Ca\textsuperscript{2+} Mobilization in Nontransformed Ciliary Nonpigmented Epithelial Cells

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To investigate the calcium second messenger system in nonpigmented epithelial (NPE) cells, we studied drug-dependent cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) transients in cultured nontransformed human and rabbit NPE cells with a fluorescent Ca\textsuperscript{2+} indicator, fura-2, and a digital video-imaging system. The main findings of this study were: (1) The basal [Ca\textsuperscript{2+}]\textsubscript{i} was 141.9 ± 1.2 nM (mean ± standard error of the mean, n = 401) in humans and 157.0 ± 1.4 nM (mean ± SEM, n = 346) in rabbits. (2) Isoproterenol (10\textsuperscript{-4} M) had little effect on [Ca\textsuperscript{2+}]\textsubscript{i} mobilization in both species. Norepinephrine (10\textsuperscript{-4} M) and epinephrine (10\textsuperscript{-4} M) increased [Ca\textsuperscript{2+}]\textsubscript{i} in 56% and 78% of rabbit NPE cells by 1.8- and 2.1-fold of basal [Ca\textsuperscript{2+}]\textsubscript{i}, respectively, but induced little [Ca\textsuperscript{2+}]\textsubscript{i} change in human NPE cells. Carbachol (10\textsuperscript{-3} M) elicited significant [Ca\textsuperscript{2+}]\textsubscript{i} increase (more than 3-4-fold of basal level) in about 60-70% of NPE cells in both species. Heterogeneity was seen in the cellular response to these agonists. (3) Norepinephrine-induced response was blocked by phentolamine (10\textsuperscript{-5} M), and the effect of carbachol was blocked by atropine (10\textsuperscript{-4} M). (4) Time course of norepinephrine-induced [Ca\textsuperscript{2+}]\textsubscript{i} change was primarily monophasic. In contrast, [Ca\textsuperscript{2+}]\textsubscript{i} transients induced by carbachol were mostly biphasic. (5) The duration of carbachol- or norepinephrine-induced responses were shortened by the chelation of extracellular Ca\textsuperscript{2+} without affecting other parameters of the reaction. This study confirms the presence of the calcium signaling system in cultured nontransformed human and rabbit NPE cells. Invest Ophthalmol Vis Sci 33:1696-1705, 1992

Adrenergic and cholinergic drugs are used widely in the treatment of glaucoma. The effects of adrenergic drugs on intraocular pressure (IOP) are exerted by decreasing aqueous humor formation in the ciliary nonpigmented epithelium or by increasing the outflow facility.\textsuperscript{1,2} Pilocarpine, a typical cholinergic drug, is considered to manifest its IOP lowering effect mainly by increasing the outflow facility.\textsuperscript{3} Although the framework of actions of these drugs has been established as such, they may reduce IOP through some complex actions on aqueous formation, outflow facility, and uveoscleral outflow. Some aspects of the mechanism of action, especially the biochemical pharmacology of these drugs on aqueous humor formation, remain to be elucidated.

The ciliary process is the site of aqueous humor formation, and adrenergic and cholinergic receptors in the ciliary process have been studied extensively to investigate the molecular mechanisms of these drugs.\textsuperscript{4-7} Cyclic AMP (cAMP) has been implicated as the second messenger that modifies aqueous humor formation by beta-adrenergic drugs.\textsuperscript{8-10} However, the precise role of cAMP in aqueous humor formation is still debatable.\textsuperscript{11-13} The calcium ion also has been established as another important second messenger that modifies a variety of cellular functions, such as secretion, contraction, and proliferation.\textsuperscript{14,15} Recently, the subject of possible roles for calcium in the ciliary epithelium has attracted the attention of many researchers. Lee et al reported calcium changes in SV40 transformed human ciliary nonpigmented (NPE; 8-SVHCE) cells with various agonists using quin2 and spectrofluorometry.\textsuperscript{16} Another report has shown that some drugs (carbachol, vasopressin, bombesin, and others) induce [Ca\textsuperscript{2+}]\textsubscript{i} transients in cultured human NPE cells.\textsuperscript{17} In addition, Ca\textsuperscript{2+}-calmodulin and protein kinase C-dependent protein phosphorylation has been reported in rabbit ciliary process and 8-SVHCE.
cells. Furthermore, aqueous humor formation possibly could be modified by ion transport through gap junctions between ciliary epithelial cells, which are known to be regulated by calcium ions. Although no direct evidence for the involvement of Ca\(^{2+}\) in the regulation of aqueous humor formation is available, investigating drug-dependent [Ca\(^{2+}\)]i changes in the ciliary epithelium is of interest.

