J. Membrane Biol. 26, 109–119 (1976)

Effects of Cd^{++} on Short-Circuit Current across Epithelial Membranes

I. Interactions with Ca^{++} and Vasopressin on Frog Skin

Stanley D. Hillyard and Harvey C. Gonick

Department of Medicine, UCLA Center for the Health Sciences, Los Angeles, California 90024

Received 4 March 1975; revised 26 August 1975

Summary. Cadmium ion (Cd^{++}) significantly increased potential difference (PD) and short-circuit current (SCC) across isolated frog skin when added to the outside Ringer's solution at 10^{-4}, 10^{-3} and 5×10^{-3} M concentration. Resistance was reduced by 10^{-4} M Cd^{++} but not significantly changed by the higher concentrations. When SCC was first stimulated by vasopressin, 10^{-4} and 10^{-3} M Cd^{++} produced additive stimulation which was reversible by washing with Cd^{++}-free Ringer's. If SCC was first stimulated by Cd^{++}, further stimulation by vasopressin was additive with 10^{-4} M Cd^{++} but completely inhibited by 10^{-3} M Cd^{++}. Elevating the calcium ion (Ca^{++}) concentration of the outer Ringer's from 10^{-3} M to 5×10^{-3} M or 10^{-2} M prior to Cd^{++} treatment did not reduce the magnitude of SCC stimulation by Cd^{++}. Removal of Ca^{++} from the outside Ringer's with 2×10^{-3} M EDTA increased SCC as predicted. Subsequent addition of 5×10^{-3} M Cd^{++} drastically reduced SCC below control levels while equimolar concentrations of Cd^{++} and EDTA reduced SCC only to control levels. These results suggest that Cd^{++} interacts with the components of the apical plasma membranes of epithelial cells which are associated with the stimulation of SCC by vasopressin and Ca^{++} removal and may be a useful probe for elucidating these components.

Divalent cations have been shown to affect the rate of sodium transport, as measured by short-circuit current (SCC), across isolated frog skin. Total removal of Ca^{++} from the solution bathing the outer surface of the skin stimulates SCC in a manner suggesting facilitated entry of sodium into the cells (Curran & Gill, 1961). This effect is rapid, with maximal elevation of SCC being observed within 10–15 min, and is reversible by subsequent replacement of Ca^{++}. Stimulation of SCC by Ca^{++} removal is additive with the elevation of SCC produced by vasopressin and is believed to involve a separate mechanism in the apical plasma membrane (Herrera & Curran, 1963). Recently, Cd^{++} added to the outside

Send correspondence to: Stanley D. Hillyard, Ph.D., Department of Life Science, The University of Texas of the Permian Basin, Odessa, Texas 79762.

of the skin has been shown to stimulate SCC across isolated frog skin (Borghraef, Stymans & Van Driessche, 1971; Banks, 1974). As with Ca^{++} removal, the maximal elevation in SCC following Cd^{++} treatment is rapid and can be reversed by removal of Cd^{++}.

The purpose of the present study was the examination of the interaction between the effects of Cd^{++}, Ca^{++} and vasopressin on SCC across the isolated frog skin, in an attempt to more clearly define the nature of Cd^{++} stimulation of SCC.

Materials and Methods

Measurement of Short-Circuit Current

Short-circuit current (SCC) was measured across the isolated ventral skin of *Rana pipiens* (Los Angeles Biologicals) with the technique of Ussing and Zerhan (1951). Three chambers were bored into the Ussing-type cell such that simultaneous measurements could be made on three adjacent regions of the same piece of skin. Each chamber had a cross-sectional area of 1 cm^2. Potential difference was measured with agar-Ringer's bridges and paired calomel reference electrodes connected to an Orion Research Model 801 digital voltmeter. SCC was applied with Ag/AgCl electrodes via agar-Ringer's bridges to the Ringer's chambers. After mounting the skin between the chamber halves, one hour was allowed for equilibration of SCC. At this time, potential (PD) was monitored constantly (open circuit). SCC was applied at 10-min intervals in all experiments. No electrical coupling was observed between the chambers as short-circuiting one chamber failed to alter the voltage across the skin in the other two chambers. When SCC varied by less than 10% during three consecutive intervals (control period), the experimental treatments were begun. The Ringer's used during the control period contained NaCl 111 mM, KCl 2.0 mM, $NaHCO_3$ 2.4 mM, $CaCl_2$ 1.0 mM and glucose 5.0 mM (standard Ringer's). The pH was 7.5–7.6 and the osmotic pressures 220 mOsm/liter. If the control SCC of the three chambers varied by more than 25% of one another or if the SCC of any of the chambers was less than 15 $\mu A/cm^2$, the preparation was discarded.

