Cholesterol Depletion with (2-Hydroxypropyl)- β -Cyclodextrin Modifies the Gating of Membrane Electroporation-Induced Inward Current in Pituitary Tumor GH₃ Cells: Experimental and Analytical Studies

Sheng-Nan Wu^{a,b,c}, Chia-Chen Yeh^a, Hsien-Ching Huang^a and Wei-Hsin Yang^a

^aDepartment of Physiology, National Cheng Kung University Medical College, Tainan City, ^bDepartment of Anatomy and Cell Biology, National Cheng Kung University Medical College, Tainan City, ^cInstitute of Basic Medical Sciences, National Cheng Kung University Medical College, Tainan City

Key Words

Membrane cholesterol • Ion current • Electroporation • Activation kinetics • Pituitary cells

Abstract

The effects of (2-hydroxypropyl)- β -cyclodextrin (HP β CD), a cyclic oligomer, on membrane electroporation-induced inward current (I_{MEP}) in pituitary tumor (GH₃) cells were experimentally and analytically characterized. Depletion of membrane cholesterol by exposing cells to HP β CD (2 mM) increased the activation time constant of delayed rectifier K⁺ current. Such maneuver resulted in a significant reduction of I_{MEP} density. 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), an initiator of free radicals, increased the magnitude of I_{MEP} . AAPH-stimulated I_{MEP} was not reversed by the blockers of Ca²⁺-activated K⁺ channels, but by LaCl₃ or MnCl₂. However, in HP β CD-treated cells, the ability of AAPH to enhance I_{MEP} was abolished. Under such maneuver, the gating charge of I_{MEP} activation was increased by 2 fold, along with a hyperpolarized shift of the activation curve by 30 mV. No change in single-channel conductance of MEP-induced channels during cell exposure to HP β CD was demonstrated.

The energy change of I_{MEP} in untreated and HP β CD-treated cells was estimated to be -17.7 and -44.8 kJ/mol, respectively, and the perturbation of free energy following HP β CD treatment was -27.1 kJ/mol. Based on an MEP model, cell exposure to HP β CD increased the edge energy of the electropore size. By use of a two barrier-one site barrier model, HP β CD treatment can increase both the peak height and well depth of the barrier profile. Taken together, depletion of membrane cholesterol by HP β CD can elevate the edge energy of pore formation, thereby decreasing the I_{MEP} magnitude. The channel-suppressing properties during membrane cholesterol depletion with HP β CD might thus contribute to the underlying mechanisms by which such maneuver alters neuronal or neuroendocrine function.

Copyright © 2011 S. Karger AG, Basel

Introduction

Membrane electroporation (MEP) is recognized to exert a considerable increase in the electrical conductivity and permeability of the plasma membrane by the use of an externally applied electrical field [1]. By applying strong

electrical pulses, it has been widely used to electrotransfer large, membrane impermeant molecules such as DNAs, anti-neoplastic drugs or antibodies into cells [2]. Oxidative modification of the cell membrane has been described to increase the formation of electropores induced by MEP [3, 4]. In GH₃ pituitary tumor cells, we have recently identified a unique type of membrane hyperpolarization-induced inward currents referred to as MEP-induced current (I_{MEP}) that is sensitive to be blocked by memantine and LaCl₃ [5]. Owing to high conductance of MEP-induced channels, even at low probability of opening, significant currents tend to flow, thereby altering the electrical behavior of cells. However, whether I_{MEP} can be influenced by either oxidative injuries or cholesterol depletion remains largely elusive.

Cholesterol is one of the principal lipid components of the plasma membrane in all mammalian cells. Changes in the level of membrane cholesterol are known to have an important impact on the physical properties of the membrane lipid bilayer, including ordering of the phospholipids, changes in membrane fluidity, and membrane elastic modulus [6-9]. Cholesterol can promote the formation of rafts that are membrane subdomains enriched in cholesterol and sphingolipids [10]. Besides that, there are several lines of evidence to indicate that changes in the amount of membrane cholesterol can directly modify the activity of membrane ion channels [11-15]. Recent work in our laboratory has shown that the challenge of NG108-15 neuronal cells with methyl- β -cyclodextrin (M β CD) was able to increase the activation time constant of delayed rectifier K⁺ current and alter the firing pattern of these cells [16]. A previous study demonstrated that the presence of cholesterol in the egg-yolk bilayer membrane increased the value of the breakdown potential induced by MEP [17], indicating that cholesterol-enriched membrane tends to increase membrane stiffness. However, cholesterol depletion was reported to enhance the stiffness of the membrane by altering the mechanical scaffold of the plasma membrane of vascular endothelial cells [7]. Therefore, the issue of how changes in membrane cholesterol interact with I_{MEP} in neurons or neuroendocrine cells remains controversial.

Similar to M β CD, 2-hydroxypropyl- β -cyclodextrin (HP β CD) is a cyclic oligosaccharide which is composed of a hydrophilic outer surface and a hydrophobic core that has a high affinity for sterols. This compound has been reported to enhance water solubility of various compounds through an inclusion complex formation [18]. As a cholesterol scavenger, it is presumed to disrupt cholesterol-rich membrane domains and sequester lipids

within their core [9, 10]. Therefore, the present study was undertaken to test the hypothesis that depletion of membrane cholesterol by HP β CD may regulate the activity of I_{MEP} present in GH₃ pituitary tumor cells. The voltage-sensitivity of I_{MEP} activation has also been compared in cells treated with and without HP β CD. Interestingly, this study indicates that by its cholesterol-depleting action, HP β CD is able to interact with the gating of MEP-induced channels to decrease the density of I_{MEP} in these cells. The results presented here suggest that the electropores induced by membrane hyperpolarization could be primarily co-localized to the cholesterol-containing membrane rafts.

