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QUANTITATION OF Ca^{2+} FLUXES IN CHICK CALVARIA

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SUMMARY

This report utilizes an Ussing-type apparatus to quantitate the unidirectional fluxes of Ca^{2+} across the periosteal and endosteal membranes of frontal bones derived from calvaria of 20-day chick embryos. The influx was found to be proportional to the concentration of Ca^{2+} and equal to 0.31 μmoles/cm^2 per h at a concentration of ultrafiltrable Ca^{2+} of 1.75 mM. There were no observable differences in the influx measured from periosteal or endosteal sides. The influx was found to be inversely proportional to decreasing temperature and increasing viscosity. The influx increased to 150% of the control flux when the incubation medium contained iodoacetate at a concentration of 1 mM and increased to 200% of control flux when the endosteum or periosteum was removed. These characteristics support the view that the influx is a passive flow with the integrity of cellular layer a controlling factor. The endosteal efflux was greater by a factor of 2 when compared to the periosteal efflux at 37 °C. When the temperature was reduced to 6 °C the endosteal to periosteal efflux ratio decreased to 1.26 indicating a temperature-sensitive component in the endosteal efflux.

INTRODUCTION

It has long been established that the skeleton is not in electrolyte equilibrium with the general extracellular fluids[1]. Moreover, exchange of Ca^{2+} between bone and the circulation must take place across at least one layer of cells lining bone[2–4]. The magnitude of such unidirectional fluxes into and out of bone far exceed the net movement of Ca^{2+} due to deposition or resorption[5].

This report utilizes an Ussing-type apparatus to quantitate the unidirectional fluxes of Ca^{2+} across the periosteal and endosteal membranes of frontal bones derived from calvaria of 20-day chick embryos. While influx was found to be a passive diffusion, the integrity of the cellular covering was a controlling factor. In contrast, endosteal efflux seemed to be very sensitive to temperature and was greater by a factor of 2 when endosteum was compared to periosteum. A preliminary report of some of these results has been given elsewhere[6].

METHODS

Fertilized eggs were obtained from Babcock Poultry Farms, Ithaca, N.Y., and

Spafas Inc., Norwich, Conn. Calvaria were dissected from 20-day embryos which otherwise would have hatched at 21 days.

Influx experiments

Calvaria were dissected along the suture lines which circumscribe the two frontal bones and then mounted in the Ussing chamber so that only the center of one of the frontal bones was exposed to the chamber opening. Lyophilized bovine serum (Nutritional Biochemical Corp., Cleveland, Ohio) equilibrated with 5% CO_2:95% O_2 (v/v) at 37 °C was used as the medium, 3 ml/side. To either the endosteal or periosteal side, 30 μl of a $^{45}Ca^{2+}$ solution was added to give a final activity of $8 \cdot 10^6$ cmp/ml. In iodoacetate experiments, 20 μl of 150 mM sodium iodoacetate was added to the same side as the radioactivity, resulting in a concentration of iodoacetate of 1 mM. The entire apparatus was maintained in an incubator at 37 °C. Stirring was accomplished by the use of a magnetic stirring bar. The medium was gassed with 5% CO_2:95% O_2 (v/v) and samples taken *via* the top ports which otherwise were sealed. At times ranging from 1 min to 6 h, the medium was removed from both sides with a syringe, the calvaria removed, rinsed in serum or synthetic buffer for 0.5 min and dissolved for 24 h in 3 ml of 0.1 M HNO_3. Subsequently, aliquots of this solution were counted for $^{45}Ca^{2+}$ and analyzed by atomic absorption spectroscopy for total Ca^{2+} (ref. 7). For short time periods (1 min and 5 min) after removal of serum, 5 ml of a synthetic buffer consisting of 120 mM NaCl–1 mM $CaCl_2$–3 mM KCl–1 mM $MgSO_4$–1 mM NaH_2PO_4–25 mM $NaHCO_3$ glucose, 3.06 mg/ml were immediately added to the side which contained the $^{45}Ca^{2+}$ to prevent further influx while the calvaria was removed. Samples for $^{45}Ca^{2+}$ determinations were removed from the chamber after initiation and just before termination of the experiments. Initial values of Ca^{2+}, K^+, and Na^+ were determined from samples taken from the serum prior to its addition to the chamber and final values from samples taken after its removal. Na^+ and K^+ were determined by flame photometry[7]. The influx was computed by the following:

$$\frac{\text{total counts in the bone}}{\text{time in h} \cdot 0.283\ \text{cm}^2 \cdot \text{av. counts in medium}/\mu\text{mole Ca in medium}}$$

All counts were corrected for background.

