I. Introduction: Multiple Control of Aldosterone Secretion—Multiplicity of Signal Transduction Pathways in the Glomerulosa Cell

II. Calcium Signal Generation in Glomerulosa Cells
   A. Receptor-mediated Ca\(^{2+}\) release
   B. Ca\(^{2+}\) transport through the plasma membrane
   C. Diacylglycerol-PKC and lipoxygenase pathways
   D. Vasopressin: a transiently acting paracrine agonist

III. Effect of Cytoplasmic Calcium on Mitochondrial Function

IV. Cross-Talk Between Calcium and cAMP-Activated Pathways
   A. Ca\(^{2+}\) influx evoked by ACTH
   B. Ca\(^{2+}\)-induced formation of cAMP

V. Regulation at the Level of Plasma Membrane Receptors
   A. Mechanisms for regulation of GPCRs
   B. Regulation of adrenal angiotensin receptors in vivo
   C. Desensitization of glomerulosa cells
   D. Intracellular trafficking of angiotensin receptors
   E. Receptor downregulation

VI. Long-Term Effects of Angiotensin II
   A. Activation of growth responses by ANG II in the glomerulosa cell
   B. Role of receptor and nonreceptor tyrosine kinases
   C. ANG II-induced activation of MAPKs

VII. The Final Action: Role of Calcium in the Control of Steroid Production
   A. Cholesterol transport
   B. Increased reduction of pyridine nucleotides in mitochondria
   C. Induction of aldosterone synthase

Spát, András, and László Hunyady. Control of Aldosterone Secretion: A Model for Convergence in Cellular Signaling Pathways. Physiol Rev 84: 489–539, 2004; 10.1152/physrev.00030.2003.—Aldosterone secretion by glomerulosa cells is stimulated by angiotensin II (ANG II), extracellular K\(^{+}\), corticotrophin, and several paracrine factors. Electrophysiological, fluorimetric, and molecular biological techniques have significantly clarified the molecular action of these stimuli. The steroidogenic effect of corticotrophin is mediated by adenylyl cyclase, whereas potassium activates voltage-operated Ca\(^{2+}\) channels. ANG II, bound to AT\(_1\) receptors, acts through the inositol 1,4,5-trisphosphate (IP\(_3\))-Ca\(^{2+}\)/calmodulin system. All three types of IP\(_3\) receptors are coexpressed, rendering a complex control of Ca\(^{2+}\) release possible. Ca\(^{2+}\) release is followed by both capacitative and voltage-activated Ca\(^{2+}\) influx. ANG II inhibits the background K\(^{+}\) channel TASK and Na\(^{+}\)-K\(^{+}\)-ATPase, and the ensuing depolarization activates T-type (Ca\(_{\text{V3.2}}\)) Ca\(^{2+}\) channels. Activation of protein kinase C by diacylglycerol (DAG) inhibits aldosterone production, whereas the arachidonate released from DAG in ANG II-stimulated cells is converted by lipoxygenase to 12-hydroxyeicosatetraenoic acid, which may also induce Ca\(^{2+}\) signaling. Feedback effects and cross-talk of signal-transducing pathways sensitize glomerulosa cells to low-intensity stimuli, such as physiological elevations of [K\(^{+}\)] \((\leq 1 \text{ mM})\), ANG II, and ACTH. Ca\(^{2+}\) signaling is also modified by cell swelling, as well as receptor desensitization, resensitization, and downregulation. Long-term regulation of glomerulosa cells involves cell growth and proliferation and induction of steroidogenic enzymes. Ca\(^{2+}\), receptor, and nonreceptor tyrosine kinases and mitogen-activated kinases participate in these processes. Ca\(^{2+}\)- and cAMP-dependent phosphorylation induce the transfer of the steroid precursor cholesterol from the cytoplasm to the inner mitochondrial membrane. Ca\(^{2+}\) signaling, transferred into the mitochondria, stimulates the reduction of pyridine nucleotides.
I. INTRODUCTION: MULTIPLE CONTROL OF ALDOSTERONE SECRETION—MULTIPlicity OF SIGNAL TRANSDUCTION PATHWAYS IN THE GLOMERULOSA CELL

The steroid hormone aldosterone, secreted by the glomerulosa cells of the adrenal cortex, controls sodium and potassium balance and also influences acid-base homeostasis of the vertebrate organism. Its major physiological targets are the epithelial cells, of which the most important are located in the distal nephron. It augments Na⁺ reabsorption as well as K⁺ and H⁺ excretion. Through changes in sodium balance, it influences the extracellular space and blood pressure. In addition to its epithelial actions, aldosterone influences the function of the cardiovascular system by acting on the heart, vessels, and central nervous system. Aldosterone secretion is increased during acute or chronic sodium depletion or fluid loss, erect postural position, dietary potassium loading, and tissue damage leading to hyperkalemia. In view of its essential role in maintaining extracellular and tissue damage leading to hyperkalemia. In view of its essential role in maintaining extracellular fluid and thereby circulation, it is not surprising that its secretion is controlled by several factors. The list of hormonal and paracrine factors reported to exert a stimulatory effect on aldosterone production in vitro is quite long (Table 1), and the number of proposed inhibitory factors is also remarkable (Table 2). However, under physiological conditions the control of secretion is probably confined to the stimulatory factors corticotrophin (ACTH), angiotensin II (ANG II), and K⁺ and the inhibitory factor atrial natriuretic hormone (ANP). In fact, most or all increases in aldosterone secretion may be attributable to increased activity of the renin-angiotensin system and/or increased plasma level of K⁺. When sodium or fluid loss is severe, ACTH is also secreted and synergizes with ANG II or K⁺ in stimulating glomerulosa cells. ANP secretion is increased in response to sodium and/or water loading, and it in turn inhibits aldosterone secretion. The roles of other factors (shown in Tables 1 and 2) in the physiological control of aldosterone secretion may not be essential.

Signal transduction in the adrenal cortex has been studied for almost half a century. The role of cAMP in the stimulation of steroid production by pituitary trophic hormones was one of the earliest discoveries in this field, leading to the concept of second messengers (232). The formation and mode of action of cAMP have been described in several reviews. Our review focuses on the signal-transducing mechanisms. The Ca²⁺ dependence of the secretory process was described by Douglas and Rubin four decades ago (142), and it is now well established that Ca²⁺ acts by inducing the exocytosis of secretory vesicles. This principle is not applicable for steroid-producing cells, which lack secretory vesicles and are devoid of an exocytotic mechanism. The steroid precursor cholesterol is stored in lipid droplets, and the rate of hormone secretion depends on the rate of hormone synthesis. However, the activation of hormone synthesis is Ca²⁺ dependent, and the regulatory mechanism involves both Ca²⁺-mediated and Ca²⁺-permitted processes.

Following the pioneering observation of Hokin and Hokin (241) of acetylcholine-induced increase in phospholipid turnover of pancreatic and brain cortical slices in 1955, systematic studies on the role of Ca²⁺ in agonist-induced biological response were initiated in 1975 by Michell's hallmark paper (355). After surveying data on the mode of action of several hormones and neurotransmitters, he hypothesized that Ca²⁺-dependent agonists activate phospholipase C and induce the hydrolysis of phosphatidylinositol and that this breakdown triggers, in turn, the influx of Ca²⁺ from the extracellular fluid. It was

<table>
<thead>
<tr>
<th>TABLE 1. Stimuli of aldosterone production in vitro</th>
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<tbody>
<tr>
<td><strong>Stimuli of Aldosterone Production</strong></td>
</tr>
<tr>
<td>Acetylcholine (239)</td>
</tr>
<tr>
<td>Angiotensin II (198)</td>
</tr>
<tr>
<td>ATP (274)</td>
</tr>
<tr>
<td>Bradykinin (469)</td>
</tr>
<tr>
<td>Cholecystokinin (336)</td>
</tr>
<tr>
<td>Corticotrophin (ACTH) (278)</td>
</tr>
<tr>
<td>β-Endorphine (529)</td>
</tr>
<tr>
<td>Enkephalins (239)</td>
</tr>
<tr>
<td>Endothelin (195)</td>
</tr>
<tr>
<td>Epidermal growth factor (282)</td>
</tr>
<tr>
<td>12-Hydroxyeicosatetraenoic acid (378)</td>
</tr>
<tr>
<td>K⁺ (369)</td>
</tr>
<tr>
<td>Melanocyte stimulating hormone (530)</td>
</tr>
<tr>
<td>Neuropeptide Y (239)</td>
</tr>
<tr>
<td>Neotensin (239)</td>
</tr>
<tr>
<td>Norepinephrine (134)</td>
</tr>
<tr>
<td>Parathormone (264)</td>
</tr>
<tr>
<td>Prolactin (279)</td>
</tr>
<tr>
<td>Prostaglandins (517)</td>
</tr>
<tr>
<td>Serotinin (370)</td>
</tr>
<tr>
<td>Substance P (393)</td>
</tr>
<tr>
<td>Vasoactive intestinal polypeptide (394)</td>
</tr>
<tr>
<td>Vasopressin (22)</td>
</tr>
</tbody>
</table>

Factors of documented physiological significance are printed in bold.

<table>
<thead>
<tr>
<th>TABLE 2. Inhibitors of aldosterone production in vitro</th>
</tr>
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<tbody>
<tr>
<td><strong>Inhibitors of Aldosterone Production</strong></td>
</tr>
<tr>
<td>Atrial natriuretic hormone (85)</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide (373)</td>
</tr>
<tr>
<td>Dopamine (353)</td>
</tr>
<tr>
<td>Nitric oxide (380)</td>
</tr>
<tr>
<td>Platelet-derived growth factor (381)</td>
</tr>
<tr>
<td>Somatostatin (4)</td>
</tr>
<tr>
<td>Transforming growth factor-β (208)</td>
</tr>
<tr>
<td>Unsaturated fatty acids (155)</td>
</tr>
</tbody>
</table>

Factors of documented physiological significance are printed in bold.
only later discovered that also the phosphatidylinositol derivative phosphatidylinositol 4,5-bisphosphate is cleaved (356). Subsequently, it was firmly established that the primary consequence of this breakdown is the formation of inositol 1,4,5-trisphosphate (IP₃), a water-soluble second messenger which primarily induces Ca²⁺ release from intracellular stores, rather than Ca²⁺ influx from the extracellular space (53, 437, 522).

Adrenal glomerulosa cells are a cell type in which Ca²⁺ and cAMP are equally significant in stimulation-secretion coupling. The effect of ACTH is mediated by cAMP, that of ANG II by Ca²⁺ and diacylglycerol (DAG), and that of K⁺ by Ca²⁺. ANP acts by antagonizing Ca²⁺ signaling. The basic framework of the IP₃-Ca²⁺-DAG system in adrenal glomerulosa cells was characterized in the 1980s and reviewed subsequently (32, 189, 503, 509). Therefore, classical data on the phosphoinositide-Ca²⁺ signaling. This system of the phosphoinositide-Ca²⁺-DAG system will only be reviewed concisely here. We will primarily deal with the control of Ca²⁺-releasing and influx mechanisms, the interaction of various signaling pathways, the long-term effects of Ca²⁺-mobilizing agonists, and the mechanism terminating Ca²⁺ signaling. Finally, we summarize current views on the basis of Ca²⁺-induced steroid secretion.

II. CALCIUM SIGNAL GENERATION IN GLOMERULOSA CELLS

The two most important physiological stimuli of aldosterone secretion, ANG II and extracellular K⁺ (reviewed in Ref. 570), exert their effects through generating cytoplasmic Ca²⁺ signal. The octapeptide ANG II is formed from the plasma protein angiotensinogen by the sequential action of two proteases, renin and angiotensin-converting enzyme. Under resting conditions its plasma concentration in humans and rats is between 10 and 60 ng/L (337), 492. ANG II level rises above 100 ng/L in response to administration of furosemide or dietary sodium depletion, whereas it may attain several hundred picomoles per liter following hemorrhage or water deprivation (337, 492). Plasma levels between 1 and 2 nM were measured under severe pathological conditions (e.g., malignant hypertension) (337).

Renin expression and ANG II production also occur in adrenal glomerulosa cells (371, 372). Within the adrenal cortex, immunolabeling for renin and prorenin is not limited to the steroid-producing cells of the zona glomerulosa but is also present in chromaffin cells, which occur singly or in groups throughout the whole cortex (45, 594). In this latter cell type the expression of angiotensinogen mRNA and the presence of ANG II in chromaffin granules were also shown (594), suggesting a paracrine action of ANG II released from vicinal chromaffin cells. The adrenal expression of renin mRNA is activated by the recognized physiological stimuli of aldosterone secretion, namely, ANG II (593), ACTH (576), and K⁺ (102, 281, 576). Transcription of the (pro)renin gene is also enhanced in dietary sodium depletion (566). In transgenic rats termed TGR(mREN2)27, the murine ren-2 renin gene, originally detected in the submaxillary gland, is expressed predominantly in the adrenal cortex. Although plasma renin and ANG II concentrations are low in these animals, aldosterone secretion is high, indicating that intra-adrenal production of renin can effectively stimulate the glomerulosa cells by stimulating local ANG II production (419). Sodium restriction also increases adrenal renin activity and mRNA in such transgenic rats (480). The observation that the AT₁-type ANG II receptor antagonist DuP 753 attenuates K⁺-stimulated and ACTH-stimulated aldosterone production (209) suggests that the intra-adrenal renin-angiotensin system functions as a local amplifier of systemic stimuli. Renin, in addition to acting as a soluble enzyme, has been found to bind to a 550-amino acid membrane protein. This binding results not only in increased catalytic activity, but also in the activation of mitogen-activated protein kinases (MAPKs) within the respective cell (388). No data are yet available as to whether this binding protein is expressed in the adrenal cortex. Additional studies are required to assess the tissue concentration of ANG II within the glomerulosa zone. Such data would be essential for the evaluation of the physiological relevance of experimental results obtained with exogenous ANG II.

ANG II-stimulated aldosterone production by incubated rat glomerulosa cells exhibits a biphasic dose-response curve. Steroidogenesis is stimulated in the physiological concentration range of 10⁻¹¹ M, maximal effect is attained at ~10⁻⁹ M, above which level hormone output is reduced (97, 230, 323). In superfusion system ANG II increases aldosterone production of isolated cells by two orders of magnitude (24, 447).

Aldosterone secretion in vivo (76) and in vitro (74, 180, 238, 563) are stimulated by increases in K⁺ concentration as small as a few tenths of millimolar. Maximal hormone production is attained by [K⁺] around 8–10 mM both in capsular (glomerulosa) tissue (225, 368) and cell suspension experiments (323, 432), whereas decreased hormone production can be observed at [K⁺] between 10 and 20 mM (33, 442). The falling phase of the K⁺-aldosterone dose-response curve is due to the rapid decay of aldosterone output, which follows in time the steep onset of the response (28).

ANG II is a classical Ca²⁺-mobilizing ligand: it induces Ca²⁺ release from IP₃-sensitive intracellular stores, and this release is followed by Ca²⁺ influx from the extracellular space. The mode of action of K⁺ is quite different, because its primary site of action is a voltage-operated Ca²⁺ channel. Nevertheless, how the exceptional sensitivity of glomerulosa cell to K⁺ is achieved is a question still only partially elucidated. In the forth-
ing sections we first describe the primary actions of the two agonists and later deal with additional actions that ensure the fine-tuning of regulation.

A. Receptor-Mediated Ca²⁺ Release

Glomerulosa cells do not constitute a homogeneous population. Expression of aldosterone synthase is confined to the two or three outer cell layers of the rat zona glomerulosa (221, 396, 418). The function of glomerulosa cells not expressing aldosterone synthase is not clear; moreover, presently no method is available for correlating aldosterone production and Ca²⁺ response at the single-cell level. Individual cells also differ in their response to different stimuli. Although the majority of the cells generate a Ca²⁺ signal in response to both K⁺ and ANG II, only a smaller percentage of the cells respond to vasopressin (AVP) (447) and still less to ACTH (551). Immuno- 
cell blot assay data suggest that 20–25% of the glomerulosa cells release ANG II (103).

This cellular heterogeneity is also reflected by the variable Ca²⁺ response of single cells to ANG II and K⁺ (119) (Fig. 1). However, some general characteristics of the response may be observed. Similar to other Ca²⁺-mobilizing agonists (47, 545), ANG II evokes an oscillating signal at physiological concentrations (usually below 300 pM). The hormone concentration affects the frequency rather than the amplitude of the single spikes. Gradual confluence of the spikes occurs at higher concentrations of the peptide, and the Ca²⁺ response becomes sustained in the nanomolar angiotensin II range (rat, Refs. 447–449, 466; bovine cells, Refs. 107, 269, 479). Also, the onset of the response exhibits a concentration-dependent delay (447, 466). Oscillatory Ca²⁺ signals show a gradual decrease in spiking frequency and/or amplitude after a few minutes (269, 433), which results in a gradually decreasing Ca²⁺ signal in cell suspension, where the response of 100,000 or more cells is averaged. (The underlying mechanism may also account for the declining [Ca²⁺] during the sustained phase of the Ca²⁺ signal in single cells exposed to higher concentration of ANG II.) Ca²⁺ spiking is maintained in the absence of extracellular Ca²⁺ for at least 20 min, indicating that oscillating Ca²⁺ release occurs from the IP₃-sensitive intracellular store (479). The frequency of Ca²⁺ oscillations is reduced by nifedipine, suggesting that voltage-activated Ca²⁺ channels have a supporting role during the process (479). Several models have been proposed for the mechanism of Ca²⁺ oscillation in other cell types (for review, see Refs. 178, 490), but

![FIG. 1. Effect of angiotensin II (ANG II) applied at a physiological concentration of 150 or 300 pM (37°C) on cytoplasmic and mitochondrial [Ca²⁺]. Cytoplasmic [Ca²⁺] was monitored with Fura Red (red), and changes in mitochondrial [Ca²⁺] were followed with rhod 2 (blue) by confocal microscopy in MitoTracker Green-preloaded single glomerulosa cells. The y-axis shows the fluorescence ratio of (F₀−F)/F₀ for Fura Red and (F−F₀)/F₀ for rhod 2, where F is the actual fluorescence intensity and F₀ is the averaged fluorescence intensity of the control period. Note the heterogeneity of the Ca²⁺ response of individual cells. (Recordings courtesy of A. Spät.)](http://physrev.physiology.org/)

Physiol Rev • VOL 84 • APRIL 2004 • www.prv.org
none of these has been thoroughly tested for steroid-secreting cells.

When cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) is examined in cell suspension, i.e., a statistical average of several hundred thousand cells is monitored, the Ca\(^{2+}\) signal induced by low concentration of ANG II is characterized by a slow increase followed by a gradual decrease, whereas that induced by supraphysiological concentration consists of an initial peak followed by a smaller plateau (24, 255, 301). If extracellular Ca\(^{2+}\) is chelated in a superfusion system after the onset of ANG II-induced aldosterone production, hormone output falls below resting levels (503). This phenomenon indicates that the maintenance of [Ca\(^{2+}\)]\(_{c}\) at a suprabasal level is essential for maintaining hypersecretion of aldosterone (209, 300, 435, 449).

1. ANG II receptors

ANG II is the major physiological regulator of aldosterone secretion, cell growth, and proliferation of glomerulosa cells. The effects of ANG II in glomerulosa cells and other target tissues are mediated by binding to heptahelical, G protein-coupled receptors. Two different binding sites of ANG II, termed AT\(_1\) and AT\(_2\), were identified in the rat adrenal cortex, and the AT\(_1\) receptor was found to be the predominant isof orm (17, 140, 579), whereas the bovine cortex contains almost exclusively AT\(_1\) receptors (17). Immunocytochemical localization of the receptors in the adult rat confirmed that glomerulosa cells contain AT\(_1\) receptors, whereas AT\(_2\) is expressed in the medulla (177, 501). Nonpeptide receptor antagonists have demonstrated that AT\(_1\) receptors mediate the ANG II-induced enhanced formation of IP\(_3\) and depolarization (217), inhibition of adenyl cyclase (17), stimulation of aldosterone production (17, 217), and the growth-promoting effect of ANG II in glomerulosa cells (see sect. viA). However, AT\(_2\) receptors appear to activate protein phosphatases, the nitric oxide-cGMP system, and phospholipase A\(_2\) (390). AT\(_2\) receptors inhibit cell growth and stimulate apoptosis, and the expression of this receptor is increased during fetal growth and tissue regeneration (132, 360). In contrast to other cloned mammalian AT\(_1\) receptors, rat and mouse AT\(_1\) receptors exist as two distinct subtypes, termed AT\(_{1A}\) and AT\(_{1B}\). They are 95% identical in their amino acid sequences and have very similar ligand binding and activation properties, but differ in tissue distribution and transcriptional regulation. Although most ANG II target tissues express predominantly AT\(_{1A}\) receptor mRNA, in the adrenal and the pituitary glands the AT\(_{1B}\) message is the major subtype (132, 192). In contrast to pituitary cells, in rat and murine adrenal cells the mRNA of AT\(_{1A}\) receptors can also be detected (144, 192).

Competition curves of ANG II in radioligand-binding assays are characterized by a slope factor significantly lower than 1. These curves were originally explained by the presence of high- and low-affinity AT\(_1\) receptor-binding sites. Depending on the experimental conditions in rat and bovine glomerulosa cells, the high-affinity sites have dissociation constant (K\(_d\)) values around or below 1 nM, whereas the affinity of the low-affinity site is ~1 order of magnitude lower (73, 133). Both uncoupling of the receptor from G proteins, by nonhydrolyzable GTP analogs (see references in Ref. 132) and desensitization of the receptor, markedly reduces the number of high-affinity sites (see sect. viC). Although the two-state model is now generally considered to be an oversimplification of the receptor activation process (257), the effects of G protein coupling and/or receptor phosphorylation on binding affinity are considered to be general features of many G protein-coupled receptors (GPCRs).

