Resolution of Intracellular Calcium Metabolism in Intact Segments of Rabbit Aorta

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SUMMARY A new method, based on computer-assisted kinetic analysis of \(^{45}\)Ca efflux data, was used to measure calcium contents and fluxes for extracellular and intracellular compartments in intact segments of rabbit aorta. After a 1-hour loading period, efflux data were collected for 8 hours using a flow-through tissue chamber. These long-term effluxes were necessary because information on intracellular calcium metabolism was concentrated in the slow components of the efflux curves while earlier components appeared to be dominated by washout of extracellular calcium. Intracellular compartments were identified as those whose calcium contents were altered by 10 \(\mu\)M phenylephrine. This method complements previous approaches by providing simultaneous estimates of compartmental calcium contents and fluxes without requiring the assumption of isotopic equilibrium and without recourse to standard wash techniques for removal of extracellular calcium. In normal, calcium-containing, bicarbonate-buffered physiological salt solution these compartments contained a total of approximately 300 nmol Ca/g wet aorta. Of this total, 55 nmol/g were associated with the slowest resolvable compartment whose turnover time was 170 minutes and whose exchange flux was 0.32 nmol min\(^{-1}\)g\(^{-1}\). Two other intracellular compartments had turnover times of 30 minutes. One of these was phenylephrine releasable and contained 145 nmol/g; it exchanged calcium at 4.9 nmol min\(^{-1}\)g\(^{-1}\). In normal physiological salt solution the plasma membrane was, surprisingly, not rate limiting for Ca efflux; and in 10 \(\mu\)M phenylephrine the membrane Ca flux was even greater, increasing 3.5-fold compared to control. (Circulation Research 1986;59:74-84)

KEY WORDS • computer model • tracer kinetics • organellar calcium • smooth muscle

IONIZED calcium concentration, an important intracellular signal, is determined by the net effect of all processes that deliver calcium to the cytosol or remove calcium from it. For vascular smooth muscle cells, many such processes have been identified. These include Ca influx across the plasma membrane, extrusion by a plasma membrane Ca ATPase, binding to and release from intracellular macromolecules, as well as uptake and release of Ca by sarcoplasmic reticulum and mitochondria. In vascular muscle, these sources and sinks and the pathways that link them are of interest because they modulate active stress development and because of the potential for Ca control of other biochemical events.

Once the sources and sinks have been identified, it is useful to obtain measures of their Ca contents and their exchange fluxes. Such measurements are required if we wish to know the relative importance of each process in controlling cytosolic Ca or if we wish to know their potential for influencing vascular tone in pathophysiological situations. Furthermore, when these measurements are obtained for various physiological circumstances, they also provide information on the mechanisms of action of vasoactive substances by identifying the altered Ca handling processes.

A major obstacle to accurate estimation of these Ca contents and fluxes has been the predominance of extracellular over intracellular Ca; extracellular Ca accounts for 90–95% of total arterial wall Ca. The problem is that small changes in intracellular Ca are difficult to detect above the extracellular background. For this reason, experimental techniques have been developed to separate cellular from extracellular Ca; these include the EGTA wash, low Ca, lanthanum wash, and the cold Ca EGTA "quench". Another approach that may be classified as cell fractionation relies on isolation of membrane fractions such as sarcoplasmic reticulum and plasma-lemma or organelles such as mitochondria. The identity and homogeneity of these fractions are established by marker enzymes or microscopy, and vesicular Ca uptake can then be examined without interference from extracellular Ca. A useful check on these techniques can be obtained using electron probe x-ray analysis although this method is best suited for static rather than dynamic measurements.

In this paper we describe an alternative method for resolving intracellular Ca contents and fluxes despite the large background contributed by extracellular calcium. This method is based on tracer kinetics and is carried out using intact arterial segments in normal, bicarbonate-buffered, calcium-containing physiological salt solution (PSS). It complements previous approaches by providing simultaneous estimates of Ca contents and fluxes without recourse to wash or quench techniques for removing extracellular Ca. We applied this method to rabbit thoracic aorta and found, within the limitations of our method, that 1) intracellular Ca...
could be resolved because it turned over more slowly than extracellular Ca and was increased by contractile agonists. 2) the plasma membrane was not rate limiting for Ca efflux, 3) extracellular Ca was bound at two kinetic sites, 4) total Ca content of the resolved compartments, in 1.5 mM Ca, was 4.13 mol/g wet weight, of which 93% was extracellular, 5) simultaneously estimated compartmental Ca contents compared favorably with individual estimates made using other techniques, and 6) plasma membrane calcium flux increased 3.5-fold when the vessel was exposed to 10 µM phenylephrine.

