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Effects of Extracellular Acidification on Intracellular pH and ATP-Induced Calcium Mobilization in Rabbit Lens Epithelial Cells

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Effects of extracellular acidification on intracellular pH (pH_i) and ATP-induced calcium mobilization were investigated in rabbit lens epithelial cells. Primary-cultured lens epithelial cells of Japanese white rabbits were used. Intracellular calcium ($[Ca^{2+}]_i$) and pH_i were measured by using fluorescent dyes, fura-2 acetoxymethylester (fura-2 AM) and 2',7'-bis (carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester (BCECF AM), respectively. The addition of 10 μmol/L ATP produced an initial peak followed by a sustained increase in $[Ca^{2+}]_i$ in a standard artificial aqueous humor at extracellular pH (pH_o) 7.40. The initial peak was abolished by pretreatment with 1 μmol/L thapsigargin, whereas the sustained increase was attenuated in a Ca^{2+}-free solution or by pretreatment with 100 μmol/L verapamil. Acidification of the pH_o from 7.40 to 6.80 decreased the pH_i from 7.21 to 7.03, and enhanced both the initial peak and sustained increase in $[Ca^{2+}]_i$. These results suggest that acidification of pH_o significantly affects the pH_i and modifies the ATP-induced $[Ca^{2+}]_i$ transient in rabbit lens epithelial cells.

Key words: ATP-induced calcium mobilization; extracellular acidification; intracellular pH; rabbit lens epithelial cells

Concentration of cytosolic free calcium ($[Ca^{2+}]_i$) modulates a variety of cell functions such as muscle contraction, secretion, phototransduction and cell proliferation. A large number of agonists are known to regulate the $[Ca^{2+}]_i$ by activating phosphatidylinositol turnover (Berridge, 1993). They include acetylcholine, histamine, adrenaline, arginine vasopressin, bradykinin and ATP. ATP has been reported to be present in the normal aqueous humor and to be released into it from injured cells (Neary et al., 1996). It is also known that ATP exhibits its action through P_{2u} purinergic receptor in human lens epithelial cells (Riach et al., 1995).

Intracellular pH (pH_i) also plays an important role in the regulation of cellular functions. Previous studies demonstrated that $[Ca^{2+}]_i$ and pH_i are linked to each other, although the direction of changes in those parameters is controversial. A parallel relationship was observed between $[Ca^{2+}]_i$ and pH_i in squid axons (Baker, 1978), rat lymphocytes (Grinstein and Goetz, 1985), cultured vascular smooth muscles (Siskind et al., 1989) and colonic carcinoma cell line HT29 (Benning et al., 1996). On the other hand, an inverse relationship between them was found in *Xenopus* embryos (Rink et al,. 1980), sheep heart Purkinje fibers (Bers and Ellis, 1982), rat vascular smooth muscles (Daugirdas et al., 1995) and rat parotid acinar cells (Nishiguchi et al., 1997). In the rat lens, however, the acidification of pH_i had no effect on $[Ca^{2+}]_i$ (Bassnett and Duncan, 1988). The effect of pH_i on agonist-induced $[Ca^{2+}]_i$ transients

Abbreviations: BCECF AM, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester; $[Ca^{2+}]_i$, concentration of cytosolic free calcium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; fura-2 AM, fura-2 acetoxymethylester; IP_3, inositol triphosphate; pH_i, intracellular pH; pH_o, extracellular pH

was also investigated in HT29 cells (Nitschke et al., 1997) and rat lacrimal acinar cells (Yodozawa et al., 1997), but not in lens epithelial cells.

Therefore, in the present study effects of extracellular pH (pH_o) on pH_i and ATP-induced calcium mobilization were investigated in rabbit lens epithelial cells by measuring $[Ca^{2+}]_i$ and pH_i simultaneously with fluorescent dyes, fura-2 acetoxymethylester (fura-2 AM) and 2', 7'-bis (carboxyethyl)-5, 6-carboxyfluorescein acetoxymethylester (BCECF AM), respectively. The main questions were: i) Are the ATP-induced $[Ca^{2+}]_i$ transients modified by an acidification of pH_i? and ii) Which calcium source is involved in such pH-dependent alteration?

