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# Block of *erg* current by linoleoylamide, a sleep-inducing agent, in pituitary $GH_3$ cells

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**Abstract**

Linoleoylamide is physiological constituent of neurons. The effects of this agent, also a sleep-inducing agent, on ion currents in pituitary $GH_3$ cells were investigated. Hyperpolarization-elicited $K^+$ currents in $GH_3$ cells bathed in a high-$K^+$, $Ca^{2+}$-free solution were studied to determine the effects of linoleoylamide and other related compounds on the $I_{K(IR)}$ that was sensitive to inhibition by E-4031 and identified as an *erg* (*ether-à-go-go*-related-gene) current. Linoleoylamide suppressed the amplitude of $I_{K(IR)}$ in a concentration-dependent manner with an $IC_{50}$ value of 5 μM. Oleamide (20 μM) inhibited the amplitude of $I_{K(IR)}$, while neither arachidonic acid (20 μM) nor 14,15-epoxyeicosatrienoic acid (20 μM) had an effect on it. In $GH_3$ cells incubated with anandamide (20 μM) or arachidonic acid (20 μM), the linoleoylamide-induced inhibition of $I_{K(IR)}$ remained unaltered. In inside-out patches, arachidonic acid (20 μM) and 14,15-epoxyeicosatrienoic acid (20 μM) stimulated large-conductance $Ca^{2+}$-activated $K^+$ channels; however, linoleoylamide (20 μM) had little or no effect on them. Under current-clamp mode, linoleoylamide (20 μM) increased the firing rate. In IMR-32 neuroblastoma cells, linoleoylamide also suppressed $I_{K(IR)}$. This study provides the evidence that linoleoylamide has a depressant effect on the *erg* current, and suggests that this effect may affect hormonal secretion.


*Keywords:* Linoleoylamide; *erg* current; $GH_3$ cell

## 1. Introduction

Linoleoylamide and related endogenous lipids are physiological constituents of neurons (Di Marzo et al., 1994; Cravatt et al., 1995) and can be produced in vivo by brain lipoxygenase (Van der Stelt et al., 1997a). These compounds were reported to be competitive inhibitors of the activity of fatty-acid amide hydrolase (Mechoulam et al., 1997). It was found that, in rat brain membrane, linoleoylamide could displace [$^3$H]CP-55,940 ((−)-*cis*-3-2-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol), a potent cannabinoid, from cannabinoid receptors (Van der Stelt et al., 1997b).

It has been reported that arachidonic acid and epoxyeicosatrienoic acid can modulate the activity of large-conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels in pituitary tumor cells (Duerson et al., 1996; Wu et al., 2000a,b). Linoleoylamide can be degraded via fatty-acid amide hydrolase to arachidonic acid or its metabolites (e.g., epoxyeicosatrienoic acids) (Cravatt et al., 1996; Patterson et al., 1996). A recent study has also demonstrated that these sleep-inducing lipids can increase cytosolic $Ca^{2+}$ in renal tubular cells (Huang and Jan, 2001). However, no reports have thus far been available regarding the effects of linoleoylamide on ion currents in neurons or neuroendocrine cells.

Pituitary $GH_3$ lactotrophs, in addition to the presence of voltage-dependent $K^+$ and $Ca^{2+}$ currents, have been shown to exhibit an inwardly rectifying $K^+$ current ($I_{K(IR)}$) (Wu et al., 1998b, 2000a; Bauer and Schwarz, 2001). There is considerable evidence to indicate that this current is inhibited by E-4031. It was thus identified as an *erg*

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(*ether-à-go-go*-related-gene) (Weinsberg et al., 1997; Schäfer et al., 1999; Bauer and Schwarz, 2001). In addition, it was shown that thyrotropin-releasing hormone reduced the *erg* current by an as yet unknown signal cascade (Schledermann et al., 2001). This current is responsible for the maintenance of the resting potential, which is less negative due to channel deactivation at membrane potentials more negative to about −50 mV. Block of this current can lead to an increase in the firing rate of action potentials, thus leading to an increase in prolactin secretion (Bauer et al., 1999).

In view of these considerations, the electrophysiological effects of linoleoylamide and other related compounds in $GH_3$ cells were investigated in this study. We sought to: (1) determine whether linoleoylamide has any effect on $I_{K(IR)}$ in $GH_3$ cells; (2) compare the potency of other related compounds in suppressing the amplitude of $I_{K(IR)}$; (3) examine the effect of linoleoylamide on other types of ionic currents, including voltage-dependent $K^+$ and L-type $Ca^{2+}$ currents, and $BK_{Ca}$ channels; and (4) ascertain whether linoleoylamide influences the membrane potential and the firing pattern of spontaneous action potentials in these cells. The present results indicate that the linoleoylamide-induced inhibition of the *erg* current is not associated with its degradation to arachidonic acid or its metabolites, and can significantly contribute to the change in membrane potential, thus affecting prolactin secretion.

## 2. Materials and methods

### 2.1. 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) (Wu et al., 2000a). $GH_3$ cells were maintained in Ham's F-12 medium (Life Technologies, Grand Island, NY), supplemented with 15% heat-inactivated horse serum, 2.5% fetal calf serum, and 2 mM L-glutamine (Life Technologies) in a humidified incubator at 37 °C with 5% $CO_2$. Cells were passaged once a week, and a new stock line was generated from frozen cells (frozen in 10% glycerol in medium plus serum) every 3 months. It has been validated that this cell line continually secretes prolactin and growth hormone. The experiments were performed 5 or 6 days after cells were subcultured (60–80% 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/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (v/v).

### 2.2. Electrophysiological measurements

Immediately before each experiment, $GH_3$ cells were dissociated, and an aliquot of cell suspension was transferred to a recording chamber mounted on the stage of an inverted microscope (Diaphot 200; Nikon, Tokyo, Japan). The microscope was coupled to a digital video camera with magnification up to 1500× to continuously monitor cell size during the experiments. Cells were bathed at normal temperature (20–25 °C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes that had a resistance of 3–5 MΩ were made from Kimax capillary tubes (Kimble, Vineland, NJ, USA), using a vertical two-step electrode puller (PP-83; Narishige, Tokyo, Japan) and fire polished with a microforge (MF-83; Narishige). Ion currents were recorded in the whole-cell or inside-out configuration of the patch-clamp technique using an RK-400 amplifier (Bio-logic, Claix, France) or an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) (Hamill et al., 1981; Wu et al., 2000a). All potentials were corrected for the liquid junction potential, which develops at the tip of the pipette when the composition of pipette solution is different from that of bath.

