Effects of Growth in Low Potassium Medium or Ouabain on Membrane Na,K-ATPase, Cation Transport, and Contractility in Cultured Chick Heart Cells

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SUMMARY. Growth of cultured cells in low potassium medium has been shown to result in an increase in the number of Na,K-ATPase sites. This phenomenon and its physiological and pharmacological consequences were examined in spontaneously beating monolayers of cultured chick heart cells. Growth of cells in 1 mM extracellular potassium, 2 μM ouabain, or 1 μM veratridine for 48 hours caused 60%, 40%, or 20% increases, respectively, in the total number of specific ouabain binding sites measurable in intact cells. Acute exposure of control cells grown in 4 mM to 1 mM extracellular potassium caused elevation of steady state [Na+] by 37%, while 1 μM veratridine exposure increased [Na+] by 12%. After 48 hours of growth in 1 mM extracellular potassium, intracellular sodium concentrations declined to near-control levels. In cells grown in low extracellular potassium and then equilibrated with 4 mM potassium for 30 minutes, the positive inotropic effects of 1 mM extracellular potassium and 0.3 μM isoproterenol, expressed as a percent of contractile response to 3.6 mM calcium, were 40 ± 6% and 37 ± 5% (means ± SEM), respectively, in low potassium-grown cells, compared with 63 ± 8% and 35 ± 4% in control cells. Growth of cells in low potassium shifted the concentration-effect curve for ouabain to the right. The rapid component of calcium uptake in zero extracellular sodium was significantly lower in low potassium-grown cells than in control cells after equilibration in 1 mM extracellular potassium for 30 minutes. These findings demonstrate that prolonged exposure of cultured heart cells to 1 mM extracellular potassium or ouabain causes induction of an additional functional sarcolemmal sodium pump sites. The increased levels of intracellular sodium caused by these interventions appear to be an important determinant of sodium pump site density. The reduced contractile response of cells grown in 1 mM extracellular potassium or ouabain (but not isoproterenol) supports the view that elevated intracellular sodium due to Na,K-ATPase inhibition mediates the positive inotropic response to low extracellular potassium and ouabain, probably via augmented transsarcolemmal sodium-calcium exchange. In addition, our results support a mechanism of inotropic action of digitalis glycosides based on inhibition of the sodium pump rather than altered calcium binding properties of sarcolemmal sites due to cardiac glycoside binding to Na,K-ATPase.


INTRACELLULAR sodium concentration in nearly all types of mammalian cells remains at a level substantially below that of extracellular fluid as a result of active extrusion of sodium from the intracellular space by plasma membrane sodium pumps characterized at a molecular level as Na,K-ATPase. Half-maximal activation of Na,K-ATPase by internal sodium occurs at a concentration close to that normally found inside the cell (Skou et al., 1965; Thomas, 1972; Cook et al., 1975; Pollack et al., 1981a). Thus, when intracellular sodium concentration is acutely increased by interventions such as increased electrical stimulation frequency in cardiac tissues (Yamamoto et al., 1979), exposure of cells to low extracellular potassium (Pollack et al., 1981b; Barry et al., 1982), or exposure to agents such as monensin or grayanotoxin (Akera et al., 1976; Honegger et al., 1977; Ku et al., 1977a; Temma and Akera, 1982), sodium pump activity is stimulated, resulting in a greater rate of sodium extrusion.

In contrast to such short-term regulation of sodium transport in which cells respond to perturbations in internal sodium by altering the turnover rate of existing pump sites, prior studies have suggested that long-term regulation of sodium transport in some tissues may be achieved by a change in the number of pump sites. For example, HeLa cells grown in low [K+]o for 24 hours showed a 2-fold or greater increase in the number of Na,K-ATPase sites, as judged by ouabain binding (Boardman et al., 1972, 1974; Lamb and McCall, 1972; Pollack et al., 1981a; Anderson et al., 1983). In these cells, the
steady state intracellular sodium concentration was maintained at a relatively low level, indicating the functional role of additional sodium pump sites (Pollack et al., 1981b). Thus, physiological regulation of sodium transport may occur by changes in the pumping (turnover) rate of existing Na,K-ATPase sites and/or changes over longer time intervals in the number of enzyme (sodium pump) units in the plasma membrane.

The experiments reported here were designed to test the hypothesis that changes in the number of sodium pump sites also occur in cardiac cells in response to long-term stress, such as exposure to low [K+]o, ouabain, or veratridine, that the pump sites are active, and that they produce predictable physiological responses.

