SUMMARY  Paired pulse stimulation increases the contractile strength of mammalian myocardium. If stimulation is discontinued the "potentiated state" takes several minutes to decay, as shown by the first contraction following resumption of stimulation. From earlier experiments it has been inferred that contractile force depends on Ca\textsuperscript{2+} released from intracellular stores rather than on an influx of Ca\textsuperscript{2+} associated with a given action potential. This view now receives support from the following findings: (1) Ni\textsuperscript{2+} and Co\textsuperscript{2+}, known to inhibit Ca\textsuperscript{2+} influx during the action potential, when applied during a 2-minute rest period, are practically without effect on the strength of the first beat but strongly depress steady state contractions. (2) Caffeine, known to enhance Ca\textsuperscript{2+} efflux from and inhibit Ca\textsuperscript{2+} uptake into intracellular stores, greatly accelerates the decay of the potentiated state during a rest period. (3) Na\textsuperscript{+}-poor solution, known to inhibit Ca\textsuperscript{2+} efflux, has a strong positive inotropic effect. Paired pulse stimulation fails to increase contractile strength in Na\textsuperscript{+}-poor solution, and a rest period of many minutes is practically without effect on the amplitude of the first beat after resumption of stimulation. The results indicate that contraction is due to Ca\textsuperscript{2+} released from internal stores whose degree of filling can be altered.

A POSITIVE staircase can be produced in mammalian myocardium by paired pulse stimulation.\textsuperscript{1} When a preparation has reached a potentiated state and is subsequently allowed to rest for many seconds, the first stimulus still gives a strong contraction. This phenomenon is generally known as "memory."\textsuperscript{1} A rest of several minutes is required for the first beat to be reduced in amplitude to control levels (steady state contractions during single pulse stimulation) or below control levels. On the basis of existing hypotheses,\textsuperscript{1,2} the phenomenon can be explained as follows. Intracellular Ca\textsuperscript{2+} is increased by paired pulse stimulation, by high frequency stimulation, or by prolonged depolarization. Under all these conditions there is a greater Ca\textsuperscript{2+} influx than Ca\textsuperscript{2+} efflux until the steady state is reached and the internal Ca\textsuperscript{2+} stores (for instance those in the lateral cisternae)\textsuperscript{3} are loaded to a greater extent. Because the strength of contraction of mammalian heart is controlled mainly by Ca\textsuperscript{2+} released from intracellular stores, the force of contraction will depend on the Ca\textsuperscript{2+} content within such stores. During a period of rest the Ca\textsuperscript{2+} content of the stores is reduced.\textsuperscript{4} Consequently, the first beat after a rest gradually diminishes in amplitude as the duration of the rest period is increased. If this hypothesis is correct the following procedures should have predictable results: (1) Substances known to decrease Ca\textsuperscript{2+} influx across the surface membrane, when applied during the rest period of the first beat, and thus on "memory." If a Ca\textsuperscript{2+}-induced release of Ca\textsuperscript{2+} occurs in heart,\textsuperscript{5} then sufficient Ca\textsuperscript{2+} still must flow into the cell to trigger Ca\textsuperscript{2+} release from the intracellular stores. (2) A reduction of transmembrane Ca\textsuperscript{2+} efflux should prolong the potentiated state. (3) An increase in Ca\textsuperscript{2+} efflux should shorten the potentiated state.

Methods

Small bundles, between 0.5 and 0.8 mm in diameter and between 8 and 15 mm in length, were removed from the right ventricle of calf and sheep hearts and stimulated by single and paired pulses in oxygenated Tyrode's solution by two Grass S4 stimulators connected in series. The volume of the perfusion chamber was 0.3 ml and solutions could be changed within 3 seconds, as shown by washout of methyl-ene blue. The mechanogram was recorded with an RCA 5734 transducer and a Tektronix 502A oscilloscope. Micro-electrodes, for recording action potentials, were filled with 3 M KCl according to the method of Tasaki et al.\textsuperscript{6} Solutions used are listed in Table 1. The experiments were carried out at a temperature of 36°C. The results from studies on sheep and calf muscle were similar; therefore the results from both species have been evaluated together.

