Neuropeptide-Induced \([\text{Ca}^{2+}]\) Transients in Cultured Bovine Trabecular Cells

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Various kinds of neuropeptides have been identified to be immunoreactive in the drainage angle of mammalian eyes. However, little is known about second messenger system involvement with these peptides. To determine whether some of these peptides are linked to a calcium signalling system in the trabecular meshwork (TM) cells, their effects on \([\text{Ca}^{2+}]\) transients in fura-2 loaded cultured bovine TM cells were studied with a digital video-imaging system. The main findings of this study were: (1) The basal \([\text{Ca}^{2+}]\) was 164.0 ± 1.0 nM (mean ± standard error of the mean, n = 668). (2) Of the neuropeptides examined, neuropeptide Y (NPY) (10^-6 M) is the most potent because it increased \([\text{Ca}^{2+}]\) by about four-fold from the basal level. Other peptides—substance P, bombesin, calcitonin-gene-related peptide, and vasoactive intestinal peptide induced smaller increases in \([\text{Ca}^{2+}]\). (3) We defined a response as positive if \([\text{Ca}^{2+}]\) increased to a value that was 1.2-fold over the basal level. The majority of the TM cells reacted to NPY, whereas only 20–30% of the cells reacted to any of the other peptides. (4) The chelation of extracellular \(\text{Ca}^{2+}\) shortened the half-life of a NPY-induced response without affecting its latency. (5) NPY (10^-6 M) significantly increased the formation of inositol triphosphate following a 15 sec exposure. The same was the case for inositol monophosphate and inositol diphosphate. The results of this study suggest that in bovine TM cells, NPY stimulation is coupled to \(\text{Ca}^{2+}\) signalling through an increase in polyphosphoinositide turnover. Invest Ophthalmol Vis Sci 33:1676–1684, 1992

Trabecular meshwork is the site for aqueous outflow and therefore plays an important role in the regulation of intraocular pressure (IOP). One approach in the treatment of glaucoma is to lower IOP with agents that increase outflow through the trabecular meshwork as well as its neighboring tissues. The action of such antiglaucoma drugs is considered to be mediated through modulation of the production of cyclic AMP (cAMP).1'2 In particular, agents such as epinephrine, forskolin, low-doses of prostaglandins, and vasoactive intestinal peptide (VIP) are thought to elicit their therapeutic effects partly through increases in cAMP in the trabecular meshwork cells.1'2'3

In addition to adrenergic and cholinergic nerve fibers, various kinds of neuropeptides, such as neuropeptide Y (NPY), VIP, substance P, calcitonin-gene related peptide (CGRP), and galanin were detected immunohistochemically in the drainage angle of mammalian eyes.4 Neuropeptides generally are known to have diverse physiological effects. Some of them include modulation of ocular tissue function. For example, CGRP increases outflow facility and decreases IOP. Substance P and cholecystokinin are known to contract iris sphincter muscles.5'6 However, relatively little is known about the physiological responses and the mode of action of neuropeptides in TM cells. Considering the numerous biologic activities of TM cells, which include contractility, extracellular matrix production, and phagocytosis,7'9 these neuropeptides may have some regulatory roles in TM cells. Previous studies from this laboratory have shown that TM cells use a calcium signalling system to elicit adrenergic and cholinergic regulation.10 \(\text{Ca}^{2+}\) is a well known second messenger,11 and NPY, substance P, and bombesin are reported to induce \([\text{Ca}^{2+}]\), transients in other systems.12'13 Accordingly, assessing whether there are any neuropeptide-induced \([\text{Ca}^{2+}]\), changes in TM cells is relevant. In the present study, we studied the effects of these neuropeptides on \([\text{Ca}^{2+}]\), transients and the sources of their changes. For evaluating \([\text{Ca}^{2+}]\), changes, we employed a sophisticated digital-video imaging system and a fluorescent \(\text{Ca}^{2+}\) indicator fura-2.
**Materials and Methods**

