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# ACTIVE CALCIUM TRANSPORT IN THE SKIN OF THE FROG *RANA PIPIENS*: KINETICS AND SEASONAL RHYTHMS

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## Summary

**The frog *Rana pipiens* takes up $Ca^{2+}$ against an electrochemical gradient from dilute external solutions that are similar to natural freshwater environments. The influx is dependent upon external [$Ca^{2+}$] and is saturable. Kinetic analysis yielded a $K_m$ of 0.625 mmol $l^{-1}$ and a $J_{max}$ of 38 nmol $cm^{-2}$ $h^{-1}$. These kinetic variables suggest that both the affinity and capacity are smaller than those for $Na^+$ and $Cl^-$ transport in the skin of the same species. They are also smaller than those for $Ca^{2+}$ transport in fish gill. A significant portion (20–25 %) of the $Ca^{2+}$ entering a frog remains in $Ca^{2+}$-rich layers of the skin, with ventral skin containing about three times as much $Ca^{2+}$ as dorsal skin. There are seasonal rhythms in $Ca^{2+}$ exchange: although $Ca^{2+}$ influx does not vary significantly over the year, efflux is minimal in July, while net flux, which is negative most of the year, appears to be positive in July. Since these fluxes do not include dietary calcium, one cannot conclude that feeding frogs are in negative $Ca^{2+}$ balance.**

Key words: active calcium transport, Amphibia, *Rana pipiens*, frog, skin, kinetics, season.

## Introduction

Amphibian skin, and frog skin in particular, have been extensively used as model epithelia for studying the mechanisms involved in NaCl transport (Kirschner, 1983). Early efforts to document the existence of $Ca^{2+}$ transport in these epithelia have met with varying success (Stiffler, 1993). While Watlington *et al.* (1968) reported significant net fluxes of calcium from isolated frog skin, two other studies did not find such net fluxes and concluded that frog skin does not transport $Ca^{2+}$ actively (Zadunaisky and Lande, 1972; Baldwin and Bentley, 1981*a*). There are a number of reasons to suspect that none of these studies is conclusive and that the question of calcium transport in amphibian skin remains open (Stiffler, 1993). Calcium metabolism in amphibians displays a strong seasonal rhythm, with atrophy of the parathyroid glands in winter (Dougherty, 1973) and decreases in plasma [$Ca^{2+}$] in late spring (Robertson, 1977, 1978). Of the previous studies of frog skin calcium transport, only one (Baldwin and Bentley, 1981*a*) reports at what season measurements were made and then for only some of the data. These early studies were often conducted with Ringer's solution bathing the apical (water-facing) surface and it is well known that such solutions obscure active chloride transport in this epithelium (Kirschner, 1983). Finally, the usual approach to measuring $Ca^{2+}$ fluxes, in isolated skin, has been to put radioactive $Ca^{2+}$ on the apical side of the isolated skin preparation and to measure its appearance in the basal solution. Since frog skin has large quantities of calcium stored in the subepithelial connective tissue (Zadunaisky and Lande, 1972; Baldwin and Bentley, 1981*a*), it is not necessarily true that all or even a majority of the $Ca^{2+}$ transported across the epithelial layers (where the electrochemical gradient is located) will ever reach the basal solution. It may be placed in storage in the dermal skin layers, without leaving the skin, so that sampling the basal medium would not detect its transport.

The objectives of the experiments described in the present paper are (1) to characterize $Ca^{2+}$ exchange with respect to the electrochemical gradient; (2) to determine the nature of the dependence of $Ca^{2+}$ fluxes on external $Ca^{2+}$ concentration; (3) to re-examine the high cutaneous calcium levels in skin, examining both its dorsal–ventral distribution and how much of the transported $Ca^{2+}$ remains in the skin after uptake; and (4) to determine whether reported seasonal rhythms in plasma and urinary [$Ca^{2+}$] are reflected by seasonal rhythms in $Ca^{2+}$ exchange.

## Materials and methods

### *Animals*

*Rana pipiens*, weighing 28.8±0.6 g (mean ± S.E.M. of all frogs, $N$=175) were purchased from Charles Sullivan, Amphibians of North America, Nashville, TN, USA. The frogs were kept at room temperature (approximately 25 °C) and with a choice of shallow water or dry perches. Frogs were used between 1 and

6 weeks of arrival, were healthy and did not show signs of excessive weight loss. In a few cases, when storage approached 6 weeks, they were fed live crickets.

*Bathing media*

The composition of the tap water in which the frogs were stored was as follows: $Na^+$, 1.5 mmol $l^{-1}$; $K^+$, 0.1 mmol $l^{-1}$; $Cl^-$, 1.2 mmol $l^{-1}$; $Ca^{2+}$, 3.8 mmol $l^{-1}$, $CO_3^{2-}$ + $HCO_3^-$, 3.5 mmol $l^{-1}$. This water contains quite high levels of calcium and carbonates; however, the plasma [$Ca^{2+}$] was normal for the species so the storage medium had no discernible effects. The composition of the artificial pond water (APW) in which experiments were performed was as follows: NaCl, 0.6 mmol $l^{-1}$; KCl, 0.1 mmol $l^{-1}$; $CaCl_2$, 0.04–0.5 mmol $l^{-1}$; $NaHCO_3$, 2 mmol $l^{-1}$.

