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Stimulatory Effects of δ-Hexachlorocyclohexane on Ca^{2+}-Activated K^+ Currents in GH_3 Lactotrophs

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ABSTRACT

δ-Hexachlorocyclohexane (δ-HCH), a lipophilic neurodepressant agent, has been shown to inhibit neurotransmitter release and stimulate ryanodine-sensitive Ca^{2+} channels. However, the effect of δ-HCH on neuronal activity remains unclear, although it may enhance the γ-aminobutyric acid-induced current. Its effects on ionic currents were investigated in rat pituitary GH_3 cells and human neuroblastoma IMR-32 cells. In GH_3 cells, δ-HCH increased the amplitude of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$). δ-HCH (100 μM) slightly inhibited the amplitude of voltage-dependent K^+ current. δ-HCH (30 μM) suppressed voltage-dependent L-type Ca^{2+} current ($I_{Ca,\ L}$), whereas γ-HCH (30 μM) had no effect on $I_{Ca,\ L}$. In the inside-out configuration, δ-HCH applied intracellularly did not change the single channel conductance of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels; however, it did increase the channel activity. The δ-HCH-mediated increase in the channel activity is mainly mediated by its increase in the number of long-lived openings. δ-HCH reversibly increased the activity of BK_{Ca} channels in a concentration-dependent manner with an EC_{50} value of 20 μM. δ-HCH also caused a left shift in the midpoint for the voltage-dependent opening. In contrast, γ-HCH (30 μM) suppressed the activity of BK_{Ca} channels. Under the current-clamp mode, δ-HCH (30 μM) reduced the firing rate of spontaneous action potentials; however, γ-HCH (30 μM) increased it. In neuroblastoma IMR-32 cells, δ-HCH also increased the amplitude of $I_{K(Ca)}$ and stimulated the activity of intermediate-conductance K_{Ca} channels. This study provides evidence that δ-HCH is an opener of K_{Ca} channels. The effects of δ-HCH on these channels may partially, if not entirely, be responsible for the underlying cellular mechanisms by which δ-HCH affects neuronal or neuroendocrine function.

Hexachlorocyclohexanes (HCHs) can reach the environment through their use as pesticides. Most human exposures also occur through ingestion of plants, animals, and dairy products (Doong et al., 1999). HCH isomers have been detected at a number of hazardous waste sites. δ-HCH, a lipophilic organochlorine pesticide, was found to stimulate ryanodine-sensitive Ca^{2+} channels in endoplasmic reticulum derived from cardiac and skeletal muscle and brain tissue (Pessah et al., 1992). This isomer has also been reported to mobilize Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores and to inhibit Ca^{2+} entry induced by the depletion of Ca^{2+} stores in basophilic leukemia cells (Mohr et al., 1995). δ-HCH was thought to be more potent than γ-HCH in increasing intracellular Ca^{2+} and producing cytotoxicity (Rosa et al., 1997b). Previous reports have demonstrated that δ-HCH enhanced the current induced by γ-aminobutyric acid (GABA) in rat dorsal root ganglion cells (Nagata and Narahashi, 1995) and, in both human embryonic kidney cells and *Xenopus* oocytes in which GABA receptors were expressed (Belelli et al., 1996; Nagata et al., 1996; Aspinwall et al., 1997; Belelli et al., 1999). On the other hand, a recent study also showed that δ-HCH induced a Ca^{2+}-dependent membrane current that was selective for K^+ ions in phospholipid bilayer membranes (Buck and Pessah, 1999).

Large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are present in neurons and can mediate spike repolarization and the early afterhyperpolarization that follows each action potential (Kaczorowski et al., 1996). Presynaptic Ca^{2+} signals and transmitters released from nerve terminals were reported to be regulated by the activity of BK_{Ca} channels (Robitaille and Charlton, 1992; Sun et al., 1999), and the activity of these channels may mediate prejunctional inhibition in peripheral nerves (Robitaille et al., 1992; Sun et al., 1999). The activity of BK_{Ca} channels was also thought to play a role in controlling the hormonal secretion by altering the duration and frequency of action potentials (Robitaille and Charlton, 1992; Kaczorowski et al., 1996).

Therefore, the goal of the present study was: 1) to examine the effect of δ-HCH on voltage-dependent K^+ and Ca^{2+} cur-

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ABBREVIATIONS: HCH, hexachlorocyclohexane; GABA, γ-aminobutyric acid; IP_3, inositol 1,4,5-trisphosphate hexasodium; $I_{K(Ca)}$, Ca^{2+}-activated K^+ current; BK_{Ca} channel, large conductance Ca^{2+}-activated K^+ channel.

rents in GH_3 cells, 2) to study the effect of δ-HCH on Ca^{2+}-activated K^+ currents ($I_{K(Ca)}$), 3) to address the issue whether δ-HCH affects the activity and kinetic properties of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, and 4) to determine whether δ-HCH can affect $I_{K(Ca)}$ and intermediate-conductance K_{Ca} channels in neuroblastoma IMR-32 cells. These results indicate that, unlike γ-HCH, δ-HCH could increase the amplitude of $I_{K(Ca)}$ and these effects could lead to a decrease in the excitability of neurons or neuroendocrine cells.

Materials and Methods

Cell Preparation. GH_3 (a cell line from a rat anterior pituitary adenoma) cells were obtained from the Culture Collection and Research Center (CCRC-60015, Hsinchu, Taiwan). Cells were routinely cultured in 50-ml Ham's F-12 medium (Life Technologies, Grand Island, NY) that was supplemented with 15% horse serum (v/v), 2.5% fetal calf serum (v/v), and 2 mM L-glutamate (Life Technologies) in a 5% CO_2 atmosphere. Cells were subcultured once a week, and a new stock line was generated from frozen cells (frozen in 10% glycol in medium plus serum) every 3 months. The experiments were performed after 5 or 6 days of subcultivation (60 to 80% confluence).

Stock cultures of human neuroblastoma IMR-32 cells were also obtained from the Culture Collection and Research Center (CCRC-60014). IMR-32 cells were maintained in Eagle's minimal essential medium (Life Technologies) supplemented with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (v/v).

Electrophysiological Measurements. Immediately before each experiment, GH_3 or IMR-32 cells were dissociated and an aliquot of cell suspension was placed into a recording chamber affixed to the stage of an inverted phase-contrast microscope (Diaphot-200; Nikon, Tokyo, Japan). The microscope was coupled to a video camera system with a magnification of up to 1500× to continually monitor cell size during the experiments. Cells were bathed at room temperature (20–25°C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Ionic currents were recorded in the whole-cell or inside-out configuration of the patch-clamp technique, using a patch-clamp amplifier (RK-400; Biologic, Claix, France) (Hamill et al., 1981; Wu et al., 1999a). Patch pipettes (3 to 5 MΩ in bathing solution) were made from borosilicated glass capillary tubes (Kimble Products, Vineland, NJ) using a two-step pipette puller (PB-7; Narishige Scientific, Tokyo, Japan), and the tips were heat-polished with a microforge (MF-83; Narishige). A programmable stimulator (SMP-311; Biologic) was used to digitally generate voltage pulses, which were rectangular- or ramp-shaped. Tested drugs were applied by perfusion or added to the bath to obtain the final concentration indicated.