For concentrations of Ca^{2+} other than the quantity present in the commercial serum, 100 ml of reconstituted serum (7 mg.%) was dialyzed twice against 0.1 M NaCl for 8 h, twice against distilled water for 8 h, lyophilized, and redissolved in the same volume of the synthetic buffer described above with the desired concentration of Ca^{2+}. In experiments involving stripped calvaria, bones were excised just inside sutures, and the periosteum or endosteum peeled off. When mounted in the chamber, the side which retained its membrane was bathed in the usual serum while the stripped side was bathed in 3 ml of a synthetic buffer consisting of 25 mM $NaHCO_3$ –0.85 mM $CaCl_2$–0.72 mM NaH_2PO_4–25 mM KCl–1 mM $MgSO_4$–159 mM NaCl and a $^{45}Ca^{2+}$ activity of $8 \cdot 10^6$ cpm/ml. It was necessary to use a buffer which was in equilibrium with the exposed bone to prevent any net changes in mineral content. An appropriate composition for this buffer was found by successive incubation of calvaria in a trial buffer until the buffer's composition stabilized. The initial trial

TABLE I

8-H LEVELS OF IONS IN EQUILIBRIUM WITH STRIPPED CALVARIUM

In addition the medium contained 1 mM $MgSO_4$, 25 mM HCO_3^- and 3.06 mg/ml glucose. See text for details.

	No. vials (5 bones/vial)	Ca^{2+}	PO_4^{3-}	K^+	Na^+
a	3	0.87	0.77	21.9	143
b	3	0.85	0.72	25.1	159

buffer, 1 mM $CaCl_2$–1 mM NaH_2PO_4–20 mM KCl–115 mM NaCl–25 mM $NaHCO_3$–3.06 mg/ml glucose was contrived from a combination of incubation studies with rat calvaria[8] and powdered bovine bone[3]. Five calvaria were incubated in 2 ml of this buffer and at the end of 8 h the concentrations of Na^+, K^+, Ca^{2+}, and PO_4^{3-} were determined. PO_4^{3-} was determined by a colorimetric method[9]. The results are listed in Table I, Line a. Then, with this buffer as the medium, an additional set of stripped calvaria were incubated for 8 h, and the results are listed in Table I, Line b which is the composition of the buffer used in all stripped-calvaria experiments.

Efflux experiments

In efflux studies, calvaria were dissected outside the suture lines and placed in flasks containing 5 ml serum at 37 °C which had been equilibrated with 5% CO_2:95% O_2 (v/v) and $^{45}Ca^{2+}$ at an activity of $1 \cdot 10^8$ cpm/ml. Flasks were agitated in a water bath at 37 °C for 4 h.

At the end of the loading period, calvaria were removed, rinsed in cold serum, clamped into the chamber, and 4 ml of serum were added to each side of the chamber. Samples, 50 μl/side, were removed at various times up to 2 h. The cumulative counts at sample point t_j was computed by the following:

$$\text{Cumulative counts } t_j = \text{Cumulative counts } t_{j-1} + (\text{activity in cpm at } t_j - \text{cpm } t_{j-1})V_j$$

where V_j is the volume prior to sampling at time t_j and t_1 is the first sample point at zero time and the associated activity treated as background (Flux $t_1 = 0$).

At the termination of the experiment, the calvaria were removed and the portion exposed to the chamber excised with a cork borer. Ca^{2+} was extracted by 24-h incubation in 3 ml 0.1 M HNO_3 and subsequently analyzed for $^{45}Ca^{2+}$ and total Ca^{2+}.

RESULTS

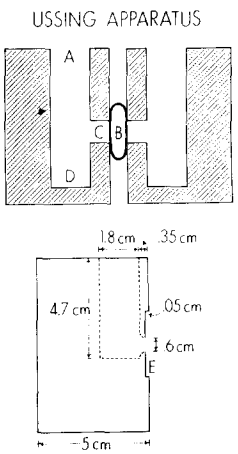
The use of an Ussing chamber (Fig. 1) obviates some of the drawbacks encountered by earlier investigators in quantitating the Ca^{2+} fluxes in calvaria[10]. A properly dissected calvarium mounted in the chamber permits independent in-

vestigation of the periosteum and endosteum, eliminates the exposure of any non-cellular lined bone, and eliminates any membrane discontinuities at the points of dissection.

