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Calcium Ion Distribution in Cytoplasm Visualized by Aequorin: Diffusion in Cytosol Restricted by Energized Sequestering

Abstract. *The distribution of Ca^{2+} in the cytoplasm following a local rise in Ca^{2+} concentration is visualized by means of aequorin luminescence and a television system with an image intensifier. Diffusion of Ca^{2+} through the cytosol is so constrained that a rise in cytoplasmic Ca^{2+} concentration produced by local Ca^{2+} entry through cell membrane or by local Ca^{2+} injection is confined to the immediate vicinity of these sites. The diffusion constraints are lifted by treatment with cyanide or ruthenium red. Thus, energized calcium sequestering, probably by mitochondria, is the dominant factor in the constraints. In cell regions where the sequestering machinery is sufficiently dense, different Ca^{2+} message functions inside a cell may be effectively segregated, permitting private-line intracellular communication.*

Among the cellular inorganic ions, Ca is rather unique in that it is rapidly sequestered by mitochondria (*1*). Probably largely because of this, a local increase in free cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_i$, such as produced by a Ca injection into a cell, falls off more steeply with distance than in simple diffusion (*2, 3*). We show here—in experiments in which the Ca distribution inside the cell is displayed by aequorin—that the spatial attenuation is so steep that a local increase in $[Ca^{2+}]_i$ in the cytosol is effectively compartmented.

Cells of isolated *Chironomus* salivary glands were injected with the Ca^{2+}-sensitive luminescent protein aequorin (*4*). The aequorin light emission, which increases with $[Ca^{2+}]_i$ (*5*), was viewed and recorded through a microscope with the aid of an image intensifier coupled to a TV camera (*6*) (Fig. 1, inset). The method has sensitivity for $10^{-6}M$ $[Ca^{2+}]_i$ over a 1-μm-diameter cytoplasmic volume (steady state), and a spatial resolution of 1 μm. The total luminescence was measured independently with a photomultiplier. Photomultiplier current, membrane potential, and Ca^{2+} injection current were displayed on a storage oscilloscope (and a chart recorder) onto which a second TV camera was focused. The two camera outputs were videotaped and displayed simultaneously on a TV screen. Test pulses of Ca^{2+} were introduced by iontophoresis into the basal region of the cells with micropipettes filled with $0.4M$ $CaCl_2$ and $0.2M$ ethylene glycol-bis(aminoethylether) tetraacetate (EGTA) (*p*H 7). The resulting $[Ca^{2+}]_i$ was estimated by deriving the unit-volume radiant fluxes in the (spherical) luminescent region and then matching these against equivalent fluxes produced by standard Ca-EGTA buffers. The buffers, aequorin, fluorescein, and ruthenium red were microinjected hydraulically.

Figure 1 illustrates an experiment in which a series of test Ca^{2+} pulses produced rather constant spherical aequorin glows. The striking feature is that the glows are confined to the vicinity of the micropipette tip. Evidently the rise in $[Ca^{2+}]_i$ does not extend much beyond this vicinity: the $[Ca^{2+}]_i$, which was $< 10^{-4}M$ within a radius of 16 ± 2 μm from the tip at the time of maximal glow spread, fell precipitously within the next 1 to 2 μm to $< 10^{-6}M$.

This Ca^{2+} confinement depends on energy metabolism. When the cell was poisoned with 2 m*M* cyanide (CN), the standard Ca^{2+} pulses produced glows which spread throughout the cytoplasm. The $[Ca^{2+}]_i$ then fell off more gradually with distance from the tip, resembling the pattern of a more freely diffusing molecule (Fig. 2). The glows then also lasted longer. At Ca^{2+} pulse repetition rates of 2.4 per minute, the glows initially faded out between pulses; but eventually they summed, as one might expect if the Ca^{2+} sequestering and extrusion capacities were depressed.

