Effects of Rapid Cooling on the Mechanical and Electrical Activities of Smooth Muscles of Guinea Pig Stomach and Taenia Coli

T. MAGARIBUCHI, Y. ITO, and H. KURIYAMA

From the Department of Physiology, Faculty of Dentistry, Kyushu University, Fukuoka, Japan

ABSTRACT The effects of rapid cooling on the mechanical and electrical activities of the guinea pig taenia coli and circular muscle of the stomach were investigated. Lowering the temperature from 32° to 10°C (cold shock) depolarized the membrane and increased the membrane resistance in both tissues. However, in the taenia coli, an initial reduction of membrane resistance was observed. In both tissues, contracture evoked by cold shock and rapid relaxation after rewarming, preceded the changes of membrane properties. Displacements of the membrane potential did not modify the amplitude of contracture under cold shock. Caffeine and thymol modified the membrane properties, but the effects of cold shock were still observed. The effects of cold shock were also observed on K-induced contracture. It was postulated that at least two different sites of sequestered bound Ca are located in these smooth muscles and are responsible for evoking the mechanical response. One component possesses a close relation to membrane and the other component is presumably sequestered within the muscle.

INTRODUCTION

Multiple actions of Ca ion in mammalian smooth muscle have been described by many investigators. For instance, the action potential of the many visceral smooth muscles is a Ca-spike, which may also provide the activator for contraction, and the membrane-bound Ca regulates the permeability of the membrane to various ions including Ca itself. However, it is uncertain whether or not the membrane-bound Ca which regulates the permeability of the membrane is the same Ca that accumulates in the sarcoplasmic reticulum-like structure, presumably distributed in the smooth muscle for excitation-contraction coupling (Somlyo and Somlyo, 1968; Brading, Bülbring, and Tomita, 1969; Axelsson, 1970; Kuriyama, 1970; Tomita, 1970; Bülbring and Tomita, 1970; Rüegg, 1971).

The present experiments were intended to analyze the possible components of bound Ca which evoke mechanical responses of the smooth muscle cells of guinea pig taenia coli and stomach circular muscle layer using the double sucrose gap method. To evoke the mechanical response, an isotonic K-Krebs solution, caffeine, and thymol were used in the presence and absence of a rapid cooling method (cold shock method).

The results suggested that there are at least two sources of Ca which may supply ionized Ca to the contractile protein. One component is closely related to the properties of the muscle membrane. The other component is related neither to actions of caffeine and thymol nor to electrical properties of the membrane. It is, therefore, postulated that the latter component is located within the organelles in the cell. The possibility of evoking the mechanical response by another mechanism in which one source of Ca shows different releasing modes, is also discussed.

METHODS

Male guinea pigs weighing 250–350 g were stunned and bled. The circular muscle of the pyloric region of the stomach and taenia coli (20–25 mm in length) were excised from the abdomen and placed in Krebs solution. The connective tissue and the mucosa of the stomach muscle were dissected carefully.

The double sucrose gap method was the same as that described by Kuriyama and Tomita (1970), i.e., a small portion in the central part of the tissue was exposed to the test solution and the remaining parts of both sides were perfused with isotonic sucrose solution (90 g of solid sucrose dissolved in 1000 cm^3 of distilled deionized water). Pulses of current were applied (through a resistor of 50 MΩ) in the sucrose solution on one side and across the tissue. Changes in membrane potential in the central part were recorded across the sucrose gap on the other side of the tissue. Changes in isometric tension were recorded with a strain gauge attached to the end of the tissue strip on the side that was stimulated.

A modified Krebs solution of the following composition was used (millimolar): Na^+ 137.4, K^+ 5.9, Mg^{++} 1.2, Ca^{++} 2.5, Cl^- 134.0, HCO_3^- 15.5, H_2PO_4 1.2, and glucose 11.5, equilibrated with 97% O_2 and 3% CO_2. The Na-free solution was prepared by replacement of NaCl and $NaHCO_3$ with Tris-Cl and the pH of the solution was kept at 7.2–7.3. Isotonic K-Krebs solution was prepared by increasing KCl and $KHCO_3$ concentrations with equivalent reduction of the concentrations of NaCl and $NaHCO_3$.

For rapid cooling, two baths including thermounits with perfusion pumps (Taiyo Co., Ltd., C 550 type) were used. In one bath, the temperature of the water was kept at 38 ± 1°C (temperature of the perfusate was dropped to 32 ± 1°C), and in another vessel, the temperature of the water was kept at any desired level. Most of experiments were done at 10 ± 1.0°C. The temperature of the perfusates started to change within 3 s after exchanging the water in the bath, and the temperature of the test solution became steady within 40–50 s.

Drugs used in the present experiment were caffeine (3–5 mM) and thymol (1 mM).

For the application of caffeine and thymol, one end of a small vinyl tubule (diameter 0.5 mm) was connected into the vinyl tube used for the perfusion of the test solutions. The other end of the tubule was attached to the injection syringe (1 cm^3). Drugs were diluted with the test solution to the required concentration. The final concentrations of drugs are described in the results. In other experiments, caffeine and thymol were applied to the tissue through a test tube connected to the vessel (200 cm^3) which was the same volume of the vessel containing the test solution.

In order to prepare the low concentrations of Ca (10^{-7} and 10^{-6} M), the Locke solution was prepared by addition of EGTA-buffer using the equation abbreviated by Imai and Takeda (1967).

$$\mathrm{PCa} = 2\mathrm{pH} - 7.28 + \log\left(\frac{[\mathrm{EGTA}]\ \text{added}}{[\mathrm{CaCl_2}]\ \text{added}} - 1\right).$$

RESULTS

Changes of Electrical and Mechanical Activities during Cold Shock

When the circular muscle of stomach and taenia coli were perfused at low temperature (10°C), the membrane was depolarized and a tonic contracture evoked. In the circular muscle of the stomach the frequency of slow potential changes was reduced and they finally ceased. During depolarization of the membrane, the membrane resistance gradually increased. When the tissue was rewarmed from 10° to 32°C, the contracture rapidly relaxed and then a rebound contraction was produced. The amplitude of this rebound response never exceeded the amplitude of the contracture evoked by the cold shock. Repolarization of the membrane occurred gradually.

In the taenia coli, additional responses of the membrane in comparison to the stomach muscle could be observed by cold shock. When the temperature was lowered from 32° to 10°C, the membrane resistance was transiently reduced and often spikes were generated. After the spikes stopped, the membrane resistance gradually increased as observed in the stomach muscle. The mechanical responses consisted of a phasic contraction during the presence of spikes which was preceded by the tonic contracture evoked by cold shock. After rewarming the tissue, the membrane was markedly hyperpolarized.