Materials and Methods

Drugs and solutions

t-Butyl hydroperoxide (*t*-BHP), (2-hydroxypropyl)- β -cyclodextrin (HP β CD), methyl- β -cyclodextrin (M β CD), and tetrodotoxin were obtained from Sigma-Aldrich (St. Louis, MO), 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH) was from Wako Pure Industries (Osaka, Japan), and iberiotoxin and apamin were from Alomone Labs (Jerusalem, Israel). E-4031 was purchased from Enzo (Plymouth Meeting, PA) and ZD-7288 was from Tocris (Bristol, UK). All culture media, fetal calf serum, horse serum, L-glutamine, trypsin/EDTA, and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA). All other chemicals, including CsCl, CdCl₂, LaCl₃, and MnCl₂, were commercially available and of reagent grade. Reagent water that was obtained using a Milli-Q Ultrapure Water Purification System (Millipore, Bedford, MA) was used in all experiments. The composition of normal Tyrode's solution is as follows (in mM): NaCl 136.5, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.53, glucose 5.5, and HEPES-NaOH buffer 5.5 (pH 7.4). To record $I_{k(DR)}$ or I_{MEP} , the patch pipette was filled with a solution (in mM): K-aspartate 130, KCl 20, KH₂PO₄ 1, MgCl₂ 1, Na₂ATP 3, Na₂GTP 0.1, EGTA 0.1, and HEPES-KOH buffer 5 (pH 7.2).

Cell preparation

GH₃ pituitary tumor cells, obtained from the Bioresources Collection and Research Center ([BCRC-60015]; Hsinchu, Taiwan), were maintained in Hams'F-12 medium supplemented with 15% horse serum, 2.5% fetal calf serum and 2 mM L-glutamine in a humidified environment of 5% CO₂/95% air [5, 12]. The experiments were performed 5 or 6 days after cells had been cultured (60-80% confluence). The colorimetric method was used in examining the cell densities in microtiter plates with a tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; WST) and an ELISA reader (Dynatch, Chantilly, VA).

The treated cells were incubated at 37 °C for one hour in the media containing different concentrations of HP β CD or M β CD [12]. Such treatment was thought to decrease membrane cholesterol contents in neuroendocrine cells [8]. For restoration

of the membrane cholesterol content, GH₃ cells were incubated in the media containing a mixture of HPβCD and cholesterol (HPβCD/cholesterol) [11, 12].

Evaluation of the HPCD effect on lipid raft

GH₃ cells or HPβCD-treated GH₃ cells were washed and fixed in 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and subsequently stored in 2% glutaraldehyde. After being washed in phosphate buffer, cells were post-fixed for 1 hour in 1% aqueous reduced osmium, and dehydrated through graded series of ethanol. Cells were then treated with hydroxypropyl metacrylate and embedded in Epon for ultramicrotomy. Sections were collected and stained with uranyl acetate. The cells were photographed in an electron microscope (Jeol JEM-1200EX, Tokyo, Japan). Treatment of cells with HPβCD (2 mM for one hour at 37 °C) resulted in the loss of morphologically recognizable invaginated caveolae [12, 16].

Electrophysiological measurements

Cells used for electrophysiological experiments were dissociated and an aliquot of cell suspension was transferred to a recording chamber positioned on the stage of an inverted DM-IL microscope (Leica, Wetzlar, Germany). Cells were bathed at room temperature (25 °C) in normal Tyrode's solution containing 1.8 mM CaCl₂. Patch electrodes were made from Kimax-51 capillaries (#34500; Kimble Glass, Vineland, NJ) using a PP-830 puller (Narishige, Tokyo, Japan), and had a resistance of 3-5 MΩ when filled with different pipette solutions described above. Patch-clamp recordings were made in cell-attached or whole-cell configurations using an RK-400 amplifier (Bio-Logic, Claix, France) [5].

Data recordings and analyses

The data were stored online in a TravelMate-6253 computer (Acer, Taipei, Taiwan) at 10 kHz through a Digidata-1322A interface (Molecular Devices, Sunnyvale, CA). The interface device was equipped with an Adaptec SlimSCSI card (Milpitas, CA) via a PCMCIA slot and controlled by pCLAMP 9.2 (Molecular Devices). The pCLAMP-generated voltage-step profiles were used to determine the current-voltage (*I-V*) relationship for *I*_{K(DR)} or *I*_{MEP}. The activation kinetics of *I*_{K(DR)} in response to depolarizing voltage pulses were measured by fitting an exponential function to the rising phase of current traces with least squares method. To compare *I*_{K(DR)} or *I*_{MEP} from cells which differ in size and are therefore expected to differ in whole-cell current, the density (pA/pF) of *I*_{K(DR)} or *I*_{MEP} was measured, assuming that the cell surface is proportional to membrane capacitance.

The normalized amplitude of *I*_{MEP} (i.e., *I*/*I*_{MEP}) was constructed against the membrane potential and the activation curves of *I*_{MEP} obtained in untreated and HPβCD-treated cells were fitted with a Boltzmann function of the following form:

$$\frac{I}{I_{\max}} = \frac{1}{1 + \exp\left[\frac{(V - V_{1/2})qF}{RT}\right]} \quad (1)$$

where *I*_{max} is the maximal amplitude of *I*_{MEP}, *V*_{1/2} is the voltage at which there is half-maximal activation, *q* is the

apparent gating charge, and *F* and *RT* have their usual thermodynamic meanings. Curve-fitting to data sets was performed with the aid of Excel 2007 (Microsoft) or Origin 8.0 (OriginLab Corp.). The free energy involved in the gating of *I*_{MEP} (ΔG_0) was calculated assuming a 2-state (i.e., closed and open) gating model. ΔG_0 for *I*_{MEP} activation at 0 mV with or without treatment of HPβCD would be equal to $q \times F \times V_{1/2}$ [19]. Standard errors of ΔG_0 ($\sigma_{qV_{1/2}}$) were calculated according to:

$$\sigma_{qV_{1/2}} = F \times \sqrt{V_{1/2}^2 \sigma_q^2 + q^2 \sigma_{V_{1/2}}^2} \quad (2)$$

where σ_q and $\sigma_{V_{1/2}}$ are the standard errors in *q* and *V*_{1/2}, respectively.

