Interaction of Cadmium, Calcium, and Amiloride in the Kinetics of Active Sodium Transport through Frog Skin

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Abstract The epidermal effects of activator (cadmium) or inhibitors (calcium and amiloride) as well as their interactions in active Na transport through bullfrog abdominal skin were analyzed kinetically. Cadmium at 1 mM increased the short circuit current (SCC) to 126%. The Hill coefficient (n) for the relation between $[Cd^{2+}]$ and SCC was 0.737, which indicated negative cooperation. Calcium at over 8 mM decreased the SCC to 80% at its maximum effect. Calcium inhibited the Cd^{2+} -induced SCC at lower $[Cd^{2+}]$, and facilitated it at higher $[Cd^{2+}]$. Calcium acted uncompetitively on the binding reaction of Na^+ with a Na entry channel. Cd^{2+} alone or Cd^{2+} under the influence of calcium also acted uncompetitively on the reaction. Amiloride at 5×10^{-5} M inhibited the SCC almost completely. The Hill coefficient in the relation between amiloride and SCC was 0.757, indicating the presence of negative cooperation. In contrast to the effect of calcium, the Cd^{2+} -induced SCC was much reduced at higher [amiloride]. Amiloride inhibited the channel-Na binding reaction in a mixed manner (competitive + noncompetitive), while Cd^{2+} activated the reaction under the influence of amiloride noncompetitively. From the SCC model based on the hypothesis that the kinetic constants are invariable even under interaction of the activator and inhibitors, the percentage of the various forms of channel-ion complex were calculated. The SCC was then calculated, assuming that it was directly proportional to the sum of the rate of decomposition of channel-Na complex and the higher rate of decomposition of channel-Na- Cd^n complex. The above assumption is supported by the fact that there is good agreement between the calculated SCC and experimentally obtained SCC in the mixture of the activator and the inhibitor.

Active Na transport by frog skin has been reported to be increased by the application of Cd^{2+} (STYMANS and BORGHGRAEF, 1969; HILLYARD and GONICK, 1976; HAYASHI *et al.*, 1977). Cd^{2+} is considered to exert its effect on the outer facing membrane of the skin (HAYASHI *et al.*, 1978; TAKADA and HAYASHI, 1978a, 1980a). The Cd^{2+} effect is not due to enhancement of Na, K-ATPase activity

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in the frog epidermis (TAKADA and HAYASHI, 1978b).

Many investigators have noted the inhibitory effect of Ca^{2+} and amiloride on Na transport through frog skin (*cf.* BENOS *et al.*, 1979; ACEVES and CUTHBERT, 1979). They found that these inhibitors acted on the outer facing membrane and decreased the Na translocation. They also carried out kinetic studies on Ca^{2+} and amiloride effects. We therefore attempted to analyze the mechanism of the Cd^{2+} effect on Na translocation in more detail by comparing the action of calcium and/or amiloride.

The following hypotheses were discussed. (1) Na translocation can be described quantitatively in terms of channel-ion complexes and their dissociation constants. (2) Furthermore, the dissociation constants appeared never to be changed by the simultaneous application of the activator or inhibitors.

Preliminary descriptions of this work have been given elsewhere (TAKADA and HAYASHI, 1980b).

MATERIALS AND METHODS

The abdominal skin of bullfrogs (*Rana catesbeiana*) of both sexes was used. The frogs were captured in Saitama Prefecture, Japan, and kept at room temperature in a bath for a week or two without feeding. The water level was maintained so that about half of the body was immersed.

The frogs were anesthetized by intrathecal injection of 1 ml per kg body weight of 0.25 g/ml urethane solution. The abdominal skin was dissected out and mounted on the open tip of a glass cannula (8–12 mm inner diameter), with the dermal side facing the inside of the cannula. Both sides of the skin were soaked in aerated Ringer's solution. The hydrostatic pressure difference between the epidermal and dermal sides was nullified by adjusting the saline levels. SO_4 -Ringer's solution (see Table 1 for various [Ca gluconate] and Table 2 for various $[\text{Na}^+]$) was used to eliminate the possible influence of passive and active transport of Cl^- (ZADUNAISKY *et al.*, 1963).

The potential difference (PD) of the skin was measured (dermal side positive) at room temperature (18–23°C) using a pair of calomel electrodes, a DC amplifier, and a millivoltmeter. A current was applied across the skin through a pair of silver electrodes. The short circuit current (SCC) was determined by adjusting the PD to zero manually (USSING and ZERAHN, 1951). The methods for measuring the PD and SCC were as described in detail previously (HAYASHI *et al.*, 1977). The skin resistance (R_M) was calculated using the equation: $R_M = \text{PD}/\text{SCC}$.

The skin, in the open-circuit condition, was allowed to equilibrate with the control bathing solution for at least 3 hr before commencing the experiments.

Cadmium and/or amiloride were applied to the epidermal side of the skin in saline containing CdSO_4 and/or amiloride HCl.

All results were normalized to values obtained with control skin (100%), and expressed as the mean value and one standard error of the mean.

Table 1. Composition of the Ringer's solution (mM) with various [Ca].

	Ca-free	1-Ca	5.25-Ca	9.5-Ca	18-Ca	35-Ca
Na ₂ SO ₄	55	55	55	55	55	55
K ₂ SO ₄	1	1	1	1	1	1
Ca-gluconate	0	1	5.25	9.5	18	35
Glucose	10	10	9	8	6	2
Sucrose	60	60	52.5	45	30	0
Tris-H ₂ SO ₄	10	10	10	10	10	10
pH	7.2	7.2	7.2	7.2	7.2	7.2

Table 2. Composition of the Ringer's solution (mM) with various [Na⁺].