The signals, consisting of voltage and current tracings, were displayed on a digital storage oscilloscope (model 1602; Gould, Valley View, OH) and on-line recorded in a digital audiotape recorder (model 1204; Biologic). After the experiments, the data were fed back and stored in a Pentium III-grade computer (Lemel, Taipei, Taiwan) at 10 kHz through an analog/digital interface (Digidata 1200; Axon Instruments, Foster City, CA) using the Clampex subroutine of the pClamp 7.0 software (Axon Instruments). Voltage-activated currents recorded during whole-cell experiments were stored without leakage correction and analyzed using the Clampfit subroutine (Axon Instruments) or pClamp module in the Origin 6.0 software package (Microcal Software, Inc., Northampton, MA) to establish a current-voltage relationship for ionic currents.

Single Channel Analysis. Single channel currents were analyzed using Fetchan and Pstat subroutines in the pClamp software (Axon Instruments). Multi-Gaussian adjustments of the amplitude distributions between channels were used to determine unitary currents. The functional independence among channels was verified by comparing the observed stationary probability with the values calculated according to the binomial law. The number of active channels in a patch, N, was taken as the maximum number of channels simultaneously open under conditions of maximum open probability. When there was a sufficiently large number of independent observations, the opening probabilities ($N{\cdot}P_o$) of unitary current were evaluated by an iterative process that was continued until the χ^2 value was no longer changed. The single channel conductance was calculated by linear regression using mean values of the current amplitudes measured at different voltages

To assess the concentration-dependent effect of δ-HCH on the activation of BK_{Ca} channels, the opening probabilities of the channel current enhanced by various concentrations of δ-HCH (3 to 300 μM) were examined. Under symmetrical K^+ (145 mM) conditions, the inside-out configuration in which bath medium contained 0.1 μM Ca^{2+} was performed, and the holding potential was set at +60 mV. The opening probability of BK_{Ca} channels in the presence of 300 μM δ-HCH was considered to be 1.0 and the channel activity produced by various concentrations of δ-HCH was compared. The curve was fitted to the Hill equation by using a nonlinear regression analysis. The following form of the Hill equation was used: $y = y_{max} \times x^n/(c^n + x^n)$, where x is the concentration of δ-HCH, y is the relative $N{\cdot}P_o$, y_{max} is the maximal relative $N{\cdot}P_o$, and c and n are the concentrations required for a 50% increase and Hill coefficient, respectively.

To determine the effect of δ-HCH on the activation curve of BK_{Ca} channels, the ramp pulses from +20 to +140 mV with a duration of 1 s were digitally applied with the aid of a programmable stimulator (SMP-311). This made the measurements of single channel conductance and channel activation more efficient (Carl and Sanders, 1990). The activation curves were calculated by averaging current responses to 20 voltage ramps and dividing each point of the averaged current by the unitary amplitude of each potential after each leakage component was corrected. The rate of change of voltage ramps was 120 mV/s, a value that was found to be not distorted by the time constants of activation or deactivation (Carl and Sanders, 1990). The number of active channels in the patch N was also counted at the end of each experiment by perfusing a high K^+ solution with 100 μM Ca^{2+}. The number was then used to normalize the opening probability at each potential. To obtain values for the slope factor of the voltage-dependent activation and half-maximal activation voltage, the activation curves obtained before and after the addition of δ-HCH were fitted with Boltzmann functions of the form: relative $N{\cdot}P_o = n/\{1 + \exp[-K(V - V_{1/2})]\}$, where n is the maximal relative $N{\cdot}P_o$, K^{-1} is the slope factor of the voltage-dependent activation [i.e., change in potential required to produce an (exponential) e-fold increase in the activation], and $V_{1/2}$ is the voltage at which there is half-maximal activation. Curve fitting to the data presented here was performed by use of Origin 6.0 software (Microcal).

Open lifetime distributions measured before and after the addition of δ-HCH were fit with logarithmically scaled bin width by using the method of McManus et al. (1987). When the square root of the number of events in a bin was plotted against the open lifetime, each component of the open lifetime distribution appeared as a clear peak and the respective time constant would fall in the vicinity of this peak.

All values are reported as means ± S.E. The paired or unpaired Student's t test and ANOVA with a least-significance difference method for multiple comparison were used for the statistical evaluation of differences among means. Differences between the values were considered statistically significant when P was $<.05$ or $<.01$.

Drugs and Solutions. δ-HCH (1α,2α,3α,4α,5α,6β-hexachlorocyclohexane), γ-HCH (Lindane: 1α,2α,3β,4α,5α,6β-hexachlorocyclohexane), 17β-estradiol, and tetraethylammonium chloride were purchased from Sigma. Paxilline and ryanodine were obtained from Biomol (Plymouth Meeting, PA). Dantrolene, ruthenium red, inositol 1,4,5-trisphosphate hexasodium (IP_3), ionomycin, and tetrodotoxin were obtained from Research Biochemicals (Natick, MA). Clotrimazole was purchased from Calbiochem (La Jolla, CA). All other chemicals were of the highest quality commercially available. The

composition of normal Tyrode's solution was as follows (in mM); NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES-NaOH buffer 5 (pH 7.4). To record K^+ currents or membrane potential, the patch pipettes were filled with solution (in mM): KCl 140, KH_2PO_4 1, $MgCl_2$ 1, EGTA 0.1, Na_2ATP 3, Na_2GTP 0.1, and HEPES-KOH buffer 5 (pH 7.2). To record Ca^{2+} current, KCl inside the pipette solution was replaced with equimolar CsCl, and the pH was adjusted to 7.2 with CsOH. For the inside-out patch-clamp recording, high K^+-bathing solution contained (mM): KCl 145, $MgCl_2$ 0.53, and HEPES-KOH buffer 5 (pH 7.4), and the pipette solution contained (mM); KCl 145, $MgCl_2$ 2, and HEPES-KOH buffer 5 (pH 7.2).

Results

Stimulatory Effect of Ca^{2+}-Activated K^+ Current ($I_{K(Ca)}$) by δ-HCH in GH_3 Cells. In these experiments, GH_3 cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Each cell was held at the level of 0 mV to inactivate other voltage-dependent K^+ currents (Wu et al., 1999b). When cells were depolarized from 0 mV to various potentials with a duration of 300 ms at a rate of 0.1 Hz, a family of large noisy outward currents were elicited. The direction of this membrane current was reversed at −80 mV. The current amplitudes were increased with greater depolarization, reduced by the removal of extracellular Ca^{2+}, and enhanced by the presence of ionomycin (10 μM). These outward currents were thus identified as Ca^{2+}-activated K^+ currents ($I_{K(Ca)}$) (Wu et al., 1999b). When the cell was exposed to δ-HCH (30 μM), the amplitude of outward current was profoundly increased throughout the entire voltage-clamp step (Fig. 1). For example, when cells were depolarized from 0 to +70 mV, δ-HCH (30 μM) significantly increased the current amplitude measured at the end of the voltage pulses from 386 ± 96 to 1221 ± 150 pA ($P < .05$; $n = 10$). However, the effect of δ-HCH was poorly reversible after 5 min of washout. The averaged current-voltage (I–V) relationships for the current amplitude in the absence and presence of δ-HCH (30 μM) are illustrated in Fig. 1B.

To assess the nature of the outward current stimulated by δ-HCH, another series of experiments were conducted in bath solution containing different extracellular K^+ concentrations. The reversal potential in each cell was measured in the presence of δ-HCH. The data were then pooled and plotted as a function of extracellular K^+ concentrations (Fig. 1C). The finding showing a best-fit line through the averaged data revealed a slope of 57 mV per 10-fold increase in extracellular K^+. These results were interpreted to indicate that the δ-HCH-stimulated outward current in these cells followed the Nernstian behavior of a K^+-selective channel.