The perturbation by HPβCD treatment of free energy involved in *I*_{MEP} gating was calculated as $\Delta \Delta G_0 = \Delta G_0^{\text{HP}\beta\text{CD}} - \Delta G_0^{\text{Ctrl}} = F(qV_{1/2}^{\text{HP}\beta\text{CD}} - qV_{1/2}^{\text{Ctrl}}) = \Delta(qV_{1/2})$, where ΔG_0^{Ctrl} and $\Delta G_0^{\text{HP}\beta\text{CD}}$ indicate the free energy of *I*_{MEP} activation obtained in untreated cells and cells exposed to HPβCD, respectively.

Values are provided as the mean values ± SEM with sample sizes (*n*) indicating the number of cells from which the data were taken. The paired or unpaired Student's *t*-test and one-way analysis of variance with the least significant difference method for multiple comparisons were used for the statistical evaluation of difference among means. To evaluate the sum of squared residuals (SSR), 99% confidence intervals were estimated with the use of Fisher's *F* distribution. A *P* value of less than 0.05 was considered to indicate statistical difference.

Evaluation of energy changes of MEP-induced conducting channels

To evaluate how HPβCD treatment alters the change in energy (i.e., *E(r)*) of MEP-induced channels. A simplified model for pore energy of *I*_{MEP} generation is given below [20, 21]:

$$E(r) = 2\pi\gamma r - \pi\sigma r^2 + \left(\frac{C}{r}\right)^4 - \frac{\epsilon_w - \epsilon_m}{2h} \epsilon_0 \pi V^2 r^2 \quad (3)$$

where *V* is the transmembrane voltage, *r* is the pore radius, γ is the energy per unit length of the pore diameter (i.e., edge energy of MEP-induced pore), σ is the energy per unit area of the intact membrane, *h* is the membrane thickness, and ϵ_w and ϵ_m represent the permittivities of water and membrane, respectively. The third term in this equation represents the steric repulsion between lipid heads lining the pore and is responsible for the increase in pore energy with shrinking radius. The value of *C* (i.e., constant) and the power in this equation were chosen to fit the experiments results [20]. The default values of these parameters used in this study were given in Table 1 of reference [20].

Results

Effect of HPβCD treatment on delayed rectifier K⁺ current (*I*_{K(DR)}) in pituitary GH₃ cells

Previous studies in our laboratory have demonstrated that membrane cholesterol depletion with methyl-β-cyclodextrin (MβCD) was able to slow down the time

course of $I_{K(DR)}$ activation in motor neuron-like NG108-15 cells [16]. In the initial set of experiments, we examined whether HP β CD treatment exerted any effect on $I_{K(DR)}$ in pituitary GH₃ cells. Cells were bathed in Ca²⁺-free solution containing 1 μ M tetrodotoxin. Figure 1A illustrates superimposed traces of $I_{K(DR)}$ obtained in the untreated cell and HP β CD-treated cell. Each current trace in response to membrane depolarization was fitted with single exponential. The relationships of activation time constant for $I_{K(DR)}$ and membrane potential in the two groups of cells was shown in Fig. 1B. The data showed that, in agreement with previous work conducted in NG108-15 neuronal cells [16], $I_{K(DR)}$ present in HP β CD-treated cells exhibited a significant slowing in activation kinetics of $I_{K(DR)}$ at the voltage ranging from -30 to +60 mV as compared with that in untreated cells. For example, when GH₃ cells were depolarized from -50 to +50 mV, HP β CD treatment significantly increased the activation time constant of $I_{K(DR)}$ from 23 ± 2 to 36 ± 3 msec ($n=7$). However, the $I_{K(DR)}$ density measured from untreated cells did not differ significantly from that in cells exposed to HP β CD. No change in the relationships for voltage versus density of $I_{K(DR)}$ between the two groups of cells was also observed. The results indicate that the depletion of membrane cholesterol by HP β CD can stabilize the gating conformations of $I_{K(DR)}$, leading to a slowing of current activation in these cells.

Effect of HP β CD treatment on the density of I_{MEP} in pituitary GH₃ cells

We next investigated the possible effect of HP β CD treatment on I_{MEP} in these cells. The cells examined were bathed in Ca²⁺-free Tyrode's solution containing 10 mM CsCl. To avoid the contamination of Cl⁻ currents, Cl⁻ ions included in the recording pipette were replaced with aspartate. When membrane hyperpolarizations from -80 to -200 mV with a duration of 300 msec at a rate of 0.1 Hz were applied to the cell, an irregular and transient inward current was elicited (Fig. 2). These inward currents with fluctuating properties were increased with greater hyperpolarizations and have been identified as I_{MEP} [5]. Interestingly, after cells were exposed to HP β CD (2 mM), the densities of these inward currents in response to membrane hyperpolarizations were greatly reduced. For example, when the voltage step from -80 to -200 mV was applied to the cells, HP β CD (2 mM) treatment significantly decreased current density from 21.7 ± 1.5 to 6.5 ± 1.2 pA/pF ($n=8$). Similar results were also obtained in seven different cells exposed to methyl- β -cyclodextrin (M β CD; 2 mM). However, the results revealed no

significant difference in the I_{MEP} density between untreated cells and cells treated with a mixture of HP β CD and cholesterol (HP β CD/cholesterol; 2 mM). Moreover, cell capacitance in untreated cells (13.4 ± 1.1 pF, $n=8$) was not found to differ significantly from that in HP β CD-treated cells (13.5 ± 1.2 pF, $n=7$).

