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Non-genomic action of 17β-estradiol on opening of Ca^{2+}- and voltage-activated K^+ channel in lacrimal acinar cells

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The effect of the sex hormone 17β-estradiol on the opening of Ca^{2+}- and voltage-activated K^+ channels (BK channels) in the basolateral plasma membrane of mouse lacrimal acinar cells was studied by patch-clamp single-channel recording and Ca^{2+}-measurement using fura-2 AM. In intact cells (the cell-attached configuration) using a pipette containing a Na^+-rich solution, estradiol was added to the bath solution, which does not have direct contact with the electrically isolated areas of membrane patch from which the single-channel currents were recorded. Estradiol increased the frequency of opening of the BK channels within a few minutes after its application. The effect of estradiol on the opening of the BK channels in acinar cells in male mice was greater than that in females. In Ca^{2+}-measurement using fura-2 AM, estradiol did not increase the level of intracellular Ca^{2+} during a 5-minute observation period. The application of estradiol with propranolol, a β-adrenergic receptor blocker, did not increase BK channel opening. The application of estradiol with Rp-cAMPS, an inhibitor of cyclic AMP-dependent protein kinase (protein kinase A), also inhibited the increase in channel opening. The addition of a catalytic unit of protein kinase A to the inside of the excised membrane patch increased the frequency of opening of the BK channels. These results suggest that estradiol interacts with β-adrenergic receptor on the basolateral membrane and regulates the opening of BK channels by protein phosphorylation via a cyclic AMP pathway, without a change in the Ca^{2+} level.

Key words : estradiol, lacrimal gland, potassium channel, tear, cyclic AMP

INTRODUCTION

It has been proposed that estrogens may play a role in the gender-associated variation in the lacrimal gland [13]. Ovariectomy has been reported to elicit regressive changes in lacrimal tissue [1, 12], whereas estrogen treatment restores the glandular appearance to that of intact females [13, 14]. Estrogen deficiency has been linked to the development of keratoconjunctivitis sicca and postmenopausal dry eye syndromes [3].

A major mode of sex steroid action takes place via the classical genomic pathway. Recent observations of steroid hormones indicate that they elicit rapid and reversible responses of membrane electrical activity via a non-genomic pathway in various tissues [2, 8, 10, 17, 27]. However, it is not clear whether sex steroids can act on the rapid membrane responses in the lacrimal gland via a non-genomic pathway.

A model accounting for NaCl-rich fluid secretion from the lacrimal acini has been proposed [25]. Intracellular Ca^{2+} is released by acetylcholine (ACh) application to the basolateral membrane. Ca^{2+}-induced increases in plasma membrane permeability are involved in the secretion process in the lacrimal gland. BK channels, which have been suggested to regulate K^+ permeability changes in the plasma membrane during fluid secretion, have been identified on the basolateral membrane of lacrimal acinar

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cells [5]. BK channels have been found in various cell types [11], which have three typical properties: large single-channel conductance, sensitivity of the open-state probability to intracellular Ca^{2+} level and membrane potential.

It has been suggested that BK channels are important for Cl^- uptake into cell.

ACh evoking an increase in cytoplasmic Ca^{2+} level activate BK channels causing an increase in the local K^+ concentration. Na^+, K^+, and Cl^- are taken up via the Na^+-K^+-$2Cl^-$ cotransporter in the basolateral membrane, and the Na^+ uptake activates the Na^+-K^+ pump. In the steady state, the three basolateral ion pathways, BK channels, the Na^+-K^+ pump, and the Na^+-K^+-$2Cl^-$ cotransporter, operate together as an electrogenic Cl^- uptake. The activation of the BK channels allows outward current flow at the basolateral membrane, which hyperpolarizes the cells and increases the driving force of Cl^- efflux into the lumen. On the other hand, elevation of the intracellular Ca^{2+} level increases Cl^- conductance in the lacrimal membrane [6], and evokes Cl^- efflux into the lumen. The lumen negativity allows Na^+ to move between the cells through the narrow intercellular spaces (tight junctions) placed at their outer end. These are generally cation-selective, and Na^+ flux dominates simply because Na^+ is the main cation in the extracellular fluid [19].

This study examines whether estrogen acts on the activity of BK channels in male and female mice via a non-genomic pathway as an index of tear production in the acinar cells.

A brief preliminary report of some of the findings described here has already appeared [26].