Results

To decrease the inward Ca\textsuperscript{2+} current, the Ca\textsuperscript{2+} channel blockers Ni\textsuperscript{2+} and Co\textsuperscript{2+} were used.\textsuperscript{1,11} However, if one is to be able to test the effect of Ca\textsuperscript{2+} blockers on "memory," the diffusion process within the bundle should be fast compared to the normal loss of the potentiated state. With bundles less than 0.8 mm in diameter Co\textsuperscript{2+} and Ni\textsuperscript{2+} abolished 90% of the contraction within 2 minutes. Calculation of diffusion profiles,\textsuperscript{12} assuming a diffusion coefficient of 10\textsuperscript{-4} cm\textsuperscript{2}/sec, suggests that the concentration within the core of such bundles was no more than 10% of that in the perfusion solution. However, 75% of the muscle mass lies within an outer 0.2-mm ring, where the concentration would be 72% or more of that in the perfusion medium.

The time course of decay of the potentiated state is shown in Figure 1A. An interval of several minutes was required for the first beat to be reduced in amplitude to or below the control level. After 2 minutes the potentiated state had declined to 65% of its initial value. Since this was also a
reasonable time for diffusion of Co\textsuperscript{2+} and Ni\textsuperscript{2+}, a period of 2 minutes was chosen as a standard rest period.

In studies on seven muscle bundles, maximal potentiation by paired pulse stimulation first was achieved in normal Tyrode's solution. Stimulation was then discontinued and the chamber perfused with a solution containing NiCl\textsubscript{2} or CoCl\textsubscript{2} (1 mmol/liter). Two minutes later, while perfusion with this test solution continued, stimulation at 0.4 Hz was resumed. The first beat had practically the same amplitude as that observed when the same sequence of stimuli had been applied to a preparation in a solution free of inhibitor (Fig. IC). The action potential duration, however, was shortened, with a less marked plateau: this was characteristic of the action of Co\textsuperscript{2+} and Ni\textsuperscript{2+} (Fig. IB). As expected, the steady state contractility in the presence of Co\textsuperscript{2+} and Ni\textsuperscript{2+} dropped to below control values. As shown in Fig. IB and D, the new steady state almost was reached after the third beat.

**INCREASE IN Ca\textsuperscript{2+} EFFLUX**

Because caffeine reduces stored Ca\textsuperscript{2+} and increases Ca\textsuperscript{2+} flux out of the cell,\textsuperscript{10} four experiments were carried out in the presence of 2 mM caffeine. As shown in Figure 1A, in the presence of caffeine the decay of the potentiated state occurred more quickly and reached a level below the steady state level of the control in less than 1 minute. In a solution containing 5 mM caffeine, the positive staircase phenomenon no longer could be produced. If the rest period was lengthened to 4 minutes in the presence of caffeine, the first beat after resumption of stimulation was smaller than the subsequent ones.

**DECREASE IN Ca\textsuperscript{2+} EFFLUX**

It has been shown\textsuperscript{4} that Ca\textsuperscript{2+} efflux from cardiac muscle is reduced when the extracellular Na\textsuperscript{+} concentration is
lowered. With solutions containing 50% or 25% of normal Na+ it was not possible to demonstrate a positive staircase phenomenon. Similar findings also have been reported by Beeler and Reuter and by New and Trautwein. The effect of increasing frequency of stimulation is shown in Figure 2A. In contrast to the curve in normal Tyrode’s solution, the contraction in Na+-poor solution decreased with increasing frequency. It also was found that it was not always possible to induce an action potential if the interval between stimuli was less than 400 msec.

Since potentiation did not result from the methods used previously, potentiation was induced by stopping stimulation and resuming it after various rest periods (four experiments). The first contraction was potentiated under these conditions, even after a rest period of 15 minutes or more; in normal Tyrode’s this occurred for periods of only up to 2 minutes (Fig. 2B). The decrease in amplitude of the first beat after a rest period of 15 minutes in Na+-poor solutions amounted, on the average, to 50% with respect to the maximal potentiation observed after ½-1½ minutes of rest (Fig. 2B).