**Culture of TM Cells**

Bovine eyes were obtained from a local slaughterhouse within 2 hr postmortem. TM cells were cultured as described elsewhere. Briefly, after the globe was washed in 70% ethanol, the anterior segment was excised and the lens and iris were removed. Trabecular meshwork was gently isolated from the sclera under a dissecting microscope. Excised tissue was allowed to settle on a collagen-coated culture dish (Corning, Corning, NY) that contained a minimum volume of culture medium. When initial outgrowths of TM cells were observed, more medium was added. TM cells were cultured under a humidified atmosphere of 5% CO₂, 95% air at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (MA Bioproducts Inc., Wakersville, MD) supplemented with 16% fetal bovine serum (MA Bioproducts Inc.), penicillin G (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). TM cells grown from an explant were subcultured, and cells of the third passage were used in this study. They had a flattened “endothelial” shape with processes that overlapped neighboring cells (Fig. 1).

**Measurement of Cytosolic Calcium Concentration ([Ca²⁺])**

As previously described, two days before experimentation, TM cells were subcultured on a thin coverglass coated with type I collagen and attached to a four-well Flexiperm Petri Dish (Haereus Biotechnologie, Hanau, FRG) at a density of 5,000 cells/well. TM cells were loaded with 2 µM fura-2 acetoxyethyl ester (fura-2/AM) (Molecular Probes, Eugene, OR) in DMEM at 37°C for 30 min and incubated at room temperature with fura-2-free DMEM for another 30 min. A four-well Petri dish was placed on the thermostat-regulated stage of an Olympus (Tokyo, Japan) IMT-I inverted microscope maintained at 37°C, and one of the wells was alternately excited at 340 nm (F340) and 380 nm (F380). Cell-associated fluorescence output was imaged at 500 nm using epifluorescence optics and a Hamamatsu SIT camera C2400-08H. Images were stored in a Hamamatsu digital image processor, Argus-100 (Hamamatsu Photonics, Hamamatsu, Japan). Excitation wavelengths were selected with automatically controlled, filters and paired recordings were made at 5 sec intervals. Neuropeptides dissolved in HEPES-buffered saline (HBS) solution (20 mM HEPES, 115 mM NaCl, 2.2 mM CaCl₂, 0.8 mM MgCl₂, 13.8 mM glucose, pH 7.4) at 37°C were applied to the cells on a coverglass by a bath application method. The volume of each of the wells was 0.7 ml and the drugs were dissolved in 2 ml of HBS. The drugs were applied using a syringe. The replacement of a buffer in the bath occurred immediately after an application of a drug. Neuropeptides used were: NPY, substance P, bombesin, CGRP, and VIP at 10⁻¹⁰ M to 10⁻⁶ M. All peptides were obtained from the Peptide Institute (Osaka, Japan). The purity of the peptides was more than 99.9%. Experiments employing Ca²⁺-free HBS contained EGTA (1 mM) in place of CaCl₂. Neuropeptides were dissolved in this Ca²⁺-
free HBS buffer and applied to the cells in the same way as the Ca\textsuperscript{2+}-containing HEPES buffer.

**Quantitation of [Ca\textsuperscript{2+}]\textsubscript{i}**

Cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) was calculated from the ratio of F\textsubscript{340}/F\textsubscript{380} on a pixel basis by using an in vitro calibration curve. The calibration curve was obtained by measuring the ratio of F\textsubscript{340}/F\textsubscript{380} of 1 \mu M fura-2 solution in a Drummond microcapillary (outer diameter, 1.09 mm; inner diameter, 0.80 mm). The Ca\textsuperscript{2+} concentrations employed were 19, 77, 141, 269, 462, 1,040, 2,150, and 7,100 nM. They were obtained with a Ca\textsuperscript{2+}/EGTA buffer system.17 Because there was no linearity in the calibration curve above 1,000 nM, we estimated [Ca\textsuperscript{2+}]\textsubscript{i} above 1 nM to be 1 \mu M.

