DETERMINATION OF CALCIUM IONS EFFLUXED FROM SMOOTH MUSCLE INTO Ca^{2+} -FREE PHYSIOLOGICAL SOLUTION BY MEANS OF Ca^{2+} -SELECTIVE ELECTRODE

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In order to measure Ca^{2+} efflux from the smooth muscle into a Ca^{2+} -free physiological solution, the method using a Radiometer Ca^{2+} -sensitive electrode was applied and compared with the method using an atomic absorption spectrophotometer to examine its usefulness. The results obtained show that the Ca^{2+} electrode used here can accurately and continuously monitor free calcium ions effluxed from the smooth muscle into a Ca^{2+} -free solution. The Ca^{2+} -sensitive electrode used here is a useful tool to investigate Ca^{2+} efflux from the smooth muscle into a Ca^{2+} -free physiological solution.

Keywords— Ca^{2+} -selective electrode; intestinal and vascular smooth muscle; atomic absorption spectrophotometry; lanthanum; Ca^{2+} efflux from smooth muscle into Ca^{2+} -free solution

INTRODUCTION

The methods which have been routinely utilized for investigating calcium movements and distribution in smooth muscle include radioisotope techniques and atomic absorption spectrophotometry. Recently, the method using Ca^{2+} -sensitive electrode for investigating calcium movements in the biological membrane systems such as sarcoplasmic reticulum, microsome and mitochondria has been described.¹⁻⁷⁾ Since a reliable and sensitive Ca^{2+} -sensitive electrode is now commercially available,^{3,8)} we tried to use a Radiometer Ca^{2+} -selective electrode for determination of Ca^{2+} effluxed from the guinea pig taenia coli and the rabbit thoracic aortae into a Ca^{2+} -free physiological solution. In addition, the residual tissue Ca content after exposure to a Ca^{2+} -free physiological solution containing La^{3+} was investigated by atomic absorption spectrophotometry and compared with that after the Ca^{2+} efflux into the Ca^{2+} -free solution.

MATERIALS AND METHODS

The Apparatus and Calibration of the Electrode

—The potentiometric set-up for Ca^{2+} determination is composed of an F2112Ca Calcium Selectrode, a K401 saturated calomel electrode, a PHM64 pH meter (Radiometer A/S, Copenhagen, Denmark) and a recorder. An amplifier (ME Commercial, Tokyo, Japan) with an offset voltage controller³⁾ was inserted in series between the pH meter output and the recorder input.

The Ca^{2+} electrode was calibrated by successive addition of 5 nmol. Ca^{2+} with a concentrated CaCl_2 stock solution and a calibration curve for the Ca^{2+} electrode was obtained by plotting changes in potential (Δ mV) against the logarithm to the added Ca^{2+} concentrations. Under the conditions used here, calibration curves became linear within the range of Ca^{2+} concentrations from 5×10^{-6} to 2.5×10^{-5} M. The calibration curve was made before and after the efflux experiments to minimize possible errors due to the electrode potential drift. Since the potential decreases with increasing Ca^{2+} concentration in the medium, changes in the potential were expressed in terms of absolute values.

Tissue Preparation — Thoracic aortae were removed from male rabbits weighing 3.0 to 4.0 kg and quickly cleaned of accessory tissue. Helically cut strips, approximately 4×25 mm, were prepared. The aorta was not separated into adventitial and media-intimal layers. Pieces of taenia coli (50 mm long *in situ*) were dissected from the caecum of male guinea pig weighing 400 to 500 g.

Ca^{2+} Efflux from Tissue, Residual Tissue Ca Content and Tissue Ca Content — Before determination of Ca^{2+} effluxes from the tissues, they were allowed to equilibrate for 120 min in a normal Tris physiological solution (hereafter called Tris-solution) (mM): NaCl 137; KCl 2.7; CaCl_2 1.4; glucose 8.0 and tris(hydroxymethyl)amino-methane (Tris) 5.0. The Tris-solution was adjusted to pH 7.5 with 6 N HCl and bubbled with 100% O_2 . After equilibration, the tissue was gently blotted on ashless filter paper (Whatman No.42) and then placed in a vessel containing a Ca^{2+} -free Tris-solution (5 ml) which was made by removing CaCl_2 from the normal Tris-solution, and Ca^{2+} effluxed from the tissue during 60-min incubation was continuously determined by the Ca^{2+} electrode system. Stirring of the efflux solution was accomplished by the bubbling of 100% O_2 .

