STUDIES ON ISOLATED SMOOTH MUSCLE CELLS. VIII. ^{45}Ca -EFFLUX FROM SINGLE SMOOTH MUSCLE CELLS ISOLATED FROM TAENIA COLI OF GUINEA PIG

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^{45}Ca -loaded single smooth muscle cells were prepared from taenia coli of guinea pig. The cells were perfused continuously with physiological salt solution on membrane filter with pore size of $12\ \mu\text{m}$ and radioactivity in the effluent was determined. The ^{45}Ca -efflux curves consisted of two straight lines in semilogarithmic figure representing loosely and tightly bound calcium compartments with half time of 1.87 ± 0.27 and 6.78 ± 0.43 min, respectively. The loosely bound calcium was supposed to be localized on plasma membrane. Size of the tightly bound ^{45}Ca was dependent on period for calcium-depletion of the preparation prior to ^{45}Ca -loading. The efflux was stimulated by acetylcholine and the stimulated efflux returned to the spontaneous efflux level instantly after removal of acetylcholine. This stimulation was abolished by atropine. Repetitive stimulation failed to increase ^{45}Ca -efflux while the rate of the spontaneous ^{45}Ca -efflux did not change significantly. These results suggested that, in taenia coli of guinea pig, at least three calcium compartments existed, loosely bound calcium, tightly bound calcium and more tightly bound calcium which can be released by acetylcholine, in addition to calcium in extracellular fluid. Advantages and disadvantages of using single smooth muscle cells for investigation of calcium movement and some technical improvement for determination of ^{45}Ca -efflux from single cells were described.

Keywords — single cell; single smooth muscle cell; taenia coli; ^{45}Ca -efflux; acetylcholine

INTRODUCTION

It is well known that calcium ions participate contraction of smooth muscle. When the muscle is excited by some stimulants, calcium ions are released from storage site(s) located inside and/or outside the muscular cells and contributes to the tension development. The extracellular calcium can be classified into two categories, calcium in extracellular fluid and superficial calcium. The intracellular calcium is also classified, according to the exchangeability, such as loosely and tightly bound calcium. Which calcium contributes to the tension development has been a major problem in smooth muscle physiology and pharmacology.

To distinguish those calcium, various techniques have been introduced. One of the most

widely applied techniques is lanthanum method introduced by van Breemen¹⁾ and Weiss and Goodman.²⁾ However, the method is not proper to follow alternation of calcium content continuously in the same preparation. Elimination of calcium from the incubation medium is a way to determine relative contribution of intracellular calcium. But, muscles exposed to calcium-free medium are physiologically abnormal and leakage of the intracellularly sequestered calcium might be possible. Quantitative determination of radioactive calcium-efflux during excitation of smooth muscle is useful to investigate contribution of intracellular calcium to the tension development. However, as far as the whole tissue is used, amount of ^{45}Ca -efflux is unable to be determined precisely because calcium effluxed

from the muscular cells can be trapped by extracellular spaces. To eliminate the effect of extracellular spaces, introduction of dispersed cells is preferable.

We have been investigating pharmacological properties of single smooth muscle cells isolated from taenia coli of guinea pig and reported that the preparation was responsive to various agonists such as acetylcholine and sensitivity to those agents were not altered during the isolation procedure.^{3,4)}

Since the preparation retains several properties of the intact tissue, we attempted to use the dispersed cells for investigation of calcium movement. In this paper, we presented spontaneous and acetylcholine-stimulated ^{45}Ca -efflux under various conditions and discussed a possible calcium origin to be released by acetylcholine. We also described advantage and disadvantage of using dispersed smooth muscle cells and some technical improvement to determine calcium-efflux. These results are in the preliminary presentation.⁵⁾

MATERIALS AND METHODS

Medium—Physiological salt solution was prepared as follows: 137 mmol sodium chloride, 2.7 mmol potassium chloride, 1.8 mmol calcium chloride, 1.0 mmol magnesium chloride, 5.6 mmol glucose and 4.2 mmol HEPES were dissolved in 950 ml of deionized and glass-distilled water. After equilibration with air and pH adjustment to 7.4 with sodium hydroxide, the volume was made up to 1.0 l. The same medium was used for preparation and perfusion of ^{45}Ca -loaded single cells and contraction of intact tissue. Where indicated, calcium-free medium was used. The medium was prepared in the same manner except no addition of calcium, but it contained approximately $2\ \mu\text{M}\ \text{Ca}^{2+}$ which was contaminated in the distilled water.

Isolation of Taenia coli from Guinea Pig—Male guinea pig, Hartley, 300–400 g, were killed by cervical dislocation and two strips of taenia coli were carefully isolated. The tissue was immediately immersed in the incubation

medium at 35°C . The following experiments were carried out at 35°C except centrifugation of cells at room temperature ($20\text{--}25^\circ\text{C}$).

