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CALCIUM, CELLS AND VIRUS—ALTERATIONS CAUSED BY PARAMYXOVIRUSES

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Abstract—Washed Lettree cells contain ~ 12 fmoles Ca/cell. When cells are exposed to $^{45}Ca^{2+}$, ^{45}Ca rapidly becomes associated with the cells: the amount increases from ~ 15 amoles/cell at 0.01 mM external Ca^{2+} to ~ 10 fmoles/cell at 10 mM external Ca^{2+}. Approximately 50 per cent of this exchangeable Ca is on the outside of the cell surface; the remainder is within the cells; at least two internal compartments, each involving bound Ca, can be distinguished. Washing pre-labeled cells at 4° releases most of the surface-bound ^{45}Ca. Washing at 37° facilitates the release of internal ^{45}Ca. These and other data confirm the view that Ca^{2+} enters cells by passive diffusion, and that the amount of internal Ca (virtually all of which is bound) is regulated by an active efflux process. The characteristics of $^{54}Mn^{2+}$ uptake and release resemble those of $^{45}Ca^{2+}$. Competition experiments show that Mn^{2+} (but not Mg^{2+}) effectively "dilutes" $^{45}Ca^{2+}$ uptake; Ca^{2+} is less effective in "diluting" $^{54}Mn^{2+}$ uptake, since $^{54}Mn^{2+}$ appears to bind to Mg sites, as well as to Ca sites, in cells. These and other data show that Mn^{2+} is a potentially useful analogue of Ca^{2+} in biological studies. Electron paramagnetic resonance measurements show that ~ 40 per cent of Mn associated with cells is on the outside of the cell surface; the spectral characteristics of the surface-bound Mn and of internal Mn are quite different. It is concluded that the binding sites for Mn^{2+}, and hence for Ca^{2+}, that are on the surface of cells are relatively weak and nonspecific. The addition of Sendai virus has little effect on the binding of Ca^{2+} or Mn^{2+} to the cell surface. Uptake into cells and efflux from cells are accelerated; the internal distribution of "recently acquired" Ca^{2+} or Mn^{2+} is different from that in the absence of virus. None of these effects is observed with "1-day" Sendai virus (which fuses with the cell surface, but does not cause permeability changes). It is concluded that altered Ca movements are a consequence, and not a cause, of the fusion of the viral envelope with the cell surface.

While Arnold Welch was at Yale, he initiated several investigations on the mechanisms of action of potential anticancer drugs. The fruitfulness of approaching the problem through the use of different mammalian systems, including one of the earliest uses of cultured cells [1], was 2-fold. Not only were some interesting drugs, chiefly nucleotide analogues [2], developed, but the study of their properties led to a better understanding of the basic pathways of nucleotide metabolism in mammals. For example, the investigation of the mechanism of action of azauridine [3], on which one of the present authors was privileged to work [4], led to a clarification of the properties of orotidylate decarboxylase [5], a key enzyme in pyrimidine biosynthesis. In short, the study of an abnormal situation is often accompanied by clarification of a normal process. The work that follows is another, modest example of such a development. It concerns the movement of Ca^{2+} into and out of cells, a subject that has become recently [6–9] best described by the words Arnold Welch helped to coin: Biochemical Pharmacology.

Our reasons for studying the effects of viruses on Ca^{2+} movements were 3-fold. First, paramyxoviruses, such as Sendai, cause a general increase in permeability of the plasma membrane to low molecular weight molecules [10, 11] and ions [12–14]. Second, external Ca^{2+} prevents the changes [13, 15]. Third, the envelope of paramyxoviruses is known to fuse with cells [16], and membrane fusion is a process said to involve Ca^{2+} [17].

The literature on Ca^{2+} uptake is extensive, and embraces roughly two approaches: a study of Ca^{2+} movement in intact cells such as HeLa [18, 19] or Ehrlich ascites cells [20–23], and a study of Ca^{2+} uptake and binding by isolated organelles such as mitochondria [24–27], sarcoplasmic reticulum of muscle [28] or the plasma membrane of red cells [29–31]. Our first aim was to study the characteristics of both movement and binding in the same system, namely Lettree cells. Next, we investigated the movement of Mn^{2+}, a potentially useful analogue of Ca^{2+} [32], the binding of which can be studied rather precisely by electron paramagnetic resonance (e.p.r.) techniques. Finally, the effects of Sendai virus on movement and binding of Ca^{2+} and Mn^{2+} were studied.

