The EMBO Journal vol.7 no.3 pp.619-624, 1988

Angiotensin II-induced stimulation of voltage-dependent Ca^{2+} currents in an adrenal cortical cell line

J.Hescheler, W.Rosenthal[1], K.-D.Hinsch[1], M.Wulfern[1], W.Trautwein and G.Schultz[1]

II. Physiologisches Institut, Universität des Saarlandes, D-6650 Homburg/Saar and [1]Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69/73, D-1000 Berlin 33, FRG

Communicated by U.Gehring

Biochemical studies suggest that stimulation of aldosterone secretion by angiotensin II involves activation of voltage-dependent Ca^{2+} channels. We used an adrenocortical cell line (Y1) to study the effect of angiotensin II on transmembranous currents. The hormone (1 nM to 1 μM) caused depolarization of the plasma membrane (from −35 to 10 mV) and elicited repetitive action potentials. Using the whole-cell clamp technique, we identified two types of voltage-dependent Ca^{2+} currents which differed with respect to their threshold potential and time course of inactivation. Angiotensin II (1 nM to 1 μM) stimulated a slowly inactivating Ca^{2+} current on average up to 1.7-fold whereas a fast inactivating Ca^{2+} current remained almost unaffected by the hormone. Ca^{2+} currents were not influenced by forskolin (1 μM) or intracellularly applied cAMP (50 μM). Pretreatment of cells with pertussis toxin abolished the hormonal stimulation of the slowly inactivating Ca^{2+} current but was without effect on control currents. The toxin ADP-ribosylated a single membranous peptide of 40 kd M_r. An antiserum raised against a synthetic peptide corresponding to a region common to all sequenced α-subunits of guanine nucleotide-binding proteins (G-proteins) and an antiserum raised against a peptide corresponding to a region of α-subunits of G_i-like G-proteins reacted with membranous 40 kd peptides, whereas an antiserum raised against a synthetic peptide corresponding to a region specific for the α-subunit of the G-protein, G_0, failed to recognize a peptide in the 39 to 40 kd region. The results demonstrate that angiotensin II depolarizes Y1 cells and stimulates voltage-dependent Ca^{2+} channels and that stimulatory functional coupling of angiotensin II receptors to Ca^{2+} channels occurs independently of cAMP but involves a pertussis toxin-sensitive G-protein of the G_i-type.

Key words: Y1 cells/voltage-dependent Ca^{2+} currents/pertussis toxin/guanine nucleotide-binding proteins

Introduction

Angiotensin II is the major stimulator of aldosterone secretion in adrenocortical glomerulosa cells. A number of observations suggests that activation of voltage-dependent Ca^{2+} channels is involved in the angiotensin II-induced steroidogenesis. Both angiotensin II- and K^+-induced aldosterone secretions depend on extracellular Ca^{2+}, are inhibited by various Ca^{2+} channel blockers (Fakunding *et al.*, 1979; Fakunding and Catt, 1980; Foster *et al.*, 1981; Kojima *et al.*, 1984b; Kojima *et al.*, 1985a; Aguilera and Catt, 1986) and are stimulated by the Ca^{2+} channel agonist, Bay K 8644 (Kojima *et al.*, 1984b; Hausdorff *et al.*, 1986). In addition, angiotensin II and K^+ have been shown to stimulate Ca^{2+} influx in a dihydropyridine-sensitive manner (Kojima *et al.*, 1985b, 1986). The assumption that angiotensin II activates Ca^{2+} channels is further supported by the identification of specific, high affinity binding sites for Ca^{2+} channel blockers (Aguilera and Catt, 1986) and by the recent electrophysiological identification of voltage-dependent Ca^{2+} channels in glomerulosa cells (Maatsunaga *et al.*, 1987).

Besides the angiotensin II-triggered Ca^{2+} influx, stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis, subsequent release of intracellularly stored Ca^{2+} and activation of protein kinase C contribute to the steroidogenesis induced by the hormone (Kojima *et al.*, 1984a). The functional relevance of angiotensin II-induced inhibition of adenylate cyclase (Woodcock and Johnston, 1984) is not clear. This inhibitory effect of angiotensin II is in contrast to that of adrenocorticotropin (ACTH), which stimulates adenylate cyclase in adrenal cells; stimulation of cAMP formation by ACTH may contribute to the ACTH-induced steroidogenesis (Graham-Smith *et al.*, 1967; Sala *et al.*, 1979; Aguilera and Catt, 1986).

Cellular responses to angiotensin II appear to be mediated by guanine nucleotide-binding proteins (G-proteins), acting as transducers between cell surface receptors and various effectors such as enzymes and ion channels (for reviews see Dolphin, 1987; Iyengar and Birnbaumer, 1987). The involvement of G-proteins in angiotensin II-induced effects is supported by several findings. Guanine nucleotides reduce the affinity of angiotensin II to its receptor (Glossmann *et al.*, 1974). In addition, stimulation of Ca^{2+} influx by angiotensin II (Kojima *et al.*, 1986) and hormonal inhibition of adenylate cyclase, in general, are abolished by pertussis toxin (PT) (Ui *et al.*, 1984), known to inhibit receptor-mediated responses by catalyzing ADP-ribosylation of α-subunits of most G-proteins. Moreover, hormonal stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in glomerulosa cells is mimicked by the GTP analogue, guanosine 5'-O-(3-thiotriphosphate) (GTPγS) (Enyedi *et al.*, 1986), but is insensitive to PT (Enyedi *et al.*, 1986; Kojima *et al.*, 1986); these findings suggest the involvement of a PT-insensitive G-protein in the angiotensin II-induced stimulation of a phospholipase C.

