

Activation by Angiotensin II of Ca^{2+} -dependent K^+ and Cl^- Currents in Zona Fasciculata Cells of Bovine Adrenal Gland

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Received: 29 May 1997/Revised: 4 November 1997

Abstract. The effects of angiotensin II (100 nM) on the electrical membrane properties of zona fasciculata cells isolated from calf adrenal gland were studied using the whole cell patch recording method. In current-clamp condition, angiotensin II induced a biphasic membrane response which began by a transient hyperpolarization followed by a depolarization more positive than the control resting potential. These effects were abolished by Losartan (10^{-5} M), an antagonist of angiotensin receptors of type 1. The angiotensin II-induced transient hyperpolarization was characterized in voltage-clamp condition from a holding potential of -10 mV. Using either the perforated or the standard recording method, a transient outward current accompanied by an increase of the membrane conductance was observed in response to the hormonal stimulation. This outward current consisted of an initial fast peak followed by an oscillating or a slowly decaying plateau current. In Cl^- -free solution, the outward current reversed at -78.5 mV, a value close to E_{K} . It was blocked by external TEA (20 mM) and by apamin (50 nM). In K^+ -free solution, the transient outward current, sensitive to Cl^- channel blocker DPC (400 μM), reversed at -52 mV, a more positive potential than E_{Cl} . Its magnitude changed in the same direction as the driving force for Cl^- . The hormone-induced transient outward current was never observed when EGTA (5 mM) was added to the pipette solution. The plateau current was suppressed in nominally Ca^{2+} -free solution (47% of cells) and was reversibly blocked by Cd^{2+} (300 μM) but not by nisoldipine (0.5–1 μM) which inhibited voltage-gated Ca^{2+} currents identified in this cell type. The present experiments show that the transient hyperpolariza-

tion induced by angiotensin II is due to Ca^{2+} -dependent K^+ and Cl^- currents. These two membrane currents are co-activated in response to an internal increase of $[\text{Ca}^{2+}]_i$, originating from intra- and extracellular stores.

Key words: Zona fasciculata cells — Angiotensin II — K^+ current — Cl^- current — Ca^{2+} stores

Introduction

Zona fasciculata cells isolated from the inner area of bovine adrenal gland are known to secrete corticosteroid hormones in response to adrenocorticotrophic hormone (ACTH). In bovine species, the secretory activity of this cell type is also stimulated by angiotensin II (Ang II) (Hepp et al., 1977). Specific receptors for Ang II identified in zona fasciculata cells (Penhoat et al., 1988; Valotton et al., 1981) are divided into 2 subtypes: angiotensin receptors of type 1 (AT1 receptors) and of type 2 (AT2 receptors). AT1 receptors, which represent 80% of angiotensin receptors in bovine zona fasciculata cells, are selectively antagonized by Losartan (Ouali et al., 1992; Ouali, Lebrethon & Saez, 1993) and appear to mediate the biological effects of the hormone.

Various studies have investigated the secretion of corticosteroids and its regulation in response to the hormonal stimulation of bovine zona fasciculata cells but the electrophysiological properties underlying these mechanisms are poorly documented. Different voltage-activated ionic currents have been identified in this cell type, namely, a transient inward Ca^{2+} current (Mlinar, Biagi & Enyeart, 1993a), a maintained inward Ca^{2+} current (Bilbaut, Chorvatova & Ojeda, 1993) and a large transient outward K^+ current (Bilbaut et al., 1996; Mlinar & Enyeart, 1993) which dominates the total membrane

current. On the other hand, Mlinar, Biagi & Enyeart (1993b) have shown that the stimulation of bovine zona fasciculata cells by ACTH or Ang II induced a dose-dependent depolarization of the cell membrane. Voltage-clamp experiments performed on isolated cells using the standard whole cell recording method indicated that this depolarization resulted from the inhibition by Ang II of a noninactivating K^+ current named (I_{AC}) displaying a time-dependent growth (Mlinar et al., 1993b). In calf adrenal zona fasciculata cells, Bilbaut et al. (1996) did not report the presence of such a K^+ current when voltage-clamp experiments were performed using either the standard or the perforated recording method. However, using the standard method, 30% of tested cells expressed an instantaneously, noninactivating DPC-sensitive outward current that developed during the course of the experiments (Bilbaut et al., 1996).

In the present work, we describe the effects of Ang II (100 nM) on membrane potential and membrane current of isolated calf zona fasciculata cells in primary culture using both perforated and standard whole cell recording methods. Our results indicate that Ang II induces a fast transient hyperpolarization followed by a slow depolarization. We showed that the transient hyperpolarization was due to the increase of a Ca^{2+} -dependent potassium conductance together with a smaller Ca^{2+} -dependent chloride conductance. Part of these results have been reported in an abstract form (Chorvatova et al., 1995).

Materials and Methods

CELL PREPARATION

Adrenal glands were removed from 4–6-month-old calves. The cortical tissue was dissociated as described by Crozat, Penhoat & Saez (1986) by sequential trypsinization of 0.5 mm thick slices obtained with a Stadie-Riggs microtome. Only the second and possibly the third slices were used for enzymatic dispersion. The dissociation medium contained: trypsin (0.16%) in HAM F12/DMEM medium (1:1), gentamycin (20 $\mu\text{g}/\text{ml}$), nystain (10 U/ml), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), glutamate (5 mM) and NaHCO_3 (14 mM) buffered with HEPES (15 mM) at pH 7.4. Dispersed cells were then washed and resuspended in culture medium containing HAM F12/DMEM (1:1), glutamate (5 mM), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), NaHCO_3 (14 mM) insulin (5 $\mu\text{g}/\text{ml}$), transferrin (10 $\mu\text{g}/\text{ml}$), and vitamin C (10^{-4} M) supplemented with foetal bovine serum (1%). To avoid confluency, the cells were seeded at low density (4000 to 6000 cells/ cm^2) in 35 mm Petri dishes and cultured in a humidified incubator at 37°C gassed with 5% CO_2 in air. After 24 hr, the culture medium was replaced with the same medium without serum.

ELECTROPHYSIOLOGY

Patch-clamp recordings were made in whole cell configuration using both standard (ruptured patch membrane) (Hamill et al., 1981) and perforated patch recording methods (Rae et al., 1991). Current- and