EXPERIMENTAL

Lettree cells were grown ascitically in MF1 mice by continuous passage. The cells were washed three times in 150 mM NaCl containing 5 mM *N*-2-hydroxyethyl piperazine *N'*-2-ethane sulfonic acid adjusted to pH 7.2 with sodium hydroxide (HEPES buffer) to remove peritoneal fluid, and were resuspended at 1–5 × 10^7/ml, as determined by a Coulter counter. $^{45}CaCl_2$ (28 Ci/g) and $^{54}MnCl_2$ (> 100 Ci/g) were obtained from the Radiochemical Centre, Amersham. Sendai virus was harvested after growth in embryonated eggs for 3 days (normal virus) [10, 13] or for 1 day ("1-day" virus) [33]; HAV = haemagglutination unit.

Uptake into cells was measured either by overlaying a 0.2 ml incubation mixture on 0.5 ml of a 2:1 mixture of di-*n*-butyl phthalate–dinonyl phthalate [34] and centrifuging for 5 sec at 95,000 *g* in a Beckman microfuge (method A), or by diluting a 0.2 ml incubation mixture with 10 ml of ice-cold HEPES buffer, centrifuging for 5 min at 500 *g*, removing the supernatant fraction and

washing a further two times in 10 ml of ice-cold HEPES buffer (method B); at each stage, cells were in contact with the washing medium for > 5 min.

The efficiency of removing free $^{45}Ca^{2+}$, which had been carried over with any medium trapped between cells, when centrifuging through oil (method A), was assessed using [^{3}H]inulin (425 mCi/m-mole; Radiochemical Centre). The amount of trapped inulin, which was proportional to cell number, was equivalant to 1.7 μl/10^7 cells. This value, which corresponds to < 5 per cent of ^{45}Ca associated with cells at the highest concentration used, has been subtracted in every case.

Release from cells was measured as described previously [10, 13] or by a continuous flow technique as follows. The incubation mixture was placed on a GF/D Whatman filter in a Swinnex filter holder (millipore) and HEPES buffer was pumped through the filter at 1 ml/min at 37°; the effluent was sampled for radioactivity.

Total Ca was measured [13] by washing cells in HEPES buffer once and digesting the cell pellet with 70% perchloric acid at 150° for 10 hr. The digest was diluted in $LaCl_3$ to give a solution containing 0.1% $LaCl_3$. Ca was measured on a Perkin Elmer 107 Atomic Absorption Spectrophotometer using an acetylene flame.

Radioactivity was measured as described previously [10] or in a 2:1 mixture of toluene–methoxyethanol containing 6 g/liter of 2,5-diphenyloxazole and 150 mg/liter of 1,4-bis[2-(5-phenyloxazolyl)]benzene.

RESULTS AND DISCUSSION

Binding and movement of Ca^{2+}. The uptake of Ca^{2+} has been measured by exposing cells to $^{45}Ca^{2+}$ and removing unbound $^{45}Ca^{2+}$ by (A) spinning through oil or (B) washing with ice-cold isotonic buffer. Method A yields values that are some 10-fold higher than method B, as shown in Fig. 1. It has to be emphasized that experiment to experiment variation, especially for method B, is quite high. Not only does the uptake of Ca^{2+} by a particular batch of cells vary during the course of the day, but there is much variation between different batches of cells, which is probably due to the number of times a cell suspension has been passaged in mice; other properties of the Lettree cell surface, such as the activity of 5′-nucleotidase and the ease of swelling and disrupting cells, are also affected by repeated passage (J. M. Graham, unpublished experiments). Another potential variable in experiments involving uptake of $^{45}Ca^{2+}$ is the buffer used; all experiments reported in this paper were carried out in the presence of HEPES buffer, which has negligible affinity for Ca^{2+} [35]. Tricine buffer, on the other hand, chelates Ca^{2+} rather well [35], and uptake of $^{45}Ca^{2+}$ is reduced approximately 20-fold by the presence of tricine.