There is convincing evidence that contractility of cardiac cells is intimately related to the level of intracellular sodium, presumably because of the Na+-Ca++ exchange mechanism present in sarcoplemmal membranes (Reuter and Seitz, 1968; Langer, 1977; Horackova and Vassort, 1977). Thus, alteration in [Na+] has been shown to affect the rate of calcium influx (Glitsch et al., 1970; Langer, 1976) and contractility (Biedert et al., 1979; Horackova and Vassort, 1977; Barry et al., 1981). If this formulation is correct, then changes in the number of sodium pump sites in the sarcoplemma should result in altered contractile responses to positive inotropic agents that elevate [Na+] and thereby augment [Ca++]

In this way, one may distinguish between a mechanism of digitalis-induced inotropy based upon inhibition of the sodium pump and alternative mechanisms such as those proposed by Lüllmann and Peters (1976) and Gervais et al. (1977), in which studies they suggest that altered Ca++ binding to sarcoplasmal sites may result directly from cardiac glycoside binding to Na,K-ATPase. Accordingly, we used the cultured chick embryo ventricular cell monolayer preparation to explore these phenomena and the underlying mechanisms, taking advantage of the relative ease with which growth conditions can be modulated, and contractility and cation fluxes and contents determined. These findings were correlated with changes in sarcoplasmal Na,K-ATPase sites as determined by [3H]ouabain-binding studies.

**Methods**

**Tissue Culture**

Monolayer cultures of spontaneously contracting chick embryo ventricular cells were prepared as previously described (Biedert et al., 1979). Briefly, hearts of 10-day-old chick embryos were removed and placed in Ca++ and Mg++-free Hanks' solution (Gibco Laboratories). Ventricular tissue was cut into small fragments (less than 0.5 mm³), and individual cells were isolated by four cycles of trypsinization with 0.025% (wt/vol) trypsin at 37°C. Cell suspensions from each dissociation cycle were placed into 10 ml of cold trypsin inhibitor solution (50% heat-inactivated fetal calf serum and 50% Ca++- and Mg++-free Hanks' solution). This cell suspension was centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in culture medium containing 6% heat-inactivated fetal calf serum, 40% M199 with Hanks' salts, 0.1% penicillin-streptomycin solution, and 54% balanced salt solution. Balanced salt solution contained 116 mM NaCl, 1 mM NaH2PO4, 0.8 mM MgSO4, 1.18 mM KCl, 26.2 mM NaHCO3, 0.87 mM CaCl2, and 5.5 mM glucose. The final concentrations of K+, Na+, and Ca++ in culture medium were 4.0, 137, and 0.97 mM, respectively. The cell suspension was diluted to 5 × 10⁶ cells/ml and placed in plastic culture dishes (100 × 20 mm, or 60 × 15 mm; Falcon). The larger culture dishes contained 25-mm circular glass coverslips and were used in ion flux studies, whereas the smaller dishes without coverslips were used in [3H]ouabain-binding studies. Cultures were incubated in a humidified 5% CO2, 95% air atmosphere at 37°C. Confluent monolayers in which an estimated 80% of cells exhibited spontaneous synchronous contractions developed by 3 days of incubation.

After 24 hours of growth in control culture medium, cultured cells were allowed to continue to grow in medium containing 4 mM [K+]o, or were switched to identical media except that 1 mM [K+]o, 0.5–2 μM veratridine, 2 μM ouabain, or 1.8 mM Ca++ were present. Low K+ culture medium was prepared from K+-free M199 and K+-free balanced salt solution. The final K+ concentration was adjusted to 1 mM, as assayed by flame emission photometry.

The period of growth in the new medium was 24, 48, 72, or 96 hours, at which times experiments were performed. Cells to be grown in the test medium for only 24 hours were allowed to grow in control culture medium for 48 hours, so that the day of the experiment was the 3rd day in culture.

**[3H]Ouabain Binding**

The number of sodium pump sites in the cell membrane was estimated from [3H]ouabain binding to intact, beating cultured ventricular cells. Specific binding of [3H]ouabain was measured by minor modification of methods described by McCall (1979) and Aiton et al. (1981). Briefly, monolayers of ventricular cells attached to culture dishes were incubated in K+-free HEPES buffer solution (pH 7.35) containing 4 μM HEPES, 0.05 mM CaCl2, 137 mM NaCl, 0.5 mM MgCl2, and various concentrations of [3H] ouabain (60–100 nM) for 15 minutes at 37°C. Stock [3H] ouabain (18 Ci/mmol, New England Nuclear) was diluted with unlabeled ouabain to give the desired final concentrations of [3H]ouabain. Cells grown in the presence of ouabain were washed three times for 5 minutes each with buffer solution to remove bound ouabain. These washing conditions were sufficient to remove more than 98% of bound ouabain, documented by a measured dissociation rate constant of 0.22/min. After [3H]ouabain exposure, the cells were quickly scraped from the dish and filtered through a microfiber filter (Whatman, pore size 1.2 μm). Filters were washed three times with ice-cold HEPES buffer solution (pH 7.35) containing 20 mM KCl and dissolved in Hydrofluor (National Diagnostics). Radioactivity on the filter was assayed using a liquid scintillation spectrometer. Specific [3H]ouabain binding was calculated as total binding in the absence of 10⁻⁷ M unlabeled ouabain minus the value obtained in its presence, and is expressed as picomoles per milligram protein. To determine protein content on each filter, cells on the plates not exposed to...
[H]Ouabain were thoroughly washed to remove all the radioactive \(^{14}C\)leucine in the extracellular space (see below) by placing the plates in three large volumes (50 ml) of bicarbonate buffer solution for 5 minutes each at 37°C. The cells then were dissolved, and aliquots of the medium containing dissolved cells were assayed for protein content by the method of Lowry et al. (1951).

