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MEASUREMENT OF FACILITATED CALCIUM DIFFUSION BY A SOLUBLE CALCIUM-BINDING PROTEIN

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The flux of calcium through an aqueous compartment was determined in a flow-dialysis cell in which two dialysis membranes separated the middle aqueous compartment from two outer compartments. The contribution of convection to the total calcium flux was large but could be removed by addition of 1% agar. The flux of calcium through the gelled aqueous compartment agreed with theoretical expectations. The self-diffusion coefficient for calcium from these results was calculated to be $0.81 \cdot 10^{-5}$ $cm^2 \cdot s^{-1}$. Carp parvalbumin significantly enhanced the calcium flux at $2.3 \cdot 10^{-6}$M free calcium. The calcium flux increased linearly with parvalbumin concentration. These observations are consistent with the hypothesis that the overall unidirectional calcium flux J is the sum of free calcium diffusion and protein-calcium diffusion: $J = D[Ca] + D'[CaPr]$. The value of D', the self-diffusion coefficient for parvalbumin, was calculated from the flux data to be $13.7 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$.

Introduction

It has recently been reported [1] that the intestinal, vitamin D-dependent calcium-binding protein can increase the unidirectional flux of calcium across an aqueous compartment separated by dialysis membranes from two outer compartments. Analysis of the fluxes in this system indicated that the dialysis membranes contributed significantly to determining the overall calcium flux. Because of the tightness of the dialysis membranes, quantitation of the enhanced diffusion was not possible. Furthermore it was not clear to what extent the inner aqueous compartment was convectionally stirred. The present study was undertaken to provide a method for measuring the enhancement of calcium diffusion by a soluble, calcium-binding protein and to determine the contribution of convection to the overall transcompartmental calcium flux.

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

Methods

Flow dialysis apparatus. The flow-dialysis apparatus described earlier [1] was used throughout the experiments reported herein. The basic components of this apparatus are shown schematically in Fig. 1. Each of the two outer compartments were separated from the inner compartment by a dialysis membrane (Spectrapor, molecular weight cutoff of 6000–8000). The membranes were prepared by rinsing for 4 h in tap water, then rinsing four times with dialysis buffer. The membranes were stored at 4°C in dialysis buffer until used. The available thicknesses of the middle chamber were varied from 0.17 to 1.29 cm. When leaks through the membranes were detected, by placing solutions of 10 mg/ml cytochrome *c* within the middle compartment, the membranes were replaced.

Parvalbumin. Carp parvalbumin was generously provided by Dr. James Potter. The concentration of parvalbumin in the middle compartment was determined after dialysis by freezing and then

thawing the collected gel, centrifuging and estimating protein content in the aqueous phase by the method of Lowry.

Flow dialysis. The left compartment of the flow-dialysis apparatus was perfused with a solution containing 10 mM Mops buffer, 140 mM KCl, 0.5 mM $MgCl_2$, 5 mM NaN_3, 1 μM added calcium, and 0.1 μCi ^{45}Ca/ml. The measured pH of the dialysis solution was 7.06. The right compartment was perfused with an identical solution except ^{45}Ca was omitted. The total calcium concentration was measured by atomic absorption spectrophotometry after acidification with 6% trichloroacetic acid and 1% HCl, and was found to be 2.3 μM. The perfusion was performed with a peristaltic pump adjusted to provide a flow rate of 0.2 ml/min through each outer compartment. The effluent from the right compartment was collected in 20-min fractions and aliquots were counted by liquid-scintillation spectrometry for determination of ^{45}Ca. The unidirectional calcium flux per unit area was calculated as:

$$J_{LR} = \frac{dCa^*}{dt} \cdot \left(\frac{Ca^*}{Ca}\right)^{-1} \cdot A^{-1} \quad (1)$$

where dCa^*/dt is the rate of appearance of ^{45}Ca in the effluent from the right compartment, Ca^*/Ca is the specific activity in the left compartment and A is the area through which calcium flux occurred. This formula was applied only for steady-state measurements of J_{LR} and is not valid except under steady-state conditions. The operational calculation was:

$$J_{LR} = \frac{[^{45}Ca]_r}{[^{45}Ca]_l} \cdot \frac{(0.2 \text{ ml/min})(2.3 \text{ nmol/ml})}{3.46 \text{ cm}^2} \quad (2)$$

where $[^{45}Ca]_r$ is the concentration of ^{45}Ca, in cpm/ml, in the effluent from the right compartment and $[^{45}Ca]_l$ is the similar variable for the left compartment. The flow rate (0.2 ml/min), total calcium (2.3 μM), and area (3.46 cm^2) were kept constant for all experiments reported herein.