Centrifugation through oil (method A) retains all bound ^{45}Ca, since the values obtained are similar to those obtained by measuring the difference between ^{45}Ca in the medium before and after cells have been spun through it (15 amoles/cell at 0.01 mM; 11 fmoles/cell at 10 mM external Ca^{2+}). Washing cells in buffer (method B) causes most of the surface-bound Ca to be removed; in addition, some intracellular Ca is also removed. The remainder (Ca_B) is almost entirely intracellular. This is shown by the following observations. First, ^{45}Ca remaining after washing is, by definition, bound to high affinity sites: during the three washes, the incubation medium becomes diluted > 10^6-fold. This suggests either that Ca_B is bound to sites having dissociation constants < 0.01 mM, or that Ca_B is separated from the suspending medium by a barrier, namely the plasma membrane. As the internal concentration of ionized Ca^{2+} is very low (< 10^{-8} M [36]), the interior of the cell clearly possesses high-affinity binding sites. Second, a large proportion of $^{45}Ca_A$ shows rapid uptake at 0° (Fig. 2). Such Ca does not appear to be restricted by a permeability barrier. Moreover, isolated plasma membranes bind $^{45}Ca^{2+}$ only weakly, and show no temperature effects (C. C. Impraim, unpublished experiments). Third, the weakly bound, labile component behaves like mobile, cell-associated Mn^{2+} (Mn_b^*, Fig. 3), which is likely for additional reasons to be entirely on the cell surface; Mn_b^* represents approximately 40 per cent of total exchangeable ^{54}Mn (see below). Fourth, Ca_B is released in a temperature-dependent manner [37]. The amount of Ca_B is optimal under conditions where passive influx is maximal and active efflux is minimal (C. C. Impraim, unpublished experiments), indicating that Ca_B is restricted by a permeability barrier, namely the plasma membrane.

The values for Ca_A and Ca_B shown in Fig. 1 were obtained after incubating for 20–30 min at 37°. That these represent "equilibrium" values is shown by the

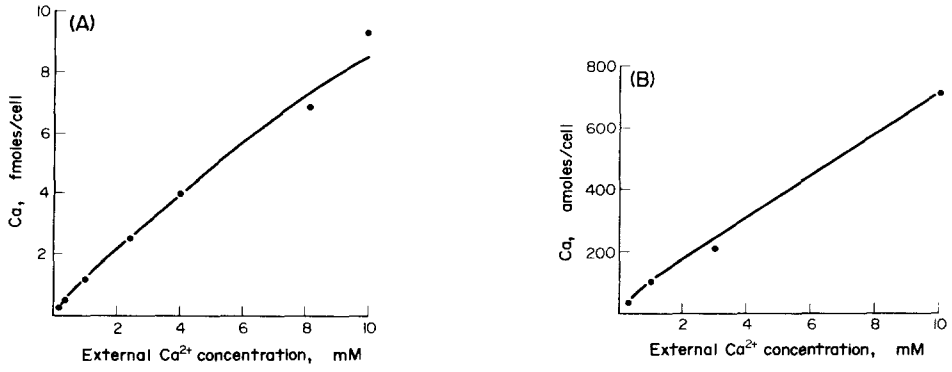

Fig. 1. Concentration dependence of $^{45}Ca^{2+}$ uptake. Cells were incubated at 37° for 30 min in the presence of $^{45}Ca^{2+}$ at the concentration indicated. Panel A: treatment by method A; panel B: treatment by method B (see Experimental section).

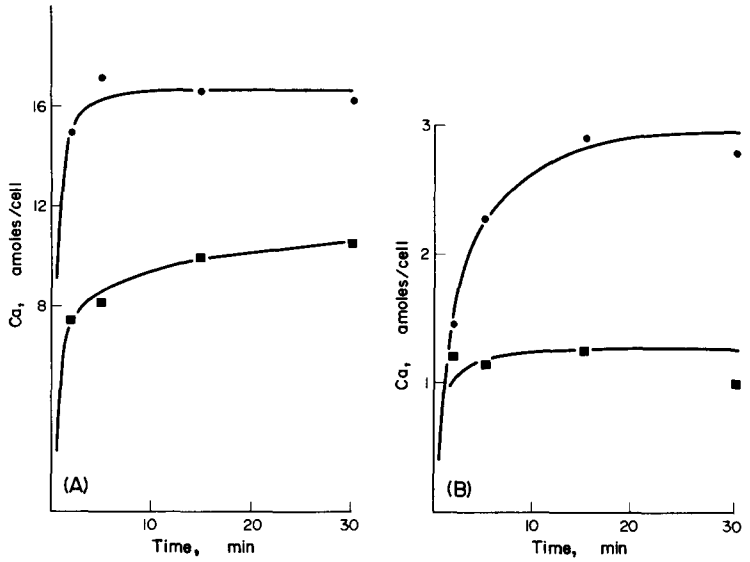

Fig. 2. Time course of $^{45}Ca^{2+}$ uptake. Cells were incubated with $^{45}Ca^{2+}$ (0.01 mM) at 4° (■——■) or 37° (●——●) for the times indicated and washed by method A (panel A) or method B (panel B).