Methods

Procedure

New Zealand white rabbits (2-3 kg) of either sex were obtained from a single source, anesthetized with sodium pentobarbital (52 mg/kg IV), and bled. The upper part of the descending thoracic aorta was excised and quickly transferred to PSS of the following composition (millimolar); 116.9 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 28.0 NaHCO3, 5.5 dextrose, 1.5 CaCl2, 0.026 CaNa2EDTA bubbled with 95% O2-5% CO2 at 37°C. Residual blood inside the lumen of the vessel was gently washed out with PSS using a Pasteur pipette. The vessel was then transferred to a dissection dish containing aerated PSS and maintained at 33°-35°C. Gross adipose tissue, connective tissue, and adventitia were removed under a dissecting microscope with minimal pulling on the vessel. The vessel was finally trimmed to a length of about 2 cm and placed in 100 ml PSS bubbled with O2-CO2 at 37°C for a 1-hour equilibration.

After the equilibration period the vessel was loaded with 45Ca in a vial containing 45Ca (approximately 5 x 107 cpm/ml), 4 ml PSS, and other chemicals depending on the experiment. After loading the vessel for 1 hour it was quickly rinsed in PSS and transferred to a flow-through chamber perfused with PSS of the same composition as the loading solution except that 45Ca was omitted. The chamber was fabricated by cutting a rectangular slot (4 cm long x 1 cm wide x 2 cm high) in one surface of a plexiglass block. Solutions were pumped in and out through two holes drilled through opposite long walls, near the bottom and near opposite ends of the chamber. The vessel was mounted on two stainless steel bars passed through its lumen so that isotopic tension could be monitored with a Grass FT.03 transducer and a Grass 7D polygraph. The volume in the chamber was 6.3 ml and was perfused at a constant flow of 7 ml/min. The PSS in the chamber was maintained at 37°C and was mixed continuously by a miniature magnetic stirrer (1.5 mm diameter, 5 mm long). Approximately 1 ml/min of the effluent was diverted to a fraction collector; the exact flow diversion and the total perfusate flow were recorded for each experiment. Fractions were collected in 2-minute intervals over the entire 8-hour efflux period. Effluent samples were collected in 7-ml polyethylene mini-vials, and 3 ml of scintillation fluid (Hydro-solv #2, Anoroc) were added to appropriately spaced vials, which were then counted in a Beckman LS 7500 liquid scintillation counter. We refer to this protocol, in which equilibration, loading, and efflux solutions all have the same chemical composition, as the steady state protocol. In some experiments we employed a "step" protocol in which chemical composition of the equilibration and loading solutions was continued for only the first 60 minutes of efflux. The efflux medium was then altered for the remainder of the efflux.

After the washout the vessel was removed from the chamber, cut open, blotted, and weighed. Radioactivity in each efflux vial was normalized for two factors before plotting: 1) the activity of the 45Ca loading solution, which decreases between experiments because of radioactive decay, and 2) the blotted wet weight of the vessel segment, which varied (range: 38-86 mg) from experiment to experiment. Chemicals used: 1-phenylephrine hydrochloride (Sigma), 45CaCl2 (New England Nuclear, ICN); all salts were reagent grade.

Characteristics of the Preparation

1) It was desirable to use thin preparations so as to minimize diffusion distances for oxygen, agonists, and ions. We chose 2-3 kg rabbits so that the half-thickness of the thoracic aorta calculated from circumference, length, weight (65.4 ± 4.5 (SEM) mg, n = 16), and density of the wall of the aorta was 178 ± 6.0 (SEM) µm. 2) Large, 2 cm long, segments were used so that even small Ca compartments contained detectable 45Ca after a 1-hour load. Large segments also minimize "edge effects" because only a small fraction of the tissue is damaged in dissection. 3) We use a high-specific-activity loading solution (compared to other investigators) to further improve the resolution of small Ca compartments. 4) Perfusate pH was maintained at 7.4 by vigorously bubbling the reservoir with 5% CO2 (95% O2). We paid attention to this because the pH sensitivity of smooth muscle is well known even though Ca kinetics are reportedly insensitive to pH changes between 6.5 and 8.0. 5) Hematoxylin and eosin stained sections of our freshly dissected aorta show virtually no adventitial layer, and smooth muscle is the predominant cell type. 6) When the vessel was mounted for efflux, initial tension was adjusted to 20 g, which decreased to 14-16 g after stress relaxation. This initial stretch was found, in preliminary experiments, to correspond to approximately 70% of optimal length. 7) Cross-sectional area of the unstretched vessel wall was 0.0603 ± 0.0038 (SEM) cm². This number, uncorrected for extracellular space, was used to calculate developed stress, yielding 9770 ± 850 (SEM) N/m² (basal) and 48100 ± 6690 (SEM) N/m² (10 µM phenylephrine). Because both walls of the segment exert force, twice the calculated area was used in stress calculations.

