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Effects of isoproterenol on Na^+ and K^+ transport in frog skin epithelium

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The acute effects of isoproterenol on Na^+ extrusion and K^+ uptake across the basolateral membrane of the isolated epithelium of the frog skin were examined. A chloride-free sulfate Ringer was used in all experiments. Isoproterenol caused an approximate doubling of the short-circuit current (I_{sc}) and the transepithelial Na^+ flux (J_{13Na}). I_{sc} remained equal to J_{13Na}. After isoproterenol treatment, ouabain inhibited I_{sc} and J_{13Na} in a manner similar to control tissues. Ouabain-sensitive K^+ uptake was also measured under comparable conditions. In two sets of experiments, K^+ uptake was increased on average by only 5 and 17 percent after isoproterenol treatment. Thus, isoproterenol caused Na^+ flux to more than double while K^+ uptake increased by only 5–17%. These data cannot be readily accounted for by a pump with a fixed Na^+/K^+ exchange ratio.

Introduction

The apical membrane is a primary site of hormonal regulation of transepithelial Na^+ flux [1,2]. With increased Na^+ influx into the cell, a corresponding increase in Na^+ exit is required to prevent changes in cell volume [3]. The primary mechanism for net Na^+ exit is the Na^+ pump. Therefore, ouabain-sensitive basolateral Na^+ exit would be expected to increase as apical Na^+ influx rises. If the Na^+/K^+ exchange ratio is fixed, there should also be an increase in ouabain-sensitive basolateral K^+ uptake directly related to the transepithelial Na^+ transport rate. While the Na^+/K^+ exchange ratio was found to be fixed at about 3:2 in some studies [4–6], this has not been found to be true in other studies using epithelia [7–12]. Indeed, it recently has been shown that there is not a linear relationship between K^+ uptake and short-circuit current (I_{sc}) where I_{sc} is equal to net transepithelial Na^+ flux [9].

The purpose of the present study was to examine the acute effects of isoproterenol on Na^+ and K^+ fluxes at the basolateral membrane of frog skin. This approach allowed us to address the following questions. Does K^+ uptake increase linearly with acute increases in transepithelial Na^+ transport rate? A linear relationship of K^+ uptake versus transepithelial Na^+ transport rate would be predicted for a fixed stoichiometry even if basal stoichiometry was not 3:2. Does Na^+ recycling across the basolateral membrane change as a function of transport rate? This could compromise estimates of pump stoichiometry if I_{sc} is no longer a good estimate of Na^+ flux through the pump.

The results show that K^+ uptake increased only slightly during a time when Na^+ transport had more than doubled. This appeared to be independent of geometry since similar results were obtained using matched pieces of skin. The use of I_{sc} as an estimate of Na^+ flux through the pump was not compromised due to changes in Na^+ recycling at the basolateral membrane. The overall results of this study cannot easily be explained by the presence of unstirred layers at the basolateral membrane or by an increase in turnover rate or number of pumps with a fixed Na^+/K^+ exchange ratio.

Methods

Tissue preparation

Isolated epithelia were prepared by the method of Aceves and Erlij [13] as modified by Fisher et al. [14]. The corium was separated from the epithelium without the use of hydrostatic pressure after incubation of the basolateral side with Ringer solution containing collagenase (0.4 mg/ml CLS II, Cooper Biochemical, Freehold, NJ) for 2–3 h, leaving the isolated sheets of tissue glued (Zipbond, Tescom Corp., Minneapolis, MN)

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to Lucite rings. Northern *Rana pipiens* (Kons, Germantown, WI) were used in all studies. The experiments were done at room temperature. A sulfate Ringer containing 56 mM Na_2SO_4, 2.4 mM $KHCO_3$, and 1.2 mM $CaSO_4$ was used in all studies. Solutions were equilibrated with room air (pH 8.1). NaCl (1 M) salt bridges were used in the chamber studies. Isoproterenol, propranolol, and ouabain were purchased from Sigma Chemical, St. Louis, MO; furosemide was a gift from Hoechst-Roussel Pharmaceuticals, Somerville, NJ; amiloride was a gift from Merck, Sharpe & Dohme Research Laboratories, West Point, PA.

Adopting the three compartment notation of Curran et al. [15], the subscripts of the unidirectional tracer fluxes J_{ij} will refer to the flux from compartment i to compartment j. Compartments 1,2 and 3 are apical, cellular, and basolateral, respectively. Accordingly, J_{13Na} is the unidirectional flux of Na^+ from the apical to the basolateral solution.

Previous studies have shown that changes in epithelial cell volume can be estimated from changes in thickness of the epithelium [16]. Using Hoffman Modulation Contrast optics and the calibrated fine focus on a Nikon microscope we were able to focus on the apical as well as the basolateral surface of the frog skin. We obtained estimates of epithelial thickness before and after treatment with isoproterenol.

