Changes in Membrane Cholesterol of Pituitary Tumor (GH_3) Cells Regulate the Activity of Large-Conductance Ca^{2+}-Activated K^+ Channels

Ming-Wei Lin[1], Adonis Z. Wu[2], Wen-Hung Ting[3], Ching-Lin Li[3], Kuo-Sheng Cheng[3], and Sheng-Nan Wu[1,2]

[1]*Institute of Basic Medical Sciences*
Medical College
[2]*Department of Physiology*
and
[3]*Institute of Biomedical Engineering*
National Cheng Kung University
Tainan 70101, Taiwan, R.O.C.

Abstract

The effects of changes in membrane cholesterol on ion currents were investigated in pituitary GH_3 cells. Depletion of membrane cholesterol by exposing cells to methyl-β-cyclodextrin (MβCD), an oligosaccharide, resulted in an increase in the density of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$). However, no significant change in $I_{K(Ca)}$ density was demonstrated in GH_3 cells treated with a mixture of MβCD and cholesterol. Cholesterol depletion with MβCD (1.5 mg/ml) slightly suppressed the density of voltage-dependent L-type Ca^{2+} current. In inside-out patches recorded from MβCD-treated cells, the activity of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels was enhanced with no change in single-channel conductance. In MβCD-treated cells, voltage-sensitivity of BK_{Ca} channels was increased; however, no change in Ca^{2+}-sensitivity could be demonstrated. A negative correlation between adjacent closed and open times in BK_{Ca} channels was observed in MβCD-treated cells. In inside-out patches from MβCD-treated cells, dexamethasone (30 μM) applied to the intracellular surface did not increase BK_{Ca}-channel activity, although caffeic acid phenethyl ester and cilostazol still opened its probability effectively. However, no modification in the activity of ATP-sensitive K^+ channels could be seen in MβCD-treated cells. Current-clamp recordings demonstrated that the cholesterol depletion maneuver with MβCD reduced the firing of action potentials. Therefore, the increase in BK_{Ca}-channel activity induced by membrane depletion may influence the functional activities of neurons or neuroendocrine cells if similar results occur *in vivo*.

Key Words: methyl-β-cyclodextrin, cholesterol, Ca^{2+}-activated K^+ current, large-conductance Ca^{2+}-activated K^+ channels, GH_3 cells

Introduction

Cholesterol, an important component of the mammalian plasma membrane, is known to promote the formation of rafts that are membrane subdomains, enriched in cholesterol and sphingolipids (3, 10, 26). There are several lines of evidence to indicate that changes in the amount of membrane cholesterol can directly modify the activity of ion channels, including cyclic nucleotide-gated channels (8), pacemaker channels (4), volume-sensitive Cl- channels (33), L-type Ca^{2+} channels (7, 19, 31), inwardly rectifier K^+ channels (32), voltage-gated K^+ channels (16, 26), ATP-sensitive K^+ (K_{ATP}) channels (29), and large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (6, 12, 13). The BK_{Ca} channel has been reported to be sorted to lipid rafts on

Corresponding author: Dr. Sheng-Nan Wu, Institute of Basic Medical Sciences, National Cheng Kung University Medical College, No. 1, University Road, Tainan 70701, Taiwan. Tel: 886-6-2353535-5334, Fax: 886-6-2353660, E-mail: snwu@mail.ncku.edu.tw
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the apical membrane of Madin Darby canine kidney cells (9). A recent report also showed that the α-subunit of the BK_{Ca} channel could be colocalized to the cholesterol-containing membrane rafts (2).

The BK_{Ca} channel is functionally expressed in many types of cells, including pituitary GH_3 cells (22). The gating of this channel can be controlled by intracellular Ca^{2+} and/or membrane depolarization. The activity of this channel is known to play a role in the regulation of neuronal excitability, and the coupling of excitation-contraction and stimulus-secretion (15, 17, 28, 46). In addition to the complex pattern of gating by voltage and intracellular Ca^{2+}, BK_{Ca}-channel activity can be modulated by changes in membrane cholesterol (6, 12, 13) and by a wide variety of molecules (15, 39). Cholesterol modulation of BK_{Ca} channels has been studied using native channels in isolated vascular myocytes (6), following the reconstitution of native channels into artificial lipid bilayers (12), and also pore-forming subunit (*hslo*) channel reconstituted in bilayers (13). In addition, the ability of changes in cholesterol cell content to affect prolactin release was demonstrated in pituitary tumor cells (21). Previous work from this laboratory has shown that cilostazol or caffeic acid phenethyl ester could directly stimulate BK_{Ca}-channel activity in GH_3 cells (23, 45). Cilostazol was recently reported to reduce plasma triglyceride concentrations and to increase HDL-cholesterol concentrations in patients with intermittent claudication (37). Caffeic acid phenethyl ester could reduce lipid peroxidation in the membrane of erythrocytes caused by burn injury or ischemia-reperfusion (1, 35). The ability of α7 nicotinic acetylcholine receptor to target to lipid rafts was reported in ciliary neurons (11). However, to our knowledge, the effects of changes in membrane cholesterol on ion currents in neurons or neuroendocrine cells have not been studied thoroughly.

Therefore, the present study was undertaken to test the hypothesis that depletion of membrane cholesterol by methyl-β-cyclodextrin (MβCD), a cholesterol scavenger, may regulate the activity of BK_{Ca} channels present in pituitary GH_3 cells (16). The voltage- and Ca^{2+}-sensitivity of BK_{Ca} channels in MβCD-treated cells have also been characterized. The effect of MβCD treatment on the firing of action potentials in these cells was also examined. Interestingly, the results indicate that by its cholesterol-depleting action, MβCD can modulate BK_{Ca}-channel activity, thus leading to the reduced firing of action potentials in these cells.

Materials and Methods

Cell Preparation

The clonal rat pituitary GH_3 cells were obtained from the Bioresources Collection and Research Center (BCRC-60015; Hsinchu, Taiwan). Briefly, cells were grown in 50-ml Ham's F-12 medium that was supplemented with 15% horse serum (v/v), 2.5% fetal calf serum (v/v), and 2 mM L-glutamine in a 5% carbon dioxide atmosphere at 37°C (22, 40). The experiments were performed 5 or 6 days after cells were subcultured (60-80% confluence). The treated cells were incubated at 37°C for 1 hour in normal Tyrode's solution containing different concentrations of MβCD or a mixture of MβCD and cholesterol (MβCD/cholesterol) (16). Such treatment was thought to decrease membrane cholesterol contents in neuroendocrine cells (18).

Electrophysiological Measurements

Immediately before each experiment, GH_3 cells were dissociated and an aliquot of the cell suspension was transferred to a recording chamber mounted on the stage of a DM-IL inverted microscope (Leica, Wetzlar, Germany). Cells were bathed at room temperature (20-25°C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes were pulled from Kimax-51 capillaries (Kimble Glass, Vineland, NJ, USA) using a two-stage microelectrode puller (PP-830, Narishige, Tokyo, Japan), and their tips were fire-polished with a microforge (MF-83, Narishige). When filled with pipette solution, their resistance ranged between 3 and 5 MΩ. Ion currents were measured in the cell-attached, inside-out, and whole-cell configurations of the patch-clamp technique, using an RK-400 patch-clamp amplifier (Bio-Logic, Claix, France) (22). The whole-cell current clamp recordings were performed to measure changes in membrane potential from individual cells (24).