After determination of the Ca^{2+} efflux by the Ca^{2+} electrode, the tissue was gently blotted on ashless filter paper, transferred into a quartz tube, dried at 100° for 12 hr, weighed to obtain its dry weight and ashed at 550° for 16 hr. This ashed tissue was dissolved in 0.1 N HCl and its residual tissue Ca was measured by atomic absorption spectrophotometry.

The total tissue Ca content and the residual tissue Ca content after incubation in the Ca^{2+} -free Tris-solution were obtained as follows.

Procedure (1): The total tissue Ca content was indirectly calculated from a summation of the Ca^{2+} concentration effluxed from the tissue during 60-min incubation, which was measured by the Ca^{2+} electrode, and the residual tissue Ca content, measured by atomic absorption spectrophotometry, at the end of the efflux period. The residual tissue Ca content at each incubation time

was expressed by subtracting the effluxed Ca^{2+} concentration at each efflux time from the total tissue Ca content summed above.

Procedure (2): After equilibrating the tissue for 120 min in the normal Tris-solution, the total tissue Ca content was directly measured by employing a procedure similar to those used in measuring the residual tissue Ca described above with an atomic absorption spectrophotometer. The residual tissue Ca content after incubation in the Ca^{2+} -free Tris-solution during given periods was also measured by atomic absorption spectrophotometry.

The effect of La^{3+} on the tissue Ca content was investigated by measuring the residual tissue Ca content after incubation in the Ca^{2+} -free Tris-solution containing 10 mM LaCl_3 (hereafter called the Ca^{2+} -free, La Tris-solution) during the given periods by atomic absorption spectrophotometry, since the Ca^{2+} electrode responded to La^{3+} . The Ca^{2+} -free, La Tris-solution was prepared by substituting 10 mM LaCl_3 for 20 mM NaCl in the Ca^{2+} -free Tris-solution and was adjusted to pH 7.2.

Mechanical Response — Contractile responses of the muscle strips suspended in a muscle bath containing 10 ml of the Tris-solution were measured as isotonic contractions. The muscle strips were under 0.5 g tension, and allowed to equilibrate for 60 min prior to obtaining control response. Stimulatory agents employed in this study were carbachol for the taenia coli and noradrenaline for the thoracic aorta. The experiments determining the effect of the Ca^{2+} -free Tris-solution on the drug-induced contractions are as follows. After reproducible contractions of identical size were obtained in the normal Tris-solution (control response), the muscle strip was exposed to the Ca^{2+} -free Tris-solution for a given period of time. The bathing solution was then switched to the Ca^{2+} -free Tris-solution containing the stimulatory agents and drug-induced contractions were obtained.

All experiments were performed at 26° to slow the rate of Ca^{2+} efflux and to minimize evaporation of the efflux solution. All solutions were

made using reagent-grade water obtained through Mill-Q systems (Millipore Corp. Bedford).

Statistical analysis for the significance of difference between means was, if necessary, done using the Student's *t*-test. A *p* value of 0.05 or less was considered to be significant.

RESULTS

Fig. 1 shows the Ca^{2+} effluxes, continuously measured by the Ca^{2+} electrode, from the guinea pig taenia coli and the rabbit thoracic aorta into the Ca^{2+} -free Tris-solution. The amounts of Ca^{2+} effluxed from the taenia coli and the thoracic aorta into the Ca^{2+} -free Tris-solution during 60-min incubation were 5.00 ± 0.21 ($n=5$) and 7.82 ± 0.36 ($n=5$) mmol. Ca^{2+} /kg dry wt, respectively. After determination of Ca^{2+} effluxes from the tissues for 60 min, their residual tissue Ca contents measured by atomic absorption spectrophotometry were 1.50 ± 0.07 ($n=5$) mmol. Ca/kg dry wt for the taenia coli and 2.47 ± 0.21 ($n=5$) mmol. Ca/kg dry wt for the

thoracic aorta. The effluxed amounts of Ca^{2+} from the rabbit thoracic aorta were significantly greater than those from the guinea pig taenia coli ($p < 0.005$), and the residual tissue Ca content was much greater in the thoracic aorta compared to that in the taenia coli ($p < 0.005$).

