for AII, the AT_2-selective ligands, CGP 42112 and PD 123177, as well as for the AT_1-selective antagonist, DuP 753, indicating the presence of a single subtype of AII receptors. The order of affinities, CGP 42112 ≥ Sar^1Ile^8 AII > AII > PD 123177 >>> DuP 753, shows that these receptors are of the AT_2 subtype [1,3,4]. We have also observed that AII does not stimulate phosphatidyl inositide hydrolysis in our non-differentiated NG108-15 cells (data not shown).

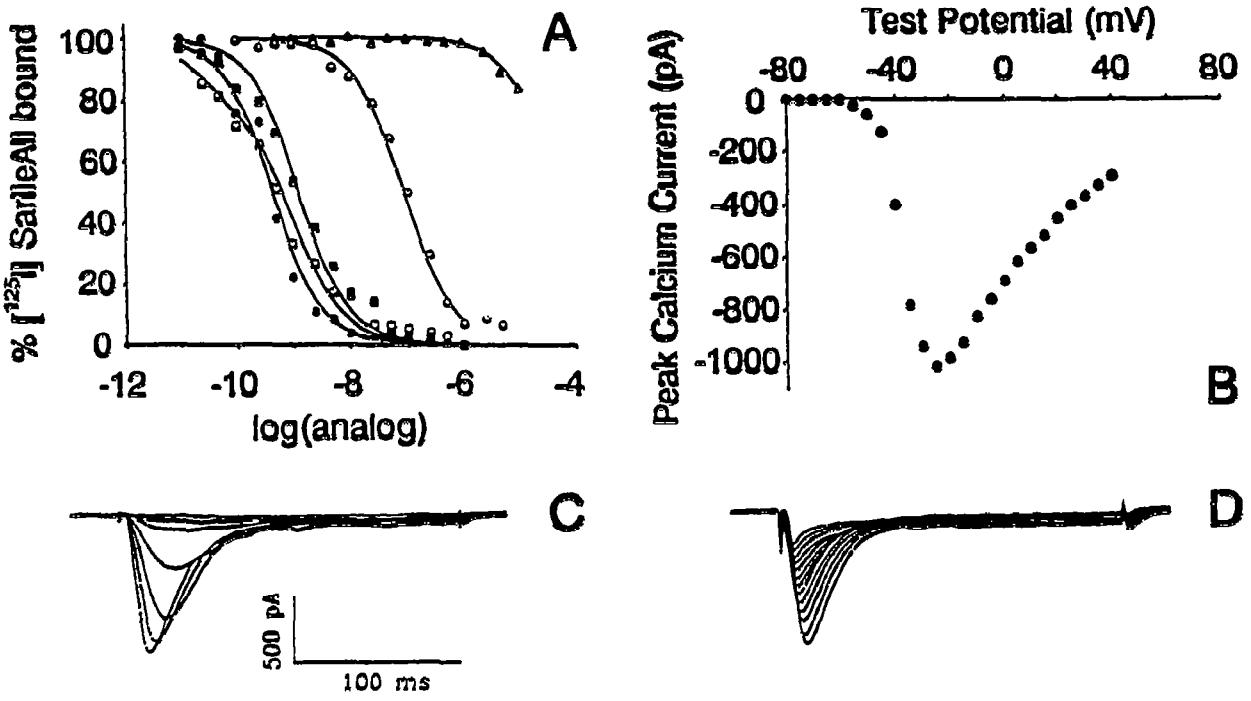

Fig. 1. (A) Pharmacology of the AII receptors in non-differentiated NG108-15 cells. Competition of [^{125}I]Sar^1Ile^8 AII binding with Sar^1Ile^8 AII (□), AII (■) or AT_1 [DUP 753 (△) or AT_2 [CGP 42112 (●) and PD 123177 (○)]-selective receptor ligands. (B) Non-differentiated NG108-15 cells exhibit mainly T-type calcium current. The plot of the peak current vs. test potential corresponding to the panels (C) and (D) displays only a low-threshold (and fast-inactivated) calcium current. (C and D) Currents recorded for test potentials from −55 tot −25 mV and from −20 to 40 mV, respectively (holding potential, −80 mV).

