Stimulatory Actions of Caffeic Acid Phenethyl Ester, a Known Inhibitor of NF-κB Activation, on Ca^{2+}-activated K⁺ Current in Pituitary GH_{3} Cells*

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Caffeic acid phenethyl ester (CAPE), a phenolic antioxidant derived from the propolis of honeybees, is known to be an inhibitor of activation of nuclear transcript factor NF-κB. Its effects on ion currents have been investigated in pituitary GH_{3} cells. This compound increased Ca^{2+}-activated K⁺ current (I_{Ca,K}) in a concentration-dependent manner with an EC_{50} value of 14 ± 2 μM. However, the magnitude of CAPE-induced stimulation of I_{Ca,K} was attenuated in GH_{3} cells preincubated with 2,2'-azo-bis-(2-amidinopropane) hydrochloride (AAPH, 100 μM) or t-butyl hydroperoxide (1 mM). CAPE (50 μM) slightly suppressed voltage-dependent L-type Ca^{2+} current. In inside-out configuration, CAPE (20 μM) applied to the intracellular face of the detached patch enhanced the activity of large conductance Ca^{2+}-activated K⁺ (BKCa) channels with no modification in single-channel conductance. After BKCa channel activity was increased by CAPE (20 μM), subsequent application of nordihydroguaiaretic acid (1 mM) did not further increase the channel activity. CAPE-stimulated channel activity was dependent on membrane potential. CAPE could also increase Ca^{2+} sensitivity of BKCa channels in these cells. Its increase in the open probability could primarily involve a decrease in the mean closed time. In current-clamp conditions, CAPE hyperpolarized the membrane potential and reduced the firing of action potentials. The stimulatory effects on these channels may partly contribute to the underlying mechanisms through which this compound influences the functional activities of neurons or neuroendocrine cells. Caution has to be used in attributing its response in the activation of NF-κB.

Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant that has been identified as one of the major components of honeybee propolis (1). It has been demonstrated to be a specific inhibitor of activation of nuclear transcript factor NF-κB (2, 3). Previous studies have shown that this compound could protect the spinal cord and brain from ischemia reperfusion injury (4, 5) and prevent neurotoxic events caused by excessive inflammatory reaction in brain (6). Several lines of evidence also indicate that CAPE may modify the redox state in transformed fibroblast cells and in leukemic HL-60 cells (7–9). Furthermore, it has been reported that this compound inhibited the contractile response to phenylephrine or to high K⁺ solution in isolated rat thoracic aorta (10). However, to our knowledge, the effects of CAPE on ion currents have not been thoroughly studied. Large conductance Ca^{2+}-activated K⁺ (BKCa) channels play important roles in controlling the excitability of nerve, muscle, and other cells by stabilizing cell membrane at negative potentials (11). Their gating is known to be controlled by intracellular Ca^{2+} and/or membrane depolarization. The challenging of cells with oxidizing agents has been found to suppress the channel activity of these channels (12). Pituitary GH_{3} cells have been demonstrated to exhibit the activity of these channels (13). Riluzole and ciglitazone, both of which were reported to prevent neuronal injuries, could enhance the activity of BKCa channels functionally expressed in these cells (14, 15). Importantly, the opener of these channels has been shown to counteract the deleterious effects of excitatory neurotransmitters following neurotoxic or ischemic injuries (16). Previous studies also revealed that the BKCa channel might be a relevant target of DNA synthesis in cultured Müller glial cells (17).

Therefore, the objective of this study was to (a) address the question of whether CAPE could affect Ca^{2+}-activated K⁺ currents (I_{Ca,K}) in GH_{3} cells; (b) determine the effects of this compound on the activity of BKCa channels; and (c) examine whether it can influence the membrane potential. Interestingly, the present results indicate that in GH_{3} lactotrophs, CAPE does not appear to affect the activation of NF-κB exclusively, despite its ability to inhibit NF-κB activation in these cells (18). The CAPE-induced increase in BKCa channel activity may account, at least in part, for its effects on cellular functions in neurons or neuroendocrine cells.

