Original Paper

Identification of Minuscule Inward Currents as Precursors to Membrane Electroporation-Induced Currents: Real-Time Prediction of Pore Appearance

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Key Words

Membrane electroporation • Ion current • Principal component analysis (PCA) • Palmitoyl-L-carnitine

Abstract

Background/Aims: The objective of this study is to examine the current signals in response to large hyperpolarizations with the aid of principal component analysis (PCA) to search for or even predict current fluctuations related to membrane electroporation-induced current (I_{MEP}).

Methods: The characteristics of principal eigenvalues generated for I_{MEP} and the current signals at 10 sec prior to the start of initial I_{MEP} (I_{Pre}) were examined. As membrane hyperpolarizations were applied at 0.1 Hz, the appearance of I_{MEP} coincided with the higher principal eigenvalues extracted in PCA. **Results:** Subsequent addition of $LaCl_3$ (100 μ M) greatly reduced I_{MEP} and associated principal eigenvalues. In real-time analysis for a single frame (i.e., 300 msec), in response to large hyperpolarization, multiple runs of heralded minuscule inward currents (I_{min}) occurring before large rise in current amplitudes were detected. With PCA, such heralded I_{min} was noted to coincide with the extreme principal eigenvalues. The duration of I_{min} together with large principal eigenvalues was influenced by different levels of membrane hyperpolarization. In GH3 cells, palmitoyl-L-carnitine (PALCAR), a long-chain acylcarnitine, effectively increased the I_{MEP} amplitude with an EC_{50} value of 2.4 μ M. However, in PALCAR-treated cells, the I_{min} together with higher principal eigenvalues disappeared, while in isoflurane-treated cells, I_{min} occurring before large rise of current amplitude remained intact. Similarly, the PCA analysis

from I_{Pre} in RAW 264.6 macrophages showed the presence of herald I_{min} accompanied by the extreme principal eigenvalues. **Conclusion:** It is clear from this study that these large principal eigenvalues are representative of MEP-associated formation of electropores. Therefore, different compositions around the surface membrane of cells may alter the appearance of I_{min} followed by I_{MEP} emergence.

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Introduction

Membrane electroporation (MEP) or electro-permealization is a cellular dynamic process that consists in a significant but transient increase of the electrical conductivity and permeability of the plasma membrane by means of an externally applied electrical field. This physical phenomenon has been described in terms of structural transitions from closed to porated states through semi-permeable membranes as the transmembrane potential exceeds a critical threshold [1-3]. Such a maneuver has been employed in cancer research, gene transfection, drug delivery, and cell fusion [4, 5]. In order to efficiently electrotransfer drugs and DNAs into the cells, quantitative predictions and descriptions of mechanical, electrical, and molecular transport aspects of MEP are potentially important [3, 4]. Additionally, 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 [1, 2, 6-8]. Recent work in our laboratory has characterized the electrical properties of MEP-induced currents (I_{MEP}) in different types of cells [9-12]. Besides that, despite numerous experimental and theoretical studies, many aspects of MEP-induced currents (I_{MEP}) remain elusive.

The detrended fluctuation analysis has been previously applied to analyze the scaling exponents in the time series of I_{MEP} [11]. However, its real-time computation capacity used to herald the coming of pore appearance is limited. Alternatively, principal component analysis (PCA) has been widely used in all forms of analysis ranging from neuroscience to computer graphics, because it is a simple, non-parametric method of extracting relevant information from confusing data sets [13, 14]. With minimal additional effort, PCA is able to provide a framework for how to reduce a complex data set to a lower dimension and then to reveal the sometimes hidden, simplified structure that often underlies it. However, whether such maneuver can be applied for either the characterization of I_{MEP} or the prediction of pore formation remains largely unknown.

Palmitoyl-L-carnitine (PALCAR) is the most abundant long-chain acylcarnitine in ischemic tissue which may accumulate rapidly due to its ability to inhibit mitochondrial β -oxidation of fatty acid and thereby to produce toxic effects on the tissue involved [15]. Earlier studies have shown that this compound could reduce electrophoretic mobility of erythrocytes [16], alter the vectorial transport in Caco-2 cell monolayers [17], elevate intracellular Ca^{2+} in vascular endothelial cells and heart cells [18-20] and increase the amplitude of late Na^+ current [21]. However, the ability of this compound to interact with I_{MEP} to influence the electrical behavior of I_{MEP} is incompletely understood.

Therefore, in this study, the physical properties of I_{MEP} in different types of cells including pituitary tumor (GH₃) cells and RAW 264.7 macrophages were characterized with the use of patch-clamp recordings. The PCA was thereafter applied to analyze the principal eigenvalues in the time series of hyperpolarization-induced inward current through MEP-induced channels in these cells. The results showed that there exist heralded minuscule inward currents (I_{min}) immediately prior to escalating rise of current amplitude in response to membrane hyperpolarization. We also found that PALCAR was effective at stimulating I_{MEP} in pituitary GH₃ cells. If this approach shown here continues to prove successful, it could be used in real-time prediction of I_{MEP} occurrence through early detection of MEP-associated current signals.