2. G proteins

Catt and co-workers (194) described the inhibition of the binding of ANG II by guanine nucleotides as early as 1974. The underlying mechanism of this phenomenon was elucidated by the discovery of heterotrimeric G proteins 6 years later (464). Of the different heterotrimeric G proteins in glomerulosa cells, G\(_i\), the inhibitory G protein of adenyl cyclase, was the first to be identified on the basis of pertussis toxin-induced ADP-ribosylation of a 41-kDa protein (162). Immunoblotting revealed G\(_i\) and the related G\(_{12}\) in bovine glomerulosa cells (344). These observations were in harmony with the previous finding that ANG II reduces, rather than enhances, ACTH-induced and serotonin-induced cAMP production in rat glomerulosa cells (43, 230, 463) and adenyl cyclase activity of membrane preparations (587). G\(_i\) seems to couple the activated ANG II receptor with T-type voltage-activated Ca\(^{2+}\) channels in both rat and bovine glomerulosa cells (see sect. viB). The primary effect of calcium-mobilizing agonists, such as ANG II, is the hydrolysis of phosphoinositides by a phosphoinositide-specific phospholipase C (PLC). Although \(\beta\gamma\)-subunits derived from G\(_{12}\) may activate the \(\beta\gamma\)-isoform of PLC (454), inhibition of G\(_{i}\) by pertussis toxin failed to influence the ANG II-induced and AVP-induced activation of phosphoinositide metabolism (36, 162, 204, 296) or aldosterone production (230). Therefore, it may be assumed that the \(\beta\gamma\)-isoform of PLC is not expressed in the glomerulosa cell; however, nonhydrolyzable GTP analogs increased the formation or potentiated the ANG II-induced formation of IP\(_3\) by permeabilized glomerulosa cells and glomerulosa membrane preparations (36, 162, 473). These observations indicated the involvement of another G protein in the action of ANG II. It was demonstrated in 1992 that the two pertussis toxin-insensitive G proteins, G\(_{q}\) and G\(_{11}\) (the G\(_{q}\) family of heterotrimeric G proteins)
proteins), mediate the effects of ANG II and AVP on PLC (210). Few data are available on Gq in adrenocortical cells. It was found that the Gq/G11 protein is associated with the cytoskeleton and that this association is essential for the translocation of cytosolic G protein to the plasma membrane (121). As shown with the application of a specific antibody, αq/α11, mediates the inhibitory effect of ANG II on Ca2+-dependent K+ (BK) channels (408).

The third heterotrimeric G protein participating in the control of steroid-producing cell types is Gs, the stimulator of adenyl cyclase. The presence of αs, the guanyl nucleotide binding subunit of Gs, in glomerulosa cells was demonstrated by Western blot analysis. This also showed that a 1-min exposure to ACTH induced a large but transient translocation of αs from the cytoskeleton to the plasma membrane (122).

The site of action of heterotrimeric G proteins in the glomerulosa cell is summarized in Figure 2.

3. Phosphoinositide metabolism and formation of inositol phosphates

Enhancement of phosphoinositide turnover by ANG II, but not by the two other physiological stimuli of the glomerulosa cell, K+ and ACTH, was described two decades ago (153, 245, 580). The effect of ANG II did not require extracellular Ca2+ (246), indicating that reduction of the radioactively labeled phosphatidylinositol pool was not secondary to ANG II-induced Ca2+ influx.

It was R. H. Michel who first presented data showing that agonist stimulation resulted in a rapid, phosphodiesterase-catalyzed breakdown of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (PIP2) that did not appear to be a response to changes in [Ca2+] (356). Subsequent work by Berridge, Irvine, Schulz, and co-workers (52, 522) definitely confirmed in 1983 that Ca2+-mobilizing agonists induce the breakdown of PIP2, resulting in the formation of the water-soluble, Ca2+-releasing second messenger IP3 and DAG. Shortly after this landmark discovery, the ANG II-induced rapid breakdown of PIP2 (161, 172, 289) and the ensuing formation of IP3 (18, 161, 188, 473) were observed in bovine and rat glomerulosa cells.

Whereas the ACTH-induced cAMP response does not evoke the formation of IP3 (161, 172), vasopressin (22, 204, 589), endothelin (588), and acetylcholine (293), all acting in a paracrine mode within the adrenal gland, enhance the formation of IP3. The hydrolyzing enzyme, the phosphoinositide-specific PLC, is activated not only by hormonal or neurotransmitter stimuli (through Gs), but also by Ca2+ (158, 366). The K+-induced moderate breakdown of PIP2 (218) is compatible with such a Ca2+ action.

Although ANG II (18, 161) and AVP-induced increases in IP3 (204) peak within 10–15 s and decrease thereafter, a second phase of enhanced PIP2 breakdown may last as long as stimulation is persisting. In the presence of lithium, an inhibitor of inositol polyphosphate-1-phosphatase and of inositol monophosphatase, labeled inositol phosphates, exhibit continuous accumulation, indicating the sustained activation of PLC (161, 289). In contrast to the initial formation of IP3 (246, 473), the sustained phase of ANG II-stimulated IP3 formation is dependent on Ca2+ influx (248, 590). This observation may be accounted for by the Ca2+ requirement of PLC activity (158, 375) and is consistent with the Ca2+ requirement of the sustained (but not the initial) phase of ANG II-stimulated aldosterone production (503).

The sustained phase of stimulation with ANG II is characterized not only by increased formation of inositol phosphates but also by a change in the ratio of different inositol phosphate fractions. Dephosphorylation by 5-phosphomonoesterase is the prevailing metabolic route of IP3 in resting cells and is also a significant pathway in ANG II-stimulated cells (18). Another metabolic route is the sequential phosphorylation of IP3 to inositol 1,3,4,5-tetrakisphosphate (IP4) by IP3 3-kinase (262) and dephosphorylation to its biologically inactive product, inositol 1,3,4-trisphosphate. Ca2+ signaling may activate IP3-kinase (59), leading to increased formation of IP4 and inositol 1,3,4-trisphosphate, as also observed in bovine glomerulosa cells (18, 23, 475). In the course of sustained stimulation, inositol 1,3,4-trisphosphate is consecutively converted into inositol 1,3,4,6-tetrakisphosphate (23, 250), inositol 1,3,4,5,6-pentakisphosphate, and inositol 1,4,5,6-...
tetakisphosphate (25). An alternative pathway of the pentakisphosphate metabolism, which seems to be significant only after several hours of stimulation, is the formation of two further inositol tetrakisphosphate stereoisomers (1,4,5,6- and 3,4,5,6-P₄) (20).

Of these compounds, increased labeling of inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate has been found in rat glomerulosa cells exposed to ANG II (19, 505) or AVP (204). The formation of an unspecified pentakisphosphate was also detected, and its labeling was not influenced by AVP within 30 min (204). The small number of glomerulosa cells that can be isolated from rat adrenal (a few hundred thousands per rat) has discouraged further physiological studies on higher inositol polyphosphate metabolism in these cells.

4. Ca²⁺ release by IP₃

Before the availability of Ca²⁺-sensitive fluorescent dyes, Ca²⁺ release from an intracellular particle into the cytoplasm in response to hormones and neurotransmitters was revealed by the application of isotope flux techniques. Similar to the effect of acetylcholine on the salivary gland (387), ANG II was found to enhance Ca²⁺ efflux from rat glomerulosa cells, irrespective of the presence of extracellular Ca²⁺ (27). These observations indicated that these agonists induce intracellular Ca²⁺ release and that their primary action does not require Ca²⁺ influx. Moreover, in ANG II-stimulated glomerulosa cells the applied isotope flux technique revealed a nonmitochondrial origin of the released Ca²⁺ (27). IP₃-induced Ca²⁺ release from a nonmitochondrial intracellular store was first demonstrated in permeabilized pancreatic acinar cells, in 1983 (522). This observation was soon confirmed in permeabilized bovine glomerulosa cells (289) and in insulinoma microsomes (436). High-affinity IP₃-binding sites were described in 1986 by Späti and coworkers in permeabilized peritoneal polymorphonuclears and hepatocytes (507) and liver microsomes (510). The concentration dependence of binding and Ca²⁺ release were comparable, showing that the binding sites represented biologically active receptors. Whereas Scatchard analysis revealed both high-affinity and low-affinity binding compartments in liver microsomes (426, 510), only a single (high-affinity) compartment of binding sites was observed in adrenocortical microsomes (37, 94, 201, 403). It is possible that the lack of low-affinity binding sites in the adrenal cortex was caused by Ca²⁺-induced conversion of low-affinity to high-affinity binding sites (367, 427).

In glomerulosa cells, in harmony with other cell types, the Kᵦ of IP₃ binding to the (high-affinity) receptor (10⁻⁹ M) is about two orders of magnitude lower than the EC₅₀ of IP₃-induced Ca²⁺ release (201). This discrepancy may be accounted for by the different experimental conditions (temperature and absence or presence of Mg-ATP) of binding and transport studies. The redox state of thiol groups also influences the binding of the ligand (203). It has also been proposed that Ca²⁺ release by IP₃ in other cell types depends on the occupancy of low-affinity binding sites (426). It is possible that the IP₃ concentration in confined regions of stimulated cells might attain 10⁻⁶ to 10⁻⁵ M, which may induce maximal Ca²⁺ release also from low-affinity receptors (reviewed in Ref. 546).

Fractionation of liver homogenates (510) as well as electron microscopic studies on cerebellar Purkinje cells (486) confirmed the localization of the IP₃ receptor in the endoplasmic reticulum (ER). That IP₃ acted on the ER was supported later by several studies, among others on adrenocortical membrane fractions (94, 201, 403). Comparison of the effect of IP₃ and thapsigargin on glomerulosa cells indicated that only a subpopulation of ER vesicles can be regarded as IP₃ sensitive (159, 465). These data were in harmony with the results of subfractionation studies on liver that suggested that the receptor may not be evenly distributed in the whole reticulum but concentrated in specialized vesicles, marked with the calcium-binding protein calreticulin (166). In intact exocrine cells (421) and neurons (51), the ER appeared to be a continuous membrane system that has a number of regional specializations. Provided that also in intact glomerulosa cells ER is luminally continuous, IP₃ receptors may cluster in confined regions that could appear as a separate population of vesicles after homogenization.

IP₃ receptors have been identified in the plasma membrane of T lymphocytes (304). However, these receptors probably differ from the ER receptor, since activation of such nonselective cation channels in the plasma membrane would cause depolarization; that is not the case. IP₃ receptors in the plasma membrane were also reported in adrenocortical cells (474). Observations on adrenocortical cells (202) and liver homogenates (166, 471) strongly suggest that IP₃ receptor detected in the plasma membrane fraction is localized in ER vesicles, loosely attached to the plasma membrane by cytoskeletal elements. Additional IP₃ binding sites, described in the nuclear envelope and in the Golgi apparatus, as well as in secretory vesicles, (presently) have no known relevance in steroid-producing cells, and therefore we refer to a recent review (405).

The IP₃ receptor type cloned first (IP₃R1) contains 2,749 amino acid residues, corresponding to a molecular mass of 313 kDa. It consists of three functional domains, the cytosolic NH₂-terminal ligand-binding domain, a COOH-terminal channel-forming domain, and an interconnecting coupling domain. The COOH-terminal 550 amino acid residues contain six membrane-spanning regions, which form the ion-conducting pore. The receptor expressed in the brain may differ from that expressed in peripheral tissues by splice variance: the former containing, and the latter missing, a 40-amino acid segment (SII).
in the coupling domain. IP₃ receptors and ryanodine receptors (RyR) share 40% amino acid sequence identity in their COOH-terminal, pore-forming domains and show some sequence identity in the NH₂-terminal, cytosolic ligand binding domains (for review, see Refs. 55, 542). Following the cloning and characterization of IP₃R₁, two further types (IP₃R₂ and IP₃R₃) have been identified. The central nervous system contains almost exclusively IP₃R₁ [SII(+) splice variant], while several peripheral cell types express more than one type of IP₃R (reviewed in Refs. 55, 405, 542). Simultaneous with the observation of the coexpression of more than one type of IP₃R in peripheral tissue, including the whole adrenal (containing both cortex and medulla) (384), we reported that rat glomerulosa cells express the mRNAs of all three types of IP₃R (165).

About four times more IP₃R₁ mRNA was expressed than IP₃R₂ mRNA, and the SI segment was present in nearly 50% of the IP₃R₁ receptors. Somewhat less than 5% of the total IP₃R was expressed as type 3. Interestingly, sodium restriction for 1 wk, a strong stimulus of aldosterone formation, failed to influence the ratio of expression of the different receptor types or spliced variants (165).

The receptor affinity for IP₃ has a rank order of IP₃R₂ > IP₃R₁ > IP₃R₃ (364, 384). However, the membrane environment may modify the properties of the receptor (425). Different receptor types may exhibit different downregulation rates or rates of receptor degradation (498, 585). The receptor has a tetrameric structure of ~1.2 kDa (for review, see Refs. 55, 174), and different receptor isoforms may constitute heterotetramers (272). Since the three receptor types differ in their affinity for IP₃ as well as in their sensitivity to controlling factors (see below), the diverse composition of these heterotetrameric receptors may provide more versatile control of IP₃ receptor function.

Physiological concentrations of Ca²⁺-mobilizing agonists usually evoke oscillatory Ca²⁺ signals (see sect. A). Although no uniformly valid model for different cell types has yet been presented, oscillations are obviously evoked by positive and negative feedback control of [Ca²⁺]c, by Ca²⁺ itself. Ca²⁺ variously activates different isoforms of PLC, protein kinase C (PKC), plasmalemmal Ca²⁺-ATPase, adenyl cyclase, and cAMP phosphodiesterase, and cell type-specific expression of these isoforms contributes to the complexity of Ca²⁺ signal generation. Nevertheless, the major factor responsible for Ca²⁺ oscillations is probably the dual effect of Ca²⁺ on IP₃R itself. Elevation of [Ca²⁺]c within the lower physiological range (up to ~300 nM) increases, and further elevation, still in the physiological range, decreases the sensitivity of IP₃R₁ to IP₃ (260). Gradual elevation of [IP₃] from 20 nM to 180 μM shifts the peak of the Ca²⁺-dependence curve of channel open probability from 100 nM to ~1 μM (275). The biphasic effect of Ca²⁺ on channel activity is extremely important in determining the pattern of Ca²⁺ signaling. The Ca²⁺-dependent enhancement of IP₃R activity implies that Ca²⁺-induced Ca²⁺ release (CICR), similar to that occurring through RyRs, may also occur through the IP₃R provided that [Ca²⁺]c is ~300 nM (55). This process may have an essential role in the generation of cytoplasmic Ca²⁺ oscillations and waves (48). The dependence of the nadir of the bell-shaped Ca²⁺-dependence curve on [IP₃] ensures the coregulation of channel function by Ca²⁺ and IP₃, allowing the generation of short-lasting Ca²⁺ transients at low levels of IP₃, while maintaining high [Ca²⁺]c during prolonged and intense stimulation of the cell. Ca²⁺ can directly facilitate IP₃-induced Ca²⁺ release (at Glu²¹⁰⁰ in IP₃R₁) (56, 224, 365, 405). Calmodulin-dependent kinase II (CaMKII), on the other hand, phosphorylates the IP₃R and potentiates the Ca²⁺-releasing effect of IP₃, thus exerting positive feedback on IP₃R function (83, 598). High [Ca²⁺]c, may reduce IP₃ binding (510). This inhibition, which may be attributed to an increase in the Kd of IP₃ binding to the receptor (367), is independent of calmodulin. However, calmodulin probably exerts tonic inhibition on the IP₃R under physiological conditions (357, 389, 405, 406). In contrast to the bell-shaped Ca²⁺ sensitivity IP₃R₁, peaking at ~300 nM Ca²⁺, IP₃R₂ displays a sigmoidal increase in open probability of the release channel up to ~1 μM, and [Ca²⁺]c exerts a moderate inhibitory effect only above 1 μM (452, but see Ref. 364). Observations on the Ca²⁺-dependence of IP₃R₃ are conflicting, since increasing (212), unaltered (364), and decreasing open probability at high [Ca²⁺]c were reported (333, 363). The predominant expression of IP₃R₁ in the glomerulosa cell is compatible with the observed high affinity of IP₃ binding; moreover, the bell-shaped Ca²⁺ dependence of IP₃-induced Ca²⁺ release is to be expected.

Simultaneous knock-out of the three types of IP₃R in B lymphocytes abolishes agonist-induced Ca²⁺ signals. However, signal generation is maintained if any of the three isoforms can function (523). Nevertheless, when only a single isoform was expressed, different patterns of Ca²⁺ signals were observed. IP₃R₂ was required for sustained Ca²⁺ oscillations, and IP₃R₁ mediated less regular Ca²⁺ oscillations, while IP₃R₃ generated monophasic Ca²⁺ transients (364).

The threshold and pattern of IP₃-induced Ca²⁺ signals are significantly modified by other signal transduction mechanisms. The receptor is phosphorylated in its coupling domain by protein kinase A (PKA), PKC, and CaMKII. The number, and even the site, of the phosphorylation consensus sites varies in the different receptor types (for review, see Refs. 405, 546). In the nonneural [SII(−)] splice variant of IP₃R₁, that is predominantly expressed in glomerulosa cells (165), low cAMP concentrations preferentially phosphorylate Ser1589 and therefore potentiate IP₃-induced Ca²⁺ release (80, 215). Sub-
stantial phosphorylation of the IP₃R with PKC, as well as augmentation of IP₃ potency in stimulating ⁴⁵Ca release, were detected after pharmacological inhibition of the Ca²⁺/calmodulin-dependent phosphatase calcineurin, known to be anchored to IP₃R (83). Phosphorylation of IP₃R by PKC and dephosphorylation by calcineurin were reported also in bovine glomerulosa cells (430). The potentiation by PKC of IP₃ action at low [Ca²⁺], and the calcineurin-mediated inhibition of IP₃ action at higher [Ca²⁺ₐ], may contribute to the bell-shaped Ca²⁺-sensitive response to IP₃. However, in bovine glomerulosa cells a persistent high-conductance state was observed in the phosphorylated state of the release channel, suggesting that PKC-induced phosphorylation causes cessation of ANG II-induced Ca²⁺ oscillations (430). Tyrosine phosphorylation and cGMP-dependent phosphorylation of IP₃R were detected in stimulated T lymphocytes and vascular smooth muscle cells, respectively (reviewed in Ref. 405), but no data are available for glomerulosa cells.

ATP exerts a concentration-dependent effect on the function of IP₃R. Applied at micromolar concentrations, it enhances the binding of IP₃ (308, but see Ref. 583) and potentiates the open probability of the IP₃-gated Ca²⁺ channel (for review, see Refs. 55, 405). IP₃R1, the dominant receptor isoform in glomerulosa cells, exhibits greater affinity for ATP and greater ATP sensitivity of IP₃-induced Ca²⁺ release than IP₃R3 (331, 332). Competitive inhibition of the binding of IP₃ was observed with supramicromolar concentrations of ATP in several cell types (301, 512, 582), including adrenocortical cells (94, 474). This competition may be an additional and significant factor in the 10⁻⁷ M EC₅₀ of IP₃-induced Ca²⁺ release (in ATP-containing media), as opposed to the 10⁻⁸ M Kₘ of IP₃ binding. The question may be raised: how can the potentiating effect of low, and the inhibitory effect of high concentrations of ATP on IP₃-induced Ca²⁺ release be reconciled? We assume that following Ca²⁺ release the activated Ca²⁺-ATPase would reduce ATP concentration around the ER. The ensuing disinhibition of IP₃R would facilitate the generation of Ca²⁺ signal.

When describing the control of the different types of IP₃R, emphasis was placed on that of IP₃R1, which is predominantly expressed in glomerulosa cells. IP₃R3 constitutes a minor fraction within the IP₃R pool (165), and its significance has yet to be studied. In this respect the Trp binding site (72) should be kept in mind, since it may be essential for capacitative Ca²⁺ influx (see sect. uB).

Significant expression of IP₃R in rat adrenal fasciculata-reticularis cells (secreting the glucocorticoid cortisol) could not be detected. The IP₃R mRNA content was <0.5 molecules/fasciculata cell, while it was ~300 copies/glomerulosa cell (524). Since the rat fasciculata cell would be unique among nucleated cells in lacking IP₃R, this unexpected result certainly requires confirmation by other laboratories. Even so, it seems to account for data showing that ANG II is capable of increasing the formation of IP₃ in these cells (468, 581) but fails to evoke Ca²⁺ signals (77, 584) or increased production of corticosterone (581).

5. Actions of higher inositol phosphates and polyphosphoinositides

There is a notable contrast between the number of identified highly phosphorylated inositol phosphates and our knowledge of their role. There are sporadic observations on the effect of a given compound in a special cell type, but confirmation of these observations in other cell types is usually missing. The most studied compound is the first metabolite of IP₃, inositol 1,3,4,5-tetakisphosphate. It potentiated the Ca²⁺-releasing effect of IP₃ in neuroblastoma (193) and L1210 lymphoma cells (126) as well as in pituitary microsomes (512). It was also found to synergize with IP₃ in inducing Ca²⁺ influx in sea urchin eggs (263) and in exocrine acinar cells (420). It was also proposed that capacitative Ca²⁺ influx is triggered by the interaction of IP₃Rs in the ER vesiicle and the vicinal, plasmalemmal IP₄ receptors (261). Adrenocortical microsomes also contain high-affinity binding sites for IP₄ (168), but their function has not been examined. Antisecretagogue (555) and hemodynamic effects of other inositol tetra- and pentakisphosphate analogs (556) have no relevance for our subject. Data on the modulation of chromatin-remodeling complexes by inositol polyphosphates (494) are not yet available for adrenocortical cells.

It was recognized only in recent years that PIP₂, in addition to being the precursor of the second messengers IP₃ and DAG, controls the function of several membrane-associated proteins. However, phosphoinositides also regulate protein targets through direct binding to specific phosphoinositide-binding domains (266). These domains include pleckstrin homology (PH) domains, Fab1P, YOTB, Vps27p, EEA1 (FYVE) domains, Phox homology (PX) domains and epsin NH₂-terminal homology (ENTH) domains, and other less well-defined lipid-binding structures.