The residual tissue Ca contents of the guinea pig taenia coli which were measured by atomic absorption spectrophotometry, after incubation in the Ca^{2+} -free Tris-solution or the Ca^{2+} -free, La Tris-solution for 1, 5, 10, 15 and 30 min are shown in Fig. 2. The tissues incubated in the Ca^{2+} -free Tris-solution had large amounts of residual Ca than those in the Ca^{2+} -free, La Tris-solution and significant differences between them were observed at all incubation times except for 1-min incubation ($p < 0.05$).

After determination of Ca^{2+} effluxes from the tissues into the Ca^{2+} -free Tris-solution for 60 min by the Ca^{2+} electrode, their residual tissue Ca contents were measured by atomic absorption spectrophotometry. In this case, the total tissue Ca content and residual tissue Ca content at each

FIG. 1. Ca^{2+} Effluxes from Rabbit Thoracic Aorta (A) and Guinea Pig Taenia Coli (B) into the Ca^{2+} -free Tris-solution measured by the Ca^{2+} Electrode

An upward movement in the Ca^{2+} trace indicates an increase in Ca^{2+} concentrations in the solution. Ca^{2+} effluxes are expressed as mmol. Ca^{2+} /kg dry wt. The curves represent a summary of 5 experiments.

FIG. 2. Comparison of Residual Tissue Ca Contents of Guinea Pig Taenia Coli after Incubation in the Ca^{2+} -free Tris-solution (open circles) or the Ca^{2+} -free Tris-solution containing 10 mM LaCl_3 (filled circles) during Given Periods

Residual tissue Ca contents were measured by atomic absorption spectrophotometry and are expressed as mmol. Ca/kg dry wt. Each point represents the mean value of 4 to 12 experiments. Significant difference from the corresponding value with 10 mM LaCl_3 at $p < 0.05$ (*) and at $p < 0.01$ (**).

incubation time were calculated according to the methods described in Procedure (1) under MATERIALS AND METHODS. The residual tissue Ca contents calculated are shown in Fig. 3. In the taenia coli (Fig. 3, A), the residual tissue Ca

FIG. 3. Comparison of the Residual Tissue Ca Contents of Guinea Pig Taenia Coli (A) and Rabbit Thoracic Aorta (B) after Incubation in the Ca²⁺-free Tris-solution (open circles) or the Ca²⁺-free Tris-solution Containing 10 mM LaCl₃ (filled circles) during Given Periods

Residual tissue Ca incubated in the Ca²⁺-free Tris-solution was measured by the method described in Procedure (1) under MATERIALS AND METHODS and that incubated in the Ca²⁺-free Tris-solution containing 10 mM LaCl₃ by atomic absorption spectrophotometry. The residual tissue Ca contents are presented as mmol. Ca/kg dry wt. Each point represents the mean value of 4 to 18 experiments and the S.E. of mean is indicated if it is larger than the size of symbols.

contents calculated according to the methods in Procedure (1) after incubation in the Ca²⁺-free Tris-solution for 10 and 30 min were 2.98 ± 0.16 ($n=5$) and 2.05 ± 0.10 ($n=5$) mmol. Ca/kg dry wt, respectively. In the case of determination by atomic absorption spectrophotometry described in Procedure (2) under MATERIALS AND METHODS, they were 2.73 ± 0.09 ($n=8$) and 2.09 ± 0.21 ($n=4$) mmol. Ca/kg dry wt, respectively. There were no significant differences between the values measured by both methods ($p > 0.05$).

Fig. 3, B. shows the residual tissue Ca contents of the rabbit thoracic aorta calculated from the effluxed Ca²⁺ concentration measured by the Ca²⁺ electrode. The residual tissue Ca content was much larger in the thoracic aorta than that in the taenia coli at all incubation times (Fig. 3, A, B).

