Effects of extracellular calcium and potassium on the sodium pump of rat adrenal glomerulosa cells

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Received 21 May 1999; accepted in final form 29 August 2000

Yingst, Douglas R., Joanne Davis, and Rick Schiebinger. Effects of extracellular calcium and potassium on the sodium pump of rat adrenal glomerulosa cells. Am J Physiol Cell Physiol 280: C119–C125, 2001.—Because the activity of the sodium pump (Na-K-ATPase) influences the secretion of aldosterone, we determined how extracellular potassium (Ko) and calcium affect sodium pump activity in rat adrenal glomerulosa cells. Sodium pump activity was measured as ouabain-sensitive 86Rb uptake in freshly dispersed cells containing 20 mM sodium as measured with sodium-binding benzozuran isothitale. Increasing Ko from 4 to 10 mM in the presence of 1.8 mM extracellular calcium (CaO) stimulated sodium pump activity up to 165% and increased intracellular free calcium as measured with fura 2. Increasing Ko from 4 to 10 mM in the absence of CaO stimulated the sodium pump ~30% and did not increase intracellular free calcium concentration ([Ca2+]i). In some experiments, addition of 1.8 mM CaO, in the presence of 4 mM Ko increased [Ca2+]i above the levels observed in the absence of CaO and stimulated the sodium pump up to 100%. Ca-dependent stimulation of the sodium pump by Ko and CaO was inhibited by isradipine (10 μM), a blocker of L- and T-type calcium channels, by compound 48/80 (40 μg/ml) and calmidazolium (10 μM), which inhibits calmodulin (CaM), and by KN-62 (10 μM), which blocks some forms of Ca/CaM kinase II (CaMkII). Stauroporine (1 μM), which effectively blocks most forms of protein kinase C, had no effect. In the presence of A-23187, a calcium ionophore, the addition of 0.1 mM CaO, increased [Ca2+]i, to the level observed in the presence of 10 mM K, and 1.8 mM CaO and stimulated the sodium pump 100%. Ca-dependent stimulation by A-23187 and 0.1 mM CaO was not reduced by isradipine but was blocked by KN-62. Thus, under the conditions that Ko stimulates aldosterone secretion, it stimulates the sodium pump by two mechanisms: direct binding to the pump and by increasing calcium influx, which is dependent on CaO. The resulting increase in [Ca2+]i may stimulate the sodium pump by activating CaM and/or CaMkII.

ouabain; signaling; Na-K-ATPase; calmodulin; calcium- and calmodulin-dependent kinase II; aldosterone

INCREASING EXTRACELLULAR POTASSIUM (Ko) has been thought to stimulate aldosterone secretion by depolarizing the membrane potential, which opens voltage-dependent calcium channels and leads to an influx of extracellular calcium (Ca2+; see Refs. 5, 6, 9, 26, 29). The depolarizing effect of Ko is in large part due to a decrease in the equilibrium potential for potassium (27). However, increasing Ko over the range that stimulates aldosterone secretion should also stimulate the sodium pump by increasing the amount of Ko that binds to the pump (14). Because sodium pump activity is ultimately responsible for most of the resting membrane potential, the ability of Ko to regulate aldosterone secretion could in part be due to its effect on sodium pump activity. In fact, it has been clearly shown that changes in sodium pump activity can both stimulate and inhibit aldosterone secretion, depending on the species studied and the experimental conditions (4, 16, 31, 35).

The addition of physiological concentrations of CaO stimulates the sodium pump even at normal levels of Ko (16). The mechanism of this stimulation is not yet known but could be due to an increase in intracellular free calcium ([Ca2+]i), which in some cells has been shown to activate sodium pump activity (1, 23, 24, 34). However, if this is the case, then one also might expect Ko to stimulate the sodium pump by increasing calcium influx through voltage-dependent calcium channels. Such an effect has not yet been reported.

Thus, in this study, we have examined how CaO and Ko affect the activity of the sodium pump under conditions that they stimulate the secretion of aldosterone. These studies will help determine how aldosterone secretion may be affected by changes in the activity of the sodium pump and will help define mechanisms that regulate sodium pump activity during cell activation.