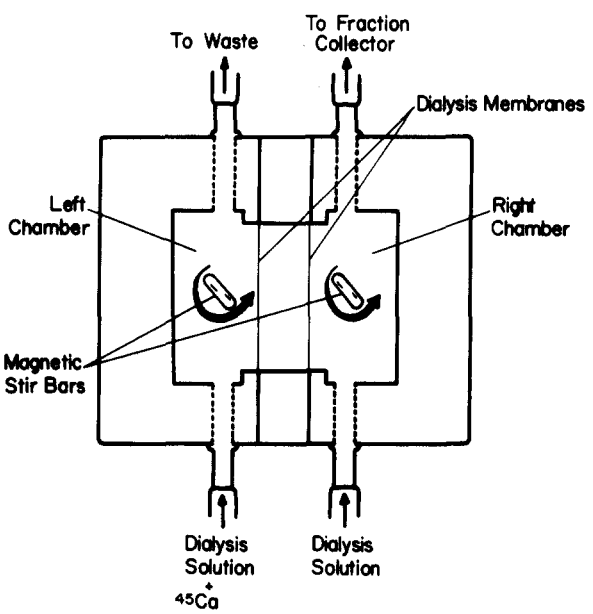

Fig. 1. Schematic diagram (top view) of the flow dialysis apparatus. The middle chamber was limited by spacers of various thickness fit between two outer chambers. The two dialysis membranes were clamped between the middle and outer chambers. A vertical hole drilled into the spacer allowed access to the middle chamber. The continuous inputs to both outer chambers were identical except that ^{45}Ca was added to the input for the left outer chamber. Both outer chambers were stirred continuously.

Composition of the middle compartment. The contents of the middle compartment varied with the experiment. For gelling the middle compartment, solutions of 2% agar (Difco Laboratories) were prepared in dialysis solution heated to 90°C. This was diluted with an equal volume of dialysis solution containing various concentrations of parvalbumin. The liquid solution was rapidly transferred to the middle compartment and allowed to gel. Preliminary experiments were performed to indicate the volume of solution necessary to completely fill the middle compartment.

Following attainment of steady-state flux, the gelled middle compartment was collected and then frozen. When thawed, it was observed that the gel had collapsed and released its contents of calcium and parvalbumin. The aqueous phase was assayed for protein by the method of Lowry, total calcium by atomic absorption spectrophotometry after extraction with 6% trichloroacetic acid and 1% HCl, and ^{45}Ca by liquid-scintillation spectrophotometry.

Results

Effect of parvalbumin on calcium flux

The effect of parvalbumin on the transcompart-

mental calcium flux is shown in Fig. 2. In this figure, the flux of ^{45}Ca was expressed as $([^{45}Ca]_r/[^{45}Ca]_l) \times 100$, which is proportional to the unidirectional flux, J_{LR}, as described by Eqn. 2.

When perfusion solution alone was added to the middle compartment of thickness 0.55 cm, the transcompartmental ^{45}Ca flux was 2.6%. This is nearly identical to the transcompartmental ^{45}Ca flux reported earlier [1] when the thickness of the middle compartment was 0.17 cm. This indicates that convection must play an important role; that is, diffusion did not appear to depend on the diffusion distance. When the middle compartment was gelled by the addition of 1% agar, transcompartmental ^{45}Ca flux was markedly reduced (Fig. 2), as would be expected if the agar prevented convectional mixing. When this 1% agar was replaced by a middle compartment containing 1% agar and 0.14 mg/ml parvalbumin, the transcompartmental ^{45}Ca flux was increased. Similar results were obtained at all diffusion distances investigated.

