Ca Flux and Action Potential in Smooth Muscle of Guinea-pig Taenia Coli

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NAGASAWA, J. and SUZUKI, T. *Ca Flux and Action Potential in Smooth Muscle of Guinea-pig Taenia Coli*. Tohoku J. exp. Med., 1970, **102**(1), 1-12. ——— Ca uptake and efflux in the smooth muscle cell of guinea-pig taenia coli were estimated in the normal Krebs solution and in Krebs solution containing MnCl_2 . MnCl_2 had no significant effect on the efflux of ^{45}Ca in the concentration range between 1.0 mM and 20 mM. In the presence of 1 mM MnCl_2 , adrenaline (up to 2.5×10^{-5} g/ml) and tetrodotoxin (up to 2.5×10^{-3} g/ml) had no significant effect on Ca efflux. But acetylcholine (up to 2.5×10^{-3} g/ml) was found to increase the ^{45}Ca efflux coincidentally with the tension development. Ca influx in normal NaCl Krebs solution was estimated to be 0.135 p-mole/cm²·sec, and it was increased by 1.5 to 2 times in Na free sucrose Krebs solution. About 15% of total Ca influx was suppressed by 1.0 mM MnCl_2 . This Ca content corresponding to 0.022 p-mole/cm²·sec was tentatively attributed to the extra entry of Ca by the generation of action potential. But this amount proved to correspond to only 2% of the theoretically calculated necessary amount of divalent cation inflow accompanying the generation of action potential. ——— smooth muscle; calcium flux; manganese

It is well known that the generation of action potential in the smooth muscle is not strictly dependent on the Na concentration of the external fluid,^{1,2} and tetrodotoxin (TTX), which is known to be a specific Na spike inhibitor in many excitable tissues,³ cannot stop the generation of action potential in the smooth muscle.⁴

On the other hand, Mn ion which is known to be an inhibitor of the Ca spike in the crustacean and barnacle muscles^{5,6} also inhibits the action potential of the smooth muscle of guinea-pig taenia coli⁷. Such evidence strongly suggests that action potential of the taenia coli is generated by the influx of Ca.

The direct way to prove this possibility is to show the increment of the intracellular Ca as a result of the evolution of action potentials. But there are many difficulties in conducting such experiments; the major one is the presence of spontaneous action potentials in this tissue. Their irregular occurrence made it almost impossible to get the so-called resting flux of the taenia coli.

As mentioned above, application of a low concentration of Mn ion could stop the action potential of taenia coli completely without causing any appreciable

change in the resting potential, and this Mn ion effect is reversible as far as its concentration is low and the application time is not too long.^{7,21,22}

So it seems possible to compare the resting Ca influx and the extra entry of Ca accompanying the generation of action potential using the Mn ion as a tool, if it could be assumed that Mn ion in a low concentration exerts no deleterious influences on the membrane characteristics and the metabolism of the smooth muscle cells.

In this study, Ca uptake in normal Krebs solution and in the Krebs solution containing 1 mM MnCl_2 was estimated, and Ca influx accompanying evolution of action potentials was tentatively calculated as a difference between them. Changes in the rate of efflux of ^{45}Ca by Mn and Zn ions and by certain drugs in the presence of 1 mM MnCl_2 were also studied.

METHODS

Guinea-pigs weighing 300 to 500 g were stunned and bled, and six to eight pieces of taenia coli were dissected. After the taenia strips were mounted on a stainless-steel spring to give a tension of approximately 2 g, they were equilibrated with fully oxygenated modified Krebs solution at 37°C for at least 1 hour before starting the experiments.