The CN effects were rapidly reversible upon washout of the poison. The CN treatment produced no detectable change in the background $[Ca^{2+}]_i$. This could be determined between earlier pulses (0 to 200 seconds; Fig. 1) in the test cell and throughout the experiment in the adjacent cell. However, at higher CN concentrations (5 m*M*), an increase in $[Ca^{2+}]_i$ due to intracellular Ca release was clearly visible.

In another set of experiments, the cells were injected with ruthenium red (0.1 m*M*), a blocker of mitochondrial Ca uptake (*7*). Here the glows of the Ca^{2+} test pulses became diffuse (Fig. 4, II) and lasted longer too.

The method was sensitive enough to detect minute inward leaks of Ca through cell membrane. Thus, for instance, while the microelectrodes were in the cells, leakage of Ca^{2+} from the medium could be seen at the regions of their insertion into the membrane. In medium containing $\geqslant 2$ m*M* Ca, such leakage was generally visible, even with electrodes with < 0.2-μm tip diameters, when cell input resistance and membrane potential were high (0.3 megohm and 30 to 40 mv). The leaks were seen as steady luminescent dots, sometimes as small as 1 μm in diameter, which were typically confined to the immediate vicinity of the membrane. As in the case of Ca injection, this confinement required metabolic energy. The glow spread upon treatment with 2 m*M* CN, eventually reaching deep into the cell, and after washout the glow returned to the original limits (Fig. 3). Here again the CN occasioned no significant internal Ca^{2+} release: upon change to a Ca-free CN medium at the peak of the glow spread, the glow ceased. The possibility of significant outward leakage of aequorin (molecular weight, 30,000) is also excluded: not only are there no a priori reasons why the glow should then have spread after CN treatment, but, in fact, the glow was always within the cell boundaries when

leak points were viewed at the periphery of optical cell-sections.

The experiments reported here demonstrate visually on a microscopic scale that the domain of free Ca^{2+} diffusion in the cytoplasm is severely restricted by rapid energy-dependent Ca^{2+} sequestering. This is consistent with results obtained on a macroscopic scale with radiotracers in squid axon (*3*). The most obvious candidates for the sequestering are the mitochondria, which are known to take up Ca rapidly when energized by electron transport or adenosine triphosphate (ATP) (*1*). The basal third of the salivary gland cells (into which we inject) contains large amounts of mitochondria, and the cytoplasm immediately below the basal cell membrane (where the leaks are observed) is literally filled with them (*8*). Besides, the abundant endoplasmic reticulum in these cells may conceivably take part in energized Ca^{2+} sequestering. Simple Ca binding to cytoplasmic proteins and other chelators is unlikely to be the predominant factor here, in view of the results of the experiments with CN and ruthenium red. This argument applies also to Ca binding by aequorin, where light emission is known to be unaffected by CN (*4*). We can also exclude the more remote possibility that the injected Ca^{2+} causes a significant increase in cytoplasmic viscosity by interfering with an energy-dependent process: when a fluorescein solution containing 2 m*M* Ca is injected into a cell, the fluorescein (molecular weight, 330) diffuses everywhere through the cytosol, as it does when Ca is omitted (Fig. 2).