Materials and Methods

Animals

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, intravenously). All procedures were reviewed and approved by the Committee for Animal Experimentation in the Faculty of Medicine, Tottori University and adhered to the ARVO Statement for the Use of Animals in Ophthalmic Vision Research.

Cell culture

The eyeballs were maintained in 50 mL of phosphate-buffered solution at 5°C without added Ca^{2+} and Mg^{2+}. The lenses were dissected out 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) 1 to 3 h later. 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 humidified atmosphere containing 5% carbon dioxide. After adding 3.5 mL of DMEM with 10% FBS to slow the 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 glass coverslip coated with poly-d-lysine (MatTek Corp., Ashland, MA) and allowed to settle. The cells were flooded with DMEM containing 10% FBS, which was changed twice a week, and cultured for 10 to 21 days during which confluent cultures were usually obtained.

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 NaCl, 5 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose and 10 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) equipped with a fluorometric system (QuantiCell 700, Applied Imaging, Sunderland, United Kingdom). Cells were observed through the coverslip of the culture dish using a 40 × 0.85 numerical aperture, dry objective lens (Fluor 40, Nikon Corp.). Fura-2 AM and BCECF AM were excited with light from a 100 W xenon lamp which was alternatively filtered to 340 or 380 nm for fura-2 AM and to 440 or 490 nm for BCECF AM excitation, respectively. The fluorescence emission was filtered between 510 nm and 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. Concentration of cytosolic free calcium was calculated from the ratio (R) of the fluorescence measured with excitation at 340 nm and 380 nm using the following equation by Grynkiewicz and co-

workers (1985): $[Ca^{2+}]_i = Kd \times (R - R_{min})/(R_{max} - R) \times \beta$, where Kd is the dissociation constant of the fura-2 AM; R_{max} and R_{min} are the ratios for the bound and unbound forms of the fura-2 AM/Ca^{2+} complex; and β is the ratio between the maximum and the minimum fluorescence intensities of fura-2 AM at 380 nm excitation. To obtain R_{max}, the cells were exposed to a solution of the following composition: 150 mmol/L KCl, 10 µmol/L ionomycin (Sigma Chemical), 10 mmol/L HEPES and 10 mmol/L $CaCl_2$. The cells were then exposed to the Ca-free solution with 1 mmol/L EGTA to obtain R_{min}. The value for the Kd increases significantly when pH falls to less than 6.50. However, acidification from 7.20 to 7.0 has a much smaller effect on the fura-2 AM Kd value (Negulescu and Machen, 1990; Battle et al., 1993) causing less than 10% underestimation of $[Ca^{2+}]_i$. In the present study, the constant value (224 nmol/L) determined by Grynkiewicz and coworkers (1985) was used since pHi did not fall below 7.0.

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 pHi with the known pH_o (Williams et al., 1992), dissolved in a potassium (150 mmol/L) buffer. The 490/440 ratios were obtained during changes in pH_o by perfusing three pH standard solutions. A pH standard (pH_i 6.50) contained 10 mmol/L piperazine-*N*,*N*,-bis(2-etaethsulfonic acid) (PIPES, Sigma Chemical Co.), and other standards (pH_o 7.0 and 7.50) contained 10 mmol/L HEPES. As the response ratio was linear in the pH_o range between 7.50 and 6.50, a simple transformation was performed to obtain the corresponding pH_i values from the ratios (Williams et al., 1992). All the experimental protocols gave pH_i values within the linear range. In order to minimize the bleaching effect, excitation of BCECF AM 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 using a peristaltic pump (EYELA MICROTUBE PUMP MP-3, Tokyo Rikakikai Co., Ltd., Tokyo) at a flow rate of 2 mL/min. The pH of the solution was adjusted at either 7.40 or 6.80 by adding 1 N NaOH. The Ca^{2+}-free solution was made by substituting 2 mmol/L EGTA for 1 mmol/L $CaCl_2$ in the standard artificial aqueous humor. Each agonist was dissolved just before use and added to the perfusate. The doses of agonists were expressed as the final organ bath concentrations.