Controls

1) A 1-hour equilibration appeared to achieve steady state since efflux curves obtained after 3 hours of equilibration were superimposable on those obtained with a 1-hour equilibration. This is a strong indicator
of stationarity; if a steady state was not established these effluxes would not be the same. Although maintenance of steady state for Ca was also indicated by recorded tension, which varied no more than ± 5% for 8 hours (and, on two occasions, for 16 hours) of efflux, the tracer criterion is much stronger. This is because it permits verification of steady state for small intracellular Ca compartments, something that cannot be assessed by, for example, measurement of whole tissue ion content. 2) Mechanical stretch of the vessel (from 12 to 40 g during the efflux) had no detectable effect on 45Ca efflux. Nor did the α$_1$-adrenergic blocker, prazosin (0.1 M) cause any alteration of the efflux. 3) We considered the possibility that the lumen of the long vessel segment might constitute a poorly mixed volume, which contributed a slow exponential to the efflux curve. Apparently this was not a problem; effuxes from two vessels, suspended as sheets after being cut open along their lengths, were superimposable on data collected with the lumen intact. 4) The corresponding possibility that the walls of the efflux chamber might bind 45Ca and contribute a slow component of efflux was also considered. The chamber itself (including the vessel mounting hardware) was loaded for 15 minutes with a solution whose specific activity was equal to the greatest specific activity recorded during an efflux. When this was washed out, no slow components were detected. Effluent activity fell to 0.09% of its initial value in 7 minutes and reached background in 15 minutes.

Limitations

Our method for resolution of intracellular Ca depends on the postulate that intracellular Ca and only intracellular Ca will change when the vessel segment is exposed to 10 μM phenylephrine or to other compounds purported to act on cellular Ca metabolism. We know of no evidence for agonist-induced extracellular Ca binding and while α-adrenergic agonists are known to release a store of Ca, that store is reportedly intracellular. Also, the release of rapidly exchanging extracellular Ca caused by the prostaglandin analog, U44069,19 has not been reported for α-adrenergic stimulation.

A compromise had to be reached between the cost of 45Ca and the resolution of small Ca compartments. The procedures employed result in samples, late in the efflux, which produce counts at only twice the background rate. Nevertheless, these counts were both reproducible and reproducibly altered by physiological and pharmacological agents. Further evidence for an effective compromise was provided by two experiments in which the loading solution activity was increased fivefold; when adjusted for initial dose the data were superimposable on previous experiments.

Very small Ca compartments could not be resolved by our method. The smallest compartment we resolved contained 44 nmol Ca/g (wet wt. of artery). This means we could not estimate cytosolic free Ca which, even at maximal contraction, is probably no more than 3 nmol/g.

Kinetic Analysis

We analyzed the data and tested models using the Simulation Analysis And Modeling (SAAM) program18 and its recent, interactive counterpart, CON-SAM.19 We ran these programs on a Digital Equipment Corporation VAX 11/780. Differential equations representing models to be tested were routinely solved using the exponential method20 because it is fast. Solutions were checked periodically for consistency with classical Runge-Kutta and Gear numerical integrators.

In all cases the goal was the smallest model compatible with the 45Ca efflux data and with other data, such as published measurements of extracellular fluid volume in rabbit aorta. Once a compatible model was found, best estimates of the rate constants, L(i,j), were obtained from a generalized, nonlinear least-squares fit of the experimental data. These estimated values for the rate constants constitute the information extracted from the experimental data. Statistical uncertainties associated with the final parameter estimates were calculated by multiplying the inverse normal equation matrix by the best estimate of the variance of the data.18

All steady state solutions (Ca contents and fluxes) were obtained by solving the equation, U + LM = 0, where U is the vector of compartmental inputs, L is the matrix of intercompartmental rate constants, and M is the vector of compartmental masses. This equation assumes only steady state and conservation of mass; it permits translation of tracer kinetic information [L(i,j)] into steady state masses and fluxes.21

Limitations

Models, formulated in this way, serve as quantitative statements of working hypotheses constrained as much as possible by currently available information. These models are necessarily incomplete and subject to change as new data become available. A further limitation is non-uniqueness of the model structure. This is a widely discussed problem, but it is no more serious for a theory expressed as a mathematical model than it is for a theory expressed as a series of paragraphs. The mathematical model developed in this paper is not proven correct; it is, however, consistent with all of the data we have collected. Moreover, completed models such as this are advantageous not only because they are demonstrably and quantitatively consistent with available information but also because they can make precise and testable predictions that can be compared directly to new experimental data.22