The residual tissue Ca contents of the taenia coli and the thoracic aorta measured by atomic absorption spectrophotometry after exposure to the Ca²⁺-free, La Tris-solution for 10, 20, 30, 40,

FIG. 4. The Effect of the Ca²⁺-free Tris-solution on 10⁻⁵ M Noradrenaline-induced Contraction of Rabbit Thoracic Aorta (filled circles) and 10⁻⁵ M Carbachol-induced Contraction of Guinea Pig Taenia Coli (open circles)

Each value is expressed as a percentage of the control response obtained in the normal Tris-solution. Each point represents the average of 5 experiments and vertical bars indicate standard error of the mean.

50 and 60 min, are also shown in Fig. 3. The exposure to the Ca^{2+} -free, La Tris-solution for 10 min induced a rapid decrease in the tissue Ca content and a gradual decrease in the tissue Ca content with an increase in the time of the exposure.

The effects of Ca^{2+} depletion in the medium on the noradrenaline (10^{-5} M)-induced contraction of the rabbit thoracic aorta and the carbachol (10^{-5} M)-induced contraction of the guinea pig taenia coli are shown in Fig. 4. The noradrenaline-induced contractions of the rabbit thoracic aorta showed a higher resistance to Ca^{2+} depletion in the medium compared with the carbachol-induced contractions of the guinea pig taenia coli.

DISCUSSION

Calcium movements and distribution in smooth muscle have usually been studied by using radioisotope techniques and atomic absorption spectrophotometry. These methods are not suitable for continuous measurement of free calcium ions. Recently, a method using a Ca^{2+} -selective electrode has been described in the biological membrane systems.¹⁻⁷⁾ By using this method the Ca^{2+} movement was continuously monitored. Since a Ca^{2+} -selective electrode has not been utilized for determination of calcium ions effluxed from smooth muscle so far, we continuously monitored the Ca^{2+} effluxed from the guinea pig taenia coli and the rabbit thoracic aorta into a Ca^{2+} -free physiological solution by using a Radiometer Calcium Ion Selectrode and compared this method with the method using atomic absorption spectrophotometry.

Under the present experimental conditions, the Ca^{2+} concentrations effluxed from the tissue into a Ca^{2+} -free medium (5 ml) for 60 min were approximately 10^{-5} M (approximately 6.00 mmol Ca^{2+} /kg dry wt in Fig. 1). The Ca^{2+} electrode responded linearly to a continuous increase in Ca^{2+} concentration in the Ca^{2+} -free Tris-solution at concentrations over 2×10^{-6} to 10^{-5} M and a significant difference in the responses of the Ca^{2+} electrode to the continuously increased Ca^{2+} concentration was not observed at the constant infusion rates of 0.3×10^{-6} and 0.6×10^{-6} M/min.⁹⁾ In

the present observation, after incubating the taenia coli in the Ca^{2+} -free Tris-solution for 10 and 30 min, there were no significant differences between the residual tissue Ca contents calculated from the effluxed amounts of Ca^{2+} measured by the Ca^{2+} electrode (Fig. 3A) and those obtained by atomic absorption spectrophotometry (Fig. 2). These results indicate that the Ca^{2+} electrode used here could accurately respond to an increase in Ca^{2+} concentration in the medium and that Ca^{2+} concentration effluxed from the smooth muscle into a Ca^{2+} -free medium could be continuously monitored by the Ca^{2+} electrode easily at micromolar levels.

The Ca^{2+} effluxed from the rabbit thoracic aorta into the Ca^{2+} -free Tris-solution during 60-min incubation was significantly greater than that from the guinea pig taenia coli ($p < 0.005$) (Fig. 1) and the residual tissue Ca measured by atomic absorption spectrophotometry after determination of Ca^{2+} efflux during 60-min incubation was much greater in the thoracic aorta than that in the taenia coli ($p < 0.005$). Both the residual tissue Ca content which was not depleted into the Ca^{2+} -free Tris-solution and the content calculated from the effluxed amounts of Ca^{2+} measured by the Ca^{2+} electrode were much larger in the thoracic aorta compared with those in the taenia coli at all incubation times (Fig. 3A, B). Thus, the Ca^{2+} electrode used here was able to differentiate Ca^{2+} effluxes from different tissue into a Ca^{2+} -free physiological solution.