Materials and Methods

Drugs and Solutions

Glibenclamide, lysophosphatidylcholine, palmitoyl-L-carnitine (PALCAR), ranolazine, riluzole and tetrodotoxin were obtained from Sigma-Aldrich (St. Louis, MO), apamin and iberiotoxin were from Alomone Labs (Jerusalem, Israel), and isoflurane (Florane®) was from Abbott Lab. (Taipei, Taiwan). Prior to the experiment, isoflurane was mixed to graduated syringes with the appropriate bath solution. All culture media, fetal calf serum, L-glutamine, trypsin/EDTA, and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA). Other chemicals including CsCl, CsOH, LaCl₃, MnCl₂ and *N*-methyl-D-glucamine⁺ (NMDG⁺) were commercially available and of reagent grade. Deionized water used throughout the experiments was made from a Milli-Q water purification system (Millipore, Bedford, MA).

The composition of normal Tyrode's solution was 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 hyperpolarization-elicited currents, the patch pipette was generally filled with a solution (in mM): K-aspartate 130, KCl 20, KH₄PO₄ 1, MgCl₂ 1, Na₂ATP 3, Na₂GTP 0.1, EGTA 0.1, and HEPES-KOH buffer 5 (pH 7.2). To preclude the possible contamination of Cl⁻ currents [22], Cl⁻ ions inside the pipette solution was replaced with aspartate. In some set of experiments, GH₃ cells were incubated with PALCAR (3 μM) or isoflurane (2.0% [v/v]) for two hours.

Cell Preparations

GH₃ pituitary tumor cells, obtained from the Bioresources Collection and Research Center ([BCRC-60015]; Hsinchu, Taiwan), were routinely maintained at a density of 10⁶/ml in Hams's F-12 nutrient media supplemented with 15% horse serum, 2.5% fetal calf serum and 2 mM L-glutamine in a humidified environment of 5% CO₂/95% air. The murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (TIB-71; Manassas, VA). RAW 264.7 cells were grown in plastic disks containing Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum [23]. The electrophysiological experiments were commonly performed 5 or 6 days after cells had been cultured (60-80% confluence). The colorimetric method was commonly used in examining the cell densities in microtiter plates with a tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-terazolio]-1,3-benzene disulfonate; WST) and an ELISA reader (Dynatch, Chantilly, VA) [24].

Electrophysiological Measurements

GH₃ or RAW 264.7 cells used for the experiments were dissociated and an aliquot of cell suspension was immediately transferred to a recording chamber positioned on the stage of an inverted CKX-41 microscope (Olympus, Tokyo, Japan). 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 either a PP-830 puller (Narishige, Tokyo, Japan) or a P-97 Flaming/Brown micropipette puller (Sutter; Novato, CA). They had a resistance of 3-5 MΩ when filled with different pipette solutions described above. Ion currents were measured with glass pipettes in whole-cell configuration of standard patch-clamp technique by means of an RK-400 amplifier (Bio-Logic, Claix, France) [9, 11].

The signals consisting of potential and current traces were stored online in a TravelMate-6253 laptop computer (Acer, Taipei, Taiwan) at 10 kHz through a Digidata-1322A interface (Molecular Devices, Sunnyvale, CA). The latter device was equipped with an Adaptec SlimSCSI card (Milpitas, CA) via a PCMCIA slot and controlled by pCLAMP 9.2 (Molecular Devices). Current signals were low-pass filtered at 3 kHz. The voltage-step profiles generated by pCLAMP were applied to evaluate the current-voltage relationships for ion currents (e.g., I_{MEP}). Current signals were commonly analyzed using Origin 8.0 (OriginLab, Northampton, MA) or custom-made macros built in Excel 2007 spreadsheet running under Windows-7 (Microsoft, Redmond, WA). When current signals elicited by membrane hyperpolarization from -80 mV were greater than 100 pA and the corresponding standard deviation (SD) was greater than 100 pA, I_{MEP} was identified.

Concentration-Response Analysis

Concentration-response data for PALCAR-induced stimulation of I_{MEP} emerging from GH₃ cells were fitted with a Hill function. That is,

$$y = (E_{\max} \times [C]^{n_H}) / (EC_{50}^{n_H} + [C]^{n_H})$$

where y is the relative amplitude of I_{MEP} ; $[C]$ is the PALCAR concentration; IC_{50} and n_H are the concentration required for a 50% increase and the Hill coefficient, respectively. The non-linear least-squares fitting was made in the Solver-add-in bundled with Microsoft Excel 2007 [9, 11, 25]. The I_{MEP} amplitudes were generally taken about 2 min after the voltage protocol was repetitively applied to the cell. The latency to I_{MEP} generation is Gaussian normal distribution with mean value of 68 ± 7 sec when cells were repetitively hyperpolarized from -80 to -200 mV [11].