voltage-clamp experiments were performed between 16 and 72 hr after plating. Membrane responses were observed at a concentration of 100 nM Ang II which is a saturating concentration known to stimulate the corticosteroid secretion of isolated cells in primary culture (Penhoat et al., 1988). For experiments, a Petri dish was transferred from the incubator onto the stage of an inverted microscope and the culture medium was replaced by the control physiological solution. Further changes of external solution were then performed using a gravity perfusion system at a rate of 0.5 ml/min placed close to the tested cell. Recording chamber was grounded using an agar-KCl bridge. Fire polished pipettes, pulled from borosilicate glass (CG 150T-15 mm o.d., Clark Electromedical Instruments, Reading, G.B.) using a horizontal puller (DMZ, Universal Puller, Zeitz Instrument, Augsburg, Germany), were connected to the headstage of a patch-clamp amplifier RK 300 (Bio-Logic, Claix, France). Patch pipettes had a tip resistance of 2–4 M Ω and using the standard recording method, series resistance was in the range of 4 to 6 M Ω . For perforated patch recordings, the tip of the pipette was dipped into the pipette solution for 15 sec then the pipette was backfilled with the solution containing amphothericin B. Experiments were started about 10 to 15 min after the seal was established when the increase of the transient capacitive current reached a steady-state value indicating a final series resistance of about 8 to 12 M Ω . Usually, the series resistance was not compensated because the voltage error introduced during the agonist-induced membrane responses was estimated to be lower than 2%. From a holding membrane potential of –10 mV, membrane conductance was monitored by injecting 20 mV hyperpolarizing pulses of 300 msec duration. Current-voltage curves were determined either from voltage pulses of 40 msec duration delivered each 5 sec between +30 and –90 or –110 mV by 20 mV increments or from voltage ramps of 2-sec duration applied between –90 and +30 mV as indicated in the text. All experiments were performed at room temperature (20–25°C) on single cells that adhered to the bottom of the Petri dish. When the control holding current recorded using the standard method displayed a time-dependent growth, the cells were discarded from the experiments.

DATA ACQUISITION AND ANALYSIS

Pulse protocols were generated using the P-Clamp software (Axon Instruments, Burlingame, CA) or a programmable stimulator SMP 300 (Bio-Logic). Current signals were filtered at 300 Hz, digitized at 1 kHz with an analogue to digital converter (Labmaster TM 40, Scientific Solutions, Solon, OH) and stored on the hard disc of a computer. For current-clamp experiments, the I_o device of the patch-clamp amplifier was used. Voltage and current traces evoked in response to hormone application were recorded on both pen (Brush 2400) and tape (DTR 1204, Bio-Logic) recorders. For data analysis, Bio-Logic softwares were used. Results are presented as means \pm SEM. Statistical data were considered as significantly different when P values obtained from t -test were lower than 0.05.

SOLUTIONS AND DRUG PREPARATION

The control external solution contained (in mM): NaCl, 130; KCl, 5; CaCl_2 , 2.5; MgCl_2 , 2; glucose, 10; HEPES, 10 at pH 7.2 buffered by NaOH. In high Ca^{2+} solution, CaCl_2 (20 mM) was isosmotically substituted for NaCl in the control solution. Nominally Ca^{2+} -free solutions were prepared in plastic flasks from deionized water originating from a double ion exchange column. Ca^{2+} -selective electrode measurements indicated that the free- Ca^{2+} concentration was lower than 0.5 μM . In these nominally Ca^{2+} -free solutions, CaCl_2 was equimolarly replaced by MgCl_2 . In K^+ -free solution, 5 mM KCl was substituted by 5 mM

Table 1. Ionic composition (mM) of pipette filling solutions used for standard patch recording

	K Aspartate	K (MSA)*	CsCl	Cs Aspartate	MgCl ₂	Mg (MSA)*	ATP (Na) ₂	EGTA	TEA Aspartate	Hepes
Solution A	130				2.5		2			10
Solution B		130				2	2			10
Solution C				110	2.5		2		20	10
Solution D			20	90	2.5		2		20	10
Solution E	130				2.5		2	5		10
Solution F				110	2.5		2	5	20	10

pH was adjusted at 7.2 by NaOH. (MSA)*: methanesulfonate acid.

CsCl while in Cl⁻-free solutions, Cl⁻ was exchanged with methanesulfonate ions. Tetraethylammonium (TEA) was added at the concentration of 5, 20 and 50 mM in the external solution in replacement of Na⁺. Cd²⁺ (300 μM) was added to the control solution. 4-aminopyridine (4-AP) stock solution (100 mM) was prepared at pH 7.2 just before use. Charybdotoxin (Latoxan, Rosans, France) and apamin (a gift of G. Romey, UPR 411 CNRS, Valbonne, France) were kept as stock solution at -20°C in control solution and distilled water respectively. Nisoldipine (a gift of Bayer-Pharma, Paris, France), DPC (diphenylamine-2,2' dicarboxylic acid, Aldrich, Steinheim, RFA) and 9-AC (anthracene-9-carboxylic acid, Sigma, St. Louis, MO) were prepared as stock solution in dimethylsulfoxide (DMSO) and kept at -20°C. The final DMSO concentration which was 0.1% did not affect the membrane properties of isolated cells. Human Ang II (Bachem, France) was prepared at the final concentration of 10⁻⁷ M from aliquots of 100 μl at 10⁻³ M frozen in acetic acid (50 mM). Successive dilution were made using the external solution. Stock solution of Losartan (DuPont Pharmaceuticals, Wilmington, Delaware) at 10⁻² M in control solution was used at a final concentration of 10⁻⁵ M.

For the standard patch recording, the ionic composition of different pipette filling solutions used in the present work is summarized in Table 1. For the perforated patch recording, the ionic composition of the pipette filling solution was similar to the solution E (Table 1) except that ATP(Na₂) was omitted. Amphotericin B at a concentration of 240 μg/ml was prepared as described by Rae et al. (1991): 6 mg of amphotericin B (Sigma, St. Louis, MO) were solubilized in 100 μl of DMSO by sonication for a few seconds and 20 μl of this solution were then added to 5 ml of internal solution.

Results

EFFECTS OF ANG II ON THE MEMBRANE POTENTIAL

Ang II-induced membrane potential changes were observed using the perforated patch recording method under current-clamp conditions. In the control solution, the resting membrane potential was very variable from cell to cell, ranging from -15 to -83 mV with a mean value of -45 ± 2 mV (*n* = 84). This membrane potential was poorly dependent upon external K⁺ changes. When the membrane potential was plotted against [K⁺]_e changes on a logarithmic scale, the relationship was linear between 5 and 135 mM K⁺ with a slope of 37 ± 3 mV (*n* = 9) per decade instead of 58 mV as expected for a K⁺ selective electrode (*not illustrated*). By contrast, Mlinar et al.

(1993b) reported in bovine adrenal zona fasciculata cells a mean resting membrane potential of -71 mV that was selectively dependent on K⁺.

Figure 1A shows the membrane response of an adrenal cell exposed to 100 nM Ang II. After an apparent delay of 25 sec, the hormone induced a transient hyperpolarization to -65 mV from a resting potential of -40 mV. This fast initial hyperpolarization was followed by large oscillations of the membrane potential then by a slow depolarization that reached a steady-state value near -35 mV, about 200 sec after the beginning of the response. In other cells, the depolarizing phase could begin immediately after the initial hyperpolarization or occur after a steady hyperpolarizing plateau of variable duration. Such membrane responses were recorded in 12 different cells whose resting membrane potential ranged from -17 to -54 mV. During the depolarizing phase or the steady depolarizing plateau, no regenerative membrane responses were detected in any of the studied cells.

These Ang II-induced membrane responses were abolished by Losartan (10⁻⁵ M) (Ouali et al., 1992), a nonpeptidic selective antagonist of AT1 receptors (Chiu et al., 1990). In the presence of this antagonist, the cell membrane potential was not modified after Ang II was added (Fig. 1B). When Losartan was removed and the hormone perfused alone, a fast hyperpolarization to -48 mV from a resting potential of -20 mV was followed by a slow depolarization (Fig. 1B). Similar results were obtained in 2 other cells.