Recently, the measurement of [Ca\(^{2+}\)]i has become much easier and more reliable through the use of fluorescent calcium dyes. One of these dyes, fura-2, has several advantages. These include wavelength shifts upon Ca\(^{2+}\) binding, a weaker affinity for Ca\(^{2+}\), an improved selectivity for Ca\(^{2+}\), a better absorption coefficient, and quantum yield. These features enabled us to evaluate [Ca\(^{2+}\)]i changes more accurately with less damage to the cells. Furthermore, advanced technology has allowed us to monitor [Ca\(^{2+}\)]i in situ by a digital video-imaging system.

In the present report, we report drug-dependent Ca\(^{2+}\) mobilization in cultured human and rabbit ciliary NPE cells using a digital video-imaging system.

**Materials and Methods**

**Cell Culture**

Primary culture of ciliary NPE cells was prepared from human fetal eyes and pigmented rabbit eyes. Cell culture was performed essentially according to the methods of Fain et al and Polansky and Alvarado. In brief, after rinsing in phosphate-buffered saline supplemented with antibiotics, the eyes were incised at the ora serrata, and the posterior segment was discarded. The lens and vitreous were gently removed. The dissected ciliary body was incubated in 0.05% trypsin and 0.02% ethylene glycol bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) for 15–30 min or in 3.46 mM EGTA for 30–60 min. Following the incubation, the nonpigmented epithelial layer was separated from the underlying pigmented layer with forceps under a dissecting microscope. The isolated nonpigmented epithelium was cut into small pieces and explanted with minimal volume of culture medium onto a collagen-coated dish (Corning, Cornig, NY). When an initial outgrowth of NPE cells was observed, medium was added (Fig. 1A). NPE cells then were cultured under a humidified atmosphere of 5% CO\(_2\), 95% air at 37°C in 1:1 mixture of Dulbecco’s modified Eagle’s medium (MA Bioproducts Inc., Walkersville, MD) and Ham’s F-12 (MA Bioproducts Inc.) supplemented with 16% fetal bovine serum (MA Bioproducts Inc.), penicillin G (100 IU/ml), streptomycin (100 \(\mu\)g/ml), and amphotericin B (0.25 \(\mu\)g/ml). The second or the third passages of rabbit and the third or the fourth passages of human NPE cells with nonpigmented epithelial features were used in the experiments (Fig. 1B).

**Fura-2 Loading**

NPE cells were plated in a four-well Flexiperm Petri dish (Heraeus Biotechnologie, Hanau, FRG) attached to a thin coverglass (Matsunami, Tokyo, Japan) at a density of 5,000 cells/well. Forty-eight hours after plating, NPE cells were labeled with 5 \(\mu\)M fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probe, Eugene, OR) diluted in culture medium for 30 min at 37°C. The cells were incubated with fura-2 free medium for another 30 min at room temperature to facilitate hydrolysis of intracellular fura-2/AM to fura-2. Then the cells were washed with HEPES-buffered saline (HBS) solution (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 2.2 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), and 13.8 mM glucose, pH 7.4) three times and used for the fluorescent studies.