	110-Na	60-Na	30-Na	10-Na	4-Na
Na ₂ SO ₄	55	30	15	5	2
K ₂ SO ₄	1	1	1	1	1
Ca-gluconate	0	0	0	0	0
Glucose	10	30	52	67	70
Sucrose	60	92	100	105	110
Tris-H ₂ SO ₄	10	35	50	60	61
pH	7.2	7.2	7.2	7.2	7.2

RESULTS

Effect of calcium on SCC

The effect of various [calcium] on the short circuit current (SCC) of bullfrog abdominal skin was investigated. The composition of the Ringer's solution in this series of experiments is shown in Table 1. The "1-Ca" saline on both sides was simultaneously replaced by a test saline about 3 hr after setting up the skin preparation. Each test saline was applied for 20 min, then it was replaced by the "1-Ca" saline. Recovery usually took about 30 min.

Figure 1 summarizes the results of 32 experiments. Each data point is nor-

Fig. 1. Relationship between [Ca] and the short circuit current (SCC) of bullfrog skin. [Na⁺]=110 mM. The SCC is normalized with respect to that in "Ca-free" saline. The vertical bar on each data point represents the standard error of the mean.

Fig. 2. Effects of 1 mM Cd^{2+} on the SCC and potential difference (PD) in "1-Ca" and "35-Ca" saline. $[\text{Na}^+] = 110$ mM.

malized with respect to the SCC in the "Ca-free" saline, and represents the average of 5 to 6 experiments. The SCC was inhibited by the addition of Ca gluconate; the higher the [calcium], the less the SCC became. Sodium transport current was not completely inhibited by calcium. Maximum inhibition was observed at a [calcium] of over 8 mM, where the SCC decreased to 80% of control. The concentration of calcium at half-maximum inhibition was 1.4 mM.

Effect of Cd^{2+} at different [calcium]

It has been demonstrated that epidermally applied Cd^{2+} increases the SCC. This Cd^{2+} -induced increase in SCC was examined at various [calcium] (see Table 1) on both sides of the skin.

Figure 2 illustrates a typical example of the effect of 1 mM Cd^{2+} on the SCC in "1-Ca" and "35-Ca" saline. The preparation was soaked in the "1-Ca" saline for about 4 hr, and Cd^{2+} was first applied epidermally for 20 min when both the SCC and PD attained a new steady level. The SCC and PD were increased to 125.0 and 120.2%, respectively, and the R_M was decreased to 95.6%. After washing the skin with the "1-Ca" saline, the bathing solution was substituted by "35-Ca" saline. After 20 min, the SCC and PD decreased to 80.3 and 80.0%, respectively. Then Cd^{2+} was added to the "35-Ca" epidermal solution to a final concentration of 1 mM. The SCC and PD increased to 144.9 and 130.5%, respectively, and the R_M decreased to 89.6% compared to the values just prior to the application of Cd^{2+} . The results of the second Cd^{2+} application in the "1-Ca" and "35-Ca" saline were similar to those of the first.

The results of 8 experiments of this type are summarized as follows. Cadmium at 1 mM increased the SCC by $27.6 \pm 5.8\%$ in "1-Ca" saline, while the Cd^{2+} -induced increase of the SCC was greatly enhanced in "35-Ca" saline ($45.1 \pm 6.7\%$). The difference in the effect of Cd^{2+} was highly significant ($P < 0.003$). The results of the second Cd^{2+} application in the "1-Ca" and "35-Ca" saline were similar to those of the first: $33.2 \pm 5.7\%$ in the "1-Ca" saline, and $50.6 \pm 5.3\%$ in the "35-Ca" saline. Again, the difference in the Cd^{2+} -induced increase of the

Fig. 3. Relationship between $[Cd^{2+}]$ and the SCC at various $[Ca]$. $[Na^+] = 110$ mM. The SCC is normalized with respect to that in "Ca-free" saline (Fig. 3A). The same data are replotted for the relationship, $(SCC_{Cd} - SCC_{Cd-free})$ vs. $[Cd^{2+}]$, in Fig. 3B. Open circles, closed circles and crosses indicate data for "Ca-free," "9.5-Ca," and "35-Ca" saline, respectively.

SCC was highly significant ($P < 0.01$) in the second "1-Ca" and "35-Ca" saline. However, no significant differences were observed between the results of the first and second Cd^{2+} application in the "1-Ca" saline ($P > 0.5$) or between those in the "35-Ca" saline ($P > 0.1$).

Figure 3A shows the relationship between $[Cd^{2+}]$ and the SCC at [calcium] of 0, 9.5, and 35 mM (see Table 1). Each data point is normalized with respect to the SCC in Cd-free "Ca-free" saline, and represents the average of 5 to 9 experiments. The SCC in the "9.5-Ca" saline was less than in the "Ca-free" saline at all $[Cd^{2+}]$ tested. In the Cd^{2+} -free condition, the SCC was decreased to almost the same extent in both the "35-Ca" and "9.5-Ca" saline. However, the SCC in the "35-Ca" saline appeared to be larger than that in the "Ca-free" saline for over 10 mM Cd^{2+} .

Fig. 4. Hill plot of the Cd^{2+} -induced increase in SCC vs. $[\text{Cd}^{2+}]$ in "Ca-free" saline. The data are taken from Fig. 3, and the linear regression line was fitted by the method of least squares.

Figure 3B shows the dose-response curve of the Cd^{2+} -induced increase in SCC, $(\text{SCC}_{\text{Cd}} - \text{SCC}_{\text{Cd-free}})$ vs. $[\text{Cd}^{2+}]$ at various [calcium], replotted from the data shown in Fig. 3A. As $[\text{Cd}^{2+}]$ increases, so the Cd^{2+} -response increase in a sigmoid manner. This curve shifts upwards as [calcium] becomes greater. The curve appears to coincide with the following equation:

$$\text{SCC} = \text{SCC}_{\text{max}} / (1 + 10^{-(k_1 \cdot \log[\text{Cd}^{2+}] + k_2)}), \quad (1)$$

where k_1 and k_2 are constants. In order to determine the values of the constants in the equation, a Hill plot was drawn from the data.

