Characterization of Myoepithelial Cells in Mammary Gland: Intracellular Ca^{2+} Signalling and ATP Receptors

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CONTENTS

SUMMARY

Mammary myoepithelial cells in mouse lactating glands were isolated and cultured to characterize the intracellular calcium (Ca_i^{2+}) signalling leading to contraction. The Ca_i^{2+} was measured using the ratio of fura-2 fluorescence (F_{340}/F_{360}) with an image analysis system, and the contraction was simultaneously monitored by the changes of fluorescence intensity (F_{360}).

The first step of this study was to establish an isolation method of myoepithelial cells from the mammary gland. The new isolation method consisted of (1) the removal of connective tissue around the alveoli by the Dispase-collagenase method, (2) the dissociation of myo- and secretory epithelial cells from the alveoli by pipetting in 0.02% EDTA phosphate-buffered saline (PBS), and (3) the isolation of myoepithelial cells by Percoll density gradient centrifugation. Isolated myoepithelial cells were clearly distinguished from secretory epithelial cells by morphological and physiological characteristics.

Cultured myoepithelial cells assumed irregular spindle shapes and contracted well in response to oxytocin. F-actin stained with NBD-phallacidin was observed in dense bundles along the longitudinal axis in the cytoplasm. At the ultrastructual level, abundant bundles of microfilaments were prominent in the cytoplasm. In myoepithelial cells thin and thick filaments existed but not arranged in regular arrays in contrast to skeletal and cardiac muscles. Caveolae, the invagination of plasma membrane, were arranged in rows parallel to bundles of microfilaments, and associated with smooth endoplasmic

reticulum. The immunofluorescence pattern of caveolin-1, a principal component protein of caveolae, was found to correspond to caveolae. Thus, using the new method I could isolate and culture myoepithelial cells that responded to oxytocin. Cultured myoepithelial cells showed well organized contractile features like smooth muscle cells.

Oxytocin (≥0.1 nM) induced an increase in Ca_i^{2+} (F_{340}/F_{360}) and contraction monitored by F_{360} in the central region of the cells. The increase in Ca_i^{2+} was transient even in the presence of oxytocin, while contraction followed the increase in Ca_i^{2+} and was persistent. The oxytocin-induced Ca_i^{2+} increase in the Ca^{2+}-free solution (0.5 mM EGTA) was nearly identical to that in normal Ringer's solution. The result suggests that oxytocin increases Ca_i^{2+} by releasing Ca^{2+} from intracellular stores and thereby induces contraction. The following results are consistent with intracellular stores being the primary source of Ca^{2+} for oxytocin-induced Ca_i^{2+} increase: (1) Nifedipine (10 μM) did not affect the oxytocin-induced increase in Ca_i^{2+}, and (2) oxytocin did not induce the increase in Ca_i^{2+} after an application of thapsigargin (1 μM), an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase. Caffeine (1 or 10 mM), an activator of Ca^{2+}-induced Ca^{2+} release (CICR), did not induce the change in Ca_i^{2+}, and ryanodine (10 μM) did not affect the oxytocin-induced increase in Ca_i^{2+}. These results suggest that CICR is not involved in myoepithelial contraction.

Various substances were examined whether they induce the Ca_i^{2+} increase and contraction in myoepithelial cells. Bradykinin (≥10 nM), acetylecholine (≥10 μM), arg-vasopressin (≥1 nM) and ATP (≥10 μM) induced the increase in

Ca_i^{2+} and contraction, while noradrenaline (100 μM), histamine (100 μM), endothelin-1 (100 nM), endothelin-3 (10 nM), substance P (100 nM) and angiotensin-II (10 μM) did not have any effect on Ca_i^{2+}. The Ca_i^{2+} responses to bradykinin, acetylcholine, arg-vasopressin and ATP were not affected in the Ca^{2+}-free solution. These results indicate that some neuroactive and vasoactive substances act on myoepithelial cells and induce the increase in Ca_i^{2+} by releasing Ca^{2+} from intracellular stores.