**Contractility Measurements**

Changes in the contractile state of individual cells in the monolayers were assessed by the use of an optical-video system as previously described (Biedert et al., 1979; Barry et al., 1981). A glass coverslip with attached monolayer of ventricular cells was continuously superfused in a chamber on the stage of an inverted phase contrast microscope with bicarbonate buffer solution (pH 7.35) containing 116.3 mm NaCl, 1 mm NaH₂PO₄, 0.8 mm MgSO₄, 4 mm KCl, 26.2 mm NaHCO₃, 0.6 mm CaCl₂, 5.6 mm dextrose, and 1% fetal calf serum at a rate of 2 ml/min. The pH of the buffer solution was maintained at 7.4 by continuously gassing the chamber with a 95% air, 5% CO₂ gas mixture. A constant temperature of 37°C was maintained by enclosing the microscope in a thermostated Lucite box. After a 15-minute equilibration period, cells were superfused with solution containing either 1 mm K⁺, 0.3 mm I-isoproterenol, 0.1-4 μm ouabain, or 3.6 mm CaCl₂, and changes in the amplitude of motion of an individual cell were monitored. Only one cell from each coverslip was used, and the change in the amplitude of contraction was expressed as a percent of the change produced by 3.6 mM Ca²⁺, a concentration which produces maximal contractile response.

**Sodium Content and Calcium Fluxes**

The procedures for estimating sodium content and calcium fluxes have been described in detail by Biedert et al. (1979) and Barry and Smith (1982). For determination of sodium content, glass coverslips with attached monolayers of cells were immersed in bicarbonate buffer solution (pH 7.35) containing \(^{24}Na\) (5 μCi/ml, [Na⁺] = 143 mm), and the cells were labeled to asymptote (30 minutes). For determination of calcium uptake, cells were exposed to medium containing \(^{45}Ca\) (5 μCi/ml, [Ca⁺] = 0.6 mm) for 0.5, 1, 2, and 5 minutes. Barry and Smith (1982) have shown that this period of exposure to \(^{45}Ca\) labels the rapidly exchangeable calcium pool, which has been shown to be affected by cardiac glycosides or low K⁺ (Barry et al., 1981; Barry and Smith, 1982). After appropriate periods of labeling with \(^{24}Na\) or \(^{45}Ca\), cells were washed twice for 8 seconds each by gently agitating the glass coverslips sequentially in two 60-ml volumes of ice-cold balanced salt solution containing no radiolabeled ions. The cells then were dissolved in medium containing 1% SDS and 10 mm sodium borate. Aliquots of the medium containing dissolved cells were assayed for radioactivity in a liquid scintillation spectrometer (Packard, model 3330).

**Cell Density Correction**

To normalize for cell density on each culture dish or coverslip, the monolayers were incubated in either \(^{14}C\)(U)-leucine (0.05 μCi/ml) or \(^{14}C\)(U)-leucine (0.1 μCi/ml) for 24 hours before the experiment. \(^{14}C\)Leucine or \[^3H\]leucine was incorporated into cell protein, and the \(^{14}C\) or \(^3H\) counts permitted estimation of cell density on each culture dish or glass coverslip, respectively. The relationship between radioactive counts and protein concentration (Lowry et al., 1951) allowed accurate estimation of protein concentration of cells in each culture dish or glass coverslips. Thus, simultaneous counting of \(^3H\) and \(^24Na\) or \(^45Ca\), \(^{14}C\) and \(^3H\), permitted normalization of sodium content, calcium flux, or \[^3H\]Ouabain binding per milligram cell protein.

**Miscellaneous**

Protein concentration was assayed by the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard. Hanks' salt solution, M199, and fetal calf serum were purchased from Gibco Laboratories. All radiolabeled compounds and ions were purchased from New England Nuclear. Veratridine was kindly provided by Dr. Tai Akera, Michigan State University. I-Isoproterenol was purchased from Sigma Chemical Co. All other chemicals used were of the highest grade commercially available. All solutions used were sterilized by filtering through Nalgene sterilization filters (Nalge Company) (pore size, 0.45 μm).

Student's t-test was used for statistical analyses.