MATERIALS AND METHODS

Cell Culture—The clonal strain GH_{3} cell line, originally derived from a rat anterior pituitary adenoma, was obtained from the Culture Collection and Research Center (CCRC-60015; Hsinchu, Taiwan). The detailed methods have been previously described (19). Briefly, the cells were cultured in Ham’s F-12 medium (Invitrogen) supplemented with 15% heat-inactivated horse serum (v/v), 2.5% fetal calf serum (v/v), and 2 mM l-glutamine (Invitrogen) in a humidified environment of 5% CO_{2}/95% air. The experiments were generally performed 5 or 6 days after cells were subcultured (60–80% confluence).

Electrophysiological Measurements—Immediately before each experiment, the cells were dissociated, and an aliquot of cell suspension was transferred to a recording chamber positioned on the stage of an inverted microscope (DM IL; Leica, Wetzlar, Germany). The cells were bathed at room temperature (20–25 °C) in normal Tyrode’s solution containing 1.9 mM Ca^{2+}. The recording pipettes were pulled from Kimax-51 capillaries (Kimble Glass, Vineland, NJ) using a two-stage microelectrode puller (PP-830; Narishige, Tokyo, Japan), and the tips were fire-polished with a microforge (MF-83, Narishige). When filled

*This work was supported by National Science Council Grants NSC-91-2320B-006-106 and NSC-92-2320B-006-041. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: CAPE, caffeic acid phenethyl ester; BKCa, large conductance Ca^{2+}-activated K⁺; AAPH, 2,2'-azo-bis(2-aminopropane) dihydrochloride.
with pipette solution, their resistance ranged between 3 and 5 MΩ. Ion currents were measured in the cell-attached, inside-out, and whole cell configurations of the patch-clamp technique, using an RK-400 patch-clamp amplifier (Bio-Logic, Claira, France) (19).

**Data Recording and Analysis**—The signals were displayed on an analog/digital oscilloscope (HM 507; Hameg, East Meadow, NY) and on a liquid crystal projector (PJ550-2; ViewSonic, Walnut, CA). The data were stored in a Pentium III grade laptop computer (Simtomate, Lemel, Taipei, Taiwan) at 10 kHz through a Digidata 1322A interface (Axon Instruments, Union City, CA). This device was controlled by a commercially available software (pCLAMP 9.0; Axon Instruments). The currents were low pass filtered at 1 or 3 kHz. Ion currents obtained during whole cell experiments were stored without leakage correction and analyzed using the pCLAMP 9.0 software (Axon Instruments) or the Origin 6.0 software (Microcal, Northampton, MA).

To calculate the percentage of stimulation of CAPE on $I_{K(Ca)}$, each cell was depolarized from 0 to +50 mV, and current amplitude during cell exposure to CAPE was measured and compared. The amplitude of $I_{K(Ca)}$ in the presence of this compound at a concentration 200 μM was taken as 100%. The concentration of CAPE required to increase 50% of current amplitude was then determined using a Hill function, $y = (E_{\text{max}} \times [C]^{n_{h}})/([C]^{n_{h}} + [C]^{n_{k}})$, where [C] is the CAPE concentration, $E_{\text{max}}$ is the concentration required for a 50% increase; $n_{h}$ is the Hill coefficient, and $E_{\text{max}}$ is the CAPE-induced maximal increase in the amplitude of $I_{K(Ca)}$.

The amplitudes of single $K_{Ca}$ channel currents were determined by fitting Gaussian distributions to the amplitude histograms of the closed and the open state. The channel open probability in a patch was expressed as $N_{P_{o}}$, which can be estimated using the following equation: $N_{P_{o}} = N_{o}(A_{1} + 2A_{2} + 3A_{3} + \ldots + nA_{n})(V_{o} + A_{1} + A_{2} + A_{3} + \ldots + A_{n})$, where $N_{o}$ is the number of active channels in the patch, $A_{n}$ is the area under the curve of an all points histogram corresponding to the closed state, and $A_{n}-A_{1}$ represent the histogram areas reflecting the levels of distinct open state for 1 to $n$ channels in the patch.

