Angiotensin II negatively modulates L-type calcium channels through a pertussis toxin-sensitive G protein in adrenal glomerulosa cells

MATURANA, Andrés, et al.

Abstract

In bovine adrenal glomerulosa cells, angiotensin II and extracellular K+ stimulate aldosterone secretion in a calcium-dependent manner. In these cells, physiological concentrations of extracellular potassium activate both T-type (low threshold) and L-type (high threshold) voltage-operated calcium channels. Paradoxically, the cytosolic calcium response to 9 mM K+ is inhibited by angiotensin II. Because K+-induced calcium changes observed in the cytosol are almost exclusively due to L-type channel activity, we therefore studied the mechanisms of L-type channel regulation by angiotensin II. Using the patch-clamp method in its perforated patch configuration, we observed a marked inhibition (by 63%) of L-type barium currents in response to angiotensin II. This effect of the hormone was completely prevented by losartan, a specific antagonist of the AT1 receptor subtype. Moreover, this inhibition was strongly reduced when the cells were previously treated for 1 night with pertussis toxin. An effect of pertussis toxin was also observed on the modulation by angiotensin II of the K+ (9 mM)-induced cytosolic calcium response in [...]
Angiotensin II Negatively Modulates L-type Calcium Channels through a Pertussis Toxin-sensitive G Protein in Adrenal Glomerulosa Cells*

(Received for publication, December 2, 1998, and in revised form, March 29, 1999)

Andrés D. Maturana‡‡, Andrés J. Casal‡‡, Nicolas Demaurex‡, Michel B. Vallotton‡, Alessandro M. Capponi‡, and Michel F. Rossier‡‡**

From the ‡Division of Endocrinology and Diabetology, Department of Internal Medicine, the ¶Laboratory of Clinical Chemistry, Department of Pathology, the §Department of Physiology, and the ¶¶Fondation pour Recherches Médicales, University Hospital, 24 rue Micheli-du-Crest, CH-1211 Geneva 14, Switzerland

In bovine adrenal glomerulosa cells, angiotensin II and extracellular K+ stimulate aldosterone secretion in a calcium-dependent manner. In these cells, physiological concentrations of extracellular potassium activate both T-type (low threshold) and L-type (high threshold) voltage-operated calcium channels. Paradoxically, the cytosolic calcium response to 9 mM K+ is inhibited by angiotensin II. Because K+-induced calcium changes observed in the cytosol are almost exclusively due to L-type channel activity, we therefore studied the mechanisms of L-type channel regulation by angiotensin II. Using the patch-clamp method in its perforated patch configuration, we observed a marked inhibition (by 63%) of L-type barium currents in response to angiotensin II. This effect of the hormone was completely prevented by losartan, a specific antagonist of the AT1 receptor subtype. Moreover, this inhibition was strongly reduced when the cells were previously treated for 1 night with pertussis toxin. An effect of pertussis toxin was also observed on the modulation by angiotensin II of the K+ (9 mM)-induced cytosolic calcium response in fura-2-loaded cells, as well as on the angiotensin II-induced aldosterone secretion, at both low (3 mM) and high (9 mM) K+ concentrations. Finally, the expression of both Go, and G; proteins in bovine glomerulosa cells was detected by immunoblotting. Altogether, these results strongly suggest that in bovine glomerulosa cells, a pertussis toxin-sensitive G protein is involved in the inhibition of L-type channel activity induced by angiotensin II.

Angiotensin II (AngII)1 and potassium ion (K+) are the major regulators of calcium influx into adrenal glomerulosa cells, a crucial step in the stimulation of aldosterone production. Both stimuli are able to maintain a sustained influx of Ca2+ in these cells. AngII induces a biphasic response of the cytosolic free Ca2+ concentration ([Ca2+]i), an initial transient rise due to inositol 1,4,5-trisphosphate-induced release of Ca2+ from the intracellular stores is followed by a sustained plateau phase, resulting from the activation of the capacitative influx triggered by the depletion of intracellular Ca2+ pools (1, 2). Angiotensin II also activates voltage-operated Ca2+ channels of both T- and L-types by inducing cell depolarization through inhibition of K+ channels. Ca2+ influx through these channels also contributes to the sustained Ca2+ entry triggered by AngII (1). Extracellular K+, by depolarizing the cells, directly activates the voltage-operated Ca2+ channels, leading to a sustained Ca2+ entry into the cell (3, 4). Paradoxically, Ca2+ entry elicited by K+ is markedly inhibited upon addition of AngII (5, 6). Although this phenomenon was observed some years ago both in rat and bovine glomerulosa cells, the mechanism of this AngII effect on cytosolic Ca2+ homeostasis has never been elucidated.

