Resveratrol inhibits Kv2.2 currents through the estrogen receptor GPR30-mediated PKC pathway

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Submitted 20 May 2013; accepted in final form 21 June 2013

Dong WH, Chen JC, He YL, Xu JJ, Mei YA. Resveratrol inhibits Kv2.2 currents through the estrogen receptor GPR30-mediated PKC pathway. Am J Physiol Cell Physiol 305: C547–C557, 2013. First published June 26, 2013; doi:10.1152/ajpcell.00146.2013.—Resveratrol (REV) is a naturally occurring phytoalexin that inhibits neuronal K+ channels; however, the molecular mechanisms behind the effects of REV and the relevant α-subunit are not well defined. With the use of patch-clamp technique, cultured cerebellar granule cells, and HEK-293 cells transfected with the Kv2.2 and Kv2.2 α-subunits, we investigated the effect of REV on Kv2.1 and Kv2.2 α-subunits. Our data demonstrated that REV significantly suppressed Kv2.2 but not Kv2.1 currents with a fast, reversible, and mildly concentration-dependent manner and shifted the activation or inactivation curve of Kv2.2 channels. Activating or inhibiting the cAMP/PKA pathway did not abolish the inhibition of Kv2.2 current by REV. In contrast, activation of PKC with phorbol 12-myristate 13-acetate mimicked the inhibitory effect of REV on Kv2.2 by modifying the activation or inactivation properties of Kv2.2 channels and eliminated any further inhibition by REV. PKC and PKC-α inhibitor completely eliminated the REV-induced inhibition of Kv2.2. Moreover, the effect of REV on Kv2.2 was reduced by preincubation with antagonists of GPR30 receptor and shRNA for GPR30 receptor. Western blotting results indicated that the levels of PKC-α and PKC-β were significantly increased in response to REV application. Our data reveal, for the first time, that REV inhibited Kv2.2 currents through PKC-dependent pathways and a nongenomic action of the oestrogen receptor GPR30.

resveratrol; Kv2.2; GPR30; PKC

RESVERATROL [trans-3‘,4‘,5‘-trihydroxystilbene (REV)] is a naturally occurring phytochemical compound that is found in >70 plant species (11). Plants synthesize REV to protect against bacterial and fungal infections, stress, and injury (2, 25). Accumulating evidence indicates that REV mediates a wide range of biological activities, including increasing life span through anti-ischemic, anticancer, antiaging, and anti-inflammatory properties (23, 35, 37). Although the chemical structure of REV indicates its antioxidant actions, previous studies suggest that REV can modify a wide variety of enzymes and signaling molecules (6, 7, 42), increases total cellular zinc (47), and blocks GLUT1-mediated hexose uptake HL-60 in U937 leukemic cell lines (38). In addition, it has been suggested that REV binds to the estrogen receptor (ER; Ref. 14).

Recent studies have shown that REV has an important protective effect on the nervous system (31, 36). In mammalian neurons, REV was reported to delay axonal degeneration after injury (1), block accumulation of Aβ-peptide in vitro (17), and provide protection from brain ischemia. Furthermore, REV has been reported to provide maximal neuroprotection (41) and prevent the progression of acute seizures into chronic epilepsy and cognitive dysfunction (39). Despite these clear neuronal effects, the molecular mechanisms underlying the neuroprotective and excitatory activities of REV have not been conclusively determined.

It is well established that voltage-gated K+ (Kv) channels determine neuronal action potential frequency, control the strength of synaptic contacts between neurons (20), and play an important role in the regulation of neuronal apoptosis and survival (45). This is especially true for the outward delayed rectifier (IK) channels, due to their high K+ conductance and their non- or slow-inactivation behavior that occurs during membrane depolarization (32). Thus the Kv channels are considered as therapeutic targets (44). Using the primary cultured rat hippocampal neurons, Gao and Hu (13) demonstrated that application of REV could reversibly inhibit IK channels. Furthermore, Gao and Hu (13) used extracellular recording techniques in hippocampal slices to demonstrate that REV inhibits neuronal discharges in the rat hippocampal CA1 area. In addition, REV suppresses epileptiform discharges in slices (12), suggesting that the K+ channel is involved in REV-induced effects on neuronal protection and excitability. However, the relevant Kvα subunit and the molecular mechanism of REV-induced modulation on IK channels is not well defined.

Previous studies indicated that neuronal IK channels are composed of α-subunits from the Kv2 subfamily and that they are particularly important in regulating neuronal excitability and survival (16). It was also reported that the Kv2.1 subunit, which is highly expressed in most mammalian central neurons, is a major contributor to IK channels and plays a crucial role in regulating neuronal excitability (8, 28). Recently, the Kv2.2 channel was detected in brain areas; in addition, it maintains high frequency action potential firing (21, 22) and was modulated by the antidepressant citalopram (46). In our present study, whole cell patch-clamp recordings of HEK-293 cells transfected with Kv2.1 and Kv2.2 α-subunits were used to examine the mechanistic effects of REV on the Kvα subunit of IK channels.

