Inhibition of Ca^{2+} Entry Caused by Depolarization in Acetylcholine-Stimulated Antral Mucous Cells of Guinea Pig: G Protein Regulation of Ca^{2+} Permeable Channels

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Abstract: The effects of depolarizing conditions resulting from increasing extracellular K^+ concentration or nystatin treatment on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were studied in guinea pig antral mucous cells following acetylcholine (ACh) stimulation. ACh stimulation evoked a biphasic increase in $[\text{Ca}^{2+}]_i$, that is, an initial transient increase followed by a plateau. Depolarizing conditions reduced the $[\text{Ca}^{2+}]_i$ in the plateau phase during ACh stimulation. However, pertussis toxin (PTX, a G protein inhibitor) treatment caused $[\text{Ca}^{2+}]_i$ in the ACh-evoked plateau

phase to increase under depolarizing conditions, while it had no effect on $[\text{Ca}^{2+}]_i$ under hyperpolarized conditions. Based on these observations, Ca^{2+} permeable channels are regulated by a G protein which is activated by depolarized conditions and inhibited by hyperpolarized conditions and PTX; activation of the G protein (depolarization) causes Ca^{2+} permeable channels to inhibit, and in turn, inhibition of the G protein (hyperpolarization) causes them to activate. [Japanese Journal of Physiology, 49, 545–550, 1999]

Key words: membrane potential, capacitative Ca^{2+} entry, pertussis toxin.

Secretory functions in exocrine glands are regulated by intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). In guinea pig antral mucous cells, the stimulation of acetylcholine (ACh) evoked cell shrinkage followed by exocytotic events, which were regulated by increases in $[\text{Ca}^{2+}]_i$ [1]. ACh evoked a biphasic increase in $[\text{Ca}^{2+}]_i$, that is, an initial transient increase followed by a plateau. The initial transient increase in $[\text{Ca}^{2+}]_i$ has been reported to be evoked by release from internal Ca^{2+} stores, and the plateau has been reported to be maintained by Ca^{2+} influx from the extracellular fluid [2]. Putney [3] has proposed a model of Ca^{2+} entry, “capacitative Ca^{2+} entry,” which is triggered by the depletion of internal Ca^{2+} stores. The capacitative Ca^{2+} entry has been reported to be regulated by several messengers such as cAMP [4] and small G proteins [5, 6].

Moreover, the membrane potential in nonexcitable cells has been reported to have a role in maintaining the $[\text{Ca}^{2+}]_i$ [7, 8]. We do not know at present whether or not Ca^{2+} entry in exocrine cells is regulated by voltage, although Ca^{2+} entry is not thought to be mediated via voltage-gated channels in exocrine cells. Many exocrine cells have large Ca^{2+} -activated K^+ channels, activation of which causes hyperpolarization of the membrane potential. In salivary acinar cells, depolarizing conditions reduced Ca^{2+} entry evoked by carbachol stimulation, and in contrast, hyperpolarizing conditions enhanced it [9, 10]. We have also reported that increasing extracellular K^+ concentrations, $[\text{K}^+]_o$, causes $[\text{Ca}^{2+}]_i$ in ACh-stimulated antral mucous cells to decrease [1]. These observations indicate that the membrane potential modulates capacitative Ca^{2+} entry. This modulation of Ca^{2+} entry by mem-

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brane potential is thought to be caused by the changing electro-chemical potential gradients across the cell membrane for Ca^{2+} entry [9]. However, it still remains uncertain whether or not the membrane potential regulates Ca^{2+} entry pathways [4].

On the other hand, we have examined effects of the pertussis toxin (PTX), a G protein inhibitor, on ACh-evoked exocytotic events in antral mucous cells. In the course of the experiments, we found that PTX treatment caused the $[\text{Ca}^{2+}]_i$ in the ACh-evoked plateau phase to increase during perfusion with a high- K^+ solution ($[\text{K}^+]_o = 150.5 \text{ mM}$). This may suggest that the Ca^{2+} entry pathways are regulated by the membrane potential. In the present study, we examined the effects of depolarizing conditions on $[\text{Ca}^{2+}]_i$ in ACh-stimulated antral mucous cells with and without PTX treatment.