In bovine glomerulosa cells, the presence of both high threshold, long lasting (L-type) and low threshold, transient (T-type) voltage-operated Ca2+ channels has been demonstrated (7). Because of their low threshold of activation, T-type channels have been first thought to be the major mediators of the Ca2+ response to physiological increases of extracellular K+ (3, 8). Various laboratories have subsequently investigated the modulation of T-type channels by AngII, but contradictory results have been published. Lu et al. (9) have observed an increase of T channel activity induced by AngII and mediated by a G; protein. In fact, they have shown that AngII shifts the activation curve of T channels to more negative voltage values, increasing the size of the permissive window of voltage of the channel and therefore allowing more Ca2+ to enter the cell in a steady state manner. In contrast, in our laboratory, we found that AngII shifts the activation curve of T channels to more positive voltages, thus reducing the steady-state current through these channels (10). In this study, Rossier et al. (10) demonstrated that AngII exerts a negative modulation on T channels and that this modulation is mediated by protein kinase C (PKC). In summary, both positive and negative effects of AngII on T channel activity have been observed in electrophysiological experiments performed under similar conditions, but no correlation with [Ca2+]i, has been ever obtained.

Recently, a clear dissociation between L- and T-type channel functions has been established. Indeed, Ca2+ entering through each channel appears to have distinct functions and destinations (11). Selective inhibition of L-type channels does not markedly affect steroidogenesis, as demonstrated by Barrett et al. (12) with the spider toxin ω-agatoxin-III-A, as well as in our own laboratory, using low concentrations of dihydropyridines (13). In contrast, in these and other studies, T-type channel activity has been shown to be more closely related to aldosterone production. For example, T channel inhibition with the divalent ion nickel or the relatively specific alkaloid tetrandrine strongly reduced aldosterone production (14). Moreover, L-type channels appear to be the major mediators of the large...
[Ca²⁺], variations observed with fluorescent probes in response to extracellular K⁺, whereas at low, physiological concentrations of the agonist, the cytosolic Ca²⁺ signal resulting from T channel activation is barely detectable (15).

In order to understand the mechanism of the inhibition by AngII of the K⁺-induced cytosolic Ca²⁺ response, we therefore have investigated the modulation of the activity of L-type channels by the hormone, using both patch-clamp and microfluorometry methods.

**Experimental Procedures**

Percoll was obtained from Amersham Pharmacia Biotech. Amphotericin B, nifedipine, nicardipine, phenylmethylsulfonyl fluoride, Triton X-100, aprotinin, leupeptin, 2-mercaptoethanol, pertussis toxin, and tetrodotoxin were purchased from Sigma; fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR); Tween-20 was from Merck (Geneva, Switzerland); glycerol and bromphenol blue were from Fluka (Buchs, Switzerland); and Cell-Tak was from BioReba (Basel, Switzerland). Losartan (DuP753) was a generous gift from Dr. R. D. Smith, DuPont Merck Pharmaceutical Co. Rabbit polyclonal antibodies selectively raised against rat Gq and G₁ proteins were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Adrenal Glomerulosa Cell Isolation and Culture—Bovine adrenal glomerulosa glands were obtained from a local slaughterhouse, and adrenal glomerulosa cells were prepared by enzymatic dispersion, purified on a Percoll density gradient, and, for the patch-clamp and microfluorometry experiments, maintained in culture for 2-4 days on CellTak-coated glass coverslips, as described previously in detail (14). Otherwise, cells were used freshly prepared, after two washes in Krebs-Ringer buffer. Cells were incubated in a 37°C 5% CO₂ atmosphere, and Ca²⁺ was determined in freshly isolated bovine glomerulosa cell populations loaded with the fluorescent probe fura-2. After isolation, cells were preincubated overnight in multiwell plates containing a modified Krebs-Ringer medium and various concentrations of AngII. At the end of the incubation period, the aldosterone content of the medium was determined by direct radioimmunoassay, using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX).