Sodium flux studies

Short-circuit current (I_{sc}) and transepithelial Na^+ influx (J_{13Na}) measurements were made according to methods described in detail previously [17]. In brief, epithelia were short circuited for 30 min, after which ^{22}Na (3 μCi/ml, New England Nuclear, Boston, MA) was added to one side of the chamber and its rate of appearance on the opposite side determined. Tracer equilibration of Na^+ in the intracellular pool is complete in 10–15 minutes [17–19].

Sodium pool studies were performed using standard techniques [20,22] as described for frog skin [19,21]. Tissues were loaded symmetrically by incubation of pieces in Ringer solution containing tracer and in some cases $9 \cdot 10^{-6}$ M isoproterenol. Loading from the basolateral side was done when the tissues were mounted in chambers. Ringer containing tracer was then placed in the basolateral chamber. In both cases, the tissues were incubated with tracer for 30–40 min, a time sufficient to reach specific activity equilibrium with the loading solution [19]. The washout protocol was the following. At the end of the incubation period, symmetrically loaded pieces were rinsed briefly (20 s) in tracer-free Ringer containing ouabain (10^{-3} M) and amiloride (10^{-4} M). This maneuver served to remove most of the adherent tracer and to prevent any rapid loss of tracer from the cells. It also defined time zero for the washout curve. The tissue was then transferred every two minutes to counting vials containing 3 ml of Ringer with ouabain and amiloride. The vials were continuously agitated on a shaker. At the end of the total washout period (about 30 min), the tissue was dried overnight at 100 °C to obtain the tissue dry weight. (The average dry weight for this set of studies was 1.3 mg/cm^2.) The tissue was then extracted by digestion with warm 0.5 M NaOH. The digested sample was neutralized with an equal volume of 0.5 M HCl. Scintillation fluor was added and tracer activity was determined using conventional methods. For the chamber experiments, the tracer incubation period was ended by rapidly flushing Ringer containing the drugs ouabain and amiloride on both sides of the tissue. The tissue was then punched out using a cork borer, rinsed briefly, and then subjected to the washout protocol described above. The washout curves represent the calculated amount of tracer remaining in the tissue as a function of time.

Potassium flux studies

Potassium uptake studies were done using methods previously described [9]. Studies were done using pieces of tissue in beakers or skins mounted in chambers. For the former, a piece of epithelium was divided into two or three parts allowing each tissue to serve as its own control. The tissues were treated with $9 \cdot 10^{-6}$ M isoproterenol or just Ringer (control). After a 30-min equilibration period, the tissues were incubated for 2–3 min in Ringer containing $^{42}K^+$ which in some cases also had isoproterenol or isoproterenol plus ouabain (10^{-3} M). At the end of this period, the tissue was rinsed for 20 s in tracer-free Ringer containing ouabain. Ouabain served to prevent any further uptake of tracer [9]. The tissue was counted in a gamma counter and then dried to determine tissue dry weight.

In chamber experiments, uptakes were determined after I_{sc} had stabilized (usually after 10–15 min) or after the tissue had been treated with isoproterenol for 30 min. Tracer-containing Ringer was perfused into the basolateral chamber completely replacing the previous solution. Other experiments have shown that uptake is linear for at least 5 min under these conditions. After 2–3 min, tracer-free Ringer containing ouabain was rapidly perfused in to terminate the uptake. The tissue was then punched out, rinsed briefly, and counted as described above. The average I_{sc} was determined during the 2–3-min uptake period.

Flux values are reported in μA/cm^2. This is for comparison purposes only and does not imply mechanism. Statistics were calculated using standard techniques [23]. When means were multiplied, errors were estimated according to techniques outlined in Ostle and Malone [24].