In order to determine whether voltage-dependent Ca^{2+} channels are possibly involved in the secretory effect of angiotensin II, we characterized the murine adrenocortical cell line, Y1 (Schimmer, 1980, 1985), using the whole-cell clamp technique. Here we report that angiotensin II depolarizes Y1 cells and stimulates voltage-dependent Ca^{2+} currents in a cAMP-independent, PT-sensitive manner. Parts

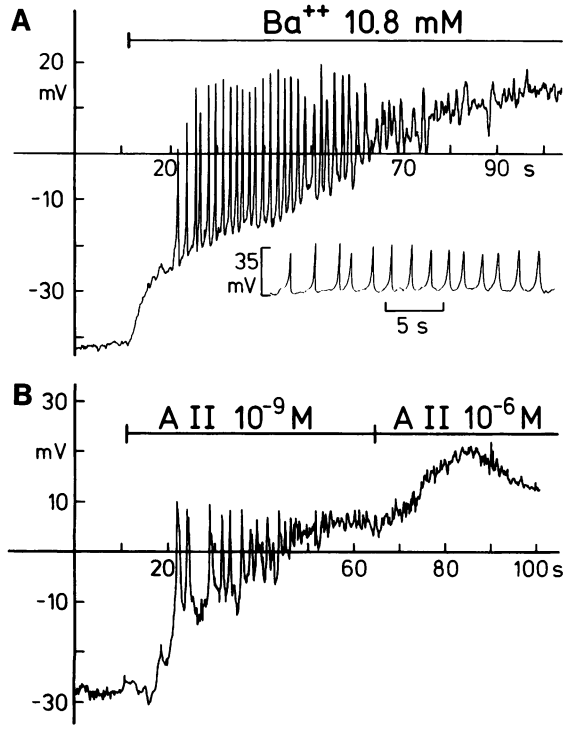

Fig. 1. Recordings of the membrane potential of a Y1 cell under current clamp conditions. The pipette was filled with solution I1. **Panel A**: At the time point 12 s, superfusion of the cell with solution E1 with 10.8 mM Ba^{2+} replacing Ca^{2+} was started. Depolarization and action potentials were observed. The inset shows action potentials 1–14 at an enlarged time scale. **Panel B**: Experimental conditions as in Panel A. Angiotensin II (A II; 1 nM) evoked depolarization and action potentials. At a higher concentration of angiotensin II (1 μM), a further transient depolarization was observed. The example shown was typical for seven cells tested.

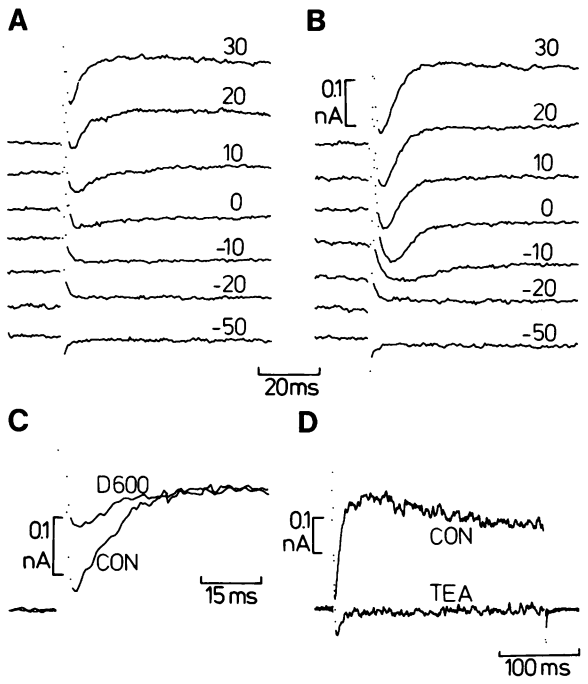

Fig. 2. Differentiation of inward and outward currents. Cells were kept at a holding potential of −40 mV. Pipettes were filled with solution I1. **Panels A** and **B**: Shown are membrane currents in response to the indicated potentials. In Panel A, solution E1 (1.8 mM Ca^{2+}) was used as extracellular medium; in Panel B, solution E1 was supplemented with 10.8 mM Ca^{2+}. **Panel C**: Solutions were as in Panel B. The test potential was 20 mV. Shown are current traces in the absence (CON) and presence of D 600 (1 μM). **Panel D**: Solutions were as in Panel B. The test potential was 30 mV. Current traces were recorded in the absence (CON) and presence of 20 mM tetraethylammonium chloride (TEA).

of the data have been presented in abstract form (Hescheler *et al.*, 1987b).

Results

Y1 cells, superfused with 5.4 mM K^+-containing solution, exhibited a resting membrane potential of −35 ± 4.2 mV (n = 7; Figure 1). Typically, we found fluctuations by about 5 mV, indicating an instability of the membrane potential higher than in cardiac or neuronal cells. As in other cells (Antoni and Oberdisse, 1965; Malecot *et al.*, 1984), Ba^{2+} (10.8 mM) caused depolarization of the plasma membrane and, above a threshold potential, elicited action potentials with an amplitude of about 30 mV. Spontaneous firing ceased after 1 min, and cells remained depolarized with fluctuations larger than those at the resting potential. Similarly, angiotensin II (1 nM) caused depolarization of the plasma membrane and action potentials; after the burst, the membrane potential remained positive with larger fluctuations. Increasing the angiotensin II concentration to 1 μM led to a further transient depolarization.