Effect of AAPH in GH₃ cells treated or untreated with HP β CD

Previous work has demonstrated that oxidative modification of the cell membrane could enhance the formation of electropores induced by MEP [3, 4]. Changes in membrane cholesterol were reported to alter the activity of different K⁺ channels [12-16]. We further investigated whether AAPH can directly modify the amplitude of hyperpolarization-induced I_{MEP} in these cells. AAPH is known to be an azo compound that can generate free radicals [22]. As depicted in Fig. 3, the challenge of untreated cells with AAPH (100 μ M) was found to increase the amplitude of I_{MEP} induced by membrane hyperpolarization. When membrane was hyperpolarized from -80 to -200 mV, addition of AAPH (100 μ M) significantly increased the I_{MEP} density from 21.7 ± 1.5 to 39.2 ± 1.6 pA/pF ($n=9$). Similar results were also obtained when the cells were exposed to 1 mM *t*-butyl hydroperoxide (*t*-BHP), a hydrophilic oxidant [23]. However, in continued presence of AAPH, the inhibitors of Ca²⁺-activated K⁺ (K_{Ca}) channels, such as iberiotoxin (200 nM) and apamin (200 nM), exerted little or no effects on I_{MEP} , while subsequent application of 100 μ M LaCl₃ or 100 μ M MnCl₂ was effective in suppressing AAPH-stimulated I_{MEP} by 75 and 72%, respectively. Similarly, neither E-4031 (30 μ M) nor ZD-7288 (30 μ M) caused any effects on AAPH-induced I_{MEP} . E-4031 is a selective inhibitor of *erg*-mediated K⁺ current, while ZD-7288 can block the amplitude of hyperpolarization-activated cationic current. Therefore, in untreated GH₃ cells, addition of AAPH has a stimulatory effect on I_{MEP} , rather than on K_{Ca} currents. It is important to note that in cells exposed to HP β CD, the I_{MEP} density between the absence and presence of AAPH (100 μ M) did not differ significantly (6.5 ± 1.2 versus 6.7 ± 1.3 pA/pF; $n=7$). Taken together, the results indicated that addition of AAPH could increase the I_{MEP} amplitude; however, in HP β CD-treated cells, AAPH-mediated increase of I_{MEP} was abolished.

Effect of HP β CD treatment on I-V relationship of I_{MEP} in GH₃ cells

To characterize the effect of HP β CD treatment on I_{MEP} , we further evaluated whether such maneuver

Fig. 1. Effect of HPβCD treatment on the density of $I_{K(DR)}$ in GH₃ pituitary tumor cells. The untreated and HPβCD-treated cells were bathed in Ca²⁺-free Tyrode's solution containing 1 μM tetrodotoxin and 0.5 mM CdCl₂. The treated cells were incubated with HPβCD (2 mM) for one hour at 37 °C. In A, the superimposed current traces were obtained in the cell without (upper) and with (lower) the exposure to HPβCD (2 mM). Each cell was depolarized from -50 mV to various potentials ranging from -50 to +60 mV in 10-mV increments. The inset indicates the voltage protocol used. B, the relationship of activation time constant for $I_{K(DR)}$ and membrane potential in untreated (●) and HPβCD-treated (○) cells (mean±SEM; n=6-9). *Significantly different from controls (i.e., untreated cells). Notably, the activation time courses of $I_{K(DR)}$ measured in HPβCD-treated cells became slowed at the voltages ranging from -30 to +60 mV as compared with those in the untreated cells.

altered I - V relationship of this current. As shown in Fig. 4A, the magnitude of I_{MEP} in response to membrane hyperpolarization was diminished in HPβCD-treated cells throughout the entire voltage range examined, as compared with that in untreated cells. Figure 4 illustrates the I - V relations in the absence and presence of HPβCD treatment. The threshold for elicitation of I_{MEP} in untreated cells was around -100 mV, while that occurring in HPβCD-treated cells became hyperpolarized to -160 mV. Moreover, cell exposure to HPβCD significantly decreased the slope of the linear fit of I_{MEP} amplitude to voltages between -160 and -190 mV from 7.9 ± 1.1 to 3.5 ± 0.6 nS (n=10). The results clearly indicated that when GH₃ cells were exposed to HPβCD, the I - V relationship of hyperpolarization-induced I_{MEP} was modified. It needs to be noted that the noise levels in some of recordings (e.g., Fig. 4A) appear to be high. The reasons could be

Fig. 2. Effect of HPβCD treatment on the density of I_{MEP} in pituitary GH₃ cells. In these experiments, cells were bathed in Ca²⁺-free Tyrode's solution which contained 10 mM CsCl. The treated cells were incubated with HPβCD (2 mM) for 1 hour at 37 °C. The cells were held at -80 mV, and the voltages from -80 to -200 mV with a duration of 300 msec at a rate of 0.1 Hz were applied. The upper parts of left and right panels, respectively, show the time course of current amplitudes in response to membrane hyperpolarizations in untreated and HPβCD-treated cells. The lower parts shown in a, b, and c in each panel denote superimposed current traces corresponding to those labeled a, b, and c in the upper part of each panel. The inset in each panel indicates the voltage protocol used. Note that there was a considerable decrease of I_{MEP} density following HPβCD treatment.