Extraction and SDS-PAGE Separation of Proteins—Bovine glomerulosa cells in primary culture, as well as cultured GH₄C₁ cells, were homogenized in lysis buffer containing 137 mM NaCl, 1.8 mM KCl, 1.2 mM KH₂PO₄, 1.25 mM MgSO₄, 5 mM NaHCO₃, 1.2 mM CaCl₂, 5.5 mM glucose, 20 mM Heps, pH 7.4) for 15 min at 4°C. Extracted proteins were quantified using a protein microassay (Bio-Rad). SDS-PAGE was performed according to the method of Laemmli (18). Extracts of total cellular protein (16 µg/lane) were solubilized in sample buffer (60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.01% (v/v) bromophenol blue) and loaded onto a 10% SDS/polyacrylamide (10% gel) (MiniProtein II system, Bio-Rad). Electrophoresis was performed at 130 V for 1 h.

Blotting and Immunodetection of G Proteins—SDS-PAGE-resolved proteins were electrophoretically transferred onto a nitrocellulose membrane (Macherey-Nagel, Düren, Germany). The membrane was then incubated in blocking buffer (phosphate-buffered saline containing 0.4% Tween-20 and 5% nonfat dried milk) overnight at 4°C and then incubated for 1 h in phosphate-buffered saline containing 0.4% Tween-20 with rabbit polyclonal antibodies raised against the α subunits of the Gq and G₁ proteins. The membrane was washed with the same buffer and then incubated for 1 h with horseradish peroxidase-labeled goat anti-rabbit IgG (CovAlb, Dullins, France). The membrane then was washed three times for 10 min with phosphate-buffered saline containing 0.4% Tween-20, and the antigen-antibody complex was revealed by enhanced chemiluminescence using a Western blot detection kit and Hyper-ECL film (Amersham Pharmacia Biotech).

**Results**

Inhibition by Angiotensin II of the Cytosolic Calcium Response Induced by Potassium—As previously observed in bovine glomerulosa cells (5) and confirmed by others in rat cells (6, 10) and AngII markedly reduced the sustained elevation of [Ca²⁺], elicited upon addition of 9 mM extracellular K⁺ (Fig. 1A). Because of this difference with AngII is normally accompanied by Ca²⁺ release from inositol, 4,5-trisphosphate-sensitive intracellular Ca²⁺ pools and by development of a capacitative Ca²⁺ influx, thapsigargin, an inhibitor of the microsomal Ca²⁺/Mg²⁺-ATPase, was added at the beginning of the experiment in order to empty Ca²⁺ stores and to allow an accurate determination of the inhibition of the Ca²⁺ signal by the hormone.

**Fig. 1.** Inhibition of the potassium-induced cytosolic calcium response by angiotensin II. A, fura-2-loaded cells were sequentially exposed to 200 nm thapsigargin (Tg), 9 mM KCl, 10 mM AngII, and 2 µM nifedipine. Cytosolic Ca²⁺ values were calculated as described under “Experimental Procedures.” Dotted lines represent maximal and minimal [Ca²⁺], values measured before and after complete inhibition of voltage-operated calcium channels, respectively. B, same experiment as in A, but the addition of nifedipine and AngII was inverted. Traces are representative of 12 experiments yielding similar results.
The amount of this inhibition was estimated to be 63 ± 15% from 12 independent experiments. The addition of 2 μM nifedipine, a dihydropyridine antagonist, at the end of the experiment achieved to reduce [Ca$^{2+}$]$_i$ down to the level corresponding to 100% inhibition of the voltage-operated Ca$^{2+}$ channels.

