Evidence for Increased Aortic Plasma Membrane Calcium Transport Caused by Experimental Atherosclerosis in Rabbits

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Several lines of evidence, including the reported ability of calcium channel blockers to prevent ath-erosclerosis in cholesterol-fed rabbits, suggest that calcium mediates one or more of the pathologic changes in atherosclerosis. Moreover, it has long been known that calcium accumulates in atherosclerotic blood vessels. To test the hypothesis that a substantial fraction of this accumulated calcium is intracellular and to identify possible causes of this accumulation, calcium fluxes and contents were determined in aortic segments from cholesterol-fed rabbits and age-matched controls. A new method, based on Ca efflux experiments and computer-assisted kinetic analysis, was used to measure intracellular and extracellular calcium contents (nmol calcium/g wet wt tissue) and fluxes. Total intracellular calcium increased from 269 ± 11.6 to 1,300 ± 352 nmol/g in cholesterol-fed animals compared with controls (p < 0.01). This change was sufficient to account for the observed increase in total tissue calcium from 4,190 ± 211 to 5,240 ± 477 nmol/g (p < 0.05). Thus, the fraction of tissue calcium that is intracellular increased significantly from 0.065 ± 0.006 to 0.223 ± 0.048 (p < 0.01) in experimental atherosclerosis. In addition, the data were quantitatively consistent with the hypothesis that these changes are brought about by a 4.8-fold increase in the plasma membrane calcium permeability of aortic smooth muscle cells. These results provide evidence that increased intracellular calcium is a possible mediator of cholesterol-induced atherogenesis. (Circulation Research 1988;62:75-80)

Many risk factors for the development of atherosclerosis have been identified, but the resulting lesions all appear to progress in the same way. Consequently, it has been suggested that all of these risk factors act through a single common pathway that leads to the development of the atheroma. During the past several years, it has been demonstrated that many cellular processes that contribute to the development of atherosclerosis, such as control of membrane permeability, secretion of extracellular matrix proteins, cell proliferation, cell migration, and cell death, are regulated by the concentration of intracellular calcium. This has led several investigators to suggest that atherogenesis is dependent on calcium movement across the smooth muscle plasma membrane into the cell. For example, hypercholesterolemia, a major risk factor, might promote this calcium entry by delivering cholesterol to the plasma membrane and thus alter the function of membrane ion channels that are sensitive to their lipid environments. Cholesterol delivery may take place by receptor-mediated endocytosis or by other means, but, in either case, once intracellular calcium is increased, it would be expected to accelerate the many cellular processes that are known to be calcium-dependent and that are characteristic of atherogenesis. Previous studies aimed at observing calcium’s involvement in atherosclerosis reported substantial increases in total aortic calcium content in cholesterol-fed rabbits. It has been demonstrated that some of this additional calcium is bound to altered extracellular proteins, but no quantification of intracellular calcium changes has been reported. Although there are a few negative reports, it has repeatedly been shown that inhibition of calcium influx inhibits the development of atherosclerotic plaques in cholesterol-fed rabbits.

Because the processes that are involved in atherosclerosis are controlled by intracellular calcium, the calcium hypothesis for atherogenesis predicts that changes in arterial cell calcium metabolism must be at least partially intracellular. By applying a technique we recently developed for the resolution of intracellular calcium metabolism in arterial segments, we sought to determine whether the changes in arterial wall calcium metabolism that occur during atherosclerosis are, in fact, intracellular. The abilities to resolve intracellular changes in calcium metabolism caused by an inhomogeneously distributed disease and to determine the mechanisms responsible for any observed intracellular calcium accumulation are significant advantages of our method.
Materials and Methods