Results

To resolve intracellular Ca kinetics we adopted long-term efflux protocols. This was because extracellular exchanges appeared to dominate early efflux data while slower and smaller intracellular Ca pools appeared to dominate later efflux data. In Figure 1 we have plotted data for two steady state protocols: one in normal PSS and the other in PSS containing 10 μM phenylephrine (PE, a relatively specific α-adrenergic agonist).
A wide dynamic range is an essential feature of any Ca measurement that purports to resolve, simultaneously, large extracellular and small intracellular compartments. This is a useful, but generally underexploited, feature of tracer methods. In Figure 1 this dynamic range is apparent in the ratio of early-to-late efflux data; the ratio is about 50,000 and allowed us to resolve Ca compartments from 44 nmol/g to 2310 nmol/g. We found this large range, in combination with our criteria for identifying intracellular compartments, was essential if intracellular Ca stores were to be resolved. In fact, the early overlap and later divergence of the PSS and PE data in Figure 1 are qualitative statements of the method’s ability to resolve small intracellular Ca compartments despite the extracellular background.

Efflux data collected during PE "step protocols" (with the step beginning after 60 minutes of efflux) are shown in Figure 2. Steps beginning at 60 minutes were chosen because, both empirically and theoretically, they maximized our ability to resolve the releasable store and to extract information that could not be extracted from the steady state efflux data described above. In fact, pioneering experiments of this kind are credited with demonstrating the existence of the noradrenaline-releasable Ca store. In our hands the PE step protocol always resulted in a pulse of released $^{45}$Ca (Figure 2).

**Kinetic Analysis**

We began with the relatively simple hypothesis shown in Figure 3. It was based on known physiological and anatomical facts including: 1) reported extracellular fluid volume,\(^23\) 2) the existence and approximate size of the cytosolic Ca pool;\(^1\) 3) the presence of extracellular bound Ca,\(^6,24\) and 4) the presence of an $\alpha$-agonist-releasable intracellular Ca pool.\(^{25}\) In addition, turnover of the tissue bath compartment, Cal, (calcium compartments are numbered as shown in Figures 3 and 4) was calculated from bath fluid volume and perfusion rate.

The first question was whether this initial model could account, simultaneously, for both the steady state data in Figure 1 and the step data in Figure 2. It could not; if the intracellular exchange compartment,
Ca4, was released by PE (to reproduce the step data) then no intracellular compartment was available to be filled during contraction and to account for the elevated "tail" of the efflux curve seen in the PE steady state protocol. Moreover, there is no evidence for PE-induced increases in extracellular bound Ca, so using an extracellular compartment to reproduce part of the elevated tail was ruled out. Furthermore, even if Ca4 was not released by PE so that it could account for the early part of the elevated tail, a second (slower) intracellular exchange, Ca7, was required to account for elevation of the slowest part of the efflux curve.

Even the early data, dominated by extracellular events, could not be accounted for with the single extracellular exchange, Ca5. Ca5 could be made to account for the very early data or for the data between 60 and 100 minutes of efflux, but not both. A second extracellular exchange, Ca6, was required if data in both periods were to be matched simultaneously. To summarize: A model without Ca6 fails to account for the early (t<100 min) data in Figures 1 and 2, a model without Ca7 fails to account for the slowest efflux component seen in Figure 1, and a model without Ca8 fails to account for the first part of the elevated tail. Ca6 must be extracellular because it is unaffected by PE; Ca7 and Ca8 must be intracellular because they contain additional 45Ca when loaded in the presence of phenylephrine.

Thus, a minimal model compatible with all the data contains three compartments in addition to those shown in Figure 3. This augmented model is shown in Figure 4; it is able to fit the steady state efflux data in normal PSS (Figure 5) and, with only two major parameter changes, can account for both the phenylephrine steady state and the phenylephrine step data (Figures 5 and 6). The two altered rate constants, L(3,2) and L(3,4), correspond to trans-plasma-membrane influx of Ca and to the release of Ca from an intracellular location.

Changing these rate constants in order to fit the PE data implies that these processes are directly or indirectly controlled by PE. Two other rate constants, L(5,2) and L(3,7), are significantly different when the two data sets are compared (Table 1). This implies that other Ca-handling processes may be controlled by PE, but the changes are small. Notice that we have treated the PE-induced changes as instantaneous; when the PE concentration increases, L(3,2) and L(3,4) are immediately changed to their new values. In effect, this treatment tests the hypothesis that a new steady state is achieved in a time that is short on the time scale of the efflux experiment. As can be seen in Figure 6, this hypothesis is supported since step changes in rate constants account for the Ca efflux transient quite precisely.