Modified Krebs solution had the following composition (mM): NaCl 120.7, KCl 5.9, CaCl_2 2.5, MgCl_2 1.2, NaHCO_3 15.5 and glucose 11.8; and was bubbled with a gas mixture of 95% O_2 and 5% CO_2 . The pH of the solution was kept at 7.4. Phosphate ion was omitted in this experiment, since it easily made precipitates with Ca or Mn ions.⁸

^{45}Ca (specific activity 8.8 mCi/mg Ca) was supplied from Nuclear Science & Engineering Corporation (U.S.A.). The final concentration of ^{45}Ca in the loading solution was between 20 and 25 $\mu\text{Ci/ml}$.

Efflux experiments

After 1 hour's equilibration in normal Krebs solution, taenia strips were transferred into ^{45}Ca Krebs solution, which had the same chemical composition as normal Krebs solution. After 2 hours' loading in ^{45}Ca solution, they were re-mounted on freshly prepared stainless-steel springs to avoid the contamination by the ^{45}Ca adsorbed to the loading springs.

Then taenia strips were washed out for 30 min in nonradioactive Krebs solution before starting the serial collection of effluent. Serial washing was performed with passing taenia strips every 5 minutes through a series of test-tubes containing 2 ml of washing Krebs solution.

Normal Krebs solution was used as a washing solution in Mn and Zn experiments. In other experiments, washing solution was Krebs solution containing 1 mM MnCl_2 to suppress the spontaneous activity of the taenia strip. The effect of drugs on the rate of loss of ^{45}Ca was tested including a suitable concentration in two successive test-tubes.

Radioactivity of the effluents and the taenia strips at the end of experiments were counted by a gas-flow counter with automatic sample changer (Aloka, Japan).

From the quantity of radioactivity which remained in the muscle at the end of experiment and the amount lost into each tube, ^{45}Ca content of the muscle at any time could be calculated. The rate of efflux was expressed as the proportion of the tracer lost in unit time.

Uptake experiments

Six to eight portions of taenia coli from the same guinea-pig as equal in weight and size as possible were separated into two groups. They were mounted on a stainless-steel

spring and equilibrated 2 hr in normal Krebs solution. Then they were transferred into ^{45}Ca loading solution; one into the control, and the other into a loading solution containing 1 mM MnCl_2 .

Normal loading solution had the same chemical composition as normal Krebs solution. In sucrose loading solution, total NaCl of the normal Krebs solution was replaced by 275 mM sucrose, and KCl concentration was raised to 11.8 mM in order to maintain the spike generation in Na free media.⁷

Loading period was exactly 15 min, and then the strips were passed through a series of washing Krebs solution which contained 1 mM MnCl_2 besides normal ionic composition.

Loss of ^{45}Ca was followed as in the efflux experiments. The amount of Ca taken up by the muscle during bathing in control and in 1 mM MnCl_2 loading solution was then compared by the intercepts of the extrapolation of the curves to zero-time. The detail will be described in the text.

RESULTS

Effect of Mn ion on the efflux of ^{45}Ca

As is well known, wash-out curve of ^{45}Ca loaded in the taenia coli is composed roughly of the sum of two exponentials. The fast phase is mainly concerned with loss of the tracer present in the extracellular space.⁹⁻¹²

To avoid the influence of this fast phase, all the efflux experiments were carried out after 30 min preliminary washing in inactive solution. And the wash-out curves of the present experiments were considered to express mainly the movement of intracellular ^{45}Ca .

Mn had no significant effect on the efflux of ^{45}Ca in the concentration range between 1.0 mM and 20 mM. Fig. 1 shows the effect of 1.0 mM Mn on the loss of Ca. In this figure, the rate coefficient for Ca loss seemed to decrease slightly just after the application of 1 mM MnCl_2 , but this proved to be not significant by the statistical analysis owing to the large standard errors of the results as shown in the figure.

With 5 mM MnCl_2 , a curve almost identical with that in Fig. 1 was obtained. Fig. 2 shows the effect of 20 mM MnCl_2 ; such high concentration had also no effect on the rate of efflux.

A transient dominant increase in the rate of efflux was observed in common, when the taenia strips were returned from the test solution to the normal washing solution. The same tendency was observed in the case of 1 mM ZnCl_2 (Fig. 3).