Fig. 3. Effect of AAPH on I_{MEP} density in GH₃ cells with or without treatment of HPβCD. A, Original current trace showing effect of AAPH on I_{MEP} density in untreated (upper) and HPβCD-treated (lower) cells. Current traces labeled a (black colors) are controls and those labeled b (red colors) were obtained after addition of 100 μM AAPH. The inset indicates the voltage protocol used. B, Comparison between the effects of AAPH on I_{MEP} density in untreated and HPβCD-treated cells (mean±SEM; n=7-10). *Significantly different from controls.

due to the possibility that the I_{MEP} in response to membrane hyperpolarizations often comprises multiple smaller currents occurring asynchronously.

Effect of HP β CD treatment on voltage-dependent activation curve of I_{MEP}

The voltage-dependence of I_{MEP} in untreated and HP β CD-treated cells was further examined. The normalized amplitudes of I_{MEP} versus the membrane potentials were constructed. Figure 5 depicts the activation curves of I_{MEP} in cells with or without treatment of HP β CD. Fitting was done using the Boltzmann function described in equation (1). In the untreated cells, $V_{1/2} = -141 \pm 11$ mV and $q = 1.3 \pm 0.2 e$ ($n=6$), whereas in HP β CD-treated cells, $V_{1/2} = -172 \pm 12$ mV and $q = 2.7 \pm 0.3 e$ ($n=6$). The plots of SSR versus the $V_{1/2}$ value obtained in the absence and presence of HP β CD treatment are illustrated in the inset of Fig. 5. The steep slope on both sides of the minimum shown in each inset indicates that the value of $V_{1/2}$ is determined with high confidence. Therefore, it is clear from these data that HP β CD treatment could shift the midpoint of the activation curve toward hyperpolarizing voltage by approximately 30 mV. A two-fold increase in the gating charge of I_{MEP} activation following HP β CD treatment was also noted.

Based on the values of q and $V_{1/2}$, the free energy involved in the gating of I_{MEP} at 0 mV (ΔG_0) in the absence and presence of HP β CD treatment was estimated to be -17.7 ± 0.4 and -44.8 ± 0.9 kJ/mol ($n=6$), respectively [19]. The perturbation by HP β CD treatment of free energy ($\Delta \Delta G_0$) involved in I_{MEP} gating was also calculated to be -27.1 ± 0.5 kJ/mol ($n=6$), a value (i.e., -6.4 kcal/mol). It is notable that this value is similar to that previously obtained for activation energy of membrane cholesterol transfer [24]. It is thus anticipated that depletion of membrane cholesterol caused by HP β CD treatment can increase the free energy needed for I_{MEP} activation.

Lack of HP β CD treatment on single-channel conductance of MEP-induced channels

Previous reports showed that the value of pore size induced by MEP could be altered when cholesterol was incorporated in the phosphatidylcholine bilayer [17]. To this aim, experiments were next carried out to investigate whether HP β CD treatment modifies single-channel conductance of MEP-induced channels in GH $_3$ cells. In this series of experiments, cells were bathed in Ca $^{2+}$ -free Tyrode's solution containing 10 mM

Fig. 4. Effect of HP β CD treatment on the I - V relationship of I_{MEP} in GH $_3$ cells. Cells were bathed in Ca $^{2+}$ -free solution containing 10 mM CsCl, and I_{MEP} was elicited from -50 mV to different potentials ranging from -190 to -80 mV with 10-mV increments. A. Superimposed current traces obtained in cells exposed to HP β CD. The upper part indicates the voltage protocol used. B. Averaged I - V relationships of I_{MEP} in the absence (upper) and presence (lower) of HP β CD treatment (mean $\pm$ SEM; $n=6-12$ for each point). Current amplitude was obtained at the end of each voltage step.

Fig. 5. Effect of HP β CD treatment on the activation curve of I_{MEP} in GH $_3$ cells. The normalized amplitude of I_{MEP} in the absence (●) and presence (○) of HP β CD treatment was constructed and plotted against the membrane potentials (mean $\pm$ SEM; $n=7-11$ for each point) and the smooth lines were fitted by the Boltzmann function described in equation (1). Insets show confidence assessment of best-fit parameter values in the absence and presence of HP β CD treatment. Purple dotted line shown in each inset marks parameter value (i.e., $V_{1/2}$) at which the sum of squared residuals (SSR) in the absence and presence of HP β CD treatment amounts to 0.0031 and 0.00939, respectively.

Fig. 6. Characterization of single-channel events induced by membrane hyperpolarization in the absence and presence of HP β CD. A. Current traces obtained when the treated cell was held at -80 mV and membrane hyperpolarization to -200 mV were applied. In these experiments, cells were bathed in Ca²⁺-free Tyrode's solution containing 10 mM Cs⁺ and 1 mM LaCl₃. Downward deflections indicate the opening event of the channels. The inset in A indicates the voltage protocol used. B. Single-channel conductances measured in the absence (■) and presence (□) of HP β CD treatment. The straight lines show the *I-V* relations of MEP-elicited channels obtained in untreated and HP β CD-treated cells (mean $\pm$ SEM; n=6-9 for each point). Notably, the single-channel conductance of MEP-induced channels between the absence and presence of HP β CD did not differ significantly.

CsCl and 1 mM LaCl₃. As illustrated in Fig. 6A, in cells treated with HP β CD, single-channel currents existing on the cell membrane were noted emerge with an open time of millisecond in the time scale. When membrane hyperpolarization was from -80 to -200 mV was applied, single-channel amplitude with 191 ± 13 pA (n=11) was clearly obtained. To construct the plots of current amplitude as a function of membrane potential, the hyperpolarizing pulses from -80 to different voltages ranging from -80 to -200 mV with a duration of 300 msec were applied at a rate of 0.1 Hz. The ohmic *I-V* relationships of MEP-induced channels with or without treatment of HP β CD are illustrated in Fig. 6B. The single-channel conductance in the absence of HP β CD treatment was calculated to be 1.09 ± 0.03 nS (n=12) with a reversal potential of -25 ± 2 mV (n=10). The value for single-channel conductance of these channels measured in untreated cells did not differ significantly from that in HP β CD-treated cells (1.08 ± 0.04 nS; n=10). Inconsistent with previous observations [17], our data showed that the challenge of cells with HP β CD did not result in any modification in single-channel conductance of MEP-induced channels, although such maneuver significantly reduced the density of macroscopic I_{MEP} (Fig. 2). Therefore, it is anticipated that the reduction of I_{MEP} activity in HP β CD-treated cells can result primarily from either a reduction of the number of active channels, a decrease in open state probability, or both.