*Calcium flux measurements*

The influx, efflux and net flux of $Ca^{2+}$ were measured using $^{45}Ca^{2+}$ by following the changes in water radioactivity and total $Ca^{2+}$ content. The method of Kirschner (1970) was used. It is of paramount concern when using this method to be certain that the disappearance of $Ca^{2+}$ and/or $^{45}Ca^{2+}$ is due to uptake by the animal and not to some non-specific process such as binding of $Ca^{2+}$ to the plastic experimental chambers or to the skin of the animals themselves. In order to test these possibilities, two control experiments were performed. In the first, which tested chamber binding, $^{45}Ca^{2+}$ was placed in the water (APW, 100 ml) but no animals were introduced into the chambers. There was no decrease in radioactivity of the water over 24 h, indicating that the $Ca^{2+}$ did not bind to the plastic walls of the chambers. In order to check for non-specific binding of $Ca^{2+}$ to frog skin, frogs were killed with an overdose of anaesthetic and placed in radioactive ($^{45}Ca^{2+}$) solutions. Again there was no decrease in radioactivity during the first 10 h and only a small decrease (about 12 %) over the final 14 h of the 24 h period. This small decrease was probably due to breakdown of the barriers to passive $Ca^{2+}$ permeation in the frog skin as decomposition became evident 10 h after death. For comparison, live frogs (in 0.1 mmol $l^{-1}$ $Ca^{2+}$) can reduce the radioactivity of 100 ml of water by 40 % over a 24 h period. It is clear from these control experiments that the decrease in radioactivity in the bathing media during the experiments was due to some specific process in the frog skin and not to non-specific binding to plastic or protein.

Two potential problems need to be considered. If external specific activity changes significantly during the experiment, error will be introduced. This has been avoided by calculating specific activity for each individual flux period, not using the 24 h average or terminal specific activity. The average change in specific activity during these flux periods was about 1.4 %. The second potential problem has to do with back flux of $^{45}Ca^{2+}$ into the water if extracellular specific activity rises to approach external specific activity. The extracellular specific activity was measured at the end of experiments (plasma cts $min^{-1}$/plasma [$Ca^{2+}$]) and was found to be about 0.8 % of the external specific activity. Back flux is not a problem, therefore.

*Experimental design*

Frogs were weighed and placed in 100 ml of APW containing approximately 8 $\mu$Ci $l^{-1}$ $^{45}Ca^{2+}$. Calcium concentrations varied from 0.04 to 0.5 mmol $l^{-1}$ in these experiments. Different groups of frogs were used at each concentration. 5 ml samples were taken at 0, 4, 8, 12 and 24 h and divided into subsamples for liquid scintillation counting and atomic absorption analysis for total $Ca^{2+}$. At the end of the experiment, the frogs were thoroughly rinsed (distilled water) and anaesthetized (0.1 % tricaine methanesulphonate, buffered to pH 7.0 with 0.1 % $NaHCO_3$) for measurement of the electrical potential difference across the skin (in media of the same ionic composition as was used for the flux measurement) and for sampling of blood and skin tissues. Eight frogs were used in each experiment.

*Urine collection*

Urine was collected from eight frogs in order to estimate the urinary fraction of total $Ca^{2+}$ efflux so that cutaneous effluxes could then be estimated by subtraction. Frogs were anaesthetized in 0.1 % tricaine methanesulphonate as above, and a cannula was fixed into the cloaca of each frog using purse-string sutures. The frogs were allowed to recover for about 18 h before experiments. At the beginning of the urine collection period, a tared balloon urine collection bag was attached to the cannula of each frog. At the end of the collection period, the volume and [$Ca^{2+}$] of the contents were measured. Urinary $Ca^{2+}$ flux was estimated as urine flow multiplied by urine [$Ca^{2+}$].

*Chemical analysis*

Total [$Ca^{2+}$] was analyzed using an atomic absorption spectrometer (Perkin-Elmer 4000). Bath and plasma samples were diluted appropriately and measured against standards ranging from 0.005 to 0.2 mmol $l^{-1}$ $Ca^{2+}$. The correlation coefficient for the relationship between the standard concentrations and the absorption of light in the flame was greater than 0.999. Skin samples were cut into 1–4 $cm^2$ pieces, weighed and dissolved in 1 ml of 36 mol $l^{-1}$ HCl. Samples of the digest were diluted and analyzed for [$Ca^{2+}$] as above.

*Counting of radioactivity*

$^{45}Ca^{2+}$ was counted in a Packard Minaxi 4000 liquid scintillation counter. 1 ml water samples were counted undiluted; skin acid-digests were diluted 10:1 with distilled water for counting. The scintillation cocktail (10 ml:1 ml sample) was Aquasol (DuPont-New England Nuclear). Samples were counted for a sufficient time to reduce the standard deviation to less than 1 % of the mean cts $min^{-1}$. Quenching was constant (counting efficiency approximately 90 %) and therefore did not present a problem.

*Transcutaneous electrical potential difference measurements*

The electrical potential differences across the skin of each

of the frogs was measured using a Tektronix DM 502 digital multimeter. Salt bridges made with Ringer/3 % agar in PE 50 tubing were placed in the water and under the skin of each anaesthetized (see above) frog at the end of the flux period. The free ends of these salt bridges were then connected to calomel half-cells, which were in turn connected to the poles of the multimeter. Liquid junction potentials were measured in the absence of the frog and these asymmetries (less than 4 % of the transcutaneous potential difference) were subtracted from the total potential difference.

