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A proton-led model of fast calcium waves

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Abstract

Fast (10–30 μm/s) calcium waves can be propagated through all nucleated eukaryotic cells that have been tested as well as certain cell-free extracts. In a widely used model, they are propagated by a reaction–diffusion cycle in which calcium ions diffuse along the outside of endoplasmic reticula and induce their own release from calsequestrin or calreticulin molecules stored within the reticulum's lumen. Here we propose a new tandem wave model in which they are also propagated by a reaction–diffusion cycle within a reticulum's lumen. In this cycle, increases in luminal $[H^+]$ induce proton release from luminal calsequestrin or calreticulin. The released protons diffuse ahead to where they release more protons from these luminal storage proteins. What might be called proton induced proton release. They also raise luminal electropositivity. The resultant luminal waves are coordinated with extrareticular ones by movements of calcium and hydrogen ions through the reticular membrane.

This model makes five testable predictions which include the autorelease of protons in solutions of calsequestrins or calreticulins as well as waves of increased $[H^+]$, of increased $[Ca^{2+}]$ and of more positive voltage within the reticula of whole cells. Moreover, under some conditions, such luminal waves should cross regions without cytosolic ones.

1. Introduction and model

Fast (10–30 μm/s) calcium waves are propagated through cells which vary from fertilizing eggs (where they were discovered) to our brains during epileptic seizures and include skeletal and cardiac muscle cells (which are discussed in the following section) and even include cell-free extracts from pig skeletal muscle [1a]. Within intact cells, they are propagated by calcium induced calcium release or CICR, a reaction–diffusion mechanism in which calcium ions diffuse along the outside of the endoplasmic reticulum or ER and induce their own release from the ER [1b]. However, it has long been conjectured that they are also propagated within the lumen of the ER [2,3]. In these first tandem wave models, the luminal mechanism involves the direct autorelease of calcium from calsequestrin or of calreticulin. However, no such mechanism has been found. Calcium increases in the physiological range do indeed induce conformational changes in calsequestrin [4–6] as well as in calreticulin [7]; however, they do not directly induce calcium release from these luminal storage proteins.

So here I propose a new model in which luminal *hydrogen* ions induce calcium-releasing as well as proton-releasing conformational changes in them. The protons that are thus released swiftly diffuse ahead within the lumen to induce further proton release as well as calcium release into the lumen. What one could call proton induced proton release or PIPR by analogy with CICR. All this is speeded by a transient rise in electropositivity within the lumen—a voltage change that is conventionally called hyperpolarization [8] Coordination with the extrareticular cycle is brought about by calcium leakage from the lumen into the cytosol as well as proton leakage from the cytosol into the lumen (Fig. 1). This model is suggested by evidence of an early fall of pH in the cytosol (which suggests an early rise of $[H^+]$ within the lumen), by evidence that calsequestrin can be induced to release calcium by hydrogen ions, by evidence of hydrogen ions' ultrahigh diffusion constant, by evidence that luminal calcium can rise swiftly by release from calsequestrin, by evidence that calcium release channels in the reticulum can be opened by luminal electropositivity and by evidence that calcium ion release into the lumen leads to rapid release into the cytosol.

Evidence of an early fall of cytosolic pH is provided by a 1976 report from David Epel's laboratory in Pacific Grove [11]. Fig. 5 of this report—which is reproduced in our Fig. 2a—shows the pH in homogenates of sea urchin eggs

