Rotenone induces K_{ATP} channel opening in PC12 cells in association with the expression of tyrosine hydroxylase

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Abstract. The activation of ATP-sensitive potassium (K_{ATP}) channels in PC12 cells play a pivotal role in protection against the neurotoxic effect of rotenone. However, it remains unclear why rotenone seems to preferentially affect activation of K_{ATP} channels and if this could affect its physiological activity. In this study, we sought to determine how the different energy states caused by various doses of rotenone affect the K_{ATP} opening state and whether the K_{ATP} opening state influences the expression of tyrosine hydroxylase (TH) which is related with DA synthesis. With patch clamp technology, results showed that treatment of PC12 cells with rotenone (0.2-1 μg/ml) for 15 min can cause K_{ATP} channel opening with significantly increased intracellular ROS production. Treatment with rotenone (2-16 ng/ml) for 24 h also caused the channels to open with gently increased ROS. In order to study if the rather long-term action on K_{ATP} channel opening states could affect the specified function of PC12 cells, the K_{ATP} channel opener pinacidil and the inhibitor glibenclamide were used to treat cells for 24 h, and the expression of TH was detected. Our results showed that treatment of PC12 cells with glibenclamide for 24 h can notably promote TH expression and can also enhance the expression of TH which were reduced by rotenone. These data indicate that the energy states in PC12 induced by various doses of rotenone could significantly influence the opening states of K_{ATP} channels. However long-term energy stress may raise the opening rate and opening sensitivity of this channel. In addition, our results demonstrate for the first time that activation of plasma membrane K_{ATP} channels induced by rotenone inhibits TH expression which influences DA synthesis in PC12 cells.

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Abbreviations: PC12, pheochromocytoma cell line; PI, propidium iodide; TH, tyrosine hydroxylase; K_{ATP} channel, ATP sensitive potassium channel; PD, Parkinson's disease; DA, dopamine; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; Mn (III) TBAP, Mn (III) tetrakis (4-benzoic acid) porphrin chloride

Key words: pheochromocytoma cell line, ATP sensitive potassium channel, patch clamp, rotenone, tyrosine hydroxylase

Introduction

Dopaminergic neurons play a crucial role in a variety of brain functions such as voluntary movement which is severely affected in Parkinson's disease (PD), a common neurodegenerative movement disorder. Metabolic stress has been identified as an important trigger factor for the neurodegenerative process of PD. In particular, it has been consistently found that the activity of mitochondrial respiratory chain complex I (CXI) is reduced 40% in PD patients. In this context of metabolic dysfunction in PD, ATP-sensitive potassium (K_{ATP}) channels are of special interest, because their open probability directly depends on the metabolic state of a cell (1). K_{ATP} channels are closed at high ATP-to-ADP ratios and open in response to decreased ATP and increased ADP levels. By this mechanism, K_{ATP} channel activity exerts a powerful control mechanism of cellular excitability and affect the cell's physiologic activity. However, for the common form of sporadic PD, it remain unclear if the metabolic dysfunction could contributed to its development through K_{ATP} channel.

Rotenone, pesticide and toxin that inhibit complex I, results in selective DA degeneration (1-3). Studies *in vivo* have shown that chronic, systemic administration of rotenone produces dopaminergic degeneration and Lewy body-like cytoplasmic inclusions, which closely mimic the pathology of PD (1). Rotenone treatment also functions as an effective PD model *in vitro*, resulting in toxicity to dopaminergic cells (4). Partial inhibition of complex I by rotenone has been shown to increase mitochondrial production of ROS (5,6), which may be the precipatory event in toxicity models. However, the basis for rotenone-induced selective toxicity to dopaminergic neurons remains ambiguous.

Recent evidence suggests that the increased oxidative stress within dopaminergic neurons, due to dopamine (DA) metabolism and oxidation, combined with a complex I inhibition-induced ROS production may lead to cell death by overloading the oxidative capacity of dopaminergic cells. Liss *et al* suggest that the K_{ATP} channels is related with the selective death of dopaminergic cells in the midbrain, but the mechanism remained unclear (7). Consequently, we wanted to know what is the relationship between DA metabolism and the

K_{ATP} channels. We supposed that rotenone, as complex I inhibition in PD model, affect DA metabolism and oxidation, which associate with K_{ATP} channel activity and then contribute to the dysfunction of the dopaminergic cells. Therefore, in this study we sought to investigate whether tyrosine hydroxylase (TH), the rate-limiting enzyme (8) of DA synthesis, was involved in open probability of K_{ATP} channel in PC12 cells induced by rotenone. We found that in PC12 cells rotenone treatment enhanced K_{ATP} channel opening and decreased the expression of TH which could be inversed by the K_{ATP} channel inhibitor glibenclamide. Thus, the present study delivers important new insights into the molecular pathways that may contribute to one of pathological mechanism on dopaminergic degeneration in dopaminergic neurons.

Materials and methods

Materials. PC12 cells were obtained from the Cell Bank in the Chinese Academy of Sciences (Shanghai, China). DMEM/F12 culture medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (Rockville, MD, USA); RNA extraction reagent (TRIzol), RNA reverse transcription reagent and PCR expansion reagent kit were from Takara Biotechnology Co., Ltd. (Dalian, China). Protein lysis buffer, rabbit anti-β-actin were from CST Co. (Danvers, MA, USA). Rabbit anti-TH was from Millipore Co. (Billerica, MA, USA). Goat anti-Kir6.2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ATP Assay kit was from Beyotime Institute of Biotechnology (Jiangsu, China). Mn (III) TBAP was from Biosense Laboratories AS (Bergen, Norway). All the other chemicals, including rotenone, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), lipophilic cationic dye JC-1, PI and fluorescent Ca^{2+}-indicator dye were obtained from Sigma (St. Louis, MO, USA).

