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STUDIES ON THE MECHANISM OF ACTION OF A NEW Ca^{2+} ANTAGONIST, 8-(*N*,*N*-DIETHYLAMINO)OCTYL 3,4,5-TRIMETHOXYBENZOATE HYDROCHLORIDE IN SMOOTH AND SKELETAL MUSCLES

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1 The rabbit aortic strip, guinea-pig ileum and rabbit skeletal muscle sarcoplasmic reticulum preparations were used to determine at which sites and in what manner 8-(*N*,*N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate (TMB-8) interferes with Ca^{2+} availability in smooth and skeletal muscles.

2 TMB-8 (50 μM) significantly inhibited equivalent responses of the rabbit aortic strip to KCl and noradrenaline.

3 TMB-8 (65 μM) produced no significant alteration in the extracellular space of the guinea-pig ileum as measured with [^{3}H]-sorbitol.

4 The resting cellular Ca^{2+} influx as well as the resting $^{45}Ca^{2+}$ efflux in the guinea-pig ileum preparation were significantly inhibited by TMB-8 (65 μM).

5 TMB-8 (5 μM and 50 μM) had no significant effect on the uptake of $^{45}Ca^{2+}$ by the sarcoplasmic reticulum preparation of skeletal muscle; however, TMB-8 (5 μM) did significantly inhibit the caffeine (20 mM)-induced release of $^{45}Ca^{2+}$ from this preparation.

6 It is concluded that TMB-8 reduces Ca^{2+} availability in smooth and skeletal muscles by stabilizing Ca^{2+} binding to cellular Ca^{2+} stores and thereby inhibits the release of this Ca^{2+} by contractile stimuli.

Introduction

Recent studies of 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) have shown this agent to be a potent inhibitor of the contractility of both smooth (Malagodi & Chiou, 1974a) and skeletal (Malagodi & Chiou, 1974b) muscles. While TMB-8 has been shown to inhibit responses to all contractile agents tested in smooth and skeletal muscle (acetylcholine, 1,1-dimethyl-4-phenylpiperazinium, nicotine, noradrenaline, K^+, Ba^{2+} and caffeine) as well as responses to indirect and direct electrical excitation, the only instances of competitive antagonism of contractile responses were in studies of Ba^{2+}-induced contractions in smooth muscle and caffeine-induced contractions in skeletal muscle. Because both Ba^{2+} and caffeine are Ca^{2+}-releasing agents (Caldwell & Walster, 1963; Karaki, Ikeda & Urakawa, 1967; Weber & Herz, 1968; Thorpe & Seeman, 1971; Saito, Sakai & Urakawa, 1972; Antonio, Rocha e Silva & Yashuda, 1973; Thorpe, 1973), the competitive antagonism of responses to these agents suggests a specific interference of Ca^{2+}-release by TMB-8. Further, the antagonism of TMB-8 inhibition of responses of smooth and skeletal muscles to K^+ by increased extracellular Ca^{2+} concentrations provides additional evidence that TMB-8 exerts its inhibitory action by interfering with the availability of Ca^{2+} in the contractile sequence.

In smooth muscle, the Ca^{2+} involved in the contractile process appears to come both from intracellular and extracellular sources (Nayler, 1966; Ebashi, Endo & Ohtsuki, 1969; Somlyo, 1972; Holman, 1973), while Ca^{2+} supplied to the contractile proteins of skeletal muscle appears to come chiefly from the intracellularly-located sarcoplasmic reticulum (Nayler, 1966; Ebashi & Endo, 1968). The present study has been conducted in order to determine at which of these sites and in what manner TMB-8 interferes with the availability of Ca^{2+} in smooth and skeletal muscles.

Methods

Materials

8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) was synthesized as described previously (Malagodi & Chiou, 1974a). Other chemical agents used in this study include noradrenaline bitartrate (Winthrop Labs), caffeine sodium benzoate (Parke, Davis & Co.), [^{45}Ca]Cl_2 (specific activity, 955 mCi/mmol) (New England Nuclear), D-sorbitol-[1-^{3}H] ([^{3}H]-sorbitol, specific activity, 6.8 Ci/mmol) (New England Nuclear), NCS Solubilizer (Amersham/Searle Corp.), Aquasol scintillation solution (New England Nuclear), naphthalene (Eastman Organic Chemicals), 1,4-dioxane (W.H. Curtin & Co.), 2,5-diphenyloxazole (PPO) (New England Nuclear) and adenosine 5′-triphosphate, disodium salt (ATP) (Sigma Chemical Co.).

