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TASK-1 Channel Promotes Hydrogen Peroxide Induced Apoptosis

Jihyun Yun, Seungtae Kim, and Hyoweon Bang

Department of Physiology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea

Hydrogen peroxide (H_2O_2) causes oxidative stress and is considered as an inducer of cell death in various tissues. Two-pore domain K^+ (K_{2P}) channels may mediate K^+ efflux during apoptotic volume decreases (AVD) in zygotes and in mouse embryos. In the present study, we sought to elucidate linkage between K_{2P} channels and cell death by H_2O_2. Thus K_{2P} channels (TASK-1, TASK-3, TREK-1, TREK-2) were stably transfected in HEK-293 cells, and cytotoxicity assay was preformed using cell counting kit-8 (CCK-8). Cell survival rates were calculated using the cytotoxicity assay data and dose-response curve was fitted to the H_2O_2 concentration. Ionic currents were recorded in cell-attached mode. The bath solution was the normal Ringer solution and the pipette solution was high K^+ solution. In HEK-293 cells expressing TREK-1, TREK-2, TASK-3, H_2O_2 induced cell death did not change in comparison to non-transfected HEK-293. In HEK-293 cells expressing TASK-1, however, dose-response curve was significantly shifted to the left. It means that H_2O_2 induced cell death was increased. In cell attached-mode recording, application of H_2O_2 (300 μM) increased activity of all K_{2P} channels. However, a low concentration of H_2O_2 (50 μM) increased only TASK-1 channel activity. These results indicate that TASK-1 might participate in K^+ efflux by H_2O_2 at low concentration, thereby inducing AVD.

Key Words: Hydrogen peroxide, Apoptosis, K_{2P} channels, TASK-1 channel

INTRODUCTION

Background K^+ conductances are major determinants of membrane resting potential and input resistance. Some kinds of background channels have been cloned and they are K^+ channel families structurally different from K_v, K_{Ca} and K_{ir} channels. They belong to the family of two pore (2P) K^+ channels with four transmembrane segments (TMS), two P domains, an extended M1P1 external loop (60~70 residues) and intracellular N- and C-termini (Lesage & Lazdunski, 1999).

These channels with two-pore domains (K_{2P} channels) are voltage-, calcium- and time-independent. They are relatively insensitive to classical potassium channel blockers such as tetraethylammonium (TEA), 4-aminopyridine (4-AP), Ba^{2+} and Cs^+ (Koh et al, 1992; Koyano et al, 1992; Shen et al, 1992). K_{2P} channels are regulated by numerous physical and chemical stimuli, including extracellular and intracellular pH, temperature, hypoxia, pressure, bioactive lipids, and neurotransmitters (Bang et al, 2000; Sirois et al, 2000; Bayliss et al, 2001; Maingret et al, 2001; Washburn et al, 2002). The regulation of these background K^+ channels alters the cellular excitability.

Apoptosis occurs in the most cell types and can be triggered by a variety of physiological and pathological stimuli. Oxidative stress activates apoptotic cell death pathway (Jacobson, 1996) and cell death is nearly always accompanied by cell shrinkage (Gomez-Angelats et al, 2000; Patel et al, 1998). Apoptotic volume decrease (AVD) precedes other better understood processes of apoptosis such as cytochrome c release, mitochondria membrane potential dissipation, caspase activation, and DNA fragmentation (Gomez-Angelats et al, 2000; Maeno et al, 2000; Patel et al, 1998; Trimarchi et al, 2000).

Hydrogen peroxide (H_2O_2), formed as a natural byproduct of enzymatic oxidase action, is an endogenous source of free radicals that contribute to the background level of cellular oxidative stress (Halliwell, 1992). Exogeneous H_2O_2 can elevate oxidative stress beyond the protective capacity of endogenous antioxidant defenses and can induce apoptosis and necrosis in cultured cortical neurons (Koh et al., 1995; Whittemore et al, 1995) and PC12 cell lines (Tong & Perez-Polo, 1996).

