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The activation by estrogen receptor agonists of the BK_{Ca}-channel in human cardiac fibroblasts

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ABSTRACT

The agonists selective for estrogen receptor (ER)-α (4,4′,4″-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl) tris-phenol, PPT) and ER-β (2,3-bis(4-hydroxyphenyl)-propionitrile, DPN) are known to stimulate ER-α and ER-β receptors, respectively. It remains unknown whether these two agents regulate the activity of ion channels via a direct stimulation. In this study, we tested the hypothesis that DPN or PPT stimulates the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in cultured human cardiac fibroblasts (HCFs). In whole-cell configuration, depolarizing pulses evoked K^+ outward currents in an outward rectification in HCFs, the amplitude of which was increased in the presence of DPN or PPT. In inside-out patches, the activity of BK_{Ca}-channel with a conductance of 167 ± 8 pS was observed in these cells. PPT or DPN applied to the intracellular face of the membrane enhanced the activity of BK_{Ca} channels with no change in single-channel conductance. DPN and PPT increased BK_{Ca}-channel activity with an EC_{50} value of 2.3 and 2.6 μM, respectively. The mean closed time of these channels during the exposure to these compounds was reduced with no change in the gating charge of the channels. Intracellular Ca^{2+} was not altered by these two compounds. RT-PCR analysis revealed that no change in the transcriptional level of the BK_{Ca}-channel α-subunit was observed in chronic treatment with these two compounds. PPT- and DPN-stimulated increase in BK_{Ca} channels reveal novel pharmacological properties attributable to the activity of these channels, and their increase in BK_{Ca} channels activity in HCFs may contribute to cell function.

1. Introduction

It was thought that biological actions of estrogen are manifested through its binding to ERs, which consist of two subtypes, i.e., ER-α and ER-β. PPT and DPN (Fig. 1) were selective ER agonists for ER-α and ER-β, respectively. DPN was reported to have a 70-fold higher relative binding affinity for ER-β, while PPT exhibits a 40-fold binding affinity for ER-α [1]. These compounds have been demonstrated to play an important role in mediating physiologic functions via genomic pathway (e.g., increased expression of progesterone receptor, increased exocytosis of luteinizing hormone, antidepressive

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Abbreviations: BK_{Ca} channel, large-conductance Ca^{2+}-activated K^+ channel; $[Ca^{2+}]_i$, intracellular Ca^{2+}; DMSO, dimethyl sulfoxide; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; PPT, 4,4′,4″-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl) tris-phenol; ER, estrogen receptor; GADPH, glyceraldehydes-3-phosphate dehydrogenase; HCF, human cardiac fibroblast; I_K, K^+ outward current; *I-V*, current-voltage; RT-PCR, reverse transcription-polymerase chain reaction

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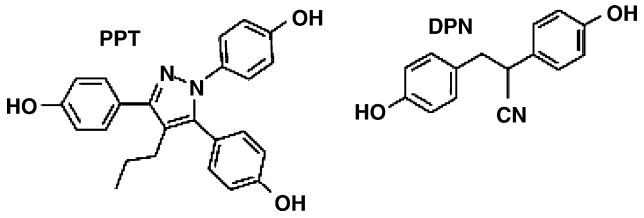

Fig. 1 – Chemical structures of PPT (left) and DPN (right).

behavior, and vascular relaxation). There are numerous reports showing that they are effective in producing cardioprotective and neuroprotective actions [2–7].

There are additional lines of information showing that both ER-α and ER-β can be localized to the plasma membrane [8–11]. It is thus possible that the rapid effects of PPT and DPN are mediated via their actions at the plasma membrane. In other words, there may be membranous receptor/binding sites for these two compounds that are responsible for their acute non-genomic actions. Overall, these two compounds were reported to elicit many pharmacological responses through both the genomic (i.e. nuclear ER-initiated) and the rapid non-genomic (i.e. membrane ER-initiated) activation in different target tissues. However, the biological actions of two compounds involved in other pathways of membrane-initiated activation remain largely unknown.

The BK_{Ca}-channel has a high single-channel conductance and is selective K^+ ions [12]. These channels, which are products of a nearly ubiquitous, alternatively spliced gene (*Slo*1 or *KCNMA*1) [13,14], are known to be distinguished from other K^+ channels in that their activation is under dual control, i.e., activated by membrane depolarization and/or by increased intracellular Ca^{2+}. These channels, which are widely distributed in a variety of cells, can control Ca^{2+} influx as well as a number of Ca^{2+}-dependent physiological processes. Previous reports have demonstrated the ability of 17β-estradiol to activate BK_{Ca} channels [15]. Additionally, the ER-α isoform has recently been shown to mediate estrogen-induced activation of BK_{Ca} channels functionally expressed in human coronary smooth myocytes [16].

In this study, we sought: (a) to determine the effect of DPN or PPT on macroscopic currents in HCFs, (b) to address the issue of whether these agents can have any effects of the activity of BK_{Ca} channels in these cells via a non-genomic pathway, (c) to examine any changes in the kinetics of BK_{Ca} channels during the exposure to these agent, and (d) to study if chronic treatment with this agent has any effects on the expression of BK_{Ca}-channel α-subunits, Based on our results, we demonstrated that DPN or PPT may interact directly with the BK_{Ca}-channel to affect I_K through a mechanism linked to a non-genomic activation.

2. Materials and methods

2.1. Cell preparation

The HCF, originally derived from normal human heart tissue, was obtained from Cell Applications, Inc. Cells were cryopreserved at the first passage, and cultured and propagated at least eight population doublings. Cells were routinely cultured in HCF growth medium (Cell Applications, Inc.) in 50-ml plastic culture flasks in a humidified environment containing 5% CO_2/95% air. At confluence, cells obtained from flasks often underwent passaged using 0.25–0.5% trypsin in 0.02% EDTA. The medium was replaced twice weekly. These cells were identified as fibroblasts, as the expression of fibroblast surface protein (e.g., matrix metalloproteinases and tissue inhibitors of metalloproteinases) has been consistently verified by immunostaining with biotin-conjugated antibody [17]. Experiments were generally performed 5–7 days after cells were subcultured (60–80% confluence).