METHODS

Primary cultures of rat cerebellar granule neurons. Sprague-Dawley female rats were purchased from the Laboratory Animal Center of Shanghai at the Chinese Academy of Sciences (Shanghai, China). The cells were derived from the cerebellum of 7-day-old pups as described previously (15). Isolated cells were plated onto 35-mm Petri dishes coated with poly-L-lysine (1 µg/ml) at a density of 106 cells/ml. Cultured cells were incubated at 37°C under 5% CO2 in DMEM supplemented with 10% fetal calf serum, insulin (5 µg/ml), KCl (25 mM), and a 1% antibiotic-antimycotic solution. After 24 h in
culture, cytosine β-D-arabinofuranoside (5 μM) was added to the culture medium to inhibit the proliferation of nonneuronal cells. The experiments were performed using primary cerebellar granule neurons (cNGNs) after 5–7 days in culture.

**DNA construction and cell transfection.** Total RNA was isolated from primary cultured rat cortical neurons according to the manufacturer’s instructions (Qiagen Mini RNeasy; Qiagen, Valencia, CA). First-strand synthesis was performed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Amplification was performed with the following primer sets: primer K2.2: forward, 5′-CCCGCTCGG ATGGCAGAAGGACACC TCCCTGGCTTGA-3′ (Xhol); reverse, 5′-GGAGATCCC GATGCTGTCGTTCA CAGATGGTGGG-3′ (EcorI; GenBank NM_054000). Primer K2.1: forward, 5′-TGGCTG CAGATGCCGGCGC CGGCCGATG 3′ (Xhol); and reverse, 5′-ATACAGAATCTGATGACTC TGGATCCCT 3′ (EcorI; GenBank NM_008420). Rat Kv2.2 cDNA or Kv2.1 cDNA was ligated into pEGFPN1 by using Xhol and EcoRI restriction sites. Each gene was fused to the NHEK terminus of enhanced green fluorescent protein to enable detection of the transfected human embryonic kidney (HEK) 293 cells. All the constructs were verified by sequencing. The plasmids were extracted using a Qiagen plasmid midi kit (Qiagen). The DNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm. HEK-293 cells were transfected using Lipofectamine 2000 (Invitrogen). The average transfection efficiency was >80%. Two days after transfection, HEK-293 cells with green fluorescence were further analyzed.

**Patch-clamp recordings.** Whole cell currents of HEK-293 cells were recorded using a patch-clamp technique. All currents were recorded using an Axopatch 200B amplifier (Axon Instrument, Foster City, CA) operated in voltage-clamp mode. A Pentium computer was connected to the recording equipment with a Digidata 1300 analog-to-digital (A/D) interface. The current was digitally sampled at 100 μs (10 kHz). The current signals were filtered by a 3-kHz, three-pole Bessel filter. Currents were corrected online for leak and residual capacitance transients with a P/4 protocol and current amplitude was analyzed by calculating the mean of the data points from 130 to 200 ms of a 200-ms pulse. This range was chosen because the Ikh amplitude was stable within this range. Data acquisition and analysis were performed with pClamp 8.01 software (Axon Instruments) and Origin7.5 (Microcal analysis software, Northampton, MA). Before Ikh current channel recording, the culture medium was replaced with a bath solution containing 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, and 1 mM MgCl2. Soft glass recording pipettes were filled with an internal solution containing 135 mM K gluconate, 10 mM KCl, 10 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, 10 mM EGTA, and 2 mM ATP (pH adjusted to 7.3 using KOH). The pipette resistance was 5–7 MΩ after addition of the internal solution. All recordings were performed at room temperature (23–25°C).

**Western blot analysis of PKC.** Cells were lysed in HEPES-NP40 lysis buffer (20 mM HEPES, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 2 mM EDTA, 100 μM Na2VO4, 50 mM NaF pH 7.5, and 1% proteinase inhibitor cocktail) on ice for 30 min. After centrifugation, the supernatant was mixed with 2× sodium dodecyl sulphate loading buffer and boiled for 5 min. The proteins were separated on a 10% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, MA). The membrane was blocked with 10% nonfat milk and incubated at 4°C overnight with two types of rabbit polyclonal antibodies against the phosphorylated PKC-α and PKC-β or PKC-δ and PKC-θ (1:4,000; Cell Signaling Technology, MA) or the mouse monoclonal antibody against GAPDH (1:10,000). After being extensively washed in TBS Tween, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:10,000; KangChen Bio-Tech) for 1 h at room temperature. Chemiluminescent signals were generated using a SuperSignal West Pico kit (Pierce) and detected by exposure to X-ray film or using a ChemiDoc XRS System (Bio-Rad Laboratories).