Either of the frontal bones of the 20-day embryonic chick adequately cover the 0.283 cm^2 opening in the chamber, yet are pliable enough to withstand the pressure exerted on its periphery. Similarly prepared calvaria have shown a linear consumption of oxygen for 6 h (ref. 10) and a linear production of lactic acid (5 μmoles/calvaria per h) which decreased to nearly zero in the presence of 1 mM iodoacetate (Neuman, W. F. and Mulryan, B. J., unpublished).

Influx results

The Ca^{2+} influx over a period of 6 h is shown in Fig. 2. The influx was linear with a rate of 0.31 μmoles/cm^2 per h. at a concentration of ultrafiltrable Ca^{2+} of 1.75 mM. There was no detectable difference in the fluxes measured at the periosteal or endosteal sides. The small rapid uptake component at 1 min could represent membrane-bound Ca^{2+} not removed in washing. Considering the system employed it is not surprising to find a linear influx for 6 h. It has been established that isotopic exchange with bone crystals occurs in successive steps: (1) from solution to the chemisorbed double layer or hydration shell, (2) from the hydration shell to the crystal surface, and (3) from the surface to successive layers of intracrystalline positions. The half time for exchange rate of Step 1 is in the order of minutes; Step 2, h; and Step 3, months[1]. In the present preparation Steps 1 and 2 are predominantly involved in any isotopic exchange and represent approx. 20% of the total Ca (ref. 1). The 20-day embryonic calvarium contains 500 μg Ca within the 0.283 cm^2



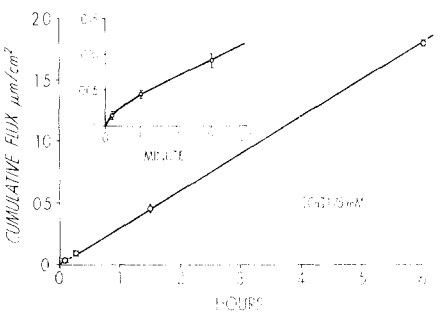


Fig. 1. Top: drawing of assembled chamber; (A) open top where the medium is added and samples removed, (B) calvarium, (C) 0.283 cm^2 circular opening where medium is in contact with calvarium, (D) location of stirring bar. Bottom: dimensions of chamber as seen from side view; (E) location of teflon washer. The calvaria are approx. 15 mm in length, 10 mm in breadth, and 0.2 mm in thickness.

Fig. 2. The cumulative uptake of Ca^{2+} as measured by $^{45}Ca^{2+}$ accumulation in calvaria. Each point is the average of from 4–9 experiments.

segment utilized in the chamber. In addition, once $^{45}Ca^{2+}$ has crossed the cellular envelope and entered the crystal surface, longitudinal exchange can occur with Ca not directly within the chamber opening. This at least doubles the total available Ca, thus a minimum of 200 μg are exchangeable. Over 6 h the cumulative unidirectional flux was 21 μg or approx. 10% of the total exchangeable Ca^{2+}. Thus the bone acted as an isotopic sink for any incoming $^{45}Ca^{2+}$ and black flux was, accordingly, negligible. Likewise, the medium contained 300 μg of Ca^{2+} and thus remained invariant with respect to total Ca^{2+} concentration. This has been found to be true for all ions investigated, Na^+, K^+, Ca^{2+} and H^+; their concentrations in the incubation medium remained unchanged for the duration of the experiment.

The influx as a function of the concentration of Ca^{2+} is displayed in Fig. 3. The flux was observed to be proportional to the concentration of Ca^{2+} from 0.2–2.35 mM ultrafiltrable Ca. Again there was no observable differences between endosteal and periosteal influxes. The ultrafiltrable Ca^{2+} was found to be 65% of the total Ca^{2+} throughout this concentration range. The failure of varying Ca levels to influence the percentage ultrafiltered over this concentration range of Ca has been reported previously[11–13].

