2007

The Endogenous Cannabinoid Anandamide Inhibits Cromakalim-Activated K+ Currents in Follicle-Enclosed Xenopus Oocytes

Murat Oz
National Institutes of Health

Keun-Hang Susan Yang
Chapman University, kyang@chapman.edu

Meral Dinc
Oncology Training and Research Hospital, Ankara, Turkey

Toni S. Shippenberg
National Institutes of Health

Recommended Citation

Oz, Murat, et al. "The endogenous cannabinoid anandamide inhibits cromakalim-activated K+ currents in follicle-enclosed Xenopus oocytes." *Journal of Pharmacology and Experimental Therapeutics* 323.2 (2007): 547-554.
doi: 10.1124/jpet.107.125336

The Endogenous Cannabinoid Anandamide Inhibits Cromakalim-Activated K+ Currents in Follicle-Enclosed Xenopus Oocytes

0022-3565/07/3232-547–554
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
U.S. Government work not protected by U.S. copyright
JPET 323:547–554, 2007

Vol. 323, No. 2
125336/3264008
Printed in U.S.A.

The Endogenous Cannabinoid Anandamide Inhibits Cromakalim-Activated K^+ Currents in Follicle-Enclosed *Xenopus* Oocytes

Murat Oz, Keun-Hang Yang, Meral Dinc, and Toni S. Shippenberg

Intramural Research Program, Integrative Neuroscience Section, Department of Health and Human Services, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland (M.O., T.S.S.); Laboratory of Neural Control, Section on Developmental Neurobiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland (K.-H.Y.); and Department of Pulmonary Diseases, Oncology Training and Research Hospital, Ankara, Turkey (M.D.)

Received May 8, 2007; accepted August 1, 2007

ABSTRACT

The effect of the endogenous cannabinoid anandamide on K^+ currents activated by the ATP-sensitive potassium (K_{ATP}) channel opener cromakalim was investigated in follicle-enclosed *Xenopus* oocytes using the two-electrode voltage-clamp technique. Anandamide (1–90 μM) reversibly inhibited cromakalim-induced K^+ currents, with an IC_{50} value of 8.1 $\pm$ 2 μM. Inhibition was noncompetitive and independent of membrane potential. Coapplication of anandamide with the cannabinoid type 1 (CB_1) receptor antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride (SR 141716A) (1 μM), the CB_2 receptor antagonist *N*-[(1*S*)endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) (1 μM), or pertussis toxin (5 μg/ml) did not alter the inhibitory effect of anandamide, suggesting that known cannabinoid receptors are not involved in anandamide inhibition of K^+ currents. Similarly, neither the amidohydrolase inhibitor phenylmethylsulfonyl fluoride (0.2 mM) nor the cyclooxygenase inhibitor indomethacin (5 μM) affected anandamide inhibition of K^+ currents, suggesting that the effects of anandamide are not mediated by its metabolic products. In radioligand binding studies, anandamide inhibited the specific binding of the K_{ATP} ligand [^{3}H]glibenclamide in the oocyte microsomal fractions, with an IC_{50} value of 6.3 $\pm$ 0.4 μM. Gonadotropin-induced oocyte maturation and the cromakalim-acceleration of progesterone-induced oocyte maturation were significantly inhibited in the presence of 10 μM anandamide. Collectively, these results indicate that cromakalim-activated K^+ currents in follicular cells of *Xenopus* oocytes are modulated by anandamide via a cannabinoid receptor-independent mechanism and that the inhibition of these channels by anandamide alters the responsiveness of oocytes to gonadotropin and progesterone.

Arachidonylethanolamine (anandamide) is an endogenous signaling lipid that binds to cannabinoid receptors and produces pharmacological effects similar to cannabinoids in several in vitro preparations (Howlett et al., 2002). Anandamide has been implicated in a number of physiological and pathophysiological processes, including drug abuse, vascular tone, obesity, and embryonic development. Although the mechanisms of action of anandamide are complex and not well established, modulation of various ion channels is thought to mediate some of its effects. For example, in excitable cells such as neurons, the activation of cannabinoid receptors by anandamide suppresses the presynaptic release of various neurotransmitters by inhibiting the function of voltage-dependent Ca^{2+} channels (Howlett et al., 2002). Other studies have shown that anandamide modulates K^+ channels (Poling et al., 1996; Van den Bossche and Vanheel, 2000; Maingret et al., 2001; Oliver et al., 2004; Sade et al., 2006).

ATP-sensitive potassium (K_{ATP}) channels form an important link between metabolic state and cell excitability. They are implicated in the control of insulin secretion, vasoconstriction, and cardiac rhythmicity (for review, see Seino and

The research in this study was supported by the Intramural Research Programs of National Institute on Drug Abuse and National Institute of Neurological Disorders and Stroke, National Institutes of Health.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.107.125336.

ABBREVIATIONS: K_{ATP}, ATP-sensitive potassium; MBS, modified Barth's solution; PTX, pertussis toxin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; SR144528, *N*-[(1*S*)endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; ANOVA, analysis of variance; PMSF, phenylmethylsulfonyl fluoride; GVBD, germinal vesicle breakdown; NEM, *N*-ethylmaleimide; FAAH, fatty acid amide hydrolase; R_o, total resistance determined by oocyte input resistance; R_j, gap junction resistance; R_f, follicular cell input resistance; SUR, sulfonylurea receptor; hCG, human chorionic gonadotropin.

Miki, 2003). K_{ATP} channel openers such as cromakalim and pinacidil or manipulations that decrease intracellular ATP levels activate these channels, whereas antidiabetic sulfonylureas such as glibenclamide suppress their activity. In earlier investigations, it was reported that follicular cells surrounding the oocytes of *Xenopus laevis* express K_{ATP} channels and that activity of these channels can be monitored electrophysiologically (Honoré and Lazdunski, 1991; Guillemare et al., 1994). K_{ATP} channels of follicular cells have been shown to play important roles in oocyte maturation, hormonal regulation of oocyte development (Honoré and Lazdunski, 1991; Wibrand et al., 1992; Arellano et al., 1996), and the growth of *Xenopus* embryos (Cheng et al., 2002; Rutenberg et al., 2002). In the present study, we have investigated the effect of anandamide on K_{ATP} channels of follicle-enclosed oocytes and on the cromakalim-induced acceleration of oocyte maturation.