time course depicted in Fig. 2. That is, binding of Ca_A and Ca_B is relatively rapid. The amount of ^{45}Ca associated with cells (Ca_B) at 10 mM external $^{45}Ca^{2+}$ is approximately 1 fmole/cell. Since the total Ca content of washed cells, measured by atomic absorption, is approximately 12 fmoles/cell, the "equilibrium" value indicates that some 8 per cent of cellular Ca is readily exchangeable (assuming that all uptake figures represent an exchange, which seems reasonable). Although this figure is an underestimate, since some internal ^{45}Ca is removed by washing at 0°, it is in the same range as the values quoted for "rapidly exchangeable" Ca in HeLa [18, 19], Ehrlich ascites [20, 21] and liver [38] cells. In order to exchange more than this amount, cells have to be exposed to $^{45}Ca^{2+}$ for much longer periods. As mentioned above, most of the Ca that is present in cells is bound: estimates [36] put the amount of free Ca^{2+} at < 0.01 amole/cell (i.e. $< 10^{-8}$ M). Electron paramagnetic resonance measurements of Mn^{2+}-treated cells do not reveal any free internal Mn^{2+} either (see below). It may be assumed, from other studies [18, 19, 38], that "non-exchangeable" or "slowly exchangeable" Ca is largely mitochondrial or other type of bound or sequestered Ca. "Rapidly exchangeable" Ca probably represents Ca that is bound to sites such as the inner side of the plasma membrane and associated cytoskeletal elements.

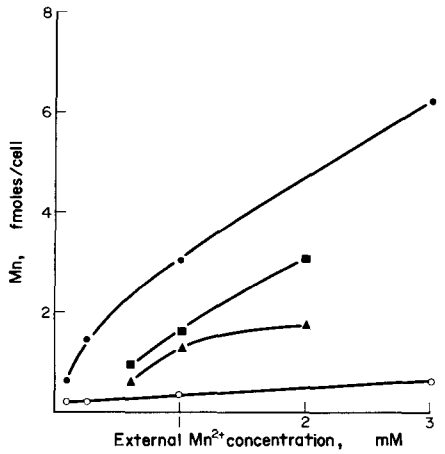

Fig. 3. Concentration dependence of Mn^{2+} uptake. Cells were incubated at 37° for 30 min in the presence of $^{54}Mn^{2+}$ at the concentrations indicated and treated by method A (●——●) or method B (○——○). Values for Mn_b (■——■) and Mn_b* (▲——▲) (taken from D. Getz, J. Gibson, R. Sheppard, K. J. Micklem and C. A. Pasternak, manuscript in preparation), are included for comparison. Mn_b refers to "immobile", largely intracellular Mn; Mn_b* refers to "mobile", loosely bound, entirely extracellular Mn.

The release of $^{45}Ca_B$ from cells is temperature dependent. This was shown in a previous report [37] in which ^{45}Ca-labeled cells were prepared by method B. (In that report we incorrectly interpreted $^{45}Ca_B$ as being on the outside of the cell surface; we now believe it to be internal, though much of it may indeed be bound to the inside of the plasma membrane.)