Several studies have also shown that K^+ efflux leads to a decrease in $[K^+]_i$ was implicated in the cell death. Conversely, cell death was found to be inhibited when K^+ efflux was inhibited (Bortner et al, 1997; Yu et al, 1997; Yu et al, 1999). Recently it has been shown that oxidative stress (200 μM H_2O_2) evokes rapid apoptotic volume decreases (AVD) and K^+ efflux from zygotes (Trimarchi et al, 2000), and that K_{2P} channels underlie K^+ efflux during AVD in mouse embryo (Trimarchi et al, 2002). Although K_{2P} channels have been shown to concern K^+ efflux by H_2O_2, a question of which K_{2P} channels participate in K^+ efflux remains to be determined.

In this study, we investigated the direct effects of H_2O_2 on K_{2P} channels and the effects of H_2O_2 on cell death in cell stably transfected with K_{2P} (TASK-1, TASK-3, TREK-1

Corresponding to: Hyoweon Bang, Department of Physiology, College of Medicine, Chung-Ang University, Heukseok-dong 221, Dongjak-gu, Seoul 156-756, South Korea. (Tel) 82-2-820-5650 (Fax) 82-2-817-7115, (E-mail) haena@cau.ac.kr

ABBREVIATIONS: K_{2P}, two-pore domain K^+; AVD, apoptotic volume decrease; TREK, TWIK-related K^+; TASK, TWIK-related Acid-Sensitive K^+; MAP, mitogen-activated protein.

and TREK-2) channels. The increase of cell death by H_2O_2 was found in only TASK-1 transfected cells. In addition we also found another clue that TASK-1 could act as a mediator for H_2O_2 induced apoptosis. At a relatively low concentration of H_2O_2 (50 μM), the activity of only TASK-1 channel was significantly increased. As a result, we suggest that TASK-1 channel among the K_{2P} channels may promote H_2O_2 induced cell death.

METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Jeil Biotechservices Inc. (South Korea). H_2O_2 was Junsei Chemical Co., Ltd. (Japan). Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies (Japan), and LipofectAmine™ 2000 (LF2000) reagent and G418 were from Life Technologies (Grand Island). Poly-L-lysine and all other chemicals were from Sigma-Aldrich Co. (St. Louis, USA).

Transfection and cell culture

HEK-293 cells were transfected with a vector (pcDNA3.1) containing a K_{2P} channel cDNA using LF2000 and were selected with 1 mg/ml G418 sulfate. Twenty-four hours before transfection, HEK-293 cells were placed at 80 % confluence. For each cell to be transfected, 0.8~1.0 μg of cDNA/vector and 1~3 μl of LF2000 were diluted into 50 μl of medium without FBS. The mixture was incubated for 20 min at room temperature to allow cDNA/vector-LF2000 reagent complexes to be formed. The complexes were directly added to the cells in the culture dish and gently mixed. The cells were incubated at 37°C in a CO_2 incubator. After 5 h, the transfection solution was removed and fresh medium was added. At 48 h after transfection, the cells were passaged at a higher dilution (around 100 cells/100 mm culture dish) into fresh medium. The next day, medium was replaced by selection medium containing 1 mg/ml G418. Selection medium was replaced every 3~4 days. After 2~3 weeks, single colonies were isolated by adhering sterile cloning cylinders to the bottom of the dishes using silicon, and cells contained within each cylinder were isolated by adding 200 μl trypsin/EDTA (1 : 25) at 37°C for 5 min. Cells were titrated and transferred into 35mm culture dish. Each clone was characterized for channel expression by patch clamp analysis. For electrophysiological experiments, cells were grown on 12 mm microscope cover glasses in 35 mm petri-dish and maintained for 30~60 min at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cover glasses were coated with poly-L-lysine for best attachment of cells. For cytotoxicity assay, cells were seeded on 96-well plates at a cell density of 9×10^3 cells/well. After 24 h, the old medium in the 96-well plates was removed from each well and replaced with new medium. And, H_2O_2 was then added to give final concentrations of 0, 1, 10, 30, 50, 100, 200, 300, 400, 500 and 1,000 μM. After exposure to H_2O_2 for 20 min, cell viability was determined by the cytotoxicity assay as described below. In all cases, H_2O_2 solutions were freshly prepared in DMEM supplemented with 10% FBS immediately before use.

