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Effect of arvanil (*N*-arachidonoyl-vanillyl-amine), a nonpungent anandamide–capsaicin hybrid, on ion currents in NG108-15 neuronal cells

Yuk-Keung Lo[a,b], Hung-Ting Chiang[c,d], Sheng-Nan Wu[d,e,*]

[a]*Section of Neurology, Kaohsiung Veterans General Hospital, Kaohsiung City, Taiwan, ROC*
[b]*National Yang-Ming University, Taipei City, Taiwan, ROC*
[c]*Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung City, Taiwan, ROC*
[d]*Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung City, Taiwan, ROC*
[e]*Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung City, Taiwan, ROC*

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Abstract

The effects of arvanil (*N*-arachidonoyl-vanillyl-amine), a structural hybrid between capsaicin and anandamide, on ion currents in a mouse neuroblastoma and rat glioma hybrid cell line, NG108-15, were examined with the aid of the whole-cell voltage-clamp technique. Arvanil (0.2–50 μM) caused an inhibition of voltage-dependent L-type Ca^{2+} current ($I_{Ca,L}$) in a concentration-dependent manner. Arvanil produced no change in the overall shape of the current–voltage relationship of $I_{Ca,L}$. The IC_{50} value of arvanil-induced inhibition of $I_{Ca,L}$ was 2 μM. Arvanil (5 μM) could shift the steady-state inactivation curve of $I_{Ca,L}$ to a more negative potential by approximately −15 mV. No effect of arvanil (20 μM) on delayed rectifier K^+ current ($I_{K(DR)}$) was observed; however, capsaicin (20 μM), glyceryl nonivamide (20 μM) and capsinolol (20 μM) suppressed it significantly. Arvanil (20 μM) caused a slight reduction in the amplitude of *erg* (*ether-à-go-go*-related)-mediated K^+ current ($I_{K(erg)}$) without modifying the activation curve of this current, while capsaicin and glyceryl nonivamide were more effective in suppressing $I_{K(erg)}$. Under current-clamp configuration, arvanil decreased the firing frequency of action potentials. Arvanil-mediated inhibition of $I_{Ca,L}$ appeared to be independent of its binding to either vanilloid or cannabinoid receptors. The channel-blocking properties of arvanil may, at least in part, contribute to the underlying mechanisms by which it affects neuronal or neuroendocrine function.

Keywords: Arvanil; L-type Ca^{2+} current; Delayed rectifier K^+ current; *erg*-Mediated current; NG108-15 cells

1. Introduction

Arvanil (*N*-arachidonoyl-vanillyl-amine), a synthesized nonpungent anandamide–capsaicin hybrid [1], was reported to behave as a ligand at both cannabinoid CB1 and vanilloid VR1 receptors, and to be a compound with therapeutic importance [1–3]. Arvanil also acts as a potent inhibitor of the anandamide transporter [1,3] and thus, is able to activate cannabinoid CB1 receptors indirectly by increasing the levels of anandamide. *In vivo* systemically administered arvanil was found to be extremely potent in the mouse "tetrad" of cannabinoid effects [4]. Several reports have also suggested the possible existence of non-cannabinoid, non-vanilloid molecular targets for arvanil and its homologues [4–7]. The neurobehavioral effects induced by arvanil appeared to be not necessarily related to the activation of either cannabinoid or vanilloid receptors [4]. In addition, it has been reported that arvanil-induced inhibition of spasticity and persistent pain was independent of activation of these two receptors [6,7].

Previous studies have demonstrated that cannabinoid CB1 receptor agonist produced inhibition of Ca^{2+} current through N-type or P/Q-type Ca^{2+} channels but not through dihydropyridine-sensitive L-type Ca^{2+} channels [8–10]. A previous report also showed that activation of the CB1

* Corresponding author. Tel.: +886-7-3422121-1507; fax: +886-7-3468056.
E-mail address: snwu@isca.vghks.gov.tw (S.-N. Wu).

Abbreviations: $I_{Ca,L}$, voltage-dependent L-type Ca^{2+} current; $I_{K(DR)}$, delayed rectifier K^+ current; $I_{K(erg)}$, *erg* (*ether-à-go-go*-related)-mediated K^+ current; *I–V*, current–voltage; TEA, tetraethylammonium chloride; ANOVA, analysis of variance.

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receptors expressed in cerebral vascular smooth muscle cells could inhibit $I_{Ca,L}$ possibly through a pertussis toxin-sensitive GTP-binding protein [11]. However, the anandamide-mediated inhibition of $I_{Ca,L}$ did not appear to be a consequence of its binding to cannabinoid receptors [12–14]. On the other hand, a number of studies have demonstrated that capsaicin and other nonpungent capsaicin-related compounds can inhibit the amplitude of $I_{Ca,L}$ and voltage-dependent K^+ current [15–20]. Such an inhibition was thought to be direct and independent of the binding to vanilloid receptors. To our knowledge, the effects of arvanil on ion currents have not been extensively studied.

Therefore, the electrophysiological effects of arvanil and other structurally related compounds in NG108-15 cells were investigated in this study. Undifferentiated NG108-15 cells have been previously reported to express dihydropyridine-sensitive L-type Ca^{2+} channels at our laboratory [21]. It has been shown that these cells could express cannabinoid receptors and the inhibition of Ca^{2+} currents caused by cannabinoid receptor ligands appeared to be pertussis toxin-sensitive [22]. In this study, we sought to (1) determine whether arvanil has any effect on $I_{Ca,L}$ in NG108-15 cells; (2) compare the potency of other related compounds in blocking the amplitude of $I_{Ca,L}$; (3) examine the effect of arvanil on other types of ion currents, including $I_{K(DR)}$ and $I_{K(erg)}$; and (4) ascertain whether arvanil can affect the firing frequency of spontaneous action potentials in these cells. The present results indicate that the underlying arvanil-mediated effect on ion currents in NG108-15 cells can significantly contribute to the change in membrane potential.

2. Materials and methods

2.1. Cell preparation

The clonal strain NG108-15 cell line, originally formed by Sendai virus-induced fusion of the mouse neuroblastoma clone N18TG-2 and the rat glioma clone C6 BV-1, was obtained from the European Collection of Cell Cultures. Cells were grown in monolayer culture in 50-mL plastic culture flasks in a humidified environment of 5% CO_2/95% air at 37°. Cells were maintained at a density of 10^6 mL^{-1} in 5 mL Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (v/v), and 2 mM L-glutamine [21]. The experiments were performed after 5 or 6 days of subcultivation (60–80% confluence). In a separate series of experiments, cells were incubated with ω-conotoxin GVIA (1 μM) at 37° for 4 hr, or with pertussis toxin (200 ng/mL) at 37° for 12 hr.