Data Recording and Analysis

The signals were displayed on an analog/digital oscilloscope (HM 507, Hameg, East Meadow, NY, USA) and on a liquid crystal projector (PJ550-2, ViewSonic, Walnut, CA, USA). The data were on-line stored in a Pentium III-grade computer (Slimnote VX_3, Lemel, Taipei, Taiwan) at 10 kHz through a Digidata 1322A interface (Axon Instruments, Union City, CA, USA). This device was controlled by a commercially available software (pCLAMP 9.0, Axon Instruments). Currents were low-pass filtered at 1 or 3 kHz. Ion currents obtained during whole-cell experiments were stored without leakage correction and analyzed using either the pCLAMP 9.0 software (Axon Instruments), the ORIGIN 6.0 software (Microcal, Northampton, MA, USA), or custom-made macros in Excel (Microsoft, Redmont, WA, USA). The pCLAMP-generated voltage step protocols were

used to examine the current-voltage relations for ion currents. The densities (pA/pF) of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) or L-type Ca^{2+} current ($I_{Ca,L}$) were calculated by normalizing the amplitude with cell capacitance. The time constants for inactivation (τ_{inact}) of $I_{Ca,L}$ obtained in untreated and MβCD-treated cells were calculated by fitting currents to a two-exponential function. The firing frequency of action potentials was obtained by dividing the number of spikes to the duration of recording period.

Single-Channel Analysis

The single-channel amplitudes were determined by fitting Gaussian distributions to the amplitude histograms of the closed and the open state. The channel open probability obtained in each patch was expressed as $N \cdot P_o$, which can be estimated using the following equation: $N \cdot P_o = (A_1 + 2A_2 + 3A_3 + \cdots + nA_n)/(A_0 + A_1 + A_2 + A_3 + \cdots + A_n)$, where N is the number of active channels in the patch, A_0 is the area under the curve of an all-points histogram corresponding to the closed state, and $A_1 \cdots A_n$ represent the histogram areas reflecting the levels of distinct open state for 1 to n channels in the patch.

The relationships between membrane potentials and the probability of channel openings in the cells treated with or without MβCD were constructed and fitted with a Boltzmann function of the form: relative open probability = $1/\{1 + \exp[-(V - V_{1/2})/k]\}$, where V = the membrane potential in mV, $V_{1/2}$ = the voltage at which there is half-maximal activation, and k = the slope factor of the activation curve (i.e., the voltage dependence of the activation process in mV per *e*-fold change). The relationships between intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the channel open probability in the absence and presence of MβCD treatment were constructed with the Hill equation: open probability = $(P_{max} \times [Ca^{2+}]_i^{n_H}) / (K_d + [Ca^{2+}]_i^{n_H})$, where K_d and n_H are the apparent dissociation constant and the Hill coefficient (slope factor), respectively; and P_{max} is the maximal increase in channel open probability.

To determine the relationships between open intervals and subsequent duration of closed intervals, time intervals from a channel opening to its closing were paired with time intervals from this channel closing to opening of any channel in inside-out patches exhibiting relatively steady activity with few multiple openings. Spearman's correlation coefficient between these two variables was then determined for each patch (27). To define the exponential components adequately, tens to hundreds of thousands of intervals were collected during stable activity.

The averaged results are presented as the mean values ± SE. Linear regression (least squares fit) and all nonlinear curve-fitting routines were performed using ORIGIN 6.0 (Microcal). The paired or unpaired Student's *t*-test was used for the statistical analyses. To clarify further the statistical difference among the two or four treatment groups, analyses of variance with Duncan's multiple-range test for multiple comparisons were also performed. Differences between values were considered significant when $P < 0.05$.

Drugs and Solutions

Methyl-β-cyclodextrin (MβCD), tetrodotoxin and tetraethylammonium chloride were obtained from Sigma Chemical (St. Louis, MO, USA), and dexamethasone, diazoxide and glibenclamide were from Sigma/RBI (Natick, MA, USA). Caffeic acid phenethyl ester (CAPE; phenethyl caffeiate) was purchased from Cayman Chemical (Ann Arbor, MI, USA), and cilostazol (6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1*H*)- quinolinone) was from Tocris Cookson Ltd. (Bristol, UK). Apamin and paxilline were purchased from Alomone Labs (Jerusalem, Israel). Tissue-culture media and trypsin/ethylenediaminetetraacetic acid (EDTA) were obtained from American Type Culture Collection (Manassas, VA, USA), and L-glutamine, penicillin-streptomycin and amphotericin B (Fungizone) were from Life Technologies (Grand Island, NY, USA). All other chemicals were obtained from regular commercial chemicals and were of reagent grade. The twice-distilled water that had been de-ionized through a Millipore-Q system (Millipore, Bedford, MA, USA) was used in all experiments.

The composition of normal Tyrode's solution was 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose and 5.5 mM HEPES-NaOH buffer, pH 7.4. To record K^+ currents or membrane potential, the recording pipette was backfilled with a solution consisting of 140 mM KCl, 1 mM $MgCl_2$, 3 mM Na_2ATP, 0.1 mM Na_2GTP, 0.1 mM EGTA and 5 mM HEPES-KOH buffer, pH 7.2. The free Ca^{2+} concentration of this solution was estimated to be 230 nM assuming that the residual contaminating Ca^{2+} concentration was 70 μM, and the ratiometric fura-2 measurement with F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) showed that this solution contained 214 ± 15 nM free Ca^{2+} for three different experiments. To measure voltage-dependent Ca^{2+} current, KCl inside the pipette solution was replaced with equimolar CsCl, and pH was adjusted to 7.2 with CsOH, while bathing solution contained 1 μM tetrodotoxin and 10 mM tetraethylammonium chloride to eliminate Na^+ and K^+ currents.

For single-channel current recordings, the high-K^+ bathing solution contained 145 mM KCl, 0.53 $MgCl_2$ and 5 mM HEPES-KOH buffer, pH 7.4, and