**Measurement of Inositol Phosphate Formation**

Inositol phosphates were measured essentially according to the method of Berridge et al.18 Confluent 2 d-cultured TM cells grown in six-well plates were labelled in 2 ml of DMEM containing 2 \mu Ci/ml myo-[2-\textsuperscript{3}H]inositol for 24 hr at 37°C. After this labelling medium was removed, the cells were rinsed twice with Krebs-HEPES-Bicarbonate (KHB) buffer (125 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 11 mM glucose, 15 mM HEPES, 15 mM NaHCO\textsubscript{3}, pH 7.4) and preincubated in 0.9 ml of KHB buffer with 10 mM LiCl for 15 min. Then 0.1 ml of KHB buffer that contained NPY (10\textsuperscript{-5} M) and 10 mM LiCl was added into each of the wells, and the incubation proceeded for the indicated times. The reaction was stopped by the addition of 1 ml of 10% ice-cold trichloroacetic acid (TCA) and was chilled on ice for 10 min. The TCA extracts were washed with 2 ml of water-saturated diethyl ether and mixed thoroughly. After diethyl ether was removed, the extracts were neutralized with 0.2 M Tris-HCl. The samples were applied to columns that contained 1 ml of a 50% slurry of Dowex AG1X8 (formate form; Bio-Rad, Richmond, CA). Inositol monophosphate (IP\textsubscript{1}), inositol diphosphate (IP\textsubscript{2}), and inositol triphosphate (IP\textsubscript{3}) were sequentially eluted with 2 \times 5 ml of 0.1 M formic acid/0.2 M ammonium formate, 0.1 M formic acid/0.4 M ammonium formate, 0.1 M formic acid/1 M ammonium formate. Radioactivities in the eluates were quantitated with a liquid scintillation counter. Proteins in individual wells were determined with the Pierce BCA protein assay kit (Pierce, Rockford, IL). The data were normalized in terms of protein content.

**Statistical Analysis**

Statistical analysis was performed using Student's t-test and Pearson's correlation coefficient.

**Results**

**Basal [Ca\textsuperscript{2+}]\textsubscript{i} and Neuropeptide-Induced [Ca\textsuperscript{2+}]\textsubscript{i} Transients in TM Cells**

Basal [Ca\textsuperscript{2+}]\textsubscript{i} in cultured TM cells was 164.0 ± 1.0 nM (mean ± standard error of the mean) (n = 668) and ranged from 100–200 nM in 77% of the cells (Fig. 2). We observed two subpopulations with basal [Ca\textsuperscript{2+}]\textsubscript{i} clustered around 150 nM and 190 nM. In individual cells, the fluctuations of the basal [Ca\textsuperscript{2+}]\textsubscript{i} in HBS buffer solution were rather small and usually within 5% of the resting value. To normalize any variation of the basal [Ca\textsuperscript{2+}]\textsubscript{i}, we used the ratio of the peak [Ca\textsuperscript{2+}]\textsubscript{i} response to the basal [Ca\textsuperscript{2+}]\textsubscript{i}, (ie, peak/basal ratio). Of the various kinds of neuropeptides, NPY induced a marked [Ca\textsuperscript{2+}]\textsubscript{i} increase (Table 1). The overall peak/basal ratio in the total cell population was 3.76 ± 0.19 (mean ± SEM, n = 108). Other peptides, such as substance P, bombesin, CGRP, and VIP, had smaller but significant effects on this ratio. The mean peak/basal ratios were 1.22, 1.37, 1.25, and 1.21.