Dependence of flux on the diffusion distance

The transcompartmental calcium flux consists of several component fluxes as shown diagrammatically in Fig. 3. The contribution of each of these fluxes to the overall transcompartmental calcium flux (J_{LR}) can be obtained by the methods of Stein [2] as described earlier [1]. The result gives

$$J_{LR} = \frac{J_{lm}^2 J_{lr}}{J_{lm}^2 + 2J_{lm}J_{lr}} \tag{3}$$

This result is based on the steady-state assumptions that $J_{lm} = J_{ml} = J_{rm} = J_{mr}$ and $J_{lr} = J_{rl}$ when the calcium concentrations have equilibrated and the dialysis membranes are identical.

According to Fick's laws of diffusion, the unidirectional flux per unit area across the inner aqueous layer will be given as

$$J_{lr} = DC/\Delta x \tag{4}$$

where D is the self-diffusion coefficient for calcium, C is the concentration of calcium, and Δx is the thickness of the layer through which flux occurs. In these experiments, C was kept constant at 2.3 μM. Eqn. 4 can be substituted into Eqn. 3 to give

$$J_{LR} = \frac{J_{lm}^2 DC/\Delta x}{J_{lm}^2 + 2J_{lm}DC/\Delta x} \tag{5}$$

which can be rearranged to

$$\frac{1}{J_{LR}} = \frac{\Delta x}{DC} + \frac{2}{J_{lm}} \tag{6}$$

According to this equation, plots of J_{LR}^{-1} against Δx should be linear with slope $(DC)^{-1}$ and an ordinate intercept of $2/J_{lm}$. The results obtained in the absence of agar, shown in Fig. 4, produced a horizontal line of slope essentially zero. The intercept gives $J_{lm} = 0.117$ pmol $\cdot$ cm$^{-2} \cdot$ s^{-1}.

The zero slope obtained in Fig. 4 suggested that there was no diffusion barrier within the enclosed aqueous compartment and therefore this compartment was convectionally stirred. To remove convectional mixing, a second series of experiments were conducted in which the inner aqueous compartment was gelled by the inclusion of 1% agar.

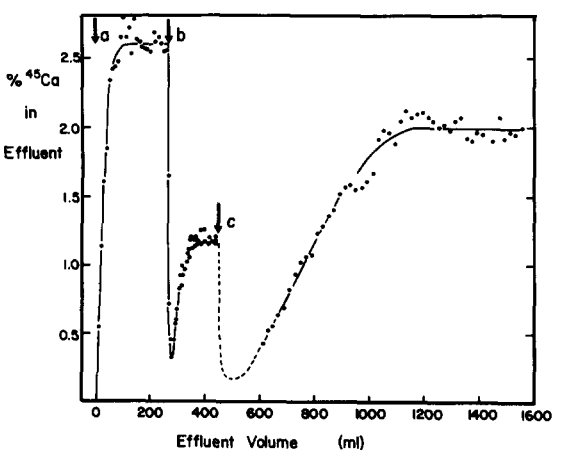

Fig. 2. Effect of 1% agar and parvalbumin on ^{45}Ca flux. The spacer was 0.55 cm wide. The middle compartment was filled with dialysis solution and ^{45}Ca flux was measured (a); the middle compartment was removed by aspiration with a syringe, washed twice and filled with hot dialysis solution containing 1% agar, which was then allowed to gel (b); the cell was disassembled and the gelled inner compartment was removed. After reassembly, the inner compartment was filled with hot dialysis solution containing 1% agar and 0.14 mg/ml parvalbumin (c).

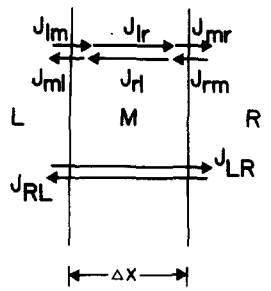

Fig. 3. Component fluxes of transcompartmental calcium flux. The overall unidirectional calcium fluxes, J_{LR} and J_{RL}, are composed of unidirectional fluxes across single dialysis membranes (J_{lm}, J_{ml}, J_{mr} and J_{rm}) and flux across the aqueous compartment (J_{lr} and J_{rl}).

The results, also shown in Fig. 4, indicated a high correlation between J_{LR} and Δx, the overall thickness of the inner compartment. The diffusion coefficient calculated from the results in 1% agar was $0.81 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1}, which is in excellent agreement with the reported self-diffusion coefficient for calcium, $0.78 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1} [3].