2.2. Electrophysiological measurements

Immediately before each experiment, NG108-15 cells were dissociated and an aliquot of cell suspension was transferred to a recording chamber positioned on the stage of a Diaphot-200 inverted microscope (Nikon). Cells were bathed at room temperature (20–25°) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The patch pipettes were made from borosilicate glass capillaries (Kimax-51) using a vertical two-stage electrode puller (PB-7, Narishige), and the tips were fire-polished with a microforge (MF-83, Narishige). Patch pipettes had resistances between 3 and 5 MΩ in normal Tyrode's solution. Ion currents were recorded with glass pipettes in tight-seal whole cell recording by means of an RK-400 amplifier (Bio-Logic) or an Axopatch 200B amplifier (Axon Instruments) [23].

2.3. Data recording and analysis

The signals consisting of voltage and current tracings were monitored with a digital storage oscilloscope (Model 1602, Gould) and simultaneously stored in a Pentium-III personal computer (TravelMate 525TE, Acer). Current traces were filtered at 1 kHz. The data were digitized at 10 kHz with a Digidata 1320A device (Axon Instruments) controlled by the Clampex subroutine of the pCLAMP 8.02 software package (Axon Instruments). Cell membrane capacitance of 15–25 pF (21.4 ± 2.5 pF, $N = 13$) was compensated. Series resistance that was in the range of 5–15 MΩ was electronically compensated. Voltage-activated currents measured during whole-cell experiments were analyzed using the Clampfit subroutine (Axon Instruments), the Origin 6.0 software package (Microcal Software, Inc.), or custom-made macros in Excel (Microsoft) to establish an I–V relationship for ion currents.

To calculate the percentage inhibition of arvanil, each cell was depolarized from a holding potential of −50 to 0 mV, and the amplitude of $I_{Ca,L}$ obtained during the exposure to different concentrations of arvanil was compared with the control value. The concentration-dependent effect of arvanil on the inhibition of $I_{Ca,L}$ was fitted with a Hill function using a nonlinear least-square fitting algorithm [24]. That is, percentage inhibition $= (E_{max} \times [C]^{n_H})/(IC_{50}^{n_H} + [C]^{n_H})$, where $[C]$ represents the arvanil concentration; IC_{50} and n_H are the concentration required for a 50% inhibition and the Hill coefficient, respectively; and E_{max} is the arvanil-induced maximal inhibition of $I_{Ca,L}$.

To determine the effect of arvanil on the steady-state inactivation curve of $I_{Ca,L}$, the relationships between the membrane potentials and the normalized amplitude of $I_{Ca,L}$ with or without addition of arvanil (5 μM) were fitted by the Boltzmann function: $I = I_{max}/\{1 + \exp[(V - a)/b]\}$, where I_{max} represents the maximal activated $I_{Ca,L}$; V is the membrane potential in mV; a is the membrane potential for a half-maximal inactivation; and b is the slope factor of the inactivation curve of $I_{Ca,L}$. To examine the effect of arvanil on the steady-state activation curve of $I_{K(erg)}$, the relationships between the conditioning pulses and the normalized amplitudes of $I_{K(erg)}$ with or without the application of

arvanil (20 μM) were also constructed with fitted by the Boltzmann function: $I = I_{max}/\{1 + \exp[(V - a)/b]\}$, where I_{max} is the maximal activated $I_{K(erg)}$, V is the membrane potential in mV, a is the membrane potential for a half-maximal activation, and b is the slope factor of activation curve for $I_{K(erg)}$. Curve fitting to data presented here was performed by means of the Origin software (Microcal).

Values are provided as means ± SEM with sample sizes (N) indicating the number of cells examined. The paired or unpaired Student's *t*-test and one-way ANOVA with a least-significance difference method for multiple comparisons were used for the statistical evaluation of differences among means. A P value less than 0.05 or 0.01 was considered to be statistically significant.

2.4. Drugs and solutions

Arvanil ($\Delta^{5,8,11,14}$-*cis* eicosatetraenoyl-*N*-acyl-vanillyl-amide) was obtained from Cayman chemicals. Anandamide (arachidonylethanolamide), capsaicin, capsazepine and tetrodotoxin were purchased from Sigma Chemical Co. E-4031, pertussis toxin and ω-conotoxin GVIA were purchased from Biomol. Tetrodotoxin was obtained from Alomone and iodo-resinferatoxin was from Tocris. Azimilide was a gift from Proctor & Gamble. Capsinolol (*N*-[4-(2-hydroxy-3-(isopropyl-amino) proxyl)-3-methoxy-benzyl]-nonanamide) and glyceryl nonivamide were general gifts from Drs. Jwu-Lai Yeh and Yi-Ching Lo, Department of Pharmacology, Kaohsiung Medical University, Kaohsiung City, Taiwan [20,25,26]. Tissue culture media, L-glutamine, penicillin–streptomycin, fungizone, and trypsin were obtained from Life Technologies. All other chemicals were of laboratory grade obtained from standard sources.

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 5.5 (pH 7.4). To record $I_{K(erg)}$, high-K^+, Ca^{2+}-free solution contained a solution (in mM): KCl 130, NaCl 10, $MgCl_2$ 3, glucose 6, HEPES-KOH buffer 10 (pH 7.4). To record membrane potential or K^+ current, the patch pipettes were backfilled with a solution (in mM): K-aspartate 130, KCl 20, 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 measure Ca^{2+} current, K^+ ions inside the pipette solution were replaced with equimolar Cs^+ ions, and the pH was adjusted to 7.2 with CsOH.

3. Results

3.1. Effect of arvanil on $I_{Ca,L}$ in NG108-15 cells

The effect of arvanil on the amplitude of $I_{Ca,L}$ was examined in this study. In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM

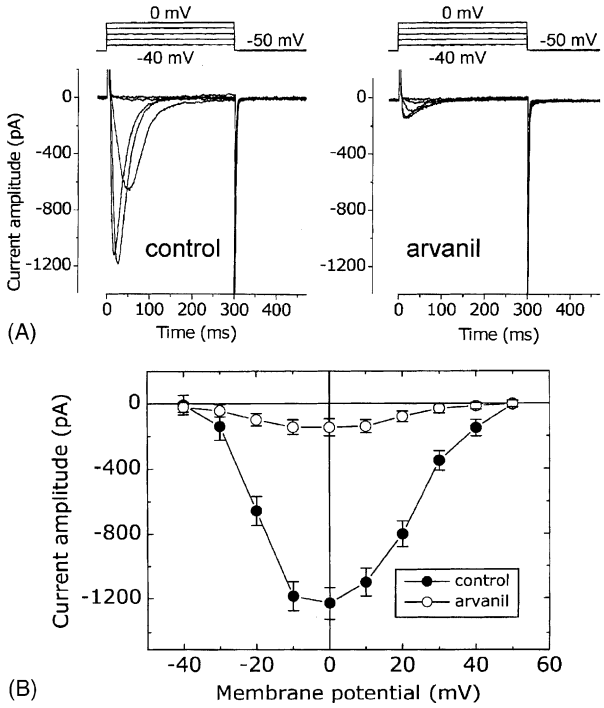

Fig. 1. The *I–V* relationships for $I_{Ca,L}$ before and during the exposure to arvanil in NG108-15 cells. Patch pipette was loaded with Cs^+-containing solution and cells were bathed in normal Tyrode's solution that contained 1.8 mM $CaCl_2$, 10 mM TEA and 1 μM tetrodotoxin. (A) Superimposed current traces obtained in the absence (left) and presence (right) of arvanil (5 μM). Ca^{2+} currents were elicited by depolarizing pulses from a holding potential of −50 mV to test potentials of −40 to 0 mV in 10 mV increments. The voltage protocol is shown in the upper part. (B) Averaged *I–V* relationship of the peak $I_{Ca,L}$ in control (filled circles) and during exposure to 5 μM arvanil (open circles). (mean ± SEM; N = 7–10 for each point). Of note, after application of arvanil, $I_{Ca,L}$ was decreased over the entire range of test potentials.