I investigated effects of nucleotides on myoepithelial cells in more detail, because extracellular nucleotides released from mechanically stimulated secretory epithelial cells are known to play a key role in propagating the intercellular Ca^{2+} wave in these cells. ATP induced an increase in Ca_i^{2+} and contraction in about 80% of myoepithelial cells. The Ca_i^{2+} response to ATP was not affected by Ca^{2+} removal but was suppressed by suramin (100 μM), an antagonist of ATP receptors. The order of potency of nucleotides to increase Ca_i^{2+} was ATP = ADP > UTP > UDP. These results suggest that ATP receptors in myoepithelial cells may be of the P_{2Y} type.

Application of ATP (1 μM) plus oxytocin (≤100 pM) induced the increase in Ca_i^{2+} and contraction, although each of the stimulants did not have any effect on Ca_i^{2+} at the doses. The result suggests that ATP may ensure myoepithelial cell contraction induced by oxytocin at physiological concentrations (about 50 pM).

In the lactating mammary gland, secretory epithelial cells suffer dual mechanical stress from the pressure of milk accumulated in the alveoli during milk secretion and the contraction of myoepithelial cells upon milk ejection.

Mechanically stressed secretory epithelial cells may provide extracellular nucleotides to myoepithelial cells and allow to initiate and potentiate oxytocin-induced milk ejection. Thus, in addition to the hormonal action of oxytocin, extracellular ATP may work as a paracrine mediator to enhance or regulate the milk ejection process.

Myoepithelial cells are flat cells located on the basal position of the glandular end-pieces and ducts of many exocrine glands such as mammary, salivary, sweat and lacrimal glands. They form network structures and embrace alveolus of secretory epithelial cells. Myoepithelial cells are differentiated from the ectoderm and lie in epithelia, although they have contractile ability. Because of their morphological appearance the role of myoepithelial cells has been expected to squeeze the glandular end-pieces as the result of contraction and expell the secretory products into ducts. This has been clearly revealed in the mammary gland. In 1955, Linzell observed that the contraction of alveoli forced milk into the ducts by direct microscopical examination of the living mammary glands. Afterwards, using an electron microscope, the deformation of the alveoli by contraction of myoepithelial cells was observed (Pitelka et al., 1973; Abe, 1979). The contraction of myoepithelial cells in the mammary gland is so impressive that the role of these cells is generalized to all glands. For example myoepithelial cells in sweat gland (Hurley and Shelley, 1954; Sato, 1977) seem to play a similar role in that in mammary gland. However, in parotid and mandibular glands, myoepithelial cells have less developed cell processes and do not cover the entire area of the glandular end-pieces. Thus, they seem to maintain the contour of the glandular end-pieces supporting as the exoskeleton (Satoh et al., 1994).

Myoepithelial cells in mammary glands excessively proliferate and differentiate from stem cells common to secretory epithelial cells during

pregnancy, and full differentiation is achieved around at parturition (Radnor, 1972a; Emerman and Vogl, 1986). The contraction of myoepithelial cells are involved in the ejection of milk from mammary glands for the nutritional support of offspring during lactation. The reflex ejection of milk from mammary glands is evoked by the offspring's suckling, which in turn dramatically increases the firing rate of oxytocin-secreting neurons in brief periods, leading to intermittent release of oxytocin from the neurohypophysis (Tindal, 1978; Crowley and Armstrong, 1992). Oxytocin-secreting neurons are concentrated as in clusters within the hypothalamic paraventricular and supraoptic nuclei (Rhodes et al., 1981). After delivering via systemic circulation, oxytocin acts on the specific receptors in myoepithelial cells and induces contraction of the cells, leading to expulsion of milk from alveoli to the duct. This response is transient and intermittent, rather than sustained even when the sucking stimulus is continually applied (Lincoln et al., 1973).

