Role of Cellular Na^+ Accumulation in Acetylcholine-Induced Desensitization of Guinea Pig Ileal Longitudinal Muscle

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ABSTRACT—The role of cellular Na^+ accumulation in acetylcholine-induced desensitization was investigated in guinea pig ileal longitudinal muscle. Desensitization was induced by the pretreatment with acetylcholine (10^{-4} M, 30 min) and was expressed by the rightward shift in the concentration-response curve for acetylcholine after the treatment. The same treatment with acetylcholine caused accumulation of cellular Na^+ that amounted to about 3.5-fold of the control level. To study the relationship between the gain of cellular Na^+ and the development of desensitization, we treated the muscle strips with acetylcholine under the condition in which the external Na^+ concentration ranged from zero to 149.2 mM. The result showed that cellular Na^+ content is closely related to the extent of desensitization; that is, desensitization was at the lowest level when acetylcholine induced no increase in cellular Na^+ , while desensitization developed in proportion to the increase in cellular Na^+ content. However, when cellular Na^+ was increased by another method (by the treatment with ouabain), the inhibition of the acetylcholine response was far less than that observed in the case of desensitization. We concluded that both muscarinic stimulation and the accompanying accumulation of cellular Na^+ are required for desensitization to occur in full. This desensitization could be the result of a muscarinic stimulated and cellular Na^+ -dependent mechanism.

Keywords: Ileum (guinea pig), Desensitization, Acetylcholine, Ca^{2+} channel, Cellular Na^+

Prolonged treatment of smooth muscles with high concentrations of agonists leads to desensitization; that is, cellular responses to the agents become reduced. Desensitization can be classified into two types, specific and nonspecific ones. The former is characterized by diminished responses only to a given agonist, probably reflecting alterations at the receptor level (1, 2). On the other hand, in the latter case, the responses to multiple agonists are affected, probably due to a change in some common process distally from the receptor. It has been well-established that treatment with muscarinic agonist leads to nonspecific desensitization in guinea pig ileum (3–6). Muscarinic stimulation opens nonselective cation channels in smooth muscle cells (7), and the cells thereby gain Na^+ . This increase in cellular Na^+ has been suggested to be responsible for this type of desensitization (8–10). Probably, increased cellular Na^+ stimulates the electrogenic Na^+ pump, hyperpolarizes the plasma membrane and leads to reduced responsiveness (9, 11). However, there is evidence showing that increased cellular Na^+ inhibits contractions without any change in membrane potential (12, 13). Moreover, Himpens et al.

(14) reported that desensitization could be the result of a G-protein-mediated inactivation of voltage-gated Ca^{2+} currents rather than the membrane hyperpolarization.

Thus in the present study, we examined in detail the relationship between cellular Na^+ accumulation and occurrence of desensitization in guinea pig ileal longitudinal muscle and verified the involvement of Na^+ in the desensitization process.

MATERIALS AND METHODS

Preparations

Male guinea pigs, weighing 250–450 g, were killed by a blow on the head and cutting the major blood vessels in the throat. Strips of longitudinal muscle from the ileum were obtained by the method of Rang (15) and immersed in Tyrode solution.

Solutions

The Tyrode solution had the following composition: 136.9 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1.05 mM MgCl_2 , 0.42 mM NaH_2PO_4 , 11.9 mM NaHCO_3 and 5.6 mM

glucose. Low- Na^+ (0–120 mM) solutions were prepared by replacing NaCl with isomolar tris(hydroxymethyl)aminomethane (Tris) chloride and contained: 0–120 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1.05 mM MgCl_2 , 5.6 mM glucose and 29.2–149.2 mM Tris-HCl, pH 7.4.