Lack of Effect of δ-HCH on Voltage-Dependent K^+ Outward Current ($I_{K(V)}$) in GH_3 Cells. To determine whether δ-HCH affects the amplitude of voltage-dependent I_K in these cells, the experiments were conducted in cells bathed in Ca^{2+}-free Tyrode's solution containing 1 μM tetrodotoxin and 0.5 mM $CdCl_2$. When the cell was held at −60 mV and various potentials ranging from −50 to +70 mV were applied, the addition of δ-HCH (30 μM) did not have effect on $I_{K(V)}$. However, a higher concentration of δ-HCH (100 μM) slightly inhibited the noninactivating component of $I_{K(V)}$ (Fig. 2A). For example, the current amplitude measured at the end of the voltage pulses from −60 to +70 mV was decreased by the presence of δ-HCH (100 μM) to 286 ± 25 pA from a control value of 333 ± 29 pA ($P < .05$, $n = 5$). Thus, these results indicate that δ-HCH at a concentration of 100 μM or above can suppress the amplitude of $I_{K(V)}$ in GH_3 cells.

Inhibitory Effect of δ-HCH on Voltage-Dependent L-type Ca^{2+} Current ($I_{Ca,\ L}$) in GH_3 Cells. The effect of δ-HCH on $I_{Ca,\ L}$ was also examined. The experiments were conducted with the Cs^+-containing pipette solution. As shown in Fig. 2B, the cell was held at −50 mV, and the depolarizing pulses (300 ms in duration) to 0 mV were delivered at 0.1 Hz. The presence of δ-HCH suppressed the amplitude of $I_{Ca,\ L}$ in a concentration-dependent manner. When cells were depolarized from −50 to 0 mV, the amplitude of $I_{Ca,\ L}$ was significantly decreased by δ-HCH (30 μM) to 195 ± 29 pA from a control value of 368 ± 26 pA ($P < .05$; $n = 7$). However, under the same voltage protocol, the presence of δ-HCH (30 μM) produced no significant change in the kinetics of activation or inactivation of $I_{Ca,\ L}$ [control: $\tau_{act} = 5 \pm 3$ ms, $\tau_{inact(f)} = 20 \pm 7$ ms, $\tau_{inact(s)} = 206 \pm 11$ ms; δ-HCH: $\tau_{act} = 5 \pm 3$ ms, $\tau_{inact(f)} = 21 \pm 6$ ms, $\tau_{inact(s)} = 208 \pm 13$ ms ($n = 6$)]. In addition, the inward tail current, which was evoked by the depolarizing pulses that activate $I_{Ca,\ L}$, was reduced by δ-HCH (Fig. 2B). However, there was no significant effect on the I–V relationship of $I_{Ca,\ L}$ in the presence of δ-HCH (data not shown). Conversely, unlike δ-HCH, γ-HCH (30 μM) did not significantly affect the amplitude of $I_{Ca,\ L}$. These results indicate that, like nifedipine or tetrandrine (Wu et al., 1998),

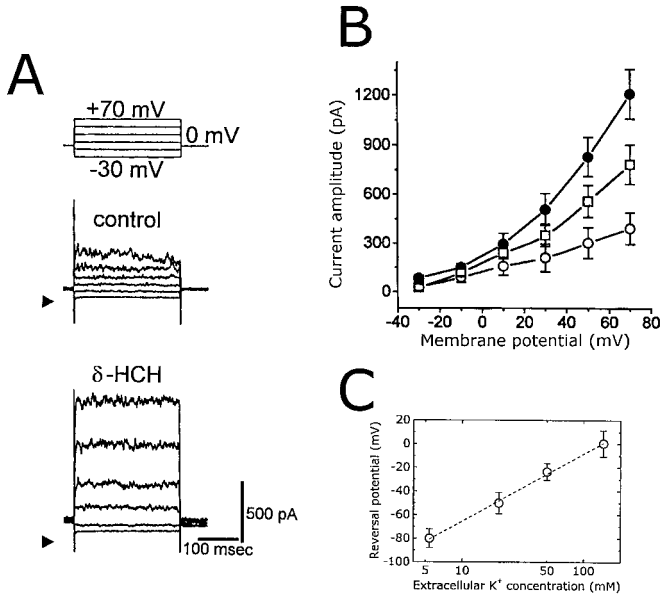

Fig. 1. Effect of δ-HCH on the current-voltage (I–V) relationships of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$). Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. A, superimposed original voltage and current traces obtained before and after the addition of δ-HCH (30 μM). Cell was held at 0 mV to inactivate other voltage-dependent K^+ currents, and the voltage pulses to various potentials in 20-mV increments were then applied at 0.1 Hz. The traces shown in the upper part are control, and those in the lower part were obtained 1 min after the addition of δ-HCH (30 μM). Arrows indicate zero current level. Voltage protocol is shown in the uppermost part. B, the averaged I–V relationships of the outward current measured at the end of voltage pulses in control (○), 1 min after the application of δ-HCH (●) and 5 min after the washout of the drug (□) are plotted (mean ± S.E.; $n = 9$ to 11 for each point). C, the relationship between the reversal potential of the δ-HCH-stimulated current and extracellular concentration of K^+ ions. Each cell was exposed to δ-HCH (30 μM) and each patch pipette was filled with K^+-containing solution. Each datum represents the mean ± S.E. ($n = 4$ to 5). The line was plotted semilogarithmically and was well fit by the linear regression analysis.

δ-HCH is capable of suppressing the amplitude of $I_{Ca,\ L}$ in GH_3 cells.

Effect of δ-HCH on the Activity of Large Conductance Ca^{2+}-Activated K^+ (BK_{Ca}) Channels in GH_3 Cells. Because $I_{K(Ca)}$ is a large, noisy, voltage-dependent, Ca^{2+}-sensitive current, and it results mainly from the opening of BK_{Ca} channels that have been previously studied (Wu et al., 1999b). Therefore, to determine whether the effect of δ-HCH on $I_{K(Ca)}$ is related to the increased amplitude of unitary current, the enhanced opening probability, or both, the activity of BK_{Ca} channels present in these cell was measured and analyzed. As shown in Fig. 3, under symmetrical K^+ (145 mM) conditions, the activity of BK_{Ca} channels can be observed in an excised inside-out patch. When the membrane patch was exposed to δ-HCH, the activity of channel opening was profoundly increased (Fig. 3). The opening probability of the channel measured at the level of +60 mV in the control (i.e., in the absence of δ-HCH) was found to be 0.015 ± 0.007 ($n = 8$). The addition of δ-HCH (30 μM) to the bath medium significantly increased the channel activity to 0.274 ± 0.015 ($P < .01$; $n = 8$). However, there was no significant difference in the amplitude of the unitary outward current between the absence and presence of δ-HCH [12.6 ± 1.2 pA ($n = 8$) versus 12.8 ± 1.4 pA ($n = 8$), $P > .05$]. Thus, it is clear that the presence of δ-HCH can increase the opening probability of BK_{Ca} channels in GH_3 cells.

Concentration-Dependent Stimulation of BK_{Ca} Channels by δ-HCH. The relationship between the concentration of δ-HCH and the opening probability of BK_{Ca} channels was further examined. These experiments were conducted with symmetrical K^+ concentration, the inside-out configuration in which bath medium contained 0.1 μM Ca^{2+} was performed, and the holding potential was set at +60 mV. As shown in Fig. 4A, δ-HCH (3–300 μM) increased the channel activity in a concentration-dependent manner. The EC_{50} value for δ-HCH-induced channel activity was 20 μM. In addition, the Hill coefficient was found to be 2.3, suggesting that there was a positive cooperativity for the stimulation of BK_{Ca} channels.

