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EXCITATION-CONTRACTION COUPLING IN SKELETAL MUSCLE[1]

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I. INTRODUCTION

Excitation-contraction (E-C) coupling is the function of the muscle fiber in which an electrical depolarization of the plasma membrane initiates a sequence of reactions that causes mechanical activation of the contractile myofibrils lying within the membrane. By far the greatest amount of work on this function has been done on fast skeletal muscle fibers. In this type of muscle, the membrane electrical change is the action potential, and present evidence indicates that this gives rise to contraction by a sequence of at least two main processes. Firstly, the action potential generates a signal, probably also electrical in nature, which is conducted inwardly by the internal membranous structures of the T system,

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and which, in effect, rapidly transforms the depolarization at the plasma membrane into an equivalent change in close proximity to the myofibrils; secondly, this signal causes release from internal stores within the sarcoplasmic reticulum of a chemical agent, very likely the calcium ion, which directly activates contraction in the myofibrils.

In this review I will be mainly concerned with these mechanisms in skeletal muscle fibers. Although E-C coupling in cardiac and smooth muscle is in some respects similar to that of skeletal muscle, there are also basic differences. But there will be no detailed coverage of such questions, and references to these other types of muscle will be quite peripheral to the discussion of skeletal muscle. In any case, various aspects of E-C coupling are discussed elsewhere for skeletal muscle (29, 135, 176, 177), for heart (18, 32, 33, 76, 191, 260, 262), and for smooth muscle (28, 249), and it will be profitable for the interested reader to refer to these publications.[2]

Throughout the discussion of our subject, I shall use the term "excitation-contraction coupling" in the very broad fashion previously defined. Thus, it will naturally apply to cases in which the membrane depolarization is caused by the action potential, and in which, therefore, the fiber undergoes excitation and contraction in the sense that these responses occur in a twitch or a tetanus. But I will use E-C coupling also to refer to the coupling sequence that occurs in contractures in which depolarization results from the endplate potentials associated with the activity of slow neuromuscular systems or from the action of increased external K^+ concentration. It has become customary to use the term, excitation-contraction coupling, to refer to all of these means for evoking a mechanical response, and this usage will be continued here. This practice evidently reflects the viewpoint that the twitch type of excitation-contraction is by far the more usual kind of activity of muscle, and that in a contracture the pertinent features, that is, the membrane depolarization and coupled mechanical activation, are in essence similar to those that occur in a contraction. When clarity and unambiguous generality are desired, however, I shall use the term "electromechanical coupling," as this obviously holds for all cases in which an electrical change at the membrane elicits a mechanical response of the contractile system.

II. ELECTROMECHANICAL FACTORS

A. General role of the action potential

When a single effective stimulus is applied to a fast muscle fiber, the ensuing reactions of E-C coupling begin with an action potential and end with the twitch mechanical response. In the study of E-C coupling in the physiologically intact living muscle, it is, in fact, only these two phenomena that ordinarily can be directly and easily recorded, and it is of interest to see what can be learned about the role of the action potential in the initiation and regulation of contraction under such living, though experimentally restricted, conditions. Analyses of this

[2] It will also be of interest that, at the time this review is being submitted, a symposium is in preparation on Excitation-Contraction Coupling in Striated Muscle under the chairmanship of Dr. Richard J. Podolsky for presentation at the 1965 meeting of the American Physiological Society and for subsequent publication in *Federation Proceedings*.

sort have already been made in several reviews (39, 69, 225, 226) and a general conclusion is that at any excited spot of a fiber, *e.g.*, in a frog skeletal muscle, the rise of the action potential spike precedes the onset of the mechanical response (as evidenced by the first sign of tension that appears during the latency relaxation) by an interval of about 2.0 milliseconds at room temperature. It is this precedence in time of the spike that, even in the absence of other findings to be mentioned later, justifies the assumption that the spike is indeed the key event of excitation initiating or "triggering" the E-C coupling sequence. The spike, however, lasts only a millisecond or two, and, as interestingly discussed by Falk (69), this raises the very important question as to the mechanism by which a depolarization of the membrane of such brevity determines a mechanical response which appears only after the indicated latency and which, moreover, lasts many times longer than the spike whether it is measured by the duration of the twitch, as such, or by its active state. Indeed, it is this sort of temporal disparity that led some of the early workers on muscle to doubt whether the "action current" had anything to do with the activation of tension development [for review see Fulton (95), Chap. 8]. As the following discussion brings out, however, there is no question that the spike does initiate E-C coupling. And, as for the mentioned time differences, evidence given later indicates that they reflect the kinetics of the processes that are set in motion by the spike and serve to link it to activation of contraction.

Under usual conditions of excitation of a muscle fiber, the action potential is a self-propagated disturbance and therefore produces not only a wave of depolarization of the membrane, but also a complex diphasic wave of longitudinal current within the fiber. I discuss in the following the possible role of each of these electrical changes in E-C coupling.

B. Membrane depolarization: fast fibers

Since the depolarization of the membrane effected by the action potential cannot be easily varied and is not in synchrony with the contractile response, it is in effect impossible to study directly the role of the degree of depolarization in the E-C coupling of a twitch (but see 61). It is possible, however, to study the effect of gradation of depolarization in producing contractures, and, since there is every reason to believe that such mechanical responses are essentially the same as contractions, we can apply the information gained by these means to the elucidation of the electromechanical relations of the twitch.

Crude studies of this sort were being made during the latter part of the last century. And as reviewed as early as 1895 by Biedermann in his voluminous book on electrophysiology (26), it was clear from both his own experiments and those of others that sufficient depolarization of a muscle causes contraction, while repolarization reverses contraction, *i.e.*, causes relaxation. Subsequently, further work was done on these problems and the developing status of knowledge in this field can be followed by reading the reviews of Gasser (96), Kruger (160), Fleckenstein (75)—who deals mainly with his own extensive research in this area—and Sandow (225, 226).

With certain outstanding exceptions to be discussed later (155, 161) this older

work was very limited in that it did not provide a quantitative correlation between membrane potential and tension output. This is indicated especially in the important segment of this work concerned with potassium depolarization contractures. Ordinary external electrode techniques were used and therefore true membrane potentials could not be measured. Furthermore, the tension output was measured on whole muscles such as the sartorius or the gastrocnemius of the frog and thus could not possibly reflect the true mechanical behavior at the single fiber level. For, in such procedures there is a great disparity between the time of diffusion of the K^+ into the depth of the muscle, which even for the thin sartorius takes about 10 minutes for completion (231a), and the time course of the contracture of a single fiber, which runs to completion in about as many seconds (124). Thus, as a muscle reacts to immersion in a K-enriched medium, the surface fibers complete their response much before the deeper ones have been even reached by the K. Hence the curve for the tension output for the whole muscle is a greatly distorted account of what occurs in any one fiber and so it is wholly inadequate for relating mechanical output to electrical change. Nonetheless, such work produced the very important result that the application of a steady depolarization to a muscle fiber (*e.g.*, by means of a given concentration of K^+) causes an automatically reversed tension output in the fast (or phasic) muscle fiber, but a prolonged output in the slow (or tonic) fiber, which does not reverse until the depolarization is removed either by restoring the muscle to Ringer solution or by applying to it a repolarizing electric current. In view of this difference, it is clear that the older findings about contractures of tonic muscles, especially as they deal with fully developed states, retain a certain degree of validity even today.

Our present basic knowledge of electromechanical coupling has been obtained from experiments in which the electrical changes were monitored, for the most part, with the internal electrode; and the mechanical output was determined on either single fibers or small bundles of them, so that especially in the studies of K-depolarization contractures there was negligible diffusion delay in establishing contact of the K ions with the surface of the muscle fibers and thus in altering the potential difference across the membrane. Such pioneering studies on slow fibers by Katz and Kuffler (155) and on fast fibers by Kuffler (161) will be discussed in the following. Kuffler's work, reflecting the general development of technique of a generation ago, still used external electrodes for electrical recording; but important technical improvements were introduced by directly applying contracture-producing substances to restricted regions of a muscle fiber (or a small bundle of them) and by microscopically observing the resultant mechanical changes evoked at such regions. This work has been reviewed elsewhere (226); but it is worth noting here that Kuffler for the first time raised the question whether the coupling of a membrane depolarization to the activation of the contractile material involves the flow of intrafibrillar electrical currents, especially as they appear as action currents in the excited and contracting fiber, and that he concluded that such currents do not provide the link between excitation and contraction (see also 154).

The definitive research relating tension output to change in membrane potential was initiated by Hodgkin and Horowicz (124) in their work on K-depolarization contractures of single frog muscle fibers. They used the internal electrode for measuring transmembrane potentials and a special method for very rapid change of solutions so that an entire fiber could be completely exposed to a new medium, *e.g.*, of altered K content, in about 0.3 second. Hodgkin and Horowicz found that, as the single fiber was exposed to a series of solutions of increasing K concentration and thus was increasingly depolarized from its resting internal membrane potential of about −90 mV, a minimal contraction first appeared at a membrane potential of about −50 mV (the mechanical threshold). The curve for peak tension output *versus* membrane potential then greatly increased along a steep S-shaped curve as the potential was reduced further, so that practically maximal output was developed at −46 mV. Unfortunately, the results reported in both this paper and in another one by Hodgkin and Horowicz (125) did not exactly determine the membrane potential at which the full maximum of tension is produced. From the results given, however, the maximal tension output seems to occur when the membrane is not quite fully depolarized, and we may take −20 mV as a tentative value of the membrane potential that presumably saturates the mechanism by which depolarization produces tension output. In any case, the maximal output was surprisingly high, the average being about 3.7 kg/cm^2 and thus some 10% greater than that produced by the single fiber in a maximal tetanus in normal Ringer solution. Similar results have been obtained by Frank (80) on very thin bundles (diameter less than 0.5 mm) of fibers (obtained either in the form of the intact extensor longus digiti IV muscle of the frog, or by dissection from a sartorius muscle), and by Edwards *et al.* (62) on the very large single muscle fiber of the barnacle.

Presumably the electromechanical relations described in the foregoing for contractures, may be used to delineate the role of the action potential in the activation of the mechanical output of a twitch. Thus, the action potential must become effective in activating the links that cause contraction as soon as it depolarizes the membrane to the level of the mechanical threshold, *i.e.*, about −50 mV. And as the action potential continues to rise and then falls, it should continue to engender mechanical activation for as long as it maintains depolarization of the membrane beyond the mechanical threshold. At room temperature, this mechanically effective period of the action potential lasts for about 1.5 milliseconds. Clearly, certain portions of the action potential do not have any mechanical effect. Thus, the early part of the action potential before the mechanical threshold is reached, which includes the electrotonic changes up to about −65 to −60 mV (149, 252) and some 10 mV further depolarization due to the earliest phase of the spike itself, is without mechanical effect; and this is proved, insofar as the electrotonic changes are concerned, by the fact that no shock can evoke a mechanical response from a normal fast muscle fiber unless it is able to cause excitation. Furthermore, the negative afterpotential is likewise without mechanical consequence since it runs its course through a range of potentials—from

about −65 to −70 mV, where it has onset, to the resting value of −90 mV—which is at all times below mechanical threshold.

During a major portion of the mechanically effective period of the action potential the membrane potential transiently takes values considerably beyond the level of about −20 mV at which depolarization contractures are supposed to involve maximal tension output. The question therefore arises whether the series of membrane potentials corresponding to the swing of the action potential from −20 mV to crest of overshoot at about +30 mV and back again to −20 mV should be considered as supramaximal, and therefore a sort of safety factor, in the generation of the processes that finally cause tension output. A positive answer to this question had been reported (225) on the basis of a series of results indicating that the spike potential could be considerably reduced by either slightly increasing the external concentration of K or decreasing the external Na, without diminishing the twitch tension, and, in fact, even possibly increasing it. In this work, however, the action potentials were recorded diphasically; and the choline used to compensate for the sodium deficiency has since been found not to be an indifferent substance but to potentiate the twitch (63). Furthermore, it is now known that various agents alter the level of the mechanical threshold and thus change the twitch output (68, 125, 234). For these reasons the earlier conclusion that the action potential normally initiates E-C coupling with a considerable safety factor is not so clear, and further work is needed to clear up this point. In any case, as discussed in detail in Sections VI C and VII, the efficacy of the action potential in eliciting the mechanical response is dependent on its duration, as well as on its magnitude and the mechanical threshold, so that the question of a "safety factor" is a more complex problem than originally throught.

The question may be raised whether it is valid to base a description of the electromechanical relations of the twitch on those that have been determined for K-depolarization contractures. In particular: does a given increase in K concentration produce only an electrical effect, *i.e.*, a depolarization, or does it also cause other changes that might independently influence the processes involved in activating contraction. Increased concentrations of K do cause striking metabolic effects on muscle fibers, as is shown by the great output in heat (118, 242, 243) and the marked increase in respiration and other metabolic reactions (76, 109, 156). The mechanism by which K causes these effects is not known, but, as stressed by Hill and Howarth (118), it is most likely that depolarization of the membrane is the essential trigger. It is noteworthy that the heat output, at least, can be activated by concentrations of K that are too low to cause a mechanical response, and that, in any case, the kinetics of the heat output and that of the contracture are entirely different. Thus, it is unlikely that the various metabolic reactions evoked by the depolarizing action of K are directly involved in the activation of the contracture. More positive indications that the electromotive effect is the critical factor in generation of K-contractures may be found in the counteraction of such contractures by the repolarizing action of an anodal current even though the increased K concentration persists, and in the contracture that can be produced purely electrically by an outward transmembrane current provided it depolarizes the membrane beyond the mechanical threshold (197, 252).

C. *Membrane depolarization: slow fibers*

True slow fibers are particularly interesting because their normal responses in the body are typically contractures. Such fibers are not present in mammals, but are found in amphibia and arthropods, where they are innervated by special nerve fibers and are organized into anatomically and functionally distinct slow neuromuscular systems. Nerve stimulation does not generally evoke action potentials and associated twitches in such slow muscles, but instead it produces at the neuromuscular junctions, local nonpropagated depolarizations which activate contractures. The magnitude of the depolarization is in general proportional to the frequency and the duration of a train of nerve impulses affecting a particular junction. Slow muscle fibers have multiple innervations and thus these junctional potentials and their resultant contractures can appear along the whole length of the muscle fiber. Katz and Kuffler (155), working with arthropod slow systems, and Kuffler and Vaughan Williams (162, 163), with frog systems, determined the effects of neurally evoked depolarizations on the mechanical responses of the respective slow muscle fibers. They obtained essentially identical results for the two types of slow fibers; the data showed that the degree of mechanical activation is proportional to the extent of depolarization. The two types differed, however, in that a clearly defined mechanical threshold found in the arthropod slow fibers is absent in the amphibian fibers. Certain exceptional features of arthropod neuromuscular responses were discussed by Hoyle and Wiersma (130) and Atwood (7).