To investigate the ionic basis of the membrane potential and its modulation by angiotensin II, we performed voltage clamp experiments, using the K^+-containing external and internal solutions, E1 and I1, respectively (see Materials and methods) (Figure 2). Negative to −20 mV, the outward current measured at the end of pulses was small (slope conductance of 0.8 ± 0.8 nS; n = 11). Positive to −20 mV, we observed increasingly large outward currents with a slope conductance of 12.7 ± 1.9 nS (n = 11). The outward currents were completely blocked by supplementing solution E1 with tetraethylammonium chloride (20 mM) or by replacing K^+ by Cs^+ in the external and internal solutions (not shown). These findings indicate that the outward currents represent ion fluxes through voltage-dependent K^+ channels which were shown to exist in glomerulosa cells (Maatsunaga *et al.*, 1987). We also observed an early transient inward current component. This component became more apparent when the Ca^{2+} concentration was increased. In the presence of 1.8 mM and 10.8 mM extracellular Ca^{2+}, the current density measured as peak inward current in reference to the current 50 ms after onset of the pulse was 2.7 ± 1.1 μA/cm^2 (n = 11) and 5.3 ± 1.2 μA/cm^2 (n = 5), respectively. The inward current component was not affected by tetrodotoxin (TTX) (not shown) but was decreased by 1 μM D 600, suggesting that it was at least in part due to activation of voltage-dependent Ca^{2+} channels.

For further characterization of inward currents, we used the external solution E2 and the internal solution I2, which increase inward currents and decrease outward currents. Figure 3 compares current traces and current–voltage relations of inward currents obtained at holding potentials of −40 mV (Figure 3A–C) and −100 mV (Figure 3D–F). Superimposed current–voltage curves were taken during 300 ms test pulses at times given in the legend to Figure 3. At a holding potential of −40 mV, the time course of inward current inactivation was slow within the recorded

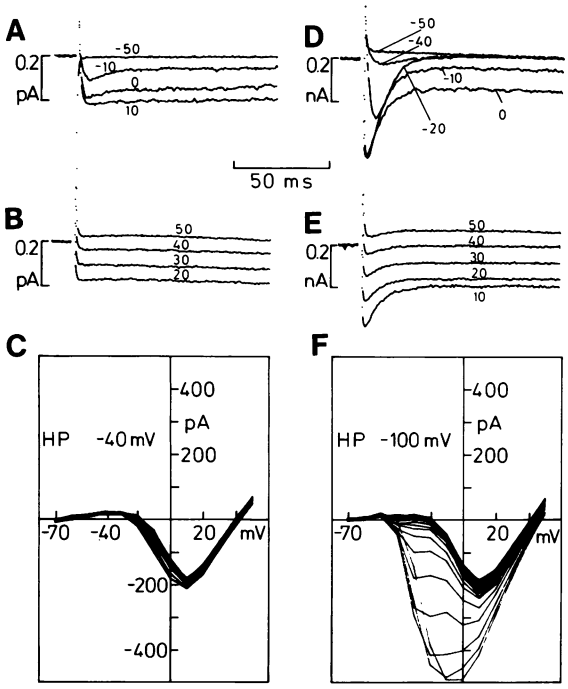

Fig. 3. Characterization of inward currents. Inward currents were measured at holding potentials (HP) of −40 mV (left panels) or −100 mV (right panels). **Panels A, B, D** and **E**: Superimposed current traces were obtained at the indicated test potentials with the external solution E2 supplemented with 0.1 μM TTX and the internal solution I2. **Panels C** and **F**: Shown are corresponding superimposed current−voltage relations. Currents were measured at 25 times within the 300 ms test pulse, i.e. 7, 9, 11, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280 and 290 ms after beginning of the pulse. In Panel F, curves taken between 7 and 25 ms after beginning of the pulse showed a rapid decrease in the current amplitude.

interval. At a test potential of 5.6 ± 2.7 mV (n = 22), the current was maximal with a mean current density of 11.2 ± 3.1 μA/cm^2 (n = 105). At a holding potential of −100 mV, current−voltage relations taken at the end of pulses were identical to those observed at a holding potential of −40 mV. In addition, a second current component was observed. This component represented a fast inactivating current. After subtraction of the slowly inactivating component, the maximal current density was 7.8 ± 2.8 μA/cm^2 (n = 22) and occurred at −24.5 ± 2.7 mV (n = 22). Although the example shown was typical for most cells tested, the contribution of either inward current to the total inward current varied from cell to cell.

To test whether activated Na^+ channels attributed to inward currents, cells were superfused with solution E2 supplemented with 0.1 μM TTX or a solution containing (in mM) tetramethylammonium chloride 90, tetraethylammonium chloride 50, $MgCl_2$ 1, $BaCl_2$ 10.8, Hepes 10 and glucose 10 (titrated to pH 7.4 by CsOH). Neither TTX nor removal of Na^+ affected inward currents obtained at holding potentials of −100 or −40 mV (not shown). Thus, under the experimental conditions, Na^+ channels were not activated.

Pharmacological characterization of inward currents (Figure 4) revealed that the slowly inactivating current was inhibited by the inorganic and organic Ca^{2+} channel antagonists, Ni^{2+} (0.1 mM) and D 600 (1 μM) respectively. The Ca^{2+} channel agonist, Bay K 8644 (1 μM) stimulated the peak current of the slowly inactivating current on the one

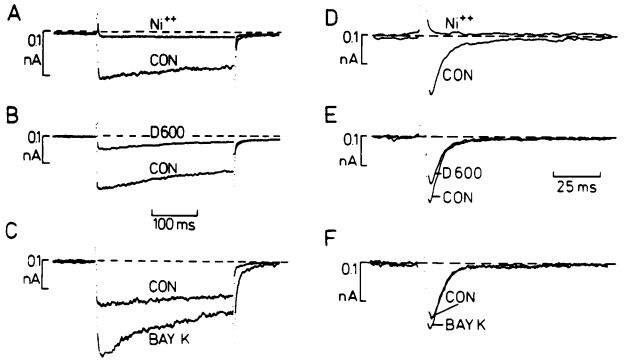

Fig. 4. Pharmacological characterization of Ca^{2+} currents. External and internal solutions E2 and I2, respectively, were used. In the experiments shown in **Panels D−F**, 0.1 μM TTX was present in the extracellular medium. Cells were clamped at −40 or −100 mV and depolarized to 0 mV or −30 mV (**Panels A−C** and **D−F** respectively). The concentrations of Ni^{2+}, D 600 and Bay K 8644 (Bay K) were 100, 1 and 1 μM respectively. CON, control.