EFFECTS OF ANG II ON THE BACKGROUND MEMBRANE CURRENT

Because we hypothesized that the Ang II-induced membrane hyperpolarization could be caused by the increase of a K⁺ membrane conductance, voltage-clamp experiments were performed at a holding membrane potential of -10 mV to strengthen the driving force (*E_m* - *E_K*) in order to amplify the whole cell membrane current.

Using the perforated patch recording method, the control membrane current (*I*_{control}) observed at this holding potential was a steady-state outward current, the

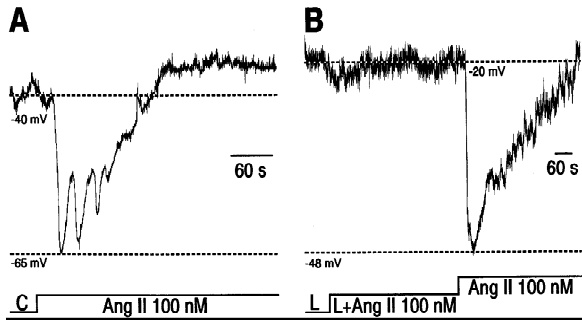


Fig. 1. (A) Typical membrane potential changes evoked in response to 100 nM Ang II in control solution. (B) Inhibition of the Ang II-induced membrane response in the presence of 10^{-5} M Losartan. This effect was reversible. The control resting membrane potential (-20 mV) was not affected by Losartan. Perforated patch recording. Dashed lines give different values reached by the membrane potential during the responses. C: control solution. L: Losartan.

mean value of which was $+15 \pm 2$ pA, $n = 77$ (Table 2). When the adrenal cells were exposed to 100 nM Ang II, the membrane response began by a fast initial peak of outward current (Fig. 2A) which was elicited after an apparent delay of 33 ± 4 sec ($n = 84$). This initial peak of current was followed by an oscillating (Fig. 2A) or a smooth (Fig. 3A) plateau current which declined more or less slowly. The maximum amplitude (I_{\max}) and the duration of the transient outward current activated by the hormone were very variable from cell to cell. The mean value of the maximum outward current calculated as $\Delta I_{\max} = I_{\max} - I_{\text{control}}$ was $+60 \pm 5$ pA, $n = 81$ (Table 2). Determined from the cell capacitance measurements, the magnitude of ΔI_{\max} was not linearly related to the cell size. The duration of the transient outward current induced by Ang II (measured between the beginning of the response and the time at which the current turned back to the holding control value) was 172 ± 37 sec ($n = 64$). This transient membrane response ended by a steady late current (I_{late}) that was more negative and less noisy than the control holding current (Fig. 2A). At the holding potential of -10 mV, the magnitude of I_{late} varied between $+10$ and 0 pA and in 7 out of 40 cells, I_{late} was slightly inward (-2 to -4 pA). The averaged decrease of the late current ($\Delta I_{\text{late}} = I_{\text{control}} - I_{\text{late}}$) obtained from 33 different cells was 7 ± 1 pA.

Figure 2B illustrates the evolution of the membrane conductance during the increase of the Ang II-induced outward current shown in Fig. 2A. From a resting mean value of 1.4 nS, the membrane conductance calculated as $G_m = \Delta i / \Delta V$ increased up to 3.1 nS. Averaged from 20 cells, the maximum membrane conductance was 2.4 ± 0.3 nS while the resting conductance was 0.8 ± 0.15 nS (Table 2).

In Figure 3A, current-voltage relationships were performed at different times during the long-lasting response of a cell exposed to 100 nM Ang II. From the

holding potential of -10 mV, membrane currents activated by voltage steps of variable amplitude did not display voltage- or time-dependence (*not illustrated*). The current-voltage relationships of the total membrane current (Fig. 3B) were established in control condition (a) then 40 (b), 400 (d), 990 (f), 1600 (h) and 2260 sec (j) after the beginning of the response. By comparison with the control (Fig. 3B, a), the reversal potential of the total membrane current activated in response to the hormonal stimulation was first shifted towards more negative values (Fig. 3B, b and d). Then, during the decay of the outward current, this reversal potential was close to (Fig. 3B, f) and finally more positive (Fig. 3B, h and j) than that of the control current. As revealed by the slopes of the current-voltage curves of the total membrane current established before (Fig. 3B, solid symbols) and during hormone application (Fig. 3B, open symbols), the membrane conductance was increased not only during the early phase of the membrane response but also during the late phase. Current-voltage relationship obtained by subtracting procedures from curves illustrated in Fig. 3B revealed that the reversal potential of the Ang II-induced membrane current decreased continuously from -67 mV at the beginning of the response to -7 mV at the end of the recording (Fig. 3A and C).

When current-voltage curves established at the maximum of the Ang II-induced transient outward current were averaged, no obvious rectification was detected. The mean value of the reversal potential of this outward current was -67 ± 4 mV ($n = 7$) (Table 2) while the reversal potential of the steady-state late current was -14 ± 3 mV ($n = 10$). The positive shift of the reversal potential from the beginning to the end of Ang II-induced membrane responses indicates that this hormone has likely multiple effects on the membrane conductances of this cell type and the next sections deal with the characterization of ionic components of the increase of the outward current in response to the hormonal stimulation.

IONIC COMPONENTS OF THE ANG II-INDUCED TRANSIENT OUTWARD CURRENT

Two organic blockers of K^+ currents, 4-AP and TEA, were tested during the hormone-induced membrane response obtained by the perforated patch recording method. The control outward current was reversibly diminished by 8 ± 2 pA ($n = 9$) in the presence of 20 mM TEA but was insensitive to 5 mM 4-AP. In 3 different cells, 4-AP (5 mM) did not modify the outward membrane current evoked by hormonal stimulation (*not illustrated*). The effects of TEA were observed for different concentrations. At a concentration of 5 mM, TEA inhibited the transient outward current by $64 \pm 8\%$ ($n = 6$). At a concentration of 20 mM, the inhibition of the plateau

Table 2. Summary of the main characteristics of the Ang II-induced transient outward current recorded in different experimental conditions

	<i>I</i> Cont (pA)	ΔI_{Ang} max (pA)	Gm control (nS)	Gm max (nS)	Erev (<i>I</i> _{Ang}) (mV)
Perforated patch Control solution	15 ± 2 <i>n</i> = 77	60 ± 5 <i>n</i> = 81	0.8 ± 0.15 <i>n</i> = 20	2.4 ± 0.3* <i>n</i> = 20	-67 ± 4 <i>n</i> = 7
Standard patch Control solution	17 ± 4.5 <i>n</i> = 12	53 ± 6 <i>n</i> = 12	0.8 ± 0.15 <i>n</i> = 12	1.9 ± 0.3* <i>n</i> = 12	-64 ± 2.5 <i>n</i> = 6
Standard patch Cl ⁻ -free solution (in-out)	12 ± 2 <i>n</i> = 26	44 ± 4.5 <i>n</i> = 26	0.6 ± 0.15 <i>n</i> = 15	1.4 ± 0.2* <i>n</i> = 15	-78.5 ± 4 <i>n</i> = 4
Standard patch K ⁺ -free solution (in-out)	13 ± 2 <i>n</i> = 21	14 ± 2 <i>n</i> = 21	0.7 ± 0.15 <i>n</i> = 12	1 ± 0.2 <i>n</i> = 12	-52 ± 2.5 <i>n</i> = 5

(* indicates that compared to Gm control, Gm Max was significantly higher with $P < 0.05$.)