Rabbit aortic strip preparation

New Zealand white rabbits, 1.5-2.0 kg, were stunned by a blow on the head and decapitated. The thoracic aorta was removed and cut into circumferential strips 2-3 mm in width according to the method of Haeusler (1972). Each strip was mounted with 1 g of initial tension on a superfusion apparatus. Tyrode solution of the following composition (mM): NaCl, 137; KCl, 2.7; $MgSO_4$, 1.0; NaH_2PO_4, 0.36; $NaHCO_3$, 11.9; $CaCl_2$, 1.35 and dextrose, 11.1 was used. It was maintained at 37° C, oxygenated with 95% O_2 and 5% CO_2 and was pumped over the preparation by a Polystaltic pump (Buchler Instruments) at a constant flow rate of 6-7 ml/minute. The tension developed by the muscle during contraction was recorded on a Physiograph (Four-A, Narco Bio-Systems, Inc.). TMB-8 (50 μM) inhibition of muscle response to KCl (90 mg) and noradrenaline (0.1 μg), which were injected into the stream of superfusate solution, was examined. These particular doses of KCl and noradrenaline were selected because they produced equivalent responses which were approximately 50% of the maximum responses of the tissue to each agent. Responses to KCl and noradrenaline were examined on the same muscle before and during superfusion with TMB-8. The muscles were superfused with TMB-8 for 20 min before the repetition of KCl and noradrenaline responses. Only the phasic portion of noradrenaline responses was measured in this study.

Guinea-pig ileum preparation

Measurement of extracellular space. Guinea-pigs of either sex weighing 200-400 g, were used. The ileum was removed, cleaned with warm Tyrode solution, opened along its longitudinal axis and cut into 40 pieces, each approximately 0.5 cm in length. Twenty pieces each were used to measure the uptake of [^{3}H]-sorbitol in the absence and in the presence of TMB-8 and were placed in two 50 ml beakers containing 25 ml of Tyrode solution. Both Tyrode solutions were maintained at 37° C and oxygenated with 95% O_2 and 5% CO_2 throughout the experiment. After an initial 10 min equilibration period, TMB-8 was added to the experimental beaker to give a final concentration of 65 μM. This concentration of TMB-8 was just sufficient to produce 100% inhibition of responses to all contractile agents in this preparation. The incubation of both the experimental and control beakers was continued for an additional 20 minutes. At the end of this 20 min period, the solutions in both beakers were carefully and simultaneously decanted. Tyrode solution (25 ml) containing [^{3}H]-sorbitol (0.12 μCi/ml, final concentration) was then added to the control beaker and Tyrode solution (25 ml) containing the same amount of [^{3}H]-sorbitol plus TMB-8 (65 μM) was added to the experimental beaker. The time of addition of the [^{3}H]-sorbitol-Tyrode solutions was designated as zero time. After 1 min, two pieces each of ileum from the control solution and from the experimental solution were removed, blotted once on filter paper and weighed. The samples were then placed in scintillation vials containing 0.5 ml of NCS Solubilizer and agitated in a water bath at 50° C overnight in order to solubilize the tissues. This procedure was also carried out after incubation times of 5, 10, 20, 30, 45 and 120 minutes. After solubilization, the vials were removed from the water bath, cooled and 0.015 ml of glacial acetic acid was added to each vial to neutralize the solubilized tissue. Ten ml of scintillation solution (naphthalene, 100 g, and PPO, 7.0 g/litre 1,4-dioxane) was then added and the samples were placed in the dark for one week to eliminate chemiluminescence of the samples. The samples were then counted in a Beckman Scintillation System (Model 1650). Also counted with the tissue samples were four vials containing 10 ml of the scintillation solution plus 0.1 ml of the control [^{3}H]-sorbitol-Tyrode solution sampled at zero time (two vials) or 0.1 ml of the experimental [^{3}H]-sorbitol-Tyrode solution plus TMB-8 sampled also at zero time (two vials). To correct for quenching produced by the solubilized tissue, two additional 0.1 ml samples, each from the control and experimental [^{3}H]-sorbitol-Tyrode solutions, sampled at zero time, were added to scintillation solution containing tissue that had been solubilized previously. The results were

expressed in terms of [^{3}H]-sorbitol spaces and were calculated according to the following equation:

$$\frac{\text{ct min}^{-1}\ \text{kg}^{-1}\ \text{(wet wt) of tissue}}{\text{ct min}^{-1}\ \text{ml}^{-1}\ \text{of solution}} = \frac{\text{ml of solution}}{\text{kg (wet wt) of tissue}}$$