Cytotoxicity assay

The viability of cells in 96-well plates treated as above was determined by cytotoxicity assay using cell cytotoxicity assay kit-8 (CCK-8). After adding various concentrations of H_2O_2 into the culture medium of the plates, 10 μl of CCK-8 solution were added to each plate and incubated for 4 hr at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The optional density of each well was measured by absorbance at 450 nm, using a Spectra Max 340 ELISA reader (Molecular Devices) with a reference wavelength at 600 nm. The amount of the yellow colored formazan dye generated by dehydrogenases in cells is directly proportional to the number of viable cells in a culture medium. Cell viability was calculated using these data. Data (mean ± SEM of five separate experiments) are expressed as a percentage of the control value. Data were fitted with Hill equation $y=V_{max}[X^n/(K^n+X^n)]$. The student's t-test was used for the test of significance and represented as p value (*: $p<0.05$, **:$p<0.01$).

Electrophysiology

For patch-clamp experiments, the cultured cells on the cover glasses were placed in a recording chamber on the stage of an inverted microscope. In the cell-attached mode of patch clamp experiments, the bath solution was Normal Tyrode solution. Normal Tyrode solution contained (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 Glucose and 5 HEPES (pH 7.3) with NaOH. The pipette solution contained (in mM) 150 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (pH 7.3) with KOH. The external solution was applied by a microperfusion (0.5 ml/min) that consisted of ten teflon tubes. All experiments were performed at room temperature. Patch pipettes were made of borosilicate glass (WPI, MTW 150F-4) with electrode puller (PP-83, Narishige, Tokyo, Japan) and had resistances of 2~4 $M\Omega$ after fire polishing with Microforge (MF-83, Narishige, Tokyo, Japan). The shank of patch pipette used for cell-attached patch clamp was coated with Sylgard and the tip was fire polished. Electrophysiological recording was performed using a patch clamp amplifier (Axopatch 200B, Axon Instruments, USA). All experiments began by recording control currents without any drug addition to the cells. Only cells with stable currents were used for analysis. K^+ currents were recorded by applying ramp pulse for 900 ms (+20~−160 mV). Data were analyzed by using Clampfit 9.0 (Axon Instruments, USA). Single-channel currents were filtered at 2 kHz and digitized at 10 kHz.

RESULTS

Effect of H_2O_2 on viability of K_{2P} transfected HEK- 293 cells

To elucidate linkage between K_{2P} channels and cell death by H_2O_2, we compared the effects of H_2O_2 on four members (TREK-1, TREK-2, TASK-1 and TASK-3) of the two-pore K^+ channel family. Cells were stably transfected with K_{2P} channels and exposed to H_2O_2 (1~1000 μM) for 20 min. Cell viability was measured with the cytotoxicity assay and plotted against the corresponding H_2O_2 concentration. As

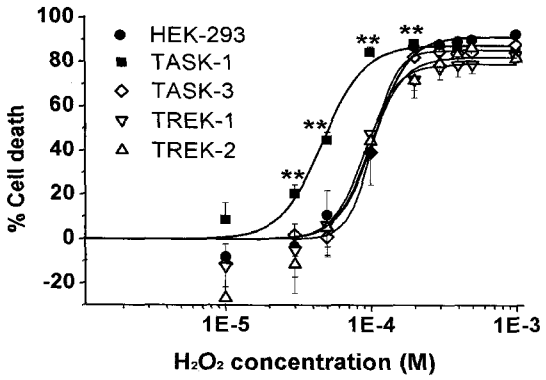

Fig. 1. Dose-response curve induced by H_2O_2 in HEK-293 and K_{2P} transfected HEK-293 cells. The cells were treated with various concentrations of H_2O_2 (1~1,000 μM) for 20 min. Half-maximal concentration (IC_{50}) of H_2O_2 is around 100 μM in TREK-1 (▽), TREK-2 (△), TASK-3 (◇) and HEK-293 (●) cells, but around 50 μM in TASK-1 (■) cells. Data are presented as means±SEM (n=5) and **denotes $p<0.01$.

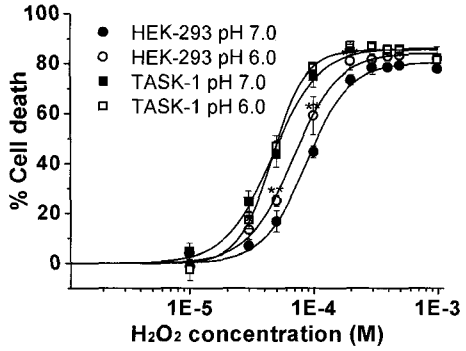

Fig. 2. Effect of lowing pH effect on H_2O_2-induced cell death of HEK-293 (●) and TASK-1 (■) cells. Dose-response curve of HEK-293 cells is shifted to the left by lowing pH (pH 6.0), however, there is no shift in curve, in case of TASK-1 cells. Data are presented as means±SEM (n=5) and ** and * denote $p<0.01$ and $p<0.05$, respectively.