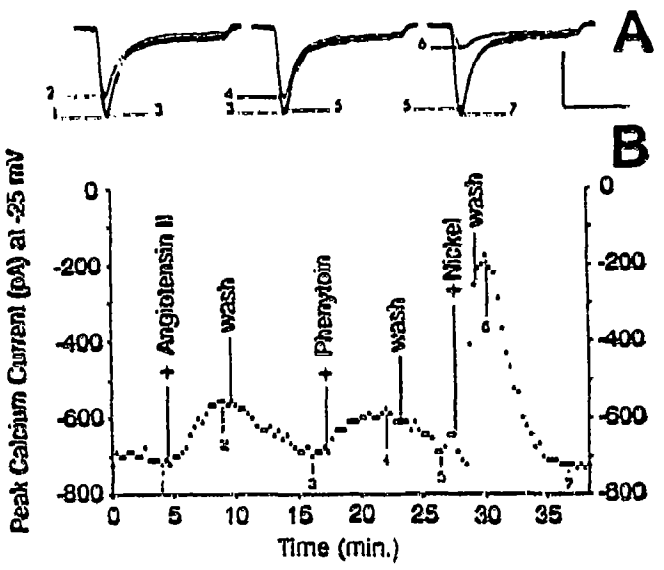

Fig. 2. AII and the selective T-type calcium current inhibitors, phenytoin and Ni^{2+}, decrease the calcium current in non-differentiated NG108-15 cells. (A) Control and currents under AII (100 nM) or modified by phenytoin (30 μM) or $NiCl_2$ (100 μM). Holding potential, −80 mV; test potential, −25 mV. The horizontal bar represents 100 ms and the vertical bar 500 pA. (B) Peak calcium current recorded at −25 mV as a function of time.

3.2. *Non-differentiated NG108-15 cells display only T-type calcium current*

Patch-clamp studies, recording in the whole-cell configuration [30], have been performed to study the effect of AII on calcium currents in non-differentiated NG108-15 cells. Most of these cells exhibit one type of calcium current [31,32], namely, one that is activated at low threshold and rapidly inactivated again. Fig. 1B shows its characteristic current–voltage relation. The calcium current is activated between −60 and −55 mV, reaches a maximal amplitude between −30 and −20 mV and inactivates within 70 ms for test potentials higher than −40 mV (Fig. 1C and 1D; n= 25). Ni^{2+} (100 μM) strongly decreases this current by 63.8 ± 5.5% at −25 mV (n= 8) in a reversible manner (Fig. 2). Phenytoin (30 μM), a more specific blocker of the low-threshold-activated calcium current in differentiated N1E-115 cells [33], also decreases the same current in our non-differentiated NG108-15 cells (Fig. 2), by 25.2 ± 3.1% at −25 mV (n= 8), with completely recovery after wash-out. Based on its activation threshold, kinetics and sensitivity to Ni^{2+} [34] and to phenytoin [33], this calcium current is thus identified as a T-type calcium current [34,35].

