0031-6997/88/4003-0189$02.00/0
PHARMACOLOGICAL REVIEWS

Vol. 40, No. 3
Printed in U.S.A.

Calcium Mobilization and Cardiac Inotropic Mechanisms

MELCHIOR REITER

Institut für Pharmakologie und Toxikologie der Technischen Universität München, 8000 München 40, Federal Republic of Germany

I. Introduction

THIS REVIEW is based on a contribution to a symposium on the role of calcium in cardiac function* which was held in honor of Otto Krayer, Pharmacologist at Harvard from 1939 to 1966 (144). Krayer had developed, in 1931, a method which allowed quantitative determination, in the heart-lung preparation, of the ability of the heart to function as a pump (224). He thereby provided a new and greatly improved means for the experimental evaluation of inotropic drugs which he used most effectively for most of the rest of a distinguished career.

The elucidation of the cellular mode of action of inotropic drugs became possible only after the intracellular messenger function of calcium in muscle became recognized in the late forties and fifties (for reviews, see refs. 337 and 405). The first evidence that calcium might transmit the signal given by the action potential on the membrane surface to the contractile material in the center of the fiber came from the injection of calcium into living muscle by Heilbrunn and Wiercinski (156). They found that calcium ions, even in low concentration,

* This article is the first of a series of articles arising from a program on Vistas in Pharmacology presented at a joint meeting of the American Society for Pharmacology and Experimental Therapeutics and the American Chemical Society Division of Medicinal Chemistry, August 18–22, 1985, in Boston. The program entitled "The Role of Calcium in Cardiac Function" was dedicated to Otto Krayer. The material has been updated by the authors and prepared for publication with the assistance of John R. Blinks whose participation is gratefully acknowledged.

cause an immediate and pronounced shortening, an effect which is not shared by any other cation in a concentration normally present in muscle. Later, a relaxing factor was identified and found to consist of calcium-accumulating vesicles formed from the sarcoplasmic reticulum which contains much of the cellular calcium content during rest (154, 95).

In asking why cardiac muscle is more susceptible to inotropic interventions than skeletal muscle, it was reasonable to look for differences in the ultrastructure of the two types of striated muscle (123). In cardiac muscle, which is much more dependent on extracellular calcium, the sarcoplasmic reticulum was found to be less extensive than that of skeletal muscle. The difference between the two kinds of muscle with respect to calcium metabolism was therefore assumed to reside primarily in the difference in their sarcoplasmic reticulum.

The lower capacity of cardiac muscle for calcium storage may be indirectly connected with a special electrophysiological feature of the cardiac cell: the long duration of its action potential (408). Its long-lasting plateau allows voltage-dependent passive movements of ions to take place which might be relevant for contraction. Influences on passive or active transport of ions through the sarcolemma may affect cellular calcium metabolism and thereby the inotropic state of cardiac muscle.

II. Control Sites of Contraction-related Calcium in the Cardiac Ventricular Cell

A. Calcium Influx

When the cytoplasmic concentration of Ca ions is raised above a threshold concentration of about 200 nmol/liter, the contraction of cardiac muscle is activated (361). In order to relax, the muscle has to reduce its sarcoplasmic Ca concentration again to values below the threshold concentration which is four orders of magnitude lower than the Ca concentration of the extracellular fluid. The contraction, therefore, is governed by rapid changes in intracellular Ca concentration (calcium transients) as can be convincingly shown by means of the calcium-sensitive bioluminescent protein aequorin (39, 41). In principle, there are two ways of modifying the strength of contraction: either by influencing the concentration (more precisely, the activity) of free Ca ions which is obtained after excitation, or by changing the Ca sensitivity of the myofilaments.

One way to increase the sarcoplasmic Ca concentration would be to open a channel in the sarcolemma during the action potential, thus allowing Ca ions to flow down their electrochemical gradient. This is probably the way in which contraction is activated in the amphibian heart (407, 287, 204). However, in the mammalian heart the mechanism is more complicated. This can be deduced from experiments in which the influence on contraction of a prolongation of the action potential by current injection was studied in both types of heart muscle (12). The frog's ventricle responded to each lengthening of the action potential duration with an increase of the strength of the accompanying beat, whereas in the mammalian ventricle the change was observed not in the accompanying beat but in the next following one. From these and other comparable experiments (421) we must conclude that, in the mammalian heart cell, most of the activator calcium does not come directly from the extracellular space but from an intracellular compartment that stores calcium in such a way that its release can be triggered by the action potential. Simultaneously, calcium influx loads this compartment from which it is available for later release.

B. Intracellular Calcium Stores

Two intracellular compartments are known to accumulate calcium, the mitochondria and the sarcoplasmic reticulum. In terms of total capacity the mitochondria represent the largest calcium reservoir in the cell. However, their maximal velocity of Ca uptake is much less than that of the sarcoplasmic reticulum (69), and they appear not to accumulate much calcium under physiological conditions (83). Considerable Ca uptake by mitochondria occurs only if the free Ca^{2+} concentration is raised to levels that cause contractures (83). At 1 μM sarcoplasmic concentration, Ca uptake was calculated to amount to only 6% of total uptake as compared with nearly 90% by the sarcoplasmic reticulum (69). This and a relatively low rate of Na-induced Ca release make the mitochondria not adequate for the beat-to-beat control of Ca movements.

The sarcoplasmic reticulum (SR) (see table 1) must be regarded as the chief regulator of the contraction-related Ca in the mammalian heart. The SR is suitably located to serve its purpose. Its dense network surrounds the myofibrils, and it is abundant in the space directly subjacent to the sarcolemma (123, 365, 282, 364, 338). The volume of SR associated with each myofibril is up to 15 times greater in the mammalian heart than in the frog ventricle (300). The SR consists essentially of two components, the free SR (or longitudinal SR) and the junctional SR, a synonym for the terminal cisterna or subsarcolemmal cisterna. In contrast to the tubules of the free SR, the junctional SR contains an electron-dense granular material which consists of a calcium binding protein referred to as calsequestrin (258). The structure of the junctional SR is characterized by the presence of junctional processes (feet) bridging the gap between the SR membrane and the inner surface of the sarcolemma (137, 261, 364, 131, 100, 336).

C. Excitation-Contraction Coupling

The regulation of intracellular Ca as related to cardiac contraction is connected with the excitation of the cell in a complex way (52). The depolarization of the membrane triggers the release of calcium from the subsarcolemmal cisternae of the sarcoplasmic reticulum (see sec-

TABLE 1
Explanation of terms

Abbreviation	Definition
a^i_{Na}	Intracellular sodium activity
BAY K 8644	Dihydropyridine derivative
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
$[Ca^{2+}]_o$	Extracellular (outside) Ca^{2+} concentration
cyclic AMP	Cyclic adenosine monophosphate
D 600	Gallopamil
dbcAMP	N^6-2′-O-Dibutyryl derivative of cyclic AMP
DCI	Dichloroisoproterenol
EC_{50}	Half-maximally effective concentration
EGTA	Ethyleneglycol- bis(β-aminoethylether)-N,N′-tetra-acetic acid
E_{NaCa}	Reversal potential of I_{NaCa}
fura-2	Ca^{2+}-sensitive fluorescent dye
IBMX	1-Methyl-3-isobutylxanthine
I_{Ca}	Ca current
I_{NaCa}	Na-Ca exchange current
$InsP_3$	Inositol 1,4,5-trisphosphate
I_{si}	Second (slow) inward current
I_{sic}	Counteraction-related component of I_{si}, probably identical with I_{NaCa}
I_{TI}	Transient inward current
$[K]_o$	Extracellular (outside) K concentration
$[Mg^{2+}]_o$	Extracellular (outside) Mg^{2+} concentration
$[Na]_i$	Intracellular Na concentration
NIEA	Negative inotropic effect of activation
OPC-8212	Phosphodiesterase inhibitor
PDE III	Phosphodiesterase subclass III
PIEA	Positive inotropic effect of activation
quin-2	Ca^{2+}-sensitive fluorescent dye
SR	Sarcoplasmic reticulum
T-system	Transverse tubular system
UD-CG-115	Pimobendane

tion III A), which leads to a rise of the sarcoplasmic Ca concentration. The calcium release is apparently influenced by muscle length (189, 84, 10). Increase in muscle length causes an immediate increase in contractility and a gradual subsequent rise. Whereas the former is most likely the result of an increase in the affinity of troponin C for calcium (159, 10), the slowly developing increase in force is probably the result of a change in the amount of calcium released by the SR (189, 84, 10). The binding of calcium by troponin C activates the sliding filament system, and the muscle contracts. Relaxation results from the active uptake of calcium by the longitudinal parts of the sarcoplasmic reticulum, from where it is returned with some delay to the release sites (junctional SR).

During the plateau of the action potential, calcium flows from the extracellular space into the cell with the second (slow) inward current (I_{si}). This current differs from the (fast) sodium current in having slower kinetics and different voltage dependence (for review, see ref. 329). The slow inward current is increased by catecholamines and produces a "slow action potential" if the fast sodium current is prevented by partial depolarization (302). The second inward current (I_{si}) may consist not of calcium current (I_{Ca}) alone, but also of a more slowly activating Na-Ca exchange current (see sections IV A and V A). I_{Ca} flows through special channels with a relatively long-lasting activity. These channels have been designated as L-type channels, in contrast to newly observed channels (20, 289, 269) with a short activity of a markedly transient time course (T-type Ca channels). T-channel current is much smaller and decays much more quickly than L-type channel current, so it contributes relatively little to Ca influx during the action potential plateau. Since T-type channels are activated at relatively negative potentials, they are thought to have functional significance mainly for pacemaker depolarization and action potential initiation in cells capable of spontaneous activity (20, 289).

Most of the inflowing calcium is taken up by the sarcoplasmic reticulum and stored, after some delay, in its release compartments (324; see section III A). The loading effect of each action potential is opposed by a net extrusion of calcium from the cell through sodium-calcium exchange (see section V A) and partly by an ATP-dependent Ca pump (see section VI). Sodium-calcium exchange plays a special role in the regulation of Ca movements through the sarcolemma (for review, see ref. 32). Since this exchange is electrogenic, Ca transport either to the outside or the inside depends on membrane potential and the intracellular concentrations of Na and Ca (see section V).

Calcium leaks during rest from the sarcoplasmic reticulum of cardiac muscle (108, 210), at a rate which is influenced by the resting membrane potential (399; section IV C). The calcium content of the store (release compartment) therefore depends, at a given resting potential, on the frequency and pattern of stimulation. In ventricular muscle from most mammalian species (there are some exceptions like that of the rat, but see section VI B), the store will be empty after a period of 5 to 10 min during which the muscle is not stimulated. Then, total calcium content of the cardiac muscle has dropped to one-fourth of its steady-state value (241), and most of the regions corresponding presumably to junctional SR are found "empty" of calcium by X-ray microanalysis (413, 412). As the muscle is continuously stimulated, the calcium release from the store and consequently the contractile force will increase until the frequency-dependent steady state is reached. As more of it is released, calcium becomes more effective in increasing the potassium conductance of the cellular membrane (185, 351, 65); this leads to an abbreviation of the action potential (184, 18) and thereby to a negative feedback in regard to calcium loading during the plateau of the action potential.

The fact that so many regulatory mechanisms are capable of influencing cardiac contractility makes it evident that a number of different cellular structures may be regarded as sites of contractile control and therefore as likely sites of pharmacological interaction. These are mainly: (*a*) the sarcolemma because of its role in cellular

Ca uptake and elimination [sodium- and potential-dependent Na-Ca exchange, potential-dependent Ca current, calcium release, and the Ca pump (Ca^{2+}-transport ATPase)]; (*b*) the intracellular compartments—the sarcoplasmic reticulum (with Ca uptake and release mechanisms) and the mitochondria (Ca buffer); (*c*) Ca-binding "modulator" proteins (*e.g.*, calmodulin); and (*d*) the myofilaments with possible changes of their responsiveness to calcium.

III. Increase of Calcium Influx through Voltage-dependent Channels by Catecholamines

Calcium uptake in electrically stimulated heart muscle is increased under the influence of epinephrine (326). This effect is exerted through a β-adrenoceptor-induced increase in I_{Ca} (327, 328, 393) which results from a lengthening of the mean open time of activated (L-type) calcium channels in the sarcolemma (331). I_{Ca} can be inhibited by various calcium channel blockers (129). The influence of the increase in Ca influx on contraction can be demonstrated very clearly when the Ca store of the sarcoplasmic reticulum is empty and, accordingly, the force of contraction is negligibly small as in rested state contractions of cardiac ventricular muscle from most mammalian species (213; regarding the exceptions, see section VI B).