The logarithmic form of the Hill equation in enzyme kinetics gives a linear relationship for $\log [v / (V_{\text{max}} - v)]$ vs. $(n \cdot \log [S] - \log K)$, where $[S]$ is the substrate concentration, v and V_{max} are the velocity and maximum velocity of the reaction, and n and K are constants (SEGEL, 1975). The same equation is used for the analysis of the relationship between the Cd^{2+} -induced increase in SCC and $[\text{Cd}^{2+}]$ (v and $[S]$ in the above equation) (Fig. 4). From the data in Fig. 3B, the V_{max} in the "Ca-free" saline was estimated to be 37%. The linear regression line for the Hill plot was fitted by the method of least squares. The slope of the line (n) is 0.737, which is clearly less than unity, suggesting that the binding reaction between Cd^{2+} -binding sites and Cd^{2+} is not of the Michaelis-Menten type, but of the sigmoid type which exhibits negative cooperation (see SEGEL, 1975). The intersection of the Hill plot and a horizontal line at $v = V_{\text{max}}/2$ ($y=0$) gives $[S]_{0.5}$ as 341 μM . The Hill coefficients (n) in the "9.5-Ca" and "35-Ca" salines were almost identical to that in the "Ca-free" saline. The constants in Eq. (1), k_1 and k_2 , were expressed in terms of the constants in the Hill plot as n and $-\log [S]_{0.5}^n$, respectively.

Effects of Ca and Cd^{2+} at different $[\text{Na}^+]$

Figure 5 illustrates the influence of various $[\text{Na}^+]$ (Table 2) on the SCC in the

Fig. 5. Eadie-Hofstee plot of (normalized SCC) vs. (normalized SCC)/[Na⁺]. The SCC was normalized with respect to that in Ca-free Cd-free 110 mM [Na⁺]. The inset shows the relationship between [Na⁺] and the normalized SCC. A, control saline in Table 2; B, +5 mM Ca; C, +2 mM Cd; D, +5 mM Ca +2 mM Cd.

presence or absence of 2 mM Cd²⁺ and/or 5 mM calcium. The Ca-containing Ringer's solution was prepared by dissolving gluconate in the Ca-free saline. All data were normalized with respect to the SCC in "110-Na" saline. The inset shows the relationship between the SCC and [Na⁺] in control (A), +5 mM Ca (B), and +2 mM Cd (C) salines. It was demonstrated that Ca inhibited the SCC (B) and Cd²⁺ activated it (C) at each [Na⁺]. The slopes of the Hill plots of log [SCC/(SCC_{max} - SCC)] vs. log [Na⁺] in the control, +5 mM Ca, +2 mM Cd, and +5 mM Ca +2 mM Cd saline, were 1.02, 1.08, 1.03, and 1.11, respectively. Since the values of the Hill coefficients are close to unity, all these relations for Na⁺ are considered to belong to the Michaelis-Menten type.

A single reciprocal plot (Eadie-Hofstee plot) was drawn of SCC vs. SCC/[Na⁺]. The equation can be expressed as follows: $SCC = -K(SCC/[Na^+]) + SCC_{max}$, where $-K$ is the slope of the line, and SCC_{max} is as extrapolated saturated value of the SCC at $[Na^+] \rightarrow \infty$. Each data point represents the average of 6 to 12 experiments. The linear regression line was fitted by the method of least squares for the data for each saline. The intercepts of the regression line on the Y-axis, SCC_{max} , in A, B, C, and D (+5 mM Ca +2 mM Cd) saline were 110.3, 91.7, 153.6, and 136.3%, respectively; *i.e.*, the order of SCC_{max} was (C) > (D) > (A) > (B). The slopes of the lines (K) can be classified into two groups; control dissociation constant, K_s , and dissociation constant with an activator or an inhibitor, apparent K_s . The K_s in A was 13.7 mM, and the apparent K_s in B, C, and D saline were 11.8, 19.5, and 16.4 mM, respectively.

All the regression lines converged to a point on the X-axis (0, 8.00). The

difference in values of the X -intercept was not significant ($P > 0.04$). This implies that Ca (B) and Cd^{2+} (C) are an inhibitor and an activator of uncompetitive type for active Na transport, respectively.

The SCC in Ca saline (B) was increased to that in (D) by the addition of Cd^{2+} , but the difference between the SCC in (D) and (B) was always greater than that in (C) and (A) for each $[\text{Na}^+]$. In other words, the effect of Cd^{2+} with Ca was greater than that without Ca. This conclusion is in accordance with that derived from Fig. 3.

Effect of amiloride on SCC

Amiloride is known to be a blocker of active Na transport. The mechanism of amiloride inhibition on the SCC in bullfrog skin was then investigated. The "1-Ca" saline in Table 1 was employed in the experiments. Amiloride was applied to the skin epidermally in a similar manner to the application of Cd^{2+} .

Figure 6 summarizes the results of 78 experiments. Each data point is normalized with respect to the SCC in amiloride-free "1-Ca" saline, and represents the average of 5 to 15 experiments. The SCC was inhibited by the addition of amiloride: the higher the [amiloride], the greater the inhibition became. The SCC was almost completely inhibited (92.1%) at 5×10^{-5} M amiloride, and the [amiloride] at half-maximum inhibition was 2.8×10^{-7} M.

By replotting the data in Fig. 6, the Hill coefficient (n') of amiloride was obtained as 0.757. This n' value, being clearly less than unity, suggests that the reaction is not of the Michaelis-Menten type, but of the sigmoid type which exhibits negative cooperation.

Effect of Cd^{2+} at different [amiloride]

The Cd^{2+} -induced increase in the SCC was examined under the influence of epidermal application of various [amiloride] in "1-Ca" saline (Table 1). Figure 7

Fig. 6. Relationship between [amiloride] and SCC. $[\text{Na}^+] = 110$ mM. The SCC is normalized with respect to that in amiloride-free "1-Ca" saline.