Results

Isoproterenol on sodium flux and I_{sc}

Beta agonists have been shown to stimulate I_{sc} in intact frog skin by increasing transepithelial Na^+ influx through the Na^+ transport pathway and transepithelial Cl efflux through the glands [25–31]. In the isolated epithelium (free from glands), isoproterenol causes no increase in I_{sc} after amiloride treatment. In intact skin, an amiloride-insensitive I_{sc} was shown to be dependent on Cl^- in the basolateral solution [26,29]. Since this study was directed at examining the effect of isoproterenol on the amiloride-sensitive Na^+ transport pathway, split skins in a chloride-free Ringer were used throughout. To ensure that I_{sc} was a good estimate of net transepithelial Na^+ transport under the conditions of this study, the relationship between Na^+ influx and I_{sc} was determined before and after isoproterenol treatment (Fig. 1). As shown, isoproterenol ($9 \cdot 10^{-6}$ M) increased I_{sc} and Na^+ influx within 1–2 minutes. After 10–15 minutes, a plateau was reached. On average I_{sc} went from 12.8 ± 1.7 to 27.8 ± 2.9 $\mu A/cm^2$ ($n = 10$) after 30 min. Sodium influx went from 13.2 ± 1.8 to 26.2 ± 2.7 $\mu A/cm^2$ ($n = 10$) during the same time interval. This represents about a 6% discrepancy between I_{sc} and Na^+ influx after isoproterenol treatment. The correlation between Na^+ influx and I_{sc} was examined more closely as shown in Fig. 2. Prior to isoproterenol treatment Na^+ influx and I_{sc} correlated well. This demonstrates that virtually all transepithelial Na^+ influx is through the cells and little passes through the shunt. After isoproterenol treatment there was still a good correlation between Na^+ flux and I_{sc}. Linear least-squares regression forced through zero of the data gave a slope 0.94, a number not significantly different from one. A small amount of chloride may have been present that diffused from the salt bridges. As a check for possible chloride secretion [25–31], furosemide (10^{-3} M) was added to the basolateral side after isoproterenol treatment. After 2–4 min of furosemide, the I_{sc} and J_{13Na} were 100.2 ± 1.1 and 100.9 ± 0.2 percent of the pre-furosemide value ($n = 3$), respectively. Therefore in subsequent calculations, I_{sc} was used as an index of transepithelial Na^+ flux before and after isoproterenol treatment.

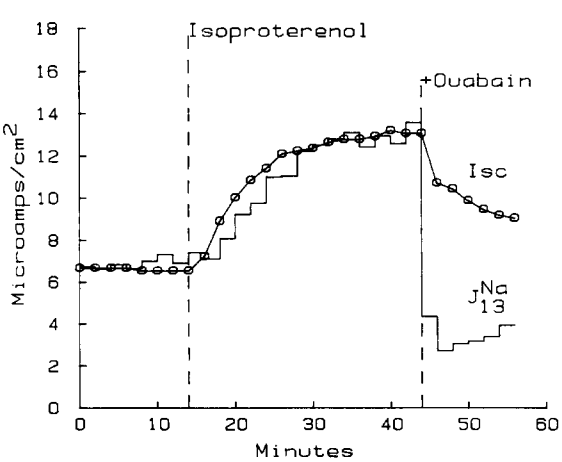

Fig. 1. Effect of isoproterenol ($9 \cdot 10^{-6}$ M) on short circuit current (I_{sc}) and transepithelial Na^+ influx (J_{13Na}). Ouabain (10^{-3} M) was added after 30 min of isoproterenol treatment.

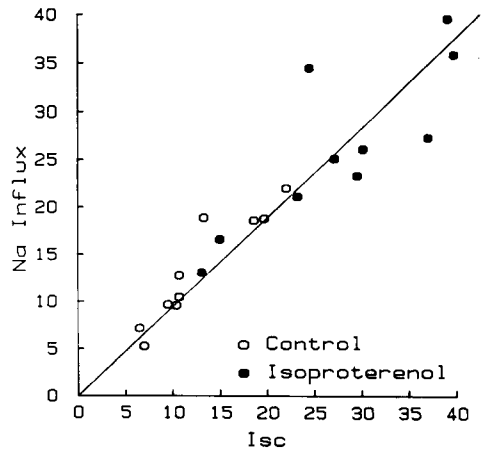

Fig. 2. Correlation of transepithelial Na^+ influx and I_{sc} before and after isoproterenol treatment. The solid line is the line of identity.

After isoproterenol treatment, ouabain was added to determine the ouabain-sensitive Na^+ influx (J_{13Na}). As shown in Fig. 1, ouabain caused substantial inhibition of I_{sc} and Na^+ influx. After 2–4 min, I_{sc} was decreased from 28.5 ± 3.4 to 19.4 ± 1.9 (7) $\mu A/cm^2$. Na^+ influx was reduced from 25.4 ± 2.6 to 6.8 ± 1.6 (7) $\mu A/cm^2$. This represents a 32 and 73 per cent inhibition of I_{sc} and Na^+ flux, respectively. These values are not different from those previously reported for control tissues under similar conditions [17,18].

To ensure that this result was via an adrenergic receptor, the concentration response relationship was examined and the effects of propranolol were determined. The data in Fig. 3 show the peak fractional increase in I_{sc} after treatment with various concentrations of isoproterenol. The peak usually occurred in 10–15 min. Each concentration was evaluated on a separate skin from the same batch of frogs. As shown here, 10^{-4} M isoproterenol was sufficient to achieve a near maximal response. The concentration that achieved a half-maximal response was $1.2 \cdot 10^{-6}$ M while $9 \cdot 10^{-6}$ M isoproterenol gave an 84% increase in this group. This was the concentration used in the remaining experiments. It should be noted that the magnitude of the response of I_{sc} to isoproterenol varied from week to week and from batch to batch of frogs. The reason for this is not clear.