A study on slow fibers subjected to transmembrane electric currents has been made by Orkand (197) on single fibers of the *contractor epimeralis* muscle of crayfish. This is a very favorable preparation because the fibers are only about 5 mm in length and have a space constant which may be as much as 2.9 mm. A current injected into the interior of such a fiber by a microelectrode will therefore spread out lengthwise in passing outward into the external medium so that the entire membrane will be fairly uniformly subjected to the flow of current and hence to changes in polarization. These crab fibers are mechanically activated by these graded, purely electrotonic membrane changes applied to the whole fiber's membrane, and this permits precise investigation of the effects of electrically produced membrane depolarization on the contractile material. The resting potential of these fibers is about −80 mV and the mechanical threshold is close to −60 mV. As the potential is reduced still further, the tension output increases very steeply, but, for technical reasons, Orkand was unable to determine the membrane potential which evokes maximal contractile output. These results are in general like those characteristic of K-depolarization contractures in fast frog fibers. But Orkand's research also showed the following: (1) Hyperpolarizing currents never activated contraction, even when 20 times as intense as threshold depolarizing currents, but they caused relaxation when applied to an already contracted fiber. (2) Depolarizing currents activated tension output in relation to the new level of membrane potential and not to the change in potential required to attain this level. (3) During a given depolarization the tension output, in general, continued to increase with time; and the rate of increase was greater

the lower the membrane potential, *i.e.*, the farther removed the membrane was initially from mechanical threshold. (4) Transmembrane and internal current, as such, had no independent ability to stimulate tension production, but did so only insofar as they depolarized the membrane to a new level of potential. (5) In some fibers, a brief, sufficiently intense depolarization elicited an action potential which could be graded with stimulus intensity. The excitation threshold for such active responses, however, was always at a very much smaller membrane potential than that for mechanical threshold, and thus would not ordinarily be reached until the membrane had passed through a series of potentials capable of arousing a contracture response of considerable magnitude. These various features of electromechanical coupling in the slow muscle fibers permit them to develop and use graded mechanical responses in the normal course of function in the intact animal. Obviously the absence of twitches, with dependence on contractures instead, establishes very clearly for slow fibers the general mechanism that depolarization of the membrane is the key membrane event that starts the train of processes finally causing normal activation of contraction. It is odd, however, that crayfish fibers under special experimental conditions producing Cl-excitatory activation, instead of the usual Na- and K-activation, develop spontaneous random contractures evidently in consequence of associated hyperpolarization of the membrane (personal communication from Dr. H. Grundfest). These contractures are attributed to special ion fluxes (98) which are supposed to activate contraction in general (see section III D). For further general information on slow muscles, refer to the review by Peachey (205a). Sugi and Kosaka (246) discussed interesting features of the electromechanical coupling in summated contractions of crayfish muscle. And specialized analyses of work on electromechanical coupling will be found for heart muscle in the reviews by Brady (32) and Fleckenstein (76), and for smooth muscle in the articles by Axelsson (9), Hurwitz (130a), and Timms (249).

D. The question of internal currents

We turn now to the previously mentioned problem of the role of internal electric currents in electromechanical coupling. During propagation, the action potential produces in a given cross section of the muscle fiber a longitudinal current, $i_{long.} = (1/r_i)\ \partial V/\partial x = (1/r_i u)\ \partial V/\partial t$, where r_i = the resistance per unit length of the internal medium of the fiber, u = velocity of conduction of the action potential, and $\partial V/\partial x$ and $\partial V/\partial t$ are the rates of change of the action potential, V, with distance, x (at a given instant), or with time, t (at a given point), respectively (153). From the way in which the derivatives of V enter into these expressions, it is evident that $i_{long.}$ flows in a given direction during the rise of the action potential and then in the reverse direction during the fall of the potential. Knowing the values of r_i and V in the above expressions, and determining the values of the derivative corresponding to the greatest rates of change of the action potential, one can calculate that, under typical conditions, the action potential produces a maximal, transient internal longitudinal electric field of about 3 V/cm with which is associated a longitudinal current density of about 15×10^{-3} A/cm², or, equivalently, about 10^{-6} A in each typical fiber.

Since the myofibrils are embedded in the medium carrying these electric disturbances, it is reasonable to suppose that the internal electrical changes, and not the depolarization in the spatially separated membrane, are responsible for activating contraction. This concept has been developed mathematically by Bay *et al.* (13) in their "window field" theory; and a new facet was then added to their supposition by Csapo and his co-workers (45, 48, 49) by the suggestion that the longitudinal current cannot alone activate contraction but requires depolarization of the membrane as a "priming step." The experimental tests of this theory have been made by subjecting various types of frog muscle preparations [sartorius muscles, Csapo and Suzuki (49); toe muscles, Sten-Knudsen (244); single fibers, Sten-Knudsen (245)] to externally originating longitudinal electric fields. Observations were then made to determine whether contraction occurs predominantly at the ends of the preparation—where the current passes through the membrane and can therefore, at the cathodal region, depolarize it; or in the middle region—where there should be internal as well as external longitudinal currents, which are, however, equipotential and therefore incapable of causing any change in membrane polarization. Membrane depolarization could also be introduced in the middle region by increasing the external K concentration. The tests of the theory, both in its original and modified forms, are not unequivocal: the results of Csapo and his associates confirmed the theory but those of Sten-Knudsen (244, 245) contradicted it.

There is not much point in attempting here a detailed analysis of these controversial results, especially as the experiments and their theoretical aspects are quite complex and cannot adequately be discussed briefly. The explanation of these conflicting findings may be found partly in Sten-Knudsen's (245) claim that some of Csapo and Suzuki's (49) critical experiments were done with currents that were too intense and therefore injured the fibers. But it is also important that while Csapo's work was done mainly on the relatively large, whole sartorius muscle, Sten-Knudsen's involved the very thin toe muscle or single fibers. This is an important difference, for only in the case of the single fiber preparation can cable theory be applied rigorously to the problem of distribution of current flow through muscle fibers. Such a theoretical analysis was made by Sten-Knudsen (245), and the fact that his results confirm his predictions lends credibility to his conclusion that longitudinal currents are not critically involved in the coupling of excitation to contraction. Meanwhile, the problem of the role of internal currents has been attacked in other ways. Thus, Hagiwara and Watanabe (100) and Watanabe (252) directly applied longitudinal currents inside the intact living fiber by means of two internal microelectrodes and found no mechanical response was elicited by currents as high as 2×10^{-6} A in normally excitable fibers, or even 3×10^{-6} A in fibers made inexcitable by procaine treatment. Natori and Isojima (191) found, however, that denuded living myofibrils [the Natori preparation (190)] can be made to contract when stimulated by directly applied electric shocks. It is also interesting that the rate of ATP-induced contraction of glycerinated muscle fibers can be somewhat increased by application of electric shocks. It is difficult to assess these results since in neither case is the value of the current given. But from the reported conditions of Natori

and Isojima (191) that a threshold effect was obtained by applying a 1.5-V shock with electrodes 1 mm apart, and the assumptions that this potential difference existed along a cylinder of myoplasm of 100-μ diameter and specific resistance of 200 ohm cm (72, 153), it can then be calculated that the total current was about 10^{-5} A, and thus some 3 to 5 times greater than that used at maximum by Watanabe. Watanabe's negative result may therefore be explained by assuming that his currents were below threshold for activation of contraction.

Normal mechanical responses can be elicited by procedures that undoubtedly depolarize the fiber membrane but do not produce internal longitudinal currents. One such procedure is massive stimulation of muscle fibers (38, 225), especially if the shock strength exceeds the highest threshold that exists along the length of a fiber and in this way throws all longitudinal elements into excitation simultaneously. Under these conditions the action potential is synchronous at all points and therefore involves no lengthwise differences of potential and hence no longitudinal currents. Nonetheless, there is activation of an essentially normal contraction (225, 226).

Another instance of this general sort is found in K-depolarization contractures (124, 161). Csapo and Suzuki (49), however, questioned whether there may not be longitudinal currents during the time in which the change in K concentration is effected. But, quoting Hodgkin and Horowicz (124): "There might be small longitudinal currents during the fraction of a second required to change solutions, but at longer times any longitudinal currents should be exceedingly small compared with those in the propagated impulse. Nevertheless, the tension generation with high K_0 in an Na-free solution can remain for several seconds at about the same level as that in a maximal tetanus." And finally it is interesting that there is at least one instance in which internal current actually leads to relaxation, namely, the case in which application of an anodal current to an already depolarized region of a fiber suppresses the contracture at that spot (161). Relaxation occurs despite the introduction of current into the fiber at this spot and its flow internally toward the cathode; but the reason for this is simply that at the anode the current repolarizes the membrane.

Hence the preponderance of the evidence strongly indicates that internal currents are not involved in coupling the depolarization of the membrane to activation of contraction. It must be emphasized, however, that the internal currents discussed above are either the local circuit currents associated with the propagated action potential or artificially injected currents (*e.g.*, in Watanabe's experiments) that simulate the longitudinal component of local currents. It will be seen in the next section (III A) that such currents are not the only type of electric process that might be involved in E-C coupling. Attention will be given there to the internal membranes especially of the T system that ramify throughout the interior of the muscle fiber and are evidently capable of conducting some special type of electrical change different from the internal currents discussed above, and very likely responsible for the inward spread of activation.

The presence of the internal membranous elements introduces a rather special feature of the problem of conduction of the local circuit type of internal current.

In the foregoing it has been assumed that such currents flow in an electrically homogeneous medium with the current density the same at all points. This assumption is obviously incorrect when proper account is taken of the internal morphology of the muscle fiber and, especially, of the high probability that the membranes of the sarcoplasmic reticulum and the T tubules offer a very high resistance to electrical current. Thus any internal current should divide into at least two main branches: the one of low intensity flowing through the high-resistance pathway including the membranous elements, and the other of relatively high intensity passing through the low-resistance branch made of sarcoplasm and myofibrils. If the internal membranes play a role in inward conduction and this is electrical in nature it is conceivable that the difference between the results of Watanabe (252) and of Natori and Isojima (191) in regard to the threshold effects of applied currents may now be referred explicitly to a threshold in the mechanism by which the membrane elements participate in E-C coupling. This has already been suggested (191) and it may also be inferred from the view of Fujino *et al.* (93) that their result—the hastening of ATP-induced contraction by applied electric shocks—is explicable by the presence in their glycerinated fibers of a residuum of internal membrane elements (Ca-containing reticular vesicles; see Section IV D).

Despite the above analysis of the course of the longitudinal current through the muscle fiber, it still holds that local circuit current of the action potential or its simulated equivalent is unable to activate contraction even when, as in Watanabe's experiments, it is several times normal strength. This is a very important conclusion, for it indicates not only that inward activation is not accomplished by longitudinal current, but, even more important, that the myofibrils cannot be directly activated to contract by electric current. It will be seen later (Section IV) that such activation is based on a chemical mechanism involving the direct effect of the calcium ion on the contractile filaments.

Thus we return to the point stressed in the foregoing that the action potential performs its essential function in E-C coupling by depolarizing the membrane during the mechanically effective period and thus touching off the coupling sequence. We will see later (Sections VI D, E and VII) how the mechanical effectiveness of this action can be varied by certain ways of modulating this function of the action potential.

III. INWARD SPREAD OF STIMULUS

Elimination of internal currents as a means of coupling the electrical change at the membrane to activation of contraction poses the problem of determining what other mechanism performs this function. It had been supposed that, under the action of a depolarization, the membrane releases a chemical activator (*e.g.*, Ca^{2+}) which then somehow exerts its effect on the myofibrils throughout the sarcoplasm (111, 124). Quite apart from the question of the specific chemical nature of the activator and of its action on the myofibrils, Hill (113, 114) showed that if any such substance were released by excitation at the membrane, its diffusion to the myofibrils throughout the interior of a fiber would be altogether

too slow to account for the abrupt development of the active state. An attempt to circumvent this difficulty was made for the specific case that Ca is the activator by the supposition that the release of this ion at the membrane sets up a very fast, automatically propagated, inward moving wave of Ca-release (124). But this was proved untenable by Niedergerke's (194) demonstration that Ca^{2+} injected into the sarcoplasm of a muscle fiber caused contraction locally at the point of injection, but did not set up a wave of activity across the fiber.

A. Internal membrane systems

At just the time in the mid 1950's when it was becoming clear that depolarization did not release the activator for contraction at the surface membrane, the sarcoplasmic reticulum was being rediscovered and studied with the electron microscope, and speculations were being advanced that this system of internal membranous cisternae and tubules might function to spread an activating influence inward from the depolarized membrane to the fibrils. Porter in his review (214) gives an excellent detailed account of the recent morphological work in this field, especially as it pertains to E-C coupling and its relation to the earliest work on the sarcoplasmic reticulum. Present evidence indicates that the complex of internal membranes is composed of two main morphologically and functionally distinct systems. One is the set of generally longitudinally arrayed, thin, irregularly shaped cisternae or vesicles located in the spaces between the myofibrillae and so arranged that each serves as if it were a "lace-like sleeve" enveloping a particular myofibril (213). Then there is the T system, *i.e.*, the set of transverse tubules about 0.03 μ in diameter (6, 215) which evidently originate at the plasma membrane and penetrate laterally, or transversely, into the depths of each fiber. [There does not seem to be uniformity in the terminology used in this field: the term sarcoplasmic reticulum is used by some to designate the entirety of the internal membrane-enclosed cavities (*e.g.*, 143), and by others it is restricted to the system of longitudinal cisternae (87). In this review, the term "internal membrane structures" will designate the whole system and this will be considered to be composed of two basic systems, namely, the sarcoplasmic reticulum, and the T system.]

A remarkable feature of these systems is their regular variation in the longitudinal direction of the muscle fiber so that they are in periodic accord with the striational pattern of the sarcomeres. This periodicity varies with the species of the muscle fiber, but a common, most striking feature is the presence of regularly spaced "triads," each of which, as seen in longitudinal section, is composed of a central rather small T tubule flanked by a pair of large lateral sacs derived from, and in communication with, the neighboring cisternae of the sarcoplasmic reticulum (215). In the case of frog skeletal muscle these triads always appear at either side of a myofibril at the level of the Z-striation, but in lizard and in crab muscle they are found near the border of the A- and I-bands [though in the case of the crab muscle, dyads instead of triads are found (136)].

As previously mentioned, it was speculated, practically from the start of the work on the internal membrane structures, that their function might be to con-

duct inwardly an impulse corresponding to the action potential in the plasma membrane, and thus couple the surface membrane events to activation of myofibrillar contraction (219). This point of view has been developed anew by many of the workers who were active in the electron microscopic rediscovery of the internal membrane systems (*e.g.*, 16, and see 214 for further references). Furthermore, on the basis of a comparison of morphological and physiological features of various types of muscle cells, evidence accumulated that supports the assumed function for the internal membrane systems. Thus, Peachey and Porter (208) pointed out that the fast and relatively thick (50 to 100 μ) twitch striated muscle fibers are richly supplied with sarcoplasmic reticulum, but the slow and very thin smooth muscles (3 to 5 μ) that could obviously depend on direct coupling of membrane excitation to activation of myofibrils, are very poor in reticulum. Also significant was the fact that the presumably fast fibers of Amphioxus myotomes were devoid of reticulum but could nonetheless perform E-C coupling rapidly since the fibers were only about 1 μ thick (205). There was a similar correspondence between function and structure in the comparison made by Peachey and A. F. Huxley (207) between twitch and slow striated muscles of the frog, and in the demonstration of exceptionally richly developed internal membrane elements in the extremely fast fibers of a fish (73) and a bat (218). Thus, there is a great amount of presumptive evidence that the internal membranes are specialized for rapid, inward stimulus conduction on the grounds that, in general, the faster the response of a fiber, the greater is the development of the reticular elements.

B. Physiological inward spread of activation

The first attempt to utilize physiological means to study inward movement of activation in relation to the internal structures of the muscle fiber was made in the laboratory of A. F. Huxley (for a brief review, see 137). This work was started just before it was realized that the internal membrane elements were important in E-C coupling. It is therefore understandable that in the preliminary reports of these studies (139, 140) such elements were not mentioned, but instead the Z- (or Krause's) membrane (or disc), which extends continuously across the muscle fibers at the level of the Z-line, was suggested as the structure that might perform inward conduction. This whole field of research was advancing very rapidly, however, and by the time that A. F. Huxley and Taylor (141) and A. F. Huxley (134) published full-length reports of their research they could implicate the triads in the performance of inward activation.

Their work dealt with changes in polarization of the plasma membrane. But such changes were impressed on extremely small chosen areas of the external membrane by means of current pulses that were made to pass between a glass micropipette, whose tip of only 1- or 2-μ diameter was in close contact with the membrane, and an indifferent electrode in the outside medium. By varying the position of the tip on the surface of a muscle fiber, the effects of these highly localized changes in polarization of the membrane were studied in relation to the distribution of the various underlying membrane elements of the sarcomere.