In sucrose gap recording, return to normal solution from 1 mM Mn solution was sometimes accompanied by a transient increase of action potentials. Except for this, a corresponding change with the increase of ^{45}Ca efflux was observed neither in the frequency of the action potentials nor in the tension development. And this transient increase of ^{45}Ca was considered to be due to the change in the physicochemical properties of the bathing solution.

Effect of Zn ion on the efflux of ^{45}Ca

Zn ion in a low concentration is also known to stop the action potentials and tension development in the taenia coli. Its effect is much stronger than Mn and is

Fig. 1. Effect of 1.0 mM MnCl_2 on the rate of loss of ^{45}Ca from taenia strips bathed in the normal Krebs solution. Each point represents mean value $\pm$ s.e. from 10 preparations. Ordinate, rate of loss of ^{45}Ca : Abscissa, time after beginning of the serial washing.

Fig. 2. Effect of 20 mM MnCl_2 on the rate of efflux of ^{45}Ca . 20 mM MnCl_2 was applied twice in the course of the experiment. Each point represents mean value $\pm$ s.e. from 7 preparations. Plotted as in Fig. 1.

irreversible.^{21,22}

Fig. 3 shows the effect of 1 mM ZnCl_2 on the efflux of ^{45}Ca . Owing to the large variance of the results, the slight increase in the rate of efflux in ZnCl_2 solution proved to be statistically not significant.

Effect of acetylcholine, adrenaline and TTX on the efflux of ^{45}Ca

In a previous paper, one of the authors (N.) reported the increase of ^{45}Ca efflux by adrenaline, but not consistent changes by acetylcholine (Ach).¹⁰ These experiments had been carried out in the normal Krebs solution where spontaneous contractions vigorously occurred and might seriously affect the results. Now, similar experiments were performed in the presence of 1 mM MnCl_2 to avoid the effect of spontaneous contraction.

Fig. 3. Effect of 1 mM ZnCl_2 on the rate of efflux of ^{45}Ca . Each point represents mean $\pm$ S.E. from 10 preparations. Plotted as in Fig. 1.

Fig. 4. Effect of acetylcholine (first time 2.5×10^{-4} g/ml; second time 2.5×10^{-3} g/ml) on the rate of efflux of ^{45}Ca from taenia strips bathed in the Krebs solution containing 1 mM MnCl_2 . Each point represents mean $\pm$ S.E. from 7 preparations.

In Krebs solution containing 1 mM MnCl_2 , Ach at lower than 1×10^{-5} g/ml had no effect, but at concentrations higher than 1×10^{-4} g/ml it consistently increased the rate of efflux of ^{45}Ca (Fig. 4). The threshold concentration of Ach for the tension development was also found to rise in the concentration range of 1×10^{-5} to 1×10^{-4} g/ml in the presence of 1 mM MnCl_2 . Thus the increase of ^{45}Ca efflux by Ach seemed to occur concomitantly with the tension development. In contrast to the previous experiments in normal Krebs solution, adrenaline caused no significant change in the rate of efflux of ^{45}Ca in the presence of MnCl_2 (Fig. 5).

Effect of TTX on the efflux of ^{45}Ca was also tested (Fig. 6). Even a very high dose of TTX (up to 2.5×10^{-3} g/ml) had no significant effect on ^{45}Ca efflux. The result is consistent with the current view that TTX affects solely Na influx accompanying the generation of action potentials. Recently it was reported that

Fig. 5. Effect of adrenaline (first time 2.5×10^{-6} g/ml, second time 2.5×10^{-5} g/ml) on the rate of efflux of ^{45}Ca from 5 taenia strips. Experimental procedures as in the legend for Fig. 4.

Fig. 6. Effect of tetrodotoxin (first time 2.5×10^{-4} g/ml, second time 2.5×10^{-3} g/ml) on the rate of efflux of ^{45}Ca . Each point represents mean $\pm$ S.E. from 12 preparations.