Measurement of Ca^{2+} movements. The experimental protocol for $^{45}Ca^{2+}$ influx was identical to that described for the study of the sorbitol space of the guinea-pig ileum, except that the radioactive isotope added after the 20-min control or drug incubation period was [^{45}Ca]Cl_2 (0.05 μCi/ml, final concentration). Tissues were incubated for periods of 5, 10, 20, 30, 45 and 120 minutes. The results for all time points were initially expressed in terms of total $^{45}Ca^{2+}$ space according to the above equation and were then converted to cellular Ca^{2+} influx (which included superficially bound Ca^{2+} and intracellular Ca^{2+}) by the following equation:

$$(\text{total } ^{45}Ca^{2+} \text{ space (ml/kg)} - [^3\text{H}]\text{-sorbitol space (ml/kg)}) \times \frac{1.35 \text{ mmol } Ca^{2+}}{1000 \text{ ml}} = \frac{\text{mmol } Ca^{2+}}{\text{kg (wet wt) tissue}}$$

For the study of Ca^{2+} efflux, the guinea-pig ileum was opened along its longitudinal axis and two pieces, each approximately 1 cm in length, were cut. Each piece was tied at both ends and mounted with approximately 1 g of tension between two hooks on a support. The two supports were then placed in two test tubes containing 10 ml of Tyrode solution which was maintained at 37° C throughout the experiment. The Tyrode solutions were oxygenated with 95% O_2 and 5% CO_2 throughout the experiment. After an initial equilibration period of 30 min, the tissues on the supports were transferred to two test tubes containing 10 ml of $^{45}Ca^{2+}$-Tyrode solution (2.0 μCi/ml) and a 2 h loading period was begun. The end of the 2 h loading period was designated as zero time and the two tissues on the supports were then simultaneously moved to two 15 ml centrifuge tubes. One tube (control) contained 5 ml of Tyrode solution and the other tube (experimental) contained 5 ml of Tyrode solution plus TMB-8 (65 μM). After 5 min, the mounted tissues were again moved to two other efflux tubes containing fresh 5 ml solutions of the identical respective compositions. Subsequent transfers of the mounted tissues occurred at 10, 20, 30, 45, 60, 90 and 120 minutes. At 120 min, the two pieces of ileum were removed from the mounts, blotted once and weighed. They were then placed in scintillation vials and prepared for scintillation counting as described in the preceding section. After thorough mixing of the contents of each efflux tube, a 0.5 ml sample was removed and added to a scintillation vial containing 10 ml of scintillation solution. Similarly prepared for counting were 0.02 ml samples (in duplicate) taken from the two $^{45}Ca^{2+}$-Tyrode loading solutions. Identical samples were added to scintillation solution plus previously solubilized tissues to correct for tissue quenching. The samples were counted in a Beckman Scintillation System. From the $^{45}Ca^{2+}$ content of the tissue at 120 min and from the total $^{45}Ca^{2+}$ released during the selected intervals over the 2 h efflux period, the $^{45}Ca^{2+}$ remaining in the tissue during the efflux period was calculated. The results were expressed as the percent of $^{45}Ca^{2+}$ remaining in the tissue at the selected times, taking the tissue $^{45}Ca^{2+}$ content at zero time as 100% and plotted as desaturation curves.

Skeletal muscle sarcoplasmic reticulum preparation

New Zealand white rabbits, weighing 1.0-1.5 kg, were stunned by a blow on the head and decapitated. The two gastrocnemius muscles were isolated and removed. The sarcoplasmic reticulum of the rabbit gastrocnemius muscle was prepared according to the procedure of Ogawa, Harigaya & Ebashi (1971). From the final sarcoplasmic reticulum suspension 0.075 ml samples were taken to study $^{45}Ca^{2+}$ uptake and release. Pilot studies indicated that a 0.075 ml sample of this preparation would take up approximately 50% of the $^{45}Ca^{2+}$ contained in the incubation medium. Fresh reticulum fractions not older than 3 h were used for each experiment.

