Relative Roles of Sodium and Calcium Ions in the Steroidogenic Response of Isolated Rat Adrenal Glomerulosa Cells

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SUMMARY To study the relative roles of sodium (Na$^+$) and calcium ions (Ca$^{2+}$) in the response of adrenal glomerulosa cells, we investigated the effects of different Na$^+$ concentrations in the incubation media and the actions of substances that interfere with Ca$^{2+}$ fluxes. Basal aldosterone secretion and response to angiotensin II (AII), adrenocorticotropic hormone (ACTH), or potassium (K$^+$) were dependent on extracellular Na$^+$ concentration. Veratridine, a Na$^+$ channel opener that dissipates Na$^+$ gradients, blocked the stimulated steroidogenic response. Mersalyl acid and tetracaine, which are potent Ca$^{2+}$ antagonists, blocked the effects of aldosterone secretagogues. Divalent cations with Ca$^{2+}$ antagonistic action such as manganese (Mn$^{2+}$), nickel (Ni$^{2+}$), and cobalt (Co$^{2+}$) blocked the aldosterone secretory response to AII, ACTH, and K$^+$. Barium (Ba$^{2+}$) and strontium (Sr$^{2+}$), known to mimic Ca$^{2+}$ effects, increased or did not affect responses of the glomerulosa cells. Sodium vanadate, an inhibitor of ATP-dependent Ca$^{2+}$ translocation, did not alter the stimulated aldosterone responses. Trifluoperazine (10$^{-5}$ M), an inhibitor of calmodulin, blocked AII and K$^+$-induced aldosterone secretion, but was partially effective on ACTH-stimulated aldosterone output only at a concentration of 10$^{-4}$ M. The actions of ouabain on aldosterone biosynthesis were similarly affected by all these drugs. Thus, both extracellular Na$^+$ and Ca$^{2+}$ appear to play a role in the steroidogenic response of isolated glomerulosa cells. The intracellular action of Ca$^{2+}$ may involve a calmodulin-like protein. The effects of ACTH are only partially dependent on Ca$^{2+}$ as a second intracellular messenger.

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EVIDENCE has accumulated that calcium (Ca$^{2+}$) plays a role as the mediator of agents that stimulate aldosterone biosynthesis by adrenal glomerulosa cells.$^{1-8}$ Some of the evidences in favor of Ca$^{2+}$ as the intracellular messenger of aldosterone secretagogues have been obtained using Ca$^{2+}$-antagonists such as verapamil and lanthanum.$^{3,4}$ Reports have suggested that Ca$^{2+}$-antagonists may also block sodium (Na$^+$) influx into the cell.$^{5}$

It has therefore appeared important to us to investigate the dependency of basal and stimulated aldosterone secretion on extracellular Na$^+$ concentration and the effects of drugs like veratridine, a Na$^+$ channel opener.$^{6}$ It has also seemed of interest to show whether other substances with Ca$^{2+}$-antagonistic action blocked the response to angiotensin II (AII), adrenocorticotropic hormone (ACTH), potassium (K$^+$), or ouabain, all of which stimulate aldosterone biosynthesis.$^{4-7}$ Among the Ca$^{2+}$ blocking agents we have investigated are tetracaine,$^{8}$ mersalyl acid,$^{7}$ and divalent cations such as manganese (Mn$^{2+}$), cobalt (Co$^{2+}$), and nickel (Ni$^{2+}$).$^{10,11}$ Adenosine triphosphate (ATP)-dependent Ca$^{2+}$ translocation may be blocked by vanadate,$^{12}$ which is apparently inactive on nonATP-dependent Ca$^{2+}$ mobilization.$^{6}$ Finally, Ca$^{2+}$ actions inside the cell have been linked to binding to a heat-stable protein, calmodulin.$^{13}$ Since phenothiazines, particularly trifluoperazine (TFP), have been shown to inhibit calmodulin,$^{14}$ we have also investigated the effects of TFP on basal and stimulated steroidogenesis.
Materials and Methods