Materials and Methods

The control solution contained (in mM): NaCl 146, KCl 4.5, MgCl_2 1, CaCl_2 1.5, HEPES 10 and glucose 5. The KCl solution contained (in mM): KCl 150.5, MgCl_2 1, CaCl_2 1.5, HEPES 10 and glucose 5. The pH values of the solutions were adjusted to 7.4 by adding 1 M NaOH or 1 M KOH. To increase $[\text{K}^+]_o$, an appropriate amount of KCl solution was added to the control solution. Acetylcholine chloride (ACh) was obtained from Daiichi Pharmaceutical Drugs (Osaka, Japan), nystatin from Sigma Chemical Co. (St. Louis, MO, USA), pertussis toxin (PTX) from Research Biochemicals International (Natick, MA, USA), and collagenase and bovine serum albumin (BSA) from Wako Pure Chemical Industries (Osaka, Japan). PTX was dissolved in 0.1 M sodium phosphate and 0.5 M NaCl (pH 7.0) and stocked at $100 \mu\text{g/ml}$. The reagents were prepared to their final concentrations just before the experiments. All of the solutions were gassed with 100 % O_2 at 37°C .

Male Hartley strain guinea pigs weighing approximately 250 g were purchased from Shimizu Experimental Animals (Kyoto, Japan), and fed a standard pellet diet and water. The guinea pigs were anesthetized by inhalation of ether and sacrificed by cervical dislocation. The experiments were approved by the Animal Research Committee of Osaka Medical College, and animals were cared for according to the guidelines of this committee.

The procedures for cell preparation have already been described in detail previously [1, 11]. Briefly, the antrum was excised, and the mucosal layer was stripped from the muscle layer using glass slides in saline at room temperature. The stripped antral mucosa was minced and incubated in a control solution

containing 0.1% collagenase and 2% BSA for 10 min at 37°C . The digested mucosa was filtered through a nylon mesh with a pore size of $150 \mu\text{m}^2$ and washed three times. The cells were resuspended in control solution (4°C) containing 2% BSA. The suspension was stored at 4°C and used for experiments within 3 h.

The isolated cells were incubated in control solution containing PTX (200 ng/ml) for 2 h to inhibit G proteins and then used for the experiments.

The isolated cells were loaded with fura 2-AM ($2.5 \mu\text{M}$, Dojindo, Kumamoto, Japan) for 25 min at room temperature ($22\text{--}24^\circ\text{C}$) and washed with the control solution. The cells were mounted on a coverslip precoated with neutralized Cell-Tak. The coverslip and cells were set in a perfusion chamber mounted on the stage of an inverted microscope (IX70, Olympus, Tokyo, Japan) connected to an image analysis system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan). The fura 2 fluorescence ratios ($340 \text{ nm} : 380 \text{ nm}$) were calculated and stored in the image analysis system. In the present study, the cell-free calibration method was used for calculating the $[\text{Ca}^{2+}]_i$.

The statistical significance of the differences between the mean values were assessed using Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results and Discussion

Effects of $[\text{K}^+]_o$ on $[\text{Ca}^{2+}]_i$. In unstimulated antral mucous cells, the $[\text{Ca}^{2+}]_i$ was approximately 60–80 nM. ACh stimulation evoked a transient increase in the $[\text{Ca}^{2+}]_i$ followed by a plateau. The concentration of ACh used was $10 \mu\text{M}$, which evoked maximum responses in Ca^{2+} -stimulated exocytotic events in antral mucous cells [1]. When the perfusion solution was switched from the control solution ($[\text{K}^+]_o = 4.5 \text{ mM}$) to the KCl solution ($[\text{K}^+]_o = 150.5 \text{ mM}$) during ACh stimulation, the $[\text{Ca}^{2+}]_i$ in the plateau phase (approximately 150 nM) decreased rapidly and reached a new plateau (approximately 100 nM) as shown in Fig. 1A. Thus, $[\text{K}^+]_o$ of 150.5 mM markedly reduced the $[\text{Ca}^{2+}]_i$ in the ACh-evoked plateau phase. When the perfusion solution was switched from the control solution to the KCl solution for the unstimulated cells, $[\text{Ca}^{2+}]_i$ decreased slightly and the cells were then stimulated with $10 \mu\text{M}$ ACh. ACh stimulation caused an initial transient increase in $[\text{Ca}^{2+}]_i$ followed by a plateau. The $[\text{Ca}^{2+}]_i$ in the initial transient increase (the peak value was approximately 180 nM) was not affected during perfusion with the KCl solution, while the $[\text{Ca}^{2+}]_i$ of the plateau phase was markedly decreased (approximately