Contraction of Whole Tissue by Acetylcholine—The isolated tissue was mounted in organ bath filled with 10 ml of the incubation medium. Contraction induced by acetylcholine was recorded by isotonic transducer (TD-112S, Nihon Kohden Ltd.) and recorder (MC6602,

FIG. 1. Preparation of ^{45}Ca -loaded Single Smooth Muscle Cells from Taenia coli of Guinea Pig
Experiment was carried out at 35°C except centrifugation of cells at room temperature ($20\text{--}25^\circ\text{C}$).

Watanabe Instrument Co.).

Preparation of ⁴⁵Ca-loaded Single Smooth Muscle Cells — The ⁴⁵Ca-loaded cells were prepared as illustrated in Fig. 1. This procedure yielded 5–10 × 10³ cells from two strips of the tissue (3–4 cm long, each). Although those cells were morphologically intact, a half of the cells were not responsive to acetylcholine. In this experiment, ⁴⁵Ca was loaded during the isolation procedure to avoid fragility of dispersed cells by suspending in calcium-free medium.

Determination of ⁴⁵Ca-efflux — Suspension of ⁴⁵Ca-loaded smooth muscle cells was placed on membrane filter (Nuclepore) mounted in a holder and the medium was removed by gentle suction. Then, 1.0 ml of perfusion medium was passed twice through the membrane filter (diameter: 25 mm) to wash the cells. The holder was immediately fixed to perfusion apparatus as shown in Fig. 2 and perfusion started.

The cells were perfused continuously at the rate of 1.0 ml/min and the effluent was collected directly into counting vials. Acetylcholine was

dissolved in the perfusion medium and administered to the cell preparation. The cells were exposed to acetylcholine for 15 s at each administration by operating the 3-way stopcock. Atropine was administered throughout the perfusion.

Prior to perfusion, the membrane filters were mounted in the holder and washed by perfusion at 35°C with distilled water for 60 min and then with the perfusion medium for 30 min.

Radioactivity in the effluent was determined by using scintillation spectrometer along with scintillation cocktail (toluene, PPO, POPOP and Triton X-100).

Materials — Collagenase (type I) and bovine plasma albumin (fraction V) were purchased from Sigma. Acetylcholine chloride was obtained from Daiichi Pharmaceutical Co. and atropine sulfate was the product of Wako Pure Chemical Ind. Radioactive calcium was obtained from Amersham (2 mCi/105 μg Ca). Other chemicals were guarantee grade. Nuclepore (N1200 RC02500) was purchased from Nomura Micro Science.

RESULTS

Single Smooth Muscle Cells on Membrane Filter

The dispersed cells were spindle-shaped and

FIG. 2. Apparatus for Perfusion of Single Cells

⁴⁵Ca-loaded cells were placed on membrane filter and washed twice with 1.0 ml perfusion medium. Then the cells were perfused continuously with the medium (1.0 ml/min) at 35°C and the effluent was collected into counting vials directly for determination of radioactivities. Agonist and antagonist were administered by operating the 3-way stopcocks.

FIG. 3. Phase Contrast Micrograms of Single Smooth Muscle Cells Placed on Nuclepore

The cells were perfused for 10 min on the filter under the same condition as shown in Fig. 2. Bar indicates 50 μm.

FIG. 4. ^{45}Ca -efflux from ^{45}Ca -loaded Single Smooth Muscle Cells

^{45}Ca was loaded as shown in Fig. 1. Prior to ^{45}Ca -loading, the preparations were exposed to calcium-free medium for 30 min (—●—) or 90 min (—○—). The preparations were perfused continuously and radioactivity in the effluent was determined.

length of the cells was less than 100 μm when suspended in the physiological saline solution. In addition to the dispersed cells, the cell suspension contained large amount of cell fragments. These fragments were easily eliminated by washing the cells on the filter and morphologically intact cells remained as shown in Fig. 3.

Perfusion of the cells for more than 60 min did not change the cells morphologically under the condition mentioned above.

^{45}Ca -efflux from Single Smooth Muscle Cells

^{45}Ca -efflux curves obtained from the single cells consisted of two straight lines in semilogarithmic figure; fast phase ($T_{1/2} = 1.87 \pm 0.27$ min, $n=7$) and slow phase ($T_{1/2} = 6.78 \pm 0.43$ min, $n=7$) as shown in Fig. 4. Turning points of the efflux curves were dependent on conditions of ^{45}Ca -loading. When the preparation was exposed to calcium-free medium for 30 min prior to ^{45}Ca -loading, the point appeared 8–9 min after the perfusion started, whereas it appeared

FIG. 5. Stimulation of ^{45}Ca -efflux by Acetylcholine

^{45}Ca -loaded cells were perfused as described in Fig. 4. Prior to ^{45}Ca -loading, the preparation was exposed to calcium-free medium for 90 min. Cells were exposed to perfusate containing 10^{-6} or 10^{-3} M acetylcholine for 15 s.