Thus, the role of myoepithelial cells in milk ejection has been recognized, but little is known about the cell physiology of these cells due to difficulties in isolation and culture. The difficulties mainly come from the fact that myoepithelial cells constitute only 10% of mammary parenchymal cells in the lactating glands (Soloff et al., 1980). Olins and Bremel (1982) used involuting mammary glands to isolate myoepithelial cells, since myoepithelial cells were resistant to glandular involution (Radnor, 1972c). They reported that oxytocin stimulated the phosphorylation of the myosin light chain (20,000 mol wt). But the involuted mammary gland can not be considered as the physiological state

in which oxytocin usually acts. Soloff et al. (1980) and Zavizion et al. (1992) isolated myoepithelial cells of lactating glands. However, they mainly described the morphological identification of the isolated cells, and did not address the intracellular signalling leading to contraction. Myoepithelial cells differ from skeletal, cardiac and smooth muscle cells that are derived from mesoderm, and express both muscle and epithelial phenotypes. For example, myoepithelial cells contain dense bundles of filaments that are composed of contractile proteins such as fibrous actin and myosin, while they express cytokeratin not desmin as major component of the intermediate filaments (Warburton et al., 1982; Gugliotta et al., 1988). It is interesting to elucidate the intracellular signalling leading to contraction in myoepithelial cells from the viewpoint of comparative muscle physiology.

Milk secretion is activated during lactation by the lactogenic hormones such as prolactin, insulin and glucocorticoid (Borellini and Oka, 1989). Milk is secreted through the constitutive secretory pathway but not regulated one. Chemical and mechanical stimulations are known to increase Ca_i^{2+} in secretory epithelial cells (Furuya and Enomoto, 1990; Enomoto et al., 1992, 1994; Furuya et al., 1993a,b). The mechanical stimulation of a secretory epithelial cell with a glass pipette caused an increase in Ca_i^{2+} and a release of nucleotides such as ATP, UTP and UDP from the cell. These nucleotides diffused through the extracellular medium, and sequentially increased the Ca_i^{2+} in adjacent cells via the activation of P_{2U}-type ATP receptors (Enomoto et al., 1996), forming an intercellular Ca^{2+} wave. As a source of mechanical stimulation, the

contraction of myoepithelial cells is considerable. In fact the contraction of myoepithelial cells is shown to stress mechanically secretory epithelial cells upon the milk ejection (Pitelka et al., 1973; Abe, 1979). Secretory epithelial cells may also suffer mechanical stresses from the pressure of milk accumulated in the alveoli. It is likely that, in the mammary gland, the intercellular communication via extracellularly released nucleotides may be activated by mechanical stimulation, and involved in mammary gland functions such as milk ejection, milk secretion and ion transport. To clarify these mechanisms, it is indispensable to characterize properties of the myoepithelial cells.

The aims of this study were to establish a method of isolation and culture of the myoepithelial cells from the mouse lactating mammary gland and to investigate Ca_i^{2+} signalling leading to contraction. I have found that oxytocin increases Ca_i^{2+} by releasing Ca^{2+} from intracellular stores and thereby induces contraction. Extracellular ATP also induced the Ca_i^{2+} increase and contraction via activation of ATP receptors (maybe P_{2Y}-type). ATP also had synergistic effects on the Ca_i^{2+} increase with oxytocin. These results suggest that there exists paracrine control of myoepithelial cell contraction in the mammary gland.

MATERIALS AND METHODS

1. Animals

Pregnant ICR mice (E12-17) were obtained from Japan SLC Inc. (Shizuoka). Mice were allowed to standard diets and water ad lib. Room temperature and humidity were controlled to 24°C and 50%, respectively. Parturition usually occurred at 19 days pregnant.

2. Preparation of mammary myoepithelial cells

Lactating ICR mice were killed by cervical dislocation. The mammary gland tissues were minced and washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The minced tissues were incubated with 2000 PU (protease unit) /ml of Dispase (Godo Shusei, Tokyo, Japan) in Dulbecco's modified Eagle's / F-12 medium (DME/F-12, Sigma, Missouri, USA) containing 100 IU/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), supplemented with 5% fetal bovine serum (ICN, Casta Mesa, USA) and 0.0001% deoxyribonuclease I (Sigma) for 90 min in a shaker at 37°C. The tissues were washed with PBS and digested with 0.5 mg/ml collagenase (Type III, Worthington, Freehold, New Jersey, USA) in the medium described above for 15 min at 37°C. The digested tissue was washed with PBS, and cells were dissociated using a Pasteur pipette in PBS containing 0.02% EDTA. The cell suspension in PBS was filtered with nylon mesh (mesh size: 150 and 25 μm), and the solution of filtrate was replaced with DME/F-12. The suspension was layered on top of Percoll discontinuous gradient (1.050, 1.060, 1.065, 1.070, 1.075, 1.080 g/ml) in