Cell culture and treatment. PC12 cell line were cultured in a DMEM/F12 medium containing 10% inactivated FBS, 100 U/ml penicillium and 100 mg/l streptomycin at the conditions of 37˚C and 5% CO_2. Pancreatin (0.125%) was used to digest for passage. Logarithmic phase cells were used for all the experiments. For the drug treatment, the cells were inoculated in a culture plate or a culture flask for 12 h, and then added with the corresponding drugs, while the blank control group was only added with the culture solution of the same volume.

Cell viability measurement. Cell viability was assessed by the MTT assay (9). Briefly, PC12 cells were seeded in 96-well plates ($2x10^3$ cells/well) and then the rotenone treatment was administered. After treatment, cells were washed with PBS and incubated with MTT (5 mg/ml) in culture medium at 37˚C for another 3 h. Then formazan blue, which formed in cells, was solubilized in 100 μl of DMSO. The absorption values were measured at a wavelength of 490 nm using a Sunrise Remote Microplate Reader (Grodlg, Austria). The viability of PC12 cells in each well was presented as the percentage of control cells.

Confocal fluorescence microscopy on Kir6.2 co-localization. After cells were cultured in culture dishes for 24 h, they were washed with ice-cold PBS and fixed in PBS-buffered 4% paraformaldehyde at room temperature for 20 min. Then, cells were washed with PBS and blocked with 10% horse serum for 10 min and incubated overnight with the goat anti-Kir6.2 antibodies (1:100) at 4˚C. Thereafter, the cells were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat IgG at 37˚C for 1 h, PI for 1 min. Finally, cells were observed under a confocal fluorescence-microscope system (TCS SP-2, Leica).

Detection of the K_{ATP} opening by patch clamp technique. The treated PC12 cells were digested by 0.125% pancreatin until deformed. Then the cells were washed and resuspend with the extracellular solution, and dripped on the slides. Within 20 min, the cells sunk and stuck to the slides and were prepared for patch clamp experiments. The whole-cell recording mode was used to detect the K_{ATP} opening state with patch clamp amplifier EPC-10 (HEKA, Germany) and the micropipette puller PUL-100 (WPI, Worcester, MA, USA). Cells of middle size and with very smooth outline were used. The intracellular solution (potassium gluconate 140 mM, KCl 10 mM, $MgCl_2$ 5 mM, EGTA 0.5 mM, ATP 0.5 mM, HEPES 10 mM, pH 7.2 with Tris-OH) was used. While the extracellular solution included NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1 mM, glucose 10 mM, HEPES 10 mM, $CaCl_2$ 2 mM, pH 7.4 (with Tris-OH) (10). All experiments were performed at room temperature. The pClamp 6.01 procedure was used to collect and analyze the data.

Intracellular calcium ion, ROS, ATP detection. When detecting the alteration of intracellular Ca^{2+} concentration in PC12 cells induced by rotenone, the cells were treated in the same way as described above and added with the Fluo-3-AM probe (75 mM) for 1 h at 37˚C, then the cells were collected and the fluorescence intensity detection was conducted by a FACSVantage SE flow cytometer at an excitation wavelength of 355 nm and an emission wavelength of 485 nm.

When intracellular ROS production in PC12 cells induced by rotenone was detected, the cells were washed by PBS and fluorescent probe DCFH-DA diluted with serum-free culture medium (1:5,000) and added in culture medium. After being cultured for 1 h, the cells were collected and the fluorescence intensity detection was conducted by flow cytometry.

The alteration of intracellular ATP concentration in PC12 cells treated with rotenone was detected by the method of luciferase bioluminescent according to the kit instruction of the manufacturer and tested by the Lmax II Luminometer (Molecular Devices, Sunnyvale, CA, USA).

Cell culture medium pH detection. The pH of cell culture medium was measured at room temperature by pH meter (pHS-3D, Shanghai, China).

Mitochondrial transmembrane potential detection by flow cytometry. The changes of mitochondrial transmembrane potential in PC12 cells were detected by flow cytometry. Briefly, the cells were stained with JC-1 (20 μg/ml) for 20 min at 37˚C. The JC-1 fluorescence intensity on cells was measured by a FACSVantage SE flow cytometer with an excitation wavelength of 490 nm and an emission wavelength of 527 nm.

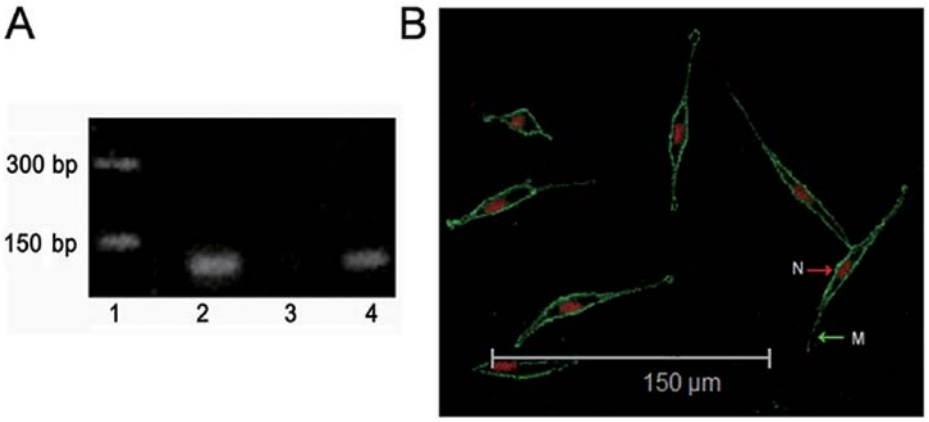

Figure 1. K_{ATP} channel subunit Kir6.2 expressed in PC12 cell line. (A) Electrophoregram of Kir6.2 mRNA PCR products (1, marker; 2, rat cardiac tissues; 3, rat intestine tissues; 4, PC12 cell line). (B) PC12 Kir6.2 cellular immunofluorescence image by laser confocal microscopy (N, cell nucleus by PI; M, cell outer membrane).