METHODS

Cells

The following procedures were performed in accordance with the Guiding Principles for the Care and Use of Animals of the Physiological Society of Japan. Mice (Jc1: ICR, 4 weeks old) were obtained from CLEA Japan (Shizuoka, Japan). The animals had free access to water and food until the day of the experiment. They were killed rapidly by stunning and cervical dislocation, and the exorbital lacrimal glands were isolated. A mixture of individual cells and small clumps of acinar cells was obtained by enzymatic dispersion of the glands in a warm bath at 37 °C. This was achieved in two stages: first, the intact glands were injected through their capsules of the gland with a solution of hyaluronidase (2 mg/ml) and incubated for 18-20 min; and second, the glands were injected with a solution of pure collagenase (200 U/ml) and incubated for 30 min. After collagenase digestion, a mixture of completely isolated cells and cell clusters remained, and these were rinsed with collagenase-free physiological solution for 10-50 min before use for a patch clamp experiment.

Cells for Ca^{2+}-imaging measurement were aseptically prepared because healthy cells were required for use for a long period. After enzymatic treatment, the cells were loaded with fura-2 AM (5 μM) for 40 min at 37 °C, and then the solution containing fura-2 AM was washed away. The cells were incubated in culture medium (F12) for 4-6 hrs in CO_2 incubator at 37 °C to promote complete hydrolysis of the AM ester.

Ca^{2+}-imaging measurement

Cells were placed on a 35-mm culture dish with a glass coverslip at the bottom of the dish. Culture medium was replaced with a Na^+-rich solution. The dish was mounted on the stage of an inverted fluorescence microscope (Nikon, Diaphot-TMD). Fura-2 was excited with light from a xenon lamp alternately filtered to 340 and 360 nm. Fluorescence emission was filtered to 510 nm, focused with a ×40 lens, and monitored with a CCD camera (Hamamatsu Photonics, C2400-08). Images were captured and processed with the software package Argus 50 (Hamamatsu Photonics, Japan). The software performed background subtraction and then calculated the 340 nm-to- 360 nm ratios for each matched pixel pair from the intensities of the 340 nm and 360 nm images by the computing method described by Grynkiewicz, G *et al.* [7], and displayed the resulting images in pseudocolor. The pseudocolor images of the ratios were converted into images of Ca^{2+} concentration according to calibration prepared in advance.

Solutions

Two types of physiological saline solutions were used: a Na^+-rich and a K^+-rich type. The Na^+-rich solution contained (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 10 glucose, and 10 *N*-2-

hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) (pH 7.2). The K^+-rich solution contained (mM): 145 KCl, 10 NaCl, 1.13 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.2). The osmolalities of the solutions were adjusted to 290 ± 5 mOsm/kg for the Na^+-rich and 300 ± 5 mOsm/kg for the K^+-rich solutions. In the experiment where the effect of an ionized Ca^{2+} concentration of 10^{-8} M was tested on the membrane inside, Ca^{2+}-EGTA buffer mixtures prepared by titration were used. The free Ca^{2+} and ATP^{4-} concentrations in the K^+-rich solution containing ATP were calculated using the stability constants as described by Fabiato [4], and adjusted to 10^{-8} for Ca^{2+}, and to 10^{-5} M for ATP^{4-} by the addition of Ca^{2+}-EGTA buffer mixture, $MgCl_2$ and ATP. The free Mg^{2+} concentration was adjusted to 1 mM in all membrane excision experiments. In the experiment using the cell-attached configuration, the bath was filled with Na^+-rich solution containing 1.2 mM Ca^{2+}.

The commercial catalytic subunit of protein kinase A was dialyzed just before use against deionized water containing the reducing agent dithiothreitol (6 mg/ml) at 4 °C for 4 hrs to remove buffer materials, and was then dissolved in the bath solution.

Single-channel current recordings

Single-channel current recording was performed by a conventional patch-clamp technique. We placed the tip of the patch-pipette on the basolateral side under visual guidance. The basolateral membrane was identified from the histological characteristics of the acinar cells: that is, the basolateral side has no granules, but it occupies a large area. All experiments were done at room temperature (20-23 °C).

Single-channel currents were recorded with a patch-clamp amplifier (Nihon Kohden CEZ2300, Tokyo) filtered at 1 kHz low pass, and stored on tape (Sony KS609, Tokyo) for subsequent replay and analysis. The taped current records were digitized using a microcomputer (NEC 9801DA, Tokyo) for the calculation of open-state probability (p) as described by Suzuki *et al*. [24], which was determined by current records lasting 5-10 s. The current amplitude was obtained by measuring the amplitude of the current on the recorder chart with vernier calipers.

