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Selective Inhibition of Potassium-stimulated Rat Adrenal Glomerulosa Cells by Ruthenium Red

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ABSTRACT. The effect of the cationic dye, ruthenium red (RR), on ionic fluxes, Ca^{2+} signal generation, and stimulation of aldosterone production was studied in isolated rat adrenal glomerulosa cells. In these cells, increased extracellular $[K^+]$ as well as angiotensin II (Ang II) elevate cytoplasmic Ca^{2+} concentration and thereupon activate steroidogenesis. However, the mode of action of the two stimuli are different: while a dihidropyridine-sensitive mechanism contributes to the response to both agonists, Ang II induces Ca^{2+} release from intracellular stores and causes capacitative Ca^{2+} influx, whereas K^+ was recently shown to activate a plasma membrane Ca^{2+} current (I_{gl}) independently of membrane depolarization. The difference is reflected in the sensitivity of the response of the cells to RR. The Ang II-induced Ca^{2+} signal and aldosterone production were not inhibited, but rather slightly potentiated by the dye. This potentiation was probably the consequence of the membrane-depolarizing effect of RR, due to the observed inhibition of the resting K^+ conductance. Conversely, Ca^{2+} signal and aldosterone production were significantly reduced by RR when the cells were stimulated by moderately elevated $[K^+]$ (6–8 mM). Our patch clamp studies suggest that this effect was related to the inhibition of different voltage-dependent and -independent inward Ca^{2+} currents and indicates the functional importance of the latter in the signal transduction of the potassium-stimulated glomerulosa cell. BIOCHEM PHARMACOL **57**;2:209–218, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. rat adrenal glomerulosa cell; aldosterone; ruthenium red; I_{gl}; voltage-dependent Ca^{2+} channel

Aldosterone production in the adrenal glomerulosa cells is under multifactorial control. Ang II† and extracellular $[K^+]$ are the two major stimuli which act by elevating cytoplasmic Ca^{2+} concentration (for review see [1]). The consequence of Ang II binding to its plasma membrane receptor is the activation of phospholipase C and the generation of diacylglycerol and $Ins(1,4,5)P_3$, which liberates calcium from intracellular stores. This is followed by the influx of Ca^{2+} from the extracellular space. Capacitative influx mechanism contributes to this process [2, 3], although VDCC are also involved according to patch clamp, fluorimetric, and pharmacological studies [2, 4, 5; for review see (6)]. Moreover, dihydropyridine-sensitive channels were also suggested to influence the early phase of the response, i.e. when the calcium signal is dependent only on the release of the ion from the intracellular stores [7].

The source of calcium which elevates cytoplasmic Ca^{2+} concentration in K^+-stimulated glomerulosa cells is exclusively the extracellular space. These cells show extreme sensitivity to even small physiological changes (<1mM) in extracellular $[K^+]$. Here again, the role of VDCC with unusually low threshold potential is well documented [8, 9]. A further mechanism, which may funnel Ca^{2+} into the cell in response to K^+, has recently been described. This current (I_{gl}) is activated by K^+ even when the cells are clamped at −100 mV [10]. Under these conditions, when all the VDCC are inactive K^+ is still able to elevate cytoplasmic Ca^{2+} as measured by combined patch clamp and fluorimetric methods [9]. This suggests a physiological role for I_{gl}; however, the lack of specific and potent inhibitors limited further studies regarding its functional significance in potassium-induced cell activation, i.e. aldosterone production.

Ruthenium red (RR), a colored trinuclear ruthenium ammine, is known to influence transmembrane cation fluxes through different membranes. When applied intracellularly or to subcellular fractions, it has been shown to block the mitochondrial Ca^{2+} uniporter, the Ca^{2+} ATPase of the sarcoplasmic reticulum, and the release of Ca^{2+} through the ryanodine receptor. In addition to these intracellular effects, RR can modify plasma membrane ion fluxes. It blocks the recently cloned capsaicin receptor, a nonspecific cation channel in primary afferent neurons with high affinity (for review see [11]). Recently, RR was also shown to interact with VDCC and influence plasma membrane $[K^+]$ conductance [12–14].

We observed that addition of purified RR to adrenal glomerulosa cells reduced the potassium-induced aldoste-

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† *Abbreviations:* Ang II, angiotensin II; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; $Ins(1,4,5)P_3$, inositol 1,4,5 trisphosphate; RR, ruthenium red; VDCC, voltage-dependent calcium channel; fura-2/AM, fura-2/acetoxymethylester; and I_{gl}, a K^+-activated Ca^{2+} current.

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rone production while failing to inhibit the effect of Ang II. We analyzed the relation of this effect to plasma membrane ion currents and cytoplasmic [Ca^{2+}] changes.

MATERIALS AND METHODS

Purification of Ruthenium Red

Commercially available RR preparations contain at least three types of contamination which can be distinguished photometrically. The absorption maximum of pure RR is 532 nm. Ru360, a dinuclear Ru–ammine complex, which has an absorption maximum of 360 nm, was found to be responsible for the mitochondrial effects [15]. Ruthenium brown is the oxidized product of RR, its absorption maximum being 460 nm [16]. Ruthenium violet has an absorption peak at 734 nm [17] and can also be used in staining procedures. To obtain RR free of contamination, we purified the commercial product (Fluka) using a modification of Luft's method [17]. RR (200 mg) was dissolved in 8 mL of 0.5 M NH_4OH heated to 60° for 30 min, chilled and centrifuged (1600 *g*, 10 min). The pellet containing the ruthenium violet was discarded. The supernatant was applied to a 25 mL CM Macroprep cation exchange column (Bio-Rad) and eluted with a linear gradient of ammonium formate 120 mL (0.1–2 M). RR eluted an AF concentration between 1.44 and 1.52 M as determined by the absorbtion maximum at 532 nm. Fractions containing pure RR were pooled and lyophilized after removing formate ions on a Bio-Rad AG 1-X8 anion exchanger column (OH^- phase). The concentration of RR was determined photometrically.