Ca^{2+} uptake into sarcoplasmic reticulum. The experimental protocol for Ca^{2+} uptake studies consisted of a 5 min preincubation period at 25° C of 0.075 ml of the final reticulum suspension with either 0.1 ml of distilled water (control) or 0.1 ml of distilled water solutions of TMB-8 (5 μM or 50 μM, final concentrations) in a 3 ml reaction mixture containing tris-maleate, 20 mM; KCl 100 mM; $MgCl_2$, 10 mM; $CaCl_2$, 0.01 mM and [^{45}Ca]Cl_2, 0.01 μCi/ml. The pH of the solution was 6.8. After this preincubation period, 0.1 ml of distilled water (control) or ATP (2.0 mM, final concentration) was added to initiate active Ca^{2+} uptake into the sarcoplasmic reticulum. After 2 min of uptake, 0.1 ml of distilled water was added. This step was included to provide appropriate controls for Ca^{2+} release experiments described below. One min after this addition, a

1.0 ml aliquot of the reaction mixture was rapidly passed through a millipore filter apparatus (0.22 μm pore size) which separated the reticulum and its associated Ca^{2+} from the free Ca^{2+} in solution. A 0.3 ml volume of the reticulum-free filtrate was placed in a counting vial containing 5 ml of Aquasol and counted in a Beckman Scintillation System. All control and experimental samples were run in duplicate. Initial observations confirmed the findings of other investigators (Scarpa, Baldassare & Inesi, 1972) that steady-state levels of Ca^{2+} uptake by skeletal muscle sarcoplasmic reticulum preparations are reached within 1 min at 25° C. A 3 min loading period was selected for Ca^{2+} uptake studies for the sake of experimental convenience.

Ca^{2+} release from sarcoplasmic reticulum. The experimental protocol of studies of Ca^{2+} release by caffeine consisted of a 5 min preincubation period identical to that described in the preceding section. After the preincubation period, ATP (2.0 mM, final concentration) was added to initiate active Ca^{2+} uptake into the sarcoplasmic reticulum. After 2 min, caffeine (20 mM, final concentration) was added. One min after this addition, a 1.0 ml aliquot of the reaction mixture was filtered as described in the preceding section and the subsequent steps also described in the preceding section were followed.

The amount of $^{45}Ca^{2+}$ taken up into the reticulum under all experimental conditions was calculated by subtracting the amount of free $^{45}Ca^{2+}$ from the total amount of free $^{45}Ca^{2+}$ in control experiments where distilled water was added in place of ATP. The results were converted into nmol of Ca^{2+}/g (wet wt) of muscle by appropriate conversions based on ct min^{-1} mol^{-1} Ca^{2+} in the unfiltered incubation medium.

Statistical analysis

All results were expressed as the mean ± s.e. mean. The significance of differences between two values was tested with Student's *t*-test and among three or more values with analysis of variance (Goldstein, 1964).

Results

Effect of TMB-8 on drug responses in the rabbit aortic strip

Table 1 shows the effects of TMB-8 (50 μM) on equivalent responses of the rabbit aortic strip to KCl and noradrenaline. It is important to note that responses to both KCl and noradrenaline were significantly inhibited by TMB-8 under the conditions of these experiments.

Table 1 The effects of TMB-8 on responses of rabbit aortic strips to equipotent doses of KCl and noradrenaline

	Response (g tension)		
Drug	*Control*	*TMB-8 (50 μM)*	*% Inhibition*
KCl, 90 mg	0.86 ± 0.09	0.18 ± 0.03*	78.5 ± 4.1
Noradrenaline, 0.1 μg	0.80 ± 0.10	0.43 ± 0.05*	46.4 ± 5.2

Values are mean with s.e. mean of 5 experiments.
* Significantly different from control responses at $P < 0.01$ as determined by Student's *t* test (Goldstein, 1964).