The relationships between the membrane potentials and the probability of channel openings obtained before and after the application of CAPE (20 μM) were fitted with a Boltzmann function of the form: $P_{o} = N_{o}/(1 + \exp(-[V_{m} - V_{1/2}]/k)))$, where $V_{1/2}$ is the membrane potential at which there is half-maximal activation, and $k$ is the slope factor of the activation curve (i.e., the voltage dependence of the activation process in mV per $k$-fold change).

The averaged results are presented as the mean values ± S.E. The paired Student’s t-test was used for the statistical analyses. To further clarify the statistical difference among the two or four treatment groups, analyses of variance with Duncan’s multiple range test for multiple comparisons were also performed. Differences between values of $n$ were significant at $p < 0.05$.

**Drugs and Solutions**—CAPE (phenethyl caffeate) was obtained from Cayman Chemical (Ann Arbor, MI). Curcumin, glibenclamide, nordihydroguaiaretic acid and bilirubin were obtained from Sigma/RBI (Natick, MA), and t-butyl hydroperoxide, dithiothreitol, and tetraethylammonium chloride from Tocris Cookson Ltd. (Bristol, UK). Tetrodotoxin and apamin were obtained from Alomone Labs (Jerusalem, Israel), and fura-2 acetoxy-methyl ester and fura-2 were from Molecular Probes (Eugene, OR). Squamocin was a gift from Dr. Yang-Chang Wu (Graduate Institute of Pharmaceutical Chemistry, Taiwan). HEPES-KOH buffer, pH 7.2. The value of free Ca$^{2+}$ concentration was calculated assuming a dissociation constant for EGTA and Ca$^{2+}$ (at pH 7.2) of 0.1 μM. To provide 0.1 μM free Ca$^{2+}$ in bath solution, 0.5 mM CaCl$_2$ and 1 mM EGTA were added.

**RESULTS**

**Effect of CAPE on Ca$^{2+}$-activated K$^{+}$ Current ($I_{K(Ca)}$) in GH3 Cells**—In the first series of experiments, the whole cell configuration of the patch-clamp technique was used to investigate the effect of CAPE on ion currents in these cells. The cells were bathed in normal Tyrode’s solution containing 1.8 mM CaCl$_2$, 1.5 mM MgCl$_2$, 5.5 mM glucose, and 5.5 mM HEPES-NaOH buffer, pH 7.4. To record the K$^{+}$ currents or membrane potential, the recording pipette was backfilled with a solution consisting of 140 mM KCl, 1 mM MgCl$_2$, 3 mM Na$_2$ATP, 0.1 mM Na$_2$GTP, 0.1 mM EGTA, and 5 mM HEPES-KOH buffer, pH 7.2. The free Ca$^{2+}$ concentration of this solution was estimated to be 250 nM, assuming that the reconstituted Ca$^{2+}$-sensitive K$^{+}$ channels had a Ca$^{2+}$ concentration of 70 μM, and the ratio-metric fura-2 measurement with F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) showed that this solution contained 205 ± 12 nM free Ca$^{2+}$ for three different experiments. To measure voltage-dependent Ca$^{2+}$ current, KCl inside the pipette solution was replaced with equimolar CaCl$_2$, and pH was adjusted to 7.2 with CsOH, whereas bath solution included 1 μM tetrodotoxin and 10 mM tetraethylammonium chloride.