MATERIALS AND METHODS

A-23187, KN-62, calmidazolium, compound 48/80, gramicidin A, and monensin were purchased from Calbiochem (San Diego, CA). 86Rb was purchased from NEN Research Products (Boston, MA). Isradipine was a gift from Sandoz Pharmaceuticals (East Hanover, NJ).

A stock solution of 500 μM monensin was prepared by adding monensin to medium 199 in 0.5% DMSO at 37°C while vortexing. The solution was maintained at 37°C and alternately was shaken and vortexed until the monensin was dissolved. KN-62 and isradipine were dissolved in DMSO.

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Adrenal cell preparation. Adrenal capsules, containing the zona glomerulosa, were removed from female Sprague-Dawley rats weighing 200–224 g and collagenase dispersed as previously described (7) in medium 199 (GIBCO, Grand Island, NY) containing modified Earle’s salts (130 mM NaCl, 4.0 mM KCl, 1.8 mM CaCl$_2$, and 0.8 mM MgCl$_2$), 10 mM HEPES, sodium salt (pH 7.4), 0.2% BSA, and no bicarbonate. After dispersion, the cells were incubated for 2 h to allow for recovery. Cells were continuously gassed with 100% O$_2$.

In experiments where Ca$_o$ was either 0 or 1.8 mM (see Figs. 3, 6, and 8), cells were prepared as usual except that they were divided into two equal groups after dispersion. One group was subsequently washed two times and resuspended in medium 199 without added calcium, and the other was suspended in normal medium 199 containing 1.8 mM CaCl$_2$. Soon thereafter, the cells were divided into the assay, and the experiments were performed as usual. In the experiments shown in Fig. 7, cells were washed two times in medium 199 without calcium and then were suspended in medium 199 containing 20 μM EGTA and either no added CaCl$_2$ (0 μM free Ca$^{2+}$), or 100 μM CaCl$_2$ (100 μM free Ca$^{2+}$). They were then immediately used to measure sodium pump activity.

Sodium pump activity. Sodium pump activity was measured as the difference in the uptake of $^{86}$Rb, a congruent for potassium, in the presence and absence of ouabain, a specific inhibitor of the sodium pump. Measurements were carried out at 37°C over a 5-min period in medium 199 as previously described (16). Quadruplicate samples were run in the presence and absence of 1 mM ouabain. Experiments were performed in a total volume of 150 μl containing 100,000 cells and from 1 to 2.5 μCi of $^{86}$Rb, depending on the experiment. Measurements were carried out in a 96-well plate (cat. no. MADCN6550; Millipore) gently agitated at constant intervals and maintained at 37°C on top of a heated sand bath. Cells were added to medium 199, except where noted, followed shortly thereafter by the addition of ouabain or an equal volume of buffer (time 0). Monensin, a sodium ionophore, was added at a final concentration of 10 μM at 20 min; $^{86}$Rb was added at 30 min. Fluxes were terminated 5 min later by rapid filtration of the cells using a Millipore Multiscreen Assay System followed by six washes (~300 μl/wash) with ice-cold medium 199. The membranes (containing the washed cells) were then punched out and separately placed in 0.5 ml of 1% SDS to which scintillation fluid was then added. The filter blank in this assay was <0.015% of the total counts.

KN-62 and isradipine were added to the assay 15 and 30 min before $^{86}$Rb, respectively. A-23187 was added 5 min before $^{86}$Rb. In the experiments when K$_o$ was increased from 4 to 10 mM, a small volume of concentrated KCl was added to the normal medium 5 min before the addition of $^{86}$Rb. In experiments where sodium pump activity was assayed at both 4 and 10 mM K$_o$, ouabain-sensitive $^{86}$Rb uptake at 10 mM K$_o$ was multiplied by 2.5 to compensate for the difference in the ratio of $^{86}$Rb to K$_o$.

Measurement of intracellular sodium. Glomerulosa cells were prepared as described above and then were incubated with shaking under oxygen at 37°C for 45 min in medium 199 (minus phenol red) containing 3 μM of the acetylomethyl ester of sodium-binding benzofuran isophthalate (SBFI) and 0.03% pluronic following previously developed procedures (17). The cells were then washed twice by centrifugation and resuspended in the same medium without SBFI and pluronic.