Effect of δ-HCH on the Activation Curve of BK_{Ca} Channels. Fig. 4B shows the activation curve of BK_{Ca} channels in the absence and presence of δ-HCH (30 μM). In these experiments, the activation curves of BK_{Ca} channels were obtained with the aid of the voltage ramp protocols. The ramp pulses were delivered from +20 to +140 mV with a duration of 1 s. The plots of opening probability of BK_{Ca} channels as a function of membrane potential were constructed and fit with Boltzmann function as described under *Materials and Methods*. In control, $n = 0.43 \pm 0.03$, $V_{1/2} = 95.3 \pm 1.3$ mV, and $K^{-1} = 11.9 \pm 0.5$ mV ($n = 6$), whereas in the presence of δ-HCH (30 μM), $n = 1.10 \pm 0.05$, $V_{1/2} = 81.2 \pm 1.1$ mV, and $K^{-1} = 12.0 \pm 0.4$ mV ($n = 6$). Thus, the

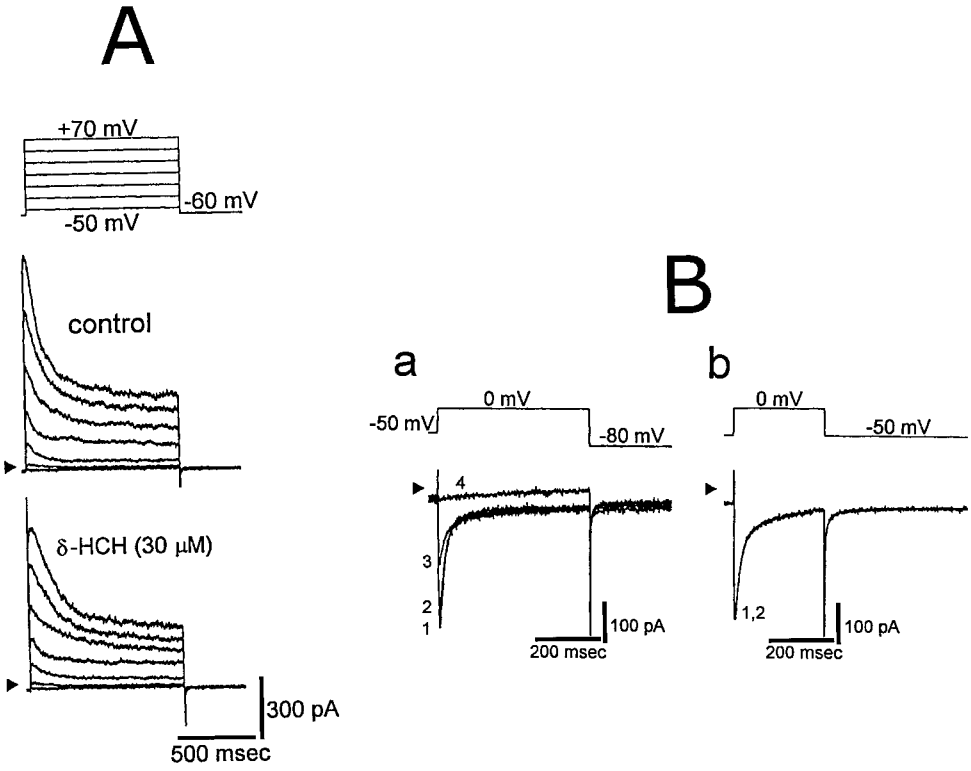

Fig. 2. Effect of δ-HCH on voltage-dependent K^+ outward ($I_{K(V)}$) and L-type Ca^{2+} inward currents ($I_{Ca,\ L}$) in GH_3 cells. A, superimposed current traces of $I_{K(V)}$ shown in the upper part are control, and those in lower part were recorded 1 min after addition of δ-HCH (100 μM). Cells, bathed in Ca^{2+}-free Tyrode's solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM), were depolarized from −60 to various potentials ranging from −50 to +70 mV in 20-mV increments at a rate of 0.1 Hz. Voltage protocol is shown in the uppermost part. Arrows indicate the zero current level. A, inhibitory effect of δ-HCH on L-type voltage-dependent Ca^{2+} currents ($I_{Ca,\ L}$) in GH_3 cells. Each patch pipette was filled with Cs^+-containing solution, and the cells were bathed in normal Tyrode's solution containing $CaCl_2$ (1.8 mM), tetrodotoxin (1 μM), and tetraethylammonium chloride (10 mM). In A (a), current traces were recorded when the cell was depolarized from −50 to 0 mV followed by a return to −80 mV. Trace 1 is control, and traces 2, 3, and 4 were obtained 1 min after the application of 10, 30, and 100 μM δ-HCH, respectively. In A (b), current traces were obtained when the cell was depolarized from −50 to 0 mV. Trace 1 is control, and trace 2 was obtained in the presence of γ-HCH (30 μM). Arrows shown in A indicate the zero current level.

presence of δ-HCH (30 μM) not only caused a 2.5-fold increase in the maximal opening probability of BK_{Ca} channels but also significantly shifted the activation curve to a less positive membrane potential by approximately 15 mV. However, there was no significant effect on the slope (i.e., K^{-1}) of the activation curve in the presence of δ-HCH. These results indicate that δ-HCH enhanced the activity of BK_{Ca} channels in a voltage-dependent fashion in GH_3 cells.

Lack of Effect of δ-HCH on Single Channel Conductance of BK_{Ca} Channels. It was examined whether δ-HCH affects the single channel conductance of BK_{Ca} channels. To construct the plots of current amplitude as a function of membrane potential, the voltage ramp pulses from +30 to +90 mV with a duration of 1 s were applied at a rate of 0.1 Hz. Figure 4C illustrates the *I–V* relationships of BK_{Ca} channels in the absence and presence of δ-HCH (10 and 30 μM). The single channel conductance of BK_{Ca} channels calculated from the linear *I–V* relationship in control (i.e., in the absence of δ-HCH) was 208 ± 8 pS (n = 12) with a reversal potential of 0 ± 1 mV (n = 12). The value of unitary conductance for these channels was found to be similar to that reported previously (Wu et al., 1999b) but not significantly different from that (209 ± 9 pS; $P > .05$, n = 10) measured in the presence of δ-HCH (30 μM). Thus, δ-HCH produced no significant change in the single channel conductance of BK_{Ca} channels, but enhanced the channel activity in these cells.

Effect of δ-HCH on Kinetic Behavior of BK_{Ca} Channels. Because it was observed that δ-HCH tended to prolong the open-time duration of BK_{Ca} channels, the effect of δ-HCH on the kinetic properties of BK_{Ca} channels was further characterized. As shown Fig. 5, in the absence of δ-HCH, the open-time histogram of BK_{Ca} channels at +60 mV could be fitted by a single-exponential curve with a mean open time of 1.9 ± 0.2 ms (n = 5). However, the presence of δ-HCH (10 μM) was found to increase the lifetime of the open state. A two-exponential function was thus needed to fit the open-time histogram obtained in the presence of δ-HCH (10 μM) (Fig. 5). When the membrane patches were exposed to δ-HCH (10 μM) intracellularly, the time constants for fast and slow components of open-time histogram were 1.9 ± 0.2 and 9.6 ± 0.5 ms, respectively (n = 5). Thus, δ-HCH can enhance the channel activity by increasing mean open time.