TTX (10^{-5} M) had no influence on the rate of ^{45}Ca efflux from the desheathed frog sciatic nerve preparation.¹³

Effect of MnCl_2 on the influx of ^{45}Ca

The plot of loss of ^{45}Ca from the taenia coli was composed of two exponential curves. The first phase is rapid and thought to represent the loss of ^{45}Ca from the extracellular space. The second phase is slow and assumed to correspond mainly to the exchange of intracellular ^{45}Ca . In some instances, a more slowly declining third component was observed well after 90 min of washing.⁹⁻¹¹ But its exact evaluation was impossible and was neglected in this experiment.

From the extrapolation of the linear portion of the second phase to zero-time axis, Ca content taken up during the load period of 15 min was calculated. Fig. 7. illustrates schematically the method of estimation.

In the early phase of the experiments, such curves were drawn for each

Fig. 7. Schematic representation illustrating the method of calculation of uptake of Ca during load period of 15 min. Two preparations from the same guinea-pig were loaded in separate loading solutions, one in control loading solution (open circles), the other in the loading solution containing 1 mM MnCl_2 besides normal ionic composition (solid circles). Extrapolation of the linear portion to 0-time gave the Ca content taken up by the muscle during the load period.

TABLE 1. *Half-time ($\text{min} \pm \text{S.E.}$) of the slow phase of ^{45}Ca loss from taenia coli*

	Control solution	1 mM MnCl_2 solution
Normal Krebs solution	$54.30 \pm 11.87 (15)$	$57.50 \pm 10.34 (15)$
Na free sucrose Krebs solution	$41.57 \pm 3.46 (16)$	$44.95 \pm 0.32 (17)$

Numerals in brackets indicate the number of observations.

TABLE 2. *Ca content taken up during 15 min soaking in control and 1 mM MnCl_2 containing normal Krebs solution ($\text{m-mole/Kg wet wt/15 min} \pm \text{S.E.}$)*

Exp. No.	Control solution	MnCl_2 solution	Rate of decrease in MnCl_2 solution
1	1.047 ± 0.041	0.734 ± 0.034	70.1%
2	0.651 ± 0.043	0.532 ± 0.036	81.6
3	0.490 ± 0.020	0.656 ± 0.035	110.9*
4	0.472 ± 0.041	0.384 ± 0.029	81.4
5	0.348 ± 0.008	0.300 ± 0.021	86.2
6	0.202 ± 0.009	0.172 ± 0.006	85.1
7	0.267 ± 0.018	0.203 ± 0.013	75.6
8	0.369 ± 0.025	0.308 ± 0.017	82.1
9	0.328 ± 0.004	0.300 ± 0.023	91.5
10	0.452 ± 0.005	0.340 ± 0.016	75.3
11	0.452 ± 0.005	0.418 ± 0.013	92.6
12	0.531 ± 0.014	0.489 ± 0.018	92.1
13	0.320 ± 0.012	0.315 ± 0.006	98.3
14	0.316 ± 0.005	0.249 ± 0.012	79.0

* This case was omitted from the calculation of means.

TABLE 3. *Mean Ca content taken up by control and $MnCl_2$ loading solution in normal and sucrose Krebs solution (m-mole/kg wet wt./15 min $\pm$ S.E.)*

	Control solution	$MnCl_2$ solution	Rate of decrease in $MnCl_2$ solution
Normal Krebs-R	$0.400 \pm 0.033 (14)$	0.386 ± 0.043	$83.91 \pm 2.16\%$
Sucrose-R (1)	$0.597 \pm 0.050 (8)$	0.557 ± 0.031	86.83 ± 2.23
Sucrose-R (2)	$0.768 \pm 0.056 (6)$	0.737 ± 0.068	86.43 ± 5.60

Numerals in brackets indicate the number of observations.

taenia strip. And half-time and the point of intercept were determined graphically. As it became clear that such a method was more seriously affected by the residual ^{45}Ca , only the residual ^{45}Ca at the end of experiment was counted exactly, and uptake of ^{45}Ca was calculated from the mean half-time obtained initially.