To calibrate the SBFI as a function of sodium, cells were loaded with SBFI as described above. Cells from one preparation were then divided into five equal groups and centrifuged, and each group was resuspended separately in one of five solutions, each containing different concentrations of sodium. Cells were then centrifuged again and resuspended in the same solution. The five solutions contained 0, 10, 20, 30, and 40 mM NaCl and the appropriate concentration of choline chloride so that the sum of the NaCl and choline chloride equaled 82 mM. In addition, all of the solutions contained a final concentration of 50 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 1 mM KH$_2$PO$_4$, 5 mM glucose, 2 mM l-glutamine, 10 mM HEPES, and 4 μM gramicidin. The gramicidin was included to equilibrate sodium and potassium concentrations across the plasma membrane (17). The fluorescence of each set of cells was then measured as shown in Fig. 1A, and the concentration of sodium was calculated as previously described (33).

Measurement of [Ca$^{2+}$]. Glomerulosa cells were prepared as described above and then were incubated with shaking under oxygen at 37°C for 45 min in medium 199 (minus phenol red) containing 3 μM of the acetylomethyl ester of sodium-binding benzofuran isophthalate (SBFI) and 0.03% pluronic following previously developed procedures (17). The cells were then washed two times in medium 199 made to contain no added CaCl$_2$. The cells were then centrifuged again. The next step depended on the type of experiment that was performed. For the experiment shown in Fig. 4A, the cells were resuspended in medium 199 containing 1.8 mM CaCl$_2$ and 10 μM monensin. Immediately thereafter, the cells were put in a cuvette, and the change in fluorescence was recorded as a function of time. For the experiment shown in Fig. 4B, the cells were suspended in medium 199 containing no added CaCl$_2$. The suspension of cells was then added to a cuvette, and the measurement of fluorescence began. As the fluorescence was measured, small volumes of concentrated KCl, EGTA, A-23187, and CaCl$_2$ were added to the cuvette as shown (Fig. 4B). The concentration of [Ca$^{2+}$], was calculated as previously described (15).

Measurements of fluorescence. Fluorescence was measured using a dual excitation monochromator spectrophuorometer (SPEX Fluorolog 1680; Spex Industries, Edison, NJ). The measurements were carried out at 37°C in a stirred cuvette containing 200,000 cells/ml. The fluorescence of the SBFI was excited at 340 and 385 nm and was measured at 500 nm (17); the fluorescence of fura 2 was excited at 340 and 380 nm and was measured at 505 nm (15).

Statistics. Each data point is the mean and SE of quadruplicate samples of an individual experiment. Each experiment is representative of at least three similar experiments. The data were analyzed by one-way ANOVA. Groups were compared using a Bonferroni multiple-comparisons test. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Intracellular sodium. To measure the concentration of intracellular sodium in adrenal glomerulosa cells, the response of incorporated SBFI was first measured as a function of sodium. (Fig. 1A). This calibration indicated that the initial sodium concentration in freshly dispersed cells suspended in medium 199 was 20 ± 3.5 (SD) mM sodium (Fig. 1B). This value is close to the value of 23.9 ± 1.8 mM measured by others in freshly dispersed cells and much less than the value of 48.5 ± 5.5 mM in cells activated by ANG II (33). Cultured glomerulosa cells have a lower sodium concentration of 9.2 ± 3.5 mM, perhaps due to recovery
from deleterious treatment during enzymatic dispersion (33). Adding monensin to freshly dispersed cells only modestly increased the intracellular sodium (Fig. 1B). Thus, under these experimental conditions, intracellular sodium was well within the physiological range for adrenal glomerulosa cells, even in the presence of monensin.

**Sodium pump assay conditions.** Sodium pump activity was measured in freshly dispersed cells in the presence of monensin, as previously described (16). Monensin was added to assure that the sodium pump activity did not change during the assay due to a reduction in intracellular sodium, which is a primary pump substrate. Under these conditions, the uptake of $^{86}$Rb was linear for at least the first 6 min in the presence and absence of ouabain (Fig. 2A). Therefore, the ouabain-sensitive uptake, which is a measure of sodium pump activity, was also linear during this period (Fig. 2B). In most of the experiments, the ouabain-sensitive $^{86}$Rb uptake accounted for >85% of the total uptake; in all experiments, it accounted for at least 70% of the total influx.