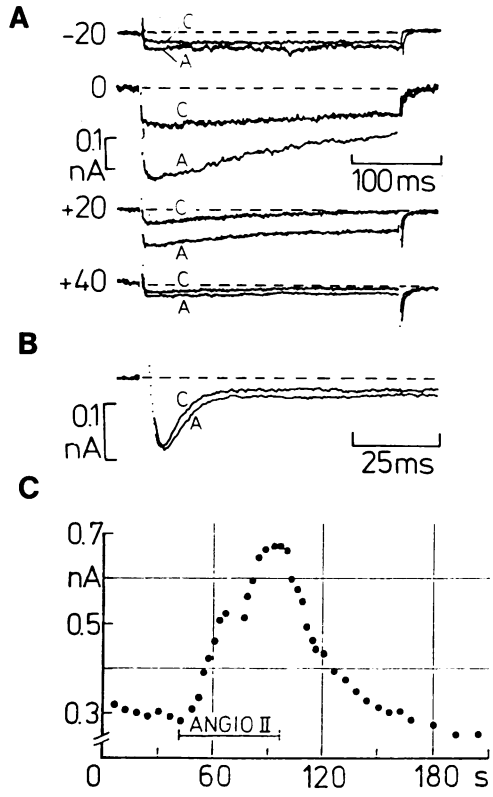

Fig. 5. Effect of angiotensin II on Ca^{2+} currents. External and internal solutions E2 and I2, respectively, were used. **Panel A**: The holding potential was −40 mV. The test potentials are indicated. Shown are current traces in the absence (C) and presence of 1 μM angiotensin II (A). **Panel B**: The holding potential was −100 mV and the test potential was −30 mV. Abbreviations and conditions are as in Panel A. **Panel C**: Shown is the time course of the inward current evoked by repetitive (0.2 Hz) voltage-clamp pulses from −40 to 0 mV. Angiotensin II (0.1 μM, ANGIO II) was present at the time indicated.

hand and accelerated its inactivation on the other hand, as was previously observed in myocytes (Hess *et al.*, 1984). The fast inactivating inward current, too, was inhibited by Ni^{2+}, but showed little sensitivity to the organic compounds, D 600 and Bay K 8644. Thus, the pharmacological properties of slowly and fast inactivating inward currents

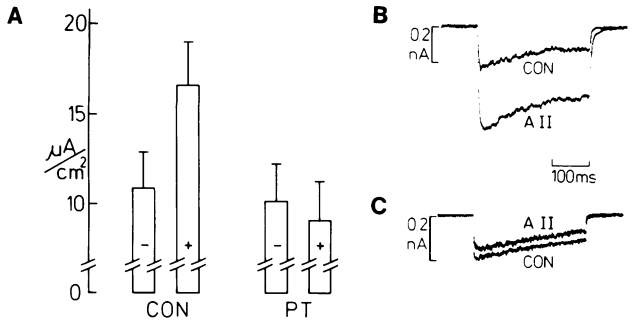

Fig. 6. Inhibition of the angiotensin II-induced increase in Ca^{2+} current by PT. Solutions E2 and I2 were used. The holding potential was −40 mV and the test potential was 0 mV. **Panel A**: Shown are mean current densities of 23 control cells (CON) and of 5 cells that were pretreated with PT (100 ng/ml) for 3–5 h. (−) and (+) indicate current densities in the absence and presence of angiotensin II (1 μM), respectively. **Panels B** and **C**: Shown are typical superimposed current traces of a control cell (Panel B) and of a cell pretreated with the toxin (Panel C). A II, angiotensin II.

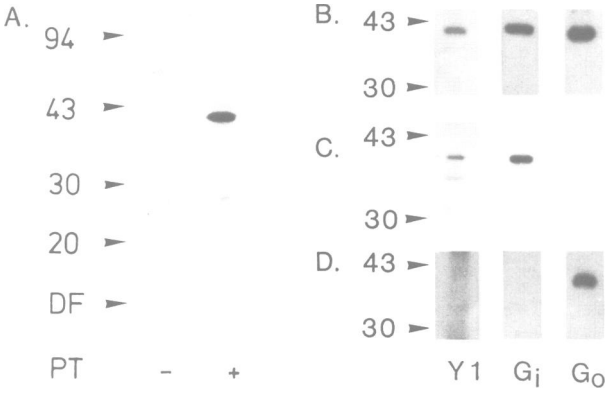

Fig. 7. G-protein α-subunits in membranes of Y1 cells. **Panel A**: Membranes (10 μg) were incubated with [^{32}P]NAD in the presence or absence of PT as described under Materials and methods; peptides were separated by SDS–PAGE (10% acrylamide). Shown is an autoradiograph of the SDS gel. **Panels B–D**: Membrane proteins (75 μg), G_i (purified from human erythrocytes, 3 μg) and G_o (purified from porcine brain, 3 μg) were subjected to SDS–PAGE (12.5% acrylamide). The separated subunits were blotted onto nitrocellulose filters. Subsequently, filters were incubated with a 1 : 300-diluted antiserum raised against the α_{common} peptide (Panel B), a 1 : 300-diluted antiserum raised against the α_i peptide (Panel C) or a 1 : 300-diluted antiserum raised against the α_o peptide (Panel D). Shown are autoradiographs obtained after incubation of filters with ^{125}I-protein A. Exposure time for lanes with membrane proteins were 36 h and for lanes with purified G-proteins 6 h. Figures on the left panel margins indicate relative molecular masses (kd).

of Y1 cells are similar to L- and T-type Ca^{2+} currents, respectively, of neuronal (Carbone and Lux, 1984; Nowycky *et al.*, 1985) and cardiac (Bean, 1985) cells.