We tried to observe the effect of La^{3+} on Ca^{2+} effluxes into the Ca^{2+} -free Tris-solution by the Ca^{2+} electrode, but failed because of La^{3+} interference for the Ca^{2+} electrode. Therefore, the residual tissue Ca contents after exposure to the Ca^{2+} -free, La Tris-solution during the selected periods were measured by atomic absorption spectrophotometry (Fig. 2, 3), and were compared with the residual tissue Ca contents calculated from the effluxed amounts of Ca^{2+} obtained by the Ca^{2+} electrode (Fig. 3). The exposure to the Ca^{2+} -free, La Tris-solution caused a rapid decrease in the tissue Ca content within 10 min and thereafter a gradual decrease in the Ca content

was observed in both the taenia coli and the thoracic aorta. In addition, the residual tissue Ca after incubation in the Ca^{2+} -free, La Tris-solution was much smaller than that in the Ca^{2+} -free Tris-solution at all incubation times (Fig. 3) and the Ca fraction which was not depleted into the Ca^{2+} -free Tris-solution but displaced by La^{3+} was much larger in the thoracic aorta compared with that in the taenia coli. It is likely that such a Ca fraction may be derived from the membrane bound Ca fraction, since it has been suggested that the primary action of La^{3+} is the displacement of Ca^{2+} at the superficial membrane Ca binding sites.¹⁰⁻¹²⁾ These results indicate that the rabbit thoracic aorta has a large membrane bound Ca fraction which is not depleted into the Ca^{2+} -free Tris-solution compared with the guinea pig taenia coli. Our results were in agreement with the suggestion that vascular smooth muscle has a large bound Ca fraction which is not readily depleted.¹³⁾

It has been reported that contractions of many types of smooth muscle caused by acetylcholine and noradrenaline seem to depend mostly on intracellular Ca stores or on both intracellular and extracellular Ca.^{10,14-19)} In a Ca^{2+} -free medium, these drug-induced contractions become diminished with time.^{17,20)} In the present study, we observed that in the aortic strips after exposure to the Ca^{2+} -free Tris-solution for 10 and 30 min, 10^{-5} M noradrenaline-induced contractions were 90.6 ± 3.8 ($n=5$) and 84.0 ± 3.1 ($n=5$) % of the control response obtained in the normal Tris-solution, respectively (Fig. 4). However, in the taenia coli after incubation in the Ca^{2+} -free Tris-solution for the same period of time, contractions induced by 10^{-5} M carbachol were 26.4 ± 2.5 ($n=5$) and 5.7 ± 0.7 ($n=5$) % of the control response, respectively (Fig. 4). The rabbit thoracic aorta showed a higher resistance not only to the depletion in tissue Ca but also to the noradrenaline-induced contractions in the Ca^{2+} -free Tris-solution compared with the taenia coli. Since Farley and Milles²⁰⁾ have indicated by using the dog tracheal smooth muscle that the decline in the acetylcholine-induced contraction which occurs in a Ca^{2+} -free medium is related to the Ca^{2+}

efflux from the muscle into a Ca^{2+} -free medium, it seems that most of the noradrenaline-induced releasable Ca which activates contractile elements to produce a contraction is not depleted into the Ca^{2+} -free Tris-solution by 30-min incubation. However, since the carbachol-induced contractions of the guinea pig taenia coli were less resistant to the exposure to the Ca^{2+} -free Tris-solution (Fig. 4) and were completely abolished after incubation in the normal Tris-solution containing 10 mM LaCl_3 for 10 min (not shown), it may be deduced that Ca related to the carbachol-induced contraction can be readily displaced by La^{3+} and depleted into the Ca^{2+} -free Tris-solution for a long exposure.

In summary, the Ca^{2+} -sensitive electrode used here may be a useful tool to investigate free calcium ions effluxed from the smooth muscle into a Ca^{2+} -free medium and can continuously and easily monitor free calcium ions effluxed from the smooth muscle into a Ca^{2+} -free medium at micromolar levels. Furthermore, the Ca^{2+} electrode may be useful to investigate the effects of drugs on the membrane bound Ca fraction which is not readily depleted into a Ca^{2+} -free medium. Usefulness of the Ca^{2+} electrode for a study of the effects of drugs on the Ca fraction which is not readily depleted into the Ca^{2+} -free medium is now under investigation.

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