All rate constants were derived from the efflux data or from other, independent, constraints such as extracellular fluid volume and tissue chamber turnover rate. Their values are listed in Table 1. The mean values shown in the table were calculated by averaging individual rate constants obtained from fitting individual efflux curves. Absence of a standard error for any entry implies that that rate constant could not be esti-
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Figure 5. Present model's predictions (solid line) compared to experimental data. Steady state protocols in normal PSS (triangles) and in PSS containing 1 E-5 M PE (squares). Individual experiments are shown; they were chosen to be representative of the mean.

All but four of the \(L(i,j)\) were determined with coefficients of variation between 7 and 30% for each experiment. \(L(2,1)\) and \(L(1,2)\) were not as well determined since only the first few efflux points contained information on them. However, the ratio of these rate constants was strongly constrained both by the known extracellular fluid volume and by the fact that increasing the ratio increases \(^{45}\text{Ca}\) loading proportionately. But, what constrains their absolute value? First, \(L(1,2)\) must be faster than all of the other resolvable compartments; if it were not, all compartments more remote from the bath than the ECF would appear well-mixed and unresolvable. Second, the turnover rate of Ca2 cannot be as slow as Ca5. If it is supposed that the fastest tissue compartment, Ca5, is not bound ECF Ca but rather is the free ECF Ca, then the required Ca content of 2300 nmol/g would imply an impossible ECF volume of 1.5 ml/g. For this reason Ca5 must be distinct from Ca2 and since Ca5 is nonetheless resolvable, Ca2 must turn over more rapidly than Ca5 [\(L(2,5) = 0.5/\text{min}\)]. This places a lower bound on \(L(1,2)\) [and therefore on \(L(2,1)\)], but what about an upper bound? If the stirred bath and the ECF volume were always in equilibrium, \(L(2,1)\) and \(L(1,2)\) would be very large or they could be eliminated by merging Ca1 with Ca2. The presence of a boundary layer and the size of the aortic interstitium make this an untenable position. We chose to set \(L(1,2)\) at 1.74/\text{min}, corresponding to an ECF diffusion coefficient of \(1.9 \times 10^{-6}\) cm²/sec similar to that measured for sheep carotid artery.

Surprisingly, the other two rate constants that were not well determined were the transmembrane constants, \(L(3,2)\) and \(L(2,3)\). This reflects their not being rate limiting for efflux; since transmembrane movement does not determine the shape of the efflux curve, little information on this process could be extracted from the curve. For example, dependence of Ca pumping on [Ca], would be unresolvable with this technique.

Figure 6. Present model's prediction (solid line) compared to efflux data (triangles) collected during a step protocol in which 10 μM phenylephrine was added to the efflux medium after 60 minutes of efflux. Individual experiment representative of the mean.
The preceding hour is the time during which the artery (and the kinetic model) are exposed to the tracer. This is an important point because it is by modeling the tracer uptake as well as the efflux that we avoid the assumption of isotopic equilibrium. Indeed, isotopic equilibrium is definitely not achieved in 1 hour of exposure to $^{45}\text{Ca}$; if it was, effluent activity would reach background in 1 hour of efflux or less. Indeed, efflux activity does not reach background even after 8 hours (or 16 hours, $n = 2$) which means that loading small and slow compartments to isotopic equilibrium is, at best, impractical. By using the same model and rate constants to account for the loading period as well as the efflux, the model is doubly constrained; it must account not only for the observed efflux data but also for the amount of $^{45}\text{Ca}$ that is loaded in 1 hour of exposure to the tracer.

Since isotopic equilibrium is not reached in 1 hour of $^{45}\text{Ca}$ loading, the model predicts elevation of the tail of the efflux curve when loading time is increased. In Figure 7 this prediction is compared to data from a PSS steady state protocol in which loading time was increased to 2 hours.

Additional predictions made by the model were: 1) any agonist or drug that increases plasma-membrane Ca permeability will cause elevation of the efflux tail, 2) any agonist or drug that slows the active extrusion of Ca from the cells to the point where it becomes rate limiting for efflux will substantially alter the tail of the efflux curve, 2) any perturbation that displaces Ca from extracellular binding sites will lower the initial slope of the efflux curve, and 4) any agonist or drug that stimulates the processes corresponding to L(3,4), L(3,7), or L(8,3) will not result in a pulse of released $^{45}\text{Ca}$ even if the compound acts quickly and even if the same amount of Ca is lost. These predictions serve a dual role; they are testable for compounds whose mechanisms of action are known and they may be used to evaluate the mechanisms of action of new vasoactive compounds.
Information on tracer metabolism would be limited value if it were not for the link between the tracer and the traced substance, or tracee. Using the known Ca concentration of the PSS and the rate constants derived from the tracer data, Ca contents and fluxes were calculated and are shown in Tables 2 and 3. Phenylephrine caused intracellular Ca to increase, but because of a concomitant decrease in extracellular bound Ca, total tissue Ca did not change.