Preparations of RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from $1x10^6$ treated PC12 cells using cold TRIzol reagent and RNA (500 ng) was used to RT-PCR with RT reagent Kit according to the manufacturer's protocol. The primers of Kir6.2 used for PCR were: sense, 5'-CCGCCAGCTTGATGAGGAC-3', and antisense, 5'-GGACCGCAACTCAGGACAAG-3', with product of 146 bp. The PCR amplification method to detect Kir6.2 among the samples was set as follows: pre-denatured at 94˚C for 4 min, 35 cycles x 30 sec at 94˚C, 30 sec at 55˚C and 30 sec at 72˚C. PCR products were detected by 2% agarose gel electrophoresis. TH and β-actin mRNA were detected by qRT-PCR with the following primers: for TH: sense, 5'-AGGGCTGCTGTCTTCCTAC-3' and antisense, 5'-GCTGTGTCTGGGTCAAAGG-3'; for β-actin: sense, 5'-AGGCCAACCGTGAAAAGATG-3' and antisense, 5'-ACCAGAGGCATACAGGGACAA-3'; with the product of 81 and 88 bp, respectively. The reaction system contains 5 μl SYBR-Green Mix, sense and antisense primers each 30 μM, cDNA 1 μl, and RNA-free H_2O supplied to 10 μl. Real-time PCR parameters were: 3 min at 95˚C, 35 cycles x 10 sec at 95˚C, 20 sec at 55˚C, then 65-95˚C for dissolved curve. The amount of TH mRNA was normalized by β-actin mRNA levels as the endogenous reference and relative to the control is then given by $2^{-\Delta\Delta Ct}$.

TH protein expression by western blot analysis. The treated cells were collected and broken down by CST Lysis Buffer (including 10% Ser/Thr inhibitor, 10% Tyr inhibitor and 10% PMSF), then centrifuged in 12,000 x g for 15 min at 4˚C, the supernatant was collected, 5X protein loading buffer was added and boiled for 10 min. After SDS-PAGE, the proteins were transferred to the PVDF membrane. Membranes were washed in water 2 times, and blocked with 5% BSA which contained 0.05% Tween-20 for 1 h, then incubated overnight in primary antibody (diluted with 5% BSA of 1:2,000) at 4˚C. Membranes were washed with TBST 3 times, and then incubated in the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (diluted with 5% BSA of 1:3,000) for 1 h at ambient temperature. The ECL chemiluminescent method was used to observe the results and then analyzed in the ChemDoc imager (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Each batch of experiments were repeated for at least 3-5 times. Statistical analyses were performed using the SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± SD of 3-5 independent experiments. ANOVA and Student's t-test were performed to determine the statistical significance. Differences between groups were considered to be significant at $P<0.05$.

Results

Functional K_{ATP} channels exist on the outer membrane of the PC12 cells. The results on functional K_{ATP} localization on PC12 is contradictory, some researchers suggest functional K_{ATP} exist on the outer membrane of the PC12, but others showed contrary results (11-13). Therefore, we first ascertained whether functional K_{ATP} channels exist in PC12 cells. In neural and dopaminergic cells the K_{ATP} channel usually is transcribed by SUR1/Kir6.2 gene which is important for the physiology of dopaminergic cells (14,15), we detected Kir6.2 expressed at mRNA levels on PC12 cells by RT-PCR methods.

While we detected the expression of Kir6.2 mRNA in PC12 cells, as a positive or negative control, we also tested the expression of Kir6.2 in rat cardiac muscle and small intestine (16). Results in Fig. 1A show that Kir6.2 was expressed in PC12 cells and rat cardiac tissues. To confirm this result, we assessed K_{ATP} channel colocalization on PC12 cells with Kir6.2 antibody by laser confocal microscopy. The expression of Kir6.2 protein on the outer membrane of the PC12 cells were observed (Fig. 1B). Both results indicated that K_{ATP} channels exist in PC12 cells.

To determine whether these K_{ATP} channels worked on PC12 cells, whole cell patch clamp technology was implemented to detect opening state of K_{ATP} channels with K_{ATP} channel selective opener, pinacidil, and the selective inhibitor, glibenclamide. Results showed that the outward current induced by pinacidil is non-voltage dependent and can be inhibited by glibenclamide (Fig. 2). This is a typical characteristic of functional K_{ATP} channel (10,17).