Fig. 1 shows typical effects of low temperature on the electrical and mechanical activities of the guinea pig taenia coli and stomach.

In the taenia coli, application of an inward current pulse (3 s) allowed measurement of changes of the membrane resistance. The reduction of membrane resistance accompanied depolarization of the membrane; this was followed by a gradual increase in membrane resistance. The reduction of membrane resistance during the initial phase of cooling was thought to be due to an increase of Na-conductance, and an increase of membrane resistance was mainly due to reduction of ionic permeability of the membrane to all ions.

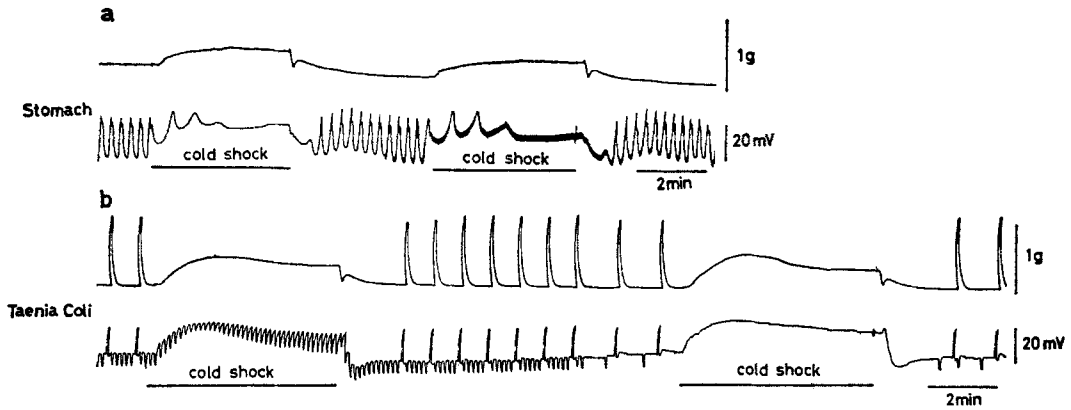

FIGURE 1. Effects of rapid cooling (cold shock) on electrical and mechanical activities of guinea pig circular muscle of the stomach (*a*) and taenia coli (*b*). Horizontal bars indicate the duration of cold shock (10°C). Inward current pulses (500 ms in the latter half of *a* and 3 s in an initial half of *b*) were applied to the tissues. In (*b*), to generate the spikes 3 s of 5×10^{-7} A of the outward current was applied to the tissue.

After rewarming the tissue, the membrane was markedly hyperpolarized. The magnitude of the hyperpolarization is a function of the reduced membrane resistance during the initial phase of rapid cooling. The hyperpolarization is thought to be due to activation of the electrogenic Na pump mechanism (Magaribuchi, Ito and Kuriyama, 1972).

In the circular muscle of the stomach, it was rather difficult to calculate the relative change of membrane resistance between the resting state (32°C) and the low temperature (10°C). When a pulse duration of more than 1 s was used to measure the changes of the membrane resistance of the muscle, an anodal break excitation and evoked slow potential changes interfered with the measurement of the amplitude of the electrotonic potential. Therefore, the electrotonic potential evoked by an inward current pulse of 500 ms did not reach a steady potential level.

Fig. 2 shows the effects of temperature on the electrical and mechanical activities of the taenia coli. The temperature was varied from 32° to 10°C. The contracture was evoked when the temperature was lowered to 21°C ($n = 5$) and rapid relaxation of the tissue was observed when the temperature was raised to 18°–19°C ($n = 5$). Repolarization of the membrane started at 23°C. The bath temperature became steady after 40–50 s under cold shock. After rewarming the temperature was restored to 32°C within 40 s.

Fig. 3 shows effects of cold shock (10.5°C) on the electrical and mechanical activities of stomach muscle. To study the changes in membrane resistance before and after applications of cold shock, pulses of 500 ms duration were applied. It was clear that the development of the contracture preceded the changes of membrane potential and membrane resistance, and that after

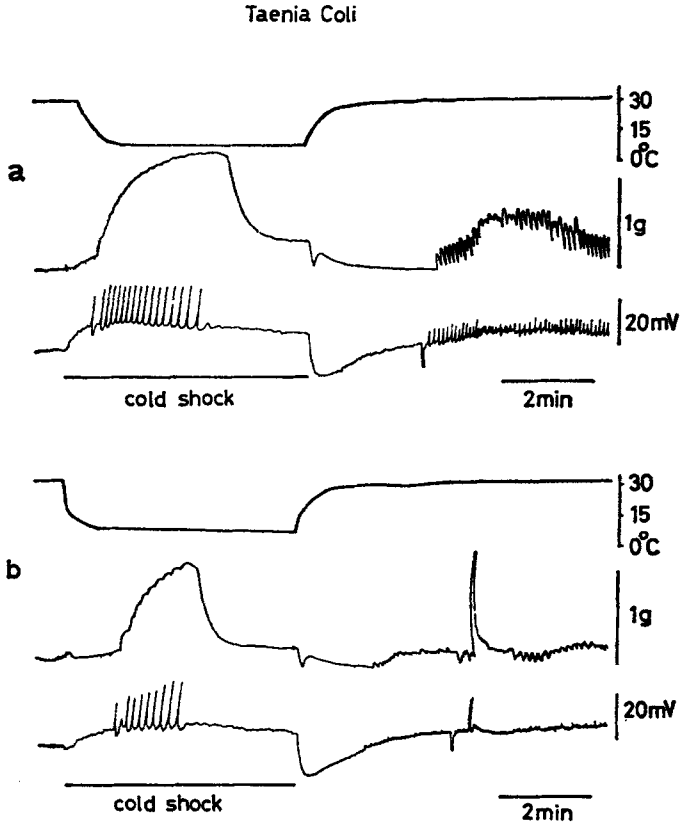

FIGURE 2. The relationship between the temperature of the test solution in which the tissue was placed and the electrical and mechanical activities of the guinea pig taenia coli. In (*a*), top record, changes of temperature; middle record, changes of mechanical activity; lower record, changes of electrical activity. (*a*) and (*b*) are recorded from the different preparations. Horizontal bars indicate the duration of cold shock.

rewarming the tissue, the rapid relaxation preceded the repolarization of the membrane and reduction of membrane resistance. The latency for the onset of rapid relaxation and repolarization was 8 s (n = 5). The rapid relaxation of the tissue was followed by rebound contraction. The rebound contraction relaxed with the time-course of 1 min.