Materials and Methods

Clusters of oocytes from *X. laevis* frogs (Xenopus I, Ann Arbor, MI) were removed surgically under tricaine (Sigma-Aldrich, St. Louis, MO) anesthesia (0.15%). Oocytes were manually dissected in a solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, and 10 mM HEPES, pH 7.5, and they were stored for 2 to 7 days in modified Barth's solution (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.9 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 10 mM HEPES, pH 7.5, supplemented with 2 mM sodium pyruvate, 10,000 IU/l penicillin, 10 mg/l streptomycin, and 50 mg/l gentamicin. They were placed in a 0.25-ml recording chamber, and then they were superfused at a constant rate of 5 to 7 ml/min. The bathing solution consisted of 95 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, and 5 mM HEPES, pH 7.5. The cells were impaled at the animal pole with two glass microelectrodes filled with 3 M KCl (1–5 MΩ) and voltage-clamped at a holding potential of −20 mV using a GeneClamp-500 amplifier (Molecular Devices, Sunnyvale, CA). Current responses were digitized by A/D converter and analyzed using pClamp 6 (Molecular Devices) run on an IBM/PC or directly recorded on a Gould 2400 rectilinear pen recorder (Instrument Systems Inc., Cleveland, OH). Current-voltage characteristics were studied using 1-s voltage steps (−120 to 20 mV). Drugs were applied externally by addition to the superfusate. Oocytes were incubated in pertussis toxin (PTX; 5 μg/ml) overnight (14–16 h). Procedures for the injections of BAPTA (50–70 nl, 100 mM) were described earlier in detail (Oz et al., 1998). BAPTA was prepared in Cs_4-BAPTA. Injections were performed 1 h before recordings using an oil-driven ultramicrosyringe pump (Micro4; WPI, Sarasota, FL). Stock solutions of anandamide were prepared in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. DMSO, alone, did not affect cromakalim-induced currents when added at concentrations up to 0.3% (v/v), a concentration 2-fold greater than the highest concentration used in the present study.

Anandamide, cromakalim, glibenclamide, *R*-(+)-methanandamide, *N*-ethylmaleimide, pertussis toxin, human chorionic gonadotropin, progesterone, and BAPTA were obtained from Sigma/RBI (St. Louis, MO). SR 141716A and SR144528 were generously provided by National Institute on Drug Abuse Drug Supply System/National Institutes of Health (Baltimore, MD). Both SR 141716A and SR144528 were originally synthesized by Research Triangle Institute (Research Triangle Park, NC) on behalf of National Institute on Drug Abuse.

Data are expressed as mean ± S.E.M. Statistical significance at the level of 0.05 was analyzed using the Student's *t* test, paired *t* test, or ANOVA. Concentration-response curves were obtained by fitting the data to the logistic equation $y = \{(E_{max} - E_{min})/(1 + [EC_{50}/x]^n)\} + E_{min}$, where x and y are concentration and response, respectively, E_{max} is the maximal response, E_{min} is the minimal response, EC_{50} is the half-maximal concentration, and n is the slope factor.

Radioligand Binding Experiments. For radioligand binding experiments, follicle-enclosed oocytes were suspended in 300 ml of buffer containing 50 mM HEPES, 0.3 M sucrose, and 1 mM EDTA at 4°C on ice. Oocytes were homogenized using a motorized Teflon homogenizer (six strokes, 15 s each at high speed). This was followed by sequential centrifugations at 1000*g* for 10 min and 10,000*g* for 20 min; each time the pellet was discarded, and the supernatant was used for the subsequent step. The final centrifugation was at 60,000*g* for 25 min. The microsomal pellet, which contains the membranes of follicular cells (Guillemare et al., 1994), was resuspended in 50 mM HEPES buffer, and it was used for the binding studies.

The radioligand binding experiments were carried out at room temperature (20–22°C) for 1 h (Oz et al., 2004), and 0.2 mM PMSF was routinely included to prevent anandamide degradation. Oocyte membranes were incubated in 1 ml of 50 mM HEPES, pH 7.5, at a protein concentration of 200 to 500 μg/ml. [^{3}H]glibenclamide was dissolved in ethanol/dimethyl sulfoxide (1:1). For each experiment, freshly made glibenclamide solution was used. At the final concentrations used in this study (0.2%), organic solvents had no effect on [^{3}H]glibenclamide binding. IC_{50} values were determined using one-site competition nonlinear curve fitting. For the nonlinear curve-fitting and regression fits of the radioligand binding data, the computer software Origin (OriginLab Corp., Northampton, MA) was used.

Maturation Experiments. Oocytes (stages V or VI according to Dumont, 1972) were manually dissected using watchmaker's forceps under a stereomicroscope, and then they were stored in MBS until used for drug or hormone treatments. Groups of oocytes (20 oocytes each) from each donor were incubated in the MBS containing hormones (200 IU/ml human chorionic gonatropin or 0.1 μM progesterone and 100 μM cromakalim) at 18°C in the absence or presence of test compounds (10 μM anandamide or 10 μM glibenclamide) up to 18 h. Germinal vesicle breakdown (GVBD) was monitored by tracking white spot (Roux) formation followed by fixation of oocytes in 2% trichloroacetic acid, dissection using watchmaker's forceps under the stereomicroscope, and examination for the presence or absence of an intact nucleus (germinal vesicle). Stock solutions of anandamide were prepared in 0.1% DMSO (v/v), and 0.2 mM PMSF was routinely included in MBS solution to prevent anandamide degradation. At these concentrations, DMSO alone and PMSF alone did not affect oocyte maturation monitored up to 16 h. The results are expressed as the percentage of the matured oocytes (% GVBD) as a function of time. The total number of oocytes in each group was considered as 100%.

Results

Figure 1A shows that application of cromakalim induces a slowly developing outward current in follicle-enclosed oocytes. In agreement with earlier studies (Honoré and Lazdunski, 1991), the maximal amplitudes of outward currents did not change during repeated applications of cromakalim every 15 min for up to 2 h. Cromakalim-activated currents are reversibly inhibited to 53 ± 6% of controls (mean ± S.E.M.; $n = 4$) by 1 μM glibenclamide, a selective blocker of K_{ATP} channels.

Treatment of follicle-enclosed oocytes with 10 μM anandamide for 30 min significantly inhibited the slow-outward current induced by 100 μM cromakalim (Fig. 1A). Recovery was incomplete during the 45- to 60-min washout period. Results of experiments demonstrating the time course of the effects of anandamide on the mean amplitudes of the cromakalim-induced currents are presented in Fig. 1B. Effects of anandamide were observed in response to a concentration