normal Ringer's solution containing 2% Ficoll, 20 mM HEPES (pH 7.5) and 2% bovine serum albumin (Sigma) and centrifuged for 30 min at 800 × g. Myoepithelial cells sedimented at 1.070 - 1.080 g/ml were separated from secretory epithelial cells (≤ 1.065 g/ml). The cells were washed in DME/F-12 and resuspended usually in DME/F-12 containing 1 mg/ml ovalbumin, 40 μg/ml conalbumin, 30 nM sodium selenate, 100 μM putrescine, 5 μg/ml insulin and 20 nM progesterone (Sigma). These additives maintain the contractility of cultured smooth muscle cells (Bowers and Dahm, 1993). The cell suspensions were plated on coverslips (Matsunami No.1, 25 mmϕ) coated with collagen (Cellmatrix type I-A, Nitta Gelatin, Osaka, Japan) and cultured at 37°C with 5% CO_2 for 1-3 days.

3. Fluorescence immunocytochemistry

Cells cultured on collagen-coated coverslips were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C. After three times washes with PBS, the cells on the coverslips were immersed in acetone at -30°C for 5 min. After air-drying, the cells were stained as follows. For F-actin staining, the cells were incubated with 20-fold diluted NBD-phallacidin (Molecular Probe, Oregon, USA) in PBS for 2 hrs at room temperature. For the staining of caveolin-1, cells were preincubated with 10% goat serum in PBS for 30 min at 4°C, and then incubated with polyclonal anti-caveolin-1 antibody (Transduction Laboratories, Kentucky, USA, 1:500 dilution in PBS containing 1% BSA) at 4°C overnight. The coverslips were washed three times and then

incubated with biotin-labeled anti-rabbit IgG (1:200 dilution in PBS containing 1% BSA) for 2 hrs at room temperature. After three times washes with PBS, the coverslips were finally incubated with Texas red streptavidin (1:200 dilution in PBS) for 2 hrs at room temperature. The coverslips were washed, mounted on slide glasses in aqueous mounting medium (PermaFluor), and then viewed using a fluorescence microscope (Olympus BHS).

4. Transmission electronmicroscopy

Cells grown on plastic dishes were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), post-fixed in 2% osmium tetraoxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h, block-stained with 1% aqueous uranyl acetate, dehydrated with an ethanol series, and embedded in Epon 812. After polymerization, the plastic dishes were removed. Ultrathin sections were cut parallel to the plane of the culture dish with a Porter-Blum MT-1 ultramicrotome. After staining with uranyl acetate and lead citrate, the sections were examined with a JEOL 1200 EX electron microscope.

5. Measurements of the intracellular Ca^{2+} (Ca_i^{2+}) responses

To introduce fura-2 into the cells, they were incubated with 2 μM fura-2 acetoxymethyl ester (Dojindo, Kumamoto, Japan) and 0.2% cremophor EL (Sigma) for 40-70 min at 37°C. Coverslips bearing the cells were then placed in a chamber on the stage of an inverted epifluorescence microscope (Olympus IMT2-RFL, with a xenon lamp). The chamber was continuously perfused.

Normal Ringer's solution consisted of NaCl 152, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.8, glucose 5.6 and HEPES 10 (in mM). The composition of the Ca^{2+}-free solution was NaCl 152, KCl 5.4, $MgCl_2$ 0.8, EGTA 0.5, glucose 5.6 and HEPES 10 (in mM). The pH of the solutions were adjusted to 7.2 with 2 N NaOH.