Two mechanisms could be responsible for the decrease of [Ca$^{2+}$]$_i$, induced by AngII: 1) an inhibition of the calcium influx through voltage-operated calcium channels, or 2) an activation of the pumping of calcium out of the cytosol through plasma membrane Ca$^{2+}$/Mg$^{2+}$-ATPases. In order to discriminate between these possibilities, we completely blocked the Ca$^{2+}$ channels opened by K$^+$ with 2 μM nifedipine, before the addition of AngII (Fig. 1B). As expected, an almost complete inhibition of the sustained [Ca$^{2+}$]$_i$, response to K$^+$ was observed upon addition of nifedipine, and AngII exerted only a very small effect on the residual [Ca$^{2+}$]$_i$, level thereafter, thus excluding a strong activation of the Ca$^{2+}$ pumps by the hormone and therefore privileging an action of AngII on the dihydropyridine-sensitive Ca$^{2+}$ channels.

Inhibition of L-type Currents by AngII—Single bovine glomerulosa cells were voltage-clamped in the permeabilized patch configuration of the patch clamp technique and both T- and L-type Ca$^{2+}$ channels were activated upon a step depolarization from −90 to 0 mV for 600 ms (Fig. 2, inset). L current was measured after 500 ms, when most T channels are inactivated, as expected according to their fast inactivation characteristics (time constant, τ = 20 ms at 0 mV) and when T current amplitude is therefore negligible.

A time-course study of L-type current amplitude (Fig. 2) confirmed its relative stability in the perforated patch configuration. Addition of 100 nM AngII induced a marked inhibition of the current in less than 1 min. Addition of a dihydropyridine antagonist, such as nifedipine or nicardipine, at the end of the experiment blocked the residual current and pharmacologically confirmed the identity of this current. The mean inhibition induced by AngII has been estimated to be 63 ± 11% (n = 14) of the maximal current, a value closely related to the inhibition of the [Ca$^{2+}$]$_i$, signal.

Moreover, it is noteworthy that the action of AngII on L current was only rarely observed in the whole cell configuration and that the cells needed to be maintained metabolically intact (in the perforated patch configuration) for AngII to exert its inhibition on L channels.

Washing out the hormone (Fig. 3) allowed recovery of 30 ± 3% of L channel activity (n = 3), whereas addition of losartan (10 μM), a specific AT$_1$ receptor antagonist, a few minutes after the hormone reversed only 19 ± 5% of the AngII effect (n = 4). This lack of complete reversibility could be attributed to AngII receptor sequestration or endocytosis, as previously suggested by Ambroz and Catt (19).

To further characterize the molecular mechanism through which AngII exerts its inhibition on L currents, we tested whether protein kinase C (PKC), which is coupled to the AT$_1$ receptor through the formation of diacylglycerol, is involved in the modulation of L-type channels. We have previously shown that in glomerulosa cells, T-type channels are negatively regulated by PKC and that aldosterone production is concomitantly reduced (10, 20); however, we have also demonstrated that activation of PKC with the phorbol ester phorbol 12-myristate 13-acetate is unable to mimic AngII action on the [Ca$^{2+}$]$_i$, signal induced by K$^+$ (13). In agreement with the latter
The observation, phorbol 12-myristate 13-acetate had no effect on L-type currents and did not prevent the inhibition induced by AngII in 4 individually tested cells (data not shown).

The inhibition of L-type Current by AngII Is Sensitive to Pertussis Toxin Treatment—In numerous cells, it has been shown that L-type calcium channels can be modulated by pertussis toxin-sensitive G proteins, such as Gi or Go (21). We therefore treated bovine glomerulosa cells overnight with 500 ng/ml pertussis toxin (Ptx) before recording L-type currents. Under these conditions, we observed a marked reduction of the inhibition of L-type Ca\(^{2+}\) channels in response to AngII. Fig. 5 shows a cell in which AngII effect on L-type current was completely abolished by Ptx treatment, although the sensitivity to dihydropyridines remained unchanged. On average, the inhibition of the current elicited by AngII, which amounted to 63% in control cells, was reduced to only 21 ± 17% after treatment with Ptx (n = 16). The effect of Ptx was statistically significant according to an unpaired Student's t test with a p value < 0.0001.