Fig. 4 shows the effects of low temperature (10°C) on the smooth muscle of taenia coli in the isotonic K-Krebs solution in comparison with the normal temperature (32°C). Excess K-Krebs solution depolarized the membrane and produced a phasic and tonic response of the contracture. When cold shock (10°C) was applied during the generation of the tonic response of the contracture, the amplitude of the tonic response was transiently enhanced initially and then it relaxed. When the tissue was rewarmed, the low amplitude of the tonic contracture was transiently relaxed further (rapid relaxation)

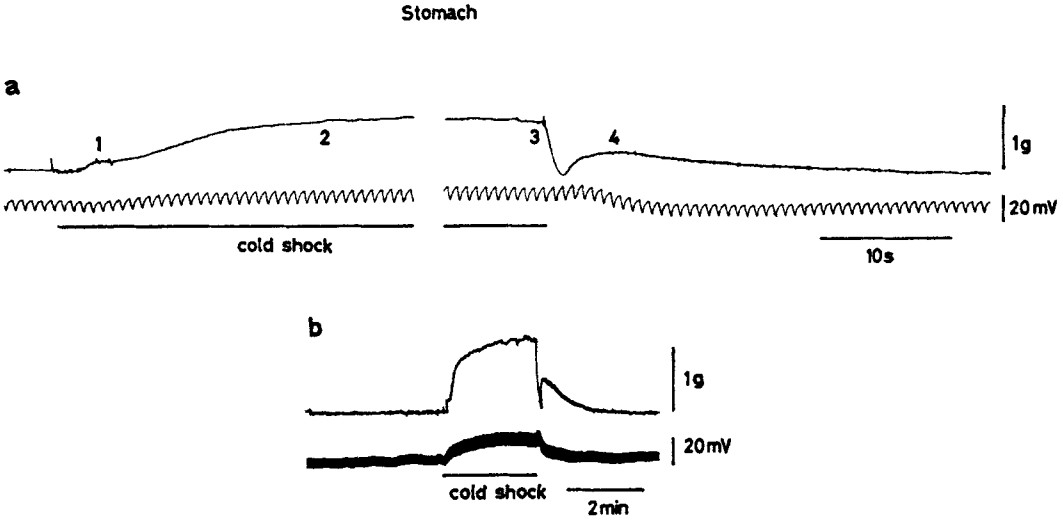

FIGURE 3. Effects of cold shock on electrical and mechanical activities of the circular muscle of the stomach. (*a*) and (*b*) are recorded from the same preparation at different speeds. Applied inward current pulse is 500 ms and 1×10^{-7} A. Horizontal bars indicate the duration of cold shock. 1, initial tension development; 2, tonic contracture; 3, rapid relaxation; 4, residual contraction.

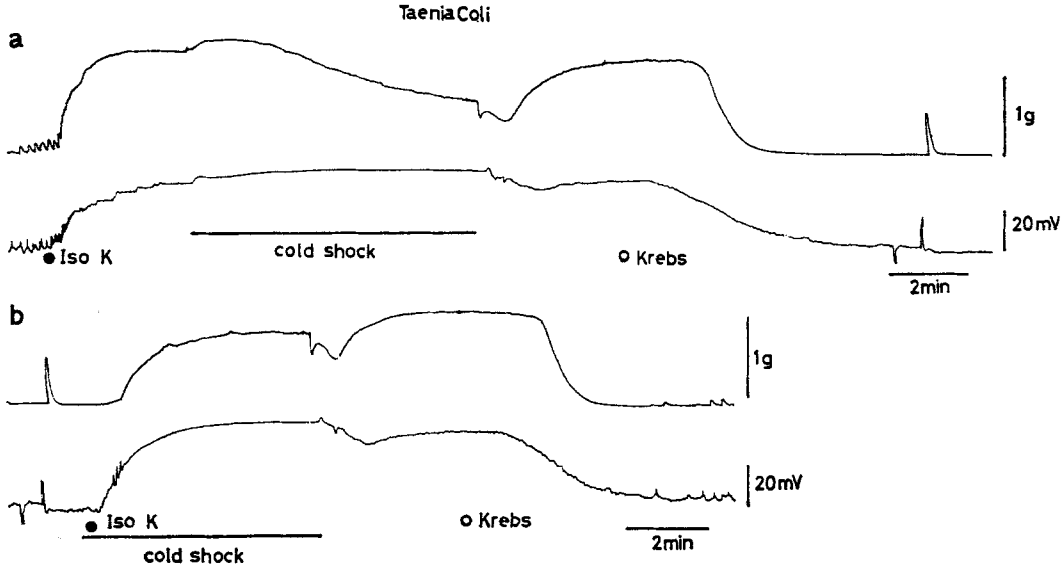

FIGURE 4. Effects of cold shock on electrical and mechanical activities of the smooth muscle of taenia coli under depolarized condition (isotonic K-Krebs solution). (*a*), during the maintenance of the tonic response of the contracture evoked by excess K-Krebs solution, cold shock was applied. (*b*), before and during application of excess K-solution, cold shock was applied. ●, application of isotonic K-Krebs solution; ○, removal of isotonic K-Krebs solution. Horizontal bars indicate the duration of cold shock.

as observed in Krebs solution. Then the contracture was redeveloped to the level expected from the amplitude of the tonic response of the contracture. Upon rewarming the tissue, the membrane was repolarized slightly and remained at the level observed before applications of cold shock. It is interesting that even when the membrane was markedly depolarized by excess K-Krebs solution at 32°C, the cold shock further depolarized the membrane. These effects must be clarified by further investigations.

When cold shock was applied to the tissue before exposure to isotonic K-Krebs solution, the phasic contracture to potassium had a very slow rate of rise. Cold shock suppressed the generation of the tonic response of the contracture. However, the tonic contracture after application of cold shock was maintained with the same amplitude as that observed in Krebs solution.