Fig. 7. Effects of 1 mM Cd^{2+} on the SCC and PD in the presence and absence of 5×10^{-7} M amiloride (Am). The effects of Cd^{2+} and amiloride were tested in "1-Ca" saline.

gives an example of the effect of 1 mM Cd^{2+} in the presence or absence of 5×10^{-7} M amiloride. Twenty min after the first application of the Cd^{2+} , the SCC and PD were increased to 126.2 and 121.3%, respectively, and the R_M was decreased to 94.8%. After washing the skin with the "1-Ca" saline, the bathing solution was substituted by 5×10^{-7} M amiloride containing "1-Ca" saline. The SCC and PD were decreased to 34.9 and 44.6%, respectively, and the R_M was increased to 127.8% in the amiloride saline. When 1 mM Cd^{2+} further was added epidermally, the SCC and PD were increased to 116.0 and 119.5%, respectively, as compared to the values just prior to the addition of Cd^{2+} .

The influence of amiloride on the Cd^{2+} -induced increase in SCC was examined in 11 cases. Cadmium at 1 mM increased the SCC by $25.5 \pm 4.2\%$ in amiloride-free "1-Ca" saline. Both calcium and amiloride thus inhibit the SCC, but the mechanism of the two inhibition processes may differ. The Cd^{2+} effect was almost doubled in "35-Ca" saline, while the Cd^{2+} -induced increase in SCC was reduced to almost half in 5×10^{-7} M amiloride saline ($11.2 \pm 1.6\%$, $n=11$). The difference in the effects of Cd^{2+} in the absence or presence of amiloride was highly significant ($P < 0.005$). The second application of Cd^{2+} yielded essentially similar results to the first: $22.5 \pm 2.5\%$ in the amiloride-free saline and $12.7 \pm 2.2\%$ in 5×10^{-7} M amiloride saline. No significant differences were observed between the results of the first and second Cd^{2+} applications in the presence or absence of amiloride ($P > 0.5$).

Figure 8A shows the relationship between $[\text{Cd}^{2+}]$ and the SCC at [amiloride] of 0, 10^{-7} , 5×10^{-7} , and 5×10^{-6} M in "1-Ca" saline (Table 1). Each data point is normalized with respect to the SCC in Cd^{2+} -free and amiloride-free "1-Ca" saline, and represents the average of 6 to 11 experiments. The SCC became smaller with higher [amiloride], and became larger with higher $[\text{Cd}^{2+}]$. No intersection of any two curves was observed which was different from the case of the Cd^{2+} -effect under the influence of Ca (cf. Fig. 3A).

Fig. 8. Relationship between $[Cd^{2+}]$ and the SCC at various [amiloride]. The SCC is normalized with respect to that in Cd^{2+} -free "1-Ca" saline (Fig. 8A). The same data are replotted for the relationship, $(SCC_{Cd} - SCC_{Cd-free})$ vs. $[Cd^{2+}]$, in Fig. 8B. Open circles, closed circles, crosses, and triangles indicate data for "0," " 10^{-7} M," " 5×10^{-7} M," and " 5×10^{-8} M" amiloride saline, respectively.

Figure 8B shows the dose-response curves of $(SCC_{Cd} - SCC_{Cd-free})$ vs. $[Cd^{2+}]$ at various [amiloride], replotted from the data in Fig. 8A. As $[Cd^{2+}]$ increases, so the Cd^{2+} -response increases in a sigmoid manner, as in Fig. 3B. The main difference in the influence of calcium and amiloride is that, contrary to the former, the latter shifts the dose-response curve downwards when the [amiloride] becomes higher.

A Hill plot was drawn from the data in Fig. 8B. The same equation as in Fig. 4 was employed, and linear regression lines were drawn using the method of least squares. The slope of the linear regression line in the amiloride-free saline was 0.653, and those in amiloride-containing saline were almost the same. Those values were clearly less than unity, suggesting that the binding reaction between Cd^{2+} -binding sites and Cd^{2+} under the influence of amiloride is not of the Michaelis-Menten type but is negative cooperation type as in the case of the reaction under the influence of calcium. The $[S]_{0.5}$ values (*i.e.*, $[Cd^{2+}]$ for $v = V_{max}/2$) at 0, 10^{-7} , and 5×10^{-7} M amiloride were 0.264, 0.910, and 1.65 mM, respectively.

Effect of Cd^{2+} and amiloride at different $[Na^+]$

Figure 9 illustrates the influence of various $[Na^+]$ (Table 2) on the SCC in relation to the presence or absence of Cd^{2+} and/or amiloride. An Eadie-Hofstee plot was drawn of SCC vs. $SCC/[Na^+]$ using the same equation as in Fig. 5. Linear regression lines were drawn from the data for control saline (A), $+10^{-7}$ M amiloride

Fig. 9. Eadie-Hofstee plot of (normalized SCC) vs. (normalized SCC)/[Na⁺]. A, control saline in Table 2; B, +10⁻⁷ M amiloride; C, +2 mM Cd; D, +10⁻⁷ M amiloride +2 mM Cd. The SCC was normalized with respect to that in amiloride-free Cd-free 110 mM [Na⁺] saline.

(B), +2 mM Cd (C), and +10⁻⁷ M amiloride +2 mM Cd (D). A and C in Fig. 9 are identical to those in Fig. 5. All the data were normalized with respect to the SCC in "110-Na" saline, and each data point represents the average of 6 to 10 experiments. The intercepts of the regression line on the Y-axis, SCC_{max}, in A, B, C, and D saline were 110.3, 97.2, 153.6, and 116.8%, respectively; *i.e.*, the order of SCC_{max} was (C)>(D)>(A)>(B). From the slope of the line, the K_s in A was 13.7 mM, and the apparent K_s in B, C, and D saline were 58.8, 19.5, and 57.1 mM, respectively. Amiloride (B) shifted the control line (A) downwards; the Y-intercept of B was significantly smaller than that of A ($P<0.001$), and the slope of B was clearly steeper than that of A. This suggests that the type of amiloride inhibition for Na entry is a mixed one. Addition of Cd²⁺ to amiloride saline (B) shifted the regression lines to the right almost in parallel (D). This shift indicates that the type of amiloride inhibition is also a mixed one even though in Cd²⁺-saline (D). It is interesting to note that although Cd²⁺ alone is an uncompetitive activator, Cd²⁺ in amiloride saline exhibits a different type of activation, *i.e.*, a noncompetitive activation.