Effect of HP β CD treatment on change in the energy of MEP-induced conducting pore

It is necessary to evaluate how HP β CD treatment alters the energy change of MEP-induced channels in

Fig. 7. Relationship between the radius and energy of MEP-induced pore (A) and two barrier-one site energy profile (B). In A, the calculation of the pore energy in the absence (thin red line) and presence (thick red line) of HP β CD treatment was based on equation (3). The initial peak at small pore size indicates the pore energy ($E(r)$) for the nonconducting hydrophobic pores. The $E(r)$ value for the conducting hydrophilic pore is elevated as the pore size progressively increases. Dotted blue lines shown in A extend to the $E(r)$ values in untreated and HP β CD-treated cells and corresponding pore radius (1.7 nm). B. Barrier profile of MEP-induced pore in untreated (solid red line) and HP β CD-treated (dashed red line) cells. The relationship of free energy versus the fraction of electrical distance (δ) was plotted. Based on this profile, translocation across the two barrier-one site channel in either direction requires a sequence of two 'hopping' events in the same direction. The vertical axis in A and B represents the magnitude of the changes in free energy normalized to RT .

GH₃ cells. A simplified model given in equation (3) was also implemented to study energy changes in I_{MEP} gating in the absence and presence of HP β CD treatment [20, 21]. The relationship of pore energy versus pore radius when there is no externally applied voltage is illustrated in Fig. 7A. Based on the relationship among the resistance, radius and thickness of MEP-induced pores, the pore radius was previously estimated to be 1.7 nm [5, 25]. This value was noted to correspond with the pore energy (3.2 RT) shown in Fig. 7A. Because of no change in single-channel conductance of MEP-induced channels, the pore size was assumed to remain the same in HP β CD-treated cells. Under the condition in which other parameter values remain unchanged, when the edge energy (i.e., γ value) of MEP-induced pore increased from 1.8×10^{-11} to 4.3×10^{-11} Joule/mol, the free energy required for I_{MEP} gating that corresponds with the peak height of the barrier was elevated to 7.7 RT (Fig. 7A). Based on the experimental values of q and $V_{1/2}$ obtained in untreated and HP β CD-treated cells, the free energy involved in the gating of I_{MEP} at 0 mV ($E(r)$), which is equivalent to the well depth of the barrier, was calculated to be -7.2 and -18.3 RT , respectively.

By use of the simple model from Eyring rate theory [26], a two barrier-one site energy barrier profile used for investigation of MEP-induced channels in untreated and HP β CD-treated cells was also constructed and plotted in Fig. 7B. This pore model consists of two barriers ($\delta=0.25$ and 0.75) and one internal well (or binding site; $\delta=0.5$), which may be occupied by only one ion at a time. The x-axis shown in Fig. 7B indicates the 'electrical distance' (δ) as measured by the fraction of the total membrane voltage which is sensed in a transition between two points rather than the actual physical distance. The y-axis represents the magnitude of the changes in free energy normalized to RT (G/RT), relative to the hydrophilic conducting path, experienced by an ion as it transverses the pore. The peak heights of the energy barrier on either side of the well at $\delta=0.25$ and 0.75 indicate the energy which the ion must overcome in order to enter or exit the microenvironment of the pore. Notably, the results revealed that the challenge of cells with HP β CD can not only increase peak height of barrier, but also produce an increase in the depth of the well. Taken together, the experimental and analytical results prompted us to propose that membrane cholesterol depletion by HP β CD can increase the edge energy of MEP-induced conducting channels with no apparent change in the pore radius of these channels.

Discussion

In this study, we observed that removal of membrane cholesterol by HP β CD, a cyclic oligosaccharide, altered the gating of I_{MEP} by changing both the midpoint and slope of voltage-dependent activation curve for this current. Because membrane cholesterol content in these cells is altered by HP β CD treatment, reduction in the magnitude of I_{MEP} will likely take place in a number of physiological or pathophysiological processes and, as a result, contribute to the underlying mechanisms through which perturbation of membrane cholesterol affects the electrical behaviors of neurons or neuroendocrine cells occurring *in vivo*.

Recent studies have demonstrated that MEP-induced channels lined by the negatively charged head groups of membrane phospholipids can be formed as a consequence of electrical field-induced perturbation of the membrane lipids [1]. In this study, we reported that in pituitary GH₃ cells, removal of membrane cholesterol by HP β CD was capable of modifying the activity of MEP-induced channels. It is important to note that the stimulation by AAPH of MEP-induced channels did not occur in HP β CD-treated cells, although AAPH was effective in increasing I_{MEP} magnitude in untreated cells. AAPH is known to be an initiator of free radicals [22]. It currently remains unclear what the significant mechanism by which the production of reactive oxygen species facilitates MEP-induced currents occurs and how these currents can be influenced by membrane cholesterol. However, it appears that stimulatory effect of oxidative injury with AAPH or *t*-BHP on I_{MEP} is dependent on membrane cholesterol content in which the electropores residue. Membrane cholesterol content and/or distribution may contribute to differential AAPH sensitivity of MEP-induced channels in neurons or neuroendocrine cells. Additionally, cholesterol and free radicals are likely to share a common target on the MEP-induced channel or in the phospholipid layer, which is important in determining the stability of the channel opened state(s).