Comparison between the Effects of δ-HCH and Those of γ-HCH, 17β-Estradiol, Ryanodine, Dantrolene, IP_3, Ruthenium Red, and Paxilline. Effects of γ-HCH, 17 β-estradiol, ryanodine, dantrolene, IP_3, ruthenium red, and paxilline on the activity of BK_{Ca} channels in GH_3 cells were also examined and compared. As shown in Fig. 6, ryanodine (10 μM), dantrolene (10 μM), or IP_3 (10 μM) applied intracellularly had no significant effect on the channel activity. These compounds can affect Ca^{2+} release from intracellular Ca^{2+} stores. However, γ-HCH (10 μM) was found to suppress the activity of BK_{Ca} channels significantly. Likewise, ruthenium red (10 μM) or paxilline (1 μM) produce a profound inhibition of channel activity. Both ruthenium red and paxilline were reported to be a blocker of BK_{Ca} channels (Sanchez and

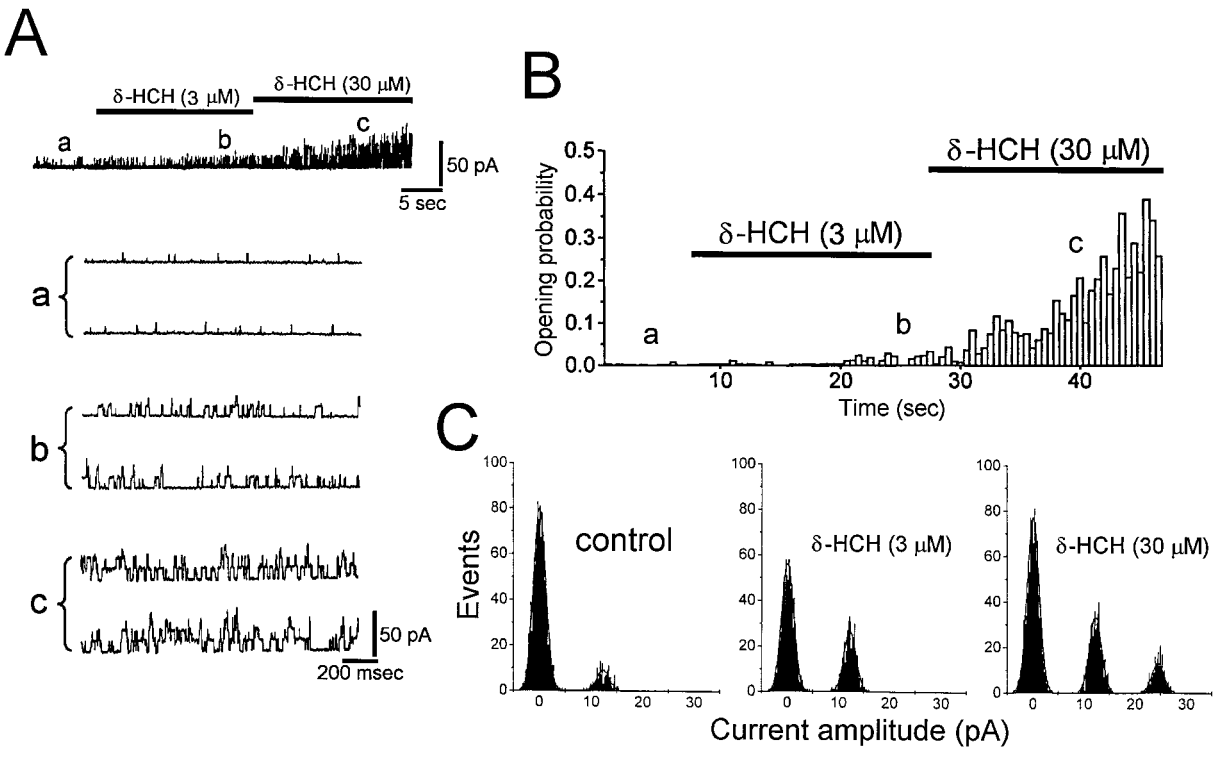

Fig. 3. Effect of δ-HCH on the activity of BK_{Ca} channels in an inside-out patch. A, original current traces showing the change in the channel activity after addition of δ-HCH. The experiments were conducted with symmetrical K^+ concentration (140 mM). The holding potential was +60 mV, and the bath medium contained 0.1 μM Ca^{2+}. The horizontal bar indicates the application of δ-HCH (3 and 30 μM). The lower parts in A show the current traces obtained in an expanded time scale. The original current traces (a, b, and c) shown in A correspond to those labeled a, b, and c in B. Channel openings are shown as an upward deflection. B, the opening probability for the activity of BK_{Ca} channels shown in A plotted against time of recording. Bin width is 0.5 s. The horizontal bars shown in the panel indicate the application of δ-HCH (3 and 30 μM). C, the amplitude histograms measured in the control and after addition of 3 and 30 μM δ-HCH. All data points shown in the amplitude histograms were fitted by one or more Gaussian distributions using the method of maximum likelihood. The closed state corresponds to the peak at 0 pA.

McManus, 1996; Wu et al., 1999a). On the other hand, like δ-HCH, 17β-estradiol was also noted to enhance the activity of BK_{Ca} channels significantly.