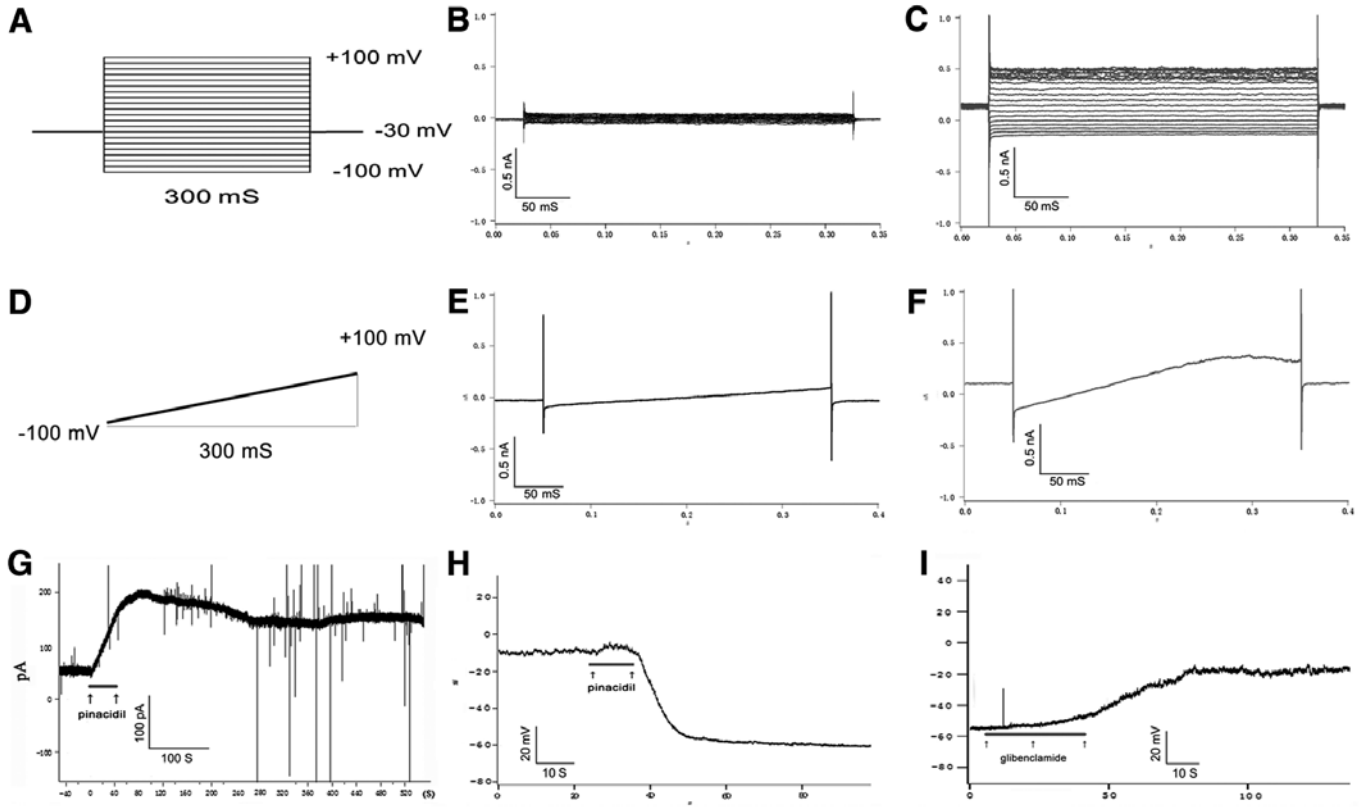

Figure 2. Identification of the PC12 K_{ATP} channel by whole-cell recording method of patch clamp technology. (A-C) Current responses to the voltage-clamp steps between -100 to +100 mV at a holding potential of -70 mV (in 10-mV increments) with 300 ms duration. (A) Protocol, (B) without pinacidil, (C) with pinacidil 100 nM. (D and F) Current responses to the voltage-clamp ramps between -100 to +100 mV at a holding potential of -30 mV with 300 ms duration. (D) Protocol, (E) without pinacidil, (F) with pinacidil 100 nM. (G) A representative trace of the membrane current at -30 mV with pinacidil 100 nM. (H and I) Continuous current clamp recording of the membrane potential; (H) with pinacidil 100 nM; (I) with hyperpolarzation induced by pinacidil 100 nM and then glibenclamide 20 μM was added.

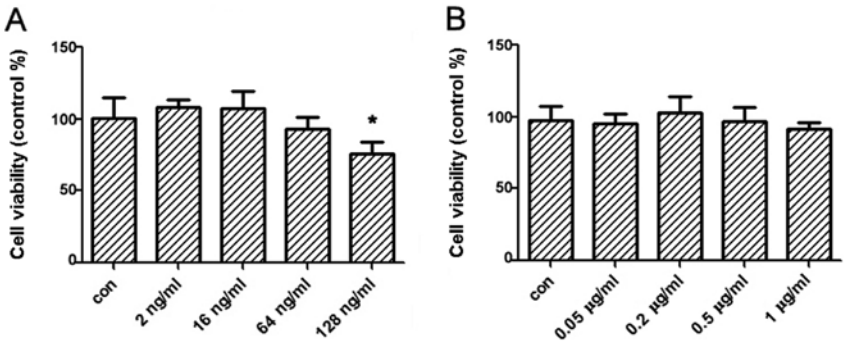

Figure 3. Effects of rotenone on PC12 cell viability by MTT method. (A) Rotenone (2-128 ng/ml) treated for 24 h. (B) Rotenone (0.05-1 μg/ml) treated for 1 h. *P<0.05 vs. the control group.

Effect of acute and chronic rotenone treatment on the cell vitality of PC12. There are various states of energic stress such as acute and chronic energic disturbance in compensated or decompensated manner in dopaminergic neurons. To understand whether various energic states influence activity of K_{ATP} channels, the treatment with different doses of rotenone on PC12 cells was implemented. Therefore, we firstly determined the concentration of rotenone treated on PC12 cells by MTT assay so that the direct toxicity on cells by rotenone was excluded. Results showed that the concentration of rotenone ≤1 μg/ml for 1-h treatment and 64 ng/ml for 24-h treatment had no effect on the cell vitality. Therefore, the suitable dose of rotenone treatment on PC12 cells was selected in further experiments (Fig. 3).

K_{ATP} channel opening state and cell energy state affected by acute rotenone treatment for 15 min. The ROS increase and ATP decrease occurred and aggravated the time of rotenone treatment (18) were the important factors to affect the states of the K_{ATP} channel, whole cell patch clamp was used to record the opening state affected by the energic state induced by rotenone and the recorded on the opening state