Contractile measurements

The longitudinal muscle strips (20–25 mm in length) were suspended in Tyrode solution at 31°C and bubbled with air under a resting tension of about 0.5 g. The strips were allowed to equilibrate for at least 1 hr. Then isotonic contractions were recorded with a lever on a smoked drum. For the measurement of desensitization, cumulative concentration-response curves for acetylcholine were measured 2–3 times on a muscle strip at intervals of about 1 hr. When the last two concentration-response curves were similar for each preparation, the last of these curves was taken as the control response curve. Then, the muscle strip was treated with a desensitizing agent (10^{-4} M of acetylcholine) for 30 min. This desensitizing treatment was done either in Tyrode solution or low- Na^+ solutions (the concentration of Na^+ ranged from 0–120 mM). After the muscle was washed out with Tyrode solution for 5 min, the concentration-response curve for acetylcholine was re-examined. The concentration-response curve shifted almost in parallel to the right after the desensitizing treatment. Then the concentration-ratio for the shifted curves was determined to assess the degree of desensitization (3, 6). Here, the concentration-ratio is the ratio of the concentration of acetylcholine required to elicit 50% of the maximal response after the desensitizing treatment to the concentration needed to elicit the same response in the control experiment. The treatment with each low- Na^+ solution for 30 min, followed by a 5-min washout, had no significant effect on the concentration-response curves for acetylcholine. Effect of ouabain on contractile responses to acetylcholine was examined as follows: After a control concentration-response curve for acetylcholine was obtained on a muscle strip, it was treated with 10^{-5} M ouabain for 30 or 60 min, washed with Tyrode solution for 5 min, and then the concentration-response curve was re-examined. Then the concentration-ratio for the shifted curves was calculated as described above.

Determination of cellular Na^+ content

Cellular Na^+ content was determined by the "Li-method" developed by Friedman (16). After incubation of the muscle strips in various test solutions, the strips were washed with Tyrode solution for 1–10 min (usually for 1 min) and then exposed to Li^+ solution at 1°C for 30 min to remove extracellular Na^+ . The Li^+ solution had the following composition: 136.9 mM LiCl, 2.7 mM KCl,

1.8 mM CaCl_2 , 1.05 mM MgCl_2 , 5.6 mM glucose and 11.9 mM Tris-HCl, pH 7.2. The strips were blotted between filter paper and weighed. Each strip was then treated in a teflon tube (Sanai Kagaku Co., Nagoya) with 0.5 ml HNO_3 overnight, dried by heating at 150°C for 1.5 hr, and then added with 0.5 ml HNO_3 and heated at 150°C for 10 min. Na^+ content in the samples was measured by a flame photometer (Type 205; Hitachi, Tokyo) and expressed as mmol/kg wet wt. of the tissue. All determinations were done in duplicate. No significant difference in cellular Na^+ contents was observed when the washout time with Tyrode solution ranged from 1–10 min.

Drugs

Ouabain was obtained from Merck (Darmstadt, FRG). Acetylcholine chloride was from Daiichi Pharmaceutical Co. (Tokyo). All other reagents were of analytical grade.

RESULTS

We previously reported that pretreatment of strips of guinea pig ileal muscle with 10^{-5} – 10^{-4} M acetylcholine led to nonspecific desensitization; that is, responses to both acetylcholine and histamine were reduced with the same time course and to the same degree (6). In the present study, to clarify the involvement of cellular Na^+ in the desensitization process, we first examined the change in cellular Na^+ content induced by the treatment

Fig. 1. Effect of acetylcholine on cellular Na^+ content in guinea pig ileal longitudinal muscle. The muscle strips were equilibrated in Tyrode solution for several hours at 31°C and then treated with 10^{-4} M acetylcholine. At the time shown, strips were removed from the incubation medium and washed with Tyrode solution and then subjected to the procedure described in the Materials and Methods for the measurement of cellular Na^+ content. Each point represents the mean $\pm$ S.E.M. of 3 experiments.

with acetylcholine. As shown in Fig. 1, the cellular Na⁺ content increased rapidly in the first 10 min and then more slowly in the next 20 min. This time course is in accord with that of acetylcholine-induced desensitization that developed rapidly within 10 min and proceeded more slowly in the next 20 min (6).