The intercept in the presence of agar gave $J_{lm} = 0.188$ pmol $\cdot$ cm$^{-2} \cdot$ s^{-1}. This value is significantly larger than that obtained in the absence of agar and points to an 'unstirred' layer adjacent to the dialysis membranes when agar is not used. The equivalent thickness of the unstirred layers can be calculated by assuming the calcium diffusion coefficient is identical in 1% agar and within the unstirred layer. The conditions evaluated are shown in Fig. 5. The flux across the dialysis membrane and the associated unstirred layer on the outside surface is combined into the flux denoted J_a. The overall transcompartmental flux in this case is

$$J_{LR} = \frac{J_a^2 J_{lr}}{J_a^2 + 2J_a J_{lr}} \tag{7}$$

when Δx becomes zero, J_{lr} becomes infinite and this equation reduces to

$$J_{LR} = \frac{J_a}{2} \tag{8}$$

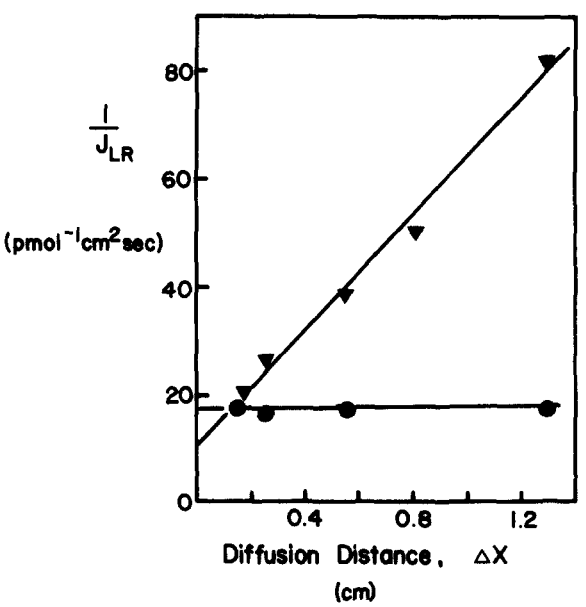

Fig. 4. Effect of gelling on unidirectional transcompartmental calcium flux. The inverse of the flux calculated by Eqn. 2 is plotted against the thickness of the middle compartment for data obtained in the absence (●) and presence (▼) of 1% agar.

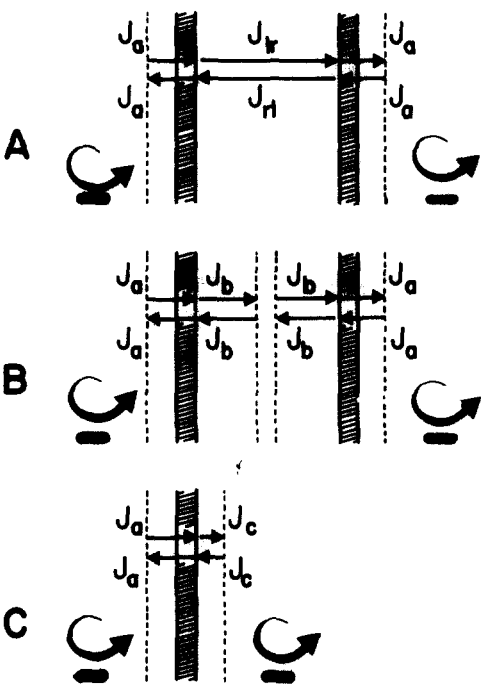

Fig. 5. Schematic diagram of the barriers to diffusion provided by the dialysis cell when the middle compartments is gelled with agar (a); when the middle compartment is not gelled by agar (b); and when a single dialysis membrane is situated between two stirred compartments (c). The hatched areas represent the dialysis membranes and the region between the vertical dashed line and the dialysis membrane represents the unstirred layer present even when the outer compartments are vigorously stirred.

The value of J_{LR} when $\Delta x = 0$ can be obtained by extrapolating the line shown in Fig. 4 for the case when agar was used. The result is $J_{LR} = 0.0941$ pmol · cm^{-1} · s^{-1}, and thus $J_a = 0.188$ pmol · cm^{-1} · s^{-1}.