Cell Isolation and Incubations

Glomerulosa cells were prepared from the adrenal capsular tissue of Wistar rats (180–300 g) using collagenase digestion as previously described [18]. For Ca^{2+} and patch clamp measurements, 2–5 $\times$ 10^4 cells were plated on fibronectin-coated coverslips and incubated in a Petri dish in 0.2 mL of a mixture of modified Krebs–Ringer bicarbonate solution and M199 (GIBCO) (solution *A*) [10]. After 3 hr incubation, the medium was replaced with 3 mL of fresh medium. The cells were kept in the same solution until used in the experiments: on the same day for Ca^{2+} measurements, and two days later for patch-clamping. For aldosterone measurements, the cells were plated in tissue culture plates, one adrenal (approx. 1.5 $\times$ 10^5 cells)/well in 0.5 mL of solution *A*. All the incubations were carried out at 37° in 5% CO_2.

Aldosterone Measurements

For aldosterone measurements 1.1–1.7 $\times$ 10^5 cells, equivalent to one adrenal, were plated on fibronectin-coated (24-well) tissue culture dishes. After 3 hr preincubation, stimuli were applied changing solution *A* to the same containing the stimulators [Ang II (SERVA), potassium, or thapsigargin (Sigma)] and/or inhibitors [ruthenium red, nifedipine (Sigma) and Ni^{2+}]. After 1 hr the medium was removed and the quantity of aldosterone measured directly by radioimmunoassay. The antibody used was raised in sheep against d-aldosterone keyhole limpet haemocyanin. Its cross-reactivity was 0.07% with corticosterone and less than 0.01% for all the other steroid hormones tested. The intraassay and interassay variation coefficients were 9% (N = 24) and 20% (N = 19), respectively.

Patch-Clamp Measurements

For ion current measurements, the whole-cell patch-clamp technique [19] was applied. The standard extracellular solution had the following composition (mM): NaCl 127, KCl 3.6, $MgCl_2$ 0.5, $CaCl_2$ 2, glucose 11, Hepes 10, pH 7.4 (NaOH), while VDCC were measured in a high-Ba^{2+} solution: NaCl 100, KCl 3.6, $MgCl_2$ 0.5, $BaCl_2$ 10, glucose 11, Hepes 10, pH 7.4 (NaOH). Pipettes were pulled from hard borosilicate glass (B120-90-10, Sutter) by a P-87 puller (Sutter) and fire-polished. Pipette resistance ranged between 2.5 and 5 MΩ when filled with the intracellular solution, containing (mM): KCl 125, $MgCl_2$ 0.1, $CaCl_2$ 0.25, EGTA 7.65, Na-ATP 5, Hepes 10, pH 7.2 (KOH). When specified, KCl in the pipette solution was replaced with equimolar CsCl. The free [Ca^{2+}] of the pipette solution was about 10^{-8} M as calculated by computer program [20]. The pipette was connected to the headstage of a patch-clamp amplifier (Axopatch-1D, Axon or RK-400, Biologic) which was mounted on a PCS-750/1000 manipulator (Burleigh). Seal resistance was about 10 GΩ. The capacitance of the selected glomerulosa cells amounted to 25–35 pF. Series resistance was about 10 MΩ. Due to a significant resting K^+ conductance in the entire voltage range applied, the calculated resistance of the configuration was ca. 100 MΩ in the K-containing pipette solution, while in the Cs-containing pipette solution the resistance increased to about 1 GΩ. Data were filtered at 2 kHz (−3 dB; 4-pole, low pass Bessel filter) and digitally sampled at 4 kHz by a Digidata 1200 interface board (Axon), stored and later analyzed by PC/AT computer. Experiments, data storage, and analysis were performed with P-Clamp software, version 6.0 (Axon). Drug solutions were applied by a gravity-driven perfusion system from a linear array of seven microcapillary plastic tubes located about 50 μm from the cell.

Fluorescent Ca^{2+} Measurements

To measure cytoplasmic [Ca^{2+}], cells plated on glass coverslips were incubated with the fluorescent calcium indicator fura-2/AM (1 μM, TEFLABS) for 30 min in solution *A* in the presence of 2.5 mM probenecid. Fluorimetric measurements were carried out at 37° in a solution containing (mM): NaCl 143, KCl 3.6, $MgCl_2$ 0.5, $CaCl_2$ 1.2, glucose 12, Hepes 20, probenecid 2.5, pH 7.6 (NaOH) (solution *B*). In the measurements, a dual wavelength Deltascan spectrophotometer (Photon Technology International) was used in combination with a Zeiss Axiovert 135 inverted microscope (Zeiss). Excitation was performed at 340 and

380 nm, and the emission wavelength was 505 nm. Intracellular [Ca^{2+}] was measured either on single cells or on cell populations. In the latter case, the iris was broadened to see about 10–20 cells. Cytoplasmic [Ca^{2+}] was calculated from the ratio of the emitted fluorescences. Calibration of cytoplasmic [Ca^{2+}] was carried out on the basis of a previously described procedure using a dissociation constant of 224 nM for fura-2 [21]. Maximum and minimum fluorescence ratios were determined by using fura-2 free acid in a cytosol-like solution. The correction factor for viscosity was measured by comparing intracellular and extracellular fluorescence changes [22]. Drug solutions were applied as in the patch-clamp measurements.

Membrane Potential Determinations

Membrane potential changes were measured in glomerulosa cell suspension using the fluorescent dye $DiSC_3(5)$ (Molecular Probes) based on a previously described method [7]. After isolation 0.5×10^6 cells were suspended in 3 mL of solution *B*, and $DiSC_3(5)$ was added in 4.6 μL of DMSO to a final concentration of 0.8 μM. Fluorescent measurements were carried out in a cuvette thermostated at 37° using continuous stirring. The excitation and emission wavelengths were 620 and 673 nm, respectively. The cells were incubated with the dye for at least 15 min before any treatment. Addition of RR caused an immediate quench in the fluorescence, which was proportional to the concentration of the dye, so the shift was corrected using the ratio of the fluorescence before and immediately after the application of the drug.

Statistical Analysis

Statistical results are expressed as means ± SEM.

RESULTS

Effects of RR on Potassium- and ANG II-stimulated Aldosterone Production

Basal aldosterone production of adrenal glomerulosa cells was 157 ± 29 pg/adrenal/hour (N = 6, mean ± SEM). Upon exposing the cells to a submaximally effective concentration of K^+ (6 mM), aldosterone production was stimulated 26.56 ± 20.8-fold (N = 3). RR (0.3–10 μM) inhibited the effect of K^+ dose dependently (Fig. 1). On the other hand, the effect of 300 pM of Ang II (11.46 ± 0.3-fold stimulation of aldosterone production, N = 3) was potentiated by RR (Fig. 1). As the discrepancy between these inhibitory and potentiating effects of RR was maximal at 3 micromolar concentrations of the dye, this concentration was used in further experiments to analyze pharmacologically the mechanisms involved in the stimulatory action of K^+ and Ang II.