RNA interference knockdown of GPR30. The plasmid used to silence the GPR30 receptor was constructed using the hU6/MCS/CMV/GFP/SV40/Neomycin vector (Genechem, Shanghai, China). The small interfering (si)RNA sense oligonucleotide sequences were as follows: GPR30 GGG UGA AGC GCC UCA GUU Auu. As a negative control, a random shRNA sequence was inserted into the hU6/MCS/CMV/GFP/SV40/Neo vector. Transfection was accomplished using Lipofectamine 2000 (Invitrogen) and Opti-mem media (GIBCO, Grand Island, NY) according to the manufacturer’s instructions.

**Quantitative RT-PCR assays.** To measure GPR30 mRNA levels following treatment of cells with RNAi, quantitative real-time PCR analysis was performed using primer pairs with the following sequences: GPR30 forward primer, 5′-CGTGCTCTTACACACTCT-3′ and reverse primer, 5′-GACGCGAGATGTGGTAGA-3′. To control for sampling errors, quantitative real-time PCR was conducted on the housekeeping gene cyclophilin D using primer pairs with the following sequences: forward primer, 5′-AAGGGCAGCTGAGCCGCAA-3′ and reverse primer, 5′-GCCATGCTGACCAACC-3′. This analysis was performed routinely on each sample and used as a control. The reaction solution consisted of 2.0 μl of diluted RT-PCR product, 0.2 μM of each primer pair, and Power SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). The annealing temperature was set at 58°C for GPR30 and 62°C for cyclophilin D, and the number of amplification cycles was set at 38 cycles. The absolute mRNA levels in each sample were calculated according to a standard curve that was constructed using serial dilutions of known amounts of specific templates and the corresponding cycle threshold (Ct) values. The results are displayed as the normalized ratio of the target gene over cyclophilin D. The specificity of the primers was verified by both agarose gel electrophoresis and sequencing of the PCR products.

**Statistical analysis.** Statistical analysis was performed using Student’s t-test with nonpaired comparisons or paired comparisons when appropriate. Values are expressed as the mean ± SE, with n as the number of cells tested. P < 0.05 was used to denote the significant difference between groups. When multiple comparisons were made, data were analyzed by a one-way ANOVA test.

**Chemicals.** REV, G15, phorbol 12-myristate 13-acetate (PMA), forskolin, cytineisole-β-arabinofuranoside, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indololo[2,3-alpypyrrolol[3,4-c]barbazole (Gö6976), guanosine 5′-[thio]triphosphate (GTP§S), cyclohexadine, Rp-cyclic 3′,5′-hydrogen phosphorothioate adenosine triethylammonium salt (Rp-cAMP), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulphonamide (H-89), poly-l-lysine, and DEME were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies against phosphorylated PKC were purchased from Cell Signaling Technology. Fetal calf serum, heat inactivated horse serum, and antibiotic-antimycotic solutions were obtained from Gibco Life Technologies. REV solutions were prepared thoroughly and gravity ejected for 1 to 2 min from MSC-200 Manual Solution Changer (Bio-Logic-Science Instruments). It was first dissolved in DMSO and then diluted in the bath with a final DMSO concentration <0.1%. The 0.1% of DMSO did not produce the significantly effect on K2,2 currents.

The drug was ejected by gravity for 40–60 s from MSC-200 Manual Solution Changer (Bio-Logic-Science Instruments). We tested the exact dilution rate by perfusion of a high concentration potassium solution. The exact dilution rate and the time for the drug to reach maximal concentration around the cell have been evaluated from the resting membrane potential shift recorded in response to perfusion of 50 mM K+ solution. By using Goldman-Hodgkin-Katz equation, we figured out that the drug was diluted ~3.4 times after it got to the cell surface, and the time for the drug to reach maximal concentration around the cell was 3 s.
RESULTS

