Selective Inhibition of Potassium Contracture in Guinea Pig Taenia Coli by Ruthenium Red

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Abstract Effects of ruthenium red on isotonic KCl induced contracture (K-contracture), cellular ^{45}Ca uptake and ^{45}Ca binding to surface membranes were examined in the smooth muscle cells of guinea pig taenia coli. These results were compared with those using lanthanum (La^{3+}).

The tonic component of the K-contracture was selectively inhibited by 1 mM ruthenium red. In contrast, 1 mM La^{3+} inhibited the phasic component of the K-contracture to a large extent. Use of 1 mM ruthenium red selectively inhibited the tonic component of K-contracture and caused a marked decrease in cellular ^{45}Ca uptake in that component of K-contracture. In contrast, 1 mM La^{3+} largely inhibited the phasic component and caused a significant decrease in cellular ^{45}Ca in that component. According to Scatchard plot analysis, there are two kinds of Ca^{2+} binding sites of high and low affinity, respectively, on the surface membrane of the taenia coli. One mM ruthenium red suppressed those of low affinity more strongly than those of high affinity. In contrast, 1 mM La^{3+} suppressed high affinity sites more markedly than low affinity sites.

Based on these results, it seems possible to conclude that ruthenium red mainly blocks the initial binding sites linked with Ca^{2+} influx which is related to the production of the tonic component while La^{3+} blocks those sites related to the phasic component of the K-contracture of guinea pig taenia coli.

The contraction of smooth muscle is mainly dependent upon extracellular Ca^{2+} for the activation of contractile protein. This function has been accepted as evidence that K-contracture of guinea pig taenia coli, in its initial rapid phase, is dependent upon the influx of Ca^{2+} during spike discharge and its subsequent sustained tonic phase is dependent upon Ca^{2+} influx during K-depolarization (MAYER *et al.*, 1972).

It is generally supposed that a primary and essential step for the influx of Ca^{2+} is the binding to surface membranes in barnacle muscle (HAGIWARA and TAKAHASHI, 1967), mammalian myocardial tissues (SANBORN and LANGER, 1970) and isolated skeletal sarcoplasmic reticulum (CHEVALLIER and BUTOW, 1971).

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The terminal group on the saccharide side chain of glycoproteins is always composed of sialic acid and is negatively charged. Calcium binding to the sialic acid residues in the surface of liver mitochondria (BOSMANN *et al.*, 1972; SHLATZ and MARINETTI, 1972), erythrocytes (LONG and MOUAT, 1971) and liver microsomes (LARSÉN *et al.*, 1972) plays an important role in the regulation of transmembrane Ca^{2+} flux. Recently, ISHIYAMA *et al.* (1975) observed that when the taenia coli was treated by neuraminidase (EC 3.2.1.18), K-contraction was suppressed with a decrease in its sialic acid content and they suggested that sialic acid residues were the first sites triggering Ca^{2+} influx.

In this study we attempted to observe the effects of ruthenium red on cellular Ca^{2+} uptake and on superficial Ca^{2+} binding in relation to its effect on K-contraction in guinea pig taenia coli. Ruthenium red is well known as a mucopolysaccharide binding agent (MOORE, 1971; LUFT, 1971). Lanthanum as well has been reported to strongly inhibit Ca^{2+} binding to Ca^{2+} binding sites of surface membranes in a competitive manner. Thus, the effects were compared with those of La^{3+} .

Based on the results obtained, the possible existence of two different systems for Ca^{2+} influx through the surface membranes of smooth muscle cells will be discussed.

Some of these results have already been reported (KAWAMURA *et al.*, 1977).

MATERIALS AND METHODS

Preparation of sample. Guinea pigs of either sex, weighing 300–500 g, were stunned and bled. The taenia coli was excised and was kept in normal solution for 1 hr before the start of an experiment. The normal solution was a Tris-buffered solution (hereafter called Na-Tris sol.) of the following composition (mM); NaCl, 139.3; KCl, 2.68; MgCl_2 , 0.5; CaCl_2 , 1.36; tris(hydroxymethyl) aminomethane (Tris), 5.0 and glucose, 8.0. It was aerated with 100% O_2 and its pH was adjusted to 7.4 with 1 N HCl. Isotonic KCl solution (K-Tris sol.) was prepared by replacing NaCl with equivalent amounts of KCl.