Effects of Cd^{++} on SCC, PD and Resistance

After a stable control period, the Ringer's bathing the outer surface of one section of the skin was replaced with fresh Ringer's containing 10^{-4} M Cd^{++} while a second section was similarly treated with 10^{-3} M Cd^{++}. The outer Ringer's bathing the third section of the skin was exchanged with fresh Ringer's containing no Cd^{++}. In a separate series of experiments the outer Ringer's was replaced with Ringer's containing 5×10^{-3} M Cd^{++}. PD and SCC were recorded for 30 min after the addition of Cd^{++}. Skin resistance was calculated as the ratio of PD/SCC in each experiment.

Since Cd^{++} will precipitate with the bicarbonate ions in the frog Ringer's it was necessary to mix Cd^{++} with Ringer's just prior to its addition. This was done by mixing 4 ml of frog Ringer's made to 5/4 strength with 1 ml of distilled water containing the appropriate amounts of $CdCl_2$ to give final concentrations of 10^{-4}, 10^{-3} and 5×10^{-3} M. No precipitation was visible during the time course of any of the experiments.

Cadmium and Calcium

Two series of experiments were conducted to examine the interaction of Cd^{++} and Ca^{++}. Since both are divalent cations, some common site of action at the outer surface of the skin might be expected. In the first series the effects of increasing the Ca^{++} concentration of the outer Ringer's from 10^{-3} M to 5×10^{-3} M and 10^{-2} M were examined on the stimulation of SCC that is normally produced by 10^{-3} M Cd^{++}. In this series, following the control period, the outer Ringer's of two of the chambers received fresh Ringer's containing 5×10^{-3} M and 10^{-2} M Ca^{++}. The third chamber was refilled with standard Ringer's and SCC was recorded for 30 min. Cd^{++}, 10^{-3} M, was then added to the three outer chambers and SCC recorded for a final 30 min.

In the second series the effect of Cd^{++} was examined in the absence of Ca^{++} in the outer Ringer's. After the control period, the outer Ringer's in two of the chambers was replaced with Ringer's having no Ca^{++} and containing 2×10^{-3} M EDTA; the pH of this solution was adjusted to 7.5 prior to use with concentrated NaOH. Thirty minutes after the removal of Ca^{++} one of the outer chambers was re-filled with a Ringer's solution containing 5×10^{-3} M Cd^{++} in addition to 2×10^{-3} M EDTA. The higher dose of Cd^{++} was used to insure that some free Cd^{++} would be present. The second chamber was re-filled with fresh 2 mM EDTA Ringer's. SCC was recorded for an additional 30 min whereupon the outer chambers were filled with standard Ringer's and SCC recorded for a final 30 min. In a separate experiment equimolar amounts (2×10^{-3} M) of Cd^{++} and EDTA were added after the initial EDTA treatment in order to assess the effects of the EDTA-bound Cd^{++}.

Cadmium and Vasopressin

Two final series of experiments were conducted to examine the relationship between the stimulation of SCC produced by Cd^{++} and that produced by vasopressin (ADH). In the first series, after a stable control period, ADH (Pitressin, Parke-Davis) was added to the inner Ringer's of all three chambers to give a final concentration of 100 mU/ml. SCC was monitored for 30 min after ADH treatment whereupon Ringer's containing 0, 10^{-4} or 10^{-3} M Cd^{++} was added to the outer chambers. SCC was measured for an additional 30 min at which time the Cd^{++} in the outer chambers was washed out with standard Ringer's; SCC was then measured for a final 30 min.

The second series of experiments was an extension of the dose-response study. Skins that had been pretreated with 0, 10^{-4} or 10^{-3} M Cd^{++} in the outer Ringer's were treated with 100 mU/ml ADH in the inner Ringer's of the three chambers. SCC was recorded for 30 min following ADH treatment at which time the Cd^{++} in the outer Ringer's was washed out with standard Ringer's and SCC recorded for a final 30 min.