2.2. Electrophysiological recordings

Immediately before each experiment, HCFs were dissociated, and an aliquot of cell suspension was placed in a recording chamber positioned on the stage of a DM-II inverted microscope (Leica). Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The recording electrodes were made from Kimax-51 capillaries (Kimble Glass) using a PP-830 microelectrode puller (Narishige). The pipette resistance was usually 3–5 MΩ, and seal resistance was at least 10 GΩ. All recordings in whole-cell, cell-attached, and inside-out configurations were performed at room temperature with the aid of an RK-400 patch-clamp amplifier (Bio-Logic) [18].

The signals were displayed on an HM-507 oscilloscope (Hameg) and a PJ550-2 liquid-crystal-display projector (ViewSonic). The data were low-pass filtered at 1 or 3 kHz and sampled into a computer at a sampling frequency of 10 kHz. The analysis of single-channel currents was off-line performed by means of the pCLAMP 9.0 software (Axon Instruments), the Origin 7.5 software (Microcal), or custom-made macros in Excel 2003 (Microsoft). Multi-gaussian adjustments of amplitude distributions among channels were often used to determine the BK_{Ca}-channel current. The activity of the channel in each patch was expressed as NP_0, which can be estimated using the following equation: $NP_0 = (A_1 + 2A_2 + 3A_3 + \ldots + nA_n)/(A_0 + A_1 + A_2 + A_3 + \ldots + A_n)$, where N is the number of active channels in the patch, A_0 is the area under the curve of an all-points histogram corresponding to the closed state, and $A_1 \ldots A_n$ represent the histogram areas reflecting the levels of distinct open state for 1 to n channels in the patch. The single-channel conductance was calculated by a linear regression using mean current amplitudes measured at different voltages.

To assess concentration-dependent effect of DPN or PPT on the activation of BK_{Ca} channels, the probability of channels openings during the exposure to different concentrations of DPN or PPT was examined. Under symmetrical K^+ concentration (145 mM), inside-out configuration with a holding potential of +60 mV was performed and bath medium contained 0.1 μM Ca^{2+}. The channel open probability in the presence of 30 μM DPN or PPT was considered to be 100%, and the channel open probability measured at different concentrations of DPN or PPT was then compared. The concentration of DPN or PPT required to stimulate 50% of channel activity was determined with the use of a Hill function, $y = E_{max}/\{1 + (EC_{50}{}^{n}{}_{H}/[C]\ {}^{n}{}_{H})\}$, where y is the percentage increase of

channel open probability; [C] is the concentration of DPN or PPT; EC_{50} and $^{n}_{H}$ are half-maximal concentration of DPN or PPT and the Hill coefficient, respectively; and E_{max} is the maximal increase in BK_{Ca}-channel activity activated by DPN or PPT.

The voltage dependence of the channel open probability in the absence and presence of DPN or PPT was determined using a Boltzmann function using a non-linear regression analysis with the Origin 7.5 (Microcal). That is, the relative open probability = $P_{max}/[1 + \exp(-(V - V_{1/2})qF/RT)]$, where P_{max} is the maximal open probability of channel openings, $V_{1/2}$ the half-activating voltage, q the gating charge, F the Faraday's constant, R the universal gas constant and T is the absolute temperature.

All values are reported as means ± S.E.M. The paired or unpaired Student's *t*-test and ANOVA with a least-significance difference method for multiple comparisons were used for the statistical evaluation of differences among means. Differences between the values were considered statistically significant when P was <0.05 or <0.01.

2.3. RT-PCR

To determine the α-subunit expression of BK_{Ca} channels in HCFs, a semi-quantitative RT-PCR assay was used in this study [21]. After being removed from 37 °C incubator, cells were thawed in TRIzol reagent (Invitrogen Life Technologies) and homogenized. Total cellular RNA was extracted and re-suspended in 20 μl of 0.1% diethyl pyrocarbonate water. Concentration and purity of RNA were measured at 260/280 nm OD. RT was performed with 2 μg total RNA as previously described [21]. The reaction was incubated at 25 °C for 10 min, at 50 °C for 50 min, at 85 °C for 5 min, at 4 °C for 2 min, and then thawed in RNase at 37 °C for 20 min. PCR was performed on 3 μl of RT product (cDNA) with specific primers designed from nucleotide sequences retrieved from the GenBank. GADPH was used as a reference gene to perform semi-quantitative RT-PCR. Because the reference gene must not be altered by experimental paradigm, the levels of GADPH mRNA were initially compared in each of the study groups. No effect of DPN or PPT on GADPH transcripts in HCFs was found. The values for BK_{Ca} α-subunit mRNA were then normalized to GADPH, in order to allow a semi-quantitative measure of changes in the α-subunit mRNA. The primers for GADPH were forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′ [19]. The primers for the BK_{Ca}-channel α-subunit were forward 5′-CAGCATTTGCCGTCAGTGTCCT-3′ and reverse 5′-CATGCCTTTGGGTTATTTTTCC-3′ [20]. Experimental conditions for the PCR reaction for the α-subunits were optimized. The cycles for DNA amplification were on the linear portion of the assay curve for each artery and run at optimal temperature. As of BK_{Ca}-channel α-subunits and GADPH, DNA amplification was carried out in 36 sequential cycles at 94 °C for 45 s, 62 °C for 1 min, 72 °C for 1 min, and followed by 72 °C for the final extension for 7 min. PCR products were size fractionated by applying 5 μl of PCR product to 1.5% agarose gel containing ethidium bromide and then visualized under ultraviolet light. Optical densities of DNA bands were scanned and quantified (Scion Image Software; Scion). When the values were compared, the targeted PCR products were often run on the same gel.

2.4. Measurement of $[Ca^{2+}]_i$

Cells were loaded with 3 μM fura-2/AM for 30 min at 25 °C in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Subsequently, cells were washed to remove non-hydrolyzed fura-2/AM. The glass coverslips on which the cells were grown were mounted in a 1-ml capacity plastic chamber and placed on an inverted fluorescence microscope (Olympus). Changes in $[Ca^{2+}]_i$ were monitored with digital imaging using a TillvisION imaging system equipped with a polychrome II high-speed monochromator (TILL Photonics). Fura-2 was excited sequentially by 340 and 380 nm light delivered from a xenon lamp via a 40×, 1.3 NA UV fluor oil objective (Olympus). Fluorescence images were collected at 510 nm at a rate of 0.5–2 frames/s by a Peltier-cooled charge-coupled device camera. The ratio of fluorescence (340/380 nm) from individual cell was analyzed with the aid of TillvisION software 4.0 (Till Photonics) [18,21,22].