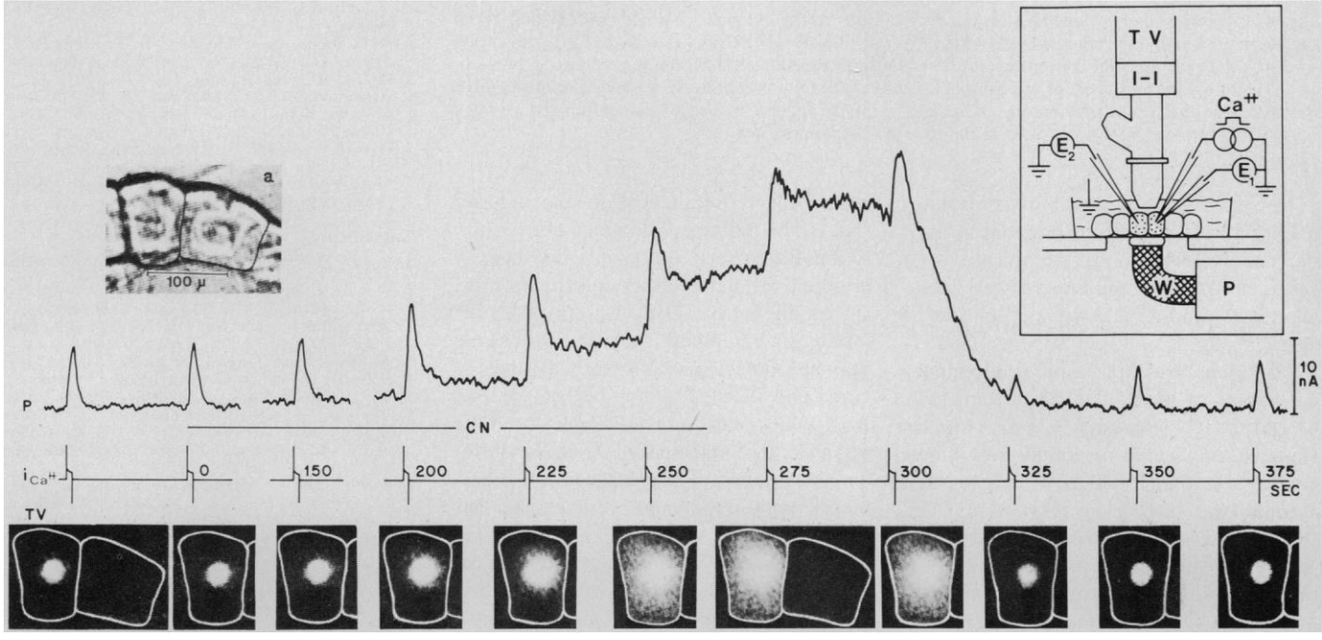

Fig. 1. Energy-dependent Ca^{2+} diffusion restriction in the cytosol. (Inset) Experimental setup. Dotted cells contain aequorin, whose luminescence displays spatial distribution of cytoplasmic Ca^{2+}. The cells' luminescence is scanned by an image intensifier–TV system (*I-I* and *TV*) and integrated separately by photomultiplier *P* (*W* = waveguide). The Ca^{2+} is pulsed out of microelectrode Ca^{2+}; microelectrodes E_1 and E_2 measure membrane potentials. The main figure shows aequorin luminescences produced by standard Ca^{2+} test pulses, (2.4 per minute): *TV*, dark-field TV pictures giving the spatial distribution of luminescence at the time of maximum spread of each luminescence pulse; *P*, chart record of photomultiplier current giving time course of pulses; *i*, Ca^{2+} injection current (1.5×10^{-8} ampere; 1 second). The bar (*CN*) signals superfusion with 2 m*M* cyanide; the numbers are the times (seconds) after start of superfusion (time for half-maximal concentration change $\approx$ 0.5 minute). Cells are in Ca-free medium (*6*). (a) Bright-field TV picture of cells.

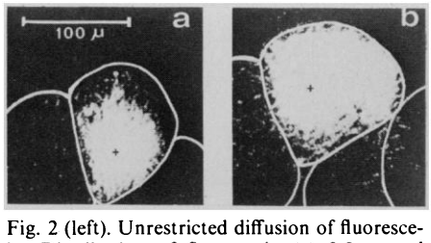

Fig. 2 (left). Unrestricted diffusion of fluorescein. Distribution of fluorescein (a) 0.5 second after start of a single iontophoretic pulse (1 second) of fluorescein and (b) 2 seconds after a hydraulic pulse of 0.2*M* fluorescein plus 1 m*M* buffered Ca^{2+}. At the time the photograph was taken in (a), fluorescein had not yet reached the cell boundaries. In (b) $[Ca^{2+}]_i$ was sufficiently elevated to block junctional cell-to-cell passage of fluorescein. Injection sites are marked by +. The TV dark-field pictures show the fluorescein fluorescence (540 nm); no aequorin is used here.