Aldosterone production by isolated rat adrenal glomerulosa cells was studied during a 90-minute incubation of cells prepared according to a technique previously described. Briefly, rats weighing approximately 250 g and kept on a normal-sodium diet were killed by decapitation. Adrenal glands were immediately removed and dissected free of fat, and capsules were separated from the fasciculata reticularis by manual compression. Capsules were minced and incubated for 40 minutes at 37°C in phosphate-buffered saline containing 80 μg/ml dispase (Boehringer Mannheim, Lot 1369304) (a neutral protease from Bacillus polymyx, EC 3.4.24.4), 3 μg/ml deoxyribonuclease (DNAse I from bovine pancreas) (Sigma, St. Louis, Missouri), 1.7 mg/ml collagenase (C2139, Sigma, St. Louis, Missouri), and 3% chicken serum (Gibco, Grand Island, New York). The cells were dispersed mechanically, filtered through a 100-μm Nitex nylon filter (Tetko Inc., Elmsford, New York), and centrifuged at 200 × g for 2 minutes. The cells were resuspended in F12 nutrient medium (Gibco, New York) containing 0.35% HEPES (Calbiochem, La Jolla, California), 0.12% sodium bicarbonate, 0.002% gentamicin (Schering, Kenilworth, New Jersey), and 0.5% bovine serum albumin (Sigma, St. Louis, Missouri).

Cells were first incubated for 2 hours at 37°C in a Dubnoff metabolic shaker. After centrifugation they were resuspended in the same medium or in media containing 7, 52, 97, or 142 mM of Na⁺. Na⁺ was replaced isoosmotically by sucrose or lithium chloride (LiCl) in a modified Hanks' salt solution, buffered with 25 mM HEPES, to which essential amino acids (0.1 mM) and MEM nonessential amino acids (0.1 mM), MEM vitamins, and sodium pyruvate (50 mg/liter) (all from Gibco, New York) were added. The cell suspension, 0.90–0.95 ml as appropriate, was pipetted into 1.5-ml Eppendorf tubes containing 0.05 ml of stimulating agent and/or antagonist dissolved in F12 medium or in the low Na⁺ medium. The tubes were stoppered and incubated for 90 minutes at 37°C in a specially designed rack to keep them in an oblique position to facilitate adequate agitation of the cell suspension. At the end of the incubation, tubes were centrifuged in an Eppendorf 3200 microcentrifuge for 2 minutes. The supernatant was decanted into glass tubes and frozen at −20°C until assayed for aldosterone by direct radioimmunoassay of the incubation medium. Mersalyl acid interfered with the direct radioimmunoassay. Samples containing mersalyl acid and controls were measured after extraction with dichloromethane and paper chromatography. Results are expressed as ng/mg deoxyribonucleic acid (DNA). DNA was measured in at least 10 tubes per experiment by dissolving the sedimented cells in 1 ml of cold 10% trichloroacetic acid; DNA was separated by its preferential solubility in hot trichloroacetic acid and quantitated colorimetrically by pentose analysis.

To test for possible cytotoxicity of the drugs employed to inhibit steroidogenesis, after the usual preincubation, cells were incubated in the presence or absence of each of the drugs used for 1 hour. After two washings, the cells were again incubated for 90 min-

![Figure 1](http://hyper.ahajournals.org/)

**FIGURE 1.** Aldosterone output in response to All, ACTH, and K⁺ at different Na⁺ extracellular concentrations. Na⁺ was replaced isoosmotically by sucrose. The means of duplicate vessels per incubation of five different experiments are shown.
Results

The effects of different Na\(^+\) concentrations in the extracellular medium (NaCl replaced isoosmotically by sucrose), on aldosterone output by isolated glomerulosa cells are shown in figure 1. A sharp reduction was found at an extracellular Na\(^+\) concentration of 7 mM, both in basal aldosterone output and in the response to All, ACTH, and K\(^+\). Aldosterone secretion was far less impaired by higher, albeit subnormal (52 and 97 mM) Na\(^+\) concentrations, with respect to normal Na\(^+\) solution (142 mM). Inhibition of responses was less marked for ACTH stimulation than for responses to All or K\(^+\). Similar results were found when LiCl was used instead of sucrose to replace NaCl. The stimulating action of 10\(^{-4}\) M ouabain on aldosterone biosynthesis was reduced when extracellular Na\(^+\) concentration was 52 mM or less (fig. 2).

Veratridine, a drug that dissipates Na\(^+\) gradients, at a concentration of 92 \(\mu\)M inhibited the responses to All, ACTH, K\(^+\), and ouabain (fig. 3). Since the action of veratridine appears to produce an opening of the Na\(^+\) channel, the reduced effects of aldosterone secretagogues shown in figure 1 do not appear to depend on reduced ion flux through a Na\(^+\) channel.