For all kinds of fibers studied, hyperpolarizing currents never produced contraction when applied at any spot of the surface. Depolarizing currents of sufficient intensity (*i.e.*, above some threshold) caused contraction which was typically unpropagated and, even locally, did not spread out very much. Moreover, these contractions developed only when the depolarization was produced at certain highly restricted areas of the membrane. Thus, in the case of the frog skeletal muscle fiber, contraction occurred when depolarization was produced only opposite an I-band, and even then a depolarization caused contraction only when applied to a set of circumferentially located discrete spots at intervals roughly 5 μ apart. Such contractions occurred in the restricted I-band region just underlying the depolarized spot of the membrane, and they appeared as a shortening of the I-band, which was symmetrical in respect to the Z-line and extended, with diminishing degree, about 10 μ away from this region in both inward and circumferential directions. It should not be construed from this shortening in the I-band that the force for contraction is generated there. According to the sliding-filament mechanism, the contractile force develops where the actin and myosin filaments overlap, *i.e.*, in the A-bands (160). Thus, the observed shortening of the I-band is a passive consequence of the action of this force that tends to drag the myosin filaments of two contiguous sarcomeres toward their common Z-line and thus shorten the I-band. This localization of the generation of force is important because it indicates that, if some structure in association with the Z-disc conducts the stimulus inward, then there must be a longitudinally oriented mechanism conveying the effect of the stimulus from the Z-line to the two adjacent regions of overlap in the neighboring A-bands and thus directly causing activation of contraction by formation of cross-bridges between the actin and myosin filaments. In fact, it is on this basis that A. F. Huxley (133) suggested that, at least for frog skeletal muscle, the functional unit of the myofibril is not the sarcomere as usually defined, *i.e.*, the structures extending between two adjacent Z-lines, but the element of length based on a particular, centrally located Z-line and its two flanking regions each consisting of one-half of an I-band and one-half of an A-band.

These results suggest that in the twitch fiber of the frog, inward conducting paths exist in the plane of the Z-disc and that they originate at a series of discrete spots distributed around the fiber circumference which is at the position of each of the Z-discs. (If such spots are on the average 5 μ apart, then there are about 60 of them, and their associated paths, for each sarcomere of a fiber of 100-μ diameter, or about 4×10^6 of them in 1 cm^3 of muscle.) An adequate depolarization of the membrane in the immediate neighborhood of one of these spots evidently starts a process that moves inward along its associated path and thus provides the signal for activation of contraction.

In corresponding studies with crab and lizard fibers (138), the results were similar to those observed in the frog fibers except for the important difference that contraction occurred only when the depolarization was produced at a spot of the membrane opposite to the boundary between the A- and I-bands—and the shortening was limited to the I-band adjacent to this particular boundary.

These results indicate that the inward conducting path in crab and lizard fibers is not a component of the Z-disc itself, and they suggest that in these fibers, and those of the frog as well, the conducting path must be in some other kind of structure. It was at this juncture that observations made with the electron microscope demonstrated that triads are a regular feature of the internal membranes and that they occur in periodic register with certain elements of the sarcomere. And, most significantly, it was discovered that the triad is the common element at the different sarcomere points of the frog and the lizard fibers at which depolarization of the membrane causes contraction. It is, therefore, generally believed at present that the triads are importantly involved in the operation of the links coupling depolarization at the membrane to activation of contraction in the contractile filaments. The crab fibers offer a special case in that the conducting elements are not triads but dyads, whose transverse tubular elements make open connection with the plasma membrane at the level of the outer part of the A-band (136).

C. Role of the T system

On the basis of earlier evidence of the type discussed above and her own extensive morphological studies, Andersson-Cedergren (6) has proposed that the T tubules are the particular structures conducting the activating impulse inward from the plasma membrane, and this view is gaining strong support. This support is derived, first of all, from the facts of the general morphological relations of the elements of the triads (and dyads). The lateral sacs are connected with the longitudinal cisternae of the sarcoplasmic reticulum (143) and it has never been observed that they have radial elements extending to the surface; they are thus inappropriately arranged to conduct the effects of an event at the plasma membrane into the interior of the fiber. The central T tube elements of the triads have just the opposite relationship, and thus they are ideally arranged for the sort of transverse conduction that must be involved in transferring effects of plasma membrane excitation to the activation of contraction. Furthermore, the very troublesome questions recently posed, whether the T tubules are continuous structures that originate at the plasma membrane (134, 141) and, if so, whether they are infoldings of this structure, can now both be answered in the affirmative for at least certain types of muscle fibers. Such morphological details have been observed directly in electron microscope pictures of skeletal muscles of the crab (98, 136, 206) and of fish and tadpole (86, 87). Although there is no direct visual evidence of such features for frog muscle, there is just as compelling physiological evidence based on the rapid diffusion into the T tubules of various substances that do not pass at all across the plasma membrane or do so extremely slowly. Thus H. E. Huxley (143) and Page (199) examined frog sartorius fibers in the electron microscope after they had been exposed, while living, to solutions of ferritin. The molecules of ferritin, which are 100 Å in diameter, were found to move very quickly into only the T tubules of the fiber. Similarly, it has been found that the fluorescent dye, lissamine rhodamine B200 (64), albumin (119a), and sucrose, and sodium sulfate (1, 2)—all of which

are impermeant to the plasma membrane—do enter the T tubules. The experiments with ferritin are especially illuminating, because they prove that the ferritin molecules that have entered the muscle fiber are only in the lumen of the T tubules. This is not only very strong evidence that the T tubule communicates freely with the external medium, but, since no other part of the triads contains ferritin, it also demonstrates that free continuity is lacking between the T system and any part of the sarcoplasmic reticulum.

D. Possible electrical mechanisms

Although it is clear that the various elements of the internal membrane systems provide a structural basis admirably suited for some sort of functional coupling of membrane to myofibril, there is no evidence definitely indicating how they operate in the performance of this function. However, this is an interesting field for speculation, and it will be of value to consider some possible mechanisms involving the T tubules as core conductors for transmitting a membrane depolarization transversely into the fiber. The lateral vesicles are evidently concerned with another function, *i.e.*, the actual control of the Ca ion serving as the activator that initiates the contractile response in the myofibrils; but the details of this function will be discussed later.

It is not known whether the internal membranes are electrically polarized. But, unless they are freely permeable to all ions of the media they separate (a supposition which is hardly likely), they must be the seat of diffusion potentials that would polarize them. In particular, the wall of the T tubule is continuous with the plasma membrane, and it separates two media which are individually continuous with counterparts, *viz.*, the external medium and the sarcoplasm, that are separated by the plasma membrane. It is thus reasonable to suppose that the tubular membrane bears a polarization like that of the plasma membrane, positive on the side facing the lumen of the tubule and negative on the sarcoplasmic side. This assumption, and certain more detailed inferences regarding the specific permeability characteristics of the T tubular membrane, have been proposed in recent publications (1, 2, 3, 4, 88, 89, 98, 123).

It is generally assumed that a depolarization which develops directly across the plasma membrane in consequence of an action potential (as in fast fibers) or an endplate potential (as in slow fibers), or because of the effect of some depolarizing agent, such as K^+, initiates a wave of depolarization that sweeps inward along the tubular membranes. It has been supposed (6, 15, 143, 213, 214) that this wave is similar to the impulse at the fiber surface, but probably moves much more slowly. As stressed by A. F. Huxley and Taylor (141) and A. F. Huxley (134), however, the limited inward spread of contraction effected by highly localized depolarizations of the plasma membrane indicates that the electrical change that moves inward along a tubule is not all-or-none, as would be the case if some sort of impulse conduction were involved. They therefore suggested that the postulated electric conduction must be graded like a passive, electrotonic pulse whose intensity falls off with distance. To explain the contradiction between the complete activation of contraction of a fiber undergoing a normal twitch or tetanus and the limited spread of activation of contraction by

locally applied currents, it has been suggested (141) that the influence of a local depolarization at one spot of the plasma membrane would be attenuated by spreading out as it progressed inward, but that this would not occur in the presence of an action potential, which would depolarize along the whole perimeter of the fiber.

If inward conduction is a passive spread of an electrotonic potential along the membrane of the T system, then it is interesting to see whether it is possible to use the theory of the electrotonic potential (127), dealing especially with speed of propagation of the potential and decline in its strength with distance, to determine some aspects of the mechanism in detail. It will be seen that there is little to be gained at present by this approach. But the problem is so important that it is worthwhile presenting an exact statement of it and an indication of the impediments that must be overcome to obtain a solution. Thus, for an ideal core conductor an index of the speed of electrotonic propagation is given by the velocity of propagation of the half-value of the electrotonic potential, $v_{0.5} = 2\lambda/\tau_m$, where λ is the length constant and τ_m the time constant of the surface membrane; and the fractional decline in strength of the potential with distance, x, is given by $\exp(-x/\lambda)$. Valuable information regarding E-C coupling obviously can be obtained if these formulae are applicable to the function of the T tubules.

Unfortunately, we are unable to do this unambiguously. In the first place, the tubules of the T system are evidently not simple cylindrical core conductors like those for which the theory of the electrotonic potential has been developed (127). In fact, the configuration of the T system has not been definitely determined, but it is likely that the "tubules" of the system are organized into a network in the plane of each of the regularly repeated cross-sectional areas of the fiber at which elements of the T system are found (*e.g.*, in frog fibers, at the level of each Z-line). From the physiological work of A. F. Huxley and collaborators (134) and other evidence (*e.g.*, 64, 119a, 143, 199) the connection of this network to the external medium is believed to be made through a series of mouths that pierce the plasma membrane at discrete spots of the fiber's perimeter. And, from the electronmicroscopic evidence, the network must ramify internally so that there is a branch encircling each myofibril. But even if we ignore these complications, and assume as a first approximation that the system is composed of a set of radially arranged cylindrical core conductors, we are then faced with a second difficulty, *viz.*, our practically total ignorance of the values of λ and τ_m of the T tubule that must be known if we wish to use the formulae mentioned above. For these reasons, it is not possible now to present a definitive calculation of the features of the possible cable behavior of the T tubules mentioned above. It is clear that in respect to the T system we need more definite information regarding (a) its configuration, (b) the theory of the electrotonic behavior of such a system, and (c) experimentally determined values of the relevant basic electrical constants.[3]

[3] Note, however, that we may consider a T tubule to be a core conductor consisting of a cylindrical membrane enclosing a medium, which is a continuation of the fiber's external medium, and surrounded by another medium, which is the rest of the myoplasm. Applying

Falk and Fatt (70) suggested another scheme for inward conduction, which, though involving the T tubules, does not depend on electrotonic propagation as discussed above but is based on their analysis of the electrical properties of the resting striated muscle fiber of the frog. They proposed that the equivalent electric circuit of the fiber includes not only membrane elements of the plasma membrane, *i.e.*, capacitance, C_m, in parallel with a resistance, R_m, but also another path made up of a resistance, R_e, in series with a capacitance, C_e, which is in parallel with the R_m-C_m arm. They inferred that the new elements corre-

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the formula for an ideal core conductor (127), the length constant of the tubule is taken to be $\lambda_T = (r_T/(r_L + r_S))^{1/2}$ (cm), where r_T = transverse resistance × unit length of the tubule membrane (Ω cm), r_L = resistance of the tubule lumen per unit length (Ω/cm), and r_M = resistance of the myoplasm per unit length (Ω/cm). Similarly, the time constant of the tubule would be $\tau_T = r_T c_T$ (sec), where c_T = capacitance of the tubule membrane per unit length (F/cm). The formula for λ_T can be simplified by noting that the radius of the tubule, a, is very small, 175×10^{-8} cm (143), compared to the dimensions of the surrounding myoplasm of a sarcomere; or, considering this otherwise, the tubules at the level of one Z-line have a volume only about 0.3% of the associated one-half sarcomere volume (206a). Thus, noting also that the specific resistance of the myoplasm is low, 200 Ω cm (153), r_L must be much greater than r_M (even if all the T tubules at the level of one Z-line be considered electrically in parallel), and r_M may be ignored relative to r_L. Taking this into account, and furthermore using pertinent conversion formulae (127), we have $\lambda_T = (R_T a/2R_L)^{1/2}$ and $\tau_T = C_T R_T$, where R_T = tubule membrane resistance × unit area (Ω cm²), R_L = specific resistance of lumenal medium of tubule (which may be assumed to be 87 Ω cm, the same as an external Ringer medium, 153), and C_T = capacitance of the tubule membrane per unit area (F/cm²).

It is now evident that values of R_T and C_T are needed to calculate λ_T, C_T and thus $v_{0.5}$ and the fractional decay of the electrotonic potential along the T tubule. Falk and Fatt (70, p. 109) present such data; however, it must be realized that they were not obtained as direct measurements but in consequence of quite complicated calculations from raw data connoting directly quite different electrical features of the muscle fiber. Nevertheless, using Falk and Fatt's data, but changing the values, given relative to fiber surface, to values relative to tubule surface by means of the conversion factor 7 (206a), we obtain R_T = 56,000 Ω cm² and C_T = 1.1 μF/cm². Making the necessary calculations, $v_{0.5}$ = 0.77 cm/second. Hence, electrotonic propagation from the surface of a fiber down a 5-μ length of all its T tubules (*i.e.*, 0.1 the distance to the axis of a fiber with radius of 50 μ and thus involving 19% of the volume of the fiber) would require 0.65 millisecond, and propagation to the axis, 6.5 milliseconds. Furthermore, the fractional value of the potential would be 0.98 at the 5-μ distance and 0.82 at the axis. Thus, the electrotonic propagation under the assumed conditions would occur with probably negligible loss of signal strength. And, if we consider only the events conceivably related to the duration of the latent period (about 2 msec at room temperature), it is clear that the 0.65-millisecond delay does not seem to be unreasonable for the time to bring the action potential signal into close proximity with about 20% of all the contractile material of the fiber. (It will be seen later, section V A, that additional time is evidently involved in the diffusion of the activator of contraction from the sarcoplasmic reticulum to the sites of cross-bridges between the thin and thick contractile filaments.) As for the interval of 6.5 milliseconds for the signal in the T tubule to get to the fiber axis, this may reflect the time required for the active state to develop to full intensity throughout the whole of the fiber volume. Despite a certain degree of reasonableness to these calculations, it must be admitted that, although they outline some of the main features of the problem that is involved, they are merely suggestive regarding the solution of this problem, and that, as stated in the main text, definitive analyses of the role of the T tubules in E-C coupling must await the exact determination of the basic physical properties of these structures.

spond to electrical properties of the internal membranes, C_e representing the capacitance of the T tubule membrane, and R_e the resistance of the lateral sac membrane. Thus, depolarization of the plasma membrane, which would change the potential across C_m, would cause current to flow through the R_e and C_e path. Tracing out this current in detail shows that positive current would originate at the inside of the depolarized plasma membrane, and then flow, in turn, through the myoplasm, R_e, C_e, and T tube channel, and so back to the outside of the plasma membrane. In this way, a depolarization of the plasma membrane would cause a depolarizing potential to build up on C_e, *i.e.*, on the membrane of the T tubule, with a lag determined essentially by the time constant R_eC_e. It is assumed that this potential across C_e is the stimulus acting in the T tubules that triggers activation of contraction. And, in accord with the graded inward activation (141), it is supposed that C_e is not part of an all-or-none excitable system, but changes potential only in graded fashion.

Falk and Fatt calculated, in particular, that when depolarization of the plasma membrane is due to the action potential, the potential across C_e, when $R_eC_e = 2$ milliseconds, has a time course which is like the original action potential but distorted by rising and falling more slowly and by peaking at a later time than, and at about half the potential of, the original spike. They pointed out that such a projection of the action potential on C_e would involve a total change in potential that would depolarize the tubular membrane by about 20 mV beyond the mechanical threshold and thus would be just capable of causing maximal activation of the contractile system. Because of the slower rate of rise of the projected potential, however, the mechanical threshold would be reached (at room temperature) after a delay of about 0.8 millisecond in respect to the corresponding moment of the original action potential; other instants of the period of mechanical activation would be delayed somewhat more. But these delays are still small, and thus the mechanism proposed by Falk and Fatt could transform a depolarization of the plasma membrane into an activating signal pervading the interior of the fiber with sufficient rapidity to be consistent with the high speed of activation of contraction.