Analysis of Results

Changes in SCC produced by the experimental treatments were normalized as the percentage change from the control period in each individual experiment. Statistical comparisons utilized Student's *t*-test, except where indicated.

Results

In Table 1, the changes in PD, SCC and resistance produced by treating the outer surface of the skins with 10^{-4}, 10^{-3} and 5×10^{-3} M

Table 1. The effect of cadmium ion on potential difference, PD (mV), short-circuit current, SSC (μA), and resistance, R (Ohms) across isolated frog skin[a]

	Cadmium Concentration							
	0		10^{-4} M		10^{-3} M		5×10^{-3} M	
	C	E	C	E	C	E	C	E
PD								
Mean	45	45	55	66	47	69	51	99
SE	±12	±12	±16	±17	±13	±10	±8	±8
Percent	0		+20		+47		+94	
p	NS		<0.025		<0.001		<0.001	
SCC								
Mean	82	80	73	102	77	128	73	133
SE	±17	±17	±12	±10	±17	±21	±14	±24
Percent	−3		+40		+66		+82	
p	NS		<0.025		<0.001		<0.001	
R								
Mean	703	702	731	627	635	579	782	809
SE	±229	±221	±153	±129	±097	±082	±148	±090
Percent	0		−14		−9		+3	
p	NS		<0.025		NS		NS	

[a] Each value represents the mean of five observations ± one standard error of the mean (SE) during a control period (C) and 30 min after cadmium addition to the outer bathing solution (E). When the change following cadmium treatment is significant, the level of significance (*p*) is given, while insignificant change is termed NS.

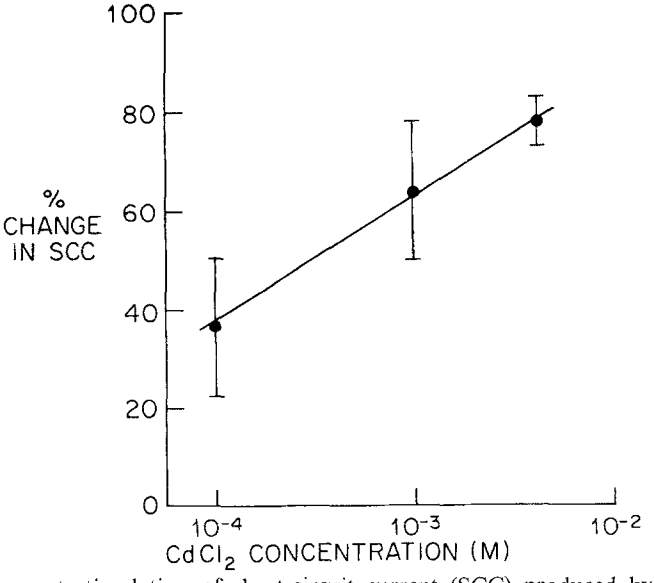

Fig. 1. The percent stimulation of short-circuit current (SCC) produced by 10^{-4}, 10^{-3} and 5×10^{-3} M cadmium chloride added to the outer surface of the skin. Each point is the mean of five experiments and the vertical bar ±1 standard error (SE)

