Membranes, calcium, and coupling[1]

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Every eukaryotic cell contains systems linking the extracellular space and internal membrane compartments. These systems allow cells to communicate and, ultimately they allow the nervous system to control most of the cytoplasmic activity. In skeletal muscle, this system is called "excitation–contraction coupling." While much is known of the early and late steps in coupling, the critical link between the cell (i.e., here the T system) membrane and sarcoplasmic reticulum membrane is not known. Electrical coupling cannot easily account for experimental results; here we show that the Ca^{2+} influx is not causally related to the excitation–contraction coupling. The most likely mechanism seems to be a variant of the "remote control model" in which a voltage change and accompanying charge movement in the T membrane activates an enzyme tethered to the cytoplasmic leaflet of the T membrane but spanning part of the T – sarcoplasmic reticulum gap.

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Chaque cellule eucaryotique contient des systèmes reliant l'espace extracellulaire et des compartiments de la membrane interne. Ces systèmes permettent aux cellules de communiquer et (enfin) au système nerveux de contrôler la majorité de l'activité cytoplasmique. Dans le muscle squelettique, ce système est appelé « couplage excitation–contraction ». Alors que les premières et dernières phases du couplage sont bien définies, on ignore quel est le lien crucial entre la membrane cellulaire (c.-à-d. ici, le système T) et la membrane du réticulum sarcoplasmique. Le couplage électrique ne peut facilement expliquer les résultats expérimentaux; dans cette étude-ci, nous montrons que l'influx de Ca^{2+} n'a pas de lien causal avec le couplage excitation–contraction. Le mécanisme le plus plausible semble être une variante du « modèle de contrôle à distance » dans lequel une variation de tension et son mouvement de charge accompagnateur dans la membrane T active une enzyme fixée au feuillet cytoplasmique de la membrane T, mais traversant une partie de l'interstice T – réticulum sarcoplasmique.

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Enzymes cannot function usefully alone; they cannot function dependably in a fluctuating environment. Membranes of the cell keep enzymes together, and the (outer) cell membrane smooths fluctuation of the external environment. The cell membrane is thus an essential functional component of cells, as well as forming its anatomical limit and semantic definition.

Enzymes must not be totally protected from their external environment, however, if they are to acquire substrates or dispose of wastes; and in eukaryotes, or colonies of prokaryotes, cells must communicate. For example, in more complex eukaryotes, the cells of the nervous system acquire information from sensory cells, interact with each other to process that information, and communicate with the output systems that allow the organism to modify its environment. In particular, the nervous system controls the movement of the animal by controlling its musculature, i.e., the cells of its skeletal muscle.

The membrane of cells is then a *selective* barrier to external disturbance. It prevents destructive permeation from outside to inside, but it allows (indeed it must mediate and amplify) the signals to pass ultimately from the nervous system to the cytoplasm that control cell activity. This variety of membrane functions is served, not surprisingly, by a mosaic membrane structure. The membrane of cells includes a variety of systems, nearly organelles, specialized for each function.

In large measure these membrane systems consist of proteins, or moieties of proteins acting together to make a supermolecular machine. It is the task of the basic scientist to determine what these proteins are and how they work, to identify, describe, and understand them. It is the task of the clinical scientist to put this understanding to work by understanding the role of these proteins in disease.

A variety of approaches are used to study membrane proteins. The biochemist tries to identify what is there, starting with individual soluble, or solubilized molecular species, studying their properties as enzymes, synthesizing systems from the membrane proteins available, and hoping the synthetic system matches the natural system of the muscle. The anatomist would define the molecule and its neighbors directly if instruments of infinite resolution were available. With actual instruments, the anatomist can often identify the structures responsible for function and only sometimes (with the help of specific molecular labelling) identify the molecule.

The physiologist takes a more direct approach to function and a more indirect approach to the membrane molecules. The physiologist identifies the membrane *systems* that perform a specific function and studies how they work. The molecular description that gives the best insight into the system is sought. And the modern physiologist expects the molecular description to provide the best insight to a problem, as it has to so many others (Alberts et al. 1983), because evolution can only determine function by modifying gene products and all gene products are proteins (Creighton 1983, pp. 93–131).

Turning more specifically to membranes, we as physiologists have had many successes. The systems governing the action potential of nerve, the radial spread of signalling in skeletal muscle, and transport in simple epithelia were all identified and often well analyzed long before their protein constituents were known. Recently, the physiological method of patch clamp has allowed the study of one membrane protein molecule at a time in its natural environment, with a resolution and sensitivity far beyond that of any chemical technique available today.

