Effect of Cadmium on Active Sodium Transport by the Abdominal Skin and the Isolated Epidermis of the Bullfrog: Differences in Effects between Epidermal and Dermal Cadmium Applications

Makoto Takada and Hideo Hayashi

Department of Physiology, Saitama Medical School, Saitama, 350-04 Japan

Abstract Epidermal application of 2 mM-cadmium (Cd\(^{2+}\)) to the SO\(_4\)Ringer's solution bathing the whole frog skin increased its short circuit current (SCC) significantly, whereas the dermal application did not, in most cases. When Cd\(^{2+}\) was applied to the epidermal side, the uptake of cadmium by the skin was approximately 150 \(\mu\)M/kg wet weight, but the uptake by the skin from the dermal side was about eight times as large. In the isolated epidermis, the epidermal application of Cd\(^{2+}\) increased the SCC, whereas the dermal application decreased it. The amount of cadmium taken up by the isolated epidermis was approximately the same when the cation was applied to either epidermal or dermal side. These seemingly contradictory results can probably be explained by the barrier-hypothesis which is supported by the results of an electron probe X-ray microanalysis. In the case of the epidermal application, the junction between s. corneum and s. granulosum may act as an outside barrier for cadmium translocation. For the dermal application of Cd\(^{2+}\), the sieve layer in the dermis, where a large amount of calcium is deposited, may probably exert its effects as an inside barrier by the mechanism of Cd-Ca exchange process. Tela subcutanea, the basement membrane and its adjacent tissue may be also inside barriers, though their role as a diffusion barrier may be considered to be weaker than that of the sieve layer.

The active transport of Na\(^{+}\) by the frog skin is known to be enhanced by application of cadmium (Cd\(^{2+}\)) into the bathing medium as evidenced by increases in the short circuit current and in the potential difference of the skin (Borghgraef et al., 1971; Hillyard and Gonick, 1976). Although Borghgraef et al. (1971) tested the effects of the epidermal and dermal application of Cd\(^{2+}\), their results were not consistent in regard to the differences in effect by the side of application.

We previously found that the epidermal application of Cd\(^{2+}\) caused an in-
crease in the short circuit current (SCC), whereas the dermal application did not. The increase in the SCC induced by epidermally applied Cd²⁺ was associated with an increase in the potential difference (PD), a decrease in the skin resistance (Rₘ), a decrease in the resistance to the active Na⁺ current (Rₙa as defined by Ussing and Zerahm, 1951), and no change in the electromotive force of active Na⁺ transport (Eₙa) (Hayashi et al., 1977a). The decrease in Rₙa appeared to coincide with the decrease in Rₘ which was located between the outer surface of the skin and s. germinativum (Hayashi et al., 1978). The increase in SCC caused by epidermally applied Cd²⁺ could not be ascribed to its effect on Na⁺, K⁺-ATPase activity (Takada and Hayashi, 1978a).

On the other hand, the reason for the ineffectiveness of dermally applied Cd²⁺ is still unknown. The present study was carried out in an attempt to clarify the nature of differences in effect of Cd²⁺ with application. For this purpose, the differences in tissue uptake of cadmium and its distribution within the skin were compared using two methods of application. Also, the difference in effect on SCC was carefully examined in both the whole skin and the isolated epidermis. The results of the study indicate that the differences can be ascribed to the difference in accessibility of Cd²⁺ to the outside and inside barriers for Na⁺ transport mechanism: the deposition of cadmium in the sieve layer of dermis may be a major factor for the ineffectiveness of the dermal application of Cd²⁺.

Preliminary accounts of this work were given elsewhere (Takada and Hayashi, 1978b).

MATERIALS AND METHODS

The abdominal skin of a bullfrog (Rana catesbeiana) of both sexes were used. The frogs were captured around Saitama Prefecture in Japan, and were kept at room temperature in a bath for a week or two without feeding. The water level was maintained so that about the half of the body was immersed in water. The frogs were anesthetized with intrathecal injection of 0.5 ml of 25% urethane solution. The abdominal skin was removed by dissection and mounted either on an open tip of a glass cannula (12 mm in inner diameter) or between Ussing-type lucite chambers (window area 1×1 cm²). 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 fluid levels. SO₄²⁻-Ringer’s solution (Na₂SO₄ 55 mM, K₂SO₄ 1 mM, calcium gluconate 1 mM, glucose 10 mM, sucrose 60 mM, and Tris/H₂SO₄ buffer 10 mM, pH 7.2) was used in order 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 by means of a pair of calomel electrodes, a DC amplifier, and a millivoltmeter. The 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 method for measuring
the PD and the SCC was previously described in detail (HAYASHI et al., 1977a).
The skin resistance \((R_d)\) was calculated by using the following equation:
\[ R_d = \frac{PD}{SCC} \]. The resistance and the electromotive force of the active \(Na^+\) transport
\((R_{Na}^+\) and \(E_{Na}^+\)\) could be obtained by the measurements of influx and outflux of
\(Na^+\) (USSING and ZERAHN, 1951).