2.5. Drugs and solutions

Paxilline, ionomycin and resveratrol were purchased from Biomol Research Laboratories and DPN, PPT and ICI-182780 (7α-[9[4,4,5,5,6-pentafluoropentyl]sulfinyl]nonyl)-estrad-1,3,5(10)-triene,3,17β-diol) were from Tocris Cookson Ltd. Iberiotoxin and glibenclamide were obtained from Alomone Labs and 17β-estradiol was from Sigma Chemical. DPN or PPT was prepared daily as a 10 mM stock solution in DMSO. The final concentration of DMSO was less than 0.03%. DMSO at a concentration less than 0.03% did not affect ion currents in these cells. Tissue culture media, L-glutamine, penicillin-streptomycin, and fungizone were obtained from life technologies.

The composition of normal Tyrode's solution was 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). In the experiments of recording K^+ currents or membrane potential, the recording pipette was backfilled with a solution (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, EGTA 0.1, Na_2ATP 3, Na_2GTP 0.1 and HEPES-KOH buffer 5 (pH 7.2). For single-channel current recordings, the high K^+-bathing solution contained (mM): KCl 145, $MgCl_2$ 0.53 and HEPES-KOH buffer 5 (pH 7.2). The pipette solution contained (mM): KCl 145, $MgCl_2$ 2 and HEPES-KOH buffer 5 (pH 7.4). The value of free Ca^{2+} concentration was calculated assuming a dissociation constant for EGTA and Ca^{2+} (at pH 7.2) of 0.1 μM.

3. Results

3.1. Stimulatory effect of I_K by DPN and PPT in HCFs

In the initial set of experiments, whole-cell configuration was used to investigate electrical properties of I_K in HCFs. Cells were bathed in normal Tyrode's solution which contained 1.8 mM $CaCl_2$. When each cell was held at −50 mV and different voltage pulses ranging from −80 to +80 mV with 10-mV increments were applied, a family of large noisy outward currents with an outward rectification was elicited. Current amplitudes were increased with greater depolarization and

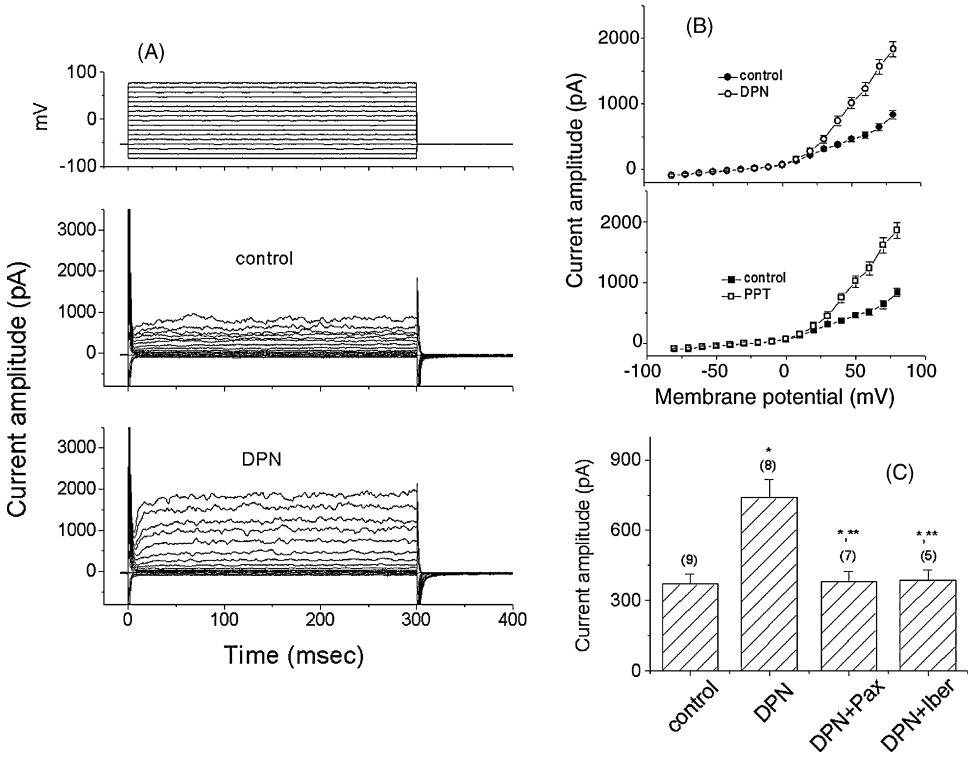

Fig. 2 – Stimulatory effect of DPN and PPT on I_K in HCF. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The cell was held at −50 mV and the voltage pulses were delivered from −80 to +80 mV in 10-mV increments. (A) Superimposed current traces in the absence and presence of DPN (10 μM). Pulse protocol is given at the top. (B) Averaged *I–V* relationships in control (filled symbols) and during the exposure (open symbols) of 10 μM DPN (upper) or 10 μM PPT (lower). Each point represents the mean ± S.E.M. (*N* = 5–8). (C) Bar graph showing the amplitude of I_K in the presence of DPN (10 μM), DPN (10 μM) plus paxilline (1 μM; Pax), and DPN (10 μM) plus iberiotoxin (200 nM; Iber). Current amplitude was measured at +40 mV. Parentheses at the top of each bar shown in this and the following figures indicate the number of cells from which the data were taken. Each bar represents the mean ± S.E.M. (*) Significantly different from control. (**) Significantly different from DPN alone group. In the experiments with DPN plus paxilline or DPN plus iberiotoxin, paxilline or iberiotoxin was subsequently applied in the continued presence of DPN.

reduced by the removal of extracellular Ca^{2+}. When the cells were exposed to DPN (3 μM) or PPT (3 μM), the amplitude of outward current was greatly increased throughout the entire voltage-clamp step (Fig. 2A). For example, when cells were depolarized from −80 to +80 mV, DPN (3 μM) significantly increased I_K amplitude from 835 ± 65 to 1833 ± 121 pA (*N* = 7). This stimulatory effect was readily reversed on the washout of DPN. After washout of the compound, current amplitude was returned to 858 ± 85 pA (*N* = 5). The averaged *I–V* relationships for current amplitudes in the absence and presence of DPN or PPT are illustrated in Fig. 2B. In addition, a further application of paxilline (1 μM) or iberiotoxin (200 nM) can reverse the increased amplitude of I_K induced by DPN (10 μM) (Fig. 2C). The results suggest that DPN and PPT have a stimulatory effect on I_K in HCFs.