### *Calculations*

Influx ($J_{in}$) was calculated using:

$$J_{in} = (CPM_i - CPM_f)/(S_0 \times M \times t), \quad (1)$$

where $J_{in}$ is influx, $CPM_i$ is the initial total radioactivity of the water, $CPM_f$ is the final total radioactivity of the water, $S_0$ is the specific activity of the water (cts $min^{-1}$ $mmol^{-1}$), $M$ is the mass of the animal (g) and $t$ is time (h).

The uptake was relatively constant, and for each animal the influx is the mean of four sequential determinations over the 24 h period.

Net flux was calculated using:

$$J_{net} = (Ca^{2+}_i - Ca^{2+}_f)/M \times t, \quad (2)$$

where $J_{net}$ is net $Ca^{2+}$ flux ($\mu$mol $kg^{-1}$ $h^{-1}$), $Ca^{2+}_i$ is the initial total $Ca^{2+}$ (mol) in the water and $Ca^{2+}_f$ is the final total $Ca^{2+}$ in the water.

Efflux was calculated as net flux minus influx.

The Ussing flux ratio equation (Ussing, 1949) was used to calculate flux ratios from measured transcutaneous potential differences and plasma calcium ion concentrations:

$$J_{in}/J_{out} = (C_{out}/C_{in})e^{-zFE/\boldsymbol{R}T}, \quad (3)$$

where $C_{in}$ is plasma [$Ca^{2+}$], $C_{out}$ is bath [$Ca^{2+}$], $z$ is valence (+2), $E$ is the potential difference across the skin (approximately 65 mV), $\boldsymbol{R}$ is the gas constant (8.314 J $K^{-1}$ $mol^{-1}$) and $T$ is absolute temperature (K).

Calculated flux ratios ($J_{in}/J_{out}$) can then be compared with isotopically measured flux ratios. A measured flux ratio exceeding the calculated flux ratio indicates that influx is occurring against the electrochemical gradient, which suggests that active transport is involved.

The surface area of the frogs was calculated from the allometric relationship reported by Rey (1938):

$$A = 7.2M^{0.66} \quad (4)$$

where $A$ is the surface area in $cm^2$.

### *Statistics*

All data are expressed as mean ± S.E.M. In the experiments in which external calcium concentration and season were the variables, one-way analysis of variance and Tukey's multiple comparisons test were used to determine statistical significance of differences. When single comparisons were made, Student's *t*-test was used.

## Results

Frogs in 0.1 mmol $l^{-1}$ $Ca^{2+}$ were able to take up about 40 % of the total available radioactivity during the 24 h period of the flux experiments. This was not due to non-specific binding of $Ca^{2+}$ to the chambers or to the outside of the frog skin since chambers containing radioactive calcium without frogs and those containing dead frogs showed no loss of radioactivity. Oral ingestion of the water will not explain the $Ca^{2+}$ uptake as frogs do not drink (Bentley and Yorio, 1979). In most cases, net $Ca^{2+}$ flux was negative. Since the [$Ca^{2+}$] in the tap water in the holding tanks was quite high, it might be suspected that the frogs were $Ca^{2+}$-loaded. However, the plasma [$Ca^{2+}$] was in the normal range for this species (Stiffler, 1993).