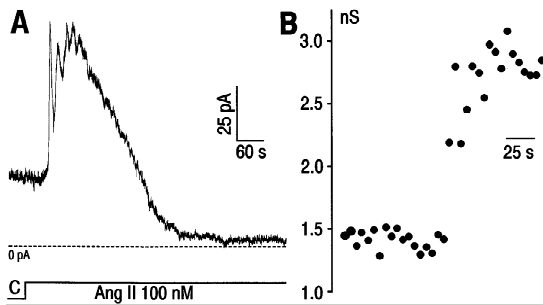


Fig. 2. (A) Membrane current changes induced in the presence of 100 nM Ang II from a holding potential of -10 mV. Perforated patch recording. In this and the following figures, C indicates control solution and dashed line gives the zero current level. (B) Membrane conductance changes induced by 100 nM Ang II during the increase of the outward membrane current illustrated in A.

current was more pronounced but was always partial as illustrated in Fig. 4 (representative of 5 cells) where 84% of the membrane current was suppressed during the application of this blocker. This figure also shows that the late current was sensitive to TEA (20 mM). No further inhibition of the plateau current was detected when the concentration of TEA was increased up to 50 mM. The effects of external potassium concentration changes were examined on the same cell during the decaying phase of the Ang II-induced transient outward current (Fig. 4). When 50 mM K⁺ were superfused in the bath, the outward current induced by Ang II was decreased as expected from the positive shift of the K⁺ equilibrium potential (E_K). Similar observations were obtained from 2 other cells.

These results reveal that Ang II would activate at least two distinct outward ionic currents, one of them being a TEA-insensitive current. To better characterize the components of the transient outward current, experiments were performed using the standard recording method that allowed control of the ionic composition of the internal medium.

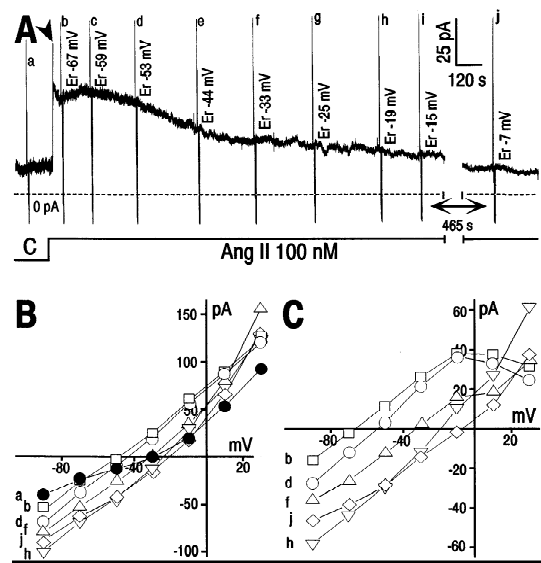


Fig. 3. (A) Changes of the reversal potential during the time course of the Ang II-induced membrane current. Large vertical lines superimposed on the current traces correspond to membrane currents evoked by voltage steps of variable amplitude applied in control condition (a) and at different times during the membrane response (b-j). Er gives the value of the reversal potential of the hormone-induced membrane current from current-voltage curves presented in C. Current traces in (i) and (j) are separated by a gap of 465 sec. Arrowhead shows the initial peak current. Perforated patch recording. (B) Current-voltage relationships of the total membrane current determined from recordings performed in a, b, d, f, h and j. (C) Current-voltage relationship of the Ang II-induced membrane current obtained by subtracting procedures from current-voltage curves illustrated in B.

POTASSIUM COMPONENT OF THE ANG II-INDUCED TRANSIENT OUTWARD CURRENT

Using the standard whole cell recording method, the membrane responses of isolated adrenal cells elicited by 100 nM Ang II from a holding potential of -10 mV were qualitatively similar to those obtained by the perforated patch recording method. A typical response to the hor-

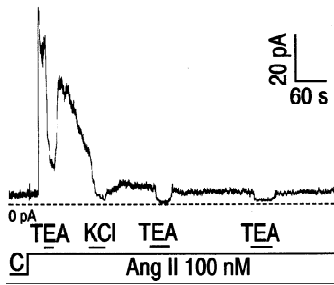


Fig. 4. Effects of TEA (20 mM) on the outward plateau current and the late current induced by 100 nM Ang II. The outward plateau current was also sensitive to external potassium concentration changes ($[\text{K}^+]_e = 50$ mM). Perforated patch recording.

monal stimulation recorded in control solution after the cell membrane was ruptured (internal solution: A, Table 1) is shown in Fig. 5A. Averaged from 12 cells, I_{control} , ΔI_{max} (Table 2) and duration of the membrane responses were $+17 \pm 4.5$ pA, $+53 \pm 6$ pA and 149 ± 26 sec respectively. Compared to data obtained by the perforated-patch recording method, statistical analysis indicated that these parameters were not significantly different ($P > 0.5$). As reported in Table 2, the membrane conductance was multiplied by a factor of 2.4 during the maximum outward current. Usually, the magnitude of the late current was lower than that of the control current by 7 ± 2 pA ($n = 8$) except in 4 out of 12 cells where this steady late current was more positive by 10 ± 3 pA than the control current. Averaged from 6 cells, current-voltage relationships of the membrane current in control condition (Fig. 5B, solid symbols) and of the maximum outward current activated by Ang II (Fig. 5B, open symbols) show that the reversal potential of this outward current was 21 mV more negative than the reversal potential of the control current. In Fig. 5C, the current-voltage relationship of the Ang II-induced outward current was determined by subtracting procedures ($I_{\text{max}} - I_{\text{control}}$) from curves presented in Fig. 5B. This curve did not display obvious rectification and intercepted the voltage axis at -64 ± 2.5 mV (Table 2). This value was close to that obtained using the perforated-patch recording method (-67 ± 4 mV, $n = 7$).

When Cl^- was omitted from the external and internal solutions (internal solution: B, Table 1), an outward current was activated in response to 100 nM Ang II as illustrated in Fig. 6A. Averaged from 26 cells, I_{control} was $+12 \pm 2$ pA and ΔI_{max} was $+44 \pm 4.5$ pA (Table 2). In 20 out of 25 cells, the magnitude of the late current was either similar or more positive than that of the control holding current and in some cells, this late current could again increase in outward direction. In 5 other cells, the late current was less positive than the control holding current. Compared to the control conductance, the mean membrane conductance determined at the peak of the outward transient current was increased by a factor

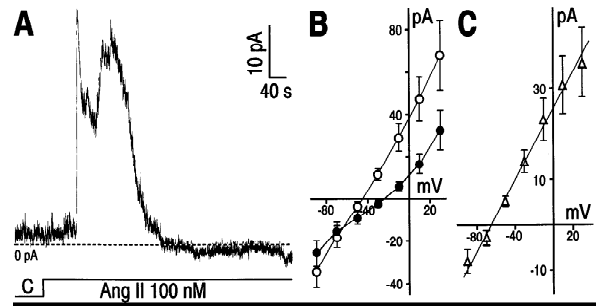


Fig. 5. (A) Ang II-induced membrane current changes recorded by the standard recording method (compare with Fig. 2A). (B) Current-voltage relationships ($n = 6$) of the total current in control solution (solid symbols) and in the presence of 100 nM Ang II (open symbols). (C) Current-voltage relationship of the Ang II-induced transient outward current obtained by subtracting procedures ($I_{\text{max}} - I_{\text{control}}$) from curves illustrated in B.