**Table 1.** The ratio of peak/basal [Ca\textsuperscript{2+}]\textsubscript{i} in response to neuropeptides

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Peak/basal [Ca\textsuperscript{2+}]\textsubscript{i}, ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropeptide Y</td>
<td>(10\textsuperscript{-8} M) 3.76 ± 0.18 (108)*</td>
</tr>
<tr>
<td>Substance P</td>
<td>(10\textsuperscript{-8} M) 1.22 ± 0.04 (68)</td>
</tr>
<tr>
<td>Bombesin</td>
<td>(10\textsuperscript{-8} M) 1.37 ± 0.13 (56)</td>
</tr>
<tr>
<td>CGRP</td>
<td>(10\textsuperscript{-8} M) 1.25 ± 0.04 (56)</td>
</tr>
<tr>
<td>VIP</td>
<td>(10\textsuperscript{-8} M) 1.21 ± 0.03 (88)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

* Number of cells studied in parentheses.
Table 2. \([Ca^{2+}]_i\) transients in reactive cells

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Reactive cells</th>
<th>Peak/basal ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropeptide Y ((10^{-6} \text{ M}))</td>
<td>82.4% (89/108)</td>
<td>4.34 ± 0.18</td>
</tr>
<tr>
<td>Substance P ((10^{-6} \text{ M}))</td>
<td>27.9% (19/68)</td>
<td>1.61 ± 0.07</td>
</tr>
<tr>
<td>Bombesin ((10^{-6} \text{ M}))</td>
<td>17.9% (10/56)</td>
<td>2.89 ± 0.47</td>
</tr>
<tr>
<td>CGRP ((10^{-6} \text{ M}))</td>
<td>28.6% (16/56)</td>
<td>2.58 ± 0.38</td>
</tr>
<tr>
<td>VIP ((10^{-6} \text{ M}))</td>
<td>17.1% (15/88)</td>
<td>2.11 ± 0.19</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

Heterogeneity of Cellular Response

With the digital video-imaging system, we observed heterogeneity in the cellular responses to the various peptides. To evaluate the heterogeneity, we designated a response to a peptide as positive if \([Ca^{2+}]_i\) in a single cell increased by more than 1.2-fold (>4 standard deviations) over its basal level. This heterogeneity is indicated in Table 2. NPY \((10^{-6} \text{ M})\) elicited a response in about 80% of the cells in this heterogeneity, whereas the percentage of cells responding to the other peptides were significantly smaller. In the reactive cells, NPY had the most potent and reproducible effects because it induced a 4.34-fold increase in the ratio, whereas substance P, bombesin, CGRP, and VIP-induced \([Ca^{2+}]_i\) in reactive cells were much smaller. Their ratios were 1.61, 2.89, 2.58, and 2.22-fold, respectively. In the cells that responded to NPY, substance P, bombesin, CGRP, and VIP-induced \([Ca^{2+}]_i\), in reactive cells were much smaller. Their ratios were 1.61, 2.89, 2.58, and 2.22-fold, respectively. In the cells that responded to NPY, substance P, bombesin, or CGRP, there was no significant difference in the basal \([Ca^{2+}]_i\) of the reactive and the nonreactive cells. On the other hand, VIP tended to increase the \([Ca^{2+}]_i\) when the cells had a lower basal \([Ca^{2+}]_i\) level (Fig. 3).

Dose-Dependent \([Ca^{2+}]_i\) Transients Induced by NPY

With \([Ca^{2+}]_i\), increases elicited by NPY, we observed dose-dependent \([Ca^{2+}]_i\), changes (Fig. 4). TM cells rarely responded to \(10^{-10} \text{ M} \) NPY, and some cells responded to \(10^{-9} \text{ M} \). However, the majority of the cells reacted to concentrations greater than \(10^{-8} \text{ M} \). The responses induced by \(10^{-7} \text{ M} \) NPY were larger than those induced by \(10^{-8} \text{ M} \).