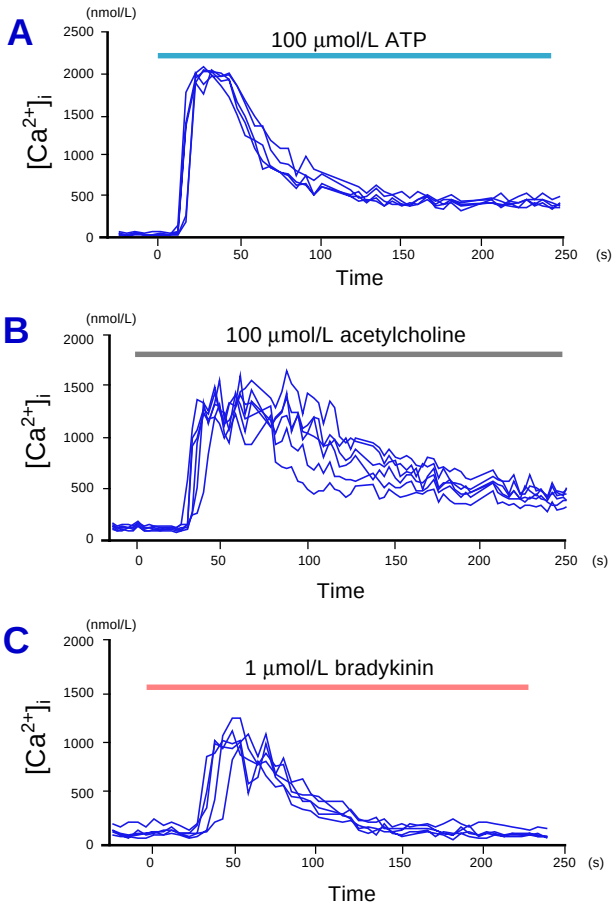

Fig. 1. Typical responses of $[Ca^{2+}]_i$ in primary-cultured rabbit lens epithelial cells during continuous perfusion with 100 µmol/L ATP (**A**), 100 µmol/L acetylcholine (**B**) or 1 µmol/L bradykinin (**C**). $[Ca^{2+}]_i$, concentration of cytosolic free calcium.

Table 1. Effects of various agonists on $[Ca^{2+}]_i$ in primary-cultured rabbit lens epithelial cells (n = 6) at pH_o 7.40

Agonist	Concentration (μmol/L)	Responded cells (%)*	Peak $\Delta[Ca^{2+}]_i$ (nmol/L)†
Acetylcholine	100	87	866 ± 264
ATP	100	100	1227 ± 218
Bradykinin	1	83	475 ± 178
Histamine	100	20	68 ± 28
Dopamine	100	0	0
Adrenaline	100	0	0
Angiotensin II	1	0	0

* Percentage of cells which responded to each agonist.
† Values are expressed as mean ± SEM.
$[Ca^{2+}]_i$, concentration of cytosolic free calcium; peak $\Delta[Ca^{2+}]_i$, peak increases in $[Ca^{2+}]_i$; pH_o, extracellular pH.

During the application of the agonist the perfusion rate was increased to 5 mL/min to achieve a quick delivery of the agonist. Changing the flow rate itself had no effect on $[Ca^{2+}]_i$. In order to avoid desensitization, consecutive agonist applications were made with intervals of longer than 30 min each. The following agonists were used in this study: acetylcholine, ATP, bradykinin, histamine, dopamine, adrenaline and angiotensin II, which were all purchased from Sigma Chemical Co. When the cells were treated with verapamil or thapsigargin (also purchased from Sigma Chemical Co.), the antagonist was added in the perfusate 15 min before the application of ATP.