The beta blocker propranolol (10^{-4} M) significantly blocked the isoproterenol response at $9 \cdot 10^{-6}$ M (Fig. 3). This is consistent with interaction with a beta receptor. Propranolol alone caused a 25 ± 8.5 (7) percent increase in I_{sc}. The value shown in Fig. 3 is the ad-

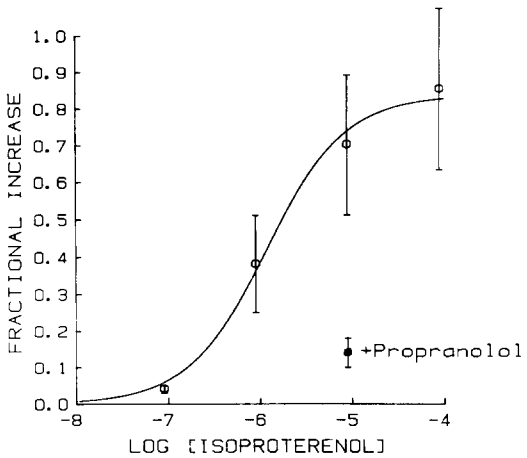

Fig. 3. Concentration response curve for the effect of isoproterenol on the fractional increase in I_{sc}. Values reported are the averge of the peak response for 6–8 skins at each concentration. The effect of isoproterenol was also determined in the presence of propranolol (10^{-4} M). The best fit of the data gave a concentration for a half-maximal response of 1.2 μM.

ditional effect that isoproterenol had on top of the propranolol response.

In four experiments, epithelial thickness was measured as described in the Methods. After treatment with isoproterenol, the ratio of experimental to control thickness was 1.05 ± 0.13. Isoproterenol does not change epithelium thickness and by inference cell volume as determined by this technique.

Determination of the Na^+ transport pool and intracellular specific activity of Na^+

If ouabain acts only to block the Na^+ pump, Na^+ extrusion from the cell by the pump can be determined from the ouabain-sensitive Na^+ extrusion if the intracellular specific activity of $^{22}Na^+$ is known. Tissues were loaded with $^{22}Na^+$ and washouts performed as described in Methods. A typical experiment where a skin was exposed to tracer on both sides under control conditions is shown in Fig. 4A. Notice the remnant of an apparent fast pool and the remaining slow pool. The data were fit to the following bi-exponential equation using a nonlinear least-squares algorithm.

$$y = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (1)$$

where A_1 and A_2 are intercepts, k_1 and k_2 are slopes, and t is time. In this experiment, the intercept of the slow pool corresponded to a Na^+ transport pool (A) of 43.7 nmol/cm^2.

A parallel set of experiments was done with isoproterenol treated tissues. As shown in Fig. 4B, there was a significant elevation in the slow pool intercept. In this case it was 103.8 nmol/cm^2. On average, the sodium transport pool more than doubled after isoproterenol treatment (Table 1). These values translate to about 8 and 18 mM intracellular Na^+ using 4.6 μl/cm^2 of tissue [19], respectively. Since there was no change in epithelial thickness after isoproterenol treatment, we used 4.6 μl for both calculations. The control values were very similar to those observed previously using this [19] and other techniques [28,34–36]. The increase in tissue Na^+ content after isoproterenol was not quite as large as that observed by Rick et al. [28] using the electron microprobe.

Isoproterenol had very little effect on the slope of the washout curve (k) which gives an index of basolateral membrane Na^+ permeability in the presence of ouabain and amiloride (Table I). We calculated the value of the unidirectional Na^+ efflux from the cells under these conditions as $k * A$. The values for control and isoproterenol treated tissues were 2.5 ± 0.3 (12) and 5.9 ± 1.1 (8) μA/cm^2.

Tissues were also loaded from the basolateral side only. A smaller 'calculated' pool size was expected due to the relatively low basolateral membrane permeability [19]. The shapes of the washout curves were essentially identical to those in Fig. 4; the intercepts were lower.