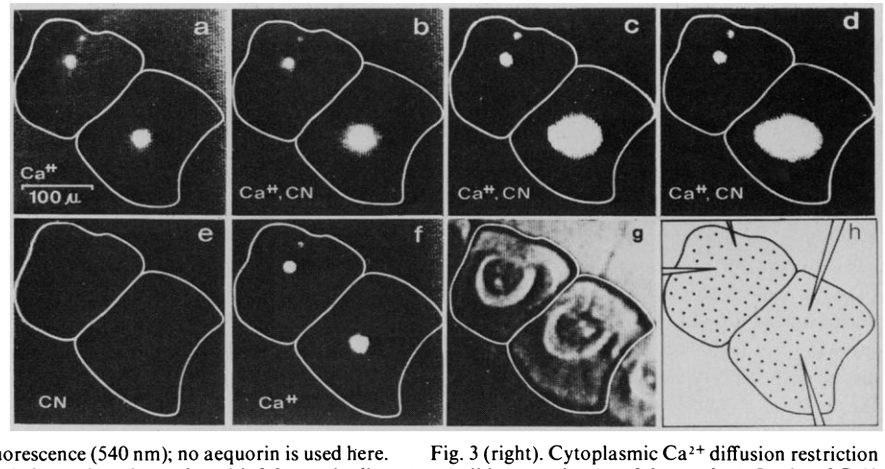

Fig. 3 (right). Cytoplasmic Ca^{2+} diffusion restriction at local membrane entry points. The cells are impaled on microelectrodes with 0.2-μm tip diameters (cell input resistance, 0.3 megohm). Leaks of Ca^{2+} are visible in dark-field TV pictures (a to f) as luminescent dots at three impalement sites. The larger leak in the second cell was produced by vibrating the microelectrode for a few seconds before (a). (a) Leaks in medium containing 4 m*M* Ca; (b to d) 1, 2, and 3 minutes after application of medium containing 4 m*M* Ca and 2 m*M* CN; (e) Ca-free medium containing 2 m*M* CN; (f) 2 minutes after return to medium of (a); (g) bright-field TV picture of the two aequorin-loaded cells; and (h) cell diagram showing electrode locations.

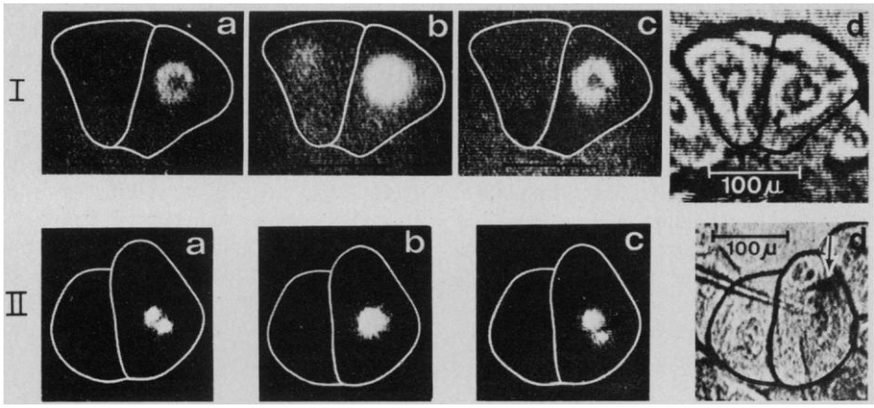

Fig. 4. Complex, energy-dependent Ca^{2+} distributions in the cytosol. Two examples (I and II) of aequorin glow figures produced by standard Ca^{2+} test pulses. (I) Before (a) and after (b) application of 5 m*M* CN (at this CN concentration there is also intracellular Ca^{2+} release); (c) after CN washout. (II) Before (a) and after (b) injection of 0.1 m*M* ruthenium red into cell; (c) several minutes later. The arrow in the bright-field picture (d) points to the cytoplasm stained by ruthenium red. Series I and II are from different preparations; cells are in Ca-free medium.