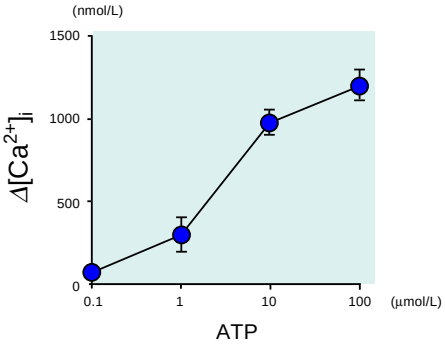

Fig. 2. Concentration-response relationship of ATP-induced $\Delta[Ca^{2+}]_i$ in primary-cultured rabbit lens epithelial cells (n = 6). $\Delta[Ca^{2+}]_i$, increases in $[Ca^{2+}]_i$.

Statistical analysis

The values are expressed as mean ± SEM. The data of $[Ca^{2+}]_i$ and pH_i were obtained by averaging the signals from 5 single cells in each culture dish. The n values reflect the number of animals used. Student's t-test (unpaired) was applied to determine the statistical difference between the 2 groups. Values of $P < 0.05$ were considered to be statistically significant.

Results

The resting $[Ca^{2+}]_i$ was 133 ± 10 nmol/L (n = 12) in primary-cultured rabbit lens epithelial cells perfused with the standard artificial aqueous humor. The pH_i was 7.21 ± 0.01 (n = 5) when pH_o was 7.40.

Figure 1 shows typical responses of $[Ca^{2+}]_i$ in the rabbit lens epithelial cells during continuous perfusion with 100 μmol/L ATP (Fig. 1A), 100 μmol/L acetylcholine (Fig. 1B) or 1 μmol/L bradykinin (Fig. 1C) in the standard artificial aqueous humor. Administration of ATP or acetylcholine produced an initial peak followed by a sustained increase in $[Ca^{2+}]_i$, whereas bradykinin caused only the initial peak. The peak increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) produced by each agonist are shown in Table 1. Adrenaline, dopamine and angiotensin II had little effect on $[Ca^{2+}]_i$ in the rabbit lens epithe-

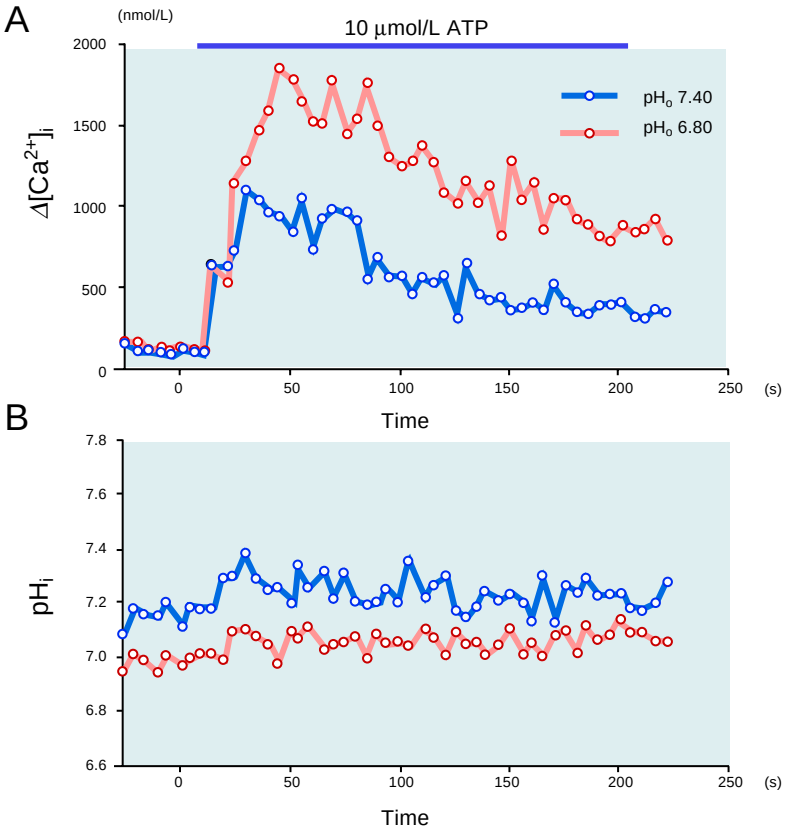

Fig. 3. Effects of extracellular acidification on 10 μmol/L ATP-induced $[Ca^{2+}]_i$ transient (**A**) and intracellular pH (pH_i) (**B**). $[Ca^{2+}]_i$, concentration of cytosolic free calcium; pH_o, extracellular pH.

lial cells. Histamine caused a small increase of $[Ca^{2+}]_i$ in limited number of the cells.