Table 2 The effects of TMB-8 on the [^{3}H]-sorbitol space of the guinea-pig ileum

Incubation time (min)	*[^{3}H]-sorbitol space (ml/kg tissue)*	
	Control	*TMB-8 (65 μM)*
5	110.8 ± 8.2	127.0 ± 24.9
10	159.6 ± 24.2	146.7 ± 7.9
20	168.2 ± 10.6	187.7 ± 24.3
30	192.3 ± 17.5	197.9 ± 17.1
45	203.7 ± 18.1	220.2 ± 13.2
120	319.6 ± 19.5	344.7 ± 21.3

Values are mean with s.e. mean of 5 experiments.

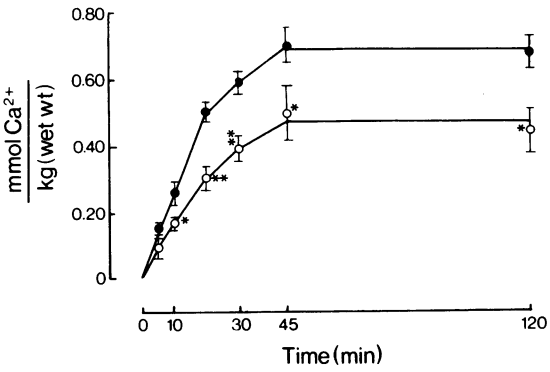

Figure 1 Effects of TMB-8 on resting cellular Ca^{2+} influx into the guinea-pig ileum preparation. (•) Control influx; (○) influx in the presence of TMB-8 65 μM. Each point is the mean of 5 values and vertical bars indicate s.e. mean.
* $P < 0.05$ compared with control value; ** $P < 0.01$.

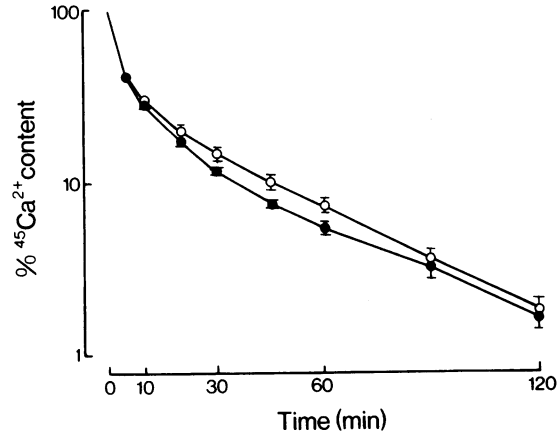

Figure 2 Effects of TMB-8 on the resting $^{45}Ca^{2+}$ efflux in the guinea-pig ileum preparation. (•) Control efflux of $^{45}Ca^{2+}$; (○) efflux of $^{45}Ca^{2+}$ in the presence of TMB-8 65 μM. Each point is the mean of 5 values and the vertical bars indicate s.e. mean.

Effect of TMB-8 on the extracellular space of the guinea-pig ileum

TMB-8 at a concentration of 65 μM had no effect on the extracellular space of the guinea-pig ileum as measured with [^{3}H]-sorbitol during the 2 h incubation period (Table 2).

Effect of TMB-8 on the resting cellular Ca^{2+} influx and efflux in the guinea-pig ileum

Figure 1 illustrates the effect of TMB-8 on the resting cellular Ca^{2+} influx in the guinea-pig ileum. TMB-8 (65 μM) significantly inhibited the influx of Ca^{2+} at 10, 20, 30, 45 and 120 minutes.

Figure 2 illustrates the effect of TMB-8 on the resting $^{45}Ca^{2+}$ efflux from the guinea-pig ileum. TMB-8 (65 μM) produced a small but significant reduction ($P < 0.05$) of the percentage of $^{45}Ca^{2+}$ lost from this tissue after 30, 45 and 60 min washing.

Effect of TMB-8 on $^{45}Ca^{2+}$ uptake and release in skeletal muscle sarcoplasmic reticulum

Table 3 shows that TMB-8 at concentrations of 5 μM and 50 μM had no effect on active $^{45}Ca^{2+}$ uptake into the sarcoplasmic reticulum of rabbit skeletal muscle. Table 4 indicates that TMB-8 at a concentration of 5 μM significantly decreased the quantity of $^{45}Ca^{2+}$ released from the sarcoplasmic reticulum of rabbit skeletal muscle by 20 mM caffeine.