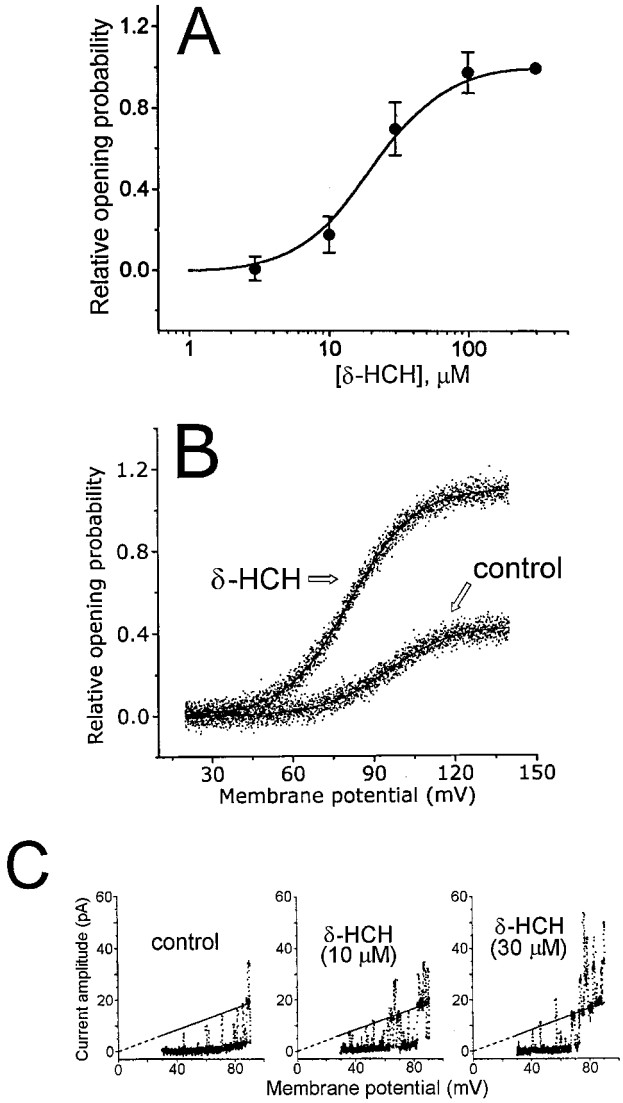

Fig. 4. The concentration- and voltage-dependent effects of δ-HCH on large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in GH_3 cells. The experiments were conducted with symmetrical K^+ concentration. Under the inside-out configuration, the holding potential was +60 mV and bath medium contained 0.1 μM Ca^{2+}. A, concentration-response curve for the δ-HCH-induced activation of BK_{Ca} channels. The opening probability of BK_{Ca} in the presence of δ-HCH (300 μM) was considered to be 1.0. The curve was fitted with the Hill equation as described under *Materials and Methods*. The EC_{50} value and maximal relative $N \cdot P_o$ were 20 μM and 1.0, respectively. The Hill coefficient was 2.3. Each point represents mean ± S.E. (n = 8 to 12). B, the effect of δ-HCH on the activation curve of BK_{Ca} channels. The activation curves were obtained when the ramp pulses were from +20 to +140 mV with a duration of 1 s applied. The smooth lines showed Boltzmann fits of the data yielding a $V_{1/2}$ of 95 mV for control and 81 mV when the membrane patch was exposed to δ-HCH (30 μM). C, lack of effect of δ-HCH on the single channel conductance of BK_{Ca} channels. Under symmetrical K^+ condition, the holding potential was +60 mV in an inside-out configuration and bath the solution contained 0.1 μM Ca^{2+}. The voltage ramp pulses from +30 to +90 mV with a duration of 1 s were used to measure single channel conductance. The straight lines with a reversal potential of 0 mV represent the *I–V* relationships of BK_{Ca} channels in the absence and presence of δ-HCH (10 and 30 μM).

Effect of δ-HCH and γ-HCH on Spontaneous Action Potentials in GH_3 Cells. The effect of δ-HCH and γ-HCH on membrane potentials was also examined. Under the current-clamp conditions, GH_3 cells, bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, had a resting membrane potential of −48 ± 7 mV (n = 26). The typical effects of δ-HCH and γ-HCH on spontaneous action potentials in these cells are illustrated in Fig. 7. About 70% of GH_3 cells were found to exhibit the repetitive firing of action potentials, which was Ca^{2+}-sensitive and inhibited by tetrandrine, a blocker of Ca^{2+} channel blocker (Wu et al., 1999b). When cells were exposed to δ-HCH (30 μM), spontaneous spiking discharge was significantly decreased to 0.4 ± 0.1 Hz from a control value of 0.9 ± 0.2 Hz ($P < .05$, n = 8). Cells were also hyperpolarized to −53 ± 9 mV from a control value of −46 ±

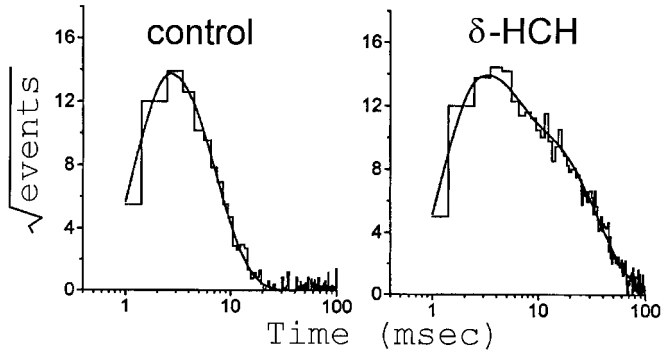

Fig. 5. Effect of δ-HCH on the kinetic properties of BK_{Ca} channels. The mean open-time histograms of the BK_{Ca} channel were obtained before and after the addition of δ-HCH (10 μM). Under a symmetrical K^+ condition, the holding potential was +60 mV in an inside-out configuration and the bath solution contained 0.1 μM. Data were obtained from a measurement of 563 channel openings with a total recording time of 2 min in the control (left), whereas data were measured from 764 channel openings with a total recording time of 30 s during the exposure to 10 μM δ-HCH (right). Open-time histograms were fitted by a one- or two-exponential function. Of note, the abscissa and ordinate show the logarithm of apparent open time (ms) and the square root of the number of events ($n^{1/2}$), respectively.

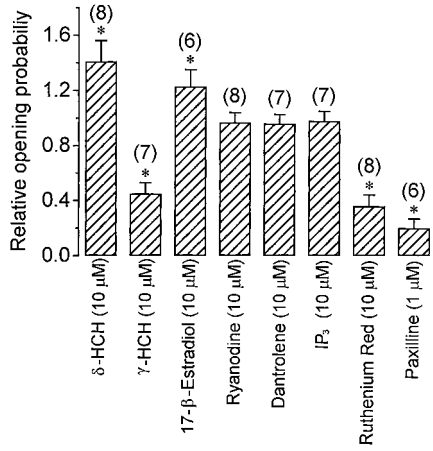

Fig. 6. Comparison between the effect of δ-HCH and those of γ-HCH, 17 β-estradiol, ryanodine, dantrolene, IP_3, ruthenium red, and paxilline on the activity of BK_{Ca} channels in GH_3 cells. Inside-out configuration was performed in these experiments. The potential held at each excised patch was +60 mV, and the bath medium contained 0.1 μM Ca^{2+}. The channel activity in the absence of each agent was considered to be 1.0, and the relative $N \cdot P_o$ after application of each agent was then plotted. The parentheses denote the number of cells examined. Mean ± S.E. * Significantly different from controls ($P < .05$).

8 mV ($P < .05$, $n = 8$). In contrast, the firing frequency of action potential was increased by the addition of γ-HCH (30 μM). The presence of γ-HCH (30 μM) increased the repetitive firing of action potentials from 0.9 ± 0.2 to 1.3 ± 0.3 Hz ($P < .05$, $n = 7$).

Stimulatory Effect of δ-HCH on $I_{K(Ca)}$ in Neuroblastoma IMR-32 Cells. Because $I_{K(Ca)}$ or BK_{Ca} channels observed in GH_3 cells may be different from those in neurons, the effect of δ-HCH in neuroblastoma IMR-32 cells was also examined. As shown in Fig. 8, when cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and the voltage pulses from 0 mV to various potentials ranging from +10 to +70 mV in 20-mV increments were applied, the addition of δ-HCH (30 μM) produced an increase in the amplitude of $I_{K(Ca)}$ throughout the entire voltage-clamp step. For example, when the voltage pulses from 0 to +70 mV were evoked, 30 μM δ-HCH significantly increased the current amplitude to 432 ± 42 pA from a control value of 202 ± 35 pA ($P < .05$, $n = 5$).

To further characterize the effect of δ-HCH on $I_{K(Ca)}$, the single channel experiments with an inside-out configuration were also performed in neuroblastoma IMR-32 cells. In these experiments, cells are bathed in symmetrical K^+ concentration, the holding potential was +60 mV and bath medium contained 0.1 μM Ca^{2+}. As shown in Fig. 9, the activity of intermediate-conductance K_{Ca} (IK_{Ca}) channels in these cells was observed. When clotrimazole (10 μM) was applied to the bath, the channel activity was significantly suppressed (data not shown). However, when the patch membrane was intracellularly exposed to δ-HCH (30 μM), the channel activity was greatly increased. The opening probability of IK_{Ca} channels measured at +60 mV in control was found to be 0.009 ± 0.001 ($n = 5$). After addition of 30 μM δ-HCH into the bath, the channel activity was significantly increased to 0.546 ± 0.008 ($P < .01$, $n = 5$). However, as shown in Fig. 9B, the single channel conductance of IK_{Ca} channels between the absence and presence of δ-HCH did not differ significantly [72 ± 2 pS ($n = 5$) versus 73 ± 2 pS ($n = 5$), $P > .05$]. These data indicate that δ-HCH is also capable of stimulating the activity of IK_{Ca} channels present in neuroblastoma IMR-32 cells.