The effect of angiotensin II on inward currents is summarized in Figure 5. The hormone increased the slowly inactivating current but left the fast inactivating current largely unaffected. Angiotensin II stimulated the slowly inactivating current at all current-inducing test potentials; at a test potential of 0 mV, an acceleration of current inactivation by the hormone was observed, similar to that observed with Bay K 8644. The hormonal response, observed in 85% of the cells tested, occurred at angiotensin II concentrations ranging from 1 nM to 1 μM. The average increase of the slowly inactivating current in hormone-responsive cells amounted to 38 and 74% at angiotensin II concentrations of 1 nM and 1 μM, respectively. The current increase was rapid in onset and completely washed out within 1 min.

In a series of experiments, we examined whether cyclic nucleotides are involved in the stimulation of inward currents by angiotensin II. When cAMP at a concentration of 50 μM was infused into cells, the peak current density of the slowly inactivating component decreased within 5 min from 9.2 ± 2.6 to 7.7 ± 2.3 μA/cm^2 (n = 9). This decrease was not significantly different from the 'run down' observed in control cells (see Materials and methods). In addition, cAMP did not affect the stimulatory effect of angiotensin II (not shown). Moreover, superfusion of cells with 1 μM forskolin, a stimulator of adenylate cyclase (Seamon and Daly, 1986), did not affect inward currents or the angiotensin II effect. Intracellular application of cGMP (100 μM) stimulated the slowly inactivating inward current within 5 min after disruption of the membrane patch on an average by 15%. Although the observed increase was statistically not significant, a stimulatory effect of cGMP could not be ruled out, taking into account the time-dependent 'run down' of inward currents observed in control cells. However, preliminary data indicate that the effect of the hormone is additive to that of cGMP.

In order to test the involvement of G-proteins in the angiotensin II-induced increase in inward currents, we pretreated cells with PT (100 ng/ml). The toxin did not affect basal inward currents but abolished the stimulatory angiotensin II effect on the slowly inactivating current (Figure 6). In fact, in most cells pretreated with the toxin, a slight inhibition of the slowly inactivating current by angiotensin II was observed. The effect of the toxin indicated that hormonal stimulation of phospholipase C cannot be assumed to provide a basis of the hormone-induced stimulation of inward currents (see Introduction). This assumption was supported by preliminary data indicating that 4β-phorbol-12-myristate-13-acetate, a potent activator of protein kinase C, did not stimulate inward currents in Y1 cells (not shown). The results demonstrate rather that PT-sensitive G-proteins are involved in functional coupling of receptors for angiotensin II and voltage-dependent Ca^{2+} channels.

Figure 7 shows the characterization of G-protein α-subunits in membranes of Y1 cells, using PT and antisera raised against synthetic peptides of confined regions of α-subunits. The toxin catalyzed the ADP-ribosylation of a single membranous peptide of 40 kd M_r. An antiserum raised against the α_{common} peptide (see Materials and methods), which reacted with the 40 kd α-subunit of G_i purified from porcine brain or from human erythrocytes or with the 39 kd α-subunit of G_o purified from porcine brain, recognized a 40 kd membranous peptide of Y1 cells. A membranous peptide of 40 kd M_r was also recognized by an antiserum raised against the α_i peptide; this antiserum reacted with the α-subunits of G_i-like G-proteins purified from brain or erythrocytes but not with the α-subunit of G_o. In contrast, an antiserum raised against the α_o peptide, which only recognized the α-subunit of G_o, did not recognize a membranous peptide in the 39 to 40 kd region. The results indicate that the PT substrate of membranes of Y1 cells corresponds to the α-subunit of a G_i-like G-protein (Suki *et al.*, 1987).

Discussion

Y1 cells exhibit two types of Ca^{2+} currents resembling those of the L- and T-types of neuronal (Carbone and Lux, 1984; Nowycky *et al.*, 1985) and cardiac (Bean, 1985) cells with respect to voltage dependence, time course of inactivation and sensitivity toward D 600 and Bay K 8644. Because of the low outward currents at negative potentials, the instability of the resting potential may reflect a partial activation/inactivation of Ca^{2+} channels, which may contribute to basal aldosterone secretion. Angiotensin II induces a strong depolarization and action potentials. The hormone-induced depolarization may, at least in part, be caused by the activation of voltage-dependent Ca^{2+} channels.

To test whether Y1 cells represent a suitable model for the physiological counterpart, the adrenocortical cells, we performed a few experiments with porcine glomerulosa cells isolated by collagenase treatment. Similar to Y1 cells, glomerulosa cells exhibited slowly and fast inactivating inward currents. Angiotensin II (1 μM) caused a 2.5-fold increase of the slowly inactivating inward current; the fast inactivating inward current was not affected by the hormone. The increase in inward current evoked by the hormone appeared to be more pronounced than in Y1 cells.