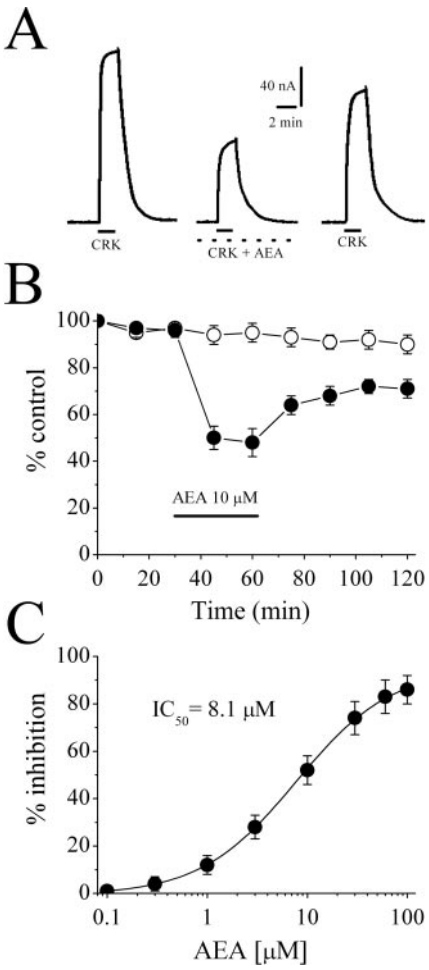

Fig. 1. Anandamide inhibits cromakalim-induced outward current in follicular cells of *Xenopus* oocytes. A, current traces induced by 100 μM cromakalim (left), during coapplication of cromakalim and 10 μM anandamide following 30-min preincubation in anandamide (middle), and 45-min recovery (right). Application times for cromakalim are presented with black lines. Continuous application of anandamide during recordings is presented with dashed lines. B, time course of the peak cromakalim-activated currents in the absence (open circles) and the presence 10 μM anandamide (closed circles). Each data point represents the normalized means and S.E.M. of four to six experiments. The duration of the anandamide application is indicated by the horizontal bar. C, concentration-response curve for inhibitory effect of 30-min anandamide treatment on 100 μM cromakalim-activated currents. Data points are expressed as mean ± S.E.M (n = 4–5). The curve is the best fit of the data to the logistic equation described under *Materials and Methods*. In all figures, AEA and CKL indicate anandamide and cromakalim, respectively.

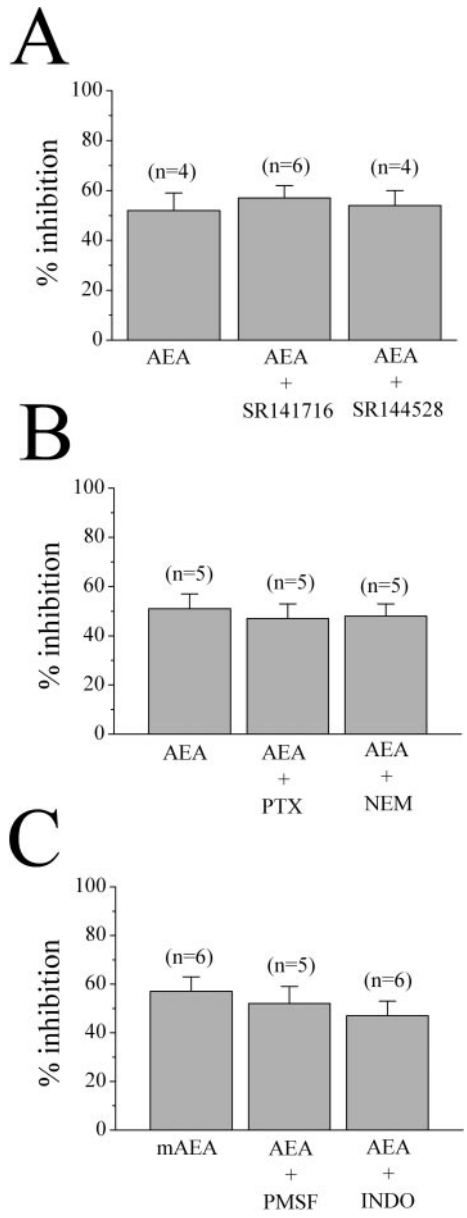

Fig. 2. Anandamide inhibition of cromakalim-activated K^+ currents is not altered by CB_1 or CB_2 receptor antagonist, and it is not mediated by pertussis toxin-sensitive G proteins. A, CB_1 receptor antagonist SR 141716A at 1 μM or the CB_2 receptor antagonist SR144528 at 1 μM was coapplied with 10 μM anandamide for 30 min, and the maximal amplitudes of cromakalim-activated K^+ currents are presented as percentage of controls. B, oocytes were incubated in 5 μg/ml pertussis toxin overnight, and the extent of anandamide inhibition was presented as percentage of controls. In experiments using NEM, 10 μM anandamide was coapplied with 10 μM NEM. C, percentage of anandamide (10 μM) inhibition of cromakalim-activated currents in the presence of *R*-methanandamide, PMSF, and indomethacin. The number of oocytes tested is presented on top of each bar. INDO, indomethacin, metAEA, *R*-methanandamide,

of 1 μM, and they reached a maximum in the range of 90 to 100 μM (84 ± 7% inhibition; n = 4–6). Figure 1C shows the effect of increasing anandamide concentrations on cromakalim-induced outward currents. The concentration resulting in 50% of maximal anandamide inhibition (IC_{50}) was obtained at 8.1 μM. The slope value was 0.9.

To determine whether endogenous cannabinoid-like receptors mediate the effects of anandamide, we tested the effects of the CB_1 antagonist SR 141716A on anandamide-induced inhibition of cromakalim-activated currents. Coapplication of 1 μM SR 141716A with 10 μM anandamide for 30 min did not alter the effects of anandamide (Fig. 2A) (ANOVA: $P > 0.05$, n = 5–7). Likewise, the CB_2 receptor antagonist SR144528 (1 μM) did not affect the inhibition of cromakalim-activated currents by anandamide (Fig. 2A) (ANOVA: $P > 0.05$, n = 5–6). Applications of 1 μM SR 141716A alone or 1 μM SR 144528 alone for 30 min did not significantly alter the amplitudes of peak currents in response to cromakalim (paired t test: $P > 0.05$, n = 3–4; data not shown).

Because the G proteins involved in the signaling of cannabinoid receptor-mediated effects are PTX-sensitive (Howlett

et al., 2002), we tested the effect of anandamide in vehicle (distilled water) and PTX-treated follicle-enclosed oocytes. No significant difference between controls and PTX-treated cells in the response to anandamide was seen (Fig. 2B) (ANOVA: $P > 0.05$, $n = 6$). *N*-Ethylmaleimide (NEM; 10 μM), a sulfhydryl-alkylating agent that blocks G protein-effector interactions by alkylating α-subunits of PTX-sensitive GTP-binding protein, is commonly used to investigate the functional roles of G_i and G_o type of G proteins (Oz and Renaud, 2002). Analogous to PTX, the extent of inhibition by 30-min treatment with 10 μM NEM and 10 μM anandamide was not significantly different from the inhibition by 10 μM anandamide alone (Fig. 2B) (51 $\pm$ 6 versus 47 $\pm$ 5%; $n = 5$, in the absence and presence of NEM treatment, respectively; ANOVA: $P > 0.05$).

