CALCIUM SOURCE FOR CONTRACTILE RESPONSE OF GUINEA PIG TAENIA CAECUM TO CARBACHOL IN A CALCIUM DEFICIENT, POTASSIUM RICH SOLUTION

Nobuo SHIBATA, Hidenori OHASHI, Tadashi TAKEWAKI and Toshiaki OKADA

Department of Pharmacology, Faculty of Agriculture, Gifu University, Gifu 504, Japan

Accepted February 3, 1978

Abstract—The Ca storing site for the carbachol-induced contraction in a high-K medium without adding CaCl₂ was further investigated in the guinea pig taenia caecum. La³⁺ (0.05-0.5 mM) caused an increase in peak tension and relaxation rate in the carbachol contraction but reduced or abolished the response to CaCl₂. Simultaneous application of carbachol and CaCl₂ produced tension development the trace of which was comparable to the curve obtained by adding graphically the respective traces of the carbachol- and Ca-induced tensions. La³⁺ (0.5 mM) abolished the late, sustained component without appreciable change in the initial, transient contraction. The carbachol contraction persisted after 5 min perfusion of solution containing 0.25 mM EGTA (estimated free Ca²⁺ concentration less than 10⁻⁸ M). The intracellular Ca content was little changed before and after exposure to carbachol. Histamine induced a transient contraction with a dose-dependent amplitude and resulted in a decrease in tension development produced by subsequently applied carbachol. There was an inversely proportional relationship in amplitude between the histamine contraction and the following carbachol contraction, while the sum of tensions raised by both drugs showed little variation. These results favour an intracellular site for the carbachol-sensitive Ca store. Histamine appears to release Ca²⁺ from the carbachol-sensitive store.

The guinea pig taenia caecum incubated in a high K-medium retains its capacity to contract in response to carbachol in the virtual absence of external Ca²⁺ (1-3). The contractile capacity disappears gradually in such a Ca-deficient environment and is restored during incubation with Ca. The rates of disappearance and restoration are much slower than the rate of diffusion of Ca ions across the cell membrane which is inferred from the time course of tension development and relaxation following application and withdrawal of Ca (3). Calcium-antagonists such as La³⁺, Mn²⁺, verapamil and D-600, have no direct effect on the carbachol-sensitive Ca store but they do interfere with Ca entry into the store during incubation with Ca, causing depletion of Ca²⁺ (4). From these results, it has been suggested that the carbachol-sensitive Ca store may be located in more internal sites than the Ca-channels where the Ca-antagonists act.

The present study was undertaken to provide further support to the hypothesis. Lanthanum ions were used to block Ca entry into the carbachol-sensitive store, since these ions have been shown to be most potent in this effect (4).
MATERIALS AND METHODS

A segment of the taenia caecum (15 mm in length), isolated from guinea pigs of either sex which weighed 300 to 400 g, was suspended in an organ bath containing 5.0 ml of Tyrode solution bubbled with air at 37°C. After equilibration with the solution for about one hour, the muscle was transferred to a K-rich solution without adding CaCl₂ (hereafter referred to the Ca-free, K-Tyrode solution) which was bubbled with air and kept at 20°C. Isometric tension was measured using a mechano-electronic transducer (Nihon Kohden, SB-1T) and recorded by a potentiometric pen recorder (Hitachi, 056). The drugs and CaCl₂ were added to the bathing solution by rapid injection in a small volume (0.05 ml) of their concentrated solutions and the final required concentration was obtained. All concentrations in the present paper refer to the final concentration in the bathing medium. Concentrations of CaCl₂ and carbachol were 0.5 mM and 1 mM, respectively throughout the experiments, thus their description has been omitted. The effect of each drug was examined in more than six preparations.

Each preparation was deprived of the carbachol-sensitive Ca-component by exposing 1 mM carbachol for 3 min in the Ca-free, K-Tyrode solution and then suspended in this solution for 10 min. The Ca-deprived muscle was incubated in the Ca-containing, K-Tyrode solution for 10 min during which a tonic contraction developed as shown in Fig. 1. The tonic contraction hereafter refers to the Ca-contraction. The muscle was rinsed three times with the Ca-free, K-Tyrode solution and was left in this solution for 10 min. During this period, the muscle completely relaxed but restored its capacity to contract in response to carbachol (Ca-loaded taenia). The Ca-loaded taenia responded by a transient and rapid contraction on exposure to carbachol (see Fig. 1) (hereafter referred to as the carbachol contraction). When the carbachol contraction fell to the initial level (about three min after the carbachol addition) the muscle was again rinsed with the Ca-free, K-Tyrode solution and left in this solution for 10 min. By repeating the sequence of procedures, the amplitude of the Ca contracture and of the carbachol contraction virtually became constant. In this study, effects of the drugs on the fifth Ca-contraction or carbachol contraction were observed and the fourth respective responses were taken as the control.