Still another suggestion of a mechanism to provide rapid inward conduction has been offered by Girardier *et al.* (98) on the basis of the inference that the membrane of the T tubules of crab fibers has a specific permeability to anions. Thus, a chloride diffusion potential would form across it which, like the K diffusion potential across the resting plasma membrane, would be polarized negatively on the myoplasmic side. At rest, the batteries of the tubular and the plasma membranes would be in series opposition, and no current would flow. In accord with other hypotheses discussed above, it has been assumed that the tubular membrane is inexcitable, so that when the action potential occurs, only the plasma membrane would be depolarized, and now the two batteries would be in series addition, and the current that consequently flows around their circuit would constitute an outward, *i.e.*, a depolarizing, current across the tubular membranes, and thus could act as the stimulus for contraction throughout the interior of the fiber.

Obviously the T system can serve as the structural basis for a variety of hypo-

thetical mechanisms which, at least in theory, can spread the activating signal inwardly and can do so with a speed consonant with the rapidity of mechanical activation. These mechanisms depend on the flow of certain types of electric disturbance through the internal substance of the muscle fiber. But, it is important to note that these disturbances are different from the local circuit type of longitudinal current discussed in section II D and therefore should not necessarily have ascribed to them the inadequacy in causing activation characteristic of the longitudinal currents. Evidently, participation of the T tubules and the sarcoplasmic reticulum in the process of inward activation introduces the possibility of conduction of special types of internal electrical signals that can fully trigger mechanical activity and that can normally be initiated by an adequate depolarization of the plasma membrane (even if this is of the massive type), or can be engendered by simulated longitudinal currents when, as in the work of Natori and Isojima (191), they are intense enough. There is no experimental basis at present for deciding which mechanism involving the internal membranes actually occurs. It should be noted, however, that if the T tubule is inexcitable but can be depolarized either by an inwardly propagated electrotonic potential or by the mechanism proposed by Falk and Fatt (70), then this depolarization would be in series opposition to that across the plasma membrane. There would thus be a reduction of the current proposed by Girardier *et al.* (98) for coupling excitation to contraction. Although there seems to be little doubt that the T tubules provide the structural basis for inward conduction of the activating signal in E-C coupling, much more experimental work is needed to determine the actual electrical and electrochemical details of the process by which this function is performed. Furthermore, the discussion has not considered the means by which the signal in the T tubule finally results in activation of contraction. This problem will be taken up in the next section, and it will be seen that this process evidently involves the Ca ion as an activator of contraction and the sarcoplasmic reticulum, especially the terminal sacs of the triads, as structures that regulate the activity of the Ca ion.

IV. ROLE OF CALCIUM IN THE CONTRACTION-RELAXATION CYCLE

A. General

Ever since Ringer (220) demonstrated that the frog heart stops beating in calcium-free saline solution, it has been recognized that this element is importantly involved in the responses of muscle. The involvement of Ca is very complex, however, and, as will be seen in the following introductory general treatment of the problem, the Ca may act at a number of different points in the E-C coupling sequence. Thus, Mines (185) observed that hearts made mechanically inactive by calcium deprivation produce essentially normal action potentials (see also 250), and this finding showed that the Ca ion is needed in the normal E-C coupling of heart muscle at a stage later than the action potential. In the case of skeletal muscle, however, the situation is more complex, for a frog sartorius or toe muscle that has been thoroughly equilibrated to a Ca-free Ringer solution does not respond either electrically or mechanically when electrically stimulated (60, 147). Hence, Ca is required in the response of skeletal muscle at

least for the generation of the action potential, and it might be concluded that the failure of the muscle to contract in the absence of Ca is due simply to the lack of the membrane depolarization that would be provided by the action potential in the normal course of E-C coupling. This conclusion is disproved, however, by the finding of Frank (78, 79), that such Ca-free skeletal muscles do not develop a mechanical response, *i.e.*, a contracture, when subjected to K-depolarization. Consequently, in skeletal muscle, Ca is involved not only in production of the action potential but also in the processes of E-C coupling that follow a depolarization. But such experiments still leave undetermined the site and mechanism of action of Ca, since it is clear that there are several general loci (*e.g.*, T tubules, sarcoplasmic reticulum, contractile filaments) at which the Ca ion might conceivably exert its effect. In the following, I make a more detailed analysis of the participation of the Ca in E-C coupling, concentrating on research that tells fairly directly about the role of the ion in the final steps causing activation of contraction.

B. Activation of contraction in the living muscle fiber

Heilbrunn (110) and Heilbrunn and Wiercinski (111), experimenting on frog skeletal muscle fibers, first clearly indicated that direct intracellular action of Ca causes shortening of the myoplasm. Heilbrunn's second report was especially significant because the tests were made by micropipette injection of solutions of the chlorides of Ca and other cations into living muscle fibers, and so there was no question that the ions were acting on the internal substance of the fiber. Of the various physiologically significant cations that were tested, only Ca elicited contraction. This response was likened to a "clotting," was considerable at 1.2 mM in the injected solution, and there was a clear threshold at about 0.2 mM. By contrast, Na^{+}, K^{+} and Mg^{2+}, in concentrations as high as 123 mM (isotonic to the myoplasm) had no contractile effect at all. Ba^{2+} acted somewhat like Ca^{2+}, but this has no physiological significance since barium is not a normal constituent of myoplasm. Thus, these results strongly suggested that the calcium ion is a physiologically significant, potent and quite specific chemical activator of contraction.

Extension of these early results has appeared in further studies involving either injection of Ca and other substances into skeletal muscle fibers of the frog (194) and the crab (41, 217), or direct application of the various cations to the denuded myofibrils of the Natori (190) preparation (210, 212). Improved techniques have been used in this more recent work and, when shortening is observed, it is clear that the myoplasm does not suffer "clotting" but responds with a localized reversible contraction that is like the graded contractions evoked by a locally applied membrane depolarization. Caldwell and Walster (41) showed that not only did K^{+}, Na^{+}, and Mg^{2+} but also adenosinetriphosphate [see also Falk and Gerard (71)], adenosinemonophosphate, potassium phosphate, and arginine hydrochloride have no activating effect. These results strengthen the conclusion that, among the physiologically significant substances, Ca^{2+} is unique as a specific activator of contraction. The investigation of Portzehl *et al.* (217) is especially significant because they used calcium buffers to stabilize the concentrations of the Ca^{2+} in the solutions they injected into the muscle fibers.

They used EGTA [ethylene glycol *bis*(β-aminoethylether)-N,N′-tetraacetate] as the buffer. This is an important technique because, as first stressed in work on contractile activity of actomyosin systems by Weber and Winicur (259), this chelator is much more specific for Ca than for other divalent cations such as Mg^{2+}. Portzehl and her co-workers (217) showed that threshold contraction effects were activated by Ca^{2+} in the very low range of concentrations between 0.3 and 1.5 μM.

Heilbrunn and Wiercinski (111) had suggested, on the basis of their results, a role for Ca in E-C coupling by postulating that "diverse types of stimulation cause a release of calcium ion from the surface or outer region of the cell and that this calcium then enters the cell and produces the response." We now realize, of course, that no activating chemical agent can diffuse speedily enough from the outer region into the interior of a muscle fiber to account for the rapidity of contractile activation (114). Therefore, the inclusion of such a diffusion mechanism for the distribution of the Ca^{2+} is untenable in this (or in any hypothesis) dealing with the action of Ca in E-C coupling. But we can now infer that the Ca ions that activate contraction are released not at the surface of the fiber but throughout its interior in response to the signal that is spread inwardly by the T system. This release would occur wherever there are T tubules, and the activating Ca ions would thus appear so close to the myofibrils that diffusion distances would be no more than about 0.5 to 1 μ, and, as will be seen later more specifically, the reactions releasing Ca^{2+} and finally initiating contraction in the myofilaments should then be rapid enough to account for the high speed of activation of contraction.

Before considering in detail such kinetic, and other, features of this action, however, we will discuss first certain special aspects of the mechanism by which Ca^{2+} activates contraction of the myoplasm. Myoplasm is a complex material containing not only the contractile myofibrils but also other material, including sarcoplasm and the various membranous structures of the sarcoplasmic reticulum and the T system. It is conceivable, therefore, that Ca^{2+} does not activate contraction by directly acting on the myofibrils but indirectly by first acting on some other internal component of the fiber. In fact, Gergely (97) has postulated that activation of contraction results from inhibition of a relaxing factor. And, in connection with the development of investigations on the sarcoplasmic reticulum as the basic element of the relaxing factor (for details, see section IV D) it had been proposed that the reticulum forms a "soluble relaxing factor" and that Ca^{2+} releases contraction by inactivating the relaxing effect of this soluble factor (*e.g.*, 90, 200, 201). Such views have now been withdrawn (37, 236), and there is, furthermore, direct evidence from the work of Weber and her co-workers (*e.g.*, 257; see also 167a) that Ca^{2+} activates contraction by a direct action on the myofilaments. The ensuing discussion will elaborate this view of the nature of activation of contraction, in particular reference to E-C coupling.

C. *Calcium-activation of actomyosin systems*

Weber and her associates (for review, see 257, 258) experimented on model systems of muscle made up basically of extracted actomyosin or myofibrils

which, in the presence of adenosinetriphosphate (ATP) and magnesium, produce contractile activity by hydrolyzing ATP and simultaneously undergoing superprecipitation (actomyosin) or syneresis and shortening (myofibrils). It will simplify our discussion to refer to such reactions of extracted muscle systems as "contractile activity," as, without doubt they are, even though no overt "contraction" like that of a living muscle is observed. Weber and her co-workers found that such contractile activity occurred, however, only when Ca^{2+} was available in the reaction medium so that it could be bound to the contractile protein and thus activate ATPase activity and contraction (253, 254, 256, 257). The bound Ca, typically in the amount of 1 to 2 moles per mole of myosin, was easily exchangeable with the free Ca^{2+} and thus the amount bound and the resultant contractile activity were sensitively dependent on the Ca^{2+} concentration in the medium (255). Threshold contractile activity appeared at a concentration of about 0.2 μM Ca^{2+} and maximal effects developed at concentrations of 5 to 10 μM (257, 259). In general, Weber and her associates found that they could make their actomyosin systems undergo the reactions which simulate either contraction or relaxation of living muscle simply by making appropriate increases or decreases, respectively, in the concentration of Ca^{2+} in the medium. It is especially noteworthy that the threshold concentration of Ca for activation of contraction is about the same in both the work of Weber *et al.* (256) on extracted actomyosin systems and that of Portzehl *et al.* (217) on the myoplasm of living muscle fibers, *i.e.*, about 0.2 μM for the former, and 0.3 to 1.5 μM for the latter. This is confirmatory evidence that results of studies on the extracted systems can be properly applied to the analysis of the behavior of the native forms of such systems in the intact fiber.

These findings strongly indicate that the mechanical state of the myofibrils in living muscle is under control of the Ca^{2+} concentration in the myoplasm. Since contractile activity occurs even when the concentration of Ca^{2+} in the medium is as low as 0.2 to 0.3 μM, the myoplasmic Ca^{2+} concentration of a muscle must be less than this to maintain the resting state. As discussed in more detail in the next section, the sarcoplasmic reticulum is evidently capable of accomplishing this for, when isolated, it can reduce the Ca^{2+} concentration to 0.1 μM or even less (103, 258). In fact, we can use some determinations of Hasselbach (104) which indicate that essentially all of the Ca in a resting muscle is in the sarcoplasmic reticulum. For he found that freshly prepared reticular microsomes of skeletal muscle take up 0.6 to 3.0 μmoles of Ca per mg of reticular N, and that there are 2 mg of such N per gram of muscle. Thus, we have 1.2 to 6.0 μmoles of Ca taken up by the reticulum in 1 gram of muscle. Since the amount of analytically determined Ca is about 1 to 2 μmoles/gram of wet tissue, we must conclude that, in a resting muscle, almost all of the Ca is sequestered in the reticulum, and that therefore the concentration of Ca^{2+} is almost zero in the sarcoplasm surrounding the myofibrils. Furthermore, it is known by direct analysis that practically all of the Ca normally present in resting muscle is bound in one form or another so that the concentration of free Ca ions is below threshold for activating contraction (101, 240, 261). We can then postulate that in E-C coupling the signal in the T tubules causes a release of Ca^{2+} from a bound

state characteristic of the resting condition and that this liberated Ca^{2+} causes contraction by acting directly on the contractile filaments of the myofibrils. This statement gives no details of the process by which Ca activates contraction. It would take us too far afield to deal with this problem, which, in any case is still unsolved [*e.g.*, see 259; and for a provocative but highly speculative theory see Davies (52)]. But the process depends on Mg^{2+} and it thus involves the ATPase activity of actomyosin (not myosin). And the Ca^{2+} acts as if it were a cofactor that activates this enzymatic action of actomyosin, thus hydrolyzing ATP and releasing the energy for contraction.

It is interesting to note that activation of ATPase activity of contractile proteins by Ca^{2+} has commonly been thought in the past to play a key role in the activation of contraction and thus provides a basis for the final link in E-C coupling (*e.g.*, 225). As recently pointed out by A. F. Huxley (135), a very perspicacious proposal of this sort was made a generation ago by Bailey (11). This proposal has a particular pertinence to our current views on E-C coupling in its suggestion that the liberation of the Ca ions takes place "in the neighborhood of the ATP-ase grouping," *i.e.*, throughout the interior of the fiber and not merely near the surface. It must be pointed out, however, that Bailey's and other of the corresponding older speculations were based on the features then known of a type of Ca-activated ATPase and breakdown of ATP that did not involve Mg^{2+}, that probably was concerned with the enzymatic action of myosin and not actomyosin, and that, above all, was not in any way capable of energizing contraction (107). (This point applies occasionally in even more recent work, *e.g.*, 30, 31.) Bailey's presented data seem to deal with a role of Ca like that in the experiments of Weber *et al.* (257) and others, in that activation was stated to occur at concentrations of Ca of the order of 1 μM. But, as pointed out to me by Dr. A. Weber, internal evidence in Bailey's paper clearly proves that such statements are erroneous, and that the actual Ca concentrations in this work were 10^3 greater than those given, and therefore were of the order to be expected for a type of ATPase that is associated with myosin (not actomyosin) and that does not couple to the mechanism causing contraction.

Thus, work such as Bailey's, although of considerable historical interest, should not divert us from realizing that current results and hypotheses regarding the role of Ca^{2+} in Mg-actomyosin systems differ from the older ones, in that they give us a correct and specific basis, not provided by the older speculations, for tracing out certain essential steps of E-C coupling, since they apply to ATPase activity directly coupled to contraction.

D. Regulation of myoplasmic Ca^{2+} by the sarcoplasmic reticulum

To return to the main thread of our account, I now point out that the discussion of the work indicating that the concentration of Ca^{2+} in the myoplasm regulates the mechanical activity of the myofibrils raises another basic question: what is the mechanism which regulates the myoplasmic concentration of Ca^{2+}? To put this in another form: what are the links by which the signal in the T tubules causes liberation of Ca^{2+}? And what is the process by which the Ca^{2+} is

removed from its bound state on the actomyosin so that the contracted myofibrils can relax? It is interesting that the available information regarding both of these reactions is obtained almost entirely from the study of the relaxation process. It will be desirable for us, therefore, to approach the question of the general regulation of the free Ca^{2+} in myoplasm by studying the findings that have been made on relaxation.

From the work of Marsh (175) and Bendall (14), it has been known that a factor exists in muscle which is capable of causing a variety of effects—such as inhibition or reversal of myofibrillar syneresis, suppression of actomyosin-ATPase activity, and direct initiation of relaxation of contracted glycerinated fibers—that correspond to relaxation of living muscle. Furthermore, it was demonstrated (a) that this relaxing factor can be isolated from muscle as the microsomal fraction (216); (b) that it is derived mainly from the membranes of the sarcoplasmic reticulum (55, 187, 188); and (c) that in the form of the microsomal vesicles (or "grana"), it has the remarkable property of acting as a powerful "calcium pump," that is, of accumulating Ca^{2+} from its external medium against extraordinarily high electrochemical gradients with the aid of energy derived from a special breakdown of ATP (54, 55, 105).