Discussion

The results presented here show that: 1) in GH_3 lactotrophs, δ-HCH can enhance the amplitude of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$); 2) δ-HCH does not affect the amplitude of voltage-dependent K^+ current; however, it suppresses voltage-dependent L-type Ca^{2+} inward current; 3) δ-HCH stimulates the activity of BK_{Ca} channels in a concentration-dependent manner, but does not change single channel conductance; 4) δ-HCH shifts the activation curve of BK_{Ca} channels to a less positive potential; 5) the δ-HCH-

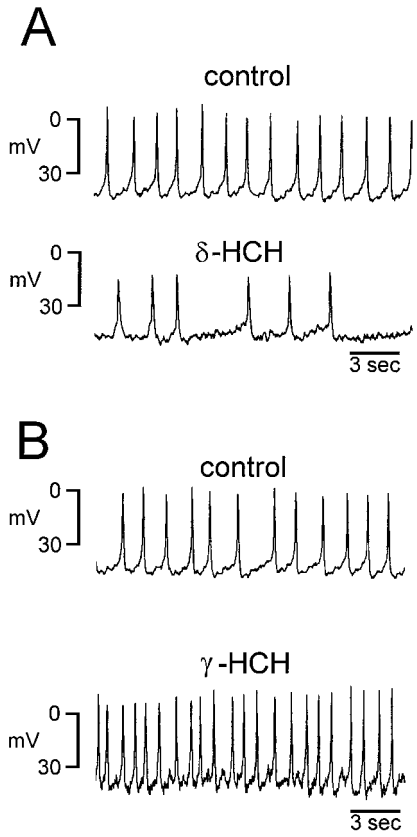

Fig. 7. Effect of δ-HCH and γ-HCH on the firing of action potentials in GH_3 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes were filled with K^+-containing solution. The change in membrane potential was measured under current-clamp condition. A, original potential traces showing the inhibitory effect of δ-HCH (30 μM) on spontaneous action potentials of GH_3 cells. B, original potential traces showing the stimulatory effect of γ-HCH (30 μM) on the firing of action potentials. Potential traces shown in the upper part of each panel are controls; those in the lower part were obtained 1 min after application of δ-HCH (A) or γ-HCH (B).

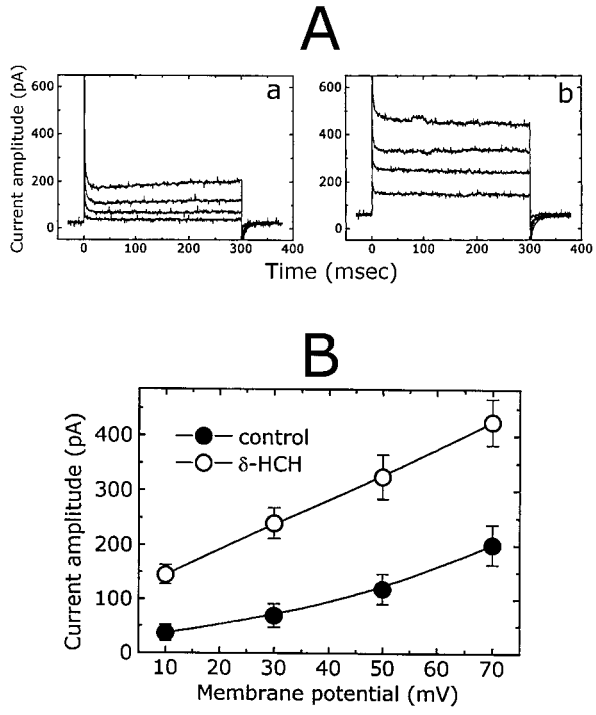

Fig. 8. Effect of δ-HCH on $I_{K(Ca)}$ in neuroblastoma IMR-32 cells. A, superimposed current traces in control (a) and during the exposure to 30 μM δ-HCH (b). Cells, bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, were held at 0 mV, and voltage pulses from +10 to 70 mV in 20-mV increments were applied at 0.05 Hz. B, the averaged current-voltage relations of $I_{K(Ca)}$ measured at the end of voltage pulses in the absence (●) and presence of 30 μM δ-HCH (○). Each point represents the mean ± S.E. ($n = 7$–11).

mediated increase in the opening probability is mainly caused by an increase in the number of long-lived openings; and 6) δ-HCH also stimulates $I_{K(Ca)}$ and enhances the activity of intermediate-conductance K_{Ca} (IK_{Ca}) channels in human neuroblastoma IMR-32 cells. This stimulatory action of BK_{Ca} and IK_{Ca} channels will cause membrane hyperpolarization, thus affecting the neuronal or neuroendocrine function, if the δ-HCH action in neurons or neuroendocrine cells in vivo is the same as those on these cells shown in this study.

Previous reports have shown that δ-HCH can modulate ryanodine-sensitive Ca^{2+} channels and stimulate Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores (Pessah et al., 1992; Rosa et al., 1997a). However, in our study performed in the inside-out configuration, δ-HCH applied intracellularly can enhance the activity of BK_{Ca} channels. Ryanodine, dantrolene, or IP_3 caused no significant change in the channel activity as compared with the control data. Therefore, it is unlikely that the δ-HCH-mediated increase in the activity of BK_{Ca} observed in GH_3 cells results from an increase in intracellular Ca^{2+} that is induced by Ca^{2+} release from internal stores, Ca^{2+} entry from the cell exterior, or both. Furthermore, the present finding, demonstrating that δ-HCH suppressed the amplitude of $I_{Ca,\ L}$, excludes the possibility that the effect of δ-HCH on BK_{Ca} channels depends on the increased Ca^{2+} influx, which is caused by the depolarizing stimuli that lead to the activation of $I_{Ca,\ L}$.

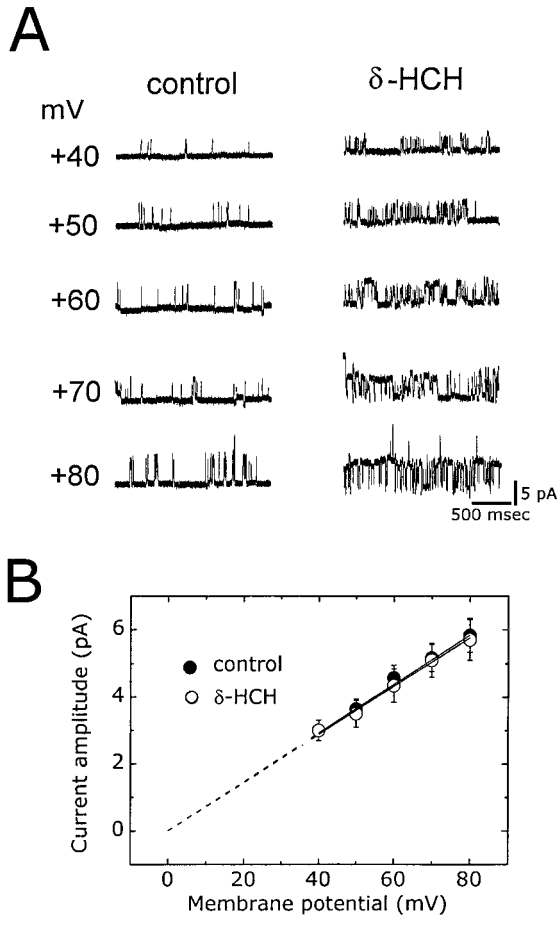

Fig. 9. Stimulatory effect of δ-HCH on intermediate-conductance K_{Ca} (IK_{Ca}) channels in neuroblastoma IMR-32 cells. Cells were bathed in symmetrical K^+ solution (145 mM) containing 0.1 μM Ca^{2+}, and the single channel experiments were conducted under the inside-out configuration. A, examples of IK_{Ca} channels in the absence (left) and presence (right) of δ-HCH (30 μM) measured from an excised patch at various membrane potentials. δ-HCH was applied to the bathing solution. The numbers shown at the beginning of each current trace mark the voltage applied to the patch pipette. Upward deflections are the opening events of the channel. B, the *I–V* relations of IK_{Ca} channels in the absence (●) and presence (○) of δ-HCH (30 μM). Note that the single channel conductance in the absence and presence of δ-HCH is nearly identical. Each point represents mean ± S.E. (n = 6–8).