Calcium determination: To estimate the cellular Ca content, the tissue was treated in a manner similar to the lanthanum method (5, 6). After blotting on filter paper (Tōyō Roshi, No. 7) the tissue was weighed on a torsion balance.

![Fig. 1. Traces of mechanical responses to CaCl₂ and carbachol in the K-rich Tyrode solution without adding CaCl₂. Left, a contracture evoked by addition of 0.5 mM CaCl₂ (Ca) to the bathing medium in the taenia which had exposed to the solution containing carbachol (1 mM) for 3 min and then suspended in the drug-free solution for 10 min; right, a contraction induced by 1 mM carbachol (Carb) applied 10 min after removal of the Ca.](image-url)
to determine the fresh wet weight. The tissue in a crucible was dried at 90°C for 18 hours and then ashed in a muffle furnace at 600°C for 3 hours. This ash was dissolved in 5 ml of 0.5 N HCl solution containing 1% LaCl₃. The Ca content of this solution was measured by an atomic absorption spectrophotometer (Hitachi, 208) and expressed as mM/kg wet weight of the tissue.

To determine the Ca content of the medium, the bathing medium was transferred into a crucible and evaporated to dryness by heating at 90°C for 18 hours, before being ashed in a muffle furnace at 600°C for 3 hours. The amount of Ca in the ash was estimated.

**Solutions:** Tyrode solution (mM); NaCl 137.0, KCl 2.7, NaH₂PO₄ 0.4, CaCl₂ 1.8, MgCl₂ 1.0, NaHCO₃ 12.0 and glucose 5.0. The Ca-free, K-Tyrode solution; KCl 137.0, MgCl₂ 1.0, tris-maleate buffer (pH=7.4) 5.0 and glucose 5.0.

**Drugs:** Carbamylcholine hydrochloride (carbachol) (Merck), histamine dihydrochloride (Ishizu), mepyramine maleate (May & Baker), Lanthanum chloride and EGTA (Wako).

**RESULTS**

**Effects of La³⁺ on the carbachol contraction**

Previous exposure of a Ca-loaded taenia to the K-rich solution containing La³⁺ (0.05–0.5 mM) resulted in an appreciable increase in the amplitude and the rate of falling phase of the carbachol contraction. This increase occurred in a dose-dependent manner. The averaged amplitude of the carbachol contractions in the presence of 0.5 mM La³⁺ was found to be 120.3±3.6% (±S.E., n=6) of the control. When a Ca-deprived taenia was exposed to La³⁺ in varied concentrations before its Ca loading, it showed reduced tension development during the Ca loading and by subsequently-applied carbachol. La³⁺ also slows the rates of tension development and of relaxation following application and withdrawal of Ca by diminishing Ca²⁺ membrane flux. These inhibitory effects of La³⁺ on the Ca-contracture were dose-dependent and virtually irreversible. Some of these observations confirm our previous findings (4).

The marked difference in the La³⁺ sensitivity between the Ca contracture and the carbachol contraction suggests that such are induced by Ca²⁺ from different sources. If

![Fig. 2. Tension development produced by simultaneous application of CaCl₂ and carbachol. a and b, Ca-contractures; a' carbachol-induced contraction following the Ca-contracture (a); b', contraction evoked by simultaneous application of CaCl₂ and carbachol (Carb+Ca) following the Ca-contracture (b).](image-url)
such is the case, both contractile responses should not fuse but rather remain independent and additive (2). Fig. 2 shows tension development produced in a Ca-loaded muscle by simultaneous application of CaCl₂ and carbachol. The response consists of an initial, transient component and a late, sustained component. The trace of this response was compared with the curve obtained by adding graphically the respective traces of the control Ca-contracture and carbachol contraction, as shown in Fig. 3. It can be seen that the two curves are almost superimposable, which suggests that the Ca and carbachol induced contractions are independent and additive. The peak tension of the contraction evoked by simultaneous application of CaCl₂ and carbachol was compared with that of the contraction elicited by carbachol alone after normalization by expressing as a percentage of the peak tension of the control carbachol contraction (the fourth carbachol response, see Methods). The mean percentage of the former in six preparations was 108.9±1.7% (±S.E.) and that of the latter in another six preparations was 103.8±2.4%. The difference between the two means was statistically significant (p<0.05). The level of the sustained tension varied in a dependent manner to Ca concentrations added, as shown in Fig. 4. Furthermore, when a Ca-loaded muscle had been exposed to 0.5 mM La³⁺, it responded to simultaneous application of CaCl₂ and carbachol by a monophasic, transient contraction which was quite similar to that elicited by carbachol alone (Fig. 5). Thus it is apparent that the initial, transient component of the response is due to intracellular Ca²⁺ release by carbachol and that the late, sustained component is due to Ca²⁺ influx.