### 2.3. Data recording and analysis

Voltage and current tracings were displayed on a digital storage oscilloscope (model 1602; Gould, Valley View, OH) and a liquid crystal device projector (AV600; Delta, Taipei, Taiwan). The data were simultaneously recorded on a digital audio tape recorder (model ZA5ES; Sony, Tokyo, Japan). Current signals were low-pass filtered at 1 kHz before digitization. A Digidata 1320A interface (Axon Instruments) was used for analog-to-digital/digital-to-analog conversion. To minimize electrical noise, this interface device was connected to a Pentium III-based laptop computer (Slimnote $VX_3$; Lemel, Taipei, Taiwan) through a universal serial bus port, and was then controlled with the aid of the Clampex subroutine in pCLAMP 8.02 software (Axon Instruments). Voltage-activated currents recorded during whole-cell experiments were stored without leakage correction and analyzed subsequently using the Clampfit subroutine of pCLAMP (Axon Instruments) or the Origin 6.0 software (Microcal Software, Northampton, MA, USA) to construct current–voltage ($I$–$V$) relationship for ion currents.

To calculate the percentage inhibition by linoleoylamide on $I_{K(IR)}$, cells were bathed in high-$K^+$, $Ca^{2+}$-free solution and each cell was hyperpolarized from −10 to −120 mV. The current amplitudes of deactivating tail currents obtained after application of linoleoylamide were compared with those measured after a subsequent application of E-4031 (10 μM). E-4031 is known to be a selective blocker of $I_{K(IR)}$ (Weinsberg et al., 1997; Wu et al., 2000a, 2001). The concentration of linoleoylamide needed to inhibit the current amplitude by 50% was calculated by fitting the data to

the Hill equation: percentage inhibition = $E_{max}/\{1+(IC_{50H}^{n}/[C]^{n}_{H})\}$, where $[C]$ represents the linoleoylamide concentration, $IC_{50}$ and $n_H$ are the concentrations of linoleoylamide required for half-maximal reduction of $I_{K(IR)}$ (i.e., E-4031-sensitive current) and the Hill coefficient, respectively, and $E_{max}$ is linoleoylamide-induced maximal inhibition of $I_{K(IR)}$.

Single-channel currents of $BK_{Ca}$ channels were analyzed with Fetchan and Pstat subroutines in the pCLAMP 8.02 software (Axon Instruments). Multigaussian adjustments of the amplitude distributions between channels were used to determine single-channel currents. The functional independence between the channels was verified by comparing the observed stationary probabilities. The open probability was evaluated using an iterative process to minimize the $\chi^2$ calculated with a sufficiently large number of independent observations.

Changes in membrane potential of $GH_3$ cells were investigated under current-clamp conditions. The frequency of spontaneous action potentials was characterized by transforming the oscillating signals from their time domain to their representation in the frequency domain with the aid of power spectral analysis. Spectral analysis was performed using a discrete Fourier transform algorithm with the aid of Origin software (Microcal) (Wu et al., 2000a). When the firing of action potentials in $GH_3$ cells exhibits a regular discharge pattern, a concentrated peak in the power spectrogram that corresponds to the mean firing rate will appear.

All values are reported as the means ± S.E. Paired or unpaired Student's *t*-test and one-way analysis of variance (ANOVA) with the least-significance difference method for multiple comparisons were used for the statistical evaluation of differences among the mean values. Differences between the values were considered significant at a value of $P<0.05$ or $P<0.01$.

### 2.4. Drugs and solutions

Linoleoylamide (9*Z*,12*Z*)-octadeca-9,12-dienoylamide) and oleamide (*cis*-9-octadecenamide) were purchased from Calbiochem (La Jolla, CA, USA), and 14,15-EET (14,15-epoxyeicosatrienoic acid) and E-4031 from Biomol (Plymouth Meeting, PA, USA). Tetrodotoxin, tetraethylammonium chloride, arachidonic acid, anandamide (arachidonylethanolamide), and nimodipine were obtained from Sigma (St. Louis, MO, USA). Azimilide was a gift from Procter and Gamble Pharmaceuticals (Cincinnati, OH, USA). All other chemicals were of analytical grade.

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). For action potential recordings, patch pipettes were filled with the following 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 5 (pH 7.2). Because of the small amplitude of the inwardly rectifying $K^+$ current ($I_{K(IR)}$) in normal Tyrode's solution, a high-$K^+$, $Ca^{2+}$-free solution containing (in mM) KCl 130, NaCl 10, $MgCl_2$ 3, glucose 6, HEPES–KOH 10 (pH 7.4) was used to increase current amplitude when $I_{K(IR)}$ was measured. To measure the $Ca^{2+}$ current, KCl in the pipette solution was replaced with equimolar CsCl and the pH was adjusted to 7.2 with CsOH.

For single-channel recordings, high $K^+$-bathing solution contained (in mM) KCl 145, $MgCl_2$ 0.53, and 5 mM HEPES–KOH 5 (pH 7.4) and pipette solution contained (in mM): KCl 145, $MgCl_2$ 2, and HEPES–KOH 5 (pH 7.2).

## 3. Results

### 3.1. Effect of linoleoylamide on hyperpolarization-elicited currents in $GH_3$ cells

The whole-cell configuration of the patch-clamp technique was used to investigate the effect of linoleoylamide on macroscopic ion currents. When $GH_3$ cells were bathed in a high-$K^+$, $Ca^{2+}$-free solution, a family of large inward currents was elicited upon membrane hyperpolarization. Examples of ion currents evoked by the 1-s-long clamp pulses to various membrane potentials from a holding potential of −10 mV are illustrated in Fig. 1. Consistent with our previous studies (Wu et al., 1998b, 2000a), hyperpolarizing voltage pulses induced an instantaneous current followed by a voltage- and time-dependent elicitation of a $K^+$ inward current, i.e., a deactivating tail $K^+$ current. These inward currents decayed at potentials below −50 mV and current deactivation became faster with greater hyperpolarization.

Within 1 min of exposing the cell to linoleoylamide (5 μM), the amplitude of the hyperpolarization-elicited currents was greatly reduced (Fig. 1). For example, when cells were hyperpolarized from −10 to −120 mV, linoleoylamide (5 μM) significantly decreased the amplitude of peak inward currents from 1502 ± 98 to 832 ± 93 pA ($n=8$). This inhibitory effect was readily reversed on the removal of linoleoylamide. However, there was no significant difference in current amplitudes at the end of the hyperpolarizing pulses from −10 to −120 mV (i.e., late sustained inward current) in the absence or presence of 5 μM linoleoylamide (432 ± 52 pA [$n=8$] versus 467 ± 48 pA [$n=8$]). In addition, the presence of linoleoylamide (5 μM) significantly decreased the slope of the linear fit of current amplitudes to voltages between −120 and −60 mV from 17.1 ± 4.4 to 5.8 ± 3.2 nS ($n=7$). These data indicate that an inwardly rectifying $K^+$ current ($I_{K(IR)}$) was expressed in $GH_3$ cells, and that the blocking effect of linoleoylamide was exerted primarily on the component of membrane currents that is responsible for inward rectification.

