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Effect of Excitability-Inducing Material on Regenerative Ca^{++} Release of Rat Ventricular Muscle

Doo Hee Kang, Young Ho Lee and Hye Jin Song

The effects of excitability-inducing materials (EIM) and electrical stimulation on Ca^{++}-induced Ca^{++} release were studied in this experiment. Direct application of three EIM (bacterial EIM, alamethicin, NaI) did not release Ca^{++} from skinned rat cardiac fibers or from an SR suspension. Direct electrical stimulation of the SR suspension had no effect on Ca^{++} release either. However, both electrical stimulation and pre-treatment with an EIM augmented Ca^{++}-induced Ca^{++} release in the skinned fiber and led to increased contractility of the fiber in a dose-dependent manner. When electrical stimulation and EIM application were superimposed, their effects were additive, suggesting that information transfer across the junction between T-tubule and SR is at least chemical in nature.

Key Words : Excitability-inducing material (EIM), sarcoplasmic reticulum, skinned muscle fiber, Ca^{++}-induced Ca^{++} release

Present understanding of excitation-contraction coupling in cardiac and skeletal muscle is as follows (Constantin and Podolsky 1967 ; Constantin 1970 ; Ebashi 1976 ; Fozzard 1977 ; Martonosi 1984). Electrical stimulation (action potential) of the surface membrane is transmitted to T-tubules in a regenerative manner. The electrical impulse is finally transmitted to terminal cisterna of the sarcoplasmic reticulum (SR) via some specialized membrane structures (feet) across the membrane gap, leading to depolarization of all SR and subsequent Ca^{++} release (depolarization-induced Ca^{++} release). The detailed mechanism of direct electrical transmission across the T-SR junction is not known but chemical transmission has been postulated to be one mode of transmission (Vergara and Asotra 1987) (see discussion).

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Department of Physiology, Yonsei University College of Medicine, Seoul, Korea
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Address reprint requests to Dr. D H Kang, Department of Physiology, Yonsei University College of Medicine, CPO Box 8044, Seoul, Korea, 120-752

Besides depolarization-induced Ca^{++} release, there are also other mechanism(s) by which Ca^{++} is released from the SR. One of the most important mechanisms is Ca^{++}-induced Ca^{++} release or regenerative Ca^{++} release (Ford and Podolsky 1970; Fabiato and Fabiato 1977).

The story of Ca^{++}-induced Ca^{++} release in skinned cardiac or skeletal muscle is well established (Ford and Podolsky 1970; Kerric and Best 1974; Endo 1977). In short, if Ca^{++} is loaded into the SR of skinned cardiac and skeletal muscle fibers, a small increase in the Ca^{++} concentration of the perfusion medium (or sarcoplasm) induces Ca^{++} release from the SR, resulting in a progressive increase in Ca^{++} concentration in the medium surrounding the myofilament. This increase in Ca^{++} concentration is manifested by an increase in the tension developed by the (skinned) fiber. This Ca^{++}-induced Ca^{++} release was postulated to be one of the possible modes of action of some positive inotropic agents such as β-adrenergic agonists.

Since a weak stimulus, which alone was not effective in releasing Ca^{++} from isolated SR or skinned fiber, was found to augment Ca^{++}-induced Ca^{++} release in this experiment, the Ca^{++}-induced Ca^{++} release mechanism is useful in studying infor-

mation transfer across the T-SR junction (see discussion). Such a weak stimulus includes changing the chemical composition of the medium to induce SR depolarization in the skinned fiber (Ford and Podolsky 1970; Endo 1977).

In 1962 Mueller *et al.* discovered excitability-inducing materials (EIM). These agents rapidly increase the conductance of artificial lipid bilayers by several orders of magnitude when they are added to the aqueous phase. EIM include alamethicin, I^-, an extract of Enterobacter cloacae ATCC 961, and other organic ions (Muller and Rudin 1963; Kushnir 1968; Bukovsky 1977).

The present experiments were carried out to study (1) the effect of electrical stimuation of the skinned fiber on Ca^{++}-induced Ca^{++} release, and (2) the effect of EIM on Ca^{++} release with or without electrical stimulation. In other words, can EIM alone release Ca^{++} from SR without an electrical impulse, and can the effect of EIM and the effect of electrical stimulation on Ca^{++} release be additive?