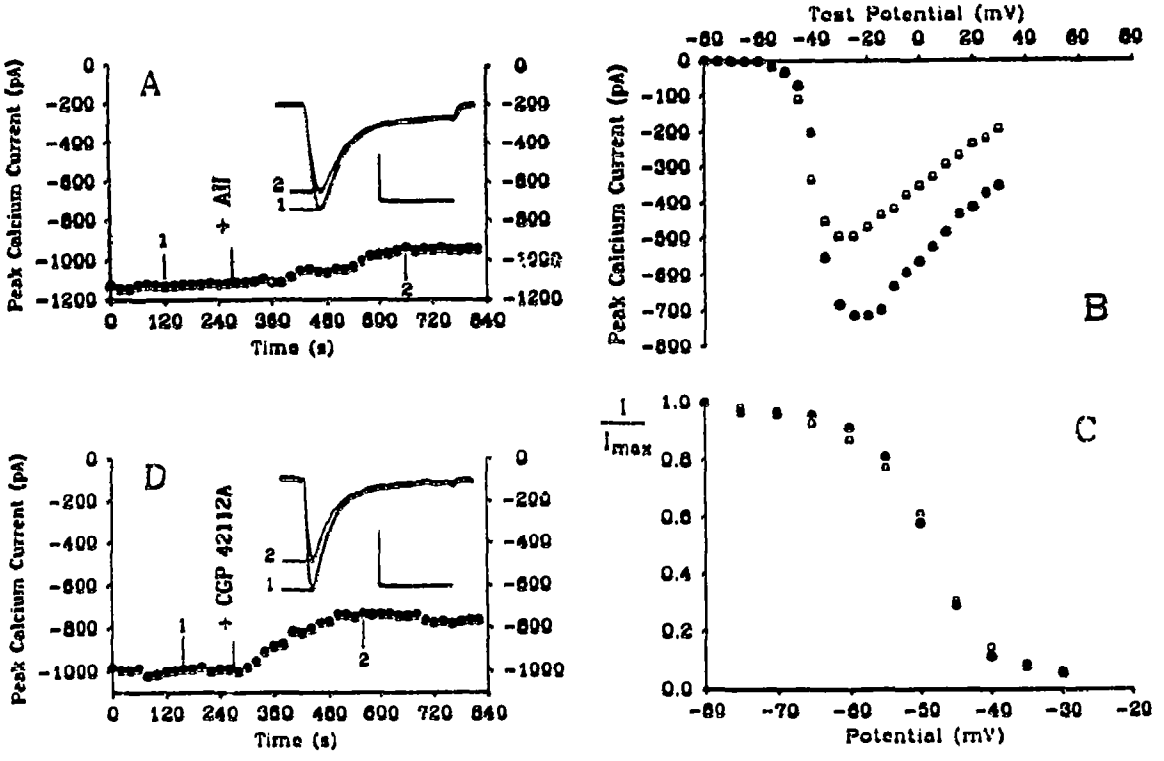

Fig. 3. Time-course and voltage effects of AII and the AT_2-selective agonist, CGP 42112, on the T-type calcium current. (A and D) AII (100 nM) and CGP 42112 (100 nM) significatively modulate T-type calcium current about 2 min after addition, with maximal effect between 5 and 10 min; (inserts) control and peptide-modified currents; the horizontal bar represents 100 ms and the vertical bar 500 pA. The currents were recorded at −25 mV (A) and −30 mV (D) (holding potential, −80 mV). (B) AII (100 nM) decreases the T-type calcium current for test potentials higher than −40 mV. For the lowest potentials (<−40 mV), a shift in the I–V curve is observed; the potential for half-amplitude current ($V_{1/2}$) shifts from −37.8 ± 0.8 mV (n = 23; ●) to −42.3 ± 1.0 mV (n = 7) after the addition of AII (□). (C) AII does not significatively modify steady-state inactivation curves: V_h= −48.4 ± 0.8 mV and k = 5.1 ± 0.2 mV (n = 10) in control conditions (●); V_h = −50.9 ± 0.8 mV and k = 5.8 ± 0.5 mV (n = 5) 10 min after addition of AII (□).

3.3. *Angiotensin II and CGP 42112 modulate T-type calcium currents through AT_2 receptors*

As shown in Fig. 3A, we find AII (100 nM) consistently inhibits (21.5 ± 2.4% at −25 mV; n= 21) this current. The effect is noticeable approximately 2 min after application of AII and peaks between 5 and 10 min. The recovery after wash-out (n= 5/7) demonstrates that the decrease observed is not a spontaneous run-down (Fig. 2). Current–voltage curves (Fig. 3B) show that AII reduces the T-type current at membrane potentials higher than −40 mV (n= 7). A shift in the I–V relationship is observed at lower potentials (−60 to −45 mV), however, AII does not significantly modify the steady-state inactivation curves (Fig. 3C). The AII peptide analog, CGP 42112 (100 nM), produces exactly the same effects as AII (n= 10; Fig. 3D), which indicates the AT_2 specificity of our observations and that CGP 42112 behaves as an agonist. Moreover when the AT_1 antagonist, DuP 753 (1 μM), is added to the extracellular medium, AII (10 nM) decreases the T-type calcium current by 21.5 ± 4.3% at −25 mV (n= 6; data not shown), which confirms the AT_2 specificity of AII's effects.

Our data provide the first evidence of a membrane conductance modulation by AII through the AT_2 receptor, and clearly show that CGP 42112 is an AT_2 receptor agonist. The transduction mechanism involved between the AT_2 receptor and the T-type calcium channels is now under investigation. It has been well established that a T-type calcium current supports pacemaker activities in neurons [34,36,37]. Looking at the pattern of AT_2 distribution in the brain [6–10] it is noteworthy that structures like the thalamus, the inferior olivary and the locus coeruleus express high levels of AT_2 receptors and have pacemaker activities resting on a low threshold-activated calcium conductance [38–40]. In the light of these observations, it is tempting to speculate that AII could act as a frequency modulator of membrane potential oscillations through AT_2 receptors.

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