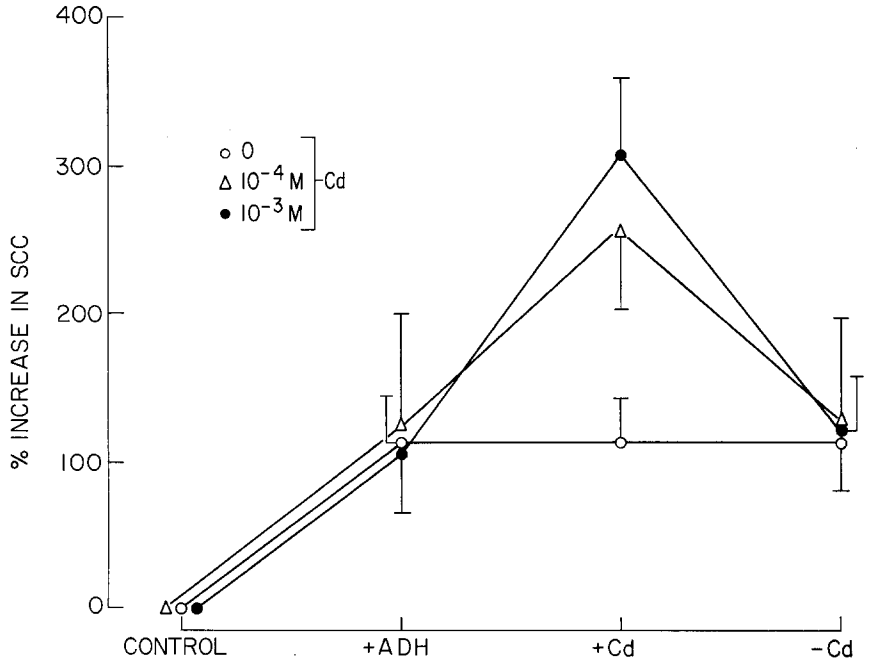

Fig. 2. The changes in SCC produced by sequentially adding vasopressin (ADH), cadmium and then removing cadmium during three consecutive 30-min periods. The data are normalized as the percent change in SCC relative to the control period for five experiments. For clarity, only one standard error bar is drawn in Figs. 2–5 and it may be in either the plus or minus direction. The cadmium concentrations are indicated in the figure legend

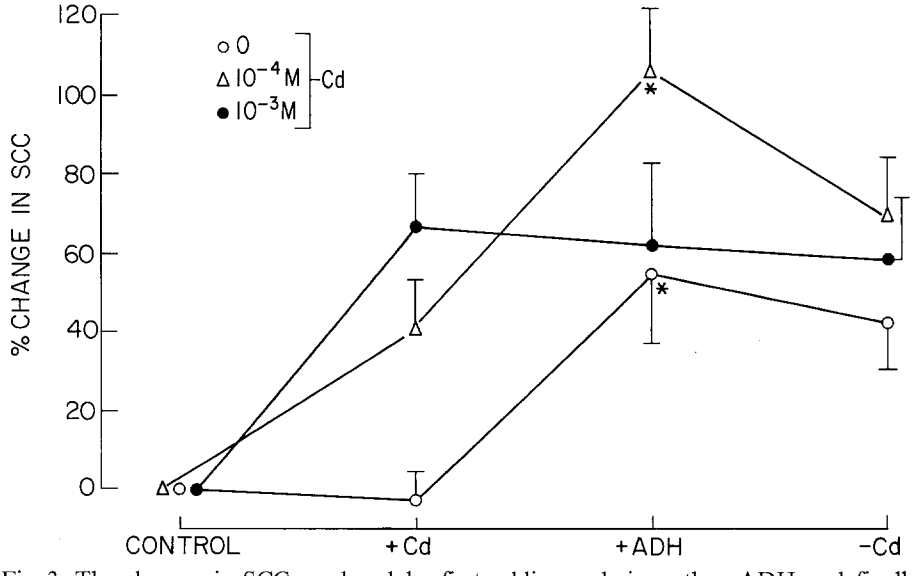

Fig. 3. The changes in SCC produced by first adding cadmium, then ADH and finally removing the cadmium. As in Fig. 2 each point represents the mean of five experiments and the cadmium concentrations are indicated in the figure legend. The asterisk (*) indicates significant elevation of SCC relative to the cadmium treatment value ($p < 0.025$)