Measurement of tension. The taenia coli was mounted in a 5 ml organ bath at 37°C. One end of strip was tied to a mechano-transducer (Nihon Kohden Ltd., model SB-IT) and the other end to a hook at the bottom of bath. The mechanical responses were recorded on an ink-writing oscillograph (Nihon Kohden Ltd., RJG-3022-IS).

Electron microscope study. The procedure for staining with ruthenium red was essentially the same as that described by LUFT (1971). Small pieces (about 1 mm³) of taenia coli were prefixed for 1 hr at 4°C in a 2.5% glutaraldehyde solution containing 10^{-3} M ruthenium red and 0.1 M cacodylate buffer (pH 7.4). The tissue blocks were washed out with a 0.1 M cacodylate buffered solution. Then they were postfixed for 3 hr at room temperature in 1% osmium tetroxide solution containing

10^{-3} M ruthenium red and 0.1 M cacodylate buffer.

^{45}Ca binding and ^{45}Ca uptake. 1) ^{45}Ca binding: Before the start of an experiment, taenia coli was immersed in the Ca^{2+} -free Na-Tris sol. for 5 min at 20°C and then was transferred into a Na-Tris sol. containing ^{45}Ca ($2\ \mu\text{Ci/ml}$). After incubation for 5 min the reaction was terminated by blotting on filter paper (No. 5, Toyo Roshi Co.) and subsequent washing for 10 sec with the Ca^{2+} -free Na-Tris sol. Blotting was repeated again and the taenia coli was digested with the solubilizer (Soluene-350, Packard Co.). The radioactivity of ^{45}Ca was thereafter counted using a liquid scintillation spectrometer (Hitachi-Horiba, LS-700). To determine the binding sites the amounts of Ca^{2+} binding were measured at two different concentrations of added Ca^{2+} ($1.36 \cdot 10^{-6}$ and $1.36 \cdot 10^{-3}$ M). The ratio of Ca^{2+} bound to free Ca^{2+} concentrations can be used to classify these Ca^{2+} binding sites of different affinities (Rossi *et al.*, 1973). 2) ^{45}Ca uptake: The taenia coli was exposed to a Na-Tris sol. containing ^{45}Ca ($2\ \mu\text{Ci/ml}$) for a specified period and then washed five times during 1 hr with a nonradioactive Ca^{2+} Na-Tris sol. containing 20 mM La^{3+} . Similarly, ^{45}Ca uptake in K-Tris sol. was observed. The subtraction of the amounts of ^{45}Ca uptake in Na-Tris sol. from those in K-Tris sol. led to an estimate of ^{45}Ca uptake during K-contracture.

Agents. Ruthenium red (hereafter called RR), LaCl_3 and RuCl_3 are commercial products of the best reagent grades available. Radioisotope Ca^{2+} was purchased from the International Chemical Nuclear Corporation as CaCl_2 in 0.5 M HCl (6.6 mCi/mg).

RESULTS

Effects of RR and La^{3+} on K-contracture

As shown in Fig. 1A, K-contracture consisted of two components, an early rapid phasic component and a late sustained tonic one. After pretreatment with 10^{-3} M RR for 10 min in Na-Tris sol. in the presence of the same concentration of RR in K-Tris sol. the phasic component of K-contracture was only slightly suppressed but the tonic component was markedly affected, being reduced to about 50% of the control level. When the bath solution containing RR was replaced with the Na-Tris sol., tension in each component returned to the control level within 10 min. With an increase of RR up to $5 \cdot 10^{-3}$ M, the inhibition of the tonic component gradually became greater than that of the phasic component (Fig. 1B).

As shown in Fig. 2A, the extent of inhibition was dependent upon the concentration of RR. At a concentration of RR, 10^{-4} , 10^{-3} and $5 \cdot 10^{-3}$ M, the effect on the phasic component was much less pronounced than on the tonic component. There was approximately 80% inhibition of the tonic component in K-Tris sol. containing $5 \cdot 10^{-3}$ M RR but less than 30% inhibition of the phasic component in the same concentration of RR. A gradual prolongation in the duration of RR (10^{-3} M) treatment caused inhibition of both components to be reinforced as shown in Fig. 2B. Throughout the altered treating period tested, the inhibition of the