Next, we examined the relationship between cellular Na⁺ content and the degree of desensitization. To obtain varied cellular Na⁺ content, we treated the muscle strips with acetylcholine (10^{-4} M) under the condition in which external Na⁺ concentration ranged from 0 to 149.2 mM. Low-Na⁺ medium would inhibit accumulation of cellular Na⁺, since it is due to influx of Na⁺ through nonselective cation channel opened by muscarinic stimulation (7). Figure 2A shows that acetylcholine did not increase cellular Na⁺ at 0–50 mM external Na⁺. Accumulation of cellular Na⁺ occurred above 50 mM external Na⁺, in proportion to the rise in the concentration of external Na⁺. Figure 2B shows the result of desensitization experiment induced by acetylcholine that was performed under various concentrations of external Na⁺ (0–149.2 mM). The desensitizing treatment with acetylcholine (10^{-4} M, 30 min) shifted the concentration-response curve for acetylcholine rightward. Then concentration-ratio was calculated as described in the Materials and Methods to assess the degree of desensitization. Those treatments (0–149.2 mM Na⁺) in the absence of acetylcholine had little effect on the concentration-response curves for acetylcholine. Desensitization developed in accordance with the accumulation of cellular Na⁺; that is, desensitization was minimum at 0–50 mM external Na⁺, while it developed in proportion as the concentration of external Na⁺ rose, just as the cellular Na⁺ content increased. It is to be noted, however, that desensitization occurred to a significant extent under the condition where no Na⁺ accumulation occurred (0–50 mM external Na⁺).

To see whether the increase in Na⁺ content obtained by acetylcholine treatment, which amounted to about 30 mmol/kg wet wt. (Fig. 1), is sufficient to account for the decreased response (desensitization), we employed another means to increase cellular Na⁺ and studied the relationship between cellular Na⁺ content and inhibition of the contractile response to acetylcholine. This was done by treating the muscle strip with ouabain (10^{-5} M). Figure 3A shows the increase in cellular Na⁺ content after the treatment with ouabain, and Figure 3B shows the decreased responses to acetylcholine after the same treatment. Cellular Na⁺ content comparable to that obtained by the acetylcholine treatment was attained after the treatment with ouabain for 30 min. Such treatment (10^{-5} M ouabain, 30 min) suppressed acetylcholine response to some extent as shown in Fig. 3B, but this inhibition was far less than that observed after the desensitizing treatment (Fig. 2B). Namely, the concentration-ratio obtained by the ouabain treatment was 2.1 ± 0.3 ,

Fig. 2. Effects of external Na⁺ concentration on acetylcholine-induced changes in cellular Na⁺ content (A) and acetylcholine-induced desensitization (B) in guinea pig ileal longitudinal muscle. A: The muscle strips were treated with 10^{-4} M acetylcholine for 30 min in Tyrode solution (containing 149.2 mM Na⁺) or low-Na⁺ (0–120 mM) solution (○). For the control experiment, the strips were treated with each low-Na⁺ solution for 30 min in the absence of acetylcholine (●). The strips were then washed with Tyrode solution and subjected to the procedure described in the Materials and Methods for the measurement of cellular Na⁺ content. Each point represents the mean $\pm$ S.E.M. of 3 experiments. B: After a control concentration-response curve for acetylcholine was obtained on a muscle strip, it was treated with 10^{-4} M acetylcholine for 30 min in Tyrode solution or low-Na⁺ (0–120 mM) solution. The strip was then washed with Tyrode solution and the concentration-response curve was re-examined. Concentration-ratios on the ordinate scale were determined from shifted concentration-response curves as described in the Materials and Methods to express the degree of desensitization. Each point represents the mean $\pm$ S.E.M. of 4–6 experiments.

sitizing treatment (Fig. 2B). Namely, the concentration-ratio obtained by the ouabain treatment was 2.1 ± 0.3 ,

Fig. 3 Effect of ouabain on cellular Na⁺ content (A) and the concentration-response curves for acetylcholine (B) in guinea pig ileal longitudinal muscle. A: The muscle strips were equilibrated in Tyrode solution for several hours and then treated with 10^{-5} M ouabain. At the time shown, strips were removed from the incubation media and washed with Tyrode solution, and subjected to the procedure described in the Materials and Methods for the measurement of cellular Na⁺ content. Each point represents the mean $\pm$ S.E.M. of 4 experiments. B: After a control concentration-response curve (○) was obtained, the muscle strip was treated with 10^{-5} M ouabain for 30 min (△) or 60 min (□). The strip was then washed with Tyrode solution and the concentration-response curve was re-examined. Each point represents the mean $\pm$ S.E.M. of 4 experiments.

while that obtained by the desensitizing treatment was 22.6 ± 1.4 . When we performed similar experiments using K⁺-free solution (simply omitting KCl from the Tyrode solution) to increase cellular Na⁺, essentially the same result was obtained (data not shown).