ANOVA for two-way classification and the *t*-test were used for statistical analysis.

Chemicals

Hyaluronidase, ATP (Na_2ATP), the catalytic subunit of protein kinase A (P2645), DL-propranolol and 17 β-estradiol were purchased from Sigma (USA). Ham's F12-medium, penicillin/streptomycin, and calf serum were purchased from GIBCO (USA). Pure collagenase and Rp-cAMPS triethylamine were purchased from Worthington (USA) and RBI (USA), respectively. Fura-2 AM was purchased from Dojindo Lab. (Japan). All other chemicals used were of reagent grade.

RESULTS

An experiment to test whether estradiol can evoke an increase in the opening of BK channels in intact cells (cell-attached configuration) of male mice was performed. In this experiment, acinar cell clusters were used and the recording pipette was filled with Na^+-rich solution containing 0.5 mM EGTA. The bath always contained Na^+-rich solution with 1.2 mM Ca^{2+}. Figure 1A shows the results of an experiment in which channel opening could be observed when the pipette voltage (Vp) was held between − 10 and − 50 mV. Before estradiol application, channel opening was seen only with a low open-state probability (p). At its final concentration of 0.5 μM, 17β-estradiol was added to the bath solution so that it had no direct contact with the electrically isolated membrane patch area from which the single-channel current was recorded. The opening at high frequency was observed at 4 min after the addition of estradiol. The observed currents were outward currents. The value of p, plotted against Vp (Fig. 1B), increased in a voltage-dependent manner. In most cases there was only one effective channel. After each experiment using the cell-attached configuration, the patch was excised in an inside-out configuration: the pipette was filled with a Na^+-rich solution, and the bath was filled with a K^+-rich solution containing 10^{-8} M Ca^{2+}. The reversed (null) potential for the current was around − 90 mV (not shown), and since the only ion with a negative reversal potential in this experimental situation is K^+ (the Cl^- concentration being the same on both sides of the membrane), this channel must be K^+-selective.

Figure 1C shows the relationships between amplitude of single-channel current and pipette voltage (Vp). Addition of estradiol