DISCUSSION

In active Na transport through frog skin, the relation between the Na transport and [Na⁺] is a hyperbolic curve which is convex upwards (CEREJIDO *et al.*, 1964). This saturable behavior is explained as follows. Na⁺ moves into the frog skin by using a Na-selective molecule (BIBER and SANDERS, 1973) or through a

pore (LINDEMANN and VAN DRIESCHE, 1977), which becomes saturated at high $[\text{Na}^+]$. These characteristics make it possible to analyze the steady-state Na transport kinetics by using similar techniques to enzyme kinetics (BENOS *et al.*, 1979; ACEVES and CUTHBERT, 1979). Specialized sites located on the outer membrane surface, which correspond to the reaction sites of an enzyme, appear to be responsible for Na saturation (ERLIJ and SMITH, 1973; MANDEL, 1978). Since Cd^{2+} was found to increase the SCC only when it was applied from the epidermal side, it appeared reasonable to apply the principles of enzyme kinetics for elucidating the mechanism of Cd^{2+} activation on Na^+ entry. A kinetic analysis of the effects of the well-known inhibitors, Ca and amiloride, and their interaction with that of Cd^{2+} was also performed. No other investigators have so far made kinetic studies on the Cd^{2+} effect.

Inhibitory effect of Ca on SCC. Calcium itself inhibits the SCC in a hyperbolic manner (Fig. 1). The limiting value of the curve in this paper was 80%. Two possibilities may explain this partial inhibitory effect. The first is that the total current is divided into two fractions, one sensitive to, and the other insensitive to Ca (CURRAN and GILL, 1962), and the second is that Ca inhibits every channel related to active Na transport by 20% at maximum. The analysis utilized in this paper followed the second assumption due to its simplicity. The maximum Ca-inhibition of 20% indicated by our results is smaller than that (60%) obtained by CURRAN and GILL (1962). This may be due to the difference in animal species employed and the composition of the Ringer's solution.

Facilitatory and inhibitory effects of Ca on the Cd^{2+} -induced increase in SCC. HILLYARD and GONICK (1976) reported that the Cd^{2+} effects was not influenced by the addition of Ca. However, we found that the effect of Cd^{2+} on the SCC was modified by changing the $[\text{Ca}]$ as follows. (1) At a constant $[\text{Ca}]$, the increase in SCC was higher at higher $[\text{Cd}^{2+}]$ than at lower $[\text{Cd}^{2+}]$ (*cf.* Fig. 3B). (2) Ca facilitated the Cd^{2+} -induced increase in SCC at higher $[\text{Ca}]$ (*cf.* Fig. 3B). (3) The Hill plot of SCC vs. $[\text{Cd}^{2+}]$ shifted to the right almost in parallel at higher $[\text{Ca}]$. The ratio of the Cd^{2+} -induced SCC at higher $[\text{Ca}]$ to that at lower $[\text{Ca}]$ increased with increasing $[\text{Cd}^{2+}]$. MATSUMOTO and CHIHARA (1958) described both facilitatory and inhibitory effects of Ca^{2+} on acetylcholine (ACh)-induced contraction of rabbit small intestine: through the coexistence of higher $[\text{Ca}^{2+}]$, the ACh-induced contraction at lower $[\text{ACh}]$ was depressed and that at higher $[\text{ACh}]$ was enhanced. The effects of Ca on the Cd^{2+} -induced increase in SCC may also consist of a facilitatory one (noncompetitive type) at higher $[\text{Cd}^{2+}]$ (upward shift in Fig. 3B) and an inhibitory one (competitive type) at lower $[\text{Cd}^{2+}]$ (shift to the right in Fig. 3B), even though Ca does not apparently depress the Cd^{2+} -induced SCC at lower $[\text{Cd}^{2+}]$.

It is interesting to note that although the effect of Ca on the SCC attained a maximum at 10 mM Ca (*cf.* Fig. 1), the effect of Ca on the Cd^{2+} -induced SCC increased even at over 10 mM Ca (*cf.* Fig. 3B).

Interaction of Ca and Cd^{2+} in relation to active Na transport. Na^+ on the

Fig. 10. Reactions related to active Na transport and their kinetic constants. B, a channel which has binding sites with either Na^+ , Cd^{2+} , Ca, or amiloride; A, activator (Cd^{2+}); I, inhibitor (Ca, amiloride=Am); k and αk , coefficients of $\text{B} \cdot \text{Na}$ and $\text{B} \cdot \text{Na} \cdot \text{Cd}^n$ complexes transformed into SCC ($\alpha=1.4$). $\text{B} \cdot \text{Na} \cdot \text{I}^{n'}$ and $\text{B} \cdot \text{Na} \cdot \text{I}^{n'} \cdot \text{Cd}^n$ are assumed never to decompose so that they are not transformed into SCC. Hill coefficient (n): $n_{\text{Cd}}=0.737$, $n'_{\text{Am}}=0.757$. Na-B, Michaelis-Menten type reaction; Cd (with or without Ca), uncompetitive activator; Ca, uncompetitive inhibitor; Am, mixed-type inhibitor; Cd (with Am), noncompetitive activator. Dissociation constants: $K_S=13.7 \times 10^{-8}$ (M), $K_{A(\text{Am}+\text{Cd})}=K'_A=8.02 \times 10^{-8}$ ($\text{M}^{0.737}$), $K_{I(\text{Ca}, \text{Ca}+\text{Cd})}=2.45 \times 10^{-2}$ (M), $K'_{I(\text{Am}, \text{Am}+\text{Cd})}=3.72 \times 10^{-5}$ ($\text{M}^{0.757}$), $K_{I(\text{Am}, \text{Am}+\text{Cd})}=1.29 \times 10^{-6}$ ($\text{M}^{0.757}$), $K'_S(\text{Am}, \text{Am}+\text{Cd})=K_S \cdot K'_I/K_I=3.96 \times 10^{-1}$ (M).