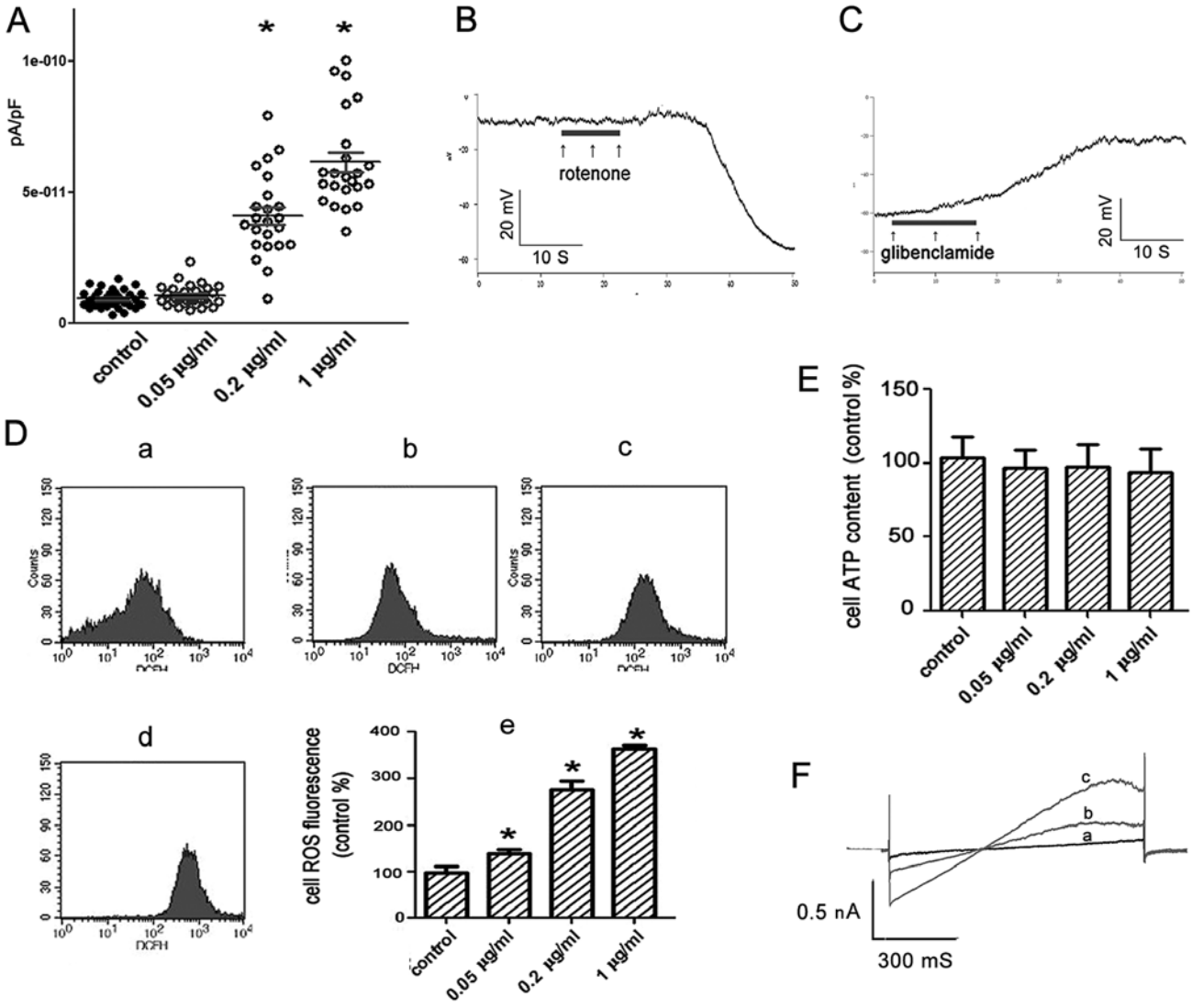

Figure 4. K_{ATP} channel opening states and physiologic states of PC12 cells treated by rotenone for 15 min. (A) Patch-clamp whole cell recording of the currents by voltage-clamp steps at depolarization of +60 mV under the rotenone treatment (n>20). Control, 9.39E-12±3.39E-12 pA/pF; 0.05 μg/ml, 1.06E-11±3.97E-12 pA/pF; 0.2 μg/ml, 4.11E-11±1.60E-11 pA/pF; 1 μg/ml, 6.15E-11±1.82E-11 pA/pF. (B and C) Continuous current clamp recording of the membrane potential. (B) Treated with rotenone 1 μg/ml, (C) with hyperpolarization induced by rotenone, glibenclamide 20 μM was added. (D) ROS level of the cells treated by rotenone and analyzed by flow cytometry. a, control; b, 0.05 μg/ml; c, 0.2 μg/ml; d, 1 μg/ml; e, general trend (control, 100±12%; 0.05 μg/ml, 137±9%; 0.2 μg/ml, 275±18%; 1 μg/ml, 363±8%). (E) ATP level of the cells treated by rotenone and analyzed by luciferase bioluminescent method. (F) Effects of Mn (III) TBAP on the voltage-clamp ramps responded currents induced by rotenone, n=3; (a, rotenone 1 μg/ml + Mn (III) TBAP 30 μM; b, rotenone 1 μg/ml + Mn (III) TBAP 10 μM; c, rotenone 1 μg/ml). *P<0.05 vs. the control group.

of K_{ATP} channels were completed within 15 min after the adding of the rotenone in this parts of the study. Results showed that treatment with various doses of rotenone (0.05-1 μg/ml) on PC12 cells elicited outward current in a dose-dependent manner (Fig. 4A). This outward current was inhibited by glibenclamide, the specific K_{ATP} inhibitor, which are commonly used to identify the current of K_{ATP} opening (Fig. 4B and C).

To understand what factors did affect the opening of K_{ATP} channel under this circumstance, we investigated the intracellular ATP and ROS concentration after treatment with rotenone on PC12 cells for 15 min. Results showed that treatment with rotenone markedly increased intracellular ROS production in a dose-dependent manner (Fig. 4D). The concentration of intracellular ATP was not obviously decreased (Fig. 4E). Consequently, one of the reasons for the opening of the K_{ATP} was the increased ROS production in PC12 cells induced by rotenone.

To confirm this assumption, PC12 cells were treated with both rotenone (1 μg/ml) and a superoxide dismutase mimetic Mn (III) TBAP (30 μM). We found that Mn (III) TBAP (30 μM) markedly prevented rotenone-induced currents on PC12 cells (Fig. 4F). Therefore, results suggest that the rotenone-induced currents was mainly caused by the increased ROS production on PC12 cells treated with rotenone for 15 min.