METHODS

Preparation of chemically skinned fibers

The rat heart was excised and transferred into a cold homogenizing medium (Table 1) immediately after the rat was sacrificed by a blow on the head. The cardiac tissue was trimmed and homogenized in a Virtis homogenizer (dial setting 20 for 40sec). The tissue was cut into strips approximately 1-2mm wide and 1.0 cm long auording to the method used before in this laboratory (Kang and Lee 1977; Cho *et al.* 1986).

Tension measurement of skinned fiber

The tissue strip was mounted on a force transducer (Grass type FT03). One end of the muscle strip was fixed to 3 pieces of platinum wires embedded in a small glass capillary tube (one of which served as an electrode when the muscle was stimulated). The other end of the glass tube was fixed in the long arm of L-shaped muscle lever. The FT03 force transducer, mounted on a micromanipulator, was connected to the short arm of the muscle lever (Fig. 1). The other end of the muscle strip was held

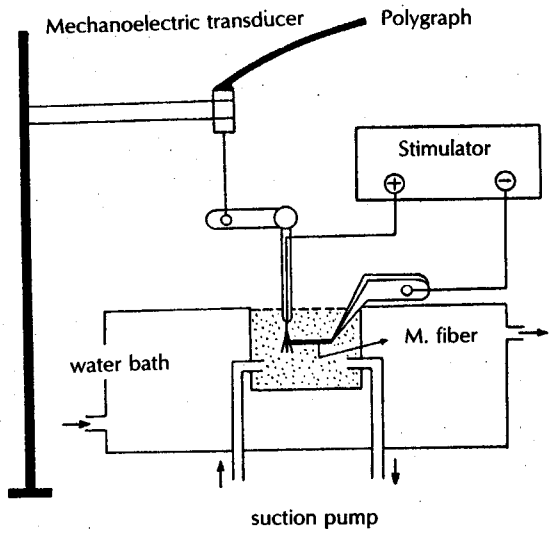

Fig. 1. *Schematic diagram of experimental setup for tension measurement in the skinned fiber. The tissue strip was mounted on a force transducer. One end of the muscle strip was fixed with 3 pieces of platinum wires, while the other end of the muscle strip was held with stainless steel forceps fixed with a micromanipulator.*

Table 1. Composition of solutions (conc. unit : mM)

Composition	Homogenizing	Loading	Washing	Test
K-propionate	70	70	70	70
$MgCl_2$	4	4	4	4
Tris-Maleate	20	20	20	20
Glucose	7	7	7	7
NaCl	3	3	3	3
NaH_2PO_4	3	3	3	3
ATP	5	5	5	5
pCa*	9	3.3	7.5	5

* pCa = negative logarithm of free Ca^{++} concentration

in stainless forceps fixed in the micromanipulator. The forceps served as another stimulating electrode when the muscle was stimulated.

The mounted fiber was eqilibrated for about 5 min in the muscle chamber maintained at 37°C, after which the solution in the muscle chamber was changed to a loading solution (pCa 3.3). Then SR was loaded with Ca^{++} at the same time as muscle tension was increased. After the tension reached the maximum (T_1), the solution in the chamber was changed sequentially to the washing solution (pCa 7.5). When the tension reached the minimum, the test solution (pCa 5) was applied, and the tension generated was recorded. The second maximum of the tension is taken as T_2 (a measure of Ca^{++}-induced Ca^{++} release). The magnitude of the tension of each preparation was expressed as a percentage of T_2 to T_1.

Electrical stimulation

The skinned muscle fiber was sometimes stimulated using a square wave pulse generator. The stimulating voltage ranged from 0 to 4.5 volts with a duration of 40 to 200 msec.

Isolation of the SR and direct electrical stimuation of SR suspension

The SR was isolated from cardiac muscle by the method of Peter and Worsford (1969).