epidermal side is considered to bind with a channel (B) to form a $\text{B} \cdot \text{Na}$ complex which is transformed to the SCC during the subsequent process of decomposition of the $\text{B} \cdot \text{Na}$ complex (MANDEL, 1978). This idea may be expanded further as follows (Fig. 10). Binding sites of Na^+ , Cd^{2+} , and Ca exist on the channel which is related to active Na transport. The SCC is produced by the decomposition of $\text{B} \cdot \text{Na}$ and $\text{B} \cdot \text{Na} \cdot \text{Cd}^n$ (n =Hill coefficient). $\text{B} \cdot \text{Na} \cdot \text{I}^{n'}$ and $\text{B} \cdot \text{Na} \cdot \text{I}^{n'} \cdot \text{Cd}^n$ (I =Ca or amiloride) never decompose, so that no SCC is produced from these complexes. The capital letter "K" represents a dissociation constant. The small letter "k" represents the decomposition velocity of the $\text{B} \cdot \text{Na}$ complex, and " αk " is that of the $\text{B} \cdot \text{Na} \cdot \text{Cd}^n$ complex, where $\alpha > 1$. K and k are termed kinetic constants.

The results of experiments on Ca, Cd^{2+} , or both in relation to the SCC at various $[\text{Na}^+]$ (cf. Fig. 5) indicate that these reactions belong to the uncompetitive type as regards the main reaction ($\text{B} + \text{Na} \rightleftharpoons \text{B} \cdot \text{Na}$): both Ca and Cd^{2+} exert their effects by reversibly binding not to a B but to a $\text{B} \cdot \text{Na}$ complex solely. Cd^{2+} may also bind with a $\text{B} \cdot \text{Na} \cdot \text{Ca}$ complex. Uncompetitive inhibition or activation is rarely observed in enzymology (DIXON and WEBB, 1979) or pharmacology (ARIÈNS, 1964). In the skin of *Rana pipiens*, MANDEL (1978) reported that Ca^{2+} noncompetitively inhibited Na^+ entry, but from the Fig. 8 of CEREJIDO *et al.* (1964), uncompetitive inhibition of a type similar to ours can be observed. Dis-

Table 3. Relative values of various forms of channel-ion complex related to active Na transport (per unit surface area of frog skin).

	Control (110-Na)	+2 mM Cd	+5 mM Ca	+Ca+Cd	+10 ⁻⁷ M Am	+Am+Cd
[B]:	11.1	5.2	9.4	4.5	7.2	3.1
[B·Na]:	88.9	41.6	75.3	36.2	57.4	25.4
[B·Na·Cd ⁿ]:	—	53.2	—	46.2	—	32.2
[B·Cd ⁿ]:	—	—	—	—	—	4.0
[B·Na·I ⁿ]:	—	—	15.3	7.4	7.7	3.4
[B·I ⁿ]:	—	—	—	—	27.7	12.2
[B·Na·I ⁿ ·Cd ⁿ]:	—	—	—	5.8	—	4.3
[B·I ⁿ ·Cd ⁿ]:	—	—	—	—	—	15.6
[B total]:	100	100	100	100	100	100
SCC _{ex} :	100	131	82.8	119	63.8	76.9
SCC _{cal} :	100	131	84.7	114	64.6	79.4

B, a channel which has binding sites with Na⁺, Cd²⁺, Ca, or amiloride; I, inhibitor (Ca, amiloride); SCC_{ex} and SCC_{cal}, experimental and calculated values of the normalized SCC, respectively, at 110 mM [Na⁺].

sociation constants for various reactions which appear in the equation of the law of mass action were obtained from the data in Fig. 5. Using these dissociation constants, the ratios of various forms of channel-ion complex were calculated. The calculated SCC (SCC_{cal}) was then compared with the experimentally obtained SCC (SCC_{ex}) (Table 3). The methods employed for the calculation are described in APPENDIX below. Assuming the kinetic constants to be invariable from those in individual agents (+Ca, and +Cd saline), even in a mixture of the activator and inhibitor, SCC_{cal} almost coincided with SCC_{ex} in (+Ca+Cd)-saline. Complete agreement was obtained if $\alpha=1.50$ was used instead of $\alpha=1.40$. If B·Na·Ca·Cdⁿ is not formed at all, SCC_{cal} (121)=SCC_{ex} (119) even under the condition of $\alpha=1.40$. However, from the type of reaction it was not possible to judge whether this complex was formed or not. In the case of (+35 mM Ca+2 mM Cd)-saline, assuming the kinetic constants to remain unchanged, some discrepancy existed between SCC_{cal} (102.5) and SCC_{ex} (125.2). If we took $\alpha=1.7$, complete agreement was obtained for both. The increase in α value at higher [Ca] may correspond to a phenomenon in which the higher [Ca] facilitates the Cd²⁺-induced increase in the SCC further, as stated previously.