Figure 2 shows the concentration-response relationship of ATP-induced $\Delta[Ca^{2+}]_i$ in the rabbit lens epithelial cells. The threshold concentration for this response was 0.1 μmol/L. The EC_{50} value, the concentration of the agonist causing half of the maximum response, was 3.25 ± 0.58 μmol/L. Figure 3 demonstrates the influence of extracellular acidification on 10 μmol/L ATP-induced changes in $[Ca^{2+}]_i$ and pH_i. Changing extracellular pH from 7.40 to 6.80 decreased pH_i by 0.18 units (Fig. 3B), but does not alter the resting $[Ca^{2+}]_i$ (Fig. 3A). This acidification enhanced the magnitudes of both the initial peak and the sustained increase in $[Ca^{2+}]_i$ induced by ATP. ATP (10 μmol/L) increased $[Ca^{2+}]_i$ by 1532 ± 101 nmol/L at the peak and 760 ± 95 nmol/L at the sustained phase (4 min after the administration of ATP) when pH_o was 6.80, which were significantly greater than the values at pH_o 7.40 (peak: 1090 ± 173 nmol/L, $P < 0.05$; sustained phase: 423 ± 67 nmol/L, $P < 0.05$) (Fig. 4).

The source of calcium mobilization induced by ATP was investigated in the rabbit lens epithelial cells. The addition of 10 μmol/L ATP produced an initial peak followed by a sustained increase in $[Ca^{2+}]_i$ in the standard artificial aqueous humor (Fig. 5A). In the Ca^{2+}-free solution, 10 μmol/L ATP produced the initial increase in $[Ca^{2+}]_i$ without the sustained increase (Fig. 5B). The sustained increase induced by 10 μmol/L ATP was also greatly attenuated after the cells had been pretreated with 100 μmol/L verapamil, a L-type calcium channel

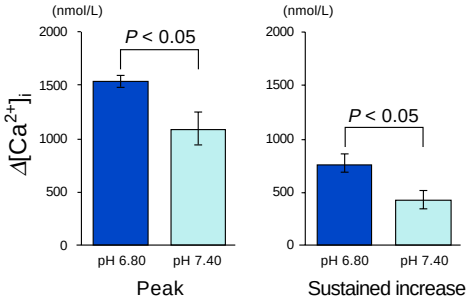

Fig. 4. Effects of extracellular acidification on 10 µmol/L ATP-induced peak and sustained increases of $[Ca^{2+}]_i$ in primary-cultured rabbit lens epithelial cells (n = 6). $[Ca^{2+}]_i$, concentration of cytosolic free calcium; $\Delta[Ca^{2+}]_i$, increases in $[Ca^{2+}]_i$.

blocker (Fig. 5C). Application of 1 µmol/L thapsigargin, a Ca^{2+} pump inhibitor, induced a gradual and sustained elevation of $[Ca^{2+}]_i$ to 874 ± 31 nmol/L (n = 6) (Fig. 5D). In the thapsigargin-treated cells, 10 µmol/L ATP failed to produce both the initial peak and the sustained increase in $[Ca^{2+}]_i$ (Fig. 5D).

Discussion

In a previous study (Duncan et al., 1996) $[Ca^{2+}]_i$ in rabbit lens cells was measured by averaging the signals from a large number of cells using a cuvette-based fluorimeter system since it was not possible to incorporate sufficient fura-2 AM into the cells to image single cells. In the present study, therefore, we loaded the cells with a high concentration (15 µmol/L) of fura-2 AM for a long period (1 h). Thus, the concentration is higher than that usually used (1–5 µmol/L), and the period is longer than usual (20–45 min) in lens cells (Riach et al., 1995; Duncan et al., 1996; Churchill and Louis, 1997).