Table 1 shows the mean half-time of the decline of ^{45}Ca of the slow phase. There was a tendency toward prolongation of half-time in the preparations soaked in the loading solution containing 1 mM $MnCl_2$. But the difference was small and not significant statistically. Therefore, the calculation was based on the half-times of the control loading solutions in every case.

Definitely shorter half-times were obtained in the preparations loaded in Na free sucrose solution. This might suggest that the smooth muscle cell membrane or the Ca binding site in the smooth muscle received prolonged effects even in a short period of soaking in Na free solution.

Table 2 shows the Ca content taken up by the taenia strips in normal Krebs solution. Though the absolute values of the uptake varied from one animal to another, the ratio of the values in control loading solution to those in $MnCl_2$ loading solution were fairly constant. This ratio obtained from individual guinea-pigs were plotted in Fig. 8, and their mean values were summarized in Table 3 as the rate of decrease in $MnCl_2$ solution. Mean Ca contents taken up by control and $MnCl_2$ loading solution were also shown in the table.

As seen in these tables and Fig. 8, sucrose R-(1) showed the values obtained directly after the transfer of taenia strips into the sucrose Krebs loading solution, and sucrose R-(2) showed the values obtained after 30 min preliminary incubation in tracer free sucrose Krebs solution. The latter experiment was performed to see the Ca influx of taenia strips which are well equilibrated in Na free environment.

It is concluded from these results that uptake of Ca was in most instances suppressed by 1 mM $MnCl_2$ of both the loading solutions containing NaCl or sucrose. The average Ca uptake in normal NaCl Krebs solution was 0.4 m-mole/kg wet wt. and it was 1.5 to 2 times higher in the Na free sucrose solution. Uptake of Ca was suppressed to 85% of its control value when 1 mM $MnCl_2$ was present in the loading solution regardless of whether the loading solution contained NaCl or sucrose.

Fig. 8. Each point represents the ratio of Ca uptake in the taenia coli strips from the same guinea-pig and bathed in the control solution and MnCl_2 containing solution. Values obtained in the normal NaCl Krebs solutions are plotted on the left column as NaCl-Ringer. Sucrose-R (1) and sucrose-R (2) represent the values obtained in Na free sucrose Krebs solution. See text.

The amount of Ca suppressed by 1 mM MnCl_2 in NaCl loading solution was calculated to be $0.40 \times (1 - 0.84) = 0.064$ m-mole/kg wet wt./15 min, and it was 0.078 to 0.10 m-mole/kg wet wt./15 min in Na free sucrose solution.

DISCUSSION

The present experiments showed that Mn ion had no significant effect on the efflux of ^{45}Ca in the concentration range between 1.0 and 20 mM. It could be deduced from this result that any change in the uptake of ^{45}Ca caused by 1 mM MnCl_2 might be attributed directly to the change of Ca influx.

On the basis of this assumption, absolute values of Ca influx in the taenia coli muscle in normal and in 1 mM MnCl_2 solutions were calculated from the values of Table 3 as follows.

The surface area of smooth muscle cell membrane of 1 kg wet wt of taenia coli was calculated as $3.3 \times 10^6 \text{ cm}^2$, using the values of Goodford,^{8,15} tissue specific gravity 1.1 and extracellular space 450 ml/kg wet wt, and assuming that the smooth muscle cell is uniform cylinder of 6μ diameter. From this, total Ca influx in control NaCl solution was calculated as $0.135 \text{ p-mole/cm}^2 \cdot \text{sec}$ and the amount suppressed by 1 mM MnCl_2 as $0.022 \text{ p-mole/cm}^2 \cdot \text{sec}$.