K_{ATP} channel opening state and cell energy state affected by chronic rotenone treatment for 24 h. To explore K_{ATP} channel opening states induced by rotenone for 24 h, PC12 cells were measured by whole cell patch clamp after treatment with various doses of rotenone (2-64 ng/ml) for 24 h. Results showed treatment with rotenone in low concentration (2 and

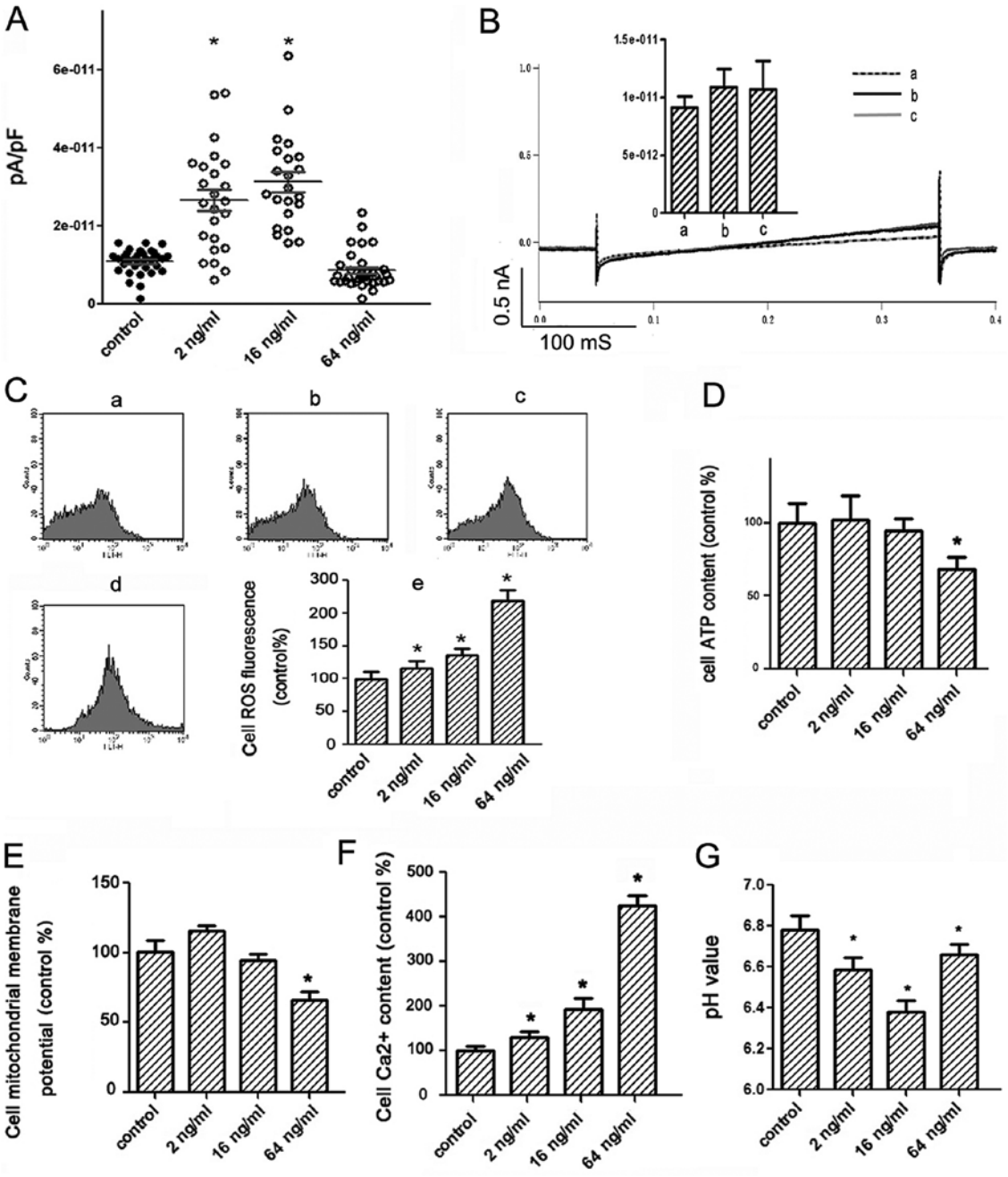

Figure 5. K_{ATP} channel opening states and physiologic states of PC12 cells treated by rotenone (2-64 ng/ml) for 24 h. (A) Patch-clamp whole cell recording of the currents by voltage-clamp steps at depolarization of +60 mV (n>20). Control, 1.09E-11±3.19E-12 pA/pF; 2 ng/ml, 2.65E-11±1.32E-11 pA/pF; 16 ng/ml, 3.12E-11±1.17E-11 pA/pF; 64 ng/ml, 8.54E-12±4.80E-12 pA/pF. (B) Patch clamp testing the K_{ATP} channel function of the cells which had been treated by rotenone (64 ng/ml) for 24 h with the channel opener (currents responded to voltage-clamp ramps were recorded and the data for the bars were the currents (pA/pF) recorded at 250 ms), n=3 (a, without channel opener; b, with rotenone 1 μg/ml; c, with pinacidil 100 nM). (C) ROS levels analyzed by flow cytometry. a, control; b, rotenone 2 ng/ml; c, rotenone 16 ng/ml; d, rotenone 64 ng/ml; e, general trend. (D) ATP levels analyzed by luciferase bioluminescent method. (E) Mitochondrial transmembrane potential analyzed by flow cytometry. (F) Ca^{2+} level analyzed by flow cytometry. (G) pH value of the culture medium measured by pH meter. $^{*}P<0.05$ vs. the control group.

16 ng/ml) on PC12 cells elicited outward current. Since the outward current induced by the 2 and 16 ng/ml treatment can be inhibited by glibenclimade, it was considered as the current of K_{ATP} channel opening. However, treatment with rotenone in high concentration (64 ng/ml) did not alter outward current on PC12 cells (Fig. 5A). Then, we further added the K_{ATP} opener pinacidil (100 nM) or another high dose of rotenone (1 μg/ml) in the PC12 cells treated with rotenone (64 ng/ml), opening of K_{ATP} channels was not observed (Fig. 5B). It was demonstrated that the inhibition of K_{ATP} channels by rotenone for long-term treatment (24 h) was not reversed by pinacidil.