Before stimulants were added, perfusion was stopped and the medium was aspirated, so that only a thin layer of medium (about 60 μl in volume) remained in the bath chamber. The stimulants (200 μl) were applied on the cells under investigation. They spread throughout the chamber in a short time. The stimulants were washed out by restarting perfusion. To study effects of extracellular Ca^{2+} removal, the Ca^{2+}-free solution was perfused for 3 or 30 min before applying the stimulants. To study effects of chelating Ca_i^{2+} on contraction, cells were incubated with 100 μM BAPTA-AM (cell-permeant Ca^{2+} chelator, Dojindo) for 10 min at 37°C before experiments. All experiments were performed at room temperature (about 24°C).

Changes in intracellular calcium concentration were measured with an image analysis system, ARGUS-50/CA (Hamamatsu Photonics). Fluorescence and phase-contrast images were detected by a CCD camera with image intensifier (C2400-87, Hamamatsu Photonics). Fura-2 fluorescence was excited at 340 and 360 nm alternately using a filter changer (C4312 Hamamatsu Photonics). Both images, after background correction, were stored in a computer (PC/AT-compatible) at intervals of 7, 30, or 60 s. The fluorescence ratios (F_{340}/F_{360}) or

intensity of some cells in the images were calculated simultaneously with or after the measurements and also stored in the computer.

The values of the fluorescence ratio in the text are expressed as average ± S.E.M. There were some variations of the responses from culture to culture. Some data were acquired in different experimental set up. Therefore, effects of given drugs were analyzed by comparing with control value of each experiment. Statistical significance was tested using Student's paired or unpaired t-test, ANOVA and Turkey-Kramer multiple comparison test. Differences were considered significant at $P < 0.05$.

Calibration converting the fluorescence ratio to Ca_i^{2+} concentration was done by cell-free calibration method as follows (Furuya et al., 1994). The fluorescence ratio of small droplet of each high K^+ solution, containing a known concentration of free Ca^{2+}, was measured under the same conditions as those for the experiment. The high K^+ solution consisted of (in mM) KCl 150, $MgCl_2$ 0.8, and MOPS 10 (pH 7.2). Free concentrations were controlled by Ca^{2+}/EGTA buffers assuming apparent dissociation constants for the Ca^{2+}-EGTA complex of 145 nM and for Mg^{2+}-EGTA complex of 15.8 mM (25°C, pH 7.2). A series of solutions with different free concentrations can be achieved from the mixing of two types of high K^+ solutions, which contained 10 mM EGTA (Dojindo) and 10 mM Ca^{2+}-EGTA, respectively, in various proportions. The concentration of fura-2 (Dojindo) in each solution was 4 μM. The calibration curve was fitted with the equation of Grynkiewcz et al. (1985).

6. Monitoring of the cell contraction

I monitored changes in the cell shape caused by contraction during Ca_i^{2+} measurements by measuring the fluorescence intensity upon 360 nm excitation (Furuya et al., 1994), which is independent from the Ca_i^{2+} change. During the contraction, the thickness of the central region of the cell visibly increased. The fluorescence intensity of this area (Fcenter) was a good indicator of cell thickness. To eliminate the effect of the fluorescence decrease due to bleaching or leaking of fura-2, I measured the fluorescence intensity of a large area that included almost the whole cell body (Fwhole) and calculated the ratio of Fcenter/Fwhole. I regarded the ratio as normalized F_{360} and used it as an indicator of cell contraction, although it was not quantitative value for contraction. The values of the normalized F_{360} in the text are expressed as average.

7. Patch clamp experiments

Membrane currents and membrane potentials of myoepithelial cells were measured in conventional whole cell recording mode (Hamill et al., 1981) using a patch clamp amplifier EPC-7 (List Electronic, Darmstadt, Germany). Patch pipettes were fabricated from borosilicate glass capillaries (o.d.=1.5 mm, G-1.5, Narishige, Japan) using a micropipette puller (Narishige, Japan). Fire-polished patch pipettes had tip resistance of between 3 MΩ and 6 MΩ. Voltage regulation and data acquisition were done by pCLAMP software (version 5.5: Axon Instruments, Forster city, CA, USA) run on IBM compatible computer.

The bath solution was normal Ringer's solution and the pipette solution consisted of (in mM) KCl 150, $MgCl_2$ 0.8, EGTA 0.1, HEPES 10 (pH 7.2). Stimulants were applied through a pipette positioned near the patched cell.

