Article

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Abstract

Angiotensin-II (AngII)-induced Ca2+ influx in adrenal glomerulosa cells, a signal necessary for the stimulation of steroidogenesis by the hormone, is believed to involve two distinct mechanisms: 1) opening of voltage-operated Ca2+ channels, and 2) activation of a capacitative Ca2+ entry pathway that is dependent on calcium release from intracellular stores. Nicardipine, a dihydropyridine calcium antagonist, has been used to investigate the role of these Ca2+ entry mechanisms in the steroidogenic response to AngII. As demonstrated with the patch-clamp technique, micromolar concentrations of nicardipine completely blocked voltage-operated Ca2+ channel activity of both T- and L-types. This agent similarly inhibited the rise of cytosolic free calcium concentration induced by potassium, but did not significantly affect the response to thapsigargin, an activator of the capacitative pathway. Nicardipine reduced by only 22% the calcium influx stimulated by AngII, and the nicardipine-insensitive part of this response was abolished after exhausting the intracellular Ca2+ stores with thapsigargin. Similarly, aldosterone secretion [...]

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Role of the Capacitative Calcium Influx in the Activation of Steroidogenesis by Angiotensin-II in Adrenal Glomerulosa Cells*

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ABSTRACT

Angiotensin-II (AngII)-induced Ca^{2+} influx in adrenal glomerulosa cells, a signal necessary for the stimulation of steroidogenesis by the hormone, is believed to involve two distinct mechanisms: 1) opening of voltage-operated Ca^{2+} channels, and 2) activation of a capacitative Ca^{2+} entry pathway that is dependent on calcium release from intracellular stores. Nicardipine, a dihydropyridine calcium antagonist, has been used to investigate the role of these Ca^{2+} entry mechanisms in the steroidogenic response to AngII. As demonstrated with the patch-clamp technique, micromolar concentrations of nicardipine completely blocked voltage-operated Ca^{2+} channel activity of both T- and L-types. This agent similarly inhibited the rise of cytosolic free calcium concentration induced by potassium, but did not significantly affect the response to thapsigargin, an activator of the capacitative pathway. Nicardipine reduced by only 22% the calcium influx stimulated by AngII, and the nicardipine-insensitive part of this response was abolished after exhausting the intracellular Ca^{2+} stores with thapsigargin. Similarly, aldosterone secretion induced by AngII was only partially inhibited (40%) by nicardipine at concentrations that completely abolished the steroidogenic response to potassium. Thapsigargin by itself was able to stimulate aldosterone production, an action highly potentiated by physiological concentrations of extracellular potassium.

These data strongly suggest that the major part of the calcium influx response to AngII, leading to aldosterone formation, involves a capacitative calcium entry pathway activated by the release of calcium from intracellular stores. This mechanism of calcium influx could be responsible for some features of aldosterone response to the hormone, such as its poor sensitivity to dihydropyridines or its potentiation by potassium. (*Endocrinology* **135:** 751–758, 1994)

THE DEPENDENCY on extracellular Ca^{2+} of the steroidogenic action of potassium ion (K^+) and angiotensin-II (AngII) has been firmly established in adrenal glomerulosa cells for many years (1–3), and a role for cytosolic calcium in aldosterone stimulation has been clearly demonstrated (4); however, the calcium entry pathways triggered by these agonists are still poorly characterized.

Potassium ion, by depolarizing the cell membrane, is believed to open voltage-operated calcium channels. In bovine glomerulosa cells, the presence of both low threshold (T-type) and high threshold (L-type) calcium channels has been demonstrated (5, 6), with properties similar to those described in other cell types (7). The extremely high sensitivity of glomerulosa cells to extracellular K^+ strongly suggests that low threshold T-type channels are involved in the response to physiological concentrations of this agonist (6, 8). On the other hand, AngII, in addition to releasing Ca^{2+} from intracellular Ca^{2+} pools through generation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (9, 10), has been shown to induce cell depolarization by inhibiting some potassium conductances (11) and, therefore, could also stimulate Ca^{2+} influx through T-channels (6).

Recently, another pathway for Ca^{2+} entry, resulting from intracellular Ca^{2+} pool depletion, has been described in bovine (12, 13) and rat (14) glomerulosa cells. This pathway is activated by thapsigargin, an agent leading to Ca^{2+} release from intracellular stores by inhibiting the associated Ca^{2+}/Mg^{2+}-ATPase. Because AngII-responsive and thapsigargin-sensitive Ca^{2+} pools are largely coincident in these cells (12), AngII has been proposed to stimulate a capacitative Ca^{2+} influx, similar to that described in several nonexcitable cell types (15).

It appears, therefore, that AngII is able to stimulate Ca^{2+} influx through at least two distinct complementary mechanisms: 1) by activating voltage-operated T-type calcium channels, and 2) through the capacitative Ca^{2+} entry pathway. However, the lack of an efficient agent to selectively block one of these pathways has so far prevented an accurate estimation of the relative importance of these mechanisms in the stimulation of steroidogenesis by the hormone.

In the present study, we show that micromolar concentrations of nicardipine, a dihydropyridine inhibiting low threshold Ba^{2+} currents in mouse embryonic dorsal root ganglion neurons (16), completely blocked T- and L-type Ca^{2+} channels in bovine glomerulosa cells. We used this agent to demonstrate that only a minor part of the Ca^{2+} influx response to AngII involves voltage-operated Ca^{2+} channels, whereas the rest of the response is thapsigargin sensitive. Either mechanism of Ca^{2+} entry is able to independently

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stimulate aldosterone synthesis, but a full steroidogenic response requires activation of both pathways.