Aldosterone production induced by a moderate elevation of [K^+] (6 and 8 mM) was inhibited by RR, while the robust stimulatory effect of higher concentrations (about 200-fold

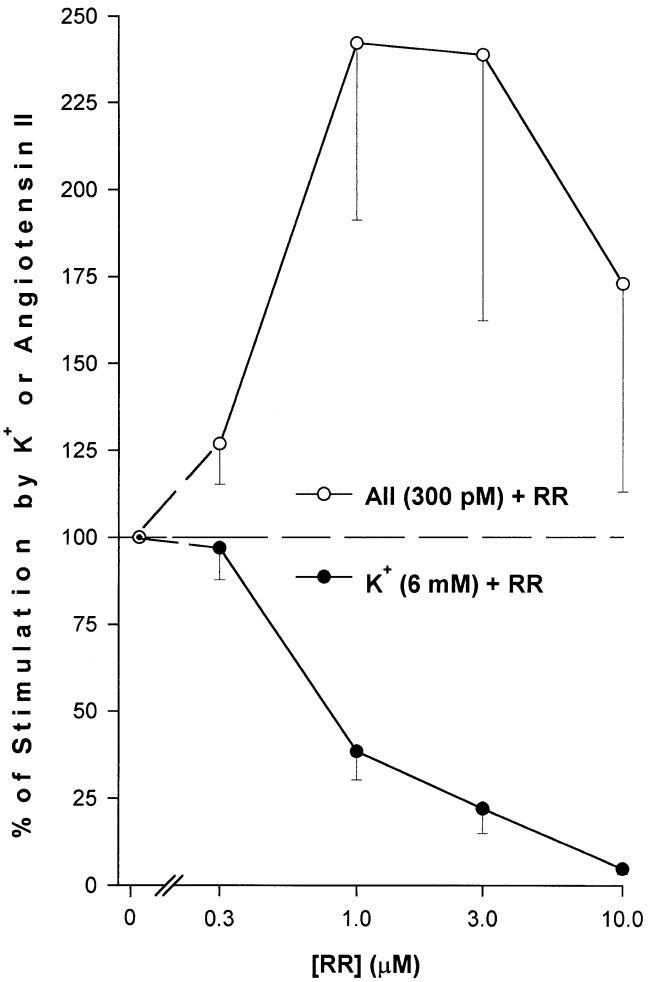

FIG. 1. Dose-dependent effect of RR (0.3–10 μM) on aldosterone production stimulated by 300 pM of ANG II (open circles) and 6 mM K^+ (filled circles). Data are normalized to the ANG II- or K^+-stimulated aldosterone production. Aldosterone production was measured after 1 hr incubation as detailed in Materials and Methods. Means ± SEM of three separate experiments, each performed in duplicate, are shown.

at 13 and 18 mM) was unaltered. Accordingly, the EC_{50} of K^+ was shifted from 6.91 to 8.59 mM (Fig. 2A). A combination of Ni^{2+} (300 μM) and nifedipine (1 μM), inhibitors of L- and T-type VDCC, respectively, reduced the K^+-stimulated aldosterone production, while RR added in combination with the other two inhibitors further diminished the steroid response to 8 mM [K^+] (Fig. 2B).

Ang II simulated aldosterone production in a concentration-dependent manner between 0.1–10 nM (maximal stimulation was 62.65 ± 12.50-fold, N = 3). RR (3 μM) failed to inhibit, but rather slightly potentiated the effect of the peptide, particularly at lower concentrations of the agonist. Contrarily to RR, inhibition of the VDCC by Ni^{2+} (300 μM) and nifedipine (1 μM) reduced the Ang II-induced steroid production (Fig. 3). To test the effect of RR on the capacitative Ca^{2+} influx pathway, which is involved in the signal transduction of Ang II, we applied thapsigargin to stimulate steroidogenesis. Thapsigargin (20–600

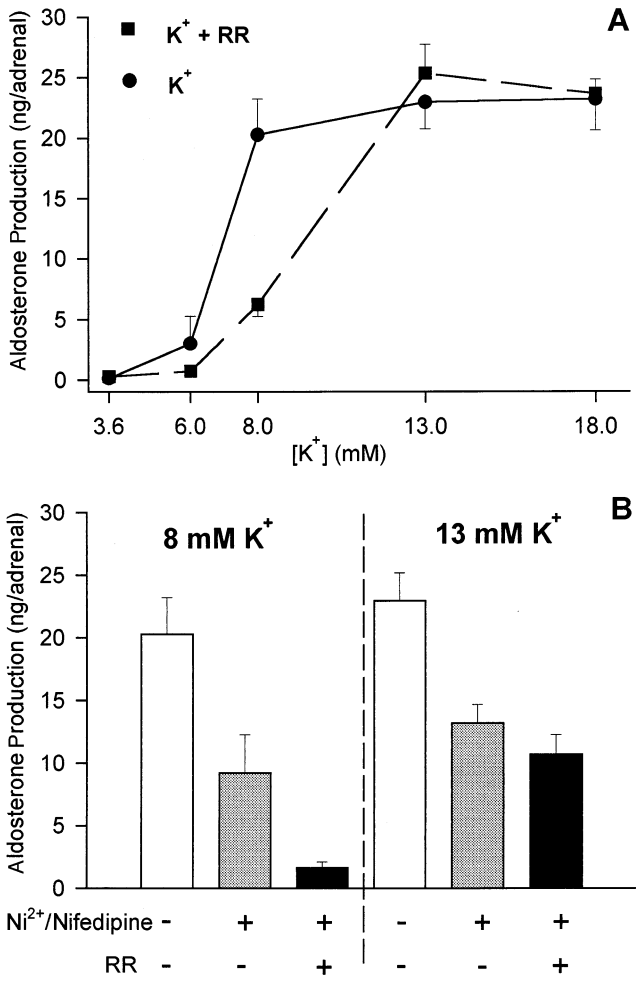

FIG. 2. Effect of 3 μM RR and the combination of 300 μM Ni^{2+}/1 μM nifedipine on K^+-stimulated aldosterone production. *A*: Dose-response curve of aldosterone production stimulated by K^+ itself (circles) and in the presence of 3 μM RR (squares). *B*: The effect of 300 μM Ni^{2+}/1 μM nifedipine (grey bars) and 3 μM RR added together with Ni^{2+}/nifedipine (black bars) on aldosterone production stimulated by 8 mM [K^+] (open bars, left) and 13 mM [K^+] (open bars, right). Aldosterone production was measured after 1 hr incubation as detailed in Materials and Methods. Data are expressed as ng steroid production per adrenal, and means ± SEM of three separate experiments' each performed in duplicate, are shown.

nM) dose dependently stimulated the aldosterone production (maximal response was 21.66 ± 5.69-fold at 600 nM concentration). Neither RR nor the combination of Ni^{2+}/nifedipine significantly affected this response (N = 3, data not shown).