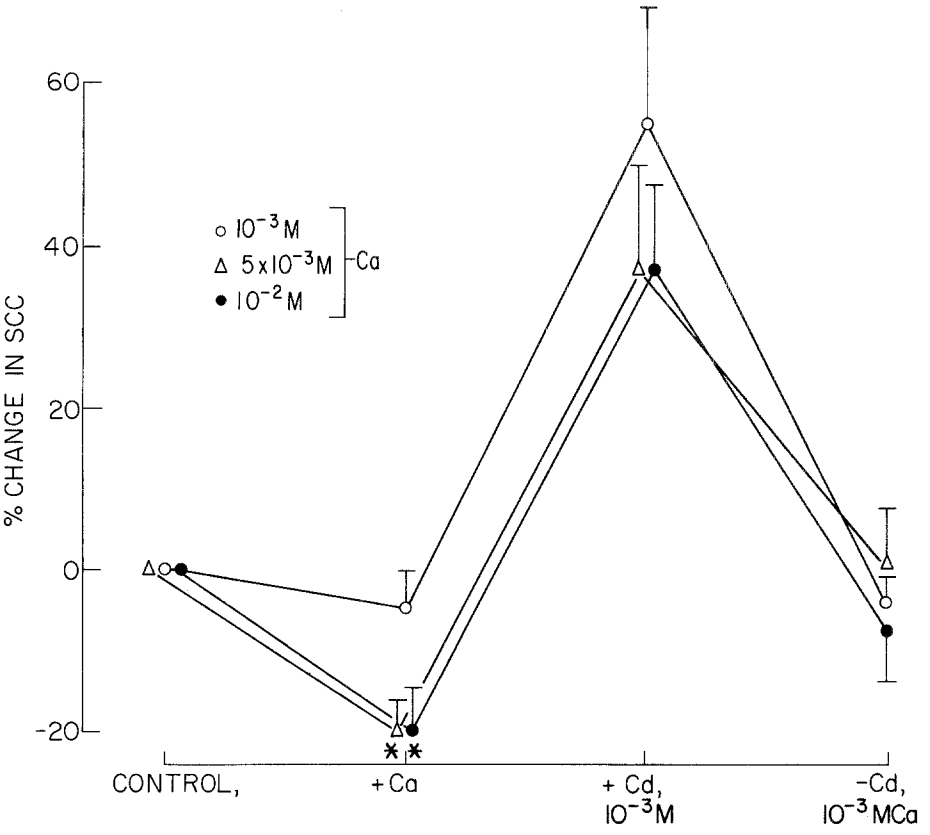

Fig. 4. The effect of increasing the external calcium ion concentration from 10^{-3} to 5×10^{-3} and 10^{-2} M on SCC and on the elevation in SCC produced by subsequent addition of 10^{-3} M cadmium. Each point is the mean of six experiments. The asterisk (*) indicates significant reduction of SCC by the elevated calcium concentrations ($p < 0.001$)

Cd^{++} are compared with the changes in these parameters occurring in untreated control skins over the same time interval. Neither PD, SCC nor resistance changed significantly during two consecutive 30-min periods in the control skins while PD and SCC were significantly elevated by Cd^{++} at all concentrations tested. The stimulation of SCC by Cd^{++}, expressed as percent elevation from the control period, increased linearly with the log of the Cd^{++} concentration in the range 10^{-4} M to 5×10^{-3} M (Fig. 1). Mean resistance (calculated from each individual experiment) was significantly reduced by 10^{-4} M Cd^{++}, but not significantly altered by 10^{-3} or 5×10^{-3} M Cd^{++}.

In skins treated with ADH, SCC was elevated by more than 100% over the control levels and remained so for 90 min (Fig. 2). When Cd^{++} was subsequently added to the outer Ringer's, SCC was further elevated