In the absence of agar, the barrier to diffusion presented by the dialysis membrane consists of the membrane itself and two unstirred layers, one adjacent to the stirred outer compartment and the other adjacent to the unstirred inner compartment. The flux across this entire barrier was given earlier as $J_{lm} = 0.117$ pmol · cm^{-2} · s^{-1}. According to the definitions shown in Fig. 5B, this flux is

$$J_{lm} = \frac{J_a J_b}{J_a + J_b} \tag{9}$$

where J_a is equivalent to the flux across the dialysis membrane when the inner unstirred layer is removed. After inserting $J_a = 0.188$ pmol · cm^{-2} · s^{-1} and $J_{lm} = 0.117$ pmol · cm^{-2} · s^{-1}, J_b is calculated as $J_b = 0.310$ pmol · cm^{-2} · s^{-1}. Using $J_b = DC/\Delta x$ where $D = 0.81 \cdot 10^{-5}$ cm^2 · s^{-1} and $C = 2.3$ nmol · cm^{-3}, one can calculate $\Delta x = 0.06$ cm as the equivalent thickness of the unstirred layer adjacent to the inner compartment when agar is not used to remove convectional mixing.

The equivalent thickness of the unstirred layer on the side adjacent to the stirred compartment can be evaluated from the flux across a single membrane, as shown in Fig. 5C. The experimental value of the single membrane flux was 0.157 pmol · cm^{-2} · s^{-1}. The overall single membrane flux, J'_{lm}, is given as

$$J'_{lm} = \frac{J_a J_c}{J_a + J_c} \tag{10}$$

inserting $J'_{lm} = 0.157$ pmol · cm^{-2} · s^{-1} and $J_a = 0.188$ pmol · cm^{-2} · s^{-1}, J_c is calculated as $J_c = 0.952$ pmol · cm^{-2} · s^{-1}, and the equivalent unstirred layer thickness is 0.02 cm. The operation of the magnetic stirrers, then, reduced the equivalent unstirred layer from 0.06 cm to 0.02 cm.

Dependence of flux on parvalbumin concentration

The transcompartment flux of ^{45}Ca was measured at steady-state when various concentrations of parvalbumin were added to the middle compartment gelled with 1% agar. The unidirectional transcompartmental flux was calculated as described in Methods, and the results are shown in Fig. 6 where J_{LR} is plotted against Δx for three different concentrations of parvalbumin. The slope of the line in this plot is $D_a C$ where

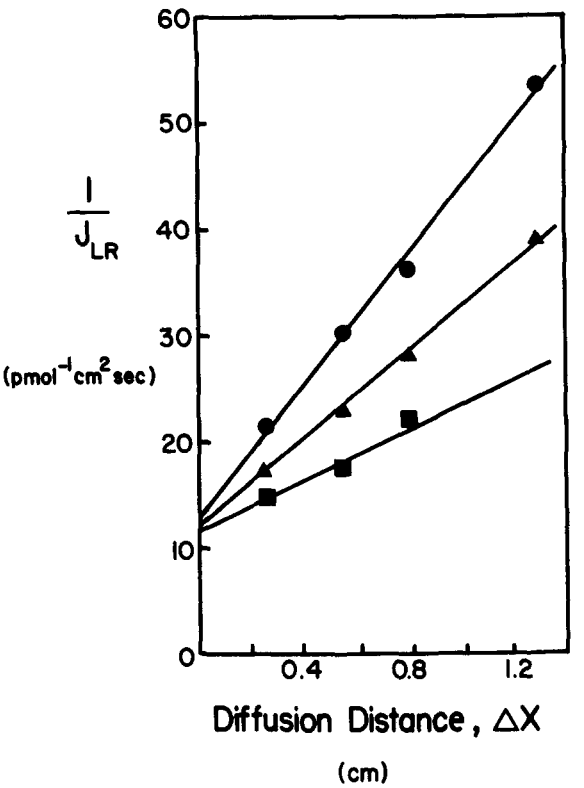

Fig. 6. Effect of parvalbumin concentration and diffusion distance on calcium flux. The inverse of J_{LR} is plotted according to Eqn. 6. The parvalbumin concentration was 0.07 mg/ml (●), 0.14 mg/ml (▲), or 0.28 mg/ml (■).

$$J_{lr} = D_a C/\Delta x \tag{11}$$

where D_a is the apparent diffusion coefficient and C is the free calcium concentration. The results in Fig. 6 clearly show that parvalbumin enhances the apparent diffusion coefficient for calcium and the degree of enhancement depends on the parvalbumin concentration.