Procedures

New Zealand white male rabbits (3-4 kg) were maintained on regular rabbit chow (Link Klein, Inc., Bacto, Maryland; n = 4) or were fed the same chow supplemented with 1% cholesterol (n = 8). All rabbits were housed for 13-17 weeks. On the day of the experiment, rabbits were anesthetized with sodium pentobarbital (52 mg/kg i.v.) and bled. Plasma cholesterol and triglyceride levels were determined in The Johns Hopkins University Lipid Research Clinic Laboratory using enzymatic methods (CHOD-PAP, Boehringer-Mannheim Diagnostics, catalog number 70412, Indianapolis, Indiana, and the Abbott A-Gent, Triglycerides Reagent Set, Abbott Laboratories, catalog number 6097, Chicago, Illinois) that have been standardized according to the Center for Disease Control-National Heart, Lung, and Blood Institute criteria for lipid standardization. The 43Ca efflux method has recently been described in detail. In the present study, the entire aorta from the descending thoracic aorta to the iliac bifurcation was quickly excised and transferred to a physiological salt solution (PSS) of the following millimolar composition: NaCl 116.9, KCl 4.7, KH2PO4 1.2, MgSO4 1.2, NaHCO3 28.0, dextrose 5.5, CaCl2 1.5, CaNa2EDTA 0.026, and bubbled with 95% O2-5% CO2 at 37°C. The adventitia was removed with care taken to not stretch the muscle. Next, the aorta was cut into 2-cm long segments. Alternate segments (usually 4-6) were placed in 100 ml PSS and equilibrated for 1 hour at 37°C. The aortic segments were then loaded with 43Ca in a vial containing approximately 5 x 106 cpm/ml and 4 ml PSS. After the loading period of 1 hour, the vessel segments were quickly rinsed in PSS and transferred to a flow-through chamber perfused with PSS. The PSS was pumped into the 9 x 1 x 3-cm Plexiglas efflux chamber through a small port near the bottom of the chamber; it exited through an orifice on the top of the opposite side of the chamber. The chamber was sealed, permitting effluxes that required only a single pump. A Plexiglas plate, mounted perpendicular to the chamber lid, provided an array of 10 stainless steel wires situated in the perfusion fluid. Each vessel segment was mounted on one of these wires by threading the wire through the vessel lumen. The 9-ml chamber volume was perfused with PSS at a constant flow of approximately 9.5 ml/min at 37°C and was mixed continuously using a small magnetic stirrer. The flow rate was chosen to ensure that the turnover rate of the chamber was at least as fast as that obtained in our original study; effluxes using the control protocol of the chamber was at least as fast as that obtained in our previous study. The flow rate was chosen to ensure that the turnover rate of the chamber was at least as fast as that obtained in our original study; effluxes using the control protocol were within one SEM of the effluxes in that report. Approximately 1 ml/min of the effluent was diverted to a fraction collector, and fractions were collected at 2-minute intervals over the entire 8-hour efflux period. 43Ca in appropriate effluent samples was measured by liquid scintillation spectrophotometry. After the 8-hour efflux, the aortic segments were removed from the chamber, cut open, blotted, and weighed. Radioactivity in each efflux vial was normalized for the activity of the 43Ca loading solution and the total blotted wet weight of the vessel segments in the efflux chamber.

Kinetic Analysis

The kinetic data were analyzed using the latest interactive version of the National Institutes of Health's Simulation Analysis and Modeling (SAAM29) program in conjunction with our recently published model of calcium metabolism in rabbit aorta. Several hypotheses, representing possible cholesterol-induced alterations in this model, were tested by examining their ability to account quantitatively for the experimental efflux data. Once a successful hypothesis was found, its rate constants and their coefficients of variation were estimated using the program's generalized nonlinear least-squares procedure.