3.2. *Effect of resveratrol and ICI-182780 on the amplitude of I_K in HCFs*

Resveratrol and ICI-182780 have been previously reported as stimulating and inhibiting the activity of BK_{Ca} channels, respectively [12,23,24]. We also examined whether the effects of DPN and resveratrol on these channels are additive. Interestingly, as shown in Fig. 3, resveratrol (10 μM) increased the amplitude of I_K; however, a subsequent application of DPN (10 μM) did not increase current amplitude further. Resveratrol (10 μM) significantly increased current amplitude at the level of +80 mV from 763 ± 59 to 1221 ± 87 pA (*N* = 6). There was no significant difference in channel activity between the presence of resveratrol alone and resveratrol plus DPN [1221 ± 87 pA (*N* = 6) versus 1232 ± 129 pA (*N* = 6)]. However, application of DPN (10 μM) could increase the amplitude of I_K when ICI-182780 (10 μM) was continuously present in the bath. Taken together, the results led us to suggest that the stimulatory actions of DPN and resveratrol on the amplitude of I_K could not be additive in HCFs, although these two compounds can be effective in increasing the amplitude of I_K. However, DPN could reverse the ICI-182780-induced decrease in the amplitude of I_K

3.3. *Stimulatory effect of DPN or PPT on BK_{Ca}-channel activity in HCFs*

To characterize the stimulatory effect of DPN or PPT on I_K, we further examined the effect of these compounds on the

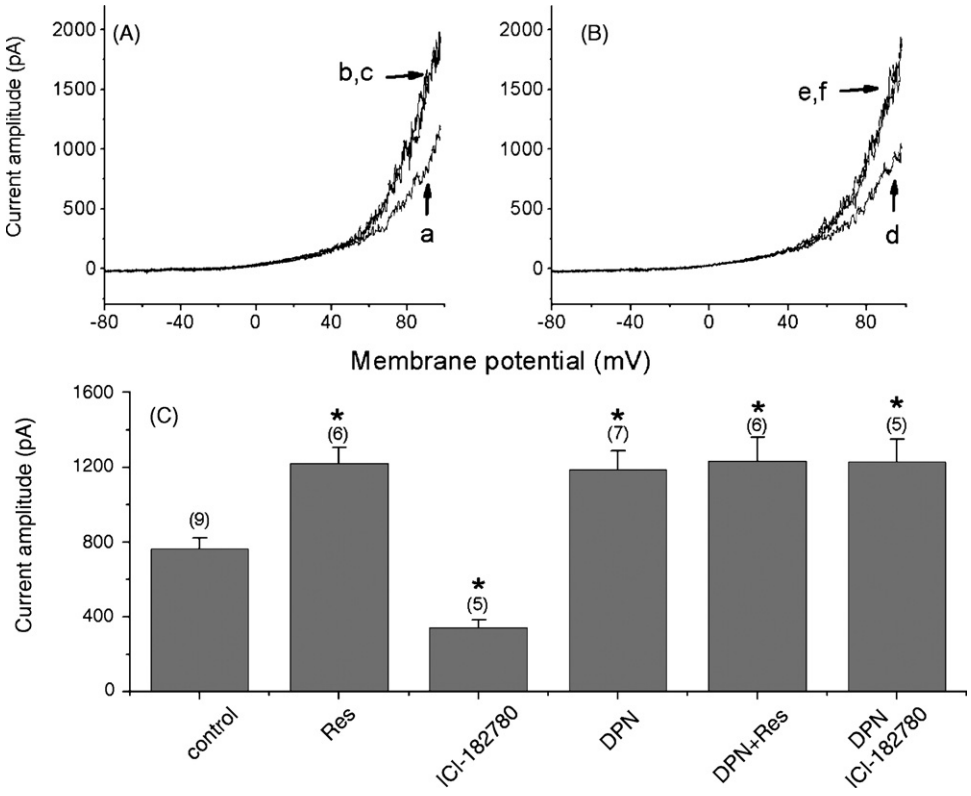

Fig. 3 – Effects of resveratrol, ICI-182780, DPN, resveratrol plus DPN, and ICI-182780 plus DPN on the amplitude of I_K in HCFs. In these experiments, HCFs were bathed in normal Tyrode's solution and ramp pulse from −80 to +100 mV at a rate of 0.05 Hz was applied to each cell. The amplitude of I_K was measured at the level of +80 mV. (A) Superimposed current traces obtained in control (a) and in the presence of 10 μM resveratrol (b) and resveratrol plus DPN (10 μM) (c). (B) Bar graph showing the effects of resveratrol, ICI-182780, DPN, resveratrol plus DPN, and ICI-182780 plus DPN on the amplitude of I_K. In the experiments with resveratrol plus DPN or ICI-182780 plus DPN, DPN was subsequently applied in continued presence of resveratrol or ICI-182780. Each point represents the mean ± S.E.M. (*) Significantly different from control group. (**) Significantly different from ICI-182780 alone group.

activity of BK_{Ca} channels. In this set of experiments, single-channel recordings with an inside-out configuration were conducted with symmetrical K^+ concentration (145 mM), and bath medium contained 0.1 μM Ca^{2+}. As shown in Fig. 4A, when the potential was held at +60 mV, addition of DPN (10 μM) to the bath caused a significant increase in BK_{Ca}-channel activity. For example, the probability of channel openings was significantly increased from 0.021 ± 0.003 to 0.132 ± 0.008 ($N = 6$) after application of DPN (10 μM). Likewise, when applied to the intracellular surface of excised patches, PPT (10 μM) significantly increased to 0.134 ± 0.011 ($N = 5$). However, no change in single-channel amplitude of these channels was demonstrated in the presence of DPN (10 μM) or PPT (10 μM), despite their ability to increase the channel open probability. Similar to the effect on I_K, paxilline at a concentration of 1 μM could reverse the effect of these two compounds on the probability of channel openings (Fig. 4B).

The relationship between the concentration of these two compounds and the channel activity was constructed and plotted (Fig. 5). The presence of DPN or PPT (0.1–30 μM) was found to increase channel activity in a concentration-dependent fashion. The half-maximal concentrations required for the stimulatory effect of DPN and PPT were calculated to be 2.3 and 2.6 μM, respectively. The Hill coefficient was 1.8, suggesting that there was a positive cooperativity for the activation of these channels caused by these compounds.