A. Late and Early Rested State Contractions

Rested state contractions are defined as contractions preceded by intervals of rest long enough that the strength of contraction is independent of previous beats (38). It is characteristic for the small rested state contractions of mammalian ventricular muscle that peak force is achieved relatively late after stimulation (7, 8). Catecholamines increase the late peak of rested state contractions (344, 345, 24). This is also true of the dibutyryl derivative of cyclic adenosine monophosphate (cyclic AMP) (379, 344, 345), 8-substituted cyclic AMP analogues (220), and phosphodiesterase inhibitors (caffeine, 40, 240; theophylline, 24; amrinone, 173; pimobendane, UD-CG-115, 168; OPC-8212, 380). As is illustrated in figs. 1*a*, 2, and 3, the rested state contraction of the guinea pig papillary muscle under the influence of a catecholamine has a time course quite unlike that of the contractions at physiological frequencies. Force starts to rise soon after stimulation and the upstroke of the action potential in both cases, but in the rested state contraction, it rises very slowly at first. Then, some 100 ms after the stimulus, there is an inflection point, after which the force rises much more rapidly to the late peak characteristic of the rested state contraction. In what follows I shall refer to the time to the inflection point as the *latent period* of the rested state contraction. There has been some debate about the mechanisms underlying the latent period and the peculiar shape of the rested state contraction. Some have suggested that the slow rise and delayed peak of the contraction may be accounted for simply on the basis of the time required for the diffusion of Ca from the surface membrane to the myofibrils. However, others (ref. 8), including the author, believe that this interpretation is not sufficient to explain either the existence of the inflection point, or the virtual constancy of the latent period (regardless of the amplitude of the delayed peak) illustrated in fig. 2. We feel that these features of the rested state contraction (and a number of others that will be discussed in the pages to follow) are most plausibly explained by assuming that most of the calcium entering the cell during the action potential is first sequestered by some intracellular store (presumably a component of the sarcoplasmic reticulum), and then released again after a mandatory delay which may represent the time required for translocation of calcium from uptake sites to release sites within the SR. (A similar delay is manifest in the restitution curve observed at higher frequencies of contraction, when the action potential is considerably briefer: very little force is developed during closely coupled extrasystoles, even though relaxation from the preceding beat is complete and therefore the SR must contain the calcium required to activate a strong contraction.) In rested state contractions the slow phase of force development before the inflection point may represent the effect of Ca^{2+} entering the cell from the extracellular space and acting directly on the myofilaments. During this phase of the contraction the sarcoplasmic reticulum will be competing with the myofilaments for the entering Ca^{2+}. The late peak of the contraction occurs only if the action potential outlasts the mandatory delay period and causes the release of some of the Ca^{2+} sequestered by the SR during the latent period. It seems likely, therefore, that the delayed release of calcium must be under the control of the membrane potential, as is the initial release.

That the calcium responsible for the rested state contraction in fig. 1*a* came from the extracellular space and not from an intracellular store can be deduced from the fact that the contraction was completely abolished by the 1,4-dihydropyridine derivative nifedipine, which specifically blocks L-type calcium channels (214, 20, 289) at concentrations more than 100-fold higher (237a) than expected from radioligand binding (23a, 165a; see section IV C). The total inhibition of the contraction proves that

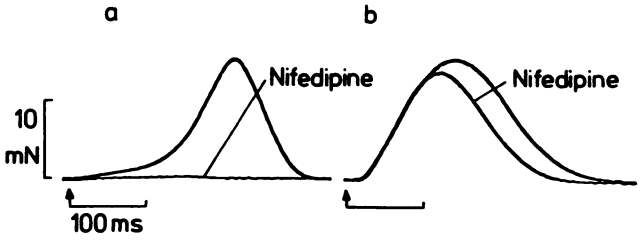

FIG. 1. Different effects of the calcium channel blocker nifedipine (1 μmol/liter) on rested state contractions of the same guinea pig papillary muscle under the influence of either norepinephrine (30 μmol/liter) (*a*) or low-sodium solution (40 mmol/liter) (*b*). The time of stimulation is indicated by *arrows*. Adapted from Reiter et al. (323).

the drug at the applied concentration was fully effective even after a long rest period (which might not have been the case with a distinctly use-dependent Ca channel blocker, such as verapamil, as shown in fig. 5 of ref. 46). Fig. 1*b* shows a rested state contraction of the same muscle in low sodium solution. Studies with radioactive tracers had shown that, at low external sodium concentrations, the calcium influx increases (286, 287, 233) while the calcium efflux diminishes (330), thus leading to a net increase of intracellular Ca (see section V) and, accordingly, to a filled Ca store of the sarcoplasmic reticulum. The first contraction after the rest period started without any delay after stimulation (see also ref. 3) and reached its peak at a time when the contraction in normal Na was just beginning. In low Na solution, nifedipine had no influence on the ascending slope of the contraction curve; it only shortened the contraction in accordance with a shortening of the duration of the action potential. The lack of influence of the calcium channel blocker on the velocity of the early contraction, in a concentration which completely inhibited the late contraction in the presence of norepinephrine, shows that the dihydropyridine-sensitive Ca current was not involved in this contraction. A comparable finding was made by Mascher (fig. 3 of ref. 252) using partially depolarized (18.9 mmol/liter of KCl) field-stimulated cat papillary muscles. These responded to threshold stimulation with either regenerative or nonregenerative (local) electrical responses which were accompanied by a weak late contraction. In low-sodium solution even nonregenerative electrical responses elicited strong contractions which appeared early after stimulation. The activation of slow (calcium-dependent) potentials, therefore, was not necessary as a trigger for calcium release. Under the influence of ouabain which causes an increase in intracellular calcium (see section V B), strong contractions could be induced in spite of an inhibition of slow potentials by the calcium channel blocker verapamil (382). With skeletal muscle, a comparable observation was made by Ildefonse et al. (181), who found that frog semitendinosus fibers developed biphasic contractions during long-lasting clamp depolarizations from a holding potential of −90 mV. The first, rapid, phase reached its maximum before the activation of an inward calcium current. A slow second phase correlated in time with the inward current. Nifedipine (10 μmol/liter) inhibited I_{Ca} and the second contraction component, but not the initial rapid one. The lack of effect of nifedipine on the initial contraction was confirmed by Neuhaus (284a), but the author observed that the plateau phase was prolonged which led him to suggest that the calcium inward current accelerates the inactivation of Ca^{2+} release from the SR.

The question of whether the latent period of the rested-state contraction under the influence of catecholamines is the consequence of a slow diffusion of Ca to the myofilaments or whether it is due to a delay between uptake into and release from the sarcoplasmic reticulum can be answered from the relation of the catecholamine concentration to both height and time of appearance of the late peak. If the late peak were the result of a simple diffusion lag, one would expect the latent period to shorten with increasing Ca fluxes (assuming linear kinetics of diffusion), since then the threshold concentration of Ca which triggers contraction should be reached considerably faster if Ca entry is enhanced by catecholamines. The tracings of fig. 2 show that the latent period of the rested state contraction was not shortened when the Ca uptake was more than quadrupled with increasing norepinephrine concentrations, as evidenced by the respective increase in force of contraction. One could argue that the increased Ca inflow was compensated for in its contraction-activating effect by various intracellular effects of a catecholamine-induced elevation in cyclic AMP levels which occurs in contracting (334) as well as in resting muscle (92). In particular, a reduction in the Ca sensitivity of the contractile apparatus (359, 250, 333) might be supposed to counteract, at least temporarily, the contraction-activating effect of the inflowing Ca and thus be responsible for the constancy of the latent period. However, such an interference seems improbable, since the late peak appears in addition to an early contraction peak in rested state contractions at low external sodium concentration. In this situation the contractile system is already highly activated as a result of calcium release from a filled storage compartment of the SR; furthermore, in this case the late, and not the early, peak is sensitive to calcium channel blockade by nifedipine (fig. 6 in Ref. 323). In two-component contractions of a guinea pig papillary muscle in the presence of norepinephrine at a stimulation frequency of 0.3 Hz, the calcium channel blocker verapamil diminished only the late contraction peak (46). Obviously, the development of the late peak of the rested state contraction is independent of an early force generation after stimulation. Furthermore, the delayed appearance of the late peak did not change if it was increased by the dihydropyridine derivative BAY K 8644 instead of a catecholamine (unpublished observation and ref. 28). This compound increases calcium current by a direct action on calcium channels without

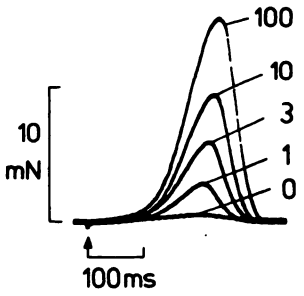

FIG. 2. Unchanged late appearance of rested state contractions under the influence of increasing concentrations of norepinephrine (1 to 100 μmol/liter). The electrical stimuli are marked by the *arrow*. Adapted from Seibel et al. (345).

increasing the intracellular cyclic AMP level (215, 60, 381).

The constancy of the latent period of the rested state contraction points to the functional involvement of the sarcoplasmic reticulum. Its dense peripheral network which is located immediately subjacent to the cell membrane, instead of only retarding calcium diffusion, seems to act as a diffusion barrier by taking up most of the inflowing Ca, especially under the facilitating effect of cyclic AMP (for reviews, see refs. 385, 342, and 378). A corresponding observation regarding extracellular calcium transients as measured with tetramethylmurexide was made by Hilgemann (161). He found that premature excitations (i.e., when the calcium release stores are depleted and the elicited contraction is weak) result in a prolonged depletion of extracellular calcium due to prolonged calcium influx. This can be explained by internal calcium sequestration sufficiently fast to prevent the cytosolic calcium accumulation that would be necessary not only for activation of contraction but also for initiation of calcium channel inactivation (237), which would terminate the influx of extracellular calcium. Consistent with this view is the finding that, in skinned cardiac cells, the SR that is wrapped around individual myofibrils accumulated calcium rapidly enough to prevent high Ca^{2+} concentrations of externally applied solutions from activating the myofilaments (fig. 10 in ref. 120; fig. 7 in ref. 115). This is analogous to the reaccumulation and movement back to release sites of Ca during an activation-relaxation cycle in skeletal (312) as well as in mammalian cardiac muscle (275). In the latter, mechanical restitution, indicating the transfer of calcium from the uptake sites of the sarcoplasmic reticulum to the release store, reaches its maximum after 0.7 to 1.0 s (164, 97, 311, 419). Accordingly, the latent period of the rested state contraction can be regarded as an expression of the delay between sequestration of inflowing calcium and its first availability as activator.

B. Two-Component Contractions

Contractions with two components, an early and a late one, sometimes occur during regular stimulation at very low frequencies (7). The two components become especially distinct if they are strengthened by the addition of drugs that increase cyclic AMP levels (328, 344, 345, 22, 24, 46, 255, 417, 111, 323, 248). Two-component contractions can also be observed when the action potential is prolonged after most of the calcium of the bathing solution has been replaced by strontium (56, 207).

The two components of these contractions should not be confused with the "phasic" and "tonic" components of contraction observed in frog cardiac muscle during prolonged depolarizing clamp pulses (146, 392, 99, 239). Since the phasic (transient) component has the same voltage dependence as the calcium current (175) and both are inhibited by calcium channel blockers (Mn^{2+}, ref. 392; D 600, ref. 99), it is assumed that the phasic contractions of the frog heart are, in contrast to those of the mammalian heart, directly activated by Ca entering via I_{Ca}. The generation of tonic (sustained) contractions is attributed to a calcium transfer mechanism different from I_{Ca} (99). The influence of sodium on the tonic force development at inside positive potentials makes it seem likely that the tonic components are activated by calcium derived from Na-Ca exchange (391, 87, 11, 176, 82).

In mammalian cardiac muscle a terminal contracture-like component is observed if the period of depolarization outlasts the triggered contraction. This is true whether the action potential is prolonged pharmacologically (203, 202, 253) or the membrane potential is controlled by voltage clamping (272, 421, 55, 294, 271, 103). It has been found that, during the prolonged depolarization, the tonic component is controlled by both intracellular sodium activity (a^i_{Na}) and membrane potential in a manner consistent with Na-Ca exchange (103; see section V). Whether the "tonic" contracture-like component is activated directly by the prolonged transmembrane uptake of calcium or via a sustained release from the intracellular store which continuously is sequestering the inflowing calcium is a matter of discussion. In heart muscle of mammals, unlike that of frogs, the contracture force of these "tonic" components is usually considerably lower than the peak force of the triggered contraction (55, 253). However, the tonic components may be followed after repolarization by aftercontractions (103), and the peak force of the first regular systole after such a "tonic" component is always considerably stronger than the contracture force (421).

In contrast, the two-component contraction of mammalian cardiac muscle under the conditions of increased I_{Ca} and low frequency stimulation consists of two distinct phasic components. Only the early component depends on the amount of previously stored calcium. There is an inflection of the force trace after about 100 ms, and we interpret this to be the point at which the inflowing calcium taken up from the longitudinal sarcoplasmic reticulum starts to be released by the release sites of the SR. The release is ended with the repolarization of the cell membrane which apparently closes the calcium release channels of the junctionally associated SR. Depending on the length of the action potential, the two contraction components may be distinctly separated, or they may fuse together into a contraction plateau (fig. 3). The duration of the action potential, therefore, controls the duration of the late contraction peak and thereby the amount of total calcium released. The time to peak of the late component is linearly related to the duration of the action potential, and this relation is preserved in the presence of catecholamines (24, 345) or cesium (323). The late component is reduced or even abolished if the duration of the action potential is decreased toward physiological values by increasing the frequency of contraction.