The PH domain is a conserved region of 100–120 amino acids that was first identified in pleckstrin but also present in many other proteins. PH domains have a central role in the formation of molecular complexes involved in signaling and trafficking of receptors. These domains can be categorized into four groups, which preferentially bind phosphatidylinositol 3,4,5-P₃ [PI(3,4,5)P₃] (e.g., Bruton tyrosine kinase, PI(4,5)P₂ (e.g., PLCβ1, pleckstrin-N, spectrin), or PI(3,4)P₀ (e.g., Akt/PKB) or show no clear ligand specificity (e.g., dynamin) (see references in Ref. 266). The affinity of such domains for specific phosphoinositide ligands was sufficient to monitor ANG II-induced changes in membrane lipid composition using GFP-tagged PH domains (558,561), and the lipid
binding of the PH domain of dynamin is required for β-arrestin-dependent AT₁ receptor endocytosis (see references in sect. V). However, the latter effect cannot be explained by the role of the PH domain in the targeting of dynamin, since the lipid binding is not required for the proper targeting of dynamin to clathrin-coated structures (531). Therefore, the interaction with phosphoinositides can regulate the function or the submicroscopical orientation of PH domain-containing proteins.

FYVE domains bind PI(3)P and are present in proteins involved in the processing of endosomal vesicles (e.g., EEA1) and regulation of actin cytoskeleton (e.g., Fgd1). Inhibition of phosphatidylinositol (PI) 3-kinase has a marked effect on the trafficking of AT₁ receptors expressed in HEK 293 cells by interfering with the proper localization of FYVE domain-containing endosomal proteins (249). PX domains, which also bind 3-phosphoinositides, were first identified in two cytosolic components of NADPH oxidase (p47phox and p40phox), but they also occur in a variety of other proteins associated with signaling (e.g., class II PI 3-kinase, phospholipase D) and membrane trafficking (e.g., sorting nexins). Finally, the ENTH domain, which was first identified in epsin, is present in proteins that participate in endocytosis via clathrin-coated pits, and the intact phosphoinositide binding of this domain was shown to be required for clathrin-mediated endocytosis (see references in Ref. 266).

In addition to these well-defined protein folds, the lipid binding domains of plasmalemmal Ca²⁺ pumps, the Na⁺/Ca²⁺ exchanger, Na⁺/H⁺ exchangers, the RyR (for review, see Ref. 237) and the background K⁺ channel TASK (see sect. IIβ) may also be relevant to the adrenergic actions of ANG II. The role of lipid rafts in the plasma membrane in the colocalization of the receptor of the Ca²⁺ mobilizing hormone, PIP₂, Gₛ₁₁, and PLC, as well as the transport proteins, is currently being elucidated.

B. Ca²⁺ Transport Through the Plasma Membrane

ANG II-induced Ca²⁺ influx was one of the most contradictory subjects of aldosterone research. Species differences, inappropriately applied experimental techniques, and application of ANG II at concentrations one or two orders of magnitude higher than required for maximal aldosterone production have all delayed the recognition of the physiological role and mechanism of ANG II action.

Several early studies, applying the ⁴⁵Ca flux technique, failed to detect increased Ca²⁺ influx in response to ANG II. This failure may be due to neglecting the significance of measuring the initial rate of isotope uptake. Yet, this technique could reveal the ANG II-induced reduction of the exchangeable Ca²⁺ pool in bovine (107, 154, 289) and rat glomerulosa cells (27). The loss of cell Ca²⁺ is due to increased efflux of Ca²⁺, also detectable in a Ca²⁺-free medium (27, 157, 176, 301, 584). This Ca²⁺ efflux is due to the extrusion of Ca²⁺, released from the IP₃-sensitive store, by the Ca²⁺-activated plasmalemmal Ca²⁺-ATPase. The subsequently verified increase of Ca²⁺ influx in ANG II-stimulated cells is not in contradiction with the sustained efflux; together they perform continuous Ca²⁺ cycling across the plasma membrane. Although a high concentration of ANG II was applied in all the aforementioned studies, in a single report on the effect of ANG II at low concentration no significant change in the exchangeable Ca²⁺ pool could be revealed (107).

The frequently observed dependence of the ANG II-induced sustained Ca²⁺ signal on extracellular Ca²⁺ (89, 301, 467) has been regarded as evidence in favor of ANG II-induced Ca²⁺ influx. The successful demonstration of ANG II-induced increase in the initial influx rate of ⁴⁵Ca confirmed this interpretation in rat (95, 503) as well as bovine glomerulosa cells (159, 290, 301).

Calcium influx immediately following Ca²⁺ release is brought about by the depletion of IP₃-sensitive calcium stores and, subsequently, the slowly developing depolarization activates voltage-operated Ca²⁺ influx. Ca²⁺ is extruded from the cytoplasm by means of the plasmalemmal and ER Ca²⁺ pumps. In addition, Na⁺/Ca²⁺ exchange may modify [Ca²⁺]c.

1. Membrane potential and Ca²⁺ transport

A major factor controlling Ca²⁺ transport through the plasma membrane is the membrane potential (Eₘ). Together with the concentration gradient it determines the driving force of Ca²⁺ transport and also regulates the activity of a set of Ca²⁺ channels. Considering that the two major physiological stimuli of aldosterone secretion, K⁺ and ANG II, depolarize the glomerulosa cell, we first discuss the control of Eₘ in these cells. Eₘ depends on the distribution of diffusible ions and their selective conductance across the membrane. The Na⁺-K⁺ and Ca²⁺ pumps are responsible for the uneven distribution of the major cations. The greater the contribution of opened K⁺ channels to the total conductance of the cell membrane, the more the membrane potential approaches the equilibrium potential of K⁺ (Eₖ), which is in the range of 70–90 mV (inside negative) in excitable cells. Accordingly, a reduction of (the absolute value of) Eₖ following elevation of extracellular [K⁺] ([K⁺]ₑ), decrease of intracellular [K⁺], inhibition of Na⁺-K⁺-ATPase, or closure of K⁺ channels will depolarize the cell. This depolarization may, in turn, activate voltage-operated channels.

[K⁺]ₑ under resting conditions in the rat is 3.5–4 mM (75, 513). At such K⁺ levels Eₘ may exceed −80 mV, as measured with microelectrodes (445) or by patch-clamp technique (321, 324, 563). This highly negative Eₘ is due to a dominant K⁺ conductance (gₖ), as confirmed by the perforated patch technique, which avoids the rundown of...
Control of Aldosterone Secretion

The primary step in the energy-dependent generation of membrane potential is the build-up of a transmembrane \( K^+ \) concentration gradient by the \( Na^+-K^+\)-ATPase ("sodium pump"). Furthermore, the exchange of three cytoplasmic \( Na^+ \) for two extracellular \( K^+ \) also contributes to the generation of intracellular electronegativity ("pump potential"). Reducing the activity of the sodium pump dissipates the \( K^+ \) gradient and results in depolarization. It was revealed a quarter of a century ago that ANG II decreased rather than increased the potassium content of rat glomerulosa cell. The isotope uptake is dependent on cytoplasmic \( Na^+ \), and it attains half-maximal rate at 10 mM, corresponding against at the \( \alpha \)-isoenzyme form. Maximal uptake rate is reached at \( \sim 25 \) mM \( Na^+ \), which corresponds to the measured physiological level of cytoplasmic \( Na^+ \) (557) and decreases again above 50 mM. The transport activity is 4 and 20 times higher than that in fasciculata cells and hepatocytes, respectively. ANG II reduces the ouabain-sensitive \( ^{86} \)Rb uptake, i.e., the activity of \( Na^+-K^+\)-ATPase, and the observed IC\(_{50} \) of ANG II (300 pM) suggests that the inhibitory effect of the peptide has physiological relevance.

ANG II exerts its inhibitory effect on \( Na^+-K^+\)-ATPase through \( AT_1 \) receptors (213). A recent observation suggests that ANG II inhibits the pump through activating a tyrosine phosphatase (596), whereas activation of the pump by \( Ca^{2+} \) through CaMKII (597) may serve as negative feedback during intense stimulation with ANG II. When ouabain is applied at submillimolar concentrations, the drug induces a continuous increase in [\( Ca^{2+} \)], without any detectable lag time, and this increase can be abolished with nifedipine (213) At similar concentrations the drug also stimulates the production of aldosterone, again requiring extracellular \( Ca^{2+} \) and activable voltage-operated \( Ca^{2+} \) channels (213, 488, 527, 595). These data suggest that during ANG II-induced stimulation of aldosterone secretion, inhibition of \( Na^+-K^+\)-ATPase contributes to the depolarization and \( Ca^{2+} \) signal generation in glomerulosa cells.

In addition to \( Na^+-K^+\)-ATPase, \( K^+ \) conductance is of great significance in setting \( E_m \). Patch-clamp studies revealed inwardly rectifying, outwardly (delayed) rectifying, \( Ca^{2+} \)-activated as well as background \( K^+ \) current in glomerulosa cells. The ion channel through which the current is conducted has been identified in only a few cases. Of major concern is the estimation of the physiological significance of the observed currents/channels.

Only ion channels that are active in the range of the resting membrane potential may play a role in its fine setting. Members of the superfamily of inwardly rectifying \( K^+ \) channels (386, 481) display a large inward current at potentials negative to \( E_K \) (occurring only under laboratory conditions) and a small and gradually diminishing outward current as \( E_m \) is shifted to more positive values. It is this latter \( K^+ \) efflux by which \( E_m \) approaches the more negative \( E_K \). Inwardly rectifying \( K^+ \) current, with characteristics of Kir 2, has been observed in the membrane patch of rat and bovine glomerulosa cells (564). Kir 2 passes through the ubiquitous IRK channels and, in contrast to other families of inwardly rectifying channels, does not require G protein for activation and is insensitive to the ADP/ATP ratio. Nonetheless, the physiological significance of Kir 2 in glomerulosa cells has been questioned by the lack of inward rectification in macroscopic current under whole cell configuration (79, 321).

The previously mysterious background (or leak) \( K^+ \) current, which, by definition, is also capable of permeating \( K^+ \) in the range of resting \( E_m \), would also be capable of generating highly negative \( E_m \). Such a current was described in glomerulosa cells by Lotshaw in 1997 (322). Since that time the respective channel has been identified and termed TASK, an acronym for TWIK (tandem of \( P \) domains in a weak inward rectifying \( K^+ \) channel)-like, acid-sensitive \( K^+ \) channel (148). The expression of this two-pore \( K^+ \) channel in rat glomerulosa cell has been demonstrated by Enyedi and co-workers in our laboratory (129). The zona glomerulosa exhibits the highest density of TASK mRNA among all tissues hitherto examined. In Xenopus oocytes injected with glomerulosa mRNA, the expressed \( K^+ \) current had a pharmacology comparable with that of the authentic TASK current (129). More recently, the mRNA of the subsequently described TASK-3 (284, 451) was also found in rat glomerulosa cells (128). Interestingly, the glomerulosa cell is exclusive among several examined cell types in expressing TASK-3. TASK-1 and TASK-3 are coexpressed (the latter in larger amount) and form heterodimers. A fine-tuning of channel control may be achieved by the formation of heterodimers by the two subtypes (127).

Several \( K^+ \) currents or channels, active in depolarizing feedback during intense stimulation with ANG II. The linear relationship between \( \log [K^+]_o \) and \( E_m \) had a slope of 53.7 mV/10-fold change in [\( K^+ \)] (323) that almost attains the value predicted by the Nernst equation for a membrane with \( K^+ \) conductance only (58 mV/decade). These findings are consistent with the lack of \( Na^+ \) channel expression in the glomerulosa cell.

The previously mysterious background (or leak) \( K^+ \) current, which, by definition, is also capable of permeating \( K^+ \) in the range of resting \( E_m \), would also be capable of generating highly negative \( E_m \). Such a current was described in glomerulosa cells by Lotshaw in 1997 (322). Since that time the respective channel has been identified and termed TASK, an acronym for TWIK (tandem of \( P \) domains in a weak inward rectifying \( K^+ \) channel)-like, acid-sensitive \( K^+ \) channel (148). The expression of this two-pore \( K^+ \) channel in rat glomerulosa cell has been demonstrated by Enyedi and co-workers in our laboratory (129). The zona glomerulosa exhibits the highest density of TASK mRNA among all tissues hitherto examined. In Xenopus oocytes injected with glomerulosa mRNA, the expressed \( K^+ \) current had a pharmacology comparable with that of the authentic TASK current (129). More recently, the mRNA of the subsequently described TASK-3 (284, 451) was also found in rat glomerulosa cells (128). Interestingly, the glomerulosa cell is exclusive among several examined cell types in expressing TASK-3. TASK-1 and TASK-3 are coexpressed (the latter in larger amount) and form heterodimers. A fine-tuning of channel control may be achieved by the formation of heterodimers by the two subtypes (127).

Several \( K^+ \) currents or channels, active in depolarizing
ized cells only, have also been described in the glomerulosa cell. Noninactivating delayed rectifier K⁺ current (inducing K⁺ efflux from depolarized cells) was described in rat, bovine, and human glomerulosa cells (79, 407, 409, 564). This current probably corresponds to KvLQT1, the slow component of the delayed outward K⁺ current (Iₖ,slow) in the heart. It is conducted by the channel KCNQ1, the regulatory subunit of which (KCNE1) has been detected in glomerulosa cells. It is conducted by the channel KCNQ1, the slow component of the delayed outward K⁺ current (Iₖ,slow) in the heart. It is conducted by the channel KCNQ1, the regulatory subunit of which (KCNE1) has been detected in glomerulosa cells (13). In view of the voltage dependence of their activation, the delayed rectifier channels may not modify the resting Eₘ but their activation could limit the extent of depolarization by allowing the efflux of K⁺. In harmony with this prediction, knock-out of the KCNE1 gene resulted in enhanced responsiveness of the plasma aldosterone concentration to K⁺ loading (13).

Transient outward (A-type) K⁺ current was observed in rat, bovine, and human glomerulosa cells (407, 409, 564). Ca²⁺-dependent maxi-K⁺ (BK) channels were also described in the rat (321, 408, 564). In view of the voltage dependence of their activation, the A-type and the BK channels may not modify the resting Eₘ. On the other hand, ANG II, probably acting through Gq/11, inhibits the Ca²⁺-dependent K⁺ channel (408), an action that could augment the depolarization triggered by some other mechanism. ANP, probably acting via cGMP, increases K⁺ conductance by facilitating the function of BK channels (191). The ensuing hyperpolarization may represent one of the means by which the peptide antagonizes the secretagogue action of Ca²⁺-mobilizing hormones.

Any agent that decreases K⁺ conductance evokes the shift of Eₘ from the strongly negative Eₘ towards the positive equilibrium potential of Na⁺ and Ca²⁺. The first evidence that ANG II induces membrane depolarization was obtained with intracellular microelectrode recording of cat adrenocortical slices as early as the 1970s (383). Later studies provided evidence that a significant component of this depolarization is caused by the reduction of gK. In microelectrode studies a biphasic Eₘ response to ANG II was observed in rat glomerulosa cells (446). A brief hyperpolarization was followed by a long-lasting depolarization, characterized by a large decrease in cell conductance. The reversal potential of the response suggested that Eₘ followed changes in gK. Subsequent ⁸⁶Rb flux measurements confirmed that ANG II inhibits gK in rat glomerulosa cells (217). The primary site of action of ANG II is the AT₁ receptor, and its effect is mimicked neither by ionomycin-induced elevation of [Ca²⁺], nor by pharmacological activation of PKC, suggesting that inhibition of K⁺ conductance by ANG II is a membrane-delimited process. An initial increase, followed by a prolonged decrease in gK, was observed by means of ⁸⁶Rb flux studies also in bovine glomerulosa cells. Surprisingly, the decrease in gK was found to be Ca²⁺ dependent (320). The significance of the ANG II-induced decrease in K⁺ conductance is shown by the observation that the K⁺ ionophore valinomycin significantly reduced ANG II-stimulated aldosterone production (495).

Subsequently, in the patch-clamp era, ANG II was found to inhibit each type of the hitherto described K⁺ channels. However, only inhibition of channels active at resting Eₘ may be responsible for triggering depolarization. Of the two channel types meeting this requirement in glomerulosa cell, Kir 2 displayed reduced single-channel activity in membrane patches exposed to ANG II (276). However, as previously mentioned, this current has not yet been found in whole cells. The background K⁺ current, corresponding to the later described TASK, was inhibited by physiological concentrations of ANG II in rat glomerulosa cells. At the moderately elevated physiological concentration of 10⁻¹⁰ M, ANG II reduced the leak membrane conductance (measured between −127 and −87 mV) by 50% and shifted Eₘ by −6 mV to positive direction. The extent of depolarization was 18 and 32 mV on average at 10⁻⁹ and 10⁻⁸ M ANG II, respectively (323). A stable level of current inhibition required −10 min at concentrations below 100 pM ANG II and at least 5 min at higher concentrations (321).

ANG II also inhibits the TASK channels responsible for background K⁺ current. ANG II added to isolated glomerulosa cells or to oocytes injected with glomerulosa mRNA inhibited the K⁺ current. ANG II also inhibited the TASK current in oocytes, coexpressing TASK and the AT₁a angiotensin II receptor. These observations provided firm evidence that TASK exerts a significant role in the generation of the strongly negative resting membrane potential in glomerulosa cells and that ANG II may depolarize the cell by inhibiting these channels (129). Although the inhibition of TASK-3 current by ANG II is smaller than that of TASK-1 current (128), it still may have physiological significance, especially in view of the modified characteristics of the TASK-1/TASK-3 heterodimers (127). Maneuvers applied to activate phospholipase C, Gₛ, and protein kinase C, or to increase the concentration of cytoplasmic Ca²⁺ and IP₃, led to the conclusion that the action of ANG II (or other Ca²⁺-mobilizing hormones) is mediated by the breakdown of PIP₂ in the plasma membrane (130).

ANG II inhibits the delayed rectifier K⁺ current (79, 276). The Ca²⁺-dependent maxi K⁺ channels are also inhibited by ANG II, and in this case the effect is mediated by a pertussis toxin-insensitive G protein (408). Considering that depolarization induced by ANG II levels occurring in vivo is probably not sufficient for the activation of these channel types, their role in the physiological action of ANG II awaits confirmation.

Activation of nonelective cation channels also results in depolarization. Such a channel, with a nearly linear slope conductance between −80 and 0 mV under quasi-physiological ionic conditions, has been described by Lotshaw and Li (324). The channel was also permeable...
to Ca$^{2+}$, exhibiting a $P_{Ca}/P_{Na}$ of 4 (with 110 mM Ca$^{2+}$ on the extracellular side). It would be worthwhile to examine the relation of this current with the dihydropyridine (DHP)-insensitive background Ca$^{2+}$ currents observed in glomerulosa cells by applying the cell-attached mode of the patch-clamp technique, with pipette solutions lacking monovalent cations (149). In cell-attached patches ANG II (1 nM) increased the opening probability of the nonselective cation channel, rendering cation influx and depolarization possible in the intact cell. In fact, using the nystatin-perforated patch technique, depolarization and depolarization possible in the intact cell. In fact, using the nystatin-perforated patch technique, depolarization began in $\approx$2 min and was characterized by a large increase in input resistance, indicating the decrease in $g_K$. However, transient depolarizations of variable amplitude were superimposed on the slow depolarization, and membrane conductance during these voltage transients exhibited large increases. Considering that Ca$^{2+}$ was omitted from the pipette solution, the increased conductance indicated the activation of nonselective cation channels. Summarizing, these channels may contribute to signal generation by ANG II by two means: cation influx contributes to the voltage-dependent activation of Ca$^{2+}$ channels; moreover, Ca$^{2+}$ influx per se may participate in Ca$^{2+}$signaling. (For further studies of channel structure, see Ref. 325.)

The participation of the Na$^+$-K$^+$-ATPase and ion channels in hormonally induced resetting of $E_m$ is summarized in Figure 3.

Because changes in Cl$^-$ conductance are involved in the depolarization or repolarization of various cell types, their function in glomerulosa cells is also summarized. Apart from a Ras-dependent chloride current activated by ACTH (105), we are not aware of any report on Cl$^-$ current in the glomerulosa cell. No significant Cl$^-$ current was detected in early microelectrode studies (445). We examined Cl$^-$ currents with the patch-clamp technique in rat glomerulosa cells. K$^+$ currents were inhibited with CsCl-containing pipette solution and 10 mM Ba$^{2+}$ and 10 mM tetraethylammonium in the extracellular bath. With the application of nearly symmetrical Cl$^-$ concentration, the current at $\approx$100 mV was less than $\approx$20 pA. Membrane resistance in the voltage range of $\approx$100 and $\approx$120 mV reached 7 G$\Omega$ on average, indicating the absence of any significant channel activity. Also, the current displayed ohmic change only during a slow ramp depolarization from $\approx$100 to $\approx$60 mV. However, in a significant fraction of the cells, the slow activation of a tiny inward current could be observed at strongly negative voltages. This was significantly accentuated by reducing extracellular pH from 7.4 to 6.9, attaining $\approx$3.5 pA/pF on average at $\approx$100 mV. Both the kinetic and pharmacological characteristics of this inwardly rectifying current suggest that it passes through ClC-2 Cl$^-$ channels (J. K. Makara and A. Spital, unpublished observations). The role of this current in the glomerulosa cells remains to be elucidated.

2. Capacitative influx

The concept of capacitative Ca$^{2+}$ influx, also termed store-operated or calcium release-activated Ca$^{2+}$ influx, was introduced by Putney and co-workers in 1989 (534, 535). They proposed that agonist-induced Ca$^{2+}$ uptake from the extracellular fluid is evoked by the depletion of IP$_3$-sensitive intracellular calcium stores. Store depletion can be experimentally elicited by pharmacological inhibition of the SERCA-type Ca$^{2+}$-ATPase (e.g., with thapsigargin). Since that time, capacitative Ca$^{2+}$ uptake has been demonstrated as a general and major Ca$^{2+}$ uptake mechanism in nonexcitable cells and may also be present in excitable cells. The current flowing through capacitative Ca$^{2+}$ entry channels was first characterized in mast cells and has been termed calcium-release activated Ca$^{2+}$

![FIG. 3. The participation of the Na$^+$-K$^+$-ATPase and ion channels in hormonally induced resetting of membrane potential ($E_m$). Nonspecific, nonspecific cation channel; delayed rect. K$^+$, delayed rectifier K$^+$ channel; big K, Ca$^{2+}$-activated maxi (BK) K$^+$ channel. The red arrows indicate stimulatory actions, and the green arrows indicate inhibitory hormonal actions.](http://physrev.physiology.org/DownloadedFrom/to/2020134onNovember6,2017)
It is highly Ca\(^{2+}\) selective, inwardly rectifying, and although its very low unitary conductance is compatible with a carrier mechanism; noise analysis indicates that \(I_{\text{CRAC}}\) passes through a channel. There are, however, several types of store-operated currents, different from \(I_{\text{CRAC}}\), in cells that exhibit store-operated Ca\(^{2+}\) influx (404).