Principal Component Analysis (PCA)

PCA is recognized to be a widely used method in data analysis and compression [13, 14]. It is a simple nonparametric method that allows the extraction of relevant data from mixed datasets. Mathematically, the data of current signals were exported to a transform matrix D of m rows (number of measurement types) and n columns (number of samples) with s being the datum points.

$$D = \begin{bmatrix} s_{1 \times 1} & \dots & s_{1 \times n} \\ \downarrow & & \\ s_{m \times 1} & \dots & s_{m \times n} \end{bmatrix},$$

For each n , u is a unit vector. $MM^T u = \lambda u$ with the largest eigenvalue being λ_1 . The eigenvalue λ_1 is called the principal eigenvalue and represents the characteristics of current signals because the first component extracted in a PCA explains as much of the variability in the data as possible. Consequently, based on PCA transformation, it is convenient to change the great number of input data (i.e., a matrix of multivariate data) into a set of components (hypothetical variables) according to their importance.

The set of principal eigenvalues generated in this study were normalized by dividing all principal eigenvalues by the maximal principal eigenvalue discovered. This allows for the relative comparisons to be made. Before current signals were analyzed, white or Gaussian noise with a duration of 300 msec was created and testified for the reliability of our measures. Principal eigenvalues of greater than 0.8 were considered large, while those for white or Gaussian noise were computed to be around 0.6 and no extreme principal eigenvalues could be clearly demonstrated.

For real-time analysis of the current signals at 10 sec prior to the start of initial I_{MEP} (I_{Pre}), the digital data for each period (i.e., 5 msec) were taken as inputs into the transform matrix described above. The resultant principal eigenvalues representing the principal characteristic of current signals for this period were then computed. In this way, about 55 principal eigenvalues were extracted for each frame of current signal, when capacitive current transients ranging usually between the twentieth and thirtieth millisecond after the beginning of voltage pulses were removed.

The data are presented as means $\pm$ standard error of mean (SEM) with sample sizes (n), indicating the number of cells examined. The paired or unpaired Student's t test and one-way analyses of variance with a least-significance difference method for multiple-group comparisons were used for the statistical evaluation of difference among means. To evaluate the sum of squared residuals (SSR) value as a function of EC_{50} value for the stimulatory action of PALCAR on I_{MEP} , the 95% confidence interval was estimated with the aid of Fisher's F distribution [9, 25]. The PCA computations were performed using PROC FACTOR procedure in SAS 9.3 application program (SAS Institute Inc., Cary, NC). Differences were considered statistically significant at $P < 0.05$.

Results

Analysis of Hyperpolarization-Induced Currents in Pituitary GH_3 Cells

By use of standard whole-cell voltage-clamp recordings, we measured hyperpolarization-induced currents existing in pituitary GH_3 cells for up to 5 min. In order to evaluate the characteristics of current signals in response to membrane hyperpolarization in these cells, the principal component analysis (PCA) was thereafter applied to current signals for calculation of the principal eigenvalues (λ_1). In these experiments, immediately after a stable whole-cell model was achieved, membrane hyperpolarizations at a rate of 0.1 Hz were repetitively applied from a holding potential of -80 mV to -200 mV. Figure 1 depicts a typical

Fig. 1. Analyses of hyperpolarization-induced currents recorded from pituitary GH₃ cells. In these experiments, cells were bathed in Ca²⁺-free Tyrode's solution containing 10 mM CsCl. CsCl was used to prevent the contamination of whole-cell K⁺ currents. The I_{MEP} was elicited in an episodic fashion as the cell examined was hyperpolarized from -80 to -200 mV with a duration of 300 msec at a rate of 0.1 Hz. (A) Time course of changes in current amplitude, standard deviation (SD) of corresponding amplitude (B), and principal eigenvalues (C) of current signals. The measurement was made immediately after whole-cell configuration was established. Red open circles and black filled circles in (A) correspond to current amplitudes measured at the beginning (10 msec) and end (295 msec) of repetitive hyperpolarizations to -200 mV, respectively. Black filled squares in (B) correspond to SD of current amplitude, and red filled circles in (C) indicate the principal eigenvalues derived from PCA. (D) Bar graph showing the correlation coefficient between each signal and the first component versus time. Correlation coefficient in each pulse was made in the first component of PCA. On note, changes in the positive correlation coefficient tend to be coincident with those in principal eigenvalues. (E) Scree plot (i.e., simple plot of eigenvalues) of hyperpolarization-induced inward currents which shows the proportion of variance for each principal component. The red dashed curve indicates the eigenvalues expected under a random model for separating the meaningful components from the trivial components. Thus, the eigenvalues under this red curve may represent non-significant components. Note that there are three components (above the red curve) representing the most important ones extracted in PCA.

example of one of these experiments showing effects of large hyperpolarization on membrane current measured from a pituitary GH₃ cell. Consistent with previous observations [10-12, 26], a population of irregular and transient inward currents occurring in a periodical fashion were clearly elicited. These inward currents occurring asynchronously were increased with greater hyperpolarizations and have been previously identified as I_{MEP} . The time courses of changes in the amplitude, standard deviation (SD) and principal eigenvalue of these currents measured at the potential of -200 mV are illustrated in Fig. 1. Notably, when current signals elicited by repetitive hyperpolarizations were pooled and transformed with PCA, the larger principal eigenvalues which are assumed to be greater than 0.8 were found to coincide with the emergence of large inward currents. As the I_{MEP} became declined, principal eigenvalues