In all preparations, the peak tension and the line of the falling phase of the transient component and of the early part of the sustained component were distorted somewhat above from the graphically-constructed curve. It was found that the peak tension and the maximum rate of tension development of Ca-contractions were appreciably increased in the presence of carbachol, as illustrated in Fig. 6. This indicates that carbachol increases Ca perme-
ability of the depolarized smooth muscle cell membrane as in the polarized membrane (7) and accelerates Ca\textsuperscript{2+} transport across the membrane. The increased tension development observed on addition of CaCl\textsubscript{2} in the presence of carbachol was still abolished by La\textsuperscript{3+} of 0.5 mM. The slight distortion from the constructed curve, therefore, may be due to an increased Ca\textsuperscript{2+} entry during exposure to carbachol.

Effects of EGTA on the carbachol contraction

To decrease free Ca\textsuperscript{2+} of the solution in the bath EGTA was added. Calcium concentration in the bathing solution was determined to be 0.024±0.001 mM (mean±S.E.,

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**FIG. 5.** Effects of La\textsuperscript{3+} on contraction evoked by simultaneous application of CaCl\textsubscript{2} and carbachol. a and a’, Control Ca-contracture and contraction evoked by simultaneous application of CaCl\textsubscript{2} and carbachol; b, Ca-contracture, followed by contraction (b’) on exposure to CaCl\textsubscript{2} and carbachol at the same time (Carb+Ca) in the presence of 0.5 mM La\textsuperscript{3+} (La), added 5 min prior to the simultaneous exposure. See the transient contraction (b’), just like that elicited by carbachol alone.

**FIG. 6.** Effects of carbachol on Ca-contraction. a, Control carbachol contraction and Ca-contracture; b, carbachol-contracture, followed by contraction evoked on exposure to CaCl\textsubscript{2} in the presence of 1 mM carbachol, added at the mark (’’) 2 min prior to the Ca exposure.

**FIG. 7.** Effects of EGTA on carbachol contraction. a and a’, Control Ca-contracture and carbachol-contraction; b, Ca-contracture, followed by contraction induced by carbachol in the presence of 0.25 mM EGTA, added 5 min prior to the carbachol exposure.
and the free Ca\(^{2+}\) concentration was reduced to less than 10\(^{-8}\) M by adding 0.25 mM EGTA. This free Ca\(^{2+}\) concentration was far lower than the concentrations required to activate the contractile elements in the skeletal muscle (8). After 5 min perfusion by the solution containing EGTA, carbachol contractions were still evoked and their amplitude was reduced only to 85% or so of the control (Fig. 7).

**Effects of carbachol on intracellular Ca content**

Twenty-four Ca-loaded preparations were divided into two groups and were used to determine the intracellular Ca content before and after exposing same to carbachol. The mean Ca contents were determined to be 1.11±0.12 mM/kg wet weight tissue before the exposition and 1.18±0.12 mM/kg wet weight tissue after the exposition. There was no statistically significant difference between the values (P>0.05).

**Effects of histamine on carbachol contraction**

Histamine was applied to determine whether or not the carbachol-sensitive Ca store is also sensitive to other smooth muscle stimulants. Exposure to a Ca-loaded muscle to histamine (2.5×10^{-6}–2.5×10^{-5} M) resulted in a contraction. The contraction was transient and completely subsided even when perfusion by the solution containing histamine was continued. The peak tension developed during exposure to histamine was increased in a

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**FIG. 8.** Effects of histamine on carbachol contraction. a, Ca-contracture, followed by contraction induced by 5×10^{-4} M histamine (Hist) and that by subsequently-applied carbachol (a'); b and b', control Ca-contracture and carbachol contraction.