To investigate which is the main reason for K_{ATP} channel opening states, we then measured the ROS and the ATP levels on PC12 cells induced by rotenone. Results showed that treatment with various dose rotenone (2-64 ng/ml) led to increase in ROS production on PC12 cells in a concentration-dependent manner (Fig. 5C). The alteration of ATP concentration on PC12 cells induced by rotenone in

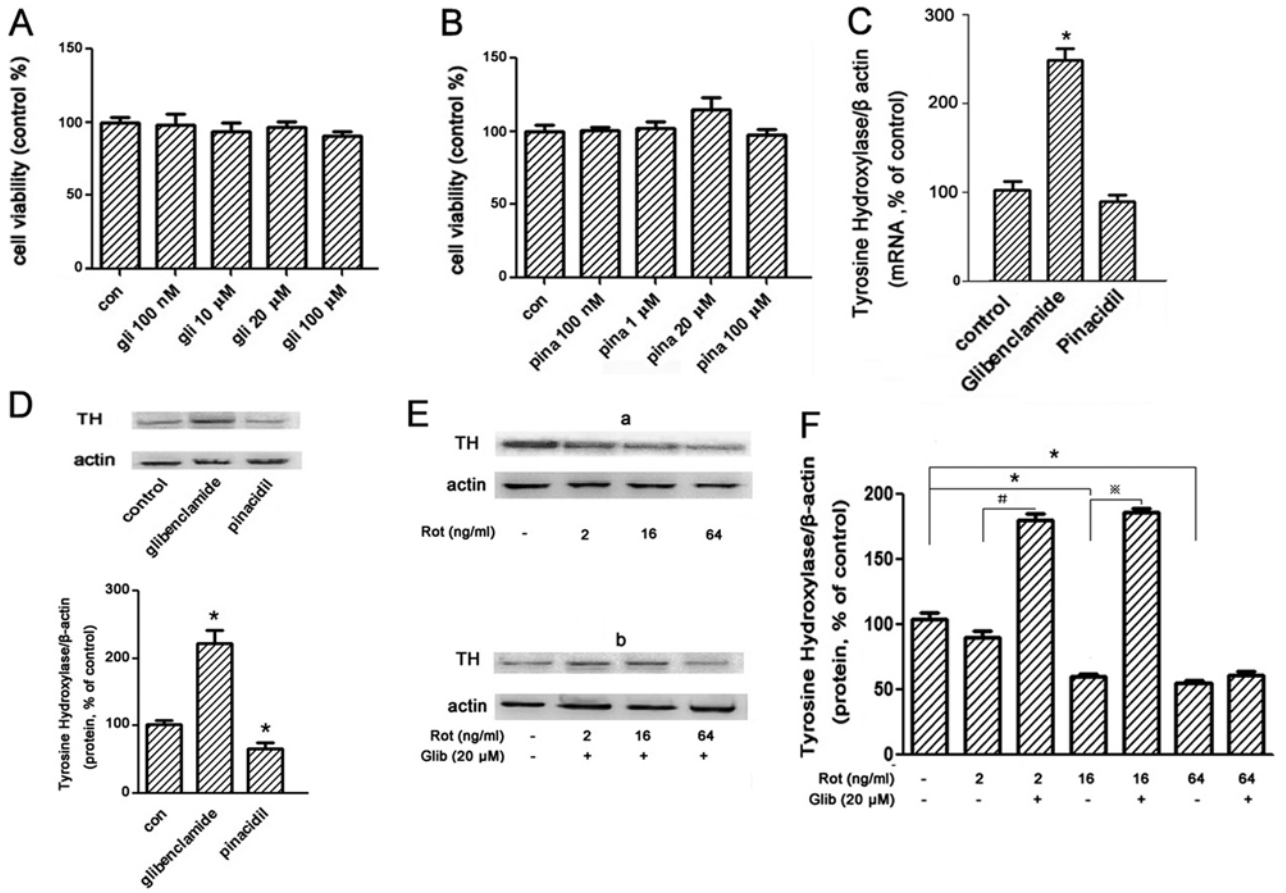

Figure 6. Effect on TH expression of PC12 cells by the K_{ATP} channel chemicals or rotenone treatment for 24 h. (A and B) Viabilities of the cells measured by MTT method with the treatment of pinacidil (A) and glimbenclamide (B) for 24 h (con, control; gli, glibenclamide; pina, pinacidil). (C) TH mRNA expression of the cells treated by pinacidil 100 nM and glibenclamide 20 μM for 24 h and analyzed by qRT-PCR. (D and F) TH protein expression of the cells treated by chemicals for 24 h and analyzed by western blot analysis. (D) Pinacidil 100 nM and glibenclamide 20-μM treatment. (E) a, rotenone (2-64 ng/ml) treatment; b, rotenone (2-64 ng/ml) + glibenclamide 20 μM. (F) Quantitative analysis of (E). Rot, rotenone; Glib, glibenclamide. *P<0.05 vs. the control group, $^{\#}$P<0.05 vs. the rotenone (2 ng/ml) group, *P<0.05 vs. the rotenone (16 ng/ml) group.

low dose was not clearly observed. Treatment with rotenone in high dose (64 ng/ml) notably reduced ATP production on the cells (Fig. 5D).

The cellular calcium ion level and the mitochondrial membrane potentials could reflect the physiological states of cells. So, we tested them by flow cytometry. Results showed the mitochondrial membrane potentials were decreased and calcium ion levels were increased by treatment with rotenone in a dose-dependent manner, respectively (Fig. 5E and F).

When cells are under energic stress, glycolysis is promoted to produce more acidic metabolites. We measured the pH value of culture medium on PC12 cells treated with various dose rotenone. The pH value of culture medium on PC12 cells treated with low dose rotenone (2 and 16 ng/ml) was 6.59±0.06 and 6.38±0.05, respectively, which was lower than the control (6.78±0.07). However, pH value in culture medium on cells treated with high dose (64 ng/ml) rotenone was 6.66±0.05, which recovered near to the control level (Fig. 5G). Results shown that treatment with rotenone in high dose might cause more injury to PC12 cells which may have lost their metabolism compensation function and induce irreversible physiologic dysfunction. It is suggested that pH might be one of the important causes to affect open state of K_{ATP} channels.