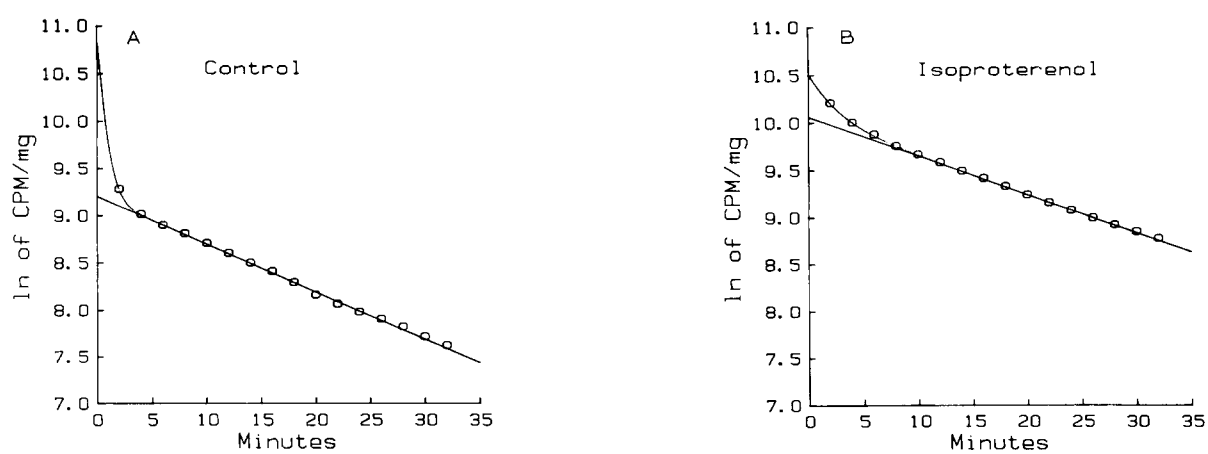

Fig. 4. Measurement of the Na^+ transport pool (loaded from both sides) under control conditions (A) and after isoproterenol treatment (B). See text for details.

TABLE I

Effect of isoproterenol on the Na^+ transport pools and washout kinetics

Values are means ± S.E. (n). A and A^b are in nmol/cm^2. k is in min^{-1}.

	Both sides		Basolateral side	
	A	k	A^b	k
Control	36.4 ± 4.4 (12)	0.044 ± 0.003 (12)	5.3 ± 0.9 (7)	0.049 ± 0.005 (7)
Isoproterenol	80.7 ± 13.3 (8) *	0.043 ± 0.004 (8)	7.4 ± 1.2 (8) *	0.046 ± 0.008 (8)

* Indicates a statistical difference from control.

Values for the pool determined from basolateral loading (A^b) and k are summarized in Table I. Isoproterenol caused a small increase in A^b. The rate constants were essentially identical to those determined when tissues were loaded from both sides. This result indicates that the basolateral membrane permeability to Na^+ was similar in both populations of frogs.

With the data in Table I we calculated the unidirectional Na^+ uptake across the basolateral membrane J_{32Na}. As shown by Stoddard and Helman [19],

$$J_{32Na} = (I_{sc} * A^b/A^a) \quad (2)$$

where I_{sc} is taken as an estimate of J_{12Na} and A^a is the difference between A and A^b. We used the values of 36.4 and 80.7 from Table I for A. The values for A^b/A^a calculated in this way were 0.13 ± .001 (7) and 0.07 ± 0.001 (8) for control and isoproterenol, respectively. I_{sc} values were from Table II. The calculated values for J_{32Na} were 1.7 ± 0.3 (7) and 1.5 ± 0.2 (8) for control and isoproterenol treated skins, respectively. Isoproterenol had no significant effect on basolateral membrane Na^+ uptake as determined by this method.

The ouabain-sensitive Na^+ flux was calculated in the following way. From the results presented in Fig. 2 it will be assumed that I_{sc} gives a measure of net Na^+ flux across the basolateral membrane. The unidirectional Na^+ efflux from the cells (J_{23Na}) was calculated by adding J_{32Na} to J_{12Na}. The results were 14.7 ± 2.4 and 22.2 ± 2.8 for control and isoproterenol conditions, respectively. These values were then multiplied by the fraction of tracer flux that remained after ouabain. The results were 3.9 ± 0.8 and 5.9 ± 1.1, respectively. If the assumption that ouabain blocks only the pump with no other effects holds, the difference between these two numbers will be equal to the unidirectional Na^+ efflux through the pump.

TABLE II

Effect of isoproterenol on I_{sc}, K^+ uptake (J_K), and the calculated pump coupling ratio (r).

I_{sc}, J_K, and J_K (corr.) are in μA/cm^2. See text for the correction factors used in the calcultion of J_K (corr.) $r = I_{sc}/J_K$ (corr.).

	I_{sc}	J_K	J_K (corr.)	r
Control (7)	13.0 ± 2.4	5.8 ± 0.5	5.2 ± 0.5	2.4 ± 0.3
Isoproterenol (7)	20.7 ± 2.8 *	6.8 ± 0.5	6.8 ± 0.5 *	3.0 ± 0.3 *

* Indicates a significant difference from control.