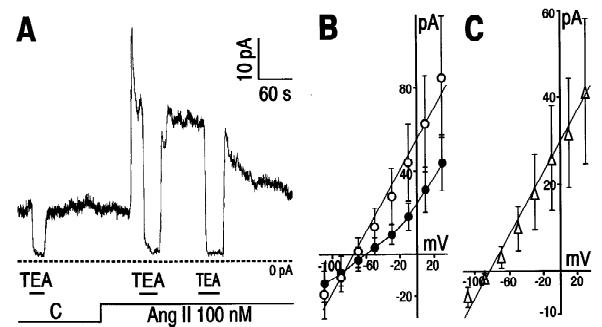


Fig. 6. (A) Effect of TEA (20 mM) on the control and the Ang II induced-outward current obtained using the standard recording method in Cl^- -free solution (compare with Fig. 4). (B) Current-voltage relationships ($n = 4$) of the total current in control solution (solid symbols) and in the presence of 100 nM Ang II (open symbols). Cl^- -free solution. (C) Current-voltage relationship of the Ang II-induced transient outward current constructed by subtracting procedures ($I_{\text{max}} - I_{\text{control}}$) from curves illustrated in B (compare to Fig. 5C).

of 2.3 (Table 2). In Cl^- -free solution, the Ang II-induced plateau current was entirely inhibited by 20 mM TEA (Fig. 6A). This record reveals that this blocker inhibits not only the hormone induced outward current but also a K^+ component of the outward background current. Current-voltage relationships averaged from 4 different cells were established in control condition (Fig. 6B, solid symbols) and at the maximum of the hormone-induced outward current (Fig. 6B, open symbols). These curves show the increase of the membrane conductance and the negative shift of the reversal potential of the membrane current activated by Ang II. The current-voltage curve of the Ang II-induced transient outward current obtained by subtracting procedures ($I_{\text{max}} - I_{\text{control}}$) (Fig. 6C) was nearly linear and intercepted the voltage axis at -78.5 ± 4 mV (Table 2). This potential was close to the calculated E_{K} (-82 mV) and was more negative by 14.5 mV

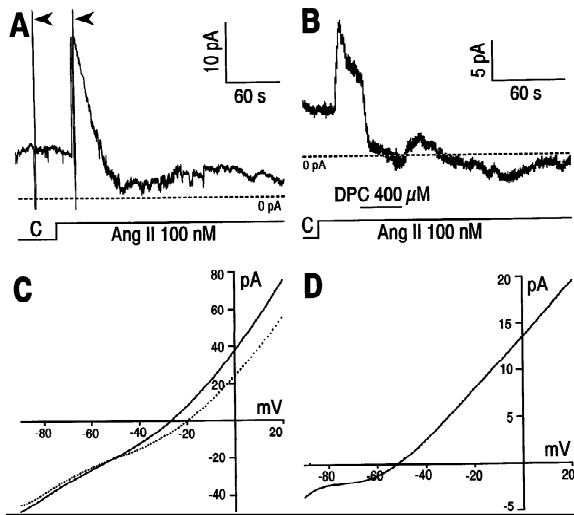


Fig. 7. (A) Transient outward current induced by Ang II (100 nM) in K⁺-free solution. Vertical lines (arrowheads) correspond to membrane currents evoked by voltage ramps. (B) Effect of DPC (400 μM) on the plateau current of the Ang II-induced transient outward current recorded in K⁺-free solution. Standard recording method. (C) Current-voltage relationships ($n = 5$) determined for the membrane current in control solution (dashed line) and for the transient outward current activated in response to the hormonal stimulation (solid line). (D) Current-voltage relationship of the Ang II-induced transient outward current obtained by subtracting procedure ($I_{\max} - I_{\text{control}}$) from current-voltage curves illustrated in C.

than that determined in control Cl⁻-containing solution (compare with Fig. 5C).

CHLORIDE COMPONENT OF THE ANG II-INDUCED TRANSIENT OUTWARD CURRENT

When the experiments were carried out in the absence of K⁺, replaced by Cs⁺ in the external and internal medium (internal solution: C, Table 1), the control membrane current averaged from 21 different cells at a holding potential of -10 mV was $+13 \pm 2$ pA (Table 2). Ang II (100 nM) induced a transient increase of the outward current as illustrated in Fig. 7A. The maximum peak current (ΔI_{\max}) averaged from 21 experiments was $+14 \pm 2$ pA (Table 2). These membrane responses which lasted 47 ± 7 sec ($n = 25$) were reversibly inhibited in 4 out of 7 cells by 400 μM DPC (Fig. 7B), a nonspecific blocker of Cl⁻ conductances, but not by 300 μM 9-AC, another blocker of Cl⁻ conductances. The control current was also sensitive to DPC (400 μM) and was diminished by about 4 pA in two cells (*not illustrated*). In all the studied cells, the transient outward current activated by Ang II ended by a late current less positive than the control current. The decrease of the membrane current ($I_{\text{control}} - I_{\text{late}}$) during this late phase was 8 ± 12 pA ($n = 17$).

The present results suggest that Ang II activates a

component of outward current that is carried by Cl⁻. The current-voltage relationships shown in Fig. 7C were averaged from 5 different experiments. These curves were constructed from voltage ramp protocols applied during the membrane responses as indicated in Fig. 7A (arrowheads). Compared to control (Fig. 7C, dashed line), the current-voltage curve determined at the peak of the transient outward current (Fig. 7B, solid line) was steeper indicating an increase of the membrane conductance as reported in Table 2. This curve intercepted the voltage axis at a potential 8 mV more negative than that of the control current. In Fig. 7D, the current-voltage curve of the Ang II-induced outward current obtained by subtracting procedure ($I_{\max} - I_{\text{control}}$) from curves illustrated in Fig. 7C crossed the voltage axis at -52 ± 2.5 mV (Table 2), a value 33 mV more positive than the calculated chloride equilibrium potential ($E_{\text{Cl}} = -85$ mV). When E_{Cl} was modified (internal solution: D, Table 1; $E_{\text{Cl}} = -44$ mV), the reversal potential of the Ang II-induced outward current was shifted in a positive direction but was more positive than E_{Cl} , -29.5 ± 8 mV ($n = 6$) instead of -44 mV. However, with $E_{\text{Cl}} = -44$ mV, ΔI_{\max} was $+7 \pm 1$ pA ($n = 8$) against $+14 \pm 2$ pA ($n = 21$) when E_{Cl} was -85 mV. Statistical analysis indicated that these two values were marginally different ($P = 0.07$).