The relationship between the concentration of linoleoylamide and the percentage inhibition of $I_{K(IR)}$ is illustrated in Fig. 2. The current amplitudes of $I_{K(IR)}$ in the presence of linoleoylamide were compared with those obtained after a

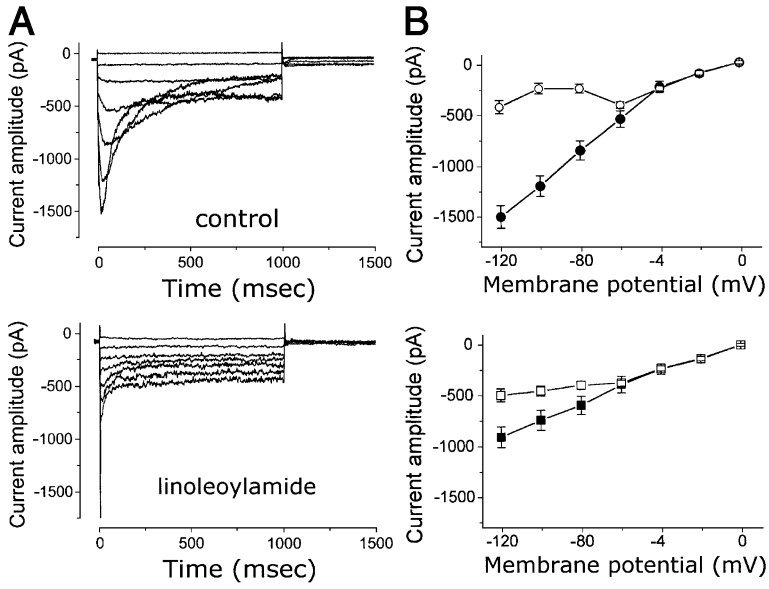


Fig. 1. Inhibitory effect of linoleoylamide on the $I-V$ relationships of the hyperpolarization-evoked currents in rat pituitary $GH_3$ cells. Cells were bathed in a high-$K^+$, $Ca^{2+}$-free solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM). (A) Superimposed current traces obtained when a cell was held at the level of −10 mV and various voltage pulses ranging from 0 to −120 mV in 20-mV increments were applied. Current traces shown in upper part of (A) are controls and those in lower part were obtained 1 min after application of linoleoylamide (5 μM). (B) Averaged $I-V$ relationships for initial (closed symbols) and steady-state (open symbols) components of ion currents in the absence (upper part) and presence (lower part) of 5 μM linoleoylamide. Each point shown in (B) represents the mean ± S.E.M. ($n=8-12$).

subsequent application of E-4031 (10 μM). E-4031 is known to be a selective blocker of $I_{K(IR)}$ (Weinsberg et al., 1997; Wu et al., 2000a). Application of linoleoylamide (0.5–100 μM) was found to suppress the amplitude of E-4031-sensitive currents in a concentration-dependent manner. The half-maximal concentration required for the inhibitory effect of linoleoylamide on $I_{K(IR)}$ was 5 μM; 100 μM linoleoylamide nearly abolished the current amplitude.

### 3.2. Effect of linoleoylamide on the deactivation of hyperpolarization-elicited transient currents

Deactivation kinetics in the absence and presence of linoleoylamide were also assessed by measuring the rate of current decay (i.e., $1/\tau$) upon stepping to the different voltages. In these experiments, the test pulses were preceded by a fixed, highly hyperpolarized prepulse (−120 mV for 30 ms) that would activate virtually all channels and uncouple the activation process from block. A range of voltage pulses between −180 and −90 mV was then applied. After addition of linoleoylamide to the bath, the rate of current decay was assessed at each voltage pulse. The current decays obtained in the absence and presence of linoleoylamide (5 μM) were fitted with a single exponential, and their reciprocal time constants ($1/\tau$) were then plotted against the test potential (Fig. 3). The difference in the value of $1/\tau$ between the absence and presence of linoleoylamide was greater with less hyperpolarization. These results suggest that linoleoylamide is able to increase the rate of channel deactivation at every voltage measured.

### 3.3. Comparison between effect of linoleoylamide and of arachidonic acid, 14,15-EET, anandamide, azimilide and E-4031 on $I_{K(IR)}$

The effects of arachidonic acid, 14,15-EET, anandamide, azilimilide and E-4031 were examined and compared. Arachidonic acid and 14,15-EET could be the degradative products of linoleoylamide (Cravatt et al., 1996; Patterson et al., 1996). Anandamide, an endogenous arachidonic acid derivative that binds to brain-type cannabinoid receptors (Devane et al., 1993), could be rapidly cleaved by intracellular fatty-acid amide hydrolase (Day et al., 2001). Azimilide and E-4031 are known to be selective blockers of $I_{K(IR)}$ (Weinsberg et al., 1997; Busch et al., 1998; Wu et al., 2000a, 2001). As shown in Fig. 4, arachidonic acid, 14,15-EET and anandamide had little or no effect on the amplitude of $I_{K(IR)}$. In contrast, similar to the effect of E-4031 and azimilide, oleamide, a structurally related compound, effectively suppressed $I_{K(IR)}$ in these cells. These findings suggest that $I_{K(IR)}$ present in $GH_3$ cells is sensitive to inhibition by E-4031 and azimilide, but not by arachidonic acid, 14,15-EET, and anandamide.