**Results**

\[^3H\]Ouabain Binding

The number of sodium pump sites within the sarcolemmal membrane of cultured chick heart cells was determined by a radioligand binding assay using \[^3H\]Ouabain. Figure 1 shows that the specific binding of \[^3H\]Ouabain to intact cells in K⁺-free medium is saturable. A Scatchard plot of the binding (inset, Fig. 1) was linear, suggesting the presence of a single class of binding sites for ouabain. The total number of ouabain binding sites at saturation, an

![Figure 1. \[^3H\]Ouabain binding to ventricular cells cultured from chick embryo hearts 10 days in ovo. Cells were incubated with graded concentration of \[^3H\]Ouabain in zero K⁺, medium for 15 minutes, and \[^3H\]Ouabain bound to the cells was assayed. Specific \[^3H\]Ouabain binding (○) is the difference in values observed in the presence (Q) and absence of 10⁻³ M nonradioactive ouabain. Each point is the mean of six determinations. Vertical lines indicate ± 1 se. Inset shows Scatchard plot of \[^3H\]Ouabain binding. B represents bound ouabain (pmol/mg protein) and B/F represents bound divided by free ouabain (× 10⁻³/liter). Each point is the mean of six values.](http://circres.ahajournals.org/doi/fig/10/1016/j.cir.1982.05.012)
estimate of the number of sodium pump sites, was therefore determined from the amount of specific ouabain bound following incubation of cells with 1 \( \mu M \) [\(^{3}H\)]ouabain.

To determine whether prolonged exposure of cultured heart cells to low \([K^{+}]_o\), causes changes in the number of sodium pump sites, cells were grown in 1 mM \([K^{+}]_o\), medium for 24, 48, 72, or 96 hours. Growth in 1 mM \(K^{+}\) did not visibly alter cellular morphology or ability to contract spontaneously for the first 3 days. However, by the 4th day, fibroblast proliferation and a reduction in the fraction of spontaneously contracting cells were observed. [\(^{3}H\)]Ouabain-binding sites on intact cells gradually increased over a 48-hour period of growth in 1 mM \(K^{+}\) medium (Fig. 2). The maximal increase observed at 48 hours was retained up to 72 hours. Further incubation in 1 mM \(K^{+}\) caused a decline in [\(^{3}H\)]ouabain binding, probably as a result of fibroblast proliferation and overgrowth. These results indicate that the number of sodium pump sites increases in cultured heart cells when stressed by growth in low \(K^{+}\) medium.

There are several physiological factors that might be involved in this induction of additional sodium pump sites. Possibilities include the low extracellular \(K^{+}\) concentration, the elevated intracellular sodium concentration produced as a result of low \(K^{+}\)-induced inhibition of Na,K-ATPase, and/or the inhibition of the enzyme itself. That the exposure of cells to low \([K^{+}]_o\) increases the contractile state of the cells also raises the question of augmented intracellular \(Ca^{++}\) as a possible mechanism. Therefore, factors potentially responsible for the induction of sodium pump sites were examined by growing cells in media containing ouabain, veratridine, or elevated calcium. Growth of cells in 2 \( \mu M \) ouabain, a concentration that produced occupancy of approximately 50% of Na,K-ATPase sites in 4 mM \([K^{+}]_o\), and produced maximal increases in contractile state of cultured heart cells without toxicity, caused increases in the number of [\(^{3}H\)]ouabain-binding sites to approximately 20 and 40% above control values at 24 and 48 hours of incubation, respectively (Fig. 3). The increase in binding occurred over a 48-hour period, similar to the time course of changes observed in cells growing in 1 mM \(K^{+}\).

Veratridine, an agent that promotes influx of sodium by prolonging the open state of fast sodium channels, also produced qualitatively similar but less marked effects on the number of sodium pump sites. As summarized in Figure 4, 0.5, or 1 \( \mu M \) veratridine produced a significant increase in the number of sodium pump sites at either 24 or 48 hours of incubation. Higher concentrations of veratridine were toxic to the cells, as manifested by a marked reduction in spontaneously beating cells and concomitant decrease in [\(^{3}H\)]ouabain binding. Growth in 1.8 mM \(Ca^{++}\), as compared to growth in control medium containing 0.97 mM \(Ca^{++}\), failed to affect the number of ouabain-binding sites (Fig. 3). The possibility that prolonged incubation with low \(K^{+}\) or ouabain alters the affinity of ouabain-binding sites to glycosides, rather than the total number of binding sites, was examined further. Scatchard plots of

\[
\begin{align*}
1.0 \text{mM [K]}^{+}_o & \quad \text{Exposure (hr)} \\
24 & 48 & 72 & 96
\end{align*}
\]

\[
\begin{align*}
\text{Relative [H] Ouabain Binding} \\
0 & 100 & 120 & 140 & 160 & 180
\end{align*}
\]

\[
\begin{align*}
\text{Relative [H] Ouabain Binding} \\
0 & 100 & 120 & 140 & 160 & 180
\end{align*}
\]

\[
\begin{align*}
\text{Ouabain or 1.8 mM Ca}\^{++}\ & \quad \text{Exposure (hr)} \\
24 & 48 & 24 & 48
\end{align*}
\]

\[
\begin{align*}
\text{Relative [H] Ouabain Binding} \\
0 & 100 & 120 & 140 & 160 & 180
\end{align*}
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\text{Ouabain or 1.8 mM Ca}\^{++}\ & \quad \text{Exposure (hr)} \\
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0 & 100 & 120 & 140 & 160 & 180
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\end{align*}
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24 & 48 & 24 & 48
\end{align*}
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\[
\begin{align*}
\text{Relative [H] Ouabain Binding} \\
0 & 100 & 120 & 140 & 160 & 180
\end{align*}
\]
binding data obtained with cells grown in control media, low [K+]o, or ouabain, were parallel, indicating that the affinity of the binding sites for ouabain did not change (Fig. 5). These results suggest that induction of additional sodium pump sites in the cell membrane occurs as a consequence of elevated intracellular sodium content, regardless of whether altered [Na+]i is caused by inhibition of Na+ extrusion mediated by a Na,K-ATPase (low [K+]o, or ouabain) or by enhanced influx of Na+ via specific sodium channels.