Effect of the Chelation of Extracellular Ca\(^{2+}\) on NPY-Ca\(^{2+}\) Transients

To identify the source of these \([Ca^{2+}]_i\), transients, TM cells were exposed to NPY \((10^{-6} \text{ M})\) with or without extracellular Ca\(^{2+}\), and their \([Ca^{2+}]_i\), responses were compared. Evaluating the effect of Ca\(^{2+}\) chelation on the same cell would be more accurate. However, we carried out these experiments separately because of the possible effect of homologous desensitization (Fig. 5). In the presence of extracellular calcium, the latency (ie, the time from the addition of the pep-
Table 3. Effect of extracellular Ca\textsuperscript{2+} on [Ca\textsuperscript{2+}]\textsubscript{i} transients in TM cells

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Reactive cells</th>
<th>Peak/basal ratio</th>
<th>Latency (sec)</th>
<th>Half life (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>77.4%</td>
<td>3.91 ± 0.29</td>
<td>20.4 ± 1.1</td>
<td>30.7 ± 2.5</td>
</tr>
<tr>
<td>NPY +Ca\textsuperscript{2+}</td>
<td>(72/93)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPY +EGTA (1 mM)</td>
<td>97.3%</td>
<td>3.15 ± 0.18</td>
<td>23.0 ± 1.8</td>
<td>13.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>(71/73)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

tide to the peak [Ca\textsuperscript{2+}], of the response) was 20.4 ± 1.1 sec, and the half life (ie, the time for the peak [Ca\textsuperscript{2+}], to decay by 50%) was 30.7 ± 2.5 sec. When extracellular calcium was chelated, the latency was not affected because it was 23.0 ± 1.8 sec, whereas the half life of the response significantly decreased to 13.5 ± 0.8 sec ($P < 0.02$; Table 3). As represented by the difference between the half lives, the time dependence of [Ca\textsuperscript{2+}], changes was influenced by the chelation of external Ca\textsuperscript{2+}. In the presence of extracellular calcium, the time course of [Ca\textsuperscript{2+}], changes showed two different patterns: (1) a biphasic pattern composed of an initial rapid rise and a subsequent sustained phase (Fig. 6A); and (2) a monophasic pattern (Fig. 6B). In most cases, the NPY-caused changes in [Ca\textsuperscript{2+}], were biphasic, but the removal of external Ca\textsuperscript{2+} changed these patterns because the sustained component was eliminated. A substitution with a Ca\textsuperscript{2+}-containing buffer induced a moderate increase in [Ca\textsuperscript{2+}], (Fig. 7).

NPY-Induced Inositol Phosphates Formation

The results of the experiments employing a Ca\textsuperscript{2+}-free buffer suggested that part of the NPY-induced increase in [Ca\textsuperscript{2+}], was from an intracellular source because the responses were not entirely eliminated by the removal of Ca\textsuperscript{2+} from the bathing solution. Because increases in polyphosphoinositide turnover elicit Ca\textsuperscript{2+} mobilization from intracellular stores in many other tissues, we examined IP\textsubscript{1}, IP\textsubscript{2}, and IP\textsubscript{3} formation following exposure to NPY (Fig. 8). At 15 sec after a NPY challenge, IP\textsubscript{3} formation increased to 163% of the control level but it returned to the basal level by 30 sec. This change was statistically signifi-

![Fig. 6. The patterns of [Ca\textsuperscript{2+}], transients associated with NPY stimulation. There were two patterns of [Ca\textsuperscript{2+}], changes: (A) a biphasic pattern composed of an initial rise and a subsequent sustained phase, and (B) a monophasic pattern without a sustained component. In most cases, NPY-induced [Ca\textsuperscript{2+}], transients were biphasic.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933167/ on 11/01/2018)