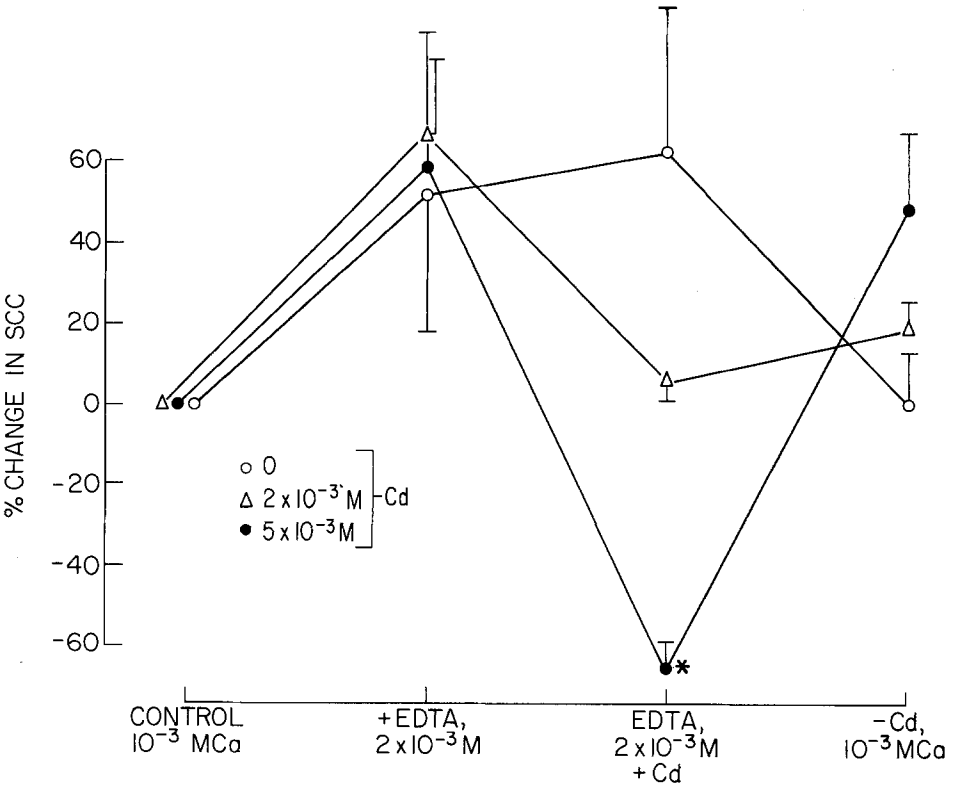

Fig. 5. The changes in SCC produced by first removing calcium in the outer solution with 2×10^{-3} M EDTA and then adding 5×10^{-3} or 2×10^{-3} M cadmium to the calcium-free preparation or continuing the EDTA (o Cd) treatment. The outer solutions of both pieces of skin then received standard Ringer's containing 10^{-3} M calcium and no cadmium. The asterisk (*) indicates a significant reduction of SCC below the control level ($p < 0.001$)

in a dose-dependent manner, 10^{-3} M stimulating SCC significantly more than 10^{-4} M ($p < 0.05$). When the skins were first treated with 0, 10^{-4} M and 10^{-3} M Cd^{++}, SCC increased as shown in Fig. 1. If ADH was then added to the inner Ringer's, SCC was significantly increased in the skins treated with 0 and 10^{-4} M Cd^{++} but not significantly changed in the skins pretreated with 10^{-3} M Cd^{++} (Fig. 3). The percentage increase in SCC produced by ADH in this series of experiments was smaller than shown in Fig. 2, probably due to the higher control levels of SCC (73–82 $\mu A/cm^2$).

The control level of SCC was significantly reduced by 20% when the Ca^{++} concentration of the outer Ringer's was elevated from 10^{-3} M to 5×10^{-3} M or 10^{-2} M while continued treatment with 10^{-3} M Ca^{++} (Standard Ringer's) did not change SCC over a period of 60 min (Fig. 4).

The stimulation of SCC produced by 10^{-3} M Cd^{++} (57–60%) was not significantly affected by increasing the Ca^{++} concentration (Fig. 4)[1]. Removal of Ca^{++} from the outer Ringer's by pretreatment with EDTA elevated SCC by over 50% (Fig. 5). In the absence of Cd^{++}, SCC remained elevated, returning to the control level when the outer chamber was refilled with standard Ringer's. If, however, 5×10^{-3} M Cd^{++} was added to the outer chamber in the continued presence of 2×10^{-3} M EDTA, SCC was markedly reduced. Replacement of this Ca^{++}-free Ringer's containing 5×10^{-3} M Cd^{++} with standard Ringer's caused SCC to rise to a level significantly above that of control and comparable to the level produced by pretreatment with EDTA. On the other hand, when equimolar amounts (2×10^{-3} M) of Cd^{++} and EDTA were added to the outer Ringer's the SCC returned to a value ($6 \pm 5\%$) not significantly different from control.

Discussion

The present study verifies the observations of Borghraef *et al.* (1971) and Banks (1974) that Cd^{++} in the outer bathing solution stimulates SCC across isolated frog skin and extends their observations to lower concentrations of 10^{-4} and 10^{-3} M. In the cited studies and in the present experiments the assumption is made that SCC reflects active transepithelial sodium transport, as originally demonstrated by Ussing and Zerahn (1951). However, as isotopic sodium fluxes were not measured, it is possible that changes in SCC may not be accompanied by equivalent changes in net sodium transport.

In both the earlier experiments and in the present study PD rose concomitantly with SCC; however, the resistance across the skin was reduced by 10^{-4} M Cd^{++} and unaffected by 10^{-3} and 5×10^{-3} M Cd^{++}. Borghraef *et al.* (1971) found resistance to increase by 11.7% following 2.5×10^{-3} M Cd^{++} exposure, while Banks (1974) found resistance to increase by 45% after exposure to 10^{-2} M Cd^{++}. Thus, at high doses there appears to be a direct relationship between the concentration of Cd^{++} and the increases in both resistance and SCC. At the lower dosage used in the present experiments, however, the increase in SCC occurred independently of changes in resistance. The effect of Cd^{++} on SCC then appears to be unrelated to a change in resistance. As we did not attempt to minimize edge damage, there may have been changes in resist-

[1] Significance evaluated by analysis of variance.

ance which were not detected. Banks (1974) has shown that the increase in resistance observed with 10^{-2} M Cd^{++} is largely related to reduced Cl-conductance. Hence, the Cd^{++} concentration required to reduce Cl-conductance, such that skin resistance is elevated, is greater than that required to stimulate SCC.