Anandamide is hydrolyzed by an intracellularly located enzyme, fatty acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2002). Because degradation products of anandamide hydrolysis can mediate the effects of anandamide, the inhibition of cromakalim-activated currents by anandamide was quantified in the presence and absence of 0.2 mM PMSF, an inhibitor of FAAH (Cravatt and Lichtman, 2002). PMSF did not alter the inhibitory actions of anandamide (Fig. 2C) (ANOVA: $P > 0.05$, $n = 6$). The effect of *R*-methanandamide, a metabolically stable chiral analog of anandamide that is resistant to hydrolytic inactivation by fatty acid amide hydrolase (Abadji et al., 1994) on cromakalim-activated currents was also tested. *R*-methanandamide at 10 μM evoked significantly greater inhibition than anandamide (52 $\pm$ 4%, $n = 6$ versus 64 $\pm$ 5%, $n = 5$ for anandamide and *R*-methanandamide, respectively; ANOVA: $P < 0.05$). Anandamide is a substrate for cyclooxygenase. To rule out the involvement of cyclooxygenated metabolites of anandamide, we tested the effects of anandamide in the presence and absence of the cyclooxygenase inhibitor indomethacin (5 μM). The extent of anandamide inhibition was not altered significantly by indomethacin (Fig. 2C) (ANOVA: $P > 0.05$, $n = 5$). Figure 3, A to D, shows the influence of anandamide on the current-voltage relationship of the cromakalim-induced net outward current (cromakalim-activated current minus resting current at given voltage). In the presence and absence of 10 μM anandamide, the reversal of the outward current is observed at -97 ± 4 and -95 ± 3 mV, respectively (paired *t* test: $P > 0.05$, $n = 4$). These findings indicate that ionic selectivity of

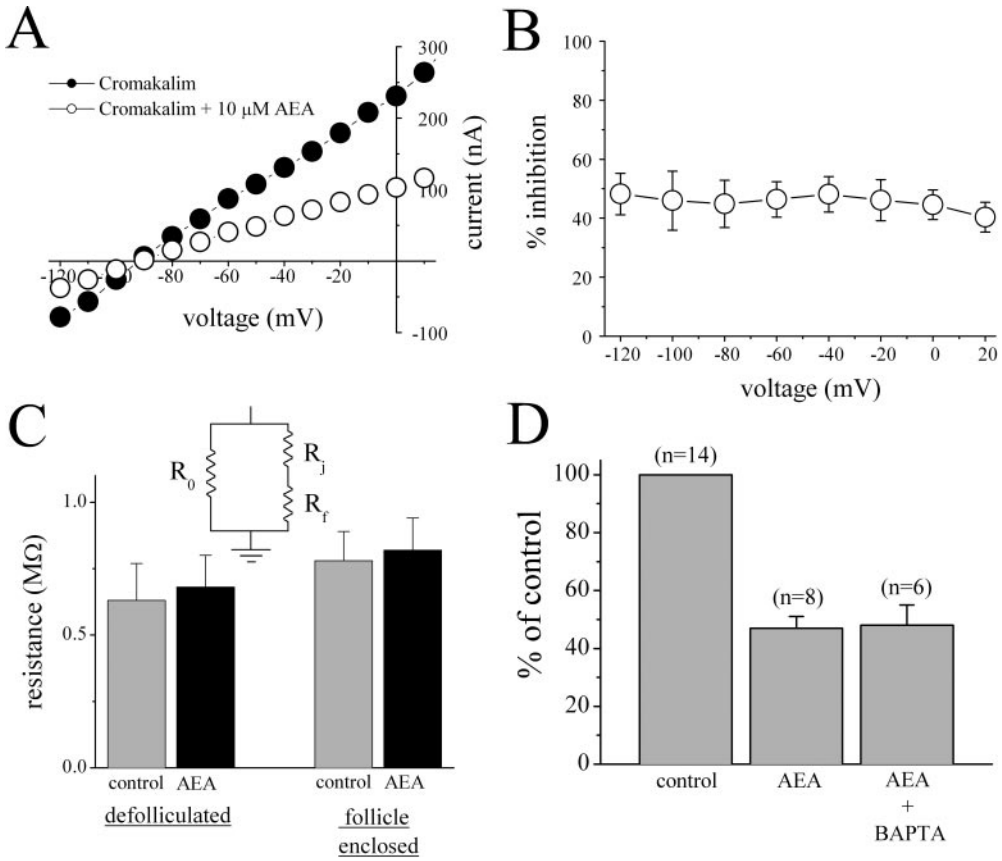

Fig. 3. Anandamide inhibition of cromakalim-activated K^+ currents is independent of voltage and intracellular Ca^{2+} levels. A, current-voltage relationship of cromakalim-activated currents recorded during 1-s voltage steps applied before (closed circles) and after application of 10 μM anandamide (open circles). Control currents were subtracted from cromakalim induced currents. B, percentage of inhibition of cromakalim-activated K^+ currents by anandamide at different membrane potentials. There were no statistically significant differences among the means of current inhibitions by anandamide at different holding potentials (ANOVA: $P > 0.05$, $n = 4–5$). C, equivalent resistive-circuit diagram is presented in inset to Fig. 2C. Before (gray bars) and after 5-min incubation with 10 μM anandamide (black bars), the mean values for the sum of R_j and R_f in follicle-enclosed oocytes are presented on the right side ($n = 8$). Values, before and after anandamide treatment, for R_o of enzymatically defolliculated oocytes are shown on the left ($n = 12$). Resistance values were calculated from current-voltages curves recorded in the range of -50 to $+10$ mV. Bars, on the right side, show mean values for resistance as a sum of R_j and R_f before and after 30-min anandamide incubation. Bars, on the left side, show mean values for R_o measurements (in the range of -50 to $+10$ mV) in defolliculated oocytes before and after 30-min incubation with 10 μM anandamide. D, percentage of anandamide (10 μM) inhibition of cromakalim activated currents in BAPTA-treated oocytes. Asterisk (*) indicates a statistically significant difference between two groups shown by the arrows in the figure (ANOVA: $P < 0.05$, $n = 6–8$).

the cromakalim-induced outward current is not affected by anandamide. Follicular cells are coupled to oocytes through gap junctions (for reviews, see Dascal, 1987; Arellano et al., 1996). Anandamide may affect gap junctions (Venance et al., 1995), thereby altering the resistance of the ionic pathway presented as equivalent resistive circuitry in the inset to Fig. 3C. In this case, total resistance would be determined by oocyte input resistance (R_o) on the one branch and by the sum of gap junction resistance (R_j) and/or follicular cell input resistance (R_f) on the other branch. We measured resistances in follicle-enclosed (to investigate the involvement of R_o) and enzymatically (2-h treatment in 0.2% collagenase A; Roche Diagnostics, Indianapolis, IN) defolliculated oocytes (to investigate the involvements of R_j and R_f) in the presence and absence of anandamide without inducing cromakalim-activated conductances in follicular cells. In the presence and absence of anandamide, there were no significant changes in resistances measured from defolliculated or follicle-enclosed oocytes (Fig. 3C) (paired t test: $P > 0.05$, $n = 7$–11).

Because anandamide releases intracellular Ca^{2+} in various cell types (Mombouli et al., 1999; Yeh et al., 2006), activation of Ca^{2+}-dependent second messenger systems or Ca^{2+}-activated Cl^- and/or K^+ channels may interfere with anandamide actions. To examine this possibility, follicle-enclosed oocytes were incubated in 5 μM BAPTA-AM for 1 h, and then they were injected with 5 nl of 100 mM BAPTA 10 min before recordings to ensure chelation of intracellular Ca^{2+} in both follicular cells and oocytes. No significant difference in the percentage of inhibition of cromakalim-induced currents by anandamide was observed in BAPTA-treated oocytes relative to controls (Fig. 3D) (Student's t test: $P > 0.05$, $n = 5$). In BAPTA-treated oocytes, current-voltage curves recorded in the absence and presence of anandamide did not show significant changes in reversal potential (-96 ± 3 versus -92 ± 4; Student's t test: $P > 0.05$, $n = 5$) or in characteristics of the curve.