**Fluorescence Measurement**

Fluorescence measurement was performed essentially according to the method previously described. In brief, NPE cell-associated fura-2 fluorescence intensity was imaged at 5 sec intervals using an Olympus IMT-I inverted microscope (Olympus, Tokyo, Japan) and a Hamamatsu silicon intensified target (SIT) camera, C2400-08H (Hamamatsu Photonics, Hamamatsu, Japan). Cells on a thermostat-controlled stage (37°C) were excited by 340 and 380 nm wavelength light. Fluorescent intensities (F) and images passing through the emission filter (500 nm) (F\(_{340}\) by 340 nm excitation and F\(_{380}\) by 380 nm excitation) reaching to the SIT camera were stored and analyzed in a Hamamatsu digital image processor, Argus-100 (Hamamatsu Photonics) and displayed on the monitor. Pre-warmed stimulants dissolved in 1 ml of HBS were applied to the well from one side and suctioned continuously from the opposite side to maintain a constant solution volume of 0.7 ml in the well. The solution in the well was replaced immediately after the stimulants were applied. Stimulants used were l-epinephrine, l-norepinephrine, and l-isoproterenol at \(10^{-4}\) and \(10^{-5}\) M and carbachol at \(10^{-3}\)–\(10^{-4}\) M. We used alpha adrenergic antagonist, phenotamine, at \(10^{-5}\) M and cholinergic antagonist, atropine, at \(10^{-4}\) M. Experiments on the chelation of extracellular calcium ions were carried out using calcium-free HBS solution containing EGTA (1 mM) instead of CaCl\(_2\). Stimulants were dissolved in this calcium-free HBS buffer and applied to the wells in the same way.
Fig. 1. Cultured rabbit NPE cells. (A) About 2 to 3 days after the explanation, initial outgrowth of NPE cells was observed. Pigmented cells were not observed in the primary culture. (B) Rabbit NPE cells of the second passage retained nonpigmented and epithelial feature.

Quantitation of Intracellular Ca\(^{2+}\) Concentration
([Ca\(^{2+}\)]\(_{i}\))

Cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) was calculated from the ratio of F\(_{340}/F_{380}\) on a pixel basis by using an in vitro calibration curve. The calibration curve was obtained by measuring the ratio of F\(_{340}/F_{380}\) of 1 \(\mu\text{M}\) fura-2 solution in a Drummond microcapillary tube (outer diameter, 1.09 mm; inner diameter, 0.80 mm) at several Ca\(^{2+}\) concentrations (29, 77, 141, 269, 462, 1,040, 2,150, and 7,100 nM) controlled by a Ca\(^{2+}\)/EGTA buffer system.\(^{27,28}\) Because there was no linearity in the calibration curve above 1,000 nM Ca\(^{2+}\), [Ca\(^{2+}\)]\(_{i}\) of more than 1 \(\mu\text{M}\) was estimated to be 1 \(\mu\text{M}\).

Statistical Analysis

Statistical analyses were performed using Student’s t-test.
Results
Basal $[\text{Ca}^{2+}]_{\text{i}}$ and Drug-Induced $[\text{Ca}^{2+}]_{\text{i}}$ Transients in NPE Cells

Basal $[\text{Ca}^{2+}]_{\text{i}}$ in cultured NPE cells was 141.9 ± 1.2 nM (mean ± standard error of the mean, n = 401) in human and 157.0 ± 1.4 nM (mean ± SEM, n = 346) in rabbit cells (Fig. 2). The majority of cells, 96% of human and 91% of rabbit NPE cells, showed basal $[\text{Ca}^{2+}]_{\text{i}}$ between 100 and 200 nM. In individual cells, the fluctuations in the resting $[\text{Ca}^{2+}]_{\text{i}}$ in the HBS buffer were rather small and usually within 5% of the mean value. To normalize the variation in the resting $[\text{Ca}^{2+}]_{\text{i}}$, we evaluated the ratio of the peak $[\text{Ca}^{2+}]_{\text{i}}$ to the basal $[\text{Ca}^{2+}]_{\text{i}}$ (peak/basal ratio). $[\text{Ca}^{2+}]_{\text{i}}$ transients by adrenergic and cholinergic drugs showed some differences between human and rabbit NPE cells. In human NPE cells, a significant response was observed only with carbachol stimulation; the mean peak/basal ratio was 2.25. On the other hand, all agonists except isoproterenol induced significant $[\text{Ca}^{2+}]_{\text{i}}$ transients in rabbit NPE cells. Their mean peak/basal ratios were 3.25 by carbachol, 1.87 by epinephrine and 1.47 by norepinephrine. Isoproterenol caused little response in NPE cells of both species (Table 1).