K^+ uptake across the basolateral membrane

Since uptake can presumably take place via the pump, the K^+ channel, and possibly other routes, uptake by the pump is often defined as that flux inhibitable by ouabain. However, ouabain may also alter uptake through other mechanisms as well, particularly through K^+ channels if ouabain causes a change in membrane potential. Under control conditions, a correction factor of 0.906 (multiplied by J_{32K}) was used to account for uptake via the K^+ channels [9]. After isoproterenol, conditions have changed significantly to warrant recalculation of this correction factor. Using values of −40 mV for intracellular voltage [32] and 80 mM for intracellular K^+ concentration [33], the new correction factor is 0.996. All uptake data reported here have been corrected by these factors. Other possible errors in uptake measurements will be considered in the discussion.

The first set of uptake analyses was done using pieces of tissue incubated in beakers. The tissue was divided into two or three pieces; one served as the control and the other was treated with isoproterenol or isoproterenol plus ouabain. The mean K^+ uptake under control conditions was 7.9 ± 0.63 (10) μA/cm^2. After isoproterenol treatment (30 min), K^+ uptake was 8.3 ± 1.07 (10) μA/cm^2. Ouabain plus isoproterenol reduced the uptake to 0.7 ± 0.36 (5) μA/cm^2. There was essentially no increase in K^+ uptake after isoproterenol even though in parallel studies I_{sc} had increased by 80–100%.

Another set of studies was done in chambers which allowed the simultaneous measurement of K^+ uptake and I_{sc}. The results of 7 control and 7 isoproterenol-treated (30 min) experiments are summarized in Table II and Fig. 5. These uptake values were not different from those determined previously using pieces. Skins were selected so there would be overlap in the I_{sc} ranges between control and isoproterenol-treated tissues. The mean I_{sc} prior to isoproterenol treatment was 8.9 ± 1.8 (7) for that group of skins. There was no noticeable

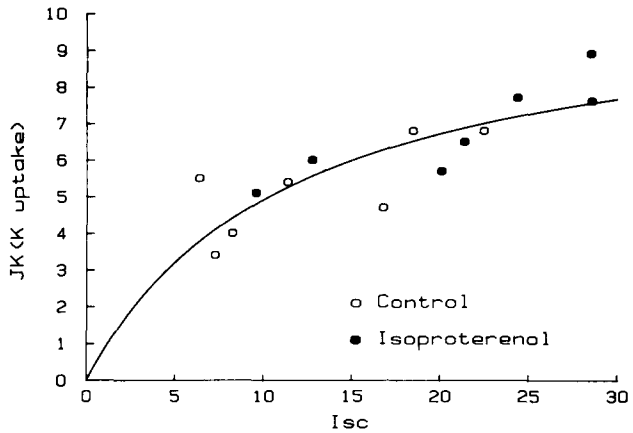

Fig. 5. Relationship of corrected K^+ uptake (see text) to short circuit current (I_{sc}) under control and after treatment with isoproterenol. The curve was fit to the equation $J_K = 1/(m + (r^0/I_{sc}))$ where m is an arbitrary contant and r^0 may be related to the coupling ratio at zero transport rate [9]. The best fit for these data was obtained with $m = 0.094$ and $r^0 = 1.09$. See text for details.

difference in the pattern of control versus experimental conditions. K^+ uptake increased with I_{sc} but appeared to saturate at the higher transport rates.

With estimates of Na^+ efflux (see above) and K^+ uptake, we can calculate the pump coupling ratio in the traditional way. Mean values are given in Table II. There was an increase in the apparent coupling ratio with an increased Na^+ transport rate. There was no apparent distinction between those tissues in which I_{sc} had been acutely increased and the controls.

Discussion

The purpose of the present study was to measure the effects of acute changes in Na^+ transport rate on the fluxes of Na^+ and K^+ across the basolateral membrane of a Na^+ transporting epithelium. The rapid increase in Na^+ transport caused by isoproterenol was accompanied by an increase in the intracellular Na^+ transport pool with little effect on basolateral membrane Na^+ permeability. There were only small increases in apparent K^+ uptake (10–20%) when the Na^+ transport rate had more than doubled.

Sodium fluxes

Recent studies of the Na^+ transport pool have shown that apical loading and basolateral washout of tracer Na^+ give essentially identical results [17,19]. In addition, apical membrane loading can be virtually eliminated by pretreatment of the tissue with amiloride [19]. These results strongly support the idea that tracer Na^+ uptake from either the apical or basolateral side loads into the same transport pool. Support for this idea comes from the present study where the rate constants for Na^+ washout were virtually identical whether the tissue was loaded from the apical or basolateral side (Table I). Studies of basolateral side loading of the transport pool probably represent a maximum estimate of this parameter due to the possibility that some Na^+ may load into nontransporting cells [19].