Tetracaine and mersalyl acid, two substances with Ca\(^{2+}\)-antagonistic action, blocked the responses to all four aldosterone-stimulating agents (fig. 3). A divalent cation such as Mn\(^{2+}\), with Ca\(^{2+}\)-blocking properties, was also an effective inhibitor of steroidogenic stimulation at concentrations of 5 \(\times\) 10\(^{-4}\) M (fig. 4). The effects of ACTH were more resistant to blockade by Mn\(^{2+}\), than the actions of All, K\(^+\), or ouabain. Ba\(^{2+}\), a divalent cation that may carry Ca\(^{2+}\) currents, blocked responses only at high concentrations (5 \(\times\) 10\(^{-3}\) M), while at 5 \(\times\) 10\(^{-4}\) M it was either ineffective or slightly agonistic (fig. 4). When the inhibitory effects of other divalent cations at a concentration of 10\(^{-4}\) M were studied, and the percent inhibition of stimulated aldosterone output plotted against their crystal ionic radii (fig. 5), it could be seen that divalent cations with radii between 0.7 and 0.8 Å were significantly inhibitory of stimulated responses. Divalent cations with larger (i.e., Sr\(^{2+}\)) or smaller radii (i.e., Mg\(^{2+}\)) were ineffective.

Ca\(^{2+}\) translocation may occur by way of an energy-dependent system that can be inhibited by vanadate.
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Figure 4. Effect of manganese (Mn⁺⁺) (●—●) and barium (Ba⁺⁺) (○—○) on stimulated responses of glomerulosa cells. Results are means ± se of triplicate vessels in a representative experiment. Similar results were observed in two other experiments.

Figure 5. Inhibition of stimulated aldosterone output by divalent cations, which were added at a concentration of 1 mM. The final concentration of manganese (Mg⁺⁺) was 1.47 mM. Results are means of close triplicates in one of two experiments with similar results and are plotted against the crystal ionic radii of each divalent cation.

Figure 6 shows that vanadate was not an effective blocker of stimulated aldosterone biosynthesis, nor did it affect basal steroid output (results not shown).

To examine the possibility that calmodulin might be involved in the actions of AII, ACTH, K⁺, and ouabain, we examined the effects of trifluoperazine (TFP) (fig. 7). Basal aldosterone output was inhibited in one of three experiments by TFP at concentrations above 10⁻⁶ M, but was unaffected in the other two experiments (results not shown). Aldosterone output stimu-
Discussion

These results demonstrate that both Na⁺ and Ca²⁺ may participate in the responses to AII, ACTH, K⁺, and ouabain. Aldosterone output in response to AII, ACTH, and K⁺ has been previously shown to be highly dependent on extracellular Ca²⁺ concentration. Extracellular Na⁺ appears now to be also equally important for the response to aldosterone secretagogues. Veratridine, a drug that opens Na⁺ channels and dissipates Na⁺ gradients, stimulates electrically excitable and endocrine cells in which the Na⁺ channel appears to participate in excitation-response coupling. Our results, in which veratridine actually inhibits responses, suggest that the Na⁺ channel is not involved. Na⁺ gradients, however, appear to play a role, since their abolition by veratridine entails an inhibition of the response. The mechanism whereby veratridine inhibits the steroidogenic response remains to be determined. It may, however, be speculated that the dissipation of the Na⁺ gradient affects Ca²⁺ fluxes or intracellular Ca²⁺ redistribution, occurring perhaps through Na⁺-Ca²⁺ exchange. We have previously suggested that the actions of ouabain imply a role for Na⁺-Ca²⁺ exchange on the cell surface in the stimulated responses of the adrenal glomerulosa. The present experiments support the view that ouabain acts via mechanisms similar to AII and K⁺. The fact that steroidogenesis is reduced in a low Na⁺ medium argues strongly against a role for a Na⁺-Ca²⁺ exchanger.

Our finding of a reduced steroidogenic response in the presence of low extracellular Na⁺ may appear at odds with the increased aldosterone secretion found after sodium depletion. However, previous evidence for a direct role of low sodium on increased aldosterone output is not clear-cut. Blair-West et al. have shown minimal direct effects of sodium on aldosterone response to AII in the sodium-replete sheep and after prolonged infusion of NaCl in the sodium-depleted sheep. A direct action of sodium in the opposite direction to the one we find was reported by Lobo et al. using dog adrenal cortex slices. The effects described were minor, however, and examined at concentrations between 160 and 120 mM.