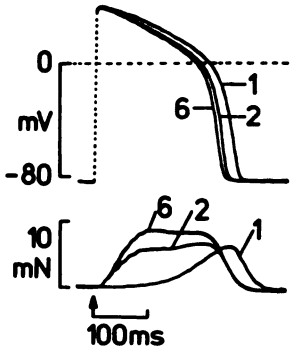

FIG. 3. Two-component contractions. Superimposed action potentials and contraction curves of a guinea pig papillary muscle in the presence of 10 μmol/liter of norepinephrine. The *numbers* indicate: *1*, rested state contraction; *2* and *6*, the first and fifth subsequent contractions at a stimulation frequency of 0.2 Hz. Adapted from Seibel et al. (345).

C. Cyclic AMP and Phosphodiesterase Inhibitors

1. Cyclic AMP. That the formation of cyclic AMP by activation of adenylate cyclase plays a decisive role in the inotropic effect of catecholamines was deduced early from the correlation between the increase in cyclic AMP and the inotropic effect (372). Isoproterenol was found to be 5 to 10 times more potent than epinephrine or norepinephrine in stimulating the formation of cyclic AMP by preparations from dog ventricle, whereas dichloroisoproterenol (DCI) behaved as an antagonist which indicates the involvement of β-adrenoceptor stimulation. It was also observed that the formation of cyclic AMP was reduced by acetylcholine and carbachol (372). From kinetic studies in the isolated heart it soon became evident that the activation of glycogen phosphorylase by cyclic AMP is not responsible for the inotropic effect (259, 334). Protein phosphorylation was found to be catalyzed by cyclic AMP-dependent protein kinase (402) in membrane particles not only from the sarcoplasmic reticulum (208) but also from the cell surface (420). The association of this phosphorylation with a cyclic AMP-dependent modulation of calcium influx (403) led to the proposal that a cyclic AMP-dependent protein kinase phosphorylates a sarcolemmal calcium channel protein, thus producing a conformational change that makes the channel available for voltage activation (366, 385). Changes in the configuration of the action potential, I_{Ca}, and contraction produced by injection of cyclic AMP and subunits of cyclic AMP-dependent protein kinase into cardiac myocytes support the hypothesis that phosphorylation of a protein within, or close to, the calcium channel by cyclic AMP-dependent protein kinase is indeed the mechanism of calcium channel modulation by β-adrenoceptor stimulation (298, 62, 193). An elegant proof of the key role of cyclic AMP formation in increasing I_{Ca} was provided by photochemically producing an intracellular concentration jump of cyclic AMP. This was achieved by applying a lipophilic photolabile adenosine cyclic 3,5-phosphoric acid triester which cleaves to yield cyclic AMP upon irradiation (332). Concentration jumps of cyclic AMP, following single brief light flashes, increased the amplitude and the duration of the action potentials, increased I_{Ca}, and simultaneously—in frog heart trabeculae—increased the force of contraction (332).

Cyclic AMP also facilitates calcium uptake into the SR (350) and calcium extrusion through the sarcolemmal calcium pump (see section VI A), thereby accelerating the relaxation of heart muscle. Several findings indicate that the modulation of the SR calcium pump parallels phosphorylation and dephosphorylation of a M_r 22,000 polymeric membrane protein, phospholamban (378). The phosphorylation of phospholamban is catalyzed by cyclic AMP-dependent protein kinase (208). Recent results suggest that, in normal cardiac SR, phospholamban in the dephosphorylated state acts as a suppressor of the calcium pump and that phosphorylation of phospholamban serves to reverse the suppression (182).

2. Cyclic AMP derivatives. Cyclic AMP applied to the extracellular fluid is unable to produce a positive inotropic effect (313) because it is destroyed intracellularly by a phosphodiesterase at a rate much faster than its rate of entry (334). However, several derivatives of cyclic AMP which are resistant to enzymatic degradation have been found to elicit catecholamine-like positive inotropic effects when applied extracellularly. The first and most widely used was the N^6-2′-O-dibutyryl derivative of cyclic AMP (dbcAMP; 226, 354, 328). The half-maximal effective concentration is about 1 mmol/liter of dbcAMP. The same potency was observed with the 8-substituted cyclic AMP derivatives 8-thio-benzyl-cyclic AMP (112).

A few of the 8-substituted cyclic AMP derivatives are of special interest. 8-(4-Chlorophenyl)thio-cyclic AMP is about 18 times as effective on protein kinase as the parent cyclic AMP (266). On isolated guinea pig papillary muscles 8-(4-chlorophenyl)thio-cyclic AMP was found to be about 25 times as potent as dbcAMP with a half-maximally effective concentration, log EC_{50}, of −4.4, followed by the 8-tertiary butyl-thio-cyclic AMP with a mean log EC_{50} of −4.0 (220). The inotropic potency of both compounds was further increased to log EC_{50} values of −5.66 and −5.38, respectively, when they were applied as benzyl esters (220). Neutralization of the phosphate hydroxyl residue of 8-substituted cyclic AMP by a benzyl group yielded lipophilic cyclic AMP benzyl esters which produced their positive inotropic effect at 20 to 100 times lower concentrations than the respective cyclic AMP salts. The lipophilic benzyl esters of cyclic AMP can be considered as transport forms for cyclic AMP across the cell membrane, which gain their biological activity through cyclic AMP released by spontaneous hydrolysis within the cell (219, 220).

3. Phosphodiesterase inhibitors. Substances which stimulate adenylate cyclase should be potentiated in their inotropic effects by drugs which inhibit the activity of

the phosphodiesterase that converts cyclic 3',5'-AMP to the inactive adenosine 5'-phosphate. Methylxanthines are competitive inhibitors of this phosphodiesterase (64); and it has been shown that theophylline and, less effectively, caffeine at concentrations at which they themselves are inotropically ineffective potentiate the cardiac inotropic response to norepinephrine (313). In higher concentrations, the methylxanthines would be expected to increase cardiac force in a catecholamine-like manner, if their activity as phosphodiesterase inhibitors were sufficiently selective. However, both caffeine (40) and theophylline (218) affect the onset and the relaxation phase of the contraction curve in a way distinctly different from that characteristic for catecholamines. This points to additional effects besides the inhibition of the phosphodiesterase (see section IV E). So far, 1-methyl-3-isobutylxanthine (IBMX), which is about 15 times as potent as theophylline as a phosphodiesterase inhibitor (21), is the only xanthine derivative that has been found to mimic the effects of isoproterenol in the extent of its inotropic effect and the abbreviation of the contraction. IBMX seems to be devoid of other effects (218, 227).

In recent years several new agents including bipyridine and pyridazinone derivatives have been developed that possess phosphodiesterase-inhibiting activity and produce positive inotropic effects in the heart (for review, see ref. 122). These agents also apparently have additional effects which might influence their therapeutic usefulness (360, 410, 343, 335, 113). It has been found that functional subclasses of the cyclic AMP-specific phosphodiesterase (PDE III) exist in ventricular muscle; these may be either membrane bound or soluble (409). Differences in their intracellular localization might be responsible for species differences that exist in the cardiotonic responses to various phosphodiesterase inhibitors.

IV. Calcium Release Mechanisms

A. Voltage- versus Current-dependent Calcium Release

In voltage-clamp experiments the relation between membrane potential and force development has been studied in an effort to determine whether Ca entering by way of the second inward current not only replenishes the intracellular calcium pools but also triggers the release of stored calcium. In addition to the methodological difficulties in obtaining homogeneous voltage control in multicellular preparations (190), these experiments have the problem of uncertainty about the extent of filling of the calcium release store, which depends upon a variety of factors, including resting membrane potential, previous stimulation pattern, outside calcium concentration, temperature, clamp duration, and the number of depolarizations. The role of the SR becomes visible if, as in the records of Beeler and Reuter (23), the developed force increases with the number of similar voltage clamp pulses. On the other hand, the intensity of the inward current itself seems to be influenced by the number of previous contractions or pattern of stimulation. Simurda and coworkers (352) observed a decrease of I_{Ca} under conditions of a mechanical staircase, reflecting the increasing amount of calcium released from the SR and its modulating effect on I_{Ca}. Therefore, it is difficult to judge from the voltage-force relationships published by various authors working under different experimental conditions whether the observed effects are directly or indirectly voltage related. In other words, it is uncertain whether parallels between I_{Ca} and force of contraction indicate a modulation by I_{Ca} of the release itself or of the filling of the release stores. Since in the mammalian heart, in contrast to the frog heart, there appears not to be direct coupling between inflowing calcium and contraction, the results are bound to depend on the extent to which the SR is filled with calcium.

In the steady state, where one might expect a rather constant filling of the SR proportional to the strength of I_{Ca} at the chosen clamp potential, almost all authors have found a parallelism between I_{Ca} and force of contraction at depolarizations up to zero potential. With further depolarizations to inside positive values, the force of contraction usually remained more or less constant, not following the decline of I_{Ca} and indicating a release of stored calcium independent of I_{Ca} (134, 23, 294, 383, 186). A few papers on multicellular preparations (271, 238, 260) and one on single heart cells (245) have presented bell-shaped voltage-force curves which run roughly parallel to the voltage-I_{Ca} curves in the positive voltage range. As a possible explanation of the divergence of their results, Morad and Goldman (271) pointed to the lower experimental temperature in contrast to that used by other authors (24–25°C, refs. 271 and 260; "room temperature," refs. 238 and 245 versus 35–37°C, the others). This could indicate that the storage capability of the release sites of the SR in the mammalian heart is reduced at low temperatures. Experimental evidence for such an assumption is provided by the rapid cooling contractures which are activated without depolarization by an abrupt Ca^{2+} leak from the SR (228, 58). If cooling significantly slows the active calcium uptake into the SR, an uncompensated calcium leak will reduce the calcium content of the release compartments. Consistent with the release sites being empty at low temperatures is the finding of Kitazawa (210) that, at 25°C, the change in force of contraction of mammalian ventricular muscle induced by a change in the outside calcium concentration is synchronous with the change in $[Ca^{2+}]$ at the cell surface. (see also ref. 6 for comparable results at 21°C). It appears, therefore, that at 25°C the contractions are activated by calcium entering the cell during depolarization via I_{Ca} and presumably consist of late components. The bell-shaped curves in the above-mentioned papers would then correspond to those obtained in single cells at 35°C under conditions in which the release sites of the

SR are empty, and the calcium that activates contraction is "directly" derived from I_{Ca} and leads to late-appearing contractions (see below, and ref. 186). In line with this reasoning is the bell-shaped voltage dependence of $[Ca^{2+}]_i$ transients observed in single guinea pig ventricular cells at 37°C by means of the fluorescent Ca^{2+} indicator fura-2 (16). The cells were voltage clamped from a holding potential of −40 mV, which is a very unfavorable condition for Ca storage in SR release compartments in spite of "conditioning" clamp pulses (186, 399; see section IV C). Therefore, it is quite possible that the transients of fluorescence were caused directly by the voltage-dependent calcium influx.

The filling state of the SR is also pertinent to the question of whether calcium release can be triggered at potentials negative to the I_{Ca} threshold. Beeler and Reuter (in their fig. 4, ref. 23) observed that appreciable force of contraction was activated at −54 mV only after the sixth depolarization to +19 mV, i.e., only after the SR was filled with releasable calcium as a result of repeated previous activation of calcium inward current. Accordingly, Gibbons and Fozzard (141) found that the contraction threshold was altered by a shift of the steady membrane voltage. The voltage-force relation was shifted to about 20 mV more positive values after a shift of the holding potential from −78 to −61 mV. This indicates that (*a*) the amount of calcium available in the storage site for release (the degree of recovery or repriming) depends on the "diastolic" membrane potential (see also section IV C), and (*b*) the release of activator calcium from a filled store upon depolarization from a membrane potential of about −80 mV is not triggered by I_{Ca} flowing through L-type channels, since strong contractions are elicited at potentials as low as −60 mV, far below the threshold for this current (see also refs. 384, 353, and 186). Simurda and coworkers (353) observed a slow inward current associated with strong contractions at voltages negative to the threshold of I_{Ca}. The current varied in parallel with the strength of contraction, both depending on the extent to which preceding activity had filled the release stores. They related this current (I_{aic}) to the calcium discharge from the stores. Evidence for a contribution of a current induced by calcium release from the SR to the second inward current in mammalian ventricular cells has also been obtained by others (254, 98, 125, 206; see sections IV B 4 and V A).