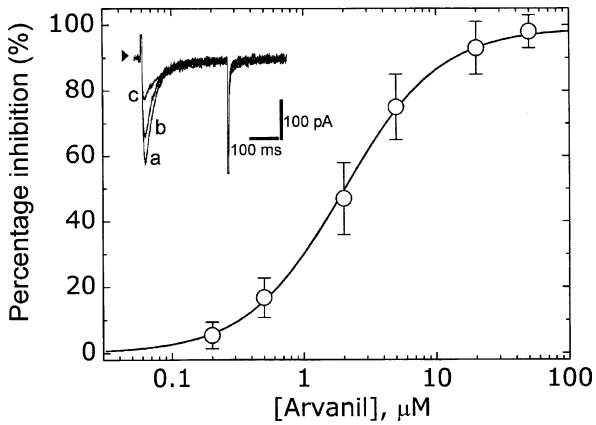

Fig. 2. Concentration–response relationship for arvanil-induced inhibition of $I_{Ca,L}$ in NG108-15 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and the depolarizing pulses from −50 to 0 mV were applied with a duration of 300 ms. Inset indicates current traces obtained in the absence and presence of arvanil. Labeled (a) is the control, and labeled (b) and (c) are results obtained after addition of 0.5 and 5 μM arvanil, respectively. Arrowhead shown in inset indicates the zero current level. The amplitude of peak $I_{Ca,L}$ obtained during cell exposure to arvanil was compared with the control value. The smooth line represents the best fit to the Hill equation as described in Section 2. The values for IC_{50}, maximally inhibited percentage of $I_{Ca,L}$, and Hill coefficient were 2 μM, 99% and 1.2, respectively. Each point represents the mean ± SEM (N = 4–8).

$CaCl_2$ and the recording pipettes were filled with a Cs^+-containing solution. As shown in Fig. 1, the effect of arvanil on $I_{Ca,L}$ was examined at different membrane potentials, and an *I–V* relationship of $I_{Ca,L}$ was constructed. When these two *I–V* curves shown in Fig. 1 were compared, it can be seen that the threshold potential (around −30 mV), the potential of maximum peak $I_{Ca,L}$ (around 0 mV), and the apparent reversal potential (around +50 mV) did not differ significantly between the absence and presence of arvanil (5 μM). Therefore, the presence of arvanil (5 μM) was capable of suppressing $I_{Ca,L}$ without modifying the overall shape of the *I–V* curves for $I_{Ca,L}$. After arvanil was removed, $I_{Ca,L}$ almost returned to the control level.

Fig. 2 shows the relationship between the concentrations of arvanil and the percentage inhibition of $I_{Ca,L}$. Arvanil (0.2–50 μM) decreased the amplitude of $I_{Ca,L}$ in a concentration-dependent manner. The half-maximal concentration required for the inhibitory effect of arvanil on $I_{Ca,L}$ was 2 μM, and arvanil at a concentration of 50 μM almost completely suppressed the amplitude of $I_{Ca,L}$. These results clearly demonstrate that arvanil has an inhibitory effect on $I_{Ca,L}$ in NG108-15 cells.

3.2. Effect of capsacin, capsazepine, capsinolol, glyceryl nonivamide, anandamide and arvanil on the amplitude of $I_{Ca,L}$

It have been reported that arvanil can bind to cannabinoid and vanilloid receptors [1–3]. Therefore, we also examined whether capsaicin-related compounds and anandamide affect the amplitude of $I_{Ca,L}$ present in NG108-15 cells. As shown in Fig. 3A, capsaicin (5 μM), capsazepine (5 μM), capsinolol (5 μM) and glyceryl nonivamide (5 μM) caused a reduction of $I_{Ca,L}$. However, anandamide