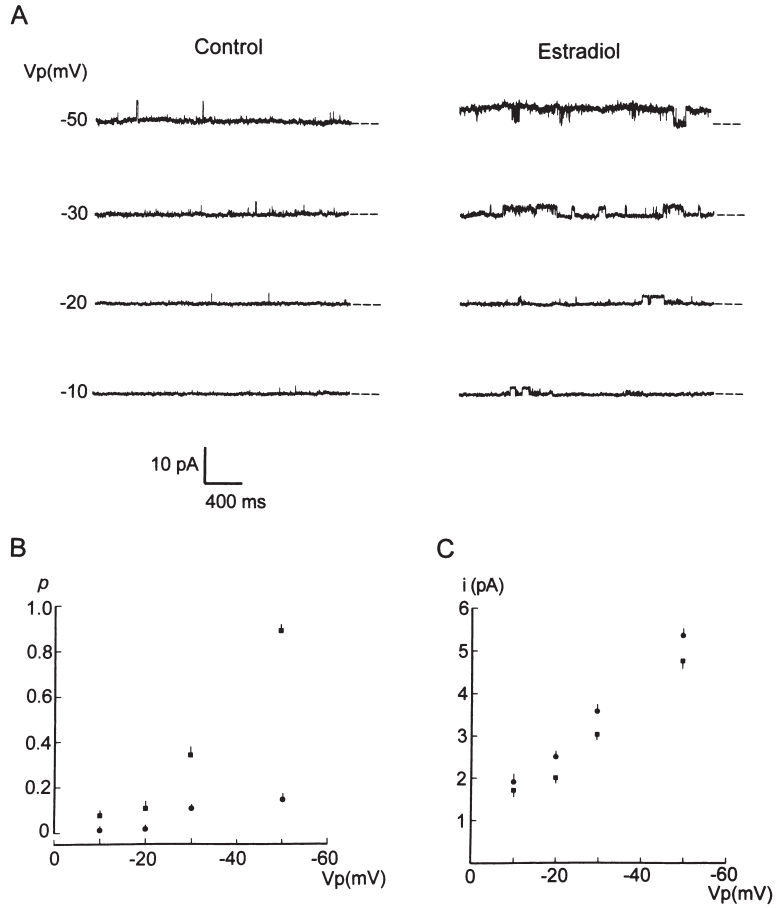

Fig. 1 Single-channel currents recorded from intact acinar cell (cell-attached configuration) at pipette potential (Vp) of −10 to −50 mV. The pipette was filled with Na^+-rich solution containing 0.5 mM EGTA without Ca^{2+}. The bath was filled with Na^+-rich solution containing 1.2 mM Ca^{2+}. A: all traces were obtained from same patch before application of estradiol (control), and 4 min after start of exposure of 0.5 μM estradiol (estradiol). ---, current level when all channels closed. Upward deflection represents outward current. B: open-state probability (p) (mean ± SEM, n = 4-6) as a function of pipette potential (Vp) in the experiment shown in Fig. 1A. Closed circles and squares represent control and estradiol application, respectively. C: the relationship between the amplitude of single-channel current and pipette potential (Vp). The ordinate shows mean ± SEM (n = 6) of amplitude of single-channel currents. Closed circles and squares represent control and estradiol application, respectively.

to the bath solution reduced the amplitude of the currents. This may be induced by hyperpolarization of the cell because of the outward current evoked by opening of the BK channel.

17β-Estradiol has been reported to inhibit calcium channel currents in rat sensory neurons via non-genomic mechanism. The rapid estogen-mediated inhibition is sex-specific [15]. To clarify the sex differences, the lacrimal acinar cells from female and male mice were examined. The p values in female and male cells for no application of estradiol (control) were 0.0051 ± 0.0014 (n = 6), and 0.0056 ± 0.0012 (n = 8) (mean ± S.E.M.), respectively. No significant difference was found between them. Table 1 shows the p values obtained from the results of experiments of the estradiol application on female and male cells. The effects of estradiol (0.1 μM to 1.0 μM) on the opening of the channel in the male cells were greater than in the female cells. Furthermore, application of 50 nM estradiol to male acinar cells slightly increased

Table 1 The mean ± SEM of the p values for concentration of 17 β-estradiol in the female and male acinar cells. The p values were obtained from experiments at − 40 mV of Vp in cell-attached configuration at 5 min after an application of estradiol. * and NS represent significant ($p < 0.05$) and no significant differences for t-test, respectively.

The p values (mean ± S.E.M.) of the concentration of 17 β-estradiol at -40 mV of Vp in cell-attached configuration

female	0.1 μM	0.045±0.017 (n=5)	*
	0.5 μM	0.120±0.023 (n=6)	*
	1.0 μM	0.708±0.030 (n=6)	*
male	0.1 μM	0.127±0.029 (n=5)	*
	0.5 μM	0.821±0.035 (n=7)	NS
	1.0 μM	0.896±0.033 (n=6)	*

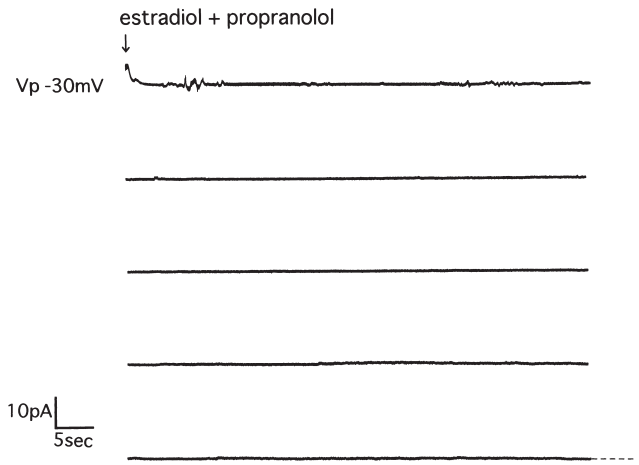

Fig. 2 Continuous single-channel currents recorded from intact acinar cell at Vp of − 30 mV. 0.5 µM estradiol was added with 50 µM propranolol to the bath as shown by arrow. The pipette and bath were filled with the same solutions as those shown in Fig. 1.

the frequency of opening of the BK channel, and the p value was 0.039 ± 0.016 (mean ± SEM, n = 6). However, 50 nM estradiol had no effect on the female acinar cells.