The absolute size of the transmembrane fluxes of various cations in the smooth muscle are not known exactly comparing with other excitable cells. Goodford and Hermansen¹⁵ reported that in the taenia coli transmembrane flux of potassium is $2\text{--}4 \text{ p-mole/cm}^2 \cdot \text{sec}$ and that of sodium is some $200\text{--}300 \text{ p-mole/cm}^2 \cdot \text{sec}$. In the estrogen-dominated rabbit myometrium, Kao *et al.*¹⁶ estimated extra entry of ^{22}Na by the electrical stimulation, and found that Na influx accompanying genera-

tion of action potential was 7–8 p-mole/cm²·impulse.

Comparing with above data, the values of Ca influx obtained in the present experiments were rather low, but they were in good agreement with those of the Ca influx of giant axon of *Loligo* reported recently by Baker *et al.*¹⁷ But this agreement may be incidental, since total Ca influx of the taenia coli in normal solution is the sum of resting Ca influx and the extra entry associated with generation of action potentials.

Baker *et al.*¹⁷ further reported that Ca influx of 0.15 p-mole/cm²·sec estimated in 460 mM NaCl sea water increased 15–20 times in Na deficient lithium or dextrose sea water. On the other hand, Na free environment only increased Ca influx by 1.5 to 2 times in the present experiments. In regard to this, Goodford⁸ reported that average Ca content of the taenia coli is 3.0 m-mole/kg wet wt. *in vitro* and it rose by 1.1 m-mole/kg wet wt, when NaCl of the bathing solution was replaced by isotonic sucrose. The present result agrees fairly well to Goodford's data, if the allowance was made for the tissue bound Ca. In this respect, Ca influx of the taenia coli behaves rather differently from that of giant axon and heart muscle cells,¹⁸ when the external Na was replaced by sucrose or other cations.¹⁹

Assuming the membrane capacity of the taenia coli to be 3 μ F/cm² and the mean resting potential to be –50 mV, the electric charges necessary to reverse the potential by 20 mV at the height of an action potential would be some 2.1×10^{-7} coulomb/cm². And the minimum quantity of divalent cations which would be necessary to carry this charge was calculated to be 1.1 p-mole/cm²·impulse.

If it is assumed that Ca influx which was suppressed by Mn ion would express the amount of Ca influx accompanying generation of action potential, these two figures could be compared directly, since the average frequency of generation of action potentials in the taenia coli is about 1 per second. Thus the Ca influx which accompanies generation of action potentials determined by the present method occupied only 2% of the calculated divalent cation influx.

Of course, there are much uncertainties that make this comparison difficult. Some of them are taken into consideration as follows. 1) That adequate length of load period is yet unknown. In this experiment, a load period of 15 min was adopted based on the previous experiments^{9,10} but there is a question whether this is suitable for measuring the influx. One way to decide the adequate time is to perform similar experiments with various length of loading time and to compare the results. 2) Whether the Ca influx suppressed by 1 mM MnCl₂ could be duly ascribed to the Ca influx accompanying action potential. If a part of the resting flux is also depressed by Mn ion, extra entry of Ca accompanying action potential would be reduced to some degree as compared with the present calculation. 3) The present calculation based on the assumption that whole muscle cells in the taenia strip uniformly and synchronously generate action potentials when bathed in the normal solution and its frequency is constant. But this does not seem to be the case, but there is a strong possibility that only a fraction of the total smooth muscle cells generates action potentials at one time. But the magnitude of this

fraction is unknown, and if we assume that 1/2 or 1/3 of the total smooth muscle cells generate action potential at one time, the estimated Ca influx would be multiplied by the factor of 2 or 3 and it will become 4-6% of the necessary divalent cation inflow.

With all these assumptions and corrections, estimated Ca influx accompanying generation of action potentials in the taenia coli is still far less than the required amount that might be expected on the basis of theoretical calculation. Thus the present experiments could not give any substantial evidence to the Ca spike hypothesis of the smooth muscle.

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