**Effects of $K_o$ and $Ca_o$ on sodium pump activity and $[Ca^{2+}]_i$.** In the absence of $Ca_o$, increasing $K_o$ from 4 to 10 mM stimulated the sodium pump 30% (Fig. 3). This is the degree of activation one would expect from increased binding of $K_o$ to its extracellular binding site on Na-K-ATPase (20). In the presence of 1.8 mM $Ca_o$, increasing $K_o$ from 4 to 10 mM stimulated sodium pump activity 165% (Fig. 3).

Increasing $K_o$ from 4 to 10 mM under these experimental conditions also increased $[Ca^{2+}]_i$ (Fig. 4A). Under similar conditions, others have found that elevating $K_o$ increases $[Ca^{2+}]_i$ from 200 to 600 nM (28). Thus $K_o$ may stimulate the sodium pump more in the presence of $Ca_o$, because it increases $[Ca^{2+}]_i$, which stimulates the sodium pump in some cells (1, 23, 24, 34).

Adrenal glomerulosa cells contain both L- and T-type calcium channels, which are both blocked by isradipine, a 1,4-dihydropyridine (2). Thus, to test if the increased activation of $K_o$ observed in the presence of $Ca_o$ could be due to the ability of $K_o$ to increase $[Ca^{2+}]_i$, through voltage-dependent calcium channels, we tested the effect of $K_o$ in the presence and absence of isradipine. For these experiments, we chose a concentration of isradipine that fully inhibits ouabain-induced aldosterone secretion (35). Isradipine significantly reduced the ability of elevated $K_o$ to stimulate the sodium pump in the presence of $Ca_o$ (Fig. 5A). In fact, in the presence of isradipine, increasing $K_o$ from 4 to 10 mM stimulated the sodium pump 36% (Fig. 5B), an amount similar to that observed in the absence of $Ca_o$ (Fig. 3). These results suggest that $K_o$ could have stimulated the sodium pump in a Ca-dependent fashion by increasing Ca influx through voltage-gated calcium channels.

Increasing $Ca_o$ from 0 to 1.8 mM stimulated sodium pump activity up to twofold at 4 mM $K_o$ in some preparations of adrenal glomerulosa cells (Fig. 6). Lower levels of stimulation were sometimes seen in
Fig. 3. Effect of increasing extracellular potassium (Ko) on the sodium pump in the presence and absence of extracellular calcium (Ca0). [Ca2+]i and [K+]o, extracellular concentration of calcium and potassium, respectively. *P<0.05 relative to corresponding control.

Other experiments (data not shown). In preparations of cells where Ca0 stimulated activity, the effect was blocked by isradipine (Fig. 6A), suggesting that Ca0 stimulated the sodium pump by increasing [Ca2+]i through voltage-dependent calcium channels.

To determine if the addition of 1.8 mM Ca0 in the presence of medium containing 4 mM Ko could stimulate the sodium pump by increasing [Ca2+]i, the concentration of [Ca2+]i was measured in the presence and absence of 1.8 mM Ca0. Cells previously loaded with fura 2 and washed in the absence of Ca0 were suspended at time 0 in normal medium 199 containing 4 mM Ko and 1.8 mM Ca0. [Ca2+]i, immediately began to increase and then reached a steady state ∼5 min later (Fig. 4A). At this point, [Ca2+]i was 140 nM, considerably above the level of 70 nM observed when the cells were suspended at 4 mM Ko in the absence of Ca0 (Fig. 4B). Thus cells suspended in normal media containing 1.8 mM Ca0 and 4 mM Ko have higher levels of [Ca2+]i than cells suspended in the same medium containing no added calcium. These measurements are consistent with the idea that the addition of Ca0 could stimulate the sodium pump by increasing [Ca2+]i in cells suspended in medium 199 containing normal levels of 4 mM K and 1.8 mM Ca. Others have shown that freshly dispersed glomerulosa cells suspended in solutions containing normal concentrations of Ko and Ca0 have resting levels of ∼200 nM [Ca2+]i (28). Given the results that correlate sodium pump stimulation with small increases in [Ca2+]i above resting levels, relatively small differences in resting [Ca2+]i could account for our observation that the addition of 1.8 mM Ca0 stimulates the sodium pump more in some preparations than in others.