It was previously demonstrated that REV could reversibly inhibit $I_K$ channels in primary cultured rat hippocampal neurons (13). Therefore, we first confirmed the effect of REV on $I_K$ channels with primary cultured CGNs that possess a rich $I_K$. The $I_K$ was evoked by 160-ms depolarizing pulses to $+50$ mV from holding potentials of $-80$ mV (Fig. 1A). The REV was ejected by gravity for 30 s, and the drug was diluted $\sim 3.4$ times after it got to the cell surface, and the time for the drug to reach maximal concentration around the cell was 3 s (see METHODS). Application of REV significantly inhibited $I_K$, and the REV-induced inhibition of $I_K$ was $50.5 \pm 2.0\%$ ($n = 6$), $46.0 \pm 1.8\%$ ($n = 8$), and $51.6 \pm 2.2\%$ ($n = 5$) in the presence of 10, 30, and $100 \mu M$ of REV, respectively (Fig. 1A). There were no significant differences among these three concentrations of REV. Next, experiments were performed with HEK-293 cells transfected with a pEGFPN1 vector encoding either the Kv2.1 or Kv2.2 $\alpha$-subunit. After a 48-h transfection, cells with green fluorescence were used for recordings. Figure 1, B and C, shows typical current recordings in the cells transfected with Kv2.1 and Kv2.2, respectively. A concentration of $30 \mu M$ REV significantly decreased the amplitude of the Kv2.2 current ($29.2 \pm 1.71\%$, $n = 5$) but only inhibited Kv2.1 current by $8.62 \pm 0.38\%$ ($n = 6$; Fig. 2C). These results indicate that REV selectively modulates Kv2.2 currents.

Thus we next investigated the mechanism of REV on Kv2.2 currents. Figure 2A shows that the inhibitory effect of REV on Kv2.2 was reversible and mildly concentration-dependent (Fig. 2A). REV at concentrations of $1 \mu M$ ($n = 5$) or $5 \mu M$ ($n = 6$) did not significantly inhibit Kv2.2 ($P > 0.05$ by Student’s $t$-test). In contrast, $10 \mu M$, and $100 \mu M$ REV inhibited Kv2.2 by $28.36 \pm 2.69\%$ ($n = 12$), $29.2 \pm 1.73\%$ ($n = 5$), and $33.47 \pm 2.08\%$ ($n = 8$), respectively (Fig. 2B). The magnitude of Kv2.2 inhibition was not significantly different between the three concentrations ($P > 0.05$, by one-way ANOVA). When the REV concentration was increased to $500 \mu M$, the inhibition of Kv2.2 by REV was $56.8 \pm 2.93\%$ ($n = 5$). Because the solubility of REV is low at concentrations $>500 \mu M$, higher REV concentrations were not tested. We also examined the effect of DMSO on Kv2.2 because REV was dissolved in 1% DMSO as the carrier solvent. Figure 2C shows typical current recordings and confirms that 1% DMSO did not affect the REV concentration was increased to $500 \mu M$, the inhibition of Kv2.2 by REV was $56.8 \pm 2.93\%$ ($n = 5$). Because the solubility of REV is low at concentrations $>500 \mu M$, higher REV concentrations were not tested. We also examined the effect of DMSO on Kv2.2 because REV was dissolved in 1% DMSO as the carrier solvent. Figure 2C shows typical current recordings and confirms that 1% DMSO did not affect the

A

B

C

Fig. 1. Effect of resveratrol (REV) on the outward delayed rectifier ($I_K$) of cerebellar granule neurons (CGNs) and Kv2.1 or Kv2.2 current in HEK-293 cells transfected with pEGFPN1 vector encoding the Kv2.1 or Kv2.2 $\alpha$-subunits. A: current traces recorded from CGNs in the absence and presence of multiple concentrations of REV. The current was evoked by a depolarizing pulse from $-80$ to $50$ mV. Statistical analysis of the REV-induced inhibitory effect with different concentrations on the $I_K$ of CGNs was obtained from 5–8 neurons ($^{*}P < 0.05$, compared with corresponding control without REV). B: time course of changes in Kv2.1 amplitudes induced by $30 \mu M$ REV. Insets: superimposed Kv2.1 traces from the initial control levels (after establishment of the whole cell configuration) and after external infusion of REV. Time points (1, 2, and 3) noted on the curves correspond to the superimposed Kv2.1 traces illustrated by the insets. C: time course of the changes in Kv2.2 amplitudes induced by REV. Kv2.1 and Kv2.2 currents were evoked by a depolarizing pulse from $-80$ to $50$ mV.
throughout the activation voltage range when the depolarizing pulse was >0 mV (Fig. 3B). We obtained the $I_K$ activation curve by plotting the normalized conductance as a function of the command potential. Data were then analyzed using the equation $G_K = I_K/(V_m - V_{rev})$, where $G_K$ is the membrane K⁺ conductance, $V_m$ is the membrane potential, and $V_{rev}$ is the reversal potential for K⁺. As shown in Fig. 3C, the activation curve shifted slightly to the left in the presence of REV. The current was half-activated at 15.4 ± 1.3 and 10.14 ± 1.7 mV in the absence and presence of 30 μM REV, respectively ($n = 14$; $P < 0.05$, using a paired Student’s $t$-test). These data suggest that REV treatment can partially modify the voltage dependence of Kv2.2 channel steady-state activation.