The mechanism by which angiotensin II stimulates voltage-dependent Ca^{2+} channels is not fully understood. Cardiac Ca^{2+} channels are stimulated by cAMP-dependent phosphorylation (Reuter, 1983; Kameyama *et al.*, 1985, 1986), and cAMP-dependent phosphorylation appears to be a prerequisite for Ca^{2+} channel activity in isolated membrane patches of pituitary cells (Armstrong and Eckert, 1987). In contrast, the inhibitory effect of angiotensin II on adenylate cyclase and the data presented above suggest that the hormonal stimulation of Ca^{2+} currents in Y1 cells is independent of cAMP. Similarly, protein kinase C, which is known to affect the activity of various ion channels (Kaczmarek, 1987) is not likely to be involved. This assumption is based on the findings that stimulation of voltage-dependent Ca^{2+} currents and influx of Ca^{2+} by angiotensin II (Kojima *et al.*, 1986) are inhibited by pretreatment of cells with PT, whereas stimulation of phospholipase C by angiotensin II is not (Enyedi *et al.*, 1986; Kojima *et al.*, 1986).

Recently, several hormones have been described that inhibit voltage-dependent Ca^{2+} channels in neuronal and pituitary cells (Holtz IV *et al.*, 1986; Lewis *et al.*, 1986; Scott and Dolphin, 1986; Hescheler *et al.*, 1987a); this inhibition is apparently independent of cAMP and mediated by a PT-sensitive G-protein which may be identical with G_o (Hescheler *et al.*, 1987a). On the other hand, angiotensin II is the only hormone that has been shown to stimulate voltage-dependent Ca^{2+} currents via a cAMP-independent mechanism in a mammalian cell. The sensitivity of this effect towards PT suggests the involvement of a G-protein. In membranes of Y1 cells, we identified a single PT substrate of 40 kd M_r. By immunological criteria, this substrate corresponded to the α-subunit of a G_i-like G-protein. A role for a G_i-like G-protein in the hormonal stimulation of voltage-dependent Ca^{2+} channels may be conceivable considering that angiotensin II inhibits adenylate cyclase and that hormonal inhibition of adenylate cyclase is generally mediated by interaction of activated cell surface receptors with a G_i-like G-protein (Dolphin, 1987; Iyengar and Birnbaumer, 1987). Thus, G_i-like G-proteins do not only mediate hormonal inhibition of adenylate cyclase and, as described recently, hormonal activation of K^+ currents (Yatani *et al.*, 1987; Codina *et al.*, 1987), but may also mediate activation of voltage-dependent Ca^{2+} channels by angiotensin II. At present, it is not clear whether one G_i-like G-protein serves several functions or whether each member of the family of G_i-like G-proteins interacts with only one effector. It also remains to be determined whether G_i-like G-proteins interact directly with voltage-dependent Ca^{2+} channels or whether as yet unknown regulatory components, e.g. protein kinases and phosphatases, are involved.

Materials and methods

PT was kindly provided by Dr Motoyuki Yajima (Kyoto, Japan). Angiotensin II and TTX were obtained from Sigma (München, FRG) and nucleotides from Boehringer Mannheim (Mannheim, FRG), Sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate was purchased from Pierce (Rodgau, FRG). Sources for other materials have been cited (Rosenthal *et al.*, 1986).

Y1 cells were seeded at a density of about 2×10^5 cells/cm^2 and cultured as monolayers at 37°C in Ham's F-10 medium containing 10% horse serum, 7.5% fetal calf serum and non-essential amino acids, and gassed with 7% CO_2 in air. Culture medium was replaced by fresh medium every 2 days.

For electrophysiological studies, cells were seeded at a density of about 10^3 cells/mm^2 in 24-well plates, each well containing 3–4 glass slides (5 × 4 mm). Cells were kept for 3–8 days and, if indicated, treated with PT (100 ng/ml) for 3–5 h prior to experiments. A glass slide with attached cells was transferred into a chamber with a volume of ~200 μl. For determination of the membrane potential and differentiation of currents, cells were superfused with a solution (E1) containing (in mM) NaCl 140, $CaCl_2$ 1.8, $MgCl_2$ 1, KCl 5.4, glucose 10, Hepes 10, pH 7.4 at 36°C. For determination of inward currents, Ba^{2+} was used as charge carrier in a solution (E2) containing (in mM) NaCl 125, $BaCl_2$ 10.8, $MgCl_2$ 1, CsCl 5.4, glucose 10, Hepes 10, pH 7.4. Patch electrodes were prepared from Pyrex glass capillaries according to Hamill *et al.* (1981) with an inside tip diameter of 1–3 μm and a resistance of 3–6 MΩ when filled with pipette solution containing (in mM) K^+ aspartate 80, KCl 50, $MgCl_2$ 1, MgATP 3, Hepes 10, pH 7.4 (solution I1, for the determination of the membrane potential and differentiation of currents) or CsCl 120, $MgCl_2$ 1, EGTA 10, MgATP 3, Hepes 10, pH 7.4 (solution I2, for the determination of inward currents). GOhm-seals were formed by suction with a negative pressure of ~ −30 cm H_2O, and whole-cell clamp configuration was achieved by disruption of the membrane patch.

Under voltage clamp conditions, cells were held at −40 or −100 mV. Test pulses of 300 ms to different potentials (for determination of current–voltage relations) were applied at a rate of 0.2 Hz. The membrane capacity was measured as current response to a ramp pulse and amounted to 17.7 ± 2.7 pF (mean ± SD; n = 46). In order to calculate current densities, a specific membrane capacity of 1 μF/cm^2 was assumed (Hescheler *et al.*, 1986; Kameyama *et al.*, 1986).