Three major hypotheses have been proposed to explain the activation of capacitative Ca\(^{2+}\) influx by store depletion. One of these hypotheses assumes that a small diffusible cytosolic factor links the IP\(_3\)-sensitive calcium store with the plasmalemmal channel. Another model attributes channel activation to the insertion of channel-containing vesicles into the plasma membrane. The third, and currently most accepted, hypothesis suggests a Ca\(^{2+}\)-dependent protein-protein interaction between the release and influx channels. The multitude of models may indicate that there is no uniform mechanism responsible for channel gating in different cell types. Since no specific data are available for the mechanism in glomerulosa cell, we refer to general reviews on this topic (49, 64, 404, 440, 565). The structure and gating mechanism of the channel responsible for Ca\(^{2+}\) entry remain to be clarified. Mammalian homologs of Trp proteins (termed after Drosophila transient receptor potential), products of at least seven genes, are presumed to constitute the channel. The channel contains four subunits, and the great number of possible heterotetramers may explain the variable function of these channels in different cell types. In fact, the channels may display selective or nonselective Ca\(^{2+}\) conductance after activation of PLC. Store depletion evoked by thapsigargin opens some but not all Trp channels (reviewed in Ref. 361).

In 1989 we observed that, in contrast to K\(^+\)-induced Ca\(^{2+}\) influx, ANG II-induced early Ca\(^{2+}\) influx was not sensitive to the DHP Ca\(^{2+}\) channel inhibitor nifedipine. This observation led us to postulate that the two agonists activate different Ca\(^{2+}\) entry mechanisms (504). A similar observation was subsequently reported in bovine glomerulosa cells (302). Further results have unambiguously demonstrated the significance of DHP-insensitive capacitative Ca\(^{2+}\) influx in ANG II-induced Ca\(^{2+}\) uptake. In rat glomerulosa cells, thapsigargin dose-dependently increases \([\text{Ca}^{2+}]_c\) and aldosterone production (219). The Ca\(^{2+}\) signal is DHP insensitive and transient only in Ca\(^{2+}\)-free medium. Readdition of Ca\(^{2+}\) induces a steeper and higher rise in \([\text{Ca}^{2+}]_c\), than that observed in thapsigargin-untreated cells (465), indicating that influx rate depends on the state of the calcium store rather than on \([\text{Ca}^{2+}]_c\). Similar results were obtained in bovine glomerulosa cells. Thapsigargin dose-dependently increases \([\text{Ca}^{2+}]_c\) (159) and stimulates aldosterone production (81).

Both mRNA and protein of Trp 4 are abundantly expressed in the bovine adrenal cortex, where the only other transcript found at a considerable level was Trp1.

Expression of antisense trp4 cDNA reduced both the endogenous capacitative current and the amount of Trp4 protein, indicating the role of this protein species in the formation of store-operated Ca\(^{2+}\) channels in bovine adrenocortical cells (424). The Trp-binding site was described in the cytosolic NH\(_2\) terminus of IP\(_3\)R3 (72). Therefore, the expression of IP\(_3\)R3 in glomerulosa cells (165) may have a special role in controlling Ca\(^{2+}\) influx in glomerulosa cells.

The role of capacitative Ca\(^{2+}\) influx in the action of ANG II has been supported by several observations. In bovine adrenocortical microsomes, the thapsigargin-releasable Ca\(^{2+}\) pool was larger than the IP\(_3\)-sensitive pool (159). Consistent with this observation, in rat glomerulosa cells ANG II did not induce any further elevation of \([\text{Ca}^{2+}]_c\) after exposure to thapsigargin, whereas a second rise in \([\text{Ca}^{2+}]_c\) could be obtained if the agonists were applied in the reverse order (159). These observations indicate that the IP\(_3\)-sensitive Ca\(^{2+}\) pool constitutes a subcompartment within a larger, thapsigargin-sensitive compartment. In bovine cells the oscillatory Ca\(^{2+}\) signal induced by ANG II at physiological (100 pM) concentration was maintained for at least 20 min in a Ca\(^{2+}\)-free medium, but was rapidly extinguished by adding thapsigargin. Nifedipine reduced the frequency of oscillations by only −30% in the Ca\(^{2+}\)-restored state; moreover, it had no effect on the plateau level of the Ca\(^{2+}\) signal induced by a high dose of ANG II (479). The Ca\(^{2+}\) signal, as measured 5 min after adding ANG II, was little affected by blocking T-type Ca\(^{2+}\) and L-type Ca\(^{2+}\) current but was eliminated by thapsigargin (81). The relatively high expression of a capacitative Ca\(^{2+}\) channel homologous to Drosophila Trp channels in bovine (whole) adrenal (423) is also in agreement with the biological relevance of capacitative Ca\(^{2+}\) influx in this organ, although the respective cell type has not yet been specified (423).

The sustainment of capacitative Ca\(^{2+}\) influx obviously requires continuous depletion of the IP\(_3\)-sensitive calcium store, which seems to be due to the continuous production of IP\(_3\). Although the postinitial level of IP\(_3\) is small, it is suprabasal (161), and this moderately elevated level may be sufficient for the continuous flow of Ca\(^{2+}\) out of the ER vesicles. If Li\(^+\) or a high concentration of wortmannin were added to the incubation medium to prevent the replenishment of the IP\(_3\) pool, the sustained formation of IP\(_3\) (18) and the sustained phase of the Ca\(^{2+}\) signal (376) were inhibited in bovine glomerulosa cells. Lithium ions also severely compromised the sustained phase of the aldosterone response to ANG II (but not to ACTH) in rat cells (21). The requirement for maintained activation of PLC, and the ensuing formation of IP\(_3\), is compatible with the essential role of capacitative Ca\(^{2+}\) influx in sustaining ANG II-induced aldosterone production.
3. Voltage-activated Ca\(^{2+}\) currents and Ca\(^{2+}\) channels

Angiotensin II induces capacitative Ca\(^{2+}\) influx and depolarization, which, in turn, reduce both the Ca\(^{2+}\) concentration gradient and electrical gradient, the driving force of Ca\(^{2+}\) flux from the extracellular space into the cytoplasm. However, depolarization also results in the activation of voltage-operated Ca\(^{2+}\) channels, and the ensuing increase in Ca\(^{2+}\) conductance may compensate for the reduced driving force. In this way, voltage-activated Ca\(^{2+}\) channels subserve the maintenance of Ca\(^{2+}\) influx.

The various members of the superfamily of voltage-activated Ca\(^{2+}\) channels give rise to Ca\(^{2+}\) currents of diverse electrophysiological and pharmacological properties. Voltage-activated Ca\(^{2+}\) currents are conventionally classified as low-voltage (or low-threshold) and high-voltage (or high-threshold) currents. In view of the voltage-activated Ca\(^{2+}\) currents that participate in the control of aldosterone secretion, this discussion will be confined to the low-voltage, T-type current and the high-voltage, L-type current. Calcium channels may exist in three distinct states, identified as the closed, activated, and inactivated states. The latter refers to a depolarization-induced state from which the opened state cannot be directly attained. T-type current is activated at more negative potentials than L-type current. In the presence of 10 mM Ca\(^{2+}\), the activation range of T-type channels is positive to \(-70\) mV, and that of L-type channels is positive to \(-10\) mV. T-type current reaches maximum amplitude and is inactivated at more negative potential than L-type current. T-current inactivates more rapidly, but deactivates (closes) and re-activates (reprimes) more slowly than L-type current. T-type channels exhibit comparable permeability for Ca\(^{2+}\) and Ba\(^{2+}\), whereas Ba\(^{2+}\) conductance through L-type channels exceeds that of Ca\(^{2+}\) conductance. The T-type channel has a unitary conductance of \(-8\) pS versus the 25 pS of an L-type channel (when the charge carrier is 100 mM Ba\(^{2+}\)). T-type current is generally more sensitive to blockade by Ni\(^{2+}\), amiloride, and nifedipine than L-type current. In contrast to L-current, T-current is not enhanced by the DHP agonist BAY K 8644 (for further details, see Refs. 41, 341, 352, 518, 552).

The first electrophysiological study on voltage-operated Ca\(^{2+}\) currents in rat glomerulosa cells was published in 1987 (445) and demonstrated that depolarizing current pulses elicited a regenerative response. The ionic mechanism underlying the regenerative response was voltage-operated Ca\(^{2+}\) current. Following these pioneering studies, three types of voltage-activated Ca\(^{2+}\) currents, T-, L- and N-type currents, have been identified by application of the patch-clamp technique to glomerulosa cells. T-type current has been observed in rat (150, 342, 562), bovine (113, 118, 259, 323, 343), and human glomerulosa cells (409). L-type current has also been described in rat (150, 323, 562), bovine (113, 343, 344), and human cells (409). N-type current was observed only in Long-Evans rats (150); no report on its expression in any other rat strain or other species has been published.

Patch-clamp studies usually employ Ba\(^{2+}\) as the charge carrier to avoid Ca\(^{2+}\)-dependent inactivation of L-type channels, and few data have been obtained using Ca\(^{2+}\) as charge carrier. The low-threshold, T-type current was characterized in the presence of 2.5 mM Ca\(^{2+}\) in rat and bovine glomerulosa cells by Quinn et al. (444). Following the elimination of K\(^{+}\) currents, the activation threshold was between \(-80\) and \(-70\) mV, peak current occurred near \(-30\) mV, and half-maximal steady-state inactivation was attained at \(-73\) mV. In our studies (563), performed with 2 mM Ca\(^{2+}\), the average activation threshold of T-type current was \(-71\) mV. The threshold of L-type current was determined with a very slow voltage ramp depolarization from \(-100\) to \(+40\) mV and also with ramp depolarization following inactivating T-type channels at \(-60\) mV. The average threshold of L-current was \(-57\) mV, applying either protocol.

Voltage-activated Ca\(^{2+}\) channels have heteromultimeric structures, and the electrophysiological characteristics of their ionic current are primarily determined by the pore-forming \(\alpha\)-subunit. This subunit consists of four homologous repeats (I–IV) of six membrane-spanning segments (termed S1–S6). Every third amino acid residue in segments IV is lysine or arginine. Changes in the electrical field induce motion of these positively charged segments, which therefore serve as voltage sensors. The intramembrane loops between S5 and S6 line the channel pore, and four glutamate residues in this pore domain are responsible for the selective Ca\(^{2+}\) permeability (352, 359, 414, 537, 540).

Molecular biological studies have identified the pore-forming \(\alpha\)-subunits of the low- and high-voltage-activated channels. Three isoforms of T-type \(\alpha\)-subunits are known, indicated as Ca,3.1, 3.2, and 3.3 (\(\alpha_{1G}, \alpha_{1H}\) and \(\alpha_{1D}\) respectively). Their intracellular loops and COOH termini exhibit significant differences in amino acid sequence, which provide opportunity for their selective pharmacological inhibition (307, 412). Half-maximal activation in the presence of 2 mM Ca\(^{2+}\) was observed for the three subtypes at \(-29\), \(-31\), and \(-25\) mV, respectively. The steady-state inactivation of \(\alpha_{1H}\) occurs at higher potentials. \(\alpha_{1G}\) activates and inactivates more rapidly. Their deactivation is relatively slow and is fastest for \(\alpha_{1H}\). All have a tiny single-channel conductance, and \(\alpha_{1H}\) is the most sensitive to Ni\(^{2+}\) (286, 311, 413).

Substantial expression of the mRNA transcript for the \(\alpha_{1H}\) subunit was found in the rat and bovine zona glomerulosa by in situ hybridization. (A much weaker expression was detected also in bovine zona fasciculata.) An extremely low level of \(\alpha_{1G}\) mRNA was found, and \(\alpha_{1H}\)
was virtually absent. In accordance with these data, the IC₅₀ of Ni²⁺ for inhibition of T-type Ca²⁺ current in these cells was 6 µM (489), corresponding to the Ni²⁺ sensitivity of the α₁H subunit (7 µM) (413). α₁H is also expressed in the H295R human glomerulosa cell line, and, unexpectedly, exposure to aldosterone for 24 h increases the expression of the channel protein as well as the density of T-type current (317). It is noteworthy that only the activity of the H isoform of the T-type Ca²⁺ channel is enhanced by Ca²⁺, through phosphorylation by CaMKII (586).

The diverse nature of L-type channels was suggested long ago by the different excitation-contraction coupling in skeletal muscle and cardiac muscle. In both cell types, activation of L-type Ca²⁺ channels results in Ca²⁺ release from the sarcoplasmic reticulum through ryanodine receptors. However, in skeletal muscle cells the L-channels (DHP receptors) in the t-tubule membrane activate type 1 RyRs through protein-protein interaction, whereas in cardiac muscle it is Ca²⁺ influx through L-channels that evokes Ca²⁺-induced Ca²⁺ release through type 2 RyRs. In addition to the structural differences in RyRs, this differential coupling is attributed to the molecular differences in the two types of L-type channels. In fact, molecular biological studies on different cell types have identified three, rather than two, isoforms of the pore-forming α₁-subunit of L-type channels. The α₁-subunit of the skeletal (S), cardiac (C), and neuroendocrine (D) isoforms of L-type channel are termed Ca₁.1, Ca₁.2, and Ca₁.3, respectively (415).

RT-PCR analysis of single rat glomerulosa cells, free of other cell types, detected mRNA of Ca₁.2 and Ca₁.3 (α₁C and α₁D, respectively) (242). The dominant expression of Ca₁.3 probably results in a depolarization shift of the activation threshold and reduced rate of Ca²⁺-dependent inactivation (298, 601). The expression of Ca₁.2 and Ca₁.3 was also observed in human H295R cells (317).

Voltage-operated Ca²⁺ channels contain several isoforms of additional (α₂δ, β, and γ-subunits), which modify the current characteristics and pharmacology of the α₁-subunit as well as its expression at the plasma membrane (137, 271, 414, 500). We are not aware of studies on these additional subunits in the glomerulosa cell.

Several laboratories have observed that cAMP, through the activation of PKA, induces marked increases in L-type current, whereas data on the effect of PKC on T-type and L-type currents are conflicting (reviewed in Refs. 93, 352). cGMP opposes the current-enhancing effect of cAMP (268). CaMKII phosphorylates α₁s and thus enhances L-type current (348). Enhancement of T-type current by CaMKII was first observed in bovine glomerulosa cells (34). HeterotrimERIC G proteins, in addition to regulating second messenger systems, may also regulate ionic channels acting via a membrane-delimited pathway. L-type current is enhanced by the α-subunit of Gₛ (352, 575), and the neuronal N-type and P/Q-type Ca²⁺ currents are inhibited by Gₛ or G, via their released βγ-subunits (235, 285). Also, Ras-related small G protein that binds CaM inhibits the L-type current (42).

4. Action of ANG II on voltage-activated Ca²⁺ channels

Aldosterone production stimulated with ANG II or K⁺ is inhibited by the Ca²⁺ channel inhibitors verapamil (26, 305, 488) and nifedipine (3, 504). The frequently observed dependence of the ANG II-induced sustained Ca²⁺ signal on extracellular Ca²⁺ (80, 301, 467) has been regarded as evidence in favor of ANG II-induced Ca²⁺ influx. In fact, data on the augmented initial influx rate of ⁴⁵Ca after adding ANG II confirmed this interpretation in rat (95, 503) and bovine cells (159, 290, 301). Several, although not all, of the studies examining the effect of channel drugs on ANG II-induced Ca²⁺ signal also supported the stimulation of voltage-activated Ca²⁺ influx by ANG II. The DHP antagonist nifedipine usually moderately reduced the Ca²⁺ signal in bovine (7, 89) and in rat cells (77, 255, 516), whereas BAY K 8644, an L-type specific agonist, enhanced ANG II-induced Ca²⁺ signal in rat (but not bovine) glomerulosa cells (229, 255). However, it should be noted that ANG II was applied at pharmacological concentration in all these experiments, giving no information on channel activity under physiological conditions. Moreover, the description of changes in [Ca²⁺], gives only an indirect indication of changes in unidirectional ion transport and the corresponding channel activity.

We observed in 1991 that the nifedipine-sensitive Ca²⁺ signal induced by 18 mM K⁺ is inhibited by physiological concentrations of ANG II in the rat glomerulosa cell (24). As later discussed, at this concentration of K⁺ the Ca²⁺ signal is evoked dominantly by L-type channels. Subsequent patch-clamp studies provided evidence that L-type Ca²⁺ current is in fact inhibited by ANG II (323). When applied at a pharmacological concentration (10 nM), ANG II also inhibited the K⁺-activated Ca²⁺ signal or current in bovine cells (107, 344). [In contrast to these observations, ANG II was found to enhance L-type Ca²⁺ current in the Y1 adrenocortical tumor cell line (236), and AVP was also reported to augment L-type current in rat glomerulosa cells (196).]

The inhibition of L-type current in the face of enhanced Ca²⁺ influx indicates that ANG II stimulates Ca²⁺ influx through T-type channels only. Furthermore, the comparable concentration dependence of inhibition of T-type current and ANG II-induced aldosterone production by nifedipin, a more-or-less specific inhibitor of T-channels, provides evidence in favor of the essential role of T-type channels in eliciting Ca²⁺ influx in the ANG II-stimulated rat glomerulosa cell (323). The same conclusion could be drawn from the report that mibebradil, applied at a concentration that inhibits T-type but not
L-type Ca^{2+} channels, reduced the [Ca^{2+}]c response to ANG II in bovine cells (476). The inhibition of Ca^{2+} current and ANG II-induced aldosterone production by tetrodotoxin, an herbal alkaloid, at concentrations that inhibit T-type channels but do not yet affect L-type channels (478), also supports the significance of T-type current in ANG II-stimulated bovine cells.

ANG II appears to enhance T-type current in rat and bovine glomerulosa cells by different mechanisms. ANG II failed to affect the function of T-type channels in Lotshaw’s experiments on rat glomerulosa cells (323), leading to the conclusion that ANG II activates the low-voltage-operated Ca^{2+} channel exclusively by depolarizing the plasma membrane. The enhancement of T-channel activity, reported in bovine cells by Barrett’s group, was explained with a hyperpolarizing shift in the voltage dependence of channel activation (97). This shift requires intracellular GTP, suggesting that the channel-activating action of ANG II is mediated by a G protein (349). Although the Ca^{2+} signal activates calmodulin-dependent kinase II, and this activation may also shift the voltage-current function to more negative values (34, 327), the ANG II-induced shift is basically due to activation of G_{i/o} (97, 326). This model is in agreement with previous reports on the pertussis toxin sensitivity of ANG II-induced Ca^{2+} influx (236, 296, 344). Activation of pertussis toxin-sensitive G protein(s) also seems to account for inhibition of L-type current by ANG II (344) or AVP (204).

Atrial natriuretic hormone, a physiological inhibitor of aldosterone secretion, attenuates agonist-induced Ca^{2+} influx (95, 186). In patch-clamp studies this inhibition was confined to T-type channels; Ca^{2+} current through L-type channels was even enhanced (33, 350). The inhibition of ANG II-induced aldosterone production may be due to the reduction of T-current, and this effect apparently is not counteracted by a concomitant enhancement of L-current because L-type channels are inhibited by a G_{i}/mediated mechanism in cells exposed to ANG II. The neurotransmitter dopamine, which may be released from local varicos axon terminals around glomerulosa cells (571), also inhibits T-type Ca^{2+} channel (143, 400) and thus inhibits aldosterone secretion (2).

As summarized in Figure 4, ANG II enhances Ca^{2+} influx through T-type channels. Depolarization as well as a negative shift in the voltage dependence of channel activation are the factors inducing enhanced Ca^{2+} influx, the latter shift being evoked by activation of a pertussis toxin-sensitive G protein, probably belonging to the G_{i/o} family. It is presently not clear whether the relative participation of these factors in current amplification depends on species or experimental conditions. ANG II, at the same time, may inhibit L-type current, probably also through the activation of G_{i/o}. This inhibition may protect the cell from Ca^{2+} overload.

5. DHP-sensitive Ca^{2+} channels and IP_{3}-induced Ca^{2+} release

In rat glomerulosa cells, the L-type channel-specific DHP agonist BAY K 8644 enhances ANG II-induced Ca^{2+} response and aldosterone production (229, 255). This suggests that even if ANG II-induced Ca^{2+} influx takes place via T-channels, some L-channels also contribute to Ca^{2+} signaling. This presumption, however, is in sharp contradiction to the previously described inhibition of L-type channels by ANG II. On the other hand, the actions of channel agonist and antagonists suggest an interaction between L-type channels and subplasmalemmal IP_{3} receptors. Such a model (516) has been based on the following experimental observations.

ANG II-induced depolarization and Ca^{2+} influx do not become detectable until after the initial peak of Ca^{2+} influx.
signal has occurred. ANG II (10^{-9} or 10^{-8} M)-induced depolarization begins at ~2 min in bovine (88) and after 30 s in rat glomerulosa cells (255). (The applied fluorimetric technique could record the immediate depolarizing effect of K^{+}.) In patch-clamp studies on rat glomerulosa cells, using the whole cell configuration, 200 nM ANG II evoked depolarization after an average lag time of 24 s (408). In patch-clamp experiments using the perforated patch technique, depolarization induced by 10^{-10} to 10^{-8} M ANG II began later than 1 min (323). These data suggest that depolarization-triggered voltage-operated Ca^{2+} influx cannot account for the generation of the initial (approximately first 15 s) Ca^{2+} signal.

In fluorimetric studies, the Mn^{2+}-quench technique is a sensitive method of detecting Ca^{2+} influx. In rat glomerulosa cells, in contrast to the rapid quenching effect of a physiological elevation of [K^{+}], 25 nM ANG II had no quenching effect within 150 s. Mn^{2+} exerted a rapid quenching effect only if added several minutes after the application of ANG II (516). This observation is in accordance with measurements of the initial 45Ca influx rate in rat (107, 504) and bovine glomerulosa cells (301), stimulated with nanomolar ANG II. Accordingly, these studies indicate that ANG II-induced Ca^{2+} influx has a lag time of >150 s. Nevertheless, the initial phase of ANG II-induced Ca^{2+} signal was modified by organic Ca^{2+} channel drugs in bovine (7, 81, 302), human (63), and rat glomerulosa cells (229). In our studies on rat glomerulosa cells, nifedipine and the benzothiazepine diltiazem reduced and BAY K 8644 augmented the amplitude of the ANG II-induced Ca^{2+} peak (attained at 13–15 s after adding the agonist) (255, 516). It is important to emphasize that nifedipine does not inhibit T-type Ca^{2+} current in these cells (502).

