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Research Paper

Effects of Honokiol on Membrane Electroporation-Induced Inward Current in Pituitary Tumor (GH_3) Cells

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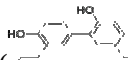
ABSTRACT

Honokiol (HNK) is a dimer of allylphenol that has been used in traditional Asian medicines (Hou p'u and Saiboku-tu(o)). It was recognized to have strong activity against a variety of tumors. In this study, the effects of HNK on membrane electroporation-induced inward currents (I_{MEP}) in pituitary tumor (GH_3) cells were investigated. HNK (0.1-10μM) increased the amplitude of I_{MEP} in a concentration-dependent manner with an EC_{50} value of 0.8μM. $LaCl_3$ or $MnCl_2$ was effective in reversing HNK-stimulated I_{MEP}; however, neither iberiotoxin, apamin, nor glibenclamide exerted any effects on it. The exposure to HNK (10μM) caused an approximately 15-mV rightward shift in voltage-dependent activation of I_{MEP}; however, it did not produce any change in the gating charge of this current in these cells. Cell exposure to AAPH (100μM), a water soluble initiator of peroxyl radicals, increased the amplitude of I_{MEP}. In continued presence of HNK (10μM), subsequent application of AAPH did not increase I_{MEP} further. Conversely, in the pretreatment of cells with AAPH, HNK still stimulated I_{MEP}. When the cells were dialyzed with HNK, a progressive increase of I_{MEP} immediately following membrane rupture was clearly observed. When the electrode was filled with HNK (1μM), the activity of MEP-induced channels was detected with single-channel conductance of 1.04 nS. The presence of HNK did not alter the single channel conductance of these channels, although it increased the probability of channel openings. In summary, our results revealed that HNK applied extracellularly or intracellularly can interact with MEP-elicited channels to increase the amplitude of I_{MEP} in pituitary tumor (GH_3) cells. Changes in reactive oxygen species could be a downstream consequence of HNK-stimulated I_{MEP}. The MEP-induced channel is proposed to be a potential target for the action of this small compound and its analogs.



Keywords: Honokiol ( ); Electroporation; Ion current; Oxidative injury; Pituitary cell.

INTRODUCTION

Honokiol (HNK) is a hydroxylated biphenyl compound obtained from *Magnolia officinalis* and from other species of the family *Magnoliaceae*, and has been used in traditional Asian medicines (Hou p'u and Saiboku-tu(o)) (Fujita, et al., 1973). Several studies have demonstrated the ability of HNK to regulate the functional activities of

neurons or neuroendocrine cells. For example, HNK was reported to inhibit acetylcholine-stimulated release of catecholamines from bovine adrenal chromaffin cells (Liu, et al., 1989; Tachikawa, et al., 2000). It could suppress glutamate-evoked Ca^{2+} influx in rat cerebellar granule cells and diminish the NMDA-induced seizure threshold (Lin, et al., 2006). An interesting study by Xu et al., (2000) showed that the mixture of HNK and magnolol possessed potent antidepressant-like properties in behaviors of the rodents with chronic mild stress. Moreover, this compound was able to inhibit NMDA receptor-mediated nociception as well as mGluR5-mediated response (Lin, et al., 2009). Previous studies in our laboratory revealed the ability of magnolol, an isomer of HNK, to stimulate the activity of large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels in smooth muscle cells (Wu, et al., 2002). However, to our knowledge, how HNK can interact with membrane ion currents to produce any effects on electrical behavior of neurons or neuroendocrine cells remains largely unclear.