Ca^{++} activity in the medium containing SR (100 mM KCl, 0.5 mM $MgCl_2$, 5mM NaN_3, 0.1 mM $CaCl_2$, 25mM Histidine, pH 7.4) was measured using a calcium-selective electrode in the presence or absence of EIM and electrical stimulation. Calcium-selective electrode was manufactured in this lab as described previously (Suh *et al.* 1987). Electromotive force generated by Ca^{++} activity was recorded between calcium electrode and 3M KCl-3% agar bridge in the bath. The solvent polymeric membranes for the calcium-selective electrode were prepared using 1% ETH 129 carrier, 66% o-nitrophenyl octylether (Fluka AG), 10% polyvinylchloride (Fluka AG) and 0.6% potassium tetrakis (p-chlorophenyl) borate (Fluka AG). Each calcium electrode was tested for response and kept in the test solution for at least one hour before use. The Ca^{++} standard solutions containing pCa 2, 3, 4, 5, 6, 7, and 8 were made from a Ca^{++} buffer containing EGTA.

Electrical stimulation was carried out was electrically stimulated with two platinum plate electrodes (about 0.8 cm in diameter) placed 1 cm apart in the medium. A stimulating voltage, ranging from 100 V/cm to 1 KV/cm, was supplied by a capacitor discharge type electrical stimulator constructed in this institute. Immediately after electrical stimulation, the stimulating electrodes were removed from the vessel, and Ca^{++} activity was measured. EIM, alamethicin, and Sodium iodide (NaI) in varied concentrations were added to the medium to see if they induced Ca^{++} release from SR suspended in the medium (mg protein/ml of medium).

Chemicals and EIM

EIMs used in this experiment were either purchased or prepared in this laboratory. EIM from Enterobacter cloacae ATCC 961 was prepared by the method employed by Bukovsky (1977) and stored at -30°C until use. The protein concentration of the final bacterial extract was measured by Lowry's method. Alamethicin was purchased from Sigma Chemicals. NaI was purchased from Osaka Yakuri Chemicals. For the sake of clarity, the extract of Euterobacter cloacae ATCC 961 is simply called the bacterial EIM, and the other EIMs will be called by their chemical or commercial names.

Statistical analysis

All values are expressed as the mean ±SD. Statistical differences between control group and experimental group were evaluated with Student t test. Two-tailed p values less than 0.05 were considered significant.

RESULTS

Ca^{++}-induced Ca^{++} release

A typical record of Ca^{++}-induced Ca^{++} release is shown in Fig. 2. As shown in this record, the test solution at pCa 5 alone could not induce Ca^{++} release if SR was not pretreated with the loading solution at pCa 3.3. However, if SR was first treated with the solution at pCa 3.3, muscle tension began to develop to the maximum. During this period of pretreatment, muscle SR is presumably loaded with Ca^{++}. Following each of two consecutive washes with the washing solution at pCa 7.5, application of the test solution (pCa 5) induced Ca^{++} release and muscle tension redeveloped. It should be noted that the repeated application of the solution still induced muscle contraction, but that the developed maximum tension gradually decreased. It may be asked why the test solution alone (pCa 5) was ineffective in inducing the contraction of the skinned fiber. This may be due to a contamination of some

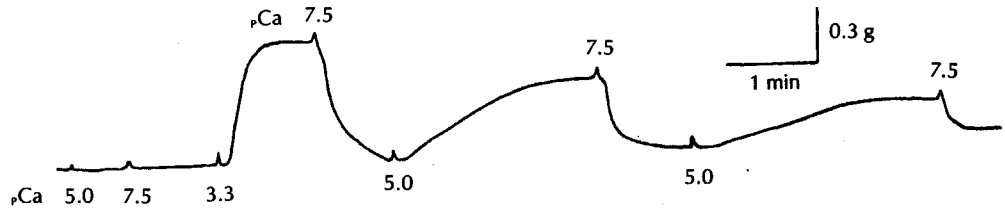

Fig. 2. *Typical record of the Ca^{++}-induced Ca^{++} release. The test solution (pCa 5) alone did not induce the Ca^{++} release. When SR was pretreated with the loading solution (pCa 3.3), the muscle tension developed to the maximum. After washing the pretreated fiber with the washing solution, an application of the test solution induced weaker tension. And repetitive application of the test solution still induced muscle contraction, while the maximum tensions developed gradually decrease.*

