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

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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 ± 2 μM. Inhibition was noncompetitive and independent of membrane potential. Coapplication of anandamide with the cannabinoid type 1 (CB₁) receptor antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride (SR 141716A) (1 μM), the CB₂ receptor antagonist N-[(1S)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 phenylmethylsulfonil 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 [²H]glibenclamide in the oocyte microsomal fractions, with an IC_{50} value of 6.3 ± 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.

The Endogenous Cannabinoid Anandamide

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 (Poli et al., 1996; van den Bosche and Vanheel, 2000; Mainget 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...
Miki, 2003). $K_{\text{ATP}}$ channel openers such as cromakalim and pinacidil or manipulations that decrease intracellular ATP levels activate these channels, whereas anti-diabetic sulfonylureas such as glibenclamide suppress their activity. In earlier investigations, it was reported that follicular cells surrounding the oocytes of Xenopus laevis express $K_{\text{ATP}}$ channels and that activity of these channels can be monitored electrophysiologically (Honore and Lazdunski, 1991; Guillemare et al., 1994). $K_{\text{ATP}}$ channels of follicular cells have been shown to play important roles in oocyte maturation, hormonal regulation of oocyte development (Honore and Lazdunski, 1991; Wirbeland 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 $K_{\text{ATP}}$ 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 NaHCO3, 0.8 mM MgSO4, and 10 mM HEPES, pH 7.5, and were stored for 2 to 7 days in modified Barth’s solution (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.9 mM CaCl2, 0.8 mM MgSO4, 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 superfused at a constant rate of 5 to 7 ml/min. The bathing solution consisted of 95 mM NaCl, 2 mM KCl, 2.4 mM NaHCO3, 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 injection of BAPTA (50–70 nl, 100 mM) were described earlier in detail (Oz et al., 1998). BAPTA was prepared in Cs4-BAPTA. Injections were performed 1 h before recordings using an oil-driven ultramicrosyringe pump (Micro4; WP1, 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 $y = (E_{\text{max}} - E_{\text{min}})/(1 + [EC_{50}/x]^n) + E_{\text{min}}$ where $x$ and $y$ are concentration and response, respectively, $E_{\text{max}}$ is the maximal response, $E_{\text{min}}$ 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 1000g for 10 min and 10,000g for 20 min; each time the pellet was discarded, and the supernatant was used for the subsequent step. The final centrifugation was at 60,000g 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. $[3\text{H}]$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 $[3\text{H}]$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 gonadotropin 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 (Honore 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 $K_{\text{ATP}}$ 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...
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 (IC₅₀) 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 CB₁ receptor antagonist SR 141716A on anandamide-induced inhibition of cromakalim-activated currents. Co-application 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 CB₂ 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 ± 6 versus 47 ± 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 ± 4%, $n = 6$ versus $64 ± 5%$, $n = 5$ 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 $R_f$ of enzymatically defolliculated oocytes are shown on the left ($n = 12$). Resistance values were calculated from current-voltage curves recorded in the range of $-50$ to $+10$ mV. Bars, on the right side, show mean values for resistance as a sum of $R_f$ and $R_j$ before and after 30-min anandamide incubation. Bars, on the left side, show mean values for $R_j$ 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_i) 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_j) and enzymatically (2-h treatment in 0.2% collagenase A; Roche Diagnostics, Indianapolis, IN) defolliculated oocytes (to investigate the involvements of R_i 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 sites 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 ± 18 μM and 1.07 versus 132 ± 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 [3H]glibenclamide in the microsomal fraction of Xenopus oocytes. Equilibrium curves for the binding of [3H]glibenclamide, in the presence and absence of anandamide, are presented in Fig. 5A. In the presence of 10 μM anandamide, the specific binding of [3H]glibenclamide decreased to 64.3 ± 7.2% of controls (n = 4). Maximum binding activity (B_{max}) of [3H]glibenclamide was 6.28 ± 0.23 and 2.86 ± 0.16 pmol/mg for controls and anandamide, respectively. The affinity (K_{d})
of [3H]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 [3H]glibenclamide from microsomal membranes were also investigated (Fig. 5B). Anandamide (1–100 μM) inhibited the specific binding of [3H]glibenclamide to 80 to 85% of control (n = 4–6). The IC50 value for anandamide was 6.3 ± 0.4 μM, with corresponding slope factors of 0.9.