Fig. 1. Effects of RR on K-contraction. Panel A shows the effect of RR at 10^{-3} M and panel B shows that at $5 \cdot 10^{-3}$ M. In each panel, a shows a control response produced by applying K-Tris sol. In each experiment, the preparation was pretreated with RR at 10^{-3} M (c in panel A) and at $5 \cdot 10^{-3}$ M (b in panel B), respectively, for 10 min. The preparation was then transferred into a K-Tris sol. containing RR at each concentration. b in panel A shows the recovery from K-contraction by Na-Tris sol. d and e in the same panel show that the inhibitory effect of RR was ceased by washing. iso-K; isotonic KCl solution, wash; the preparation and organ bath were washed with normal solution. The experimental solution was aerated with 100% O_2 and maintained at $37^\circ C$.

Fig. 2. Inhibitory effects of RR on K-contraction. A; Dose-inhibition curve of RR. B; Relationship between duration of RR pretreatment and inhibition of K-contraction. The preparation was pretreated with 10^{-3} M RR for specified periods in Na-Tris sol. and immersed in K-Tris sol. containing the same concentration of RR. The ordinate of each figure shows inhibition by RR (Control tension of K-contraction was taken as 100%). Each point represents the average for 6-9 experiments and vertical bars indicate SE ($\pm$). $\circ$; phasic component of K-contraction, $\triangle$ and $\square$; tonic components at 10 and 15 min after initiation of K-contraction, respectively.

tonic component was more marked than that of phasic one.

The effects of RR and La^{3+} on K-contracture were compared. As shown in Fig. 3, 10^{-3} M La^{3+} suppressed the phasic component more markedly than the tonic component of K-contracture. In the presence of 10^{-3} M RR and La^{3+} , both the phasic and tonic components of K-contracture were largely suppressed. These facts might indicate that specifically RR and La^{3+} , respectively, affect the tonic and phasic components of K-contracture.

Fig. 3. Effects of various agents on K-contracture. The K-contracture shown in upper-left side hand of figure, transcribed from Fig. 1, served as the control. Records except "Control" show the effects of RR, La^{3+} , RR + La^{3+} and ruthenium trichloride (Ru^{3+}). At each observation, the preparation was pretreated with the agent for 10 min in Na-Tris sol. and after transferred to K-Tris sol. containing the same concentration of its agent (at the time indicated by the arrow). The concentration of each agent was 10^{-3} M, respectively.

In order to investigate whether these inhibitory effects of RR were due to its characteristic chemical structure (a polynuclear ionic complex between oxygen-bridged 3- and trivalent ruthenium atoms and ammonia) or to its polyvalent cationic sites, the effect of ruthenium trichloride (Ru^{3+}) on K-contracture was examined. As shown in the lower left-hand corner of Fig. 3, 10^{-3} M Ru^{3+} more markedly suppressed the phasic component than did La^{3+} (10^{-3} M). This effect of Ru^{3+} obviously differs from that of RR.

Effects of RR and La^{3+} on cellular ^{45}Ca uptake

Cellular ^{45}Ca uptake during K-contracture in the presence of 10^{-3} M RR, 10^{-3} M La^{3+} and in the coexistence of 10^{-3} M RR and 10^{-3} M La^{3+} was measured

by the La method (UEMURA, 1973).

The time relation of ^{45}Ca uptake, illustrated by the upper curve in Fig. 4, served as control for the other tests. This curve consisted of two components, an early rapid phase up to about 5 min and then a subsequent slow phase. Following the early phase, the amount of ^{45}Ca uptake further increased, coming saturated with about 0.12 mEq/kg wet wt about 20 min after the initiation of K-contraction.

In the presence of 10^{-3} M RR, the early rapid phase was unaffected but the subsequent slow phase was strongly suppressed, reaching a suppressed plateau about 20 min after the initiation of the K-contraction. In contrast, in the presence of 10^{-3} M La^{3+} , the ^{45}Ca uptake in the early phase was completely inhibited but this inhibition was followed by a gradual increase of ^{45}Ca uptake about 1 min after the initiation of the K-contraction.

During coexistence of 10^{-3} M RR and La^{3+} , ^{45}Ca uptake in the early phase was completely blocked. After about 10 min exposure to K-Tris sol. containing RR (10^{-3} M) and La^{3+} (10^{-3} M), there was a slight increase in ^{45}Ca uptake.