Inhibition of K_{ATP} channel increased expression of TH. To determine if the opening states of K_{ATP} could affect the TH expression on PC12 cells, which could help us to understand the function of this channel in DA synthesis, K_{ATP} inhibitor glibenclamide and opener pinacidil were used to treat cells for 24 h to manipulate the opening state of this channel, then the TH expression was assayed. First, we tested the effect of these chemicals to the viability of PC12 cells by MTT assay to find the suitable concentration to be used in the follow experiment which would have no adverse effect. Results showed that it was best for both at ≤100 μM (Fig. 6A and B).

We then measured TH expression on PC12 cells treated with pinacidil 100 nM and glibenclamide 20 μM for 24 h, respectively. Results showed that TH expression at mRNA level increased notably (2.47-fold vs. control) by glibenclamide, whilst no alteration of TH expression was observed after treatment with pinacidil (Fig. 6C). But for TH expression at protein level measured by western blot analysis, treatment with glibenclamide increased expression 2.2-fold more than control and decreased 0.35-fold by pinacidil less than control (Fig. 6D). This suggests that treatment with glibenclamide (20 μM) for 24 h markedly promote the translation and expression of TH on PC12 cells.

Since rotenone leads to the opening of K_{ATP} channel on PC12 in our experiment, we next determined whether it can affect the TH expression. Results showed that treatment with various rotenone (2-64 ng/ml) 24 h decreased the TH expression in a dose-dependent manner on PC12 cells (Fig. 6Ea and F). But glibenclamide (20 μM) inhibited the decrease of TH expression by rotenone (Fig. 6Eb and F). This suggess that the TH expression reduced by rotenone related with the opening of K_{ATP} channel because it was notably inversed by the K_{ATP} inhibitor glibenclamide.

Discussion

In this study we tested the K_{ATP} channel on PC12 cells to explore how the different energic states caused by various doses of rotenone to affect the K_{ATP} opening state and whether the K_{ATP} opening state influences the expression of TH which is related with DA synthesis.

The PC12 cell line derived from the rat pheochromocytoma is considered close to the dopamine terminal neurons compared with other cell lines. It is often used as an *in vitro* model to study the physiology of central dopamine neurons. Recent evidence indicates that activation of K_{ATP} channels in PC12 cells confers protection against mitochondrial complex-I inhibition-induced cell death (19) and could have potential beneficial effects in Parkinson's disease. Further understanding of the mechanisms that underlie this interesting phenomenon may lead to the new insight for the treatment of neurodegenerative diseases. K_{ATP} channels are reportedly present in both plasma and mitochondrial membranes. In this study we sought to determine if the K_{ATP} channel in plasma membranes contributes to neuroprotection. Our data showed that PC12 cells distributed functional K_{ATP} channels on the plasma membranes which was discrepant in different research (11-13). The reason is that the cells in the different states and culture conditions may lead to different energic and redox states which affect expression and function of the K_{ATP} channel.

Our results suggest that treatment with rotenone in different doses could affect the K_{ATP} opening states differently. The treatment with various dose of rotenone (0.05-1 μg/ml) on PC12 cells within 15 min elicited outward current in dose-dependent manner which was inhibited by glibenclamide, the specific K_{ATP} inhibitor. In this circumstance, intracellular ROS increased by rotenone for 15 min was also observed and could be the main factor to cause the opening of this channel. In the rotenone treatment (2-64 ng/ml) for 24 h, cells with 2-16 ng/ml treatment with mild intracellular ROS increase could elicited outward current, while cells with 64-ng/ml treatment with more serious intracellular ROS increase could not. There is evidence that rotenone treatment could cause serious oxidative environment and exhaust the glutathione of dopaminergic cells (20,21), and this may cause the oxidation of the channel hydrosulfide. When the hydrosulfide of the channel was oxidized, it becomes closed (22). Therefore, we supposed that increasing lactic acid, ROS, might contribute to promote the opening of K_{ATP} channel (23). However, treatment with rotenone (64 ng/ml) for 24 h upregulated ROS production until overload, so that it led to inactivation of the K_{ATP} channel.

In PD patient, there is not only a serious decrease of the number of the dopaminergic cells, but also notably reduced dopamine synthesis in the remaining cells (8,24). We sought to determine the relationship between K_{ATP} channel and TH expression which is dopamine synthesis rate-limiting enzyme. We then correlated with detection of K_{ATP} channel subunits using specific antibodies and valuation of the presence of functional K_{ATP} channels in the plasma membrane of PC12 cells induced by rotenone using the patch-clamp technique. TH expression in PC12 cells induced by rotenone were valuated by means of western blot analysis. The overall results suggest that opening state of the K_{ATP} was related with the TH expression in PC12 cells, which would affect the synthesis of dopamine. Treatment with K_{ATP} inhibitor, glibenclamide, notably enhanced the TH expression in PC12 cells, but K_{ATP} channel opener, pinacidil, did not reduce TH expression markedly in cells. The reasons probably is that TH expression is low in the normal cells, and a part of the K_{ATP} channel of PC12 is usually quite open (about 40% of the cells). In addition, a previous study indicated that the inhibition of K_{ATP} may enhance the TH expression *in vivo*. They found that the use of glibenclimade after infarction promoted the myocardial TH expression and the sympathetic reinnervation, which is harmful under this circumstance (25), but may be a potential target for the PD therapy. The mechanism of the inhibition of the K_{ATP} that promoted the TH expression remains unclear. Because the inhibition of the K_{ATP} conduced to membrane depolarization, a consideration is the expression of the transcription factor Nurr1 might be enhanced by prolonged membrane depolarization, TH is a downstream gene of this transcription factor (26). TH expression can also be induced by the outer membrane depolarization (27). This may suggest the intimacy between the state of K_{ATP} and the occupation of dopamine turnover in these cells.

In conclusion, we have demonstrated for the first time that activation of plasma membrane K_{ATP} channels induced by rotenone inhibits TH expression which influence the DA synthesis in PC12 cells. Further elucidation of the elements up- and downstream of K_{ATP} channels may open novel therapeutic strategies for the treatment of various neurodegenerative diseases.

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