Anandamide may also compete with cromakalim binding site(s) located on the K_{ATP} channel complex, thereby causing inhibition of cromakalim-activated currents. Thus, the concentration-response curve of cromakalim activation was examined in the absence and presence of 10 μM anandamide. As shown in Fig. 4, anandamide inhibited maximal cromakalim-induced currents without altering EC_{50} values for cromakalim ($n = 4$–5). The EC_{50} and slope values in the presence and absence of 10 μM anandamide were 127 $\pm$ 18 μM and 1.07 versus 132 $\pm$ 21 μM and 1.02. These results suggest that anandamide inhibits cromakalim-activated K^+ currents in a noncompetitive manner.

The K_{ATP} channel is formed from four Kir6.2 subunits, each of which is associated with a larger regulatory sulfonylurea receptor (SUR) subunit (for review, see Seino and Miki, 2003). Because the binding site for sulfonylureas and cromakalim is located in the SUR subunit of the channel, we tested the effect of anandamide on the specific binding of [^{3}H]glibenclamide in the microsomal fraction of *Xenopus* oocytes. Equilibrium curves for the binding of [^{3}H]glibenclamide, in the presence and absence of the anandamide, are presented in Fig. 5A. In the presence of 10 μM anandamide, the specific binding of [^{3}H]glibenclamide decreased to 64.3 $\pm$ 7.2% of controls ($n = 4$). Maximum binding activity (B_{max}) of [^{3}H]glibenclamide was 6.28 $\pm$ 0.23 and 2.86 $\pm$ 0.16 pmol/mg for controls and anandamide, respectively. The affinity (K_d)

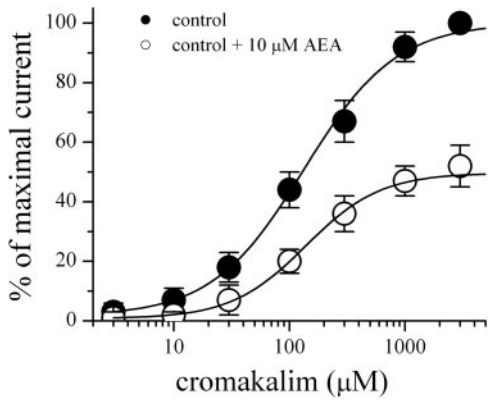

Fig. 4. Concentration-response curves for cromakalim-activated current in the absence (closed circles) and presence (open circles) of 10 μM anandamide. Anandamide was applied for 30 min, and cromakalim and anandamide were then coapplied for 2 min. Data points are the mean $\pm$ S.E.M. ($n = 4$–5; error bars not visible are smaller than the size of the symbols). The curves are the best fit of the data to the logistic equation described under *Materials and Methods*. The concentration-response curves are normalized to the percentage of maximal control cromakalim response. In absence and presence of anandamide, EC_{50} values were 163 $\pm$ 21 versus 154 $\pm$ 24 μM, respectively.

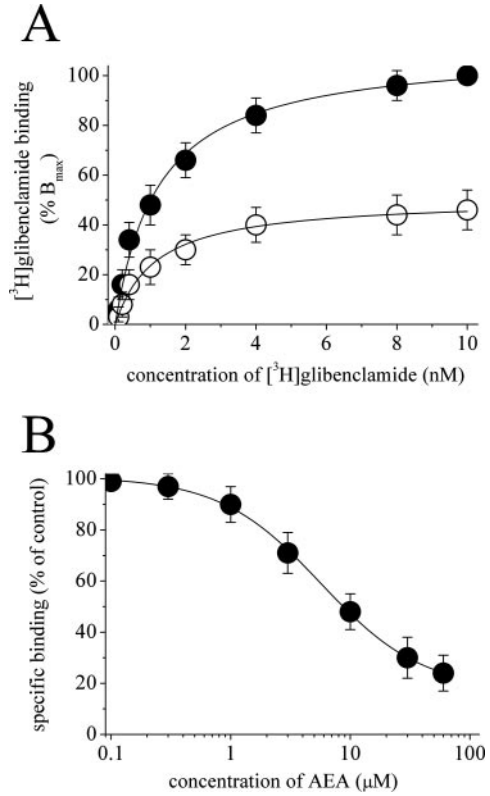

Fig. 5. Effects of anandamide on the specific binding of [^{3}H]glibenclamide to microsomal membrane fraction of *Xenopus* oocytes. A, specific binding as a function of the concentration of [^{3}H]glibenclamide. Data are presented as the arithmetic means of four experimental measurements in the absence (●) and presence of 10 μM anandamide (○). The incubation time was 60 min at 22°C, pH 7.5. Equivalent samples were incubated with 10 nM of unlabeled glibenclamide to determine nonspecific binding. B, effects of increasing concentration of anandamide on the specific binding of [^{3}H]glibenclamide. Microsomal membranes were incubated with 1 nM [^{3}H]glibenclamide at a concentration of 0.3 to 0.5 mg/ml for 60 min with increasing concentrations of anandamide in the medium. Bound and free [^{3}H]glibenclamide were separated by filtration. Symbols are the means of at least five experiments. The results present data from four to five experiments. Data points indicate mean $\pm$ S.E.M.

of [^{3}H]glibenclamide was 1.3 ± 0.1 and 1.1 ± 0.2 nM for controls and anandamide, respectively. The effect of anandamide on the displacement of specific binding of [^{3}H]glibenclamide from microsomal membranes were also investigated (Fig. 5B). Anandamide (1–100 μM) inhibited the specific binding of [^{3}H]glibenclamide to 80 to 85% of control (n = 4–6). The IC_{50} value for anandamide was 6.3 ± 0.4 μM, with corresponding slope factors of 0.9.