Effects of pharmacological agents. To help determine the mechanism by which increasing [Ca2+]i might stimulate the sodium pump, we tested the effects of pharmacological agents known to mediate the effects of Ca2+ in adrenal glomerulosa cells. Ca-dependent stimulation of the sodium pump by both Ko and Ca0 was blocked by compound 48/80 (40 μg/ml; see Ref. 13) and calmidizolium (10 μM; see Ref. 21), which inhibit calmodulin (CaM; data not shown). These results prompted us to test the effects of drugs that inhibit more specific CaM-dependent proteins. We found that KN-62, which inhibits Ca/CaM-dependent kinase II (CaMKII; see Ref. 32), blocked the Ca-dependent stimulation of the sodium pump by both Ko (Fig. 5B) and Ca0 (Fig. 6B). These data suggest that the Ca-dependent stimulation of the sodium pump could be mediated by a CaM-dependent mechanism, perhaps by a form of CaMKII.

Because KN-62 can inhibit calcium influx through voltage-dependent Ca channels (22), one explanation for the ability of KN-62 and the other CaM antagonists to inhibit Ca-dependent stimulation of the sodium pump is by limiting calcium influx. To test this hypothesis, we measured the effect of Ca0 in the presence of A-23187, a calcium ionophore that would increase [Ca2+]i independent of calcium channels. In designing these experiments, we first determined how much Ca can had to be added in the presence of 10 μM A-23187 to achieve the same [Ca2+]i observed in the presence of 10 mM Ko and 1.8 mM Ca0. For instance, in the experiment shown in Fig. 4B, adding 140 μM Ca0 (in the presence of 10 μM EGTA) gave the same [Ca2+]i as observed in the presence of 10 mM Ko and 1.8 mM Ca0 (Fig. 4A). In other experiments, we found that under the same conditions we had to add as little as 100 μM Ca0 (data not shown). Using this information, we...
tested the effect of KN-62 on the ability of Ca o to stimulate the sodium pump in the presence of A-23187. Increasing Ca o from 0 to 100 μM in the presence of A-23187 stimulated the sodium pump over twofold (Fig. 7A). This Ca-dependent stimulation was completely inhibited by KN-62 (Fig. 7A) without blocking the influx of calcium (data not shown). These data suggest that KN-62 blocks the Ca-dependent stimulation of the sodium pump independent of its ability to inhibit calcium influx through voltage-dependent calcium channels.

Finally, to test if isradipine could be blocking Ca-dependent stimulation of the sodium pump by inhibiting protein kinase C (8, 18), we tested if isradipine would block Ca-dependent stimulation of the sodium pump when Ca influx was mediated by A-23187. The results show that isradipine had no effect on the ability of 100 μM Ca o to stimulate the sodium pump in the presence of A-23187, which is when calcium could enter the cells independent of endogenous calcium channels (Fig. 7B). Likewise, isradipine had no effect on the ability of KN-62 to block the Ca-dependent stimulation seen in the presence of A-23187 (Fig. 7, A vs. B). Furthermore, staurosporine, which at least partially inhibits all known forms of protein kinase C (19, 25), had no effect on the ability of Ca o to stimulate the sodium pump (Fig. 8). Thus it is unlikely that isradipine was blocking the ability of calcium to stimulate the sodium pump by inhibiting protein kinase C.