In general, ligand-binding proteins might enhance ligand transport by acting in concert as a 'bucket brigade' or by translational diffusion of ligand-laden protein [4]. Experiments and theoreti-

cal results concerning facilitated diffusion of oxygen by myoglobin indicated that the bucket brigade mechanism is unimportant and that enhanced diffusion derives principally from translational diffusion of oxygen-laden myoglobin [5,6]. If this mechanism is also operating in the case of calcium and parvalbumin, then the flux will be given as

$$J_{lr} = (DC + D'[\mathrm{CaP}])/\Delta x \quad (12)$$

where D is the self-diffusion coefficient for calcium ($= 0.81 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1}), D' is the self-diffusion coefficient for the calcium-parvalbumin complex and [CaP] is the concentration of calcium bound to parvalbumin. Combining Eqns. 11 and 12, we have

$$D_aC = DC + D'[\mathrm{Cap}] \quad (13)$$

The concentration of calcium bound to parvalbumin is

$$[\mathrm{CaP}] = [\mathrm{P}]\nu \quad (14)$$

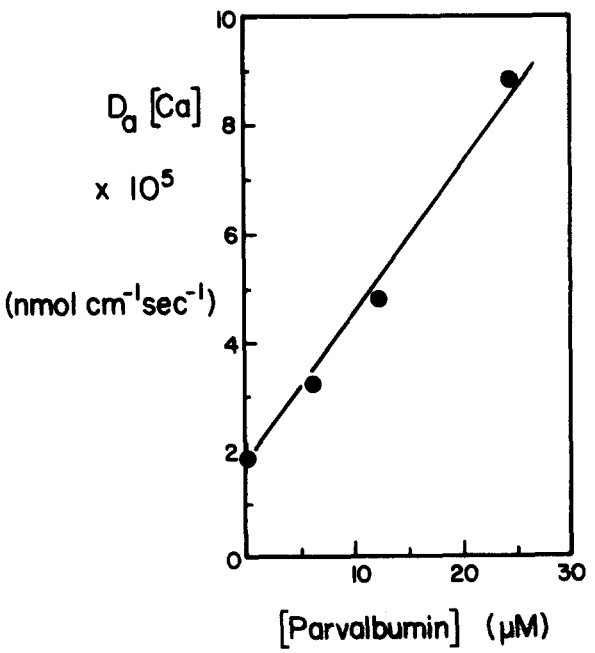

Fig. 7. Relation between D_a[Ca] and parvalbumin concentration. Parvalbumin concentrations were calculated assuming a molecular weight of 11500. The value of D_a[Ca] was obtained from the inverse of the slope of the lines shown in Fig. 6.

where [P] is the total concentration of parvalbumin and ν is the fractional saturation. Parvalbumin has two calcium-binding sites of high affinity [7,8] which would be saturated at the concentration of calcium, 2.3 μM, used in this study. The predicted value of $\nu = 2$ was confirmed by measuring the total protein and total calcium concentration in the middle compartment. The mean of three determinations of ν was 2.1. Thus we may take $\nu = 2$ and Eqn. 13 becomes

$$D_aC = DC + 2D'[\mathrm{P}] \quad (15)$$

The plot of D_aC against [P] gives an ordinate intercept of DC and a slope of $2D'$. Such a plot is shown in Fig. 7. The value of D' calculated from this plot was $13.7 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}.

Discussion

The results from this study show that the earlier flux analysis was incomplete because unstirred layers and the contribution of convection were ignored. Inclusion of 1% agar, however, appears to completely remove the effects of convection and does not significantly alter the self-diffusion coefficient of calcium, determined here to be $0.81 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1}. This value is within 5% of other published values [3].