The leakage conductance determined as ohmic conductance after blocking ion channels with a solution containing TTX, Ni^{2+} and Cs^+ was 1.5 ± 1.3 nS (mean ± SD; n = 26). In the experiments selected for presentation in the figures, the leakage conductances of cells were below 0.5 nS and, therefore, not corrected for. For calculation of mean values, all experiments performed under comparable conditions were taken into account and corrected for leakage, if the leakage conductance was above 0.5 nS. Values are given as mean values ± SD.

With the external solution E2 and the ATP-containing internal solution I2, inward currents measured during voltage clamp pulses from −40 to 0 mV were observed for about 5 min. If solution I2 was supplemented with adenosine-5'-O-(3-thiotriphosphate) (ATPγS; 1 mM) in addition to ATP, the decrease of inward currents was delayed and amounted to ~30% within 5 min. Under this condition, current densities at the beginning and 5 min after the beginning of the intracellular infusion were 10.4 ± 3.5 μA/cm^2 and 7.1 ± 4.6 μA/cm^2, respectively (n = 20). Therefore, experiments were performed with the internal solution I2 supplemented with 1 mM ATPγS. Except for its stabilizing effect, ATPγS did neither affect Ca^{2+} currents nor the stimulatory effect of angiotensin II on Ca^{2+} currents. Outward currents were stable for 15–30 min.

For preparation of membranes, Y1 cells were harvested with a cell scraper, collected by centrifugation at 1600 × g (4°C, 20 min) and disrupted by

nitrogen cavitation in a buffer consisting of (in mM) NaCl 100, EDTA 3, 2-mercaptoethanol 15, KH_2PO_4 50, pH 7.0. Nuclei were removed by short centrifugation at 1000 × *g* (4°C) and sedimentation of membranes was achieved by centrifugation at 30 000 × *g* (4°C, 15 min). Membranes were stored at −70°C in 10 mM triethanolamine−HCl buffer, pH 7.4. PT-catalyzed [^{32}P]ADP-ribosylation of membrane proteins was performed as described (Rosenthal *et al.*, 1986) with the following modifications: PT was preactivated in the presence of 0.1 mM ATP, and Lubrol PX (0.3%) and NADP (0.3 mM) were included in the reaction mixture (Rosenthal *et al.*, 1987). SDS−PAGE and autoradiography of gels were performed as described (Rosenthal *et al.*, 1986).

Antisera were raised against synthetic peptides corresponding to confined regions of G-protein subunits. Two peptides, which were identical to those used by Mumby *et al.* (1986), were a gift of Dr A.Herz, Max-Planck-Institut für Psychiatrie, München (FRG). One of these peptides corresponded to a region common to all sequenced α-subunits (α_{common} peptide). The other peptide (α_o peptide) corresponded to a region specific for the α-subunit of G_o. A third peptide with the sequence NLREDGEKAAREV (one-letter code) corresponding to a region of α-subunits of G_i-like G-proteins (α_i peptide) was kindly synthesized by Dr Heinrich Gausepohl and Dr Rainer Frank, European Molecular Biology Laboratory, Heidelberg (FRG). All synthesized peptides contained a cysteine residue at the amino terminus to facilitate crosslinking to keyhole limpet hemocyanin. Crosslinking was performed as described by Green *et al.* (1982) with the exception that sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate was used as crosslinking reagent instead of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. The protocol for the immunization of rabbits with crosslinked peptides was also adopted from Green *et al.* (1982). Antisera were tested for cross-reactivity by the immunoblotting technique, using purified G-proteins (Codina *et al.*, 1985; Rosenthal *et al.*, 1986). The α_{common} peptide antiserum used in this study reacted with the α-subunits of G_i, G_o and with that of the retinal G-protein, transducin, and less strongly with the α-subunit of G_s. The α_i peptide antiserum reacted with the α-subunit of G_i-like G-proteins purified from bovine brain and human erythrocytes; it did not recognize the α-subunits of G_o, G_s or transducin. The α_o peptide antiserum reacted with the α-subunit of G_o but did not recognize α-subunits of other purified G-proteins. A detailed characterization of the peptide antisera will be published elsewhere.

[α-^{32}P]ATP was synthesized according to Johnson and Walseth (1979), [^{32}P]NAD as described by Cassel and Pfeuffer (1978), and [^{125}I]protein A using Iodogen as oxidizing agent (Salcinski *et al.*, 1979). Protein was assayed according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Acknowledgements

We thank Sabine Bastuck, Monika Bigalke, Susanne Brendel, Karolin Dorst and Eva Retfalvi for valuable technical assistance in these studies, Rosemarie Krüger for typing the manuscript and Dr Klaus Peper for providing tissue culture facilities. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 246, and the Fonds der Chemischen Industrie.