Materials and Methods

Materials

Nicardipine, HEPES, insulin, transferrin, sodium selenite, tetrodotoxin, ATP, and GTP were purchased from Sigma Chemical Co. (St. Louis, MO). Thapsigargin was obtained from Anawa (Zürich, Switzerland), and [Ile^5]AngII from Bachem AG (Bubendorf, Switzerland). Dispase (grade II) was obtained from Boehringer Mannheim (Indianapolis, IN), Percoll from Pharmacia (Piscataway, NJ), and ascorbate from Merck (Darmstadt, Germany). Horse serum, fetal calf serum, and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco (Grand Island, NY). Metyrapone was purchased from Aldrich Chemical Co. (Milwaukee, WI), and fura-2 and 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetracesium salt (Cs_4BAPTA) from Molecular Probes (Eugene, OR).

Isolation and culture of bovine glomerulosa cells

Bovine adrenal glomerulosa cells were prepared by enzymatic dispersion with dispase and purified on a Percoll density gradient, as described in detail previously (17). Cells were then either used the same day for cytosolic free calcium concentration ($[Ca^{2+}]_c$) and aldosterone measurements or cultured on small glass coverslips for patch-clamp experiments. In the latter case, cells were transferred in antibiotics-containing DMEM supplemented with 1 mM ascorbate, 1 μg/ml insulin, 1 μg/ml transferrin, 1 ng/ml sodium selenite, 5 μM metyrapone, 2 mM glutamine, 2% (vol/vol) fetal calf serum, and 10% (vol/vol) horse serum and incubated overnight at 37 C in 5% CO_2. The next day, the medium was removed and replaced with serum-free DMEM until the cells were used for patch-clamp experiments.

Patch clamp measurements

The activity of voltage-activated calcium channels was recorded under voltage clamp in the whole cell configuration of the patch clamp technique, essentially as described previously (17). The bath solution contained 117 mM tetraethylammonium chloride, 20 mM $BaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 32 mM sucrose, and 200 nM tetrodotoxin and was buffered to pH 7.5 with 10 mM HEPES-CsOH. The patch pipette (Clark 150T/F-10, Clark Electromedical Instruments, Reading, United Kingdom) contained 85 mM CsCl, 10 mM tetrabutylammonium chloride, 6 mM $MgCl_2$, 5 mM sodium ATP, and 0.04 mM sodium GTP, and pH was buffered to 7.2 with 20 mM HEPES/CsOH. The pipette solution also contained 0.9 mM $CaCl_2$ and 11 mM Cs_4BAPTA to buffer free calcium below 50 nM and to stabilize the low access resistance from pipette to cell. The Ba^{2+} currents were filtered (1–2 kHz) and automatically leak-subtracted.

Measurement of $[Ca^{2+}]_c$

The determinations of $[Ca^{2+}]_c$ in cell populations were performed with the fluorescent probe fura-2. For this purpose, cells purified on Percoll density gradients were washed twice and resuspended in a Krebs-Ringer medium (containing 136 mM NaCl, 1.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 1.2 mM $CaCl_2$, and 5.5 mM D-glucose and buffered to pH 7.4 with 20 mM HEPES) at a concentration of 10^7 cells/ml, then incubated at 37 C for 60 min before being washed and incubated again for 30 min in the presence of 2 μM fura-2 acetoxymethyl ester. The dye excess was washed away, and the cells were kept at ambient temperature in the same medium. Aliquots of 2×10^6 cells were sedimented just before use and resuspended in 2 ml Krebs-Ringer medium in a thermostated cuvette at 37 C. Fura-2 fluorescence (excitation at 340 nm, or 360 nm when measuring Mn^{2+} influx, and emission at 505 nm) was recorded in a Perkin-Elmer LS-3 fluorescence spectrometer (Norwalk, CT), and $[Ca^{2+}]_c$ was calibrated as previously described for quin2 (18), using a value of 224 nM for the K_d of fura-2.

Determination of aldosterone formation

After dispersion and washing, the glomerulosa cells were resuspended in 50 ml Krebs-Ringer medium, preincubated for approximately 1 h at 37 C before being washed twice and distributed at a final concentration of 500,000 cells/ml in test tubes containing various agonists. Cells were then incubated for 90 min at 37 C. At the end of the incubation period, the cells were sedimented by centrifugation, and the aldosterone content in the medium was measured by direct RIA, using a commercially available kit (Diagnostic Product Corp., Los Angeles, CA).

In one experiment (see Fig. 6A), cells were cultured for 3 days, as described for patch-clamp experiments, before being stimulated in petri dishes. In this case, the serum-free DMEM was replaced by Krebs-Ringer medium at least 1 h before starting the experiment, and cells were preincubated at 37 C. Cells were then incubated in the same medium supplemented with various concentrations of thapsigargin or KCl for 90 min. After 30 min of incubation, media were discarded and replaced with fresh media, and aldosterone was determined in the supernatant after 60 min of additional incubation. Cellular protein was measured in each dish using the Coomassie blue method of Bradford (19).

Statistics

Statistical significance of differences was determined by Student's *t* test and is indicated by the two-tail *P* value.