Calcium release cannot be expected under conditions which favor the empty state of release sites. A rational method to provide empty release compartments in voltage-clamp experiments at a temperature of 35°C was used by Isenberg et al. (186). They elicited contractions of single myocytes isolated from guinea pig or bovine ventricles by clamp steps from a holding potential of −45 mV (solution containing 20 mmol/liter of KCl). These contractions were of slow onset, lagging the start of depolarization with a delay of 80 ms, and thus represented pure late contraction components. Under these conditions the ventricular cell is unable to produce early contraction components presumably because, as a result of partial diastolic depolarization of the sarcolemma, the calcium has leaked from the release stores. The curve describing the voltage dependence of these contractions is bell shaped and parallel to that of I_{Ca}, presumably because it is the calcium carried by I_{Ca} which, after its uptake into the longitudinal part of the SR, is released after some delay into the cytosol. Early (fast) contraction components were obtained in myocytes when, from a potential of −80 mV during the intervals between beats at 0.5 or 1.0 Hz, increasing clamp steps were induced from an intermediate −45 mV holding potential lasting 20 ms. These contractions, elicited by calcium release from presumably filled stores of the sarcoplasmic reticulum, must be regarded as normal contractions whose voltage dependence should be representative for physiological excitation-contraction coupling. The contraction threshold of these early contractions was obviously more negative than −45 mV (extrapolation of the voltage-shortening velocity curve in fig. 4 of ref. 186 to zero shortening velocity gives an estimated value of −60 mV), and the voltage dependence of these contractions was not bell shaped; maximal early contractions were obtained at strongly positive potentials (+100 mV) where there should be no calcium current through the calcium channels (20, 289).

B. Calcium-induced Calcium Release

1. Oscillatory contractions in skinned fibers. Ford and Podolsky (132) and Endo et al. (109) reported independently that, under certain facilitating conditions, calcium ions can actually induce a release of stored calcium from the SR of skeletal muscle fibers which have been mechanically deprived of the sarcolemma (skinned fibers, Natori, ref. 280). Endo et al. (109) observed spontaneous oscillatory contractions suggesting that calcium release is a regenerative process in which Ca itself causes the release of Ca from the SR. Essentially the same observations have been made in mammalian heart muscle cells with disrupted or removed sarcolemma but not in those of the frog heart (42–44, 118, 119, 83). The cyclic contractions were inhibited in the presence of high concentrations of a Ca buffer, and they were abolished if the SR was destroyed by a detergent.

2. Aftercontractions. The independence of oscillatory contractions from the stimulation of the sarcolemma had been observed earlier in intact multicellular preparations in which damped force oscillations (aftercontractions) appeared without accompanying action potentials after electrically triggered contractions under conditions of high calcium load (318). These aftercontractions were regarded as an example of electromechanical dissociation, of decoupling of contraction from excitation of the sarcolemmal membrane, and they were envisaged as being the result of an oscillatory Ca^{2+} release from over-

loaded stores of the SR (318–320, 57). By increasing diastolic mechanical activity they change myocardial diastolic compliance (53, 212, 126). While aftercontractions are not elicited by action potentials they may be accompanied, under certain conditions, by oscillations of the membrane potential (oscillatory afterpotentials) which are thought to be secondary events caused by intracellularly released calcium (see section IV B 4). Because of their resemblance to the oscillatory contractions of skinned muscle fibers, aftercontractions are widely considered to represent a manifestation of calcium-induced calcium release in the intact cardiac muscle (94, 118, 142, 107, 79, 47, 297, 369, 4).

3. Asynchronous calcium release. Under conditions of high Ca^{2+} loading in a sodium-free solution, Glitsch and Pott (142) observed spontaneous fluctuations of an increased resting force of guinea pig atrial trabeculae. The fluctuations were thought to result from spontaneous oscillations with asynchronous cycles in different parts of the muscles which contributed to the increased resting force. Since caffeine inhibited the fluctuations and simultaneously reduced the mean resting force, it seems likely that asynchronous Ca^{2+} release from different parts of the SR was responsible for the random motion. The latter could be interrupted by an electrically triggered twitch which was followed by aftercontractions and "hyper-relaxations" (200, 309). This was probably due to the synchronization of the spontaneous oscillations in different cells as suggested from similar observations with rat papillary muscles by Stern et al. (369).

That $[Ca^{2+}]_i$ can fluctuate in intact, unstimulated cardiac muscle preparations has been shown by means of the photoprotein aequorin (297, 415, 4) and in single heart cells by both fura-2 fluorescence (414) and aequorin (105). Such oscillations of $[Ca^{2+}]_i$ had previously been inferred from observations of laser light scattering from quiescent muscle (231). The spontaneous Ca^{2+}-dependent oscillations vary with species in a manner similar to that for Ca^{2+}-induced release of Ca^{2+} from the SR of mechanically skinned cardiac cells (120): Unstimulated rat and canine tissues exhibit spontaneous oscillations even when extracellular calcium is as low as 2 mmol/liter, whereas in rabbit ventricle the calcium concentration must be considerably higher for oscillations to occur; frog cardiac tissues do not exhibit oscillations even under high Ca^{2+}-loading conditions (230)—a fact that presumably reflects the paucity of their SR. The general observation that oscillations of cytosolic Ca^{2+} are abolished by caffeine and ryanodine, both inhibitors of sarcoplasmic reticulum function, supports the hypothesis that the oscillations of muscle force arise from a Ca-dependent release of Ca from the SR (415, 4, 387). However, in regard to the discussion on the mechanism of excitation-contraction coupling, it is noteworthy that there are distinct differences in the influence of the two agents on the function of the SR; whereas caffeine depresses the mechanical oscillation amplitude, it enhances the spontaneous frequency of Ca^{2+} release, but ryanodine suppresses both frequency and amplitude (230).

4. Oscillatory afterpotentials. The oscillatory calcium release, besides inducing oscillatory contractions, also leads under appropriate experimental conditions to oscillatory afterpotentials or afterdepolarizations of the sarcolemma (51, 201, 188, 322, 279, 127, 195, 101, 257, 244, 194). In cardiac Purkinje fibers, this may grow into sustained rhythmic activity (390, 128, 88). The membrane current underlying these afterdepolarizations is thought to be the calcium-activated transient inward current (I_{TI}) (236). The finding in Purkinje fibers that I_{TI} showed a reversal potential at about −5 mV supported the suggestion of a calcium-activated nonselective cation channel (196, 66). However, in studies on ventricular and atrial tissue, other workers have not observed a reversal in I_{TI} and have suggested that I_{TI} may result from an electrogenic sodium-calcium exchange (13, 262, 292, 140, 124). Lipp and Pott (242) found a reversal of I_{TI} to be abolished after blockade of the inward calcium current (I_{Ca}) by D 600. The authors assumed that the apparent reversal of I_{TI} is caused by intracellularly released calcium which inactivates I_{Ca}. This would be in accordance with an earlier observation by Bogdanov and coworkers (47) of an inhibition of the slow-response action potential during the aftercontraction. Consistent with the interpretation that afterdepolarizations are caused by electrogenic sodium-calcium exchange (section V A) is the observation that they are totally absent in spite of powerful aftercontractions if cardiac muscles are kept for a sufficiently long time in sodium-free solution (252, 70).

5. Oscillatory calcium release from isolated SR. Oscillations in calcium release from isolated SR vesicles of skeletal muscle and Ca^{2+}-induced release of Ca^{2+} from SR vesicles of cardiac muscle have been observed by several investigators (198, 197, 76). Although the Ca^{2+} release rates from isolated canine cardiac SR at 37°C (76) were several orders of magnitude lower than the rate of Ca^{2+} release which occurs in muscle cells in vivo, this Ca^{2+} release phenomenon may be related to the Ca^{2+}-induced release of Ca^{2+} in skinned cardiac cells. The Ca^{2+} release from the SR vesicles (containing both subpopulations, i.e., from longitudinal and junctional SR) is specifically inhibited by ruthenium red, with an EC_{50} of 80 nmol/liter (77). Calmodulin has no effect on the rate or extent of the release, although reuptake of the released Ca^{2+} is faster in the presence of calmodulin, presumably as a result of enhanced Ca^{2+} transport activity due to calmodulin-dependent phosphorylation of the cardiac SR (75).

In regard to the molecular mechanism of Ca^{2+}-induced release of Ca^{2+} it is important that the Ca^{2+} release is not accompanied by a reduction in ATP hydrolysis and that Ca^{2+} influx proceeds during the period of net Ca^{2+} release (76). Therefore, Ca^{2+} release does not involve

reversal or cessation of inward Ca^{2+} pumping. This in turn suggests that Ca^{2+} release is not mediated through the Ca^{2+} pump protein, but occurs through a separate efflux pathway. The Ca^{2+} efflux is elicited by relatively low Ca^{2+} concentrations and is significantly inhibited by elevations of $[Ca^{2+}]_o$. Both the rate and the extent of net Ca^{2+} release from SR vesicles loaded to approximately the same total Ca^{2+} content depend on the $[Ca^{2+}]_o$ at the onset of release. Since the Ca^{2+} transport ATPase of the SR, which accounts for more than half the SR membrane mass, is the only known Ca^{2+}-binding protein with a Ca^{2+} affinity high enough to bind much Ca^{2+} at the concentrations that trigger release (91), one might assume that it is the active uptake of Ca^{2+} into a Ca^{2+}-loaded SR which evokes the release and not an effect of Ca^{2+} at a channel gate at the outside of the SR. However, for an understanding of the elementary processes of Ca^{2+}-induced release of Ca^{2+} the variables involved—the extent of Ca^{2+} loading and the relation between the Ca^{2+} concentrations inside and outside of the SR—will have to be independently controlled, since they all change as the $[Ca^{2+}]$ outside the SR is varied (115, 67).

6. Does the depolarization-induced contraction result from calcium-induced calcium release? Although it seems very likely that aftercontractions result from a Ca^{2+}-induced Ca^{2+} release, it is an open question whether the regular contraction of mammalian heart muscle is triggered by the fast initial component of transsarcolemmal Ca^{2+} flux (114, 116). What seems to be consistent, at first sight, with such a mechanism is that in the skinned fiber, which consists of myofilaments surrounded by SR, a rapid increase in $[Ca^{2+}]$ produces a contraction (116). However, if the $[Ca^{2+}]$ in this preparation is not reduced to the original loading concentration after the twitch, a new contraction will now appear a few seconds later (at 22°C) and, according to the prevailing conditions, an oscillating cycle of Ca^{2+} uptake and release may be induced as seen in fig. 4 of Fabiato (ref. 116). The appearance of such a cyclic repetition of the contraction is inhibited only if, as in Fabiato's usual experimental procedure, the cytosolic $[Ca^{2+}]$ is reduced immediately after the Ca^{2+}-induced contraction, i.e., after the first part of the oscillation cycle. Therefore, it seems unlikely that there are fundamentally different release mechanisms between the first contraction which is "triggered" by a fast increase in $[Ca^{2+}]$ and the consecutive spontaneous contractions. The situation seems quite similar as in calcium release from isolated SR vesicles (see previous section), and in isolated cardiac myocytes under conditions of electrochemical shunting across the external membrane (83). In these myocytes, phasic contractile activation occurs independently of sarcolemmal excitation at Ca^{2+} concentrations sustaining calcium accumulation in the SR, as proven by electron probe analysis. Ca^{2+} transport by the SR was found to be a requirement and a rate-limiting factor for the occurrence of phasic contractile activation (83).

That the actual Ca^{2+} uptake into the SR might play a decisive role in Ca^{2+}-induced Ca^{2+} release can be inferred from the time-dependent appearance of the Ca^{2+}-induced contractions in figs. 6, 10, and 12 of Fabiato (116). With the increase in the number of mixed fast and slow Ca^{2+}-loading pulses, the contraction response is not only increased ("graded response"), but also the latency of its appearance is drastically reduced from an original value of about 1000 ms. These results have a striking similarity to the behavior of the intact guinea pig papillary muscle under rested state conditions described in section III A. They illustrate very clearly that, when Ca^{2+} enters the cell at a rate similar to that during the plateau of the action potential, the SR is capable of taking it up fast enough to prevent much force development. And they are a strong argument against the idea that, in the rested state contraction, force development results from the diffusion of Ca^{2+} directly from the surface membrane to the myofibrils. Fabiato's results also show that, when the SR is relatively poorly loaded with Ca^{2+}, Ca^{2+}-induced release occurs with a long latency, whereas when loading has been increased by a few simulated twitches, the latency is greatly reduced.

The wide variation in latency of the Ca^{2+}-induced contraction in skinned fibers seems not to be consistent with the mechanical behavior of the intact cell. Repetitive electrical stimulations usually induce contractions after a latency which remains constant irrespective of the inotropic state which is determined by the amount of released calcium and evidenced by the velocity of force development (fig. 4). Therefore, it would be difficult to believe that calcium fluxes through the sarcolemma during the action potential, regardless of whether they occur

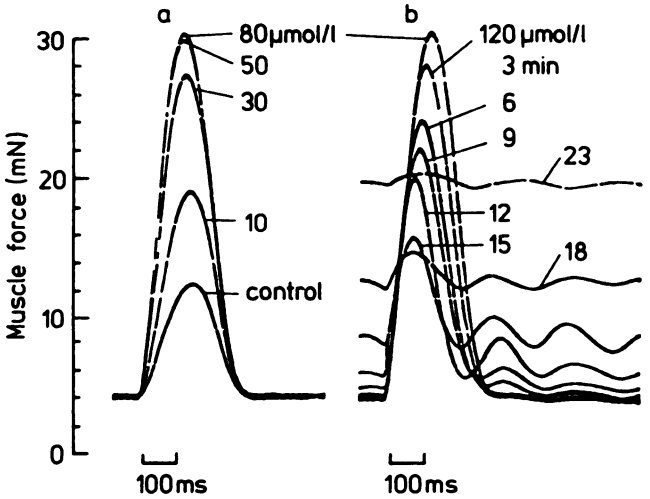

FIG. 4. The effect of increasing calcium load on the contractile behavior of mammalian ventricular muscle. Superimposed force records of a guinea pig papillary muscle in the presence of dihydroouabain. *a*, steady-state effects; *b*, effects of 120 µmol/liter of dihydroouabain as a function of time. Stimulation frequency, 1.0 Hz. Adapted from Reiter (321).

through calcium channels or via Na-Ca exchange, are primarily involved in the depolarization-induced Ca^{2+} release.