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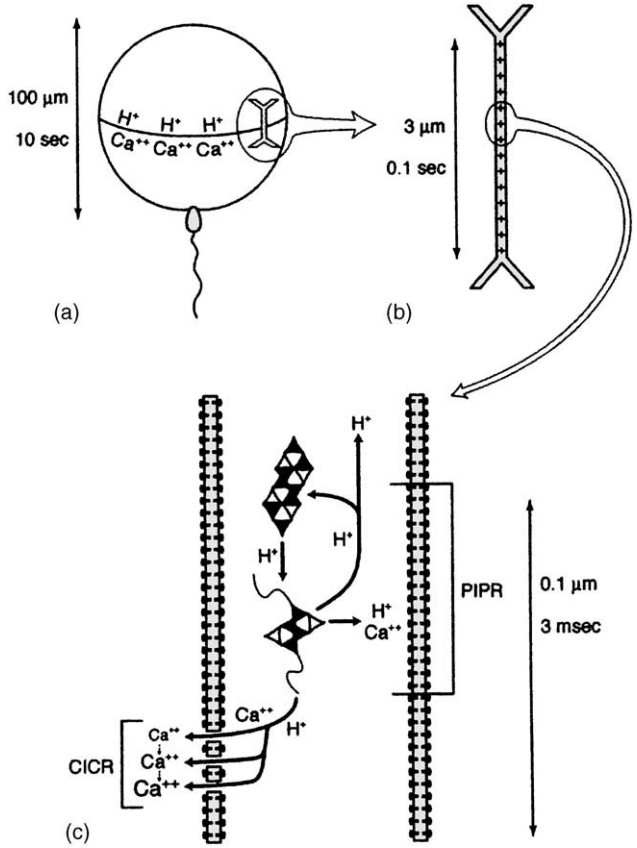

Fig. 1. A proton-led model of fast calcium waves. (a) As seen traversing a whole sea urchin egg during fertilization—from the sperm fusion point below to its antipode above. Protons within its reticulum lead the way. Intra and then extrareticular protons and calcium ions soon follow. (b) As seen at a 30-fold higher magnification between reticular branch points in the egg's cortex. The distance between such points is shown as 3 µm since this is the value seen in the ER of the cortices of live sea urchin eggs [9]. Moreover, the distances between SR branch points are comparable as reconstructed from electron micrographs (see plate #50 of reference [10]). (c) As seen at a thousand-fold higher magnification in the leading edge of the reticulum. The images of the ion-releasing macromolecular oligimers represents calsequestrin as shown in Fig. 6 of reference [6]. CICR propagates the wave outside of the reticulum while PIPR propagates it within the reticulum.

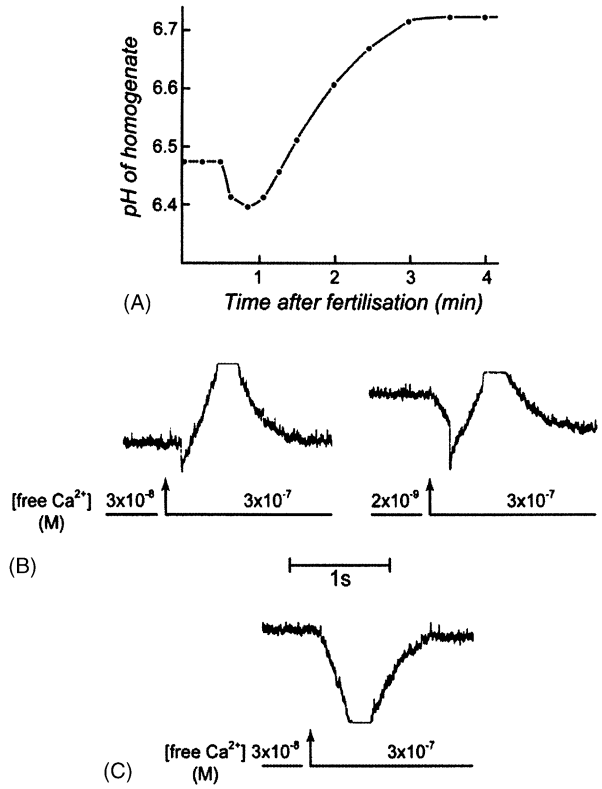

Fig. 2. Some old observations that may be best explained by the proton-led model of fast calcium waves. (A) The pH of sea urchin egg homogenates at various times after insemination. Note the early and abrupt fall in pH. The far better studied, subsequent rise in pH did not start until a minute later which was well after the waves should have crossed the eggs (modified from [11]). (B) Changes in SR membrane potential in skinned cardiac cells after the external calcium concentration was raised to 0.3 µM observed via the voltage sensitive dye NK 2367 on the left and di-S-C 3 (5) on the right. The abrupt early falls in the signal indicate luminal hyperpolarizations which occurred within milliseconds (modified from [23]). (C) Changes in cytosolic free calcium after the external free calcium was likewise raised to 0.3 µM. Observed via the calcium reporting absorption dye, arsenazo III (likewise modified from [23]).

at various times after fertilization and thus at various times after the initiation of a fast calcium wave. It shows a sharp fall in pH which long precedes the later and better studied as well as far better known rise in pH after fertilization in sea urchin eggs [12]. It is true that the rises were seen with methods—including insertion of electrodes [13,14] and immersion in permeating pH dyes [14]—that could have shown a preceding fall and, in fact, did not. However, these methods were applied with far less temporal resolution than had homogenization and, indeed, with too little to have shown a transient fall.