The present results showed that ATP, acetylcholine and bradykinin caused a marked increase of $[Ca^{2+}]_i$ in the cultured rabbit lens epithelial cells, whereas histamine, dopamine, adrenaline and angiotensin II had little or no effect on the $[Ca^{2+}]_i$ (Table 1). Duncan and coworkers (1996) demonstrated that histamine but not acetylcholine produced a marked elevation of $[Ca^{2+}]_i$ in a rabbit lens cell line (NN1003A), which is opposite to our results.

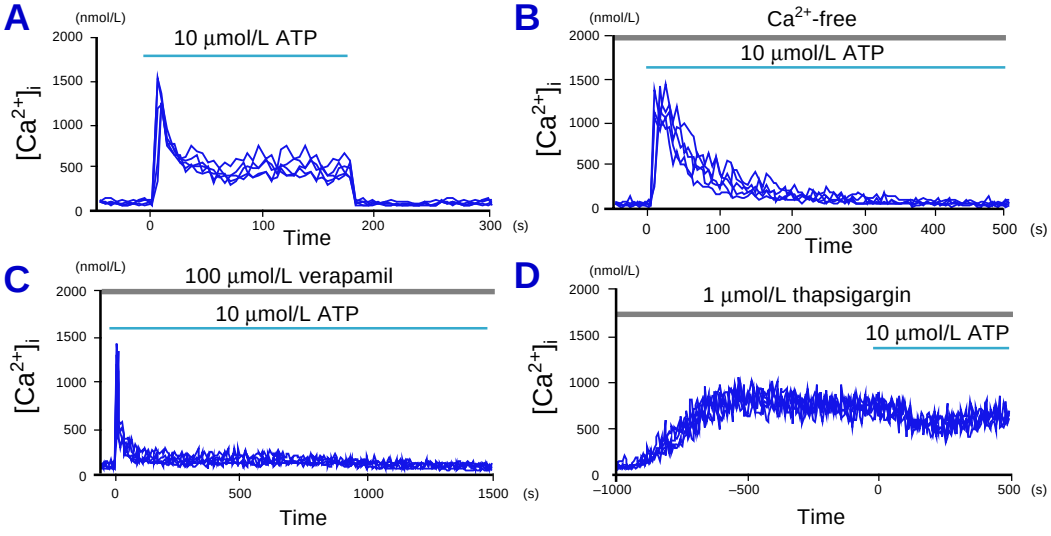

Fig. 5. Responses of $[Ca^{2+}]_i$ in primary-cultured rabbit lens epithelial cells to 10 µmol/L ATP in a standard artificial aqueous humor (**A**), in a Ca^{2+}-free solution (**B**) or by treatment with 100 µmol/L verapamil (**C**) or with 1 µmol/L thapsigargin (**D**). $[Ca^{2+}]_i$, concentration of cytosolic free calcium.

The reason for this discrepancy is not clear. Changes in type and/or number of receptors, which may occur as a result of adaptation in culture medium, may explain the difference. Both studies, on the other hands, revealed that ATP induced a marked increase of $[Ca^{2+}]_i$ in rabbit lens cells.