In considering the possibility of a mitochondrial role, it is interesting that some of our Ca^{2+} injections into the apical half of the cell (which contains fewer mitochondria) produced glows in the form of rings, bridged multiple spheres, and so forth, rather than the simple single spherical figures of most of our injections (Fig. 4). After CN treatment, these complex shapes transformed reversibly into simple single spherical ones and spread thus through the cytoplasm (Fig. 4, I). Ruthenium red injection produced a similar transformation, but here reversibility was rare (Fig. 4, II). Perhaps the complex figures reflect space with more sparse sequestering mechanisms. Fittingly also, the restoration of the complex forms and, in general, the return to constrained diffusion is faster after CN washout (1.5 to 2.5 minutes) than the lifting of the constraints after CN application (4 to 10 minutes) (Figs. 1 and 4). Such an asymmetry may be expected from the known mitochondrial property that in vitro Ca uptake stops only after exogenous ATP is sufficiently depleted, but resumes nearly as soon as electron transport (*1*, *9*).

What may be the functional adaptations of this restricted cytoplasmic Ca^{2+} diffusion? In a variety of physiological processes, information about a cell membrane event is thought to be communicated to the cell interior by Ca. For example, Ca is thought to mediate between membrane excitation and myofilament contraction in muscle cells (*10*); between nerve impulse and transmitter release at synaptic nerve terminals (*11*); and between membrane receptor mechanisms and the release of secretory products in gland cells (*12*); and to be involved in the excitation of visual cells (*13*). Thus, on the basis of the present results, we suggest that in some intracellular communications the Ca^{2+} messages may be effectively segregated in the cytosol; that is, the functional domains of Ca^{2+} are so small that several domains may coexist in a cell without message interference; or, stated differently, in cells with fast Ca^{2+} sequestering equipment, cellular constituents may be connected by discrete Ca^{2+} lines of communication. The number of possible lines will depend on the local density of the sequestering machinery. With a mitochondria population as dense as in the basal cell regions here, we suspect that this number is high.

Another corollary concerns the physiology of the cell junctions instrumental in intercellular communication by diffusible molecules (*14*). The permeability of these junctions falls drastically when the $[Ca^{2+}]_i$ at a junction rises above 5×10^{-5} to 8×10^{-5} *M* (*6*, *15*). Nonetheless, the junctional permeability may not be significantly perturbed by segregated cytoplasmic Ca^{2+} signals, as, in fact, has just been shown for the salivary gland cell (*6*). Thus, given the sufficient sequestering capacity, intracellular communication by Ca^{2+} signals and junctional intercellular communication are compatible. Heart and smooth muscle come most readily to mind.

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Minor Salivary Glands as a Major Source of Secretory Immunoglobulin A in the Human Oral Cavity

Abstract. *Secretory immunoglobulin A is the predominant immunoglobulin in labial minor salivary gland secretions. Its mean concentration is four times higher in these secretions than in parotid gland secretion. The minor salivary glands can produce 30 to 35 percent of the immunoglobin A that enters the oral cavity. This, together with the potential accessibility of these glands to antigenic stimulation, suggests that they may be an important source of the immune factors that are involved in the regulation of the microorganisms in the oral environment.*

Saliva may function to regulate microorganisms in the oral environment (*1*). Experiments with human salivary secretions (*2*) and with animal models (*3*) suggest that certain aspects of this control can be mediated by the secretory immune system. Secretory immunoglobulin A (IgA) is the principal functional component of this system and is the predominant immunoglobulin of both whole saliva and individual secretions from the major salivary glands (*4*, *5*). In addition to these major glands, minor salivary glands (MG) are present in all soft tissues of the oral cavity except for the