RR (3 μM) by itself slightly stimulated the basal aldosterone production (2.12 ± 0.20-fold, N = 6), but this effect was marginal if compared with the degree of stimulation attained by either K^+ or Ang II.

Effects of RR on Potassium and Calcium Currents

Rat glomerulosa cells possess high K^+ conductance, which is important in settling their highly negative membrane potential [9, 23]. Upon stimulation with extracellular K^+ and Ang II, different Ca^{2+} influx pathways are activated. The effect of RR on these currents was studied by different patch-clamp protocols.

K^+ currents were measured in standard extracellular solution with a patch pipette containing standard intracellular solution. Consecutive hyperpolarizing steps were applied from −70 to −120 mV. These values are above and below the reversal potential of K^+ (−92 mV; calculated according to the Nernst equation), demonstrating that K^+ moves in the opposite direction at the two potentials. When added, RR rapidly reduced both the inward and outward currents (Fig. 4A), indicating the inhibition of K^+ conductance (N = 3). The effect of RR was also tested using a voltage ramp protocol from −120 to 40 mV (N = 6). The dye reduced both the inward and the outward—predominantly potassium—current (Fig. 4B).

The effect of RR on the voltage-independent, K^+-activated Ca^{2+} current, (I_{gl}), which is neither sensitive to dihydropiridines nor to Ni^{2+}, was examined by two different protocols. In the first experimental setup, a CsCl-based pipette solution was used and the extracellular solution was supplemented with tetraethylammonium chloride (20 mM) in order to eliminate K^+ currents. The membrane potential was clamped at −100 mV. Elevation of the extracellular [K^+] to 8.6 mM induced an inward current under these conditions, which was significantly reduced by 3 μM RR (N = 5, Fig. 5A). However, in spite of the presence of potassium channel inhibitors, the intracellular Cs^+, and extracellular tetraethylammonium chloride, the contribution of an increased K^+ influx to this current cannot be unequivocally excluded. Therefore, the effect of RR on the inward current was also addressed in another way. Here, we used standard solution both in the pipette and extracellularly. The cells were clamped at the equilibrium potential of potassium calculated with an extracellular concentration of 8.6 mM (−72 mV). When the concentration of K^+ is elevated extracellularly from 3.6 to 8.6 mM under these conditions, the driving force for the ion is eliminated, and the effect of RR can thereby be regarded exclusively as an effect of the drug on I_{gl} (N = 3, Fig. 5B).

The effect of RR on the high voltage-activated (L-type) and low voltage-activated (T-type) calcium channels was studied with Ba^{2+} (10 mM) instead of Ca^{2+} in the extracellular solution. This ion is widely used as charge carrier in studies of VDCC, because it does not induce inactivation as does Ca^{2+}. Ba^{2+} was previously shown to abolish I_{gl} and potassium currents, which was an additional advantage when testing the effect of RR on VDCC. The patch pipette contained CsCl instead of KCl so as to eliminate outward potassium current. During the ramp depolarization from −100 mV to 40 mV, two types of currents were observed: a low threshold, transient inward current and a long-lasting current with smaller amplitude which activated at higher voltage. These currents correspond to the previously characterized T- and L-type currents, respectively. The T-type current was affected by RR

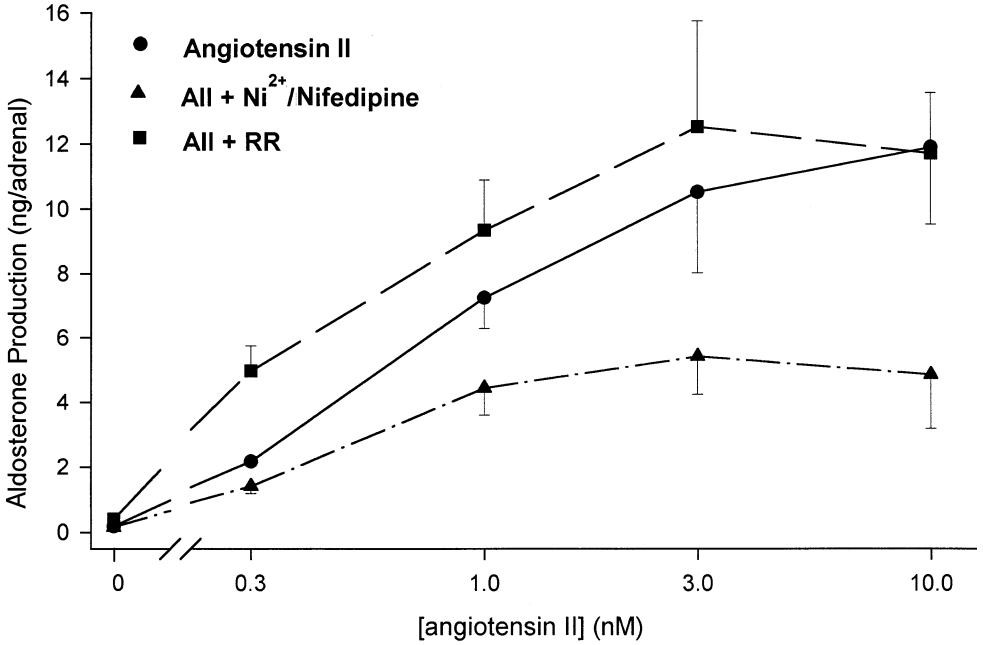

FIG. 3. Effect of 3 μM of RR (squares) and the combination of 300 μM Ni^{2+}/1 μM nifedipine (triangles) on the dose-dependent stimulation of aldosterone production by ANG II (circles). Aldosterone production was measured after 1 hr incubation as detailed in Materials and Methods. Data are expressed as ng steroid production per adrenal, and means ± SEM of three separate experiments, each performed in duplicate, are shown.