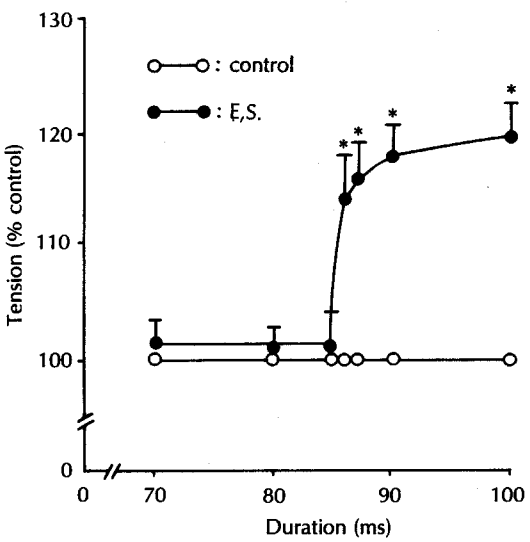

Fig. 3. *Effect of varying duration of electrical stimulus on Ca^{++}-induced Ca^{++} release. The ordinate shows tension relative to the control and the abscissa shows electrical stimulus duration. Each point represents the mean ± S.D. of 7-9 determination. Asterisks represent significant difference from control value, $p<0.05$.*
When an electrical stimulation (E.S.) was applied to the skinned fiber at the moment of application of the test solution, the development of muscle tension was increased compared to the tension without the electrical stimulation.
The strength of electrical stimulus was 4.5 volts.

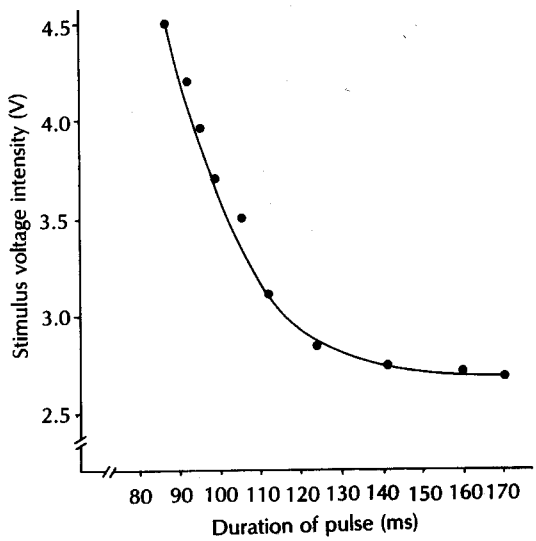

Fig. 4. *Strength-duration curve of electrical stimulation which augments Ca^{++}-induced Ca^{++} release.*
This curve was constructed by changing duration and strength of electrical stimulation. The curve is taken as the threshold above which the Ca^{++}-induced Ca^{++} release is augmented.

EGTA trapped in ECF or ICF from the washing solution.

Effect of electrical stimulation on Ca^{++} release

The effect of electrical stimulation on Ca^{++}-induced Ca^{++} release is shown in Fig. 3. When an electrical stimulation was applied to the skinned fiber at the same time as the test solution, the developed muscle tension was increased when compared to the tension without the electrical stimulation. As can be seen in Fig. 3, the stimulation intensity with 4.5 volts for a duration longer than 85 msec significantly augmented the tension developed by Ca^{++}-induced Ca^{++} release ($p<0.05$). Similar findings were obtained when the strength