Results

Nicardipine blocks both L- and T-type calcium channels

The inhibitory action of nicardipine, a dihydropyridine Ca^{2+} antagonist, on voltage-activated Ba^{2+} currents in bovine adrenal glomerulosa cells is illustrated in Fig. 1. Currents flowing through T- and L-type Ca^{2+} channels were measured in isolated glomerulosa cells voltage-clamped in the whole cell configuration of the patch-clamp technique. When the cell was depolarized from a holding potential of −90 mV to −30 mV, under conditions where Na^+ and K^+ conductances had been completely inhibited, a net inward Ba^{2+} current was observed, with the characteristics of a mixture of transient and sustained components (Fig. 1A, trace a). As described previously (5, 17), this biphasic response is believed to reflect the presence in bovine glomerulosa cells of both T- and L-type channels, the early phase being mainly due to the rapid activation and inactivation of T-channels. The addition of increasing concentrations of nicardipine into the bath (b = 0.1, c = 0.2, d = 1, and e = 2 μM), markedly reduced the Ba^{2+} currents elicited by depolarization. As shown by the current-voltage relationship (Fig. 1B), the inhibition by nicardipine occurred at each voltage. More specifically, slowly deactivating tail currents, exclusively due to T-type channels (8), were elicited by repolarizing the cell to −65 mV after a short period of depolarization at 20 mV (Fig. 1C). The same concentrations of nicardipine similarly reduced the amplitude of the tail current, and this effect was attributed to blockade of T-channels. To determine the time course of inhibition of tail currents by nicardipine, the same voltage protocol as that described in Fig. 1C was repeatedly performed every 15 sec, and the amplitude of the current, measured 8 msec after cell repolarization, was expressed as a function of time (Fig. 1D). The T-type current was completely abolished in the presence of 2 μM nicardipine. Interestingly, nifedipine, another dihydropyridine Ca^{2+} antago-

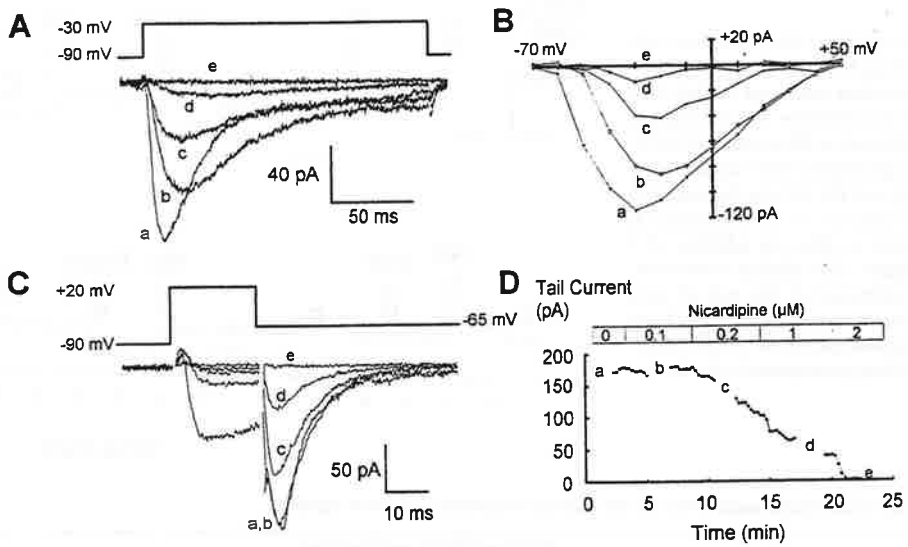

FIG. 1. Inhibition of voltage-activated Ba^{2+} currents by nicardipine. Voltage-activated Ba^{2+} currents were recorded from isolated bovine adrenal glomerulosa cells with the patch-clamp technique in the whole cell configuration, as indicated in Materials and Methods. A, Superimposed traces of Ba^{2+} currents elicited by a depolarization of the cell from a holding potential of −90 to −30 mV, before (a) or after various nicardipine concentrations (b = 0.1, c = 0.2, d = 1, and e = 2 μM). B, Current-voltage relationship of the Ba^{2+} current upon inhibition by nicardipine. Barium currents were elicited by depolarization to various voltages, as described for A, and the maximal current amplitude was measured at each potential and for each nicardipine concentration (a–e, same meaning as in A). C, Effect of nicardipine on slowly deactivating tail currents. Cells were depolarized for 20 msec to 20 mV, and then repolarized to −65 mV to evoke tail currents. The first 6-msec recording after cell repolarization (during the large capacitative transient and L-channel deactivation) were not included for current analysis. The decay of the current was best described by a single exponential function. Cells were exposed to the same nicardipine concentrations (a–e) as indicated in A. D, Time course of tail current inhibition by nicardipine. Slowly deactivating (T-type) currents were evoked every 15 sec, as described in C, and the amplitude of the current, measured 8 msec after repolarization, was plotted as a function of time. The *upper panel* indicates the concentration of nicardipine present in the bath, and recording was interrupted at various times (a–e) for generating the current *vs.* voltage curves presented in B. pA, Picoamps.

nist, was less efficient for blocking these channels (data not shown).