Membrane electroporation (MEP) is known to produce a considerable increase in the electrical conductivity and permeability of the plasma membrane by the use of an externally applied electrical field (Tsong and Su, 1999; Wang, et al., 2010). Such maneuver has been commonly used to electrotransfer large, membrane impermeant molecules such as DNAs, anti-neoplastic drugs or antibodies into cells (Rols, 2006). Several studies have demonstrated that oxidative modification of the cell membrane could be associated with the formation of electropores induced by MEP (Tsong and Su, 1999; Vernier, et al., 2009; Freikman, et al., 2011). By use of a modeling approach, it was shown that under certain circumstance, MEP was capable of disrupting intracellular organelles (Esser, et al., 2010). In GH_3 pituitary tumor cells, we have 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 $LaCl_3$ (Wu, et al., 2011). Previous studies have demonstrated that the increase of intracellular reactive oxygen species (ROS) by HNK mediated necrotic cell death through activation of the mitochondrial permeability transition pore (mPTP) (Li, et al., 2007). HNK-induced cell death was reported to be associated with the increase of intracellular ROS (Li, et al., 2007; Fried and Arbiser, 2009). Magnolol has been recently described to interact with liposomes of different fatty acyl chain lengths to form homogeneous lipid bilayer (Chen, 2009). However, whether I_{MEP} can be regulated by HNK is unknown.

Therefore, the purpose of this work was to evaluate whether HNK exerts some effects on ion currents in pituitary tumor (GH_3) cells. Importantly, findings from our study indicate that HNK is effective in increasing the amplitude of I_{MEP} in a concentration-dependent fashion in these cells. The HNK-stimulated effects on I_{MEP} could be closely linked to its actions on apoptotic or necrotic cell death (Li, et al., 2007) if similar results occur *in vivo*.

MATERIALS AND METHODS

Drugs and solutions: *t*-Butyl hydroperoxide (*t*-BHP) and honokiol [HNK, $C_{18}H_{18}O_2$, 2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol] 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). Glibenclamide was from Tocris (Bristol, UK). Part of HNK was provided kindly by Professor Chien-Chich Chen, National

Institute of Chinese Medicine, Taipei City, Taiwan. HNK was prepared as 10-30 mM stock solutions in dimethyl sulfoxide (DMSO) and added to bath solution at the indicated final concentration. All culture media, fetal calf serum, horse serum, L-glutamine, trypsin/EDTA, and penicllllin-streptomycin were obtained from Invitrogen (Carlsbad, CA). All other chemicals, including CsCl, $CdCl_2$, $LaCl_3$, and $MnCl_2$, 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 used in this study is as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES-NaOH buffer 5.5 (pH 7.4). To record I_{MEP}, the patch pipette was filled with a solution (in mM): K-aspartate 130, KCl 20, KH_4PO_4 1, $MgCl_2$ 1, Na_2ATP 3, Na_2GTP 0.1, EGTA 0.1, HEPES-KOH buffer 5 (pH 7.2). In some experiments, the pipette was filled with solution which contained HNK (1 or 10μM).

Cell preparation: GH_3 pituitary tumor cells, obtained from the Bioresources Collection and Research Center ([BCRC-60015]; Hsinchu, Taiwan), were routinely maintained in Ham's F-12 medium supplemented with 15% horse serum, 2.5% fetal calf serum and 2 mM L-glutamine in a humidified environment of 5% CO_2/95% air (Wu, et al., 2011). The medium was consistently refreshed every 2 days to provide adequate nutrition. The experiments were generally performed 5 or 6 days after cells were subcultured (60-80% confluence).

Measurement of cell proliferation: GH_3 cells (5×10^4 per ml) were cultured at 37°C in a 96-well microplate and treated with different concentrations of HNK (0.1-10μM). The rate of cell growth was calculated among day 0, day 1, and day 2 of culture. Cell viability was assessed by a colorimetric method with the use of an enzyme-linked immunoadsorbent assay reader (Dynatech, Chantilly, VA) (Liu, et al., 2003).