INVOLVEMENT OF INTERNAL Ca²⁺ IN THE OUTWARD CURRENT ACTIVATION

In bovine zona fasciculata cells, Viard et al., (1990) have reported that Ang II activated the metabolic pathway of phospholipase C which produced an increase of inositol trisphosphate (InsP₃) correlated with an increase of the cytosolic free Ca²⁺ concentration. To determine whether changes in the internal Ca²⁺ concentration could be involved in the increase of the outward current in response to the hormonal stimulation, experiments were performed using the standard recording method in the presence of EGTA added to the pipette filling solution (internal solution: E, Table 1). We remind that the membrane responses to the hormonal stimulation described in the above sections using the standard recording method were obtained in the absence of EGTA in the pipette filling solution (zero Ca²⁺ nominal). In the 6 tested cells where 5 mM EGTA was added to inner solution, Ang II never induced a transient increase of outward current. In 4 of these 6 cells no significant change in the membrane current was observed while in the 2 other cells, a transient decrease of the outward current was detected as illustrated in Fig. 8A.

Other evidence of the internal Ca²⁺ involvement on the Ang II-induced outward membrane current was obtained using pharmacological tools. In many cell types, Ca²⁺ concentration changes are known to activate differ-

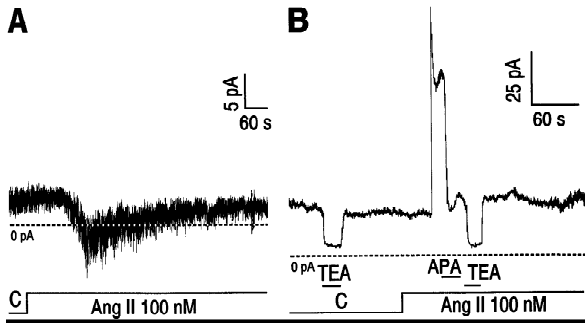


Fig. 8. (A) Membrane current activated in response to the hormonal stimulation in the presence of 5 mM EGTA in the pipette filling solution (compare with Fig. 5A). (B) Effect of apamin (50 nM) on the Ang II-induced transient outward current recorded in Cl⁻-free solution. Note that 20 mM TEA blocked also a K⁺ component of the control holding membrane current. Standard recording method. APA: apamin.

ent classes of Ca²⁺-sensitive K⁺ channels which can be selectively inhibited by different toxins (Latorre et al., 1989). Two toxins, charybdotoxin known to selectively block Ca²⁺-dependent K⁺ channels of large unitary conductance (Miller et al., 1985) and apamin known to selectively inhibit Ca²⁺-dependent K⁺ channels of small unitary conductance (Romey & Lazdunski, 1984), were tested on our preparation. Charybdotoxin did not affect the Ang II-induced plateau current (*not illustrated*) of isolated adrenal cells when used to a concentration of 100 nM (3 cells). The control current was insensitive to 50–100 nM apamin (8 cells) while, used at a concentration of 50 nM in Cl⁻-free solution, this toxin irreversibly inhibited the transient outward current activated by the hormonal stimulation (6 cells). This is shown in Fig. 8B where the effects of apamin on the Ang II-induced plateau current are illustrated together with the effects of 20 mM TEA on the control and late currents. Apamin blocks only the membrane current activated by the hormone while the TEA-sensitive component of the background current was not affected.

INVOLVEMENT OF EXTERNAL Ca²⁺ IN THE OUTWARD CURRENT ACTIVATION

To discriminate between different Ca²⁺ stores that could be involved in the Ca²⁺ mobilization, the effects of the hormonal stimulation on the adrenal cells were recorded in the absence of Ca²⁺ in the external solution (zero Ca²⁺ nominal) or in the presence of different blockers of Ca²⁺ entry. In zero Ca²⁺ solution, two types of response were observed during the hormonal stimulation. In 8 out of 15 cells, the membrane responses were similar to those recorded in control solution. An initial peak of current was followed by a decaying plateau current, the mean duration of which was 126 ± 26 sec. In the remaining 7 cells,

the membrane responses consisted only of a rapidly decaying peak current (Fig. 9A). The mean amplitude of this outward peak current (ΔI_{\max}) was +91 ± 12.5 pA ($n = 6$) while its duration was 29 ± 5 sec ($n = 7$). By comparison, only 3% of the cells produced such a signal in control solution against 47% in zero Ca²⁺ solution. In this situation, the re-exposure of the preparation to 2.5 mM Ca²⁺-containing solution provoked a large increase in the outwardly directed membrane current which could again be suppressed by Ca²⁺ withdrawal (Fig. 9A). The Ang II-induced plateau current was also reversibly inhibited in 4 out of 5 cells by 300 μM Cd²⁺ (Fig. 9B) while used at a similar concentration, this blocker did not modify the control holding current (11 cells).

When the cells were exposed to a control solution containing 100 nM Ang II and 150 or 300 μM Cd²⁺, the membrane response consisted only of a rapidly decaying peak of outward current while the late current did not seem to be modified (Fig. 9C). Such an effect was observed in 2 out of 3 cells experimented using the perforated-patch recording method and in 3 cells experimented using the standard recording method in the absence of EGTA in the pipette filling solution. In these 5 cells, the averaged peak current (ΔI_{\max}) was +35 ± 9.6 pA while its duration was 48.4 ± 17 sec suggesting that the effects of Cd²⁺ on the membrane response induced by Ang II were similar to those observed in 7 out of 15 cells experimented in Ca²⁺-free solution. In these two situations, the initial peak current was present while the plateau current failed to be produced.

The fact that the plateau current of responses induced by Ang II was blocked by Cd²⁺ leads us to suppose that voltage-gated Ca²⁺ channels described in this cell type (Bilbaut et al., 1993; Mlinar et al., 1993a) could be the pathway by which Ca²⁺ entered the cell. Although no exhaustive study was made, two types of voltage-gated inward Ca²⁺ currents, maintained and transient (Fig. 10A), were identified in our preparation. These two different types of inward current were characterized using the standard recording method after the voltage-gated transient outward current was blocked in the presence of Cs⁺ and TEA in the pipette filling solution (internal solution: F, Table 1). In 20 mM Ca²⁺-containing solution, both voltage-gated inward current, maintained (Fig. 10A, left record) and transient (Fig. 10A, right record), were sensitive to 300 μM Cd²⁺ (*not illustrated*) and to 0.5–1 μM nisoldipine (Fig. 10A). However, in 5 tested cells, this dihydropyridine (DHP) used at the same concentration did not affect the Ang II-induced transient outward current (Fig. 10B). As illustrated on this figure, when nisoldipine was applied for 90 sec during the Ang II-induced transient outward current, there was no change in the time course of the decaying plateau current. This result was clearly different from the effect of Cd²⁺ shown in Fig. 9B.