### 3.4. Effect of linoleoylamide on $I_{K(IR)}$ in cells preincubated with anandamide or arachidonic acid

It is possible that the inhibitory effect of linoleoylamide on $I_{K(IR)}$ is related to that of its degradative products. Anandamide could be taken into cells and competitively inhibit the activity of fatty-acid amide hydrolase (Day et al.,

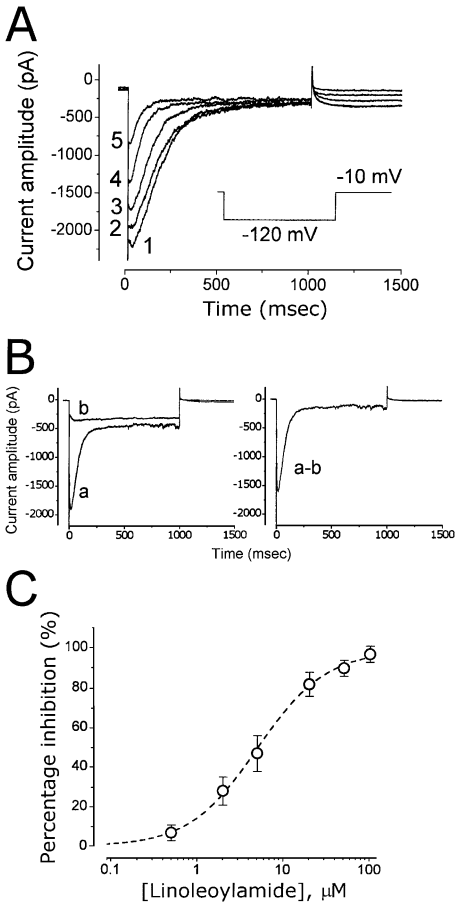


Fig. 2. Concentration-dependent inhibition of $I_{K(IR)}$ by linoleoylamide in $GH_3$ cells. (A) Superimposed current traces obtained in the absence and presence of linoleoylamide. Cells were bathed in a high-$K^+$, $Ca^{2+}$-free solution and the hyperpolarizing pulses from −10 to −120 mV were applied with a duration of 1 s. The current trace labeled 1 is the control, and traces labeled 2, 3, 4, and 5 were obtained after the addition of 0.5, 2, 5, and 20 μM linoleoylamide, respectively. Inset indicates the voltage-clamp protocol. (B) Inhibitory effect of E-4031 on $I_{K(IR)}$. The cell was hyperpolarized from −10 to −120 mV. The current trace labeled a on the left side is the control and that labeled b was obtained 2 min after application of E-4031 (10 μM), and that on the right side is an E-4031-sensitive current (i.e., a−b). (C) Concentration–response relationship for linoleoylamide-induced inhibition of $I_{K(IR)}$ (i.e., E-4031-sensitive current). Each point represents the mean ± S.E.M. ($n$ = 5–9). The curve represents the best fit to the Hill equation as described under Materials and methods. The values for $IC_{50}$, maximally inhibited percentage of E-4031-sensitive currents and the Hill coefficient were 5 μM, 99% and 1.1, respectively.

2001). Thus, the effect of linoeoylamide on $I_{K(IR)}$ was assessed in cells treated with anandamide or arachidonic acid. However, as depicted in Fig. 5, in $GH_3$ cells preincubated with anandamide (20 μM) or arachidonic acid (20 μM) for 5 h, the inhibitory effect of linoleoylamide on the $I$–$V$ relationship of $I_{K(IR)}$ remained unaltered. There was no significant difference in the magnitude of the linoleoylamide-induced inhibition of $I_{K(IR)}$ between control cells and cells treated with anandamide or arachidonic acid. Thus, the results indicate that the inhibitory effect of linoleoylamide on this current does not seem to be associated with the production of its degradative products.

### 3.5. Inhibitory effect of linoleoylamide on voltage-dependent $K^+$ current ($I_K$) in $GH_3$ cells

We also attempted to examine whether linoleoylamide affected other types of $K^+$ currents. The experiments were performed in cells bathed in $Ca^{2+}$-free Tyrode's solution. The cells were held at −50 mV and various depolarizing pulses (1 s in duration) were applied at a rate of 0.05 Hz. The presence of linoleoylamide (50 μM) caused a slight reduction of $I_K$ in $GH_3$ cells (Fig. 6). For example, when the cells were depolarized from −50 to +60 mV with a duration of 1 s, linoleoylamide (50 μM) significantly

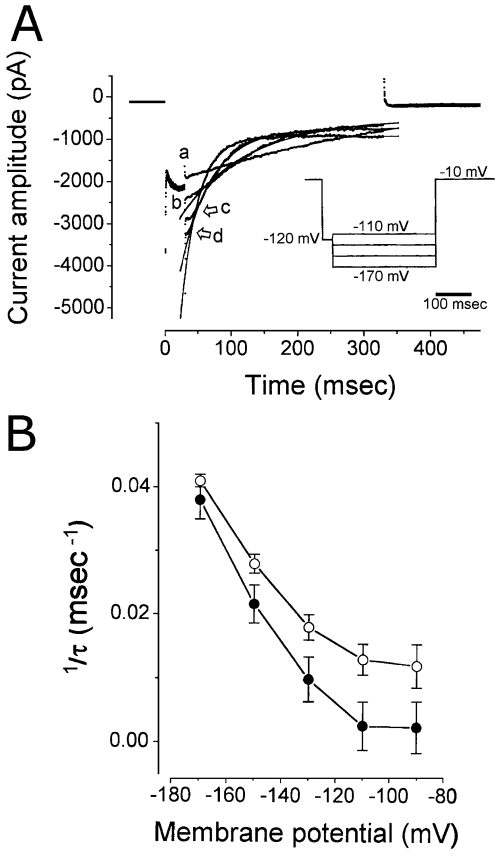


Fig. 3. Effect of linoleoylamide on $I_{K(IR)}$ deactivation in $GH_3$ cells. Cells were bathed in high-$K^+$, $Ca^{2+}$-free solution. The conditioning pulses were hyperpolarized from −10 to −120 mV with a duration of 30 ms. After each conditioning pulse, various membrane potentials ranging from −170 to −90 mV in 20-mV increments were applied. In (A), the time courses of current decay at the level of −170, −150, −130, and −110 mV in the control were fitted by single exponential with a value of 26.3, 46.2, 124.8 and 394.8 ms, respectively. Inset indicates the voltage protocol. (a) −110 mV; (b) −130 mV; (c) −150 mV; and (d) −170 mV. In (B), the reciprocal of the time constant obtained by single-exponential fit of current decay in the absence (●) and presence (○) of linoleoylamide (5 μM) was plotted against the membrane potential. Each point represents the mean ± S.E.M. ($n$ = 5–7).