We calculated the unidirectional Na^+ uptakes across the basolateral membrane (J_{32Na}) from pooled kinetic data. These values were less that 2 $\mu A/cm^2$ for both control and isoproterenol-treated tissues. These values are significantly less that the 6 $\mu A/cm^2$ observed previously [19]. This difference may be due to the use of a chloride-free sulfate Ringer in the present study. This will greatly reduce any chloride-coupled basolateral membrane Na^+ transport mechanisms.

The unidirectional efflux of Na^+, J_{23Na}, from the cell was calculated from the net flux (I_{sc}) and basolateral uptake (J_{32Na}). This was found to average 1–2 $\mu A/cm^2$ larger than I_{sc}. Using this number and the fractional inhibition of tracer flux by ouabain, the ouabain-sensitive Na^+ flux was calculated. The ouabain-sensitive flux calculated in this way was significantly less than I_{sc} for both control and isoproterenol treated skins. Since I_{sc} gives a reliable estimate of net Na^+ flux across the basolateral membrane and no other mechanisms of net Na^+ flux have been identified, this discrepancy is likely to be due to ouabain effects on cell parameters other than the pump. Ouabain is known to depolarize the basolateral membrane voltage [17,34]. This probably had little effect because Na^+ flux has been shown to be independent of voltage [17,35]. However, there could be some ouabain-induced fluxes. In a previous study it was shown in chloride Ringer that there is a significant furosemide-sensitive, chloride-dependent, neutral Na^+ flux after ouabain treatment [17,19]. We have used sulfate Ringer to minimize this component. There are still additional ouabain and furosemide-insensitive fluxes that persist even in sulfate Ringer [17]. This represents a leak through as yet undefined mechanisms. An analysis of the flux ratio for Na^+ may provide some insight into this. If J_{32Na} is neutral and coupled to an ion that can easily redistribute itself across the membrane, then flux through this pathway will be proportional to the cis concentration of Na^+. Therefore, Na^+ flux through this path would be

$$J_{23Na}(\text{passive}) = J_{32Na}[Na^+ \text{ cell}]/[Na^+ \text{ out}] \quad (3)$$

Using the data for J_{32Na} of 1.7 and 1.5 $\mu A/cm^2$, J_{23Na} (passive) would be 0.1 and 0.3 $\mu A/cm^2$ for control and isoproterenol conditions, respectively. These numbers apporach zero and represent a minimal estimate of the nonpump Na^+ extrusion from the cell. Therefore, the ouabain 'induced' portion of this nonpump flux ranges from near zero to the 2 to 6 $\mu A/cm^2$ measured in the washout studies. In the absence of a separate extrusion mechanism in the basolateral membrane, we must conclude that ouabain either does not completely block

Na^+ flux through the pump or that it simultaneously activates other mechanisms that mask the ouabain-sensitive pump flux. The idea of a ouabain induced flux is also supported by the fact that ouabain inhibited over 90% of K^+ uptake (suggesting almost complete pump inhibition) but only 75% of the Na^+ efflux (Ref. 9, present study). While our data support the idea that isoproterenol does not change basolateral membrane Na^+ permeability prior to ouabain leading to an increased load on the Na^+ pump not reflected as a corresponding change in I_{sc}, complications with ouabain inhibition of the pump do not allow calculation of the precise Na^+ load under these conditions. If we assume that all of the uptake across the basolateral membrane is extruded by the pump, then J_{23Na} is within 2 $\mu A/cm^2$ of I_{sc}. Therefore, I_{sc} remains a good estimate of pump flux.

Potassium uptakes

Quantification of K^+ uptake via the pump into the tissue potentially may be compromised by at least two sources of error. The first source comes from uptake via the K^+ channel. The fact that ouabain inhibited over 90% of K^+ uptake in this study suggests that uptake via the channel is small. However, it is possible that the voltage change caused by ouabain significantly reduced uptake through the channel. This was considered in a previous study and it was concluded that about 90% of the K^+ uptake measured under control conditions was via the pump [9]. We recalculated this factor for isoproterenol conditions and corrected all K^+ uptakes. Second, it is possible that there is an unstirred layer at the basolateral membrane such that the rate of flux is limited by the diffusion gradient across this layer. At high rates of transport the gradient across and unstirred layer may be large enough such that tracer concentration at the membrane is significantly decreased. We can get rough estimates of how an unstirred layer might compromise the data by using Fick's Law. The equation is