Figs. 5 and 6 show the effects of the displacement of membrane potential under applications of cold shock. During generation of the tonic contracture in Krebs solution, inward and outward current pulses did not modify the shape of the tonic contracture even though the membrane potential was displaced to a more hyperpolarized level than the resting level (Fig. 6 *a*). On the other hand, during the tonic response of the contracture evoked by isotonic K-Krebs solution at 32°C, the current pulse modified the shape of the responses as expected from the membrane potential level (Fig. 5 *b*). Although, in isotonic K-Krebs solution during application of cold shock, only the

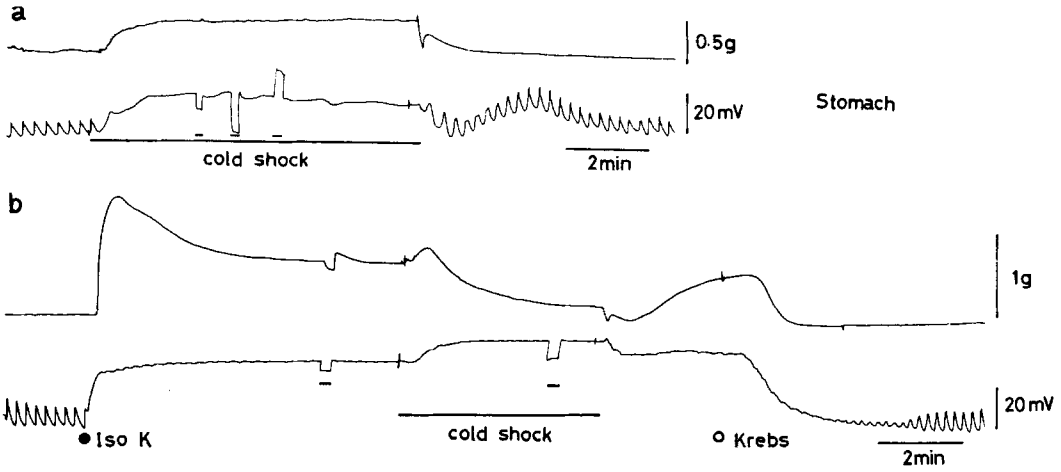

FIGURE 5. Effects of displacement of membrane potential in normal and depolarized smooth muscle of the stomach circular muscle. (*a*), applications of inward (2×10^{-7} A and 5×10^{-7} A) and outward current (5×10^{-7} A) to displace the membrane potential during application of cold shock. (*b*), applications of inward current (8×10^{-7} A) to displace membrane potential during the generation of tonic response of the contracture and tonic contracture evoked by cold shock. Short horizontal bars indicate the application of inward and outward current. ●, ○, and the horizontal bars are the same as in Fig. 4.

phasic response of the contracture was modified by the application of a current pulse to the membrane (Fig. 6 *b*).

From the above results, it is concluded that the amplitudes of phasic and tonic responses of the contracture evoked by isotonic K-Krebs solution at normal temperature, and the phasic response evoked by isotonic K-Krebs solution at low temperature, are related to membrane potential level. However, the amplitude of the tonic contracture evoked by cold shock in Krebs and in the isotonic K-Krebs solution are independent of membrane potential levels. Therefore, the tonic response of the contracture evoked by excess

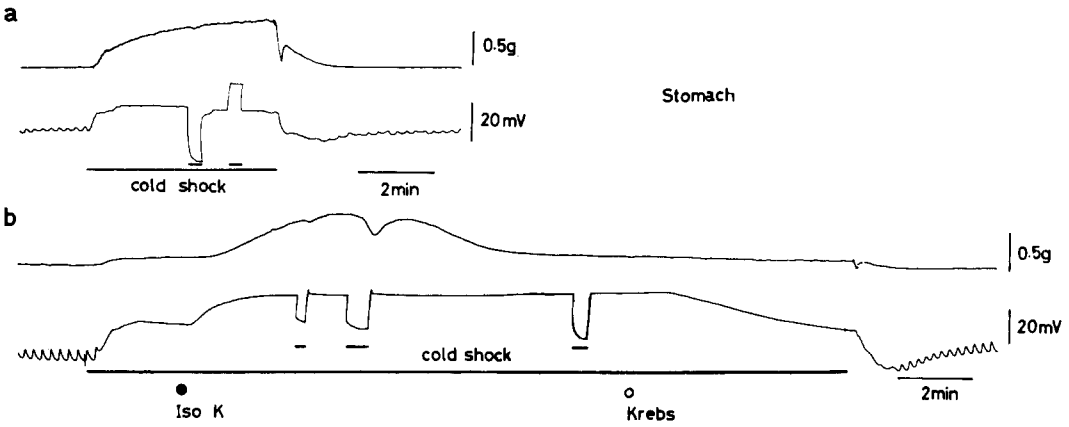

FIGURE 6. Effects of displacement of membrane potential in normal and depolarized smooth muscle of the stomach under applications of cold shock. (*a*), inward current (5×10^{-7} A) and outward current (5×10^{-7} A) are applied. (*b*), inward current (6×10^{-7} A) was applied with three different durations at the different stages of cold shock. The symbols in the figure are the same as those in Fig. 5.

K-Krebs solution and the tonic contracture evoked by cold shock might be generated from different mechanisms.

Effects of Caffeine and Thymol on Guinea Pig Taenia Coli and Stomach Circular Muscle during Application of Cold Shock

The effects of caffeine on the guinea pig taenia coli have been studied by Ito and Kuriyama (1971). It was concluded that caffeine released bound Ca from the membrane thus increasing the Na-conductance. As a consequence the membrane was depolarized and spikes were evoked. Mechanical activity was evoked by initial release of bound Ca. After this the appearance of spikes further enhanced the amplitude of mechanical responses.

The effects of caffeine (5 mM) at low temperature were observed in both taenia coli and stomach circular muscle. As shown in Fig. 7, caffeine reduced

membrane resistance, depolarized the membrane, and evoked spikes in the taenia coli at 32°C. During cold shock, caffeine (5 mM) further depolarized the membrane, reduced the membrane resistance, and evoked contraction.

Spike generation by caffeine was caused not only by depolarization of the membrane, but also by mobilization of the bound Ca sequestered in the membrane. Fig. 8 shows the effects of caffeine (10 mM) on the electrical and mechanical activities of the guinea pig taenia coli in low Ca-Krebs solution (10^{-7} M). When Ca concentration was reduced to 10^{-7} M by addition of EGTA buffer (Ito and Kuriyama, 1971), the membrane was depolarized and transiently generated spikes during the course of the depolarization. After depolarization, the membrane potential reached a steady level. Often inward current pulses triggered oscillatory potential changes. These oscillatory graded responses of the membrane did not evoke mechanical activity. After treatment with caffeine (10 mM) under treatment with the low Ca-Krebs

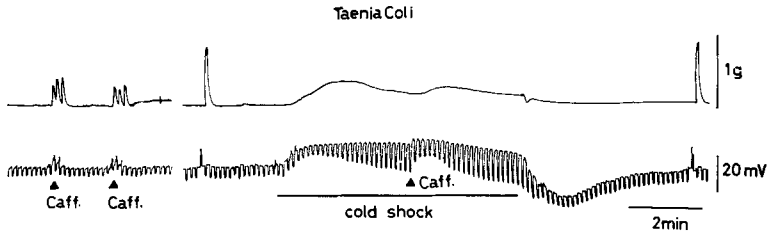

FIGURE 7. Effects of caffeine (5 mM) on electrical and mechanical activities of the taenia coli at normal (32°C) and low temperatures (10°C). Inward current pulses are applied successively (3 s in duration and 2×10^{-7} A in intensity). The outward current pulses evoking muscle activity were applied with the duration of 3 s and intensity of 5×10^{-7} A. ▲, application of caffeine (10 s).