Effects of RR and La^{3+} on superficial ^{45}Ca binding

The amount of Ca^{2+} binding in the control solution, determined according to the procedure outlined in METHODS AND MATERIALS, is about 23 ± 0.7 SE nmoles/100 mg wet wt (Table 1). In order to ascertain whether almost all of the bound

Table 1. ^{45}Ca binding to surface membrane of muscle cell. The preparations were incubated in a control solution (Na-Tris sol. containing $2 \mu\text{Ci } ^{45}\text{Ca}/\text{ml}$) for 5 min under the conditions listed. After blotting, preparations were rinsed in Ca^{2+} -free Na-Tris sol. for 4 min and blotted again. The tissue was assayed for radioactivity according to the procedure described in the text. Each value represents the mean $\pm$ SE for 6–12 experiments.

Experiments	Calculated amount of Ca^{2+} bound to surface membrane (nmol/100 mg wet wt)
Incubated in control solution at 20°C	23.0 ± 0.7 (12)
Prior treatments for 15 min	
with 10^{-3} M RR	16.5 ± 1.1 (6)
with 10^{-3} M La^{3+}	6.0 ± 0.5 (9)
After incubation in control solution, Ca^{2+} binding was measured with the La method (UEMURA, 1973)	2.3 ± 0.5 (6)
Incubated in control solution at 37°C	22.5 ± 1.0 (6)

Ca^{2+} measured was on the outer surface of the membrane or whether any amount was in intracellular space, the La method (UEMURA, 1973) was applied. Since La^{3+} has a much higher affinity for Ca^{2+} binding sites than Ca^{2+} itself, almost all the extracellular bound Ca^{2+} was displaced from tissue by La^{3+} while the intracellular Ca^{2+} remained locked inside the cells. With the use of this method, it was found that the amount of ^{45}Ca entering the cells during tissue exposure to a control solu-

tion was 2.3 ± 0.5 SE nmoles/100 mg wet wt. This indicates that the large amounts of Ca^{2+} measured in the control solution of this study must be bound on the outer surface membrane. There was a difference between these two figures determined in each control experiment, the value was 56.6 ± 4.1 SE nmoles/100 mg wet wt in the report of ISHIYAMA *et al.* (1975), whereas it was 23 ± 0.7 SE nmoles/100 mg wet wt in the present study. Similar but not exactly identical experimental procedures were used in both experiments. One explanation for the discrepancy is that different durations were used in rinsing the tissue in Ca^{2+} -free Na-Tris sol. In the work of Ishiyama *et al.*, the duration of rinsing was 10 sec, whereas it was 4 min in the present study. In general, it is evident from the ^{45}Ca efflux experiment that much more ^{45}Ca was effluxed from the tissue previously loaded with ^{45}Ca by prolongation of the rinsing. Because a more exact calculation in the analysis of Ca^{2+} binding is possible when a much more Ca^{2+} is bound, all Ca^{2+} binding experiments, other than those shown in Table 1, were done according to the method of Ishiyama *et al.*

The difference between control values at 20°C and 37°C was not statistically significant. Prior treatment with RR (10^{-3} M) decreased the amount of Ca^{2+} bound to the surface membrane from 23.0 ± 0.7 to 16.5 ± 1.1 SE nmoles/100 mg wet wt. Similarly, pretreatment with La^{3+} (10^{-3} M) decreased bound Ca^{2+} to 6.0 ± 0.5 SE nmoles/100 mg wet wt.

Determination of high and low affinity sites of Ca^{2+} binding to the surface membrane

A Scatchard plot of the Ca^{2+} binding to the surface membrane revealed the existence of at least two classes of Ca^{2+} binding sites, of high and low affinity, respectively (ISHIYAMA *et al.*, 1975).

In the present experiment, as shown in Table 2, 13.2, the ratio of bound Ca^{2+}

Table 2. Determination of high and low affinity sites of Ca^{2+} binding to surface membrane of muscle cell. The preparation was incubated in Na-Tris sol. and then washed in Ca^{2+} -free Na-Tris sol. for 5 min. Following this, the preparation was further incubated in 5 ml of either $1.36 \cdot 10^{-5}$ or $1.36 \cdot 10^{-3}$ M Ca^{2+} containing Na-Tris sol. with $2 \mu\text{Ci } ^{45}\text{Ca}/\text{ml}$ for 5 min, at 20°C , blotted, rinsed in Ca^{2+} -free Na-Tris sol. for 10 sec, and then blotted again. Two binding sites with different affinities were defined by the ratio of amounts of bound Ca^{2+} (nmoles/100 mg wet wt) to the concentration of added Ca^{2+} (M). Then the ratios obtained were multiplied by 10^{-4} . Each value represents the mean $\pm$ SE for 12 experiments.