To study the effects of REV on steady-state inactivation of the Kv2.2 channel, cells were held at −80 mV and a 1-s conditioning prepulse from −60 to +60 mV in 10-mV increments was applied. The conditioning prepulse was returned to −80 mV for 20 ms before the test pulse to +60 mV (Fig. 4A).
Steady-state inactivation curves of Kv2.2 current were obtained by peak current amplitudes plotted against the prepulse potential (Fig. 4B). After normalizing each current peak to the maximal current amplitude obtained from the −80-mV prepulses as a function of the conditioning prepulse potential, we obtained an inactivation curve of I_K and calculated the Vh 50 (the voltage at which the current amplitude was half-inactivated). As shown in Fig. 4C, the inactivation curve shifted to the right by the application of REV. The half-maximal inactivation voltage obtained was 17.1 ± 2.7 and 23.7 ± 2.2 mV in the absence and presence of 30 μM REV, respectively (n = 11; P < 0.05, using a paired Student’s t-test), suggesting that REV treatment partially modified the voltage dependence of Kv2.2 channel steady-state inactivation.

Our previous study indicated that Kv2.1 was significantly modified by the cAMP/PKA pathway (19). Thus we studied the effects of REV on Kv2.2 current in the presence of forskolin, an adenylate cyclase activator, or Rp-cAMP, a specific inhibitor of cAMP-dependent protein kinase (PKA) activation. Perfusion of cells with 20 μM forskolin or 10 μM Rp-cAMP alone did not affect Kv2.2 current amplitude; furthermore, in the presence of these compounds, REV still significantly inhibited Kv2.2 currents by 31.96 ± 1.68% (n = 5) and 29.56 ± 1.44% (n = 5), respectively (Fig. 5, A and B). Likewise, when the selective PKA antagonist H-89 was added into the patch pipette solution, 30 μM REV inhibited Kv2.2 currents by 29.28 ± 2.0% (n = 5), indicating that the PKA pathway could not abolish the inhibitory effects of REV on Kv2.2 current (Fig. 5, C and D). Our results suggest that the cAMP/PKA pathway does not contribute to REV-mediated inhibition of Kv2.2 currents.

We next studied the effects of REV on Kv2.2 current in the presence of a PKC activator or inhibitor to address whether the PKC pathway was involved in REV-mediated inhibition of Kv2.2 currents. Perfusion of cells with 50 μM PMA, an activator of PKC, in the bath solution provoked a gradual decrease in Kv2.2 current amplitude (28.6 ± 0.82%, n = 5) similar to that produced by REV. Moreover, in the presence of PMA, the REV-induced inhibition of Kv2.2 current was reduced to 1.2 ± 1.3% (Fig. 6A). REV-induced inhibition of Kv2.2 current was reduced to 3.76 ± 0.86% (n = 5) when 10 μM bisindolylmaleimide (Bis), a broad-spectrum PKC inhibitor, was used in the pipette solution (Fig. 6B). Gö6976, a selective PKCα antagonist, was used to explore the role of this PKC isoform in the inhibitory effect of REV on Kv2.2 currents. Figure 6C shows that 30 μM REV inhibited Kv2.2 currents by 2.48 ± 1.2% (n = 6) in the presence of 1 μM Gö6976 in the pipette solution, indicating that PKC-α is involved in the REV-induced inhibitory effects on Kv2.2 currents. Statistical analysis of these results (Fig. 6D) suggests that inhibition of the PKC pathway suppressed the REV-induced Kv2.2 current decrease. In addition, the steady-state activation and inactivation properties of Kv2.2 channels in the presence of PMA were similar to those observed following treatment with REV. After addition of PMA (50 μM) to the bath solution, the steady-state activation curve was shifted to the left, from 15.48 ± 1.35 to

![Image](http://ajpcell.physiology.org/)

**Fig. 4.** REV alters the steady-state inactivation of Kv2.2 currents. A: Kv2.2 currents were recorded in the absence (top) or presence (bottom) of 30 μM REV. Cells were held at −80 mV and then a 1-s conditioning prepulse from −60 to +60 mV in 10-mV increments was applied. Conditioning prepulse was returned to −80 mV for 20 ms before the test pulse at +60 mV. Voltage protocol is shown below the current recordings. B: steady-state inactivation curves of Kv2.2 current in the absence or presence of REV. Abscissa shows the conditioning prepulse potential. C: peak current amplitudes normalized to the maximal currents was plotted against the prepulse potential. Normalized current values were fitted to a Boltzmann function. Steady-state inactivation curve was significantly shifted by REV towards the hyperpolarization potential. Data shown are the means ± SE from 11 cells.
5.0 ± 1.4 mV (n = 14), and the inactivation curve was shifted to the right, from 17.07 ± 2.7 to 23.75 ± 1.12 mV (n = 12; Fig. 6, E and F).