K_{ATP} channels play an important role in hormonal activation of the maturation process in *Xenopus* oocytes (Woodward and Miledi, 1987; Honoré and Lazdunski, 1991; Wibrand et al., 1992; Arellano et al., 1996). During maturation, the large oocyte nucleus (germinal vesicle), which resides in the darkly pigmented animal part of the oocyte (Fig. 6A, top left) falls apart and the appearance of a white (Roux) spot on the animal pole (Fig. 6A, top right) correlates with GVBD, a process indicating the resumption of meiosis in oocytes (Smith, 1989). Pharmacological agents such as cromakalim or hormones such as gonadotropins and insulin are potent activators of K_{ATP} channels in follicular cells (Woodward and Miledi; 1987; Honoré and Lazdunski, 1991; Sakuta, 1994; Arellano et al., 1996). Activation of K_{ATP} channels by gonadotropins induces oocyte maturation and GVBD that can be effectively blocked by K_{ATP} channel blockers such as glibenclamide (Woodward and Miledi, 1987; Wibrand et al., 1992; Arellano et al., 1996). Thus, we have tested the effect of anandamide on human chorionic gonadotropin (hCG; 200 IU/ml)-induced GVBD process in follicle-enclosed *Xenopus* oocytes. In agreement with earlier findings (for reviews, see Smith, 1989; Murakami and Vande Woude, 1997), incubation of oocytes in hCG containing MBS solution caused a time-dependent increase of the maturation response (Fig. 6A). In the presence of 10 μM anandamide, hCG-induced maturation of oocytes from three different donors was significantly inhibited (Fig. 6A) (ANOVA: $P < 0.05$, $n = 60$). Similarly, incubation of oocytes in MBS containing 10 μM glibenclamide inhibited the hCG-induced maturation process (ANOVA: $P < 0.05$, $n = 60$ from three different donors). In agreement with earlier findings (Honoré and Lazdunski, 1991; Wibrand et al., 1992), tolbutamide, an antidiabetic sulfonylurea with approximately 1000 times less affinity than glibenclamide on pancreatic β cells (K_d values are in the range of 0.5–20 nM and 1–17 μM for glibenclamide and tolbutamide, respectively), was not active on cromakalim-activated currents in follicle-enclosed oocytes at 10 μM (n = 4), and it was also ineffective on maturation ($n = 60$). Compared with control oocytes kept in MBS, anandamide alone at 10 μM caused a small (14.2 ± 4.6%) but statistically significant increase in the maturation rate of follicle-enclosed oocytes (ANOVA: $P < 0.05$, $n = 60$). Glibenclamide alone at 10 μM ($n = 60$) did not alter maturation process (Fig. 6A).

In *Xenopus* oocytes, gonadotropins stimulate surrounding follicle cells, causing them to secrete the steroid hormone progesterone, which induces maturation in the oocyte (for review, see Murakami and Vande Woude, 1997). Thus, in defolliculated oocytes, progesterone, but not gonadotropins, can induce maturation, and K_{ATP} channel activators can potentiate progesterone-induced maturation only in follicle-enclosed oocytes (Woodward and Miledi, 1987; Wibrand et al., 1992). In the presence of cromakalim, progesterone, at concentrations that do not induce maturation when applied alone, can stimulate the maturation process in follicle-enclosed oocytes (Wibrand et al., 1992). Thus, we tested the effect anandamide on cromakalim potentiation of progesterone-induced maturations process in follicle-enclosed oocytes. Similar to an earlier result (Wibrand et al., 1992), although cromakalim at 100 μM alone did not induce GVBD ($n = 20$), it caused a significant acceleration of progesterone-induced GVBD (Fig. 6B) (ANOVA: $P < 0.05$, $n = 60$ from three different donors). Both, anandamide at 10 μM ($n = 60$) and glibenclamide at 10 μM ($n = 60$) significantly inhibited the cromakalim potentiation of progesterone-induced GVBD (Fig. 6B) (ANOVA: $P < 0.05$).

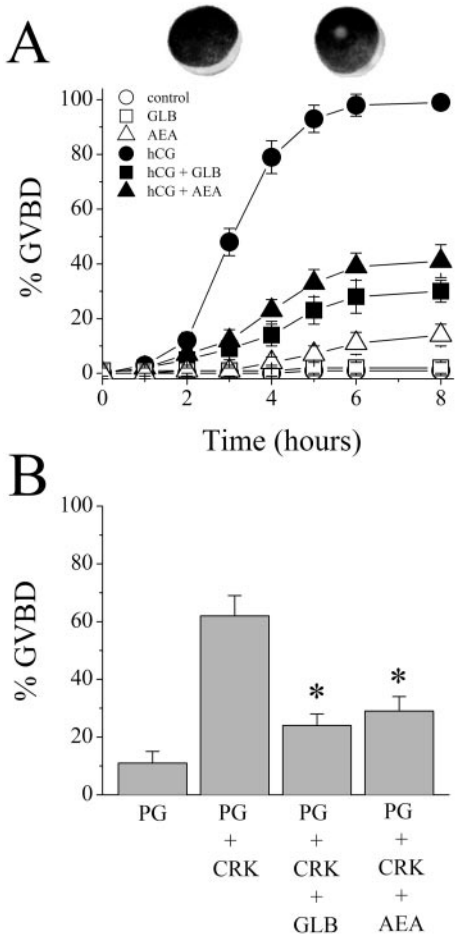

Fig. 6. Effects of anandamide on the oocyte maturation induced by the either gonadotropin alone or by cromakalim and progesterone in follicle-enclosed oocytes. A, effect of anandamide on hCG (200 IU/ml)-induced maturation in follicle-enclosed oocytes. The kinetics of maturation was assayed in oocytes exposed continuously to hCG alone (●), in the presence of hCG and 10 μM glibenclamide (■), and in the presence of hCG and anandamide (▲) were examined for GVBD process during a 8-h exposure. Control group (○) was incubated in MBS alone. The results for 10 μM glibenclamide alone and 10 μM anandamide alone were presented by □ and △, respectively. Data for each group are from total of 60 oocytes from three donors (mean ± S.E.M.). B, effect of anandamide on the cromakalim potentiation of progesterone-induced maturation in follicle-enclosed *Xenopus* oocytes. Oocytes were incubated in MBS in the presence of 0.1 μM progesterone or progesterone and 100 μM cromakalim for 16 h. Maturation responses induced by cromakalim and progesterone were tested in the presence of 10 μM anandamide or 10 μM glibenclamide. Each bar represents means ± S.E.M. of 60 oocytes from three donors. Asterisk (*) indicates a statistically significant difference between treatment groups and cromakalim + progesterone group (ANOVA: $P < 0.05$). AEA, anandamide; CKL, cromakalim; GLB, glibenclamide; PG, progesterone.

Discussion

In this study, we have demonstrated that anandamide inhibits cromakalim-induced K^+ currents in follicle-enclosed oocytes in a noncompetitive manner. The CB_1 receptor antagonist SR 141716, the CB_2 receptor antagonist SR144528, pertussis toxin, and NEM treatments did not affect the anandamide inhibition of cromakalim-induced K^+ currents. Thus, it is unlikely that the observed effects of anandamide on cromakalim-induced K^+ currents are due to the activation of cannabinoid receptors or other G_i/G_o-dependent G protein-coupled receptors. In presence of PMSF, amidohydrolase inhibitor, or indomethacin, cyclooxygenase inhibitor anandamide-induced inhibition of cromakalim-induced K^+ currents was not significantly altered, suggesting that hydrolization and cyclooxygenation products of anandamide are not involved in anandamide inhibition of cromakalim-induced currents. Furthermore, *R*-methanandamide, a metabolically stable chiral analog of anandamide that is resistant to hydrolytic inactivation by fatty acid amide hydrolase (Abadji et al., 1994), also inhibited cromakalim-induced currents, further indicating that the metabolites of anandamide degradation do not contribute significantly to the observed effect of anandamide.