$$J = (D * (C_o - C_i))/L \quad (4)$$

where J is K^+ uptake, D is the diffusion constant for K^+ in free solution ($1.67 \cdot 10^{-5}$ cm^2/s), L is the thickness of an unstirred layer, and $(C_o - C_i)$ is the gradient required to support the flux J. We have used 200 μM for L [13], a value that is about four times the thickness of the tissue. This value was derived using the split skin and represents the measured average thickness of the apparent unstirred layer. If we assume that the coupling ratio is in fact 3 : 2, then the expected K^+ uptake by the pump would be approximately two thirds of I_{sc}. Using a value of 20 $\mu A/cm^2$ for I_{sc}, this gives a K^+ uptake of 13.4 $\mu A/cm^2$. Plugging these numbers into Eqn. 4 gives a gradient of 2.4 to 2.23 mM from the bulk solution to the membrane to support a net flux of abundant K^+. Similar reasoning can be applied when considering the required gradient for tracer flux. This implies that the specific activity of tracer at the membrane is about 93% of that in the bulk solution. Even looking at maximum values for I_{sc} (about 30 $\mu A/cm^2$) this calculation gives only a 10% error. This calculation may even over estimate the error if one considers that K^+ uptake should be normalized to actual membrane area [39], which would decrease its value by anywhere from 3- to 10-fold. Since we have made our measurements at steady state and there is no net K^+ flux across this membrane, there can be no gradient in abundant K^+ if the pumps and K^+ channels are near each other on the membrane [40]. Therefore, it is not likely that an increase in abundant K^+ concentration at the membrane would dilute out tracer specific activity in addition to the unstirred layer effect. These calculations suggest that our errors are on the order of 10–15%; we would have to be in error by 2–3-fold for our data to be consistent with fixed 3 : 2 coupling. In addition to the arguments presented above, protocols were used that should minimize geometric differences between experimental and control tissues. Paired studies were done using two of three pieces of tissue from the same skin. Presumably they would have the same unstirred layer, tortuous path, etc. The results of these paired studies were essentially identical to those obtained from populations.

Stoichiometry of the Na^+ pump

Estimates of K^+ uptake and Na^+ efflux have been made under control and after isoproterenol treatment. To the extent that these values represent K^+ uptake and efflux through the pump, we can draw some conclusions about pump stoichiometry. As shown in Fig. 5, K^+ uptake increased with I_{sc} but appeared to saturate at the higer Na^+ transport rates. Under control conditions, the relationship of K^+ uptake to I_{sc} was essentially the same as previously observed [9]. We hypothesized that pump coupling ratio varied and was larger at higher rates of spontaneous transport rate. Those data did not eliminate the idea of a pumps with fixed stoichiometry; a skin with a high transport rate and therefore a large coupling ratio might retain this ratio if transport rate was slowed down. The converse might also be considered. Our data may offer some insight into this question. If pump coupling ratio is fixed but has different values depending on the initial rate of transport, then r can be determined from the equation describing the smooth curve in Fig. 5. This value of r (determined at the I_{sc} prior to isoproterenol addition) was used to calculate K^+ uptake at the new I_{sc} after isoproterenol. This can be compared with the measured uptake after stimulation with isoproterenol. The calculated K^+ uptake was 10.9 ± 1.5 (7) for the tissue treated with isoproterenol. This was significantly larger than the corre-

sponding measured values in Table I. This result does not support the idea that r is fixed during acute changes I_{sc}. Apparently isoproterenol regulates transport and pump coupling ratio in a fashion similar to the spontaneous variation. This might be expected since isoproterenol is believed to increase intracellular levels of cAMP, the same second messenger used by the naturally occurring hormone arginine vasotocin. The increased throughput of Na^+ through the cell may be caused by an increased turnover rate of individual pumps due to increased intracellular levels of Na^+ and perhaps due to an increased number of active pumps. Evaluation of these possibilities is beyond the scope of the present study.

The literature contains a wide range of reports on the modes of the pump [41] with very little agreement as to whether pump stoichiometry remains fixed under all conditions or can vary as conditions warrant [1,3–10, 37,41]. Under many circumstances (perhaps at lower transport rates), the pump stoichiometry approaches 3 : 2 [4–6,41,42]. However, there have also been recent reports of variable stoichiometry in epithelia [7–12]. Indeed, a recent study by Sansom and O'Neil [11] using an entirely different approach than that used here showed that when Na^+ transport was increased with DOCA (deoxycorticosterone), pump stoichiometry increased from 3 : 2 to about 3 : 1, a result almost identical to the present study. With the known variety of modes of operation of the pump [41–44], it is not surprising that macroscopic variability in pump coupling ratios is observed. Whether this is brought about by changes in individual pump units or shifts in the fraction of pumps in a particular mode has yet to be determined.

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