	High affinity binding site	Low affinity binding site
Added Ca^{2+} (M) (a)	1.36×10^{-5}	1.36×10^{-3}
Bound Ca^{2+} (nmol/100mg wet wt) (b)	1.79 ± 0.12 (12)	80.7 ± 4.4 (12)
Ratio (b/a)	13.2	5.9

to added Ca^{2+} ($1.36 \cdot 10^{-5}$ M), was used to define high affinity sites. In contrast, 5.9, the ratio determined in a high concentration of added Ca^{2+} ($1.36 \cdot 10^{-3}$ M), was

used to define low affinity sites. The high and low affinity site values thus determined were nearly equal to those reported by ISHIYAMA *et al.* (1975).

As shown in Table 3, 10^{-3} M RR produced only a 15.1% decrease in Ca^{2+} binding to high affinity sites. On the other hand, it decreased Ca^{2+} binding to low affinity sites by 25.5%. The effect of La^{3+} was contrasted with that of RR. One mM La^{3+} produced a 61.2% decrease in Ca^{2+} binding to high affinity sites, while it produced a 50.9% decrease in binding to low affinity sites.

The depressant effects of RR and La^{3+} on Ca^{2+} binding to both sites in isotonic

Table 3. Effects of RR and La^{3+} on high and low affinity sites of Ca^{2+} binding. The preparation was incubated in Na-Tris sol. either containing 10^{-3} M RR or La^{3+} for 15 min. Subsequent procedures are the same as described in the legend of Table 2. Each value represents the average for 6–9 experiments.

Experiments	High affinity site		Low affinity site	
	Ratio	Inhibition (%)	Ratio	Inhibition (%)
In Na-Tris sol.				
Control	13.2		5.9	
RR (10^{-3} M)	11.2	15.1	4.4	25.5
La^{3+} (10^{-3} M)	5.1	61.2	2.9	50.9
In K-Tris sol.				
Control	12.7		5.8	
RR (10^{-3} M)	10.9	14.5	4.3	25.7
La^{3+} (10^{-3} M)	4.4	65.4	3.1	46.2

Fig. 4. Effects of RR, La^{3+} and $\text{RR} + \text{La}^{3+}$ on cellular ^{45}Ca uptake during K-contraction. Cellular ^{45}Ca uptake was measured by the La method. Details of the experimental procedure are described in the text. $\circ$; control, $\bullet$; in the presence of 10^{-3} M RR, $\blacktriangle$; in the presence of 10^{-3} M La^{3+} , $\blacksquare$; in the coexistence of 10^{-3} M RR and La^{3+} . The left ordinate represents ^{45}Ca space and right ordinate Ca^{2+} contents which were calculated from specific activities of radioisotope calcium and ^{45}Ca space. Each point represents the average for 6–9 experiments.

KCl solution were similar to those in normal solution.

Electron micrograph of muscle cell stained with RR

Ruthenium red has often been used as a histochemical staining dye because it selectively reacts with mucopolysaccharide to produce a stable RR-complex.

An electron micrograph (Fig. 5) of muscle cell exposed to 10^{-3} M RR in glutaraldehyde as well as in OsO_4 shows that RR apparently did not penetrate into the muscle cell and only reacted with the outer surface of the muscle membrane and the surface vesicles.

Fig. 5. Electron micrograph of muscle cell from guinea pig taenia coli stained with RR. Note the accumulation of RR exclusively on surface and vesicles of the cell membrane (arrows). Surface (S), vesicle (V) and extracellular space (ECS) are indicated. $\times 30,000$.

DISCUSSION

It is generally accepted that both the phasic and tonic components of K-contraction of the taenia coli largely depend upon Ca^{2+} influx (MAYER *et al.*, 1972; UEMURA, 1973).

In the present study, RR inhibited K-contraction of guinea pig taenia coli and its effect on the tonic component was found to be more marked than that on the phasic component (Figs. 1 and 2). This finding shows that in the case of the taenia coli the inhibitory effect of this agent on K-contraction is more specific to the tonic component than the phasic component.