Intracellular Sodium Content

The effects of acute and chronic exposure of cells to low [K+]o, elevated [Ca2+]o, or veratridine on intracellular sodium content were examined to define the functional role of additional sodium pump sites identified by [3H]ouabain binding. Cells grown in medium containing 4 mM K+ (control), 1 mM K+, 1.8 mM Ca2+, or 1 μM veratridine for 48 hours were then incubated in medium containing 24Na and either 4 mM K+, 1 mM K+, 1.8 mM Ca2+, or 1 μM veratridine for 30 minutes, and cellular sodium content was determined. As summarized in Table 1, exposure of cells grown in 4 mM K+ (control) to 1 mM K+ for 30 minutes resulted in marked elevation in [Na+]i, as expected, in response to inhibition of Na,K-ATPase. Sodium content in cells grown in 1 mM K+ for 48 hours and then incubated in 1 mM K+ with 24Na for 30 minutes, however, had returned to near-control [Na+]i values (Table 1). Exposure of

**Table 1**

<table>
<thead>
<tr>
<th>48-Hr growth medium</th>
<th>30-Min incubation medium</th>
<th>Intracellular sodium content (nmol/mg protein)</th>
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<tbody>
<tr>
<td>K+ 4 mM (control)</td>
<td>K+ 4 mM</td>
<td>129.5 ± 8.9</td>
</tr>
<tr>
<td>K+ 4 mM</td>
<td>K+ 1 mM</td>
<td>178.6 ± 13.5*</td>
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<tr>
<td>K+ 1 mM</td>
<td>K+ 4 mM</td>
<td>122.2 ± 7.8</td>
</tr>
<tr>
<td>K+ 1 mM</td>
<td>K+ 1 mM</td>
<td>139.7 ± 8.8†</td>
</tr>
<tr>
<td>K+ 4 mM (control)</td>
<td>K+ 4 mM</td>
<td>125.9 ± 7.7</td>
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<tr>
<td>K+ 4 mM</td>
<td>K+ 4 mM + veratridine 1 μM</td>
<td>148.8 ± 7.2*</td>
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<tr>
<td>K+ 4 mM</td>
<td>K+ 4 mM + veratridine 1 μM</td>
<td>116.2 ± 5.5</td>
</tr>
<tr>
<td>K+ 4 mM</td>
<td>K+ 4 mM + veratridine 1 μM</td>
<td>127.1 ± 7.5†</td>
</tr>
</tbody>
</table>

Cells were grown on glass coverslips in either 1 mM K+, 4 mM K+, or 1 μM veratridine for 48 hours. Glass coverslips with attached monolayers of cells were immersed in medium containing 24Na and 4 mM K+, 1 mM K+, or 1 μM veratridine for 30 minutes. After brief washing to remove extracellular 24Na+, cells were dissolved and radioactivity was assayed. The sodium content was calculated for each coverslip and expressed as nanomoles per milligram protein. Each value represents the mean ± SE of observations made on eight coverslips.

* Significantly different from the control value (P < 0.05).
† Significantly different from the value for cells grown for 48 hours in 4 mM K+, then exposed for 30 minutes to 1 mM K+ (P < 0.05).
‡ Significantly different from the value for cells grown for 48 hours in 4 mM K+ without veratridine, then exposed for 30 minutes to 1 μM veratridine (P < 0.05).
cells grown in low K+ to 4 mM K+ tended to reduce further the sodium content, but the decrement was not statistically significant. The effects of veratridine on sodium content were analogous to those observed with low [K+]. Thus, acute veratridine exposure caused a significant elevation of sodium content in control cells but not in cells previously grown in veratridine. However, the extent of change in [Na+], was less with veratridine than low K+. Exposure of cells to an elevated [Ca++]o of 1.8 mM for 30 minutes or 48 hours did not alter significantly the intracellular sodium content. Sodium contents in cells grown in 0.97 mM Ca++ and exposed to either 0.97 or 1.8 mM Ca++ for 30 minutes were 91.7 ± 8.0 and 90.9 ± 8.6 nmol/mg protein, respectively. Sodium contents in cells grown in 0.97 mM Ca++ (control) and 1.8 mM Ca++ for 48 hours were 110.8 ± 4 and 119.6 ± 5.5 nmol/mg protein, respectively. Thus, intracellular sodium content rose following acute exposure to low [K+]o, or veratridine, but fell to normal levels at 48 hours, and was associated with an increased number of sodium pump sites, indicating that additional sodium pumps are physiologically functional and maintain relatively normal intracellular sodium levels.