Fig. 2. Analyses of I_{MEP} recorded from pituitary GH₃ cells. (A) Original I_{MEP} trace corresponding to the data indicated by asterisk in Fig. 1B (at 300 msec). The current signal was elicited by hyperpolarization to -200 mV from -80 mV. Panel (Ba) is original current trace at the potential of -200 mV, whereas panel (Bb) represents the time course of principal eigenvalues derived from current signal in (Ba). The dashed green line in (Bb) indicates the principal eigenvalue of 0.8. (C) Original I_{MEP} obtained in the presence of LaCl₃ (100 μ M). Panel (Da) was obtained in the presence of LaCl₃ (100 μ M), whereas panel (Db) represents the time course of resultant principal eigenvalues from (Da). The dashed blue line in (Db) indicates the principal eigenvalue of 0.6. Inset in (A) and (C) indicates the voltage protocol used. Notably, as cells were exposed to LaCl₃, besides that the amplitude of hyperpolarization-induced current was greatly decreased, the resultant principal eigenvalues derived from these current signals became diminished and was around 0.6 (indicated by the blue dashed line), suggesting that no clear extreme principal eigenvalues can be demonstrated in the presence of LaCl₃.

derived from PCA were greatly reduced to be around 0.5. It is noted that large principal eigenvalues of greater than 0.8 (in a normalized set) are closely linked to the large deflections of I_{MEP} (indicated by asterisk in Fig. 1B). Similarly, changes in correlation coefficient of the first principal component extracted in a PCA (Fig. 1D) tended to coincide with clear extreme principal eigenvalues in Fig. 1C. Similar results were obtained in 17 different cells examined. The correlation (r) between changes in the correlation coefficient of the first principal component extracted and the extreme principal eigenvalue is 0.78 ± 0.02 ($n=17$). Based on the scree plot by PCA conducted on hyperpolarization-elicited current signals (Fig. 1E), there exhibit to be the first three principal components which are well retained owing to a fairly large size of the eigenvalues directly related to these ones. Notably, the magnitude of the eigenvalues falls off quickly, such that about 70% of the value is contained in the first three eigenvectors which tend to account for a large proportion of the variability. This implies that a good approximation of the full matrix derived from current signals in response to membrane hyperpolarization can be computed using only a subset of the eigenvectors and eigenvalues.

Analysis of I_{MEP} Signals from GH₃ Cells

We further attempted to examine whether or not PCA can fairly discriminate the MEP-associated currents elicited by each pulse on the basis of the characteristics of principal

Fig. 3. Real-time analysis of I_{Pre} signal in response to membrane hyperpolarization. (A) Original trace of I_{Pre} (i.e., hyperpolarization-induced current occurring 10 sec prior to the start of initial I_{MEP}) in a GH_3 cell was shown. Inset in (A) indicates the voltage protocol used. (B) I_{Pre} signal at the potential of -200 mV. In inset of (B), blue current trace was amplified from (B) (indicated by dashed box). Note that there were minuscule inward currents (I_{min}) immediately before the rise of large inward current indicated by a large downward deflection. (C) Principal eigenvalues using PCA applied to I_{Pre} signal derived from (B). Note that there are two

clear extreme principal eigenvalues indicated by asterisk in (C) which coincide with the occurrence of herald minuscule inward currents (I_{min}) prior to the rise of large MEP-associated inward current. The green dashed line indicates the principal eigenvalue at the level of 0.8.

eigenvalues. Previous studies have shown the ability of LaCl_3 or MnCl_2 to suppress I_{MEP} [9, 11]. The single I_{MEP} elicited by large hyperpolarization with subsequent addition of LaCl_3 or MnCl_2 was then analyzed on a real-time basis. As shown in Fig. 2, as current signal elicited by large hyperpolarization was elicited, the corresponding principal eigenvalues extracted in PCA were found to be progressively elevated over time. Multiple peaks with principal eigenvalues of greater than 0.8 can be noted (Fig. 2Bb). A subsequent application of LaCl_3 (100 μM) to cells, the I_{MEP} magnitude was considerably reduced (Fig. 2), as evidenced by the significant decrease in current amplitude to 203 ± 12 pA ($n=12$, $P<0.01$) from a control value of 2317 ± 245 pA ($n=12$). Accordingly, the principal eigenvalues extracted in PCA from such current signal over time were significantly declined to be around 0.6 (Fig. 2Db). Similar results were also obtained as cells were exposed to MnCl_2 (100 μM). Subsequent application of LaCl_3 or MnCl_2 to GH_3 cells can not only decrease I_{MEP} amplitude, but also diminish principal eigenvalue extracted in PCA. These interesting results suggest that the extreme principal eigenvalues derived from current signals are well correlated with the emergence of I_{MEP} elicited by large hyperpolarizations.