Electrophysiological measurement: Before each experiment, cells were dissociated and an aliquot of cell suspension was subsequently transferred to a recording chamber mounted on the stage of an inverted DM-IL microscope (Leica, Wetzlar, Germany). The cells were immersed at room temperature in normal Tyrode's solution. Patch pipettes were pulled from Kimax-51 glass capillaries (Kimble, Vineland, NJ) using a two-stage electrode puller (PP-830; Narishige, Tokyo, Japan) and their tips were fire-polished with an MF-83 microforge (Narishige). The pipette filled with different solutions described above had a resistance of 3-5 MΩ when immersed in normal Tyrode's solution. Ion currents were measured with glass pipettes in whole-cell or cell-attached configuration of standard patch-clamp technique by use of an RK-400 amplifier (Bio-Logic, Claix, France) (Wu, et al., 2011). All potentials were corrected for liquid junction potential, which develops at the tip of the electrode when the composition of the internal solution differs from that in the bath.

Data recordings: The data 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 1 or 3 kHz. pCLAMP-generated voltage-step profiles were used to evaluate the *I-V* relationships for ion currents (e.g., I_{MEP}). Afterwards, signals were analyzed using Origin 8.0 (OriginLab, Northampton, MA) or custom-made macros built in Excel 2007 spreadsheet running under Windows-7 (Microsoft, Redmond, WA).

Data analyses: To calculate concentration-dependent effect of HNK on the amplitude of I_{MEP}, each cell was held at -80 mV, the hyperpolarizing pulses from -80 to -200 mV were applied and current amplitudes at the end of each pulse was measured during the exposure to different concentrations (0.1-10μM) of HNK. The I_{MEP} amplitude during cell exposure to 10μM HNK was taken as 1.0. The HNK concentration required to stimulate 50% of current amplitude was determined by means of a Hill function. That is,

$$relative\ amplitude = \frac{E_{\max} \times [C]^{n_H}}{EC_{50}^{n_H} + [C]^{n_H}},$$

- Where [C] is the HNK concentration, EC_{50} and n_H are the concentration required for a 50% stimulation and the Hill coefficient, respectively, and E_{max} is the maximal increase in the amplitude of I_{MEP} caused by HNK.

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 the absence and presence of HNK (10μM) were fitted with a Boltzmann function of the following form:

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

- 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 commonly performed with the aid of Excel 2007 (Microsoft) or Origin 8.0 (OriginLab Corp.) (Kemmer and Keller, 2010).

The data of macroscopic or single-channel currents are presented as means±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 values of sum of squared residuals (SSR), the 95% confidence intervals were estimated with the use of Fisher's *F* distribution (Kemmer and Keller, 2010). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of HNK on I_{MEP} in Pituitary GH_3 Cells: In the initial set of experiments, whole-cell configuration was used to investigate electrical properties of macroscopic I_{MEP} in these cells. Cells were bathed in Ca^{2+}-free Tyrode's solution containing 10 mM CsCl. When the cell was held at -80 mV, the hyperpolarizing pulse from -80 to -200 mV with duration of 300 msec was applied. As shown in Figure 1A, when the cells were exposed to HNK, the amplitude of I_{MEP} was greatly enhanced. For example, at the level of -200 mV, HNK (10μM) significantly increased the I_{MEP} amplitude from 225±21 to 937±65 pA (n=9). After washout of the compound, current amplitude returned to 487±32 pA (n=5). When K^+ ions in the pipette solution were replaced with equimolar concentrations of $NMDG^+$, this current could still be induced by HNK (10μM), although the current amplitude was relatively smaller.