**FIG. 9.** Relationship between the peak tension of contractions evoked by histamine in varied concentrations and that of the following carbachol contractions. The graph plotting the peak tension of the histamine-induced contractions (○---○), the peak tension of the following carbachol contractions (○○) and the total tension obtained by adding the former two (○•••••○), versus concentration of histamine. Ordinate: the respective peak tensions expressed as a percentage of that of the control carbachol contraction; abscissa: concentration (M). Each point of the graph is the mean ± S.E. of 11–14 experiments.
dose-dependent way. The effect of histamine could be specifically blocked by mepyramine. The amplitude of the contraction evoked by carbachol applied at 2 min after the histamine exposure was reduced (Fig. 8). Fig. 9 shows the relationship between the peak tension of contractions evoked by histamine in varied concentrations and that of the following carbachol contractions. It can be seen that the amplitude of the carbachol contractions decreased in an inversely proportional manner to that of the preceding histamine contractions, while the total tensions raised by both drugs showed little variation over the range of the histamine concentrations used. To exclude a possible incomplete recovery of the contractile elements, the interval between their applications was prolonged up to 8 min. This manner of the inhibitory effect of histamine on the carbachol contraction was little affected by varying the interval. These results are what would be expected if histamine can in fact mobilize Ca\(^{2+}\) from the carbachol-sensitive site and reduce the quantity of Ca\(^{2+}\) available for inducing the subsequent carbachol contraction.

DISCUSSION

The present results favour an intracellular site for the carbachol-sensitive Ca store and thus support the hypothesis proposed previously (3). In addition to the marked difference between the La\(^{3+}\) sensitivity of the Ca contracture and that of the carbachol contraction (4), both responses were found to be independent and additive and the carbachol contraction persisted after 5 min perfusion by the solution containing EGTA (estimated free Ca\(^{2+}\) concentration less than 10\(^{-8}\) M). The perfusion time of 5 min would expectedly be longer than the time required for simple diffusion of EGTA through the extracellular space of the tissue, since it has been demonstrated in cardiac muscle that the time constant for simple diffusion of EGTA in the extracellular space is in the sec order (9, 10). Since the Ca amount of the carbachol-sensitive store has been shown to be in a dynamic equilibrium with the Ca concentration in the medium (3, 11), it is possible that extreme reduction of free Ca\(^{2+}\) concentration in the medium by EGTA may result in an increase in the rate of loss of Ca from this store. This possible effect of the agent may be responsible for the slight decrease in the amplitude of the carbachol contraction in the presence of 0.25 mM EGTA.

An interesting feature of the present experiments is the observation that carbachol still produced a transient rise in tension in the presence of 0.5 mM La\(^{3+}\) which blocks the Ca contracture. This suggests that Ca\(^{2+}\) mobilized from the carbachol-sensitive store activate the contractile elements and then are bound to or taken up by the cellular elements which are insensitive to carbachol. No significant change in the cellular Ca amount before and after exposure to carbachol is consistent with this idea. However, if the ratio of the drug-dependent Ca to total Ca is extremely small, the method employed may not be sufficient to detect the presumed small amount of difference.

The present results suggest that histamine can also release Ca\(^{2+}\) from the carbachol-sensitive Ca store through activation of the specific receptors.

It has been suggested that calcium-binding sites may be present in the membrane of smooth muscle cells, which are more internal to the outside surface (12). Release of Ca\(^{2+}\)
from the sites is controlled by Ca\(^{2+}\) bound in the surface of the membrane (12, 13). Goodman and Weiss (14) observed dissociation of K-induced and ACh-induced contractions of the rat uterine muscle by La\(^{3+}\) whose primary action is on the superficial sites, and they suggested that ACh can directly mobilize Ca\(^{2+}\) in the less superficial sites. The contraction induced by carbachol in the depolarized taenia caecum closely resembles the ACh-induced contraction of uterine muscle in so far as it has a greater resistance to the action of La\(^{3+}\), and it seems probable that this similarity is achieved by mobilization of Ca\(^{2+}\) from the same source, the less superficial sites.

It may be considered that ACh, carbachol or probably histamine can release Ca\(^{2+}\) from the less superficial sites of smooth muscle membrane, the Ca\(^{2+}\) activating the contractile proteins. The displacement of Ca\(^{2+}\) also serves to reduce the amount of Ca\(^{2+}\) in the superficial sites which is responsible for effecting changes in the permeability to ions.

REFERENCES

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