Analysis of I_{Pre} Signals at the Potential of -200 mV in GH_3 Cells

To evaluate whether PCA can be used to predict the appearance of MEP-associated currents, the current signals at 10 sec preceding the start of I_{MEP} rise (i.e., I_{Pre}) which were accompanied by the elevated SD of current amplitude were further analyzed. As shown in Fig. 3B and 3C, the escalating rise of current amplitudes indicated in a downward deflection tends to be coincident with the increased principal eigenvalues. Interestingly, concomitant with occurrence of herald minuscule inward currents (I_{min}), the extreme principal eigenvalues as denoted by asterisks in Fig. 1B were also noted to precede the abrupt emergence of I_{MEP} in response to membrane hyperpolarization, although the amplitude of I_{min} was small and ranged between 100 and 300 pA. The results reflect that PCA is effective at distinguishing MEP-induced currents from randomly noisy ones.

Effects of Changes in Membrane Polarization on the Principal Eigenvalues of I_{Pre} Signals

Previous analysis with detrended fluctuation analysis showed that the scaling exponent of I_{MEP} signals remained unaffected by the magnitude of membrane hyperpolarization [11]. We further evaluated if the level of membrane hyperpolarization can influence I_{Pre} or the principal eigenvalues of this current signals. As illustrated in Fig. 4, when the cell was hyperpolarized to -160 mV from a holding potential of -80 mV, multiple runs of I_{min} with a

Fig. 4. Effect of different membrane hyperpolarizations on $I_{\min}$ and corresponding principal eigenvalues. (A) Original I_{Pre} trace obtained as the hyperpolarization pulse from -80 to -160 mV was applied. Inset in (A) indicates the voltage protocol used. (B) I_{Pre} signal obtained at the potential of -160 mV. Blue current trace in inset was amplified from (B) (indicated by the dashed box). (C) Time course in changes of principal eigenvalue of an I_{Pre} signal derived from (B) which was measured at -160 mV. The asterisks in (C) indicate the extreme principal eigenvalue before the rise of large current amplitude. (D) Summary of the data depicting effect of membrane potentials on duration of $I_{\min}$ (mean $\pm$ SEM, n=6 for each bar). The duration of herald $I_{\min}$ elicited by membrane hyperpolarization was taken to be the time ranging between the initial appearance of extreme principal eigenvalue and the large rise in current amplitude

(i.e., greater than 500 pA). Notably, under our whole-cell recordings, with greater membrane hyperpolarizations, the duration for $I_{\min}$ occurrence became progressively shortened. *Significantly from the values at -200 mV ($P<0.05$).

Fig. 5. Characterization of I_{Pre} and corresponding principal eigenvalues in isoflurane-treated GH₃ cells. In this set of experiments, GH₃ cells were treated with isoflurane (2.0 %) for two hours. (A) Original I_{Pre} trace at -200 mV measured from an isoflurane-treated GH₃ cell. (B) Principal eigenvalues of I_{Pre} signal derived from (A). The green dashed line indicates the principal eigenvalue at the level of 0.8. Note that, in cells exposed to isoflurane, the duration of herald $I_{\min}$ in response to membrane hyperpolarization remained unaltered, although the demarcation line between $I_{\min}$ and the rise of large current amplitude becomes slightly unclear.

longer duration tended to appear before large rise in downward deflection. Additionally, when the membrane became more hyperpolarized, the duration of both heralded $I_{\min}$ emergence and principal eigenvalues existing higher values was significantly shortened (Fig. 4D). For example, the duration of $I_{\min}$ measured at -200 mV was 56 ± 12 msec (n=6)

Fig. 6. Characterization of I_{pre} and corresponding principal eigenvalues in PALCAR-treated GH₃ cells. In this set of experiments, GH₃ cells were treated with PALCAR (3 μ M) for two hours. (A) Original I_{pre} trace from a GH₃ cell. The upper part of (A) indicated the voltage protocol used. (B) I_{pre} signal at -120 mV. Blue current signal in Inset was amplified from (B) (indicated by the dashed box). (C) Corresponding principal eigenvalues of I_{pre} signal. Notably, the amplitude of herald I_{min} tends to be smaller together with no clear extreme principal eigenvalues which were less than 0.8.

which was significantly shorter than that at -160 mV (103 ± 21 msec [$n=6$]). The frequency of extreme principal eigenvalues greater than 0.8 during I_{min} occurrence was noted to increase as membrane became more hyperpolarized. The results demonstrate that, unlike the scaling exponent of I_{MEP} signals [11], the duration of both I_{min} and corresponding higher principal eigenvalues may rely on the different levels of membrane hyperpolarization.

Analysis of I_{pre} Signals Recorded from Isoflurane-Treated GH₃ Cells

In the next set of experiments, we tested if inhalational anesthetics isoflurane can alter the biophysical properties of I_{MEP} . In these experiments, GH₃ cells were pretreated with 2.0 % (v/v) isoflurane for two hours. Membrane hyperpolarizations under the same protocol described above were repetitively applied to the cell and PCA was used for analyzing the principal eigenvalues. As shown in Fig. 5, the heralded I_{min} together with the large principal eigenvalues was clearly shown. The results suggest that pretreatment of cells with isoflurane did not significantly alter both duration of heralded I_{min} and generation of extreme principal eigenvalues extracted in PCA, although this agent may influence the biophysical properties of membrane ion channels [27].