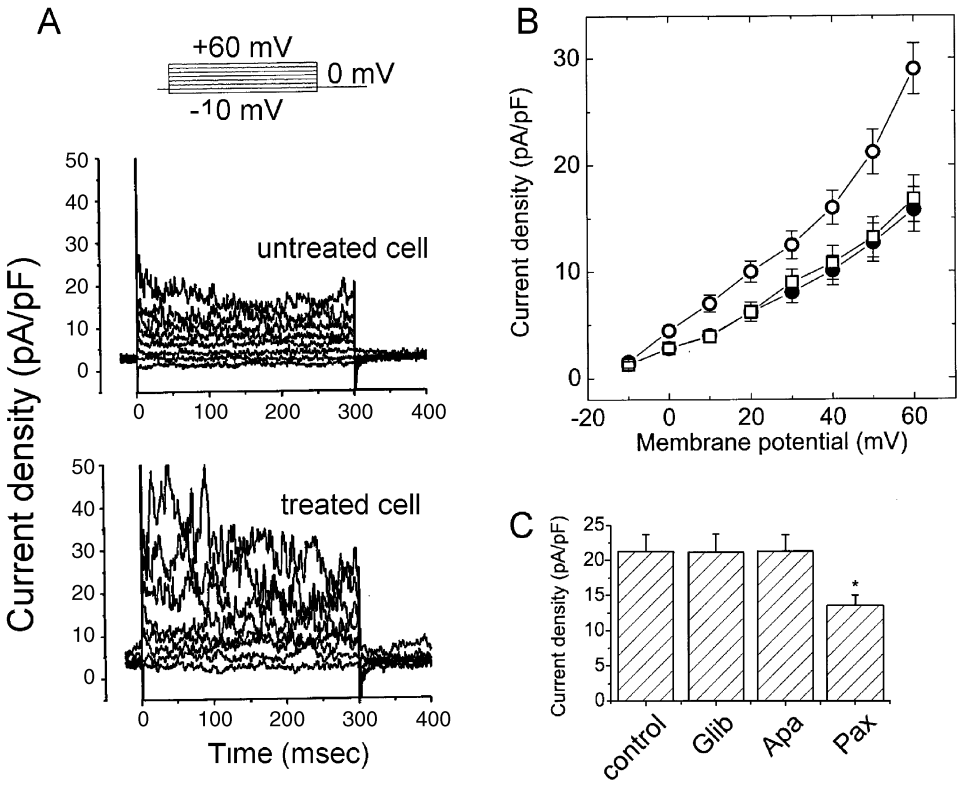

Fig. 1. Effect of MβCD treatment on the density of $I_{K(Ca)}$ in pituitary GH_3 cells. In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The treated cells were incubated with MβCD (1.5 mg/ml) for 1 hour at 37°C. **A**: Superimposed voltage and current traces obtained in untreated (upper) and MβCD-treated (lower) cells. The cells were held at 0 mV, and the voltages from -10 to +60 mV in 10 mV increments were applied. The uppermost part indicates the voltage protocol used. **B**: Current density versus membrane potential relationships of $I_{K(Ca)}$ in untreated cells (●) and in cells treated either with 1.5 mg/ml MβCD (○) or with 1.5 mg/ml MβCD/cholesterol (□). MβCD/cholesterol-treated cells were incubated with 1.5 mg/ml MβCD and 1.5 mg/ml cholesterol. Each point represents the mean ± SE (n = 8-11). C: Summary of $I_{K(Ca)}$ densities obtained in MβCD (1.5 mg/ml)-treated cells (control) and after the addition of 10 μM glibenclamide (Glib), 200 nM apamin (Apa), or 1 μM paxilline (Pax). Each cell was depolarized from 0 mV to +50 mV and the density of $I_{K(Ca)}$ was measured. Each point represents the mean ± SE (n = 6-8). *Significantly different from control group (i.e., MβCD-treated cells without addition of each agent) ($P < 0.05$; unpaired *t*-test).

the pipette solution contained 145 mM KCl, 2 mM $MgCl_2$ and 5 mM HEPES-KOH buffer, pH 7.2. The value of free Ca^{2+} concentration was calculated assuming a dissociation constant for EGTA and Ca^{2+} (at pH 7.2) of 10^{-7} M (30). For example, to provide 0.1 μM free Ca^{2+} in bath solution, 0.5 mM $CaCl_2$ and 1 mM EGTA were added.

Results

Effect of MβCD Treatment on the Density of Ca^{2+}-Activated K^+ Currents ($I_{K(Ca)}$) in GH_3 Cells

In the first series of experiments, the whole-cell configuration of the patch-clamp technique was used to investigate the effect of MβCD on ion currents in these cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, and the pipette solution contained a low concentration (0.1 mM) of EGTA and 3 mM ATP. ATP (3 mM) contained in the pipette solution can block the activity of ATP-sensitive K^+ (K_{ATP}) channels (41). To inactivate other types of voltage-dependent K^+ currents, each cell was routinely held at the level of 0 mV (43). As illustrated in Figure 1, when the cell was held at 0 mV and different potentials ranging from -10 to +60 mV with 10 mV increments were applied, a family of large, noisy, outward currents was elicited. The densities of these currents were increased with greater depolarization in an outward-rectifying manner. These outward currents have been identified as $I_{K(Ca)}$ (42). Interestingly, after the cells were exposed to MβCD (1.5 mg/ml), the densities of these outward currents were increased throughout the entire range of voltage-clamp step. For example, when the voltage step from 0 to +50 mV was evoked, MβCD (1.5 mg/ml) treatment significantly increased current density from 12.7 ± 1.8 to 21.2 ± 2.1 pA/pF ($P < 0.05$; n = 8). However, there was no

significant difference in the density of $I_{K(Ca)}$ between untreated cells and cells treated with a mixture of MβCD and cholesterol (MβCD/cholesterol; 1.5 mg/ml). In addition, no significant difference in cell capacitance between untreated (11.2 ± 1.3 pF, n = 6) and MβCD-treated (11.1 ± 1.4 pF; $P > 0.05$, n =6) cells could be found. Rectification properties of the current were also unaffected by MβCD treatment. Figure 1B illustrates the averaged current density versus voltage relationships for $I_{K(Ca)}$ obtained in untreated, MβCD-treated cells and MβCD/cholesterol-treated cells. Furthermore, when cells were depolarized from -50 to 0 mV with a duration of 300 msec, MβCD treatment caused no significant change in the inactivation kinetics of $I_{Ca,L}$ [untreated cells: $\tau_{inact(f)}$ = 9 ± 3 msec, $\tau_{inact(s)}$ = 35 ± 7 msec (n = 7); MβCD-treated cells: $\tau_{inact(f)}$ = 9 ± 3 msec, $\tau_{inact(s)}$ = 36 ± 6 msec (n = 7); $P > 0.05$].

Comparison of the Effects of Glibenclamide, Apamin and Paxilline on $I_{K(Ca)}$ Density in MβCD-Treated Cells

It was further examined if the density of $I_{K(Ca)}$ in MβCD-treated cells could be altered by the presence of glibenclamide, apamin or paxilline. Glibenclamide is an antagonist of ATP-sensitive K^+ channels, apamin is a blocker of small-conductance calcium activated potassium channels and paxilline is a BK_{Ca} channel blocker. As shown in Figure 1C, when MβCD-treated cells were depolarized from 0 to +50 mV, paxilline (1 μM) inhibited current density significantly. Paxilline (1 μM) significantly decreased the density of $I_{K(Ca)}$ from 21.3 ± 2.4 to 13.6 ± 1.4 pA/pF ($P < 0.05$; n = 6). However neither glibenclamide (10 μM) nor apamin (200 nM) had any effects on the density of $I_{K(Ca)}$ in 1.5 mg/ml MβCD-treated cells. Thus, $I_{K(Ca)}$ found in MβCD-treated cells is sensitive to inhibition by paxilline, yet not by glibenclamide or apamin.