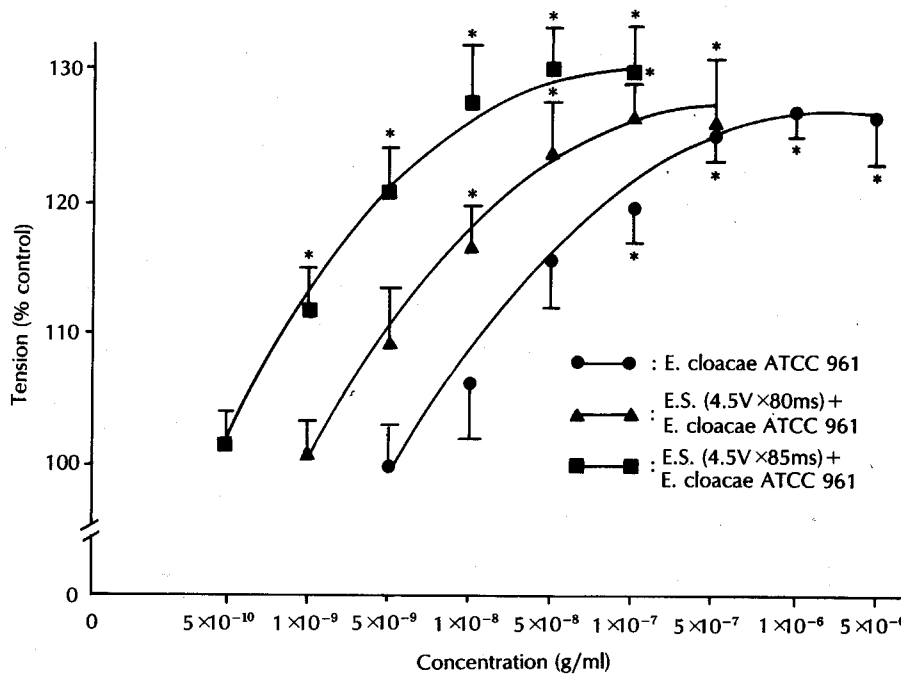

Fig. 5. *Effect of bacterial EIM on Ca^{++}-induced Ca^{++} release with or without subthreshold electrical stimulations. The ordinate shows tension relative to the control. Each point represents the mean ± S.D. of 7-9 determination. Asterisks represent significant difference from control value, $p<0.05$.*

When bacterial EIM was applied to the skinned fiber, Ca^{++}-induced Ca^{++} releases was increased in a dose-dependent manner. This dose response curve shifted to the left in the presence of electrical stimulation (E.S.), depending on duration of the stimulation.

and duration of the stimulation were varied independently, and the strength-duration so obtained is shown in Fig. 4.

Effect of bacterial EIM on Ca^{++}-induced Ca^{++} release

The effect of bacterial EIM on Ca^{++}-induced Ca^{++} release, with or without electrical stimulation, is shown in Fig. 5. When bacterial EIM was applied to the skinned fiber, Ca^{++}-induced Ca^{++} release was increased in a dose-dependent manner. When an electrial stimulation with subthreshold intensity was superimposed on an application of bacterial EIM, the response curve to the bacterial EIM was shifted to the left. The degree of the shift was also dependent on the duration of stimulation (Fig. 5). Increasing the duration of electrical stimulation from 80 msec to 85 msec caused a further shift to the left of the response curve so that a lower concentration of the bacterial EIM was required to obtain the same tension.

Effect of alamethicin on Ca^{++}-induced Ca^{++}release

The effect of alamethicin on Ca^{++}-induced Ca^{++} release, with or without electrical stimulation, is shown in Fig. 6. When alamethicin was applied to the skinned fiber, Ca^{++}-induced Ca^{++} release was increased, and the response was dose-dependent in a manner similar to that observed with the bacterial EIM. When electrical stimulation of a subthreshold intensity was superimposed on the alamethicin application, The response curve to alamethicin application again shifted to the left.

Effect of sodium iodide on Ca^{++}-induced Ca^{++} release

The effect of NaI on Ca^{++}-induced Ca^{++} release, with or without electrical stimulation, is shown in Fig. 7. The effects of NaI were similar to those of bacterial EIM and alame-thicin.