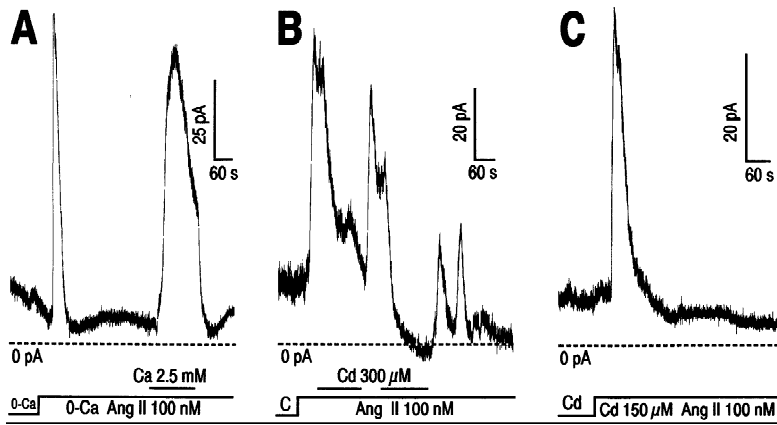


Fig. 9. (A) Ang II-induced membrane current in the absence of Ca²⁺ in the external solution. An increase of the outward current was observed when external Ca²⁺ was re-admitted which stopped rapidly after Ca²⁺ was withdrawn. (B) Blocking effect of Cd²⁺ (300 μM) on the Ang II-induced outward plateau current recorded in control solution. This effect was reversible. (C) Effect of Ang II (100 nM) on the membrane current in the presence of 150 μM Cd²⁺ added to the control solution. Perforated patch recording.

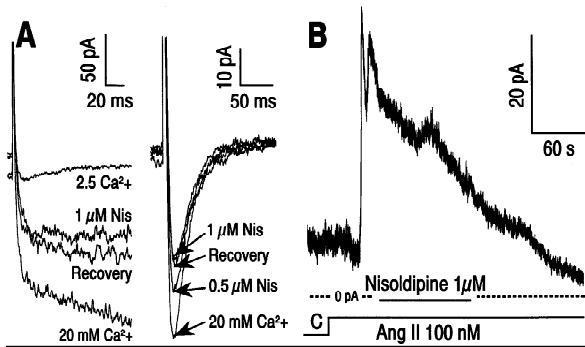


Fig. 10. (A) Effect of nisoldipine on the voltage-activated Ca²⁺ currents in response to voltage steps at -10 mV applied from a holding potential of -90 mV. In the left panel, the inward current was drastically increased after 2.5 mM Ca²⁺ was replaced by 20 mM Ca²⁺. This maintained Ca²⁺ inward current was diminished in the presence of 1 μM nisoldipine. In the right panel, the transient Ca²⁺ current recorded in 20 mM Ca²⁺-containing solution was sensitive to 0.5 μM nisoldipine. Further current inhibition was obtained in the presence of 1 μM nisoldipine. In these two records, the blocking effect of the inward current was partially reversible. Standard recording method. Nis: nisoldipine. (B) The decaying plateau current induced by 100 nM Ang II was not modified in the presence of nisoldipine (1 μM) (compare with Fig. 9B). Perforated patch recording.

Discussion

The above results show that the isolated zona fasciculata cells in primary culture respond to Ang II (100 nM) by a biphasic membrane potential change. These effects are mediated by the binding of the hormone to AT1 receptors since Losartan (10⁻⁵ M) blocks membrane responses evoked by hormonal application. The combination of both current- and voltage-clamp recordings reveals that the hyperpolarizing phase is caused by the activation of a transient outward current while the steady depolarizing phase is correlated with a late current less positive than the control holding current.

At a holding potential of -10 mV, the control current was always outwardly directed. This current was sensitive to TEA and DPC indicating the existence of a membrane permeability to K⁺ and Cl⁻. On the other hand, in all tested cells, no detectable effect was observed on the control current in the presence of Cd²⁺ which blocked the two types of voltage-activated Ca²⁺ currents identified in this cell type. Such a result indicates that the participation of these Ca²⁺ channels in the control current can be discarded and that the membrane responses elicited by the hormonal stimulation at this holding potential were not contaminated by a steady influx of Ca²⁺. This was strengthened by the fact that the control current was a steady current that was not modified by apamin while, as discussed in another section, Cd²⁺ and apamin inhibited the Ang II-induced plateau current. Furthermore, in contrast with other reports (*see* for example Akaike & Uneyama, 1994 or Ward & Giles, 1997), the present results indicated that, in our experimental conditions, the Ang II-induced membrane currents were not dependent on the recording method used.

IONIC NATURE OF THE ANG II-INDUCED TRANSIENT OUTWARD CURRENT

Our observations reveal that the hormone-induced transient outward current consists of two distinct ionic currents, K⁺ and Cl⁻ currents sensitive to changes of cytosolic free Ca²⁺.

In Cl⁻-free solutions, this outward current which reversed at -78.5 mV, a value close to E_K , was completely inhibited by 20 mM TEA and by apamin. The blocking effect of this toxin indicates that this Ang II-induced outward current is likely caused by the activity of small conductance K⁺ channels which are dependent on cytosolic Ca²⁺ changes (SK_{Ca²⁺} channels). Apamin-sensitive K⁺ current activated by various agonists have been described in other gland cells as bovine adrenal glomeru-

losa cells (Lobo & Marusic, 1986), bovine chromaffin cells (Artalejo, Garcia & Neher, 1993), or pituitary gonadotrophs (Kukuljan et al., 1992). In the present work, the current-voltage relationship of the Ang II-induced apamin-sensitive K^+ current did not display voltage dependence, in agreement with the properties of $\text{SK}_{(\text{Ca}^{2+})}$ channels characterized in other cells (Capiod & Ogden, 1989). The sensitivity of this current to TEA agrees with different reports (Cook & Haylett, 1985; Lang & Ritchie, 1988) indicating that the apamin-sensitive K^+ currents can be inhibited by high concentrations of K^+ channel blocker.

In K^+ -free-solutions, the only permeant ion whose the equilibrium potential was more negative than the holding potential (-10 mV) was Cl^- . The Ang II-induced outward current was sensitive to DPC, a non-specific inhibitor of Cl^- channels and its magnitude changed as the driving force for Cl^- ions changed. However, in such solutions, the reversal potential of the Ang II-induced transient outward current was more positive than E_{Cl} . The reasons for this discrepant observation were not elucidated but can be at least related to the presence of the late current. In the control solution, this late current that reversed at about -10 mV, was correlated with an increase in the membrane conductance. As in rat zona glomerulosa cells where an Ang II-induced cationic current has been characterized by Lotshaw & Li (1996), the late current described in this study could be caused by the activation of an inward current. The fact that, in Cl^- -free solution, most of the tested cells did not display a late current suggests that it could correspond to a Cl^- -sensitive Ca^{2+} current as reported by Hosoki & Iijima (1994) in endothelial cells.

The present results do not agree with those reported by Mlinar et al. (1993b) who described, in bovine zona fasciculata cells, the inhibition by Ang II of a noninactivating K^+ current (I_{AC}). This current was not detected in our experimental conditions using either the standard or the perforated patch recording method (Bilbaut et al., 1996). However, the absence of hyperpolarization during the Ang II-induced membrane responses obtained by Mlinar et al. (1993b) can be explained by the resting cell polarization which was -71 mV, a value close to E_{K} . In voltage-clamp conditions, the presence of BAPTA in the pipette solution (Mlinar et al., 1993b) could prevent the internal calcium concentration from raising. Concerning I_{AC} , a possible explanation which would conciliate these discrepant observations is the age of the cattle used. Indeed, bovine adrenal glands were obtained from calves (4–6 months) in the present experiments while Mlinar et al. (1993b) worked on adrenal glands obtained from steers (1–3 years). In these conditions, a difference in the cell maturation of adrenal glands cannot be discarded.