Evidence for proton-induced release of calcium from calsequestrin and of proton entry to compensate for the resultant calcium release into the cytosol comes from studies of rabbit skeletal muscle in the laboratory of Cecilia Hidalgo and Paulina Donoso in Santiago [15–17]. First, their pioneering study of calsequestrin solutions showed dramatic changes in calsequestrin's conformation as the pH was lowered from 7.0 to 6.0 [15]. Second, their studies of sarcoplasmic reticulum vesicles showed large steady decreases in calsequestrin-bound calcium as the pH within the vesicles was reduced from 8.0 to 5.5. They therefore proposed that luminal protons can induce calcium-releasing "conformational changes in calsequestrin that in turn promote activation of the calcium release channels" [16]. Third, their further studies of sarcoplasmic reticulum vesicles (which also included intravesicular measurements of pH via fluorescence) showed a very rapid entry of protons—one with a half time of about 40 ms—into vesicles as calcium leaves them at what seemed to be physiological ATP levels [17].

The ultrahigh diffusion constant of hydrogen ions in acqeous media is well known. Thus a recent reference work gives a value for the diffusion constant of hydrogen ions

in such media of about 9 cm^2/s which is more than ten times larger than the value for calcium ions [18]. Moreover, the value for D_{H+} within the ER may well be much higher than 9 cm^2/s as a consequence of surface conductance along calsequestrin molecules which are known to have a very high concentration of negative charges at their surface [19] and to be extensively polymerized in their native state within the sarcoplasmic reticulum [20]. Moreover, calreticulin molecules likewise have such a high concentration of negative charges [21].

Evidence that luminal calcium can rise swiftly and greatly by release from calsequestrin is provided by a 1991 paper from Noriaki Ikemoto's laboratory in Boston [22]. Fig. 7 of this paper shows a massive release of calcium from open pieces of sarcoplasmic reticulum membrane by caffeine addition in tenths of a second. Since the membrane pieces were open, the caffeine could not have acted by opening calcium channels; rather it must have done so via a conformational change in calsequestrin.

Further evidence of a gross, ultrafast rise in luminal calcium is found in a 1982 paper by Alexandre Fabiato [23]. Fig. 6b and 6f (here reproduced in Fig. 2) shows a large change in the voltage of cardiac reticula *in milliseconds*. One can now attribute this abrupt change to a conformational change in calsequestrin which abruptly released calcium ions (as well as hydrogen ions?) into the lumens of the reticula. These in turn would have neutralized negative charges on the inner surfaces of the reticula and thereby produce the observed ultrafast rise in luminal electropositivity. The author rightly called this his 'most important finding'.

Moreover, evidence that the increase in luminal electropositivity induces a rapid flow of calcium from the lumen into the cytosol is provided by comparing Fabiato's Fig. 6c (also shown in Fig. 2) with his Fig. 6b. It shows a rapid rise in myoplasmic calcium via the absrption dye, arsenazo III and one can reasonably infer that the swift voltage change acted by opening voltage-sensitive reticular calcium channels within tens of milliseconds.

It is also true that a 1973 study of a toad skeletal muscle preparation showed that making the lumen electronegative induces gross calcium release from the reticulum [24]. However, these old observations had a temporal resolution of less than a *second*; so one can now reasonably interpret them as reflecting calcium reentry into the reticulum which in turn induced its own release.

I should also point out that the predicted ultrafast rises in luminal electropositivity or hyperpolarization would be expected to induce action potentials along the reticula that would be somewhat comparable to those known to move along 0.1–0.2 μm thick nerves or nerve nets since these are about as thin as reticula [25]. However, one would expect them to be propagated by the voltage-sensitive chloride channels which are found in reticula of skeletal muscles [26] rather than voltage-sensitive calcium channels. Moreover, such action potentials would only be expected to move along the 3–5 μm long sections of reticula between branch points.