Effect of MβCD Treatment on Voltage-Dependent L-Type Ca^{2+} Current ($I_{Ca,L}$) in Pituitary GH_3 Cells

$I_{K(Ca)}$ can be functionally coupled with Ca^{2+} influx through plasmalemmal voltage-dependent Ca^{2+} channels in GH_3 cells (40). To this aim, we further investigated if the increase in $I_{K(Ca)}$ density observed in MβCD-treated cells could be accompanied by changes in the density of $I_{Ca,L}$ described previously in GH_3 cells (24, 40). In these experiments, untreated or 1.5 mg/ml MβCD-treated cells were bathed in normal Tyrode's solution that contained 1.8 mM $CaCl_2$. As illustrated in Figure 2, current density versus membrane potential relationships of $I_{Ca,L}$ in untreated and MβCD-treated cells were constructed and compared. The results showed that the density of $I_{Ca,L}$ obtained in cells exposed to MβCD (1.5 mg/ml)

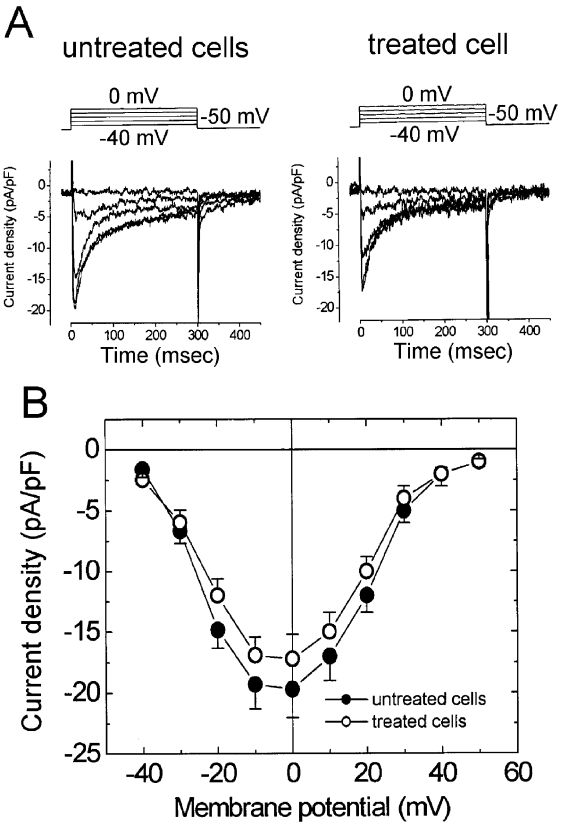

Fig. 2. Effect of MβCD treatment on $I_{Ca,L}$ in GH_3 cells. The untreated and MβCD-treated cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. **A**: Superimposed voltage and current tracings obtained in cells without (left) and with (right) the exposure to MβCD (1.5 mg/ml). Each cell was depolarized from -50 mV to various potentials ranging from -40 to 0 mV in 10 mV increments. The voltage protocol used is shown in the upper part. **B**: Current density versus membrane potential relationships of $I_{Ca,L}$ obtained in untreated (●) and MβCD-treated (○) cells. Each point represents the mean ± SE (n = 5-9).

was significantly lower than that in untreated cells. For example, when cells were depolarized from -50 to 0 mV, the density of $I_{Ca,L}$ in untreated cells was 19.7 ± 2.3 pA/pF (n = 8), while current density was reduced to 16.9 ± 1.8 pA/pF ($P < 0.05$; n = 8). However, we found no change in the configuration of current density versus membrane potential relations for $I_{Ca,L}$ in MβCD-treated cells. Therefore, the results indicate that depletion of membrane cholesterol by MβCD increases the density of $I_{K(Ca)}$ in a manner conceivably unlikely to be linked to the enhanced density of $I_{Ca,L}$.

Comparison of BK_{Ca}-Channel Activity in Untreated and MβCD-Treated Cells

The studies from our whole-cell experiments

suggest that $I_{K(Ca)}$ may be K^+ flux primarily through the BK_{Ca} channel present in GH_3 cells (22, 42), because the density of $I_{K(Ca)}$ was suppressed by paxilline, yet not by glibenclamide or apamin. To elucidate further how MβCD treatment could affect $I_{K(Ca)}$, the activity and gating of BK_{Ca} channels was investigated. In the following experiments, the single-channel recordings with inside-out configuration were performed in symmetrical K^+ concentration (145 mM). The bath solution contained 0.1 μM Ca^{2+}, and the potential was held at +60 mV. As shown in Figure 3, the activity of BK_{Ca} channels could be readily observed in excised patches from untreated and 1.5 mg/ml MβCD-treated cells. More interestingly, the probability of channel openings was greater in MβCD-treated cells, as compared with that in untreated cells. By its cholesterol depleting action, MβCD could enhance BK_{Ca}-channel activity in a concentration-dependent manner. The addition of paxilline could reduce channel activity in MβCD-treated cells. However, no significant change in single-channel amplitude of BK_{Ca} channels could be demonstrated in cells exposed to MβCD (1.5 mg/ml).

Lack of Effect of MβCD Treatment on Single-Channel Conductance of BK_{Ca} Channels

A previous study showed that in addition to the decrease in the activity of BK_{Ca} channels reconstituted into lipid bilayers, cholesterol depletion might reduce their single-channel conductance (12). Therefore, the BK_{Ca}-channel activity measured at different membrane potentials was also studied in untreated and MβCD-treated cells (Fig. 4). The unitary conductance of the channels, calculated as the slope of the unitary current-voltage relationship, was 176 ± 8 pS (n = 8). However, this value did not significantly differ from that (177 ± 7 pS; $P > 0.05$, n = 8) measured in cells treated with MβCD (1.5 mg/ml). Unlike those described previously (12), the data presented here indicate that BK_{Ca} channels may increase their activity in response to cholesterol depletion with MβCD without changing their single-channel conductance in GH_3 cells.

Effect of MβCD Treatment on the Activation Curve of BK_{Ca} Channels

Figure 4 shows the activation curve of BK_{Ca} channels in cells with and without the treatment of MβCD (1.5 mg/ml). The relationships between membrane potential and relative open probability of BK_{Ca} channels were plotted and adequately fit by the Boltzmann equation using a nonlinear regression analysis (Fig. 4B). In untreated cells, $V_{1/2}$ = 66.1 ± 1.7 mV, and k = 4.6 ± 0.5 mV (n = 7), whereas in