On the basis of these results there is general agreement that the sarcoplasmic reticulum in intact muscle is intimately involved in causing relaxation. A definite mechanism for such action is indicated by the important demonstration of Weber *et al.* (256) that extracted sarcoplasmic reticulum, in the form of the microsomal fraction of muscle, removes bound Ca from various actomyosin systems and thereby causes relaxation. In other words, by exerting its Ca-pump activity, the reticulum reduces the free Ca^{2+} concentration to 0.02 μM or less and thus causes the Ca-actomyosin to dissociate and the system to exhibit the typical relaxation phenomena of suppression of ATPase activity, and reversal of superprecipitation or syneresis.

Of further great significance are other findings about the role of the sarcoplasmic reticulum in regulating the behavior of Ca in the contraction-relaxation cycle. Hasselbach (103, see footnote 1) reported that in relaxed glycerinated frog muscle fibers Ca (in the form of Ca oxalate) is precipitated mainly in the lateral sacs of the triads. And Costantin *et al.* (47), in a thoroughly controlled investigation involving living, but denuded, frog muscle fibers, concluded that applied Ca (also precipitated as oxalate by subsequent perfusion with Na oxalate) is also taken up only by the lateral sacs. These observations are critically important because they are the first demonstrations that the calcium-pumping property of the extracted and fragmented sarcoplasmic reticulum operates in the fully organized reticulum of the essentially intact muscle fiber. And, moreover, they prove that the calcium taken up is localized in only the lateral sacs of the triad. These results are very significant for our comprehension of the mechanism of E-C coupling. For, as proposed by Costantin *et al.* (47), the proximity of the Ca-loaded lateral sacs to the transverse tubules which, as we have seen, are possibly involved in intracellular stimulus conduction, "suggests that they might also be regions from which calcium is released to trigger contraction." Indirect con-

firmation of this view is found in Winegrad's (263) autoradiographic studies of internal distribution and translocation of Ca^{45} during rest and contraction of frog skeletal muscle fibers. At rest, the Ca was preponderantly localized at the center of the I-band, *i.e.*, at the position occupied by the lateral sacs. But the greater the contractile tension of the fibers at the moment of their fixation for microscopic study, the greater was the proportion of labeled Ca localized in the region of overlap of the myofilaments within the A-band. These results indicate that, in connection with contraction, there is a movement of Ca from the I-band region of the sarcomere to the A-band region. Such a movement is completely consistent with the supposition that the Ca which activates contraction originates in the lateral sacs, which are juxtaposed to the central part of the I-band, and then moves, presumably by diffusion, to the A-band, where it is bound to the overlapping actin and myosin filaments and thus activates the interaction between them that constitutes the sliding filament mechanism of contraction (142).

E. Activity fluxes of calcium

We have just seen that there is evidence that Ca is internally translocated in performing its role in E-C coupling as activator of contraction. In the present section we are concerned with the changes in distribution and flux of Ca between the inside and outside of the muscle fiber in relation to the general function of Ca in E-C coupling.

That Ca metabolism is altered during activity was strikingly demonstrated by Weise (261), who determined the Ca content of ultrafiltrates of minced rat skeletal muscle. In resting muscle before filtration there was a total of about 1.3 mmoles of Ca/kg of wet tissue, but in the filtrate no Ca could be detected. This is proof that the Ca is very strongly bound to the relatively insoluble components of the resting muscle. Similar filtrates from highly exercised muscle contained about 0.6 mmole/kg muscle. The result indicates that activity converts Ca from a bound to a free form. Corroboratory evidence for such a conversion was then obtained by demonstrating the occurrence of actual efflux of free Ca^{2+} from various intact activated muscles in the work of Lánczos (167) on frog hearts, and, especially in the experiments utilizing tracer Ca^{45}, of Woodward (264) and Shanes and Bianchi (241) on frog skeletal muscle, and of Cosmos (46) on rat skeletal muscle. All of these results indicated that activity causes a release of intracellular bound Ca and that at least some of this free Ca then passes out of the cell into the external medium. Certain problems concerned with the transport of the free Ca^{2+} are raised by these results (see, *e.g.*, 240). But more pertinent to our special interest in E-C coupling is that these findings are consistent with the mechanism outlined in the preceding section. For it is reasonable to expect that, especially after an intense bout of activity, the Ca that is released from the triadic lateral sacs and is not bound to the actomyosin at any moment would be free to diffuse out of the muscle during the time these sacs are engaged in reabsorbing the Ca.

There are other results, however, obtained especially by Bianchi and Shanes (24), indicating that activity causes an influx of Ca, as well as an efflux. These workers found that the frog sartorius muscle fiber has a resting influx of about

0.1 μμmole per second per cm^2 of fiber surface, and that, in a series of twitches, the average influx was increased to 0.2 μμmole/cm^2 per twitch. These results are not directly comparable since the former measures the influx per second while the latter does so per twitch. But, if we take the twitch time of 0.1 second, as a more pertinent time unit, then the resting entry would be only 0.01 μμmole/cm^2, and thus the entry of Ca during the twitch would be 20 × greater than it is during an equal period of rest. Even more striking was Bianchi and Shanes's finding that, in association with a 0.1 M K-contracture, the influx was 0.013 μmole/gram of muscle or [using the conversion factor, 300 cm^2 of fiber surface per gram of muscle (24)], 44 μμmoles/cm^2, and thus, some 220 × greater than the amount of Ca influx of the twitch. Presumably, this much greater influx during the contracture is due to the much longer time of the depolarization caused by the prolonged exposure to the KCl (of the order of 60 sec) than that occurring during the mechanically effective period of the action potential (at room temperature, only about 1.5 msec).

In the development of the research based on these findings many other interesting results were obtained dealing with the fluxes of Ca (in addition to the above cited papers of Shanes and Bianchi, see, *e.g.*, 20, 21, 23, 24, 25, 168, 239, 240). The general inference that has been drawn from this work is that the observed influx of Ca is "consistent with the view that calcium entry initiates and may exert control of contraction" (24, see also 78, 79). It has been implied from the presence of this influx that the Ca^{2+} that enters the myoplasm upon excitation directly makes contact with and thus activates contraction in the contractile fibrils. Thus, in respect to theories of activation of contraction, the above "Ca-entry" mechanism is in contrast to the previously discussed mechanism which proposes an intracellular release and translocation, and not an "entry," of Ca^{2+}. It is therefore of interest to discuss whether the Ca-entry theory is adequate to explain activation of contraction. It should be noted that as new results appeared, some of which are discussed in the following, the proponents of the Ca-entry theory modified it, mainly in respect to the role in E-C coupling assigned to the Ca ions which enter the responding muscle fiber. An indication of the current position has been presented by Frank (85), who, in conclusion to a review of principally his own results bearing on this problem, stated: "It is unfortunate that none of these studies give any indication as to how the calcium ions which enter the fiber initiate the mechanical response. However, these studies support the concept that the entrance of calcium ions serves as an essential link between the electrical and mechanical events in the contraction of skeletal muscles." Nevertheless, it will be of some value to discuss in the following the general problem of the significance of Ca entry in relation to the various possibilities ascribed to it in the mechanism of E-C coupling.

This question has already been considered, in part, elsewhere (227) and the conclusion was reached that there are certain difficulties in the theory as developed in detail, particularly in the assumption that the Ca which enters during a response and activates contraction originates at the fully saturated Ca-binding sites in the surface membrane (24, 25). But more critically important for our

present interest are the following other considerations which show that the theory, at least as originally proposed, is not valid. In the first place, the Ca-entry mechanism suffers from the previously discussed defect that characterizes any activation process depending on movement of a substance from the periphery of the fiber into its interior: the process is too slow to account for the actual speed of activation. This difficulty was overcome, in theory, by the assumption that the release of the Ca might take place not at the surface of the fiber but all along the T tubules (24, 240). This does not seem likely, however, in view of the evidence presented in the last section, which indicates that the source of the activating Ca^{2+} is in the lateral sacs and not in the T tubules.

Secondly, there are certain quantitative disparities between the measured amount of the Ca influx and the amount needed to activate contraction. Winegrad (262) has calculated that the number of Ca ions entering a fiber during a twitch could supply only 0.4 % of all the myosin molecules present in a fiber with at least the one Ca ion each needs to activate its contractile response. And Frank (82), making a similar calculation, concluded that only 0.05 % of the myosin molecules would be activated. It is most unlikely that normal twitch tension could be developed in muscle fibers if such small fractions of their myosin content are activated. We must therefore conclude that activation of contraction is not mediated by the Ca that enters a muscle fiber during activity.

This conclusion may be reached by another type of calculation that does not depend explicitly on a count of the number of myosin molecules in a given muscle fiber, but involves only certain phenomenological features of muscular response. We know from the work of Weber *et al.* (254, 257) and Portzehl *et al.* (217) that the concentration of Ca^{2+} for threshold activation of contraction is in the range of 0.2 to 1.5 μM. From Bianchi and Shanes's (24) determination that the influx of Ca^{2+} is 0.2 $\mu\mu$mole/cm^2 per twitch we can calculate the resultant increase in myoplasmic concentration of free Ca^{2+}, if we assume that the Ca which enters is uniformly distributed within the fiber (as it might be if we adopt the proposed view that this Ca^{2+} is released along the T tubules). For a fiber radius of 50×10^{-4} cm, the volume corresponding to a surface area of 1 cm^2 is 25×10^{-7} liters and thus the desired concentration is given by 2×10^{-13} moles/25×10^{-7} liters = 0.08×10^{-6} M. Thus, it is clear that the observed entry of Ca^{2+} is too small by a factor of about 10 to cause even threshold effects, and it would be too small by a factor of about 100 to elicit maximal activation. This result is therefore in accord with those of Winegrad and Frank in showing that the observed influx of Ca is much too small to activate all of the myosin of a fiber. Thus, our various considerations prove that the Ca that enters a stimulated muscle fiber is inadequate both in respect to speed and quantity to serve as the activator of contraction, and we must therefore reject the Ca-entry theory in accounting for activation of contraction in the mechanism of E-C coupling.

But, then, the question arises as to the function of the influx of calcium. Hodgkin and Keynes (126) demonstrated that there is a Ca influx in excited giant axons of the squid, and they postulated that it might be associated with the electrochemical processes determining the entry of sodium during the action potential. Bianchi and Shanes (24) stressed that the Ca influx was 30 $\times$ greater

in the sartorius muscle fiber than in the squid axon, and they thought this justified their inference that Ca influx into the muscle fiber was associated not only with excitation, but also with activation of contraction. But the total Na influx during excitation is, itself, about 6 $\times$ greater in the muscle fiber than in the nerve fiber (122), so that the above cited difference factor of 30 $\times$ should be reduced to only 5 $\times$. Furthermore, it is conceivable that some of the Ca influx in the excited muscle fiber results from the depolarization of the T tubules, so that the factor of 5 $\times$ would be further reduced, since the area of the T tubules, as well as that of the plasma membrane, would have to be taken into account in calculating the flux. Thus, it is evident that, if Ca and Na influxes are coupled in the mechanism of excitation, then the entry of Ca in muscle could occur simply in consequence of the mechanisms determining the action potential and the conduction of the signal along the T tubules.

In retrospect of our discussion of the Ca fluxes of the activated muscle, we can conclude that the efflux of this ion may reflect the release of bound Ca from the lateral sacs that is postulated to provide the Ca^{2+} which activates the myofilaments to contract. As for the Ca that enters the fiber, we can be quite certain that this does not supply the Ca^{2+} that directly activates contraction, because such influx would be, by far, both too slow and too small in amount to account for the characteristics of actual contraction. Furthermore, in any case, this influx can be related, at least hypothetically, with other reactions of the E-C coupling sequence. Thus, our conclusion from these flux studies is that they do not support the "Ca-entry" theory and therefore they make more certain the view that internal translocation of Ca^{2+} is the basis for activation of contraction.

It is important to note that the foregoing conclusion refers to the function of Ca^{2+} in fast skeletal muscle. As the work of Niedergerke (195, 196) showed, the situation in heart muscle is different, for the Ca^{2+} which enters during activity is evidently directly involved in activating contraction. And this is indicated also in that, unlike the behavior of fast skeletal muscle, contraction of heart muscle depends on an antagonism between Ca^{2+} and Na^{+}, the magnitude of a response being proportional to the ratio $[Ca^{2+}]/[Na^{+}]^2$ for these ions in the external medium (174). Furthermore, it should be noted that slow skeletal muscles are also different from the twitch type, and as seen, for example, in the work of Schaechtelin (235) they show some of the characteristics of heart muscle.

V. OUTLINE OF THE CONTRACTION-RELAXATION CYCLE

On the basis of the foregoing, it is possible to outline a mechanism for E-C coupling in the fast skeletal muscle fiber. This mechanism, however, is so intimately connected with relaxation reactions that consideration of E-C coupling is easily extended so as to give an outline of an entire contraction-relaxation cycle of the twitch.

A. Excitation-contraction coupling

The initial stimulus is an adequate depolarization of the surface membrane of the muscle fiber, which results from an action potential in fast fibers, a widely

distributed set of endplate potentials (or an action potential, in certain conditions) in slow fibers, or, under relatively artificial experimental conditions, a K-depolarization. The surface depolarization produces a signal that is somehow conducted either directly in the tubules of the T system very likely as an electrotonic pulse, or otherwise (see section III D), and is thereby spread throughout the interior of the cell. The theoretical analysis of such propagation suggests that it should be speedy and strong enough to perform the function by which the T tubules presumably convey an activating signal into the interior of the fiber. It is now presumed that, on arrival at a triad (or dyad in the case of crab fibers), the signal in the tubules stimulates the neighboring lateral sacs to liberate Ca^{2+} from their store of this activator. A coupling of this sort between the central and the lateral elements of the triad has been postulated by several authors (*e.g.*, 143, 206a, 240). Nothing is actually known of this process, but its existence seems to be a necessary corollary of all that has been discussed indicating that the activating signal is spread inward by T tubules and that Ca is stored in, and thus is ready to be released from, the lateral sacs. Franzini-Armstrong (86) and H. E. Huxley (143) point out that there are special membranous elements at the junctions of a given T tubule and its two lateral sacs, and it is possible that these may function in causing the T tubular signal to stimulate release of Ca^{2+} from the neighboring lateral sacs.

Apart from structural considerations, we can postulate that, if the signal in the T tubule involves a depolarization, then the release of Ca^{2+} from the lateral sacs of the triad may be caused by an increase in permeability induced by the change in potential produced by the tubule signal at the triadic junctional membranes. It is not ruled out that such a junctional change in permeability is like those occurring during transmission across neuromuscular junctions. Or it might be possible that it is comparable to the increase in permeability characterizing excitation of a membrane. The main effect, however, would seem to be that the electrical change in the central element of the triad increases the permeability of the lateral sacs and thus causes them to release their Ca^{2+}. Such a release should occur with very great speed because the Ca^{2+} would be moving out of the lateral sacs under an enormous concentration gradient (see, *e.g.*, 103).

In any case, the next and final steps in E-C coupling are diffusion of the Ca^{2+} into the neighboring myofibrils, and the resultant activation of contraction. A rough estimate of the time taken for at least the diffusion involved in these events may be made by assuming the somewhat simplified conditions that the Ca^{2+} is released near the surface of myofibrils of 1-μ diameter, and then calculating the time required to raise its concentration at the axis of the myofibrils to 0.9 of that at the surface. For such a case of cylindrical diffusion, the details have been worked out by Hill (113), and, from his Figure 2, the time is given approximately by $t = 0.2\ a^2/k$, where the radius of the myofibril, $a = 0.5\ \mu$, and k is the diffusion coefficient for $CaCl_2$, which I assume for the relevant milieu and room temperature to be 10^{-6} cm^2/second. It then turns out that $t = 0.5$ millisecond, and thus the diffusion time is brief enough to be consistent with the high speed of activation. This determination neglects the possibility

that the Ca^{2+} may have to diffuse lengthwise within each sarcomere, but the inclusion of such a process should not add much to the time required for the Ca^{2+} to make contact with the filaments where contractile cross-bridges are formed, since this diffusion distance is so small, *i.e.*, about the same as, or even less than, that assumed in the foregoing calculation.