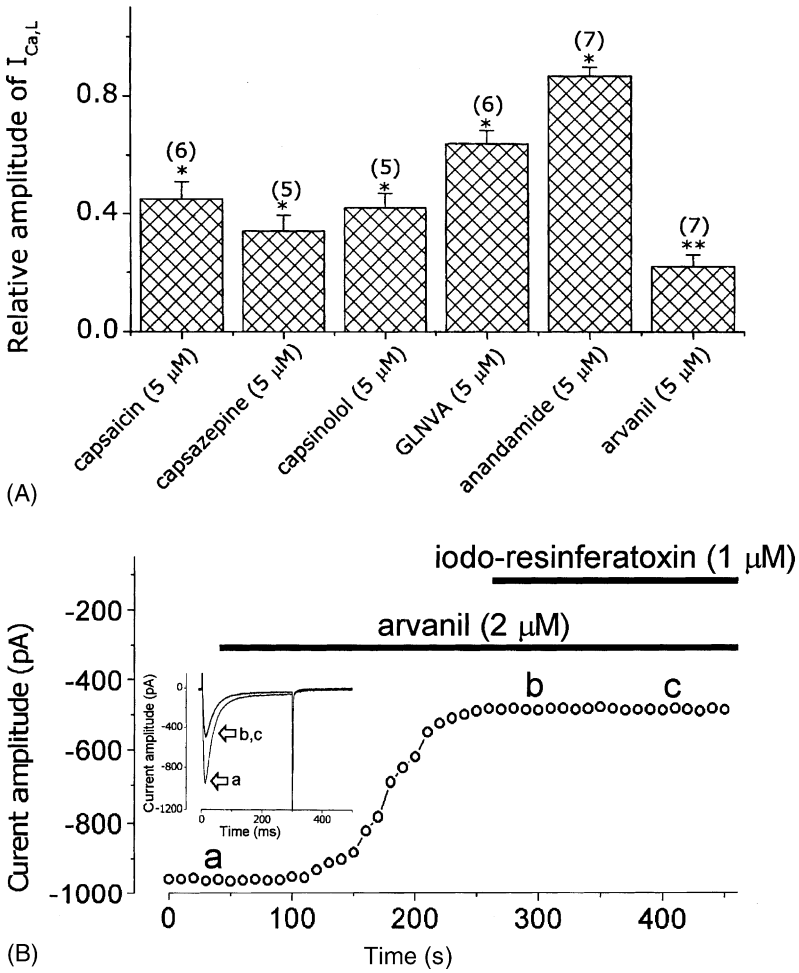

Fig. 3. Comparison between the effect of arvanil and those of capsaicin, capsazepine, capsinolol, glyceryl nonivamide (GLNVA), anandamide on the amplitude of $I_{Ca,L}$. NG108-15 cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Each cell was depolarized from −50 to 0 mV with a duration of 300 ms. (A) Bar graph showing the effect of various related compounds on the amplitude of $I_{Ca,L}$. The amplitude of $I_{Ca,L}$ in the control was considered to be 1.0 and the relative amplitude after application of each compound was plotted. The parentheses shown in each bar indicate the number of cells from which the data were taken. Mean ± SEM. (*) Significantly different ($P < 0.05$ by unpaired *t*-test) from control. (**) Significantly different ($P < 0.01$ by unpaired *t*-test) from control. (B) Lack of effect of iodo-resinferatoxin on arvanil-mediated inhibition of $I_{Ca,L}$ in NG108-15 cells. The cell was depolarized from −50 to 0 mV at a rate of 0.1 Hz. Time course in the change of the amplitudes of $I_{Ca,L}$ was plotted. Horizontal bars denote the application of arvanil (2 μM) and iodo-resinferatoxin (1 μM). The inset shown in (B) indicates original current traces marked by labels a, b and c in figure.

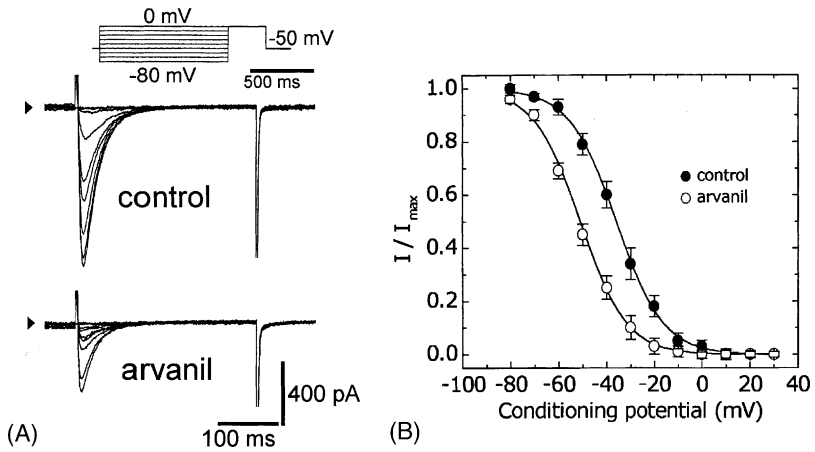

Fig. 4. Steady-state inactivation curve of $I_{Ca,L}$ in the absence and presence of arvanil. With the aid of a double-pulse protocol, the steady-state inactivation parameters of $I_{Ca,L}$ were obtained in the absence and presence of arvanil (5 μM). The conditioning voltage pulses with a duration of 1 s to various membrane potentials were applied from a holding potential of −50 mV. Following each conditioning pulse, a test pulse to 0 mV with a duration of 300 ms was applied to evoke $I_{Ca,L}$. (A) Superimposed current traces in the absence (upper part) and presence (lower part) of arvanil (5 μM). The upper part indicates the voltage protocol. Arrowheads indicate the zero current level. (B) Steady-state inactivation curves of $I_{Ca,L}$ obtained in the absence (filled circles) and presence (open circles) of arvanil (5 μM). The normalized amplitude of $I_{Ca,L}$ (I/I_{max}) was constructed against the conditioning potential and the curves were fitted by the Boltzmann equation as described in Section 2. Each point represents the mean ± SEM (N = 5–7).

(5 μM) caused a small but significant decrease in the amplitude of $I_{Ca,L}$ by approximately 13%. The results indicate that the inhibitory effect of arvanil on $I_{Ca,L}$ was more potent than that of capsaicin and its structurally related compounds. Capsazepine, a blocker of vanilloid receptors, also blocked $I_{Ca,L}$ [19] and anandamide had less inhibitory effect on it than arvanil. In addition, a further application of iodo-resinferatoxin (1 μM), a potent

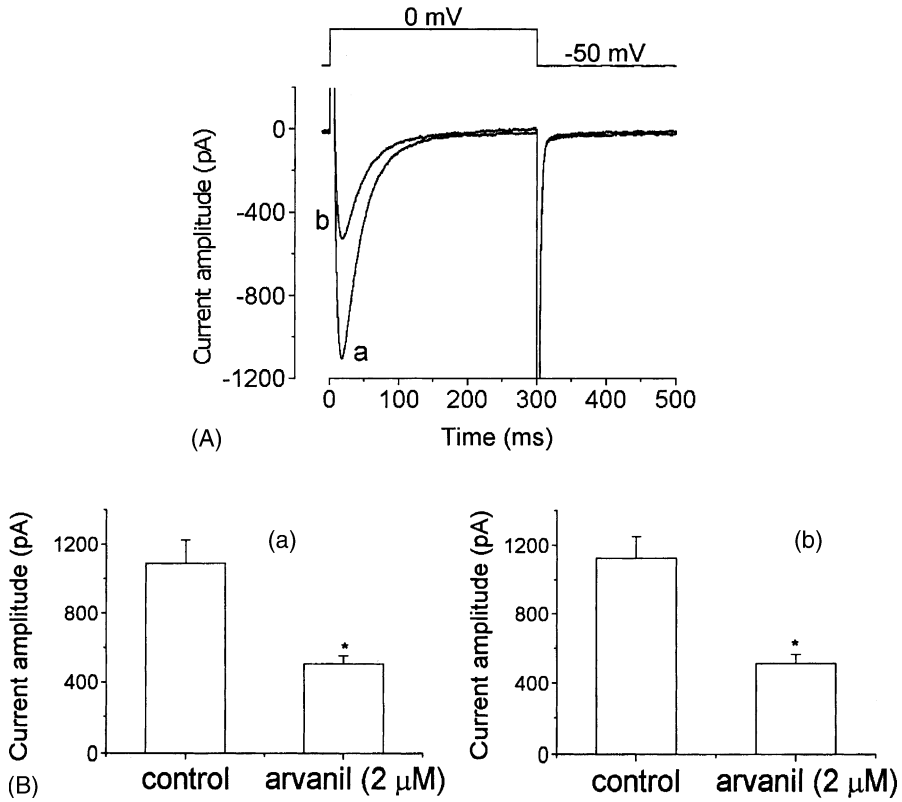

Fig. 5. Inhibitory effect of arvanil on $I_{Ca,L}$ in NG108-15 cells treated with pertussis toxin or ω-conotoxin GVIA. NG108-15 cells were preincubated with pertussis toxin (200 ng/mL) for 12 hr or with ω-conotoxin GVIA (1 μM) for 4 hr. The cell was depolarized from −50 to 0 mV. (A) Superimposed current traces obtained in the absence and presence of arvanil. In these experiments, pertussis toxin-treated cells were depolarized from −50 to 0 mV. Labeled (a) is the control and labeled (b) was obtained 2 min after application of arvanil (2 μM). (B) Bar graph showing the effect of arvanil (2 μM) on the amplitude of $I_{Ca,L}$ in NG108-15 cells treated with pertussis toxin (a) or ω-conotoxin (b). Each cell was depolarized from −50 to 0 mV. Each point represents the mean ± SEM (N = 5). (*) Significantly different from controls ($P < 0.05$ by paired t-test).