Limitations

The limitations of these experimental and kinetic analysis procedures were detailed previously. Here, the major problems and solutions are itemized. First, it is important to establish that the aortic preparations remain in steady state for the duration of the experiment. This was confirmed by extending the period of preexperiment equilibration to 3 hours (to magnify any change in state) and demonstrating that the subsequent efflux was identical to those obtained with a 1-hour equilibration. If the tissue were not in steady state, the efflux patterns would be different, indicating that tissue calcium compartments were changing with time. Second, all methods that purport to resolve changes in intracellular calcium must confront the problem of a small signal on a large background. This is because intracellular calcium constitutes less than 10% of total vessel wall calcium. Our method distinguishes between intracellular and extracellular calcium on kinetic grounds. Because it does not rely on physical removal of extracellular calcium, the method can be carried out in normal, calcium-containing media. This is a useful feature of the technique. Third, it was important to recognize that a 1-hour load with 43Ca is insufficient to achieve isotopic equilibrium, especially in intracellular compartments with slow turnover. This problem was solved by using our kinetic model to analyze both the load and the efflux. In this way, the extent to which isotopic equilibrium was attained during the 43Ca load was incorporated directly into the efflux analysis. Fourth, because atherosclerotic plaques are not uniformly distributed in the diseased aorta, the original method was modified by using four to six 2-cm segments of the aorta in an enlarged efflux chamber; this allowed resolution of tissue-average changes that might go undetected in studies of single segments.

An additional limitation arose in experiments using atherosclerotic aortic segments. It is possible that new calcium compartments were present in the diseased vessel that were not present in the control animals. For example, foam cells, derived from smooth muscle cells or macrophages, could metabolize calcium differently. Other candidates for new calcium compartments in-
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Calcium effluxes from atherosclerotic (○, n = 8) rabbit aortas and from age-matched control (△, n = 4) aortas. Ordinate represents activity released to the perfusate in cpm min⁻¹ mg⁻¹. Time during efflux is represented on abscissa. Symbols represent mean data; lines represent ± SEM. Kinetic analysis of the individual efflux curves allowed estimation of the individual rate constants, compartmental calcium contents, and intercompartmental calcium fluxes. Mean values for these parameters are in Table 1.

Results

Rabbits on the 1% cholesterol diet had serum cholesterol and triglyceride concentrations (1,180 ± 217 [SEM] and 330 ± 132 [SEM] mg/dl) that were 11.2 and 8.1 times greater (p<0.005, p<0.05) than controls, respectively. Routine histologic examination revealed extensive atheromatous involvement of the aortas from the cholesterol-fed group. No lesions were found in the age-matched controls.

The mean efflux data for 4 control and 8 cholesterol-fed animals are shown in Figure 1. The slow components of the effluxes from atherosclerotic arteries are substantially more prominent than the corresponding components of the control efflux. Because this alteration is essentially the same as that produced by loading a vessel with "Ca in the presence of a contractile agonist" and because the data could be fitted with a single major change in the plasma membrane calcium permeability, kinetic analysis strongly suggests that the increase reflects increased calcium content of intracellular calcium stores. Therefore, the experimental efflux appears as it does in Figure 1 because there is more intracellular calcium in the diseased blood vessel, and consequently, there is more "Ca loaded into the cells than is loaded in the control vessels. Then, since more label is loaded into the cells, there is more label to emerge during the efflux period. This theory, represented by the compartmental model shown in Figure 2, is both qualitatively and quantitatively consistent with the efflux data from a typical experiment, as shown in Figure 3.

Least-squares fitting of the individual efflux experiments permitted estimation of the rate constants represented by the arrows in Figure 2. When the data require changes in specific rate constants, we have putative evidence that the corresponding physical process is specifically modified in the experimental atherosclerotic group. Furthermore, knowledge of the rate constants combined with the known concentration of calcium in the medium permits calculation of calcium contents for every compartment as well as steady-state calcium fluxes on every intercompartmental pathway. This calculation is based on the fundamental principles of tracer kinetics. Calcium contents and fluxes measured in this way for atherosclerotic and control aortas are reported in Table 1.