Binding and movement of Mn^{2+}. The uptake of $^{54}Mn^{2+}$ has been compared with that of $^{45}Ca^{2+}$. The relative amounts of ^{54}Mn associated with cells after spinning through oil (method A) and after washing in buffer (method B) are similar to those for ^{45}Ca (Fig. 3). That Mn indeed binds to the same sites as Ca is shown by competition experiments: unlabeled Mn^{2+} competes with $^{45}Ca^{2+}$ as effectively as Ca^{2+} itself dilutes out $^{45}Ca^{2+}$; Sr^{2+} behaves like Mn^{2+}, whereas Mg^{2+} hardly competes at all (Fig. 4, panel A). Competition experiments between $^{54}Mn^{2+}$ and unlabeled cations show that Mn^{2+} binds not only to Ca sites, but to some Mg sites as well. Thus, (a) the total amount of $^{54}Mn^{2+}$ bound is greater that that of $^{45}Ca^{2+}$ bound, and (b) the binding of $^{54}Mn^{2+}$ is reduced by Ca^{2+}, Sr^{2+} or Mg^{2+} (Fig. 4, panel B). Note that once inside cells, isotope (i.e. $^{45}Ca_B$ or $^{54}Mn_B$) is displaced only slightly by added "cold" Ca^{2+}

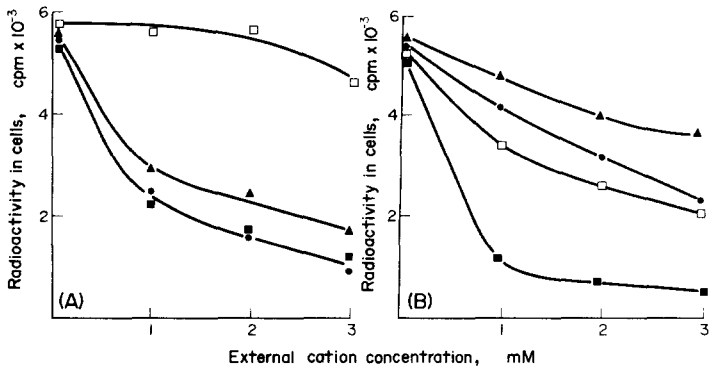

Fig. 4. Effect of unlabeled cations on uptake of $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$. Cells were incubated at 37° for 30 min in the presence of 10 μM $^{45}Ca^{2+}$ (panel A) or 10 μM $^{54}Mn^{2+}$ (panel B) in the presence of unlabeled Mg^{2+} (□——□), Ca^{2+} (●——●), Sr^{2+} (▲——▲) or Mn^{2+} (■——■) at the concentrations indicated and treated by method A. The uptake of $^{45}Ca^{2+}$ in the absence of added unlabeled cations corresponds to 5.2 amoles/cell; the uptake of $^{54}Mn^{2+}$ in the absence of added unlabeled cations corresponds to 7.2 amoles/cell.

or Mn^{2+}. In other words, "exchange" does not result in release of internally bound cation into the medium; rather the cation is displaced to other sites within the cell, from which (after dilution with endogenous cation) it leaks, or is pumped, out.

Removal of ^{54}Mn from cells resembles that of ^{45}Ca. Release of predominantly surface-bound Mn, by washing at 4°, and release of internal Mn, by incubating 4°-washed cells at 37°, are shown in Fig. 5. Mn appears to be somewhat more tightly associated with cells than Ca; this could be due either to the presence of some additional binding sites in cells (see above) or to a decreased affinity of Mn^{2+} for the Ca^{2+} exit mechanism.

A characteristic feature of Ca^{2+} is its inhibition of virally mediated permeability changes [13, 15]. Table 1 shows that Mn^{2+} is as good an inhibitor as Ca^{2+}; the transition metals such as Tb^{3+} or La^{3+}, which are known to have Ca^{2+}-like properties [39], act similarly. The point to note, in terms of biologically important cations, is that in this system Mn^{2+} resembles Ca^{2+}, not Mg^{2+} (Table 1).

Mn^{2+} is thus a potential analogue of Ca^{2+}. Its usefulness is 2-fold. First, it gives a characteristic e.p.r. spectrum. Second, it affects the n.m.r. spectrum of

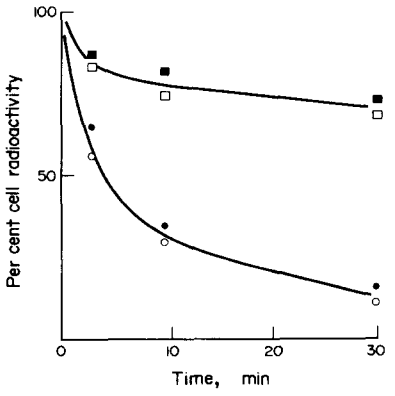

Fig. 5. Temperature-dependence of ^{45}Ca and ^{54}Mn release. Cells were incubated with $^{45}Ca^{2+}$ (open symbols) or ^{54}Mn (closed symbols) (each at 10 μM) for 30 min at 37°, washed by method B, and reincubated at 4° (squares) or 37° (circles) for the times indicated.