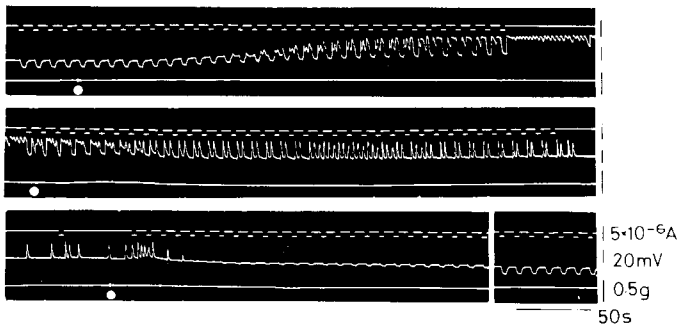

FIGURE 8. Effects of caffeine (10 mM) on electrical and mechanical activities of the taenia coli in Ca-deficient solution (10^{-7} M). Dot in top record, application of Ca-deficient Krebs solution; in middle record, application of caffeine in Ca-deficient solution; in lower record, replacement with Krebs solution. Top trace, middle trace, and lower trace in the individual records show the applied current intensity, electrical activity, and mechanical activity, respectively.

solution, the membrane was slightly repolarized and spikes occurred. As postulated for striated muscle, if the internal free Ca ion concentration in sarcoplasm is about 10^{-7} M, the Ca ion gradient across the sarcoplasmic membrane should be very small. Although caffeine evoked spikes, release of bound Ca from the membrane might be involved. Caffeine also evoked spikes in 10^{-6} M Ca-Krebs solution in the same way as observed in 10^{-7} M Ca-Krebs solution.

The effects of caffeine (5 mM) on stomach smooth muscle were also observed in Krebs and in isotonic K-Krebs solution under application of cold shock (10°C). As shown in Fig. 9, treatment with caffeine suppressed the

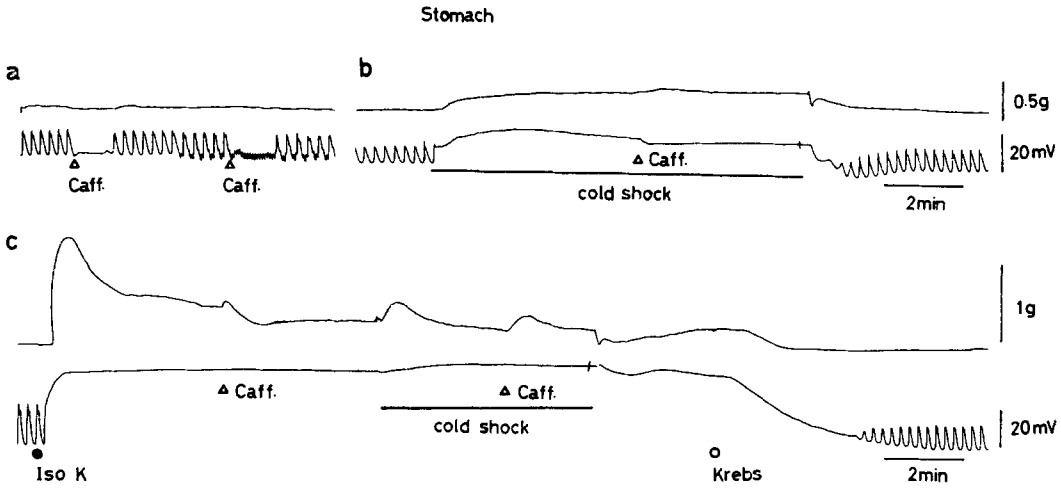

FIGURE 9. Effects of caffeine (5 mM) on electrical and mechanical activities of the circular muscle of stomach in normal and depolarized conditions. (*a*), effect of caffeine in Krebs solution. The latter half of the record is recorded during successive applications of inward current pulses (500 ms). (*b*), effect of caffeine during cold shock. (*c*), effect of caffeine during generation of tonic response of the contracture evoked by isotonic K-Krebs solution and also during cold shock. Symbols are the same as in Fig. 4.

generations of slow potential changes and reduced membrane resistance in Krebs solution at 32°C. During cold shock, caffeine also evoked a mechanical response superimposed on the tonic contracture (Fig. 9 *b*). In isotonic K-Krebs solution at 32°C, treatment with caffeine (5 mM) during the maintenance of the tonic response of the contracture transiently enhanced the amplitude of the mechanical response, then relaxed the tissue, but not to the resting level.

By application of cold shock, the phasic response was evoked. Then it relaxed. Reapplication of caffeine again produced the phasic response, but the amplitude was small. The amplitude of the tonic contracture remained the same before and after application of caffeine (Fig. 9 *c*).

In the presence of caffeine (5 mM), isotonic K-Krebs solution evoked the phasic response of the contracture. The tissue then rapidly relaxed to the resting tension level, and the generation of the tonic response of the contracture was suppressed. During application of isotonic K-Krebs solution with caffeine, cold shock still evoked a tonic contracture. The amplitude of the contracture remained the same even when the isotonic K-Krebs solution was replaced by Krebs solution and caffeine was removed (Fig. 10 *b*). Therefore, the tonic contracture evoked by the above conditions was related neither to the presence of caffeine nor to the isotonic K-Krebs solution.

The above effects of caffeine on the stomach circular muscle and taenia coli

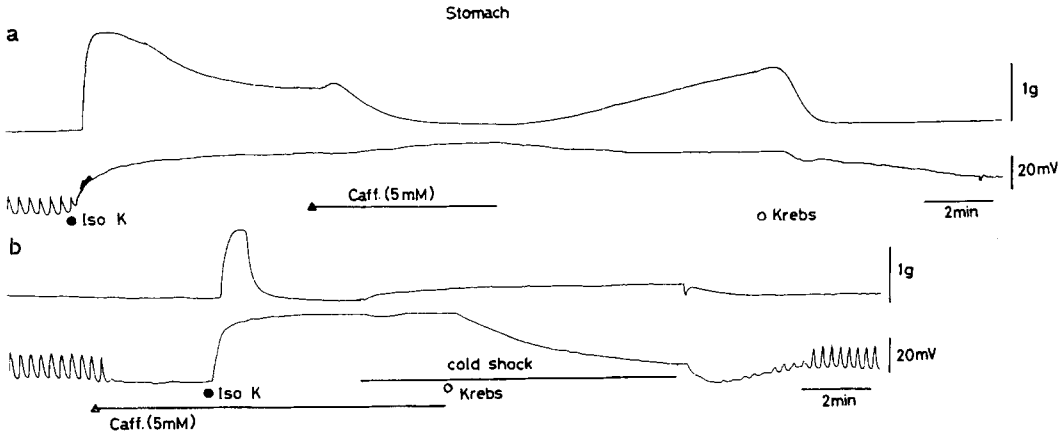

FIGURE 10. Effects of cold shock (10°C) on depolarized smooth muscle of the stomach in relation to the actions of caffeine (5 mM). (*a*), effects of caffeine (5 mM) on depolarized muscle produced by isotonic K-Krebs solution. (*b*), effects of pretreatment with caffeine (5 mM) and cold shock on smooth muscle depolarized by isotonic K-Krebs solution. Symbols are the same as in Fig. 4.