In the presence of 1 mM iodoacetate the 6-h influx was observed to increase to 150% of the control value (Table II). Iodoacetate is known to bind to the sulfhydryl group of cysteine which tends to increase the Ca^{2+} leak in membranes as evidenced in the chorioallantoic membrane[14]. In the present system, we believe iodoacetate disrupts the integrity of the endosteal and periosteal cells lining bone allowing a larger influx. Furthermore, when the cellular layer is stripped from the calvarium the 90-min influx increases to 200% of the control value (Table II). As described in the Methods section, in the buffer which incubated the stripped calvaria, the concentrations of Ca^{2+} and PO_4^{3-} were stable so that the $^{45}Ca^{2+}$ accumulation in stripped calvaria represented the exchange rate with the bone surface. The influx of Ca^{2+} into intact calvaria using this same buffer was not significantly different from the control value (Table II).

Table II shows that the transcellular influx into intact calvaria at 24 and 6 °C is reduced to 76 and 50%, respectively of the control values. When the correction

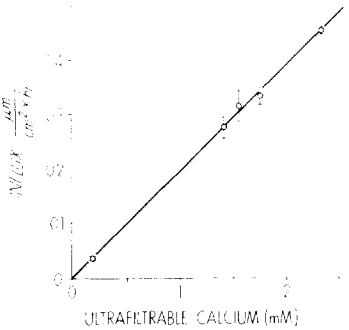
TABLE II

THE INFLUX UNDER VARIOUS CONDITIONS

All influxes are based on ultrafiltered concentration of Ca^{2+} of 1 mM.

Conditions	*No. of expts*	*Influx μmole/cm^2 per h*
Control	9	0.187 ±0.015*
IAA (1 mM)	5	0.286 ±0.017
Stripped	10	0.385 ±0.017
Intact with bone buffer	3	0.219 ±0.028*
Control at 24 °C	5	0.145 ±0.002
Control at 6 °C	6	0.0957 ±0.004

* No significant difference by means of a Student *t* test.



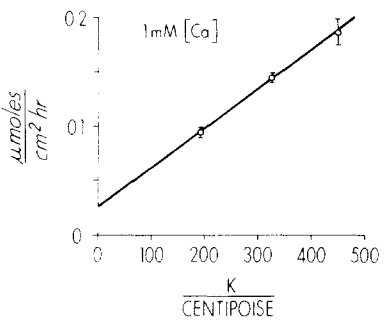


Fig. 3. The influx of Ca^{2+} as a function of the concentration of Ca^{2+}. Each point is the average of from 3–9 experiments.

Fig. 4. The influx of Ca^{2+} as a function of absolute temperature/viscosity of water at the corresponding temperature (K/cP). Each point is the average of from 5–9 experiments.

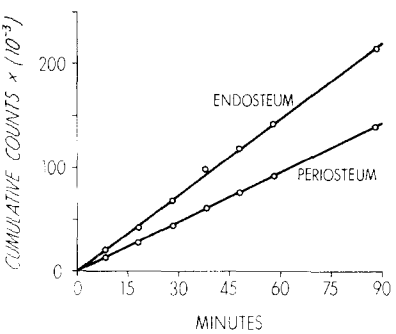


Fig. 5. The appearance of $^{45}Ca^{2+}$ in the medium after preloading the calvaria.

for reduction of diffusion due to increasing viscosity is considered as in Fig. 4, the data obey the Stokes–Einstein relationship: $\phi = aT/\eta$ for diffusion of an isotopically labeled molecule in a liquid[15] where $\phi = Ca^{2+}$ flux, T = absolute temperature, η = viscosity of water at temperature T, a = constant. There is a slight deviation from the Stokes–Einstein relationship in that the y intercept did not extrapolate to zero. This probably represents the degree to which this system deviates from the ideal system of diffusion in pure liquid, particularly at low temperatures.

Efflux results

Fig. 5 shows the efflux kinetics displayed after preloading a calvaria for 4 h with $^{45}Ca^{2+}$. The bone extracellular fluid from which the Ca^{2+} is effluxing is inaccessible for chemical analysis thus preventing the determination of the specific activity and consequently the calculation of absolute fluxes. Nonetheless, as shown above, influx into calvaria was identical through periosteal and endosteal cell layers. Thus, during loading, the bone fluid on the inner face of both the periosteal and endosteal sides should have reached the same specific activity. The ratio of endosteal to periosteal $^{45}Ca^{2+}$ efflux is therefore a valid measurement of the endosteal efflux relative to the periosteal efflux. The averaged results at 37 °C gave a ratio of 2.09 ± 0.23 endosteal–periosteal effluxes and at 6 °C gave a ratio of 1.26 ± 0.09 endosteal–periosteal effluxes (Table III).