Thus, these final steps in E-C coupling should be essentially complete, at room temperature, in at most a millisecond, if we assume that the consumption of time in these processes is mainly connected with the diffusion of the Ca^{2+} to its binding sites on the myofilaments. Adding this to the millisecond or so presumably taken up with the earliest inward spread of the activating signal, we see that a total of about, or somewhat less than, 2 milliseconds should elapse in the living muscle between the rise of the action potential and the first sign of mechanical response of the contractile system as manifested in the latency relaxation. It is satisfying to note that studies of the latent period of living frog muscle (224, 225, 232) indicate that the observed latencies are close to the predicted value of 2 milliseconds, and this similarity suggests that the delay between the spike and the onset of mechanical activity, which was mentioned in Section II A, can be reasonably accounted for by the time taken for the above mentioned coupling processes to run their course. For a discussion of a somewhat similar mechanism of inward activation and other possible mechanisms see Close (43).

I now consider an important point regarding the mechanical threshold, namely, the identification of the E-C coupling process that reacts in threshold manner for activating mechanical output as a function of increasing depolarization (or decreasing potential) of the membrane.

Contraction of the myofibrils occurs, of course, only when the Ca^{2+} concentration reaches a threshold value of about 0.2 to 1.5 μM, and it increases to saturation level as the Ca^{2+} concentration increases up to 5.0 μM (see Sections IV B and C). Thus, it is possible that the processes that link membrane potential to increase of free Ca^{2+} in the myoplasm are continuously graded and that the critical threshold in question is simply in respect to Ca^{2+} activation of contractile activity in the myofibrils. As to the links, one of these is signal conduction along the T tubules, and if, as seems likely, this conduction is electrotonic, it would be graded and therefore always directly proportional to the change in potential across the plasma membrane. A different type of process might exist, however, in connection with release of Ca^{2+} from its storage locus in the lateral sacs. Though purely speculative at present, the hypothesis is not ruled out that Ca^{2+} release from the lateral sacs occurs only when a minimal change in potential is developed across the membrane of the T tubules where they make junction with the lateral sacs in the triads. It is of greatest interest to determine if such a threshold mechanism operates in connection with the Ca^{2+} release or whether it is continuously graded in relation to the potential change in the T tubule membrane.

At any rate, we can be definite in concluding that the mechanical threshold denotes a membrane potential at which the E-C coupling sequence causes re-

lease of sufficient Ca^{2+} just to initiate contraction. This provides a basis for a reconsideration of the nature of the mechanically effective period—previously defined as the period during which the action potential maintains the membrane potential beyond mechanical threshold (Section II B). From various features of our previous discussions, it is clear that as the action potential runs through its course during the mechanically effective period, it reversibly shifts the membrane potential to values far beyond the mechanical threshold and thereby must cause a continuous outpouring of Ca^{2+} from the lateral sacs, which at any moment is probably proportional to the change in membrane potential. Thus, the mechanically effective period must be considered as an interval during which the action potential causes liberation of an amount of Ca^{2+} greatly exceeding that needed for threshold activation and sufficient to activate the entire twitch response.

B. Relaxation

In Section II A it was indicated that the mechanical activity of a twitch lasts much longer than the mechanically effective period of the action potential. This was puzzling because it seems to have been tacitly assumed by some that under all conditions actual mechanical activity is not only switched on by depolarization but also maintained by this or some other state of the plasma membrane, and then switched off by repolarization; or, to put it another way, the view was held that the entire course of the mechanical response was under the direct control of the surface membrane (see, *e.g.*, 119). This is now obviously fallacious, for, although a contraction is started in the myofilaments by the Ca^{2+} that is released in consequence of a plasma membrane depolarization, it is terminated by the loss of that Ca^{2+} from the myofilaments that is determined by the reticular Ca-pump activity. Thus, as stressed by Podolsky and Costantin (211), contraction is initiated under the control of the plasma membrane, but it is ended under the influence of another membranous structure, *i.e.*, the sarcoplasmic reticulum.

It is clear, therefore, that an important factor (not necessarily the only one) determining the duration of a mechanical response, *e.g.*, a twitch, must be the kinetics of the uptake of Ca^{2+} by the sarcoplasmic reticulum. This has been ingeniously studied in the Natori preparation by Podolsky and Costantin (211), and they found, (a) the first clear evidence that a "calcium sink operates in living muscle," determined by the Ca-accumulating function of the sarcoplasmic reticulum; and (b) that, as regulated by the kinetics of this process, 25 milliseconds is the half-time for inactivation at room temperature of the stimulating action of Ca^{2+} on the myofilaments. This agrees fairly well with the half-time of the relaxation period of the twitch of the same muscle fiber when electrically excited, although it is probably somewhat too long for the corresponding half-time of decay of the active state of this fiber. Roughly similar results have been obtained on crab fibers (217), although this study did not employ the sophisticated experimental and analytical procedures of Podolsky and Costantin (211). And certain speculations (103, 257) indicated that the isolated sarcoplasmic reticulum accumulates Ca at a rate adequate to account for the speed of actual

relaxation. It is also noteworthy that Hodgkin and Horowicz (124) theorized from their results on K-contractures of single fast muscle fibers of the frog, that "depolarization releases an activator which is destroyed in a first-order reaction with a rate constant of about 30 sec^{-1}." If for "activator" we read "Ca^{2+}" and for "destroyed" we read "taken up by the Ca pump," and note that the indicated rate constant corresponds to a half-time of about 23 milliseconds, we then see that the mechanism for relaxation of a contracture of a fast fiber is at least kinetically quite similar to that proposed by Podolsky and Costantin. These results thus strongly support the conceptions that in the mechanical response of living muscle, (a) the removal of Ca^{2+} from the myofilaments by the Ca-pumping action of the sarcoplasmic reticulum is the essential process determining relaxation, and (b) the kinetics of this action regulates the speed of actual relaxation.

The puzzle of the disparity between the brevity of the action potential and the relatively long duration of the consequent contraction is, therefore, easily explained by the difference in kinetics of the two distinct mechanisms working in tandem to regulate the free Ca^{2+} concentration in the myoplasm. The presence of a special means for causing relaxation also bears on the question whether relaxation is an active or passive process. Hill (115) had concluded from his measurements of certain mechanical and thermal properties of activated muscle that relaxation is passive and that it depends merely on the disappearance of the active state of the contractile component (116). In a later paper, Hill (117) presented other results concerned with the same muscle outputs which suggested that some sort of thermomechanical interaction can occur during relaxation and that this indicates that relaxation may not be wholly passive. The knowledge we now have about the sarcoplasmic reticulum indicates that relaxation is a very active process since it depends on the operation of the reticular Ca pump, which expends energy derived from the hydrolysis of ATP (*e.g.*, 103). It is still correct to state that relaxation involves disappearance of the active state, but it must be recognized that this is controlled by the energetically driven relaxing activity of the sarcoplasmic reticulum.

Although the preceding discussion presents a reasonable outline of the contraction-relaxation cycle of the twitch, it is not possible to give an equally satisfactory analysis of a depolarization contracture. The act of depolarizing the membrane presumably sets up contraction by an electromechanical coupling sequence like that of the twitch. But it is not clear how the relaxation system operates, especially since, under a maintained depolarization, twitch fibers spontaneously relax but slow fibers remain contracted. Hodgkin and Horowicz (124) have applied to the problem of the course of phasic contracture their previously discussed hypothesis regarding the kinetics of production and "destruction" of the activator for contraction. Their analysis provides an adequate explanation of the time course of the phasic contracture. But, in terms of the operation of the sarcoplasmic reticulum outlined in the foregoing, their scheme requires operation of the prime condition for relaxation, *i.e.*, the activity of the Ca pump, even though depolarization persists. It is not ruled out that this may occur. But if it does, then it would seem that under the influence of a long depolariza-

tion, the coupling system of the twitch fiber reacts in a biphasic fashion: initially to cause release of Ca^{2+} from the lateral sacs; and then subsequently to become accommodated to the continued action of the depolarization and thus permit the reticulum to reactivate its Ca-pump function and cause relaxation. As for the slow fibers, which maintain contracture tension as long as the depolarization lasts, it would be necessary to assume that accommodation of the coupling mechanism does not occur, or that continued depolarization exerts a maintained inhibitory action on the Ca-pump function of the reticulum. It is noteworthy that, during a depolarization contracture, the Ca influx is phasic in twitch fibers (24) but sustained in slow fibers (239a). Though not fully understood, this difference is undoubtedly a reflection of the distinction between the electromechanical coupling systems of the two types of muscle. Further research on this question should elucidate the general mechanisms which regulate the contraction-relaxation cycle.

VI. EFFECTS OF CHEMICAL AND OTHER AGENTS ON E-C COUPLING

In this section I consider modifications in intact living muscles which result from various altered conditions that cause changes in E-C coupling. Chemically produced effects are obviously interesting pharmacologically. But, as already mentioned in Section IV A regarding effects of calcium lack, such alterations may also elucidate some aspect of E-C coupling provided it is possible to identify their underlying basis or, at least, their site of origin. Since this is frequently impossible, great caution is necessary in attempting to interpret the meaning of an "effect" produced by some experimental agent. In connection with this phase of our subject, several reviews will be found useful for further consideration of the kind of effect to be discussed (8, 27, 124, 239).

A. Calcium

Caution of the sort just mentioned must be exercised especially in evaluating studies involving calcium. In fact, it is this that prompts me to consider such effects in this section of the review, rather than in the preceding one, even though this also dealt with calcium. The experiments discussed previously were designed to study quite directly the role of Ca^{2+} in the function of certain comparatively isolated systems of E-C coupling. But in the work considered in the following, the variations in external Ca^{2+} concentration provoke changes in responses of the whole muscle that are difficult to ascribe to particular reactions of E-C coupling.

Mention was previously made that certain responses are abolished in a muscle equilibrated to a Ca-free medium. Brecht and his co-workers have done many other experiments of this sort [for review of some of this work see Brecht (34)] and they find that, in consequence of soaking in Ca-free Ringer solution, frog sartorius muscles do not produce fatigue contracture (203), Tiegel's contracture (36), or acid and ether "contractures" (165). These workers have also shown that the various contractures are more pronounced when the sartorii are exposed to Ringer solution containing 10 times the normal content of Ca. These results

agree with those of other workers (*e.g.*, 5, 53, 79, 235) in showing that the size of a contracture is more or less proportional to the external concentration of Ca. Special mention should be made of the elegant experiments of Lüttgau (172) on single muscle fibers, showing that the greater the external Ca^{2+} concentration, the greater is the duration of contracture. With twitches, however, contrary results appear (178, 202), for increase in external Ca from the normal of 1.8 mM to 36 mM causes a decrease in peak tension of the frog sartorius. In fact, Brecht *et al.* (35) proposed that the muscle's capacity to develop contractions (twitches and tetani) depends on a source of fiber Ca which is internal and more tightly bound, in contrast to Ca for contracture, which is relatively loosely bound at superficial sites. These ideas are provocative, but they must be considered as speculative, especially since the experiments involved the relatively large frog sartorius in which fibers at different depths in the muscle may react differently to an external change of Ca (35, 61).

However, Frank (83, 85) found that the K-contracture can be restored in Ca-deprived frog toe muscles after they have been exposed to 1 to 2 mM solutions of Cd^{2+}, Be^{2+}, Ni^{2+} and Co^{2+}. These ions evidently do not themselves substitute for the missing Ca^{2+}, but they act by releasing Ca^{2+} from some tightly bound state and thus provide the free Ca^{2+} needed for the K-contracture. Frank (84) found further evidence for such tightly bound Ca^{2+} in relation to acetylcholine contractures of frog toe muscles. The source and site of action of this released calcium have not been specified, but the possibility that the experimental ions must at least be able to make contact with the T tubules as well as with the plasma membrane suggests that these structures may be involved.

Other aspects of the effects of Ca lack appear in the changes in membrane potential. It will be recalled that Frank (78, 79) found that, after exposure to Ca-free media, toe muscles were depolarized by excess K but did not develop contracture; this was supposed to indicate that the Ca deprivation had removed a source of superficially located Ca that would normally serve "as a link between electrical and mechanical events in contraction." Reasons have already been presented for discarding such a "Ca-entry" mechanism in E-C coupling. There are other possibilities for Ca involvement in coupling processes, however, and one of these is suggested by the decrease in resting membrane potential that occurs in a Ca-free medium (58, 147, 148, 204), or even in a medium containing some, though much lowered, Ca, *e.g.*, 5% of normal (40). The results show that under Ca-free conditions the membrane potential may fall by about 10 mV in a minute or less and then continue to decrease at a much smaller rate. But there are great variations in this behavior from fiber to fiber, depending on whether they are tested in the intact muscle or after isolation (see especially 204). These results, however, have raised the question whether the failure of a muscle to develop a K-contracture in a Ca-free medium might not be due to the fall in membrane potential induced by the Ca deprivation. In new experiments, Frank (85) showed that the membrane potential of fibers of frog toe muscle in a Ca-free solution does not decrease at all during the few minutes required for complete elimination of the K-contracture. The tests for contracture were made, however,

only with 25 mM K^+, which is just above the threshold for normal fibers. A more secure conclusion would be drawn from these experiments if the concentration of K^+ was much higher than that used. In any case, somewhat discordant results in this respect have been obtained by other workers (204).

It is interesting, furthermore, that contractile responses can be restored in nonreactive fibers by subjecting them to repolarizing electric pulses (35, 50, 148). This suggests that the significant factor causing mechanical irresponsiveness of muscle fibers under Ca deprivation is not the lack of Ca^{2+}, as such, but the associated fall in the membrane potential. It is known that the muscle fiber does not contract in response to twitch stimuli (61, 148, 149) when the membrane potential has been previously reduced to about −60 mV. This is due, however, to the failure of the fibers to develop action potentials. In the case of fibers whose resting potentials are lower than normal, and in which extra depolarization is effected with very high K concentration (*e.g.*, 190 mM), mechanical irresponsiveness is graded, beginning when the resting potential is about −60 mV and becoming maximal (indicative of complete "mechanical refractoriness") at about −30 mV (124, 192). Since extra depolarization occurs but contraction does not, the original low membrane potential, say −30 mV, has completely inactivated some link necessary for electromechanical coupling. Since the fibers that exhibit such refractoriness are completely relaxed (after having performed a contracture), we must conclude that the Ca^{2+} that had been bound to the myofilaments during activation of contraction has been given up and reaccumulated in the sarcoplasmic reticulum. It therefore is inferred that maintained depolarization of a muscle fiber causes mechanical refractoriness by inactivating some Ca-dependent electromechanical coupling link in the plasma membrane or the T tubules. Support for this inference will appear in the next section dealing with the effects of caffeine.