It needs to be mentioned that treatment of GH₃ cells with HP β CD caused the activation curve of I_{MEP} to shift leftward along the voltage axis accompanied by an increase of gating charge. These results indicated that the free energy needed for I_{MEP} activation was elevated following HP β CD treatment [19]. In other words, depletion of membrane cholesterol by HP β CD tends to shift the gating equilibrium of I_{MEP} toward the closed state. Therefore, such maneuver is mostly likely to stabilize the

closed state relative to opened, or it could destabilize the open relative to the closed. Finding from these data can be interpreted to mean that depletion of membrane cholesterol by HP β CD shifts the gating equilibrium of this current toward the closed state, because it is easier to destabilize the opened conformation of the channel with such maneuver.

Following HP β CD treatment, I_{MEP} present in GH $_3$ cells could be activated at more hyperpolarized voltages in comparison with that from untreated cells. Moreover, the steepness of the voltage-activated I_{MEP} became significantly greater in cells exposed to HP β CD, indicating that the effective number of elementary charges during channel activation in HP β CD-treated cells was significantly increased. Therefore, the experimental results are of particular importance, because they led us to estimate the energy change ($\Delta G_0^{\text{HP}\beta\text{CD}}$) for generation of I_{MEP} in HP β CD-treated cells with a value of -44.8 kJ/mol. This value was noted to be significantly greater than that (ΔG_0^{ctrl}) (-17.7 kJ/mol) in untreated cells [19]. Membrane cholesterol content is apparently involved in voltage-sensitive gating functions of MEP-induced channels. In this study, a model designed for investigation of two barrier-one site pore [26] was also implemented, in an attempt to provide an important mechanism through which membrane cholesterol depletion by HP β CD can potentially alter the barrier profile of MEP-induced pore. The results strongly suggest that the challenge of cells with HP β CD can increase both the peak height and well depth of energy barrier for MEP-induced channels occurring in GH $_3$ cells.

In our study, following the cholesterol-depleting treatment with HP β CD, no discernible change in single-channel conductance of MEP-induced channels was observed, suggesting that such maneuver did not alter the main parts of permeation pathway for these channels. It is thus tempting to speculate that the cholesterol depletion maneuver with HP β CD may primarily alter the gating kinetics of I_{MEP} together with the decreased number of functional channels on plasma membrane.

Previous studies demonstrated that the presence of cholesterol in the egg yolk phosphatidylcholine bilayer membrane could produce an increase in the value of the breakdown potential elicited by MEP [17]. On the other hand, cholesterol enrichment was found to have little effect on membrane stiffness in vascular endothelial cells [7]. In our study, although HP β CD treatment was apparently found to decrease the density of I_{MEP} , HP β CD/cholesterol treatment which supposedly results in membrane

cholesterol enrichment failed to modify I_{MEP} in GH $_3$ cells. The reason for this discrepancy is currently unclear; however, it could be due to the possibility that these cells naturally contain a cholesterol fraction in their membranes that is already saturated.

HP β CD or M β CD has been reported to deplete membrane cholesterol and to sequester lipids within their core [9, 14, 27]. In our study, the value of free energy for I_{MEP} gating perturbed by HP β CD treatment ($\Delta\Delta G_0$) was estimated to be -6.4 kcal/mol. This value is well matched to that required for activation energy of membrane cholesterol transfer [24]. It is thus possible that electropores induced by membrane hyperpolarization are associated with cholesterol-rich membrane domains (i.e., lipid rafts) in GH $_3$ cells. Membrane cholesterol primarily in lipid rafts can potentially interact with the negative charged head groups of membrane phospholipids to facilitate generation of I_{MEP} . In other words, when electrical field-induced perturbation of membrane lipid, electropores lined by the negatively charged head groups of phospholipids would not be easily formed under the conditions in which lipid rafts were disrupted with HP β CD treatment.

β -Cyclodextrins were demonstrated to clear cholesterol in Chinese hamster ovary cell lines overexpressing the human NPC1 protein [28]. However, these compounds have been described to create “holes” in human and simian immunodeficiency virus membrane as well as in artificial lipid bilayer [6, 9]. Previous study showed that β -cyclodextrins could extract sphingomyelin from lipid bilayers [10]. β -Cyclodextrins were recently found to induce Ca $^{2+}$ -dependent lysosomal exocytosis [29]. It is also important to note that cholesterol is present not only in cholesterol-rich lipid rafts but also in the rest of the membrane and that β -cyclodextrins can remove cholesterol from both types of membrane domains. Therefore, sensitivity of MEP-induced currents to cholesterol presented herein cannot be interpreted as proof of their location in lipid rafts. To what extent other additional mechanisms through which β -cyclodextrin treatment may act on its inhibitory effects on I_{MEP} gating also remains to be further explored. It also needs to be noted that M β CD has been found to be in general more effective and more commonly used to deplete membrane cholesterol. In our study, the effect of HP β CD and M β CD on the I_{MEP} density is comparable. However, the difference in the gating of electroporation-induced current in cells treated with these two agents needs to be further evaluated.

Acknowledgements

This work was supported by a grant from National Science Council (NSC-98-2320-B-006-MY3), Taiwan. The authors are grateful to the comments from Dr. Yen-

Chin Liu, Department of Anesthesiology, National Cheng Kung University Hospital, Tainan City, Taiwan. Competing interests: The authors declare no competing interests in this work.