It was previously reported that REV may act as a ligand of steroid receptors to activate intracellular signal transduction. In addition, GPR30 is an oestrogen-receptor involved in the rapid actions elicited by estrogen (26). Thus we sought to identify whether the GPR30 receptor might mediate the REV-induced inhibition of K_2,2 currents. Application of G15, GPR30 receptor antagonist, abolished the REV-induced inhibitory effect on the K_2,2 current (Fig. 7A). In the presence of 1 μM G15, the inhibition of K_2,2 produced by REV was reduced to 1.4 ± 0.92% (n = 5) and was significantly different from the effect induced by REV alone (Fig. 7D). GPR30 receptors are G protein-coupled receptors that mediate multiple signal transduction pathways (10). This pathway was tested by adding GTPγS (10 μM) to the pipette solution. Internal infusion of GTPγS (10 μM) resulted in the gradual suppression of K_2,2 current amplitude to 29.31 ± 1.02% (n = 6) after membrane rupture (Fig. 7B). Moreover, following the reduction in K_2,2 current by internal application of GTPγS, REV induced average reductions in K_2,2 current of 1.29 ± 0.84% (n = 6). E2, an oestrogen that activates the ER, modulated the K_2,2 current. The data obtained from five cells indicated that 10 μM E2 mimicked REV and reduced the K_2,2 current by 33.69 ± 1.87% (Fig. 7, C and D). We also tested if the HEK-293 cells expressed the GPR30 receptor with PCR techniques. Figure 7E shows that GPR30 mRNA was detected with PCR in HEK-293 cells. These data suggest that the GPR30 receptor is required for REV-induced inhibition of K_2,2 current.

To further ascertain whether GPR30 mediates the REV-induced inhibition of K_2,2 currents, shRNA-mediated knockdown of GPR30 expression was performed. Concurrently, the efficiency of the RNAi knockdown of GPR30 was determined in HEK-293 cells, which endogenously express this receptor. Quantitative RT-PCR data showed that transfection of GPR30 shRNA significantly reduced GPR30 mRNA expression in the HEK-293 cells (Fig. 8A). Moreover, after knockdown of GPR30 expression, the effect of REV on K_2,2 was attenuated, while the current amplitude in untreated cells was not significantly different at 97.26 ± 3.68% (n = 7) of the control (Fig. 8B). Statistical analysis showed that the effect of REV following RNAi knockdown of GPR30 was significantly different from that induced by REV alone (Fig. 8C). This result supports the role of GPR30 in the modulation of Kv2.2 currents by REV.

Lastly, we confirmed that the PKC signaling pathway was activated by REV and the ER by measuring the levels of phosphorylation of PKC (pPKC) with two types of antibodies, one specific for PKC-α and PKC-β and another selective for PKC-δ and PKC-θ. Western blotting showed that phosphorylation of PKC-α and PKC-β was significantly increased by REV. After treatment of cells with REV for 2, 5, and 10 min, the levels of pPKC-α and pPKC-β relative to untreated control were increased by 18.18 ± 1.24, 36.63 ± 2.42, and 36.88 ± 2.41%, respectively. Moreover, the effect of PKC-α and PKC-β activation by REV was inhibited by G15. In the presence of G15 for 5 min, REV-induced pPKC levels were decreased to 0.8 ± 1.06% (n = 3) and significantly different from the effect induced by REV alone when compared with untreated controls (Fig. 9A). Consistent with the current recording, the level of pPKC-α and pPKC-β activated by REV could be enhanced by E2 (Fig. 9B). In contrast, the levels of phosphorylation of PKC-δ and PKC-θ were not affected by

Fig. 5. cAMP/PKA pathways did not mediate REV-induced inhibition of K_2,2 current. 

**A-C**: time course of changes in K_2,2 current amplitudes induced by 30 μM REV in the presence of extracellular 20 μM forskolin (A); intracellular 10 μM Rp-cAMP (B), and intracellular 10 μM H-89 (C). D: statistical analysis of the effects of forskolin, Rp-cAMP, and H-89 on K_2,2 currents and REV-induced inhibition of K_2,2 currents. Data shown are means ± SE from 6–7 cells (*P < 0.05 vs. control).
REV (Fig. 9C). After treatment of cells with 30 or 100 μM REV, the levels of pPKC-β and pPKC-α were lightly increased by 3.46 ± 0.03 and 4.87 ± 0.04% (n = 3), without significant different from the untreated controls.

DISCUSSION

Although REV exists as both cis- and trans-isomeric forms, the trans-isomer is the stable form of REV that plays a role in nearly all of its biological actions (9, 11, 13, 17). Therefore, we studied only the effect of the trans-isomer of REV.