**Table 1. The ratio of peak/basal $[\text{Ca}^{2+}]_{\text{i}}$ in response to adrenergic and cholinergic agonists**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Human</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>1.13 ± 0.07 (82)*</td>
<td>1.47 ± 0.09 (81)</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>1.06 ± 0.01 (114)</td>
<td>1.87 ± 0.11 (96)</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>1.06 ± 0.02 (110)</td>
<td>1.13 ± 0.07 (74)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>2.25 ± 0.19 (95)</td>
<td>3.25 ± 0.23 (95)</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM.
* Number of cells studied given in parentheses.

Fig. 2. Population distribution of the resting $[\text{Ca}^{2+}]_{\text{i}}$. (A) Human NPE cells. (B) Rabbit NPE cells. Basal calcium level was 142 ± 1.2 nM (mean ± SEM, n = 401) in human and 157 ± 1.4 nM (mean ± SEM, n = 346) in rabbit NPE cells. Resting $[\text{Ca}^{2+}]_{\text{i}}$ in the majority of the cells, 96% of human, and 91% of rabbit NPE cells, ranged from 100 to 200 nM.

Fig. 3. Effect of various concentrations of carbachol on rabbit NPE cells. A variety of reactivities were observed for $10^{-6}$ M to $10^{-3}$ M carbachol stimulation. Cells showing the responses of types A, B, and D were of small population. Most of the cells responded to carbachol at more than $10^{-4}$ M (type C).
Heterogeneity of Cellular Response

Using a digital video-imaging system, we could observe responses in individual cells. In the evaluation of cellular heterogeneity, we used a maximal drug concentration based on the dose-dependent manner of cellular response. For example, NPE cells rarely responded to $10^{-6}$ M carbachol. At $10^{-3}$ M, some cells showed a small response. At more than $10^{-4}$ M, most of the cells showed a marked $[Ca^{2+}]_{i}$ increase. A small number of cells were reactive only to $10^{-3}$ M carbachol (Fig. 3).

Figure 4 shows pseudocolor images of $[Ca^{2+}]_{i}$ changes in human NPE cells stimulated with $10^{-3}$ M carbachol. Dark blue color represents the lowest $[Ca^{2+}]_{i}$, and pink and red show higher $[Ca^{2+}]_{i}$. The highest $[Ca^{2+}]_{i}$ is shown in white. Some cells responded to the drug within 10 sec, but other cells did not show a response. At 240 sec after stimulation, a large number of cells exhibited a decline in $[Ca^{2+}]_{i}$, while the $[Ca^{2+}]_{i}$ level of some cells remained high. It is difficult to know exactly what magnitude of $[Ca^{2+}]_{i}$ increase is meaningful in NPE cells. To evaluate this heterogeneity, we defined a response as positive when the peak $[Ca^{2+}]_{i}$ in a single cell showed a more than 20% increase (≥4 standard deviations) above the resting level (Table 2). Isoproterenol produced a positive calcium response only in a small number of NPE cells in both species. Human and rabbit NPE cells responded differently to norepinephrine and epinephrine. About 56% (45 out of 81 cells) and 78% (75 out of 96 cells) of rabbit NPE cells showed a positive response to norepinephrine and epinephrine, respectively, whereas only about 7% of human NPE reacted to these agonists. Norepinephrine and epinephrine induced peak/basal ratios of 1.79 and 2.10, which corresponded to peak $[Ca^{2+}]_{i}$ of 269 nM and 304 nM, respectively. Carbachol induced a positive response in 54 out of 95 human cells (57%) and in 72 out of 95 rabbit cells (76%), but 43% of human and 34% of rabbit cells did not show significant response. The average magnitude of response in reactive cells was 3.16 in human and 3.94 in rabbit cells as expressed by the peak/basal ratio. Peak $[Ca^{2+}]_{i}$, as calculated from the in vitro calibration curve, was 522 nM and 635 nM, respectively. Heterogeneity in response was found not only among the cultured cells but also among experiments.
Table 2. [Ca^{2+}], transients in reactive cells