The lack of any contribution of enhanced Ca^{2+} influx to the generation of the initial Ca^{2+} signal was also confirmed by examination of the signal when Ca^{2+} influx was blocked by 5 mM Ni^{2+}. Nifedipine was capable of diminishing the ANG II-induced cytoplasmic Ca^{2+} signal even under such conditions (516).

These observations suggest that activation of L-type channels by a G protein (e.g., G_{i}, cf. Refs. 196, 236, 296, 344) modifies IP_{3}-induced Ca^{2+} release. Because nifedipine did not decrease ANG II-induced formation of IP_{3} (516), an effect of L-type channels on the function of IP_{3}R had to be assumed (516). In this respect it should be recalled that a population of the IP_{3}-sensitive calcium-releasing vesicles is cytoskeletally attached to the plasma membrane (166, 471). Moreover, considering the protein-protein interaction between type 1 RyRs and L-type Ca^{2+} channels (Ca_{1, 1.1}) in skeletal muscle, as well as the significant amino acid identity between the cytoplasmic NH_{2}-terminal loops of RyR and IP_{3} receptors (181, 536), coupling between L-type channels (Ca_{1.2} and 1.3) and subplasmalemmal IP_{3}Rs can be hypothesized.

The assumption of an interaction between L-type channels and IP_{3} receptors is compatible with observations on both smooth and skeletal muscle cells. Ca^{2+} release from IP_{3}-sensitive stores in coronary myocytes, induced by acetylcholine (but not by supramaximal concentrations of IP_{3}), was potentiated by depolarization through a mechanism that did not require extracellular Ca^{2+} (190). Spontaneous membrane depolarizations (termed slow waves) in visceral smooth muscle also did not require Ca^{2+} influx, but were dependent on Ca^{2+} release from IP_{3}-sensitive stores. Nevertheless, their frequency increased at more depolarized potentials (559). The response of peeled skeletal muscle fibers to exogenous IP_{3} was significantly larger when t tubules were in a depolarized state (138). Although the authors emphasized the role of E_{m} in controlling the effect of IP_{3}, it should be recalled that depolarization changes the conformation of L-type channels.

Sustained formation of IP_{3}R, leading to prolonged IP_{3}-induced Ca^{2+} release, is a prerequisite for sustained enhancement of aldosterone production (21). Therefore, modulation of IP_{3}-dependent Ca^{2+} release by dihydropyridines should influence both Ca^{2+} signal and aldosterone production throughout the duration of stimulation with ANG II. Although our model still requires direct evidence, it presently offers an explanation for the effects of channel-regulating drugs on ANG II-induced Ca^{2+} and aldosterone responses in the face of an inhibited state of L-type channels.

6. Ca^{2+} influx induced by K^{+}

As discussed in section II, the glomerulosa cell is characterized by a dominant K^{+} conductance. The high density of the background (TASK) K^{+} channel makes it an excellent sensor for [K^{+}]_{i}. K^{+}-induced depolarization, which almost perfectly follows the Nernst equation, activates voltage-operated Ca^{2+} channels. Raising [K^{+}]_{i} gradually to 20 mM evokes a gradual increase of [Ca^{2+}]_{i} (432, 444, 445, 450). The K^{+}-induced Ca^{2+} signal is nonoscillating and long-lasting (28, 89, 448, 450, 466).

In rat glomerulosa cells, the activation threshold of T-type Ca^{2+} channels in the presence of 2–2.5 mM Ca^{2+} is between −70 and −80 mV, and half-maximal steady-state inactivation occurs at −73 mV (444, 563). At membrane potentials where T-type channels can be activated and inactivation is incomplete, as indicated by the intersection of peak current and steady-state inactivation curves (in function of E_{m}), a small but sustained Ca^{2+} current (termed window current) should occur. Because window currents were usually analyzed using a high concentration of Ca^{2+} or Ba^{2+} as charge carrier, no precise data are available for physiological conditions. Assuming that window current begins at −80 mV at physiological extracellular [Ca^{2+}], T-type channels may participate in eliciting...
the Ca$^{2+}$ signal during stimulation with physiologically elevated [K$^+$].

The question still remains whether the high voltage-activated L-type channels also contribute to Ca$^{2+}$ influx. Because Ca$^{2+}$ response to $-6.0$ mM K$^+$ (28, 33, 229) is enhanced by the L-type specific DHP agonist BAY K 8644, it may be assumed that a fraction of L-type channels is already open under moderately depolarized conditions. Consistent with this possibility are observations on the effect of mebafradil, which inhibits T-type channels much more effectively than L-type channels. The effect of mebafradil on K$^+$ induction of the aldosterone production by rat glomerulosa cells was gradually attenuated as a function of [K$^+$] (i.e., with increasing depolarization), indicating that above 6 mM [K$^+$] a shift occurs from T-type to L-type channels in the generation of Ca$^{2+}$ influx (323). Rossier et al. (472) examined the effect of nifedipine and calcizeptin on Ca$^{2+}$ currents, [Ca$^{2+}$], and aldosterone production in bovine glomerulosa cells. Their results support the previous data, indicating that in K$^+$-stimulated cells aldosterone production correlates with T-type channel activity.

Barrett et al. (33) examined aldosterone production by bovine glomerulosa cells over a wide range of [K$^+$]. From 3 to 20 mM K$^+$ the concentration-response curve was similar to that described for rat glomerulosa cells, a biphasic function peaking at 10 mM. However, there was a second increase in aldosterone production between 20 and 30 mM. The voltage dependence of the aldosterone production rate and that of the predicted Ca$^{2+}$ influx through T- and L-type channels were similar, suggesting that both types of Ca$^{2+}$ channels may support steroid production. Within the potential range studied (−76 to −23 mV) no Ca$^{2+}$ influx would be carried exclusively via one or the other channel population, but their contribution would depend on $E_m$. The calculations show that at 6 mM K$^+$ T-type channel influx would be 90% of maximal, whereas that of L-type channel influx would attain 10% of maximal.

Rat glomerulosa cells may respond to an elevation of [K$^+$] as small as 0.5 mM with Ca$^{2+}$ signals (563) and increased activity of mitochondrial NAD(P)H formation (432). Raising [K$^+$] from 3.6 to 4.6 mM may double aldosterone production (432). Although window current (see above) may account for the activation of T-type channels despite the tiny shift of $E_m$, the phenomenon still seems to be disproportionate and requires more complete elucidation. A possible mechanism for increasing the sensitivity of glomerulosa cells to K$^+$ is an increase in cell volume. Hyposmosis-induced swelling enhances T-type Ca$^{2+}$ current and, hence, K$^+$-induced Ca$^{2+}$ signal (334). The enhanced Ca$^{2+}$ response is not sensitive either to cytochalasin D or colchicine (335). When [K$^+$] is raised from 3.6 to 5 mM, a steep initial rise in [Ca$^{2+}$]$_c$ is followed by a slower further rise, and several cells display a second phase of rapid rise, superimposed on the already elevated [Ca$^{2+}$]$_c$. This second phase coincides with the detectable onset of swelling. The postinitial elevation of [Ca$^{2+}$]$_c$ may be prevented by keeping cell volume constant, with application of KCl in a hyperosmotic medium (335). This observation indicates that increased cell volume sensitizes T-type channels to depolarization. Raising [K$^+$]$_c$ from 4 mM to the supraphysiological level of 7 mM also induces swelling in bovine glomerulosa cells (231). However, the mechanism sensitizing bovine cells to elevated [K$^+$] seems to differ from that observed in rat glomerulosa cells. The Ca$^{2+}$ signal evokes a CaMKII-mediated shift of the voltage dependence of the activation of T-type channels (34, 327), more specifically that of the pore-forming subunit $\alpha_{III}$ (but not that of $\alpha_{Ic}$) (586) to more negative potential values. Therefore, it appears that increased cell volume exerts a positive feed-forward effect in rat, and elevated [Ca$^{2+}$]$_c$ exerts a positive feedback effect on the voltage threshold of T-type channels in bovine glomerulosa cells, and these mechanisms contribute to the generation of Ca$^{2+}$ responses to elevation of [K$^+$].

Summarizing the mechanism of K$^+$-induced Ca$^{2+}$ signaling, increased K$^+$ in the physiological concentration range enhances Ca$^{2+}$ influx through T-type channels. This response may be amplified by K$^+$-evoked cell swelling and/or the developing Ca$^{2+}$ signal itself. At high [K$^+$], which still occurs in vivo, activation of some L-type channels contributes to Ca$^{2+}$ signaling, rendering Ca$^{2+}$ signal generation and aldosterone secretion DHP sensitive. At pharmacological K$^+$ concentrations, T-type channels are inactivated and continuous Ca$^{2+}$ influx takes place through L-type channels. (The role of cAMP in K$^+$-elicited cell response is discussed in section nB.)

7. Na$^+$/Ca$^{2+}$ exchange

The sodium-calcium antiporter, which exchanges three Na$^+$ for one Ca$^{2+}$ and is therefore electrogenic, is an important regulator of [Ca$^{2+}$]$_c$ (for review, see Ref. 496). In most cell types, at least under resting conditions, it brings about net Ca$^{2+}$ efflux. Its presence in rat glomerulosa cells was indicated by the Na$^+$ dependence of Ca$^{2+}$ transport. The efflux rate of $^{45}$Ca was decreased when extracellular [Na$^+$] was reduced, whereas $^{45}$Ca influx was accelerated by ouabain-induced increase in intracellular [Na$^+$] (253). [Ca$^{2+}$]$_c$ also depends on extracellular [Na$^+$] in bovine glomerulosa cells (295).

Benzamil derivative inhibitors of the antiporter attenuated the effects of extracellular (295) and cytoplasmic (Na$^+$) (253) on Ca$^{2+}$ uptake and also inhibited ANG II-induced aldosterone production (253, 557). The drug reduced the initial influx rate of $^{45}$Ca and the exchangeable (60-min) pool size of calcium in control cells and inhibited the stimulatory effect of 25 nM ANG II, added 10 min earlier, on $^{45}$Ca uptake (504). The driving force for Ca$^{2+}$
ANG II reduces the exchangeable Ca\textsuperscript{2+}/H\textsuperscript{+} exchange and thus raises [Na\textsuperscript{+}]\textsubscript{c}, in several cell types, and such an effect of PKC has also been suggested in glomerulosa cells (117). These observations indicate the participation of the antiporter in the calcium metabolism of glomerulosa cells, and also suggest that it contributes to Ca\textsuperscript{2+} influx during exposure to ANG II (382). Benzamil derivatives compete with Na\textsuperscript{+} for its binding site. However, since they accumulate inside the cell where they have to compete with Na\textsuperscript{+} present at low concentration, they may inhibit Ca\textsuperscript{2+} influx more efficiently than Ca\textsuperscript{2+} efflux. Therefore, although these drugs provide a means to reveal the participation of the antiporter in an examined mechanism, their effect on Ca\textsuperscript{2+} influx may be masked by a more pronounced inhibition of Ca\textsuperscript{2+} influx. Thus the data on their effect on Ca\textsuperscript{2+} exchange and to prolong the sustained phase of the Ca\textsuperscript{2+} signal (519). However, the fact that ANG II reduces the exchangeable Ca\textsuperscript{2+} pool (27, 107, 154, 289) in spite of the enhanced Ca\textsuperscript{2+} influx requires further consideration. Extrusion of Ca\textsuperscript{2+} from the cytoplasm by the plasmaembral Ca\textsuperscript{2+}-ATPase and the Na\textsuperscript{+}-Ca\textsuperscript{2+} antiporter is enhanced by the increase in [Ca\textsuperscript{2+}]\textsubscript{c} and is partly opposed by reduction of the membrane PIP\textsubscript{2} pool, which attenuates the activity of the Ca\textsuperscript{2+} pump. The proposed inhibition of the antiporter by ANG II could mean a further blunting of Ca\textsuperscript{2+} loss. In view of the potential significance of such a mechanism, direct evidence in favor of the inhibition of the antiporter by the 12-HETE-p38 MAPK pathway should be provided by unidirectional radioflux measurements, at low ANG II concentrations.

8. Ca\textsuperscript{2+}-eliminating mechanisms

Mechanisms eliminating Ca\textsuperscript{2+} from the cytoplasm include the plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA), the sarcoplasmic/endoplasmatic Ca\textsuperscript{2+}-ATPase (SERCA), and the Na\textsuperscript{+}-Ca\textsuperscript{2+} antiporter. The function of the latter has been discussed in the previous subsection. In view of the current topic, it should be emphasized that PMCA is activated by acidic phospholipids (e.g., PIP\textsubscript{2}) and CaM (91). For lack of studies on the glomerulosa cells, in all other respects we refer to general reviews (e.g., Refs. 200, 330).

C. Diacylglycerol-PKC and Lipoxygenase Pathways

Rasmussen and co-workers (294) proposed that sustained responses to Ca\textsuperscript{2+}-mobilizing agonists would be initiated by Ca\textsuperscript{2+}, while protein phosphorylation by PKC (or PKA) would account for the sustained phase of steroid production. Their postulation was based mainly on studies on bovine glomerulosa cells, in which stimulation of PKC with tetradecanoylphorbol acetate (TPA) slightly increased aldosterone production per se and efficiently potentiated the effect of a Ca\textsuperscript{2+} ionophore. The kinetics of hormone production stimulated by the combined application of TPA and the ionophore were similar to that of ANG II-induced production (294). In view of the cell-damaging effect of prolonged elevation of [Ca\textsuperscript{2+}]\textsubscript{c}, this dual system was considered to have advantages over a simple, Ca\textsuperscript{2+}-mediated system (32, 289, 292). Occasionally, phosphorylation by PKA would serve a similar purpose (291).

PKC was originally regarded as a Ca\textsuperscript{2+} and phospholipid-dependent protein-phosphorylating enzyme. Today, the large family of PKCs is known to consist of at least a dozen isoenzymes with various activation characteristics and substrate specificities. While the classical forms are activated by DAG, high [Ca\textsuperscript{2+}] may attenuate the DAG requirement of some isoforms and arachidonate may also be an efficient activator (135, 170, 388).

DAG is produced simultaneously with IP\textsubscript{3} from PIP\textsubscript{2} by the action of PLC. In rat glomerulosa cells ANG II evokes a biphasic increase in DAG production, with an early peak at 30 s and a later one at 10 min (378). In the
bovine glomerulosa cell an early peak (at ~30 s), detected if sampling is frequent enough (248, 297), is followed by a second phase, maintained for several tens of minutes (67, 248, 297). DAG may also derive from phosphatidylcholine by the combined activity of phospholipase D (PLD) and phosphatidic acid phosphohydrolase (60). It has been suggested by Bollag and co-workers (66, 68, 273) that, during the sustained phase of stimulation with ANG II, the activation of PLD contributes to the formation of DAG. It is worth mentioning that in K⁺-stimulated cells, weak activation of PLC by Ca²⁺, obviously through the formation of DAG, results in the moderate activation of PKC (218).

In spite of the convincing logic of the dual-control system, the role of PKC in the control of aldosterone secretion is highly controversial. The criteria for accepting such a role are the following: 1) translocation of PKC from the cytosol into the membrane fraction in response to the respective stimulus; 2) induction of the biological response with DAG analogs or an active phorbol ester; and 3) inhibition of the biological response with a specific inhibitor of the enzyme (e.g., staurosporine) or by means of depleting the cell of activable PKC (with several hours of exposure to a high concentration of an active phorbol ester). We will group the data in the literature according to these criteria.

ANG II brings about the translocation of PKC from the cytosol into the particulate fraction in bovine (226, 291, 308) as well as rat glomerulosa cells (377). The enzyme also translocates to the membrane fraction in response to high [Ca²⁺]c (171), and ANG II-induced activation of PKC may depend on concomitant Ca²⁺ influx (288).

At variance with the reports of Rasmussen’s group, TPA or 1-oleoyl-2-acetylgllycerol, a DAG analog, added to bovine glomerulosa cells failed to increase basal aldosterone production (187). These drugs also inhibited the effect of ANG II and K⁺ on T-type current, [Ca²⁺]c, and the aldosterone response (12, 187, 470). TPA augmented aldosterone production in freshly isolated human adrenocortical cells (305) but failed to do so in the H295R human adrenocortical cell line (110). In the rat, TPA-stimulated aldosterone production was observed exclusively in adrenal capsular tissue (569). In contrast, TPA inhibited basal as well as ANG II-stimulated, K⁺-stimulated, and ACTH-stimulated hormone production by isolated glomerulosa cells (218). TPA induced the phosphorylation of the cholesterol carrier protein StAR (see sect. viA) in bovine glomerulosa cells (54, 226) but not in rat cells (226). TPA, in contrast to ANG II, also failed to increase the steady-state StAR mRNA level in human (H295R) glomerulosa cells (110). PKC, at least in the hamster glomerulosa cell, also depresses the expression of aldosterone synthase (314, 315). Overall, these data are not compatible with the proposed secretagogue action of PKC.

In one report the PKC inhibitor Ro31–8220 reduced ANG II-stimulated aldosterone production in rat glomerulosa cells (277). On the other hand, in our studies the PKC antagonist staurosporine potentiated the aldosterone response to K⁺ and enhanced the initial (30-min) phase of ANG II-induced aldosterone production, but failed to influence the effect of ACTH. The enzyme inhibitor enhanced the initial (30 min) phase of the ANG II-induced aldosterone production (218).

A more straightforward means of preventing PKC effects is by depletion of the enzyme. When PKC activity in both the cytosol and the membrane fraction was reduced below the control level by long-lasting exposure to TPA, aldosterone production induced by physiological stimuli (300 pM ANG II or 5.4 mM K⁺) was significantly enhanced (218). [In K⁺-stimulated cells the activation of PKC was secondary to a weak activation of PLC by Ca²⁺ (218).] PKC depletion failed to alter ANG II-induced aldosterone production in the experiments of Aguilera and co-workers (377), who also refuted the involvement of PKC in the stimulation of aldosterone production by ANG II.

The contradictions in the PKC literature may be attributed to several factors. Determination of enzyme activity has several pitfalls, including lack of appropriate substrate specificity as well as limited proteolysis of the enzyme during homogenization, which leads to a Ca²⁺ and phospholipid-independent kinase activity. TPA or other DAG analogs may be anchored in the whole cell membrane rather than at confined domains around the (probably clustered) ANG II receptors. Therefore, PKC may translocate to nonspecific sites of the membrane, resulting in artifacts. In view of the moderate specificity of kinase C blockers, their inhibitory effects may be inconclusive about the role of PKC. The species-dependent and cell type-dependent expression of different isoforms of the enzyme, often exerting opposing effects (135), should also be kept in mind. In summary, the bulk of evidence indicates that PKC has an inhibitory, rather than stimulatory, role in the acute control of aldosterone secretion.

The hydrolysis of phosphoinositides by PLA₂ results in the release of arachidonate, a precursor of eicosanoids. Since PLA₂ is not activated in ANG II-stimulated rat glomerulosa cells (244), any arachidonate released should originate from DAG. This means that the role of DAG, formed by the hydrolysis of PIP₂, may not be confined to the activation of PKC, but may also serve as an inducer of additional signaling pathways. The arachidonate derivatives, prostaglandins E₂ and A₂, stimulate aldosterone production (511, 517). Nevertheless, cyclooxygenase inhibitors do not counteract the induction of aldosterone production by ANG II, K⁺, or ACTH. Therefore, we must
reject the physiological role of prostaglandins in the control of aldosterone secretion (164). However, the observation showing that the aldosterone-stimulating effect of arachidonate cannot be blocked by cyclooxygenase inhibitors (164) suggests a role for some other arachidonate derivative in the control of steroid production (164).

Rat glomerulosa cells metabolized arachidonate to hydroxyeicosatetraenoic acids (HETEs) by lipoxygenases and epoxyeicosatrienoic acids (EETs) by a cytochrome P-450 epoxygenase, but no leukotrienes were detected (84). A DAG lipase inhibitor, which prevents the release of arachidonate, blocked ANG II-induced 12-HETE and aldosterone formation. In contrast, a DAG kinase inhibitor, which prevents the conversion of DAG to phosphatidic acid, potentiated 12-HETE and aldosterone production (84). A DAG kinase inhibitor, which prevents the release of arachidonate from DAG is activated by a separate mechanism, should be further studied.

12-HETE itself induced Ca\(^{2+}\) release from intracellular stores as well as Ca\(^{2+}\) influx from the extracellular fluid, whereas lipoxygenase inhibitors inhibited the effect of ANG II on [Ca\(^{2+}\)]\(_{i}\) and aldosterone production (520). 12-Lipoxygenase products activate p38 MAPK (382), which, in turn, was reported to reduce Ca\(^{2+}\) efflux (519) (see sect. uB7). More recent experiments applying molecular biological techniques (199) gave strong support to these data, and also indicated that activation of p38 MAPK leads to increased expression of the CYP11B2 gene product, aldosterone synthase (cytochrome P-450\(_{aldo}\)). Overall, these data support the assumption that 12-HETE is required for a full response of the glomerulosa cells to ANG II. Nevertheless, it does not seem to be sufficient to evoke maximal aldosterone response, since 12-HETE production in cells overexpressing 12-lipoxygenase was several-fold higher than that in ANG II-exposed cells transfected with empty vector, yet aldosterone production attained comparable (submaximal) levels in the two groups. The participation of 12-HETE in ANG II-induced aldosterone secretion is shown in Figure 6.

Whether the formation of 12-HETEs should be regarded as a consequence of PIP\(_2\) hydrolysis only, or the release of arachidonate from DAG is activated by a separate mechanism, should be further studied.

In view of the presumably increased formation of arachidonate, another important issue worth examination is whether ANG II activates an arachidonic acid-dependent non-store-operated Ca\(^{2+}\) channel before the activation of store-operated (capacitative) Ca\(^{2+}\) channels, as recently observed in other cell types (reviewed in Ref. 69).