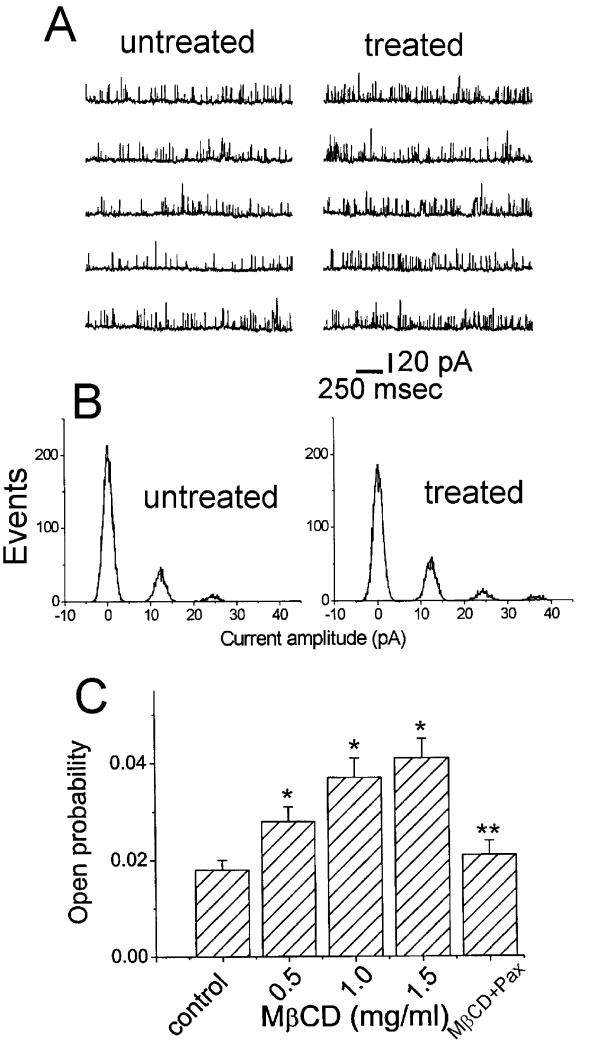

Fig. 3. Comparison of BK_{Ca}-channel activity between untreated and MβCD-treated cells. **A**: Original current traces showing the activity of BK_{Ca} channels recorded from untreated (left) and MβCD-treated (right) cells. The inside-out configuration was performed, bath medium contained 0.1 μM Ca^{2+}, and holding potential was +60 mV. Upward deflection indicates the opening events of the channel. **B**: Current amplitude histograms obtained from inside-out patches of untreated (left) and MβCD-treated (right) cells. Of note, no change in single-channel amplitude was observed in cells exposed to MβCD (1.5 mg/ml). **C**: Summary of BK_{Ca}-channel activity obtained in cells exposed to 0.5, 1 and 1.5 mg/ml MβCD. Channel activity was measured at +60 mV. Each point represents the mean ± SE (n = 5-9). MβCD+Pax: addition of paxilline (1 μM) to MβCD (1.5 mg/ml)-treated cells. *Significantly different from control group (i.e., untreated cells) ($P < 0.05$; ANOVA and Duncan's test). **Significantly different from MβCD (1.5 mg/ml)-treated group ($P < 0.05$; unpaired *t*-tests).

MβCD-treated cells, $V_{1/2}$ = 54.2 ± 1.9 mV, and k = 4.5 ± 0.6 mV (n = 6). Therefore, MβCD treatment not only caused an increase in the maximal open

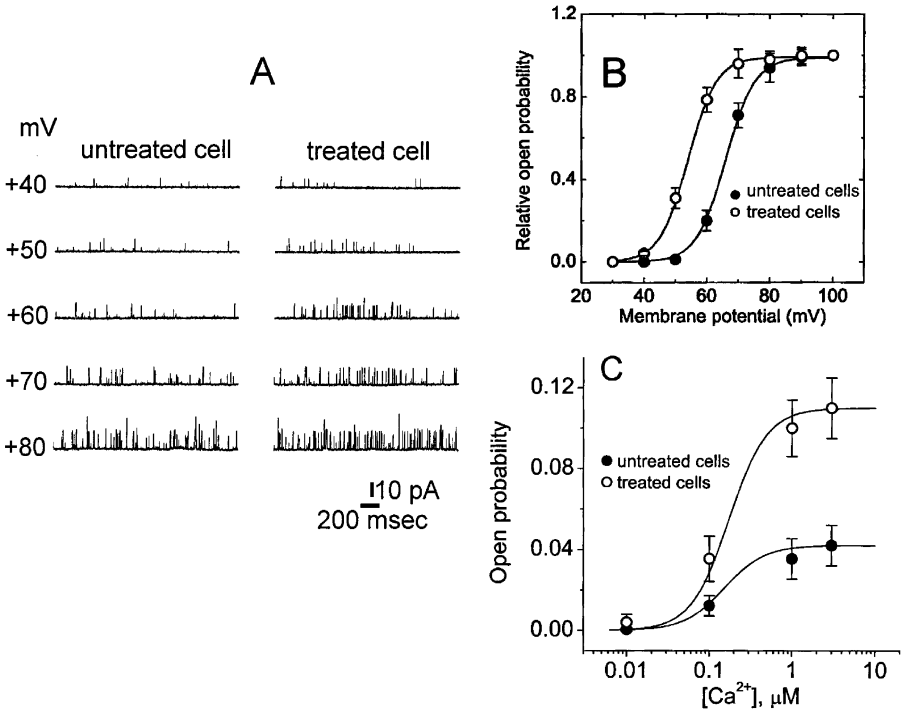

Fig. 4. Voltage- and Ca^{2+}-sensitivity of BK_{Ca} channels in untreated and MβCD-treated cells. **A**: Examples of BK_{Ca} channels in untreated (left) and MβCD-treated (right) cells measured from inside-out patches at different membrane potentials ranging from +40 to +80 mV. The numbers shown at the beginning of each current trace indicate the voltage applied to the patch pipette. Cells were bathed in symmetrical K^+ (145 mM) solution and bath medium contained 0.1 μM Ca^{2+}. **B**: Relationship between relative open probability of BK_{Ca} channels and membrane potential in untreated (●) and MβCD-treated (○) cells. The probability of channel openings at +100 mV was considered to be 1.0. The smooth lines represent best fit to the Boltzmann equation described in the text. **C**: Effect of intracellular C ($[Ca^{2+}]_i$) on BK_{Ca}-channel activity recorded from inside-out patches of untreated and MβCD-treated cells. The relationships between the open probability and $[Ca^{2+}]_i$ in the absence (●) and presence (○) of MβCD treatment was illustrated. Each patch was held at +60 mV. The smooth lines represent best fit to a Hill function described in the text. Each point represents the mean ± SE (n = 5-7).

probability of BK_{Ca} channels, but also shifted the activation curve toward less positive voltages by about 12 mV. However, no significant difference in the slope (i.e., *k*) of activation curve could be observed between the two groups of cells. Taken together, these results indicate that depletion of membrane cholesterol with MβCD can alter the voltage-sensitivity of BK_{Ca} channels in GH_3 cells. However, no change in the effective number of charges (i.e., gating particles) during channel activation can be observed in MβCD-treated cells.

Effect of Internal Ca^{2+} Concentration ($[Ca^{2+}]_i$) on BK_{Ca}-Channel Activity in Untreated and MβCD-Treated Cells

Because BK_{Ca}-channel activity also depends on $[Ca^{2+}]_i$, whether the increase in channel activity found in MβCD-treated cells is associated with internal Ca^{2+} concentration was investigated. In these experiments, when a detached patch was formed, various concentrations of Ca^{2+} in the bath were applied. The relationships between membrane potential and the probability of channel openings were plotted and fit by the Hill equation (Fig. 4C). In untreated cells, $P_{max} = 0.042 \pm 0.002$, $K_d = 0.034 \pm 0.005$ μM, and $n_H = 1.8 \pm 0.2$ (n = 6), whereas in MβCD-treated cells, $P_{max} = 0.11 \pm 0.003$, $K_d = 0.036 \pm 0.004$ μM, and $n_H = 1.9 \pm 0.2$ (n = 6). Therefore, although MβCD treatment caused an increase in the maximal open probability of BK_{Ca} channels, no significant difference in the slope (i.e., n_H) and the dissociation constant (i.e., K_d) of the relationships between $[Ca^{2+}]_i$ and open probability could be observed between the two groups of cells. Taken together, these results indicate that depletion of membrane cholesterol with MβCD can not modify the Ca^{2+}-sensitivity of BK_{Ca} channels in GH_3 cells.