antagonist of vanilloid receptors [27], did not reverse arvanil-mediated inhibition of $I_{Ca,l}$ (Fig. 3B). There was no significant difference in the amplitude of $I_{Ca,L}$ between arvanil (2 μM) alone group and arvanil plus iodo-resiniferatoxin (1 μM) group (493 ± 13 pA [N = 5] versus 494 ± 15 pA [N = 5]). It is thus unlikely that the inhibitory effect of arvanil on $I_{Ca,L}$ is necessarily associated with its binding to either cannabinoid or vanilloid receptors.

3.3. Voltage-dependence of arvanil-induced inhibition of $I_{Ca,L}$

To characterize the inhibitory effect of arvanil on $I_{Ca,L}$, we also studied the voltage-dependence of the effect of arvanil on $I_{Ca,L}$. Fig. 4 shows the steady-state inactivation curve of $I_{Ca,L}$ obtained in the absence and presence of arvanil (5 μM). A two-step voltage pulse protocol was applied to the cells. In these experiments, a 1 s conditioning pulse to different membrane potentials preceded the depolarizing pulse (300 ms in duration) to 0 mV from a holding potential of −50 mV. The interval between two sets of voltage pulses was 60 s to prevent incomplete recovery of $I_{Ca,L}$. The relationships between the membrane potentials and the normalized amplitude of $I_{Ca,L}$ with or without addition of arvanil (5 μM) were plotted and fitted with the Boltzmann equation as described in Section 2. In control, $a = -36.1 \pm 1.3$ mV, $b = 9.8 \pm 0.9$ mV (N = 6), whereas in the presence of arvanil (5 μM), $a = -50.8 \pm 1.4$ mV, $b = 9.6 \pm 1.0$ mV (N = 6). Thus, arvanil cannot only suppress the maximal conductance of $I_{Ca,L}$, but also shift the inactivation curve to a hyperpolarized potential by approximately −15 mV. Conversely, no significant change in the slope (i.e. b value) of the inactivation curve was seen in the presence of arvanil. Taken together, these results indicate that arvanil is able to decrease the amplitude of $I_{Ca,L}$ in a voltage-dependent fashion in NG108-15 cells.

3.4. Effect of arvanil on $I_{Ca,L}$ in NG108-15 cells preincubated with pertussis toxin or ω-conotoxin GVIA

It has been shown that the activation of the cannabinoid CB1 receptors could inhibit the amplitude of $I_{Ca,L}$ and that this effect was associated with the coupling to a pertussis toxin-sensitive G protein [11,22]. The component of Ca^{2+} currents that is blocked by arvanil may be sensitive to inhibition by ω-conotoxin [22,28]. The effect of arvanil on $I_{Ca,L}$ was also assessed in cells treated with pertussis toxin (200 ng/mL) for 12 hr or with ω-conotoxin (1 μM) for 4 hr. However, as shown in Fig. 5, in these cells preincubated with pertussis toxin or ω-conotoxin GVIA, the inhibitory effect of arvanil on the amplitude of $I_{Ca,L}$ was unaltered. When the cells preincubated with pertussis toxin or ω-conotoxin GVIA were depolarized from −50 to 0 mV, arvanil (2 μM) reduced the amplitude of $I_{Ca,L}$ by about 50%. Thus, the inhibition effect of arvanil on this current does not appear to involve a pertussis toxin-sensitive G protein. The treatment of cells with ω-conotoxin GVIA did not prevent the inhibition of $I_{Ca,L}$ caused by arvanil.

3.5. Lack of effect of arvanil on $I_{K(DR)}$

We also examined whether arvanil affects the amplitude of $I_{K(DR)}$ present in these cells. In these experiments, cells were 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 different potentials ranging from –50 to +40 mV in 10 mV increments were applied, no significant difference in current amplitudes at each depolarizing step between the absence and presence of arvanil (20 μM) could be observed (Fig. 6A). Similar results were obtained in eight different cells. However, glyceryl nonivamide (20 μM), capsinolol (20 μM), capsaicin (20 μM) and capsazepine (20 μM) produced a significant reduction in the amplitude of $I_{K(DR)}$, while anandamide (20 μM) had little or no change in it (Fig. 6B). Glyceryl nonivamide, capsaicin and capsazepine have been shown to block

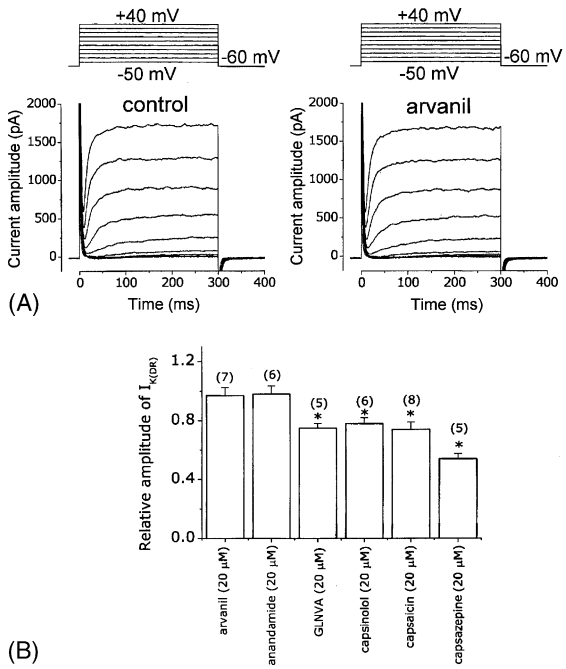

Fig. 6. Lack of effect of arvanil on $I_{K(DR)}$ in NG108-15 cells. In these experiments, cells, bathed in Ca^{2+}-free Tyrode's solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM), were held at the level of −60 mV, and the depolarizing pulses from −50 to +40 mV in 10 mV increments were applied at a rate of 0.05 Hz. In (A), superimposed current traces shown in the left are control, and those in the right are result obtained 2 min after application of arvanil (20 μM). The voltage protocol is shown in the upper part. (B) Comparison between the effect of arvanil (20 μM) and those of anandamide (20 μM), glyceryl nonivamide (GLNVA; 20 μM), capsinolol (20 μM), capsaicin (20 μM), and capsazepine (20 μM) on the amplitude of $I_{K(DR)}$. Each cell was depolarized from −60 to +40 mV. The amplitude of $I_{K(DR)}$ measured at the end of the depolarizing pulses in the control was considered to be 1.0, and the relative amplitude of $I_{K(DR)}$ after addition of each agent was plotted. The parentheses shown in each bar indicate the number of cells examined. (*) Significantly different from control ($P < 0.05$ by unpaired t-test).

voltage-dependent K^+ current [17–20]. Both glyceryl nonivamide and capsinolol are nonpungent capsaicin-related compounds, and capsazepine is a blocker of vanilloid receptors. Thus, it is clear that unlike $I_{Ca,L}$, $I_{K(DR)}$ present in NG108-15 cells was not subject to inhibition by arvanil.