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Reactive cells</th>
<th>Peak/basal ratio</th>
<th>Calculated peak [Ca^{2+}], [nM]</th>
<th>Reactive cells</th>
<th>Peak/basal ratio</th>
<th>Calculated peak [Ca^{2+}], [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>6/82 (7.3%)</td>
<td>2.40 ± 0.79</td>
<td>353.5 ± 123.1</td>
<td>45/81 (55.6%)</td>
<td>1.79 ± 0.15</td>
<td>269.2 ± 23.4</td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>8/114 (7.0%)</td>
<td>1.28 ± 0.02</td>
<td>169.8 ± 8.9</td>
<td>75/96 (78.1%)</td>
<td>2.10 ± 0.12</td>
<td>304.1 ± 24.7</td>
<td></td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>4/110 (3.6%)</td>
<td>1.88 ± 0.32</td>
<td>247.8 ± 48.0</td>
<td>5/74 (6.8%)</td>
<td>2.36 ± 0.90</td>
<td>399.0 ± 134.4</td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>54/95 (56.8%)</td>
<td>3.16 ± 0.28</td>
<td>521.7 ± 44.5</td>
<td>72/95 (75.8%)</td>
<td>3.94 ± 0.25</td>
<td>635.1 ± 40.2</td>
<td></td>
</tr>
</tbody>
</table>

Values obtained in reactive cells are expressed as mean ± SEM.

Effect of Alpha Adrenergic and Cholinergic Antagonists on [Ca^{2+}], Transients

Of the calcium mobilizing agents, carbachol was the most reproducible and potent. Carbachol-induced response was blocked by atropine (10^{-4} M). Norepinephrine elicited a smaller but more reproducible response than epinephrine (10^{-4} M), and the response was blocked by phentolamine (10^{-5} M) (Fig. 5).

Effect of the Chelation of Extracellular Ca^{2+}

To investigate the source of the [Ca^{2+}], transients, NPE cells were stimulated with carbachol or norepinephrine in the presence and absence of extracellular Ca^{2+}, and the cellular responses were compared (Table 3). Although evaluating the effects of extracellular Ca^{2+} chelation on the same cell may be desirable, we had to carry out these experiments separately because the effect of homologous desensitization cannot be ignored (Fig. 6). When stimulated by carbachol in the presence of extracellular calcium, the latency (the time from the addition of the agonist to the peak [Ca^{2+}]i) of the response was 10.5 ± 0.7 sec and the half life (the time from the peak [Ca^{2+}], to 50% decay of the response) was 50.4 ± 5.1 sec. [Ca^{2+}], changes induced by carbachol were mostly biphasic, composed of an initial rise and a subsequent prolonged component, but occasionally monophasic cells were observed (Fig. 7A). When the extracellular calcium was chelated, the latency was 11.3 ± 0.8 and the half life of the response was 22.3 ± 1.5 sec. The difference between the half lives was statistically significant (P < 0.02). In the calcium containing buffer, only 18% of the reactive cells showed a half life less than 20 sec. Without extracellular Ca^{2+}, the half life was within 20 sec in 70% of the reactive cells, and the time course of [Ca^{2+}], changes became monophasic (Fig. 7B). Other parameters of the response, such as the percentage of reactive cells and the peak/basal ratio, were not influenced by the chelation of extracellular Ca^{2+}. Similar results also were obtained in human NPE cells. Even in the presence of external Ca^{2+}, the time course of norepinephrine-induced [Ca^{2+}], was different from that of carbachol and showed mostly a spike-like pattern. Occasionally, a prolonged pattern was observed (Fig. 7C). The removal of external Ca^{2+} also reduced the half life significantly from 22.3 ± 1.9 to 8.5 ± 1.0 sec (P < 0.02), but had little effect on the ratio and the transfer of the response.
Table 3. Effect of extracellular Ca²⁺ on [Ca²⁺], transients in rabbit NPE cells