The physiological approach in each of these cases emphasized the electrical properties of the system of interest, both for biological and technological reasons. Biologically, a surprisingly large amount of function is fundamentally electrical in nature: the physics relevant to the conduction (if not generation) of the action potential is that of electricity, whether the action potential

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spreads longitudinally (in axons) or radially (in the T system of skeletal muscle). Technologically, electrical signals have been emphasized because they are by far the easiest to study. Indeed, they are so easy to study and their technology is so advanced that essentially any other signal of interest is now converted to voltage (whether as an analog or digital measure of the original signal) at the first opportunity.

The electrical measurement of membrane properties has been used extensively to study the mechanism I am describing today: the mechanism allowing communication of an action potential across one membrane to the (electrically) distant membrane of an intracellular organelle, the sarcoplasmic reticulum (SR).

Excitation–contraction coupling in skeletal muscle

Many workers sought the mechanism of excitation–contraction (EC) coupling (the name coined for the overall process linking action potential to contraction) in the membrane properties of the T wall, reviewed in several articles in Peachey and Adrian (1983). A popular approach was to seek a single electrical property of the T membrane and identify that as the key link of EC coupling. Thus, some workers thought current might flow directly from T system to SR, with the depolarization of the action potential spreading intracellularly as it spreads axonally; other thought that the current carried just by Ca^{2+} ions (i.e., Ca^{2+} influx) might be the link; still others thought that nonlinear capacity current was the key step.

The electrotonic spread of potential (i.e., the flow of nonspecific electrical current) has had a complex history as a candidate for the link between T and SR. A simple spread of current, like that responsible for axonal conduction, seemed implausible, particularly after high resolution impedance measurements of electrical structure showed no sign of the extensive membrane of the SR, even though it is far larger than that of the outer membrane (reviewed in Eisenberg 1983). Later work showed that current flow into the SR can in fact be reconciled with the measured electrical properties of skeletal muscle (Mathias et al. 1980), but only under rather certain stringent conditions, namely, if calcium release from the sarcoplasmic reticulum occurs without net current flow at any time, because the release mechanism is an anti- (or sym-) porter obligatorily swapping Ca^{2+} for ions with a precisely equal but oppositely moving charge. Others have now shown that Ca^{2+} release is not obligatorily coupled to other charge movement (Kitazawa et al. 1984; Huang 1981), and so it would take a model of unappealing and artificial complexity to reconcile electrical measurements and electrical coupling. It is probably safe to conclude that electrical coupling does not occur.

When workers turned to study the nonlinear electrical properties of skeletal muscle, however, they found something close to what they had always sought (Schneider and Chandler 1973): a signal arising across the outer membrane with many of the characteristics of excitation–contraction coupling. This signal, called nonlinear charge movement (or charge movement for short), has been shown to share a wide range of properties, including an unlikely pharmacology (Hui et al. 1984; Frank 1986) with EC coupling, and it must now be considered an essential link, or at least an electrical sign of that link, in the chain of molecular mechanisms that comprise EC coupling.

The relationship of charge movement and the mechanism of coupling remains obscure. The first suggestion was that charge movement is a *direct* sign of the movement of a rigid rod linking T and SR membrane, proportional at any instant to the calcium release of the SR. Although viewed originally with humorous skepticism by admiring colleagues, the hypothesis has grown in stature and is still a central one in the field, whether in its original form (as a rigid mechanical link from membrane to membrane) or in its molecular renaissance. In this appealing version, Franzini-Armstrong and Nunzi (1983) have suggested that the conformational change underlying charge movement might act directly to control the activity of an enzyme located on the cytoplasmic face of the T membrane, in the T–SR gap. Charge movement would directly control the product of this enzyme's catalysis; that product would in turn (in an unspecified way) span the T–SR gap and control Ca^{2+} release from the SR.

Calcium influx in skeletal muscle fibers

An alternative physiological explanation of EC coupling has always involved Ca^{2+} influx across the outer membrane, an influx linked to calcium release from the SR by another mechanism, for example, Ca^{2+}-induced Ca^{2+} release (Fabiato 1985). This hypothesis has had many incarnations, but it is the purpose of the experimental work presented here (previously published by Curtis and Eisenberg 1985) to show how untenable it is in skeletal muscle.