Fig. 1. Effects of the $[\text{K}^+]_o$ on $[\text{Ca}^{2+}]_i$ during ACh stimulation. **A:** Cells were perfused with control solution ($[\text{K}^+]_o=4.5$ mM) and stimulated with 10 μM ACh. ACh stimulation evoked a transient followed by a sustained increase in $[\text{Ca}^{2+}]_i$. The perfusion solution was switched to KCl solution ($[\text{K}^+]_o=151$ mM) 5 min after the start of ACh stimulation. Increases in $[\text{K}^+]_o$ immediately decreased $[\text{Ca}^{2+}]_i$, which reached a plateau. Thus, the sustained increase in $[\text{Ca}^{2+}]_i$ evoked by ACh stimulation was suppressed by perfusion with KCl solution. **B:** First, cells were perfused with control solution and the perfusion solution was switched to KCl solution. Switching to KCl solution decreased the $[\text{Ca}^{2+}]_i$ slightly. The cells were then stimulated with 10 μM ACh. ACh stimulation immediately increased the $[\text{Ca}^{2+}]_i$, which then decreased to a plateau. The plateau value during perfusion with the KCl solution was much less than that in the sustained increase during perfusion with the control solution. When the perfusion solution was switched back to the control solution during ACh stimulation, the $[\text{Ca}^{2+}]_i$ increased immediately and became sustained. The sustained value was almost the same as that observed in A.

90 nM). When the perfusion solution was switched back to the control solution during ACh stimulation, the $[\text{Ca}^{2+}]_i$ increased rapidly (approximately 180 nM) as shown in Fig. 1B.

Effects of PTX on the $[\text{Ca}^{2+}]_i$. The effects of $[\text{K}^+]_o$ on $[\text{Ca}^{2+}]_i$ in the ACh-stimulated plateau phase were examined (Fig. 2A). Stimulation with 10 μM ACh caused an initial transient increase in $[\text{Ca}^{2+}]_i$ followed by a plateau during perfusion with the KCl solution. The $[\text{Ca}^{2+}]_i$ s in the initial transient peak and plateau phase were 183 ± 7.7 and 76 ± 3.5 nM ($n=6$ cells), respectively. As the $[\text{K}^+]_o$ was reduced from

Fig. 2. Effects of PTX on the sustained increase in $[\text{Ca}^{2+}]_i$ evoked by 10 μM ACh. Experiments were performed in both cells treated and not treated with PTX. **A:** In cells not treated with PTX, the $[\text{Ca}^{2+}]_i$ s increased as $[\text{K}^+]_o$ decreased from 151 to 4.5 mM. On the other hand, in cells treated with PTX, the $[\text{Ca}^{2+}]_i$ s also increased as $[\text{K}^+]_o$ decreased from 151 to 4.5 mM. However, during perfusion with the KCl solution, the value of $[\text{Ca}^{2+}]_i$ in the sustained increase was much higher in cells with PTX treatment than without PTX treatment. **B:** The sustained increases evoked by ACh stimulation were plotted against $[\text{K}^+]_o$. PTX treatment caused the $[\text{Ca}^{2+}]_i$ in sustained increases to shift to a higher concentration, especially with high $[\text{K}^+]_o$.

150.5 to 117, 78, 39 and 4.5 mM, $[\text{Ca}^{2+}]_i$ s in the plateau phase immediately increased to 84 ± 5.5 , 91 ± 6.6 , 117 ± 10 and 178 ± 1.0 nM, respectively (Fig. 2A). On the other hand, cells treated with PTX (200 ng/ml) were first perfused with the KCl solution and then stimulated with 10 μM ACh. The unstimulated $[\text{Ca}^{2+}]_i$ level increased slightly in PTX-treated cells and the value was approximately 72 ± 8.2 nM (5 experiments). However, this value was not significantly different from that of non PTX-treated cells (55 ± 6.3 nM, 6 experiments). Stimulation with ACh (10 μM) evoked an initial transient increase in $[\text{Ca}^{2+}]_i$ followed by a plateau. The $[\text{Ca}^{2+}]_i$ s in the initial transient peak and plateau phase were 178 ± 3.0 and 146 ± 6.8 nM ($n=5$ cells), respectively. Thus, the PTX treatment increased $[\text{Ca}^{2+}]_i$ in the ACh-evoked plateau phase markedly during perfusion with KCl solution, while it had no effect on the initial transient peak. As the $[\text{K}^+]_o$ was