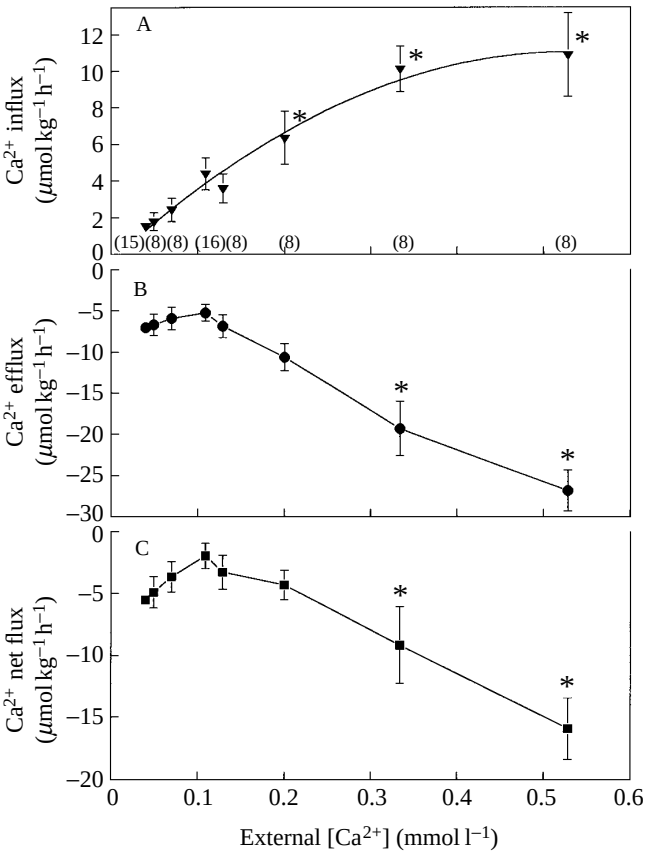


Fig. 1. $Ca^{2+}$ exchange with frog skin as a function of external [$Ca^{2+}$]. (A) Influx; (B) efflux; (C) net flux. Numbers in parentheses are numbers of frogs examined at each concentration. All measurements were made between July and September 1993. Influx is proportional to external [$Ca^{2+}$] up to about 0.3 mmol $l^{-1}$, where it levels out in a manner that appears to be explained by saturation of a carrier-assisted process. Efflux increases between 0.1 and 0.5 mmol $l^{-1}$, possibly as a result of $Ca^{2+}/Ca^{2+}$ exchange at higher [$Ca^{2+}$]. Net flux measurements indicate that the frogs are in negative $Ca^{2+}$ balance across the range of external [$Ca^{2+}$] experienced in the experiments, but approach neutral balance near 0.1 mmol $l^{-1}$. *Influxes and effluxes are significantly different ($P$<0.05) from their values at 0.04−0.13 mmol $l^{-1}$; net fluxes are significantly different from the value at 0.1 mmol $l^{-1}$ (Tukey's test for multiple comparisons). The mean mass of the frogs used in these experiments was 32.8±0.8 g. Values are mean ± S.E.M.

*Concentration-dependence of calcium fluxes*

Exchange of calcium between frogs and the environmental water is dependent upon the external concentration of calcium (Fig. 1). $Ca^{2+}$ influx increases as water $[Ca^{2+}]$ increases between 0.04 and 0.5 mmol $l^{-1}$. The influx appears to saturate between 0.3 and 0.5 mmol $l^{-1}$. Efflux also is dependent upon external $[Ca^{2+}]$. Efflux is minimal at about 0.1 mmol $l^{-1}$. Below this concentration, efflux tends to increase somewhat; above 0.1 mmol $l^{-1}$, there is a large increase in $Ca^{2+}$ efflux that is directly proportional to water $[Ca^{2+}]$. The rise in efflux with increasing water $[Ca^{2+}]$ is greater than the rise in influx, leading to an increasingly negative $Ca^{2+}$ balance as shown by the relationship between net $Ca^{2+}$ flux and water $[Ca^{2+}]$.

*Flux ratios*

In order to acquire a better understanding of the mechanisms involved in the movement of $Ca^{2+}$ across frog skin (i.e. passive *versus* active transport), the Ussing flux ratio criterion (Ussing, 1949) was applied to the calcium exchanges (Fig. 2). The flux ratio of the intact animal [cutaneous influx/(cutaneous + renal efflux)] was compared with the flux ratios expected from passive movement of $Ca^{2+}$ along its electrochemical gradient calculated from water and plasma $[Ca^{2+}]$ and the transcutaneous electrical potential difference (see Calculations). At all concentrations of external $[Ca^{2+}]$ used in the experiments, the measured flux ratio exceeded the calculated flux ratio by 2–3 orders of magnitude. This clearly shows that $Ca^{2+}$ is out of thermodynamic equilibrium and can be considered to be actively transported.

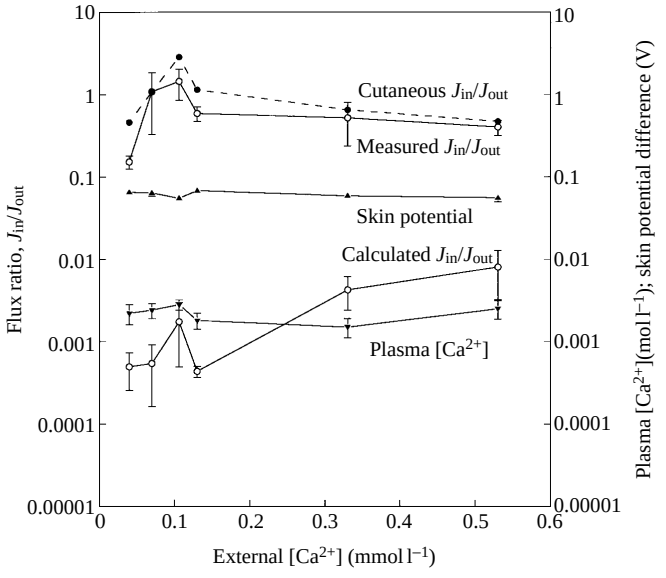


Fig. 2. Observed and calculated $Ca^{2+}$ flux ratios. Across the range of $[Ca^{2+}]$ used in the experiments, the measured flux ratios (upper trace, open circles) exceeded the calculated flux ratios (lower trace, open circles) by 2–3 orders of magnitude. When urinary efflux is subtracted from total efflux, the estimated cutaneous flux ratios (filled circles) exceed 1.0 at 0.1 mmol $l^{-1}$ $Ca^{2+}$. $N$=8 at each point. Plasma $[Ca^{2+}]$ (filled triangles) averaged 2.2±0.2 mmol $l^{-1}$ for all animals; skin potential difference averaged 61±2 mV for all animals; urinary $Ca^{2+}$ efflux was $-3.71\pm0.11$ $\mu$mol $kg^{-1}$ $h^{-1}$. Values are mean ± S.E.M.