The relationship between the HNK concentration and the relative amplitude of

I_{MEP} was constructed (Figure 1B). The half-maximal concentration required for its stimulation of I_{MEP} was 0.8±0.1μM (n=11). In the SSR plot shown in inset of Figure 1B, there was a horizontal line at SSR=0.027 which was used to determine the two EC_{50} values. For a 95% confidence interval, the lower and upper values were then calculated to be 0.54 and 1.10μM, respectively. Notably, there was a steep slope on both sides of the minimum, indicating that the EC_5 value for HNK-stimulated I_{MEP} was determined with high confidence. Similarly, magnolol (10μM), an isomer of HNK, was able to increase the I_{MEP} amplitude in these cells. Subsequent application of 100μM $LaCl_3$ or 100μM $MnCl_2$ reversed the increased amplitude of I_{MEP} induced by HNK (10μM) (Figure 2). In contrast, the inhibitors of K_{Ca} channels such as iberiotoxin (200nM) and apamin (200nM) produced no effects on HNK-stimulated I_{MEP} (Figure 2), and glibenclamide (10μM) did not reverse it either. Therefore, results from these observations reflect that, in GH_3 cells, HNK has a stimulatory effect on I_{MEP}, rather than on K_{Ca} currents (Wu, et al., 2002).

The Current-Voltage (I-V) Relationship of I_{MEP} with or without Addition of HNK in GH_3 Cells: To characterize the effect of HNK on I_{MEP}, we evaluated whether the presence of HNK altered *I-V* relationship of this current. Following the addition of 10 μM HNK, the amplitude of I_{MEP} in response to membrane hyerpolarization was increased throughout the entire voltage-clamp steps examined. Figure 3A illustrates the *I-V* relationships in the absence and presence of HNK. It was noted that the threshold for elicitation of I_{MEP} in control cells was around -100 mV, while that occurring during cell exposure to HNK (10μM) became depolarized to -80 mV. Specifically, cell exposure to HNK significantly increased the slope of the linear fit of I_{MEP} amplitude to voltages between -80 and -200 mV from 10.1±0.9 to 21.2±1.1 nS (n=8). The results indicated that when GH_3 cells were exposed to HNK, the *I-V* relationship of hyperpolarization-induced I_{MEP} can be modified.

Modification of Voltage-Dependent Activation Curve of I_{MEP} in the Presence of HNK: The voltage-dependence of I_{MEP} with or without addition of HNK was further examined. The normalized amplitudes of I_{MEP} (i.e., I/I_{max}) versus the membrane potentials were constructed. Figure 3B depicts the activation curves of I_{MEP} obtained in cells with or without exposure to HNK. Fitting was done using the Boltzmann function described in Materials and Methods. In control, $V_{1/2}$=-140±9 mV and q=1.1±0.2 e (n=7), whereas in the presence of HNK (10μM), $V_{1/2}$=-125±11 mV and q=1.3±0.2 e (n=7). Therefore, it is clear from these data that, in addition to the increase of I_{MEP} amplitude, cell exposure to HNK could shift the midpoint of the activation curve toward a less hyperpolarizing voltage by approximately 15 mV. Conversely, no significant difference in the gating charge of I_{MEP} activation between the absence and presence of HNK was demonstrated.

Changes in I_{MEP} Amplitude in the Presence of AAPH and HNK plus AAPH: As shown in Figure 4, AAPH at a concentration of 100μM significantly increased the amplitude of I_{MEP} at the level of -200 mV from 265±22 to 485±78 pA. Similarly, the challenge of cells with 1 mM *t*-butyl hydroperoxide (*t*-BHP) elevated the I_{MEP} amplitude by 55%. However, when cells were preincubated with 10μM HNK, AAPH-induced stimulation of I_{MEP} amplitude was abolished. When, the cells were hyperpolarized from -80 to -200 mV, there was no significant difference in I_{MEP} amplitude between HNK and HNK plus AAPH (100 μM) (655±57 pA versus 666±71 pA, n=7). These results reflect that oxidizing agents have some effect on activation of

I_{MEP} in these cells, and that pretreatment with HNK can attenuate the stimulatory effects of oxidizing agents on I_{MEP}.