Fabiato points out that the observation of a well-developed Ca^{2+}-induced release of Ca^{2+} in skinned cardiac cells in which all superficial couplings are removed proves that the release was not from the terminal cisternae (116). The absence or paucity of junctional SR in the skinned preparations can also be deduced from their low sensitivity to ruthenium red and ryanodine (117), substances which specifically act on Ca^{2+} channels of the junctional SR (356, 130, 307; see section IV D). Therefore, the Ca^{2+} release in skinned fibers must be considered to occur from the free longitudinal SR through other Ca^{2+} channels than those specific for the junctional SR.

There are two essentials of a normal action potential-triggered release mechanism which apparently necessitate the junctional connection between the sarcolemma and the adjacent junctional SR, and which therefore cannot be met by Ca^{2+}-induced Ca^{2+} release as demonstrated either in skinned fibers or in aftercontractions of intact cells. (*a*) The release has to be switched on instantaneously with a short and constant latency. (*b*) The release mechanism has to be switched off again with the repolarization of the sarcolemma. I have already discussed the question of the mechanical latency. The influence of the repolarization on the duration of the release and consequently on the time to peak force becomes evident when the duration of the action potential is either prolonged, as in rested state contractions by catecholamines (24, 345) or cesium (323; see section III B), or shortened with increasing Ca^{2+} load. Consequently, as shown in fig. 4*b*, a digitalis-induced increase in Ca^{2+} load causes a progressive shortening of the time to peak force despite an unchanged rate of force development as evident from the unchanged steepness of the isometric contraction curve, indicating that the initial Ca^{2+} release is probably not inhibited. This is in accordance with the finding that the diminution of force development under comparable conditions of Ca overload is not accompanied by a decrease of the systolic Ca signal as estimated from the peak systolic light signal measured with aequorin (5). The unabated maximal rate of force development precludes a reduced Ca sensitivity of the contractile apparatus as a cause for the shortening of the time to peak force and the resulting decline of the contraction amplitude. The abbreviation of the ascending slope of the contraction curve very likely results from an earlier repolarization-dependent closing of the Ca release channels which would accelerate the impact of the fast Ca uptake system of the SR on the Ca release from the contractile proteins. A shortening of the action potential duration in mammalian heart muscle leads generally to an abbreviation of the time to, and consequently to a reduction of, the contraction peak as was demonstrated by Morad and Trautwein (272) in voltage-clamp experiments. The rate of force development in fig. 4*b* begins to be reduced only if the resting force is increased by about 100%, indicating that the increase in diastolic $[Ca^{2+}]_i$ reduces the rate of Ca^{2+} release and/or the Ca sensitivity of the contractile proteins.

Considering the available evidence, one obtains the following picture of the different modes of Ca^{2+} release from the SR in the intact cell. At normal resting membrane potentials, spontaneous contractions or aftercontractions (i.e., contractions not induced by depolarization) are the result of Ca^{2+}-induced release of Ca^{2+} from a heavily loaded SR, which probably takes place mainly through channels other than those involved in the depolarization-induced release of Ca^{2+}. These contractions are usually smaller than the electrically triggered contraction (418). It is only by depolarization of the sarcolemma that the Ca channels of the release compartments are effectively opened by a mechanism which is still not understood in detail (see section IV C), and the calcium is released through these channels according to the state of filling of the junctional SR.

C. Voltage-dependent Calcium Release

More insight into the mechanism by which the release store of the sarcoplasmic reticulum releases calcium can be obtained if, by varying the extracellular potassium concentration, one studies the relation between resting membrane potential and early contraction peak. In solutions with a potassium concentration higher than 8 mmol/liter and at low contraction frequencies (10- or 5-s intervals), the contractions of ventricular muscle have only a late and no early peak (346, 255, 417, 186). However, when these contractions are followed by a stimulus after a 1-s interval, the resulting test beat shows an early contraction peak in addition to the late one, as does a regular low frequency contraction at the normal potassium concentration of 5.9 mmol/liter (fig. 5). This indicates that the release store was filled (reprimed) with calcium shortly after the Ca^{2+} influx of the preceding

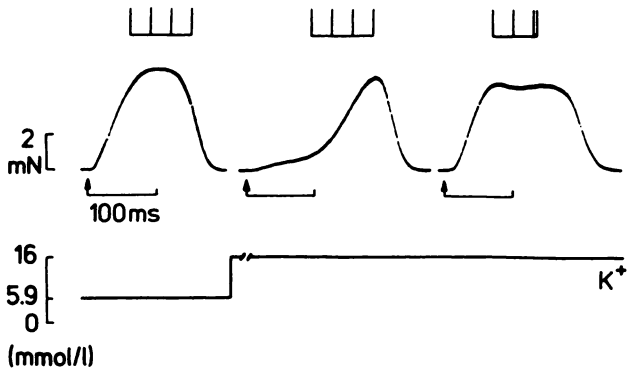

FIG. 5. Influence of potassium and stimulation frequency on calcium stored in release compartments. Contractions of a guinea pig papillary muscle in the presence of 3 μmol/liter of norepinephrine at 0.2 Hz frequency; test contraction after an interval of 800 ms (see stimulation pattern at *top*). Potassium concentration as indicated. Adapted from Vierling et al. (399).

contraction. Test contractions at various intervals between regular beats at low frequency showed that the ability of the muscle to produce early contraction components is lost in a few seconds (346, 255, 417) and that the rate of loss depends on the extracellular potassium concentration, i.e., on the resting membrane potential (in the range between −77 mV and −59 mV at $[K]_o$ of 8 or 16 mmol/liter, respectively; ref. 399). Increases in $[Mg^{2+}]_o$ or $[Ca^{2+}]_o$ prevent the potassium-induced loss of the early contraction component without altering the effect of potassium on the transmembrane potential (399). These results have been interpreted as follows. At low extracellular K^+ concentrations, i.e., during hyperpolarization, there is only a small leakage of calcium from the store of the SR in cardiac muscle. Therefore enough calcium remains in the SR to cause a distinct early contraction component. As $[K^+]_o$ is elevated and membrane potential is made less negative, a voltage sensor is affected by the membrane potential in a manner that opens some sarcomplasmic reticulum channels. This reduces the amount of stored calcium available for the early contraction component. The effect of the divalent cations is to change a surface potential (163) of the sarcolemma and thus alter the electric field sensed by the voltage sensor. Consistent with this hypothesis is the observation made by Mascher (252) in partially depolarized (18.9 mmol/liter of KCl) cat papillary muscles which were kept in low sodium solution and, therefore, had Ca^{2+}-filled release stores. These muscles had lost the ability to respond with action potentials (and slow inward currents) to electrical stimuli and responded to stimuli of increasing strength with graded electrotonic responses. Small increments in the electrotonic displacement of the membrane potential yielded marked increases in the magnitude of the associated early contraction.

It points to the similarity of the release mechanisms in skeletal and in cardiac muscle that, in skeletal muscle, a "slow" release of calcium has also been observed at potassium concentrations above 8 mmol/liter but still below the contracture threshold. Elevations of $[K^+]_o$ cause an increase in oxygen consumption (155) and in heat production (358). These effects have been attributed to an enhanced calcium sequestration secondary to an augmentation of voltage-dependent calcium release from the sarcoplasmic reticulum (293, 31) and a resultant increase in the intracellular calcium concentration (357). Furthermore, these effects were also inhibited by various divalent cations (388).

Since the coupling mechanism between membrane potential and calcium leak from intracellular stores seems to be as well developed in skeletal as in mammalian cardiac muscle, one is inclined to assume that this mechanism serves, in both types of muscle, for the rapid release of calcium that is triggered by the fast depolarization of the action potential. This release is difficult to reconcile with a mechanism other than passive diffusion through activated calcium release channels of the junctional SR. The kinetic studies by Ikemoto et al. (180) on Ca^{2+} release (induced by ionic replacement) from isolated SR/T-tubule complexes from skeletal muscle suggest that the linkage between sarcolemma and SR is required for triggering rapid calcium release, whereas a direct activation of the SR membrane by (released) calcium or drugs (caffeine and quercetin) leads to a relatively slow calcium release. Putative calcium release channels in heavy SR vesicles, derived from the terminal cisternae of both skeletal (356) and cardiac (334a) muscle, which were incorporated into planar lipid bilayers and identified on the basis of their activation by adenine nucleotides, blockade by ruthenium red, and sensitivity for divalent cations, have been shown to exhibit a very large conductance. A large conductance is certainly a prerequisite for a calcium channel that can mediate large ion fluxes on a millisecond time scale. Ryanodine (see section IV D) was found to act as a specific ligand for the Ca^{2+} release channels of the junctional SR (130). This led to the isolation, from skeletal as from cardiac muscle, of the Ca^{2+} release channel. It is a high-molecular-weight protein whose structure is identical with that of the feet bridging the gap between the sarcolemma and the junctional sarcoplasmic reticulum (183, 183a, 229a, 229b).

Several mechanisms for a voltage-dependent activation of calcium release in skeletal muscle have been suggested. The main hypotheses are electrical, chemical, and mechanical (for reviews, see refs. 368, 251, and 362). Electrical coupling was thought to occur by a flow of ionic current from the tubular space through pores (pillars) of the bridging structures into the SR, thereby inducing an electrical potential change across the SR membrane which would cause calcium release (256). This hypothesis could not be supported experimentally, since evidence for large changes in membrane potential of the SR during calcium release was found neither in the ionic composition of the SR nor in relevant optical signals (363, 295, 211, 19). A chemical excitation-contraction coupling could possibly be accomplished by a diffusible messenger substance which enters the fiber through the tubular membrane (or is released by the membrane) and activates the SR. Ca^{2+} has long been considered as a likely candidate for such messenger substance (for reviews, see refs. 30 and 135). According to this hypothesis, small quantities of Ca^{2+} entering during depolarization should trigger the release from the SR of the much larger quantity of Ca^{2+} required for contraction (Ca^{2+}-induced release of Ca^{2+}). As discussed in the previous sections (IV A to IV B 6), it was found to be rather unlikely that the normal, depolarization-induced, contraction of mammalian cardiac muscle results from Ca^{2+}-induced Ca^{2+} release. In skeletal muscle, the hypothesis appears incompatible with the fact that excitation-contraction coupling persists in calcium-free media (247, 265, 246). Ca^{2+}

release signals have even been recorded from fibers which had been bathed in 1 mmol/liter of EGTA for 2 days, a treatment which certainly should have caused them to lose their tubular calcium content (265). The experimental analysis of the influence of extracellular Ca^{2+} on excitation-contraction coupling in skeletal muscle led to the view that bound calcium is a requisite for the voltage-sensing and force-controlling system residing in the tubular membrane (247, 147, 246, 63, 61). Nickel ions can apparently substitute for Ca^{2+} in this function (389, 68, 31). The removal of external Ca caused an acceleration of force inactivation in skeletal muscle leading to a shift of the steady-state potential dependence of force inactivation to more negative potentials (247, 147, 246). Similar potential shifts were observed of the inactivation curves of Ca^{2+} release (63) and intramembrane charge movement (61). Lüttgau and coworkers (246) explained the influence of Ca^{2+} on the potential dependence of the inactivation curve by assuming a potential-dependent binding of Ca^{2+} to the potential sensor of force activation in the T-tubular membrane, with a low affinity in the depolarized inactivated state. A dissociation of Ca^{2+} is assumed to turn the system into a secondary inactivated (paralyzed) state from which it only slowly recovers after repolarization. This model would explain the failure, in skinned skeletal muscle fibers, to induce Ca^{2+} release from the SR by depolarization of sealed-off transverse tubules after application of EGTA to their cytosolic side (401). Since the membrane potential of these tubules and, therefore, their binding affinity for Ca^{2+} are presumably relatively low, it seems to be feasible that chelation of dissociating Ca^{2+} by EGTA renders the potential sensor into a paralyzed state.

As to the nature of the voltage sensors, the likelihood exists that they are identical with the high-affinity 1,4-dihydropyridine binding sites (332a) which are abundant in skeletal transverse tubular membranes (47a). Although most binding sites for the dihydropyridines are not functional Ca channels (343a), it is possible that the high-affinity receptors are channel-like proteins that perform the voltage-sensing function and are coupled to the calcium release channel by an unknown mechanism. In the intact cell, the high-affinity binding depends on depolarization; negative potentials inhibit the binding (343a). Dihydropyridines in nanomolar concentrations were found to inhibit charge movements and SR calcium release in parallel (332a). The effect has a dependence on membrane voltage analogous to that of specific binding of dihydropyridines. Since the blockade of sarcolemmal calcium channels requires more than 100-fold higher concentrations (see section III A), Ca^{2+} currents through the sarcolemma remain uninhibited at dihydropyridine concentrations high enough to bind nearly all high-affinity sites (343a).