The preparation, under the open-circuited conditions, was allowed to equili-
brate with the bathing solution for at least 1 hr before the application of \(Cd^{2+}\).

Isolated epidermis were employed in some experiments which were prepared
by a modified method of ACEVES and ERLIJ (1971). The tela subcutanea of an
excised frog skin was first eliminated by microdissection, and then several incisions
(3 mm apart in parallel) were made in the dermis (about 600 \(\mu m\) in thickness)
with a razor blade with care not to injure the epidermis (about 60 \(\mu m\)). The
skin was fixed on one end of a cannula (25 mm in tip diameter) with dermal side
facing the inside of the cannula, without adding excess strain. The epidermal
side was immersed in the \(SO_4^-\)-Ringer’s solution, whereas the dermal side was
filled with \(SO_4^-\)-Ringer’s solution containing collagenase (0.1 mg/ml, P-L Bio-
chemicals). The dermal solution was aerated for 15 hr at room temperature.
Then, the epidermis of the skin was dissected with a razor blade and forceps,
with great care not to injure the epidermis. The epidermis thus obtained was
fixed between the Ussing-type lucite chambers. After completion of electrical
measurements, the isolated epidermis was immediately freezeed with a liquid freon-
liquid nitrogen system, sectioned in 10 \(\mu m\) thickness by using a cryostat. Figure 1
shows the cross-section of the preparation, which indicated that the dermis was
completely removed and the structure of the epidermis was well maintained.

Fig. 1. Cross section of an isolated abdominal epidermis of a bullfrog. c, stratum corneum;
g, s. germinativum.
The uptake of cadmium into the whole skin of isolated epidermis was measured as follows. Cadmium sulfate was applied to the bath of either the epidermal or dermal side at 2 mM. After incubation at various times, the preparation was washed with the SO$_4^{2-}$-Ringer's solution for several seconds, blotted on filter paper, weighed, then fixed with 50% ethanol for 1 min and with pure ethanol for 5 min. The fixed skin was ashed in a plasma reactor (Yamato PR-302) at about 100°C for 6–7 hr (O$_2$ supply, 40 ml/min; power, 120 W). The ashed skin was dissolved in 0.3 ml of 0.5 N HCl, and the resulting solution was diluted up to 10 ml with distilled water. With an atomic absorption spectrophotometer (Hitachi 508A), the Cd$^{2+}$ concentration of dissolved solutions was determined using a standard additional method.

The distribution of cadmium in a cross section of the frog skin was studied by using an electron probe X-ray microanalyzer (XMA), after exposure of the skin, mounted on a glass cannula, to 2 mM-Cd$^{2+}$ in either epidermal or dermal solution. After the exposure, the skin was washed with the SO$_4^{2-}$-Ringer's solution for several seconds, and fixed with oxine-saturated 2% glutaraldehyde-1/14 M veronal buffer (pH 7.2) for 2 hr at 0–4°C. The whole skin was frozen and sectioned as stated above. The section was freeze-dried in a cryostat chamber, and mounted on a carbon plate (5 mm$^2$) coated with chloroprene containing adhesive. Pt-Pd coating was performed on the surface of the dried sections. Elements in the cross section of the abdominal skin were analyzed with a scanning electron microscope (JEOL JEM-U3S) with a wavelength dispersive analyzer for line analysis of cadmium.

RESULTS

1. Effects of Cd$^{2+}$ on the SCC, PD, and $R_M$ of the whole frog skin

Epidermal application of Cd$^{2+}$ to the whole skin caused an increase in the SCC and the PD, whereas the application to the other side of the skin caused no appreciable change as described in the previous paper (HAYASHI et al., 1977a). Figure 2 shows a typical example of such experiments. When 2 mM-Cd$^{2+}$ was applied to the epidermal side, SCC increased by about 36%, and PD also increased by about 22%. The skin resistance ($R_M$) decreased by about 6%. On the other hand, the dermal application at the same concentration did not cause any change in SCC, PD, or $R_M$.