Relative importance of voltage-activated calcium channels and of the capacitative calcium influx pathway in the response to AngII

We have used the pharmacological properties of nicardipine on T- and L-type Ca^{2+} channels to assess the role of the capacitative (nonvoltage-operated) calcium influx in the $[Ca^{2+}]_c$ response as well as in the aldosterone stimulation by various agonists. Addition of 1 μM nicardipine not only completely abolished the sustained rise in $[Ca^{2+}]_c$ induced by 9 mM K^+, but even decreased $[Ca^{2+}]_c$ slightly below the resting levels (Fig. 2A). When nicardipine was introduced first (Fig. 2B), an immediate drop of $[Ca^{2+}]_c$ occurred, suggesting that nicardipine inhibited a basal activity of T-channels present in resting cells, and a subsequent addition of potassium was totally inefficient in raising $[Ca^{2+}]_c$. Surprisingly, nicardipine seemed to inhibit the response to thapsigargin, an agent stimulating exclusively the capacitative Ca^{2+} influx in adrenal glomerulosa cells (Fig. 2C). However, the decrease in $[Ca^{2+}]_c$ observed upon nicardipine addition was, in fact, due to inhibition of the basal Ca^{2+} influx through T-channels, which was superimposed to the capacitative influx. Indeed, when nicardipine was added before thapsigargin (Fig. 2D), the response to the latter agent was not significantly different from that observed in naive cells (Fig. 2C). The same protocol was applied to the response to AngII (Fig. 2, E and F). In this case, the presence of nicardipine during stimulation by the hormone reduced the rise of $[Ca^{2+}]_c$ by approximately one third, as measured during the plateau phase of the response. This finding strongly suggests that a major part of the response to AngII is nicardipine insensitive and, therefore, involves pathways distinct from the voltage-activated Ca^{2+} channels. Similar results have been obtained from several cell preparations and are summarized in Table 1. The inhibitory action of nicardipine (1 μM) has been evaluated by comparing the Ca^{2+} responses to the various agonists in the presence and absence of that agent. Whereas nicardipine completely blocked the response to potassium in each preparation, this dihydropyridine did not significantly affect the response to thapsigargin. In the case of AngII, the inhibition of the response by nicardipine was relatively constant (on the average, 22.5%) and was statistically significant.

The presence in resting glomerulosa cells of a basal Ca^{2+} influx through nicardipine-sensitive channels was confirmed by measuring the rate of manganese influx into these cells. As shown in Fig. 3, a rapid quenching of the fura-2 fluorescence (excitation at 360 nm) resulted from Mn^{2+} entry into resting cells. Manganese influx was markedly accelerated by extracellular K^+ (9 mM) added at the same time as Mn^{2+}. The slight reduction of basal influx observed in the presence of 1 μM nicardipine strongly suggests that voltage-operated Ca^{2+} channels are partially activated in resting cells.

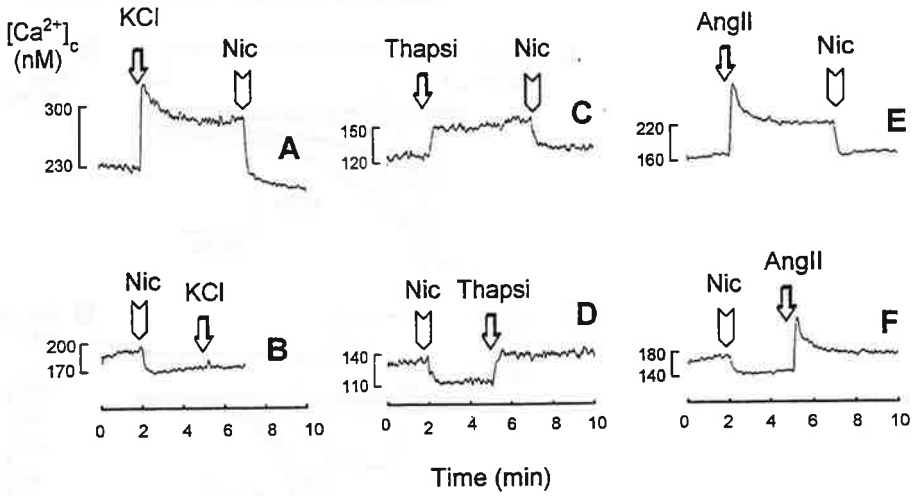

FIG. 2. Nicardipine (Nic) effect on calcium signaling by various agonists. Isolated glomerulosa cells were loaded with fura-2, and fluorescence was recorded at 37 C, as indicated in *Materials and Methods*. Cell populations were exposed to 9 mM KCl (A and B), 100 nM thapsigargin (Thapsi; C and D), or 10 nM AngII (E and F) before or after the addition of 1 μM nicardipine. The calcium concentration was calibrated at the end of each trace, as described previously (18). Each trace is representative of 3–13 experiments, yielding quantitatively similar results.

TABLE 1. Nicardipine sensitivity of the calcium response to various agonists

Stimulus	Sustained $[Ca^{2+}]_c$ response (nM)		% Inhibition	Significance (P)
	Without Nic	With 1 μM Nic		
KCl (9 mM)	125 ± 18.1[a] (3)	0 (3)	100	<0.001
Thapsi (100 nM)	49.5 ± 4.4 (13)	45.3 ± 3.9 (10)	8.5	NS
AngII (10 nM)	62.8 ± 3.5 (12)	48.5 ± 3.3 (11)	22.5	<0.01

The calcium responses to KCl (9 mM), thapsigargin (Thapsi; 100 nM), and AngII (10 nM) were determined in the presence and absence of nicardipine (Nic; 1 μM), as described in Fig. 2. The sustained $[Ca^{2+}]_c$ response was measured as the difference between the mean $[Ca^{2+}]_c$ immediately before the stimulus and the mean $[Ca^{2+}]_c$ after a plateau induced by the agonist had been reached. The number of traces (in *parentheses*) recorded for each condition is indicated, as well as the percent inhibition of the response by nicardipine and the statistical significance according to Student's t test.