3.4. *Effect of DPN on the activation curve of BK_{Ca} channels in HCFs*

The effect of DPN on BK_{Ca} channels at different voltages was further investigated. In these experiments, HCFs were bathed in symmetrical K^+ concentration (145 mM), and bath medium contained 0.1 μM Ca^{2+}. Fig. 6 shows the activation curve of BK_{Ca} channels obtained in the absence and presence of DPN (3 μM). The plots of probability of channel openings as a function of membrane potential were constructed and fitted with a Boltzmann function shown in the text. In control, $P_{max} = 1.0 \pm 0.01$, $V_{1/2} = 72.1 \pm 0.5$ mV, $q = 3.6 \pm 0.2$ e ($N = 5$), whereas, in the presence of DPN (3 μM), $P_{max} = 1.55 \pm 0.02$, $V_{1/2} = 66.9 \pm 0.6$ mV, $q = 3.5 \pm 0.2$ e ($N = 5$). Thus, the application of DPN not only increase the maximal open probability of BK_{Ca} channels, but also caused an approximately 5-mV left shift in voltage-dependent activation of BK_{Ca} channels. However, no difference in the gating charge (i.e., q) of the BK_{Ca}-channel between the absence and presence of DPN was observed. The

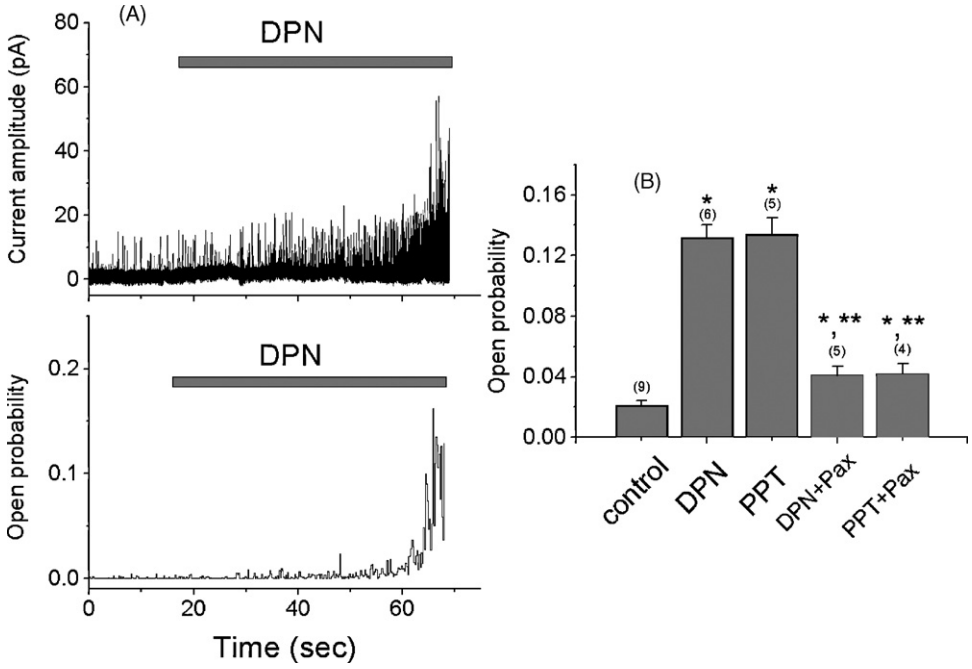

Fig. 4 – Effect of DPN or PPT on the activity of BK_{Ca} channels found in HCFs. In these experiments, cells were bathed in symmetrical K^+ (145 mM) solution and bath medium contained 0.1 μM Ca^{2+}. Holding potential was set at +60 mV and inside-out configuration was made. In (A), upper panel is the original current trace and lower panel is the time course showing effect of DPN (10 μM) on the channel open probability. Horizontal bar shown in each panel indicates bath application of DPN (10 μM). Of note, when DPN (10 μM) was added to the bath, the probability of channel openings was greatly increased. (B) Bar graph showing the stimulatory effect of DPN or PPT on BK_{Ca}-channel activity in the absence and presence of paxilline (1 μM). Potential was held at +60 mV in each excised patch and each agent was applied to the bath. Each bar represents the mean ± S.E.M. (*) Significantly different from control. (**) Significantly different from DPN or PPT alone group.

same response was also observed when HCFs were exposed to PPT. These results indicate that in HCFs, DPN can shift the voltage-dependence of BK_{Ca} channels without a change in the gating charge.

3.5. *Lack of effect of DPN on single-channel conductance of BK_{Ca} channels in HCFs*

The effect of DPN on BK_{Ca} channels at different voltages was also investigated. The plots of current amplitude as a function of holding potential were constructed. Fig. 6C illustrates averaged *I–V* relationships of BK_{Ca} channels in the absence and presence of DPN. The single-channel conductance of BK_{Ca} channels calculated from a linear *I–V* relationship in control was 167 ± 8 pS ($N = 8$) with a reversal potential of 0 ± 3 mV ($N = 8$). The value of single-channel conductance for these channels was found to be similar to those reported previously [20]. However, single-channel conductance (167 ± 8 pS, $N = 8$) remained unaltered in the presence of DPN (3 μM). It is thus clear that DPN does not modify the single-channel conductance, although it can enhance channel activity in these cells.

3.6. *Effect of internal Ca^{2+} concentrations on DPN-stimulated BK_{Ca}-channel activity*

We next examined whether the DPN-induced increase in the activity of these channels is associated with the level of internal Ca^{2+} concentrations in HCFs. In these experiments, when an excised patch was formed, various concentrations of Ca^{2+} in the bath before and during exposure to DPN (10 μM) were applied. As shown in Fig. 6D, the extent of DPN-stimulated BK_{Ca} channels was not altered by changes in the level of intracellular Ca^{2+} concentration. For example, at the level of +60 mV, DPN (10 μM) increased the probability of channel openings at an internal Ca^{2+} concentration of 0.1, 0.3 and 1 μM to a similar magnitude (i.e., 1.5-fold).