The effect of Cd^{++} on SCC closely resembles that of Ca^{++} removal with EDTA (Curran & Gill, 1961; Herrera & Curran, 1963) in that the rise in SCC is very rapid and completely reversible by washing with Ca^{++}-containing Ringer's. Owing to the rapid onset of the stimulation of SCC by Cd^{++} and the reversibility of this effect, it seems reasonable to assume that the site of action of Cd^{++} is at the apical membranes of the epithelial cells where increased sodium entry will promote an increase in transepithelial transport. Cd^{++} may also have some effect on the paracellular shunting of ions across the skin as suggested by the experiments of Ferreira (1969) with Cu^{++}, which increases the passive outfluxes of both sodium and chloride across the isolated skin of *Rana ridibunda*. In these same experiments sodium fluxes increased proportionally with SCC following treatment with Cu^{++}, indicating that the entry of sodium across the apical membranes into the transport compartment was increased and that SCC provided an accurate estimate of transepithelial sodium transport despite alterations in the shunt pathways.

It might be anticipated that Cd^{++} and Ca^{++}, as divalent cations, have some common site of action on the apical membranes. Elevated Ca^{++} levels, however, failed to diminish the effect of Cd^{++} (Fig. 4) suggesting that these ions do not compete for binding sites. The study of the effects of Cd^{++} in the absence of Ca^{++} was initially attempted with standard Ringer's containing no calcium chloride. Experimentally, we found it necessary to add EDTA to the outer Ringer's in order to produce the elevation in SCC observed by Curran and Gill (1961). EDTA presumably acts by removing sufficient Ca^{++} from the apical plasma membranes of the epithelial cells to increase the entry of sodium into the cells. EDTA (2×10^{-3} M) was, therefore, retained in the outer Ringer's to assure that minimal Ca^{++} was present and an excess of Cd^{++} (5×10^{-3} M) was added. It was then necessary to control for the effect of the chelated Cd^{++}-EDTA complex by adding equimolar (2×10^{-3} M) quantities of Cd^{++} and EDTA to a separate piece of skin.

When Cd^{++} and EDTA were added to the outer Ringer's in equimolar amounts, SCC returned from EDTA-stimulated values to control values. The stability constants (Martell, 1964) of Ca^{++} and Cd^{++} for EDTA show Cd^{++} to have a much higher affinity for the chelator.

Therefore, effectively no Ca^{++} would be bound by EDTA, and Ca^{++} diffusing from the inner Ringer's would be free to bind to the apical plasma membranes and reduce sodium entry. When Cd^{++} was added in excess of EDTA, SCC was reduced by 66% of the control value, demonstrating that free Cd^{++} inhibits SCC across the EDTA-pretreated skin. Thus, EDTA treatment reverses the stimulating effect of Cd^{++} on SCC. On the other hand, when both Cd^{++} and EDTA are washed out and replaced with standard Ringer's (Fig. 5), SCC returns to stimulated levels, indicating a residual effect of Cd^{++} on the apical surface of the skin.

When the skin was first treated with ADH and Cd^{++} subsequently added to the outer surface, the stimulatory effects on SCC were additive, implying that under these circumstances these substances acted independently. ADH also stimulated the SCC of skins pretreated with 10^{-4} M Cd^{++}. When Cd^{++} was added first at a concentration of 10^{-3} M, however, further increase in SCC produced by ADH was prevented. Thus, at this higher concentration Cd^{++} may have interacted with the apical surface of the skin in such a way as to prevent the increase in sodium permeability normally produced by ADH (Biber & Cruz, 1974). It is of interest that Zn^{++} added to the apical surface of the isolated toad bladder also inhibits the effect of ADH on SCC (Bentley, 1967). Also, Zn^{++} prevents the dephosphorylation reaction of the 49,000 dalton protein which Walton *et al.* (1975) have found coincides with the stimulation of SCC by ADH in toad bladder. As Cd^{++} is believed to be highly reactive with sulfhydryl groups of proteins (Kagi *et al.*, 1974) this ion could also bind directly to a membrane protein which affects permeability. It seems less likely, under the conditions of our experiments, that Cd^{++} interacts with intracellular enzymes associated with permeability changes or active transport.