For single-channel current recordings, the high K$^{+}$ bathing solution contained 145 mM KCl, 0.53 mM MgCl$_2$, and 5 mM HEPES-KOH buffer, pH 7.4, and the pipette solution contained 145 mM KCl, 2 mM MgCl$_2$, and 5 mM HEPES-KOH buffer, pH 7.4, and the pipette solution contained 145 mM KCl, 2 mM MgCl$_2$, and 5 mM HEPES-KOH buffer, pH 7.4, and the pipette solution contained 145 mM KCl, 2 mM MgCl$_2$, and 5 mM HEPES-KOH buffer, pH 7.4, and the pipette solution contained 145 mM KCl, 2 mM MgCl$_2$, and 5 mM HEPES-KOH buffer, pH 7.4, and the pipette solution contained 145 mM KCl, 2 mM MgCl$_2$, and 5 mM HEPES-KOH buffer, pH 7.4, and the pipette solution contained 145 mM KCl, 2 mM MgCl$_2$, and 5 mM HEPES-KOH buffer, pH 7.4.
This stimulatory effect was readily reversed after the removal of CAPE (Fig. 1). Fig. 1B illustrates the averaged I-V relationships for $I_{KCa}$ in control, during cell exposure to CAPE (20 μM) and washout of the compound.

The relationship between the CAPE concentration and the percentage increase of $I_{KCa}$ has been constructed (Fig. 1C). This compound could increase the amplitude of $I_{KCa}$ in a concentration-dependent manner with an EC50 value of 14 ± 2 μM. At a concentration of 200 μM, it fully increased $I_{KCa}$. The Hill coefficient was found to be 1.8, suggesting that there was a positive cooperation for its stimulation of $I_{KCa}$. These results indicate that CAPE can produce a stimulatory action on $I_{KCa}$ in these cells.

Effect of CAPE on Voltage-dependent L-type Ca2+ Current ($I_{Ca,L}$) in GH3 Cells—$I_{Ca,L}$ can be functionally coupled with Ca2+ influx through plasmalemmal voltage-dependent Ca2+ channels (21). A recent report also demonstrated that the action of CAPE on vasorelaxation in rat thoracic aorta could be due to the blockade of Ca2+ movement through the cell membrane (10). For these reasons, we further investigated whether it could exert any effect on $I_{Ca,L}$ that was previously described in these cells (21, 22). These experiments were conducted with a Ca2+-containing solution. The exposure to CAPE (20 μM) was found to have little or no effect on $I_{Ca,L}$. However, this compound at a concentration of 50 μM slightly suppressed $I_{Ca,L}$ although it did not modify the I-V relationship of $I_{Ca,L}$ (Fig. 2).

For example, CAPE (50 μM) decreased the amplitude of $I_{Ca,L}$ to 42 ± 3 pA from a control value of 51 ± 6 pA ($p < 0.05$; $n = 7$), when cells were depolarized from −50 to 0 mV. Therefore, this compound stimulated $I_{KCa}$ in a manner conceivably unlikely to be linked to an increase in the amplitude of $I_{Ca,L}$.

Effect of CAPE on $I_{KCa}$ in Cells Preincubated with 2,2′-Azobis(2-aminopropane) Dihydrochloride AAPH or t-Butyl Hydroperoxide—CAPE is known to be an antioxidant flavonoid (1, 23). Therefore, we next evaluated whether changes in reactive oxygen species can influence CAPE-induced stimulation of $I_{KCa}$ in GH3 cells. Interestingly, the results showed that CAPE-stimulated $I_{KCa}$ was attenuated in GH3 cells preincubated with either 100 μM AAPH or 1 mM t-butyl hydroperoxide (Fig. 3). t-Butyl hydroperoxide is an oxidative agent, whereas AAPH is known to be an azo compound that can generate free radicals (24). A subsequent application of diethiothreitol (10 μM) increased $I_{KCa}$ in cells treated with AAPH or t-butyl hydroperoxide. When the AAPH-treated cells were depolarized from 0 to +50 mV, CAPE (200 μM) increased the density of $I_{KCa}$ by about 15%. Conversely, in control cells, CAPE (200 μM) nearly fully increased the density of these currents. These results suggest that the stimulation of $I_{KCa}$ caused by this compound can be modified in the presence of these oxidizing agents.