The discovery that inositol 1,4,5-trisphosphate ($InsP_3$) mobilizes Ca^{2+} from the endoplasmic reticulum of many different cells including skinned muscles fibers (see, however, ref. 274) led to the suggestion that $InsP_3$ may be the postulated chemical messenger for excitation-contraction coupling (400, 395). Interestingly, the ability of $InsP_3$ to release calcium from the endoplasmic reticulum is unaffected by ruthenium red (25), a very potent inhibitor of the Ca^{2+} release channels localized in the junctional SR of both skeletal and cardiac muscle (130, 307). The hypothesis was tested by injection into intact skeletal muscle fibers of either $InsP_3$ (36, 152) or heparin, an inhibitor of $InsP_3$-induced Ca^{2+} release in smooth muscle and nonmuscle cells (301). No contraction was ever observed in an intact fiber, and there was no rise in aequorin luminescence after injection of $InsP_3$, whereas injections of $CaCl_2$ or caffeine produced obvious sarcomere shortening. High concentrations of heparin did not affect Ca^{2+} release elicited by the normal action potential mechanism as monitored by both fura-2 and an intrinsic birefringence signal. $InsP_3$-induced releases of Ca^{2+} in skinned skeletal muscle fibers which had been reported previously by several investigators were explained as being induced by the depolarization of sealed-off T-tubules (152). The results argue against a major physiological role of $InsP_3$ as a chemical messenger of excitation-contraction coupling in skeletal muscle.

In the so-called mechanical hypothesis of Schneider and Chandler (341), a voltage-dependent movement of fixed electrical charges in the surface membrane provides the means by which the potential across the wall of the tubular system is sensed by the junctional attachments of the SR. In the extended model (78), the charge is linked by a molecular entity to a calcium release channel of the SR which thereby is mechanically opened or closed, depending on the potential of the tubular membrane. The model is consistent with the physiological results that (*a*) depolarization of the tubular membrane can increase Ca^{2+} flux across the SR membrane, and (*b*) repolarization can rapidly shut off Ca^{2+} release. Compatible with the charge movement concept is the finding that the perchlorate anion at low concentrations rather specifically improves excitation-contraction coupling of frog skeletal muscle fibers by shifting the voltage dependence of force activation towards more negative potentials parallel with the voltage dependence of intracellular charge movement (145, 246a). Likewise, the linear relation of the calcium release rate to the charge movement, which was observed in several investigations (311a, 264, 332a, 61), points to a tight control of activation of SR calcium release by intramembrane charge movement. The hypothesis would be in accordance also with the potential dependence, in both skeletal and mammalian heart muscle, of the calcium leakage from the junctional SR at potentials more negative than the threshold for contraction, where the rate of release is apparently smaller than the rate of Ca^{2+} sequestration (see above and fig. 5). Since the isolated calcium release channel

has been found to be identical with the foot structure between the junctional SR and the tubular membrane (see following section IV D), the control of the opening state of the calcium release channel by a conformational change of a sarcolemmal membrane protein may not be as remote as originally suggested in the model of Chandler et al. (78).

D. Ryanodine

The alkaloid ryanodine has been found to alter the function of skeletal and cardiac muscle in nanomolar concentrations (187). This potent drug specifically inhibits the early contraction component of cardiac muscle (fig. 6), an effect that resembles that of increasing the extracellular potassium concentration to 16 mmol/liter (fig. 5). This effect is documented in the contraction tracings of a number of authors, although some of them did not especially mention it or described the effect as not being substantial (305, 394, 303, 167, 377, 375, 207, 268, 416, 26, 422, 248). The effect has been ascribed to a decrease in the extent of SR calcium release (374) which is consistent with the view that, in mammalian cardiac muscle, calcium release from the SR is indispensable for an early contraction component. A decrease of SR calcium release might, in principle, be achieved by either of two opposing mechanisms—by inhibition of calcium release from a filled store or by interference with the closing of the calcium release channels in the junctional SR. The latter would prevent the accumulation of calcium, so that there would be no calcium in the release store to be released. That the latter and not the former mechanism is responsible for the loss of the early contraction component is evident from its dependence on contraction frequency. The early component returns immediately with a reduction of the stimulation interval (fig. 6). This indicates that calcium release from a refilled store of the SR is not impaired by ryanodine; rather the store, as a result of its leakiness, lost its capability to keep the calcium load for the usual period of time. The leakage rate, as measured by the rate of loss of the early component, is not diminished by Mg^{2+} (397). This contrasts with the effect of Mg^{2+} on the potassium-induced loss of the early component (see section IV C and ref. 399) and is clear evidence that ryanodine does not affect the sarcolemmal voltage sensor but acts intracellularly at the junctional SR. In accordance with these findings, it was observed in early experiments on frog skeletal muscle (45) that an increase in potassium concentration to 12.4 mmol/liter intensified the response to ryanodine, an effect which was antagonized by magnesium ions. And it has been shown in suspensions of single rat cardiac myocytes by the fluorescent dye, quin-2, that ryanodine causes a slow discharge of Ca^{2+} from the SR into the myoplasmic space (153).

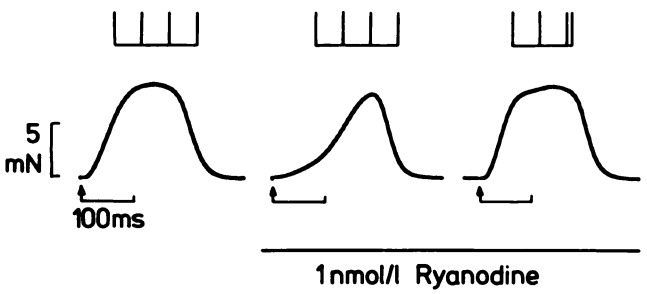

FIG. 6. Frequency-dependent effect of ryanodine on the early contraction component. Guinea pig papillary muscle in the presence of 3 μmol/liter of norepinephrine. Stimulation frequency, 0.2 Hz; test contraction after an interval of 800 ms (see stimulation pattern at *top*). One nmol/liter of ryanodine as indicated. Adapted from Vierling (397).

The ryanodine-induced increase in calcium leakage from the SR leads to an acceleration of the decay during rest of ventricular contractility which, accordingly, reaches its rested state much earlier than normal as shown for papillary muscles of the rat (376), rabbit (373), and ferret (249). In atrial muscle, which is normally distinguished by a strong rested state contraction, low concentrations of ryanodine selectively reduce the strength of contraction at low frequencies, turning the frequency-force relationship into one resembling that of ventricular muscle (355, 139, 149, 162, 160, 367). That the calcium leak from the SR results in a significant increase in calcium efflux from the muscle has been observed in skeletal (29, 150) and smooth muscle (179) and in ventricular muscle of the dog (283) and rabbit (27), and in guinea-pig atria (136). Ryanodine caused the depletion of a contraction-relevant calcium pool in the rat heart (178).

Calcium efflux from a heavy sarcotubular fraction of skeletal muscle was stimulated by ryanodine, but that of a light fraction was not (121). The enhancement of the calcium loading rate of terminal cisternae by ruthenium red, an inhibitor of the calcium release channel, can be blocked by the previous addition of ryanodine. This indicates that the alkaloid locks the Ca^{2+} release channel in the "open state," so that the terminal cisternae remain leaky to calcium (130). The inhibition constant is in the nanomolar range (20 to 180 nmol/liter; ref. 130) which corresponds to the dissociation constant of [^{3}H]ryanodine binding both in skeletal (130) and in cardiac muscle (307). Earlier studies had shown that the uptake of [^{3}H] ryanodine by rat atria exposed to nanomolar concentrations of the drug correlated with the effects on contraction (85). The binding studies localized the receptors on the junctional and not on the longitudinal SR (130). The ryanodine receptor has been purified from junctional terminal cisternae of fast skeletal muscle SR and from cardiac SR (183, 183a). The affinity for the purified cardiac receptor was 4- to 5-fold higher than that of skeletal muscle (183a). Electron microscopy of the purified receptors showed square structures comparable in size and shape to the "feet" of junctional SR (336), indicating that ryanodine binds directly to the foot structures, with a stoichiometry of ryanodine binding sites to

"feet" of about 2. These findings suggest that the ryanodine receptor and Ca^{2+} release channel represent a functional unit, the structural unit being the foot structure which, in situ, is junctionally associated with the transverse tubules (183). According to some studies (191, 192, 347, 263, 278), the effect of ryanodine on the calcium channels of isolated junctional SR is reversed at ryanodine concentrations four orders of magnitude higher (100 to 300 μmol/liter) than the dissociation constant for specific binding, and there it resembles the more specific inhibitory effect of 0.08 to 0.5 μmol/liter of ruthenium red (347, 77, 263, 278). At high concentrations of ryanodine, the paradoxical effect of the drug on ATP-dependent calcium accumulation by isolated SR vesicles is determined in part by the experimental environment (235).

Ryanodine was found to be ineffective in cardiac muscles from summer toads, from fetal mammalian hearts (human, cat, rabbit), and from newborn kittens and rabbits. The characteristic adult sensitivity to the alkaloid appears within a few days after birth at the same time as the transverse tubular system (T-system) develops (303). Since, in accompanying studies of the ultrastructure by electron microscopy, an apparent evagination of the sarcolemma at the level of the Z lines was found, it was suggested that ryanodine acts in adult mammalian heart muscle by dissociating the T tubules from the sarcoplasmic reticulum system and thus uncoupling excitation from contraction (304). These effects on the ultrastructure could not be reproduced in a later study in which ryanodine-treated muscles were found in electron micrographs to show normal T tubules and an unaltered morphology of the couplings between the sarcolemma and the SR (167, 148). The previously published observations were explained as artifacts arising from muscle contracture during the fixation process (148). Nevertheless, the specific binding of ryanodine to and actions on the foot structures of the calcium release compartments associated with the transverse tubules might facilitate an artificial contracture-induced spatial separation.

On the basis of experiments with bundles of myofibrils containing sarcoplasmic reticulum around each myofibril as obtained by microdissection of cardiac cells, it was postulated that ryanodine, instead of promoting calcium release, inhibits calcium release from the SR (117). It was observed in these experiments that ryanodine in millimolar concentrations did not uniformly influence caffeine-induced calcium release (as judged from contraction traces of the bundles). From this is was deduced that ryanodine does not decrease the calcium content of the SR and that, therefore, a ryanodine-induced depression of calcium release from the SR could not be the consequence of an impairment of calcium accumulation by the SR. However, whereas the ryanodine-sensitive calcium channels are restricted to the junctional SR (130), specific caffeine-sensitive Ca^{2+} gates have been found by various authors (270, 370) to reside not only in the heavy (junctional) but also in the lighter vesicular fractions of the SR. Although the skinned cardiac cell preparations seem to contain a considerable quantity of longitudinal (free) SR surrounding the myofibrils, it is uncertain how much junctional SR survives the skinning procedure (116, 117). Therefore, whereas the skinned cardiac cell preparation may be useful for the demonstration of caffeine-induced calcium release from the longitudinal parts of the SR, its usefulness for the study of calcium release from junctional release stores is questionable.

In view of the highly specific effect of ryanodine on the calcium channels of the junctional SR, one wonders whether the alkaloid also affects the calcium channels of the sarcolemma. In a careful study on the effect of ryanodine on the contractile performance of guinea pig papillary muscles, it was found that, in a rather high concentration (2 μmol/liter), the alkaloid exerts a biphasic effect (148). Immediately after the addition of the substance, the contraction force declined as a result of the loss of the early contraction component. However, after 4 min, the force of the remaining late component began to increase, with a continuous increase of the time to peak force until, 50 min after the addition of ryanodine, the force that developed in the late component of contraction was equal to that of the early component before addition of the drug. Similar observations have been made by others on cardiac muscle of the cat and dog (281, 377, 207). The transmembrane action potential was found to be prolonged (14); the "slow" potential at 24 mmol/liter of K^+ was also prolonged, and its velocity of depolarization was slightly increased (148, 339). A prolongation of the calcium current was also obtained in rat ventricular muscle cells (267). However, in cesium-dialyzed guinea pig ventricular myocytes the amplitude, time course, and voltage dependence of I_{Ca} were not affected by ryanodine (290). This indicates that ryanodine has no direct influence on the sarcolemmal calcium channel and that the observed prolongation of the calcium current (with the effect on the late contraction peak) is probably the consequence of the lack of an early increase in cytosolic calcium concentration and its influence on the ionic conductance of the sarcolemma.

E. Caffeine

Caffeine has, besides its inhibiting action on phosphodiesterase (section III C 3), a special effect on the SR of skeletal and cardiac muscle which is independent of and functionally antagonistic to that of cyclic AMP. From the standpoint of the positive inotropic action of a pure (selectively acting) phosphodiesterase inhibitor like IBMX; this second action of caffeine (and of theophylline) is an unsought side effect which occurs in the millimolar concentration range, whereas the phosphodiesterase-inhibiting action occurs at a concentration an order of magnitude lower.