Table 1 shows that the aortas from the atherosclerotic rabbits contained significantly more calcium than those from the age-matched control group. Moreover, the data could be fitted by increasing L₃, the rate constant that corresponds to the plasma membrane calcium permeability (Figure 2), by a factor of 4.9. Note that L₄ represents the rate constant governing transfer into

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**Figure 1.** Calcium effluxes from atherosclerotic (○, n = 8) rabbit aortas and from age-matched control (△, n = 4) aortas. Ordinate represents activity released to the perfusate in cpm min⁻¹ mg⁻¹. Time during efflux is represented on abscissa. Symbols represent mean data; lines represent ± SEM. Kinetic analysis of the individual efflux curves allowed estimation of the individual rate constants, compartmental calcium contents, and intercompartmental calcium fluxes. Mean values for these parameters are in Table 1.

**Figure 2.** Kinetic model of calcium metabolism in rabbit aorta. Heavy arrows represent those processes that are accelerated in atherosclerotic aortas. Compartments Ca₅, Ca₄, Ca₃, and Ca₂ accumulate extra calcium in atherosclerotic aortas.

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FIGURE 3. "Ca efflux data from a typical individual experiment on a cholesterol-fed rabbit aorta (C). —. Model solution that gave least-squares fit based on changes indicated by bold arrows in Figure 2. Failure of two alternative hypotheses to account for the experimental "Ca efflux data is also indicated in figure. Neither hyperlipidemia-induced decrease in cellular calcium extrusion (A) nor hyperlipidemia-induced increase in organelar calcium uptake (B) could be made to fit the observations. Although the full range of solutions for these alternative theories cannot be displayed in a single figure, the plotted curves illustrate the systematic inconsistencies that cannot be eliminated by simply adjusting the values of rate constants.

Two alternative hypotheses that might have explained the data from atherosclerotic segments were also tested. The first alternative was decreasing L23, which corresponds to decreasing the cell's ability to extrude calcium. The second alternative tested was a simultaneous activation of calcium transport into both organelles (Ca4 and Ca6) rather than a single increase in plasma membrane calcium permeability. However, neither hypothesis could account for the experimental data as is shown in Figure 3. This appears to be because both hypotheses effectively reduce the probability that a cytosolic calcium ion will be extruded into the extracellular space. This results in model predictions, for both hypotheses, that are systematically below the early (<2 hours) experimental data and systematically above the late (>2 hours) experimental data (Figure 3). No combination of parameter values can resolve both of these inconsistencies. Thus, our data refute both alternative hypotheses.

Discussion

Our principal finding is that efflux data from atherosclerotic aortas are consistent with a fivefold increase in plasma membrane calcium permeability, which increases cytosolic calcium and, by mass action, increases organelar calcium content. The data are quantitatively inconsistent with two alternative explanations: 1) decreased plasma membrane calcium extrusion and 2) activated organelar calcium uptake.

Table 1. Calcium Contents* (nmol/g wet wt) and Intercompartmental Calcium Fluxes† (nmol min⁻¹ g⁻¹) in Control and Atherosclerotic Aortic Segments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age-matched controls</th>
<th>Cholesterol-fed</th>
<th>Significance‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1*</td>
<td>155 ± 7.17</td>
<td>755 ± 205</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>M7</td>
<td>94.2 ± 7.85</td>
<td>569 ± 131</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>Total intracellular calcium§</td>
<td>269 ± 11.6</td>
<td>1,300 ± 352</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>M3</td>
<td>2,960 ± 257</td>
<td>2,420 ± 202</td>
<td>NS</td>
</tr>
<tr>
<td>M6</td>
<td>284 ± 51.1</td>
<td>865 ± 246</td>
<td>p&lt;0.025</td>
</tr>
<tr>
<td>Total extracellular calcium§</td>
<td>3,240 ± 219</td>
<td>3,280 ± 274</td>
<td>NS</td>
</tr>
</tbody>
</table>
| Total tissue calcium|| 4,190 ± 211 | 5,240 ± 477 | p<0.05
| Fraction intracellular     | 0.065 ± 0.006        | 0.223 ± 0.048        | p<0.01        |
| R3,2†                     | 11.7                 | 56.6 ± 15.3          | p<0.01        |
| R1,3                      | 0.378 ± 0.034        | 3.39 ± 1.45          | p<0.01        |
| R4,3                      | 4.35 ± 0.039         | 19.5 ± 6.13          | p<0.01        |
| R5,2                      | 2,290 ± 425          | 1,670 ± 220          | NS            |
| R6,2                      | 20.9 ± 5.63          | 54.3 ± 16.1          | p<0.05        |