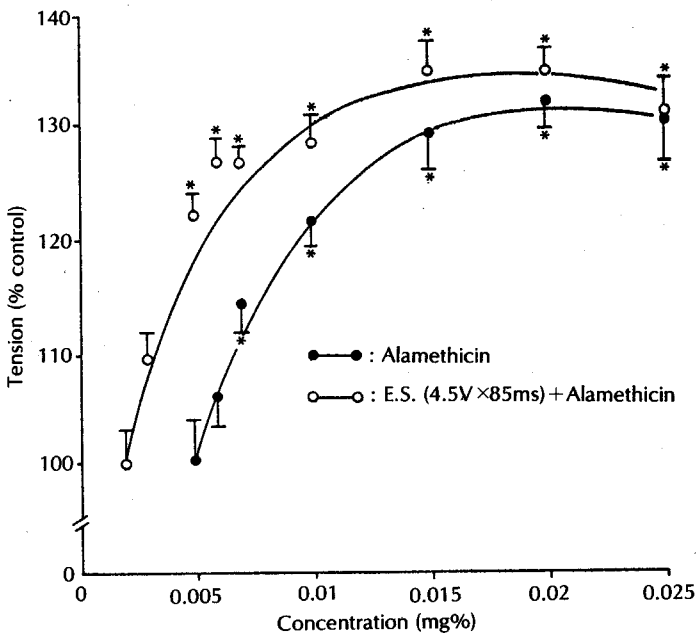

Fig. 6. *Dose response curve of Ca^{++}-induced Ca^{++} release to alamethicin with or without subthreshold electrical stimulations. The ordinate shows tension relative to the control. Each point represents the mean ± S.D. of 7-9 determination. Asterisks represent significant difference from control value, $p<0.05$.*
When electrical stimulation (E.S.) with subthreshold intensity was superimposed on alamethicin application, the response curve to alamethicin shifted to the left.

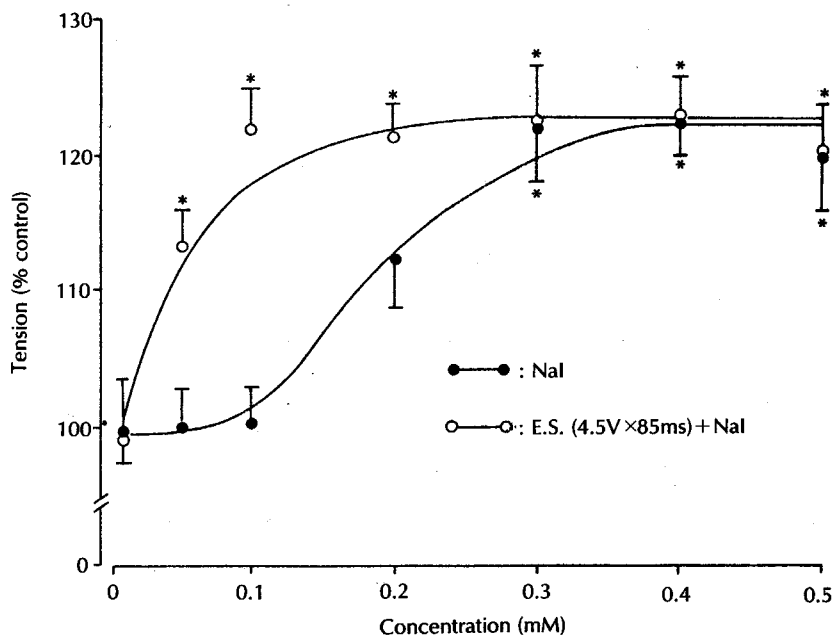

Fig. 7. *Dose reoponse curve of Ca^{++}-induced Ca^{++} release to iodide with or without subthreshold electrical stimulation. The ordinate shows tension relative to the control. Each point represents the mean ± S.D. of 7-9 determination. Asterisks represent significant difference from control value, $p<0.05$.*
When electrical stimulation (E.S.) with subthreshold intensity was superimposed on the iodide application, Ca^{++}-induced Ca^{++} release was increased.

Effect of direct electrical stimulation and EIM on a change of Ca^{++} activity in SR suspension

There was no change in Ca^{++} activity in the SR suspension even with a stimulation intensity of 1 kV/cm. Direct application of bacterial EIM, alame-thicin, or NaI did not release Ca^{++} from SR suspended in the medium even at the highest concentration which caused the maximum effects in the experiment on Ca^{++}-induced Ca^{++} release.