3.7. *Effect of DPN on the mean closed time of BK_{Ca} channels*

Because DPN or PPT was found to have no effect on single-channel conductance, we further investigated whether these compounds had any effects on the gating kinetics of these channels. As shown in Fig. 7, in a detached patch of control cell, closed-time histograms at the level of +60 mV can be fitted by a two exponential curve. The time constants for the close-time histogram were 63 ± 5 and 11 ± 3 ms, respectively ($N = 6$). Interestingly, DPN at a concentration of 3 μM added to the bath decreased the time constants of the closed state to 27 ± 4 and 4 ± 1 m s, respectively ($N = 6$). Similar results were also obtained in the presence of PPT (3 μM). However, no any significant effect of DPN or PPT on mean open time for these channels was observed. Thus, the presence of these compounds is able to produce a decrease in the closed time of BK_{Ca}

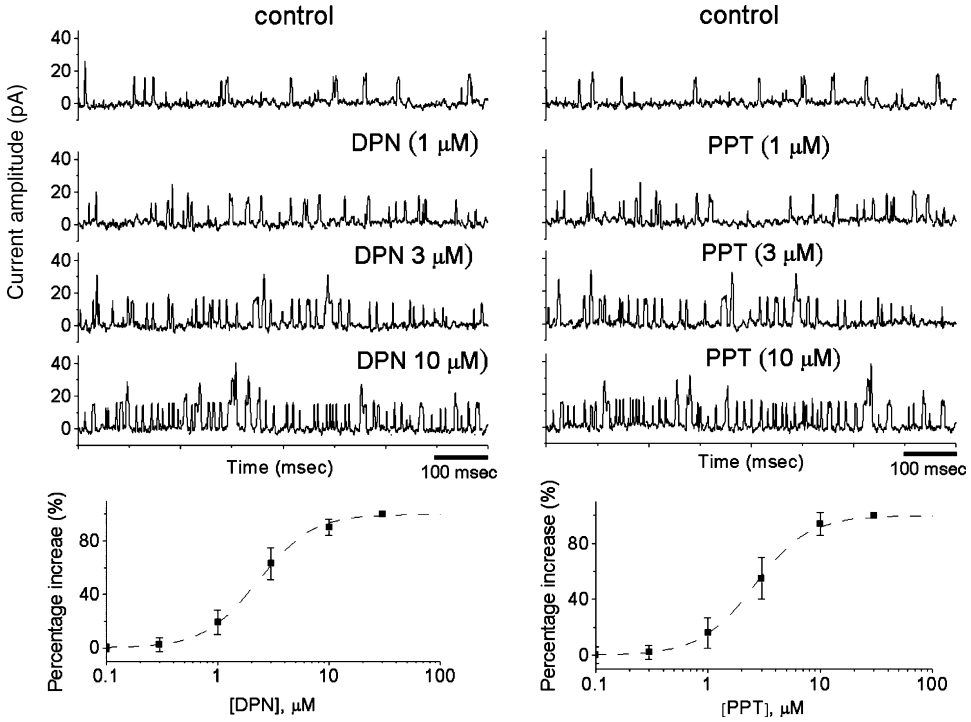

Fig. 5 – Concentration-dependent effects of DPN and PPT on BK_{Ca}-channel activity in HCFs. Inside-out configuration was made in these experiments and bath medium contained 0.1 μM Ca^{2+}. The potential was held at +60 mV at each detached patch. (A) Original current traces showing the activity of BK_{Ca} channels in the absence and presence of DPN (left) or PPT (right). The concentration of each compound is shown in the upper side of each current trace. Notably, channel openings shown in this and the following figures are shown as an upward deflection. (B) Concentration-response curves for the DPN- (left) and PPT-induced (right) activation of BK_{Ca} channels. DPN or PPT at various concentration (0.1–30 μM) was added to the bath. The probability of channel openings in the presence of DPN (30 μM) or PPT (30 μM) was considered to be 100%. Dashed smooth line shown in each panel was fitted with the Hill equation as described under Section 2. The EC_{50} values of DPN and PPT were 2.3 and 2.6 μM, respectively. The Hill coefficient was 1.8. Each point represents mean ± S.E.M (N = 6–12).

channels. It is anticipated that the binding of these compounds to the channel in HCFs causes the channel to spend less time in the closed state. Changes in mean closed times caused by these two compounds could primarily explain its stimulatory effect on the activity of BK_{Ca} channels expressed in HCFs because of no change in single-channel conductance.

3.8. *RT-PCR analysis for mRNA expression for the BK_{Ca}-channel α-subunit in HCFs*

Our findings clearly demonstrated the functional expression of BK_{Ca} channels in HCFs. The question arises whether DPN or PPT affects BK_{Ca}-channel activity in a genomic fashion. We further investigated the effect of DPN or PPT on the mRNA expression of BK_{Ca}-channel α-subunit with the aid of conventional RT-PCR. In these experiments, immediately before reaching confluence (80–90%) in culture medium, HCFs were incubated with DPN or PPT (10 μM) for 3 days. As shown in Fig. 8, the treatment of cells with DPN or PPT for 3 days was not found to alter the expression of BK_{Ca}-channel α-subunit mRNA significantly, as compared with the internal control (i.e., GAPDH). Similar results were obtained in six different experiments. These results led us to propose that chronic treatment of DPN or PPT is unable to modify the level of the expression of BK_{Ca}-channel α-subunit mRNA in HCFs. The stimulation by these agents of BK_{Ca} channels in HCFs may not be associated with changes in the mRNA expression of BK_{Ca}-channel α-subunit.

4. Discussion

In this study, we have shown for the first time that acute application of estrogen receptor agonist (e.g., DPN and PPT) is able to interact with the BK_{Ca} channels to stimulate I_K in HCFs. The stimulatory effect of these compounds was observed at concentrations greater than 0.1 μM. The stimulatory action of these compounds on I_K may play a role in the regulation of cellular function.

The EC_{50} value of DPN and PPT required for the stimulation of BK_{Ca} channels in HCFs was approximately 2 μM in this study. This value appears to be of the same order of magnitude as the concentration used for the activation of ER [1,25]. Therefore, BK_{Ca} channels present in cells like fibroblasts may be a relevant '1target' for the action of these ER agonists. Since the responses to both DPN and PPT were rapid, their actions on BK_{Ca} channels are unlikely to be genomic in nature.