Further very interesting effects of Ca^{2+} are the following: (a) In the complete absence of the ion, the magnitudes of both the twitch and the action potential fall progressively before the resting membrane potential becomes so low that inexcitability develops, and this suggests that the degree of mechanical activation is proportional to the magnitude of the action potential (61). This is further evidence that Ca^{2+} plays a role in E-C coupling at the plasma membrane level. (b) Lüttgau (172) found that variation in the external Ca^{2+} concentration from 0.5 mM to 5.0 mM causes the curve relating tension output to membrane potential induced by K-depolarizations to shift to higher ranges of potential; the mechanical threshold is altered from about −60 mV at 0.5 mM Ca to −20 mV at 5.0 mM Ca. Such a shift in threshold was first described in work on whole muscles by Fleckenstein and Hertel (77), and it has also been observed in crayfish fibers (198). Furthermore, Lüttgau (172) showed that, as the Ca^{2+} ion concentration is increased, a generally similar shift develops in the mechanical refractoriness curve (see also 51). Lüttgau pointed out that these Ca-induced changes occur very rapidly—within a few seconds after altering the concentration of Ca^{2+} in the medium containing the single muscle fiber—and this rapidity again indicates the involvement in E-C coupling of Ca ions at relatively super-

ficial sites either at the plasma membrane or the T tubule. (Lüttgau's paper should be read also for the valuable discussion of the differences between heart and twitch skeletal muscle fibers, and for the comparison of the effects of Ca^{2+} on the systems concerned with the carriage of Na in excitation and with the coupling of excitation to contraction.) (c) Special mention should be made of Jenden and Reger's (148) proposal that under Ca deprivation, the decline of the resting potential is not the only factor causing decrease in the mechanical response. The other is the increase in the potential for mechanical refractoriness (172). Accordingly, mechanical unresponsiveness to a depolarization appears when the potential for refractoriness becomes equal to or greater than the resting potential. (d) Finally, the report should be noted that immersion of a frog slow (rectus) muscle in Ca-free media surprisingly causes development of a slow contracture which is spontaneously reversible; the contracture is not dependent on spontaneous firing of the fibers, and it can be quickly reversed by restoration of Ca^{2+} (144). Such contracture was not reported in other work on the frog rectus (*e.g.*, 235), and it seems that there should be more investigation of this aberrant result, *e.g.*, in regard to the role of depolarization possibly set up by the lack of Ca.

It should now be quite clear that the effects of Ca^{2+} on the properties of the muscle fiber are extremely varied, complex, and bewildering. It should also be evident that these effects must signal important alterations in the series of linked reactions that couple membrane depolarization to contractile activation. We have yet to comprehend the specific nature of these alterations. But it is valid to conclude that the calcium ion plays a role in E-C coupling not only in connection with the various reticular mechanisms that control its concentration in the myoplasm and its ability to regulate the contraction-relaxation cycle of the myofibrils (as discussed in Section IV), but also by affecting the resting and action potentials and the mechanical threshold and refractoriness potentials, and thus entering into the functions of the plasma membrane and the T tubules in electromechanical coupling.

B. Dissociation of electromechanical coupling

Since E-C coupling consists of a sequence of linked processes, it should not be surprising that certain treatments of muscle suppress the functioning of some link and thereby dissociate the electrical and mechanical responses that, respectively, begin and end the sequence. A partial dissociation is apparently involved in the already noted result that, as Ca deprivation begins to act on a fiber, the twitch tension falls more rapidly than the spike potential (61). It may also occur in the various cases in which the so-called coupling efficiency (the size of the twitch tension relative to spike height) is reduced (178, 180). In all such cases, however, the proper characterization of the action potential should include more than a mere reference to the spike height since the triggering activity of the action potential is evidently a function of its mechanically effective period, which is determined by the duration as well as the height of the spike and by the level of the mechanical threshold.

There are several cases in which the action potential persists without significant change while the mechanical response is abolished or is very greatly reduced. Such complete, or nearly complete, electromechanical dissociation is caused by hypertonicity of the medium (121), 2,4-dinitrophenol (164), sodium cyanide and iodoacetate (173), urea (in 1 M concentration) (12), fatigue (57, 180, 184, 238), denervation (158), and prolonged immersion in Ringer solution (157). At least under the action of urea, the effect of the agent is evidently directly to prevent the actin and myosin filaments to interact (12, but also see 11a, 166, 222), and so here it may not be a question of an action on E-C coupling. But, in certain cases dissociation occurs although the myofilaments are still able to contract, as is shown (a) by the development of caffeine contracture following dissociation after long soaking in Ringer solution (157), denervation (158), or hypertonicity (42), (b) by the large tension output in K-contractures under a dissociation caused by fatigue (57), and (c) by the spontaneous reappearance of the twitch in muscles that have been soaked in a solution made hypertonic by glycerol (92, 265). Another instance of interest is the prevention of the dissociation ordinarily produced by hypertonicity by a preliminary exposure of the muscle to isosmotic KCl (94).

Thus, clear electromechanical dissociation can occur in muscles whose contractile system is evidently in working order. In such cases we must conclude that a break has developed in some link of the sequence that couples excitation (*i.e.*, the action potential) to contraction. None of the quoted work gives any indication as to the nature of the inhibited link. Work directed to its identification should be of great interest, and in this connection studies should be made when possible on the mechanical threshold and the fluxes and translocation of Ca in the affected muscles.

C. Potentiators: general actions

Many substances of chemically diverse groups potentiate skeletal muscle contraction by causing changes in E-C coupling. These potentiators include lyotropic anions like NO_3^- and I^-, divalent metal cations like Zn^{2+} and UO_2^{2+}, at least one substance, caffeine, which at physiological pH is uncharged, and a number of miscellaneous substances. I first present some general features of the actions of these potentiators (for an extensive review, see 227), and then in succeeding sections will discuss special aspects of the particular kinds of potentiators that are important in relation to E-C coupling.

The easily observable and very striking features of potentiators are mechanical: the potentiators all affect the twitch by markedly augmenting the peak tension and prolonging the contraction and relaxation periods; as for the tetanus, they do not affect the plateau tension, but they reduce the fusion frequency. These effects indicate that potentiators prolong the active state of contraction, but, in view of the absence of change in maximal tetanus tension (P_0), it is evident that they do not affect the first of the active state's intensity factors, the capacity to bear a load, which, at maximum, is measured by P_0. Potentiators also increase the rate of both shortening in isotonic contractions and tension development in isometric contractions. These increases may result merely from

the prolongation of the active state (227). But, under certain conditions, they seem to occur because the potentiators increase the second of the active state's intensity factors, the capacity to shorten (232, 233). Thus, in general, potentiators do not alter the maximal capacity of the active state to produce tension (P_0) (for interesting exceptional cases, see 38a), but they prolong the duration of the active state and may increase its intrinsic capacity to cause shortening; and these respective modifications of the active state are most directly manifested by augmentation in twitch tension and by certain specialized increases in rate of tension development, *e.g.*, in the earliest part of the isometric contraction period.

The potentiators produce these mechanical alterations, not by acting directly on the contractile material (119, 131, 145, 150, 152, 221, 227), but by affecting one or another process in the E-C coupling sequence. It is this that makes their effects on muscle of interest in this review, and it will be seen in the following that comprehension of the various changes caused by the potentiators helps in elucidating certain electromechanical and other aspects of E-C coupling (see also 129, 227, 234a). It is noteworthy that the lyotropic anions (193, 209), UO_2^{2+} (237), and Zn^{2+} (146) do not potentiate, but rather depress heart muscle contraction. These effects need further investigation, especially for their significance regarding differences in E-C coupling between cardiac and skeletal muscle.

D. Lyotropic anions

The anions Br^-, NO_3^-, I^-, SCN^-, and $CH_3SO_3^-$ cause typical potentiating effects in concentrations of the order of 0.01 to 0.1 M and are therefore generally studied by substitution for an equivalent amount of the chloride, the anion normally present in Ringer solution (119, 125, 131, 150, 151, 152). The anions are especially noteworthy in that the increase in rate of tension development they cause to appear in frog skeletal muscle has onset at the very start of the contraction period, *i.e.*, at room temperature, at the end of the latent period of about 2 milliseconds (232). Close (43), however, found a decrease and not an increase in rate of tension development in frog sartorii at 0°C, but his result could not be repeated (232). The reason for this discrepancy is at present undetermined.

As in the case of other agents that affect E-C coupling it is very important to determine the site of direct action of the anions. The effects of these ions are produced very quickly in muscles, and they are as rapidly reversed (119, 150, 152). Especially informative are the results of single fiber experiments of Hodgkin and Horowicz (125) showing that the time constant of both the "on-effect ($Cl \rightarrow NO_3$)" and the "off-effect ($NO_3 \rightarrow Cl$)" is about 1.5 to 2.0 seconds. Similar effects are obtained with the other abnormal anions. Although these time constants are indeed small, they would have been still smaller had the test anions acted directly at plasma membrane sites. The timing of these events suggests that the site of action is at some deeper locus, probably in the T tubules (125), into which the anions should be able to diffuse (see Section III C), but hardly in the myoplasm because penetration into this compartment of a muscle is a much slower process (119, 152).

The anions cause significant changes in electromechanical coupling. Most

important, they shift the curve relating contracture tension and membrane potential to lower values of potential (125; see also 81, 223). Thus, the mechanical threshold occurs at −65 mV instead of −50 mV, and, in general, the potential for saturation of tension is changed from the neighborhood of −20 mV to larger values (*e.g.*, for SCN^- to about −50 mV, but for NO_3^- it is not certain whether such an effect occurs). Although changes of the action potential have also been reported (67, 102), they are very likely not electromechanically significant (for details, and certain possible exceptions, see 69, 102, 131, 171, 227, 234, 234a). Hence, it is inferred that, in the anion-potentiated twitch, the decrease in the mechanical threshold is a major (125), and possibly the sole (234, 234a), electromechanical alteration determining the various potentiation effects. Thus, under this condition the mechanically effective period starts as soon as the rising spike depolarizes the membrane to the lowered mechanical threshold at −65 mV (instead of to the normal one at about −50 mV) and then this period is maintained for the rest of its duration in relation to this lower mechanical threshold. It is this relationship to the new mechanical threshold that evidently enhances some feature of E-C coupling that causes the mechanical consequences of potentiation, which are observable as the increases in both peak value and rate of earliest development of twitch tension. The fact that the rate of tension development begins to show the anionically determined increase at so early a moment as the end of the latent period signifies that a corresponding early event at the beginning of this period is responsible for this increase. And this event is evidently the attainment, very early during the rise of the spike, of the membrane potential corresponding to the decreased level of the mechanical threshold (232). Substances such as NO_3^- (and, as will be seen below, caffeine) that produce potentiating effects as just described are referred to as type A potentiators (232).

Present knowledge does not permit identification of the E-C coupling process that is affected by the anions (125, 227). But since the mechanical threshold is lowered, and since Ca^{2+} plays such a decisive role in direct activation of the myofibrils, it is attractive to suppose that there might be a greater-than-normal liberation of internal Ca^{2+} which could determine both the increased rate of contraction and the augmented peak twitch tension (232). Bianchi and Shanes (24) reported that NO_3^- causes an increased influx of Ca^{2+} during the potentiated twitch. But, for the general reasons previously discussed (Section IV E), this does not represent the Ca^{2+} that directly activates contraction, although it might reflect the activity of some earlier link in E-C coupling. As to other aspects of the mechanism of action of the anions, there obviously is no involvement of the action potential as such, as previously explained, nor, therefore, of the plasma membrane, even though there is evidence that the anions induce certain excitatory and other membrane changes in the muscle fiber (132, 150, 227). As previously stated, there is great likelihood that the abnormal anions enter the T tubules, replacing the normally present chloride. Thus they may increase the resistance and the length constant of the tubule membrane, in accord with their action on the plasma membrane (132), and consequently affect E-C coupling. It is also conceivable that the change in anion would alter the process that is

supposed to occur at the triad junctions and that directly causes the lateral sacs to release Ca^{2+}. Probably the reported effect (56) that the calcium-pump activity of the isolated sarcoplasmic reticulum is inhibited by the anions has some bearing on this problem, for this action represents an inhibition of a relaxation process which might be tantamount to prolonging the active state and thus enhancing contraction.

E. Divalent metal ions

The outstanding members of this group of potentiators, referred to as type B, are Zn^{2+} and UO_2^{2+}, or the equivalent hydrolytic complex ions they form in water solution (145, 227, 229, 230). Be^{2+}, Sr^{2+}, Cd^{2+}, Ba^{2+}, Ni^{2+}, Cu^{2+} and Pt^{2+} (but strikingly not Ca^{2+} or Mg^{2+}) also are potentiators, though not so effective as zinc and uranyl (146). Zinc causes a 2 to 3 $\times$ augmented twitch tension at a concentration of 50 μM, and UO_2^{2+} does so at only about 0.5 μM. The following discussion will be based mainly on the effects of these two heavy metal ions on frog skeletal muscle.

Zinc and uranyl ions produce all the typical effects of potentiators. But they cause an early increase in rate of tension development which has onset (at room temperature) at about 3.5 milliseconds following stimulation, and therefore some 1.5 milliseconds later than the corresponding increase caused by the anions. This difference becomes significant in terms of the electrical changes caused by the cations. Zn^{2+} and UO_2^{2+}, acting in concentrations for maximal effects, do not change the mechanical threshold (234), but they prolong by 3 to 4 $\times$ the entire repolarization phase of the action potential (58, 159, 181, 234). This change is evidently the means by which the cations cause potentiation, for slowing repolarization prolongs the action potential and thereby increases the mechanically effective period by an equal factor. Similar proposals involving prolongation of the action potential to account for prolonging the active state by potentiators in general, have been made previously by several authors (17, 63, 65, 66, 67, 69, 171), but without clear reference to the role of the mechanical threshold in determining the mechanically effective period of the action potential.

The rising phase of the action potential is not changed under the action of Zn^{2+} at relatively low concentrations, up to about 0.1 mM, and of UO_2^{2+} up to about 1 to 2 μM. Therefore, the alteration in E-C coupling caused by these ions does not begin until some moment during the falling phase of the action potential when the deceleration in repolarization of the membrane becomes significant in prolonging the mechanically effective period. According to Sandow and coworkers (234) this moment occurs about 1.5 milliseconds after the initiation of the spike. Therefore, this potentiating event is delayed by 1.5 milliseconds relative to the onset of the potentiating influence caused by the anions, which arises at the start of the spike and which reflects their capacity to lower the mechanical threshold. It is this that explains the fact that the onset of the increase in tension rate induced by the cations appears about 1.5 milliseconds later than that caused by the anions (232, 234a).

The site of direct action of Zn^{2+} and UO_2^{2+} is difficult to determine (145, 181,

227). These ions should get into the T tubules, and therefore it is conceivable that they might act on the membrane of the tubules or at the junctional regions of the triads. However, there is the definite evidence as discussed above that they greatly alter the action potential, and this suggests that they act at least on the plasma membrane, probably in some topochemical reaction with a membrane ligand (227, 230) that presumably slows repolarization by delaying Na inactivation or K activation, or both, in the electrochemical events that determine excitation according to the Na theory (*e.g.*, 120). Much more work should be done on these questions, and studies are especially needed on single muscle fibers in order to obtain the actual kinetics of development of the cationic effects and thus a basis for definite localization of their site of action. As for the final effects in the altered E-C coupling engendered by the heavy metal cations, we must again suppose that there is some sort of augmentation of the Ca^{2+} release (232, 234a). But more research is required to test this assumption, and it should be informative to apply to this problem the autoradiographic techniques used by Winegrad (263) to study activity-induced internal translocation of calcium.

F. *Caffeine*

This alkaloid is very interesting because it produces two rather distinct effects on muscle mechanics that are both pertinent to the problem of E-C coupling. In low concentrations, up to about 2 to 4 mM for frog muscle, its sole effect is potentiation. In higher concentrations, greater than about 5 mM, its predominant effect is contracture (10), though potentiation may also still occur. The effects of caffeine vary widely with the species of muscle, mammalian muscle being especially resistant to its contracture-inducing capacity (108). After denervation, however, such muscle readily produces caffeine contracture (99a).

As to the site of action of caffeine, Axelsson and Thesleff (10) originally reported that the drug did not cause contracture when injected into a living muscle fiber. Since it did evoke contracture when applied to the outer surface of the fiber, they concluded that it acted directly only on the plasma membrane. Miyamoto's (186) findings are concordant with this conclusion, and several workers have used this view as a basis for further attempts to analyze the action of caffeine (79, 83, 91, 183). But it is clear from the experiments of Caldwell and Walster (41) that caffeine causes contracture when injected into crab muscle fibers. Furthermore, caffeine quickly penetrates into the whole frog muscle fiber (22) and therefore when applied only externally, it can come into contact with and act upon internal sites throughout the myoplasm. Studies on glycerinated muscle fibers show that the drug does not affect the contractile system unless relaxing factor is present, and then relaxing activity is abolished and the contractile material contracts (107, 189). The mechanism of this action of caffeine is now apparent in the finding of Herz and Weber (112) that the alkaloid causes Ca release from, and inhibits uptake of Ca by, the isolated sarcoplasmic reticulum. The consensus seems to be that caffeine affects muscle by entering into the fibers and at least antagonizing the relaxing activity (the Ca pumping) of the sarcoplasmic reticulum. Corroborative evidence for this view is found in the

demonstration (19) that caffeine, at least in 5 mM concentration, increases the outflux of Ca^{2+} from resting frog muscle. It also increases the influx of Ca^{2+} and this is ascribed to an increase in permeability of the surface membrane to Ca^{2+} (19).