DISCUSSION

It is widely accepted that in skeletal muscle, and presumably also in cardiac muscle, depolarization of the T-tubule following depolarization of the plasma membrane induces Ca^{++} release from the terminal cisternae of sarcoplasmic reticulum.

The mechanism of information transfer across the T-SR junction (T-tubule and sarcoplasmic reticulum) is not well known, but two different mechanisms are thought to be responsible: electrical transmission through specialized structured (feet) and chemical transmission involving Inositol 1, 4, 5-trisphosphate (IP_3) at this junction.

However, no direct proof of electrical transmission has been reported; only indirect evidence is available (Ford and Podolsky 1970; Kerric and Best 1974; Donaldson 1985). One interesting view is that a conformational change of voltage sensors in the T-tubular membrane at the T-SR junction, which in turn leads to Ca^{++} channels in the SR membrane at the T-SR junction, which in turn leads to Ca^{++}-induced Ca^{++} release from other SR Ca^{++} channels (Schneider and Chandler 1973; Rio and Pizzaro 1988). As for the chemical transmission, several laboratories found that IP_3 induces Ca^{++} release from SR (Hirata *et al.* 1984; Vergara *et al.* 1985; Volpe *et al.* 1985), but other laboratories have found negative results (Adunyah and Dean 1985; Movsesian *et al.* 1985; Scharer and Ferguson 1985; Lea *et al.* 1986). So, there is presently no unanimous agreement on what mechanism(s) is responsible for this information transfer. This may arise from difficulties in separating two presumably interrelated phenomena. If the depolarization of T-tubules trigggers a liberation of chemical messengers such as IP_3, it would be difficult to separate the two phenomena.

Microinjection of IP_3 into intact muscle cells induces Ca^{++} release, eliciting contracture; but, in skinned fibers or isolated SR, the effect of IP_3 is not reproducible. Several investigators speculate that loss of important modulatory co-factors during the skinning or SR isolation procedures in responsible for the loss of the IP_3 effect (Lea *et al.* 1986).

On the other hand, it would be worth employing Ca^{++}-induced Ca^{++} release as an index of T-SR information transfer. This could determine if electrical stimulation or application of EIM or IP_3 with the test solution (see methods) had any effect on the Ca^{++}-induced Ca^{++} release mechanism, even if each alone has no effect on Ca^{++} release from isolated SR or skinned fibers. In other words, can either an electrical or chemical messenger alone augment Ca^{++}-induced Ca^{++} release, even in the skinned fiber, thereby indicating that Ca^{++}-induced Ca^{++} release is a more sensitive way to study the information transfer across the T-SR junction? Furthermore, if their effects were additive, it would be tempting to consider whether that electrical transmission across T-SR junction is mediated by chemical messenger.

In the present experiments on skinned cardiac fiber, both application of EIMs and electrical stimulation were found to augment Ca^{++}-induced Ca^{++} release. Their effects were additive if the two stimuli were combined. Therefore, information transfer across the T-SR junction is likely to be chemical in nature.

However, the possibility still exists that the effects of EIM and electrical stimulation are independent phenomena each having its own effect. The additive effect is merely a summation of different phenomenon and should not be given any special meaning.

As mentioned in the introduction, EIM which induce and increase the conductance of artificial lipid layer when they are added to the aqueous phase, includes many agents. However, in this experiment, alamethicin, I^-, and bacterial extract of enterobacter cloacae ATCC 961 were used. The chemical natures of all EIM are not well known, but some bacterial extracts are reported to contain protein and a small amount of nucleic acid (nucleoprotein).

Finally, the above electrical stimulation could be repeated with IP_3, particulary with or without a phospholipase C inhibitor such as neomycin, to see if the additive effect is still demonstrable. This experiment is currently in progress. Preliminary results show that IP_3 also augments Ca^{++}-induced Ca^{++} release from skinned fiber.

In conclusion, in the rat skinned cardiac fiber electrical stimulation or pre-treatment with some

EIM increases the contractility or the fiber (or increases Ca^{++}-induced Ca^{++} release in the fiber) in a dose dependent manner. However, if the electrical stimulation and EIM application are superimposed, their effect are additive, suggesting that the information transfer across the T-SR junction is at least chemical in nature.

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