Effect of CAPE on the Activity of BKCa Channels in GH3 Cells—The results from our whole cell experiments suggest that $I_{KCa}$ may be K+ flux through the BKCa channel (13, 19), because CAPE-induced increase in $I_{KCa}$ was suppressed by paxilline yet not by glibenclamide or apamin. To elucidate how it could act to affect $I_{KCa}$, the effect of this compound on BKCa channels was further investigated. In these experiments, the single-channel recordings with inside-out configuration were performed in symmetrical K+ concentration (145 mM). The bath solution contained 0.1 mM Ca2+, and the potential was held at +60 mV. As shown in Fig. 4, the activity of BKCa channels could be readily observed in an excised patch. An increase in channel activity could also be obtained in cell-attached patches when cells were exposed to ionomycin (10 μM) or squamocin (10 μM). These two agents were previously reported to be Ca2+ ionophores (25). When CAPE (20 μM) was applied to the intracellular face of the detached patch, the channel open probability was increased (Fig. 4). The open probability obtained at the level of +60 mV in the control was 0.112 ± 0.005 ($n = 6$). The application of CAPE (20 μM) significantly increased channel activity to 0.289 ± 0.035 ($p < 0.05$;
When this compound was washed out, the open probability returned to the control level. However, the single-channel amplitude remained unaltered in the presence of 20 μM CAPE (Fig. 4C). Moreover, curcumin (20 μM) applied to the intracellular face of the excised patch was not found to have any effects on the probability of channel openings, whereas cilostazol (20 μM) could increase the channel activity effectively. Similar to CAPE, curcumin has been shown to inhibit the activation of NF-κB (26). Cilostazol has been recently found to stimulate IK(Ca) in human neuroblastoma SK-N-SH cells (27).

Effect of Nordihydroguaiaretic Acid on BK Ca Channels in GH3 Cells—Nordihydroguaiaretic acid was previously reported to stimulate BK Ca channels (28). We also examined whether the stimulatory effects of CAPE and nordihydroguaiaretic acid on these channels are additive. Interestingly, as shown in Fig. 5 (A and B), nordihydroguaiaretic acid (20 μM) increased the channel open probability; however, a subsequent application of CAPE (20 μM) did not increase the channel activity further. Nordihydroguaiaretic acid (20 μM) significantly increased the open probability from 0.106 ± 0.006 to 0.286 ± 0.034 (p < 0.05; n = 6). There was no significant difference in the channel activity between the presence of nordihydroguaiaretic acid alone and CAPE plus nordihydroguaiaretic acid (0.286 ± 0.034 (n = 6) versus 0.284 ± 0.032 (n = 6), p > 0.05). Conversely, the addition of riluzole (20 μM) could increase the open probability further in the continued presence of CAPE or nordihydroguaiaretic acid. Riluzole was reported to be an opener of BK Ca channels (14). Taken together, the results indicate that the stimulatory effects of CAPE and nordihydroguaiaretic acid on a single BKCa channel are not additive in GH3 cells.