Figure 3 shows a dose-response (%) curve of Cd$^{2+}$ effect on SCC in the case of the epidermal application. The maximal response was observed at 10 mM. The half maximal response (21%) was seen at 160 $\mu$M. A detectable response appeared from 20 $\mu$M-Cd$^{2+}$.

2. The amount of cadmium taken up by the whole frog skin

The effects of Cd$^{2+}$ application on the SCC were quite different by the side of application (Fig. 2). In order to examine whether this phenomenon can be
Fig. 2. Effects of epidermal and dermal application of 2 mM Cd\(^{2+}\) on the short circuit current (SCC) (upper trace), the potential difference (PD) (middle trace), and the skin resistance (RM) (lower trace) of frog skin mounted between Ussing-type lucite chambers. epi, epidermal application of Cd\(^{2+}\); derm, dermal application of Cd\(^{2+}\).

Fig. 3. The relationship between percent increase in SCC induced by epidermal application of Cd\(^{2+}\) and its concentration. The frog skin was mounted on the top of the glass cannula with the epidermal side facing the inside of the cannula. Vertical bar represents the standard error of the mean.

explained in terms of a difference in cadmium uptake by the skin, we analyzed the amount of cadmium taken up by the whole skin after soaking the skin in the 2 mM-Cd\(^{2+}\)-Ringer's solution for various times. As shown in Fig. 4, cadmium uptake was nearly constant (about 150 \(\mu M/kg\) wet weight) when Cd\(^{2+}\) was applied to the epidermal side for 2–20 min. In the case of the dermal application, however, the uptake was much larger than that in the case of the epidermal application. This was unexpected since much less uptake was expected from the dermal side because no appreciable change in SCC in the case of dermal application. Cadmium uptake as a function of time was a two-step fashion: i.e., the uptake was
Fig. 4. Relationship between the amount of Cd uptake and time of application of 2 mM Cd\(^{2+}\). Open and closed circles represent epidermal and dermal application of Cd\(^{2+}\), respectively. The numbers in parentheses and vertical bars indicate the numbers of experiments and ± S.E.M.

about 400 \(\mu M/kg\) wet weight after 2–6 min, but it attained to 1,200 \(\mu M/kg\) wet weight after 20 min, the value being 8 times as much as the amount of uptake in the case of the epidermal application.

3. Distribution of cadmium in the cross section of the skin

The ineffectiveness of the dermal application of Cd\(^{2+}\), despite a large uptake of cadmium, could probably be ascribed to the existence of possible barriers for cadmium somewhere in the skin. Cadmium is considered to bind with some sites in the skin and increases the SCC. The barriers for cadmium may prevent the access of this cation to the sites.

Figure 5 illustrates the line analyses of Cd-specific X-ray (L\(_x\) 3.13 keV) in the cross sections of cadmium-treated skin and of control. When 2 mM-Cd\(^{2+}\) was administered to the epidermal side for 20 min, cadmium was found to be distributed in the stratum corneum and the s. granulosum, but not in the other layers (Fig. 5A). On the other hand, in the case of the dermal application, cadmium was demonstrated in the sieve layer and a small amount of it was also observed in the s. spongiosum, basement membrane, s. germinativum, and s. granulosum. Cadmium was not demonstrated in the s. corneum in this case.

Fig. 5. Line analyses of Cd-specific X-ray in the cross section of abdominal frog skin. White linear lines indicated by arrows in each ordinary scanning electron micrograph show the positions of the line analyses of Cd-specific X-ray. A: The skin treated with epidermally applied Cd\(^{2+}\) (2 mM, 20 min). B: The skin treated with dermally applied Cd\(^{2+}\) (2 mM, 20 min). C: The control skin. e, epidermis; p, s. spongiosum; s, sieve layer; c, s. compactum. 

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Fig. 5.
4. Effects of Cd$^{2+}$ on SCC of an isolated frog epidermis

Effects of 2 mM-Cd$^{2+}$ on SCC were demonstrated on the isolated frog epidermis which was obtained by using the collagenase method (Fig. 6). When Cd$^{2+}$ was applied to the preparation from the epidermal side for 12 min, SCC increased up to about 130%. On the other hand, the dermal application of Cd$^{2+}$ for 14 min decreased the SCC. The repeated dermal application of Cd$^{2+}$ on SCC seemed to be more effective than those of a single application; the decrease in SCC(%) (mean ± S.E.M.) of the first and second application were −6.0 ± 4.3 and −16.8 ± 6.3, respectively (Table 1).

| Table 1. Effects of repeated application of Cd$^{2+}$ on the short circuit current (SCC) of isolated epidermis. Differences in effect by the side of application. |
|---|---|
| | Change in SCC (%) |
| | 1st application | 2nd application |
| Epidermal Cd* | +22.9 ± 7.4** | +21.8 ± 6.1** |
| Dermal Cd* | −6.0 ± 4.3*** | −16.8 ± 6.3*** |

Values are mean ± S.E.M. (6 cases).
* 2 mM, 2 min, ** not significant, *** significant (P < 0.01).