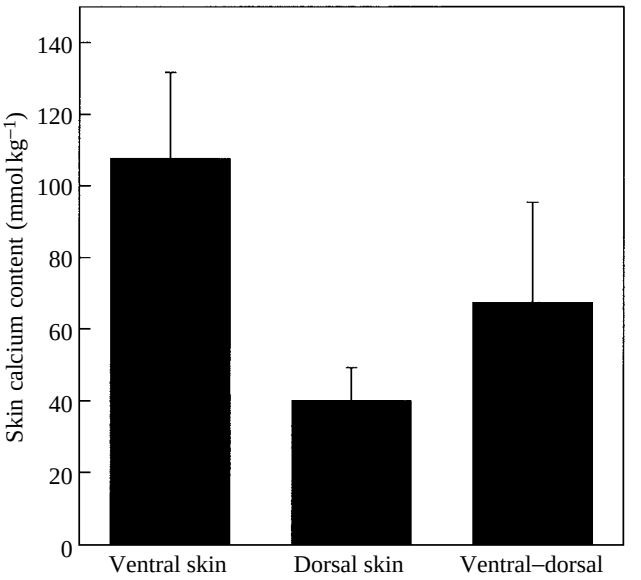


Fig. 3. A comparison of the amount of calcium deposited in ventral and dorsal skin ($N$=8 samples each of dorsal and ventral skin) of *Rana pipiens*. The ventral skin contains significantly ($P$<0.05) more calcium than dorsal skin (Student's paired $t$-test). Values are mean + S.E.M.

The observed flux ratios are flux ratios for the intact animal in that efflux is the sum of urinary and cutaneous efflux. We are more interested in cutaneous flux ratios. The urine flow of eight frogs in 0.03 mmol $l^{-1}$ $Ca^{2+}$ averaged (mean ± S.E.M.) 17.5±0.5 ml $kg^{-1}$ $h^{-1}$ and urine $[Ca^{2+}]$ was 0.21±0.01 mmol $l^{-1}$. Since plasma $[Ca^{2+}]$ did not change as water $[Ca^{2+}]$ increased (Fig. 3), and since urine $[Ca^{2+}]$ can be assumed to be more closely related to plasma $[Ca^{2+}]$ than to water $[Ca^{2+}]$, urine $[Ca^{2+}]$ is assumed to be constant in all groups. The renal $Ca^{2+}$ efflux was thus $-3.71\pm0.11$ $\mu$mol $kg^{-1}$ $h^{-1}$ (mean ± S.E.M.). When renal efflux is subtracted from total efflux, the flux ratio exceeds 1.0 at an external $[Ca^{2+}]$ of about 0.1 mmol $l^{-1}$, indicating net uptake of $Ca^{2+}$ across the skin. In terms of the intact animal, net $Ca^{2+}$ balance is negative, indicating that other sources of $Ca^{2+}$ are required by the animals. Since the animals were held in high-$Ca^{2+}$ tap water prior to experiments, it is possible that acclimation processes had slowed $Ca^{2+}$ transport; however, since the frogs showed typical plasma $Ca^{2+}$ concentrations, this explanation is unlikely. These cutaneous flux ratios would be even higher if urinary $[Ca^{2+}]$ were to increase with external $[Ca^{2+}]$.

*Kinetics*

Kinetic analysis (Lineweaver–Burk) shows that $Ca^{2+}$ influx in *R. pipiens* has an affinity ($1/K_m$) and capacity lower than those for $Na^+$ and $Cl^-$ transport in the same species (Table 1). Lineweaver–Burk analysis was selected in order to compare our $Ca^{2+}$ data with published kinetic analyses of $Na^+$ and $Cl^-$ influx (Mullen and Alvarado, 1976). The data analyzed for

Table 1. *Ionic transport kinetics: Lineweaver–Burk analysis for $Na^+$, $Cl^-$ and $Ca^{2+}$ influxes in* Rana pipiens

| Ion | Capacity, $J_{max}$[a] (nmol cm$^{-2}$ h$^{-1}$)* | $K_m$[b] (mmol l$^{-1}$) | Source |
|---|---|---|---|
| $Na^+$ | 90 | 0.135 | Mullen and Alvarado (1976) |
| $Cl^-$ | 49 | 0.167 | Mullen and Alvarado (1976) |
| $Ca^{2+}$ | 38 | 0.625 | Present study |

[a]The values for $J_{max}$ from the present study have been converted from $\mu$mol kg$^{-1}$ h$^{-1}$ to nmol cm$^{-2}$ h$^{-1}$ using the allometric equation given in Materials and methods.
[b]Affinity is inversely proportional to $K_m$.

determination of kinetic variables were taken in the summer. The Lineweaver–Burk relationship was highly linear, with all points falling close to the regression line and a correlation coefficient of 0.989.

### *Calcium storage in the skin*

A comparison of calcium deposits in ventral and dorsal skin (Fig. 3) shows that ventral skin contains almost three times as much calcium per unit mass as does dorsal skin. The fraction of transported calcium that remains in the skin was estimated by analyzing the amount of $^{45}Ca^{2+}$ that remained in the skin and comparing that with the total taken from the water (Fig. 4). This was converted to total calcium uptake using the specific activity of the water. The total uptake by ventral skin (the dorsal skin had minimal contact with the bathing medium as a result of the posture of the frogs during the experiments) was calculated by estimating $Ca^{2+}$ uptake per square centimetre of skin samples and calculating half of the surface area from the allometric relationship between body mass and surface area (see Calculations). It is clear that a sizable portion of $Ca^{2+}$ taken up by the frogs is taken up by the skin. This is not simple adsorption onto the skin as it does not occur in dead animals.