Lack of Effect of CAPE on Single-channel Conductance of BKCa Channels—In the next series of experiments, the effect of CAPE on BKCa single-channel conductance was investigated. In inside-out configuration, the cells were bathed in symmetrical K+ concentration (145 mM), and the bath solution contained 0.1 μM Ca2+. The experiments were conducted with symmetrical K+ concentration (145 mM). Under inside-out configuration, the holding potential was set at +60 mV and the bath medium contained 0.1 μM Ca2+. A, the activity of BKCa channels recorded before (left panel) and during exposure (right panel) to 20 μM CAPE. The lower part in A shows current traces obtained in an expanded time scale corresponding to those labeled a and b in B and in the upper part of A. Upward deflection indicates the opening events of the channel. B, the time course of change in the channel open probability (0.5-s bin width) before and during the application of CAPE (20 μM) to the intracellular surface of the channel in an excised patch. C, amplitude histograms measured in the absence (left panel) and presence (right panel) of 20 μM CAPE.
Effect of CAPE on the Activation Curve of BKCa Channels—Fig. 5E shows the activation curve of BK<sub>Ca</sub> channels in the absence and presence of CAPE (20 μM). The plot of open probability of BK<sub>Ca</sub> channels as a function of membrane potential was fitted with a Boltzmann function as described under "Materials and Methods." In control, m<sub>p</sub> = 0.35 ± 0.04, V<sub>1/2</sub> = 75.4 ± 1.6 mV, and k = 10.7 ± 0.4 mV (n = 6), whereas in the presence of CAPE (20 μM), m<sub>p</sub> = 0.71 ± 0.07, V<sub>1/2</sub> = 61.2 ± 1.9 mV, and k = 10.9 ± 0.6 mV (n = 6). The data showed that the activation curve was shifted along the voltage axis to less positive potentials in the presence of CAPE. In contrast, no significant change in the slope (i.e. k value) of the activation curve was detected in the presence of this compound. Taken together, these results indicate that CAPE applied to the intracellular surface of the channel is capable of increasing the open probability in a voltage-dependent fashion.

Effect of Internal Ca<sup>2+</sup> Concentration on CAPE-stimulated BK<sub>Ca</sub> Channel Activity in GH<sub>3</sub> Cells—Whether the CAPE-induced increase in the activity of these channels is associated with internal Ca<sup>2+</sup> concentration was also studied. In these experiments, when an excised membrane patch was formed, various concentrations of Ca<sup>2+</sup> in the bath before and during exposure to CAPE (20 μM) were applied. As shown in Fig. 5F, the stimulatory effect of CAPE on BK<sub>Ca</sub> channel activity was affected by changes in the level of intracellular Ca<sup>2+</sup> concentration. For example, at the holding potential of +60 mV, the presence of CAPE (20 μM) caused a 2-fold increase in the open probability at internal Ca<sup>2+</sup> concentration of 0.1 μM. However, at an internal Ca<sup>2+</sup> concentration of 10 μM, CAPE at the same concentration stimulated channel activity by 4-fold.

Effect of CAPE on Spontaneous Action Potentials of GH<sub>3</sub> Cells—The effect of CAPE on mean open and closed time of BK<sub>Ca</sub> channels was examined and analyzed during recordings from patches showing only single-channel openings. As shown in Fig. 6, in control cells, the closed time histogram of BK<sub>Ca</sub> channels at +60 mV can be fitted by a two-exponential curve with a mean closed time of 13.2 ± 2.8 and 55.8 ± 9.8 ms (n = 6). CAPE (20 μM) decreased the lifetime of the closed state to 10.9 ± 2.2 ms (n = 6). However, little or no change in mean open time was seen in the presence of CAPE (20 μM). Thus, the data demonstrate that its effect on BK<sub>Ca</sub> channel activity in GH<sub>3</sub> cells is primarily due to a decrease in closed time.

Effect of CAPE on Spontaneous Action Potentials of GH<sub>3</sub> Cells—The effect of CAPE on the repetitive firing of action potentials was further examined. In these experiments, the cells were bathed in normal Tyrode's solution containing 1.8 mM CaCl<sub>2</sub>, and current-clamp configuration was performed with a K<sup>+</sup>-containing pipette solution. When the cells were
that CAPE can increase inhibition of spontaneous action potentials, the results indicate that CAPE can increase peak outward currents from 203 ± 15 to 506 ± 34 pA (p < 0.05; n = 6). A subsequent application of paxilline (1 μM) could decrease the CAPE-stimulated \( I_{K(Ca)} \) from 506 ± 34 to 312 ± 26 pA (p < 0.05; n = 6). Thus, consistent with its inhibition of spontaneous action potentials, the results indicate that CAPE can increase \( I_{K(Ca)} \) that is active during normal action potentials.