[a] Mean ± SEM.

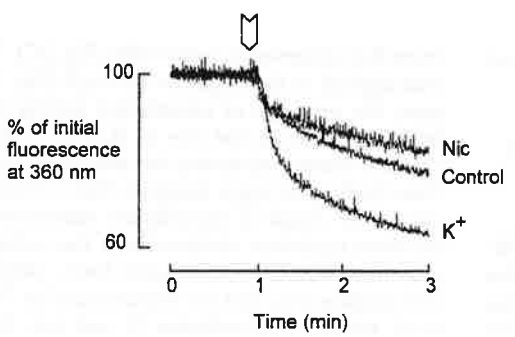

FIG. 3. Inhibition of manganese influx by nicardipine (Nic). Fura-2-loaded cells were exposed at the time indicated by the *arrow* to 0.5 mM $MnCl_2$ alone (control) or to $MnCl_2$ with either K^+ (9 mM) or nicardipine (1 μM). The Mn^{2+}-induced decrease in fura-2 fluorescence was recorded at the isosbestic (Ca^{2+}-independent) excitation wavelength (360 nm). Traces were obtained by averaging three independent experiments.

Dependence of AngII-induced calcium influx on intracellular calcium stores

To further characterize the nicardipine-insensitive Ca^{2+} influx induced by AngII, we determined the role of intracellular Ca^{2+} stores in the activation of this response. In the absence of extracellular Ca^{2+} and in the presence of nicardipine, a maximal AngII concentration (50 nM) was unable to mobilize the totality of the intracellular Ca^{2+} stores releasable by thapsigargin (Fig. 4A). This fact was not due to a rapid metabolism of Ins(1,4,5)P_3 produced upon hormone stimulation, because a second pulse of AngII before thapsigargin was without effect, although a rapid desensitization of the response to AngII, as shown in these cells by Ambroz and Catt (20), cannot be excluded. In contrast, after depletion of the pools with thapsigargin, AngII could not further release intracellular Ca^{2+} (Fig. 4B), suggesting either that a part of the thapsigargin-sensitive Ca^{2+} stores is insensitive to Ins(1,4,5)P_3, or the concentration of Ins(1,4,5)P_3 reached upon maximal hormone stimulation is not sufficient for a complete emptying of the pools.

In the presence of extracellular Ca^{2+} (1.2 mM) and nicardipine (1 μM), both AngII and thapsigargin induced a sustained Ca^{2+} influx, independent of voltage-operated Ca^{2+} channels. These Ca^{2+} influxes were not additive (Fig. 4, C and D), therefore demonstrating that both agonists share a common mechanism for stimulating Ca^{2+} entry.

Calcium entry pathways involved in aldosterone biosynthesis

The effect of nicardipine on aldosterone production induced by AngII or K^+ has been investigated. The steroidogenic response to 12 mM K^+ was inhibited in a concentration-dependent manner, with a half-maximal effect at 150 nM nicardipine and complete inhibition at 1 μM (Fig. 5A). This result is in agreement with the concept that this agonist exclusively employs voltage-activated Ca^{2+} channels for

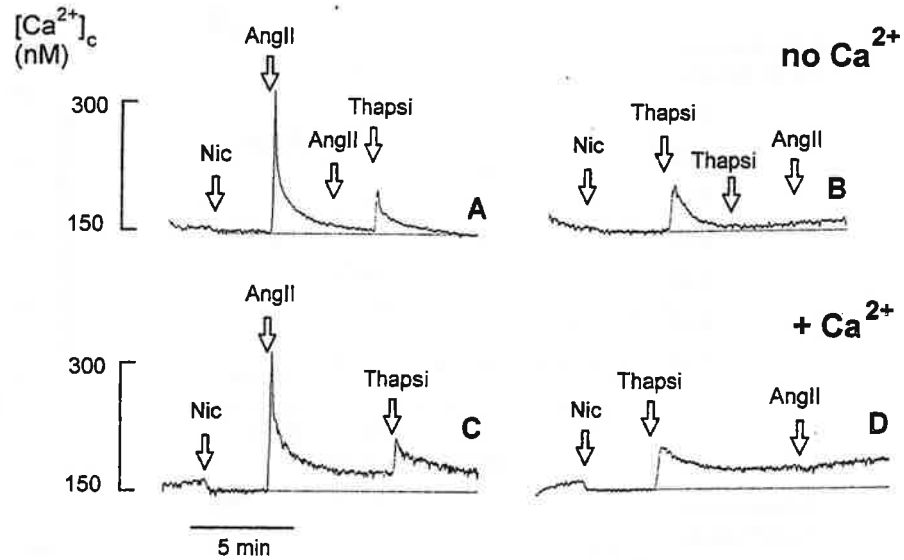

FIG. 4. Role of intracellular calcium stores in AngII-induced calcium influx. Fura-2-loaded cells were exposed to 1 μM nicardipine (Nic) in the absence (A and B) or presence (C and D) of extracellular calcium (1.2 mM), and then stimulated with 50 nM AngII and 400 nM thapsigargin (Thapsi). Traces are representative of two experiments from two cell preparations that gave similar results.

mediating its steroidogenic effect. In contrast, only 40% of the response to AngII (10 nM) was affected by micromolar concentrations of nicardipine (Fig. 5A).

