Cytoplasmic free calcium ([Ca\(^{2+}\)]_{i}) plays an important role in a variety of cell functions, such as muscle contraction, secretion, cell proliferation, and phototransduction. In lens cells, an increase in [Ca\(^{2+}\)]_{i} modulates cell growth [1]. On the other hand, it is also known that an increase in lens calcium ([Ca\(^{2+}\)]_{c}) plays a certain role in the development of cataracts. An elevation of [Ca\(^{2+}\)]_{i} seems to modify membrane permeability [2] and stability of the lens cytoplasmic gel [3], and leads to lens opacification [4, 5]. Many agonists increase [Ca\(^{2+}\)]_{i} through the activation of G-coupled or tyrosine kinase receptors on lens cells. These agonists include acetylcholine, ATP, bradykinin, histamine, and PDGF [1, 6]. ATP has been shown to be present in the normal aqueous humor and to be released into it from injured cells [7].

Extracellular and intracellular pH also play a role in the regulation of cell functions. Effects of changes in pH on both resting [Ca\(^{2+}\)]_{i} and agonist-induced Ca\(^{2+}\) mobilization have been extensively investigated in many tissues. In rat lens cells, intracellular acidification had no effect on the resting [Ca\(^{2+}\)]_{i} [8]. On the other hand, we demonstrated that ATP produced an initial peak followed by a sustained increase in [Ca\(^{2+}\)]_{i} in rabbit lens epithelial cells, and that extracellular acidification enhanced both the initial peak and the sustained increase in [Ca\(^{2+}\)]_{i} in rabbit lens epithelial cells. One possible mechanism of the ATP-induced Ca\(^{2+}\) influx seems to be a capacitative Ca\(^{2+}\) entry pathway. [Japanese Journal of Physiology, 51, 81–87, 2001]
sustained increase in [Ca\(^{2+}\)] \[6\]. In these studies, the acute effects of changing pH were examined. To our knowledge, the long-term effects of pH alteration on [Ca\(^{2+}\)] in lens cells have not been reported in literature. It is interesting to know the chronic effects of changing pH from a clinical point of view, since the acidification of aqueous humor occurs slowly and lasts for a long time in patients with chronic disease such as diabetes mellitus.

In the present study, ATP-induced Ca\(^{2+}\) mobilization was investigated in cultured rabbit lens epithelial cells. The cells were cultured in a medium with pH of 7.4, 7.2, or 7.0 for 10 to 21 d and kept in a physiological salt solution (PSS) with the corresponding pH throughout the experiments. We found that long-term extracellular acidification decreased intracellular pH (pH\(_{i}\)) and enhanced the ATP-induced Ca\(^{2+}\) mobilization in rabbit lens epithelial cells.

**MATERIALS AND METHODS**

**Cell preparations.** Japanese white rabbits weighing 2.5 to 3.5 kg were used in this study. The eyeballs were removed within 1 h after the animals were killed by an injection of an overdose of sodium pentobarbital (100 mg/kg, I.V.). All procedures were reviewed and approved by the Committee for Animal Experimentation in the Faculty of Medicine, Tottori University, and conformed to the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences” set forth by the Physiological Society of Japan.

The eyeballs were maintained in 50 ml of phosphate-buffered solution at 5°C without adding Ca\(^{2+}\) and Mg\(^{2+}\). The lenses were dissected and placed in 3 ml of Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS). The capsules with the epithelium were removed and digested with 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) for 3 min at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 3.5 ml of DMEM with 10% FBS was added to slow digestion, the medium with the capsule was triturated 4 to 5 times to disperse cells from the capsules. The cell suspension (0.5 ml) was placed into a 35 mm culture dish containing a 10 mm diameter cover glass coated with poly-d-lysine (MatTek Corp., Ashland, MA) and allowed to settle.

**Cell culture.** Cells were cultured in DMEM supplemented with 10% FBS and 20 mmol/l NaHCO\(_3\) at 37°C in a fully humidified atmosphere. The culture medium was changed every 2 d. Confluent cultures were obtained in 10 to 21 d, and the cells were then used for experimentation. The pH of culture medium (pH\(_{c}\)) was kept at a certain level throughout the culture period by adjusting CO\(_2\) concentration in the incubator according to our preliminary experimental data: group 1, pH\(_{c}\) 7.4 with 2.5% CO\(_2\); group 2, pH\(_{c}\) 7.2 with 5.0% CO\(_2\); and group 3, pH\(_{c}\) 7.0 with 7.5% CO\(_2\).