**Discussion**

The masses and fluxes displayed in Tables 2 and 3 are important because they allow us to begin to assess the relative importance of membrane and organelar processes in controlling cytosolic [Ca]. For example, our results are consistent with the widely held view that α-adrenergic agonists increase plasma-membrane Ca permeability and release Ca from an intracellular store; no other major changes are required to account for the data in this paper. We can also say that the two intracellular pools, Ca7 and Ca8, are kinetically quite distinct even though their Ca contents are similar in PSS. We found that the steady state exchange flux is about ten times greater for Ca8 than for Ca7. This means that even if the plasma membrane flux is as small as possible (fluxes must be at least 1.5 nmol min⁻¹g⁻¹ to be consistent with the tracer data), the basal Ca transport of Ca7 cannot substantially alter the timecourse of cytosolic [Ca] following receptor activation and the entry of ECF Ca. Conversely, the physiologic Ca pool corresponding to Ca8 exchanges Ca rapidly enough to buffer incoming Ca and damp the resulting transients in cytosolic [Ca].

Thus, Ca8 has several features in common with the sequestration system (S2) described by van Breemen's group; indeed the functional overlap between their S2 and S1 sinks may be reflected in the kinetics of our Ca8 and Ca4 pools: Ca8 has exactly the same turnover rate as Ca4. Tentatively, then, we would identify their agonist releasable store, S1, with our Ca4; their S2 has

**Table 2. Steady State Calcium Contents (nmol/g wet weight)**

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Normal PSS (n = 4)*</th>
<th>PSS + 10 μM phenylephrine (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca4</td>
<td>145 ± 29†</td>
<td>85.6 ± 9.7‡</td>
</tr>
<tr>
<td>Ca8</td>
<td>89.7 ± 5.4</td>
<td>369 ± 34§</td>
</tr>
<tr>
<td>Ca4 + Ca8</td>
<td>235</td>
<td>455</td>
</tr>
<tr>
<td>Ca7</td>
<td>54.9 ± 3.2</td>
<td>177 ± 20§</td>
</tr>
<tr>
<td>Total intracellular</td>
<td>290</td>
<td>632</td>
</tr>
<tr>
<td>Ca5</td>
<td>2310 ± 210</td>
<td>1840 ± 150§</td>
</tr>
<tr>
<td>Ca6</td>
<td>853 ± 144</td>
<td>950 ± 184</td>
</tr>
<tr>
<td>Total tissue</td>
<td>4128</td>
<td>4097</td>
</tr>
</tbody>
</table>

* n corresponds to the number of rabbits; one proximal aortic segment was obtained from each animal.
† Mean results ± standard error are shown. § Significantly different from PSS (p < 0.025).

**Table 3. Steady State Calcium Fluxes (nmol min⁻¹ g wet weight⁻¹)**

<table>
<thead>
<tr>
<th>Flux</th>
<th>Normal PSS (n = 4)</th>
<th>PSS + 10 μM phenylephrine (n = 5)</th>
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<tbody>
<tr>
<td>Ca5→Ca2*</td>
<td>1070 ± 170†</td>
<td>716 ± 90‡</td>
</tr>
<tr>
<td>Ca6→Ca2</td>
<td>67.8 ± 10.1</td>
<td>73.4 ± 16.6</td>
</tr>
<tr>
<td>Ca3→Ca2</td>
<td>13.5</td>
<td>47.7 ± 5.4‡</td>
</tr>
<tr>
<td>Ca4→Ca3</td>
<td>4.86 ± 0.97</td>
<td>17.2 ± 1.9§</td>
</tr>
<tr>
<td>Ca8→Ca3</td>
<td>3.02 ± 0.18</td>
<td>12.2 ± 1.0§</td>
</tr>
<tr>
<td>Ca7→Ca3</td>
<td>0.324 ± 0.037</td>
<td>1.22 ± 0.13§</td>
</tr>
</tbody>
</table>

* This notation designates the steady state flux into Ca5 from Ca2.
† Mean results ± standard error are shown.
§ Significantly different from PSS (p < 0.05).
most in common with our Ca8. Our estimate of Ca4's Ca content is larger than reported by others in part because our estimate of 145 nmol/g includes Ca, which is not actually released by PE. Additional investigation is required to establish the physiological identities of these kinetically and functionally identified compartments.