DISCUSSION

Nonspecific desensitization induced by acetylcholine in smooth muscles has been suggested to involve a gain of Na⁺ by the cells (8, 9). However, the role of Na⁺ in desensitization is yet not well-clarified, nor it is not clear whether this desensitization is solely attributed to the mechanism concerned with cellular Na⁺.

Thus in the present study, we examined in detail the relationship between the gain of cellular Na⁺ and the development of desensitization both induced by the same treatment with acetylcholine in guinea pig ileal longitudinal muscle. The result showed that cellular Na⁺ content is closely related to the extent of desensitization; i.e., desensitization was at the lowest level when acetylcholine induced no increase in cellular Na⁺, while desensitization developed in proportion as the cellular Na⁺ content increased (Fig. 2). This data strongly supports the idea that increased cellular Na⁺ participates in desensitization. The result that the time course of accumulation of cellular Na⁺ was in accord with that of desensitization induced by acetylcholine (ref. 6 and this study) also supports this idea.

On the other hand, when cellular Na⁺ was increased by another method (by the treatment with ouabain), the decrease in acetylcholine response was far less (Fig. 3) than that observed after the desensitizing treatment. This result indicates that the increase in cellular Na⁺ alone is not sufficient, but the treatment with a muscarinic agonist is indispensable for the desensitized response to occur. Thus both muscarinic stimulation and the accompanying accumulation of cellular Na⁺ are required for desensitization to occur in full.

Acetylcholine-induced increase in cellular Na⁺ has been suggested to stimulate electrogenic extrusion of Na⁺ (11), and thus to oppose the depolarizing effect of the cholinergic agent, leading to desensitization (9). However, this seems rather unlikely as pointed out by Himpens et al. (14) because desensitization occurs under conditions that diminish the activity of the Na⁺,K⁺-pump, e.g., in low-K⁺ medium (14) or at reduced temperature (6), and desensitization is inhibited under the conditions that stimulate the pump, e.g., in high-K⁺ medium (5). Moreover contractile responses to high-K⁺ in smooth muscles are reduced by the treatments that increase cellular Na⁺ without any change in membrane potentials (12, 13). Our results give additional evidence against the above idea since accumulated Na⁺ that may activate the Na⁺,K⁺-pump has only a minor role in desensitization if it is not accompanied by muscarinic stimulation. It is probable that accumulated cellular Na⁺ inhibits Ca²⁺ influx without affecting membrane potential (13, 17) and thus induces desensitization.

However, this action also may take no significant part in producing desensitization without muscarinic stimulation.

Muscarinic stimulation inhibits voltage-gated Ca²⁺ channel currents in smooth muscles (18–20). Although the precise mechanism for this inhibition is not well-clarified, Mitsui and Karaki (18) supposed that muscarinic stimulation produces endogenous inhibitory substances of the Ca²⁺ channels, and Himpens et al. (14) suggested such inactivation of the Ca²⁺ channel currents is responsible for the desensitization. Putting these and our data together, we can suppose that cellular Na⁺ may participate in desensitization either by activating the production of these inhibitory substances or by augmenting the inhibitory action of them.

It is to be noted that a significant extent of desensitization developed under the condition in which no increase in cellular Na⁺ occurred (Fig. 2). This observation is in accord with that of Mitsui and Karaki (18) showing that muscarinic stimulation under the Na⁺-free condition reduces high-K⁺-induced contractions. There are two explanations for such a component of desensitization that is insensitive to cellular Na⁺ change. One possibility is that a slight amount of Na⁺ contained in the cell (about 8 mmol/kg wet wt.) is sufficient for inducing this component of desensitization in concert with muscarinic stimulation. The other possibility is that some mechanism independent of the accumulation of Na⁺ is responsible for this desensitization. Since there exists several mechanisms that may inhibit voltage-gated Ca²⁺ channel currents through muscarinic activation (14, 20, 21), some inhibitory mechanisms may depend on cellular Na⁺ and others may not, both of them participating in desensitization.

In conclusion, our evidence suggests that both muscarinic stimulation and the accompanying accumulation of cellular Na⁺ are required for nonspecific desensitization to occur in full. This desensitization could be the result of a muscarinic stimulated and cellular Na⁺-dependent mechanism.

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