The inhibitory action of nicardipine (1 μM) was measured at various AngII concentrations (Fig. 5B). Interestingly, nicardipine appeared to be a more potent inhibitor of aldosterone synthesis at lower AngII concentrations (75% inhibition at 100 pM *vs.* 40% inhibition at 100 nM; Fig. 5B, *inset*). This suggests that the requirement for T-channel activation is stronger at lower AngII concentrations.

Thapsigargin induced a small but significant stimulation of aldosterone formation, with a maximal effect at 100–300 nM (Fig. 6A). This steroidogenic response was markedly potentiated after raising the extracellular potassium concentration from 3 to 8 mM, without any change in the sensitivity to thapsigargin, suggesting that the two agonists stimulate different mechanisms. This potentiating action of thapsigargin and potassium was further investigated by keeping a constant concentration of thapsigargin (400 nM) and varying the amount of steroidogenic potassium (Fig. 6B). The most pronounced potentiation was observed between 6 and 9 mM K^+, a range of potassium concentrations where almost exclusively T-type Ca^{2+} channels are activated.

Discussion

Calcium transport across the plasma membrane of adrenal glomerulosa cells appears unusually complex, because two different mechanisms are simultaneously responsible for the Ca^{2+} influx in these cells. On the one hand, voltage-activated Ca^{2+} channels, sensitive to dihydropyridines and particularly well documented in excitable cells, provide the glomerulosa cell with its extraordinary sensitivity to slight increases in physiological concentrations of extracellular potassium and are responsible for part of the response to AngII that is mediated by a depolarization of the plasma membrane. This hormone-induced depolarization has been shown to result from an inhibition of potassium conductances by protein kinase-C (21, 22). On the other hand, a capacitative Ca^{2+} entry mechanism (15), present in many nonexcitable cells and triggered by the release of Ca^{2+} from intracellular stores, induced either physiologically in response to hormone-dependent Ins(1,4,5)P_3 formation or pharmacologically by thapsigargin, appears to mediate the part of the response to AngII that is not sensitive to dihydropyridines.

In the present study, we have used the pharmacological properties of nicardipine to discriminate between these two Ca^{2+} pathways and to estimate the relative importance of each mechanism in the steroidogenic response to AngII. Nicardipine appeared to be a very efficient tool for inhibiting both T- and L-type channels. Indeed, this agent, at micromolar concentrations, completely blocked slowly inactivating, L-type and slowly deactivating, T-type Ba^{2+} currents; in addition, nicardipine completely prevented the $[Ca^{2+}]_c$ response triggered by K^+, and it completely inhibited the steroidogenic response to the same agonist. In this regard, nicardipine appeared more efficient in blocking T-type channels than nifedipine, another dihydropyridine more selective for L-type channels. Indeed, in our hands, similar concentrations of nifedipine reduced slowly deactivating currents by only 30% and K^+-stimulated aldosterone by 50% (data not shown). This is in agreement with the recently reported stronger inhibition of neuronal T-type channels by nicardipine than by nifedipine (16). In addition, nicardipine action appeared rather selective on voltage-activated Ca^{2+} channels and did not significantly affect the thapsigargin-induced $[Ca^{2+}]_c$ response, allowing the use of this dihydropyridine for discriminating between the Ca^{2+} entry pathways present in glomerulosa cells.

The role of the voltage-activated Ca^{2+} influx in AngII action on these cells may have been overestimated in the past (23, 24) because of the apparent sensitivity of the hormone-induced $[Ca^{2+}]_c$ and aldosterone responses to various dihydropyridines. However, a careful look at the inhi-