Analysis of I_{pre} Signals Recorded from PALCAR-Treated GH₃ Cells

Previous work has demonstrated that PALCAR could reduce electrophoretic mobility of erythrocytes [16], elevate intracellular Ca^{2+} in vascular endothelial cells [20] and increase the amplitude of late Na^+ current [21]. Therefore, we further investigated the biophysical properties of I_{MEP} in cells exposed to PALCAR (3 μ M) for 2 hours. Interestingly, as membrane hyperpolarization was applied to the cell, the I_{pre} signal emerging before the rise of initial I_{MEP} was not noted to exhibit the presence of heralded I_{min} (Fig. 6). Moreover, the principal eigenvalues derived from I_{pre} did not show the value greater than 0.8 in current signals immediately prior to the escalating inward current elicited by membrane hyperpolarization.

Stimulatory Effect of PALCAR on I_{MEP} in Pituitary GH₃ Cells

Because the characteristics of I_{MEP} were altered in PALCAR-treated cells, we next explored whether PALCAR could directly modify the magnitude of hyperpolarization-induced I_{MEP} in GH₃ cells. In these experiments, whole-cell configuration was used to investigate electrical

Fig. 7. Stimulatory effect of PALCAR on I_{MEP} in pituitary GH₃ cells. In these experiments, cells were bathed in Ca²⁺-free Tyrode's solution containing 10 mM CsCl. The cells were held at -80 mV and hyperpolarizing pulses to -200 mV with a duration of 300 msec at a rate of 0.05 Hz were applied. (A) Superimposed current traces in response to membrane hyperpolarization. a: control; b: 1 μM PALCAR; c: 3 μM PALCAR; d: 10 μM PALCAR. The upper part of (A) indicates the voltage protocol used. (B) The relationship between the relative amplitude of I_{MEP} and the PALCAR concentration. At -200 mV, current amplitude in the presence of 30 μM PALCAR was considered to be 1.0. The smooth line represents the best fit to the Hill function as described in "Materials and methods". The EC_{50} value and Hill coefficient for PALCAR-induced stimulation of I_{MEP} was 2.4 μM and 1.3, respectively. Each point represents the mean $\pm$ SEM ($n=7-13$). The inset in (B) shows confidence assessment of best-fit parameter values. The parameter range corresponds to the approximate 95% confidence intervals. Gray line marks parameter value (i.e., EC_{50}) at which the sum of squared residuals (SSR) amounts to 0.0052 (see text).

properties of macroscopic I_{MEP} with or without the addition of different concentrations of PALCAR. Similar to the experimental profile described above, cells were bathed in Ca²⁺-free Tyrode's solution. When the cell was held at -80 mV, the hyperpolarizing pulse from -80 to -200 mV with a duration of 300 msec was applied. As shown in Fig. 7A, as cells were exposed to different concentrations of PALCAR, the I_{MEP} amplitude was greatly increased (traces b to d in Fig. 7A). For example, at the potential of -200 mV, PALCAR (3 μM) significantly increased the I_{MEP} amplitude from 245 ± 34 to 657 ± 67 pA ($n=8$, $P<0.05$). After washout of the compound, the current amplitude partially returned to 425 ± 38 pA ($n=6$). When K⁺ ions in the pipette solution were replaced with equimolar concentrations of NMDG⁺, this current could still be elicited by PALCAR (3 μM), although the I_{MEP} amplitude became relatively smaller. Moreover, PALCAR-elicited increase of I_{MEP} in GH₃ cells was significantly reversed by subsequent addition of LaCl₃ or MnCl₂, but not by iberiotoxin (200 nM), apamin (200 nM) or glibenclamide (30 μM) (data not shown).

The relationship between the PALCAR concentration and the relative amplitude of I_{MEP} was then derived and plotted (Fig. 7B). The half-maximal concentration required for its stimulation of I_{MEP} was 2.4 ± 0.3 μM ($n=13$). In the SSR plot shown in the inset of Fig. 7B, there was a horizontal line at SSR=0.0052 which was used to determine the two EC_{50} values. For a 95% confidence interval, the lower and upper values were then computed to be 1.43 and 1.53 μM , respectively, indicating that the EC_{50} value computed is precise and significant. It is also noted that there was a steep slope on both sides of the minimum, indicating that the EC_{50} value for PC-stimulated I_{MEP} was determined with high confidence [25]. Similarly, lysophosphatidylcholine (10 μM), a glycerol-based lysophospholipid, was able to increase the I_{MEP} amplitude in these cells (data not shown). Lysophosphatidylcholine may mediate its stimulatory effect on I_{MEP} through a pathway similar to or identical to PALCAR. However, neither tetrodotoxin nor ranolazine produced any effects on PALCAR-stimulated I_{MEP} found

Fig. 8. Characterization of I_{Pre} and corresponding principal eigenvalues in RAW 264.7 macrophages. (A) Original I_{Pre} trace at -200 mV. Blue current trace in Inset was amplified from (A) (indicated by the dashed box). (B) Principal eigenvalues of I_{Pre} . The green dashed line indicates the principal eigenvalue at the level of 0.8. The I_{min} along with high principal eigenvalues was noted to emerge before the start of large rise in current amplitude.

in these cells. Taken together, results from these observations lead us to propose that, in GH_3 cells, PALCAR has a stimulatory effect on I_{MEP} , rather than on late Na^+ currents [21, 28, 29].