**Contractility Measurements**

The positive inotropic response to low [K+]o or cardiac glycosides in cultured heart cells occurs in association with inhibition of cardiac Na,K-ATPase and consequent augmentation of intracellular sodium concentration and enhancement of intracellular calcium via sodium-calcium exchange (Aker and Brody, 1977; Langer, 1977; Biedert et al., 1979; Barry et al., 1981; Burt and Langer, 1982). The results presented thus far indicate that additional sodium pump sites in low K+-grown cells actively extrude internal sodium, suggesting that the contractile response of these cells to interventions that tend to elevate intracellular sodium should be modified. Therefore, the extent to which the additional sodium pumps affect the inotropic effects of low [K+]o or ouabain was examined. Cells grown in 1 mM K+ for 48 hours were equilibrated in 4 mM K+ for 30 minutes and subsequently challenged with 1 mM [K+]o, 0.1-4 μM ouabain, or elevated [Ca++]o, (3.6 mM). For each cell, the maximal changes produced by 1 mM [K+]o, or ouabain were determined as the percent of change induced in the same cell by 3.6 mM [Ca++]o. Exposure of cells to 1 mM K+ solution caused immediate and prominent increases in contractile state. The maximal effects were observed within 10 minutes in both control and low K+-grown cells. The percent increase in the amplitude of contraction induced by 1 mM [K+]o, as compared to the 3.6 mM [Ca++]o, response was significantly lower in low K+-grown cells than in control cells (Fig. 6). Similarly, the concentration-effect curve for ouabain was shifted to the right for cells grown in low K+ (Fig. 7). In control cells, the maximal positive inotropic effect was observed with 2 μM ouabain. A higher concentration of ouabain (4 μM) was toxic to these cells, as indicated by a marked negative inotropic effect, probably related to early contracture development. In contrast, cells grown in 1 mM K+ tolerated 4 μM ouabain without toxic phenomena occurring; this concentration produced a positive inotropic effect indistinguishable statistically from that observed in control cells treated with 2 μM ouabain.

To determine whether the reduced inotropic response observed in cells grown in low K+ was specific for interventions thought to act through a step involving increased [Na+], rather than, for example, a change in slow calcium channels and, therefore, altered calcium influx via these channels, contractile response to a β-adrenergic agonist, I-isoproterenol, was studied. At 0.3 μM, I-isoproterenol produced a positive inotropic effect with rapid onset in both types of cells. However, percent changes in amplitude of contraction, as related to the response to 3.6 mM [Ca++]o, were indistinguishable in control and low K+-grown cells (Fig. 6). These results indicate that cells with 60% greater numbers of sodium pump sites have reduced sensitivity specifically to
Kim et al. / Effect of Low K⁺ on Na,K-ATPase

**Calcium Uptake**

Having found reduced [Na⁺]ᵢ levels in low K⁺-grown cells with increased numbers of sodium pump sites compared to control cells acutely exposed to low K⁺, we postulated that the reduced contractile response to ouabain observed would be the consequence of decreased delivery of Ca²⁺⁺ to the myofilaments during contraction, ultimately due in turn to reduced calcium influx, enhanced Ca²⁺⁺ efflux, or both. Calcium uptake into the rapidly exchangeable pool via sodium-calcium exchange was therefore examined by measuring the rate of calcium uptake of cells with abrupt exposure to zero extracellular sodium following equilibration in 1 mM [K⁺].

In both control and low K⁺-grown cells, calcium uptake was rapid during the first 60 seconds. As expected (Barry and Smith, 1982), this rapid phase was essentially complete within 2 minutes (Fig. 8). However, the rate of calcium uptake declined earlier in cells grown in low K⁺, leading to a diminished calcium uptake into the rapidly exchangeable pool as compared to that in control cells. The calcium uptake rate in low K⁺-grown cells over the first 2 minutes under the experimental condition employed was approximately 80% of that in control cells. These findings are consistent with the hypothesis that the reduced inotropic response to ouabain or 1 mM K⁺ observed in low K⁺-grown cells is due to decreased calcium entry via sodium-calcium exchange.

**Discussion**

The studies reported here demonstrate that when cultured heart cells are stressed by growth in low K⁺ medium or in medium containing sublethal concentrations of ouabain, cells respond by producing a greater number of sodium pumps in the sarcolemmal membrane and, consequently, maintaining a lower level of intracellular sodium. More important, results also show that these cells with elevated numbers of sodium pumps are in an altered physiological state such that they respond differently to changes in cellular environment as compared to responses observed in normal cells.