Table 1. Effect of cations on virally mediated permeability changes *

Cation	Concn required to inhibit leakage by 50% (mM)
Mg^{2+}	>5
Ca^{2+}	0.1
Sr^{2+}	0.5
Mn^{2+}	0.1
La^{3+}	0.03
Tb^{3+}	0.03

* Cells were incubated with [^{3}H]choline, washed, reincubated with or without Sendai virus and leakage, in the presence of the chlorides of the cations indicated at different concentrations, was assessed as described previously [10, 13].

protons in water [40]; by carrying out proton-n.m.r. measurements in the presence of Mn^{2+}, information concerning the location of Mn may be obtained. In collaboration with others (D. Getz, J. Gibson, R. Sheppard, K. J. Micklem and C. A. Pasternak, manuscript in preparation), we have been able to distinguish two types of Mn that become associated with Lettree cells: "mobile" or loosely bound Mn, that is predominantly on the outer surface of cells (Mn_b*) and "immobile", more tightly bound Mn, much of which is within cells (Mn_b). Note that Mn_b* plus Mn_b is approximately equal to total exchangeable Mn($^{54}Mn_A$).

Effect of virus on binding and movement of Ca^{2+} *and* Mn^{2+}. In a previous report [37], we showed that Sendai virus increases the "exchangeability" of cell-associated ^{45}Ca. The experiments to be described confirm and extend this finding with regard to $^{45}Ca^{2+}$, to $^{54}Mn^{2+}$ and to Mn^{2+} measured by e.p.r. and n.m.r. of protons. Figure 6 shows that the uptake of $^{54}Mn^{2+}$, like that of $^{45}Ca^{2+}$ [14, 41], into washed cells is increased by virus. The effect of virus is stabilized if cells are chilled (Table 2); this is compatible with the supposition that entry of $^{45}Ca^{2+}$—whether in the absence or presence of virus—is largely through passive "pores". At 37°, especially in the presence of glucose, the effects of virus are quite transient (Fig. 7); the release of ^{45}Ca follows the same

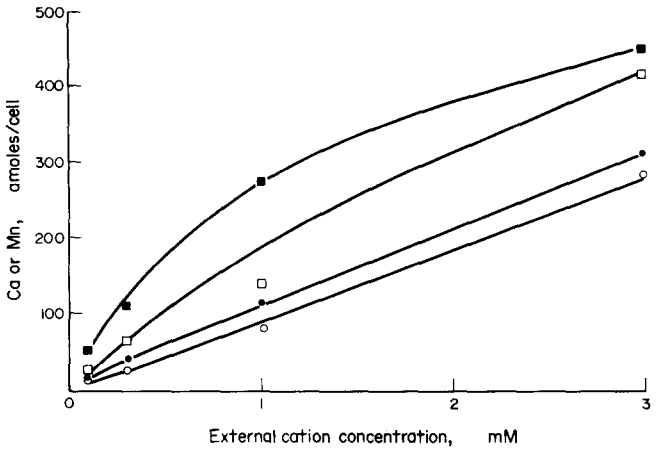

Fig. 6. Effect of virus on uptake of $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$. Cells were incubated with $^{45}Ca^{2+}$ (open symbols) or $^{54}Mn^{2+}$ (closed symbols) at the concentrations indicated at 37° with (squares) or without (circles) virus (200 HAU/ml) and sampled by method B.

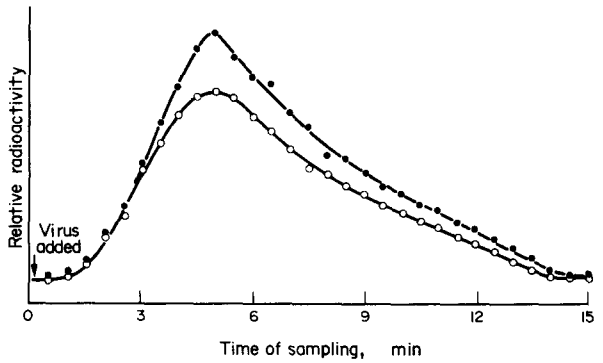

Fig. 7. Duration of virally mediated permeability changes. Cells were incubated with $^{45}Ca^{2+}$ (0.01 mM) and [^{3}H]choline (8 μM) for 20 min at 37° and release of isotope was measured by the continuous flow technique, as described in the Experimental section. After 15 min, the washing medium was replaced by medium containing virus (300 HAU/ml); 3 min later the original washing medium was restored. ^{3}H (closed symbols) and ^{45}Ca (open symbols) in the effluent fractions, collected at 30-sec intervals, are shown. Viral treatment did not deplete cells of ^{3}H or ^{45}Ca (approximately 30 per cent of cellular isotope released in each case), since further addition of virus was able to stimulate further release of isotope.