I_{MEP} Amplitude Stimulated by Loading the Cells via the Patch Pipette with HNK: Another set of experiments were conducted in cells that were loaded with HNK (10μM) by including the compound in the pipette solution. As shown in Figure 5, when the pipette was filled with 10μM HNK, the magnitude of I_{MEP} was progressively increased immediately after rupture of the membrane. Intracellular dialysis of HNK (10μM) with a duration of 2 min significantly increased the amplitude of I_{MEP} at -180 mV from 375±55 to 1352±82 pA (n=8). A subsequent application of HNK (10μM) caused a slight increase of I_{MEP} from 1352±82 to 1674±97 pA (n=7). In continued presence of extracellular and intracellular HNK, further application of $LaCl_3$ (100μM) reduced the I_{MEP} amplitude to 602±68 pA (n=7). However, when subsequent application of AAPH (100μM) to the bath was applied to the cells dialyzed with HNK, no further effects on the amplitude of I_{MEP} were seen. These results allowed us to propose that the stimulatory effect of HNK on I_{MEP} in these cells could be associated with alteration in the level of ROS. The observed stimulation of I_{MEP} in the GH_3 cells loaded with HNK tends to be related to the effect of oxidative injury on I_{MEP}.

Electric Properties of HNK-Induced MEP-Induced Channels Recorded from GH_3 Cells: The effects of HNK on the activity of MEP-induced channels were further investigated. In these experiments, GH_3 cells were bathed in Ca^{2+}-free Tyrode's solution, and the recording pipette was filled with a solution containing 1 μM HNK. The ramp pulse from -200 to +100 mV with 1.5 sec at a rate of 0.05 Hz was applied to the cell. Immediately after rupture of the patch membrane, a few activities of MEP-induced channels at the hyperpolarizing potentials were observed, although there was a pronounced outward current elicited by such long-lasting ramp pulse. However, as illustrated in Figure 6, about one minute after membrane rupture, there was a progressive increase in the activity of MEP-induced channels which occurred at the level of hyperpolarizing potentials ranging from -80 to -200 mV. The single-channel amplitude at -150 mV was calculated to be 171.6±6.8 pA (n=8). As shown in Figure 6, a fit of the data using a linear *I-V* relationship yielded the single-channel conductance and reversal potential of 1.04±0.05 nS and -25.1±0.9 mV (n=8), respectively. The values for these channels in the presence of 1μM HNK did not differ significantly from those obtained in the pipette filled with 10μM HNK (1.06±0.05 nS and -25.2±1.0 mV, n=6). Similarly, in whole-cell configuration, HNK (1μM) applied to the bath, the activity of MEP-induced channels occurring at hyperpolarizing potentials could be also increased (data not shown). The results indicated that the presence of HNK was not observed to alter the single-channel conductance of MEP-elicited channels induced by long-lasting ramp pulses and that this compound could activate MEP-induced channels by interacting with extracellular or intracellular site of the electropore.

DISCUSSION

In agreement with previous studies made in many types of cancerous cell lines (Li, et al., 2007), it is plausible that intracellular ROS was elevated as cells like pituitary tumor cells were exposed to HNK. It also needs to be noted that pretreatment of GH_3 cells with AAPH did not prevent HNK-induced stimulation of I_{MEP}. HNK was capable of activating I_{MEP} by increasing an interaction of ROS with the head groups of the phospholipids in the hydrophilic conducting pore. A significant number of studies have

demonstrated that HNK exerted an anti-oxidative action in dermatologic disorders, acute lung injury, and brain damage (Lin, et al., 2006; Hoi, et al., 2010; Kim, et al., 2010; Shen, et al., 2010; Weng, et al., 2011). AAPH is an azo compound that can create free radicals and these radicals is responsible for the formation of ROS (Huang, et al., 2005; Kim, et al., 2010). Taken together, the results reflected that the elevation by HNK of ROS production was an upstream consequence of its increase in MEP-induced channels. The stimulatory effect on I_{MEP} caused by HNK and AAPH tends to share the same mechanisms involved.