The design and interpretation of any experiment involving a macroscopic piece of membrane proceeds best from a microscopic perspective. We must presume that the membrane is a mosaic of distinct transporting systems, each a protein or moiety of proteins performing a specific function. Each function presumably represents an evolutionary adaptation to the needs of the organism at one time or another. Macroscopic measurements can be interpreted in terms of individual (genetically determined) transport systems *only* if the experimental information far exceeds the number of unknown variables. This situation occurs naturally in membranes dominated by one or two channel types (e.g., axonal membranes and red blood cells) and it is no coincidence that such membranes are examples of successful macroscopic analysis of molecular function. In the far more common situation, where many channel types are present, macroscopic measurements determine distinct molecular transport systems only if specific agonists or antagonists are known. Otherwise, the attempt to analyze such systems into molecular components is underdetermined, leading to a bewildering diversity of interpretations, as numerous as the investigators!

In this context, we turn to classical macroscopic measurements of Ca^{2+} current and flux across the cell membrane of skeletal muscle. These measurements, made before there was any hint of the diversity of channel and transport systems in most membranes, interpret the entire Ca^{2+} current or influx as if it arose in one channel and seek to identify the biological role of that entire influx. Recent voltage clamp experiments have shown clearly that the great majority of Ca^{2+} current does not play a direct role in EC coupling (see the review by Stefani and Chiarandini 1982). Thus, classical measurements of Ca^{2+} influx in the literature are likely to include, even be dominated by, Ca^{2+} movements through channels irrelevant for EC coupling. Unless those channels are blocked, Ca^{2+} influx from the entire membrane could not be used to detect the small component of Ca^{2+} influx that might be the link between action potential and SR Ca^{2+} release.

It seems clear then that an adequate experiment seeking Ca^{2+} influx associated with EC coupling requires a pharmacological agent that blocks irrelevant Ca^{2+} current and a procedure that controls EC coupling, i.e., that allows direct comparison of Ca^{2+} influx in a fiber with and without normal EC coupling. The experiment should best be done on single fibers, if diffusion problems are to be avoided.

For many years, there seemed no way to perform such experiments; but suddenly (and, need I add, fortuitously!) a pharmacological treatment was discovered (Eisenberg et al. 1983) that seemed heaven sent for such experiments. We found that the Ca^{2+} entry blocker D-600 (i.e., methoxyverapamil), when applied in the cold, produced reversible paralysis. After a prolonged soak in the drug, a muscle fiber gives one contracture in response to depolarization by K^+, and this so-called conditioning contracture is indistinguishable from normal. After this contracture, however, the fiber is unable to contract in response to depolarization (although it gives a normal response to caffeine). Any measurement made during the conditioning contracture would be expected to describe a normal fiber with blocked Ca^{2+} channels because voltage clamp measurements (Stefani and Chiarandini 1982) show that D-600 blocks Ca^{2+} current but does not alter contraction under similar conditions.

Paralysis by D-600 is a result of its action on nonlinear charge movement (Hui et al. 1984): after the fiber is exposed to K^+ and the drug, charge movement is blocked and later steps in EC coupling do not occur. If the fiber is simply exposed to the drug without the conditioning K^+, charge movement is unaffected. The reversible paralysis by D-600 thus is a useful tool to control EC coupling. Measurements can be made of the initial response of the contracting fiber to K^+ (in the presence of D-600) and another measurement can be made of the corresponding response of the paralyzed fiber. Subtracting the two responses gives a greatly improved estimate of the contribution of the EC coupling mechanism, particularly compared with classical measurements, because components irrelevant to EC coupling are common to both responses and so they are removed by subtraction (Curtis and Eisenberg 1985, Fig. 9, p. 400). For example, consider measurements of Ca^{2+} influx. The difference measurement is not affected by components of Ca^{2+} influx uncorrelated with EC coupling, if they are the same in both the contracting and paralyzed fiber, even if the uncorrelated components occur in the presence of D-600. The difference record only includes those components of Ca^{2+} influx both correlated with EC coupling and insensitive to D-600.

The strange temperature dependence of D-600 treatment also permits an elegant experimental design: contractility can be restored by warming the fiber, thus allowing bracketed controls of each experimental condition. A fiber can be tested (as just described), warmed, then recooled, and reexamined again in essentially its initial state. Such bracketed controls dramatically reduce variance, give estimates of drift and run-down in each fiber, and allow "used" fibers to be reused, thereby decreasing the demands for painstakingly dissected single fibers. Other workers had studied D-600 under more physiological conditions (see references in Frank 1986) and found many interesting effects, but only protocol like ours allows the reversible control of EC coupling so useful in investigation of mechanism.