Table 2. Effect of pretreatment on viral stimulation of $^{45}Ca^{2+}$ uptake*

Pretreatment	Amount of $^{45}Ca_B$ taken up (amoles/cell)
Control; 37° for 3 min	0.14
for 20 min	0.06
4° for 3 min	0.14
for 20 min	0.16
Virus; 37° for 3 min	1.6
for 20 min	0.67
4° for 3 min	2.0
for 20 min	9.6

* Cells were incubated with or without Sendai virus (1000 HAU/ml) for 5 min at 37°, chilled to 4° and either kept chilled, or raised to 37°, for the times indicated ("pretreatment"). Both sets of cells were then chilled to 4°, exposed to $^{45}Ca^{2+}$ for 5 min and washed by method B.

time course as the permeability change (measured release of ^{3}H from cells pre-labeled with [^{3}H]choline [10, 13]). It has been reported previously that viral antigens diffuse away from the site of fusion more rapidly at 37° than at 4° [42]. The present results (Table 2) confirm observations [15, 43] that the integrity of the plasma membrane is repaired rapidly by metabolically favorable conditions.

The increased amount of ^{45}Ca associated with cells as the result of viral treatment is small in comparison with the total amount of Ca measured by atomic absorption (Table 3); in any case, virus causes a slight though significant (Fig. 8 and Ref. 37) release of ^{45}Ca, and ^{54}Mn, from cells. This is in contrast to cellular K^+ [12, 13] and Na^+ [14] which are affected markedly by virus, to the point of causing cells to take up water and swell [44].

Nevertheless, the virally mediated influx of Ca^{2+} does

Table 3. Ca content of Lettree cells*

Treatment	Ca content (fmoles/cell)
Control; buffer	13
Control; 10 mM Ca^{2+}	11
Virus; buffer	12
Virus; 10 mM Ca^{2+}	12

* Cells (2.5×10^7/mole) were incubated with or without Sendai virus (400 HAU/ml) in HEPES buffer with or without Ca^{2+} for 20 min at 37°, washed and analyzed by atomic absorption, as described in the Experimental section.

appear to alter the distribution of intracellular Ca. Thus, the amount of unbound Ca^{2+} is transiently increased (unpublished experiments of G. Poste, cited in Ref. 14) and this, or another reason, leads to an increased association of Ca with the "non-rapidly exchangeable compartment" of Ca. This is shown by the fact that $^{45}Ca^{2+}$ or $^{54}Mn^{2+}$ taken up by cells in the presence of virus is less readily released by incubation

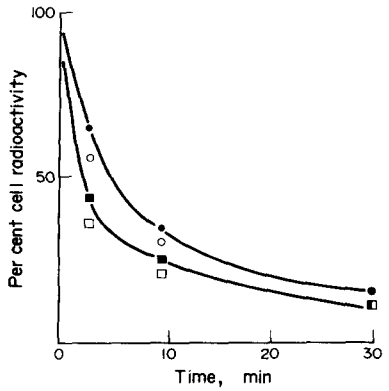

Fig. 8. Effect of virus on release of ^{45}Ca and ^{54}Mn. Cells were incubated with $^{45}Ca^{2+}$ (open symbols) or $^{54}Mn^{2+}$ (closed symbols) (each at 0.01 mM) for 30 min at 37°, washed by method B, and reincubated with (squares) or without (circles) virus (400 HAU/ml) at 37° for the times indicated.