(3 μM) only slightly, with the activation threshold being shifted toward a positive direction by 3.7 ± 0.7 mV and the current amplitude reduced by 13.0 ± 1.0% (N = 3, Fig. 6). RR substantially (by 60.6 ± 5.3%) inhibited the current through L-type channels (N = 3, Fig. 6). All the effects of RR on ion fluxes were reversible (data not shown).

Effects of RR on Cytoplasmic [Ca^{2+}]

Intracellular [Ca^{2+}] was measured on Fura-2-loaded single cells or cell populations. Ca^{2+} oscillations were induced by RR in about 50% of the single cells tested, the ratio being variable in different cell preparations (N = 14, data not shown). This effect was partially sensitive to Ni^{2+}/nifedipine (N = 7, Fig. 7). The sustained $[Ca^{2+}]_i$ elevation on single cells evoked by K^+ (8 mM) was inhibited by RR (3 μM, N = 3, Fig. 8). A similar effect was detected at a 6 mM K^+ concentration (N = 3, data not shown). On single cells stimulated with 8 mM of K^+ in the presence of Ni^{2+} (300 μM) and nifedipine (1 μM), the elevation of $[Ca^{2+}]_i$ was markedly reduced. This residual Ca^{2+} signal was further inhibited by RR (N = 8), and the effect of RR was also found to be reversible with this approach (Fig. 8).

Ang II induced an oscillatory Ca^{2+} signal on single glomerulosa cells; therefore, to test the effect of RR we measured the integrated Ca^{2+} response of cell populations. The integrated Ca^{2+} signal induced by Ang II was potentiated by RR (3 μM, N = 4). The potentiating effect was more prominent at the response to a lower (300 pM) concentration of the peptide than at the Ca^{2+} signal evoked by a higher concentration (3 nM, Fig. 9).

Effect of RR on Membrane Potential

To study the mechanism by which RR evokes Ca^{2+} signal and stimulation of aldosterone production by itself, we measured the membrane potential change evoked by RR and compared the effect to alterations induced by elevation of extracellular [K^+]. Membrane potential changes were followed by measuring the fluorescence of $DiSC_3(5)$, which increases during depolarization due to release of the dye into the extracellular space [24]. The membrane potential change in response to RR (3 μM), as indicated by 12.00 ± 0.56% Δfluorescence (N = 3, Fig. 10), was between the response to 8 mM [K^+] (Δfluorescence 7.97 ± 0.71%, N = 3) and 13 mM [K^+] (Δfluorescence 14.23 ± 1.09%, N = 3). Six mM [K^+] caused a 5.4 ± 0.57% (N = 3) change in the fluorescence (Fig. 10, inset).

DISCUSSION

RR has been reported to affect different transport mechanisms through the plasma membrane as well as ion fluxes between intracellular compartments. The interpretation of results obtained with RR should be treated with caution because of the following reasons. While intact membranes are impermeable to the dye, it may penetrate the cell during long-term incubation or when the cell membrane is damaged; however, when applied extracellularly it is excluded from intact cells during short-term applications (as reviewed in [11]). Commercially available preparations of RR are often contaminated with different inorganic complexes, which differ from RR in their oxidation level [16] and in

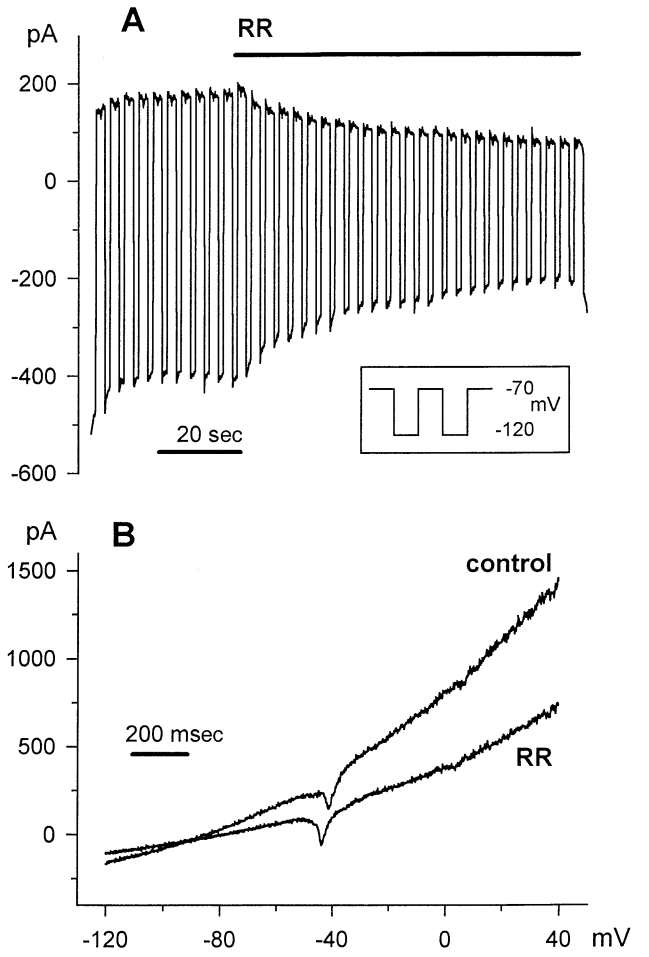

FIG. 4. Effect of 3 μM RR on the inward and outward potassium currents of glomerulosa cells. Cells were voltage-clamped at −120 or −70 mV (panel *A*), or a voltage ramp protocol was applied from −120 to 40 mV (panel *B*), in the absence or presence of 3 μM RR. The voltage protocol used in *A* is shown in the inset. The pipette contained standard intracellular solution (see Materials and Methods). The traces are representative of 3 *(A)* or 6 *(B)* similar experiments.

the number of Ru atoms involved in the complex [15]. These contaminations may also affect different cellular functions.

The rapid onset together with the rapid and full reversibility of the effect of purified RR in the present experiments suggests that within the studied time frame the dye did not enter glomerulosa cells. In the aldosterone experiments, preincubation of the cells for 30 min with RR did not influence the inhibitory effect of the drug on potassium stimulation (data not shown). Unimpaired aldosterone response to Ang II in the presence of RR also suggests that the mitochondrial Ca^{2+} transport mechanism (which is necessary for continuous steroid production) remained intact, indicating that RR is excluded from the cells. Thus, the site of action of the drug must be at the cell membrane.