can be summarized as follows: (*a*) Caffeine evoked a mechanical response, reduced membrane resistance at normal and low temperatures. (*b*) Caffeine mobilized the bound Ca and the labilized Ca ion evoked spike generation. (*c*) The amounts of the labilized Ca ion needed to evoke the phasic response in excess K-Krebs solution were modified by caffeine and low temperature. However, caffeine did not modify the amplitude of the tonic contracture evoked at low temperature. (*d*) Caffeine suppressed the tonic response of the contracture evoked by excess K-Krebs solution.

The effects of thymol on normal and depolarized muscle during application of cold shock were also investigated for comparison with the effects of caffeine.

Fig. 11 shows the effects of thymol (1 mM) on the electrical and mechanical activities of the taenia coli in Krebs solution at 32°C. Thymol reduced membrane resistance without any marked change in membrane potential. The

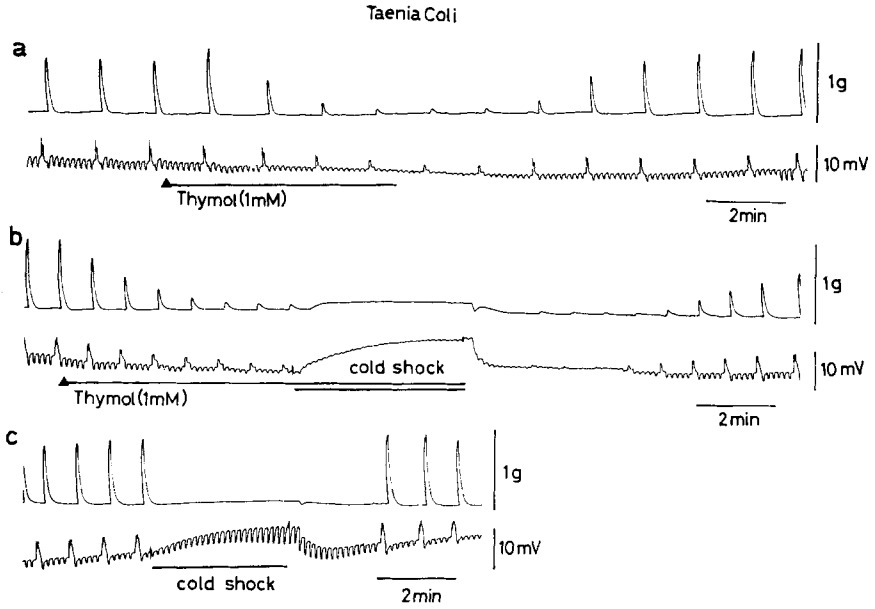

FIGURE 11. Effect of thymol (1 mM) on electrical and mechanical activities of the taenia coli in normal temperature (32°C) and low temperature (10°C). (*a*), effects of thymol (1 mM); (*b*), application of cold shock in the presence of thymol; (*c*), effect of cold shock in Krebs solution. Inward current (3 s in duration and 2×10^{-7} A in intensity) and outward current (3 s in duration and 5×10^{-7} A in intensity) were applied periodically. Symbols are the same as in Fig. 4.

reduced membrane resistance suppressed the generation of the spike evoked by an outward current pulse (*a*). After application of cold shock to the tissue in the presence of thymol (1 mM), it was still possible to evoke depolarization and a tonic contracture (*b*). The membrane resistance was increased less during cold shock than was observed in the Krebs solution (*c*).

Effects of isotonic K-Krebs solution in the presence of thymol (1 mM) were also observed. As shown in Fig. 12, the membrane of the taenia coli was depolarized by excess K-Krebs solution. However, generation of the phasic response of the contracture was suppressed and only a small sustained contracture was developed. When cold shock was applied during application of the excess K-Krebs thymol-containing solution, the tonic contracture was generated with slight depolarization of the membrane. Upon rewarming the tissue the contracture was rapidly relaxed, as observed in normal and in depolarized muscle, and the sustained contracture evoked by excess K-Krebs with thymol was reduced (*b*).

The above effects of thymol in normal and depolarized muscles under cold shock were also observed in stomach circular muscle. Fig. 13 shows the effects of thymol (1 mM) on normal (*a* and *b*) and on depolarized muscle (*d*) during

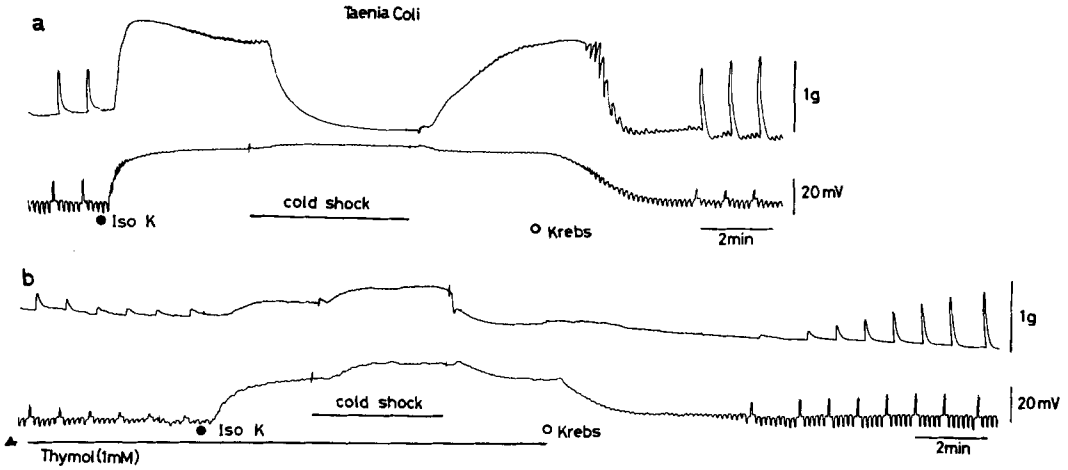

FIGURE 12. Effects of cold shock (10°C) on depolarized smooth muscle of the taenia coli in the presence and absence of thymol (1 mM). (*a*), effect of cold shock in depolarized muscle produced by isotonic K-Krebs solution; (*b*), effect of cold shock in depolarized smooth muscle in the presence of thymol (1 mM). Applied inward and outward current pulses are the same as in Fig. 11. Symbols are the same as in Fig. 4.