In agreement with our results, several earlier studies indicated that different types K^+ channels are modulated by anandamide in a cannabinoid receptor-independent manner (Poling et al., 1996; Van den Bossche and Vanheel, 2000; Maingret et al., 2001; Oliver et al., 2004; Sade et al., 2006). Anandamide belongs to a class of signaling lipids consisting of amides of long-chain polyunsaturated fatty acids (Howlett et al., 2002). Thus, several fatty acid-based lipids have also been shown to modulate the functions of K^+ channels in various expression systems (Baukrowitz and Fakler, 2000; Oliver et al., 2004; Klein et al., 2005). In addition to K^+ channels, anandamide modulates the functions of other ion channels such as serotonin type 3 (Oz et al., 2002), nicotinic acetylcholine (Oz et al., 2003) and glycine (Lozovaya et al., 2005; Hejazi et al., 2006) receptors, and voltage-gated Ca^{2+} (Oz et al., 2000; Fisyunov et al., 2006) and Na^+ (Nicholson et al., 2003; Kim et al., 2005) channels in a manner independent of known cannabinoid receptors (for recent review; see Oz, 2006).

Follicular cells of oocytes endogenously express cromakalim-activated K_{ATP} channels that have similar pharmacological and biophysical properties to those found in other tissues (Honoré and Lazdunski, 1991; Guillemare et al., 1994). These cells are electrically coupled to oocytes through gap junctions (for review, see Arellano et al., 1996). Thus, the effect of anandamide on gap junctions (Venance et al., 1995) might be expected to affect membrane resistance (through oocyte, gap junction, and follicular cells). However, in both follicle-enclosed and defolliculated oocytes, anandamide did not cause a detectable change in cell input resistance, which was calculated near −20 mV (the holding potential for cromakalim-activated currents). These results suggest that when cromakalim-activated channels are closed, ionic conductances are not altered by 10 μM anandamide in either follicle-enclosed or defolliculated oocytes and that anandamide does not affect conductances other than those activated by cromakalim.

Anandamide previously was shown to increase intracellular Ca^{2+} levels (Mombouli et al., 1999; Yeh et al., 2006). The changes in intracellular Ca^{2+} levels could affect the function of Ca^{2+}-activated Cl^- channels and might interfere with the effect of anandamide on K^+ currents. However, in BAPTA-treated oocytes, anandamide continued to suppress cromakalim responses to the same extent in untreated oocytes. Furthermore, there was no apparent change of the reversal potential of cromakalim-induced currents, suggesting that Ca^{2+}-activated conductances are not significantly involved in the effect of anandamide on K^+ currents. In addition, because we voltage-clamped near the reversal potential (−20 mV) for Ca^{2+}-activated Cl^- channels in oocytes (Dascal, 1987), it is unlikely that the changes in intracellular Ca^{2+} concentrations would interfere with the effect of anandamide on K^+ conductance. In agreement with earlier studies (Honoré and Lazdunski, 1991; Guillemare et al., 1994), the current-voltage relationship for cromakalim-activated current was linear within the voltage range studied (−120 to 20 mV). In the presence of anandamide, neither the characteristics nor the reversal potential for cromakalim-activated K^+ currents was altered (Fig. 2A).

The pore of the K_{ATP} channel is formed from four Kir6.2 subunits, each of which is associated with a larger regulatory SUR subunit, which is the primary target for K_{ATP} blockers and openers (Seino and Miki, 2003). Anandamide did not alter EC_{50} values for cromakalim activation, and it inhibited the maximal cromakalim-induced currents, suggesting that it does not compete with the cromakalim binding site. The ability of anandamide to inhibit the maximal specific binding of the radioligand [^{3}H]glibenclamide to oocyte microsomal membranes without altering its affinity to [^{3}H]glibenclamide suggests that anandamide interacts with the glibenclamide binding site on the SUR in a noncompetitive manner. Subunit identification of endogenous K_{ATP} channels in follicular cells remains currently unknown. A recent study suggested that a novel type of Kir6.1/SUR2A combination comprises endogenous K_{ATP} channels (Fujita et al., 2007). Some preliminary findings indicate that anandamide inhibits [^{3}H]glibenclamide binding to Kir6.2/SUR1 and SUR1 expressed in human embryonic kidney-293 cells, with IC_{50} values ranging 3 to 5 μM (Dr. Ulrich Quast, unpublished results), and Kir6.2/SUR2A subunit combination expressed in *Xenopus* oocytes is inhibited by 10 μM anandamide (Dr. Thomas Baukrowitz, unpublished results).

For many years, *Xenopus* oocytes have served as a model system for studying intricate mechanisms of follicle maturation and cell cycle control (Brown, 2004). In vivo, stage VI immature oocytes are physiologically arrested in the first meiotic prophase at the G_2/M border, and they resume meiosis when gonadotropins stimulate surrounding follicle cells. This causes progesterone secretion (for reviews, see Smith, 1989; Murakami and Vande Woude, 1997) and the initiation of oocyte maturation, a crucial process transforming the immature oocyte into a fertilizable egg. In *Xenopus* oocytes, gonadotropins and insulin activate K_{ATP} channels (Woodward and Miledi, 1987; Honoré and Lazdunski, 1991; Sakuta, 1994) and they facilitate progesterone-induced oocyte maturation (Wibrand et al., 1992). It was previously shown that suppression of cromakalim-activated channels by glibenclamide inhibits gonadotropin-induced maturation in follicle-enclosed oocytes (Wibrand et al., 1992). The results of the present study indicate that the gonadotropin-induced, glib-

enclamide-sensitive oocyte maturation process is inhibited by anandamide. Furthermore, both glibenclamide and anandamide inhibited the cromakalim-induced acceleration of progesterone-induced oocyte maturation, suggesting that the inhibition of K_{ATP} channels by anandamide modulates the hormonal maturation process in oocytes.

K_{ATP} channels are also expressed in early embryonic cells, and their functional modulation prevents the hatching of the *Xenopus* embryos (Cheng et al., 2002; Rutenberg et al., 2002), resulting in defective development. The effect of anandamide on the hormonal induction of oocyte development has not been studied. Because K_{ATP} channels in follicular cells modulate the actions of several hormones implicated in oocyte maturation, the present results provide suggestive evidence that inhibition of K_{ATP} channels by anandamide can modulate the hormonal maturation process in *Xenopus* oocytes.

Acknowledgments

We are grateful to Drs. U. Quast and T. Baukrowitz for comments and helpful discussions.

References

Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG, and Makriyannis A (1994) (*R*)-Methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. *J Med Chem* **37:**1889–1893.

Arellano RO, Woodward RM, and Miledi R (1996) Ion channels and membrane receptors in follicle-enclosed *Xenopus* oocytes. *Ion Channels* **4:**203–259.

Baukrowitz T and Fakler B (2000) K_{ATP} channels: linker between phospholipid metabolism and excitability. *Biochem Pharmacol* **60:**735–740.

Brown DD (2004) A tribute to the *Xenopus laevis* oocyte and egg. *J Biol Chem* **279:**45291–45299.

Cheng SM, Chen I, and Levin M (2002) K_{ATP} channel activity is required for hatching in *Xenopus* embryos. *Dev Dyn* **225:**588–591.

Cravatt BF and Lichtman AH (2002) The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chem Phys Lipids* **121:**135–148.

Dascal N (1987) The use of *Xenopus* oocytes for the study of ion channels. *CRC Crit Rev Biochem* **22:**317–387.