Ruthenium red is reported to be a selective inhibitor of active Ca^{2+} uptake by microsomes from guinea pig taenia caecum (TOMIYAMA *et al.*, 1975), by mitochondria from rat liver (MOORE, 1971) and by mitochondria from blowfly flight muscle (CARAFOLI and SACKTOR, 1972) and of the Ca^{2+} -ATPase of erythrocyte membrane (WATSON *et al.*, 1971). GILLIS (1972) showed that RR inhibited the relaxation of red muscle fiber which has few sarcoplasmic reticula, and interpreted this function in terms of inhibition of Ca^{2+} uptake by mitochondria. In addition, though this agent has been known as an extracellular marker in the field of light and electron microscope studies, HOWELL (1974) showed that when skeletal muscle was exposed to RR overnight a complete pattern of intracellular staining appeared.

Thus, if RR penetrates into the muscle cell of the taenia coli and acts as an inhibitor of Ca^{2+} uptake of some intracellular organella, *i.e.*, sarcoplasmic reticulum or mitochondria, it might be expected that the tonic component of K-contraction would be facilitated by treatment with this agent. However, as shown in the photograph (Fig. 5) and in Fig. 1, RR neither penetrated into the muscle cells nor potentiated the tonic component of K-contraction. Therefore, in our experiment, it was concluded that RR did not act on intracellular membraneous organella but only on the outer surface of the muscle cell membrane.

It has been reported that Ca^{2+} binding to the negatively charged sites of surface membrane may be the first step for the influx of Ca^{2+} in barnacle skeletal muscle (HAGIWARA and TAKAHASHI, 1967), taenia coli smooth muscle (BRADING and WIDDICOMBE, 1977) and skeletal sarcoplasmic reticulum (CHEVALLIER and BUTOW, 1971). ISHIYAMA *et al.* (1975) also reported that a number of possible Ca^{2+} binding sites on the surface membrane of guinea pig taenia coli muscle might be sialic acid residues generated from mucopolysaccharide. Calcium is bound to a variety of cell membrane by coulombic interaction with negatively charged surface groups from sialic acid. In addition, RR is considered a polyanionic stain specific for sialic acid (MOORE, 1971; LUFT, 1971).

It has been reported that, in addition to the inhibitory effect of RR on the active Ca^{2+} uptake, RR also inhibits Ca^{2+} binding to mitochondrial membrane (VASSINGTON *et al.*, 1972), cardiac sarcolemma (SCARPA and WILLIAMSON, 1974), skeletal muscle sarcolemma (MADEIRA and ANTUNES-MADEIRA, 1974) and the membrane of skeletal sarcoplasmic reticulum (VALE and CARVALHO, 1973). In the present

experiments, a clear inhibitory effect of RR on Ca^{2+} binding to the surface membrane was also observed. As shown in Fig. 4, in the presence of RR, cellular ^{45}Ca uptake in the early phase of K-contracture was unaffected but that in its subsequent late phase was largely suppressed reaching a plateau level about 20 min after the initiation of K-contracture. It is interesting to note that the time relation of cellular ^{45}Ca uptake in the presence of RR coincides with that of tension development in high K solution containing RR (Fig. 2A).

From the data presented, it appears that the effect of RR on the Ca^{2+} binding to the surface membrane is closely related to the inhibition of cellular Ca^{2+} uptake in the late phase of K-contracture.

Recently, ISHIYAMA *et al.* (1975) reported the existence of two types of Ca^{2+} binding sites on the surface membrane of the taenia coli smooth muscle cell, one of high affinity and the other of low affinity. It was suggested that, the low affinity sites of Ca^{2+} binding might be sialic acid residues, thus being the first step in Ca^{2+} influx during K-contracture.

In our experiments, RR more markedly decreased the amount of Ca^{2+} bound to the low affinity sites than those of high affinity (Table 3).

Therefore, a possible explanation is that RR competes with Ca^{2+} binding sites of low affinity, causing a release of bound Ca^{2+} and a concurrent inhibition of Ca^{2+} influx. As a result of this decrease in Ca^{2+} influx, the tonic component of K-contracture is suppressed.