Studies on the effects of caffeine on ATPase activity, calcium transport (404), and calcium accumulation of the SR isolated from skeletal muscle (406, 199) suggest that caffeine acts by increasing the permeability of the SR membrane to calcium (199). The increased leakage of calcium from the SR is modulated by the relative internal and external calcium concentrations (198). The entire surface of the SR must be involved, since specific caffeine-sensitive Ca^{2+} gates have been found not only in the heavy (junctional) SR, but also in the lighter vesicular fractions of the free or longitudinal SR (270, 370).

The effects of caffeine on ATPase activity and calcium uptake have been studied in SR vesicles from cardiac muscle of guinea pigs (284), rats (284), and rabbits (35). In all species it was found that caffeine impaired calcium accumulation by isolated SR vesicles, but paradoxically it could increase the rate constant for calcium accumulation and the Ca^{2+}-activated ATPase activity (35). It inhibited calcium accumulation to a greater extent when external (cytosolic) calcium concentration was low, and to a smaller extent if the internal free calcium concentration of the SR was kept low by oxalate. These findings imply that caffeine increases the passive efflux of calcium from the SR vesicles when the outward gradient is high and thus that caffeine also acts in mammalian cardiac muscle by making the SR membrane more permeable to calcium (35). Functional evidence for an increased rate of release of activator calcium from the SR was obtained in intact ventricular muscle preparations of various mammalian species (81) and in skinned canine cardiac Purkinje cells (115). The effect resembles that of ryanodine (see section IV D) in that it leads to an increase of the spontaneous rate of calcium leakage from the SR with an accompanying reduction of the calcium content of the release compartments and, consequently, to a diminution or total suppression of the early contraction component (40, 157, 218, 46). For the increased loss of calcium from the release compartments of the SR, it is apparently unimportant whether calcium leaks (as under the influence of ryanodine) through the specific Ca^{2+} release channels at the foot structures connecting the junctional SR with the sarcolemma or through specific caffeine-sensitive Ca^{2+} gates distributed over the entire SR. However, this difference becomes noticeable if, under high calcium loading conditions, spontaneous mechanical oscillations occur in unstimulated cardiac muscle preparations. The frequency of these oscillations, which are caused by spontaneous calcium release from the SR (section IV B), is decreased by ryanodine, whereas it is increased by caffeine (230; see section IV B 3). The increased frequency of spontaneous cyclic contractions was found, in skinned guinea pig cardiac fibers, to be prevented by procaine (209). This points to procaine-caffeine antagonism at the caffeine-sensitive calcium gates of the SR, an assumption which is supported by the observation that the suppression of the early contraction component of an intact kitten papillary muscle by caffeine was antagonized by procaine (40). The reversal by procaine of the caffeine-induced release of calcium from the SR of skeletal muscle had been reported earlier by Weber and Herz (406).

The action of caffeine and theophylline under rested state conditions is virtually uninfluenced by an effect on the SR. The action consists of a late-appearing contraction peak (fig. 5 of ref. 40; 24) which resembles that produced by catecholamines in all respects (345; see also fig. 1*a*) and probably results from a cyclic AMP-dependent increase in I_{Ca} into a cardiac cell whose SR contains no releasable calcium (see section III A). The late rested state contraction under the influence of norepinephrine is strengthened by caffeine (46). The increased calcium leakage from the SR becomes noticeable by the delay in contraction development if the muscle is stimulated regularly at relatively low frequencies (40). The effect is most obvious if caffeine acts on a two component contraction in the presence of norepinephrine: it eliminates the early and strengthens the late contraction component (46). Activity-dependent inotropic effects which operate through increased calcium loading of the SR are inhibited by caffeine, such as post-extrasystolic potentiation in the cat heart (157), the progressive increase in the mechanical responses to successive depolarizing pulses under voltage clamp, and the increase in peak force that follows a period of depolarization in the dog papillary muscle (296). Likewise, in dog ventricular muscle, post-stimulation potentiation is inhibited by theophylline (110). Understandably, in frog ventricular muscle, the action of caffeine consists predominantly of an increase in calcium influx through the sarcolemma (205, 288).

As in the case of ryanodine, the effect of caffeine on the early contraction component can, at least to a great extent, be overcome by decreasing the contraction interval (40) and thus shortening the time available for calcium leakage from the SR. Under such conditions, theophylline increases force of contraction of dog ventricular muscle at low concentrations (0.1 to 0.6 mmol/liter) by increasing the rate of force development, but with higher concentrations (1 to 20 mmol/liter), a further increase of force is due, at unchanged contraction velocity, to a prolongation of the time to peak force (37). A similar action of theophylline had been observed in the guinea pig papillary muscle (218).

Together with the isometric contraction curves, Blinks and Endoh (37) recorded the light signals of intracellularly applied aequorin which disclosed the influence of the methylxanthine on the cytosolic calcium transients. The amplitude of the calcium transient increased at the lowest inotropically effective concentrations (0.1 to 0.3 mmol/liter), but it decreased at higher concentrations. At concentrations above 1 mmol/liter, the amplitude of the aequorin signal was below the control level, and it

continued to fall as the drug concentration was increased. Concomitantly with the decline of its amplitude there was a progressive prolongation of the aequorin signal until a second light peak became distinguishable which was finally higher than the remnant of the early control signal. The second peak of the calcium transient started about 100 ms after stimulation of the muscle, culminated about 50 ms later, and then declined with a half-time considerably longer than in the control record. The effects of the low concentrations of the drug were thought by the authors to result from an elevation of cytoplasmic cyclic AMP concentration due to the inhibition of phosphodiesterase, whereas they regarded the effects of higher concentrations on the calcium transient as reflecting the action of theophylline on the SR (37). Indeed, it seems very likely that the concentration-dependent decrease of an early aequorin light signal reflects the progressive reduction of the content of activator calcium in the release compartments of the SR caused by the drug-induced increase of the rate of calcium leakage from the SR. This brings the muscle functionally into the situation of the rested state (section III A), in spite of the relatively high contraction frequency. Consistent with the reduction of the early part of the cytosolic calcium transient is the observation of a concentration-dependent increase in the duration of the transmembrane action potentials produced by caffeine in guinea pig and kitten cardiac muscle (89, 86). This is presumably because the stimulating effect of the initial intracellular calcium transient on the potassium conductance of the sarcolemma is reduced (see section II C).

As in rested state contractions under the influence of catecholamines, the methylxanthine-dependent elevation of cyclic AMP will increase I_{Ca}. In the light of biochemical reports of an increased rate constant of calcium accumulation into the SR (see above and ref. 35), there is no reason to assume that the inflowing calcium is not taken up by the SR before it is released after some delay into the cytosol, giving rise to a pronounced late calcium transient and a prolonged contraction. Whether calcium is released only through voltage-dependent gates or whether calcium leakage through caffeine-sensitive gates is also involved in the course of the calcium transient is not clear.

The same pattern of effects of theophylline and caffeine on intracellular calcium transient and contraction was observed in cat papillary muscles (9, 273, 41), and compatible results have been obtained with rat ventricular muscle (216) and canine Purkinje fibers (158). The precise relative importance for the overall effects of the methylxanthines of an increased calcium sensitivity of the myofilaments as observed in skinned cardiac muscle preparations (411) or in voltage-clamped sheep cardiac Purkinje fibers (104) remains to be elucidated.

V. Sodium-Calcium Exchange

A. Calcium Extrusion

Calcium entering the cell during rest or activity has ultimately to be extruded again in order to maintain the large concentration gradient which is necessary for its regulatory function. Part of this uphill movement has been found to depend on extracellular sodium in heart muscle (330) as in nerve (33) and attributed to exchange diffusion in which calcium efflux is coupled to sodium entry. In this sodium-calcium exchange the energy for extruding calcium is provided by the downhill movement of sodium. Consistent with a bidirectional carrier-mediated sodium-calcium exchange system is a calcium influx component that depends on $[Na]_i$ (15, 143). The activity of this transport system has been demonstrated in a preparation of cardiac sarcolemmal vesicles in which transmembrane Ca^{2+} movements in either direction could be induced by generating oppositely directed concentration gradients of Na^+ (316). From such sarcolemmal vesicles, the sodium-calcium exchanger has been partially purified and identified as a glycoprotein (151). The affinity of the exchanger for Ca^{2+} (apparent K_m 1.5 μmol/liter; ref. 73) is lower than that of another system involved in the extrusion of Ca^{2+} from the cell—the specific Ca^{2+}-pumping ATPase of the sarcolemma (K_m 0.3 μmol/liter; ref. 72; see section VI). However, the maximal velocity of Ca^{2+} pumping by the exchanger is 5 (17) to 30 times (73) as high.

The stoichiometry of the Na-Ca exchange determined by Pitts (308) from measurements of tracer fluxes in cardiac sarcolemmal vesicles was 3 Na^+ to 1 Ca^{2+}. Similar values have been obtained by other authors with sarcolemmal vesicles (315), frog atrial tissue (176, 82), mammalian cardiac muscle (348, 80, 59), and squid axons (34). A stoichiometry of more than 2 Na^+ per Ca^{2+} implies that the Na-Ca exchange must be electrogenic. Indeed, the operation of the exchanger in cardiac sarcolemmal vesicles has been shown to generate an electric current (317, 73). Since the Na-Ca exchange can move Ca either outward or inward depending on the direction and magnitude of the electrochemical gradients for Na^+ and Ca^{2+}, both inward and outward carrier currents can be generated during cardiac action potentials (276). However, the actual reversal potential of the carrier current is rapidly displaced toward positive values when $[Ca]_i$ rises during the action potential as a result of calcium release from the SR (291, 102, 292, 140). The Na-Ca exchange process then should carry inward current (i.e., Na moving inward) during part of the action potential, even at positive membrane voltage. Actually, an inward plateau current that activates more slowly than the inward Ca current (I_{Ca}) and contributes to the second inward current (I_{si}) has been found in work on single cells (268, 291, 125). Unlike I_{Ca} this slower current is not immediately inhibited by Cd ions, and the suggestion has been made that it is a Na-Ca exchange current (I_{NaCa}). This current strongly resembles the transient inward current (I_{TI}), which is correlated with the aftercontractions (see sections IV B 4 and IV A). Since during a normal twitch the intracellular calcium may rise to values even greater

than that occurring during aftercontractions, it is not surprising that a similar current is activated during normal electrical activity and contributes to the slower phase of I_{si}, corresponding to a net outward flow of calcium from the cell. Thereby the Na-Ca exchange mechanism can contribute both to electrical activity and to the maintenance of calcium balance during rhythmic activity of the heart (292; see also section VI B).

B. Calcium Uptake

Ca uptake by the exchanger will take place if the electrochemical gradient for Na is reduced, especially if the Ca gradient is not reduced as during the intracellular Ca transient after Ca release from the SR (see above, section V A). Therefore, the interval between the contractions will be favorable for Ca uptake through the exchanger in the case of an increased intracellular sodium activity, a^i_{Na}. Lipp and Pott (243) observed, in isolated cardiac cells, I_{NaCa} in the outward direction during rest at a holding potential of −50 mV, indicating a net Ca^{2+} influx. The concentration of intracellular sodium was kept constant at 20 mmol/liter and that of Ca_i^{2+} at 50 nmol/liter; the calculated reversal potential, E_{NaCa}, under these conditions was −86 mV. Ca uptake by the exchanger opposes the spontaneous Ca leak from the SR and leads to an increase in stored Ca, to a greater amount of Ca released during depolarization, and to an increased rate of force development of the early contraction component (see section III A). The importance of the reduction of both the concentration (chemical) gradient and the electrical gradient of Na is illustrated in fig. 7 which shows the inotropic effectiveness of changes in a^i_{Na} at different resting membrane potentials (96). The increase in a^i_{Na} was obtained through Na pump inhibition by ouabain. The inotropic effect in rested state contractions produced at comparable values of a^i_{Na} was increased by a factor of approximately ten after the cell membrane depolarized from −102 V to −65 mV with the increase of K from 2.4 to 12 mmol/liter. While Na influx and the counter-current Ca efflux are reduced with decreases of the resting membrane potential, the opposite fluxes (Na efflux-Ca influx) are stimulated. With the alterations of flux ratios, more intracellular Ca becomes available in the release sites of the SR for the subsequent contraction. The dependence of the inotropic effectiveness of the intracellular sodium activity on the membrane potential is unlikely to be influenced by Na^+/H^+ exchange across cardiac muscle membranes, since this is electroneutral (90). During hyperpolarization (2.4 mmol of K/liter), Ca influx via Na-Ca exchange is presumably small. A change in extracellular Ca, therefore, was quite ineffective in contrast to the situation at high K (fig. 7).