Inhibitory effect of amiloride on the Cd²⁺-induced increase in SCC. Amiloride has been found to inhibit the SCC by blocking Na⁺ entry through the outer facing membrane of toad bladder (BENTLEY, 1968). SALAKO and SMITH (1970) obtained similar findings for frog skin. Amiloride inhibits not only the SCC but also the Cd²⁺-induced SCC. The effect of Cd²⁺ varied with the change in [amiloride] as follows. (1) At a constant [amiloride], both the SCC and Cd²⁺-induced SCC were higher at higher [Cd²⁺] than at lower [Cd²⁺] (*cf.* Fig. 8). (2) Amiloride inhibited the Cd²⁺-induced increase in SCC at higher [amiloride] (*cf.* Fig. 8B). (3) The Hill

plot of SCC vs. $[Cd^{2+}]$ shifted to the right at higher [amiloride]. According to the classification of MATSUMOTO *et al.* (1955), such a non-Michaelis-Menten type of reaction (SCC vs. $[Cd^{2+}]$ under the influence of amiloride) should be considered to represent inhibition of the mixed type (competitive and noncompetitive). The main differences between the effect of Ca and that of amiloride were that (1) at most only 20% inhibition was obtained with the former, and (2) there was no facilitation of the Cd^{2+} -induced SCC with the latter.

Interaction of amiloride and Cd^{2+} in relation to active Na transport. A similar kind of kinetic analysis to that for Ca and Cd^{2+} in Na transport was performed for the interaction of amiloride and Cd^{2+} . From the results in Fig. 9, amiloride inhibition was found to belong to the mixed type (both competitive and noncompetitive). In other words, amiloride exerts its effect by binding with a Na-selective channel (B) as well as a B·Na complex with different dissociation constants. The binding site of amiloride on B is spatially distinct from the Na binding site.

Some discrepancies exist among other investigators concerning the type of amiloride inhibition. In toad bladder, BENTLEY (1968) claimed it to be a noncompetitive one, but SUDOU and HOSHI (1977) asserted that it was a competitive one based on an analysis of the potential difference. In frog skin, from the data of SALAKO and SMITH (1970, Fig. 4), the inhibition appeared to be of the mixed type. BENOS *et al.* (1979) described the amiloride inhibition as noncompetitive in bullfrog skin and as of the mixed type in toad skin. The differences in type of amiloride inhibition may arise from differences in the species of experimental animals employed, rearing conditions, Ringer's solution, and type of chambers. The mode of Cd^{2+} activation changed to the noncompetitive type under the influence of amiloride, which is different from the uncompetitive type of only Cd^{2+} or Cd^{2+} under the influence of Ca. In other words, the data in Fig. 9 suggest that the conformation of the amiloride site is never affected by the addition of Cd^{2+} (compare $A \rightarrow B$ with $C \rightarrow D$). On the other hand, the conformation of the Cd^{2+} site is modified by amiloride (compare $A \rightarrow C$ with $B \rightarrow D$).

The Hill coefficient ($n' = 0.757$) of amiloride in "1-Ca" saline was obtained by replotting the data in Fig. 6, and was estimated to be the same as that in "Ca-free" saline from preliminary experiments. This corresponds with the results for bullfrog skin where the amiloride effect at 110 mM $[Na^+]$ was not affected by the addition of 1 mM Ca^{2+} (BENOS *et al.*, 1979). The n' value is clearly less than unity, suggesting that this reaction is not of the Michaelis-Menten type, but of the sigmoid type which exhibits negative cooperation. Our n' value is intermediate between the 0.86 of ACEVES and CUTHBERT (1979) and 0.54 of BENOS *et al.* (1979). The ratios of various forms of channel·ion complex were calculated from the data in Fig. 9. Assuming the kinetic constants to be invariable from those in individual agents (+amiloride, and + Cd^{2+}), even in a mixture of the inhibitor and activator, SCC_{ca1} almost coincided with SCC_{ex} in (+amiloride + Cd^{2+})-saline (Table 3). Complete agreement was obtained if $\alpha = 1.33$ was postulated instead of

$\alpha=1.40$.

Interaction of amiloride and Ca in relation to active Na transport. When 10^{-7} M amiloride and 5 mM Ca were administered to the frog skin simultaneously, the calculated ratio of [B]: [B·Na]: [B·Na·Ca]: [B·Am^{n'}]: [B·Na·Am^{n'}]: [B·Na·Ca·Am^{n'}] at 110 mM [Na⁺] was 6.3: 50.7: 10.3: 24.5: 6.8: 1.4%, where *n'* represents the Hill coefficient of amiloride, assuming the dissociation constants to be invariable. The calculated SCC was 57.0, which is in fairly good agreement with the experimentally obtained SCC (59.9). This also supports the assumption that the dissociation constants of the reaction were scarcely influenced by inhibitors.

Confirmation of the existence of various forms of channel-ion complex will require further detailed investigations.

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APPENDIX

This section indicates how to calculate the numerical data in Table 3. The abbreviations used are the same as those in Table 3.

In control saline, we obtained the dissociation constant (K_s) of the B·Na complex as 13.7 mM from the slope of Fig. 5 (line A). According to the law of mass action, $[B][Na]/[B \cdot Na]=13.7$ mM. Hence, $[B]=0.125[B \cdot Na]$, where $[Na^+]=110$ mM. As $[B]+[B \cdot Na]=[B \text{ total}]$, $[B]: [B \cdot Na]=0.125: 1.00=11.1: 88.9\%$. It is assumed that $[B \cdot Na]$ is directly proportional to the SCC. Since the SCC is normalized with respect to that in the control saline at 110 mM $[Na^+]$, the SCC becomes $k[B \cdot Na]=100$ (arbitrary units), where *k* is a constant. Then, $k[B \text{ total}]=112.5$.