I shall now discuss in detail the potentiating effects of caffeine. Both the mechanical changes (221, 228, 232) and the evident electromechanical alterations (65, 234) are in general the same as those produced by the anions. Caffeine in 1 mM concentration (a) augments the twitch by prolonging the active state, (b) causes an increase in the rate of tension rise, which begins very early (at the end of the latent period), (c) lowers the mechanical threshold, and (d) does not significantly alter the action potential. Thus, caffeine produces the effects of a type A potentiator and much of the previous discussion regarding the mechanism of action of the anions applies to the possible influence of caffeine on E-C coupling. We must take into account, however, the previously discussed indications that caffeine causes release of Ca^{2+} from the sarcoplasmic reticulum of living muscle fibers. It seems reasonable to suppose that the drug in low concentration releases some Ca^{2+} into the resting myoplasm but that this is insufficient to cause contracture. It would, however, provide an internal pool of free Ca^{2+} adding to that released in an excited fiber, and thus increasing the myoplasmic free Ca^{2+} available for activating contraction. It is conceivable that the alkaloid also increases the sensitivity of the lateral sacs to the Ca^{2+}-releasing action of the depolarizing signal in the T tubules. Such actions, at any rate, would account for the decrease in mechanical threshold caused by caffeine and for the various resultant potentiating effects of the drug on the course and the magnitude of the twitch.

I note again that caffeine causes the same final changes in the mechanical response as the anions and also the same electromechanical change, namely reduction in mechanical threshold. This is significant since it strengthens the conclusion previously drawn only for the anions, that reduction in mechanical threshold is an electromechanical alteration in E-C coupling that can result in the characteristic type A potentiating effects. This identification also suggests that, despite possible differences between the effects of caffeine and the anions on certain intermediate reactions in E-C coupling, the end results at the final link—the release of Ca^{2+} and its action on the myofilaments—are the same.

The contracture produced by caffeine occurs evidently because the drug in relatively high concentration releases Ca^{2+} into the myoplasm in excess of the threshold for activating the myofibrils. Proof that this release of Ca^{2+} activates the contracture is found in the fact that procaine, which suppresses the Ca^{2+} release, also prevents the caffeine from producing contracture (74). That the Ca^{2+} which is released comes from the sarcoplasmic reticulum, as was previously suggested (see 112), is further indicated by the striking result that the caffeine contracture occurs just as well in a completely depolarized fiber as in a normal one—in fact, the depolarized fiber is even more sensitive to caffeine (10, 182, 183). Furthermore, the presence of the depolarization affects neither the caffeine-induced fluxes of Ca^{2+} (19, 74) nor the inhibition of such fluxes due to procaine

(74). These various results indicate that the drug acts at a stage in the E-C coupling sequence which follows the depolarization at the plasma membrane or the T tubule membrane. This leaves the sarcoplasmic reticulum as at least one remaining possibility for the site of action of the caffeine, a conclusion in accord with the other evidence previously discussed, and this suggests that the sensitization of mammalian muscle to caffeine contracture caused by denervation involves an effect on the sarcoplasmic reticulum (99a).

Caffeine contracture occurs in a muscle equilibrated to a Ca-free medium (10, 79, 83, but see 186), and Ca^{2+} deprivation does not affect the caffeine-induced fluxes of Ca^{2+} (19). Since the lack of Ca^{2+} in the external medium is most likely to affect the Ca reactions of the easily accessible membranes of the surface and the T tubules, the occurrence under these conditions of normal caffeine contractures and associated Ca fluxes suggests, again, that the caffeine acts at some more deeply located structure such as the sarcoplasmic reticulum. The store of Ca at such a locus is evidently irreversibly released in a caffeine contracture, since only 3 or 4 successive responses can be evoked by the drug in a Ca-free medium, even though a Ringer wash follows each caffeine contracture (83). Return of such an irresponsive muscle to normal Ca-Ringer restores the caffeine effect. Further studies should be made in relation to these effects, especially to determine whether several caffeine treatments significantly lower or redistribute the Ca content of the muscle, and whether the effects of caffeine on the isolated reticulum (112) are reversible.

Although there is obviously much evidence that caffeine produces its effects by increasing the capacity of the sarcoplasmic reticulum to release Ca^{2+}, we should take note of several aspects of its action that suggest the participation of other factors. Caffeine contracture is not generally a reversible mechanical change; on that account it might better be referred to as a rigor, especially when the drug has acted in high concentration and the irreversibility is complete. Furthermore, in even rather low concentration (*e.g.*, about 0.5 mM), the drug causes certain disruptive changes in fine structure of the muscle fiber, visible at both the optical and electron microscopic levels (44), which are evidently the basis of the irreversibility of the "contracture." It is of great interest to determine how these changes are related to the assumed effects of caffeine on the sarcoplasmic reticulum. A second point is that even though procaine inhibits the contracture effect of caffeine on living muscle, it does not produce an equivalent effect on glycerinated fibers treated with the relaxing factor (189). This contradiction requires further study, but its resolution may be found in the fact (unpublished results of Dr. A. Weber) that procaine antagonizes the ability of caffeine to cause release of Ca^{2+} from vesicles of the extracted sarcoplasmic reticulum. Finally, it has been found that the lyotropic anions enhance caffeine contracture, as they potentiate the twitch (183). The puzzling feature of this is that the anions do not seem to have free access to the sarcoplasmic reticulum, and it is therefore difficult to see how their influence could be exerted on the caffeine effect which is supposed to be mediated by an action on the reticulum. It may be possible that the anions cause their effect by entering the T tubules

and exerting an influence across the triad junctions that connect the tubules to the lateral sacs.

G. Other effects

As reviewed elsewhere (227), there are many other substances and agents, besides those discussed in the foregoing, that potentiate contraction, and there are other ions and drugs that alter contraction in other ways. In all such cases, modifications in E-C coupling must be involved unless the agent or substance in question causes its change by acting directly on the contractile function of the myofibrils. There is no need for us to discuss these other instances, since, like the previously discussed Ca effects, they are difficult to interpret in respect to definite mechanisms of E-C coupling (see, in respect to the action of various ions, 178, 251, temperature on heart, 179, ouabain, 128, acetylcholine on slow muscle, 247, eserine and imidazole, 231, and epinephrine, 99).

VII. REGULATION OF CONTRACTION BY THE ACTION POTENTIAL: GENERAL CONCLUSION

In this closing section, I take up again the role of the action potential in E-C coupling. This problem arose at the very start of this review and it reappeared at various later points. In view of much material that has been given in the foregoing, a fresh analysis of the function of the action potential is possible. As in the initial consideration of this function, the new discussion will be concerned essentially with directly observable activities of the physiologically intact muscle fiber, *i.e.*, the action potential and the correlated mechanical activity. These responses mark the initiation and the completion of E-C coupling and they thus specify the basic problem of E-C coupling, *i.e.*, the determination of the mechanism by which the electrical change causes the mechanical one.

Evidence was adduced in Sections II A and B that initiation of E-C coupling occurs in consequence of the events of the mechanically effective period of the action potential. The effects of the potentiators discussed above demonstrate that the ability of the events of this period to cause contractile activity can be increased in either of two ways, by lowering the mechanical threshold (type A potentiators) or by decelerating the fall of the spike and thus prolonging it (type B). Certain potentiators cause both of these effects to develop together, *e.g.*, caffeine in relatively high concentration (65, 234) and quinine (234). Furthermore, certain potentiators (and possibly other agents, *e.g.*, Na deficiency and Ca excess) affect the role of the action potential in E-C coupling in still another way, *i.e.*, by reducing the rate of rise of the spike (227, 232). This is produced, *e.g.*, by zinc in relatively high concentration (*e.g.*, 0.25 mM) and by eserine (0.5 mM) (248), which, however, act otherwise like type B potentiators in that they prolong the spike and thereby cause an augmented twitch tension and a relatively delayed increase in rate of tension development (234a). The reduction in rate of rise of the spike is reflected in the mechanical output as a reduction in rate of rise of the very earliest development of twitch tension (232). Thus, the two successive modifications caused by these agents in the action

potential have a corresponding dual effect on the rate of rise of twitch tension: *i.e.*, the slowed rise of the spike and the later prolongation of it project their respective effects into the contraction period of a given twitch as an initial decrease followed by an increase in the rate of tension development.

It is evident from the foregoing that the action potential is not a simple trigger in E-C coupling of skeletal muscle. We think of a trigger as a device whose intensity develops to some threshold and then explosively sets off a discharge in a given system to the maximal magnitude it is capable of producing. The action potential may be likened to a trigger in that it abruptly initiates the process that generates the cycle of events of the twitch. But it is unlike a trigger in that its function during the mechanically effective period is not fixed but can be modulated by altering the level of the mechanical threshold and the rates of rise and fall of the spike and thus causing changes in E-C coupling that ultimately generate corresponding modifications in the course of the twitch. For similar considerations regarding flexibility in the role of the action potential in the control of cardiac muscle contraction, see Fleckenstein (76).

The various mechanical changes in the twitch of skeletal muscle have their origin, of course, in alterations of the active state (227) and these present a more pertinent indication of the role of the action potential in regulating mechanical activity. Thus, either the lowering of the mechanical threshold or the prolongation of the spike prolongs the active state (thus increasing the peak twitch tension), and intensifies the factor of the active state which determines the velocity of shortening of the contractile component (thus increasing the rate of early contraction); and the reduction in rate of rise of the spike reduces the contractile component's velocity of shortening during the very earliest moments of development of the active state (thus reducing the rate of contraction during a corresponding very early part of the twitch). Hence, variations in the action potential regulate mechanical activity by changing the duration of the active state and the factor of that state that determines the speed of shortening. It should be noted that, according to this analysis, the action potential has no control over the active state's capacity to bear a load. This function of contractile activity is evidently determined by the properties of the myofibrils themselves, and it may reflect the maximal intrinsic strength of the cross-bridges that are formed between the actin and myosin filaments when the sliding-filament mechanism of contraction is fully activated.

It is now apparent that the solution of the problem of E-C coupling involves not only the very important question of determining the nature of the series of links that couple membrane depolarization to contractile activity. It is also necessary to elucidate the quantitative variations of these mechanisms that are presumably engaged in altering the function of the action potential in regulating contraction. It seems evident that prolongation of the action potential must increase the duration of some key process in E-C coupling that, in turn, causes prolongation of the active state. Decrease in the mechanical threshold, as in twitch potentiation by nitrate or caffeine, also prolongs the active state, but it is not so clear how this occurs. Nevertheless, it would seem, in view of the common end effect, that this change must also prolong the action of the same link

as is involved in potentiation produced by extending the duration of the action potential. It is also noteworthy that both of these variations in the major factors of the mechanically effective period cause an increase in the rate of contraction, and this mechanical effect may also be ascribed to the same common effect that prolongs the active state and potentiates the twitch.

It is obviously of great interest to determine the mechanisms by which the mentioned changes in E-C coupling produce their mechanical effects, for this information should elucidate the basic processes of normal E-C coupling. There is at present no means for attempting this in terms of the activities of all of the E-C coupling links, since our knowledge of these is too rudimentary. But there seems to be a firmer basis for a speculation regarding at least one of these links, *viz.*, the role of Ca^{2+} in activating contraction, and it is of interest to study its possible involvement as a key process mediating the regulatory function of the action potential. Much evidence has been presented above which indicates in some detail how the calcium ion must function in activating contraction. Further evidence of this sort is adduced by considering that the speed of contraction of the sliding-filament mechanism is inherently dependent on the rate of breakdown of adenosinetriphosphate (142) and that this depends, in turn, on the concentration of free Ca^{2+} (257, 259). Thus, it may be inferred that, in the living fiber, the rate of contraction (at least in the earliest part of the contraction period when the Ca^{2+} has just been released from the lateral sacs) is determined by the magnitude of the Ca^{2+} concentration; and that, conversely, the observed rate of contraction can be taken as a measure of the concentration of activator Ca^{2+}.

Hence, it can be postulated that, in potentiated twitches, the observed increase in rate of early development of tension signifies a greater-than-normal release of Ca^{2+}. This greater release of Ca^{2+} would also account for prolongation of the active state if it is assumed that removal of the activator Ca^{2+} from the myofilaments by the Ca-pump activity occurs with normal time constant. For, owing to starting from an initially higher level, the fall of the free Ca^{2+} concentration would be delayed in respect to that occurring from the normal level of released Ca^{2+} concentration. Thus, the increase in the amount of released Ca^{2+} would not only increase the rate of contraction but it would also cause the concentration of Ca^{2+} to persist at activating levels for a longer time than normal and thus prolong the active state. If these considerations are now projected backwards in time of a muscle response, they indicate that the reference previously made to the efficacy of the action potential in initiating E-C coupling can be explained in terms of the release of Ca^{2+} as quantitatively controlled by the factors of the mechanically effective period. The lower the mechanical threshold and the longer the action potential maintains the membrane potential beyond that threshold, the greater is the amount of Ca^{2+} released in E-C coupling, and the greater, therefore, is the efficacy of the action potential in E-C coupling.

Taking into account the facts of the actions of potentiators, it is clear that the mechanisms that determine the mechanical threshold and the effective duration of the action potential are independently capable of variation. Evidently, the attainment of mechanical threshold by a depolarization of the membrane acts

as a sort of switch that turns on the process that releases Ca^{2+} from the lateral sacs into the myoplasm. This process continues as long as depolarization is maintained beyond mechanical threshold, and it is switched off when depolarization falls below threshold. This switching action as performed by the action potential, however, is evidently not an all-or-nothing affair. This was suggested in the previously discussed variations in the mechanical effectiveness of the overall performance of the action potential. But it is especially evident in the twitch in terms of the increase in mechanical output in K-contractures with increase in depolarization. For this indicates that, as the action potential runs its course, the effectiveness in releasing Ca^{2+} at each moment must vary as a function of the instantaneous value of the spike potential, and, thus, the intensity of the "triggering" action due to the action potential will rise and then fall during the mechanically effective period. (For a further elaboration of these concepts regarding the role of the action potential in E-C coupling, see 234a.)

The preceding discussion of electromechanical coupling in the twitch is based on various correlations of facets of the coupling process which are observable in the living muscle—the initial events that inhere in the role of the action potential and the causally related terminal events of the mechanical output. An attempt has been made, in addition, to account for several special cases of coupling excitation to contraction by postulating the occurrence of certain specific intermediary functions involving the Ca ion. Although there is good support for such assumptions, much of the evidence is indirect and, except for the results obtained with the Natori preparation (*e.g.*, 190, 211), it is derived from experiments on model systems reconstructed from extracts of muscle. Furthermore, the attempt to explain the regulatory function of the action potential has been based on the intermediation of only one of the E-C coupling links, namely, the one directly involving participation of the Ca ion as activator of contraction. It is obvious that a full account of the role of the action potential must also include a detailed exposition of the presumably electrical and electrochemical events along the T tubules and in the sarcoplasmic reticulum that cause the liberation of activator Ca^{2+} into the myoplasm. There is no doubt that important advances will continue to be made in our knowledge of E-C coupling by means of all the various techniques that have hitherto been used, such as electron microscopy, autoradiography, studies of extracted model systems, and so on. Much more should be done, however, by studying the responses of the physiologically intact living muscle. This is, of course, because there are so many links in E-C coupling and some of these are almost completely inaccessible for direct physiological investigation. But it is in this area of research that results obtained on other types of systems can be tested for physiological relevance and in which definitive developments in the solution of the problem of E-C coupling must finally be obtained.

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