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Reactive cells</th>
<th>Peak/basal ratio</th>
<th>Latency (sec)</th>
<th>Half time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol +Ca²⁺</td>
<td>62/78</td>
<td>3.41 ± 0.22</td>
<td>10.6 ± 0.7</td>
<td>50.4 ± 5.1</td>
</tr>
<tr>
<td>Carbachol +EGTA (1 mM)</td>
<td>55/64</td>
<td>4.02 ± 0.19</td>
<td>11.3 ± 0.8</td>
<td>22.3 ± 1.5</td>
</tr>
<tr>
<td>Norepinephrine +Ca²⁺</td>
<td>45/81</td>
<td>1.79 ± 0.15</td>
<td>15.1 ± 1.1</td>
<td>22.3 ± 1.9</td>
</tr>
<tr>
<td>Norepinephrine +EGTA (1 mM)</td>
<td>22/54</td>
<td>2.06 ± 0.18</td>
<td>14.8 ± 1.6</td>
<td>8.5 ± 1.0</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *P < 0.02.

Discussion

Signal transduction of adrenergic and cholinergic drugs has been extensively studied. Their effects on [Ca²⁺], change in many cells have been documented. It is generally thought that alpha 1 adrenergic and muscarinic receptors are linked to the calcium signalling system, whereas beta adrenergic receptors are linked to the adenylate cyclase. As expected from this general rule, rabbit NPE cells reacted to norepinephrine, epinephrine, and carbachol but not to isoproterenol. Very little response to isoproterenol (a beta stimulant) and blockade of the norepinephrine-induced response by phentolamine (an alpha antagonist) indicates that alpha adrenergic receptor stimulation mobilizes [Ca²⁺]. The inhibitory effect of atropine on carbachol-induced response is the evidence for muscarinic receptor-mediated [Ca²⁺], mobilization. Interaction between cAMP and Ca²⁺ pathway in protein phosphorylation has been reported in 8-SVHCE cells. However, the direct effect of cAMP on Ca²⁺ increase may be absent from our primary cultured NPE cells.

In human NPE cells, we failed to show [Ca²⁺], transients by norepinephrine and epinephrine. Only with carbachol were positive responses observed. Lee et al also reported there was no [Ca²⁺], increase with epinephrine and norepinephrine using SV40 transformed human NPE cells. The lack of a positive response can be considered to be a result of the following. (1) Human NPE cells may have other messenger systems for alpha adrenergic receptor stimulation. It has been reported that rat ventricular myocytes also show no response to norepinephrine and epinephrine.
Fig. 7. Effect of extracellular Ca\(^{2+}\) on [Ca\(^{2+}\)]\(_i\) transients in rabbit NPE cells. (A) and (B), carbachol-induced [Ca\(^{2+}\)]\(_i\) changes. (C) and (D), norepinephrine-induced [Ca\(^{2+}\)]\(_i\) changes. (A) and (C), with external Ca\(^{2+}\), (B) and (D), without external Ca\(^{2+}\). With external Ca\(^{2+}\), carbachol-caused [Ca\(^{2+}\)]\(_i\) transients were mostly biphasic. Chelation of the external Ca\(^{2+}\) diminished sustained component of the reaction. Norepinephrine induced chiefly a spike-like pattern, and occasionally cells showing prolonged pattern were observed. Removal of external Ca\(^{2+}\) shortened the duration of response without altering its shape.