reduced from 150.5 to 117, 78, 39 and 4.5 mM, the $[Ca^{2+}]_i$ in the plateau phase immediately increased to 151 ± 4.4 , 158 ± 5.0 , 171 ± 7.5 and 195 ± 20 nM, respectively (Fig. 2A). The $[Ca^{2+}]_i$ s in the ACh-evoked plateau phase were plotted against the $[K^+]_o$ in a logarithmic scale (Fig. 2B). The $[Ca^{2+}]_i$ s in the plateau phase decline as the $[K^+]_o$ increases from 4.5 to 150.5 mM. Thus, the $[Ca^{2+}]_i$ s in the ACh-evoked plateau phase depend on $[K^+]_o$. This suggests that hyperpolarized conditions increase $[Ca^{2+}]_i$ in the ACh-evoked plateau phase, and in contrast, depolarized conditions decrease it. The $[Ca^{2+}]_i$ s in the ACh-evoked plateau phase are less dependent on $[K^+]_o$ in PTX-treated cells than in non PTX-treated cells (Fig. 2B). The PTX treatment caused the ACh-evoked plateau $[Ca^{2+}]_i$ to increase significantly under depolarizing conditions, while it had no effect on the plateau $[Ca^{2+}]_i$ under hyperpolarized conditions. This indicates that hyperpolarized conditions inhibit the PTX-sensitive G protein, which regulates $[Ca^{2+}]_i$ in the plateau phase.

Effects of nystatin. To reduce the membrane potential differences in antral mucous cells, cells were perfused with the control solution containing nystatin ($50 \mu\text{M}$), a monovalent cation and anion ionophore, and stimulated with $10 \mu\text{M}$ ACh. Experiments were performed with and without PTX treatment (Fig. 3). Treatment with nystatin ($50 \mu\text{M}$) did not change the $[Ca^{2+}]_i$ s in unstimulated antral mucous cells. In non-PTX treated cells, ACh stimulation evoked an initial transient increase in $[Ca^{2+}]_i$ followed by a plateau. The $[Ca^{2+}]_i$ s in the initial transient peak and plateau phase were approximately 150 and 80 nM, respectively. Thus, nystatin treatment markedly reduced the $[Ca^{2+}]_i$ in the plateau phase during $10 \mu\text{M}$ ACh stimulation ($\blacklozenge$ in Fig. 3). In PTX-treated cells, stimulation with ACh ($10 \mu\text{M}$) increased the $[Ca^{2+}]_i$ transiently, then $[Ca^{2+}]_i$ increased gradually and reached a new plateau. The $[Ca^{2+}]_i$ s in both the transient peak and plateau were approximately 150 nM. Thus, in the presence of nystatin, PTX treatment caused $[Ca^{2+}]_i$ in the ACh-evoked plateau phase to increase ($\blacklozenge$ in Fig. 3).

Based on these observations, depolarizing conditions cause the activation of PTX-sensitive G proteins, which decreases $[Ca^{2+}]_i$, and in contrast, hyperpolarized conditions cause the inhibition of PTX-sensitive G proteins, which increases $[Ca^{2+}]_i$.

The sustained increase in $[Ca^{2+}]_i$ evoked by ACh stimulation was reduced under depolarizing conditions resulting from perfusion with KCl solution or nystatin treatment, while the initial transient increase in $[Ca^{2+}]_i$ was not. The sustained increase in $[Ca^{2+}]_i$ is well known to be maintained by Ca^{2+} influx from the

Fig. 3. Effects of nystatin on the increases in $[Ca^{2+}]_i$ evoked by $10 \mu\text{M}$ ACh. Experiments were performed in cells with and without PTX treatment. Cells were perfused with the control solution containing $50 \mu\text{M}$ nystatin, a monovalent cation and anion ionophore, for 5 min prior to ACh stimulation. In the absence of PTX treatment, ACh stimulation evoked only transient increases in $[Ca^{2+}]_i$. However, in the presence of PTX treatment, ACh stimulation evoked a transient followed by a sustained increase in $[Ca^{2+}]_i$. Thus, PTX treatment increased the $[Ca^{2+}]_i$ in the sustained increase evoked by ACh stimulation during perfusion with control solution containing $50 \mu\text{M}$ nystatin.

extracellular fluid [2] and we did not observe any increases in the $[Ca^{2+}]_i$ in the ACh-evoked plateau in cells perfused with Ca^{2+} -free solution (data not shown) even in the presence of PTX. Therefore, reduction of the plateau $[Ca^{2+}]_i$ is thought to be caused by a decrease in Ca^{2+} influx. These observations suggest that Ca^{2+} influx is modulated by the membrane potential. The question here is whether changes in membrane potential could modulate Ca^{2+} influx by altering the electrochemical potential gradients for Ca^{2+} entry or by changing the regulation of Ca^{2+} entry pathways.