Previous studies associated with ion channel regulation indicated that REV modified ion channels by different mechanisms that were dependent on the cell model used. In human umbilical vein endothelial cells, REV significantly activated large conductance Ca2+ (BK) channels in both whole cell and inside-out configurations (6). However, REV has a concentration-dependent inhibitory effect on I\textsubscript{K} channels in primary cultured rat hippocampal neurons and L-type Ca2+ channels in A7r5 vascular myocytes (5, 13). Alternatively, the antiallo- dynic action of REV was associated the nitric oxide-cyclic GMP-protein kinase G-K channel (3). Although these data indicated the effect of REV on multiple channels, the molecular mechanisms of this compound are not well clarified. Our data presented here indicate that REV inhibits Kv2.2 α-subunits, the main contributor of the I\textsubscript{K} channel, through the nongenomic actions of the oestrogen receptor and by activating the PKC pathway.

REV was reported to be a phytoestrogen, and it binds to the oestrogen receptor and activates transcription in different test systems (14). Recently, an orphan G protein-coupled receptor, GPR30 was identified as a new estrogen-receptor involved in the rapid actions elicited by oestrogen (26). In our study, REV-induced inhibition of Kv2.2 currents occurred rapidly, suggesting that this was a nongenomic effect that occurred independently of gene transcription. However, this effect might result from the activation of intracellular signaling pathways.

Fig. 6. PKC pathway mediates REV-induced inhibition of K\textsubscript{v}2.2 current. A: time course of changes in K\textsubscript{v}2.2 current amplitudes induced by 30 μM REV in the presence of extracellular phorbol 12-myristate 13-acetate (PMA). A concentration of 20 μM PMA provoked a gradual decrease in K\textsubscript{v}2.2 current amplitude and abolished the effect of 30 μM REV on K\textsubscript{v}2.2 current. B and C: time course of the changes in K\textsubscript{v}2.2 current amplitudes induced by REV in the presence of intracellular 20 μM bisindolylmaleimide (Bis; B) and 10 μM G06972 (C). D: statistical analysis of the effects of PMA, Bis, and G06972 on K\textsubscript{v}2.2 current and REV-induced K\textsubscript{v}2.2 current inhibition. Data shown are the means ± SE from 5–9 cells (*P < 0.05 vs. control without REV; #P < 0.05 vs. the REV-induced inhibitory of K\textsubscript{v}2.2 without Bis or G06972). E and F: K\textsubscript{v}2.2 voltage-dependent activation and inactivation curves in the absence or presence of PMA. Data points were fitted using the Boltzmann function. Data shown are the means ± SE from 14 and 12 cells.

Even though the α-subunits of K\textsubscript{v}2.2 and K\textsubscript{v}2.1 channels share many properties, previous studies suggest the K\textsubscript{v}2.1 α-subunit is more biologically relevant based on its function, modulation, and phosphorylation sites (19, 27, 33, 48) when compared with the K\textsubscript{v}2.2 α-subunit (46). Although recognition of K\textsubscript{v}2.2 functionality has substantially increased in the last several years, previous studies mainly focused on the subunit contribution to the formation of the action potential (21, 22). We report for the first time that K\textsubscript{v}2.2 can be inhibited by REV through the surface membrane receptor of the oestrogen and that it can be significantly modified by the PKC pathway rather than the PKA pathway. Moreover, it is highly likely that the neuroprotective activities and neuron excitability of REV (39, 43) may be due to oestrogen receptor activation and Kv2.2 current inhibition. Thus our data have important significance for the recognition of the neurobiological role of REV and for the understanding of K\textsubscript{v}2.2 channel functioning and modulation.
Quantitative RT-PCR data indicated that GPR30 was expressed in HEK-293 cells. Despite this, Filardo et al. (10) used HEK-293 cells overexpressing GPR30 to permit detection of GPR30 at the cell surface using confocal microscopy. In this study, pharmacological antagonists and shRNA-mediated knockdown of GPR30 completely abolished the effect of REV on Kv2.2 currents, suggesting that GPR30 activation is required for REV-induced Kv2.2 inhibition. However, we also observed that treatment with pharmacological antagonists or ER knockdown in HEK-293 cells interfered with the REV-induced

Fig. 7. Effects of GPR30 receptor antagonists and GTPyS on REV inhibition of Kv2.2 current. A: time course of changes in Kv2.2 current amplitudes induced by REV in the presence of 1 µM G15, an antagonist of GPR30 (B), in the bath solution. B: time course of changes in Kv2.2 current amplitudes induced by REV in the presence of 10 µM GTPyS in the pipette solution. C: time course of changes in Kv2.2 current amplitudes induced by the application of 10 µM E2 in the bath solution. A concentration of 10 µM E2 mimicked the effect of REV inhibition of Kv2.2 current. D: statistical analysis of the effects of G15, GTPyS, and E2 on Kv2.2 current amplitudes and REV-induced Kv2.2 current inhibition. Data shown are the means ± SE from 6–7 cells (*P < 0.05 vs. corresponding control; #P < 0.05 vs. REV treatment). E: GPR30 mRNA expression in HEK-293 cells.