**DISCUSSION**

This study shows that CAPE (a) increases the amplitude of \( I_{K(Ca)} \) in a concentration-dependent manner in pituitary GH3 cells, (b) enhances the activity of BK\(_{Ca} \) channel in a voltage-dependent manner, and (c) reduces the repetitive firing of action potentials. This compound increased the probability of these channels in a mechanism unlikely to be linked to its inhibition of activation of NF-κB. The stimulation by CAPE of \( I_{K(Ca)} \) conceivably could be one of the mechanisms underlying CAPE-induced actions, if similar results occur in neurons or neuroendocrine cells in vivo.

**FIG. 6. Effect of CAPE on mean closed time of BK\(_{Ca} \) channels in GH3 cells.** Under symmetrical K\(^+\) condition, inside-out configuration was performed and potential was held at 0 mV. Closed time histogram in control (upper panel) was fitted by a two-exponential function with a mean closed time of 13.2 and 55.8 ms. The closed time histogram obtained after application of 20 μM CAPE to the bath (lower panel) was fitted by a single-exponential function with a mean closed time of 10.9 ms. The data were obtained from a measurement of 324 channel openings with a total recording time of 1 min in the control, and those obtained during the exposure to CAPE were measured from 421 channel openings with a total recording time of 30 s. The dashed lines shown in each lifetime distribution are placed at the value of the time constant in the closed state.

exposed to CAPE (20 μM), the membrane became hyperpolarized, and the repetitive firing of action potentials was gradually decreased (Fig. 7, A and B). CAPE (20 μM) decreased the firing frequency from 1.05 ± 0.08 to 0.36 ± 0.05 Hz (p < 0.05; n = 6). Paxilline (1 μM), a known blocker of BK\(_{Ca} \) channels, reversed the CAPE-induced decrease of firing frequency to 0.86 ± 0.07 Hz (p < 0.05; n = 6). Thus, it is clear that this compound can regulate the firing of action potentials in these cells.

**Effect of CAPE on \( I_{K(Ca)} \) That Is Active in Normal Action Potential Waveforms**—To determine whether CAPE affects \( I_{K(Ca)} \) that is active during normal action potentials, each cell was held at −50 mV, and the ramp hyperpolarization pulses from +20 to −50 mV with a duration of 100 ms at a rate of 0.05 Hz were delivered to mimic action potential-like waveforms of GH3 cells (22). As shown in Fig. 7 (C and D), when cells were bathed in normal Tyrode’s solution containing 1.8 mM CaCl\(_2\), the current traces representing the I-V relationships of \( I_{K(Ca)} \) were observed in response to a voltage ramp protocol ranging from +20 to −50 mV. The application of CAPE (20 μM) increased peak outward currents from 203 ± 15 to 506 ± 34 pA (p < 0.05; n = 6). A previous study showed that activation of NF-κB results in the enhanced Ca\(^{2+}\) influx through voltage-de-
pendent Ca\(^{2+}\) channels, because it was not found to increase the amplitude of \(I_{\text{Ca,L}}\). These observations are compatible with a recent report showing the inability of CAPE to alter the decrease in intracellular Ca\(^{2+}\) induced by low K\(^{-}\) solution in cerebellar granule cells (30). Our results demonstrating that this compound at a concentration of 50 \(\mu\)M produced a slight reduction in the amplitude of \(I_{\text{Ca,L}}\) can also account for its ability to inhibit high K\(^{-}\)-induced vasoconstriction in isolated rat aorta (10).