Long-term regulation of cardiac monovalent cation active transport capacity appears to be achieved mainly by changes in the number of transporter molecules. This is analogous to regulation of Na,K-ATPase sites observed in other cell types such as HeLa and Girardi cells. For example, HeLa cells grown in medium containing 20 nM ouabain or 0.5 mM K⁺ for 24 hour had significantly elevated numbers of specific ouabain-binding sites (Board-...
man et al., 1972, 1974; Vaughan and Cook, 1972; Pollack et al., 1981a). Girardi cells grown in medium containing various concentrations of ouabain also showed additional sodium pump sites (Boardman et al., 1972). Aiton et al. (1981), however, described a down-regulation of sodium pump sites after a 24-hour exposure of HeLa and chick embryo heart cells to ouabain. These investigations may have measured less than the total number of Na,K-ATPase sites with labeled ouabain, since unlabeled ouabain bound during growth was not removed in their study. Thus, it appears that, in several cell types, including spontaneously beating chick heart cells, the number of sodium pump sites is altered in response to interventions that increase [Na+]i.

The long-term effects of low [K+]o or digitalis glycosides on the Na,K-ATPase system have also been studied in heart tissues of intact animals. In guinea pigs fed a potassium-deficient diet or given digitalis drugs, myocardial Na,K-ATPase activity was significantly higher than that of control animals (Erdmann et al., 1971; Bluschke et al., 1976). In contrast, Ku et al. (1977b) found no change in enzyme activity in dog heart preparations following chronic digoxin treatment. The reasons for these conflicting results are not clear, but may relate to differences in species or experimental procedures.

The observation that the number of sodium pump sites is altered by growth in low K+ or ouabain has led several investigators to study the mechanism of control of sodium pump density. Boardman et al. (1974) examined the effects of partial replacement of extracellular sodium with sorbitol on maximal ouabain binding in HeLa cells. This procedure lowered intracellular potassium concentration without affecting sodium content. The total number of ouabain binding sites remained unchanged. These results, together with the observation that intracellular sodium concentration is elevated following incubation with low [K+]o, led to suggestions that the signal for the increase in the number of sodium pump sites was intracellular sodium (Boardman et al., 1974; Pollack et al., 1981b). It is possible, however, that the inhibition of Na,K-ATPase itself or elevated [Ca++]i, rather than [Na+], is the factor responsible for the stimulation of additional sodium pumps. To test the first of these possibilities, the intracellular sodium level was elevated without concurrent inhibition of Na,K-ATPase by growing the cells in the presence of 1 μM veratridine. It has been well demonstrated that veratridine prolongs the open state of fast sodium channels and, therefore, promotes influx of sodium (Sperelakis and Pappano, 1969; Ulbricht, 1969; Romey et al., 1980). Maximal tolerated doses of veratridine produced a moderate increase in the number of sodium pump sites in cultured heart cells. The increase was not as prominent as that produced by 1 mM [K+]o, an observation that is of interest in light of the finding that veratridine only mildly elevated intracellular sodium, to a level substantially lower than that produced by 1 mM [K+]o (Table 1). Indeed, examination of the results suggests that there may be a positive correlation between the level of [Na+], and the number of sodium pump sites in the cell membrane.

The possibility remained that elevation of [Ca++]i, secondary to elevated [Na+]i, rather than increased [Na+], itself, was responsible for the enhanced number of sodium pumps. To exclude this possibility, we grew cells in an elevated [Ca++]i of 1.8 mM. This resulted in a 70% increase in [Ca++]i in the rapidly exchangeable pool compared to control cells grown in 0.97 mM Ca++ (5.13 ± 0.23 vs. 3.35 ± 0.10 nmol/mg protein, respectively, P < 0.05). The total Ca++ content of cells grown in 1.8 mM Ca++ was also significantly increased to 53% above control levels, whereas there was no significant change in the intracellular Na+ content and no effect on the number of sodium pump sites. Acute exposure of cells grown in 0.97 mM Ca++ to 1.8 mM Ca++ also failed to alter significantly the cellular Na+ content measured after 30 minutes of exposure to 1.8 mM Ca++.

These results indicate that induction of Na,K-ATPase in cultured heart cells does not require inhibition of this enzyme, and is independent of alterations in [Ca++]i over the range of values encountered in response to ouabain, low [K+]o, veratridine, or elevated [Ca++]i. Our findings thus support the hypothesis that [Na+] is an important regulator of sodium pump density, as others have suggested (Boardman et al., 1974; Pollack et al., 1981b).