PTX treatment caused $[Ca^{2+}]_i$ s in the ACh-evoked plateau to increase under the depolarizing conditions, which reduced the plateau $[Ca^{2+}]_i$ s in non-PTX-treated cells. This may suggest that the Ca^{2+} entry pathways are regulated by the membrane potential. Moreover, even in PTX-treated cells, $[Ca^{2+}]_i$ is still reduced during perfusion with KCl solution, although the extent of reduction is small. This suggests that Ca^{2+} influx is also related to the electrochemical potential gradients for Ca^{2+} entry [9]. Thus, Ca^{2+} flux is modulated by both voltage-regulated mechanisms of the Ca^{2+} entry pathway and electrochemical potential gradients for Ca^{2+} entry in antral mucous cells. Moreover, voltage-regulated mechanisms are regulated by G proteins since PTX is a well known G protein inhibitor. The Ca^{2+} entry pathways may be regulated by the G proteins, which are activated by depolarized conditions, and in turn, inhibited by hyperpolarized

conditions or PTX; activation of the G proteins inhibits Ca^{2+} entry pathways and inhibition of the G proteins activates them.

In epithelial cells, Ca^{2+} is thought to enter through Ca^{2+} permeable channels. In antral mucous cells, the exocytotic events were inhibited by the addition of Ca^{2+} channel blockers (Cd^{2+} , Ni^{2+} and nifedipine) [1]. In mouse distal convoluted tubules cells, Ca^{2+} channels were reported to be activated by hyperpolarization [12]. These observations suggest that Ca^{2+} permeable channels may be activated by hyperpolarization in antral mucous cells.

The capacitative model has been proposed as a Ca^{2+} entry mechanism [3]. However, the signal pathways following depletion of the internal stores, which regulate the capacitative Ca^{2+} entry channels, are still not fully understood. cAMP or small G proteins are reported as candidates [4–6]. The present study suggests that the membrane potential may play an important role in the capacitative Ca^{2+} entry. Unfortunately, we have no available data of membrane potential measurements in antral mucous cells. However, our previous report proved that antral mucous cells have Ca^{2+} -activated K^{+} channels which are activated by ACh stimulation [1]. Ca^{2+} -activated K^{+} channels have also been reported in salivary acinar cells, and their activations have already been reported to evoke hyperpolarization [9]. Based on these observations, activation of the K^{+} channels may also cause hyperpolarization in antral mucous cells.

Evidence for depolarization-induced activation of G proteins in the membrane of rat synaptoneurosomes has been reported by Cohen-Armon and Sokolovsky [13], and Anis *et al.* [14] have reported that depolarization-induced activation of voltage-gated Na^{+} channels was involved in depolarization-induced activation of G_o proteins. In antral mucous cells, Ca^{2+} permeable channel proteins may also bind to a G protein which is inhibited by hyperpolarization and PTX and activated by depolarization.

In antral mucous cells, ACh stimulation first causes the Ca^{2+} release from internal stores, which increases $[\text{Ca}^{2+}]_i$ immediately and activates Ca^{2+} -activated K^{+} channels. This activation of K^{+} channels results in hyperpolarization, which increases Ca^{2+} entry by inhibiting the PTX-sensitive G protein. In exocrine cells, K^{+} release which is evoked by activation of K^{+} channels is thought to be important to evoke Cl^{-} secretion. Moreover, the present study appears to indicate that the activation of K^{+} channels also activates Ca^{2+} permeable channels mediated via hyperpolarizing membrane potential.

In conclusion, a schematic of the Ca^{2+} entry regula-

Fig. 4. A schematic of Ca^{2+} entry regulation in antral mucous cells. **A:** The depolarizing conditions activate the voltage-sensitive G protein. The G protein activation causes Ca^{2+} permeable channels to inhibit. **B:** The hyperpolarizing conditions and PTX treatment inhibit the voltage-sensitive G protein. The G protein inhibition causes Ca^{2+} -permeable channels to activate.

tion of antral mucous cells stimulated by ACh is shown in Fig. 4. The Ca^{2+} permeable channels are regulated by the G proteins which are activated by depolarization and inhibited by hyperpolarization or PTX treatment; activation of the G protein (depolarization) inhibits Ca^{2+} permeable channels, and in turn, inhibition of the G protein (hyperpolarization) activates them.

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