### *Seasonal rhythms*

Calcium flux measurements were conducted monthly between July 1993 and September 1994 in APW (0.1 mmol l$^{-1}$) in order to evaluate the influence of seasonal changes on cutaneous fluxes (Fig. 5). $Ca^{2+}$ influx appears to be minimal in May and June and relatively constant for the rest of the year. Tukey's multiple comparison test did not find these differences to be significant, however. $Ca^{2+}$ efflux was minimal in July ($P$<0.05 compared with January). Intact animal net flux is negative for most of the year and the net loss is greatest in June. The July net flux was significantly greater than the June net flux ($P$<0.05). This net flux appears to be positive; however, it is not significantly different from zero.

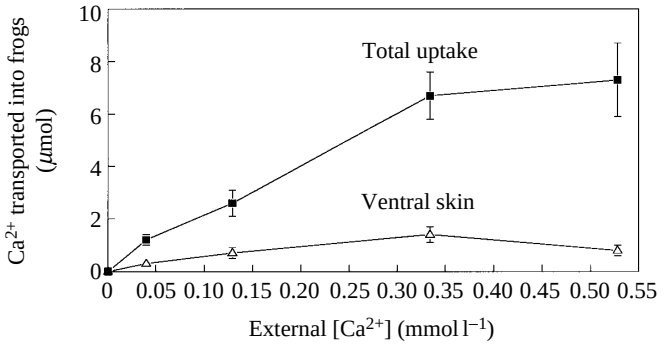


Fig. 4. Cutaneous $Ca^{2+}$ uptake compared with the total $Ca^{2+}$ taken up in 24 h. The ventral skin takes up about 20–25 % of the total $Ca^{2+}$ taken up by the animals. The remaining 75–80 % is probably partitioned between bone, extracellular fluid, soft tissues and endolymphatic sacs. $N$=8 at each point. Values are mean ± S.E.M.

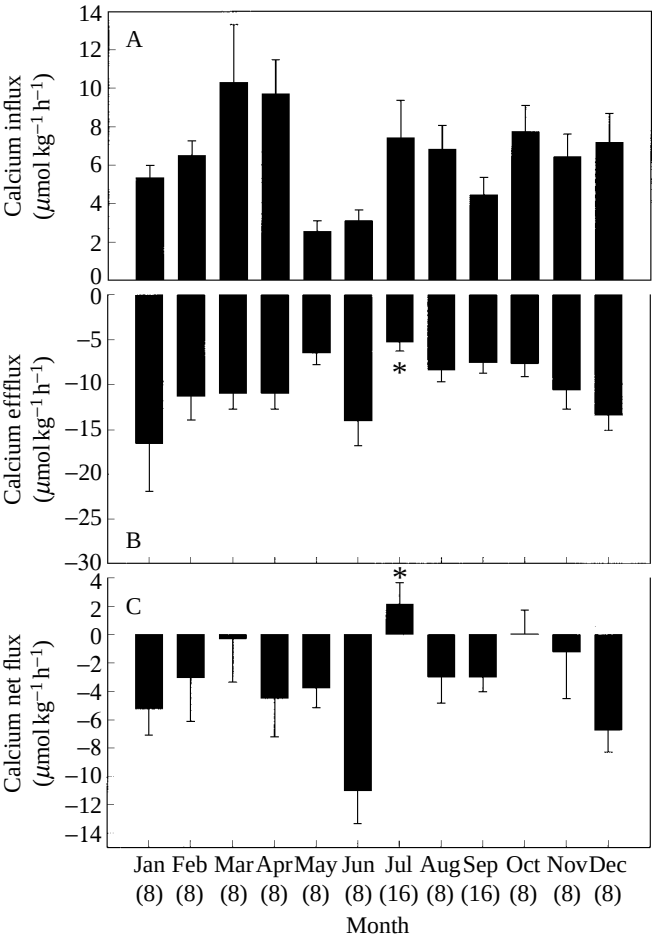


Fig. 5. Seasonal rhythm of $Ca^{2+}$ fluxes in frog skin. (A) Influx; (B) efflux; (C) net flux. All measurements were made in 0.1 mmol l$^{-1}$ $Ca^{2+}$. Numbers in parentheses are numbers of frogs examined each month. There were no significant differences in influx (Tukey's test). *The efflux in July is significantly less than the efflux in January ($P$<0.05). The net flux in July is significantly different from the net flux in June ($P$<0.05) but not significantly different from zero ($P$>0.05). The mean mass of the frogs used in these experiments was 26.9±0.7 g. Values are mean + S.E.M.

## Discussion

Calcium uptake by *Rana pipiens* occurs against an electrochemical gradient, is dependent upon [$Ca^{2+}$] in the