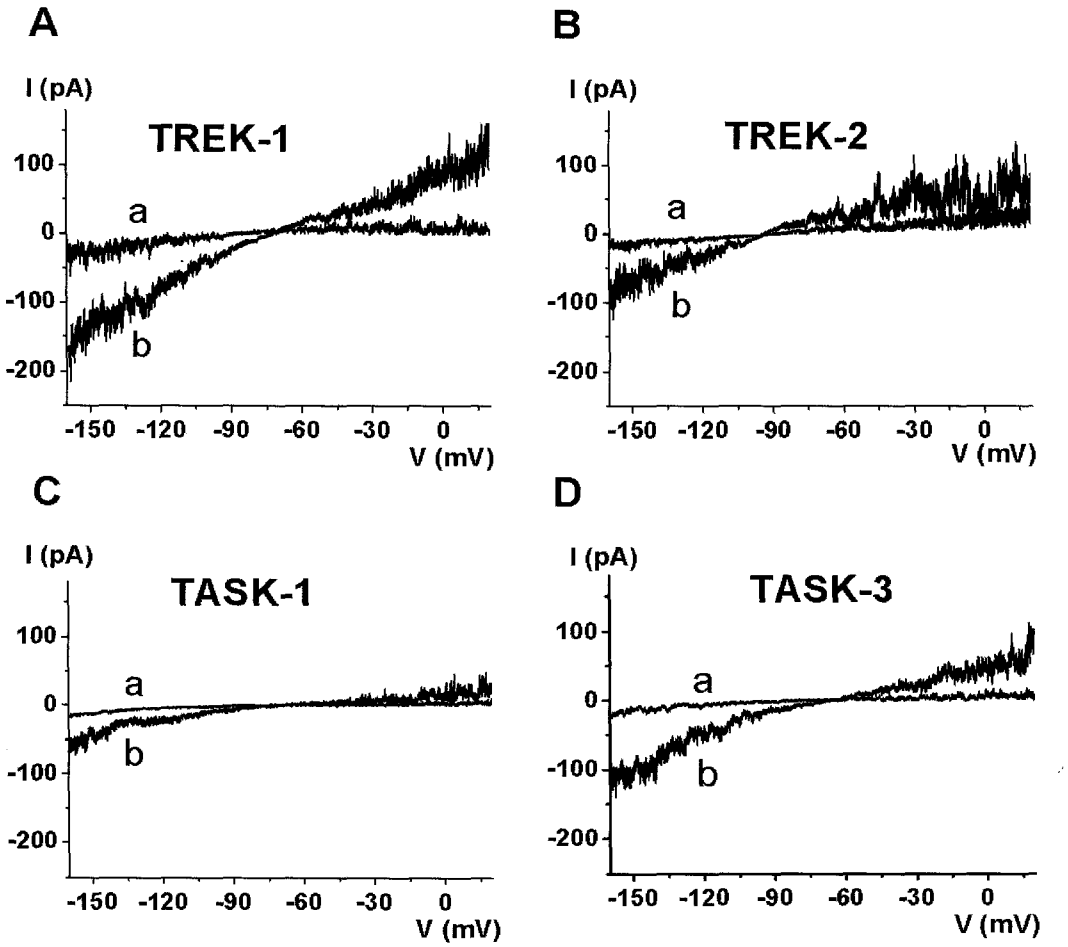

Fig. 3. Effect of H_2O_2 (300 μM) on the K_{2P} channels activity in cell-attached patch. Representative traces of membrane currents in an untreated cell (a) and in a cell exposed to 300 μM H_2O_2 for 20 min (b). (A) TREK-1. (B) TREK-2. (C) TASK-1. (D) TASK-3. Currents were elicited by voltage ramp pulses applied from 20 to −160 mV for 900 ms. The traces are represented as the summation of 5 sweeps.