A; 10^{-3} M acetylcholine was administered after the stimulated efflux curve by 10^{-6} M acetylcholine returned to the original level. B; 10^{-3} M acetylcholine was administered during the declining phase of increased efflux induced by 10^{-6} M acetylcholine.

after 4 min when the preparation was exposed to calcium-free medium for 90 min. Although the turning points varied according to the preincubation time, half-times of the fast and slow phases were not variable. Prolonged perfusion did not reveal much slower phase of the ^{45}Ca -efflux.

Effect of Acetylcholine on ^{45}Ca -efflux

^{45}Ca -loaded single smooth muscle cells were prepared after 90 min exposure to calcium-free medium and effect of acetylcholine on the ^{45}Ca -efflux was examined.

Administration of 10^{-6} M acetylcholine increased the efflux quickly and significantly but magnitude of the increase in the efflux varied with preparations. The stimulated efflux re-

turned instantly to spontaneous efflux level after removal of acetylcholine. Neither significant decrease in the efflux nor change in slope of the efflux curve was observed after application of acetylcholine. Subsequent addition of 10^{-6} M acetylcholine, 5 min after the previous stimulation, did not increase ^{45}Ca -efflux, while the first stimulation at this point (10 min after the perfusion started) increased the efflux of ^{45}Ca (data is not shown). A higher concentration of acetylcholine, 10^{-3} M, was examined but the increase in ^{45}Ca -efflux was much smaller than that induced by the first stimulation as shown in Fig. 5A. Stimulation during the declining phase of the increased ^{45}Ca -efflux resulted in a slight increase in the ^{45}Ca -efflux as shown in Fig. 5B. The effect of the third stimulation was negligible. False administration, operating 3-way stopcock to add perfusion medium without acetylcholine, did not increase the ^{45}Ca -efflux.

Effect of atropine on the increase in stimulated efflux by acetylcholine was examined. Since

FIG. 6. Effect of Atropine on the Increase in ^{45}Ca -efflux induced by Acetylcholine

The ^{45}Ca -loaded cell suspension was divided into two fractions. Both were placed on the membrane filter and perfused with (solid circle) or without (open circle) 1×10^{-6} M atropine sulfate. Acetylcholine was added as indicated by arrows.

FIG. 7. Contraction of *Taenia coli* of Guinea Pig by Acetylcholine in Calcium-free Medium

The intact tissue was suspended in organ bath filled with 10 ml physiological saline solution. Acetylcholine, 10^{-2} M, was added every 15 min until response became constant. Then the medium was replaced by calcium-free medium and response to acetylcholine was determined. The medium was replaced by fresh medium every 5 min.

the repetitive administration of acetylcholine did not induce reproducible increase in the ^{45}Ca -efflux, a paired test was carried out. The cell preparation was divided into two fractions and both were perfused simultaneously with and without 10^{-6} M atropine. As shown in Fig. 6, atropine abolished increase in the ^{45}Ca -efflux induced by acetylcholine. While the degree of increase varied in each preparation, atropine inhibited it almost completely in every preparation.

Contractile Response of Whole Tissue to Acetylcholine in Calcium-free Medium

Influence of calcium-depletion on contractile response of whole tissue to acetylcholine was examined. The degree of response in 10^{-2} M acetylcholine, which induced maximum response in normal medium containing 1.8 mM Ca^{2+} , became 68, 45 and 5% of normal response, 15, 30 and 45 min after the incubation medium was replaced by calcium-free medium, respectively (Fig. 7).

DISCUSSION

In the preliminary experiment, we used the membrane filter made of teflon fibers.⁶⁾ As previously reported, the single cell preparation was accompanied by a large amount of cell fragments which were unable to be eliminated by ordinary technique such as centrifugation.⁷⁾ These fragments were trapped in the intertwined fibers of the filter resulting in difficulties in the perfusion. Besides, cell fragments leak phosphate ions which precipitate with calcium resulting in variable ^{45}Ca -efflux.⁸⁾ To avoid these problems, we used Nuclepore with pore size of 12 μm which permitted the fragments to pass through but trapped intact cells.