**DISCUSSION**

Our results show for the first time how sodium pump activity is affected during K o-induced aldosterone secretion. This is significant, because the activity of the sodium pump has been repeatedly shown to alter agonist-stimulated aldosterone secretion. We have shown that increasing K o and Ca o stimulate the sodium pump and increase [Ca2+] i in the range that stimulates aldosterone secretion. From our measurements, we deduce that increasing [Ca2+] i from ~70 to ~300 nM stimulated the sodium pump approximately twofold. K o also stimulated the sodium pump ~30% in the absence of Ca o, probably due to increased binding of K o to the sodium pump. Thus increasing K o over the range that stimulates aldosterone secretion activates the sodium pump.
pump by two mechanisms: one that is Ca-dependent and the other that is not. It is not yet known how the stimulation of the sodium pump by \(K_o\) and \([Ca^{2+}]_i\) affects the membrane potential and alters the secretion of aldosterone. Nevertheless, it is interesting to note that both of the physiological agents that stimulate aldosterone secretion alter sodium pump activity: ANG II inhibits (16) and \(K_o\) stimulates. Perhaps these opposing effects may one day help explain the apparently conflicting reports that inhibiting sodium pump activity can both stimulate and inhibit aldosterone secretion, depending on the preparation and the experimental conditions (4, 16, 31, 35).

Stimulation of the sodium pump by \([Ca^{2+}]_i\). Our results confirm that \(Ca_o\) stimulates the sodium pump in rat glomerulosa cells (16). Hajnoczky et al. (16), however, concluded that \(Ca_o\) did not stimulate by increasing \([Ca^{2+}]_i\) because the calcium ionophore ionomycin failed to stimulate sodium pump activity. However, they added ionomycin in the presence of 1.2 mM CaCl\(_2\) (16), which may have increased \([Ca^{2+}]_i\) beyond its stimulatory range. In contrast, we observed stimulation when A-23187 was added at much lower concentrations of \(Ca_o\) (Fig. 4B).

Mechanism of stimulation. Because the stimulatory effects of \(Ca_o\), \(K_o\), and \([Ca^{2+}]_i\) were blocked with 48/80 and calmidazolium, which inhibit CaM, and by KN-62, which inhibits CaMKII, we suggest that the stimulatory effects of \([Ca^{2+}]_i\) could be mediated by CaMKII or a related kinase. CaMKII is present in bovine glomerulosa cells and is activated by \(Ca_i\) under conditions that stimulate sodium pump activity (10). In principle, Ca-CaM or Ca-CaMKII could enhance sodium pump activity directly via changes in phosphorylation or by altering how the pump interacts with other proteins. For example, in guinea pig ventricular myocytes, the relative level of \([Ca^{2+}]_i\) determines if stimulation of protein kinase A by isoproterenol either inhibits or stimulates sodium pump activity (11, 12).

Ca-dependent stimulation of the sodium pump in adrenal glomerulosa cells could be due to an increase in the affinity for sodium, as previously observed in the rat proximal tubules (1), because the measured concentration of intracellular sodium under our experimental conditions (Fig. 1) was close to the concentration that half-maximally stimulates the \(\alpha_1\)-isoform of Na-K-ATPase (3, 30). In this case, we have assumed that \(\alpha_1\) is the predominant isoform in these cells based on the concentration of ouabain required to inhibit activity (16). It is unlikely that calcium is stimulating the sodium pump by increasing its affinity for \(K_o\), as previously observed in HeLa cells (24), because both 4 and 10 mM \(K_o\) are well above the half-maximal constant for \(K_o\) to bind to the sodium pump (20). In other cells, changes in physiological levels of \([Ca^{2+}]_i\) can either stimulate or inhibit sodium pump activity, depending on the relative expression of regulatory proteins (23, 24, 34).

In conclusion, increasing \(Ca_o\) and/or \(K_o\) in the presence of \(Ca_o\) stimulated the sodium pump by increasing
Ca\textsuperscript{2+}. The mechanism by which Ca\textsuperscript{2+} stimulated the sodium pump may involve CaM and either CaMKII or a related kinase. The concentrations of K\textsubscript{o} and Ca\textsuperscript{2+} that stimulated the sodium pump are in the same range that stimulate aldosterone secretion. Therefore, it is likely that the sodium pump is stimulated by Ca\textsuperscript{2+} during K\textsubscript{o}-stimulated aldosterone secretion. How Ca-dependent stimulation of the sodium pump alters aldosterone secretion is not yet known but could involve changes in the membrane potential.

This work was supported by a grant from the American Heart Association of Michigan, National Heart, Lung, and Blood Institute Grant HL-48885, Veterans Association Research Funds, and by the Vascular Biology Program of the Department of Internal Medicine at Wayne State University.

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