![Fig. 7. Effect of extracellular Ca\textsuperscript{2+} on NPY-induced [Ca\textsuperscript{2+}], transients. The removal of extracellular Ca\textsuperscript{2+} shortened the duration of the response and changed the shape of the response rather monophasically. The substitution of Ca\textsuperscript{2+}-free with Ca\textsuperscript{2+}-containing buffer elicited a gradual and sustained increase of [Ca\textsuperscript{2+}].](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933167/ on 11/01/2018)
Fig. 8. Effect of NPY on inositol phosphates accumulation. NPY-induced IP₁ (A), IP₂ (B), and IP₃ (C) accumulation were measured according to the method of Berridge et al. IP₃ showed a transient increase 15 sec following exposure to 10⁻⁶ M NPY. IP₂ also reached a peak level at 15 sec and then declined, but remained at a level higher than the control after 5 min. IP₁ demonstrated a different time course from those of IP₂ and IP₃. It began to increase by 15 sec, and then continued to increase up to 5 min (* P < 0.05).

Discussion

This study shows that NPY induced concentration-dependent marked increases in [Ca²⁺]ᵢ in cultured bovine TM cells. NPY, a 36 amino acid peptide first isolated from pig brain, is widely distributed in the central and the peripheral nervous systems. NPY has diverse effects on blood vessels, heart, and hormone release as well as behavior such as food intake and drinking. Coexistence of NPY with catecholamines also suggests a possible role in the modulation of the release of these neurotransmitters. The signal transduction systems underlying these functions were previously investigated in human erythroleukemia cells and porcine vascular muscle cells. The possible mechanisms that were identified include [Ca²⁺]ᵢ mobilization and adenylate cyclase inhibi-
tion. However, there is scant information on the physiological role of NPY in the eye. Furthermore, little is known about the second messengers associated with NPY. Only the inhibitory effects of NPY on adenylate cyclase has been demonstrated in rabbit ciliary processes, while its effect on [Ca\(^{2+}\)]\(_i\) mobilization remains obscure. To our knowledge, this is the first report that shows NPY can induce a [Ca\(^{2+}\)]\(_i\) increase in cultured cells from the anterior segment of the eye.

Compared to the other neuropeptides we studied, NPY-induced [Ca\(^{2+}\)]\(_i\), mobilization in TM cells was different in that the majority of the cells reacted to NPY, whereas only about 20–30% of the cells responded to any of the other neuropeptides. Furthermore, the magnitude of the response to NPY was much greater than the response to the other peptides. Heterogeneity in reactivity or in time course of agonist-induced [Ca\(^{2+}\)]\(_i\), transients was reported in other cell systems. These heterogeneities are attributed to cell-to-cell variation in receptor number or the effect of culture. However, why substance P and bombesin—also believed to have a calcium signalling system—induced less pronounced response in bovine TM cells is unclear. This discrepancy may be explained by the following.

1. The presence of other second messenger systems. Bovine TM cells also may have other second messenger systems mediating the effects of substance P or bombesin. In iris sphincter, it has been demonstrated that there are species differences in the second messenger systems associated with substance P. That the substitution of a Ca\(^{2+}\)-free with a Ca\(^{2+}\) containing buffer elicited a small and gradual [Ca\(^{2+}\)]\(_i\) increase also supports the idea that TM cells depend on extracellular Ca\(^{2+}\) for the later sustained phase of a Ca\(^{2+}\) response. The dependency of an NPY-induced [Ca\(^{2+}\)]\(_i\), increase on an external Ca\(^{2+}\) source is consistent with a previous study on the isolated rat kidney. Our results, however, do not agree with several investigations that use other cells. Most of these reports suggested that extracellular Ca\(^{2+}\) did not contribute to a Ca\(^{2+}\)-free extracellular Ca\(^{2+}\); changes. Therefore, our observation of heterogeneity in neuropeptide-induced [Ca\(^{2+}\)]\(_i\), changes could be evidence for subpopulations of TM cells, even though there were no distinguishable morphologic differences between the cells.