Parvalbumin enhanced the calcium flux in proportion to the parvalbumin concentration. If it is assumed that translational diffusion of calcium-laden parvalbumin is the primary mechanism for enhanced calcium diffusion, then the proportionality constant relating parvalbumin and the calcium flux should be related to the self-diffusion coefficient of parvalbumin, D', as described in Eqn. 15. The calculated self-diffusion coefficient for parvalbumin was $13.7 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}. The diffusion coefficient for hake parvalbumin has been determined by ultracentrifugation to be $14.8 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1} at 20°C [9]. It is known, however, that agar reduces the diffusion of proteins and that the reduction depends upon the size of the protein [10]. This reduction in the diffusion coefficient disappears if correction is made for the volume occupied by the gel and its obstructive effects [11]. Such corrections were not made in this work; therefore, 1% agar should reduce the apparent

diffusion coefficient by 10–15% [10]. The expected value for D', then, would be about $13.3 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$. This compares well with the value of $D' = 13.7 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ calculated from the data of Fig. 7. Thus, the observed data are consistent with the hypothesis that parvalbumin enhances diffusion of calcium principally by translational diffusion of the laden protein and not by a 'bucket brigade' mechanism. These conclusions are in agreement with those concerning enhanced diffusion of oxygen by myoglobin [4–6].

From the above discussion and Eqn. 15, it should be clear that calcium diffusion is not enhanced when it is bound to parvalbumin. The self-diffusion coefficient for calcium is $0.81 \cdot 10^{-5}$ $cm^2 \cdot s^{-1}$, as determined by this work, whereas the self-diffusion coefficient for calcium bound to parvalbumin is about $0.27 \cdot 10^{-5}$ $cm^2 \cdot s^{-1}$, because two calcium atoms are bound per molecule of parvalbumin and the diffusion coefficient for parvalbumin is $0.137 \cdot 10^{-5}$ $cm^2 \cdot s^{-1}$. However, at a given free calcium concentration in equilibrium with parvalbumin, the total calcium flux is enhanced. This point may be made more clear by referring to Fig. 2. The initial calcium flux is depressed by parvalbumin as indicated by the rate of rise of ^{45}Ca concentration in the effluent. This is due to the fact that the free calcium concentration is reduced by parvalbumin, or the specific activity of ^{45}Ca has not yet reached steady state. When steady state is reached, the total calcium concentration consists of free calcium and calcium bound to parvalbumin. At nearly physiological calcium levels (2.3 μM in this case), the free calcium concentration may be small compared to the concentration of calcium bound to parvalbumin. Since the total calcium flux is due to diffusion of both free and bound calcium, the total flux is enhanced.

An important question is the physiological implications of the results reported herein. It is known that carp parvalbumin is one of a class of parvalbumins found in a variety of species including man [9,12–14]. They have a molecular weight of about 12000 and bind 2 mol calcium per mol protein with high affinity. There is a good correlation between the quantity of parvalbumin in a muscle fiber and its rate of relaxation [14,15]. Parvalbumin readily diffuses out of skinned muscle fibers [16], suggesting that it is a soluble cytoplasmic protein. These observations support the hypothesis that parvalbumin acts as a buffer and shuttle for calcium between the myofibrils and sarcoplasmic reticulum [17,18]. Computer simulations suggest that the kinetic constants for magnesium desorption and calcium sorption to parvalbumin are too slow for it to act as a shuttle for calcium [19], but another computer simulation study supports the argument that parvalbumin acts as a soluble relaxing factor [20]. Neither of these simulation studies have considered the spatial separation of components of the muscle and thus have provided somewhat incomplete models.

The vitamin D-dependent calcium-binding protein is another soluble protein which may facilitate calcium diffusion. Two general types of Ca^{2+}-binding protein have been identified. The avian type has a molecular weight of about 28000 [21,22] whereas the mammalian type has a molecular weight of 9000–10000 [21,23]. There is a high correlation between calcium absorption and Ca^{2+}-binding protein content in a variety of physiological conditions [24–26] and thus it has been inferred that Ca^{2+}-binding protein plays some role in calcium absorption. One hypothesis, based on the consensus that most of the Ca^{2+}-binding protein is located in the enterocyte's cytosol [27,28], is that Ca^{2+}-binding protein increases the flux of calcium through the cytosol [1,29]. In vivo concentrations of Ca^{2+}-binding protein reach 0.17 mM [1] which is seven times as high as the highest concentrations of parvalbumin used in the present study. Thus it is expected that Ca^{2+}-binding protein would markedly enhance diffusional flux of calcium through the enterocyte's cytosol.

Acknowledgements

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