K<sub>ATP</sub> channels play an important role in hormonal activation of the maturation process in Xenopus oocytes (Woodward and Miledi, 1987; Honore and Lazdunski, 1991; Wirbrand 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<sub>ATP</sub> channels in follicular cells (Woodward and Miledi; 1987; Honore and Lazdunski, 1991; Sakuta, 1994; Arellano et al., 1996). Activation of K<sub>ATP</sub> channels by gonadotropins induces oocyte maturation and GVBD that can be effectively blocked by K<sub>ATP</sub> channel blockers such as glibenclamide (Woodward and Miledi, 1987; Wirbrand 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), inhibition 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 (Honore and Lazdunski, 1991; Wirbrand et al., 1992), tolbutamide, an antidiabetic sulfonylurea with approximately 1000 times less affinity than glibenclamide on pancreatic β cells (K<sub>d</sub> 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 (12.4 ± 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<sub>ATP</sub> channel activators can potentiate progesterone-induced maturation only in follicle-enclosed oocytes (Woodward and Miledi, 1987; Wirbrand 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 (Wirbrand et al., 1992). Thus, we tested the effect anandamide on cromakalim potentiation of progesterone-induced maturation responses induced by cromakalim and progesterone in follicle-enclosed 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.

**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).
Discussion

In this study, we have demonstrated that anandamide inhibits cromakalim-induced K⁺ currents in follicle-enclosed oocytes in a noncompetitive manner. The CB₁ receptor antagonist SR 141716, the CB₂ 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 G₁/G₂-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 cannabinoïd 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 Ca²⁺ (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 cannabinoïd receptors (for recent review; see Oz, 2006).

Follicular cells of oocytes endogenously express cromakalim-activated KᵥATP channels that have similar pharmacological and biophysical properties to those found in other tissues (Honore and Lazdunski, 1991; Guillemer 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 Ca²⁺ levels (Mombouli et al., 1999; Yeh et al., 2006). The changes in intracellular Ca²⁺ levels caused the function of Ca²⁺-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 Ca²⁺-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 Ca²⁺-activated Cl⁻ channels in oocytes (Dascal, 1987), it is unlikely that the changes in intracellular Ca²⁺ concentrations would interfere with the effect of anandamide on K⁺ conductance. In agreement with earlier studies (Honore and Lazdunski, 1991; Guillemer 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 KᵥATP 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 KᵥATP blockers and openers (Seino and Miki, 2003). Anandamide did not alter EC₅₀ 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 [³H]glibenclamide to oocyte microsomal membranes without altering its affinity to [³H]glibenclamide suggests that anandamide interacts with the glibenclamide binding site on the SUR in a noncompetitive manner. Subunit identification of endogenous KᵥATP channels in follicular cells remains currently unknown. A recent study suggested that a novel type of Kir6.1/SUR2A combination comprises endogenous KᵥATP channels (Fujita et al., 2007). Some preliminary findings indicate that anandamide inhibits [³H]glibenclamide binding to Kir6.2/SUR1 and SUR1 expressed in human embryonic kidney-293 cells, with IC₅₀ 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 G₂/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 KᵥATP channels (Woodward and Miledi, 1987; Honore 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 anan-
damide inhibited the picrotoxin-induced acceleration of progesterone-induced oocyte maturation, suggesting that the
inhibition of K<sub>ATP</sub> channels by anandamide modulates the hormonal maturation process in oocytes.

K<sub>ATP</sub> 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<sub>ATP</sub> channels in follicular cells mod-
ulate the actions of several hormones implicated in oocyte maturation, the present results provide suggestive evidence that inhibition of K<sub>ATP</sub> channels by anandamide can modu-
late the hormonal maturation process in *Xenopus* oocytes.

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References


Cravatt BF and Lichtman AH (2002) The enzymatic inactivation of the fatty acid


Dumont JN (1972) Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte develop-

Fisyunov A, Tsintsadze V, Min R, Burnashev N, and Lozovaya NA (2006) Cannabi-
soids modulate the P-type high voltage-activated calcium currents in Purkinje


Guillemaire E, Lazdunski M, and Honore E (1994) CGP-induced activation of KATP


Holzmann S, Kostner GM, and Graier WF (1999) Anan-
damide-induced mobilization of cytosolic Ca<sup>2+</sup> in endothelial cells. *Br J Pharmacol* 126:1593–1600.

anandamide is a direct and selective blocker of the background K<sup>-</sup> channel TASK-1. *AMJ* 260:47–54.

Mombouli JV, Schaeffer G, Holzmann S, Kostner GM, and Graier WF (1999) Anan-
damide-induced mobilization of cytosolic Ca<sup>2+</sup> in endothelial cells. *Br J Pharmacol* 126:1593–1600.


Oz M, Soldatov NM, Melia MT, Abernethy DR, and Morad M (1998) Functional
channel of human T-type Ca<sup>2+</sup> channel and angiotensin AT<sub>1</sub> receptor coex-

Oz M, Tchugunova Y, and Dunn SMJ (2000) Endogenous cannabinoid anandamide

Oz M, Zavharova IO, Dine M, and Shippenberg T (2004) Cannabinoids inhibit picro-


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<sup>2+</sup>-activated K<sup>-</sup> channels by cannabinoids. *Am J Physiol Cell Physiol* 290:C77–C86.

Sakuta H (1994) Potentiation by insulin and insulin-like growth factor-I of giben-

Seino S and Miki T (2003) Physiological and pathophysiological roles of ATP-


Woodward RM and Mileti R (1997) Hormonal activation of ion currents in follicle-


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