VIP is a well known stimulant of adenylate cyclase, which also was reported in TM cells. A beta-adrenergic agonist, which elevates cAMP content, also mobilizes [Ca\(^{2+}\)]\(_i\), in rat parotid acinar cells. In our experiments, VIP induced a positive response in a small percentage of the cells, which had lower basal [Ca\(^{2+}\)]\(_i\)]. This relationship suggests that we may be able to select for VIP sensitivity based on basal [Ca\(^{2+}\)]\(_i\)]. CGRP had limited effects on [Ca\(^{2+}\)]\(_i\), transients in TM cells. The second messenger system associated with this peptide is thought to be cAMP, but its interrelationship with [Ca\(^{2+}\)]\(_i\) is still obscure.

In general, a [Ca\(^{2+}\)]\(_i\), increase elicited by Ca\(^{2+}\) mobilizing agents can be resolved into two phases: the initial Ca\(^{2+}\) release phase from intracellular calcium stores followed by subsequent Ca\(^{2+}\) entry through the plasma membrane. However, in the NPY-induced response, the contribution of extracellular Ca\(^{2+}\) to a [Ca\(^{2+}\)]\(_i\), increase is still not clear. Therefore, to investigate the source of an NPY-induced [Ca\(^{2+}\)]\(_i\), increase, we exposed TM cells to NPY in the presence and absence of external Ca\(^{2+}\). In the presence of external Ca\(^{2+}\), the NPY-induced [Ca\(^{2+}\)]\(_i\), transients in TM cells were heterogeneous as shown in other cells because a biphasic and a monophasic response was observed. Consistent with the previous explanation of a biphasic response, the removal of extracellular Ca\(^{2+}\) decreased the magnitude of a [Ca\(^{2+}\)]\(_i\), response and also eliminated the second sustained component. That the substitution of a Ca\(^{2+}\)-free with a Ca\(^{2+}\) containing buffer elicited a small and gradual [Ca\(^{2+}\)]\(_i\), increase also supports the idea that TM cells depend on extracellular Ca\(^{2+}\) for the later sustained phase of a Ca\(^{2+}\) response. The dependency of an NPY-induced [Ca\(^{2+}\)]\(_i\), increase on an external Ca\(^{2+}\) source is consistent with a previous study on the isolated rat kidney. Our results, however, do not agree with several investigations that use other cells. Most of these reports suggested that extracellular Ca\(^{2+}\) did not contribute to a [Ca\(^{2+}\)]\(_i\), changes. Therefore, our observation of heterogeneity in neuropeptide-induced [Ca\(^{2+}\)]\(_i\), changes could be evidence for subpopulations of TM cells, even though there were no distinguishable morphologic differences between the cells.

2. Metaplasia and decreased receptor density. The characteristics of cells may change in the process of culture. For example, ciliary nonpigmented cells are reported to change their electrical membrane potential during culture.

3. Subpopulations of TM cells. In meshwork cells, heterogeneity in cellular responses have been reported. Therefore, our observation of heterogeneity in neuropeptide-induced [Ca\(^{2+}\)]\(_i\), changes could be evidence for subpopulations of TM cells, even though there were no distinguishable morphologic differences between the cells.
In conclusion, we demonstrated dose-dependent increases of \([\text{Ca}^{2+}]_i\) by NPY in cultured bovine TM cells. The mechanisms that account for the \([\text{Ca}^{2+}]_i\) transients consist of two components—the release from an intracellular \([\text{Ca}^{2+}]_i\) store by IP_3 and the influx of extracellular \([\text{Ca}^{2+}]_i\) across the plasma membrane. NPY is known to have physiological effects, such as modulation of prostaglandin synthesis and muscle contraction. It is also possible that NPY has significant physiological effects in TM cells.

**Key words:** cultured bovine trabecular cells, digital video-imaging system, \([\text{Ca}^{2+}]_i\), transients, neuropeptide Y, inositol phosphate

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Peter S. Reinach critically read the manuscript.

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