It has been demonstrated that dithiothreitol could stimulate \(I_{\text{KCa}}\) in GH3 cells (31). In AAPH-treated cells, the stimulatory effect of CAPE on \(I_{\text{KCa}}\) was attenuated, and a subsequent application of dithiothreitol effectively increased the amplitude of \(I_{\text{KCa}}\). These results suggest that the sulfhydryl oxidizing and reducing agents can produce an effect on \(I_{\text{KCa}}\) in GH3 cells. It will be interesting to determine to what extent the decreased production of reactive oxygen species caused by CAPE affects the stimulatory effect of CAPE on \(I_{\text{KCa}}\), because this compound is known to be a potent flavonoid antioxidant (1, 23). Moreover, it seems likely that a decrease in the production of reactive oxygen species caused by CAPE is upstream of its stimulation of \(I_{\text{KCa}}\). Direct activation of BK\(\text{Ca}\) channels and indirect inhibition of production of reactive oxygen species may synergistically contribute to the underlying cellular mechanisms through which this compound modifies the repetitive firing of these cells. In addition, like NS004 (32), CAPE was found to increase Ca\(^{2+}\) sensitivity of BK\(\text{Ca}\) channels observed in GH3 cells. Its ability to increase Ca\(^{2+}\) sensitivity of BK\(\text{Ca}\) channels suggests that the CAPE molecule may modify the cysteine residues near the carboxyl-terminal, Ca\(^{2+}\) bowl domain of these channels (33).

Our study demonstrated that CAPE could not modify single-channel conductance of BK\(\text{Ca}\) channels, but it did increase the channel open probability. The increase in the amplitude of \(I_{\text{KCa}}\) caused by CAPE is primarily thought to be a result of a decrease in mean closed time. It was also seen that CAPE shifted the activation curve of BK\(\text{Ca}\) channels to the left with no modification in the slope factor of this curve. This compound thus appears to produce the stimulation of BK\(\text{Ca}\) channels by a direct effect on the channel or closely associated site, although the precise mechanisms of its action remain to be further elucidated. However, our data demonstrated that CAPE applied to the intracellular face of the excised patch produced a fraction of channel closings to shift to short-lived closings, resulting in one closed kinetic state.

It is worth mentioning that unlike the molecules of NS004, NS1619, or riluzole, the CAPE molecule has the juxtaposition of two aromatic rings, the unique structure of which is similar to those of some BK\(\text{Ca}\) channel openers, such as nordihydroguaiaretic acid and resveratrol (Fig. 8) (34). The present results demonstrated that the stimulatory effect of CAPE and nordihydroguaiaretic acid on the BK\(\text{Ca}\) channel was not additive. It is thus tempting to speculate that these two compounds, which are structurally related, may interact with the same binding site in the channel.

CAPE has been recently reported to induce the release of cytochrome c from mitochondria to cytosol in C6 glioma cells (35). Cytochrome c was found to activate K\(^{-}\) channels (36). However, in inside-out configurations, we showed that CAPE applied to the intracellular face of the excised patches enhanced BK\(\text{Ca}\) channel activity. It is unlikely that the ability of CAPE to increase the amplitude of \(I_{\text{KCa}}\) is primarily due to the release of cytochrome c from mitochondria.

Curcumin, another inhibitor of NF-\(\kappa\)B activation, was not found to have effects on BK\(\text{Ca}\) channels, when it was applied to intracellular face of the excised patch. The results lead us to suggest that CAPE could induce the change in the activity of BK\(\text{Ca}\) channels in GH3 cells in a mechanism unlikely to be linked to its inhibition of NF-\(\kappa\)B activation. However, its change in membrane potential can be explained by the stimulatory effect on these channels. Such an effect may be responsible for its actions on neurons or neuroendocrine cells in vitro, despite the ability of this compound to inhibit activation of NF-\(\kappa\)B in GH3 cells (18). Furthermore, CAPE and other structurally related compounds seem to be intriguing pharmacological tools used to characterize the properties of the BK\(\text{Ca}\) channels. Elucidation of the structure of the binding site for CAPE or other structurally related compounds might provide a structural basis for the pharmacological modulation of BK\(\text{Ca}\) channels.

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