Kinetic Behavior of BK_{Ca} Channels in Untreated and MβCD-Treated Cells

The kinetic gating of these channels was analyzed because of no change in single-channel conductance between the two groups of cells. In an excised patch of control cell, open time histograms at

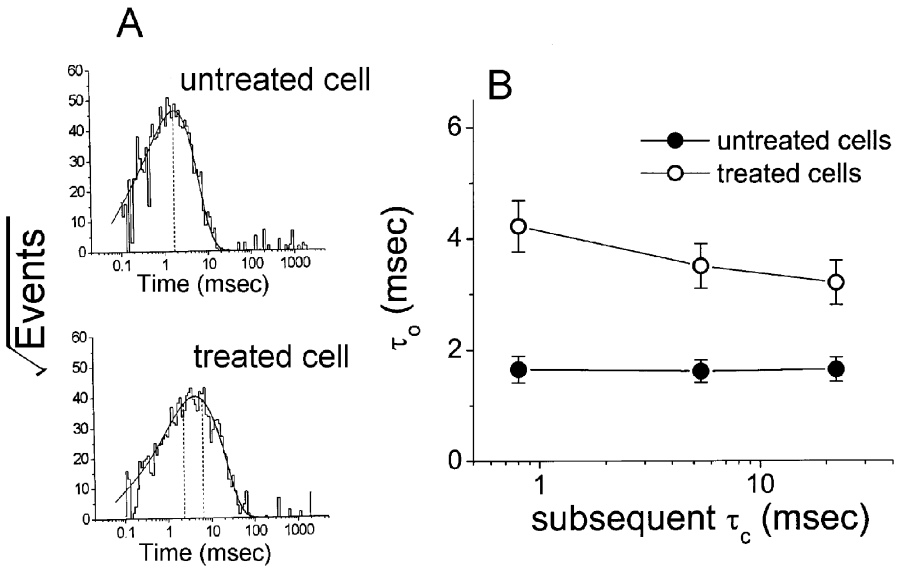

Fig. 5. Mean open time of BK_{Ca} channels in untreated (upper) and MβCD-treated (lower) cells (**A**), and relationship between open intervals (τ_o) and subsequent duration of closed intervals (τ_c) from single inside-out patches of untreated (●) and MβCD-treated (○) cells (**B**). Inside-out configuration was performed in these experiments and the potential was held at +60 mV. Cells were bathed in symmetrical K^+ solution (145 mM) and bath medium contained 0.1 μM Ca^{2+}. Open time histogram in untreated cells was fitted by a single-exponential function with a mean open time of 1.7 msec. The open time histogram obtained in MβCD-treated cells was fitted by a two-exponential function with a mean open time of 2.3 and 6.4 msec. The data were obtained from a measurement of 296 channel openings with a total recording time of 1 min in untreated cells, and those obtained in MβCD-treated cells were measured from 259 channel openings with a total recording time of 30 sec. The dashed lines shown in each lifetime distribution are placed at the value of the time constant in the open state. **B**: Relationships between τ_o and subsequent τ_c in untreated and MβCD-treated cells. The values of τ_c were sorted into three logarithmic bins. For each bin, the value of τ_o in the preceding open interval was calculated. Each point represents the mean ± SE (n = 4-5 pairs). The total number of paired intervals in this patch was 834, and the Spearman correlation coefficient was -0.172. Of note, long channel openings tend to be followed by short closings in MβCD-treated cells.

the level of +60 mV can be fitted by a one or two exponential curve (Fig. 5A). The time constant for the open time histogram was 1.8 ± 0.6 msec (n = 6). It is important to note, however, that in inside-out patches recorded from MβCD-treated cells, a two-exponential function was required to adequately fit the open time histogram. The time constants for fast and slow components of the open state were 2.3 ± 0.3 and 6.4 ± 1.1 msec, respectively (n = 6). Therefore, these results showed that depletion of membrane cholesterol by MβCD could enhance the channel activity by increasing mean open time.

To obtain more information on the channel kinetics, we analyzed the relationship between the duration of the open interval and the duration of the subsequent closed interval in inside-out patches of untreated and MβCD-treated cells (27). Each patch used for this study generally displayed a steady level of activity with few double openings. Spearman's correlation coefficients between open and subsequent closed intervals were calculated. Interestingly, as shown in Figure 5B, the duration of the channel opening was negatively correlated with the duration of subsequent channel closing in MβCD-treated cells. Similar results can be obtained in five different patches of MβCD-treated cells. The results can be interpreted to mean that when GH_3 cells were treated with MβCD, the briefer openings of these channels often occur adjacent to the longer closed intervals, whereas the longer openings may occur adjacent to the briefer closed intervals. This correlation implies that the channel has multiple open and closed states in MβCD-treated cells. Such a negative correlation may also impose further constraints on the kinetic schemes of BK_{Ca} channels when cells were exposed to the cholesterol scavenger, MβCD.

Effect of Caffeic Acid Phenethyl Ester (CAPE), Cilostazol or Dexamethasone on BK_{Ca} Channels in GH_3 Cells Treated with MβCD

Caffeic acid phenethyl ester or cilostazol has been previously reported to stimulate BK_{Ca} channels (23, 45). Glucocorticoids (e.g., dexamethasone) were also shown to increase BK_{Ca}-channel activity in a non-genomic manner (25). These findings lead us to

investigate possible changes in the sensitivity to these compounds when membrane cholesterol was depleted in the treatment of MβCD (1.5 mg/ml). As shown in Figure 6, the application of either cilostazol (30 μM) or CAPE (30 μM) increased channel open probability in MβCD-treated cells. Our observations showed, however, that addition of dexamethasone (30 μM) was found to have little or no effect on the probability of channel openings in MβCD-treated cells, despite its ability to stimulate BK_{Ca}-channel activity present in untreated cells. In untreated cells, dexamethasone (30 μM) significantly increased the open probability from 0.121 ± 0.022 to 0.34 ± 0.032 ($P < 0.05$; n = 6). In cells treated with MβCD (1.5 mg/ml), there was no significant difference in channel activity between the absence and presence of dexamethasone (30 μM) (0.321 ± 0.031 [n =6] versus 0.322 ± 0.025 [n = 6]; $P > 0.05$). Conversely, addition of cilostazol (30 μM) or CAPE (30 μM) could increase channel open probability in both untreated and MβCD-treated cells to a similar magnitude (Fig. 6). Taken together, the experimental results suggest that unlike the actions of cilostazol or CAPE, the stimulatory effects of dexamethasone on single BK_{Ca} channel is related to changes in membrane cholesterol content.

Effect of Diazoxide on the Activity of ATP-Sensitive K^+ (K_{ATP}) Channels in Untreated and MβCD-Treated Cells

We also investigated if MβCD treatment could modify the activity of K_{ATP} channels functionally expressed in GH_3 cells (41). In these experiments, untreated or MβCD-treated cells were bathed in symmetrical K^+ (145 mM) solution. In cell-attached configuration, each cell was held at the level of -60 mV. As described previously (41), when diazoxide (30 μM) was applied to the bath, K_{ATP}-channel activity was readily increased in both untreated and 1.5 mg/ml MβCD-treated cells. Diazoxide is a known opener of SUR1/Kir6.2 K_{ATP} channels. However, the magnitude of diazoxide-induced increase in K_{ATP}-channel activity did not differ significantly between untreated and MβCD-treated cells (Fig. 7). At the holding potential of -60 mV, the presence of diazoxide (30 μM) caused a 2.4-fold increase in the probability of channel openings in untreated and MβCD-treated cells. In addition, diaxozide enhanced K_{ATP}-channel activity in MβCD (1.5 mg/ml)- and MβCD (3 mg/ml)-treated cells to a similar extent. Therefore, the cholesterol depletion maneuver with MβCD was found to have no alterations in K_{ATP}-channel activity in GH_3 cells.