bathing medium and appears to be saturable. These observations all provide evidence for active $Ca^{2+}$ transport in the skin of this frog. A number of earlier studies have presented us with mixed opinions about the nature of $Ca^{2+}$ uptake in amphibian skin. An early study (Watlington *et al.* 1968) examining isolated, short-circuited pieces of frog skin reported significantly greater $Ca^{2+}$ influx than efflux and concluded that $Ca^{2+}$ must be actively transported across the skin. A later study (Zadunaisky and Lande, 1972) could not duplicate these findings, although a subepithelial layer of skin was found to be rich in calcium. These latter workers suggested that the earlier study observed significant flux ratios (influx/efflux>1) because the studies were too short (4–5 h) for equilibration of free $Ca^{2+}$ with the calcium-rich connective tissue layer. This was possible because, under the conditions of Ringer's solution bathing both sides of a short-circuited skin, there would be no electrochemical gradient opposing $Ca^{2+}$ diffusion across the epithelium. Zadunaisky and Lande (1972) also showed that when the epithelium was isolated from the underlying $Ca^{2+}$-rich layer it did not generate flux ratios significantly greater than 1. Yet another study of *Rana pipiens* (Baldwin and Bentley, 1981*a*) also failed to observe significant $Ca^{2+}$ flux ratios in isolated frog skin. All of these studies used Ringer's solution to bathe the outside of the frog skin. This unphysiological condition has been shown to obscure active chloride transport in frog skin (Kirschner, 1983) and I observed greatly enhanced efflux when external [$Ca^{2+}$] was elevated to 25 % of normal Ringer [$Ca^{2+}$] (Fig. 1). Interestingly, Baldwin and Bentley (1981*a*) did see pronounced uptake of $Ca^{2+}$ from dilute media bathing intact animals, which was similar to the uptake that I observed in the present experiments. Baldwin and Bentley (1981*b*) also observed robust $Ca^{2+}$ uptake in intact *Ambystoma tigrinum* and *Necturus maculosus* bathed by dilute media; however, flux ratios in isolated short-circuited skin bathed with Ringer's solution on the apical (outside-facing) surface did not suggest active transport. Pronounced $Ca^{2+}$ uptake has also been demonstrated for intact *Ambystoma mexicanum* bathed in dilute media; however, effluxes were not measured (Kingsbury and Fenwick, 1989).

Almost as striking as the concentration-dependence of influx was the observation that the efflux of $Ca^{2+}$ was also strongly influenced by the external concentration of $Ca^{2+}$. At very low external [$Ca^{2+}$], $Ca^{2+}$ efflux was minimal. As external [$Ca^{2+}$] increased, there was a corresponding increase in $Ca^{2+}$ efflux. This might be due to the presence of $Ca^{2+}/Ca^{2+}$ exchange. There is a good deal of evidence for a $Ca^{2+}/Na^{+}$ exchanger, which pumps $Ca^{2+}$ out of the cells across the basal membranes in exchange for $3Na^{+}$, in fish intestine (Schoenmakers *et al.* 1993) and gill (Verbost *et al.* 1994) and in rabbit renal distal tubule (Bindels, 1993). Similar $Ca^{2+}/Na^{+}$ exchangers occur in cardiac sarcolemma, and these exchangers switch from $Ca^{2+}/Na^{+}$ exchange to $Ca^{2+}/Ca^{2+}$ exchange in a $Ca^{2+}$-dependent way that can be enhanced by other cations such as $Na^{+}$ and $K^{+}$ (Philipson and Nishimoto, 1981). Such $Ca^{2+}/Ca^{2+}$ exchange cannot explain all of the increased $Ca^{2+}$ efflux, however. Even after accounting for urinary losses, efflux still exceeds influx, so some route other than urinary $Ca^{2+}$ excretion or $Ca^{2+}/Ca^{2+}$ exchange must be involved. Perhaps passive $Ca^{2+}$ permeability increases. Irrespective of the exact mechanism, it is clear that increased external [$Ca^{2+}$] strongly influences $Ca^{2+}$ efflux. This has important consequences for interpretations of flux ratios from frog skin bathed with Ringer (approximately 2 mmol $l^{-1}$ $Ca^{2+}$; approximately 100 mmol $l^{-1}$ $Na^{+}$) on both sides.

The conditions under which the present data were collected are quite different from the previous studies of isolated short-circuited skin bathed with Ringer's solution on both sides. In my experiments, the animals maintain spontaneous transcutaneous electrical potential differences of 61 mV (inside positive). They are also immersed in dilute $Ca^{2+}$ solutions that are 4–50 times as dilute as the extracellular plasma. In order for $Ca^{2+}$ to enter the animal across the epithelial layer of the skin, it must cross a steep electrochemical gradient. This is best illustrated by comparing observed flux ratios (influx/efflux) with those calculated using the Ussing flux ratio equation (see above). The calculated flux ratio is the flux ratio that one would expect if $Ca^{2+}$ were moving passively with the electrochemical gradient. When the observed flux ratio exceeds the calculated flux ratio, as in the present results, the movement of the ion must be occurring by active transport. Kinetic aspects of calcium exchange have not been described for any amphibian tissue yet investigated (Stiffler, 1993). Although the kinetics of ion transport systems bears little resemblance to the kinetics of enzyme–substrate interactions (Boutilier *et al.* 1992), the same kinetic analyses can be used to compare different ion transport systems. The fluxes used to determine the affinity and capacity of $Ca^{2+}$ influx here were all measured in the summer in APW with [$Ca^{2+}$] ranging from 0.04 to 0.5 mmol $l^{-1}$. I have chosen to use Lineweaver–Burk analysis as this method has been used to describe the kinetics of $Na^{+}$ and $Cl^{-}$ exchanges in intact animals of the same species (Mullen and Alvarado, 1976). By comparison, it seems clear that *R. pipiens* $Ca^{2+}$ influx proceeds at a lower affinity and capacity than either $Na^{+}$ or $Cl^{-}$ influx (Table 1). The $Ca^{2+}$ transport affinity and capacity in *R. pipiens* skin are also much lower than those of rainbow trout (*Oncorhynchus mykiss*) gill for $Ca^{2+}$ (Perry and Flick, 1988; Hogstrand *et al.* 1994).