**Dye loading and fluorescence measurement.** Cells were loaded with the fluorescent dyes 15 μmol/l fura-2 AM (Dojindo Laboratories, Kumamoto, Japan) and 1.5 μmol/l BCECF AM (Dojindo Laboratories) in artificial aqueous humor at 37°C in the dark for 1 h. The composition of standard artificial aqueous humor in mmol/l was as follows: 124.0 NaCl, 5.0 KCl, 1.0 CaCl\(_2\), 0.5 MgCl\(_2\), 5.0 glucose, and 10.0 HEPES. The cells were washed 3 times and continuously perfused at a rate of 2 ml/min for more than 40 min with artificial aqueous humor. The culture dish was mounted on the heated stage of an inverted epifluorescence microscope (TMD-300; Nikon Corp., Tokyo, Japan) equipped with a fluorometric system (QuantiCell 700, Applied Imaging, Sunderland, United Kingdom). The cells were observed through the cover glass of the culture dish by using a 40×0.85 numerical aperture, dry objective lens (Fluor 40, Nikon Corp.). Fura-2 and BCECF were excited with light from a 100 W xenon lamp that was alternately filtered to 340 or 380 nm for fura-2 and to 440 or 490 nm for BCECF excitation, respectively. The fluorescence emission was filtered from 510 to 535 nm and monitored with an intensified CCD camera.

The images were analyzed with the software package Graphical User Interface (Applied Imaging), which performed a background subtraction. Geometric regions matching individual cells were defined and analyzed for changes in fluorescence ratio. A concentration of cytosolic free Ca\(^{2+}\) was calculated from the equation by Grynkiewicz and co-workers \[9\]:

\[
[Ca^{2+}] = K_d \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times \beta,
\]

where \(K_d\) is the dissociation constant of the fura \(-2; R_{\text{max}}\) and \(R_{\text{min}}\) are the ratios for the bound and unbound forms of the fura-2/Ca\(^{2+}\) complex; and \(\beta\) is the ratio between the maximum and the minimum fluorescence intensities of fura-2 at 380 nm excitation. To obtain \(R_{\text{max}}\), the cells were exposed to a solution of the following composition: 150.0 mmol/l KCl, 10.0 μmol/l ionomycin (Sigma Chemical Co.), 10.0 mmol/l HEPES, and 10.0 mmol/l CaCl\(_2\). The cells were then exposed to the Ca\(^{2+}\)-free solution with 1.0 mmol/l EGTA to obtain \(R_{\text{min}}\).

Intracellular pH was estimated as a 490/440 ratio of the fluorescence and calibrated as follows: At the end
of each experiment the cells were exposed to 10 μmol/l nigericin, which equilibrates the pH with the known extracellular pH [10], dissolved in a potassium (150 mmol/l) buffer. The 490/440 ratios were obtained during perfusion with three pH standard solutions. A pH standard (pH 6.50) contained 10 mmol/l piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES, Sigma Chemical Co.), and other standards (pH 7.0 and 7.50) contained 10 mmol/l HEPES. Because the response ratio was linear in the pH range from 7.50 to 6.50, a simple transformation was performed to obtain the corresponding pH values from the ratios [10]. All experimental protocols gave pH values within the linear range. To minimize the bleaching effect, the excitation of BCECF was not carried out in some experiments.

Solution and agonist application. During the experiment, cells were covered with 3 ml artificial aqueous humor and perfused with the same solution by using a peristaltic pump (EYELA MICROTUBE PUMP MP-3, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at a flow rate of 2 ml/min. The pH of the solution (pH_{E}) was adjusted to 7.4 (group 1), 7.2 (group 2), or 7.0 (group 3) by adding 1 N NaOH. The Ca^{2+}-free solution was made by substituting 1 mmol/l EGTA for 1 mmol/l CaCl_{2} in the standard artificial aqueous humor. ATP (Sigma Chemical Co.) was dissolved just before use and added to the perfusate. The doses of ATP were expressed as the final organ bath concentrations. During the application of ATP the perfusion rate was increased to 5 ml/min to achieve a quick delivery of the agonist. When the cells were treated with thapsigargin (Sigma Chemical Co.), the inhibitor was added to the perfusate 15 min before the application of ATP.