HCH (β-isomer) was reported to exert an estrogen-like effect in human breast cancer cells (Steinmetz et al., 1996). In our study, 17 β-estradiol (30 μM) did also stimulate the activity of BK_{Ca}, when it was applied intracellularly. This result is consistent with a previous study indicating the direct stimulation of BK_{Ca} channels in endothelial cells (Rusko et al., 1995). It remains to be clarified whether δ-HCH and 17 β-estradiol might act on the same recognition site to interact with the BK_{Ca} channels expressed in GH_3 cells. However, the effect of δ-HCH on BK_{Ca} channels might be direct and independent of its binding to estrogen receptors, because the single channel experiments were performed in an excised inside-out membrane patch. In addition, the present result showing that no significant effect of IP_3 on the activity of BK_{Ca} channels was found suggests that the action of δ-HCH does not appear to be relevant to its structural similarity to IP_3 (Mohr et al., 1995).

The δ-HCH concentration used to produce a neurodepressant effect was found to be close to its EC_{50} value for the stimulation of BK_{Ca} channels (Pomes et al., 1994; Nagata and Narahashi, 1995; Aspinwall et al., 1997; Rosa et al., 1997a; Belelli et al., 1999). δ-HCH was also effective in stimulating the activity of IK_{Ca} channels in neuroblastoma IMR-32 cells. On the other hand, the EC_{50} value for the potentiation of GABA-evoked currents in oocytes expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination and the mutant ρ_{11307S} receptor was 3.4 and 38 μM, respectively (Belelli et al., 1996, 1999). Thus, there might be a link between the effects of δ-HCH on neurons and its stimulating effect on BK_{Ca} channels. The present experiments also found that δ-HCH is independent of the presence of internal Ca^{2+} (data not shown). It is likely that δ-HCH does not exert its effect via an increase in the affinity of Ca^{2+} ions for the Ca^{2+} binding site in the membrane. However, in our study, the presence of δ-HCH could produce a shift of 15 mV to a less positive potential in the activation curve of BK_{Ca} channels. Therefore, δ-HCH can enhance the activity of BK_{Ca} channels in a voltage-dependent fashion, and its interaction with these channels would be dependent on the pre-existing level of membrane potential or the concentration of δ-HCH used.

A recent study (Silvestroni et al., 1997) showed that the γ-isomer of HCH produced membrane depolarization in human sperm. The present results showing the inhibitory effect of γ-HCH on the activity of BK_{Ca} channels can account for this finding. However, in our study, there is no evidence showing that γ-HCH can increase the amplitude of Ca^{2+} inward current in GH_3 cells. It is thus possible that the effects of γ-HCH on the cell viability or the c-*fos* expression (Vendrell et al., 1992; Tusell et al., 1994; Barron et al., 1995; Silvestroni et al., 1997) are related to its indirect stimulation of Ca^{2+} channels that can be evoked by membrane depolarization.

Of interest, our data demonstrated that HCHs showed the stereospecificity in their interactions with BK_{Ca} channels.

Unlike δ-HCH, γ-HCH was found to suppress the activity of BK_{Ca} channels. Indeed, a number of studies have demonstrated that γ-HCH suppressed the amplitude of GABA-induced current, whereas δ-HCH enhanced it in cortical neurons (Pomes et al., 1994), in rat dorsal root ganglion neurons (Nagata and Narahashi, 1995), and in a human embryonic kidney cell line in which GABA receptor subunits were expressed (Nagata et al., 1996). It was also reported that the cytotoxic or cardiostimulatory effects of δ-HCH and γ-HCH could result from the differential mechanisms through which these two agents act on the Ca^{2+} release from internal stores (Pessah et al., 1992; Rosa et al., 1997a,b). More importantly, in addition to its interaction with the $GABA_A$ receptor Cl^- channel complex (Bloomquist, 1992; Cristofol and Rodriguez-Farre, 1993; Pomes et al., 1994; Narahashi, 1996; Narahashi et al., 1998), γ-HCH may produce an inhibitory effect on BK_{Ca} channels. This effect might also contribute to its action on the reduction of noradrenaline release (Cristofol and Rodriguez-Farre, 1993, 1994), given that there would be an increase in hormonal secretion in the presence of BK_{Ca} channel blockers.

Previous reports have shown that δ-HCH may disorganize the lipid bilayer in erythrocytes (Verma and Singhal, 1991; Bhalla and Agrawal, 1998), in testicular plasma membrane (Srivastava et al., 1995), and in human sperm (Silvestroni et al., 1997). Similarly, in our study, δ-HCH at a concentration of 100 μM produced an initial large increase in channel activity in inside-out patches; however, a disruption of the membrane and loss of membrane patch always accompanied this. Furthermore, the stimulation of $I_{K(Ca)}$ by δ-HCH was found to be slowly developing and not easy to fully wash out. These observations could be interpreted to mean that this compound might be able to partition into the membrane to produce its actions. The lipophilic nature of δ-HCH seemed to explain the present finding that the degree of reversibility of δ-HCH was time-dependent. Furthermore, the present results demonstrated that δ-HCH applied intracellularly produced a fraction of channel openings to shift to longer-lived openings, resulting in two open kinetic states. It would be of interest to determine whether the lipophilicity of δ-HCH or its effect on membrane disorganization is related to its prolongation in open-time duration of BK_{Ca} channels.

In our study, a steep Hill slope of 2.3 for the δ-HCH-stimulated activity of BK_{Ca} channels was found. This result suggests that the binding of more than one molecule is required for its stimulatory effect on the BK_{Ca} channel activity. Previous reports have demonstrated that the δ-HCH is a potent positive allosteric modulator of GABA-evoked currents (Belelli et al., 1996). The Hill coefficient for the δ-HCH-mediated potentiation of GABA-evoked currents recorded from oocytes expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination or the splice variant of the *Rdl* subunit was about 4 (Belelli et al., 1996). However, δ-HCH was recently found to have no effect on GABA-evoked currents in oocytes expressing the wild type ρ_1 receptor (Belelli et al., 1999). In the present study, because we measured the activity of single channel current in the inside-out configuration, it is possible that δ-HCH interacts with the channel protein per se. It thus remains to be clarified whether, in addition to binding to the distinct sites on the $GABA_A$ receptor protein, δ-HCH directly regulates the channel protein of the $GABA_A$ receptor Cl^- channel complex, although δ-HCH alone did not induce an inward current (Belelli et al., 1996).

In summary, the present study provides evidence that δ-HCH induced the change in the activity of BK_{Ca} channels in GH_3 cells. This finding will be of great help in the study of the underlying mechanisms through which HCHs interact with BK_{Ca} or IK_{Ca} channels expressed in neurons or neuroendocrine cells.

Acknowledgment

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