The EC_{50} value of HNK required for the stimulation of I_{MEP} was 0.8 μM in the present study. In addition, the sensitivity of cell proliferation to HNK was noted to be comparable to that of I_{MEP} in GH_3 cells and in many types of tumor cells (Li, et al., 2007). It appears that the pharmacologically relevant concentration would be similar to the concentration noted in our study (Fried and Arbiser, 2009). Unlike other poyphenols, HNK can readily cross the blood-brain barrier. It is thus anticipated that there should be a link between the actions of HNK on neurons or neuroendocrine cells and its observed effects on MEP-induced channels.

In this study, we provide evidence to show that HNK-mediated increase of I_{MEP} in GH_3 cells is not due to an increase in single-channel amplitude of MEP-elicited channels, because there was no significant difference in single-channel conductance of these channels between the presence and absence of HNK. Therefore, HNK-stimulated current of I_{MEP} could be due to increased probability of channel openings, the increased number of MEP-elicited pores, or both.

By the use of a simulated model, a recent work made by Esser et al. (2010) showed that conventional MEP might be large enough to porate cytoplasmic organelles inside the cells. Notably, $LaCl_3$ was recently reported to prevent the mitochondrial morphology transition induced by chemical injury with ROS in Arabidopsis (Scott and Logan, 2008). Therefore, it is tempting to speculate that HNK-induced stimulation of MEP-induced channels observed in this study contributes to its activation of mitochondrial permeability transition pore (mPTP).

It remains to be further investigated to what extent the presence of HNK can enhance MEP-elicited channels which indirectly activate the intrinsic pathway to apoptotic or necrotic changes by inducing changes in mPTP. Another interesting function of MEP-elicited channels presented here could act as a component of trans-plasma membrane electron transport (Principe, et al., 2011). Whatever the function(s) of MEP-elicited channels at the plasma membrane during the exposure to HNK, targeting mPTP as described previously (Li, et al., 2007) might be involved in the function of these channels at the plasma membrane.

It is tempting to speculate that the cytotoxic effect of HNK described previously (Li, et al., 2007; Fried and Arbiser, 2009) may result from generation of ROS through its stimulatory effect on the activity of MEP-induced channels. The ability of HNK to activate the mPTP was reported to be dependent on the p53 status (Li, et al., 2007). Indeed, GH_3 cells were recently reported to induce the expression of p53 in the challenge of GH_3 cells with hydrogen peroxide (Yoo and Jeung, 2010). Therefore, it remains to be further warranted to what extent tumor cells with high expression of the tumor suppressor p53 have greater susceptibility to HNK-stimulated I_{MEP}.

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Competing interests: The authors declare no competing interests in this work.

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Figure Legends

Figure- 1: Stimulatory effect of HNK on I_{MEP} in pituitary GH_3 cells.

●In these experiments, cells were bathed in Ca^{2+}-free Tyrode's solution containing 10 mM CsCl. The cells examined were held at -80 mV and hyperpolarizing pulses to -200 mV with duration of 1 sec at a rate of 0.05 Hz. (A) Superimposed current traces in response to membrane hyperpolarization. a: control; b: 1μM HNK, c: 3μM HNK; d: 10μM HNK. The upper part of (A) indicates the voltage protocol used. (B) The relationship between the relative amplitude of I_{MEP} and the HNK concentration. At the level of -200 mV, current amplitude in the presence of 10μM HNK was considered to be 1.0. The smooth line represents the best fit to the Hill function as described in the Experimental section. The EC_{50} value and Hill coefficient for HNK-induced stimulation of I_{MEP} was 0.8μM and 1.3, respectively. Each point represents the mean±SEM (n=8-13). 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 0.027.

Figure- 2: Inhibitory effect of $LaCl_3$ and $MnCl_2$ on HNK-stimulated I_{MEP}.