shown in Fig. 1, the exposure of cells to increasing concentrations of H_2O_2 induced a concentration-dependent decrease in cell viability. IC_{50} was estimated from the best fit of the Hill plots in HEK-293, TREK-1, TREK-2, TASK-1 and TASK-3 transfected cells, and it's values were 80 ± 4.8, 90 ± 7.9, 100 ± 4.8, 50 ± 2.6 and $100\pm6.0\,\mu M$ (n=10, TASK-1; n=20), respectively. The cell death rates induced by H_2O_2 were similar among the four groups (HEK-293, TREK-1, TREK-2 and TASK-3: around $100\,\mu M$), while TASK-1 (IC_{50}: around $50\,\mu M$) was more sensitive than others. As shown in the dose-response curves of five cell lines, the presence of TREK-1, TREK-2 and TASK-3 channels did not affect H_2O_2 induced cell death. But, in case of TASK-1, it was significantly shifted to the left (n=20, **: $p<0.01$). At concentration higher than $300\,\mu M$ H_2O_2, the peaks of cell death reached plateau. In case of TASK-1 transfected cells, the 50% reduction in viability produced by $50\,\mu M$ and $100\,\mu M$ H_2O_2 made $84.29\pm2.41\%$ viability reduction. The cell death reached maximum at around $100\,\mu M$ H_2O_2.

Considering that TASK-1 is an acid-sensitive K^+ channel, the activity of TASK-1 channel could be inhibited by acidic condition. Therefore we investigated whether H_2O_2 induced cell death might be blocked by pH 6.0 (Fig. 2). In HEK-293 cells, dose-response curve was shifted to the left by lowing pH, and IC_{50} was altered by pH 6.0 (pH 7.0: 90 ± 3.1, pH 6.0: $70\pm2.9\,\mu M$). H_2O_2 induced cell death was significantly increased by pH 6.0 (n=10, *: $p<0.05$, **: $p<0.01$). However, H_2O_2 induced cell death of TASK-1 transfected cells did not further increase by pH 6.0. Neither dose-response curve of TASK-1 nor IC_{50} were altered (pH 7.0: 50 ± 1.90, pH 6.0: $50\pm1.6\,\mu M$). Because TASK-1 channel was blocked by pH 6.0, further cell death may not occur.

Effect of H_2O_2 on ionic currents of two-pore K^+ channels

We investigated the effects of H_2O_2 on the single channel currents of TREK-1, TREK-2, TASK-1 and TASK-3 channels. To investigate the changes of channel activity, currents were recorded in cell attached patch clamp recording. Application of $300\,\mu M$ H_2O_2 increased activity of all K_{2p} channels (Fig. 3). TREK-1, TREK-2, TASK-1 and TASK-3 channel activities increased 5.3 ± 1.75, 5.4 ± 2.03, 7.8 ± 2.12 and 8.1 ± 3.24 times at 0 mV, and 9.4 ± 3.19, 9.3 ± 3.85, 7.2 ± 2.96 and 6.3 ± 2.87 times at -150 mV, respectively (n=5, mean$\pm$SEM). As shown by the I-V representative traces, $300\,\mu M$ H_2O_2 increased the currents through whole command voltage range. The effect occurred around 20 min after exposure to H_2O_2. Increased activity of all kinds of channels by $300\,\mu M$ H_2O_2 was non-specific phenomenon, because it was not restricted to only TASK-1 transfected cells.

To examine whether low concentration of H_2O_2 can increase the channel activity to sufficiently induce the apoptosis, we treated TREK-1, TREK-2, TASK-1 and TASK-3 expressing cells with $50\,\mu M$ H_2O_2. The $50\,\mu M$ H_2O_2 was the concentration that induced 50% cell death in TASK-1 transfected cells, but less than 20% cell death in TREK-1, TREK-2 and TASK-3 transfected cells. So we decided to use $50\,\mu M$ H_2O_2. In this condition, application of $50\,\mu M$ H_2O_2 did not increase the activity of TREK-1, TREK-2 and TASK-3 channels (Fig. 4). However, TASK-1 channel activity was increased 10.6 ± 3.52 times at 0 mV and 3.1 ± 0.86 times at -150 mV by $50\,\mu M$ H_2O_2 (n=5, mean$\pm$SEM) (Fig. 5), suggesting that the H_2O_2 effect at low concentration was restricted to TASK-1 channels. Also, we tried $100\,\mu M$ H_2O_2