Effect of Pertussis Toxin Treatment on AngII Action on the Cytosolic Ca\(^{2+}\) Signal and on Aldosterone Secretion—In order to determine whether the effect of Ptx on the AngII-induced inhibition observed on Ba\(^{2+}\) currents is also exerted on [Ca\(^{2+}\)]\(_{c}\), homeostasis, we performed Ca\(^{2+}\) microfluorometry experiments in single fura-2-loaded glomerulosa cells. Fig. 6 compares the [Ca\(^{2+}\)]\(_{c}\), fluctuations obtained in a control cell (A) and in a cell treated overnight with Ptx (500 ng/ml) (B). Cells were successively exposed to thapsigargin, K\(^+\), and AngII, as described in Fig. 1A. After establishment of an elevated plateau of [Ca\(^{2+}\)]\(_{c}\), at approximately 400 nM with K\(^+\), the addition of AngII led to a dramatic reduction of the [Ca\(^{2+}\)]\(_{c}\), signal in the control cell (Fig. 6A), but had a much less pronounced effect in the Ptx-treated cell (Fig. 6B). The response obtained in control individual cells (n = 3) was similar to those obtained in control cell populations (Fig. 1A); AngII-induced inhibition of the [Ca\(^{2+}\)]\(_{c}\), signal amounted on average to 60 ± 5%. In contrast, in the Ptx-treated single cell populations, the inhibition of the K\(^+\)-induced [Ca\(^{2+}\)]\(_{c}\), elevation by AngII was reduced to only 19 ± 8% in the five tested cells.

In a separate series of experiments, we have assessed the effect of Ptx treatment on the [Ca\(^{2+}\)]\(_{c}\), response to AngII at a low concentration of K\(^+\) (3 mM). Under these conditions, the [Ca\(^{2+}\)]\(_{c}\), rise induced by AngII, measured during the plateau phase 3 min after hormone addition, was slightly more pronounced in Ptx-treated cells (93 ± 16 nM, n = 10) as compared with control cells (69 ± 11 nM, n = 12, data not shown), probably reflecting a lack of L channel inhibition in Ptx-treated cells.

Because treatment with Ptx maintains [Ca\(^{2+}\)]\(_{c}\), at markedly higher levels upon stimulation with AngII, we have investigated the effect of Ptx on the steroidogenic response. As illustrated in Fig. 7, pretreatment with the toxin reduced by approximately 20% the aldosterone secretion evoked by AngII, without significantly changing the EC\(_{50}\) value for the hormone. Similar results were obtained when aldosterone was stimulated with increasing concentrations of AngII in the presence of 9 mM instead of 3 mM K\(^+\) (not shown).

Identification of G Protein Isoforms Expressed in Bovine Glomerulosa Cells—Because both Gi and Go proteins are known to be sensitive to Ptx, we have investigated which isoform is expressed in bovine adrenal glomerulosa cells. The presence of Gi\(_{a}\) and Go in cellular extracts was analyzed by SDS-PAGE and immunoblotting with specific antibodies raised against the α subunit of each isoform. Results were compared with those obtained with the GH4C1 rat pituitary cell line, in which the expression of these isoforms has been well characterized (22).

As expected, both Gi\(_{a}\) and Go isoforms were detected in GH4C1 cells as strong bands at approximately 40 kDa (Fig. 8). Bands of similar intensity were also observed in proteins extracts of bovine adrenal glomerulosa cells, strongly suggesting that both Gi and Go proteins are constitutively expressed in these cells.

**DISCUSSION**

The aim of the present study was to characterize the molecular mechanism by which AngII exerts its inhibitory action on...
**Fig. 7. Effect of pertussis toxin on the steroidogenic response to AngII.** Cultured cells were incubated overnight in the presence or in the absence (control) of 500 ng/ml Ptx, before being exposed for 1 h at 37°C to increasing concentrations of AngII. Potassium concentration in the medium amounted to 3 mM. Aldosterone production was determined in the medium by direct radioimmunoassay as described under "Experimental Procedures" and is expressed per mg of cell protein. Aldosterone secretion was significantly reduced in Ptx-treated as compared with control cells at AngII concentrations above 1 nM (p < 0.05, according to a paired Student's t test). Data are the mean values from four independent experiments performed in duplicate and were fitted to four parameter logistic functions; half-maximal stimulation was obtained at 1.5 and 3.0 nM AngII for control and Ptx-treated cells, respectively.