Analysis of I_{Pre} Emerging from RAW 264.7 Macrophages

Because the occurring events of I_{MEP} in response to membrane hyperpolarization might vary among different types of cells [11, 12], I_{Pre} measured from RAW 264.7 macrophages were further investigated and analyzed with PCA. Consistent with previous observations in RAW 264.7 cells [11], the present results clearly showed that the I_{MEP} can be readily elicited by repetitive large hyperpolarizations. As illustrated in Fig. 8, the heralded I_{min} existing in I_{Pre} signal can be found immediately before the start of initial large inward current. As the I_{min} was extracted in PCA, the high principal eigenvalues were noted to appear prior to large downward deflection in response membrane hyperpolarization. The I_{min} duration elicited by membrane hyperpolarization to -200 mV from a holding potential of -80 mV was measured to be 39 ± 4 msec ($n=9$). This value was significantly lower as compared with that from pituitary GH_3 cells. These results reflect that by use of PCA, the I_{Pre} signals observed in the two types of cells were consistently found to display heralded I_{min} accompanied by the appearance of extreme principal eigenvalues. The observed difference in I_{min} duration between these cells could be explained by either different elastic properties of phospholipid bilayers or later stress of the hydrocarbon chain region.

Discussion

In this study, we established an important association between times when extreme principal eigenvalues are given and the occurrence of large current amplitude rise. The heralded I_{min} together with high principal eigenvalues present in I_{Pre} current signals is likely to be followed by the rise of large current amplitude. PCA is applied to hyperpolarization-induced inward currents to detect heralded I_{min} associated with the emergence of I_{MEP} in GH_3 and RAW 264.7 cells. Therefore, it is anticipated from this study that MEP-induced opening of membrane pores could occur earlier and be coincident with the emergence of heralded I_{min} . By using PCA, we provided information not only on the process of pore formation, but also suggestive of the kinetics of pore generation related to MEP. This is potentially important because we might define the optimal conditions for the electroporation of cells, design systems for MEP, and protection against serious cell damage during this process.

The hyperpolarization-induced activation and irregular time course observed in these cells strongly suggested that I_{MEP} was produced by transient rupture of cell membrane due to changes in the electrical field associated with large hyperpolarization [12, 26]. It is possible that repetitive application of large hyperpolarizations creates the momentum necessary to influence the phospholipid bilayers. Large hyperpolarizations from -80 to -200 mV cause significant current variations, which were attributable to changes in the electrical field. More importantly, PCA presented herein can not only discriminate between MEP-related current signals and background noises, but also give a mathematical representation of hyperpolarization-elicited current signals without the need for prior observational analysis, as long as we understand how such signals are characterized by principal eigenvalues. Our results also prompted us to indicate that although change in the level of membrane polarization did not alter the correlation properties of I_{pre} [11], the events of herald I_{min} along with higher eigenvalues became larger with greater hyperpolarizations.

Compatible with earlier studies [11], our results indicate that the higher principal eigenvalues extracted in PCA are not simply due to an increase in the magnitude of I_{MEP} elicited by large hyperpolarizations, but also attributable in part to some other physiological phenomena. In other words, memory may arise from the internal dynamics of MEP-induced channels and is not clearly described by Markovian processes [11]. It also needs to be noted that PCA method used in this study is effective at distinguishing the noises of large currents from the noises of small currents; however it would not be an expected difference for all kinds of the noises inherent in large currents.

Previous reports showed that in response to membrane hyperpolarization, the translocation may occur on two disparate time scales, namely, the electrophoretic time (~msec), and the diffusive time (~sec) [5]. If the pulse is sufficiently strong and long to complete the translocation via electrophoretic drifting, then the electrophoretic time scale is observed. As the whole data were analyzed with PCA, although the emergence of I_{MEP} elicited by repetitive pulses was coincident with the higher principal eigenvalues, no prediction of pore formation was demonstrated, suggesting the diffusive time (~sec) for MEP-associated channels could not be evaluated with PCA effectively. However, our study showed that, distinguishable from the detrended fluctuation analysis [11], PCA could be used in real-time prediction of I_{MEP} emergence (i.e., electrophoretic time) through early detection of MEP-associated current signals.

Consistent with previous studies [10, 11, 26], the externally applied electrical field directs ion movement and influences the kinetics of I_{MEP} . PCA presented herein is capable of detecting high principal eigenvalues in the series of current traces elicited by membrane hyperpolarization. If this analysis is valid, findings from this study indicate that, for larger I_{MEP} , clear extreme principal eigenvalues generated by PCA of herald I_{min} can potentially act as the I_{MEP} precursors. It is also anticipated that PCA has the capacity to discern such precursors within 50-100 msec prior to the escalating rise in large current amplitude. Therefore, the present results provide the evidence to show the existence of different principal eigenvalues extracted from current signals, which might be applied to predict the initiation of I_{MEP} formation in different types of cells. However, whether PCA can be specific for I_{MEP} remains to be further determined.