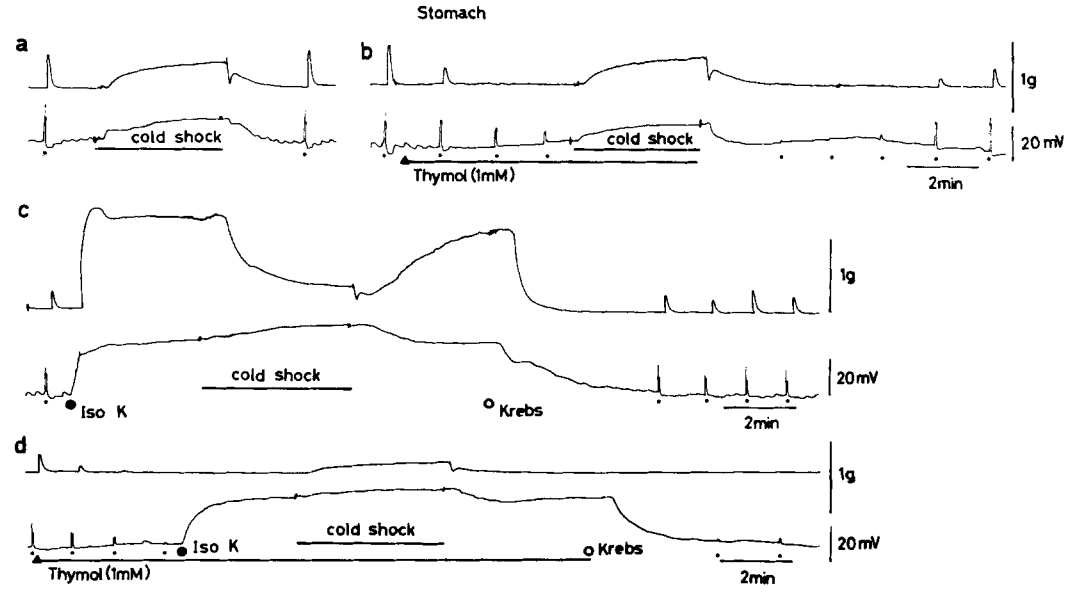

FIGURE 13. Effects of cold shock on normal and depolarized smooth muscle of the stomach in the presence and absence of thymol (1 mM). (*a*), effects of cold shock (10°C); (*b*), effects of cold shock (10°C) in the presence of thymol (1 mM); (*c*), effects of cold shock (10°C) on depolarized smooth muscle produced by isotonic K-Krebs solution; (*d*), effects of cold shock (10°C) on depolarized muscle in the presence of thymol (1 mM). Outward currents (3 s in duration and 5×10^{-7} A in intensity) were applied to the tissue to evoke electrical and mechanical responses. ●, application of isotonic K-Krebs solution; ○, removal of isotonic K-Krebs solution; •, application of outward currents.

application of cold shock (*b* and *d*). Treatment with thymol (1 mM) reduced membrane resistance, yet cold shock produced depolarization of the membrane and tonic contracture. The amplitude of the tonic contracture was not modified by the presence or absence of thymol. Under pretreatment with thymol (1 mM), isotonic K-Krebs solution depolarized the membrane to the same extent expected from the effects of isotonic K-Krebs solution in the absence of thymol. However, the phasic and tonic responses of the contracture were not developed. Application of cold shock produced tonic contracture of the muscle with the further depolarization of the membrane.

The above effects of thymol on the stomach circular muscle and taenia coli could be summarized as follows: (*a*) Thymol reduced membrane resistance without changing membrane potential, but after cold shock the tonic contracture remained the same as that observed in Krebs solution. (*b*) Thymol suppressed both the phasic and the tonic responses of the contracture evoked by excess K-Krebs solution. However, cold shock still evoked the tonic contracture. (*c*) The difference in the effect of caffeine and thymol was that thymol suppressed the phasic response of the contracture.

DISCUSSION

The discussion which follows is based on the assumption that the magnitude and duration of the mechanical response recorded from smooth muscle is a function of free Ca in the myoplasm. Furthermore, it is also assumed that the minimum concentration of Ca ion needed to evoke the mechanical response is around 10^{-6} M, a figure taken from the estimate made in frog myoplasm (2×10^{-6} M; Ebashi and Endo, 1968). It must also be stated here that changes of the properties of the contractile protein due to lowering the temperature to 10°C are not mentioned in the discussion of the effects of cold shock.

Sakai and his coworkers extensively studied the effects of rapid cooling on normal and drug-treated (caffeine and thymol) striated muscle of the frog, and concluded that rapid cooling of the tissue evoked a contracture and lowered the threshold necessary to evoke contracture by treatments with thymol and caffeine. The above actions were postulated to be due to mobilization of bound Ca from the sarcoplasmic reticulum (Sakai, 1962, 1965; Sakai, Fujii, and Takemoto, 1967; Sakai, Fujii, and Schimizu, 1968). On the other hand, Ludin, Lüttgau, and Oetliker (1966) and Lüttgau and Oetliker (1968) confirmed the development of contracture after lowering the temperature and presented the hypothesis that the rise of tension is due to some alteration in the characteristics of the mechanical contraction-activation system-tubular structure (T-tubule). Recent experiments by Sakai, Geffner, and Sandow (1970), however, favor the view that the action of caffeine on rapid cooling

contracture does not depend on intact T-tubules, since osmotic shock did not modify the above effects of caffeine and low temperature.

Hasselbach and Makinose (1961) and Hasselbach (1964) have described that ATPase activity of the fragmented sarcoplasmic reticulum is reduced at low temperature, and at temperatures near 0°C the capacity to sequester Ca ceased. Therefore, the action of the sarcoplasmic reticulum might be markedly suppressed at low temperature.

The postulations made on the effects of rapid cooling applied to the striated muscle cannot be applied strictly to smooth muscle, since the structure of the sarcoplasmic reticulum is less developed in smooth muscle. Hence the role of the sarcoplasmic reticulum is thought to be taken by the muscle membrane or structures distributed just beneath the membrane (Somlyo and Somlyo, 1968; Burnstock, 1970; Rüegg, 1971).