In our experiments, RR modified both K^+ and Ca^{2+} currents through the plasma membrane. RR diminished the K^+ conductance of the plasma membrane, which contributes to the highly negative membrane potential of glomerulosa cells [23, 25]. The effect of RR on different K^+ channel classes has been addressed previously under different conditions in several cell types. When applied intracellularly, RR has been shown to inhibit calcium-dependent K^+ channels in rat hippocampal neurons and smooth muscle cells [12, 13]. However, when applied extracellularly, RR activated rapid and Ca^{2+}-dependent K^+ currents at mouse motor terminals [14]. In glomerulosa cells, the K^+ channels which conduct around the resting membrane potential are apparently different from the previous ones, in that they are inhibited by RR added to the extracellular side. The observed inhibition of K^+ conductance by RR leads to depolarization, which may enhance the opening of VDCC, thereby provoking the observed cytoplasmic calcium response and minor stimulation of aldosterone production by RR per se.

In previous studies, RR inhibited the activation of and ω-conotoxin GVIA binding by N-type VDCC [26]. On the other hand, RR failed to influence the activation of (and

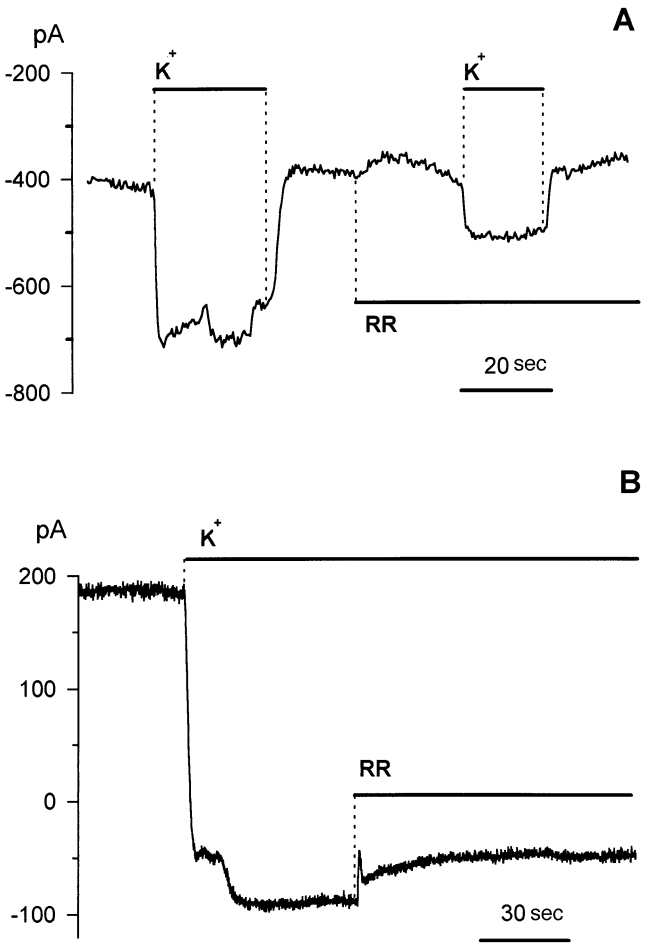

FIG. 5. Effect of 3 μM of RR on the inward current of glomerulosa cells evoked by 8.6 mM of $[K^+]$. (A) Cells were clamped at −100 mV and measurements were carried out with CsCl-containing pipette solution and standard extracellular solution. (B) Cells were clamped at the equilibrium potential of K^+ at 8.6 mM $[K^+]_{ec}$ (72 mV), using standard pipette solution. The composition of solutions is detailed in Materials and Methods. The traces are representative of 3 similar experiments.

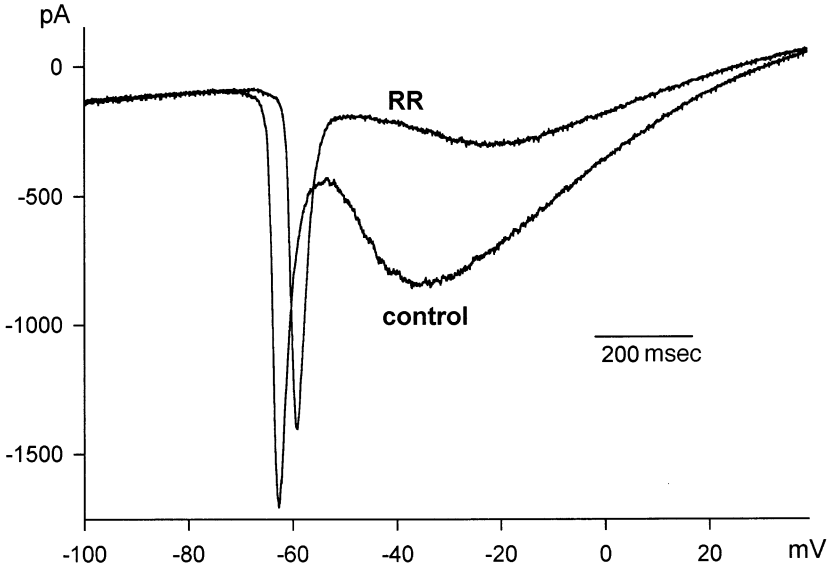

FIG. 6. Effect of 3 μM of RR on VDCC of glomerulosa cells. Currents were measured during ramp depolarization from −100 to 40 mV. The traces are representative of 3 similar experiments.

the dihydropiridine binding to) L-type Ca^{2+} channels [26–30]. Our electrophysiological results obtained in the glomerulosa cells are at variance with these latter observations, as RR reduced the amplitude of the high voltage-activated current. In previous molecular biological studies, we observed the expression of D and C class α_1 subunits [31] in accordance with the electrophysiological and pharmacological observations [10, 32] that only nifedipine-sensitive L-type channels are responsible for the high voltage-activated Ca^{2+} currents in rat glomerulosa cells. Thus, the high voltage-activated Ba^{2+} current measured in the present experiments reflects the ion flux through L-type calcium channels, and certainly this is the voltage-dependent channel inhibited by RR. RR exerted a further, moderate inhibitory effect on low voltage-activated T-type Ca^{2+} channels. The peak amplitude of the T-type current was reduced, and its activation threshold was shifted slightly towards more positive values. We are not aware of previous reports on inhibition of T- and L-type Ca^{2+} currents by RR.