Statistical analysis. The values were expressed as mean±SEM. The data of [Ca^{2+}], and pH, were obtained by averaging the signals from 5 single cells in each culture dish. The n values reflect the number of animals used. The results were statistically analyzed by Student's t-test between 2 groups or by ANOVA with repeated measures followed by a Fisher's PSLD test. The values of p<0.05 were considered statistically significant.

RESULTS

Table 1 shows the effect of extracellular pH on intracellular pH (pHi) in cultured rabbit lens epithelial cells. The extracellular pH (pHe and pHo) was maintained at 7.4, 7.2, and 7.0 in the cells of group 1, group 2, and group 3, respectively, throughout the period for culture and pH measurement. The measured pH_{E} was 7.22±0.01 in group 1 (n=5), 7.15±0.01 in group 2 (n=5), and 7.09±0.01 in group 3 (n=5).

Figure 1 shows typical responses of [Ca^{2+}], during continuous perfusion with 10 μmol/l ATP in the rabbit lens epithelial cells of group 1 (Fig. 1A) and group 3 (Fig. 1B). An administration of 10 μmol/l ATP produced an initial peak followed by a sustained increase of [Ca^{2+}], in both groups. The chronic acidification enhanced the ATP-induced transient and sustained increase in [Ca^{2+}], without changing the resting level of [Ca^{2+}] (Table 1). Figure 2 demonstrates the effects of extracellular pH on the concentration-response relationship of the ATP-induced Ca^{2+} mobilization (n=8). An administration of 0.1 to 100 μmol/l ATP caused a concentration-dependent increase in [Ca^{2+}]. Both the peak (Fig. 2, left) and sustained (Fig. 2, right)
increases were enhanced significantly by extracellular acidification in a pH-dependent manner.

In a Ca\(^{2+}\)-free solution, the sustained increase in [Ca\(^{2+}\)], induced by 10 \(\mu\)mol/l ATP was abolished in the epithelial cells of group 1 (Fig. 3A) and group 3 (Fig. 3B). The ATP-induced initial peak, however, was not affected by the removal of extracellular Ca\(^{2+}\). It was greater in group 3 (1,930 ± 70 nmol/l) than in group 1 (1,650 ± 70 nmol/l) (Fig. 3C). An application of 1 \(\mu\)mol/l thapsigargin, an ER Ca\(^{2+}\) pump inhibitor, induced a gradual and sustained elevation of [Ca\(^{2+}\)], in the cells of group 1 (Fig. 4A) and group 3 (Fig. 4B). The administration of 10 \(\mu\)mol/l ATP failed to produce both the initial peak and the sustained increase in [Ca\(^{2+}\)], in the lens epithelial cells of both groups. The thapsigargin-induced sustained increase in [Ca\(^{2+}\)], was significantly greater in group 3 (940 ± 50 nmol/l) than in group 1 (600 ± 70 nmol/l) (Fig. 4C).
DISCUSSION

The main findings of the present study were as follows: (1) long-term acidification of the extracellular environment decreased the intracellular pH without changing the resting \([\text{Ca}^{2+}]_i\) in cultured rabbit lens epithelial cells; and (2) the acidification enhanced both the initial peak and the sustained increase in \([\text{Ca}^{2+}]_i\) that were induced by ATP.

In the present study, the lens cells were cultured for 10 to 21 d in a medium with pHc of 7.4, 7.2, or 7.0. Although the effects of more severe acidification (pH of 6.5 to 6.8) have been investigated in previous studies [6, 10], these conditions hardly occur in vivo. Becker [11] reported that the average pH of aqueous humor was 7.21 in 13 patients with cataracts, and it is among the lowest pH values found in clinical research on chronic diseases. The effects of short-term exposure to acidification on cell functions have been extensively investigated in many tissues, including the eye. In most of these experiments, the duration of changing pH was less than an hour. It is suggested that the acute effects of altered pH is often different from the chronic effects [12, 13]. Thus the effects of moderate, long-term acidification on lens epithelial cells were examined in the present study.