Discharge Pattern of Spontaneous Action Potentials in Untreated and MβCD-Treated GH_3 Cells

The presence of repetitive firing of action

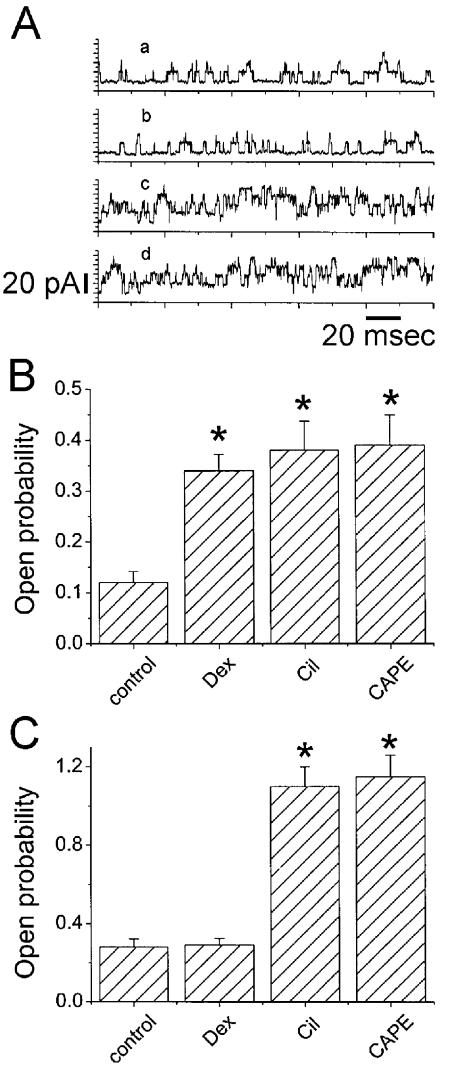

Fig. 6. Dexamethasone, cilostazol and CAPE on BK_{Ca}-channel activity in untreated and MβCD-treated cells. **A**: Original current traces showing the effect of dexamethasone, cilostazol and CAPE on BK_{Ca} channels in MβCD-treated cells. Inside-out configuration was made and cells were bathed in symmetrical K^+ (145 mM) solution. The holding potential was +60 mV and bath medium contained 0.1 μM Ca^{2+}. a: control; b: dexamethasone (30 μM); c: cilostazol (30 μM); and d: CAPE (30 μM). Bar graphs in **B** and **C** are the summary of data showing the effect of dexamethasone, cilostazol and CAPE on BK_{Ca}-channel activity in untreated (**B**) and MβCD-treated (**C**) cells, respectively. Dex: 30 μM dexamethasone; Cil: 30 μM cilostazol; and CAPE: 30 μM CAPE. Each point represents the mean ± SE (n = 5-10). *Significantly different from control groups ($P < 0.05$; unpaired *t*-test).

potentials has been thought to be of major importance in controlling basal and stimulated secretion of prolactin in pituitary tumor cells and native lactotrophs (28, 34, 38). It will be of importance to determine if the opening of BK_{Ca} channels following MβCD

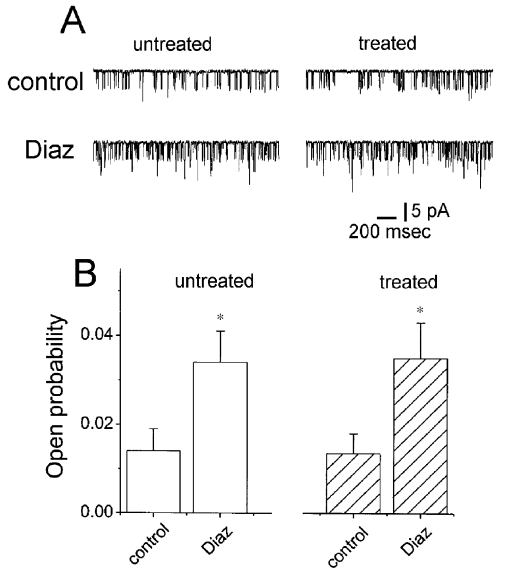

Fig. 7. Effect of diazoxide on K_{ATP}-channel activity in untreated and MβCD-treated GH_3 cells. The experiments were performed in symmetrical K^+ concentration (145 mM). Under cell-attached configuration, cells were held at -60 mV. **A**: Original current traces obtained in untreated (left) and MβCD-treated (right) cells without (control) or with addition of 30 μM diazoxide (Diaz). Channel openings are shown as a downward deflection. **B**: Bar graphs showing the effect of 30 μM diazoxide (Diaz) on channel open probability in untreated (left) and MβCD (1.5 mg/ml)-treated (right) cells. *Significantly from control groups ($P < 0.05$; paired t-test). Each point represents the mean ± SE (n = 4-8).

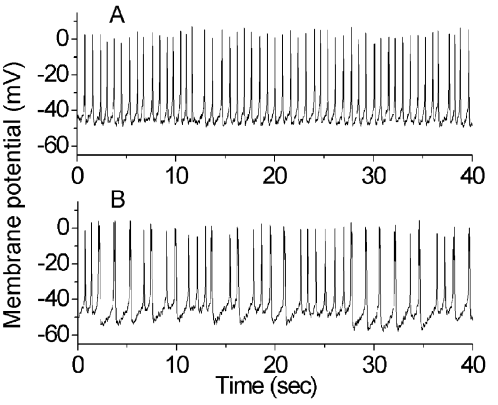

Fig. 8. Repetitive firing of action potentials in an untreated (**A**) and an MβCD-treated (**B**) cells. GH_3 cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The patch pipettes were filled with a K^+-containing solution. Changes in membrane potential were measured under the whole-cell current clamp configurations. Of note, the firing of action potentials was reduced in MβCD-treated cell.

treatment can underlie any modification in the firing of action potentials. In a final series of experiments, changes in membrane potential were thus examined in cells treated with or without MβCD (1.5 mg/ml). Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The whole-cell current clamp recordings were performed with a K^+-containing pipette solution. The typical effect of MβCD treatment on spontaneous action potentials of an untreated and an MβCD-treated cell is illustrated in Figure 8. The firing rate of action potentials recorded from MβCD-treated cells (0.6 ± 0.1 Hz, n = 5) was significantly less than that measured from untreated cells (1.1 ± 0.2 Hz; $P < 0.05$, n = 5). In MβCD-treated cells, the firing frequency was increased after application of paxilline (1 μM). The resting potentials (-52 ± 4 mV, n = 6) of untreated cells was significantly different from those (-59 ± 4 mV; $P < 0.05$, n =6) recorded from MβCD-treated cells. However, treatment with MβCD/cholesterol (1.5 mg/ml) did not affect spontaneous activity. Thus, it is plausible to think that depletion of membrane cholesterol by MβCD treatment regulates the firing of action potentials in pituitary GH_3 cells. These changes are due mostly to the increase in BK_{Ca}-channel activity induced by the cholesterol-depletion treatment with MβCD.