An increase of intracellular sodium concentration can be achieved not only by inhibiting the sodium pump with cardioactive steroids or by reducing extracellular potassium concentration (106), but also through an increase in sodium uptake by Na channel gate toxins (59a). The

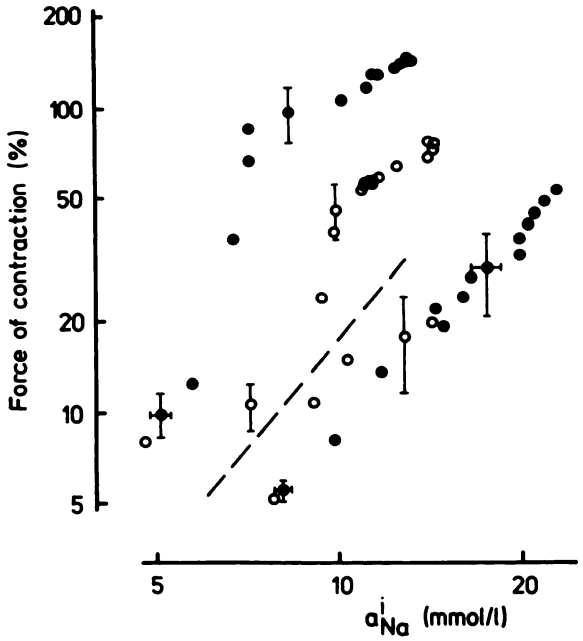

FIG. 7. Dependence of the inotropic effectiveness of a^i_{Na} on K and Ca. *Values below* the *dashed line* were obtained in the presence of 2.4 mmol/liter of K and 0.3 μmol/liter of ouabain; *values above* correspond to 12.0 mmol/liter of K, 3 μmol/liter of ouabain, and 1.2 (○) or 3.2 mmol/liter of Ca (●). *Ordinates*, force of test contractions every 16 min as the percentage of the value after the initial equilibration period at 1-Hz stimulation frequency, log scale; *abscissae*, a^i_{Na} in mmol/liter, log scale. Adapted from Ebner et al. (96). *Bars*, SE.

inotropic effects of a number of these toxins have been analyzed: ceveratrum alkaloids (veratrine, ref. 174; veratridine, ref. 170; germitrine, ref. 171); batrachotoxin, ref. 172; aconitine, ref. 169; the grayanotoxins (2, 177); and certain polypeptide animal toxins (314). The evidence in support of the theory that the sodium-dependent inotropic effects are coupled to the increase in a^i_{Na} has been compiled in a series of reviews (325, 234, 32, 1, 321, 166). It has been found recently that stimulation of low-affinity cardiac muscarinic receptors produces a positive inotropic effect parallel to an increase of intracellular Na^+ activity (221, 222) and leading to a rise of free intracellular Ca^{2+} concentration (223).

VI. The Sarcolemmal Calcium Pump

A. Ca^{2+}-Transport ATPase of the Sarcolemma

A plasma membrane-localized Ca^{2+} transport system in heart muscle mediates an active efflux of Ca^{2+} (for reviews, see refs. 371 and 306). This ATP-dependent Ca^{2+}-pumping system (like that of the red cell membrane, ref. 340) requires the presence of Mg^{2+} in the medium (71). The Ca^{2+} transport ATPase of dog heart sarcolemma possesses an apparent K_m (Ca^{2+}) of 0.3 to 1.0 μmol/liter when saturated with the activator calmodulin (72, 232, 229). Its depletion results in the transition of the Ca^{2+}-pumping ATPase to a low Ca^{2+} affinity state ($K_m \sim 20$ μmol/liter; ref. 74). The catalytic subunit of the cyclic AMP-dependent protein kinase stimulates the Ca^{2+}-ATPase, primarily by increasing its affinity for

Ca^{2+} (232, 396, 285). The Ca^{2+} affinity of the Ca^{2+} transport ATPase of the sarcolemma is higher than that of the Na-Ca exchanger, but its maximal velocity of Ca^{2+} translocation is considerably lower (see section V A). It thus seems especially suitable for ejecting Ca^{2+} under resting conditions when the intracellular $[Ca^{2+}]$ is low. Given enough time, the ATP-dependent Ca^{2+} pump is capable of securing a $[Ca^{2+}]_i$ lower than that which would be thermodynamically appropriate to a 3 Na^+ to 1 Ca^{2+} exchange (100 nmol/liter) if $[Ca^{2+}]_i$ were dependent only on the Na electrochemical gradient (277). The Ca^{2+}-ATPase represents only a minor fraction, less than 1%, of the total protein content of the cardiac sarcolemma (72). In highly purified sarcolemmal vesicles from canine ventricular muscle, the Ca^{2+} transport ATPase (measured as Ca^{2+}-dependent phosphoenzyme) amounted to a very small percentage of the (Na^+, K^+)-ATPase content (229). The rate of ATP-dependent Ca^{2+} uptake by these vesicles was much lower than in the isolated vesicles of the cardiac SR.

B. Relation to the Calcium Pump of the Sarcoplasmic Reticulum in Ventricular and Atrial Muscle

To evaluate the effectiveness of the sarcolemmal calcium pump in reducing cellular calcium in the resting muscle, some quantitative aspects should be considered in relation to the Ca^{2+}-ATPase of the SR which competes with the sarcolemmal calcium pump for Ca^{2+} leaking into the cytoplasm from both the SR and the extracellular space. In contrast to that of the sarcolemma, the Ca^{2+} pump protein of canine cardiac SR constitutes 35 to 40% of the total SR protein (75). In reconstituted vesicles (182), it is half-maximally activated at 0.5 μmol/liter of Ca^{2+}, a value quite similar to that reported for the sarcolemmal pump in situ. The amount of available Ca^{2+} ultimately achieved in cardiac muscle at rest will then depend on the quantitative relation between the two competing Ca^{2+} ATPases in the cell.

The existing stereometric measurements show (table 2) that there are great differences in the volume fractions and surface areas of the SR not only between different animal species but also between ventricular and atrial tissues of the same species. As has been pointed out by Sommer and Johnson (364), the SR is remarkably prominent in atrial fibers. The relation of total SR volume to the myofibril fraction is considerably higher in the atria than in the ventricles. This is in contrast to the mitochondrial volume fraction which amounts, in mouse atria, to only one-half that of the ventricle (50). The higher volume fraction of the SR in atrial muscle corresponds to the finding that the total calcium content of the atria of all species studied (guinea pig, rat, and cat) is significantly higher than that of the ventricles (138). When $[Ca^{2+}]_o$ is increased, the cellular calcium content of atrial muscle varies in proportion to $[Ca^{2+}]_o$, whereas that of ventricular muscle remains fairly constant (138). This indicates that the SR in the atria effectively competes in calcium sequestration with the sarcolemmal Ca^{2+} pump. The higher SR fraction with a greater loading capacity for calcium is apparently essential for the special function of the atria whose contractions precede the onset of ventricular contractions. This is achieved by greater velocities and briefer durations of atrial muscle contractions (54, 217, 386) for which a greater calcium release and uptake capacity of the SR seem to be a prerequisite. The inequality of their cellular calcium content is probably responsible for other functional differences between the two kinds of cardiac muscle, such as the duration of the action potential and the magnitude of rested-state contractions. Consistent with the importance of intracellular calcium for the short plateau duration of the atrial action potential is the demonstration that, in Ca-poor solutions, the atrial action potential becomes quite similar to that of a ventricular fiber (165). Unlike cardiac ventricles of most species, mammalian atria are distinguished by strong rested state contractions (225, 213). Since these contractions, in contrast to the late appearing rested state contractions of ventricles (see section III A), appear without delay after stimulation, they are presumably activated by calcium previously stored in the release sites of the junctional SR.

TABLE 2
Stereology of cardiac cell components

	Mouse*			Rat, left ventricle†	Lizzard‡		Frog‡	
	Right atrium	Left atrium	Left ventricle		Atrium	Ventricle	Atrium	Ventricle
SR volume (total %)	1.76	1.73	0.88§	3.5	1.22	0.69§	0.56	0.38
SR surface area ($\mu m^2/\mu m^3$ cell)	1.687	1.576	0.896§	1.22	0.914	0.503§	0.459	0.277§
Plasmalemma Surface + T system Plasmalemma ($\mu m^2/\mu m^3$ cell)	0.694	0.747	0.667	0.39	1.254	1.143	1.319	1.193
Myofibrils (%)	52.56	52.95	54.32	47.6	41.14	50.05§	42.38	46.15

* Refs. 49 and 50.
† Ref. 299.
‡ Ref. 48.
§ Difference significant from atrium ($P < 0.05$).

The calcium content of the atrial SR will probably not be completely uninfluenced by calcium leakage during long rest periods. But the remaining calcium content is very likely restricted to the release compartments of the junctional SR, whereas the larger parts of the SR (free or longitudinal SR) probably contain relatively little calcium. This may be deduced from the general experience that the strong, early appearing, rested state contraction is, at a higher stimulation frequency, followed by contractions which rapidly (in 1 to 3 beats) decline in strength, before the force of contraction gradually increases again until its frequency-dependent steady state is reached (225, 38; for review, see ref. 213). The rapid loss of contractile strength after the rested state contractions has been described by Blinks and Koch-Weser (38) as a consequence of the predominance of a large negative inotropic effect of activation (NIEA) over a small positive inotropic effect of activation (PIEA). According to these authors, the steady-state force of contraction is determined by the cumulation of these two opposing effects, of which the small PIEA disappears slowly, and the larger NIEA disappears rapidly after each contraction.

An explanation for the rapid decline of the contractile strength after the rested stated contraction (and thereby for NIEA) would be a calcium extrusion from the cell through the sarcolemma via Na-Ca exchange. If a considerable part of the amount of Ca^{2+} released in the rested state contraction is indeed extruded during the calcium transient (see section V A), the remaining part of the released calcium may not suffice to refill an empty sarcoplasmic reticulum with a great uptake capacity. Activator calcium would then become available again only according to the slowly cumulating activity-dependent Ca^{2+} uptake from the extracellular space (PIEA). Evidence in support of the idea that Ca^{2+} extrusion through Na-Ca exchange is partly responsible for the loss in contractile strength was obtained in experiments with guinea pig papillary muscles in magnesium-free solution (399a). These muscles have a distinct atrium-like frequency-force relationship with strong rested state contractions (see below). The duration of the transmembrane action potential accompanying the rested state contraction was longer than that of the following low strength contractions. The difference became more prominent (25% at 60% repolarization of the action potential, $n = 4$) after the sarcolemmal calcium channels had been inhibited with 1 μmol/liter of nifedipine. These results are interpreted to show that the second inward current (I_{si}) responsible for the action potential plateau during the strong rested state contraction was carried to a great extent by I_{NaCa}, indicating calcium extrusion through Na-Ca exchange.

A notable exception to the usual frequency-force relationship of mammalian ventricular muscle is that of the rat, which shows strong rested state contractions (213, 133). This may be causally related to an exceptionally high SR volume fraction in the rat ventricle (table 2) with a consequent high intracellular calcium activity. The reported values of the intracellular free Ca^{2+} concentration in resting rat ventricular myocytes, as measured with the fluorescent Ca^{2+} indicator quin-2 (in nmol/liter: 121 ± 11, ref. 93; 137.1 ± 2.6, ref. 310; 181 ± 18, ref. 349), are considerably higher than those in ventricular myocytes of the cat (57 ± 4, ref. 93) and the guinea pig (99.9 ± 10, ref. 310). Accordingly, the magnitude of the rested state contraction of the rat ventricle declines when $[Ca]_o$ is reduced, and at low Ca^{2+} concentrations, the rat myocardium has properties similar to those of other species with respect to inotropic effects of stimulation (133). The reverse is seen with guinea pig ventricular muscles kept in magnesium-free solutions. They develop strong rested state contractions without a delay after stimulation and an atrium-like frequency-force relationship (398), probably as a result of an unfavorable shift in the relation between sarcolemmal calcium pump capacity and passive calcium leak into the cell.

VII. Conclusions

The review has focused on the central role of an intracellular calcium store, the sarcoplasmic reticulum, in the regulation of mammalian cardiac contraction. It is the amount of calcium released from the SR after depolarization which determines the degree of activation of the contractile apparatus. The property of leaking calcium at a considerable rate during rest makes the calcium content of the SR extremely labile. On the other hand, the ability of the SR to accumulate calcium leads to the refilling of its stores to an extent that depends on the amount of calcium made available from the extracellular space. This offers the common mechanistic basis for a variety of inotropic agents that act in quite different ways.

According to our present knowledge, calcium enters the cell mainly during depolarization through voltage-dependent channels whose functional availability is regulated by cyclic AMP. Since the cellular content of cyclic AMP depends on a series of enzymatic steps, initiated by receptor stimulation and ending in cyclic AMP degradation, an increase in cellular calcium uptake may be achieved by interventions that act on any of them. A major mechanism for calcium transport through the sarcolemma in both directions consists of an electrogenic Na-Ca exchange which depends on the electrochemical gradient for Na. A reduction of this gradient leads to increased Ca uptake by the exchanger. Therefore, a great variety of agents causing, in one way or another, an increase in intracellular sodium activity will produce positive inotropic effects.

While we believe we understand, more or less thoroughly, the mechanisms responsible for an increase in cellular calcium uptake, we know, at present, relatively little about possible pharmacological influences on Ca-

binding modulator proteins and their quantitative contributions to inotropic effects on cardiac muscle.

Acknowledgments. I am indebted to Drs. John R. Blinks, F. Ebner, P. Honerjäger, M. Korth, K. Seibel, and W. Vierling for reading the manuscript and suggesting improvements. For careful secretarial help I thank Ms. Brigitte Dick and Ms. Christa Baumgärtel.

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