- Original current traces showing the amplitude of I_{MEP} in the presence of HNK and HNK plus $LaCl_3$ or $MnCl_2$ in GH_3 cells. Each cell was hyperpolarized to -200 mV from a holding potential of -80 mV. Current traces labeled a in (A) are controls, those (blue colors) labeled b were obtained during exposure to 10μM HNK, and those (red colors) labeled c were in the presence of 10 μM HNK plus $LaCl_3$ (upper), and 10μM HNK plus $MnCl_2$ (lower), respectively. (B) Bar graph showing summary of the effects of HNK, HNK plus $LaCl_3$ (100μM), HNK plus $MnCl_2$ (100μM), HNK plus iberiotoxin (Iber, 200 nM), HNK plus apamin (Apa, 200 nM), and HNK plus glibenclamide (Glib, 10μM) on the amplitude of I_{MEP} in GH_3 cells (mean±SEM; n=6-12 for each bar). In the experiments with HNK plus each agent (e.g., $LaCl_3$, $MnCl_2$, iberiotoxin, apamin and glibenclamide), each compound was subsequently applied after addition of HNK (10μM). *Significantly different from control and **from HNK (10μM) alone group.

Figure- 3: Effect of HNK on *I-V* relationship (A) and activation curve (B) 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) Averaged *I-V* relationships of I_{MEP} obtained in the absence (●) and presence (○) of 10μM HNK (mean±SEM; n=6-12 for each point). Current amplitude was obtained at the end of each hyperpolarizing step. (B) Effect of HNK on the activation curve of I_{MEP} in GH_3 cells. The normalized amplitude of I_{MEP} (i.e., I/I_{max}) in the absence (●) and presence (○) of 10μM HNK was constructed and plotted against the membrane potentials (mean±SEM; n=7-11 for each point) and the smooth lines were fitted by the Boltzmann function described in Materials and Methods.

Figure- 4: Effect of AAPH or *t*-BHP on HNK-induced I_{MEP} in GH_3 cells.

- Original current traces showing the effect of HNK and HNK plus AAPH on the amplitude of I_{MEP}. a: control; b: HNK (10μM); c: HNK (10μM) plus AAPH (100μM). Inset indicates the voltage protocol used. (B) Bar graph showing summary of the effect of HNK (10μM), HNK plus AAPH (100μM), and HNK plus *t*-BHP on the amplitude of I_{MEP} in GH_3 cells (mean±SEM; n=9-14 for each bar).

Figure- 5: Effect of intracellular dialysis with HNK on the amplitude of I_{MEP} in GH_3 cells.

- In these experiments, the upsloping ramp pulse from -200 to +100 mV with duration of 2 sec at a rate of 0.05 Hz was delivered to the cell, and the pipette was filled with 10 μM HNK. (A) Original traces of I_{MEP} elicited by a long-lasting ramp pulse. Current traces labeled a, b, c correspond to those shown in (B). (B) Time course of change in the I_{MEP} amplitude obtained immediately after rupture of the membrane. Current amplitudes were measured at the level of -180 mV. The horizontal bar shown above indicates application of HNK (10μM) or $LaCl_3$ (100μM). (C) Bar graphs showing summary of the effect of intracellular dialysis with HNK on I_{MEP} (mean±SEM; n=6-9 for each bar). 1: I_{MEP} amplitude obtained within 1 min after membrane rupture; 2: I_{MEP} amplitude obtained 2 min after membrane rupture; 3: I_{MEP} amplitude after subsequent application of HNK into the bath in continued dialysis with HNK; 4: further application of $LaCl_3$ (100μM). *Significantly different from group (1) and **different from group (3).

Figure- 6: Single-channel conductance of MEP-induced channels in the presence of HNK.

- The experiment was conducted in a recording pipette filled with 1μM HNK. Single-channel events were elicited by long-lasting ramp pulse ranging between -200 and +100 mV with duration of 1.5 sec at a rate of 0.05 Hz. Downward deflection indicates the opening events of the channel. The Inset in the upper part indicates the voltage protocol examined. The lower panel indicates amplified current trace corresponding to that appearing in the upper panel (red dashed box). The straight blue line with a reversal potential of -25 mV illustrates a linear *I-V* relation of MEP-elicited channels in the presence of HNK.