The addition of LaCl_3 to the cell caused the principal eigenvalues extracted in PCA to become significantly lowered, while the I_{MEP} signal elicited by membrane hyperpolarization showed multiple peaks of high principal eigenvalues. However, in isoflurane-treated GH_3 , the clear extreme principal eigenvalues coincident with appearance of herald I_{min} remained intact, although isoflurane may alter the activity of ion channels [27]. Moreover, the resealing of electropores induced by large membrane hyperpolarization in these cells tends to be associated with the constant lowering of eigenvalues in these cells.

A recent paper by Nesin et al. [30] reported that MEP with nanosecond pulsed electric field caused inhibition of I_{Na} as well as an increase in a noninactivating leak current, which was likely due to the formation of nanoelectropores or larger pores in the plasma membrane. Another study described the mechanism of electrical field-induced inhibition

of I_{Na} and demonstrated that it was not mediated by electrical field-induced Na^+ influx or altered Ca^{2+} signaling in response to pulsed electrical field [28]. The PALCAR-stimulated I_{MEP} observed in GH₃ cells is unlikely to be due to any changes in I_{Na} , although it was reported to alter the kinetics of I_{Na} [21]. As reported previously [9, 10, 12, 26], membrane ruptures created by MEP have large pores allowing permeation of large molecules and, therefore, are not selective for any particular organic and inorganic ions. To what extent pulsed electrical field-induced generation of noninactivating leak currents [30] is related to the formation of I_{MEP} observed here remains to be further elucidated.

Previous reports have demonstrated the ability of PALCAR to increase the amplitude of late Na^+ currents and to decrease peak Na^+ currents in heart cells [31]. However, in our study, neither tetrodotoxin, ranolazine nor riluzole was demonstrated to have any effects on PALCAR-stimulated I_{MEP} in GH₃ cells. Those compounds are recognized to suppress the amplitude of late Na^+ currents [32, 33]. PALCAR produced a progressive increase of I_{MEP} in GH₃ cells with an EC_{50} value of 2.4 μM . It thus seems unlikely that the PALCAR-mediated stimulation of I_{MEP} presented here is primarily associated with its excitatory effects on the amplitude of late Na^+ current. Although PALCAR is reported to increase late Na^+ current [21], to what extent its stimulation of I_{MEP} in heart cells contributes to the generation of cardiac arrhythmias [6, 31] remains to be further investigated.

As an amphiphilic compound, PALCAR possesses a polar head-group that contains regions of positive and negative charge that are in close spatial proximity in certain molecular conformation [16]. When cells are exposed to PALCAR, it might readily incorporate into the phospholipid bilayer of the surface membrane. The PALCAR molecule contains a single aliphatic hydrocarbon chain which can be esterified to the polar head-group of the membrane. There are several indications showing that the pores of relatively large size induced by MEP are hydrophilic and that their inner surface is covered with polar heads of phospholipids. Additionally, by use of PCA in I_{MEP} fluctuations described here, it was found that the presence of PALCAR resulted in a drop without the appearance of heralded I_{min} . Therefore, the incorporation of PALCAR into the membrane phospholipid bilayer may result in a significant perturbation of the orderly packing of adjacent phospholipid molecules which influence the I_{MEP} formation, resealing, or both. As the electrical field perturbs membrane lipid, electropores lined by the negatively charged head groups of phospholipids would be readily formed during cell exposure to long-chain acyl carnitines (e.g., PALCAR). The ability of PALCAR treatment to reduce the appearance of herald I_{min} observed in this study also lead us to propose that changes in the compositions of membrane phospholipid bilayers may alter the biophysical properties of MEP-associated currents in these cells. It also remains to be further investigated whether endogenous PALCAR or lysophosphatidylcholine [15, 34] can be a substrate used to enhance MEP-elicited channels which indirectly activate the intrinsic pathway to apoptotic or necrotic changes by inducing changes in the mitochondrial permeability transition pores [2, 3, 35, 36].

A recent report showed the suitability of patch-clamp experiments to assess I_{MEP} in plant cells [37]. The magnitude of I_{MEP} can control Na^+ or Ca^{2+} influx to increase with membrane hyperpolarization and fall with membrane depolarization owing to changes in electrochemical driving force. Activation of these channels can thus induce the firing of action potentials in excitable cells, or it regeneratively couples in a positive feedback relationship with Na^+ - or Ca^{2+} -activated K^+ currents [6, 7, 26, 38]. PALCAR has been previously reported to elevate the level of intracellular Na^+ or Ca^{2+} [20, 31]. Therefore, taken together, our study indicates that the increased activity of MEP-induced channels caused by PALCAR could be an important element in the modulation of functional activities of cells.

Conflict of Interest

The authors declare that they have no competing interests which are directly relevant to this study.

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