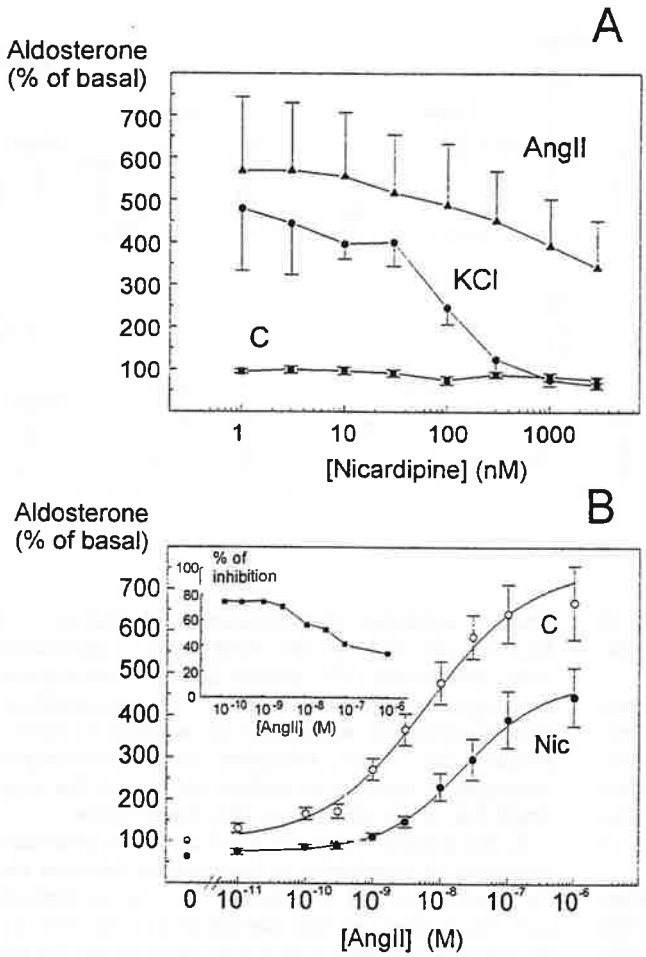

FIG. 5. Effects of nicardipine (Nic) on AngII- and potassium-induced aldosterone secretion. A, Glomerulosa cells were incubated for 90 min at 37 C, as described in *Materials and Methods*, in the presence of AngII (10 nM; ▲) or KCl (12 mM; ●) and increasing concentrations of nicardipine. Control unstimulated cells (■) were treated in the same manner. Aldosterone levels in the medium were determined in duplicate and expressed as percentage of the control values, which amounted to an average of 6 ng aldosterone/10^6 cells·90 min. Data are the mean ± SEM from three independent experiments, performed in duplicate. An IC_{50} of 150 nM for the inhibition by nicardipine of the aldosterone response to KCl was estimated by fitting the experimental data to a four-parameter logistic function (31). B, Aldosterone formation was stimulated, as described in A, with various concentrations of AngII (10^{-11}-10^{-6} M) in the presence (Nic) or absence (C) of nicardipine (1 μM). Data (mean ± SEM from five independent experiments) were fitted to a four-parameter logistic function, and IC_{50} values were 6.23 and 21.6 nM for control and nicardipine-treated cells, respectively. *Inset*, Nicardipine-induced inhibition of the steroidogenic responses to various AngII concentrations. The percent inhibition by nicardipine (1 μM) was determined by comparing the net increase in aldosterone (above basal) induced by AngII in the presence or absence of nicardipine.

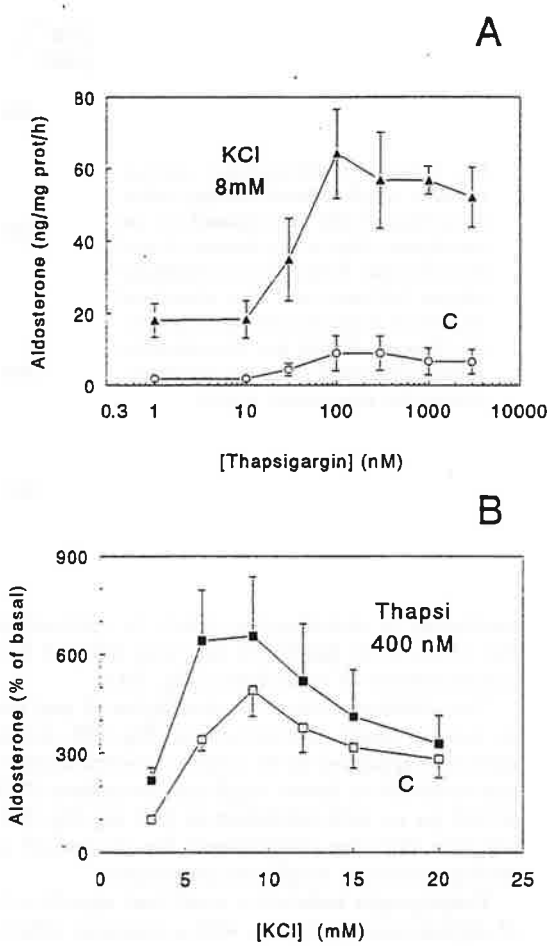

FIG. 6. Potentiation of thapsigargin (Thapsi)-induced aldosterone secretion by potassium. A, Cultured bovine glomerulosa cells were exposed to increasing concentrations of thapsigargin in a medium containing either 3 (control) or 8 mM KCl. Cells were incubated as described in *Materials and Methods*, and aldosterone released in the medium was determined and expressed as the amount produced per h and per mg cellular protein. Data are the mean ± SEM from three independent experiments performed in triplicate. B, Freshly prepared cells were incubated for 90 min at 37 C in the presence of increasing concentrations of extracellular K^+ and in the presence or absence of thapsigargin (400 nM). Aldosterone production was expressed as the percentage of basal release, which amounted, on the average, to 4.1 ± 0.6 ng/10^6 cells·90 min. Seven independent experiments were averaged, and the SEMs are indicated.

bition by nicardipine revealed that most of its inhibitory action was on a basal Ca^{2+} influx through voltage-activated channels, presumably of the T-type, and that this agent affected, on the average, only 22% of the actual response to AngII. This finding is in agreement with a recent report showing that most of the AngII-stimulated Ca^{2+} influx occurs through channels that are insensitive to dihydropyridines (20).

The presence of a basal Ca^{2+} influx through T-channels in resting glomerulosa cells, confirmed by measurement of Mn^{2+} influx, is not surprising in cells so exquisitely sensitive to extracellular K^+ and bearing Ca^{2+} channels with thresholds of activation so close to their resting potentials (25); however, it is not clear at the present time whether this basal Ca^{2+} influx is physiologically relevant or results from an artefact due to some damage during cell preparation.