The effects of RR on ionic fluxes through the plasma membrane may affect the mechanisms involved in the stimulus–secretion coupling in cells exposed to K^+ or Ang II. Although T-type and L-type Ca^{2+} currents are involved in both the action of Ang II and K^+, their mode of action differs significantly. While the Ang II-induced Ca^{2+} signal is brought about by Ca^{2+} release from $InsP_3$-sensitive stores as well as Ca^{2+} influx from the extracellular space, the action of K^+ is confined to this latter mechanism. The mechanism of Ca^{2+} influx induced by the two agonists is also different. Although high concentrations of Ang II may activate T-type current not only via depolarization but probably also via a G-protein-mediated process [5, 33] and/or by phosphorylation [34] and the dihydropyridine-sensitive L-type channels are also involved in the action of Ang II [1], a capacitative influx mechanism plays the major

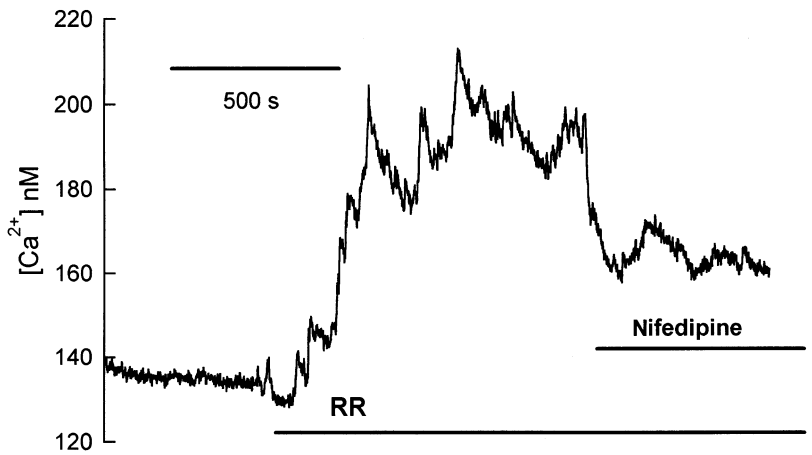

FIG. 7. Effect of 1 μM of nifedipine on the Ca^{2+} signal evoked by 3 μM RR itself on a population of glomerulosa cells. Measurements were carried out on a group of about 10–20 fura-2-loaded cells. Calibration of $[Ca^{2+}]$ is detailed in Materials and Methods. The curve is representative of 7 similar experiments.

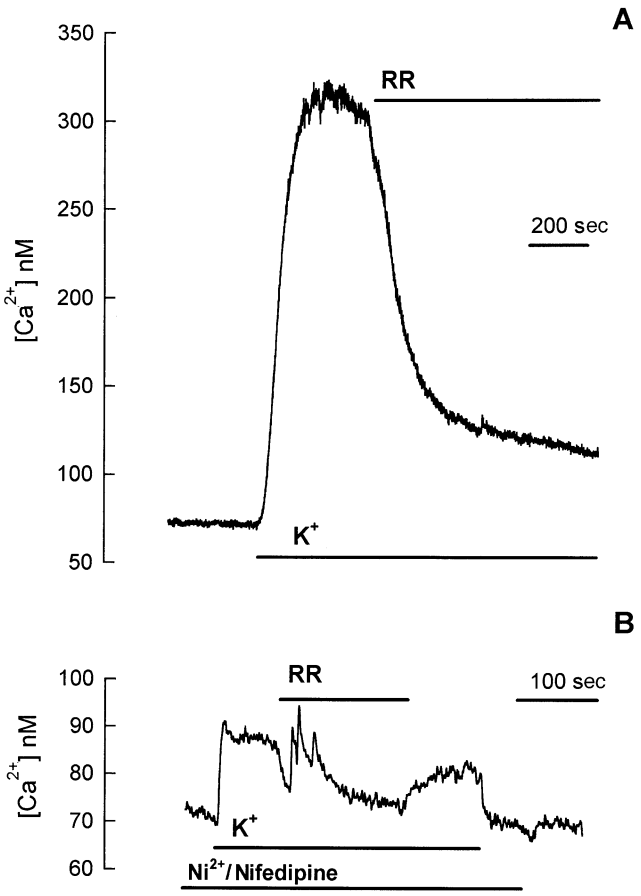

FIG. 8. Effect of 3 μM RR itself (panel *A*) and RR added after the application of the combination of 300 μM Ni^{2+}/l μM nifedipine (panel *B*) on the cytoplasmic Ca^{2+} signal evoked by 8 mM K^+. Measurements were carried out on single fura-2-loaded cells. Calibration of [Ca^{2+}] is detailed in Materials and Methods. The curve is representative of 8 similar experiments.

role in the sustained phase of the Ca^{2+} signal [2, 3]. We found that both types of VDCC are inhibited by RR. At the same time, aldosterone production stimulated by the Ca.ATPase inhibitor thapsigargin was not influenced by RR, indicating that the drug does not inhibit the store depletion-induced capacitative Ca^{2+} influx. Considering that this latter mechanism plays a dominant role in Ang II-induced Ca^{2+} influx, no considerable reduction of Ang II-induced Ca^{2+} signal and aldosterone production may be expected in RR-exposed cells. In fact, RR enhanced rather than reduced the Ca^{2+} signal and the hormone production. This enhancement may have two explanations. Recalling that RR inhibits K^+ channels and thereby depolarizes the cell, this depolarization may have overcome the direct inhibitory effect of the drug on VDCC. The alternative explanation assumes a coupling of the dihydropyridine and $InsP_3$ receptors. It was reported that voltage- or agonist-induced activation of the dihydropyridine receptor potentiates the Ca^{2+}-releasing effect of $InsP_3$ [35]. A transfer of the RR-induced conformational change of the dihydropyridine receptor to the intracellular Ca^{2+} release channel may also account for the observed potentiation of Ang II-induced Ca^{2+} and aldosterone response by RR.

Elevated extracellular K^+ concentration depolarizes the cell and activates VDCC. We observed for the first time that RR inhibited T- and L-type Ca^{2+} currents and, in harmony with this inhibition, reduced the K^+-induced Ca^{2+} signal as well as aldosterone production. It should be mentioned that although at higher, supraphysiological [K^+] (13–18 mM), the calcium signal was reduced, aldosterone production was maintained. It may be assumed that, in spite of channel inhibition, cytoplasmic [Ca^{2+}] reached the level sufficient for maximal steroid production. Therefore, the effect of RR on hormone production can be characterized by a shift in the sensitivity of the cell to potassium.