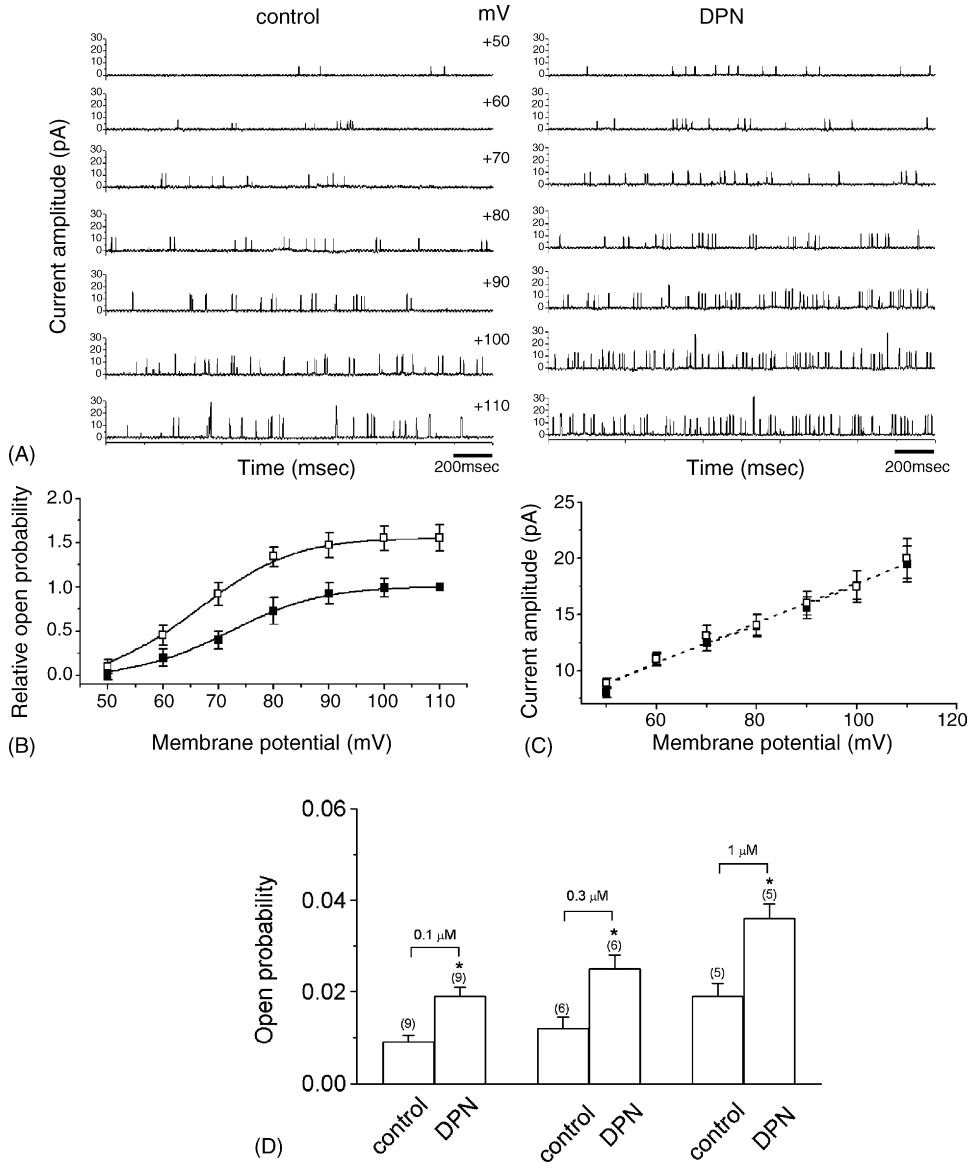

Fig. 6 – Effect of DPN on the *I–V* relationship and the Ca^{2+}-sensitivity of BK_{Ca} channels in HCFs. (A) Examples of BK_{Ca} channels in the absence (left) and presence (right) of DPN (10 μM) measured from an inside-out patch at various potentials. DPN was applied to the bath medium. The number shown at the beginning of each current trace indicates the voltage applied to the patch pipette. (B) Relationships between relative open probability of BK_{Ca} channels and membrane potential in the absence (filled symbols) and presence (open symbols) of DPN (3 μM). The smooth lines represent best fits in a Boltzmann function described in the text. Each point represents the mean ± S.E.M. Notably, the presence of DPN not only increased the probability of channel openings, but also shifted the voltage sensitivity to the leftward. (C) Averaged *I–V* relationships of BK_{Ca} channels in the absence and presence of DPN (10 μM). Current amplitude was measured at the different voltages. Values are mean ± S.E.M. (*N* = 6–9). Notably, the presence of DPN has no change in single-channel conductance of BK_{Ca} channels. (D) Lack of DPN effect on Ca^{2+} sensitivity of BK_{Ca} channels. Potential was held at +60 mV, and various concentrations (0.1, 0.3 and 1.0 μM) of Ca^{2+} in the bath were applied before and during exposure to DPN (10 μM). Data are the mean ± S.E.M. (*) Significantly different from controls (i.e., in the absence of DPN).

The binding of 17β-estradiol to cytosolic ER-α receptors has recently been reported to mediate the activation of BK_{Ca} channels in cultured smooth muscle cells of human coronary artery [16]. However, in our study, in cell-attached configuration, 30 min after application of 17β-estradiol (10 μM) was found to have no effects on the activity of BK_{Ca} channels in HCFs. In continued presence of 17β-estradiol, a subsequent application of DPN (10 μM) or PPT (10 μM) significantly increased the probability of channel openings. In addition, in an inside-out configuration, when applied to the intracellular surface of excised patches, these two compounds also enhanced BK_{Ca}-channel activity, and in continued presence of DPN (3 μM), a subsequent application of PPT (3 μM) did not increase the probability of channel openings further. Taken

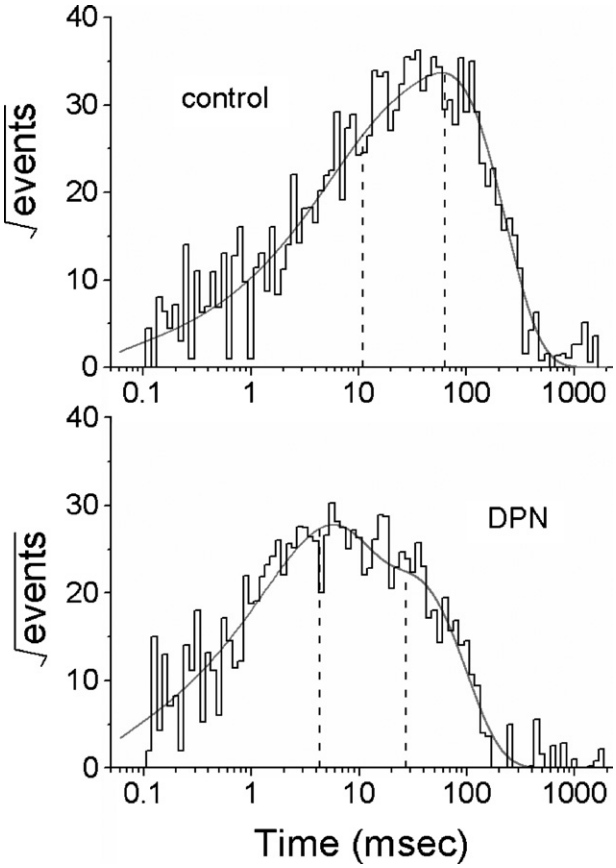

Fig. 7 – Effect of DPN on mean closed time of BK_{Ca}-channel in an HCF. Inside-out configuration was performed in these experiments, and the potential was held at +60 mV. Cells were bathed in symmetrical K^+ concentration (145 mM). DPN (3 μM) was added to the bath. Closed-time histogram obtained in the control (upper) and during the exposure to 3 μM DPN (lower) is shown, respectively. The dashed lines shown in each lifetime distribution are placed at the value of the time constant. The abscissa and ordinate show the logarithm of mean closed times and the square root of the number of event, respectively.