TABLE III

THE ENDOSTEAL TO PERIOSTEAL EFFLUX RATIOS AT 37 AND 6 °C

Temperature (°C)	*Periosteal efflux cpm/50 μl per h*	*Endosteal efflux cpm/50 μl per h*	*Endosteal:periosteal ratio*
37	1280	3340	2.60
	1610	2350	1.46
	1380	2060	1.49
	1500	5500	3.66
	1590	3350	2.10
	1080	2400	2.22
	1150	2400	2.06
	1650	2400	1.45
	1400	2500	1.78
			Av. 2.09 ± 0.23 *
6	1160	1670	1.44
	1110	1300	1.17
	1360	1600	1.18
			Av. 1.26 ± 0.088 *

* $P < 0.01$ for difference between means by Student t test.

DISCUSSION

There have been many observations that the extracellular water associated with bone is of different composition than the bulk extracellular fluids[3,5,16,17]. Some barrier must exist to contain and control these ionic gradients. There seems to be too much K^+ in bone extracellular fluid compared to serum levels[3]. Sr^{2+} appears to concentrate in bone water[16]. The rates of *in vitro* mineralization are higher in chick tibia stripped of the cellular envelopes than similar tibia with intact envelopes[18], and the 8-h levels of Ca^{2+} in the incubation media of stripped calvaria are lower than in serum as evidenced with embryonic chick calvaria (Table I) and with embryonic rat calvaria[8]. These phenomena have led several investigators[2–4,19,20] to hypothesize a cellular barrier containing these ionic gradients, possibly the layer of cells lining bone.

The present studies quantitate the influx rates across this cellular layer at 0.31 μmoles/cm^2 per h and establishes that the periosteal and endosteal rates are equal. The influx rate was proportional to the concentration of Ca^{2+} in the incubation medium and proportional to the absolute temperature/viscosity. These characteristics support the view that the Ca^{2+} flux into bone is a passive flow across the cellular envelope. Furthermore, the sensitivity to low concentrations of iodoacetate and cell removal imply that the flux is controlled by the continuity of the cellular layer. Modification of these cellular membranes or more important, modification of the cell junction alters the rate of Ca^{2+} entry into bone.

The skull grows outward by appositional growth on the outside and resorption of the inner surface. We have found the endosteal efflux to be twice the periosteal efflux at 37 °C, indicating the assymetric growth is continuing in the *in vitro* preparation. Though the absolute magnitude of effluxes cannot be measured, assuming

continued assymetric growth, an approximate calculation can be made. The endosteal efflux must be greater than the endosteal influx of 0.31 μmole/cm^2 per h at the serum level of Ca^{2+} since resorption of bone is occurring at the endosteal surface. The periosteal efflux must be less than the periosteal influx of 0.31 μmole/cm^2 per h since there is deposition of bone occurring. Thus we have:

$$\phi_E > 0.31 \qquad \phi_E = \text{endosteal efflux}$$
$$\phi_p < 0.31$$
$$\phi_E = 2\phi_p \qquad \phi_p = \text{periosteal efflux}$$

Furthermore, since there is net growth of the skull, total influx must exceed total efflux.

$$2(0.31) > \phi_E + \phi_p$$

Solving these equations we find:

$$0.15 < \phi_p < 0.21$$
$$0.31 < \phi_E < 0.42$$

Note that there is no overlap in the permissible ranges. Within 20% the endosteal efflux becomes 0.36 μmole/cm^2 per h and the periosteal efflux becomes 0.18 μmole/cm^2 per h.

In reducing the temperature from 37 to 6 °C, the ratio of endosteal to periosteal efflux decreases from 2.09 to 1.26, implying a temperature-sensitive component in the endosteal efflux. Over this temperature range any passive flux should decrease with decreasing temperature and increasing viscosity, but with decreasing temperature the solubility of hydroxyapatite increases[1] thus producing a higher free concentration of Ca^{2+} in the bone extracellular fluid and a larger passive efflux component. In addition any metabolic events associated with deposition or erosion of bone should cease destroying the origin of the difference in efflux rate between endosteal and periosteal surfaces. Some temperature-sensitive metabolic event must be involved in either bone remodeling or Ca^{2+} efflux.

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