D. Vasopressin: A Transiently Acting Paracrine Agonist

Several neurotransmitters may reach the glomerulosa cells, either from splanchic nerve endings (528, 571, 572) or from the chromaffin cells, located in medullary rays within the zona glomerulosa (185). The most studied Ca\(^{2+}\)-mobilizing paracrine agonist, vasopressin, binds to the pressor (V\(_1\)) type receptor in glomerulosa cells. Activation of the V\(_1\) receptor elicits the breakdown of PIP\(_2\), induces Ca\(^{2+}\) signaling, and stimulates aldosterone production in rat (22, 205, 589) and human glomerulosa cells (206).

In rat glomerulosa cells, ANG II and AVP, applied at concentrations equipotent in terms of the phosphoinositide response, stimulated initial aldosterone production to the same extent, but only ANG II elicited a sustained response. AVP does not maintain aldosterone production at a high level, in spite of activating phosphoinositide turnover for at least 2 h. The early decay in AVP-induced aldosterone production is not due to some form of inhibition, since AVP does not attenuate ANG II-stimulated aldosterone production (160). The Ca\(^{2+}\) signals, elicited by the two agonists, have different kinetics. AVP-induced oscillations decay within 15 min, while ANG II (evoking a similar phosphoinositide response) induces sustained Ca\(^{2+}\) oscillations as well as prolonged and significantly greater aldosterone production (447). In contrast to G\(_i\)-mediated inhibition of L-type Ca\(^{2+}\) channels in ANG II-stimulated rat glomerulosa cells (see sect. uB), AVP was reported to stimulate pertussis toxin-sensitive (i.e., G\(_{160}\)-mediated) Ca\(^{2+}\) influx through such channels (196, 204).
AVP exerts a weak inhibition on T-type \( \text{Ca}^{2+} \) channels (196). In view of the significance of T-type \( \text{Ca}^{2+} \) current in the stimulatory action of ANG II, the AVP-induced inhibition of T-type channels may be a factor responsible for the transient character of vasopressin action. Activation of PKC (183) may also result in a gradually developing inhibition of aldosterone secretion.

### III. EFFECT OF CYTOPLASMIC CALCIUM ON MITOCHONDRIAL FUNCTION

The adrenal cortex and the gonads do not store steroids; they secrete de novo synthesized hormones. Side-chain cleavage of cholesterol and all except one (C-21) of hydroxylation steps take place in mitochondria which occupy 25–30% of the cytoplasmic volume in glomerulosa cells (392). The mitochondrial hydroxylations require NADPH, which is generated from NAD\(^+\) by the mitochondrial nicotinamide nucleotide transhydrogenase (222, 240). The role of \( \text{Ca}^{2+} \) in the control of mitochondrial function in glomerulosa cells has been a neglected area of research.

Following the earlier assumption that the role of mitochondria in intracellular calcium metabolism is confined to situations of calcium overload, it became firmly established that physiological changes in \([\text{Ca}^{2+}]_c\) also influence mitochondrial metabolism. The renaissance of mitochondrial studies (431) began by the observations of Denton, McCormack, and co-workers (351), who demonstrated in mitochondrial homogenates and suspensions that \( \text{Ca}^{2+} \) activates three mitochondrial dehydrogenases: the pyruvate, isocitrate, and oxoglutarate dehydrogenase. It was also found in suspended cardiac mitochondria that physiological fluctuations in their calcium content alters \([\text{Ca}^{2+}]_m\) in the range that regulates the matrix dehydrogenases (328). We provided evidence for the physiological significance of the cytoplasmic-mitochondrial \( \text{Ca}^{2+} \) relationship in intact cells in 1992, observing that in rat glomerulosa cells \( \text{K}^+ \) stimulates the formation of mitochondrial pyridine nucleotides, NADH plus NADPH (designated as NAD(P)H) in a \( \text{Ca}^{2+} \)-dependent manner (432). Activation of voltage-dependent \( \text{Ca}^{2+} \) channels in sensory neurons also increased the formation of NAD(P)H (145). These data suggested the transfer of cytoplasmic \( \text{Ca}^{2+} \) signal into the mitochondrial matrix, an assumption that was directly demonstrated in the same year following the successful targeting of the \( \text{Ca}^{2+} \)-sensitive luminescent protein aequorin into the mitochondria (461). Since that time, the mitochondrial \( \text{Ca}^{2+} \) and/or NAD(P)H response to cytoplasmic \( \text{Ca}^{2+} \) signal has been described in several cell types (reviewed in Refs. 147, 214, 458). Mitochondria, by buffering the changes in \([\text{Ca}^{2+}]_c\), often reshape the \( \text{Ca}^{2+} \) signal and may also modify the function of IP\(_3\)R (146). In glomerulosa cells the mitochondrial response to ANG II (78, 433, 466) and AVP (466) have also been reported, but no data on signal reshaping are available.

The question may be raised whether stimuli of physiological intensity, i.e., physiological increases in \([\text{Ca}^{2+}]_c\), are also effective in eliciting the mitochondrial response. Elevating \([\text{K}^+]\) from 3.6 to 4.1 mM slowly induces a small cytoplasmic \( \text{Ca}^{2+} \) signal (563) and enhances the formation of NAD(P)H (432). However, this observation was in conflict with the low affinity of the mitochondrial \( \text{Ca}^{2+} \) uptake mechanism, which predicts that submicromolar \( \text{Ca}^{2+} \) signals would fail to affect net \( \text{Ca}^{2+} \) uptake by the mitochondria. Mitochondria contain calcium at millimolar concentration; however, its major fraction is complexed with phosphate and ATP or bound to matrix protein. Under resting conditions the concentration of ionized calcium in the matrix of glomerulosa cells is comparable to that in the cytoplasm (428). The mitochondrial outer membrane is assumed to be permeable to \( \text{Ca}^{2+} \) (but see Ref. 125), and the barrier of \( \text{Ca}^{2+} \) diffusion is the inner mitochondrial membrane. The transport of \( \text{Ca}^{2+} \) through this membrane has been reviewed by Gunter et al. (207). Briefly, \( \text{Ca}^{2+} \) uptake via the ruthenium-sensitive \( \text{Ca}^{2+} \) uniporter is driven by the 150–180 mV (inside negative) mitochondrial membrane potential. The half-maximal transport rate is attained in the range of \( 10^{-6–10^{-4}} \text{ M} \) \( \text{Ca}^{2+} \), and external \( \text{Ca}^{2+} \) exerts a positive cooperative effect with a Hill coefficient of 2. Efflux of \( \text{Ca}^{2+} \) occurs by means of a \( \text{Na}^+-\text{Ca}^{2+} \) and/or a \( \text{H}^+-\text{Ca}^{2+} \) antiporter, the former having been detected also in the adrenal cortex (123, 477). The capacity of the efflux mechanism is low, and it saturates under conditions when \([\text{Ca}^{2+}]_c\) reaches \( \sim 0.5 \text{ mM} \). At this \([\text{Ca}^{2+}]_c\), the plasmalemmal \( \text{Ca}^{2+} \) pump also saturates, and therefore further elevation of \([\text{Ca}^{2+}]_c\) results in steeply increasing net \( \text{Ca}^{2+} \) flux into the mitochondria. These data led to the still-prevailing view that net mitochondrial \( \text{Ca}^{2+} \) uptake occurs only if \([\text{Ca}^{2+}]_c\) exceeds the micromolar, but at least the half-micromolar level. Excessive uptake of \( \text{Ca}^{2+} \) may activate the mitochondrial permeability transition pore, resulting in the, often fatal, loss of ions and molecules smaller than 1,600 Da (reviewed, e.g., in Ref. 46). Due to the lack of relevant data in regard to glomerulosa cells, we do not discuss this issue in more detail.

The global \( \text{Ca}^{2+} \) signal, as measured in the cytoplasm, is the average of highly focused, transient, elementary changes of \( \text{Ca}^{2+} \) (50), limited by the diffusion of \( \text{Ca}^{2+} \) in the cytoplasm (e.g., Ref. 6). Therefore, the uptake of \( \text{Ca}^{2+} \) by, and the ensuing activation of, mitochondria will depend primarily on the distance between the \( \text{Ca}^{2+} \) source and the mitochondrion. Since ER vesicles may form contacts with mitochondria (338, 457), the narrow space around such contact points could be presumed to be an optimal site for the activation of mitochondria. The discovery of rapidly forming but short-lived high-\( \text{Ca}^{2+} \) microdomains in the perimitochondrial cytoplasm near
the IP$_3$-sensitive calcium stores (460) provided an explanation for the large mitochondrial Ca$^{2+}$ signal (216, 459) evoked by IP$_3$-mediated agonists. In fact, activation (125) or overexpression (453) of the Ca$^{2+}$-permeable voltage-dependent anion channel in the outer mitochondrial membrane enhances the IP$_3$-induced (but not the thapsigargin-induced) mitochondrial Ca$^{2+}$ signal, indicating the special role of high-Ca$^{2+}$ microdomains at the ER-mitochondrial contact sites. Similarly, the abrupt increase in subplasmalemmal [Ca$^{2+}$] during voltage-activated Ca$^{2+}$ influx may be responsible for the activation of mitochondrial metabolism in insulin-producing INS-1 cells (280) and neurons (429). The observation that in rat glomerulosa cells IP$_3$-induced Ca$^{2+}$ release more effectively induces the formation of mitochondrial NAD(P)H than Ca$^{2+}$ influx (467) may also be attributed to the IP$_3$-induced formation of perimitochondrial high-Ca$^{2+}$ microdomain. The low affinity of the Ca$^{2+}$ uniporter, and the formation of high-Ca$^{2+}$ microdomains, led to the general view that the rapid formation of micromolar [Ca$^{2+}$] in the perimitochondrial space is essential for the activation of mitochondria.

In steroid-producing cells, physiological stimuli induce submicromolar Ca$^{2+}$ signals. The activation of mitochondrial dehydrogenases is not, however, confined to the action of the IP$_3$-induced Ca$^{2+}$ release in glomerulosa (432, 433, 466) or luteal cells (525), capacitative Ca$^{2+}$ influx also induces mitochondrial response in either cell type (467, 525). The increase in [Ca$^{2+}$]$_{\text{im}}$ and the enhanced formation of NAD(P)H occur in spite of the obvious absence of high-Ca$^{2+}$ microdomains, since [Ca$^{2+}$]$_{\text{c}}$ increases gradually (giving time for Ca$^{2+}$ diffusion from the plasma membrane to more remote sites in the cytoplasm) and does not attain 200 nM (219, 525). Therefore, the transfer of submicromolar Ca$^{2+}$ signals into the mitochondrial matrix had to be assumed. We have measured the effect of buffered [Ca$^{2+}$]$_{\text{c}}$ on [Ca$^{2+}$]$_{\text{im}}$ with the fluorescent dye rhod 2 in permeabilized rat glomerulosa cells (428). [Ca$^{2+}$]$_{\text{im}}$ correlated with [Ca$^{2+}$]$_{\text{c}}$ in the examined range of 60–740 nM, and an increase in [Ca$^{2+}$]$_{\text{im}}$ could be detected already on raising [Ca$^{2+}$]$_{\text{c}}$, from 60 to only 140 nM. The mitochondrial response was small but reproducible and was associated with an increased level of NAD(P)H. In rat luteal cells [Ca$^{2+}$]$_{\text{im}}$, as measured either with the fluorescent dye rhod 2 or with the mitochondrially targeted luminescent protein aequorin, showed similar dependence on [Ca$^{2+}$]$_{\text{c}}$ (428, 515). Moreover, the increased formation rate of NAD(P)H during capacitative Ca$^{2+}$ influx has also been documented in this cell type (525). This unexpected responsiveness is not unique to steroid-producing cell types in which the formed NADPH is a cofactor of steroid hydroxylation. It also occurs in insulin-producing INS-L/EK-3 cells, where increased formation of NADH, via ATP, induces the specific biological response of the cell, the exocytosis of insulin (428).

The mitochondrial response to submicromolar increases in [Ca$^{2+}$]$_{\text{c}}$ seems to be a hitherto neglected, yet more-or-less general phenomenon. Our data are in harmony with the results of direct measurements of cytoplasmic-mitochondrial Ca$^{2+}$ relationship in excitable cells such as chromaffin cells (15) and ventricular myocytes (602), as well as in the nonexcitable HeLa cells (115) and osteosarcoma cells (428). Indirect measurements of [Ca$^{2+}$]$_{\text{im}}$ (114, 179, 429) in chromaffin and neural cells also support the view that submicromolar Ca$^{2+}$ signal can be transferred into the mitochondrial matrix. The RBL-2H3 cell line with a response threshold of 2 $\mu$M [Ca$^{2+}$]$_{\text{c}}$ seems to be an exception; however, in this cell type even the outer mitochondrial membrane exhibits restricted permeability to Ca$^{2+}$ (125).}

A comparison of data from different laboratories indicates that glomerulosa and luteal cells, the two hitherto examined steroid-producing cell types, exhibit the greatest mitochondrial sensitivity to cytoplasmic Ca$^{2+}$ signals. Interestingly, this sensitivity is reflected not only by the response threshold (below 200 nM [Ca$^{2+}$]$_{\text{c}}$) but also, at least in the case of glomerulosa cells, by kinetic data. Similar to other cell types, oscillating Ca$^{2+}$ signals often evoke an oscillating mitochondrial Ca$^{2+}$ (Fig. 1) and NAD(P)H response in glomerulosa cells (433, 466). What makes a difference from several other cell types is that the sustained cytoplasmic Ca$^{2+}$ signal, induced by K$^+$, elicits sustained mitochondrial Ca$^{2+}$ (514) and NAD(P)H (432) level. Similarly, elevated [Ca$^{2+}$]$_{\text{c}}$, evoked by high concentrations of ANG II (466), or achieved by cell permeabilization (466), is associated with sustained rises in the mitochondrial NAD(P)H level. These observations contrast with the behavior of hepatocytes, in which only oscillatory Ca$^{2+}$ signals, but not sustained rises in [Ca$^{2+}$]$_{\text{c}}$, can induce sustained mitochondrial Ca$^{2+}$ and NAD(P)H responses (216, 433, 462). The special coupling between [Ca$^{2+}$]$_{\text{c}}$ and mitochondrial metabolism in the glomerulosa cell reflects adaptation to physiological demands. Physiological stimuli of hepatocytes induce oscillating Ca$^{2+}$ signals, whereas glomerulosa cells respond to physiological K$^+$ stimuli with a sustained Ca$^{2+}$ signal. HeLa cells may represent the other extreme of mitochondrial kinetics, since not even trains of repetitive Ca$^{2+}$ oscillations can maintain elevated [Ca$^{2+}$]$_{\text{im}}$ (115). The physiological significance of the ability to sustain mitochondrial response in the glomerulosa cell is obvious, the adrenocortical responsiveness to sustained exposure to hyperkalemia is essential for vertebral homeostasis, and the sustained enhancement of mitochondrial NADPH formation may efficiently support the long-term hypersecretion of aldosterone under such a condition (see sect. vii). The significance of cell type-specific mitochondrial Ca$^{2+}$ management has been recently demonstrated also in synaptic transmission (61).
IV. CROSS-TALK BETWEEN CALCIUM AND cAMP-ACTIVATED PATHWAYS

A. Ca\(^{2+}\) Influx Evoked by ACTH

Although ACTH acts primarily through cAMP, as originally observed in the adrenal fasciculata zone (232), it also stimulates Ca\(^{2+}\) influx in glomerulosa cells. Both ACTH and 8-bromo-cAMP induce a sustained Ca\(^{2+}\) response after a lag time of ~10 min in rat glomerulosa cells (551). ACTH also elicits a Ca\(^{2+}\) signal, with a lag time of ~2 min, in human glomerulosa cells (184). Such responses are caused by activation of L-type Ca\(^{2+}\) channels (151, 184). The ability of β-adrenergic stimulation to enhance ANG II-induced Ca\(^{2+}\) responses is caused by activation of L-type Ca\(^{2+}\) channels in rat adrenal capsules (434) may result from the same mechanism. The potentiation of ANG II-induced Ca\(^{2+}\) influx and aldosterone secretion in rat adrenal capsules (434) may result from the same mechanism. The potentiation of ANG II-induced Ca\(^{2+}\) influx may exert positive feedback on the formation of cAMP itself and could also support stimulated steroidogenesis (Fig. 7) by the means discussed in section VII. cAMP, via PKA, may also potentiate IP\(_3\)-induced Ca\(^{2+}\) release (80, 215), an effect that has not been examined in the glomerulosa cell.

Serotonin, potentially released by neighboring chromaffin or mast cells, also stimulates aldosterone production (370) and enhances ANG II-induced Ca\(^{2+}\) influx (463). In rat glomerulosa cells, serotonin via the 5-HT\(_7\) receptor increases both cAMP and Ca\(^{2+}\) levels. The primary action of serotonin is on the Gs-mediated activation of adenylyl cyclase, leading to cAMP formation and a slowly developing Ca\(^{2+}\) signal. T-type Ca\(^{2+}\) current was enhanced both by 8-bromo-cAMP and serotonin, and the latter effect was reduced by a PKA inhibitor. Serotonin also induced a slow and sustained high-threshold current, probably via L-type Ca\(^{2+}\) channels (Fig. 7a in Ref. 316). This activation resembles the L-channel activating action of ACTH. Although activation of T-type channels by cAMP is an unprecedented observation, it is noteworthy these studies were performed in freshly isolated cells, whereas other laboratories have examined glomerulosa cells cultured for 1–2 days.

B. Ca\(^{2+}\)-Induced Formation of cAMP

Although the messenger role of cAMP has been well established, the steroidogenic action of ACTH also requires Ca\(^{2+}\). In its major target organ, the adrenal zona fasciculata, chelation of extracellular Ca\(^{2+}\) prevents the corticosterone response to even high doses of ACTH, but only reduces the response to dibutyryl cAMP. This disparity indicates that the Ca\(^{2+}\) requirement in the action of ACTH is more important for events before the formation of cAMP (220).

Studies on glomerulosa cells have also demonstrated the Ca\(^{2+}\) dependence of ACTH action. After an initial increase, the sustained phase of ACTH-stimulated aldosterone secretion by superfused rat glomerulosa cells is completely abolished in the absence of Ca\(^{2+}\) (503). The question can be raised again whether Ca\(^{2+}\) is required for the formation or the effect of cAMP. Studies with the organic Ca\(^{2+}\) channel inhibitor verapamil (26, 488) indicate that the activation of adenylyl cyclase has a higher requirement for Ca\(^{2+}\) than activation of steroid synthesis by cAMP. This conclusion was also supported by measurements showing that ACTH-induced cAMP production is highly Ca\(^{2+}\) dependent, and only a very small and transient increase in cAMP is observed in the absence of Ca\(^{2+}\) (184).

Although extracellular Ca\(^{2+}\) is essential for the binding of ACTH to its receptors (92), Ca\(^{2+}\) also exerts postreceptorial effects. Studies by Taits’ group (258) revealed that stepwise elevation of K\(^+\) from 3.6 to 8.9 mM progressively increased cAMP secretion from rat glomerulosa cells. This response required extracellular Ca\(^{2+}\) and was prevented with nifedipine, indicating that adenylyl cyclase was stimulated by Ca\(^{2+}\) entering through voltage-operated Ca\(^{2+}\) channels (258). In contrast, K\(^+\) did not induce Ca\(^{2+}\) signaling and did not influence cAMP output in fasciculata cells (5). While these observations indicated an interaction between

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**Diagram:**

- **depolarization**
- **T-type Ca\(^{2+}\) channel**
- **L-type Ca\(^{2+}\) channel**
- **Ca\(^{2+}\) influx**
- **ACTH**
- **adenylyl cyclase**
- **G\(_s\)**
- **cAMP**
- **PKA**
- **IP\(_3\) receptor**
- **cholesterol transport & metabolic effects**

**FIG. 7.** Interaction of the Ca\(^{2+}\)-cAMP signaling pathways. The red arrows indicate stimulatory actions.
the cAMP and Ca$^{2+}$ signaling pathways in glomerulosa cells, there were also conflicting reports on this proposal. More recently, Tait and Tait (532) provided a critical review of the literature, considering the methodological problems arising from the various biological preparations, sample for analysis, and artifacts related to phosphodiesterase inhibition by isobutyl methylxanthine. Their conclusion was consistent with the original concept that Ca$^{2+}$, entering the cell in response to K$^+$, activates adenylyl cyclase and cAMP formation that prolongs the stimulation of steroid production. Considering the slight but reproducible activation of phospholipase C by K$^+$ (218), Tait and Tait also proposed that “the action of increased extracellular K$^+$ can potentially involve all known mechanisms for the stimulation of steroidogenesis in endocrine cells.”

Tait's observation suggests that a Ca$^{2+}$-dependent isoform of adenylyl cyclase is expressed in rat glomerulosa cells. Out of the nine well-characterized isoforms of adenylyl cyclase, types 1, 3, and 8 are stimulated by Ca$^{2+}$, provided that $G_{i\alpha}$ is present. The PKC-activated cyclases AC2 and AC7 may be also subject to Ca$^{2+}$ control through Ca$^{2+}$-dependent PKC (10). Type AC3 is expressed in bovine (82) and human glomerulosa cells (120). AC1 could not be detected (493), and no data are available on the expression of other Ca$^{2+}$-dependent isoforms in rat glomerulosa cells.

In rat glomerulosa cells, ANG II reduces, rather than enhances, cAMP production (43, 587), and inhibition of PKA by H-89 does not affect ANG II- and AVP-induced aldosterone production (184). The failure of the Ca$^{2+}$ signal to activate adenylyl cyclase may be due to activation of the inhibitory G protein, $G_i$, a phenomenon observed in bovine cells (97, 326, 344).

ANG II also inhibits adenylyl cyclase activity in purified membranes from the bovine zona glomerulosa (339), yet increases in basal and potentiation of ACTH-induced adenylyl cyclase activity by ANG II have been observed in intact cells (39, 82). Pharmacological data suggest that the enhancement of the cAMP response by ANG II involves the Ca$^{2+}$-dependent and PKC-dependent phosphatase calcineurin (39). This finding may account for the positive interaction of ANG II and ACTH.