Calcium-efflux into calcium-free medium can be determined by the calcium-sensitive electrode without using the hazardous radioactive calcium.^{9,10)} However, since the single cells were fragile in calcium-free medium, we determined ^{45}Ca -efflux into normal medium containing physiological concentration of calcium. Thus, the adsorption of radioactive calcium to the

membrane filters, holders, tubings and other equipments could be negligible.¹¹⁾

The ^{45}Ca -efflux curves consisted of two straight lines, suggesting the existence of two ^{45}Ca compartments, loosely and tightly bound calcium. There are several papers concerning ^{45}Ca -efflux from intact tissue of taenia coli.^{8,12,13)} By comparing these curves, it was found that ^{45}Ca -efflux from single cells showed sharp turning points. Since extracellular spaces were eliminated in this preparation, ^{45}Ca -efflux from the muscle cells could be detected directly.

Karaki and Urakawa¹⁴⁾ reported that the exchange of calcium in taenia coli of guinea pig consisted of three components: initial rapid component, the second component with half-time of approximately 2 min and the third component with half-time of 7 min. Half-times of the second and the third components were consistent with our results, while the initial rapid component was not observed in our preparation. The rapid component was supposed to be completed during the washing of labelled preparation or, the rapidly exchangeable calcium originated from extracellular space might be undetectable with the dispersed cell preparation.

Chujo¹¹⁾ and Sekiguchi *et al.*¹⁵⁾ reported that a calcium components with half-times of less than 3 min were originated from extracellular spaces. Schatzman speculated that calcium exchanged with half-time of 3 min existed in extracellular spaces and was bound to connective tissues.⁹⁾ Thus the loosely bound calcium in single cell preparation might be located outside the dispersed cells. Scanning electron microscopic observation showed that the surface of single smooth muscle cells isolated from taenia coli of guinea pig was smooth and almost free from connective fibers.¹⁶⁾ Therefore, we suppose that these calcium ions bind to anionic sites on plasma membrane. Thus the loosely bound calcium might be correspond physiologically to the so called "superficial calcium".

The ^{45}Ca -efflux from single cells was increased by acetylcholine significantly. Since the stimulant was added 5 min after the perfusion

started, we assumed that the loosely bound calcium does not contribute to the increased ⁴⁵Ca-efflux induced by acetylcholine.

Nasu and Urakawa reported that every repetitive administration of acetylcholine to ⁴⁵Ca-loaded taenia coli increased ⁴⁵Ca-efflux.¹⁷⁾ In the present experiment, second stimulation was scarcely effective to increase ⁴⁵Ca-efflux. In the case of intact tissue, ⁴⁵Ca released could be trapped in the extracellular spaces and taken up to the binding sites. However, since the single cells were perfused continuously with physiological salt solution, ⁴⁵Ca released from the binding sites was eliminated and could not be taken up and released by the subsequent stimulation. It is also possible that a part of ⁴⁵Ca is shifted by acetylcholine to more slowly exchangeable compartment and these are not released by the following stimulation.¹⁴⁾

If the tightly bound calcium compartment is the primary target of acetylcholine, acetylcholine might decrease the subsequent ⁴⁵Ca-efflux since the initial stimulation depletes ⁴⁵Ca in the compartment. However, decrease in a spontaneous ⁴⁵Ca-efflux after stimulation was rare. Therefore, we assumed that the tightly bound calcium is different from that which contributed to stimulated increase in calcium exchange. There might be more tightly bound calcium which was difficult to be detected by measuring spontaneous exchange.

A half-time of decrease in maximum response of intact tissue in calcium-free medium is longer than 15 min. This half-time is longer than that of tightly bound calcium component (6.78 ± 0.43 min). Thus, the more tightly bound calcium might contribute to the development of muscle contraction.

Considering the present results, we concluded that, in taenia coli of guinea pig, there were at least four calcium compartments: calcium in the extracellular fluid, membrane bound calcium, tightly bound calcium and more tightly bound calcium which is released by acetylcholine.

As shown in the present paper, clear efflux curves and quick response to stimulant are

available with dispersed cells. Thus, the preparation is beneficial to investigate contribution of those calcium components to the tension development. However, there are some disadvantages with the single cell preparation, such as failure in the repetitive response. Besides, the magnitude of the increase in ⁴⁵Ca-efflux induced by acetylcholine varied with preparations. Taking a small aliquot from the cell suspension obtained by centrifugation¹⁸⁾ might be useful to measure the change in ⁴⁵Ca-efflux in the same preparation. But, the amount of single cell was not enough to apply this procedure. Low yield of the preparation is also to be improved.

A fact that some cells were not responsive to agonists³⁾ should be considered when the single smooth muscle cells were examined as the mass. It has been assumed that some smooth muscle cells in the tissue are innervated by autonomic nerves, but others are not.¹⁹⁾ And the latter cells are contracted by electrical stimuli propagated from adjacent cells.^{3,19)} Preparation of physiologically homogeneous cells with large scale is required to proceed investigation of calcium movement.

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