The nicardipine-insensitive part of the Ca^{2+} influx re-

sponse to AngII appeared to be due exclusively to activation of a capacitative pathway, resulting from the release of Ca^{2+} from intracellular stores. Indeed, this part of Ca^{2+} influx could not be activated with K^+ and was completely prevented by previously exhausting the stores with thapsigargin. The presence of a third type of Ca^{2+} influx in these cells, involving, for example, the Na^+/Ca^{2+} exchanger as proposed in rat glomerulosa cells (26), could not be demonstrated under these experimental conditions. In addition, the fact that after nicardipine treatment, the $[Ca^{2+}]_c$ response to AngII becomes indistinguishable from the response to thapsigargin speaks against the presence of an additional mechanism for hormone-induced Ca^{2+} entry.

Interestingly, a new potential mediator of Ca^{2+} release upon stimulation by AngII has been recently proposed in glomerulosa cells (27). Hormone-dependent activation of the 12-lipoxygenase pathway leads to the formation of 12-hydroxyeicosatetraenoic acid, an agent able to stimulate Ca^{2+} release from intracellular pools. This metabolite would, therefore, appear to be a possible activator of the capacitative influx.

The role of the capacitative calcium influx in the stimulation of steroidogenesis by AngII has been investigated according to various approaches, either by inhibiting voltage-activated Ca^{2+} channels with nicardipine before hormone stimulation or by directly stimulating this influx with thapsigargin. Whereas aldosterone production in response to K^+ was completely abolished by 1 μM nicardipine, only 40% of the response to AngII was affected by the same treatment, clearly demonstrating that AngII-induced aldosterone formation is less dependent on the activity of voltage-activated Ca^{2+} channels than the response to KCl. A similar conclusion had been previously obtained with verapamil and nifedipine (28), but the correlation between steroidogenesis and calcium influx had not been clearly demonstrated. At least two obvious reasons may account for the different sensitivities of these agonists to nicardipine: 1) AngII is able to activate Ca^{2+} influx through an alternative pathway, namely the nicardipine-insensitive capacitative Ca^{2+} entry; and 2) AngII stimulates a Ca^{2+}-independent mechanism for steroidogenesis. Indeed, in agreement with this second hypothesis, it has been shown that aldosterone formation triggered by AngII is less crucially dependent on extracellular Ca^{2+} than the response to KCl (2), and recently, we have demonstrated, using glomerulosa cells with clamped cytosolic Ca^{2+}, that a fraction of the steroidogenic response to AngII is Ca^{2+} independent (Python, C. P., O. P. Laban, M. F. Rossier, M. B. Vallotton, and A. M. Capponi, submitted for publication).

Recently, Cirillo *et al.* (29) suggested that Ca^{2+} transport pathways in glomerulosa cells are modulated differently depending on the AngII concentration. Our finding that hormone-induced aldosterone synthesis is more sensitive to nicardipine at lower AngII concentrations seems to confirm their conclusion. However, the fact that aldosterone formation is less dependent on Ca^{2+} entry through T-channels at higher hormone concentrations could result from either a stronger activation of the capacitative influx or a decrease in the Ca^{2+} requirement of hormone-induced steroidogenesis. In favor of the second hypothesis, we found that the $[Ca^{2+}]_c$ response to 1 nM AngII (25.1 ± 3.4 nM; n = 8) was only inhibited 17% by the presence of 1 μM nicardipine (data not shown).

A more direct approach consisted in the activation of aldosterone secretion with thapsigargin. Maximal activation was observed at 100–300 nM thapsigargin, concentrations that maximally activate Ca^{2+} influx in these cells (12, 13). This steroidogenic response was inhibited by micromolar concentrations of nicardipine (not shown), a fact attributed not to an action of the dihydropyridine on the capacitative pathway, but on the basal influx through T-channels. Because thapsigargin-induced aldosterone synthesis exclusively depends on the cytosolic Ca^{2+}, a reduction of $[Ca^{2+}]_c$ is expected to be accompanied by a concomitant inhibition of aldosterone production. Interestingly, the steroidogenic response to thapsigargin was highly potentiated by K^+, a phenomenon recently described in rat cells (14), which is reminiscent of the well known potentiation by K^+ of the response to AngII (30). This observation could suggest that various Ca^{2+} entry pathways into glomerulosa cells act synergistically to regulate aldosterone biosynthesis. In addition, we observed that this potentiation was more marked at physiological K^+ concentrations (<9 mM), where almost exclusively T-type Ca^{2+} channels are activated. The mechanism for this potentiation is still unclear. In the absence of a specific inhibitor of the capacitative Ca^{2+} influx able to dissociate Ca^{2+} release from Ca^{2+} entry, it will be difficult to appreciate whether either Ca^{2+} release from intracellular stores or stimulation of the capacitative Ca^{2+} influx is more important for stimulating steroidogenesis.

In conclusion, AngII-stimulated Ca^{2+} influx into glomerulosa cells, leading to aldosterone secretion, is a complex process involving at least two distinct mechanisms: activation of voltage-operated Ca^{2+} channels and stimulation of a so far underestimated capacitative Ca^{2+} influx. This latter pathway appears to be responsible for some features of the response specific of this hormone, such as the potentiation of aldosterone production by AngII and K^+ or the poor efficiency of dihydropyridine Ca^{2+} antagonists on AngII-induced aldosteronogenesis.

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