*M1 is the mass (nmol/g wet wt) of calcium in the ith compartment. Compartments are numbered as shown in Figure 2. †Rij is the steady-state flux of calcium (nmol min⁻¹ g⁻¹) into the ith compartment from the jth compartment. Since the tissue is in steady state, Rij = Rji. Intercompartmental rate constants, Lij, estimated from the tracer data are not shown but may be estimated from the calcium contents and fluxes shown in the table using Lij = Rij/Mij. ‡Significance was assessed using Student’s t statistic. NS, not significant (p>0.05).

†Total intracellular calcium includes a small contribution from the cytosolic compartment, Ca3. Total extracellular calcium includes free calcium in the extracellular fluid, Ca2.

§Total tissue calcium is the sum of the calcium contents of all compartments resolved in an 8-hour experiment. Additional, slower compartments may exist. If there are additional compartments, the value shown for total tissue calcium will be an underestimate.

‖No SEM is shown for R3,2 because our method allows us to estimate only the ratio of control and experimental fluxes.
Our contention that organellar uptake is not activated may appear inconsistent with the organellar fluxes reported in Table 1 since these fluxes are shown to be significantly increased in the cholesterol-fed animals. However, the reported increases reflect only the increased cytosolic calcium concentration; they are consequences of the principle of mass action. In contrast, the increased plasma membrane calcium flux results from a specific change in the inward transport of calcium. This assertion is based on our finding that the data are consistent with a change in $L_{a23}$, the rate constant corresponding to inward transmembrane calcium transport. This distinction between a modulated transport process and simple mass action is made possible by the tracer kinetic approach and is one of its most useful features.

Since plasma calcium concentration in cholesterol-fed rabbits has sometimes been found to be 5–15% above normal, we considered the possibility that our findings were secondary to this hypercalcemia. However, this hypothesis was rejected because the maximum reported increase is much too small to account for the almost fivefold increase in intracellular calcium content reported here.

Thus, detailed kinetic analysis of the data from these hypercholesterolemic animals leads us to suggest that increased cholesterol delivery may cause increased cholesterol uptake by low density lipoprotein receptors or scavenger pathways and incorporation of the free sterol into cellular membranes, especially the plasma membrane. Since increased membrane cholesterol content is known to alter integral protein function and has been shown to increase calcium influx into erythrocytes, it appears possible that hypercholesterolemia leads to a perilous cycle of increased membrane cholesterol content and increased calcium entry. During this cycle, other calcium-dependent cell processes involved in atherogenesis, such as migration, proliferation, and stimulus-secretion coupling, might also be promoted.

Hypertension, another major risk factor for atherosclerosis, is thought by some investigators to be caused by abnormal arteriolar smooth muscle function and increased cytosolic calcium concentration causing an increased total peripheral vascular resistance. Recently, it has been shown that the cytosolic calcium concentration of blood platelets correlates with blood pressure and that a serum factor from hypertensive patients causes increased cytosolic calcium in platelets. If extrapolation of the platelet model to vascular smooth muscle is appropriate, our work suggests the additional hypothesis that hypertension may be a risk factor for atherosclerosis precisely because this change in smooth muscle membrane function occurs in large vessels as well as small ones, leading to calcium entry, stimulation of cellular processes that are calcium-dependent, and in time, development of the atherosclerotic lesion.

In summary, these data support the hypothesis that atherogenesis is accompanied by an increase in aortic smooth muscle cell membrane calcium permeability.

The resulting increase in intracellular calcium may play a causal role in atherogenesis by initiating or accelerating those calcium-dependent intracellular processes that are known to be involved in this disease.

References


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