3.6. Effect of arvanil on $I_{K(erg)}$ in NG108-15 cells

When cells were bathed in a high-K^+, Ca^{2+}-free solution, a family of large inward current on membrane hyperpolarization could be readily seen in NG108-15 cells. Examples of the currents elicited by a 1-s pulse to different voltages from a holding potential of −10 mV are illustrated in Fig. 7. These hyperpolarizing voltage pulses were found to induce an instantaneous current followed by a voltage- and time-dependent decay of K^+ inward current. These currents decayed at potentials more negative to −50 mV, and the decay became faster with greater hyperpolarization. The currents evoked by membrane hyperpolarization cells were sensitive to inhibition by azimilide (20 μM) and E-4031 (20 μM), and thus correspond to those described previously as an *erg* (*ether-à-go-go*-related)-mediated K^+ current (i.e. $I_{K(erg)}$) [29,30]. Arvanil (2 μM) did not affect the amplitude of $I_{K(erg)}$; however, arvanil at a concentration of 20 μM decreased $I_{K(erg)}$ significantly (Fig. 7). For example, when cells were hyperpolarized from −10 to −120 mV,

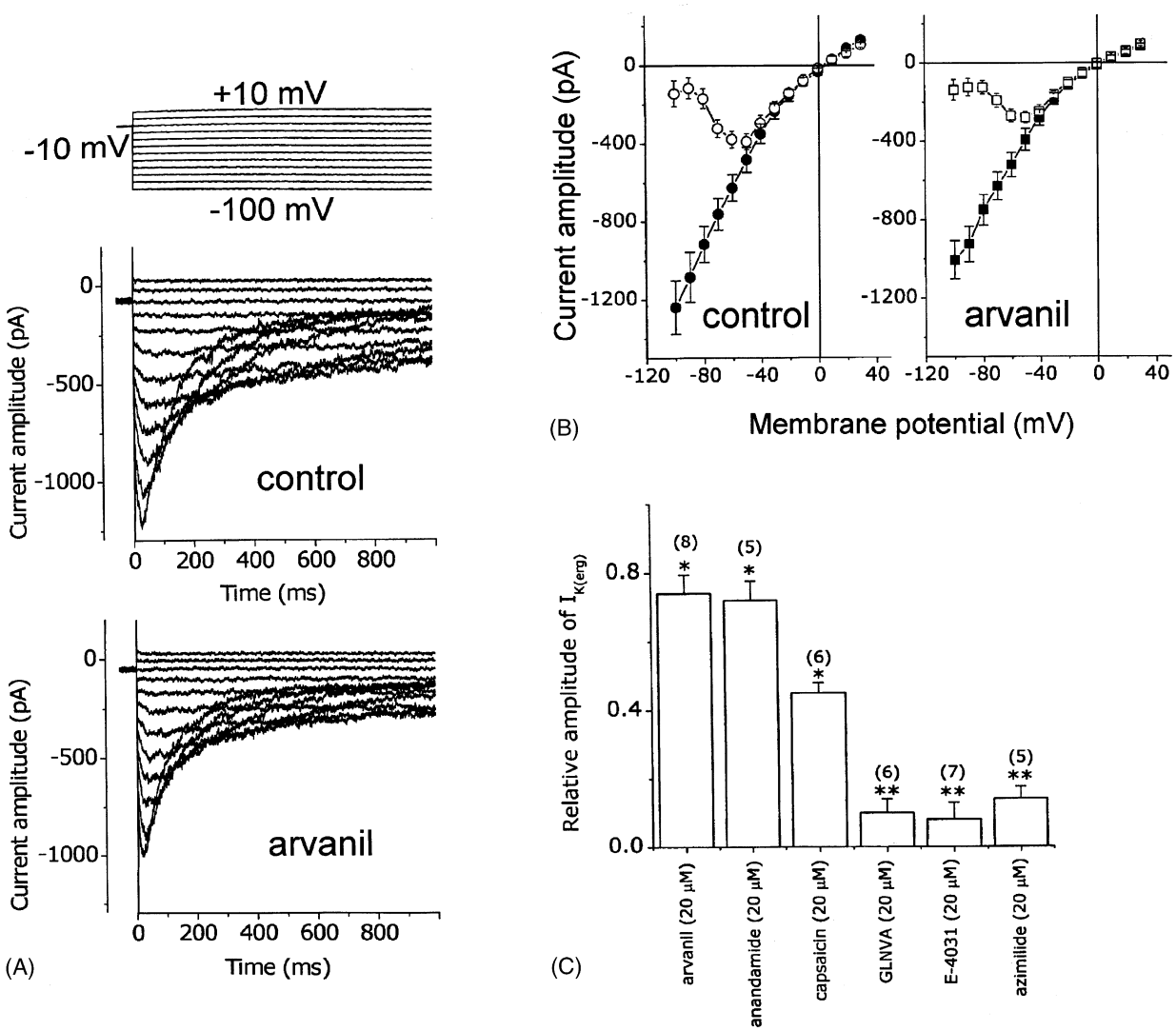

Fig. 7. Inhibitory effect of arvanil on $I_{K(erg)}$ in NG108-15 cells. Cells were bathed in a high K^+, Ca^{2+}-free solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM). (A) Superimposed current traces obtained when a cell was held at the level of −10 mV, and various voltage pulses ranging from −100 to +10 mV in 10 mV increments were applied. Current traces shown in upper part are controls, and those in lower part are results obtained 2 min after application of arvanil (20 μM). The uppermost part in (A) indicates the voltage protocol. (B) Averaged *I–V* relationships for initial (filled circles) and steady-state (open circles) components of $I_{K(erg)}$ in the absence (left) and presence (right) of 20 μM arvanil. Each point represents the mean ± SEM (N = 7–12). (C) Comparison between the effect of arvanil (20 μM) and those of anandamide (20 μM), capsaicin (20 μM), glyceryl nonivamide (GLNVA; 20 μM), E-4031 (20 μM), and azimilide (20 μM) on the amplitude of $I_{K(erg)}$. The amplitude of peak component of $I_{K(erg)}$ measured at the beginning of the hyperpolarizing pulses in the control was considered to be 1.0, and the relative amplitude of $I_{K(erg)}$ after addition of each agent was plotted. The parentheses shown in each bar indicate the number of cells examined. (*) Significantly different from control ($P < 0.05$ by unpaired *t*-test). (**) Significantly different from control ($P < 0.01$ by unpaired *t*-test).

the peak component of deactivating $I_{K(erg)}$ was significantly reduced to 1065 ± 118 pA (N = 6) from a control value of 1239 ± 92 pA (N = 7). However, no significant difference in steady-state component between the absence and presence of arvanil was observed (145 ± 48 pA [N = 6] versus 142 ± 23 pA [N = 6]).