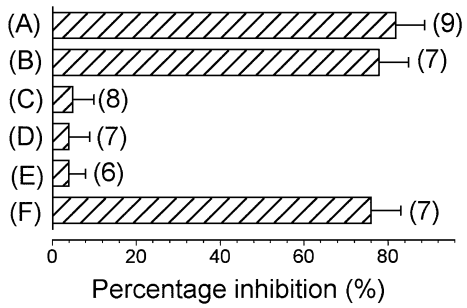


Fig. 4. Comparison between the effect of linoleoylamide and those of oleamide, anandamide, arachidonic acid, 14,15-epoxyeicosatrienoic acid (EET), and azimilide on the amplitude of $I_{K(IR)}$ (i.e., E-4031-sensitive current). In these experiments, cells were bathed in high $K^+$, $Ca^{2+}$-free solution and hyperpolarizing pulses from − 10 to − 120 mV were applied with a duration of 1 s. The peak amplitude of $I_{K(IR)}$ in the control condition was considered to be 1.0 and the relative amplitude of $I_{K(IR)}$ after application of each agent was plotted. The parentheses shown in each bar indicate the number of cells examined. Mean ± S.E.M. (A) linoleoylamide (20 μM); (B) oleamide (20 μM); (C) anandamide (20 μM); (D) arachidonic acid (20 μM); (E) EET (20 μM); (F) azimilide (20 μM).

decreased the amplitude of the sustained component of $I_K$ to 602 ± 44 pA from a control value of 1210 ± 42 pA ($n$ = 7). Linoleoylamide (50 μM) produced a downward shift, particularly when the potential was more positive to − 20 mV. These findings indicate that a higher concentration of linoleoylamide (50 μM) can suppress outward $K^+$ currents in these cells.

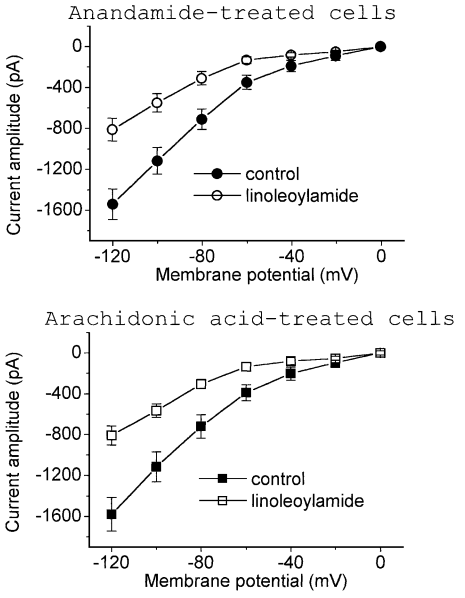


Fig. 5. Effect of linoleoylamide on the averaged $I$–$V$ relations for the peak components of $I_{K(IR)}$ in $GH_3$ cells treated with anandamide (upper part) or arachidonic acid (lower part). In these experiments, $GH_3$ cells were preincubated with anandamide (20 μM) or arachidonic acid (20 μM) for 5 h. Each cell was held at − 10 mV and various potentials ranging from 0 to − 120 mV in 20-mV increments were applied. Each point represents the mean ± S.E.M. Closed symbols: control. Open symbols: in the presence of linoleoylamide (20 μM).

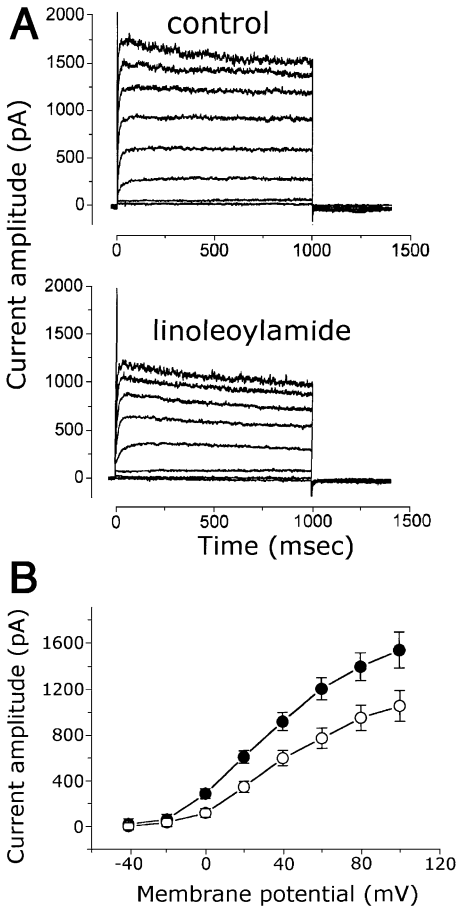


Fig. 6. Inhibitory effect of linoleoylamide on voltage-dependent $K^+$ outward currents ($I_K$) in $GH_3$ cells. Cells, bathed in $Ca^{2+}$-free Tyrode's solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM), were held at − 50 mV and depolarizing pulses from − 40 to + 100 mV in 20-mV increments were applied. Superimposed current traces shown in the upper part of (A) are controls, and those in lower part were recorded 2 min after addition of linoleoylamide (50 μM). The $I$–$V$ relationships of $I_K$ between the absence (●) and presence (○) of 50 μM linoleoylamide are illustrated in (B). Each point represents the mean ± S.E.M. ($n$ = 5–7).

### 3.6. Lack of effect of linoleoylamide on L-type $Ca^{2+}$ current ($I_{Ca,L}$) in $GH_3$ cells

These experiments were conducted with $Cs^+$-containing pipette solution. The cells were depolarized from a holding potential of − 50 mV to various potentials ranging from − 40 to + 60 mV in 20-mV increments. Linoleoylamide (20 μM) had little or no effect on the amplitude of $I_{Ca,L}$. Evidence for this appears in Fig. 7. When cells were depolarized from − 50 to 0 mV, there was no significant difference in the amplitude of $I_{Ca,L}$ between the absence and presence of linoleoylamide (20 μM) [867 ± 45 pA ($n$ = 6) versus 865 ± 43 pA ($n$ = 6)]. However, a subsequent application of tetrandrine (5 μM) or nimodipine (1 μM) significantly suppressed $I_{Ca,L}$ without altering the $I$–$V$ relationship of $I_{Ca,L}$. Tetrandrine and nimodipine were previously reported to be inhibitors of $I_{Ca,L}$ in $GH_3$ cells (Wu et al.,