The pHi value was 7.22 when the pHc was maintained at 7.40, which is consistent with previous reports as a result of using human [14] and bovine lens cells [10]. Exposure to the pHc of 7.2 and 7.0 for 10 to 21 d decreased the pHi to 7.15 and 7.09, respectively. These changes in pHi are similar to those mentioned in our previous report that acute change in extracellular pH to 6.80, from 7.40, decreased the pHi to 7.03, from 7.21, in rabbit lens cells [6]. In both cases, pHi decreased by 0.06 to 0.07 unit for a 0.20 unit drop in extracellular pH. Chronic exposure to an acidified medium did not change the resting \([\text{Ca}^{2+}]_i\) (Table 1), which is also consistent with the results in acute experiments using rat [8] and rabbit lens cells [6].

The administration of ATP produced typical responses, an initial peak followed by a sustained increase in \([\text{Ca}^{2+}]_i\), in rabbit lens epithelial cells, even after 10 to 21 d exposure to the acidified culture medium. The peak increase is due to \([\text{Ca}^{2+}]_i\) release from the intracellular stores because it was not affected by the removal of extracellular \([\text{Ca}^{2+}]_c\) (Fig. 4) and was abolished by pretreatment with thapsigargin (Fig. 5). The ATP-induced initial peak was enhanced by the chronic acidification. ATP increases inositol triphosphate (IP3), which in turn releases the calcium ions from endoplasmic reticulum, through the activa-
tion of P_{2u} purinergic receptor in human lens cells [15]. We are not certain where the action site of acidification is. The enhancement of the action of ATP is possibly due to the increase of agonist affinity by the extracellular acidification. In acute experiments it has been shown that the agonist-induced production of IP3 was not affected by acidification [16]. If it is also true in chronic acidification, the action site of the long-term acidification seems to be downstream from the IP3 production.

The influx of Ca^{2+} from the extracellular space is related to the sustained increase in [Ca^{2+}], produced by ATP, since the removal of extracellular Ca^{2+} completely suppressed it (Fig. 4). Our previous study showed that the ATP-induced sustained increase in [Ca^{2+}], was abolished by pretreatment with 100 μmol/l verapamil [6], suggesting an involvement of the L-type Ca^{2+} channel in the Ca^{2+} influx. Another possible mechanism for the ATP-induced Ca^{2+} influx is a capacitative Ca^{2+} entry pathway, which is activated by the depletion of intracellular Ca^{2+} stores [17, 18]. The finding that thapsigargin, a Ca^{2+}-pump inhibitor, caused a sustained increase in [Ca^{2+}], (Fig. 5) suggests the existence of the capacitative Ca^{2+} entry pathway in rabbit lens epithelial cells. Since chronic acidification increased the thapsigargin-induced elevation in [Ca^{2+}], Ca^{2+} influx through the capacitative Ca^{2+} entry pathway is one of the possible action sites of the acidification. This is the opposite of an effect observed in smooth muscle cells in which acidosis decreases the capacitative Ca^{2+} entry [19]. This is not surprising, since acidification reduces a contractile response to agonists in smooth muscles [20, 21]. The other possibility for the ATP-induced sustained increase in [Ca^{2+}], is Ca^{2+} release from endoplasmic reticulum triggered by Ca^{2+} influx, since treatment with thapsigargin suppressed the sustained increase. We need further quantitative analyses to evaluate which mechanism plays a major role in the ATP-induced sustained increase in [Ca^{2+}].

The acidification of aqueous humor occurs during several pathological conditions, including diabetes mellitus [22], the wearing of contact lens [23], and treatment with β-blocker [24]. Extracellular acidification causes a fall in intracellular pH, which activates some endonucleases, indicating a direct involvement of pH in triggering apoptosis [25]. ATP seems to be secreted from damaged cells [7] and increases [Ca^{2+}], in lens epithelial cells. This increase in [Ca^{2+}], is enhanced by acidification. These findings suggest that acidification exerts direct and indirect effects on cellular function. The indirect effect through the increase in [Ca^{2+}], may be related to cataract formation in patients with diabetes mellitus. Thus our data will provide clinically important information and advances in physiological knowledge.

In conclusion, chronic acidification did not change the resting [Ca^{2+}], but it enhanced ATP-induced Ca^{2+} mobilization in cultured rabbit lens epithelial cells. Both Ca^{2+} release from the intracellular store and Ca^{2+} influx from the extracellular space seemed to be increased by the acidification.

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