To further characterize the inhibitory effect of arvanil on $I_{K(erg)}$, we also investigated the voltage dependence of the effect of arvanil on $I_{K(erg)}$. Fig. 8 shows the steady-state activation curve of $I_{K(erg)}$ in the absence and presence of arvanil (20 μM). A two-step voltage protocol was applied in these experiments. A 1-s conditioning pulse to different membrane potentials preceded a test potential (300 ms in duration) to −100 mV from a holding potential of −10 mV.

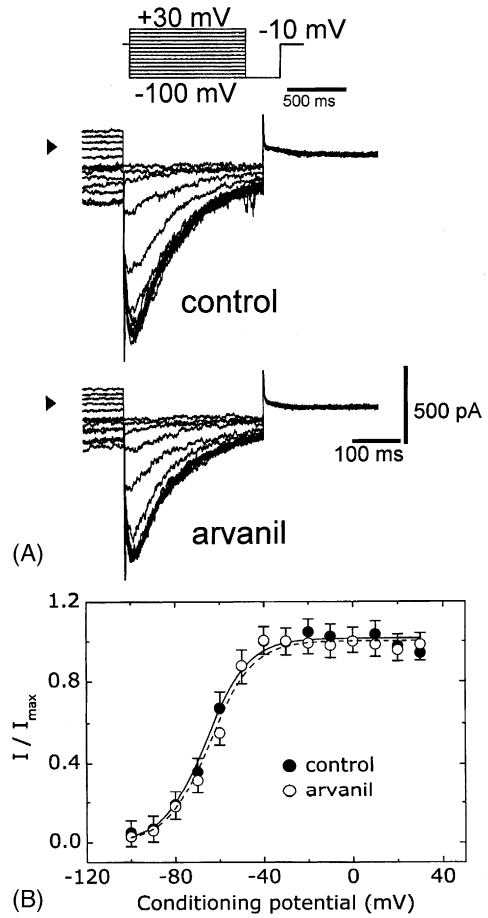

Fig. 8. Lack of effect of arvanil on steady-state activation curve of $I_{K(erg)}$. By use of a two-step protocol, the steady-state activation parameters of $I_{K(erg)}$ were obtained in the absence and presence of arvanil (20 μM). The conditioning voltage pulses with a duration of 1 s for various membrane potentials were applied from a holding potential of −10 mV. After each conditioning pulse, a test pulse to −100 mV with a duration of 300 ms was applied to evoke $I_{K(erg)}$. (A) Superimposed current traces obtained when the cell was hyperpolarized from a holding potential of −10 to −100 mV with the conditioning potentials ranging from −100 to +30 mV in 10 mV increments. Arrowheads indicate the zero current level. The voltage protocol is shown in the uppermost part. The records shown in upper part of (A) are controls, and those in lower part were obtained 2 min after addition of arvanil (20 μM). Smooth lines shown in (B) represent best fits to Boltzmann distribution as described in Section 2. Solid line: control; dash line: arvanil (20 μM). Each point represents the mean ± SEM (N = 4–6).

The relationships between the conditioning pulses and the normalized amplitudes of $I_{K(erg)}$ with or without the application of arvanil (20 μM) were constructed and fitted by the Boltzmann function as described in Section 2. In control, $a = -66.3 \pm 3.6$ mV, and $b = -9.7 \pm 1.1$ mV (N = 5), whereas in the presence of arvanil (20 μM), $a = -64.6 \pm 3.2$ mV, $b = -9.8 \pm 0.9$ mV (N = 5). Thus, the value for a and b was not modified in the presence of arvanil. The results demonstrate no change in the activation curve of $I_{K(erg)}$ when cells were exposed to arvanil.

3.7. Effect of arvanil on spontaneous action potentials in NG108-15 cells

In a final series of experiments, the effect of arvanil on repetitive firing of action potentials was investigated. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Current-clamp configuration was performed with a K^+-containing pipette solution. The typical effect of arvanil on spontaneous action potentials in these cells is illustrated in Fig. 9. When cells were exposed to

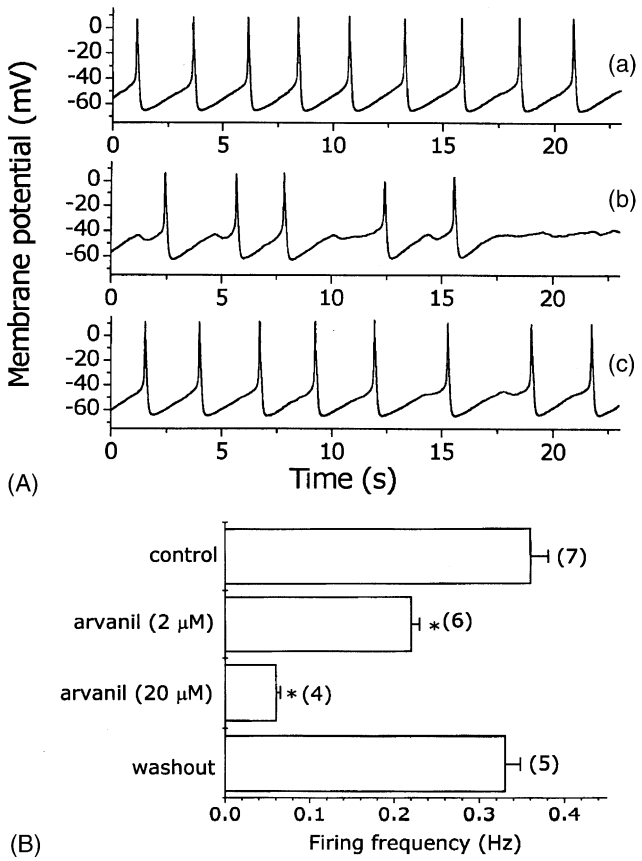

Fig. 9. Effect of arvanil on spontaneous action potentials in NG108-15 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The experiments were performed under current-clamp conditions. (A) Original traces obtained in the control (a), during the exposure to 2 μM arvanil (b), and after washout of arvanil (c). (B) Bar graph showing the effect of arvanil on firing frequency of action potentials. The parentheses shown in each bar are the number of cells examined. Values are the mean ± SEM. (*) Significantly different from control ($P < 0.05$ by unpaired t-test).

arvanil, the repetitive firing of action potentials was readily decreased. For example, arvanil (2 μM) significantly reduced the firing frequency to 0.22 ± 0.01 Hz (N = 6) from a control value of 0.36 ± 0.02 Hz (N = 7). The firing of spontaneous action potentials was almost returned to the control value after washout of arvanil. However, anandamide (5 μM) had no effect on spontaneous action potentials, while capsaicin (5 μM) reduced the frequency from 0.34 ± 0.03 to 0.24 ± 0.02 Hz (N = 5). Thus, it is clear that arvanil can regulate the firing of action potentials in NG108-15 cells. Such an effect could be closely associated with inhibition of $I_{Ca,L}$.