5. Cadmium-uptake into an isolated epidermis

When 2 mM-Cd$^{2+}$ was applied to the isolated epidermis for 20 min, cadmium-uptake by the epidermis was analyzed in relation to SCC. Table 2 summarizes the effects of epidermal and dermal Cd$^{2+}$ in the isolated epidermis. The SCC was increased about 57% on the average in the case of the epidermal application,
while the dermal application decreased the SCC slightly (about 6% on the average). Though there were remarkable differences in effect between the epidermal and dermal application of Cd²⁺ on SCC, no significant difference was observed in the amount of cadmium taken up between the sides of Cd²⁺ application (P > 0.5).

Table 2. Effects of epidermal and dermal Cd²⁺ application on short circuit current (SCC) and Cd uptake of an isolated frog epidermis.

<table>
<thead>
<tr>
<th>% of control SCC</th>
<th>Cd uptake</th>
<th>% of control SCC</th>
<th>Cd uptake</th>
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<td>4,968</td>
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<td>157.4±16.2</td>
<td>2,868±523**</td>
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<tr>
<td></td>
<td></td>
<td>94.5±9.5</td>
<td>3,053±355**</td>
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Values are mean±1 S.E.M.
* Application of CdSO₄, 2 mm for 20 min, ** μM Cd/kg wet weight.

DISCUSSION

The present study was undertaken in order to elucidate the reason for the difference in Cd²⁺ effect by the side of its application. The epidermal application of Cd²⁺ enhanced the active Na⁺ transport as evidenced by an increase in SCC in both whole skin and isolated epidermal preparations. The dermis was approximately 10 times as thick and weighty as the epidermis. Assuming cadmium evenly distributed in the whole skin, cadmium uptake per wet weight in the whole skin should be the same as that of the isolated epidermal preparation. But, this is not the case. The cadmium uptake per wet weight by the whole skin preparation treated with Cd²⁺ from the epidermal side was about 1/19 of that by the isolated epidermis treated similarly. These findings suggest that cadmium may be distributed in the epidermis exclusively and that there may be an outside barrier in the epidermis for penetration of Cd²⁺ from the epidermal to the dermal side.

We obtained the association constant (Kₐ) of cadmium to hypothetical binding sites within the epidermis by replotting the data of Fig. 3 (Fig. 7). A similar analysis was carried out on an ATP-cardiolipin system by Hyman (1977). In Fig. 7 the highest rate of SCC increase obtainable is assumed to relate full binding of Cd with hypothetical Cd sites in the epidermis, and the remaining points are
Fig. 7. The increase in SCC induced by 8 mm Cd²⁺ is assumed to be due to approximate maximal binding of Cd with Cd site. The SCC's of the remaining points in Fig. 3 are replotted according to the following equation: log [Cd] = log [bound Cd site]/[free Cd site] − log Kₐ, where Kₐ is the association constant.

replotted according to the mass action equation: log [Cd] = log [bound Cd site]/[free Cd site] − log Kₐ. “Cd site” refers to the effective site which increases the SCC when it binds to cadmium, though its chemical nature is unknown at present. A straight line was obtained with the zero intercept at log [Cd] = − log Kₐ = log 160 μM. This gives a Kₐ of 6.25×10³ M⁻¹, which is about 5 times as low as the Kₐ value found for the binding of Cd to Na⁺, K⁺-ATPase, 3×10⁴ M⁻¹ (cf. Figs. 3 and 5 in TAKADA and HAYASHI, 1978a), and about 10 times as high as the value of binding of ATP to ATPase from frog skin epidermis calculated from KAWADA et al. (1975). From these Kₐ values, it might be inferred that if Cd acted simultaneously on both locations where ATPase and Cd sites existed, Cd was bound to ATPase strongly and decreased the active Na transport. But this was not the case for epidermal Cd²⁺ which increased the SCC actually. This implies that epidermal Cd²⁺ does not seem to attain to the ATPase sites because of hindrance of Cd invasion by an outside barrier. The ATPase is located along all cell membranes facing the intercellular space of epidermis except s. corneum (FARQUHAR and PALADE, 1965; MILLS et al., 1977).