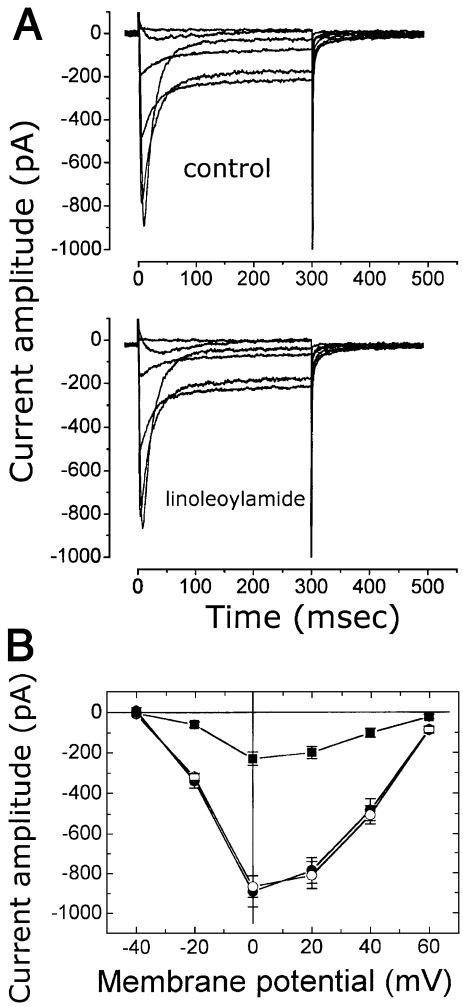


Fig. 7. Lack of effect of linoleoylamide on L-type $Ca^{2+}$inward current ($I_{Ca,L}$) in $GH_3$ cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, 1 μM tetrodotoxin, and 10 mM tetraethylammonium chloride. The recording pipette was filled with $Cs^+$-containing solution. Cells were depolarized from a holding potential of −50 mV to various potentials ranging from −40 to +60 mV in 20-mV increments. Original current traces shown in the upper part of (A) indicate the control, and those shown in the lower part indicate the samples obtained after addition of 20 μM linoleoylamide. (B) The *I–V* relationships in the absence (●) and presence of 20 μM linoleoylamide (○) or 20 μM linoleoylamide plus 5 μM tetrandrine (■). Each point represents the mean ± S.E.M. ($n$ = 5–9).

1998a). These results indicate that unlike $I_{K(IR)}$, $I_{Ca,L}$ present in $GH_3$ cells was not affected by the presence of linoleoylamide.

### 3.7. Effect of linoleoylamide on large-conductance $CA^{2+}$-activated $K^+$ ($BK_{Ca}$) channels recorded from $GH_3$ cells

The effect of linoleoylamide on the activity of $BK_{Ca}$ channels was further tested. In these experiments, the single-channel recordings with an inside-out mode were performed in 145 mM $K^+$ solution (Wu et al., 2000a). Bath medium contained 0.5 μM $Ca^{2+}$ and the holding potential was set to +60 mV. The probability of channel openings at the level of +60 mV in the control condition was found to be 0.038 ± 0.002 ($n$ = 9). When linoleoylamide (20 μM) was applied to the bath, no significant change in channel activity was found (0.038 ± 0.002 [$n$ = 9] versus 0.039 ± 0.003 [$n$ = 8]). However, 14,15-epoxyeicosatrienoic acid and arachidonic acid significantly enhanced the activity of $BK_{Ca}$ channels expressed in $GH_3$ cells (Fig. 8), although they did not modify the single-channel conductance of $BK_{Ca}$ chan-

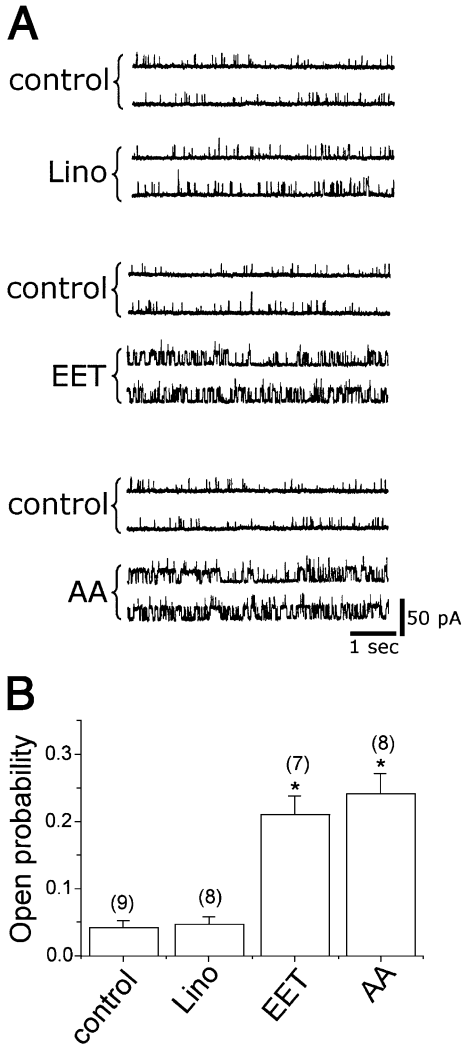


Fig. 8. Effect of linoleoylamide (Lino), 14,15-eicosatrienoic acid (EET), and arachidonic acid (AA) on the activity of large-conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels in $GH_3$ cells. (A) Original current traces showing the activity of $BK_{Ca}$ channels in the absence and presence of 20 μM linoleoylamide, 20 μM EET, or 20 μM AA. The inside-out configuration was used and bath medium contained 0.5 μM $Ca^{2+}$. Cells were bathed in $K^+$ (145 mM) solution. The holding potential was +60 mV. The detached membrane patch was intracellularly exposed to 20 μM linoleoylamide, 20 μM EET or 20 μM AA. Upward deflections are due to channel opening. (B) Bar graph showing the effects of linoleoylamide, EET, and AA on $BK_{Ca}$ channel activity. The numbers in parentheses denote the number of cells examined. Mean ± S.E.M. *$P$ < 0.05 versus control.

nels. Thus, unlike 14,15-epoxyeicosatrienoic acid or arachidonic acid, linoleoylamide did not affect the activity of $BK_{Ca}$ channels.

### 3.8. Effect of linoleoylamide on the discharge pattern of spontaneous action potentials in $GH_3$ cells

To determine whether linoleoylamide causes any changes in the membrane potential of $GH_3$ cells, the experiments were conducted with a $K^+$-containing solution and cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Fig. 9 illustrates the effect of linoleoylamide on the firing of action potentials in $GH_3$ cells. When linoleoylamide (20 μM) was added to the bath, the membrane potential was significantly depolarized from $-48 \pm 6$ to $-41 \pm 6$ mV ($n = 7$). The firing rate of action potentials was significantly increased to $0.54 \pm 0.03$ Hz from a control value of $0.18 \pm 0.04$ Hz ($n = 7$).

The effect of linoleoylamide on the firing pattern of action potentials was further investigated. To analyze the discharge pattern of spontaneous action potentials, power spectral analyses of the change in membrane potential that convert the time domain to the frequency domain were performed (Wu et al., 2000a). As shown in Fig. 9, the majority of $GH_3$ cells in the control condition exhibited a scatter power density in the spectrogram. These data indicate that there was an irregular pattern of repetitive firing in these cells. However, when cells were exposed to linoleoylamide (20 μM), the frequency-domain analysis demonstrated that the discharge pattern of these cells exhibited a concentrated power density at approximately 0.54 Hz, with its subsequent harmonic components appearing at multiples of 0.54 Hz, e.g., 1.08 Hz. Similar results were obtained in six different cells. Therefore, the results indicate that exposure of $GH_3$ cells to linoleoylamide was able to produce an increase in the firing frequency that was accompanied by conversion from an irregular to a regular discharge pattern.