4. Discussion

The major findings of the present study are as follows. First, in NG108-15 neuronal cells, arvanil directly inhibits $I_{Ca,L}$ in a concentration- and voltage-dependent manner. Second, the inhibitory effect of arvanil on $I_{Ca,L}$ appears to be independent of the binding to either cannabinoid or vanilloid receptors. Third, arvanil at a concentration greater than 20 μM suppresses the amplitude of $I_{K(erg)}$. Fourth, arvanil decreases the repetitive firing of action potentials in NG108-15 cells. The present findings suggest that the effects on ion channels could be one of the mechanisms underlying arvanil-induced actions, if similar results occur in neurons or neuroendocrine cells *in vivo*.

This study provides the direct evidence that arvanil can inhibit the amplitude of $I_{Ca,L}$ in NG108-15 neuronal cells in a concentration-dependent manner. The IC_{50} value of arvanil required for the inhibition of $I_{Ca,L}$ was 2 μM. Arvanil could inhibit the transport of anandamide into cells with an IC_{50} value of 3.6 μM [1]. Arvanil was also reported to displace the binding of [^{3}H]SR141716A to cannabinoid CB_1 receptors with a K_i value of 2.6 μM in rat brain membranes [4]. However, little or no change in the amplitude of $I_{K(DR)}$ was found in the presence of arvanil. Therefore, in addition to cannabinoid and vanilloid receptors, L-type Ca^{2+} channels functionally expressed in neurons or neuroendocrine cells appear to be the targets of arvanil, because the effect of arvanil observed in this study may occur at a concentration achievable in rat plasma [4].

In this study, we found that arvanil (5 μM) not only reduced the maximal conductance of $I_{Ca,L}$, but also shifted the steady-state inactivation curve to the left alone the voltage axis about 15 mV. This result is different from that obtained after application of capsaicin in gastric smooth muscle cells [31], but similar to that observed in A7r5 smooth muscle cells [15]. Nevertheless, the magnitude of arvanil-induced block on $I_{Ca,L}$ in NG108-15 cells may be influenced by different levels of membrane potential. Therefore, the sensitivity of neurons or neuroendocrine cells to arvanil would rely on the preexisting level of resting membrane potential, the firing rate of action potential, or the concentration of arvanil examined, if the arvanil action in cells *in vivo* is the same as those on NG108-15 cells shown herein. Indeed, we also found that arvanil was effective in suppressing repetitive firing of action potentials in NG108-15 cells.

NG108-15 neuronal cells have been reported to express at least two types of Ca^{2+} channels contributing to high-threshold Ca^{2+} current: an ω-conotoxin GVIA-resistant, dihydropyridine-sensitive type, and an ω-conotoxin GVIA-sensitive, dihydropyridine-resistant type [22,28]. The major part of arvanil-inhibited Ca^{2+} current in undifferentiated NG108-15 cells observed in this study seems to be primarily due to block of dihydropyridine-sensitive L-type Ca^{2+} channels, not to that of neuronal N-type Ca^{2+} channels. Indeed, in cells preincubated with ω-conotoxin GVIA, arvanil was also able to block $I_{Ca,L}$ effectively.

Previous studies have demonstrated that activation of the cannabinoid CB1 in neurons produced inhibition of Ca^{2+} current through N- or P/Q-type Ca^{2+} channels, but not through L-type Ca^{2+} channels [8–10]. On the other hand, previous reports have shown that activation of the cannabinoid CB1 receptors in NG108-15 cells and in vascular smooth muscle cells could inhibit Ca^{2+} current, and that these effects appeared to be coupled through a pertussis toxin-sensitive G protein [11,22]. However, in NG108-15 cells preincubated with pertussis toxin, the inhibitory effect of arvanil on $I_{Ca,L}$ remained unaffected. Thus, under our experimental conditions, the inhibition of $I_{Ca,L}$ by arvanil in NG108-15 cells did not involve a pertussis toxin-sensitive G protein. Indeed, $Ca_V1.3\alpha_1$ L-type Ca^{2+} channels have been reported to be resistant to modulation by G protein-coupled receptors [32].

Although capsaicin, capsinolol, and glyceryl nonivamide produced the inhibition of $I_{Ca,L}$, they were also capable of blocking another types of ion currents, e.g. $I_{K(DR)}$ or $I_{K(erg)}$ [15,17–20,30,33]. Arvanil at a concentration of 20 μM could suppress the amplitude of $I_{K(erg)}$ in NG108-15 cells; however, the decrease in current amplitude was not accompanied by alterations in the activation curve of $I_{K(erg)}$. In addition, there is no evidence to show that arvanil can apparently block $I_{K(DR)}$. Therefore, the blockade of ion currents by arvanil observed in NG108-15 cells appears to be direct and relatively selective for L-type Ca^{2+} channels. Arvanil may interact with L-type Ca^{2+} channels at the 1,4-dihydropyridine binding site, since this seems to be the case for anandamide, which is structurally related to arvanil [2,5,12–14]. However, the binding domain for this compound remains to be further characterized.

Four genes have recently been shown to encode L-type Ca^{2+} channel-forming subunits ($Ca_V1\alpha_1$) in mammalian cells [34]. Among them, $Ca_V1.2\alpha_1$ and $Ca_V1.3\alpha_1$ are thought to be the widely expressed L-type channel subunits. NG108-15 cells were previously reported to express $Ca_V1.3\alpha_1$ subunit [35]. It seems likely that arvanil interacts with this subunit to block this channel, although the exact mechanism by which arvanil interferes with the channel function still remains to be elucidated. It will also be of

interest to clarify whether arvanil or capsaicin-related compounds can interact with other $Ca_V1\alpha_1$ subunits to affect channel activity.

The results presented herein show a selective block of $I_{Ca,L}$ by arvanil in NG108-15 cells. Such an effect is not necessarily associated with its activities at either cannabinoid or vanilloid receptors. The block of $I_{Ca,L}$ by arvanil will influence neuronal excitability. Ca^{2+} channel blockers have been previously found to potentiate opioid antinociception [36,37]. Arvanil and anandamide have been shown to inhibit pain and spasticity by acting in part through the same, as yet unidentified, site [4–7]. The pharmacological actions of these compounds that are unrelated to their binding to cannabinoid and vanilloid receptors may thus be partly due to the inhibition of L-type Ca^{2+} channels.

Acknowledgments

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