Some investigators believe the agonist-sensitive store is filled by a direct pathway from the extracellular fluid rather than from the cytosol. We cannot rule this out, but the data in Figures 1 and 2 do not require a transmembrane process that would correspond to L(4,2). However, preliminary data on effluxes carried out in low Ca (0.15 mM) PSS do suggest the need for this pathway.

There are no previous reports with which to compare the characteristics of Ca7. This is largely because Ca7 is so small and slow that its influence on measured effluxes does not become dominant until about 230 minutes of washout. Most efflux experiments are carried out for 200 minutes or less.

It has been cogently demonstrated that additional information on intracellular Ca metabolism is obtained from vascular Ca kinetics in saponin (0.1 mg/ml, 60 minutes) treated arteries. These investigators have identified a Ca component whose turnover time is 50–100 minutes (similar to Ca4 and Ca8) and that is identified as sarcoplasmic reticulum based on ATP-dependence of Ca uptake, demonstration of oxalate stimulated uptake, as well as caffeine (30 mM) induced Ca release.

Preliminary work in our laboratory has demonstrated that Ca4 is caffeine sensitive as well as PE sensitive. This suggests that our Ca4 corresponds to the releasable part of the slow component identified by Stout and Diecke and that our Ca8 corresponds to the unreleasable part. These correspondences were supported by a simulation of the basic Stout and Diecke experiment (their Figure 5) using the model shown in Figure 4. Their data were collected for vessels loaded for 30 minutes with 45Ca at 0.45 μM free Ca and washed out for 36 minutes in either low Ca (4.24 mM) or high Ca (1.08 μM). Of course, the extracellular bound Ca sites are almost completely unloaded under these conditions so L(5,2) and L(6,2) had to be reduced accordingly. Also Ca2, the extracellular Ca pool, was adjusted to account for the approximately three-fold increase in its Ca content due to the presence of 5 mM EGTA. With these changes the model prediction (obtained by summing the activity remaining in Ca2, Ca3, Ca4, Ca7, and Ca8 and allowing a constant multiplier to account for differences in specific activity of our loading solutions) was within one standard error of the mean low-Ca desaturation data reported by Stout and Diecke.

With the additional postulate that washout in high Ca caused release of Ca4 (calcium induced calcium release) the model prediction was within two standard errors of the mean high-Ca desaturation data reported by these workers. These results are consistent with our conclusion that the plasma membrane is not rate limiting for Ca efflux.

Another approach to smooth muscle Ca kinetics has recently been reported. By the use of both uptake and efflux experiments, these investigators developed a compartmental model for calcium metabolism in collagenase-isolated toad stomach cells in which the plasma membrane, rather than an organellar membrane, is rate limiting for Ca efflux. This contrasts with our result for rabbit aortic smooth muscle, but given the differences in species, tissue, and experimental technique, such a contrast is perhaps to be expected. Further investigation will be necessary to reconcile these results.

The cold lanthanum wash technique employed by Weiss and his collaborator has been used to estimate Ca contents and dissociation constants of L-sensitive (extracellular) Ca compartments. By examining a very large range of extracellular Ca concentrations and by performing Scatchard analysis of the binding data, these investigators discovered two kinetically distinct extracellular binding sites. Their data have been reported in detail. The two sites show Kd's of 23.7 μM and 6.10 mM and capacities of 1310 and 8200 nmol/g, respectively. At an extracellular [Ca] of 1.5 mM these sites contain 1310 and 1620 nmol/g respectively. By comparison our extracellular sites, Ca6 and Ca5, contain 853 nmol/g and 2310 nmol/g in 1.5 mM Ca PSS. These results are comparable; the differences of 500–700 nmol/g may be due to the different ionic composition of the buffers used in the two experiments, or they could reflect an altered population of binding sites as extracellular [Ca] is varied.

These extracellular binding sites are the fastest processes we have tried to resolve. Consequently, we must consider whether diffusion through the extracellular space limits our ability to characterize them. The influence of diffusion on efflux parameters has been carefully examined. This analysis demonstrates that diffusion must be accounted for in analysis of Na, K, and Cl effluxes. While diffusion through interstitium is explicitly included in our model, our treatment is limited to a single, well-mixed, compartment and does not include a solution of the partial differential diffusion equation. In part, this is because of limitations imposed by our computer software, but an experiment using an extracellular marker suggests that a single compartment interstitium is an adequate model. We find that efflux data from an aortic segment loaded (1 hour) with 4C-sucrose are readily fitted to a simple two-compartment model (corresponding to Ca1 and Ca2) in spite of the larger molecular weight and slower diffusion coefficient exhibited by sucrose.