Table 4. Effect of virus on nature of $^{45}Ca_B$*

Treatment	Amount of Ca_B released (% of total) 3 min	7 min	15 min	30 min
In vitro uptake	60	73	70	83
In vitro uptake plus virus				57
In vivo uptake	20	27	49	

* For *in vitro* uptake, cells were incubated with $^{45}Ca^{2+}$ (0.01 mM) for 30 min with or without Sendai virus (1000 HAU/ml); for *in vivo* uptake, mice bearing Lettree cells were injected with $^{45}Ca^{2+}$ and cells isolated 6 hr later, as described in the Experimental section. Cells were washed by method B and reincubated in HEPES buffer at 37° for the times indicated.

at 37° than is isotope taken up in the absence of virus; the similarity between $^{45}Ca^{2+}$ taken up in the presence of virus and $^{45}Ca^{2+}$ taken up by the cells that have been exposed to the isotope for 6 hr is to be noted (Table 4). In other words, virus causes $^{45}Ca^{2+}$ or $^{54}Mn^{2+}$ to reach sites that are not normally labeled by brief exposure to extracellular isotope. Nuclear magnetic resonance experiments confirm this conclusion. In the absence of virus, cells exposed to Mn^{2+} display two T_1 (spin-lattice relaxation) values; in the presence of virus, a third T_1 value is obtained (D. Getz, J. Gibson, R. Sheppard, K. J. Micklem and C. A. Pasternak, manuscript in preparation). Since the third T_1 value is not caused by virus alone, it is clear that virus makes Mn^{2+} become associated with a component to which it is not accessible, during brief incubation, in the absence of virus. Whether this component is mitochondrial [22–27], nuclear or another organelle such as endoplasmic reticulum [45], remains to be established; using procedures designed to stimulate mitochondrial binding of Ca^{2+} [22, 23], we have shown an enhanced uptake of $^{45}Ca^{2+}$ by virally-treated Lettree cells. What is clear is that the use of Mn^{2+} coupled with proton n.m.r. provides a new approach for studying the influx of Ca^{2+} into cells; it is currently being used to clarify the effects of hormones [46, 47] on Ca^{2+} movements.

Table 5. Effect of virus on ^{45}Ca movements*

Uptake	External Ca^{2+} concn (mM)	Amount of $^{45}Ca_B$ taken up (amoles/cell) Control	One-day virus	Three-day virus
	0.1	10	8	41
	1.0	29	33	63
	3.0	48	62	140
Release	**Time of incubation (min)**	**Amount of $^{45}Ca_B$ released (% of total)**		
	3	36	37	48
	8	41	45	52

* For measurement of uptake, cells were incubated with $^{45}Ca^{2+}$ at the concentrations indicated for 30 min at 37°, and cells were washed in ice-cold HEPES buffer (method B). For measurement of release, cells were incubated with $^{45}Ca^{2+}$ (0.01 mM) at 37° for 30 min, washed by method B, and reincubated at 37° for the times indicated.

The addition of Sendai virus probably causes little change in surface-bound Ca. This is difficult to establish by the washing procedure, since virus makes cells leaky, and therefore affects the washing procedure. But isolated plasma membranes show no significant change in binding or release of ^{45}Ca. Moreover, the amount of Mn_b*, i.e. predominantly surface-bound Mn, is not increased by virus. Therefore our previous conclusion that virus increases the exchangeability of ^{45}Ca bound to the outside of the cell surface [33], has to be modified: it is the exchangeability of internal ^{45}Ca (some of which is undoubtedly bound to the inside of the cell surface) that is increased by virus. In short, the effects of virus are primarily to increase the permeability of the surface membrane.

Several authors (reviewed in Ref. 48; see also Ref. 41) have suggested that an increased influx of Ca^{2+} may be involved in the process of membrane fusion. In order to test this hypothesis, we have studied $^{45}Ca^{2+}$ movements in the presence of "1-day" Sendai virus. Sendai virus is normally harvested after 3 days; if harvested after only 1 day, it differs in several of its properties. It is non-haemolytic [33], presumably because it does not cause permeability changes [49], but it is infective [33] and its envelope undergoes fusion with the plasma membrane of cells to which it becomes attached [50]. One-day virus causes no change in influx or efflux of $^{45}Ca^{2+}$ (Table 5). Therefore, it is clear that any virally mediated increases in Ca^{2+} movement that occur are a consequence, and not a cause, of membrane fusion. Moreover, an increase in intracellular Ca^{2+} is unlikely to be necessary for the formation of cell-cell polykaryons caused by 3-day virus (S. Knutton, unpublished experiments).

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