In contrast, La^{3+} prevented the phasic component of K-contracture, leaving the tonic component unaffected (Fig. 3) and largely decreased cellular Ca^{2+} uptake in the early phase of K-contracture (Fig. 4). Furthermore, it more markedly decreased the amount of Ca^{2+} bound to high affinity than low affinity sites (Table 3). In addition, it has been reported that La^{3+} can displace extracellularly bound Ca^{2+} and blocks the transmembrane Ca^{2+} movements of the taenia coli (MAYER *et al.*, 1972; ADACHI, 1972; UEMURA, 1973).

Therefore, we would like to speculate that La^{3+} displaces Ca^{2+} from high affinity sites and blocks the influx of Ca^{2+} which might be related to the generation of the Ca^{2+} spike. Thus the phasic component of K-contracture is suppressed.

The effects of RR and La^{3+} on K-contracture, cellular Ca^{2+} uptake and superficial Ca^{2+} binding are summarized in Table 4 and a conceptional model consistent with this summary is illustrated in Fig. 6, *i.e.*, Ca^{2+} influx appears to involve the binding of Ca^{2+} to negatively charged sites of the outer side of the membrane, followed by the transfer of Ca^{2+} from outer sites to inner sites in the membrane phase with the ultimate release of Ca^{2+} into myoplasm. Calcium binding sites may be composed of two classes with different affinity constants. Each site can be blocked selectively by differential inhibitors, *i.e.*, RR and La^{3+} . The low affinity Ca^{2+} binding sites, which are sensitive to RR, are preferentially used for producing tonic component of K-contracture, the high affinity sites which are accessible to La^{3+} for the phasic component.

Table 4. Summary of RR and La^{3+} effects on contractility, on Ca^{2+} uptake and on Ca^{2+} binding. Figures of *, ** and *** are transcribed from Fig. 2, Fig. 4 and Table 3, respectively.

Experiments	Agents	Inhibition by	
		RR (%)	La^{3+} (%)
K-contracture*			
Phasic comp.		10	80
Tonic comp.		65	5
Ca^{2+} uptake**			
Rapid phase		12	100
Slow phase		41	24
Ca^{2+} binding site***			
High affinity		14.5	65.4
Low affinity		25.7	46.2

Fig. 6. Schematic illustration of possible Ca^{2+} movement across cell membrane of guinea pig taenia coli. Binding of Ca^{2+} to the outer surface of the cell membrane is the first step in Ca^{2+} influx. Ca^{2+} binding sites are composed of two classes with different affinity constants, i.e., high and low affinity sites. Each site can be blocked selectively by differential inhibitors, i.e., ruthenium red (RR) and lanthanum (La^{3+}). Ca^{2+} bound to the low affinity sites, which are sensitive to RR, is preferentially available for producing the tonic component of K-contracture. Conversely, the high affinity sites are more accessible to La^{3+} . HAS and LAS indicate high and low affinity sites, respectively. ---; inhibition by RR or La^{3+} .

According to the work of Ishiyama *et al.*, the removal of sialic acid residues by neuraminidase produced not only inhibition of both phasic and tonic components of K-contracture but abolition of Ca^{2+} binding sites of low affinity. From these facts, they proposed that Ca^{2+} binding to sialic acid residues plays a crucial role in the regulation of transmembrane Ca^{2+} flux in taenia coli. On the other hand, we used RR for perturbation of sialic acid residues. Ruthenium red decreased the amount of Ca^{2+} bound on the binding sites of low affinity and more markedly inhibited the tonic component than the phasic component. Our results

indicate that Ca^{2+} binding sites of low affinity are generated by sialic acid residues and are consistent with the concept that sites for control of Ca^{2+} flux to form the tonic component are sialic acid residues. While both we and Ishiyama *et al.* studied two perturbations in investigating the crucial role of sialic acid residues in Ca^{2+} flux; both experiments brought about different results concerning the inhibition of K-contracture; *i.e.*, neuraminidase suppressed both the phasic and tonic components, whereas RR selectively inhibited the tonic component. The reason for this discrepancy is unclear at present due to the fact that the chemical nature of the high affinity sites has not yet been elucidated. MELA (1968) suggested that in mitochondrial membrane, phospholipid residues have a high affinity for La^{3+} . Phospholipase C suppressed both the tonic and phasic components (ISHIYAMA *et al.*, 1975). In the present study La^{3+} decreased the Ca^{2+} binding to high affinity sites and inhibited the phasic component more markedly than the tonic one. Therefore, the inhibition of both components by neuraminidase might partly be due to the associated decomposition of membraneous phospholipid.

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