Fig. 8. Effects of GPR30 short hairpin (sh)RNA on REV-induced inhibition of Kv2.2 currents. A: effect of GPR30 shRNA on GPR30 mRNA levels in HEK-293 cells. Cells were transfected with empty vector as a negative control. Data were obtained from 3 independent experiments (*P < 0.05 vs. the untransfected control). B: time course of the change in Kv2.2 current amplitude induced by REV in cells transfected with GPR30 shRNA. Kv2.2 currents were recorded from cells exhibiting green fluorescence that had been transfected with shRNA against the GPR30. Cells exhibiting red fluorescence had been transfected with Kv2.2. C: statistical analysis of the effects of shRNA-mediated GPR30 knockdown on REV-induced Kv2.2 current inhibition. Data shown are the means ± SE from 8 cells (*P < 0.05 vs. corresponding control, #P < 0.05 vs. REV in control, which had not been transfected with shRNA).
levels measured in the presence of 30 and 100 μM REV in human umbilical vein endothelial cells (6), in contrast to previous reports of the effect of REV on BK channel activity. PKC activity is known to be involved in the modulation of ion channels (34), suggesting that REV might activate or inhibit PKC activity. However, our results are in contrast to the report by Han et al. (17) that describes a significant increase in PKC phosphorylation after treatment of hippocampal neurons with trans-REV. In the present study, we provide evidence that REV blocked Kv2.2 channels by activating PKC, a result similar to that observed in A7r5 vascular myocytes (5).

Interestingly, our data indicated that REV-induced inhibition of Kv2.2 currents and activation of PKC required a threshold REV concentration of 10 μM. Moreover, the inhibitory effect of REV was not significantly different between the concentrations of 10 and 100 μM. This effect is similar to that observed for REV-induced inhibition of Ik in rat CGNs. Our results are in contrast to previous reports of the effect of REV on BK channels in human umbilical vein endothelial cells (6), and L-type Ca2+ channels in primary cultured rat hippocampal neurons (13), and L-type Ca2+ channels in A7r5 vascular myocytes (5).

However, Chen et al. (6) showed that REV could activate BK channels in inside-out patches and that blockade of the oestrogen receptor with tamoxifen had no effect on REV-stimulated BK channel activity. The role of GPR30 in REV-induced inhibition of Ik in hippocampal neurons and of L-type Ca2+ channels in A7r5 vascular myocytes has not been elucidated. The diverse effects of REV might reflect variations in its mechanism of action. It is possible that REV-induced Kv2.2 inhibition in HEK-293 cells involves activation of GPR30 receptors rather than direct channel blockade. Differences in the effect of REV might also arise due to the differential action of REV on cell surface vs. intracellular transmembrane GPR30 receptors.

Another interesting finding is that REV only modulated Kv2.2 and not Kv2.1. Previous reports indicated Kv2.1 possesses a 68 score in the cytoplasmic COOH terminus as a strong consensus phosphorylation site (27, 33) modified by the cAMP/PKA pathway (19, 24) but not PKC. However, activating or inhibiting cAMP/PKA alone did not modify Kv2.2 current; moreover, activating PKC reduced Kv2.2 current implying that the channels might be phosphorylated. A study by Blaine et al. (4) using chimeric transcripts between Kv2.1 and Kv2.2 showed that a carboxyl tail domain, proxC, is found in Kv2.1. ProxC is produced from Kv2.1 in developmental post-translational modifications. Interestingly, the primary sequence of proxC predicts consensus sites for PKC, casein kinase 2, and tyrosine phosphorylation (4). Consistent with this hypothesis, REV can significantly stimulate PKC activity but does not alter PKA activity (data not shown); indeed, this may be the reason why REV significantly inhibited the Kv2.2 current rather than Kv2.1 current.

Direct evidence, such as crystal structure analysis or proxC mutation, will be necessary to support this hypothesis.
Our present study provides the first evidence that REV inhibits K\textsubscript{v}2.2 current, a component of the I\textsubscript{K} channel in rat CGNs and cortical neurons. We showed also that REV promoted PKC activation through GPR30 receptors. Although some recent studies have revealed the potential neuroprotective and neuroexcitability action of REV, the relevance of our findings to this function needs to be demonstrated in future studies.

REFERENCES