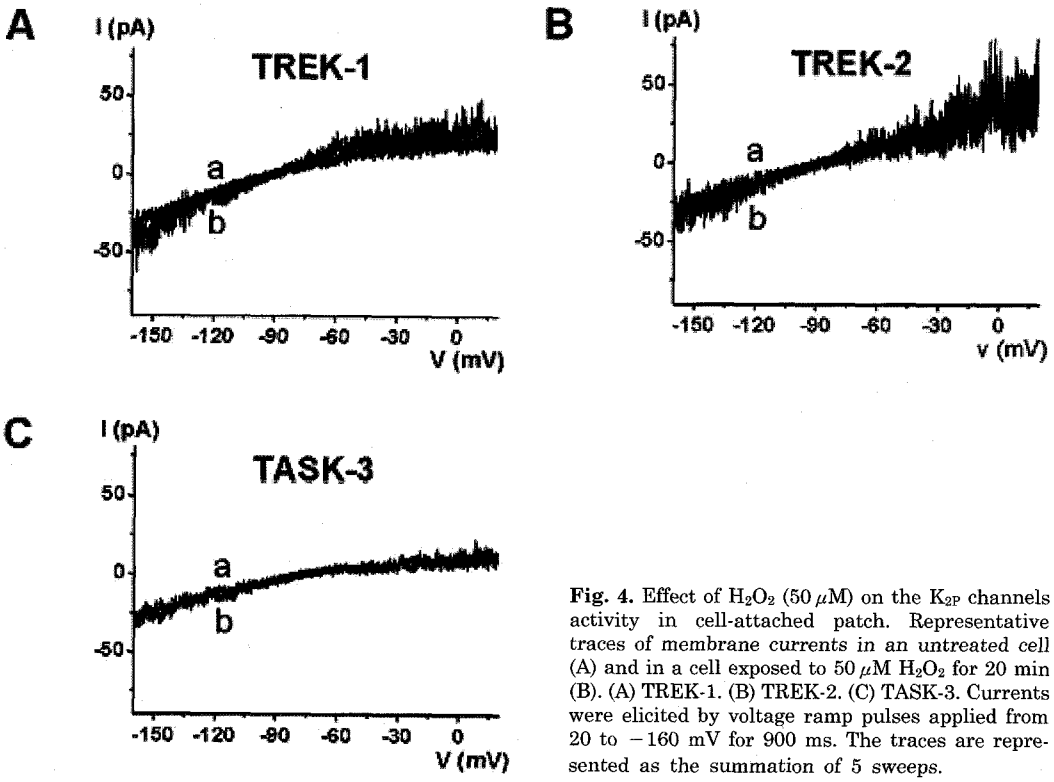

Fig. 4. Effect of H_2O_2 ($50\,\mu M$) on the K_{2P} channels activity in cell-attached patch. Representative traces of membrane currents in an untreated cell (A) and in a cell exposed to $50\,\mu M$ H_2O_2 for 20 min (B). (A) TREK-1. (B) TREK-2. (C) TASK-3. Currents were elicited by voltage ramp pulses applied from 20 to -160 mV for 900 ms. The traces are represented as the summation of 5 sweeps.

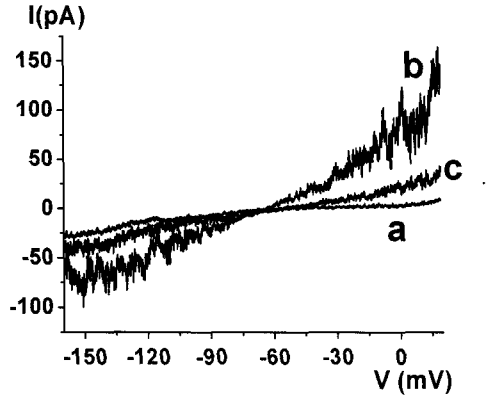

Fig. 5. Effect of H_2O_2 (50 μM) on the TASK-1 channel activity in cell-attached patch. Representative traces of membrane currents in an untreated cell (A), in a cell exposed to 50 μM H_2O_2 for 20 min (B) and in a cell exposed to 10 μM methanandamide (C). Methanandamide (10 μM) reversibly blocked H_2O_2 increased TASK-1 currents. Currents were elicited by voltage ramp pulses applied from 20 to −160 mV for 900 ms. The traces are represented as the summation of 5 sweeps.

in TREK-1, TREK-2 and TASK-3 channels, however, there were no differences between the control and experimental groups (data not shown).