### 3.9. Inhibitory effect of linoleoylamide on $I_{K(IR)}$ in neuroblastoma IMR-32 cells

In a final series of experiments, we examined the effect of linoleoylamide in neuroblastoma IMR-32 cells to determine whether linoleoylamide can affect $I_{K(IR)}$ in other types of neuroendocrine cells. As shown in Fig. 10, when cells were bathed in high-$K^+$, $Ca^{2+}$-free solution, hyperpolarization-elicited currents were detected in IMR-32 cells. As expected from previous studies (Wu et al., 2000a, 2001), the application of azimilide (20 μM) or E-4031 (20 μM) significantly suppressed these currents. Thus, it is clear that $I_{K(IR)}$ is present in IMR-32 cells. The application of linoleoylamide (5 μM) resulted in a decrease in hyperpolarization-elicited currents. For example, when the cells were hyperpolarized from $-10$ to $-120$ mV, the presence of linoleoylamide (5 μM) significantly decreased the peak component of $I_{K(IR)}$ from $274 \pm 15$ to $158 \pm 13$ pA ($n = 8$). The results also indicate that linoleoylamide can

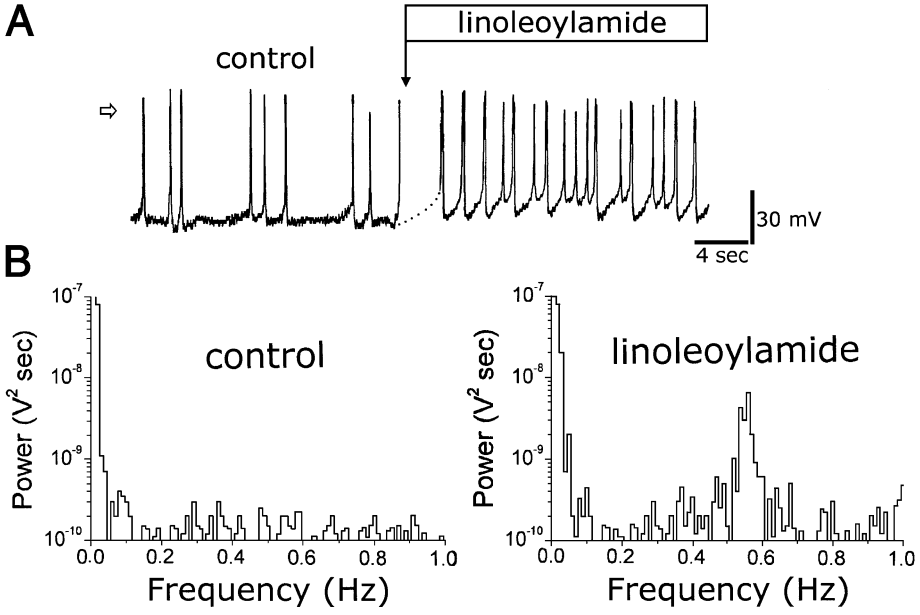


Fig. 9. Effect of linoleoylamide on spontaneous action potentials in $GH_3$ cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. These experiments were performed under current-clamp conditions. Horizontal bar indicates the application of linoleoylamide (20 μM). Note that the presence of linoleoylamide (20 μM) depolarized the cell and caused an increase in the frequency of action potentials. Open arrow indicates the 0-mV potential. The dashed line indicates a 2-min period. (B) Spectral patterns of spontaneous action potentials in the absence (upper part) and presence (lower part) of linoleoylamide. When cells were exposed to linoleoylamide (20 μM), the repetitive firing was converted from an irregular (upper part) to a regular (lower part) pattern.

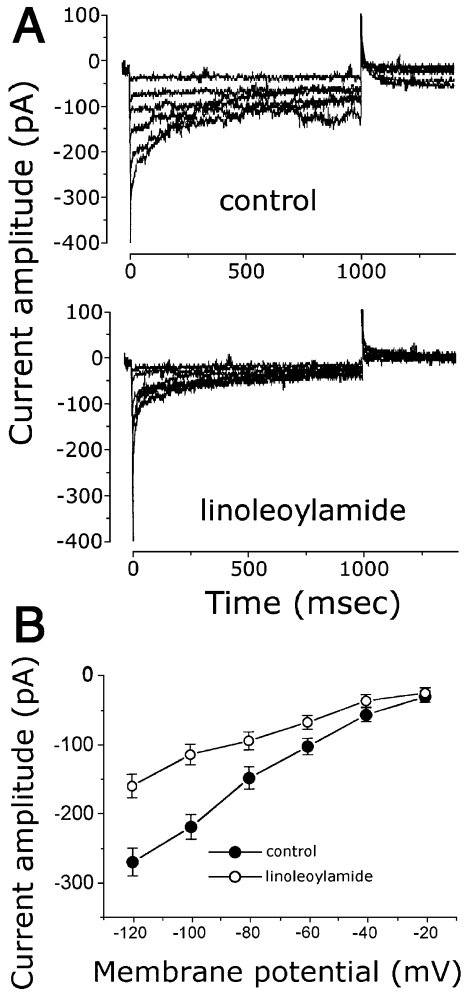


Fig. 10. Inhibitory effect of linoleoylamide on the $I-V$ relationships of hyperpolarization-evoked currents in human neuroblastoma IMR-32 cells. Cells were bathed in high-$K^+$, $Ca^{2+}$-free solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM). (A) Superimposed current traces obtained when a cell was held at −10 mV and various voltage pulses ranging from 0 to −120 mV in 20-mV increments were applied. Current traces shown in upper part are controls and those in lower part were obtained 1 min after addition of linoleoylamide (5 μM). (B) Averaged $I-V$ relationships for initial component of $K^+$ inward currents in the control (●) and during the exposure to 5 μM linoleoylamide (○). Each point represents the mean ± S.E.M. ($n=8-11$).

suppress the amplitude of $I_{K(IR)}$ present in neuroblastoma IMR-32 cells.

## 4. Discussion

The studies presented here show that: (1) linoleoylamide can suppress the inwardly rectifying $K^+$ current ($I_{K(IR)}$) in a concentration-dependent manner in pituitary $GH_3$ cells; (2) linoleoylamide can increase deactivation of $I_{K(IR)}$; (3) linoleoylamide can increase the repetitive firing of action potentials; and (4) linoleoylamide can suppress $I_{K(IR)}$ in neuroblastoma IMR-32 cells. The linoleoylamide-induced inhibition of $I_{K(IR)}$ could result in depolarization and affect the firing frequency and pattern of spontaneous action potentials in these cells.