Previous experiments with verapamil and lanthanum have suggested an activation of a "Ca²⁺ channel" in the response of the glomerulosa cell. Our experiments using tetracaine and mersalyl acid, two potent antagonists of Ca²⁺ fluxes, further strengthen the implication of Ca²⁺ as a second messenger in the glomerulosa cell. Divalent cations such as Mn²⁺, Co²⁺, and Ni²⁺ have been shown to possess Mn²⁺-blocking properties, whereas Ba²⁺ or Sr²⁺ may substitute for Ca²⁺. Due to different hydration energies and ionic radii, Ba²⁺ and Sr²⁺ act as permeant species and may carry Ca²⁺ currents, and antagonize these only at high concentrations (5 × 10⁻³ M). Mn²⁺ may act as a permeant ion or an antagonist, and Co²⁺ and Ni²⁺ are nonpermeant species that exert only blocking effects. This is in agreement with our finding of an inhibition by Co²⁺, Ni²⁺, and Mn²⁺ at 5
$10^{-4}$ M or more. Sr$^{2+}$ and Ba$^{2+}$ are ineffective or slightly agonistic at concentrations of $10^{-8}$ M or less and antagonistic only at high concentrations of $5 \times 10^{-8}$ M (figs. 2 and 3). Thus, the dependency on extracellular Ca$^{2+}$, blockade by verapamil, lanthanum,$^{1,3,4}$ and our present findings with tetracaine, mersalyl acid, and divalent cations serve to define the activation of a "Ca$^{2+}$ channel" as one of the main mechanisms involved in the response of adrenal glomerulosa cells to stimulation.$^{4}$ Since veratridine (a Na$^+$ channel opener) does not stimulate or potentiate responses to aldosterone secretagogues, and it has been mentioned that tetrodotoxin is incapable of inhibiting responses in isolated adrenal cells,$^{8}$ a Na$^+$ channel does not appear to be involved. The Na$^+$ dependency we demonstrate may imply passage of Na$^+$ through the Ca$^{2+}$ channel.

Ca$^{2+}$ extrusion from the cell and into the Ca$^{2+}$ store is accomplished mainly by a "calcium pump" that is ATP-dependent. Ca$^{2+}$-Mg$^{2+}$ ATPase has been identified with the "calcium pump" in different tissues.$^{11,12}$ It has been shown that vanadate may inhibit Ca$^{2+}$-Mg$^{2+}$ ATPase.$^{13}$ From our data, it appears that this mechanism of Ca$^{2+}$ mobilization is not an important component in the response of glomerulosa cells.

Ca$^{2+}$ effects within the cell may be mediated by Ca$^{2+}$ binding to an intracellular protein, calmodulin,$^{18}$ which interacts with numerous enzymes (phosphodiesterase, ATPase, protein kinase, etc.). Research in this field has been advanced by the finding that phenothiazines such as chlorpromazine or trifluoperazine (TFP) inhibit calmodulin.$^{14}$ In our experiments, TFP at a concentration of $10^{-4}$ M blocked All and ouabain-stimulated steroidogenesis. K$^+$-induced steroid output was blocked by TFP $10^{-4}$ M. TFP was less effective on ACTH actions. Basal aldosterone production was reduced in one of three experiments by the higher but not the lower concentrations of TFP. Calmodulin may thus be involved in the response to All and K$^+$, but not in basal cell activity or in the response to ACTH. Together with evidence of the relative resistance of ACTH-stimulated aldosterone biosynthesis to verapamil$^{14}$ and Mn$^{2+}$, it appears that mechanisms other than Ca$^{2+}$ fluxes are also involved in mediating the actions of ACTH, as has previously been suggested.$^{14}$ Finally, these findings confirm immunohistochemical evidence of the presence of calmodulin in adrenal cells.$^{18}$

The possibility that some of our findings may be explained by actions other than interference with Na$^+$ or Ca$^{2+}$ fluxes cannot be eliminated. The fact that effects were similar on All and K$^+$ responses suggests that agonist-receptor interaction was not affected by the blocking agents. Since ACTH still produced a degree of stimulation sometimes superior to the maximal response observed with All, it is unlikely that some of the results may be explained by cytotoxicity of the drugs. Furthermore, we have shown that after exposure to veratridine, tetracaine, Ni$^{2+}$, and trifluoperazine, the cells will respond to All, ACTH, and K$^+$ as cells unexposed to these drugs. The inhibitory effect of Mn$^{2+}$, Co$^{2+}$, and mersalyl cannot be washed off. The results obtained with the latter substances may thus have to be interpreted with some caution. The residual response to ACTH is not the result of contamination by fasciculata cells since: 1) this is negligible in rat adrenal capsules as shown by histological examination and the response of corticosterone to All and K$^+$, which is not found in the case of corticosterone of fasciculata origin;$^{17}$ and 2) the cross-reactivity of the antibody to aldosterone with corticosterone is 0.0006%, and therefore the corticosterone present cannot account for the aldosterone measured.

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