Also, species difference in the second messenger system to the same agonist has been shown in iris sphincter muscle.\(^{35}\) (2) Alpha adrenergic receptors or associated messenger systems are not yet present in human NPE cells because we used fetal eyes as a source.\(^{36}\) (3) The normal physiology of these cells may be modified because of the use of cultured cells. An optimal culture condition for NPE cells has not been established, and good cell proliferation is difficult to achieve. It is possible that NPE cells lose their original characteristics during culture.\(^{37}\) There were differences in the cellular responses between experiments, and we sometimes observed very little response even with epinephrine and norepinephrine in rabbit NPE cells. However, the positive responses, even if not always observed, are considered to indicate the existence of the calcium signaling system in situ. This is because the reactivities of cells more likely would be suppressed under culture conditions rather than cryptic traits being newly expressed. Therefore, in this study, we present a summary of the best responses, using a maximal drug concentration. Of the agents tested, carbachol was the most reproducible and potent agonist for calcium mobilization in NPE cells of both species, which is consistent with the results of previous studies.\(^{16,17}\)

One of the advantages of this digital imaging system is the detection of differences in cellular responses between individual cells. Hence, the heterogeneity in the cellular responses was a prominent feature of this study. These heterogeneities include: (1) differences in reactivities to the same agonist; and (2) differences in the dose-dependency and time course of Ca\(^{2+}\) response by the same or different agonist (Figs. 3, 7). Carbachol-induced [Ca\(^{2+}\)]\(_i\) increase was mostly biphasic and occasionally monophasic, while norepineph-
rime-induced response was mostly monophasic and occasionally biphasic. Such heterogeneities also are reported in other types of cells. \(^{23-40}\) Cell-to-cell variation in receptor number, calcium mobilizing mechanism, and the effect of long culture are suggested as the reasons for the heterogeneity. Receptor subtypes may provide additional explanations for the heterogeneity in response. \(^{6,41}\)

The time course of \([\text{Ca}^{2+}]_i\), transients and the effect of the chelation of extracellular \(\text{Ca}^{2+}\) indicated the mechanism of \(\text{Ca}^{2+}\) mobilization. \([\text{Ca}^{2+}]_i\) increase by calcium mobilizing agents generally is believed to consist of two phases. The first phase is due to \(\text{Ca}^{2+}\) release from intracellular \(\text{Ca}^{2+}\) store. The second phase is due to the entry of \(\text{Ca}^{2+}\) through the plasma membrane. \(^{42}\) With carbachol stimulation, the removal of the extracellular \(\text{Ca}^{2+}\) had no effect on the initial rise but eliminated the sustained component of the rise. Norepinephrine stimulation in the absence of extracellular \(\text{Ca}^{2+}\) also shortened the duration of monophasic response (Fig. 7, Table 3). The shortening of the duration of \([\text{Ca}^{2+}]_i\), rise with the chelation of external \(\text{Ca}^{2+}\) suggests that NPE cells have two \(\text{Ca}^{2+}\) mobilizing mechanisms: an initial intracellular \(\text{Ca}^{2+}\) release and a subsequent extracellular \(\text{Ca}^{2+}\) entry, for both alpha adrenergic and muscarinic receptor stimulation.

The present study demonstrates the existence of \([\text{Ca}^{2+}]_i\), transients induced by cholinergic drugs in human and rabbit NPE cells. In rabbit NPE cells, alpha adrenergic drugs as well as carbachol induced \([\text{Ca}^{2+}]_i\), transients. As shown by the differences in reactivity, the time course of \([\text{Ca}^{2+}]_i\), transients, and dose dependency, there were a variety of cellular responses. We believe the data obtained here strongly support the idea that NPE cells have the calcium second messenger system. However, its role in regulating aqueous humor formation needs further investigation.

**Key words:** cultured ciliary nonpigmented epithelial cells, fura-2, digital video-imaging system, \([\text{Ca}^{2+}]_i\), transients, adrenergic and cholinergic drugs

**References**