If sodium pump density is increased in the sarcolemmal membrane, what physiological role do the additional sodium pump sites play? Since the function of Na,K-ATPase is to maintain normal transmembrane gradients of Na+ and K+, it may be predicted that an increase in [Na+]i induced by low [K+]o or ouabain will gradually decline, associated with increased numbers of sodium pump sites. Indeed, this was observed in cultured chick heart cells (Table 1). Exposure of cells to 1 mM [K+]o, or 1 μM veratridine caused an immediate increase in intracellular sodium content. With prolonged incubation, however, the elevated sodium level declined to near-control values. This is in agreement with the findings reported by Boardman et al. (1974) in HeLa cells in which a low [K+]o-induced rise in intracellular sodium content was followed by a gradual fall to a lower level, associated with increased numbers of sodium pump sites in the cell membrane. In contrast, Lamb and McCall (1972) observed in Girardi cells that growth in the presence of ouabain caused a gradual increase in cellular sodium content, which remained elevated throughout the 5-day incubation period. Although these investigators did not determine the number of ouabain-binding sites in their study, it was subsequently shown that sodium pump sites in Girardi cells increased in number following growth in ouabain (Boardman et al., 1972). The differing nature of the stress (low [K+]o vs. ouabain)
or differing physiological characteristics of these cells from those of HeLa or cultured heart cells [resting membrane potential in Girardi cells of -20 mV (Lamb and McCall, 1972)] may account for the difference in [Na\textsuperscript{+}] adaptation. In cultured chick heart cells, however, the additional sodium pumps clearly are capable of active transport of sodium, in agreement with the findings of Pollack et al. (1981a) that HeLa cells grown in low [K\textsuperscript{+}]\textsubscript{o} were capable of transporting greater numbers of monovalent cations than control cells.

In cardiac cells, changes in transport capacity for monovalent cations might be expected to have profound effects on the contractility of the cells in response to an altered electrochemical gradient for sodium, and associated changes in calcium flux via the sodium-calcium exchange mechanism. Our results show that when cells with normal or increased numbers of sodium pumps were equilibrated in 4 mM K\textsuperscript{+} and subsequently challenged with 1 mM [K\textsuperscript{+}]\textsubscript{o}, which causes marked inhibition of Na,K-ATPase activity, the cells with higher numbers of sodium pumps were less inotropically responsive. This reduced contractility response can be attributed to a decreased calcium influx via sodium-calcium exchange (Fig. 8). Although increased [Na\textsuperscript{+}], might be expected to increase Ca\textsuperscript{2+} influx by Na-Ca exchange or to reduce Ca\textsuperscript{2+} efflux by this mechanism (or both), prior studies from this laboratory (Barry and Smith, 1982) have demonstrated that the predominant effect is enhancement of Ca\textsuperscript{2+} influx, with no measurable effect on Ca\textsuperscript{2+} efflux under the conditions of the experiments described here. Thus, cells with elevated numbers of sodium pump sites are less sensitive to low [K\textsuperscript{+}]\textsubscript{o} due to extrusion of a greater amount of internal sodium by the additional sodium pumps, and consequently reduced stimulation of calcium influx via sodium-calcium exchange.

The reduced inotropic sensitivity of low K\textsuperscript{+}-grown cells to low [K\textsuperscript{+}]\textsubscript{o} appears to be due specifically to alterations in the number of sodium pump sites, and not to alterations in other modulators of inotropic state such as calcium flux via slow calcium channels. Inotropic responses to isoproterenol were indistinguishable in control and low K\textsuperscript{+}-grown cells. Furthermore, the number of \beta-adrenergic receptors remained unchanged following 48 hours of growth in low [K\textsuperscript{+}]\textsubscript{o} (data not shown), indicating that low [K\textsuperscript{+}]\textsubscript{o} affected Na,K-ATPase specifically, and did not merely produce a nonspecific effect on sarcosomemal structures.

Since ouabain appears to have a similar mechanism of inotropic action to 1 mM [K\textsuperscript{+}]\textsubscript{o}, that is, inhibition of sodium pump activity, it is not surprising that the cells with increased numbers of sodium pump sites were also less responsive to the inotropic effects of this cardiac glycoside. These observations support the proposed mechanism of cardiac glycoside action (Akera and Brody, 1977; Langer, 1977; Biedert et al., 1979; Barry et al., 1981) that inhibition of sodium pump activity is followed by elevation of intracellular sodium and subsequent promotion of calcium influx via sodium-calcium exchange. If the alternative proposal that binding of digitalis to Na,K-ATPase causes release of calcium from enzyme-associated sarcosomemal sites (Lüllmann et al., 1969; Peters et al., 1974; Lüllmann and Peters, 1976; Gervais et al., 1977; Schwartz and Adams, 1980) were true, and if binding of one molecule of ouabain causes the release of a constant amount of Ca\textsuperscript{2+} per site irrespective of the number of binding sites present, then one might expect a greater amount of calcium mobilization in cells with increased numbers of Na,K-ATPase complexes and, consequently, a greater inotropic response, contrary to what was observed.

In conclusion, cultured chick heart cells grown for prolonged periods in low [K\textsuperscript{+}]\textsubscript{o} ouabain, or veratridine possessed greater numbers of sodium pump sites in the sarcosomemal membrane. This long-term effect on Na,K-ATPase appears to be mediated by intracellular sodium. Cells grown in low K\textsuperscript{+} were less sensitive to inotropic effects of 1 mM [K\textsuperscript{+}], or ouabain as a result of active extrusion of internal sodium by the additional sodium pumps and, therefore, reduced calcium uptake via sodium-calcium exchange.

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INDEX TERMS: Na,K-ATPase • Sodium pump • Ouabain • Digitalis • Cultured heart cells
Effects of growth in low potassium medium or ouabain on membrane Na,K-ATPase, cation transport, and contractility in cultured chick heart cells.

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