Discussion

The present studies show that: [1] in pituitary GH_3 cells, cholesterol depletion with methyl-β-cyclodextrin (MβCD), a cyclic oligosaccharide, strongly influences the density of $I_{K(Ca)}$, while exposure to MβCD/cholesterol has little or no effect on it; [2] MβCD treatment can modify the sensitivity of BK_{Ca}-channel activity to membrane depolarization, but does not alter single-channel conductance; [3] no change in K_{ATP} channels can be found by MβCD treatment; and [4] the repetitive firing of action potentials following cholesterol depletion by MβCD was reduced. Because membrane cholesterol content in these cells is altered by MβCD treatment, such an increase in BK_{Ca}-channel activity may reduce cell excitability, thus affecting the functional activities of neurons or neuroendocrine cells.

Manipulation of the cholesterol content and lipid composition in the cell membrane can directly affect the activity of BK_{Ca} channels (6, 12). MβCD is an agent known to perturb raft organization (10, 14). The modulation of the BK_{Ca} channels by MβCD treatment presented here did not occur by the gene regulation of these channels, because significant regulation of the BK_{Ca} channels appeared within a short time course. In addition, the increased density of $I_{K(Ca)}$ in MβCD-treated cells does not depend on the increased availability of intracellular Ca^{2+} resulting

from the enhanced Ca^{2+} influx through voltage-dependent Ca^{2+} channels, because the density of $I_{Ca,L}$ was reduced in treated cells.

A recent report showed that the α-subunit of the BK_{Ca} channel could be localized to sphingomyelin- and cholesterol-containing raft membranes (2). Our results demonstrated that cholesterol depletion by MβCD enhanced the activity of BK_{Ca} channels in a concentration-dependent manner. It is thus possible that changes in membrane cholesterol not simply redistribute the BK_{Ca} channel spatially within the membrane, but also alter both channel open probability and gating. A direct cholesterol-protein interaction appears to be required for the normal functioning of the BK_{Ca} channels in GH_3 cells. Inconsistent with a previous report by Chang *et al*. (12), no difference in single-channel conductance was found between untreated and MβCD-treated cells. However, Crowley *et al*. (13) found no modification in the conductance of BK_{Ca} channels following cholesterol manipulation in lipid bilayers. It thus appears that the main parts of ion channel permeation pathway (i.e., the S5 and S6 regions) in the BK_{Ca} channel are not affected by cholesterol-depleting treatment with MβCD.

In this study, neither the slope factor (n_H) nor the dissociation constant (K_d) for intracellular Ca^{2+}-BK_{Ca}-channel activity relationships recorded from GH_3 cells was altered in MβCD treatment. These results suggest that such a treatment does not modify the sensitivity of BK_{Ca} channels to $[Ca^{2+}]_i$. It thus appears that depletion of membrane cholesterol with MβCD may not alter the binding of Ca^{2+} to the long C-terminal tail regions of the channel (5). In addition, after MβCD treatment, BK_{Ca} channels present in GH_3 cells could activate at more depolarized voltages than in untreated cells. However, no significant difference in the slope factor for the relationship of voltage-BK_{Ca} channel activity was found by MβCD treatment. These results can be interpreted to mean that the effective number of elementary charges during channel activation in the absence or presence of MβCD is the same. There thus appears to have little or no effect of membrane cholesterol content on transmembrane voltage sensor (i.e., S0 through S4 regions) of BK_{Ca} channel, since these regions have been implicated in voltage-sensitive gating functions of these channels (36).

An earlier study demonstrated that in hypercholesterolemic rabbits, the K_{ATP}-channel activity appeared to be reduced in coronary arteries (29). However, no change in K_{ATP}-channel activity following the exposure to MβCD was observed in our study. This discrepancy is currently unknown; however, it could be related to the possibility that different subtypes of K_{ATP} channels are functionally expressed among cells examined. Additionally, pinacidil used in previous studies to be an opener of K_{ATP} channels (29), could also be effective in stimulating BK_{Ca}-channel activity (44).

In our study, paxilline at a concentration of 1 μM suppressed whole-cell K^+ outward currents by about 36%. However, neither glibenclamide nor apamine could block K^+ current significantly. In addition, in MβCD-treated cells, paxilline at a concentration of 10 μM could block the density of $I_{K(Ca)}$ by 93 ± 4 % (n = 6). There thus appears to be a decrease in $I_{K(Ca)}$ sensitivity to paxilline when cells were treated with MβCD.

In this study, we found that MβCD treatment might shift the BK_{Ca} channel to longer open state(s). It was also noted that the duration of the open intervals for the BK_{Ca} channel was negatively correlated with the duration of the subsequent closed intervals. These results suggest the ability of variations of membrane cholesterol content to modify the gating mechanism of BK_{Ca} channels. It is also possible that membrane cholesterol depletion leads to transition of the channel to long closed state, linked to a long open state. Moreover, additional constraints on the kinetic models could be imposed by exposing the cells to MβCD.

We demonstrate that in pituitary GH_3 cells, removal of membrane cholesterol by MβCD may modify the activity of BK_{Ca} channels. It is important to note, however, that the stimulation by glucocorticoids of BK_{Ca} channels did not occur in MβCD-treated cells, despite the ability of caffeic acid phenethyl ester or cilostazol to increase the probability of BK_{Ca} channels in both untreated and treated cells. It appears that the binding of dexamethasone to the channel protein(s) is dependent on membrane cholesterol content, in which the channel resides. Membrane cholesterol content and/or distribution thus appear to contribute to differential glucocorticoid sensitivity of BK_{Ca} channels present in neurons or neuroendocrine cells. Our study also suggests that cholesterol and glucocorticoids might share a common target on the BK_{Ca} channel or in the phospholipid layer, which is important in determining the stability of the channel open state(s).

Although MβCD treatment was found to increase the density of $I_{K(Ca)}$, MβCD/cholesterol treatment which supposedly leads to membrane cholesterol enrichment failed to modify $I_{K(Ca)}$ density and the firing of action potentials in GH_3 cells. The reason could be due to the possibility that these cells naturally contain a cholesterol fraction in their membranes that is already maximal. It is also likely that BK_{Ca}-channel activity shown here is relatively lower in untreated GH_3 cells, so that in MβCD/cholesterol-treated cells, the $I_{K(Ca)}$ density may not be reduced.

The BK_{Ca} channels are known to play important roles in the repolarization of single spike and/or after

trains of spikes (46). Previous reports suggest that these channels do not appear to contribute to the resting conductance because of a low Ca^{2+} sensitivity at the level of resting potential (20). However, a recent report clearly demonstrates that BK_{Ca} channels in clonal GH_4C_1 cells are active at the resting potential (17). It is thus likely that ion currents flowing through BK_{Ca} channels present in pituitary tumor cells or native lactotrophs significantly contribute to the level of resting potential (17). The observed difference in resting potential in control versus MβCD treatment could be primarily explained by changes in BK_{Ca}-channel activity in these two groups of cells. The ability of MβCD treatment to reduce the firing of action potentials observed in this study also lead us to propose that changes in membrane cholesterol may alter the functional activities of these cells.

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