Table 3 The effects of TMB-8 on Ca^{2+} uptake into the sarcoplasmic reticulum of rabbit gastrocnemius muscle

Treatment	*Ca^{2+} uptake (nmol/g muscle)*
None	16.38 ± 1.62
TMB-8 (5 μM)	15.82 ± 0.76
TMB-8 (50 μM)	16.30 ± 0.98

Values are mean with s.e. mean of 5 experiments.

Table 4 The effects of TMB-8 on caffeine-induced release of Ca^{2+} from the sarcoplasmic reticulum of rabbit gastrocnemius muscle

Treatment	*Ca^{2+} content (nmol/g muscle)*
None	16.38 ± 1.62[a]*
Caffeine (20 mM)	11.34 ± 0.49[b]
Caffeine (20 mM) plus TMB-8 (5 μM)	13.46 ± 0.56[c]

Values are mean with s.e. mean of 5 experiments.
* The presence of a different letter in the superscripts of the means ± s.e. mean indicates a significant difference at the $P < 0.05$ level among these values as determined by analysis of variance (Goldstein, 1964).

Discussion

It has been shown that responses of the rabbit aorta to KCl are dependent upon Ca^{2+} influx into the smooth muscle cell, while phasic responses of vascular smooth muscle to noradrenaline are independent of extracellular Ca^{2+} concentrations and are due to the direct release of Ca^{2+} from intracellular stores by this agent (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969). The inhibition of KCl and phasic noradrenaline responses of the rabbit aorta produced by TMB-8 in the present study suggests that this agent may (1) act at the membrane to inhibit the influx of extracellular Ca^{2+}; (2) act at intracellular Ca^{2+} storage sites to block the release of Ca^{2+} from these stores; or (3) act at both of these sites to reduce the availability of Ca^{2+} to the contractile apparatus.

The inhibition of resting cellular $^{45}Ca^{2+}$ influx in the guinea-pig ileum by TMB-8 suggests that TMB-8 does have a significant inhibitory effect on the movement of Ca^{2+} into the smooth muscle cell. The decrease in the percentage of $^{45}Ca^{2+}$ which effluxes from the guinea-pig ileum suggests that TMB-8 might also slow the release of Ca^{2+} from cellular stores in smooth muscle. However, it is not possible from this study to state at which site or sites of Ca^{2+} storage in smooth muscle TMB-8 exerts this inhibition.

In the sarcoplasmic reticulum preparation of skeletal muscle, TMB-8 caused potent inhibition of caffeine-induced Ca^{2+} release at a concentration (5 μM) that was well within the range of doses of TMB-8 required to block responses of skeletal muscle *in vitro* (1.8 μM to 470 μM) (Malagodi & Chiou, 1974b). However, TMB-8 had no effect on the uptake of $^{45}Ca^{2+}$ by this preparation, indicating that TMB-8 did not inhibit Ca^{2+} availability by enhancing Ca^{2+} uptake by the sarcoplasmic reticulum.

It is possible that TMB-8 may have produced all of its inhibitory effects on smooth and skeletal muscle by stabilizing Ca^{2+} binding to functionally important cellular Ca^{2+} sequestering sites. In so doing, TMB-8 could antagonize Ca^{2+} release from cellular Ca^{2+} sequestering sites regardless of whether it is elicited via depolarization by KCl or direct release by caffeine or noradrenaline. The inhibition of $^{45}Ca^{2+}$ influx into the guinea-pig ileum may be the result of an inhibition of the displacement of unlabelled Ca^{2+} by labelled Ca^{2+} at sarcolemmal and/or microsomal sites, an effect that may have been due to a TMB-8-induced stabilization of Ca^{2+} binding. The decrease in the percentage of effluxing $^{45}Ca^{2+}$ from the guinea-pig ileum and the inhibition of $^{45}Ca^{2+}$ release from the skeletal muscle sarcoplasmic reticulum preparation also support this proposed mechanism. An agent affecting such a late step in the common pathway of all contractile stimuli as the release of Ca^{2+} (Daniel, 1964) may prove to be a valuable tool in further studies of the basic mechanism of contraction and relaxation in muscles of all types.

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