Ca^{2+} -DEPENDENCE OF THE ANG II-INDUCED TRANSIENT OUTWARD CURRENT

As reported in other preparations (*see* for example Nilius et al., 1997), the transient increase of the outward current observed in response to the hormonal stimulation of adrenal cells failed to be produced in the presence of EGTA in the pipette-filling solution indicating that the activation of both K^+ and Cl^- component of the outward current was dependent on internal free Ca^{2+} changes. This conclusion is in concordance with the observations of Viard et al. (1990) which described in isolated bovine zona fasciculata cells, an Ang II-induced rise of the cytosolic Ca^{2+} . Pharmacological data strengthened the involvement of cytosolic Ca^{2+} changes in the activation of the Ang II-induced transient outward current since the K^+ component of this current was sensitive to apamin. However, it remains to be determined whether K^+ and Cl^- channels are directly activated by Ca^{2+} or via Ca^{2+} -dependent enzymes such PKC or PLC. Ca^{2+} -activated K^+ and Cl^- currents are commonly co-expressed in many cell types. For example, in isolated hepatocytes Capiod & Ogden (1989) have described K^+ and Cl^- currents induced by various agonists such as noradrenaline, ATP and Ang II. In bovine zona fasciculata cells, the Ang II-induced transient outward current was largely dominated by the apamin-sensitive Ca^{2+} -dependent K^+ current. This current was co-activated with a small component of Ca^{2+} -dependent Cl^- current which represented about 30% of the total membrane current.

Ca^{2+} -dependent outward currents were regarded as a biological indicator of changes in $[\text{Ca}^{2+}]_i$ (Akaike & Uneyama, 1994; Kukuljan et al., 1992). Although in the present study, $[\text{Ca}^{2+}]_i$ was not measured during hormonal application, we could consider that the Ca^{2+} -dependent outward current reflected $[\text{Ca}^{2+}]_i$ increase induced by the binding of Ang II to AT1 receptors. Nevertheless, we cannot ascertain that the transient time course of this Ang II-activated outward current closely paralleled changes in $[\text{Ca}^{2+}]_i$ because other mechanisms can exist. Thus, at least three distinct ionic currents were induced in response to the hormonal stimulation, $I_{\text{K}(\text{Ca}^{2+})}$, $I_{\text{Cl}(\text{Ca}^{2+})}$ and an inward current. All these currents have different equilibrium potentials and probably a different time course of activation that could explain the complex time course of the total membrane current. Finally, another mechanism such as the inhibition of Ca^{2+} -dependent outward currents by an intracellular metabolic pathway activated in response to Ang II stimulation cannot be excluded.

In many cell types, the Ca^{2+} mobilization in response to hormonal exposure occurs in two distinct phases, an initial transient release of Ca^{2+} sequestered in internal compartment followed by an entry of extracellular Ca^{2+} . In our preparation, the plateau current but not the initial peak current was reversibly suppressed in

minimally Ca²⁺-free solution or in the presence of Cd²⁺ added to the control solution before Ang II was applied. As far as the transient outward current is assumed to reflect changes in the [Ca²⁺]_i, these results confirm that mechanisms similar to those described above govern the Ca²⁺ movement induced in response to the hormonal stimulation. Because the outward current was activated by cytosolic Ca²⁺ changes, the long-lasting membrane responses recorded in about half the tested cells in zero Ca²⁺ solution could be dependent on the amount of Ca²⁺ stored in internal compartments prior to hormonal stimulation which could vary from cell to cell.

These observations also reveal that the plateau of the outward current was produced by a Cd²⁺-sensitive influx of Ca²⁺. While Cd²⁺ and nisoldipine reduced both transient and maintained voltage-activated Ca²⁺ currents identified in our preparation, the time course of the Ca²⁺-dependent outward plateau current evoked by hormonal application was not modified by nisoldipine. In addition, Chorvatova et al. (1995) have described in bovine zona fasciculata cells exposed to 100 nM Ang II, a transient increase of the membrane conductance for K⁺ at a potential as negative as -60 mV. This suggests that if voltage-gated Ca²⁺ channels participate in the influx of Ca²⁺ during the hormonal stimulation, their role could be not preponderant. Thus, we propose that a class of Cd²⁺-sensitive Ca²⁺ channels (Hoth & Penner, 1993) distinct from voltage-gated DHP-sensitive Ca²⁺ channels is likely to be involved in the entry of Ca²⁺ that sustains the plateau phase of the transient outward current evoked in response to hormone application. We thought that one of the pathways by which Ca²⁺ enters the cell during this plateau phase could correspond to voltage-insensitive channels activated by the depletion of Ca²⁺ stores (Berridge, 1997).

PHYSIOLOGICAL RELEVANCE

From observations reported by Capponi et al. (1988), the hormone-stimulated secretory activity of zona fasciculata cells needs a high level of internal free Ca²⁺. One way by which these cells could increase their [Ca²⁺]_i, could be the hormone-triggering of repetitive Ca²⁺ action potentials. However, a striking feature drawn from the present results was the absence of action potentials evoked during the hormonal response while voltage-gated Ca²⁺ channels were characterized in this cell type. In pituitary gonadotrophs Tse & Hille (1993) have shown that the hyperpolarization induced by the activation of I_{K(Ca²⁺)} in response to agonist application, removes the inactivation of voltage-dependent Ca²⁺ and Na⁺ channels that allows action potentials to fire and Ca²⁺ to enter the cell. Such a mechanism cannot be applied to isolated bovine zona fasciculata cells because if

hyperpolarization removes the inactivation of the voltage-dependent Ca²⁺ channels, the inactivation of the I_A-like K⁺ channels is also removed and the net inward current may be too weak to elicit action potentials. In addition, the activation of the voltage-dependent channels can be hindered because of the very slow time course of the membrane depolarization induced by Ang II in these cells.

The physiological role of the complex membrane potential changes induced by Ang II stimulation of isolated bovine zona fasciculata cells is yet hypothetical. As reported in other cell types (Manabe, Matsuda & Noma, 1995), the agonist-induced hyperpolarization may provide an electrochemical driving force to enhance the Ca²⁺ influx participating in the replenishing of Ca²⁺ stores and in the internal Ca²⁺ homeostasis. However, in these adrenal gland cells, this hyperpolarization is only transient and the question of a functional role of the voltage-dependent Ca²⁺ and K⁺ channels and of the Ang II-induced sustained membrane depolarization may be raised.

The authors wish to thank the staff of the INSERM U 418 from the Hôpital Debrousse, Lyon, for helpful discussions and their help in preparing isolated cells. We are grateful to Dr. B. Allard for a critical reading of the manuscript, L. Rothage-Jacquemond for reviewing the English manuscript and J.-L. Andrieu for technical assistance. This work was supported by an INSERM-Merck Sharp & Dohme-Chibret grant.

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