Since Cd^{2+} is an uncompetitive activator of active Na transport (Fig. 5), the dissociations of the $[B \cdot Na]$ and $[B \cdot Na \cdot Cd^n]$ complexes are related to two dissociation constants, K_s and K_A' . K_s is assumed to be the same as that in the control. K_A' is determined as follows: $K_A'=[B \cdot Na][Cd^n]/[B \cdot Na \cdot Cd^n]$. From Fig. 4, the Hill coefficient (*n*)=0.737. Then, $[B \cdot Na \cdot Cd^n]=(0.0103/K_A') [B \cdot Na]$ at 2 mM $[Cd^{2+}]$. $SCC_{Cd([Na^+]=110 \text{ mM})}=k\{[B \cdot Na]+\alpha[B \cdot Na \cdot Cd^n]\}=131$, where $\alpha=SCC_{Cd}/SCC_{control([Na^+] \rightarrow \infty)}=154/110=1.40$ (cf. Fig. 5 and SEGEL (1975, p. 229)). $[B \text{ total}]=[B]+[B \cdot Na]+[B \cdot Na \cdot Cd^n]$. Hence, $K_A'=8.02 \times 10^{-3}$ ($M^{0.737}$). Then,

$$[B]: [B \cdot Na]: [B \cdot Na \cdot Cd^n]=0.125: 1.00: 1.28=5.2: 41.6: 53.2\% ;$$

and,

$$SCC_{cal([Na^+]=110 \text{ mM})}=(41.6+53.2 \times 1.40) \times 100/88.9=131 .$$

Since Ca is an uncompetitive inhibitor of active Na transport (Fig. 5), the inhibition constant (K_I') or the dissociation constant of $[B \cdot Na \cdot Ca]$ in Ca saline is

obtained as follows: $SCC_a = SCC_{max}/(1 + [Ca]/K_I')$, where SCC_a = apparent $SCC_{max}([Na^+] \rightarrow \infty)$ in the Ca saline (*cf.* Fig. 5 and SEGEL (1975, p. 139)). $K_I' = [B \cdot Na][Ca]/[B \cdot Na \cdot Ca]$ at 5 mM $[Ca] = 2.45 \times 10^{-2}$ (M). Hence, $[B \cdot Na \cdot Ca] = 0.204[B \cdot Na]$. Assuming K_s to be the same as that in the control, $[B]:[B \cdot Na]:[B \cdot Na \cdot Ca] = 0.125:1.00:0.204 = 9.4:75.3:15.3\%$. Then, $SCC_{cal}([Na^+] = 110 \text{ mM}) = 75.3 \times 100/88.9 = 84.7$.

In (+Cd+Ca)-saline, assuming K_s , $K_A' = K_A'''$, K_I' , and α to be the same as those obtained previously,

$$[B]:[B \cdot Na]:[B \cdot Na \cdot Cd^{0.737}]:[B \cdot Na \cdot Ca]:[B \cdot Na \cdot Ca \cdot Cd^{0.737}] \\ = 0.125:1.00:1.28:0.204:0.159 = 4.5:36.2:46.2:7.4:5.8\%.$$

The $SCC_{cal}([Na^+] = 110 \text{ mM}) = (36.2 + 46.2 \times 1.40) \times 100/88.9 = 114$. The calculated SCC agrees closely with the experimental value. If $[B \cdot Na \cdot Ca \cdot Cd^{0.737}]$ complex may not be formed ($K_A''' = \infty$), then

$$[B]:[B \cdot Na]:[B \cdot Na \cdot Cd^{0.737}]:[B \cdot Na \cdot Ca] = 4.8:38.4:49.0:7.8\%,$$

and the $SCC_{cal}([Na^+] = 110 \text{ mM}) = 120$.

Since amiloride (Am) is a mixed-type inhibitor (Fig. 9), both K_I' and K_I should be determined. $SCC_a = SCC_{max}/(1 + [Am]^{n'}/K_I')$. The Hill coefficient (n') = 0.757. $[B \cdot Na][Am^{n'}]/[B \cdot Na \cdot Am^{n'}] = K_I'$. $[B \cdot Na \cdot Am^{n'}] = 0.135[B \cdot Na]$ at 10^{-7} M $[Am]$. Hence, $K_I' = 3.72 \times 10^{-5}$ ($M^{0.757}$). $(K_{Am}/SCC_{Am})/(K_{control}/SCC_{control}) = 1 + [Am^{n'}/K_I]$, where K_{Am} and $K_{control}$ are the slope of the B and A lines, and SCC_{Am} and $SCC_{control}$ are those at $[Na^+] = \infty$ (*cf.* SEGEL (1975, p. 171)). $[B][Am^{n'}]/[B \cdot Am^{n'}] = K_I = 1.29 \times 10^{-8}$ ($M^{0.757}$). Hence, $[B \cdot Am^{n'}] = 3.88[B] = 0.483[B \cdot Na]$ at 10^{-7} M amiloride. Then,

$$[B]:[B \cdot Na]:[B \cdot Na \cdot Am^{n'}]:[B \cdot Am^{n'}] \\ = 0.125:1.00:0.135:0.483 = 7.2:57.4:7.7:27.7\%;$$

and

$$SCC_{cal}([Na^+] = 100 \text{ mM}) = 57.4 \times 100/88.9 = 64.6.$$

In (+Am+Cd)-saline, Cd^{2+} behaves as a noncompetitive activator for the Am-saline (Fig. 9). Therefore, $[B][Cd^n]/[B \cdot Cd^n] = K_A = K_A'$. $K_s' = K_s \cdot K_I'/K_I = 3.96 \times 10^{-1}$ (M). Assuming that the K_s , K_A' , K_I , K_I' , and α in the (+Am+Cd)-saline are the same as those obtained previously,

$$[B]:[B \cdot Na]:[B \cdot Na \cdot Cd^n]:[B \cdot Cd^n]:[B \cdot Na \cdot Am^{n'}]:[B \cdot Am^{n'}]:[B \cdot Am^{n'} \cdot Cd^n]: \\ [B \cdot Na \cdot Am^{n'} \cdot Cd^n] = 0.125:1.00:1.28:0.159:0.135:0.483:0.618:0.172 \\ = 3.1:25.2:32.2:4.0:3.4:12.2:15.6:4.3\%.$$

The $SCC_{cal}([Na^+] = 110 \text{ mM}) = (25.2 + 32.2 \times 1.40) \times 100/88.9 = 74.9$. This value agrees closely with $SCC_{ex}(76.9)$.

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