The following experiments were carried out using male acinar cells. To clarify whether estradiol would alter a cytoplasmic free-Ca^{2+} concentration, Ca^{2+} imaging with fura2 was examined. Before estradiol application, the Ca^{2+} concentration was about 3 nM in the center of the cell. No increase in Ca^{2+} concentration was seen at 3 min after estradiol application. No increase in the Ca^{2+} concentration was observed during the 5 min period following estradiol, and no such increase was seen in a series of identical experiments. The average Ca^{2+} concentration in the center of the cell was 9.2 ± 2.2 nM (mean ± SEM, n = 5) for the control, and 8.8 ± 1.9 nM (n = 5) after estradiol application, but no significant difference was found between them.

An experiment was performed to clarify on which receptor of the plasma membrane estradiol would act. A blocker of the β-receptor, propranolol (50 µM), was added to the bath solution with estradiol. No opening of the channel was seen, even several minutes after additions of the two substances (Fig. 2). This experiment was repeated five times with similar results. The p value was 0.0058

± 0.0014 (mean ± SEM, n = 5), no significant difference was found between these experiments and control. When the effect of adrenalin on the opening of the K^+ channel was tested in a cell-attached configuration, its application to the bath (2 μM, final concentration) increased the frequency of opening of the K^+ channel, and the *p* value at 4 min after addition was 0.322 ± 0.041 (mean ± SEM, n = 5) at −30 mV of Vp. The application of adrenaline together with propranolol did not evoke frequent opening of the K^+ channel. This experiment was repeated five times, with similar inhibition each time.

To test whether cyclic AMP directly evokes the opening of the BK channel, 50 μM forskolin, a potent activator of adenyl cyclase, was added to the bath solution. The *p* value was increased up to 0.313 ± 0.035 (mean ± SEM, n = 6) at a Vp of −30 mV.

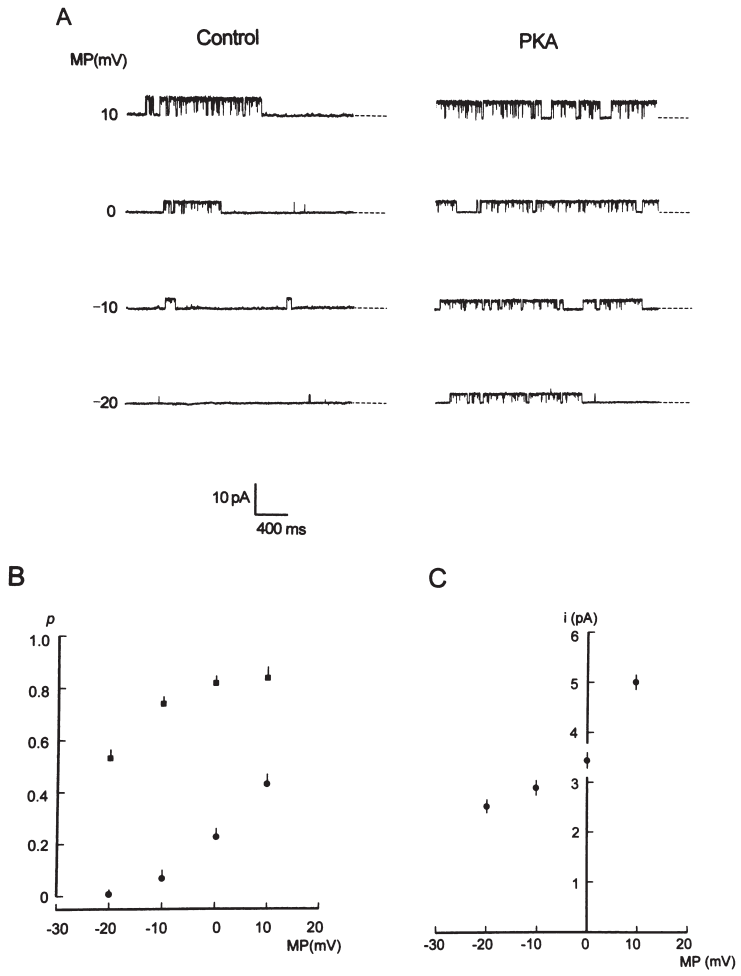

Fig. 3 Effect of PKA on the current recorded from excised inside-out membrane patch at membrane potential (MP) from − 20 to 10 mV. The pipette was filled with Na^+-rich solution containing 0.5 mM EGTA without Ca^{2+}. The bath was filled with K^+-rich solution containing 10^{-8} M Ca^{2+}, 10^{-3} M Mg^{2+} and 10^{-5} M ATP^{4-}. A: all traces were obtained from same membrane patch before PKA application (control), and 4 min after start of exposure of 25 U/ml PKA (PKA). B: open-state probability (*p*) (mean ± SEM, n = 4-6) as a function of membrane potential in the experiment shown in Fig. 3A. Closed circles and squares represent control and PKA application, respectively. C: relationship between single-channel current and membrane potential in the experiment shown in Fig. 3A. The ordinate shows mean ± SEM (n = 6-8) of amplitude of single-channel currents.