References

Aguilera,G. and Catt,K.J. (1986) *Endocrinology*, **118**, 112−118.
Antoni,H. and Oberdisse,E. (1965) *Pflügers Arch*, **284**, 259−272.
Armstrong,D. and Eckert,R. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2518−2522.
Bean,B.P. (1985) *J. Gen. Physiol.*, **86**, 1−30.
Carbone,E. and Lux,H.D. (1984) *Nature*, **310**, 501−502.
Cassel,D. and Pfeuffer,T. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2669−2673.
Codina,J., Rosenthal,W., Hildebrandt,J.D., Birnbaumer,L. and Sekura,R.D. (1985) *Methods Enzymol.*, **109**, 446−465.
Codina,J., Yatani,A., Grenet,D., Brown,A.M. and Birnbaumer,L. (1987) *Science*, **236**, 442−445.
Dolphin,A.C. (1987) *Trends Neurosci.*, **10**, 53−57.
Enyedi,P., Mucsi,I., Hunyadi,L., Catt,K.L. and Spät,A. (1986) *Biochem,. Biophys. Res. Commun.*, **140**, 941−947.
Fakunding,J.L. and Catt,K.J. (1980) *Endocrinology*, **107**, 1345−1353.
Fakunding,J.L., Chow,R. and Catt,K.J. (1979) *Endocrinology*, **105**, 327−333.
Foster,R., Lobo,M.V., Rasmussen,H. and Marusic,E.T. (1981) *Endocrinology*, **109**, 2196−2201.
Glossmann,H., Baukal,A. and Catt,K.J. (1974) *J. Biol. Chem.*, **249**, 664−666.
Graham-Smith,D.G., Butcher,R.W., Ney,R.L. and Sutherland,E.W. (1967) *J. Biol. Chem.*, **242**, 5535−5541.
Green,N., Alexander,H., Olson,A., Alexander,S., Shinnick,T.M., Sutcliffe,J.G. and Lerner,R.A. (1982) *Cell*, **28**, 477−487.
Hamill,O.P., Marty,A., Neher,E. and Sakmann,B. (1981) *Pflügers Arch.*, **391**, 85−100.
Hausdorff,W.P., Aguilera,G. and Catt,K.J. (1986) *Endocrinology*, **118**, 859−874.
Hescheler,J., Kameyama,M. and Trautwein,W. (1986) *Pflügers Arch.*, **407**, 182−189.
Hescheler,J., Rosenthal,W., Trautwein,W. and Schultz,G. (1987a) *Nature*, **325**, 445−447.
Hescheler,J., Wulfern,M., Trautwein,W. and Schultz,G. (1987b) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **335**, (Suppl.), R134.
Hess,P., Lansman,J.B. and Tsien,R. (1984) *Nature*, **311**, 538−544.
Holtz,IV,G.G., Rane,S.G. and Dunlap,K. (1986) *Nature*, **319**, 670−672.
Iyengar,R. and Birnbaumer,L. (1987) *ISI Atlas of Science: Pharmacology*, **1**, 213−221.
Johnson,R. and Walseth,T. (1979) *Adv. Cyclic Nucleotide Res.*, **10**, 135−167.
Kaczmarek,L.K. (1987) *Trends Neurosci.*, **10**, 30−34.
Kameyama,M., Hofmann,F. and Trautwein,W. (1985) *Pflügers Arch.*, **405**, 285−293.
Kameyama,M., Hescheler,J., Hofmann,F. and Trautwein,W. (1986) *Pflügers Arch.*, **407**, 123−128.
Kojima,I., Kojima,K., Kreutter,D. and Rasmussen,H. (1984a) *J. Biol. Chem.*, **259**, 14448−14457.
Kojima,I., Kojima,K. and Rasmussen,H. (1985a) *Am. J. Physiol.*, **248**, E36−E43.
Kojima,I., Kojima,K. and Rasmussen,H. (1985b) *J. Biol. Chem.*, **260**, 9171−9176.
Kojima,I., Shibata,H. and Ogata,E. (1986) *FEBS Lett.*, **204**, 347−351.
Kojima,K., Kojima,I. and Rasmussen,H. (1984b) *Am. J. Physiol.*, **247**, E645−E650.
Lewis,D.L., Weight,F.F. and Luini,A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9035−9039.
Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol.. Chem.*, **193**, 265−275.
Maatsunaga,H., Maruyama,Y., Kojima,I. and Hoshi,T. (1987) *Pflügers Arch.*, **408**, 351−355.
Malecot,C., Coraboeuf,E. and Coulombe,A. (1984) *Am. J. Physiol.*, **247**, H429−H439.
Mumby,S.M., Kahn,R.A., Manning,D.R. and Gilman,A.G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 265−269.
Nowycky,M.C., Fox,A.P. and Tsien,R.W. (1985) *Nature*, **316**, 440−443.
Reuter,H. (1983) *Nature*, **301**, 569−574.
Rosenthal,W., Koesling,D., Rudolph,U., Kleuss,C., Pallast,M., Yajima,M. and Schultz,G. (1986) *Eur. J. Biochem.*, **158**, 255−263.
Rosenthal,W., Binder,T. and Schultz,G. (1987) *FEBS Lett.*, **211**, 137−141.
Sala,G.B., Hayashi,K., Catt,K.J. and Dufau,M.L. (1979) *J. Biol. Chem.*, **242**, 3861−3865.
Salcinski,P., Hope,J., McClean,C., Clement-Jones,V., Sykes,J., Price,J. and Lowry,P.J. (1979) *J. Endocrinol.*, **81**, 131.
Schimmer,B.P. (1980) *Adv. Cyclic Nucleotide Res.*, **13**, 181−214.
Schimmer,B.P. (1985) *Methods Enzymol.*, **109**, 350−356.
Scott,R.H. and Dolphin,A.C. (1986) *Neurosci. Lett.*, **69**, 59−64.
Seamon,K. and Daly,J.W. (1986) *Adv. Cyclic Nucleotide Prot. Phosph. Res.*, **20**, 1−150.
Suki,W.N., Abramowitz,J., Mattera,R., Codina,J. and Birnbaumer,L. (1987) *FEBS Lett.*, **220**, 187−192.
Ui,M., Katada,T., Murayama,T., Kurose,H., Yajima,M., Tamura,M., Nakamura,T. and Nogimoro,K. (1984) *Adv. Cyclic Nucleotide Prot. Phosph. Res.*, **17**, 145−151.
Woodcock,E.A. and Johnston,C.I. (1984) *Endocrinology*, **115**, 337−341.
Yatani,A., Codina,J., Brown,A.M. and Birnbaumer,L. (1987) *Science*, **235**, 207−211.

Received on November 16, 1987; revised on December 23, 1987