**Fig. 8. Identification of G protein subtypes expressed in bovine adrenal glomerulosa cells by immunoblotting analysis.** Proteins from cultured bovine adrenal glomerulosa (BAG) cells and from GH3 cells were extracted and separated by SDS-PAGE, as described under "Experimental Procedures." Proteins (16 µg/lane) were then transferred onto a cellulose membrane to be analyzed for the presence of specific G proteins with rat polyclonal antibodies raised against the α subunits of the Go, Gi, and Gq proteins. Arrows indicate the position of markers with relative molecular masses of 29 and 45 kDa. Similar results were obtained in four independent preparations.

the cytosolic Ca²⁺ response to K⁺ (Fig. 1A), a phenomenon already observed several years ago in adrenal glomerulosa cells (5). Because this effect disappeared after voltage-operated calcium channel inhibition with dihydropyridines (Fig. 1B), it is deduced that the reduction of [Ca²⁺], evoked by AngII reflects the hormonal modulation of these channels rather than an activation of Ca²⁺ extrusion from the cytosol. In previous work (10), we have studied the modulation by AngII of the low threshold T-type calcium channels, which appeared, at this time, to be the main effectors of Ca²⁺ entry under very small depolarizations resulting from physiological K⁺ increases. More recently, we have focused our attention on the high threshold L-type channels for two reasons: 1) the inhibition of the steady-state current flowing through T-type channels with phorbol 12-myristate 13-acetate is not accompanied by a reduction of the [Ca²⁺], levels; and 2) the [Ca²⁺], response to low concentrations of extracellular K⁺ is mainly due to L-type channels, the contribution of T channels to the signal being negligible (13).

The main finding of the present work is that AngII clearly modulates negatively L channels in bovine adrenal glomerulosa cells, to the same extent as it reduces [Ca²⁺]. This inhibition does not result from a shift of the sensitivity of the channel to voltage and is observed at any membrane potential; however, further investigation will be required, particularly at the single channel level, in order to determine whether total current inhibition is due to a decrease of channel open probability, to a change of channel conductance or to a modulation of channel insertion within the plasma membrane. In any case, these results strongly suggest that the inhibition of L-type channels by AngII is responsible for the decreased [Ca²⁺].

The AT₁ receptor subtype has been shown to be the major AngII receptor expressed in adrenal glomerulosa cells and to mediate most of the hormone actions in these cells (23). The AngII-induced inhibition of L-type Ca²⁺ channels appears to be also mediated by the AT₁ receptor. Indeed, the presence of the AT₁-selective antagonist losartan completely abolished AngII action (Fig. 4). This result is in agreement with the previous observation that losartan also prevents the action of AngII on the sustained [Ca²⁺], response to K⁺ (10).

Various cellular mechanisms of L-type Ca²⁺ channel modulation have been extensively investigated (21). From these studies, two major pathways have been highlighted: these involve either GTP binding proteins or channel phosphorylation by specific kinases. Whereas a role for PKC or tyrosine kinases, enzymes known to be linked to AT₁ receptor activation, could be recently excluded (24), we show here (Fig. 5) that the modulation of the L-type current by AngII is much reduced after pertussis toxin treatment. This finding strongly suggests that the transduction of the AngII signal from the AT₁ receptor to the L-type channels involves a Ptx-sensitive G protein of the Gi/Go family.

A modulation of L channels by AngII through G proteins has been previously observed in other cell types, but with contrasting results. For example, Hescheler et al. (25) have demonstrated that AngII, through a Gq protein, stimulates L-type current in the murine adrenocortical cell line Y1. More recently, an inhibition by AngII of L-type currents has been reported in rabbit sinoatrial node cells (26) as well as in guinea pig cardiomyocytes (27), and these negative modulations have been proposed to be mediated by Gq through an inhibition of the cAMP-dependent pathway. Moreover, in each of these studies, pretreatment of the cells with Ptx abolished AngII action on L currents. It is, however, noteworthy that in contrast to what we observed in glomerulosa cells, in the above studies, AngII affected L currents only after they had been enhanced by β-adrenergic stimulation, the hormone being inefficient on basal currents.