References

- Wang M, Orwar O, Olofsson J, Weber SG: Single-cell electroporation. *Anal Bioanal Chem* 2010;397:3235-3248.
- Rols MP: Electroporabilization, a physical method for the delivery of therapeutic molecules into cells. *Biochim Biophys Acta* 2006;1758:423-428.
- Vernier PT, Levine ZA, Wu YH, Joubert V, Ziegler M, Mir LM, Tieleman DP: Electroporating fields target oxidatively damaged areas in the cell membrane. *PLoS One* 2009;4:e7966.
- Freikman I, Ringel I, Fibach E: Oxidative stress-induced membrane shedding from RBCs is Ca flux-mediated and affects membrane lipid composition. *J Membr Biol* 2011;240:73-82.
- Wu SN, Huang HC, Yeh CC, Yang WH, Lo YC: Inhibitory effect of memantine, an NMDA-receptor antagonist, on electroporation-induced inward currents in pituitary GH3 cells. *Biochem Biophys Res Commun* 2011;405:508-513.
- Popik W, Alce TM, Au WC: Human immunodeficiency virus type 1 uses lipid raft-colocalized CD4 and chemokine receptors for productive entry into CD4+ T cells. *J Virol* 2002;76:4709-4722.
- Byfield FJ, Aranda-Espinoza H, Romanenko VG, Rothblat GH, Levitan I: Cholesterol depletion increases membrane stiffness of aortic endothelial cells. *Biophys J* 2004;87:3336-3343.
- Hosaka M, Suda M, Sakai Y, Izumi T, Watanabe T, Takeuchi T: Secretogranin III binds to cholesterol in the secretory granule membrane as an adapter for chromogranin A. *J Biol Chem* 2004;279:3627-3634.
- Zidovetzki R, Levitan I: Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* 2007;1768:1311-1324.
- Giocondi MC, Milhiet PE, Dosset P, Le Grimellec C: Use of cyclodextrin for AFM monitoring of model raft formation. *Biophys J* 2004;86:861-869.
- Hajdú P, Varga Z, Pieri C, Panyi G, Gáspár R: Cholesterol modifies the gating of Kv1.3 in human lymphocytes. *Pflugers Arch* 2003;445:674-682.
- Lin MW, Wu AZ, Ting WH, Li CL, Cheng KS, Wu SN: Changes in membrane cholesterol of pituitary tumor (GH3) cells regulate the activity of large-conductance Ca²⁺-activated K⁺ channels. *Chin J Physiol* 2006;49:1-13.
- Romanenko VG, Roser KS, Melvin JE, Begenisich T: The role of cell cholesterol and the cytoskeleton in the interaction between IK1 and maxi-K channels. *Am J Physiol Cell Physiol* 2009;296:C878-C888.
- Levitan I, Zidovetzki R: Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* 2007;1768:1311-1324.
- Bukiya AN, Belani JD, Rychnovsky S, Dopico AM: Specificity of cholesterol and analogs to modulate BK channels points to direct sterol-channel protein interactions. *J Gen Physiol* 2011;137:93-110.
- Huang CW, Wu YJ, Wu SN: Modification of activation kinetics of delayed rectifier K⁺ currents and neuronal excitability by methyl- β -cyclodextrin. *Neuroscience* 2011;176:431-441.
- Koronkiewicz S, Kalinowski S: Influence of cholesterol on electroporation of bilayer lipid membranes: chronopotentiometric studies. *Biochim Biophys Acta* 2004;1661:196-203.
- Rojas-Aguirre Y, Yépez-Mulia L, Castillo I, López-Vallejo F, Soria-Arteche O, Hernández-Campos A, Castillo R, Hernández-Luis F: Studies on 6-chloro-5-(1-naphthyl-2-(trifluoromethyl)-1H-benzimidazole-2-hydroxypropyl)- β -cyclodextrin association: characterization, molecular modeling studies, and *in vivo* anthelmintic activity. *Biorg Med Chem* 2011;19:789-797.
- Yifrach O, MacKinnon R: Energetics of pore opening in a voltage-gated K⁺ channel. *Cell* 2002;111:231-239.
- Neu JC, Krassowska W: Asymptotic model of electroporation. *Phys Rev E* 1999;59:3471-3482.
- Joshi RP, Hu Q, Schoenbach KH, Hjalmarson HP: Improved energy model for membrane electroporation in biological cells subjected to electrical pulses. *Phys Rev E* 2002;65:041920.
- Huang MH, Wu SN, Shen AY: Stimulatory actions of thymol, a natural product, on Ca²⁺-activated K⁺ currents in pituitary GH3 cells. *Planta Medica* 2005;71:1093-1098.
- Sheu SJ, Wu SN: Mechanism of inhibitory actions of oxidizing agents on calcium-activated potassium current in cultured pigment epithelial cells of the human retina. *Invest Ophthalmol Vis Sci* 2003;44:1237-1244.
- Yancey PG, Rodriguez WV, Kilsdonk EP, Stoudt GW, Johnson WJ, Phillips MC, Rothblat GH: Cellular cholesterol efflux mediated by cyclodextrins. Demonstration of kinetic pools and mechanism of efflux. *J Biol Chem* 1996;271:16026-16034.
- Krassen H, Pliquett U, Neumann E: Nonlinear current-voltage relationship of the plasma membrane of single CHO cells. *Bioelectrochemistry* 2007;70:71-77.
- Mansoura MK, Dawson DC: 2 Barrier-1 site pore: an interactive spreadsheet model for two permeating ions. *Comp Biol Med* 1998;28:255-273.
- Levitan I, Fang Y, Rosenhouse-Dantsker A, Romanenko V: Cholesterol and ion channels. *Subcell Biochem* 2010;51:509-549.
- Millard EE, Srivastava K, Traub LM, Schaffer JE, Ory DS: Niemann-Pick type C1 (NPC1) overexpression alters cellular cholesterol homeostasis. *J Biol Chem* 2000;275:38445-38451.
- Chen FW, Li C, Ioannou YA: Cyclodextrin induces calcium-dependent lysosomal exocytosis. *PLoS ONE* 2010;5:e15054.