When Cd^{++} is injected into the circulation of dogs *in vivo* proximal tubular reabsorption of sodium is increased (Vander, 1962*a,b*). Thus, Cd^{++} acutely increases sodium transport across the proximal kidney tubule as well as the frog skin, although it is not known whether the renal effect is produced by an increased entry of sodium from the tubular lumen into the cells or an increased transport of sodium out of the cells into the peritubular space. With chronic injection, Cd^{++} produces Fanconi syndrome, a disorder in which proximal tubular reabsorption of sodium and other ions is inhibited, possibly by the inhibition of Na-K-ATPase activity (Indraprasit & Gonick, 1972). Neither Banks (1974) nor ourselves were able to show consistent inhibition of SCC

when Cd^{++} was applied to the inside of the frog skin even though it is a powerful inhibitor of the transport enzyme Na-K-ATPase *in vitro* (Rifkin, 1965). Banks (1974) was able to demonstrate inhibition of SCC when Cd^{++} and cysteine were added simultaneously, suggesting that this ion can reach the transport mechanism when associated with cysteine. The frog skin may thus provide a useful model for evaluating the acute and chronic effects of Cd^{++} on the kidney.

We are grateful to Kristen Hall and Ruby McCarty for their secretarial assistance in preparing the manuscript. This work was supported by Grant No. 435IG5 from the Americal Heart Association, Greater Los Angeles Affiliate, and by USPHS Grant No. ES-852-01.

References

Banks, R.O. 1974. Cadmium stimulation and cadmium-cysteine inhibition of active Na transport by the frog skin. *Physiologist* **17**:175

Bentley, P.J. 1967. Mechanism of action of neurohypophyseal hormones: Actions of manganese and zinc on the permeability of the toad bladder. *J. Endocrinol.* **39**:493

Biber, T.U.L., Cruz, L.J. 1974. Effect of antidiuretic hormone on sodium uptake across outer surface of frog skin. *Am. J. Physiol.* **225**:912

Borghraef, R., Stymans, A., VanDriessche, W. 1971. The action of divalent cations on the electrical resistance of frog skin. *Arch. Int. Physiol. Biochem.* **79**:171

Curran, P.F., Gill, J.R. 1961. The effect of calcium on sodium transport in frog skin. *J. Gen. Physiol.* **45**:625

Ferreira, K.T.G. 1970. The effects of Cu^{2+} on isolated frog skin. *Biochim. Biophys. Acta* **203**:557

Herrera, F.C., Curran, P.F. 1963. The effect of Ca and antidiuretic hormone on Na transport across frog skin. I. Examination of interrelationships between Ca^{++} and hormone. *J. Gen. Physiol.* **46**:999

Indraprasit, S., Gonick, H.C. 1972. Cadmium-induced experimental Fanconi syndrome. *Clin. Res.* **20**:596

Kagi, J.H.R., Himmelhock, S.R., Whanger, P.D., Bethune, J.L., Vallee, B.L. 1974. Equine hepatic and renal metallothioneins. Purification, molecular weight, amino acid composition and metal content. *J. Biol. Chem.* **249**:3537

Martell, A.E. 1964. Stability Constants of Metal Ion Complexes. Second Edition. Chemical Society, London

Rifkin, R.J. 1965. *In vitro* inhibition of $Na^{+}-K^{+}$ and Mg^{2+} ATPases by mono, di and trivalent cations. *Proc. Soc. Exp. Biol. Med.* **120**:802

Ussing, H.H., Zerhan, K. 1951. Active transport of sodium as the source of electric current in the short-circuited, isolated frog skin. *Acta Physiol. Scand.* **23**:110

Vander, A.J. 1962*a*. Cadmium enhancement of proximal tubular sodium transport. *Am. J. Physiol.* **203**:1005

Vander, A.J. 1962*b*. Effect of cadmium on renal tubular sodium transport. *Am. J. Physiol.* **203**:1

Walton, K.G., DeLorenzo, R.J., Curran, P.F., Greengard, P. 1975. Regulation of protein phosphorylation and sodium transport in toad bladder. *J. Gen. Physiol.* **65**:153