Ca^{2+} influx in K^+-stimulated glomerulosa cells may not be confined to voltage-dependent mechanisms. We presented evidence that glomerulosa cells voltage-clamped at −100 mV and that Ni^{2+} plus nifedipine responded to K^+ with the Ca^{2+} signal [9]. This phenomenon has been attributed to the activation of I_{gl}, a K^+-activated, nonvoltage-operated, noninactivating, presumably Ca^{2+} current [10]. In the present experiments the inhibition of VDCC by the simultaneous administration of Ni^{2+} and nifedipine reduced both the Ca^{2+} signal and aldosterone response at

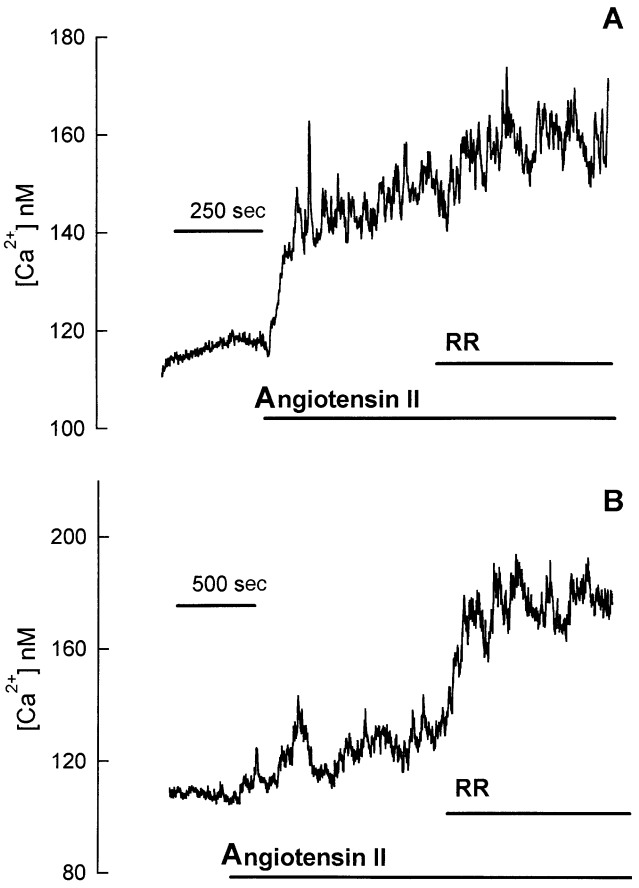

FIG. 9. Effect of 3 μM of RR on the 3 nM (panel *A*) and 300 pM (panel *B*) angiotensin II-evoked cytoplasmic Ca^{2+} signal. Measurements were carried out on a group of about 10–20 fura-2-loaded cells. Calibration of [Ca^{2+}] is detailed in Materials and Methods. The curves are representative of 4 similar experiments.

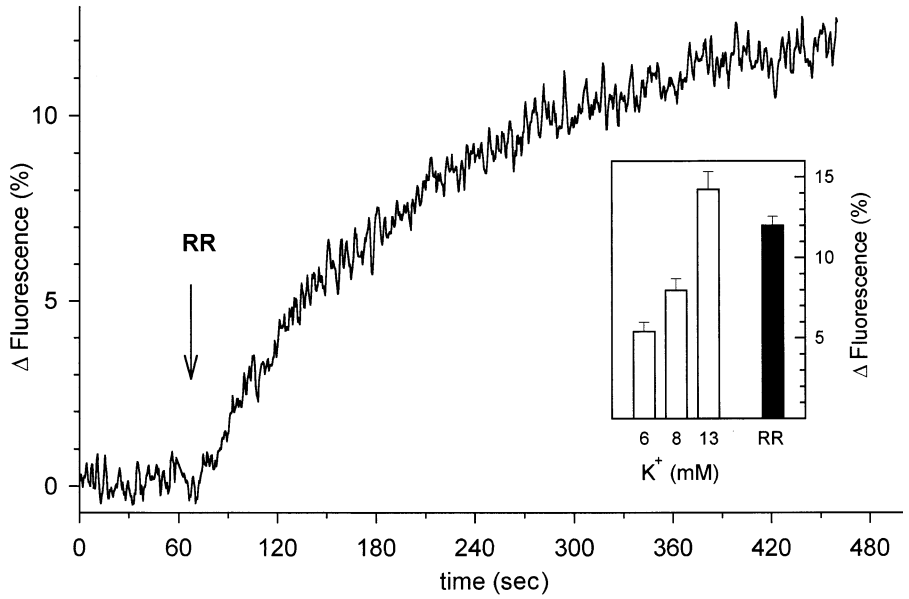

FIG. 10. Effect of 3 μM RR on the membrane potential of glomerulosa cells measured by the potential sensitive dye $DiSC_3(5)$. Experimental conditions are described in Materials and Methods. Membrane potential changes are shown as fluorescence change of the dye normalized to the control period. *Inset*: maximal fluorescent changes caused by 6, 8, and 13 mM K^+-stimulation (open bars) compared to the effect of 3 μM RR (filled bar). Data are expressed as means ± SEM. (N = 3).

all the examined K^+ concentrations, although additional inhibition was achieved by adding RR. We have examined the effect of RR on K^+-induced current in cells voltage-clamped under different conditions. When the patch pipette contained Cs^+, at −100 mV the K^+-induced inward current was significantly reduced by RR. While this observation is compatible with the inhibition by RR of a nonvoltage-operated Ca^{2+} current, it should also be kept in mind that inward potassium currents may be incompletely blocked by intracellular Cs^+. In other experiments where the membrane potential was held at the calculated equilibrium potential of K^+, an inward current was observed this again being reduced by RR. Provided that the equilibrium potential of K^+ and the membrane potential were truly identical, the charge carrier of this current could not be K^+. Even if this assumption should be confirmed by further experiments in view of the observation that RR further augmented the inhibitory effect of Ni^{2+} and nifedipine on K^+-induced Ca^{2+} signal and aldosterone production, it is reasonable to assume that RR also inhibits a K^+-activated, nonvoltage-operated Ca^{2+} uptake mechanism.

In summary, we showed for the first time that RR inhibits the resting K^+ currents and different Ca^{2+} currents in rat glomerulosa cells. The application of RR together with conventional Ca^{2+} channel blockers contributed to the elucidation of the importance of the different influx routes during the activation of these cells.

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