As expected, in bovine adrenal glomerulosa cells, Ptx treatment also markedly reduced the AngII-induced inhibition of the sustained [Ca²⁺], response to K⁺ (Fig. 6) and slightly increased the [Ca²⁺], response to AngII itself (not shown). This sensitivity of the [Ca²⁺], signal to Ptx confirms the previously described close relationship existing between [Ca²⁺], and L-type channel activity (13) and strongly reinforces the hypothesis that L channels are principally responsible for the varia-
tions of [Ca\(^{2+}\)]\(^1\), observed upon stimulation of glomerulosa cells with physiological concentrations of extracellular K\(^+\). Surprisingly, despite the fact that Ptx treatment maintained higher levels of [Ca\(^{2+}\)]\(^1\) upon stimulation with AngII, aldosterone production was significantly reduced (Fig. 7). This result suggests that either the negative feedback exerted by AngII on the [Ca\(^{2+}\)]\(^1\) signal induced by K\(^+\), or another factor controlled by a Ptx-sensitive G protein, is required for an optimal steroidogenic response. Relevant to this point is the suggestion by Barrett et al. (12) that Ca\(^{2+}\) influx through L-type channels could exert a negative action on the steroidogenic function of bovine glomerulosa cells.

Nevertheless, because pertussis toxin also negatively affects aldosterone production at relatively low [Ca\(^{2+}\)]\(^1\) (expected to occur at basal extracellular K\(^+\) and concentrations of AngII as low as 0.1 nm; see Fig. 7), a bell-shaped dependence of steroidogenesis upon calcium is probably not the only explanation, and some still undetermined steroidogenic factor, controlled by a pertussis toxin-sensitive G protein, could also be involved in this phenomenon.

In rat glomerulosa cells, it has been shown that Ptx treatment helps AngII to maintain an optimal aldosterone secretion at supramaximal concentrations of the hormone but has no effect at lower AngII concentrations (28). This specific effect of Ptx in rat cells is presumably due to prevention of adenyl cyclase inhibition by AngII through a G\(_i\) protein, although contradictory results in this species have been published by others (29, 30). In bovine glomerulosa cells, Barrett and Isales (31) have previously observed a 20% inhibition of the steroidogenic response to 10 nM AngII after treatment with Ptx, a result that is in complete agreement with our own observation (Fig. 7).

Both G\(_i\) and G\(_o\) proteins appear to be expressed in bovine adrenal glomerulosa cells (Fig. 8), but pertussis toxin does not allow to discriminate between the respective functions of each of these proteins. Nevertheless, in other cell types, G\(_i\) proteins have been generally shown to negatively modulate L-type channels, whereas in contrast, G\(_o\) protein activation is often associated with a direct (cAMP-independent) increase of the activity of these channels (21, 25, 32). Furthermore, we and others have recently shown that in bovine glomerulosa cells, AngII does not inhibit but rather potentiates cAMP production (33, 34), suggesting a poor coupling between the AT\(_1\) receptor and some G\(_o\) protein in these cells. These two facts would therefore indirectly favor the involvement of a G\(_o\) protein in the negative modulation of L-type channels by AngII in bovine glomerulosa cells.

In conclusion, the negative modulation of [Ca\(^{2+}\)], homeostasis exerted by AngII in bovine adrenal glomerulosa cells reflects the inhibition of L-type Ca\(^{2+}\) channels by this hormone. Moreover, the inhibition of these channels involves a Ptx-sensitive G protein, presumably of the G\(_i\) family, linked to the AT\(_1\) receptor subtype (Fig. 9). It therefore appears that the same hormone, through the same receptor, is able to inhibit different types of Ca\(^{2+}\) channels in the cell through distinct mechanisms:

T channel activity is negatively modulated through PKC activation, whereas L channels are partially closed via a Ptx-sensitive G protein. This differential control by AngII of Ca\(^{2+}\) entry occurring through various channels should probably represent an advantage for the cell in view of the growing evidence that different types of Ca\(^{2+}\) channels exert distinct functions in glomerulosa cells.

Acknowledgments—We are particularly grateful to G. Dorenter and W. Dimeck for excellent technical assistance.

REFERENCES