The addition of ATP ranging from 0.1 to 100 μmol/L caused a concentration-dependent increase in $[Ca^{2+}]_i$ (Fig.2). When the effect of pH on the ATP-induced $[Ca^{2+}]_i$ transient was examined, 10 μmol/L of ATP, a concentration which caused a submaximum response (Fig. 2), was used. Continuous perfusion with 10 μmol/L ATP produced an initial peak followed by a sustained increase in $[Ca^{2+}]_i$ (Fig.5A). The initial peak was abolished by pretreatment with 1 μmol/L thapsigargin but was not affected in a Ca^{2+}-free solution or by pretreatment with 100 μmol/L verapamil, indicating that release of Ca^{2+} from intracellular stores was involved. On the other hand, the influx of extracellular calcium through the L-type Ca^{2+} channel is probably responsible for the sustained increase in $[Ca^{2+}]_i$ because it was abolished in the Ca^{2+}-free solution and significantly attenuated by pretreatment with verapamil. Duncan and coworkers (1996) reported that 1 μmol/L ATP produced only a transient increase in $[Ca^{2+}]_i$ in rabbit lens cells. The failure for ATP to produce a sustained increase may be explained by the differences in concentration and application of ATP. A lower concentration (1 μmol/L) of ATP was added by a single injection in their experiments, whereas a higher concentration (10 μmol/L) of ATP was continuously applied over several minutes in the present study. Both transient and sustained increases in $[Ca^{2+}]_i$ were elicited in human (Riach et al., 1995) and sheep (Churchill and Louis, 1997) lens epithelial cells which were continuously exposed to high concentrations (10–100 μmol/L) of ATP.

The pH_i value measured in the present study was 7.21 ± 0.01 when the pH_o was maintained at 7.40. This is consistent with the previous reports using human (Sophie et al., 1988) and bovine (Williums et al., 1992) lens cells. Lowering the pH_o to 6.80 decreased the pH_i to 7.03. This change in pH_i is smaller than that observed in rat lens (Bassnett and Duncan, 1988) or in canine tracheal smooth muscle (Yamakage et al., 1995) but is comparable to that in rat portal vein (Taggrart et al., 1994) or guinea-pig vas deferens (Aickin, 1984). Changes in pH_i may be buffered by various proteins and phosphates. In addition, Na^+-H^+ and Cl^--HCO_3^- exchange systems may provide further pH_i regulation in lens epithelial cells (Williums et al., 1992). The small decrease of pH_i observed in the present study may imply that such buffer and exchange systems are well preserved.

The acidification of pH_i significantly enhanced both the initial peak and the sustained increase induced by 10 μmol/L ATP in rabbit lens epithelial cells (Fig. 4). This is consistent with the result obtained in HT29 cells that an intracellular acidification enhanced the peak and plateau $[Ca^{2+}]_i$ transients elicited by carbachol (Nitschke et al., 1997). The initial peak in ATP-induced $[Ca^{2+}]_i$ transient is probably due to Ca^{2+} release from intracellular stores activated by a second messenger, inositol triphosphate (IP_3). It has been shown that rabbit lens epithelial cells have a functional phosphoinositide cycle (Vivekanandan and Lou, 1989). The action site of pH_i seems to be distal to the IP_3 production because the IP_3 production caused by carbachol was unaltered by the acidification of pH_i (Nitschke et al., 1997). Intracellular acidification also seems to modify the Ca^{2+} influx through the L-type Ca^{2+} channel since the sustained increase in $[Ca^{2+}]_i$ caused by ATP was enhanced by the acidification. The similar finding, that cytosolic acidification stimulates an influx of Ca^{2+}, was observed in *Chlamydomonas* (Quarmby, 1996)

The relationship between calcium and cataract has been discussed for a long time (Duncan and Jacob, 1994). It is generally accepted that an increase in lens calcium plays a certain role in the development of cataract. An elevation of $[Ca^{2+}]_i$ seems to change membrane permeability (Bernardini and Perrachia, 1981) and stability of the lens cytoplasmic gel (Duncan and Jacob, 1994). Thus, clarifying the mechanism for $[Ca^{2+}]_i$ regulation may provide clinically important information as well as advances in physiological knowledge.

The present results demonstrated that ATP produced an initial peak followed by a sustained increase of $[Ca^{2+}]_i$ in the cultured rabbit lens epithelial cells, and that extracellular acidification reduced pH_i and enhanced both the peak and sustained $[Ca^{2+}]_i$ transients. These results suggest that pH_o acidification reduces pH_i and may modify intracellular signal transduction and membrane property which in turn affect cellular function in lens cells.

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