A significant portion of the $Ca^{2+}$ taken up by the skin is stored in the subepithelial calcium-rich connective tissue. Since the electrochemical gradient resides in the epithelial layer and the calcium storage depots reside in the subepithelial connective tissue (Zadunaisky and Lande, 1972), the $Ca^{2+}$ that enters the calcium deposits in the connective tissue must still cross against that gradient. Therefore, a simple model of physicochemical binding with the connective tissue is not tenable and active transport is still required for $Ca^{2+}$ to reach the connective tissue. This was not true in the experiments of Watlington *et al.* (1968) because the skin was short-circuited (potential difference 0 mV) and the presence of Ringer's solution on both sides removed the concentration gradient for $Ca^{2+}$ between the outside and inside; in other words, an electrochemical gradient did not exist and $Ca^{2+}$ was free to

diffuse into the connective tissue and to bind to the calcium matrix. The role of the cutaneous calcium storage site in overall $Ca^{2+}$ metabolism has not been established. It might serve as a depot from which $Ca^{2+}$ could be mobilized during hypocalcaemia.

Seasonal rhythms, which have been reported for plasma and urine [$Ca^{2+}$] (Robertson, 1977, 1978; Stiffler, 1993), may enter into the $Ca^{2+}$ exchange picture as well. The seasonal flux measurements reported here were all conducted in APW with a [$Ca^{2+}$] of $0.1\,mmol\,l^{-1}$. My data show that $Ca^{2+}$ influx is depressed in May and June; however, there are no significant differences in the influx values between the months. A low influx in May and June would correspond to a depressed plasma [$Ca^{2+}$] during the same season in the same species (Robertson, 1977). Efflux was fairly constant, with apparent minima in May and July. The efflux in July was significantly less than the January efflux ($P<0.05$). $Ca^{2+}$ balance is negative for most of the year (in non-feeding frogs), with maximal losses in June and an apparent net uptake in July. The July value is not significantly different from zero; however, it is significantly greater than the net flux in June ($P<0.05$). The period of maximal net loss follows closely after the reproductive season, when demands on $Ca^{2+}$ may be severe. That this would be followed by a period of maximal net uptake seems understandable.

Most of the previous studies fail to mention the season during which flux measurements were made. The season for isolated skin fluxes in *R. pipiens* (Baldwin and Bentley, 1981*a*) was reported and fluxes were greater in the autumn than in the winter; however, the fluxes were measured with Ringer's solution bathing the outside surface of the skin, obscuring the picture. The fluxes reported for *A. mexicanum* (Kingsbury and Fenwick, 1989) were measured in the summer and show robust $Ca^{2+}$ influx.

Temporal rhythms in physiological variables and particularly in calcium metabolism are well known in amphibians. Plasma calcium concentration follows a seasonal rhythm in *R. pipiens*, with minimal concentrations occurring in the winter and spring and maxima in summer and autumn (Robertson, 1977, 1978). There are also lunar and diurnal rhythms in duodenal $Ca^{2+}$ transport (Robertson, 1976). Urinary [$Ca^{2+}$] also shows seasonal rhythms (McWhinnie and Lehrer, 1972; Robertson, 1978). The seasonal rhythm of plasma [$Ca^{2+}$] is paralleled by seasonal fluctuations in parathyroid gland activity (McWhinnie and Lehrer, 1972; Dougherty, 1973).

Seasonal rhythms occur in many other amphibian physiological functions related to hydromineral balance and endocrine function. The osmotic permeability of the skin of ranid frogs increases during the winter (Share and Ussing, 1965). Renal glomerular filtration in ambystomatid salamanders is greater during the summer than during the winter (Stiffler and Alvarado, 1974). Interrenal corticosteroid levels fluctuate predictably over the seasons (Jolivet-Jaudet *et al.* 1984), as do numbers of steroid receptors (Lange and Hanke, 1988). Seasonal fluctuations also occur in plasma insulin and glucagon activities in *Rana esculenta* (Schlaghecke and Blum, 1981).

In conclusion, *Rana pipiens* seems to take up $Ca^{2+}$ across the cutaneous epithelium, against an electrochemical gradient, in a $Ca^{2+}$-dependent manner that appears to be saturable. A portion of this $Ca^{2+}$ remains in storage in the dermal layers of the skin, where it may become available as the need arises. Kinetic analyses reveal a low-affinity low-capacity transport system in the skin of this frog. There are minimal seasonal changes in $Ca^{2+}$ exchange. An apparent depression of $Ca^{2+}$ influx in late spring that corresponds to a low plasma [$Ca^{2+}$] (Robertson, 1977) is not statistically significant. Two changes in efflux and net flux are significantly different from data from only one other month. Non-feeding frogs are in negative $Ca^{2+}$ balance across most of a range of external calcium concentrations extending from 0.04 to about $0.5\,mmol\,l^{-1}$, approaching neutral balance only at concentrations approximating $0.1\,mmol\,l^{-1}$. Since the natural environments they inhabit can display $Ca^{2+}$ concentrations very different from $0.1\,mmol\,l^{-1}$, it seems that dietary calcium is an important factor in maintaining calcium homeostasis. This should be quite easily accommodated by the intestinal transport system possessed by this species (Robertson, 1975, 1976).

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