To determine whether increased currents were really TASK-1 currents, methanandamide, a selective TASK-1 channel blocker, was used to confirm the TASK-1 currents (Fig. 5). On applying 10 μM methanandamide, single channel currents were reversibly inhibited by 66.5±4.61% at 0 mV (n=5, mean±SEM). These results showed that H_2O_2-induced current increases must be due to TASK-1.

DISCUSSION

Linkage between K^+ efflux and apoptosis has been suggested by experiments, showing that the K^+ ionophore valinomycin could induce apoptosis (Ojcius et al, 1991; Inai et al, 1997) and gained strength with demonstrations that apoptotic volume decrease (AVD) in eosinophils could be attenuated by K^+ channel blockers, such as 4-aminopyridine, sparteine and quinidine (Beauvais et al, 1995). Oxidative stress (200 μM H_2O_2) evokes rapid AVD and K^+ efflux from zygotes (Trimarchi et al, 2000). Although it is shown that the K^+ channels underlying AVD exhibit properties consistent with K_{2P} channels in mouse embryos (Trimarchi et al, 2002), which of K_{2P} channels participate in K^+ efflux has not been characterized. In our experiments, prolonged (20 minutes) treatment with H_2O_2 activated K_{2P} channels, and this resulted in the increased cell death.

The present study examined the effect of H_2O_2 on the cell viability and K_{2P} channels activity in stably transfected HEK-293 cells. Viability of TREK-1, TREK-2, TASK-1, TASK-3 stable-transfected HEK-293 cells and non-transfected HEK-293 cells were decreased by H_2O_2. However, IC_{50} concentration for TASK-1 stable-transfected HEK-293 cell was 2 times lower than that of other cells. Only TASK-1 dose-response curve was shifted to the left. Dose-response curve of TREK-1, TREK-2 and TASK-3 cells were same as non-transfected HEK-293 cells. Inhibition of TASK-1 channel by pH 6.0 prevented left shift of dose-response curve. So, we hypothesized that TASK-1 channel might participate in K^+ efflux by H_2O_2, thereby modulating AVD. Next, we focused on channel activity changes in response to H_2O_2, which occurred within 20 minutes after application of H_2O_2 to the bath solution. To examine whether K_{2P} channels are involved in apoptosis induced by extreme oxidative stress, we treated the cells with 300 μM H_2O_2, and found that treatment with 300 μM H_2O_2 increased all K_{2P} channels activity, resulting from oxidative stress. As shown in cytotoxicity assay, 300 μM H_2O_2 were the concentration to induce 80~90% cell death. Thus, we used low concentration of H_2O_2 (50 μM). In contrast to 300 μM H_2O_2, treatment with 50 μM H_2O_2 increased only TASK-1 channel activity. 50 μM H_2O_2 did not change TREK-1, TREK-2 and TASK-3 channel activities. We induced oxidative stress using H_2O_2 and recorded K^+ currents. The present study showed that H_2O_2 induced cell death was further increased by TASK-1 channels. This findings indicate that TASK-1 channel, rather than K_{2P} (TREK-1, TREK-2 and TASK-3) channels, may play a role in the H_2O_2 induced cell death.

Many studies have shown that bolus addition of exogenous H_2O_2, such as menadione, lead to activation of the mitogen-activated protein (MAP) kinases (Guyton et al, 1996; Lo et al, 1996; Wang et al, 1998; Kamata & Hirata, 1999). The MAP kinases are major components of signaling pathways that control proliferation, differentiation, embryogenesis and cell death. Furthermore, many studies have implied involvement of reactive oxygen species (ROS) in MAP kinase activation after cells were stimulated with various agents based on inhibition by catalase or compounds with antioxidants properties (Hashimoto et al, 2001). The mechanisms by which exogenously or endogenously produced ROS activate the MAP kinases are not well defined. Future work will focus on which intracellular mechanisms are involved in the H_2O_2 induced AVD, and how they increase K_{2P} channels activity.

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