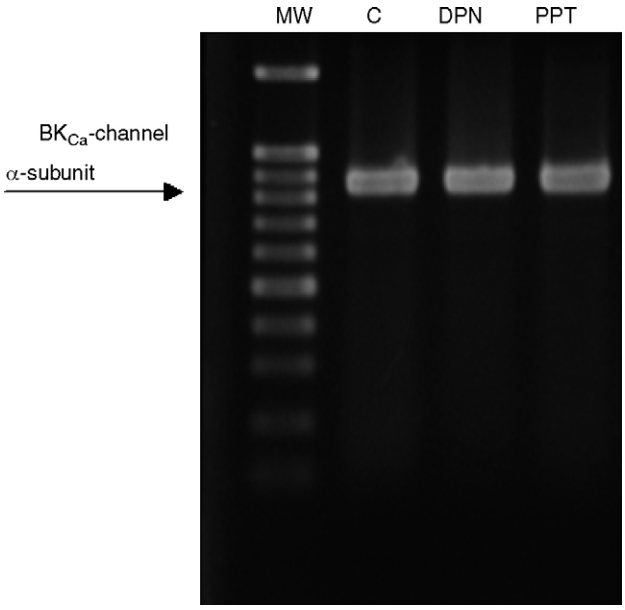

Fig. 8 – Results of RT-PCR for BK_{Ca}-channel α-subunit mRNA in HCFs obtained in the presence and absence of DPN (10 μM) or PPT (10 μM). The BK_{Ca}-channel α-subunit mRNA was present in HCFs. The densitometry level of RT-PCR for α-subunits mRNA in the presence of DPN or PPT did not differ from control (C).

together, the results led us to suggest that the stimulatory effects of these two compounds are unlikely to be mediated by the bindings to cytosolic ER-α or ER-β.

A recent study showed that the ER-α located on the cell membrane of vascular myocytes was a potential target for treatment of coronary artery diseases [16]. In addition, the ER-α isoform was proposed to mediate estrogen-induced activation of BK_{Ca} channels in coronary smooth myocytes [16]. For this reason, with the aid of the LALIGN program (http://fasta.bioch.virginia.edu/fasta/lalign.htm), we have further examined the similarity of amino acid sequence among BK_{Ca} α-subunit, ER-α and ER-β. The major portion of BK_{Ca} α-subunit (human; Q12791), to which the sequence of ER-α (40% identity) and ER-β (43% identity) shares the similarity, was found to be located at 643–652 (RILINPGNHL), which is the S8–S9 region. Thus, it is tempting to speculate that these two agents can bind to this region to activate the activity of BK_{Ca} channels, although no experimental evidence for direct activation of the BK_{Ca} channel caused by a direct interaction of these compounds with the channel proteins was provided in the present study. Interestingly, this region is also found to overlaps with either heme-binding or tetramerization domain in the BK_{Ca}-channel. Heme was previously found to bind to *Slo*1 BK_{Ca} channels, thereby suppressing the activity of these channels [26]. Hence, it is possible that these compounds modulate I_K by interacting with the ion channel protein itself or by acting at lipid sites near the channels after their partition in the lipid bilayer. As a result, the activity of BK_{Ca}-channel could be increased within 1 min after addition of DPN or PPT to the bath. The BK_{Ca}-channel is likely to be an important target molecule of these compounds via a non-genomic mechanism, although these findings still need to be verified in other types of cells or tissues. Nevertheless, these structurally-related agents are likely to share the same binding site occurring in ER-α, ER-β and BK_{Ca}-channel.

The single-channel conductance of BK_{Ca} channels was 167 ± 8 pS (N = 7), a value that is similar to that of typical BK_{Ca} channels in other types of cells [12,18,23,24], but different from the small-conductance Ca^{2+}-activated K^+ channel described in H9c2 myoblasts or in rat cardiomyocytes [27,28]. In addition, no change in the gating charge in the presence of these two compounds makes it unlikely that they act on the voltage sensor (S4) of the BK_{Ca}-channel. In our study, no modification of single-channel conductance in the presence of these compounds suggests that their binding sites should not be located in the pore region of the channel. Furthermore, despite the presence of a heterogeneous population of cardiac fibroblasts occurs, with the aid of RT-PCR, the expression of BK_{Ca}-channel α-subunits could be

detected in HCFs irrespective of passage number. The mRNA level of BK_{Ca}-channel α-subunit was not altered by the treatment with either DPN or PPT. Taken together, the electrophysiological, pharmacological and molecular analyses presented here suggest that the DPN- or PPT-stimulated activity of BK_{Ca}-channel may occur through a non-genomic mechanism.

The present results also demonstrate that the stimulatory effect of DPN and resveratrol on the amplitude of I_K is not additive. Resveratrol has been previously reported as an opener of BK_{Ca} channels in endothelial cells [23]. These two components, which are structurally related, may interact with the same binding motif in the channel. In addition, the Ca^{2+} binding is considered to allosterically modulate the voltage-dependent activation [29]. In our study, intracellular Ca^{2+} measurements in HCFs revealed little or no change of $[Ca^{2+}]_i$ in the presence of DPN or PPT (data not shown). These compounds were not found to modify Ca^{2+} sensitivity of BK_{Ca} channels. The results suggest that the increase by these two compounds in the open probability of BK_{Ca} channels in HCFs involves an mechanism independent of the level of $[Ca^{2+}]_i$. The rapid stimulatory effect of these two compounds on BK_{Ca} channels may have potential implications in the regulation of cellular function linked to a non-genomic mechanism.

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