What is the significance of Ca$^{2+}$-induced or Ca$^{2+}$-amplified cAMP production in the glomerulosa cell? As shown in section vA, cAMP-dependent phosphorylation may exert positive feedback on Ca$^{2+}$ influx. Enhancement of cholesterol transport into mitochondria is discussed in section vA. Activation of cell metabolism by cAMP also supports increased hormone production, but the details of this effect are beyond the scope of this review.

The interaction of the cAMP and Ca$^{2+}$ signaling pathways is shown in Figure 7.

V. REGULATION AT THE LEVEL OF PLASMA MEMBRANE RECEPTORS

Continuous stimulation of cells with hormones and other stimulators very rarely evokes a continuous biological response. Although some agonists cause increased sensitivity, most activate mechanisms that reduce the biological response of the cell and protect it from excessive stimulation. Desensitization is defined as the tendency of responses to reduce in intensity despite the continued presence of the stimulus (313). Agonist-induced cellular desensitization may occur at several different levels. In this section, changes in cellular responsiveness that occur at the level of plasma membrane receptors will be discussed. Mechanisms that regulate more distal elements of the signal transduction pathway have been discussed previously. After a brief introduction to the regulation of GPCRs, special attention will be given to the regulation of angiotensin receptors in adrenocortical cells.

A. Mechanisms for Regulation of GPCRs

The accepted paradigm of GPCR regulation involves their homologous and heterologous desensitization, receptor internalization, and downregulation. Desensitization and internalization are rapid phenomena that develop in minutes, or in some cases seconds, after the stimulation of GPCRs, whereas downregulation is a much slower decrease in the total cellular receptor pool.

Homologous desensitization of a GPCR involves the uncoupling of the receptor from its cognate G protein, after stimulation of the receptor with its agonist. This process is associated with agonist-induced phosphorylation of the receptor. In most cases, specific GPCR kinases (GRKs) appear to be responsible for this phosphorylation. Receptor phosphorylation promotes the binding of $\beta$-arrestin molecules, rather than the respective G protein, to the receptor (108, 303). However, in some receptors other kinases, such as casein kinases or PKA, might serve as receptor kinase (16, 549, 574). Furthermore, recent studies on metabotropic glutamate receptors suggested that binding of GRKs to the activated receptor can cause phosphorylation-independent desensitization (136). After prolonged stimulation, significant accumulation of desensitized receptors may occur, depending on the kinetics of receptor internalization and recycling (see sect. vD), but pharmacological agonist concentrations are often required to detect cellular desensitization induced by this mechanism. However, the importance of homologous desensitization should not be underestimated since it selectively affects agonist-activated receptors, which undergo rapid uncoupling from G proteins by this mechanism limiting the duration of the signal generation after recep-
tor activation. Thus this mechanism is more important in desensitizing signaling of the receptor in time than in desensitizing the hormonal responsiveness of the tissue. However, if continuous supply of plasma membrane receptors (e.g., via new receptor synthesis or receptor recycling) is not available, prolonged agonist stimulation may lead to depletion of the nondesensitized plasma membrane receptor population, and homologous desensitization of the tissue may occur.

Heterologous desensitization is caused by phosphorylation of GPCRs during stimulation of the cell with agonist(s) that target different receptors. This process is mediated by second messenger-induced stimulation of protein kinases, such as PKA or PKC. Heterologous desensitization typically occurs at lower hormone concentrations than homologous desensitization, since full activation of the second messenger generation may occur, when receptor occupancy is still low. Activated second messenger-regulated kinases can phosphorylate and effectively desensitize both active and inactive receptors, in which the consensus site for the kinase is present. Because desensitization of a large receptor population may occur at relatively low hormone concentrations, this mechanism is an important regulator of the agonist sensitivity of hormone target tissues (reviewed in Ref. 313).

Agonist binding also stimulates internalization of the receptor from the plasma membrane into intracellular vesicles. Although clathrin-independent mechanisms (e.g., via caveolae) have also been reported, endocytosis via clathrin-coated vesicles is the most well-defined mechanism of internalization of GPCRs. The process is preceded by agonist-induced phosphorylation and \( \beta \)-arrestin binding to the activated receptor. \( \beta \)- Arrestin, in addition to its role in homologous desensitization, serves as an adapter molecule to connect the receptor to clathrin and \( \beta_2 \)-adaptin, two main components of the clathrin coat (108, 252, 303). During this mechanism, internalization follows rather than precedes desensitization because the internalized receptors are already uncoupled from the G protein by \( \beta \)-arrestins.

Intracellular trafficking of internalized receptors occurs via acidic endosomal compartments, in which the ligand can dissociate and the receptor can be dephosphorylated by intracellular phosphatases (312). After these steps the resensitized receptor may recycle to the plasma membrane. Some internalized receptors also move from the endosomes to the lysosomes for degradation. Whereas receptor recycling mediates receptor resensitization after homologous desensitization, lysosomal degradation causes downregulation of the receptor (see below). Some GPCRs, such as the AT1 receptor, are preferentially targeted for recycling, whereas others, such as the thrombin receptor, are destined for degradation (550). Such targeting is determined by amino acid sequences, usually in the cytoplasmic tail of the receptor, that appear to interact with specific proteins that affect intracellular sorting (86, 550, 578).

Whereas the term internalization is applied to the translocation of cell surface receptors into intracellular compartments with no major change in total receptor number, downregulation signifies a decrease in the total cellular receptor pool. This decrease may result from both increased receptor degradation and reduced receptor synthesis, and usually develops over hours or days. Although receptor degradation, preceded by agonist-induced endocytosis, appears to be a major mechanism of downregulation, mutations that affect receptor internalization do not always interfere with the extent of downregulation (228). This finding indicates the potential importance of impaired receptor synthesis in the process. Long-term agonist stimulation frequently reduces receptor transcription, or induces mechanisms that destabilize receptor mRNA. These mechanisms cause internalization-independent downregulation by decreasing the steady-state level of the receptor population (reviewed in Ref. 487).

**B. Regulation of Adrenal Angiotensin Receptors In Vivo**

AT1 receptors are present in very high density in glomerulosa cells. Because the biological response correlates with the formation of a defined number of hormone-receptor complexes, consistent with the law of mass action, the higher the number of surface receptors the lower the agonist concentration required for maximal stimulation. Thus, due to the high receptor density, maximal steroidogenic response occurs at ANG II concentrations that only partially saturate the available receptors. The higher the number of excess ("spare") receptors, the more left-shifted is the dose-response curve for the biological response in comparison to the receptor saturation curve. The presence of a high receptor reserve explains the finding that the EC\(_{50}\) of ANG II-induced aldosterone response is about one order of magnitude lower than the \( K_d \) of the receptor in glomerulosa cells (411). This consideration is also valid for in vivo conditions. Because circulating levels of ANG II (see the first paragraph of sect. ii) are well below the \( K_d \) of AT1 receptors (see sect. iv), physiological elevations of ANG II levels that only partially saturate the receptor can elicit full stimulation of steroid secretion. Regulation of ANG II receptors in the zona glomerulosa also frequently affects the magnitude of the receptor reserve. Upregulation of AT1 receptors creates a larger receptor reserve, enhances the potency of ANG II to stimulate aldosterone secretion, consequently increasing the sensitivity of the tissue. However, a reduction of the number of functional surface receptors (e.g., by downregulation) decreases receptor reserve and causes a right-shift in the dose-response curve for steroi-
Striatal dependence. In this situation, desensitization of the tissue does occur because aldosterone secretion will be reduced at any given circulating ANG II level.

In most ANG II target tissues, prolonged agonist activation causes desensitization of its biological response and downregulation of its receptors. However, in vivo studies in rats have shown that long-term (1- to 2-day) infusion of low doses of ANG II causes upregulation of adrenal ANG II receptors and increases the aldosterone response to ANG II (141, 227). The hormonal response was similarly enhanced by ANG II infusion in humans (395). It was also observed several decades ago that sodium depletion (known to stimulate renin secretion) enhances the aldosterone secretory response to ANG II in humans, rats, and dogs (reviewed in Refs. 369, 570). Because low-sodium diet increases AT1A and AT1B receptor mRNAs in rat adrenal glands, and this increase is inhibited by an AT1 receptor blocker (144), it is likely that ANG II induces upregulation of AT1 receptors during sodium deficiency. Increased receptor binding of ANG II during sodium depletion and ANG II infusion was originally attributed to a positive feedback effect of ANG II on its receptors (227). Although molecular biological studies supported the receptor-inducing effect of ANG II (267), and this mechanism seems to explain upregulation of AT1B receptors (573), the effect of sodium depletion is primarily induced via an ANG II-AT1 receptor-independent mechanism (63, 573). Furthermore, in extra-adrenal tissues of other species, and even in the zona glomerulosa of dogs, prolonged low-dose infusion of ANG II reduces the responsiveness of the target tissue to ANG II (40). Downregulation of adrenal angiotensin receptors was also observed in rats following in vivo long-term administration of pharmacological doses of ANG II (416). Because ANG II also exerts extra-adrenal effects and, in the sodium-depleted rat, changes in potassium balance (75, 541) can also modulate adrenal ANG II responsiveness (140), the upregulation of adrenal AT1 receptors in sodium deficiency may be predominantly due to an indirect effect of the hormone. In agreement with this, as detailed in section vC, studies with isolated glomerulosa and fasciculata cells found no evidence of ANG II-induced upregulation of angiotensin receptors. Overall, these findings suggest that the in vivo upregulation of angiotensin receptors is an indirect effect of agonist treatment or requires the presence of cofactors, such as ACTH or other circulating hormones, which are absent in experiments with isolated cells.

C. Desensitization of Glomerulosa Cells

Desensitization of the receptor for several hormones, including ANG II, ACTH, bradykinin, and vasopressin, has been shown in mammalian glomerulosa cells (106, 167, 183), and similar mechanisms operate in the adrenal glands of lower vertebrates (70). Early studies showed that ANG II-induced steroid secretion is subject to homologous and heterologous regulatory mechanisms in glomerulosa (167) and fasciculata cells (411). These experiments demonstrated that ANG II directly reduces adrenal responsiveness and does not cause upregulation of ANG II-induced aldosterone responses, even in cells pretreated with low physiological concentrations of ANG II. In isolated rat glomerulosa cells, superfusion of ANG II for 6 h reduced the aldosterone responses to ANG II, potassium ions, and ACTH. This effect was maintained when superfusion with ANG II was performed in the presence of aminogluthethimide to prevent steroid synthesis, suggesting that the inhibitory effect was caused by factors other than substrate depletion or a metabolite of steroid synthesis (e.g., free radical). The ANG II-induced heterologous desensitization of the aldosterone response was attributed to inhibition of the late stage of aldosterone biosynthesis. Preincubation with ANG II evoked homologous desensitization of the ANG II-induced inositol phosphate generation, suggesting that this process involves mechanisms proximal to signal generation, such as receptor desensitization or downregulation. Phorbol ester treatment did not elicit such desensitization, suggesting that it was independent of PKC activation (163).

In other studies on the mechanism of ANG II-induced desensitization of bovine adrenocortical cells, short-term (30-min) pretreatment of glomerulosa cells with 10 nM ANG II caused rapid loss of125I-ANG II binding. Scatchard analysis revealed that exposure to ANG II induced the transformation of high-affinity ANG II binding sites (Kd ~0.2 nM) to a low-affinity state (Kd ~2 nM) with no change in the total number of extracellular binding sites (73). This homologous desensitization was suggested to be caused by PKC- and calmodulin kinase-independent uncoupling of AT1 receptors from G proteins. ANG II binding to AT2 receptors did not show similar regulation. In analogy to the β2-adrenergic receptor and other GPCRs (313), the most likely mechanism for ANG II-induced rapid homologous desensitization of AT1 receptors is their phosphorylation by receptor kinases, such as GRKs. Phosphorylation-induced uncoupling of the receptor from its cognate G protein(s) can explain the observed reduction in receptor affinity, since interaction with G proteins is believed to stabilize the high-affinity conformation of the receptor (484). In fact, ANG II rapidly induces phosphorylation of the AT1 receptor in bovine glomerulosa cells. Such phosphorylation was correlated with the degree of agonist occupancy of the receptor and was not affected by tyrosine kinase inhibitors. Also, it was stimulated, rather than reduced, by PKC inhibitors (501). These data suggest that receptor phosphorylation, which possibly causes early homologous desensitization of the receptor and may be responsible for the above-mentioned ef-
fect of ANG II on the affinity of AT_1 receptors, is mediated by receptor kinase(s).

Although the receptor kinase that mediates this effect in glomerulosa cells has not been identified, studies with AT_1 receptors expressed in tumor cell lines suggest that GRKs have an important role in the ANG II-induced rapid phosphorylation of AT_1 receptors. Coexpression of a kinase-deficient GRK2 exerted dominant negative inhibitory effect on ANG II-induced receptor phosphorylation in HEK293 (399) and COS7 cells (398). Oppermann et al. (399) also observed that in HEK293 cells the kinase-deficient GRK2 prevented homologous desensitization of the AT_{1A} receptor, whereas inhibition of PKC failed to affect this process (399). Nevertheless, studies in other cell types question the general role of GRK2 in homologous desensitization of AT_1 receptors. In COS7 cells, inhibition of receptor phosphorylation by coexpression of a kinase-deficient GRK2 did not affect the desensitization of the receptor (398). Also, a study in CHO cells suggested that homologous desensitization of AT_{1A} receptors is mediated by a heparin-sensitive kinase that is different from GRKs (538). In the latter cell line, desensitization of AT_{1B} receptors is mediated by PKC (539). These data indicate that the identification of the receptor kinase responsible for this process in glomerulosa cells requires additional studies. Nevertheless, it is likely that β-arrestin binding to the phosphorylated receptor has an important role in the uncoupling of the receptor molecule from the G protein, since it has been firmly established that ANG II induces association of these molecules with the cytoplasmic tail of AT_1 receptors (8, 173, 443).

Much less is known about the desensitization of AT_2 receptors. It has been reported that no homologous desensitization of AT_2 receptors occurs in bovine adrenocortical cells, but heterologous desensitization of AT_2 receptors caused by stimulation of AT_1 receptors has been reported (402). The latter effect was mimicked by activation of PKC, which is consistent with the PKC-mediated stimulation of AT_2 receptor phosphorylation observed in transfected COS-7 cells (397).

D. Intracellular Trafficking of Angiotensin Receptors

ANG II-induced angiotensin receptor internalization in glomerulosa cells was first described by Bianchi et al. (58), who showed in the rat that injected ^{125}I-ANG II first accumulates on the cell surface, then clusters within coated pits, is internalized in coated vesicles, and appears in lysosomes within 20 min. Internalization of radiolabeled ANG II was also reported in cultured bovine adrenocortical cells (124). Immunocytochemical localization of AT_1 receptors using receptor specific antibodies has also been reported in glomerulosa cells (501, 567). Detailed analyses of the mechanism of intracellular trafficking of AT_1 receptors have been performed in expression systems using epitope-tagged (233) or green fluorescent protein (GFP)-labeled (96, 249, 362) receptors. Endocytosis of a GFP-tagged rat AT_{1A} receptor after stimulation with rhodamine-labeled ANG II is demonstrated in Figure 8. Endocytosis of surface-bound radiolabeled ANG II occurs very rapidly in bovine glomerulosa cells (254, 455), which express predominantly AT_1 receptors (see sect. vA). Incubation of adrenocortical cells with ANG II causes progressive loss of surface angiotensin receptors (402, 411). In fasciculata cells, half-maximal loss of surface receptors occurred at an ANG II concentration (~3 nM) similar to the K_d (2 nM) of the receptor, which resembles the dose dependence of homologous desensitization (see sect. vB). The EC_{50} of ANG II to stimulate this response was much higher than that to induce steroidogenesis (411). ANG II is unable to induce internalization of AT_2 receptors in cells that selectively express AT_2 receptors (233, 251, 397, 438). In accordance with these findings, no internalization of AT_2 receptors was detected in bovine fasciculata cells (402).

Most of the available data suggest that the main mechanism of agonist-induced endocytosis of AT_1 receptors in glomerulosa cells is endocytosis via clathrin-coated vesicles. In rat glomerulosa cells, morphological studies detected internalized angiotensin receptors in coated pits (58). Disruption of the clathrin coat by potassium depletion or phenylarsine oxide treatment markedly inhibits AT_1 receptor endocytosis in bovine glomerulosa cells and other ANG II target tissues (252, 254). In COS-7 cells the kinetics of AT_{1A} receptors are somewhat faster than that of AT_{1B} receptors (256). Internalization of expressed AT_1 receptors is independent of G protein coupling (247) and appears to be regulated by agonist-induced phosphorylation of the receptor molecule (251, 502, 543, 544). The receptor kinase that regulates the internalization process has not been identified, since PKC inhibitors and/or coexpression of a kinase-deficient GRK markedly reduced agonist-induced phosphorylation of AT_1 receptors without affecting its internalization kinetics (30, 31, 398). Nevertheless, β-arrestins associate with the ANG II-stimulated and phosphorylated receptors in tumor cells expressing AT_1 receptors (8, 443, 599). Coexpression of functionally deficient, mutant β-arrestins and dynamin with the AT_1 receptor exerts dominant negative inhibitory effect on the internalization of the receptor at physiological hormone concentrations (182, 443), and internalization of AT_1 receptors is strongly impaired in cells derived from mice deficient in β-arrestins (287). These data provide a mechanism for endocytosis of the AT_1 receptor via clathrin-coated pits, and it is likely that endocytosis of AT_1 receptors in glomerulosa cells is mediated by a similar mechanism. However, at higher ANG II concentrations, when the AT_1 receptor population becomes rapidly
saturated with the agonist, this mechanism apparently becomes saturated and a β-kinase- and dynamin-independent mechanism becomes predominant (182, 600). Although caveolae have been suggested as another possible mechanism for AT<sub>1</sub> receptor endocytosis (265), this requires further studies, since endocytosis via caveolae is also dynamin dependent (234). The exact mechanism of β-arrestin- and dynamin-independent endocytosis of AT<sub>1</sub> receptors and its importance in adrenal cells has yet to be elucidated.

Studies in HEK 293 cells using fluorescent markers have demonstrated that early endosomes, recycling endosomes, and multivesicular bodies related to late endosomes participate in the intracellular trafficking of internalized AT<sub>1</sub> receptors (249, 491). Acidification of the endosomal compartments is required for efficient recycling of adrenocortical and expressed AT<sub>1</sub> receptors to the cell surface (124, 233, 411, 455). Acidification causes agonist dissociation from many GPCRs, including angiotensin receptors, and the resulting conformational change is believed to promote the dissociation of β-arrestins from internalized receptors and dephosphorylation of the receptor molecule (312). The rapid ANG II-induced loss of plasmalemmal AT<sub>1</sub> receptors in adrenocortical cells treated with monensin, an inhibitor of vesicular acidification, suggests that recycling of the receptor to the plasma membrane is a highly efficient process (411).

It was initially assumed that receptor internalization mediates agonist-induced desensitization of the cells by reducing the number of surface receptors. However, at physiological hormone concentrations many activated GPCRs, including AT<sub>1</sub> receptors, use β-arrestins as adapter proteins to connect with clathrin-coated structures (182, 303). Because β-arrestins also uncouple the receptors from their G proteins, the internalized receptors are already desensitized. Thus endocytosis of AT<sub>1</sub> receptors, similar to that of other GPCRs, could serve to maintain signal generation, since it leads to intracellular dephosphorylation, resensitization, and recycling of receptors (312). This mechanism may explain the previously reported importance of receptor internalization for the sustained phase of ANG II-induced inositol phosphate and Ca<sup>2+</sup> signal generation in bovine glomerulosa cells (254). Some of the internalized AT<sub>1</sub> receptors are also targeted to lysosomes for degradation, and during prolonged ANG II stimulation this process could eventually cause degradation of ANG II and downregulation of the AT<sub>1</sub> receptors in bovine adrenocortical cells (124, 411). It has also been proposed that endocytosis of AT<sub>1</sub> receptors is required for ANG II-induced PKC translocation (568). There is no ev-
idence in adrenal cells for a role of β-arrestins and/or receptor internalization in ANG II-induced MAPK activation (see also sect. viC). Intracellular accumulation of ANG II also prolongs the half-life of the peptide, which is rapidly degraded in the circulation, and may be utilized for paracrine and autocrine actions, or could exert intracellular actions by stimulating cytoplasmic or nuclear angiotensin receptors. However, in adrenocortical cells the importance of these mechanisms has not been established. This topic was reviewed in more detail previously (252).

E. Receptor Downregulation

Downregulation of receptors is defined as a decrease of total number of receptors (313), which typically requires agonist exposure for at least 1 h and may involve modulation of the rate of receptor synthesis or degradation. Long-term incubation of adrenocortical cells with high concentrations of ANG II has been suggested to cause downregulation of angiotensin receptors (402, 411). However, since the available studies determined surface binding of the receptor, and the decrease during the first 3 h was caused by internalization of surface receptors, it is unclear whether true downregulation of total cellular binding sites occurred during this period. More prolonged (more than 3 h) stimulation with pharmacological concentration of ANG II reduces the level of AT1 receptor mRNA (402, 456). This decrease in mRNA levels is caused by inhibition of transcription, without significant change in the half-life of the mRNA (402). Interestingly, in the same study stimulation of AT1 receptors also reduced the binding sites and the mRNA of AT2 receptors, but this effect was mediated by a decrease in AT2 receptor mRNA stability. AT1 receptor-mediated regulation of AT2 receptors was also observed in expression systems, where it has been shown to cause PKC-mediated phosphorylation of AT2 receptors (397).

Downregulation of AT1 receptors may also be caused by increased receptor degradation, which is preceded by internalization of the receptor into endosomal compartments. It has been shown in bovine adrenocortical cells that after internalization, cellular processing of the hormone-receptor complex leads to degradation of the ligand. The acidic pH of endosomal compartments, which facilitates hormone dissociation, is also essential for ligand degradation (124). Studies in HEK 293 cells have suggested that most internalized AT1 receptors recycle to the plasma membrane, whereas the hormone dissociates in acidic compartments and enters lysosomes for degradation (233). However, it is likely that a small fraction of receptors undergoes lysosomal degradation, and after prolonged incubation with ANG II, this leads to increased receptor loss. It is presently unclear whether ubiquitylation of the AT1 receptor has a role in its targeting to lysosomes, but recent studies indicated that elimination of the lysine residues that serve as potential ubiquitylation sites causes increased accumulation of labeled ANG II in CHO cells expressing AT1A receptors (358). This finding raises the possibility that ubiquitylation has a role in the regulation of intracellular targeting of the receptor.