The results of an electron probe X-ray microanalyzer (XMA) verified the existence of this outside barrier; i.e., epidermal application of Cd²⁺ invaded only into s. corneum and s. granulosum, but no further invasion was observed.

The distribution of epidermally applied La³⁺ resembled that of Cd²⁺; i.e., epidermally applied La³⁺ invaded through s. corneum to the outer membrane of the cells of the s. granulosum which were sealed together by occluding zonules. On reaching the outside barrier, La³⁺ increased the SCC (MARTINEZ-PALOMO et al., 1971). Epidermal application of Ca²⁺ decreased the active Na transport. The mechanism was likely to be relatively non-specific decrease in permeability of the outward facing membrane of the transporting cells. But the exact location of the permeability change was not identified (CURRAN and GILL, 1962).
The increase of the SCC by epidermal Cd$^{2+}$ can not be ascribed to the activation of Na$^+$, K$^+$-ATPase activity (Takada and Hayashi, 1978a). The increase might be rather due to the activation of Na$^+$ entry process as evidenced by decrease in the resistance of the outer surface of the skin with little change in $E_Na$ (Hayashi et al., 1978). Amiloride is considered to selectively block the Na$^+$ entry process. Further observations are needed to examine whether Cd$^{2+}$ may counteract with amiloride on the same channel.

In contrast to the epidermal application of Cd$^{2+}$, its dermal application caused no appreciable change in SCC of the whole skin. The Cd uptake into the whole skin from the dermal side was much higher than that from the epidermal side. Therefore, we cannot explain the ineffectiveness of the dermally applied Cd$^{2+}$ only in terms of the amounts of cadmium uptake. The two-step increase in cadmium uptake from dermal side (Fig. 4) suggested that there were multilayer-barriers for Cd in the whole skin; i.e., Cd uptake was relatively small for initial 6 min of incubation with 2 mM-Cd$^{2+}$ on the dermal side, but it increased about 3 times after 20 min incubation. The above deduction was supported by the results of XMA; namely, there were Cd peaks corresponding to the inner most layer, tela subcutanea (the first inside barrier, Takada et al., 1978), the sieve layer in dermis in which there was a large amount of cadmium in exchange of normally existed calcium deposits (the second inside barrier, Hayashi et al., 1977b), and the junction of dermis and epidermis (basement membrane and its adjacent tissues, the third inside barrier) in the cross section of the whole skin.

The activity of Na$^+$, K$^+$-ATPase was completely inhibited by 10$^{-4}$ M Cd$^{2+}$ (Takada and Hayashi, 1978a). The average concentration of cadmium by the dermal application of 2 mM-Cd$^{2+}$ to the isolated epidermis was approximately 3 mM (Table 2). If we assume that Cd$^{2+}$ does not invade into the inside of the cell, then the concentration of cadmium which may binds to outside of the cell should be much higher than 3 mM. This concentration of cadmium is enough for the complete inhibition of Na$^+$, K$^+$-ATPase (Takada and Hayashi, 1978a). In this experiment, however, dermal application of Cd$^{2+}$ to the isolated epidermis did not completely inhibit the SCC (Fig. 6). This suggests the concentration of cadmium was not actually so high around the location of Na$^+$, K$^+$-ATPase inside the epidermis that a fraction of SCC still remains because of partial impediment of cadmium by the third inside barrier in the isolated epidermis.

Repeated dermal application of Cd$^{2+}$ to the isolated epidermis destructed the third inside barrier so heavily (collagenase treatment may help Cd$^{2+}$ to pass through the third barrier), that some Cd$^{2+}$ would probably invade into the ATPase-site (Farquhar and Palade, 1965); thus the SCC became less (Table 1).

Dermal application of La$^{3+}$ caused no change in SCC, but in contrast to dermal application of Cd$^{2+}$, La$^{3+}$ attained to the outside barrier from inside (Martinez-Palomo et al., 1971). Dermal application of Cu$^{2+}$ increased the SCC which was almost a half of the percent increase in SCC caused by epidermal ap-
Application of Cu²⁺ (Ferreira, 1970). This suggests there is a weak inside barrier for Cu²⁺ in epidermis.

The concept of the outer and inner barriers was first introduced by Koefoed-Johnsen and Ussing (1958) which was concerned with the K⁺ and Na⁺ permeability. The outside and inside barriers are also assumed to explain the mechanism of amiloride and ouabain (cf. Moreno et al., 1973). These hypothetical barriers remain to be compared with Cd barriers in respect to their nature, numbers, and location in the skin.

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REFERENCES


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