2. How to test this model

2.1. How to look for the predicted induction of proton release by protons

The idea would be to add a known amount of acid to an unbuffered calsequestrin or calreticulin solution and to see if additional acid is released. This should be practical if enough calsequestrin and/or calreticulin could be obtained. The somewhat unstable calsequestrin is not commercially available. However, Chul-Hee Kang's laboratory in Pullman could make enough if the test chamber could be sufficiently miniaturized. This, in turn, should be possible with the aid of the miniaturized pH/reference electrodes that are sold by World Precision Instruments and which are only 0.7 mm in diameter; while calreticulin can be obtained from Professor Marek Michalak at the University of Alberta in Edmonton, Canada.

2.2. How to image the predicted wave of pH reduction within the reticular lumen

In doing this—as well as other efforts to image fast, 30 μm/s waves—one would have the great advantage that such waves have been seen in an extremely wide variety of cells [1]. Having chosen a cell, one would then have to find a calcium-insensitive indicator of pH within the reticular lumen that can be imaged. The predicted pH wave would be in the 4.5–6.0 range that is typical of intracellular compartments like lysosomes. So one could use so-called lysosensor dyes which increasingly fluoresce when pH's fall to such levels. Precedents for the use of such dyes to indicate steady pHs in this range within small intracellular compartments have been reported in the lysosomes of Chinese Hamster Ovary cells [27] and in the 0.1 μm interface found between mycorrhizal fungi and plant root cells [28].

2.3. How to image the predicted wave of free calcium increase within the lumen

Ideally, one would introduce appropriate aequorins into the reticular lumens. For these luminescent indicators have been widely used to image fast waves in the extrareticular space, are unaffected by pH or voltage, involve no background signals, are not calcium buffers, yield signals that rise with about the 2.5 power of free calcium [29] and have been introduced into the reticular lumen by genetic means in a variety of cells [30].

2.4. How to image the predicted voltage wave within the lumen

This may be best accomplished by injecting the widely used voltage sensitive dye, merocyanine oxazolone or NK-2367 and then looking for waves of absorption. For this was the dye that was used by Alexandre Fabiato to obtain

the results shown in Fig. 2. It was first used by the Fabiato's to study voltages in the sarcoplasmic reticulum of rat ventricles [31] and has been effectively used as an absorption dye to indicate membrane potentials in systems that vary from Xenopus muscle reticula [32] to developing chick hearts [33] and adult squid axons [34]. Reticular voltages seen via NK-2367 absorption waves should be distinguishable from voltage waves among mitochondria or along the plasmalemma simply by their patterns in space. As Fig. 2 indicates, absorption by NK2637 can show changes that happen in microseconds [23,34].

Moreover, NK-2637's use as an absorption dye should involve minimal disturbance and minimal confusion by non-dye absorption or by light scattering since it only involves the use of weak red light. Used to image possible waves, it could be used at a single wavelength of maximal response—namely 720 or 670 nm (26). Effective use could be made of the fact that this dye's absorption is far stronger when the light is polarized parallel to the membranes which contain it than light that is polarized perpendicular to them [35]. Moreover, NK-2367 is available from Molecular Probes.

2.5. How to look for the predicted luminal wave in the absence of an extrareticular one

Our model suggests that a 10–30 μm/s wave might traverse a cellular region in which an extrareticular calcium wave was blocked with a calcium buffer. After steps 2–4 were completed, one could plan experiments in which one tested the effects of locally injecting such buffers into the cytosol. Large cells such as fish eggs should be favorable ones for such experiments.

3. Conclusions

Fast calcium waves are propagated by endoplasmic reticula and it is widely understood that crystallography, electron microscopy etc. have radically increased the available spatial resolution of their overall structure and of their macromolecular components. However, the literature also contains some remarkable observations which provide ultrahigh temporal resolution of reticular actions. Indicators which enable one to begin thinking in the millisecond rather than the second to minute range. Such high speed data are critical for understanding the mechanisms of fast calcium wave propagation since a 30 μm/s wave moves 70 nm (the reticulum's internal diameter) in only two milliseconds. When these fast phenomena *are* considered, one is naturally led to consider models which are led by proton diffusion and this paper proposes the first such model.

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