Dumont JN (1972) Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J Morphol* **136:**153–180.

Fisyunov A, Tsintsadze V, Min R, Burnashev N, and Lozovaya NA (2006) Cannabinoids modulate the P-type high voltage-activated calcium currents in Purkinje neurons. *J Neurophysiol* **96:**1267–1277.

Fujita R, Kimura S, Kawasaki S, Watanabe S, Watanabe N, Hirano H, Matsumoto M, and Sasaki K (2007) Electrophysiological and pharmacological characterization of KATP channel involved in the K^+ current responses to FSH and adenosine in the follicular cells of *Xenopus* oocyte. *J Physiol Sci* **57:**51–56.

Guillemare E, Lazdunski M, and Honore E (1994) CGRP-induced activation of KATP channels in follicular *Xenopus* oocytes. *Pflugers Arch* **428:**604–609.

Hejazi N, Zhou C, Oz M, Ye HJ, and Zhang L (2006) Δ^9-Tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. *Mol Pharmacol* **69:**991–997.

Honoré E and Lazdunski M (1991) Hormone-regulated K^+ channels in follicle-enclosed
oocytes are activated by vasorelaxing K^+ channel openers and blocked by antidiabetic sulfonylureas. *Proc Natl Acad Sci U S A* **88:**5438–5442.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, et al. (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54:**161–202.

Kim HI, Kim TH, Shin YK, Lee CS, Park M, and Song JH (2005) Anandamide suppression of Na^+ currents in rat dorsal root ganglion neurons. *Brain Res* **1062:**39–47.

Klein A, Lichtenberg J, Stephan D, and Quast U (2005) Lipids modulate ligand binding to sulphonylurea receptors. *Br J Pharmacol* **145:**907–915.

Lozovaya N, Yatsenko N, Beketov A, Tsintsadze T, and Burnashev N (2005) Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. *J Neurosci* **25:**7499–7506.

Maingret F, Patel AJ, Lazdunski M, and Honore E (2001) The endocannabinoid anandamide is a direct and selective blocker of the background K^+ channel TASK-1. *EMBO J* **20:**47–54.

Mombouli JV, Schaeffer G, Holzmann S, Kostner GM, and Graier WF (1999) Anandamide-induced mobilization of cytosolic Ca^{2+} in endothelial cells. *Br J Pharmacol* **126:**1593–1600.

Murakami MS and Vande Woude GF (1997) Mechanisms of *Xenopus* oocyte maturation. *Methods Enzymol* **283:**584–600.

Nicholson RA, Liao C, Zheng J, David LS, Coyne L, Errington AC, Singh G, and Lees G (2003) Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. *Brain Res* **978:**194–204.

Oliver D, Lien CC, Soom M, Baukrowitz T, Jonas P, and Fakler B (2004) Functional conversion between A-type and delayed rectifier K^+ channels by membrane lipids. *Science* **304:**265–270.

Oz M (2006) Receptor-independent actions of cannabinoids on cells membranes: focus on endocannabinoids. *Pharmacol Ther* **111:**114–144.

Oz M, Ravindran R, Zhang L, and Morales M (2003) Endogenous cannabinoid, anandamide inhibits neuronal nicotinic acetylcholine receptor-mediated responses in *Xenopus* oocytes. *J Pharmacol Exp Ther* **306:**1003–1010.

Oz M and Renaud LP (2002) Angiotensin II induced depolarizations are mediated through two different ionic conductances in ventral horn neurons of neonatal rat spinal cord. *J Neurophysiol* **88:**2857–2863.

Oz M, Soldatov NM, Melia MT, Abernethy DR, and Morad M (1998) Functional coupling of human L-type Ca^{2+} channel and angiotensin AT_{1A} receptor coexpressed in *Xenopus* oocytes. *Mol Pharmacol* **54:**1106–1112.

Oz M, Tchugunova Y, and Dunn SMJ (2000) Endogenous cannabinoid anandamide directly inhibits voltage-dependent calcium fluxes in rabbit T-tubule membrane preparations. *Eur J Pharmacol* **404:**13–20.

Oz M, Zakharova IO, Dinc M, and Shippenberg T (2004) Cocaine inhibits cromakalim activated K^+ currents in follicle-enclosed oocytes. *Naunyn Schmiedeberg Arch Pharmacol* **369:**252–259.

Oz M, Zhang L, and Morales M (2002) Endogenous cannabinoid, anandamide, acts as a noncompetitive inhibitor on 5-HT_3 receptor-mediated responses in *Xenopus* oocytes. *Synapse* **46:**150–156.

Poling JS, Rogawski MA, Salem N, and Vicini S (1996) Anandamide, an endogenous cannabinoid inhibits Shaker-related voltage-gated K^+ channels. *Neuropharmacology* **35:**983–991.

Rutenberg J, Cheng SM, and Levin M (2002) Early embryonic expression of ion channels and pumps in chick and *Xenopus* development. *Dev Dyn* **225:**469–484.

Sade H, Muraki K, Ohya S, Hatano N, and Imaizumi Y (2006) Activation of large-conductance, Ca^{2+}-activated K^+ channels by cannabinoids. *Am J Physiol Cell Physiol* **290:**C77–C86.

Sakuta H (1994) Potentiation by insulin and insulin-like growth factor-1 of glibenclamide-sensitive K^+ currents in follicle-enclosed *Xenopus* oocytes. *Eur J Pharmacol* **268:**375–380.

Seino S and Miki T (2003) Physiological and pathophysiological roles of ATP-sensitive K^+ channels. *Prog Biophys Mol Biol* **81:**133–176.

Smith LD (1989) The induction of oocyte maturation: transmembrane signaling events and regulation of the cell cycle. *Development* **107:**685–699.

Van den Bossche I and Vanheel B (2000) Influence of cannabinoids on the delayed rectifier in freshly dissociated smooth muscle cells of the rat aorta. *Br J Pharmacol* **131:**85–93.

Venance L, Piomelli D, Glowinski J, and Giaume C (1995) Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes. *Nature* **376:**590–594.

Wibrand F, Honore E, and Lazdunski M (1992) Opening of glibenclamide-sensitive K^+ channels in follicular cells promotes Xenopus oocyte maturation. *Proc Natl Acad Sci U S A* **89:**5133–5137.

Woodward RM and Miledi R (1987) Hormonal activation of ionic currents in follicle-enclosed *Xenopus* oocytes. *Proc Natl Acad Sci U S A* **84:**4135–4139.

Yeh JH, Cheng HH, Huang CJ, Chung HM, Chiu HF, Yang YL, Yeh MY, Chen WC, Kao CH, Chou CT, et al. (2006) Effect of anandamide on cytosolic Ca^{2+} levels and proliferation in canine renal tubular cells. *Basic Clin Pharmacol Toxicol* **98:**416–422.

Address correspondence to: Dr. Murat Oz, National Institute on Drug Abuse/Intramural Research Program Integrative Neuroscience Section, 333 Cassell Dr., Baltimore, MD 21224. E-mail: moz@intra.nida.nih.gov