The $IC_{50}$ value of linoleoylamide required for the inhibition of $I_{K(IR)}$ was 5 μM in this study. This value was lower than that found to increase intracellular $Ca^{2+}$ in Madin-Darby canine kidney cells (Huang and Jan, 2001). There thus appears to be a link between the actions of linoleoylamide on neurons or neuroendocrine cells and its observed effects on $K_{ir}$ channels. However, evidence suggests that an increase in intracellular $Ca^{2+}$ concentrations can stimulate the formation, cleavage, and release of anandamide from a membrane phospholipid precursor, *N*-arachidonoyl phosphatidylethanolamine (Cadas et al., 1996). Indeed, linoleoylamide-induced depolarization and increase in the repetitive firing of action potentials may increase intracellular $Ca^{2+}$ via the activation of voltage-dependent $Ca^{2+}$ currents (Secondo et al., 2000), although it did not influence $I_{Ca,L}$ directly.

One may argue that the linoleoylamide-induced inhibition of $I_{K(IR)}$ is due either to its inhibition of the activity of fatty-acid amide hydrolase or to the effects of its degradative products, e.g., arachidonic acid. However, in $GH_3$ cells preincubated with anandamide or arachidonic acid, linoleoylamide-mediated inhibition of $I_{K(IR)}$ remained unaltered. Therefore, the linoleoylamide-induced block of $I_{K(IR)}$ appears to be direct and does not involve the activity of fatty-acid amide hydrolase. Previous studies have shown that oleamide at a concentration of 100 μM inhibits the activity of fatty-acid amide hydrolase by 50% (Mechoulam et al., 1997); however, our data showed that oleamide (20 μM) suppressed the amplitude of $I_{K(IR)}$ by about 80%. The present finding is further supported by an interesting report showing that the increased intracellular $Ca^{2+}$ in Madin-Darby canine kidney cells caused by linoleamide was apparently not associated with the activity of fatty-acid amide hydrolase (Huang and Jan, 2001). Neither arachidonic acid nor 14,15-EET produced any effect on $I_{K(IR)}$. Therefore, it seems that the inhibitory effect of linoleoylamide on $I_{K(IR)}$ in $GH_3$ cells does not result primarily from the rapid conversion of linoleoylamide to arachidonic acid or its metabolites.

Linoleoylamide at a high concentration (50 μM) was found to suppress voltage-dependent $K^+$ outward currents. However, $K^+$ outward currents upon depolarization in normal Tyrode's solution containing 5.4 mM KCl may contain $I_{K(IR)}$, which is also voltage-dependent. Our data showing linoleoylamide-induced inhibition of other types of voltage dependent $K^+$ currents could have been overestimated. Linoleoylamide had a minimal effect on $I_{Ca,L}$. Linoleoylamide was also effective in suppressing $I_{K(IR)}$ in neuroblastoma IMR-32 cells. The blockade of $K^+$ channels by linoleoylamide in these cells may thus be selective for *erg*-mediated channels, which are encoded by a rat counterpart of human *ether-à-go-go*-related gene (HERG) (Schäfer et al., 1999). However, further study is needed to find out whether linoleoylamide affects $I_{K(IR)}$ in a variety of cells or different types of *erg* currents (Schledermann et al., 2001).

The present study revealed a difference in reciprocal time constants of current decay seen at different voltages in the absence and presence of linoleoylamide, suggesting that linoleoylamide may increase the rate of deactivation of *erg* currents (Meves, 2001). Thus, the sensitivity to linoleoylamide will depend on the preexisting resting membrane potential, the firing rate of the action potential, or the concentration of linoleoylamide. Furthermore, under current-clamp conditions, linoleoylamide produced an increase in the firing of action potentials and a concomitant conversion from an irregular to a regular pattern. This finding is in agreement with a previous observation showing that the *erg*-mediated $K^+$ current is responsible for the adaptation of spike frequency (Chiesa et al., 1997; Wu et al., 2000a).

A recent study has shown that anandamide could block a TWIK-related acid-sensitive $K^+$ (TASK)-1-like background $K^+$ channel (Maingret et al., 2001). We found no effect of anandamide on the amplitude of $I_{K(IR)}$ in $GH_3$ cells. In addition, in cells preincubated with anandamide, the linoleoylamide-induced block of $I_{K(IR)}$ remained unaltered. Therefore, the linoleoylamide-mediated inhibition of $I_{K(IR)}$ seems to be independent of the presence of anandamide. However, lipoxygenase-generated products of anandamide can competitively inhibit the activity of fatty-acid amide hydrolase (Maccarrone et al., 1998, 2000), which may give rise to an elevated intracellular concentration of linoleoylamide. It will thus be of interest to determine whether linoleoylamide-induced inhibition of $I_{K(IR)}$ and/or arachidonic acid-stimulated $BK_{Ca}$ channel modulates the firing of spontaneous action potentials when intracellular levels of anandamide are so high.

Because *erg*-like $K^+$ channels are functionally expressed in heart cells, it will be important to examine whether linoleoylamide produces a similar action in heart cells. Indeed, a prolongation of the QT interval during sleep has been detected in the patients with ventricular arrhythmia (Gillis et al., 1998). Selective blockade of *erg*-like $K^+$ channels is known to manifest as a prolongation of action potential duration in cardiac myocytes and of its electrocardiographic surrogate, the QT interval (Hondeghem and Synders, 1990; Bauer and Schwarz, 2001; Vandenberg et al., 2001).

Similar to previous observations, unlike linoleoylamide, both arachidonic and epoxyeicosatrienoic acids can stimulate the activity of $BK_{Ca}$ channels in $GH_3$ cells (Duerson et al., 1996; Wu et al., 2000b). Because linoleoylamide can be degraded into arachidonic acid or its metabolites (Cravatt et al., 1996; Patterson et al., 1996), the linoleoylamide-mediated blockade of $I_{K(IR)}$ and arachidonic acid-induced stimulation of $BK_{Ca}$ channels may synergistically act to affect the functional activity of pituitary cells in vivo. In other words, it is likely that the activity of two different $K^+$ channels can reciprocally be modulated in tandem in the presence of linoleoylamide in $GH_3$ cells. Inwardly rectifying $K^+$ and $BK_{Ca}$ channels may be functionally coupled in a dynamic equilibrium driven by linoleoylamide and other sleep-inducing lipids. This seems to represent a unique mechanism for transducing biochemical signals to the ionic events involved in hormonal secretion.

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