With the D-600 treatment in hand, it seemed worthwhile to Brian Curtis and me to revive the rather arduous measurements of tracer influx we had both done as postdoctoral fellows in the laboratory of Paul Horowicz (some 20 years earlier). Fortunately, Brian had his original (plywood) set-up and lives reasonably close by, in Peoria. Just as fortunately, he is a good friend and genial companion who quickly became a house guest welcomed by all the family during his numerous visits to Chicago.

Our basic experimental plan was simple. We would measure Ca^{2+} influx in a fiber exposed to D-600 and K^+ (and thus with little irrelevant influx); we would measure influx again in a paralyzed fiber; finally, we would warm, then cool the fiber and repeat the original measurement. This paradigm was rather more straightforward than its execution, given the substantial amount of isotope needed (nearly 1 mC per fiber) and the concomitant dangers, given the difficulties of cleanly dissecting hundreds of fibers.

The experimental results were at first quite exciting. They showed that Ca^{2+} influx in contracting fibers (exposed to D-600 and thus without irrelevant Ca^{2+} current) was about 1.5 ± 0.4 pmol/(min·fiber) (mean $\pm$ standard error of the mean, 16 averaged experiments) larger than the Ca^{2+} influx in a paralyzed fiber. This averaged result was highly significant statistically but not as convincing as an additional 15 paired experiments, where the difference in influx in one fiber was measured, averaging 0.7 ± 0.1. Thus, we clearly had found a Ca^{2+} influx closely linked to the EC coupling mechanism. And it had the characteristics of the mechanism linking T and SR membranes.

Our initial pleasure soon gave way to an intrinsic and extrinsic anxiety. Intrinsically, we knew the result was inherently implausible because EC coupling in skeletal muscle is well known to be present even if the extracellular solution contains vanishing $[Ca^{2+}]$. Extrinsically, our colleagues and mutual mentor made it clear that all conceivable controls might not suffice to convince. With these pressures we undertook the fairly mindless approach of doing every control we could think of and seeing what turned up.

After many such measurements, which strained the patience of the technician doing the dissections and the safety officer overlooking our irradiation, we planned our last group of measurements. We though it worthwhile to add our isotope to the muscle fiber after it had already been exposed to high K^+, i.e., after it had contracted if it could. We expected to find the same (background) influx of Ca^{2+} in the paralyzed and contracting fibers, showing that noninactivating processes did not contribute to our measurement, reinforcing the relation of the influx and EC coupling.

But that is not what we found. Rather, we found a larger influx in the contracting fiber. In fact, we found that the entire influx of Ca^{2+} (operationally defined by the difference protocol) could be measured even in experiments in which isotope was added after contraction. The influx is tightly associated with EC coupling but occurs after the contracture. It can not be an essential link in the causal chain of mechanisms of EC coupling. The influx might be the result of EC coupling but other experiments show that it is not the result of contraction: neither force generation nor Ca^{2+} release produce the Ca^{2+} influx (Curtis and Eisenberg 1985, pp. 402–403). The flux is very tightly coupled to the EC coupling mechanism and deserves further investigation, but it is not causal.

Dismayed at first by this finding, we later came to realize its significance. While seeking a component of Ca^{2+} influx causally related to EC coupling, we had carefully accounted for all the Ca^{2+} influx of a contracture. None was left to link excitation and Ca^{2+} release from the SR! If a causal Ca^{2+} influx exists, it has to be less than 6 pmol·cm^{-2}·min^{-1} or less than 1 Ca^{2+} ion/s for each foot process of the T–SR junction, a figure far too small to be a reasonable candidate for a reliable mechanism that occurs physiologically in milliseconds.

It seems safe to conclude then that Ca^{2+} influx is not the process that links T and SR membranes.

We also realized what has not yet percolated into the general literature of excitation–secretion coupling: a Ca^{2+} influx can be very tightly associated with coupling and not be the cause of it. Perhaps an internal store has to be refilled (from outside at least

in the conditions of these experiments) before the T–SR system can rest, recover from inactivation, and be ready to initiate the next contracture (Graf and Schatzmann 1984). The experiments necessary to show the causal role of Ca^{2+} influx (i.e., to rule out the tightly coupled noncausal role we had found) are very difficult and have not been done in any of the many other tissues where Ca^{2+} influx is said to be involved in membrane cytoplasmic coupling.