**Discussion**

Our results are in agreement with the working hypothesis stated in the introduction, i.e., the amount of Ca²⁺ in the internal stores plays a central role in electromechanical coupling.

It has been shown that Ni²⁺ and Co²⁺ reduce the inward Ca²⁺ current. Thus it must be assumed that in the presence of these ions a release of Ca²⁺ from the appropriate stores still is possible and in an amount practically identical to that released under control conditions. If, in addition, it is assumed that the inward Ca²⁺ current triggers Ca²⁺ release, then a reduced amount of trigger Ca²⁺ still must be able to cause sufficient Ca²⁺ release.

The assumption has been made that Ni²⁺ and Co²⁺ are similar in their action to La³⁺ in that they block only the inward Ca²⁺ current. Although an intracellular action of these ions cannot be excluded, they have not been shown to cause changes in tension similar to that produced by verapamil, where an intracellular action has been demonstrated (unpublished observation). Because a markedly lower steady state level is reached quickly in the presence of Co²⁺, the inward Ca²⁺ current is not directly responsible for determining the force of the first beat, but for the following beats after resumption of stimulation. Thus inward Ca²⁺ current through the surface membrane must play a major part in loading the stores rather than in directly mediating contraction. This would adequately account for the potentiated first beat after a rest period as well as for the lower steady state level of contraction with blockers of the inward Ca²⁺ current.

When the efflux of Ca²⁺ during the rest period was increased, the loss of Ca²⁺ from the stores occurred more rapidly. This was brought about by adding caffeine (2 mmol/liter) to the perfusing solution. As expected, the potentiated state decayed more rapidly. It also has been reported that caffeine increases inward Ca²⁺ current during the action potential. Thus the present finding of a shortened “memory” may seem surprising but is not contradictory since, as mentioned above, the trigger Ca²⁺ does not play a major part in relation to the first beat. Also, in the presence of caffeine this Ca²⁺ cannot be stored. If Ca²⁺ efflux during the rest period is reduced, a condition that can be brought about by superfusion with Na+-poor solutions,
contractions are strong during slow stimulation with single pulses. Paired stimulation, as well as high frequency stimulation, fails to lead to a further increase in force of contraction. This occurs for two reasons: first, because an action potential no longer is produced when the interval between stimuli is shortened below 400 msec (in 25% Na+ solution), and second, at a faster rate of stimulation there is a decrease in the strength of contraction. The model for excitation-contraction coupling proposed by Bassingthwaighte and Reuter provides an explanation for this phenomenon. These authors proposed that release and uptake of Ca2+ occurred at different sites in the sarcoplasmic reticulum and that, after uptake, Ca2+ had to diffuse back to the releasing site and this was a time-dependent process. Thus, if the Ca2+ efflux in an Na+-poor solution is reduced, the loading of the internal stores will be more complete after a long interstimulus interval than after a short one, because there is more time for the diffusion process to charge releasing sites when there is only a small loss of Ca2+. The potentiated first systole after rest periods can be explained in the same way. The extremely slow loss of "memory" under conditions of low extracellular Na+ strengthens the concept that Ca2+ efflux is strongly coupled to Na+ influx. Thus, these experiments again point to the importance of internal stores for electromechanical coupling.

In summary, these results suggest that the staircase and its decay can be explained as follows. Paired stimulation, as well as stimulation at high rates, increases the intracellular Ca2+ concentration because Ca2+ influx outweighs Ca2+ efflux. This extra Ca2+ is sequestered by the sarcoplasmic reticulum and transported to the cisternae. Each single stimulus which occurs in the phase of optimal loading must lead to a greater release of Ca2+ and therefore to a stronger contraction. During a period of rest, Ca2+ escapes very slowly either directly from the cisternae to the outside or via the myoplasm. Thus the duration of the "memory" is determined by the loading of the cisternae at the beginning of the rest period as well as by the rate of Ca2+ efflux from it.

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