The present experiments elucidated that displacement of the membrane potential, and pretreatment with caffeine and thymol, did not modify the amplitude of the tonic contracture evoked by low temperature (10°C). Furthermore, changes of ionic environments changed the membrane property but did not modify the amplitude of the tonic contracture. Even in Ca-free Krebs solution, rapid cooling evoked tonic contracture with the same amplitude as that observed in Krebs solution in spite of the marked change of membrane properties which were observed (Magaribuchi, Ito, and Kuriyama, 1972).

Depolarization of the membrane and a marked increase of membrane resistance during cooling were thought to be due to the reduction of the ionic permeability of the membrane to all ions, i.e., the membrane potential measured by microelectrode lowered from -59 to -39 mV and the effective resistance increased to 2.6 times the control value. Depolarization of the membrane measured by the double sucrose gap method was 16 mV and membrane resistance increased to 2.2 times the control value. It was also observed that low temperature not only increased membrane resistance but also increased the longitudinal resistance of the tissue to 6.8 times the control value. Presumably intercellular junction resistance increased more than the myoplasmic resistance, because the length constant of the tissue was reduced to half the control value of 1.8 mm (Kurihara, Magaribuchi, and Kuriyama, unpublished observations).

An interesting observation concerning the effects of caffeine on the taenia coli is modification of the spike generation mechanism, i.e. the spike was evoked in the presence of 10^{-7} M Ca ions under pretreatment with caffeine. Presumably the bound Ca in the muscle membrane might be released. This result agreed with an observation made on crustacean muscle by Chiarandini, Reuben, Brandt, and Grundfest (1970). They reported that an increased

permeability to Ca induced by caffeine was evidenced by the transformation of the normally graded electrical response to Ca spikes. The overshoot is a function of both external Ca and caffeine.

Ito and Kuriyama (1971) observed that caffeine had dual actions on the mechanical responses evoked by isotonic K-Krebs solution, i.e., the amplitude of the contracture was enhanced, the cause being acceleration of Ca-releasing mechanism, and generation of the tonic response of the contracture was suppressed. Similar effects of caffeine on striated muscle were observed at normal and low temperatures (Sakai, 1965; Lüttgau and Oetliker, 1968). According to Lüttgau and Oetliker's (1968) explanation for this rapid relaxation of the phasic response by treatment with caffeine, relaxation after contraction is due to the faster reduction of free Ca by acceleration of the Ca-pump postulated by Hasselbach (1964).

If uptake and release of Ca ion to and from the bound sites are different, the dual actions of caffeine on smooth muscle might be more easily explained as follows: caffeine releases the bound Ca from the sequestered site and also increases Ca uptake. However, transformation mechanisms of Ca from inactive to active form might be suppressed, thus readily producing relaxation of the phasic response and suppression of the generation of tonic response and suppression of the generation of tonic response of the contracture. These postulations were partly introduced from the facts observed on striated muscle, that the release of the bound Ca occurred in the terminal cisterna, and the uptake of free Ca in the myoplasm took place in the remaining sarcoplasmic reticulum (Winegrad, 1968).

The effects of low temperature and caffeine on K-induced contracture were phenomenologically the same. Pretreatment with caffeine evoked only the phasic response of the contracture and low temperature evoked the phasic response and tonic contracture. When caffeine or low temperature was applied during generation of the tonic response of the contracture, dual actions were observed, i.e., when the tonic response showed high amplitude, caffeine and low temperature transiently produced the phasic response. Then it relaxed to a certain level below the tonic response. On the other hand, when the tonic response showed low amplitude, both procedures produced the small phasic response and then relaxed to the same or higher level than the tonic response of the contracture. The final muscle tones were, however, consistently higher at low temperature than in the presence of caffeine.

The sources of Ca producing the phasic and tonic responses of the K-induced contracture in the smooth muscles are not yet fully understood (Urakawa and Holland, 1964; Imai and Takeda, 1967). However, from the present experiments the phasic response evoked by excess K solution in the presence of caffeine and at low temperature might be mainly due to release of bound Ca sequestered in the sarcoplasmic reticulum or in part of the

muscle membrane which possesses the properties of the sarcoplasmic reticulum.

It must be mentioned here again that the amplitude of tonic contracture evoked by low temperature remained the same throughout the treatments with caffeine, thymol, excess K, and various ionic environments. Repetitive applications of caffeine and excess K-Krebs solution at low temperature gradually lowered the amplitude of the phasic response of the contracture, but the above treatments. Therefore, the sites where Ca is sequestered in the cell to produce the phasic response of the contracture and the tonic contracture might be different.

Alternatively, generations of both the tonic contracture and the phasic response of the contracture might also be explained by releasing Ca from one sequestered site. At low temperature, presumably, Ca sequestered in the cell, especially in the reticulum, can be mobilized by different modes from that appearing at the physiological temperature. For example, cold shock mobilizes Ca from reticulum by reducing the Ca-dependent ATPase activity for sequestration. This mobilization of Ca would not be influenced by treatments with excess K-Krebs and caffeine, and by displacements of the membrane potential using the current injection method.

If the latter alternative is preferable, the amplitudes of both phasic response and tonic contracture should be correlated with the amounts of Ca sequestered in the reticulum. However, as described in the results, there were discrepancies between both responses.

At 36°C, caffeine did not potentiate but suppressed the mechanical response of the taenia coli evoked by electrical stimulation, although at 10°C it did potentiate the mechanical response (Ito and Kuriyama, 1971). The amplitude of the tonic contracture was, however, not enhanced. On the contrary, in the smooth muscle of the urinary bladder caffeine enhanced the amplitude of the tonic contracture evoked by cold shock (Kurihara et al., unpublished observations). The membrane specificities can be elucidated; in fact, the responses of the muscle membrane of the urinary bladder to various ionic environments were different from those observed in the taenia coli (Creed, 1971).

It is tentatively postulated from the present experiments that the bound Ca sequestered in the smooth muscle which is released by lowering the temperature might be mainly located in the organelles of the cell; these ions might be located either in the mitochondria, nucleus, or other organelles. The possibility that the permeability of the squid axon membrane is controlled by Ca located in the mitochondria was presented by Blaustein and Hodgkin (1969). Therefore, it may be reasonable to postulate two sites of Ca binding in the cell which are involved in the development of tension in smooth muscles of the taenia coli and stomach.

The mammalian smooth muscle cells show marked species and tissue variations in response to the environmental changes. Therefore, these postulations may not be applicable directly to the phenomena observed in the other smooth muscles.

Received for publication 28 January 1972.

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