Estradiol application with 100 μM Rp-cAMPS, a potent inhibitor of protein kinase A, did not evoke an opening of the channel throughout observation for 4 min. A similar inhibition was seen in six experiments. The p value was 0.0075 ± 0.0018 (mean ± SEM, n = 6).

To test whether the catalytic subunit of protein kinase A (PKA) can evoke an increase in the frequency of opening of the channel, 25 U/ml PKA was applied to the inner surface of membrane in excised inside-out patches. The patch pipette was filled with Na^+-rich solution, and the bath was filled with K^+-rich solution containing 10^{-8} M Ca^{2+}, 10^{-3} M Mg^{2+} and 10^{-5} M ATP^{4-}. After identification of the BK channels, PKA was applied to the inner surface of the membrane. Figure 3A shows a typical trace of single-channel currents obtained from the same membrane patch at 4 min after PKA application. The PKA application increased the frequency of opening of the channel and increased the p value (Fig. 3B). Figure 3C shows the relationship between membrane potential and amplitude of the single-channel current and an outward current was observed at membrane potentials from − 20 to 10 mV. A similar increase in the p value was seen in six out of eight patches in which the experiment was attempted.

DISCUSSION

It has been reported that no Cl^- channel exists on the basolateral membrane, and that the Cl^- equilibrium potential is about − 25 mV in the lacrimal acinar cells [20, 21]. No Cl^- channel current could be seen around a Vp of −15mV (corresponding to a membrane potential of − 25 mV), since the spontaneous membrane potential is about − 40 mV [9, 25]. The fact that the application of estradiol brought about an outward current at a Vp from − 10 to − 50 mV in the cell-attached configuration (Fig. 1) and the results obtained from the experiment to find the reversal potential together suggest that these channel currents induced by estradiol must be due to the opening of the K^+ channel.

It was thought that there is no specific membrane receptor for steroids, but recent studies have revealed that steroids interact with the receptors of neurotransmitters in kidney cells, namely, the purinoceptor [2], and the $GABA_A$ receptor in the dorsal root ganglion cells [8]. A conventional microelectrode experiment has reported adrenaline-evoked membrane hyperpolarization similar to that of ACh in the lacrimal acinar cells, the β -receptor stimulant isoprenaline (isoproterenol), had no effect on membrane potential [9]. However, the present study shows that estradiol evokes no increase in the intracellular Ca^{2+} level, and that the β-receptor blocker propranolol inhibits the estradiol-induced opening of BK channels (Fig. 2). These results suggest that estradiol could act on BK channels by interacting with the β -receptor of the membrane.

When the concomitant application of estradiol with protein kinase A inhibitor (Rp-cAMPS) was studied for comparison, the results suggested that the mechanism of estradiol-induced opening of BK channels is involved in protein kinase A activity. The results of the experiment using PKA are consistent with the fact that PKA facilitates an opening of Ca^{2+}-activated K^+ channels via phosphorylation in canine pancreatic acinar cells [23], and that cyclic AMP-dependent protein phosphorylation initiates the Ca^{2+} -sensitivity of the Ca^{2+}-activated K^+ channel in guinea-pig pancreatic acinar cells [22].

Phosphorylation by protein kinase A activates BK channels in smooth muscle cells [18] and neurons [16], but inhibits channel activity in endocrine cells [29]. The pore-forming of BK channels depends on a single gene that undergoes extensive alternative splicing to produce channels with distinct phenotypes [30]. Alternative splicing switches BK channel sensitivity to protein phosphorylation by protein kinase A [28].

Although protein phosphorylation of the BK channels was not examined, the results obtained with estrogen raise the possibility that the activity level of BK channels is enhanced by protein phosphorylation of the channel by protein kinase A. Thus, tear production from the lacrimal glands may be potentiated in the presence of estrogen.

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