A recent study has questioned the importance of short-term AT1 receptor desensitization and long-term receptor downregulation in ANG II-induced regulation of glomerulosa cells, because desensitization of the steroidogenic action of ANG II required 24-h preincubation with a pharmacological concentration (1 μM) of ANG II, whereas 10 nM ANG II had no effect on the steroidogenic response (456). However, the steroidogenic response was analyzed using only maximally effective concentrations of ANG II, which may not reveal changes in cellular responsiveness in cells with excess receptors. In fact, earlier studies showed that ANG II pretreatment had a much larger effect on ANG II-induced steroidogenesis in rat and bovine glomerulosa cells (167, 411). Also, rapid desensitization of second messenger production was observed after ANG II treatment in AT1 receptor-transfected cells (399, 539). Thus the available data indicate that ANG II-induced desensitization or reduction of plasma membrane AT1 receptors causes desensitization of adrenocortical cells.

VI. LONG-TERM EFFECTS OF ANGIOTENSIN II

Long-term effects of ANG II at the cellular level include regulation of cell growth, proliferation, differentiation and apoptosis, induction of steroidogenic enzymes, modulation of cell migration, and extracellular matrix deposition. These effects are mediated by complex and highly cell-specific signaling pathways, involving G proteins, receptor and nonreceptor tyrosine kinases, MAPKs, and cytokine-like signaling pathways (132). In this paper we focus on mechanisms that have been reported to be present in aldosterone-producing cells, and we refer to recent reviews for mechanisms identified in other ANG II target cells (132, 152, 483).

A. Activation of Growth Responses by ANG II in the Glomerulosa Cell

Rat glomerulosa cells respond to sodium deficiency with hypertrophy and hyperplasia and undergo regression in animals on a high-sodium diet. These changes are dependent on the level of circulating ANG II, which has proven to be a major determinant of trophic changes in the zona glomerulosa (see references in Refs. 132, 372). These studies provided the first evidence that ANG II stimulates cell growth and/or proliferation in its target
tissues, a phenomenon which has since been shown in many other cell types, including vascular smooth muscle cells, cardiac myocytes and fibroblasts, and renal mesangial cells (132). Pharmacological studies have revealed that the growth-promoting actions of ANG II are mediated by the AT\(_1\) receptor and that ANG II binding to AT\(_2\) receptors has an opposing effect in many tissues (390).

The growth-promoting effects of ANG II are exerted directly by the hormone, because the stimulation observed in vivo can be demonstrated in isolated glomerulosa cells. However, lipoygenase-mediated formation of arachidonic acid metabolites (see sect. nC) has been suggested as a contributing factor in this process (379). In rat (345) and bovine (547) glomerulosa cells, ANG II stimulates cell growth and increases thymidine incorporation and cell proliferation. Activation of PKC, tyrosine kinases, and MAPKs, leading to increased expression of several early genes, including c-fos, c-jun, and c-myc, is believed to play a central role in these processes (29, 109).

**B. Role of Receptor and Nonreceptor Tyrosine Kinases**

In rat glomerulosa cells, parallel roles of tyrosine kinase and PKC activation in ANG II-mediated stimulation of DNA synthesis and cell proliferation have been reported and are independent of PLA\(_2\), cyclooxygenase, and lipoygenase activation (345). Endothelins, acting via the ET\(_{A}\) endothelin receptor, also stimulate proliferation of glomerulosa cells by activating tyrosine kinases and PKC (347). Endothelins, ghrelin, and proadrenomedullin-derived peptides exert proliferative actions on glomerulosa cells by stimulating tyrosine kinase-dependent activation of the p42/p44 MAPK pathway (9, 44, 346). The specific tyrosine kinases and their mode of action during ANG II-induced proliferation of adrenal glomerulosa cells have not yet been identified. It has also been proposed that ANG II promotes capacitative calcium entry and aldosterone biosynthesis in these cells via tyrosine kinases (11, 65, 277). Furthermore, tyrosine kinases have long-term effects on the synthesis of steroidogenic enzymes, and in the H295R cell line Src tyrosine kinase was reported to have a role in maintaining the glomerulosa-like phenotype (499).

In smooth muscle and other target cells, ANG II also promotes cell migration and induces changes in cell shape and volume by activating focal adhesion kinase. It stimulates the closely related tyrosine kinase, Pyk2, which is an important link between ANG II-mediated Ca\(^{2+}\) signal generation and the growth factor-regulated signaling pathways (152, 211). ANG II can also activate the Jak family of tyrosine kinases, which mediate the actions of cytokine-type receptors on transcription via phosphorylation of STAT (signal transducers and activators of transcription) proteins (57, 340). However, additional studies are required to demonstrate the roles of these mechanisms in adrenal glomerulosa cells.

Transactivation of receptor tyrosine kinases, such as the epidermal growth factor receptor and platelet-derived growth factor receptor or Axl, has been proposed to have a major role in ANG II-induced proliferation of vascular smooth muscle and other target cells (152, 482) (Fig. 9). EGF receptors are present in glomerulosa cells, as evidenced by the stimulatory effect of EGF on the aldoste-

![Fig. 9. Mechanism of ERK1/2 activation in glomerulosa cells. The thickness of the arrows reflects the quantitative importance of the pathways in glomerulosa cells, as described in text. R, receptor.](http://physrev.physiology.org/)
rone production of porcine glomerulosa cells (282), but
the relevance of EGF receptor transactivation to the pro-
liferative action of ANG II has not been elucidated in
these cells.

C. ANG II-Induced Activation of MAPKs

MAPKs are serine/threonine kinases that have a cen-
tral role in the regulation of gene expression by plasma
membrane receptors. Receptor tyrosine kinases and
GPCRs can stimulate phosphorylation cascades leading
to concomitant phosphorylation of MAPKs on adjacent
threonine and tyrosine residues. Activated MAPKs trans-
locate to the nucleus and regulate transcription factors
gene expression, resulting in cell-specific long-term
cellular responses such as cell growth, apoptosis, and
differentiation (270). The functional outcome of MAPK
activation is dependent on the availability of downstream
substrates.

Mammalian subtypes of MAPKs are grouped into six
major subfamilies, such as ERK1/2 (also known as p42/p
44 MAPKs), c-Jun NH2-terminal protein kinases (JNKs,
or stress-activated protein kinases, SAPKs), p38 MAPKs,
ERK6, ERK3, and ERK5 (or Big MAPK). Typically, recep-
tor-mediated activation of ERK1/2 regulates cell growth
and differentiation. JNKs and p38 MAPKs are activated by
cellular stress and inflammatory cytokines, which regu-
late cellular processes leading to inhibition of cell prolif-
eration and induction of apoptosis, whereas ERK5 has
been proposed as a redox-sensitive MAPK (1). The ability
of ANG II to activate ERK1/2, JNK, p38 MAPK and ERK5
is consistent with its growth-promoting, cytokine-like,
and redox-sensitive actions (132).

In glomerulosa and H295R cells, ANG II-induced ac-
tivation of the ERK, JNK, and the p38 MAPK pathways
has been reported (199, 354, 548, 577). Activation of the ERK
pathway is believed to be the major mediator of the effect
of ANG II on the proliferation of these cells (Fig. 9). As in
other cell types, ANG II causes tyrosine kinase-dependent
activation of the Ras small G protein in bovine and rat
glomerulosa cells, leading to stimulation of Raf-1, a MAPK
kinase, which phosphorylates MEK, a MAPK ki-

nase, and to phosphorylation and activation of ERKs (354,
548). More detailed studies on the mechanism of
ANG II-induced Raf-1 kinase activation have demonstrated
that the PKC-independent stimulation of this kinase is
mediated partly by pertussis toxin-sensitive G_{i/o} proteins
and phosphatidylinositol 3-kinase (500). The proposed
mechanisms of MAPK activation in the glomerulosa cell
are shown in Figure 9.

In H295R cells, high concentrations of ANG II in-
duced prolonged stimulation of ERK and JNK (SAPK)
activity with a maximal response at 5 and 30 min, respec-
tively (577). However, the effect of ANG II on JNK acti-
vation in these cells is controversial (199, 577). ANG II
also caused ras and ERK-dependent increases in cyclin
D1 promoter activity as well as enhanced cyclin D1 mRNA
levels and cyclin D1 protein levels, and promoted cell
cycle progression (577). c-Fos and c-Jun have a role in the
stimulation of the cyclin D1 promoter activity, and the
induction of JNK activity by ANG II may also contribute to
the enhanced transactivation by c-Jun. Since the induc-
tion of cyclin D1 serine/threonine kinase activity pro-
motes cell cycle progression and cellular proliferation,
these effects participate in the mitogenic action of ANG II
in aldosterone-producing cells.

ANG II also stimulates the transcription of the aldo-
sterone synthase (CYP11B2) gene, and the details of this
process are described in section viIC.

VII. THE FINAL ACTION: ROLE OF
CALCIUM IN THE CONTROL OF
STEROID PRODUCTION

Neurons and skeletal muscle cells may exhibit Ca^{2+}
signals that far exceed 10^{-5} M in well-confined microdo-

mains. Such a large Ca^{2+} signal may be required for the
extremely rapid biological response, often occurring
within milliseconds. The release of steroid hormones
takes place by a much slower rate by diffusion across the
plasma membrane. There are no data indicating the gen-

eral mechanism leading to ERK activation was pertussis
toxin insensitive, although a small contribution from per-
tussis toxin-sensitive G_{i/o} proteins could not be excluded.
Although PKC depletion significantly reduced ANG II
induced ERK activation in these cells, ras- and ras-f1 ki-

nase-mediated ERK activation was independent of PKC.
This difference suggests that ras/raf-1 activation repre-
sents a PKC-independent pathway, whereas the major
pathway of ERK activation is mediated by PKC via an
unidentified target downstream to raf-1 kinase, possibly
MEK (548). More detailed studies on the mechanism of
ANG II-induced raf-1 kinase activation have demonstrated
that the PKC-independent stimulation of this kinase is
mediated partly by pertussis toxin-sensitive G_{i/o} proteins
and phosphatidylinositol 3-kinase (500). The proposed
mechanisms of MAPK activation in the glomerulosa cell
are shown in Figure 9.
The amplitude of the \( \text{Ca}^{2+} \) signal is an essential, but not the only, factor controlling \( \text{Ca}^{2+} \)-induced steroid responses. Coapplication of ANG II or thapsigargin, on the one hand, and \( \text{K}^+ \), on the other, stimulates aldosterone production at a rate that far exceeds that attained by simple additive effects. To stimulate aldosterone production with \( \text{K}^+ \) alone, \([\text{Ca}^{2+}]_c\) has to attain a much higher value than that measured in the cells stimulated with two agonists simultaneously (81, 219). This observation led us to postulate (509) that it is not only the size, but also the subcellular localization of the \( \text{Ca}^{2+} \) signal that determines the rate of aldosterone production. It should be recalled in this regard that \( \text{Ca}^{2+} \) released from IP$_3$-sensitive stores is more efficient in activating mitochondrial dehydrogenases than that entering from the extracellular space (467). Also, \( \text{Ca}^{2+} \) entering through voltage-activated \( \text{Ca}^{2+} \) channels into the subplasmalemmal space may be more efficient in activating adenyl cyclase (5, 258).

\( \text{Ca}^{2+} \) exerts a feedback effect on the initial steps of various signal-transducing pathways, but its major action is on the stimulation of steroidogenesis. Its best-elucidated effect is the stimulation of cholesterol transport into the mitochondria, the process that determines the rate of hormone secretion during acute stimulation in each steroid-producing cell type.

**A. Cholesterol Transport**

Steroid-secreting cells do not store hormones; the rate of hormone secretion depends on the de novo synthesis of the cell-specific steroid. The precursor of steroid hormones is cholesterol, which may be synthesized intracellularly from acetyl CoA. However, increased hormone secretion requires its uptake from plasma lipoproteins. Glomerulosa cells utilize both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) as a source of cholesterol (374), and ANG II stimulates the uptake of HDL-cholesterol. This effect of ANG II is due to enhanced expression of scavenger receptor class B type 1 (SR-B1) (98). Cholesterol is stored in esterified form in cytoplasmic lipid droplets and released in response to hormonal activation of cholesterol ester hydrolase. ANG II activates the enzyme via the MAPK extracellular signal-regulated kinase (ERK 1/2)-mediated phosphorylation (87).

The rate-limiting step in steroidogenesis is the conversion of the precursor compound cholesterol to pregnenolone. This step, which results in the removal of a six-carbon unit from cholesterol by the cholesterol side-chain-cleaving enzyme, cytochrome P-450sc (the gene product of \text{CYP11A1}), takes place at the matrix side of the inner mitochondrial membrane. The transport of cholesterol from the cytoplasmic lipid droplets to the mitochondria requires an intact cytoskeleton and is facilitated by specific proteins. While the sterol carrier protein-2 (SCP2) (554) may transport cholesterol to the outer mitochondrial membrane, the limiting step of steroid synthesis is the rate of cholesterol transfer from the outer to the inner mitochondrial membrane through the aqueous phase of the intermembrane space. This transfer is dependent on a “cycloheximide-sensitive, highly labile protein,” the level of which is raised by ACTH via cAMP-dependent phosphorylation (for reviews, see Refs. 497, 521). \( \text{Ca}^{2+} \) also activates the transport of cholesterol into mitochondria and its subsequent side-chain cleavage (223, 299). Elevation of \([\text{Ca}^{2+}]_c\) by either ANG II or via cell permeation results in increased cholesterol content in the inner mitochondrial membrane, and especially in the contact sites between the outer and inner mitochondrial membranes. This effect of \( \text{Ca}^{2+} \) is also sensitive to cycloheximide (100, 101).

Although the previously described “steroidogenesis activating polypeptide” (SAP) (410) has characteristics similar to those of the “labile protein,” the 37-kDa protein (p37) termed steroidogenic active regulating (StAR) protein (169) currently meets the requirements for being identical with the labile protein. StAR is expressed in all steroid-secreting cell types, and its mutation is the cause of congenital lipoid adrenal hyperplasia, an autosomal recessive disorder characterized by impaired synthesis of adrenal and gonadal steroid hormones (318). The half-life of StAR is 3–5 min, which corresponds to that of the labile protein. After its transport into the mitochondrial matrix, its mitochondrial leader sequence is cleaved off to yield the stable 30-kDa intramitochondrial StAR (p30). StAR mRNA and protein are induced via a cAMP-mediated mechanism that causes the enhancement of StAR transcription and/or mRNA stability. ACTH, acting via PKA, phosphorylates and activates both the putative “labile protein” and StAR. Phosphorylation of StAR itself may not be required for mitochondrial import, but phosphorylation is directly linked to the steroidogenic response of the cell to stimulation (521). The crystal structure of its lipid transfer domain suggests that StAR acts by shuttling cholesterol molecules one at a time through the intermembrane space of the mitochondrion (553). However, the experimental data on its site of action are controversial. Some observations suggest that phosphorylated StAR (pp37) exerts its action at the outer mitochondrial membrane and that its further transport into the matrix and conversion to pp30 are irrelevant to cholesterol transport (71). Other observations suggest that phosphorylation of newly synthetized StAR p37, as well as its mitochondrial import and processing to pp30, are essential for enhanced steroidogenesis (14).

Expression and phosphorylation of StAR in glomerulosa cells are enhanced by ACTH as well as by \( \text{Ca}^{2+} \)-mediated stimuli such as ANG II and \( \text{K}^+ \) (54, 101, 111, 156, 226). Elevated \([\text{Ca}^{2+}]_c\), induced by the \( \text{Ca}^{2+} \) channel agonist BAY K 8644 (111) or by application of a \( \text{Ca}^{2+} \) iono-
phore (101), also induced StAR expression. The transcriptional effect of ANG II involves the activation of MAPK ERK 1/2, which represses DAX-1 (401), a transcription factor known to repress the StAR protein (306). Atrial natriuretic hormone, a physiological antagonist of aldosterone secretion, in addition to increasing K⁺ conductance and inhibiting T-type Ca²⁺ channels (see sect. III), also inhibits the transcription of the StAR gene (99). Accordingly, ANF prevents the mitochondrial import of StAR protein and the accumulation of cholesterol in mitochondrial contact sites (99, 156).

**B. Increased Reduction of Pyridine Nucleotides in Mitochondria**

Whereas the action of CaM and CaMKII in supporting aldosterone production is confined to the transport of cholesterol to the inner mitochondrial membrane (422), the cytoplasmic Ca²⁺ signal is transferred into the mitochondrial matrix (see sect. III), and intramitochondrial Ca²⁺ contributes to the stimulation of hormone production. In cycloheximide-treated adrenocortical mitochondria (which are depleted of the cholesterol-transporting labile protein), the conversion of cholesterol (present in the inner mitochondrial membrane) to pregnenolone is stimulated by Ca²⁺. This effect is completely inhibited by ruthenium red, an inhibitor of mitochondrial Ca²⁺ uptake (299). In electropermeabilized bovine glomerulosa cells, the Ca²⁺-induced aldosterone response in the presence of NADP⁺ is also blocked by ruthenium red (90). The mitochondrial Na⁺/Ca²⁺ exchanger extrudes Ca²⁺ from the mitochondria, and its inhibition also augments Ca²⁺-induced steroid production (78). These observations indicate that Ca²⁺ has an intramitochondrial site of action, and this could suggest that the underlying mechanism of this action is the activation of cholesterol transport again. However, intramitochondrial Ca²⁺ does not affect the function of StAR transiently located in the outer mitochondrial membrane (71), and no data are available to support a role of intramitochondrial Ca²⁺ in the transport of cholesterol.

Intramitochondrial NADP⁺ is reduced by transhydrogenation at the expense of NADH. All of the major mitochondrial steps of aldosterone biosynthesis, namely, the side-chain cleavage of cholesterol (yielding pregnenolone), the hydroxylation of 11-deoxycorticosterone to corticosterone, and the conversion of the latter compound to aldosterone, require NADPH. Tricarboxylic acid cycle intermediates support not only the reduction of pyridine nucleotides (439) but also steroid hydroxylation in (mixed) adrenocortical cells (310, 319, 417), including glomerulosa cells (197). Inhibition of the electron transport chain at site 1 by rotenone stimulates the side-chain cleavage of cholesterol, indicating an effect of the accumulated NADPH (222). Both NAD(P)H level and aldosterone production rate exhibit a biphasic response to K⁺, with a maximum at 8.4 mM K⁺ (432). Increased aldosterone production is associated with increased utilization of NADPH, as shown by the finding that the reoxidation of NADPH, formed in response to K⁺, is delayed by the addition of aminoglutethimide, which inhibits the conversion of cholesterol to pregnenolone (466). Although stimulation of mitochondrial metabolism by Ca²⁺ may not be essential for supporting submaximal formation of pregnenolone (441), the bulk of the evidence supports the significance of enhanced NADPH formation during stimulation of hormone secretion.

Utilizing NADH for increased formation of ATP is, obviously, essential during cell stimulation with Ca²⁺-mobilizing agonists. ATP is required for protein phosphorylation by different protein kinases. Furthermore, Ca²⁺ signaling alters not only the Ca²⁺ content of the cytoplasm and intracellular Ca²⁺ stores, but also induces secondary alterations in sodium, potassium, and proton balance. The restoration of intracellular ionic composition by PMCA and SERCA as well as Na⁺-K⁺-ATPase may cause a rapidly developing and prolonged energy demand.
C. Induction of Aldosterone Synthase

Long-term stimulation of the adrenal cortex results in cell proliferation (see sect. vA) and increased synthesis of steroidogenic enzymes. With regard to aldosterone secretion, the increased expression of the enzyme system converting deoxycorticosterone to aldosterone has special significance. In humans and rodents, this conversion is carried out by the zona glomerulosa-specific aldosterone synthase (CYP11B2, also termed cytochrome P-450aldo, or P-450c11AS), the gene product of CYP11B2. [An isofrom of aldosterone synthase, 11β-hydroxylase (P-450c11β), the gene product of CYP11B1, is responsible for 11β-hydroxylation in the zona fasciculata, and also acts as aldosterone synthase in bovine and porcine glomerulosa cells (533, 591).] It has been amply verified in rat, human (H295R), and hamster glomerulosa cells that increases in [Ca2+]i, evoked by ANG II, or K+, or a Ca2+ ionophore, induce the expression of CYP11B2 (62, 112, 315, 485, 592). This effect of Ca2+ is mediated by calcium/calmodulin-dependent protein kinase I (CaMKI) (116). The lag time of Ca2+-induced expression of aldosterone synthase in H295R cells is <6 h, as examined by real-time RT-PCR (W. E. Rainey, personal communication). This lag time accounts for the observation of increased activity of the late stage of aldosterone biosynthesis (conversion of corticosterone to aldosterone) in the chronically, but not in the acutely, sodium-depleted rat (506).

Both CYP11B2, encoding aldosterone synthase in glomerulosa cells, and CYP11B1, encoding 11β-hydroxylase in fasciculata cells, contain a CAMP-response element (CRE) in its promoter region. CaMKI and CaMKIV can phosphorylate the activating transcription factor ATF-1 and CRE-binding protein (CREB) that bind to CREs, but this binding is not sufficient to support CYP11B2 expression. CYP11B2 contains at least two additional cis-acting regulatory elements (Ad-5 and NBRE-1) that are not present in CYP11B1. The ability of these elements to bind two members of the NGFIB family of orphan nuclear receptors, NGFIB and NURR1, suggests that these proteins enhance the expression of CYP11B2 (35). The role of CaMKs in the activation of these factors is still to be elucidated.

PKC, which under acute conditions inhibits rather than stimulates aldosterone production (see sect. nC), depresses the expression of CYP11B2, as observed in the hamster glomerulosa cell (314, 315).

The sites of steroidogenic Ca2+ action are shown summarized in Figure 10.

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Address for reprint requests and other correspondence: A. Spát, Dept. of Physiology, Semmelweis University, Faculty of Medicine, PO Box 259, H-1444 Budapest, Hungary (E-mail: Spat@Puskin.SOTE.Hu).

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