Conclusions

The previous section shows that one can detect no calcium influx causally related to EC coupling in frog skeletal muscle. Are we then left only with the remote control model as a possibility? I would say yes, at least if that model is interpreted broadly (as in Mathias et al. 1980; and Franzini-Armstrong and Nunzi 1983) and not narrowly. A rigid rod seems to me hard to swallow, whether macroscopic or molecular; far more appealing is a molecular mechanism by which charge movement is linked fairly directly (i.e., monotonically) to a Ca^{2+} release channel in the SR membrane. Such a mechanism has the essential feature of the original rigid rod model (a monotonic control of Ca^{2+} release by charge movement) without its molecular implausibility, the requirement for microscopic rigidity in a size domain where flexibility is the rule (Cooper 1980; Ringe and Petsko 1985).

The difficulty with all the hypotheses defined above is that they are essentially functional, they are theories linking function to function, but they have little or no specific molecular basis. In the present molecular era, when so many functions are found to reside in single proteins, or small groups of proteins (Alberts et al. 1983), it is perhaps necessary, and certainly wiser, to use models containing specific molecules with definite roles. In other words the elements of the model should be proteins, enzymes, or even lipids, not processes. Processes are less useful elements of a model because the relation of a process and a protein is not definite. Any model using processes as elements has an uncertain relation to its protein components, a relation that must be determined once the proteins are discovered, no matter how well known the model is itself. The structure of the model as experimentally determined may also depend on the properties assumed for the elements. If these properties do not correspond to the properties of the actual biological elements (e.g., proteins), the entire model may be incorrect, in the sense that it does not describe the actual biological mechanism, even if the model fits the physiological data perfectly.

The molecules at the T–SR junction are just now being defined. It might be sensible to take the biochemical approach, simply waiting until these proteins are available "in gram quantities" and then trying to rebuild the system. It also seems worthwhile, and is certainly in character, to explore molecular speculations, using the general framework of the remote control model. Here I suggest certain principles and then risk speculations.

I suspect all models of excitation–contraction and excitation–secretion coupling will seem naive within a few decades. These mechanisms, so central to the life of the cells in which they reside, are unlikely to be simple chains of reactions. Rather, the cell is more likely to use specialized mechanisms for different aspects of what we now define as a single mechanism. After all, what better signal to use for controlling metabolism (or protein synthesis in general, or the synthesis of specific proteins) than the number of action potentials, or the charge movement signal itself? Thus, I expect that a number of the proteins seen in the T membrane are parts of parallel systems, one protein acting as the sensor of action potentials for the direct path of excitation–contraction coupling, others acting as sensors for each specific long-term (seconds to hours) cell function.

This speculation is not entirely empty, since it suggests the importance of studying the time scale of the coupling mechanism. One must directly show that the putative mechanism occurs on the physiological time scale (Vergara et al. 1985) before it becomes a viable candidate for reality. The direct consideration of the likely complexity of the T membrane has a number of implications, in fact, for the proposed inositil trisphosphate (IP_3) coupling mechanism in which IP_3 is produced in and released from the T membrane to diffuse to the SR membrane, where it initiates Ca^{2+} release. Given the widespread role of the inositol residue as a lipophilic anchor for membrane proteins (Low et al. 1986, albeit so far chiefly for proteins on the external side of the membrane), one must expect turnover of inositol if any anchored proteins are cut loose (become "deanchored") during EC coupling or contraction, even on a slow (i.e., seconds) time scale.

Indeed, these anchored proteins may be a plausible representation of some of the proteins in the T–SR junction. Perhaps a protein exists in the T–SR junction bound by its inositol residue to the T membrane, nearly invisible in the resting state, appearing as a pillar once it is activated by nonlinear charge movement in a sensor protein (coupled to the inositol residue) in the T membrane. My colleague Eduardo Rios has suggested that the sensor may be a Ca^{2+} channel, slightly modified so it does not normally conduct much current or flux. My colleague and wife Brenda and I have suggested that the appearance of pillars may reflect activation, although this questionable suggestion (Fig. 11, Eisenberg and Eisenberg 1982) certainly deserves the questioning it has received. Fabiato (1985) has made a strong case that the Ca^{2+}-induced Ca^{2+} release process (as observed on a millisecond time scale) has the appropriate characteristics for the final release step from the SR. What is missing then from this speculative composite is even a guess concerning the link between the activated "enzyme" of the T–SR junction and the Ca^{2+}-induced Ca^{2+} releasor of the SR.

This is embarrassingly close to our situation nearly 15 years ago, except we know now what we did not know in much greater detail.

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