Another possible interpretation of our data is that a distribution of rate constants, for example, log normal, resulted in a polyexponential efflux whose source was not multiple kinds of compartments but rather an inhomogeneous collection of similar compartments. We cannot rule it out, but we consider it unlikely because 1) the variance of the log normal distribution would have to be very large in order for any two of the compartments in Figure 4 to come from the same distribution since adjacent compartment turnover times are always different by a factor of between 2 and 10, 2)
Ca5 and Ca6 are differentially sensitive (data not shown) to changes in extracellular [Ca] and are therefore not likely to be the "same" in any functional sense, and 3) intracellular compartments (Ca4, Ca7, and Ca8) are also differentially sensitive, in this case, to a vascular compound.

Having discussed the intracellular and extracellular Ca compartments we will conclude with a few points related to the barrier that separates them — the vascular smooth muscle cell membrane. The tracer kinetic method described here takes advantage of the relatively large Ca permeability of the cell membrane to resolve multiple intracellular Ca pools. Conversely, it is the presence of multiple intracellular, agonist-altered Ca pools that leads to the conclusion that membrane extrusion is not the slowest process in the efflux pathway. While the relative speed of the membrane is advantageous in our experiments, it presents substantial difficulties for interpreting short-term (eg, 30–90 seconds) uptake experiments. Such experiments are useful for determining net uptake but may seriously underestimate transmembrane Ca flux because of the large backflux of labelled calcium. To estimate the magnitude of the underestimate we simulated a 90-second uptake protocol and compared the one-way Ca flux obtained by the simulated protocol to the flux actually used in the simulation; under the assumptions of this protocol there was a nine-fold difference. Thus, we can be fairly certain that short-term uptake will underestimate this flux, but because our own method provides no absolute value, we can neither confirm nor deny the flux estimates reported by other investigators.

Stimulation of this transmembrane Ca flux is generally thought to be the mechanism of action of contractile agonists, but there is little data on vascular smooth muscle that excludes the possibility that such agonists act, instead, by slowing the extrusion of Ca. However, an argument against the pump-inhibition hypothesis can be found in our kinetic data; when pump inhibition is the postulated mechanism of agonist action, more 45Ca must be released from Ca4 to account for the pulse in Figure 2. Consequently, more 45Ca must have entered Ca4 during the the loading period, and this, in turn, means L(4,3) must be larger while L(8,3) must be smaller. In other words, inhibition of the pump makes it more difficult for released 45Ca to reach the measuring site so a greater portion of the fast intracellular pool must be releasable in order to match the data. However, when this hypothesis is used to fit the step data, Ca8 becomes too small to account for the data obtained using the steady state PE protocol, so the pump inhibition hypothesis must be rejected. This complex argument could be avoided if step protocols were carried out to the full 8 hours of efflux. Our model predicts a 30% elevation of the last efflux component if PE inhibits Ca extrusion; it predicts no elevation if PE acts to increase Ca permeability. This difference should allow straightforward discrimination between the hypotheses.

Another consequence of our finding that the smooth muscle plasmalemma is relatively permeable to Ca is that organellar Ca sequestration and release cannot regulate the steady state cytosolic Ca concentration. Indeed, it is theoretically impossible for an exchange compartment (no matter what its kinetic description) to alter steady state cytosolic Ca content. A powerful Ca extrusion mechanism in the smooth muscle membrane is required to balance the large Ca permeability; in the face of this extrusion mechanism, a finite organellar supply of Ca can regulate cytosolic Ca only transiently. Of course, this transient may still be quite long since its duration is largely controlled by the size of the Ca store and the magnitude of the plasma membrane Ca flux.

Finally, preliminary work in our laboratory has demonstrated that the model can also account for 45Ca effluxes in potassium stimulated (40 mM) rabbit aorta. This suggests that the model has broader significance than it would if it were correct for only a single agonist.

The importance of the approach we have described derives from its ability to provide simultaneous measurements of intracellular and extracellular Ca contents and Ca fluxes in intact arterial segments. Of course, these measurements will become more useful when the kinetically defined compartments can be assigned physiological identities. With the aid of putative specific inhibitors, tentative identifications can be made, and an initial example of this approach is presented in the companion paper.9 Once the compartmental identities are established, the kinetic technique we have described can be used to investigate the cellular mechanism of action of a variety of physiological, pharmacological, and pathological stimuli.

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