8. Drugs

Oxytocin, arg-vasopressin (Peptide Institute, Osaka, Japan), ATP, ADP, UTP, UDP, bradykinin, noradrenaline, histamine (Sigma), acetylcholine (RBI, Massachusetts, USA) and atropine (Wako, Osaka, Japan) were dissolved in water as stock solutions and diluted more than 1000 times in the corresponding medium. Nifedipine (RBI), ryanodine and thapsigargin (Sigma) were dissolved in dimethyl sulfoxide as stock solutions and diluted in the corresponding medium. Suramin (BIOMOL, Pennsylvania, USA), an antagonist of ATP receptor, was dissolved in water as a stock solution (10 mM) and diluted in the corresponding medium.

RESULTS

1. Isolation and culture of mammary myoepithelial cells, and the morphological characteristics

The first step of this study was to obtain physiologically well functioning myoepithelial cells from the mouse mammary gland. Myoepithelial cells were located at basal portion of the alveoli wrapped with the connective tissue, so the strategy to isolate myoepithelial cells was as follows. The alveoli were uncovered using Dispase and collagenase (Fig. 1A), and then treated with pipetting in 0.02% EDTA PBS to dissociate myo- and secretory epithelial cells. Myoepithelial cells were isolated by means of Percoll density gradient centrifugation, since the buoyant density of the myoepithelial cells was higher than that of the secretory epithelial cells (Soloff et al., 1980; Zavizion et al., 1992). Isolated mammary myoepithelial cells were small, thick and round (about 10 μm in diameter) (Fig. 1B). They were clearly distinguishable from secretory epithelial cells, which were larger than myoepithelial cells and contained many fat droplets in the cytoplasm (Fig. 1C).

After one day in culture, the myoepithelial cells assumed an irregular spindle shape with an oval nucleus (Fig. 2A). The myoepithelial cells in the tissue of lactating mammary gland have highly dense bundles of microfilaments (F-actin) in the cytoplasm (Radnor, 1972b; Emerman and Vogl, 1986). I confirmed this property in the cultured myoepithelial cells by fluorescence microscopy with NBD-phallacidin, a specific probe bound to F-actin. F-actin stained with NBD-phallacidin was observed in long, straight fibrils along the

longitudinal axis (Fig. 2C). Oxytocin (100 nM) induced cell contraction (Fig. 2B), and F-actin in the cells stained with NBD-phallacidin became concentrated (Fig. 2D). The response to oxytocin revealed proof of myoepithelial cells, since oxytocin receptors were localized on these cells but not on secretory epithelial cells in the mammary gland (Soloff et al., 1975).

Abundant bundles of microfilaments in the cytoplasm of myoepithelial cells were prominent at the ultrastructure level (Fig. 3A). Thin and thick filaments existed, but the latter was scarcely observed (Fig. 3B). The filaments were not arranged in regular array in contrast to skeletal and cardiac muscles. Glycogen granules and microtubules were also evident (Fig. 3C). At the basal surface of the cells, caveolae were arranged in rows parallel to bundles of microfilaments, and were associated with smooth endoplasmic reticulum (Fig. 3C). Caveolin-1, a 21-24 kDa integral membrane protein, is a principal component of caveolae (Rothberg et al. 1992). Immunofluorescence labeling with the anti-caveolin-1 antibody was observed as dots (Fig. 4). The dots seen by immunofluorescence microscopy most likely corresponded to caveolae.

2. Oxytocin-induced Ca_i^{2+} responses and contraction

Oxytocin (100 nM) induced an increase in Ca_i^{2+} (F_{340}/F_{360} ratio) and contraction of myoepithelial cells (Fig. 5A) accompanied by an increase in fura-2 fluorescence intensity at 360 nm excitation (F_{360}) in the central region of the cells (Fig. 5B). Fig. 5C shows changes in Ca_i^{2+} and the normalized F_{360} by application of oxytocin (100 nM) on a cell (indicated by arrow in Fig. 5A).

The basal Ca_i^{2+} concentration was about 40 nM and the peak Ca_i^{2+} concentration induced by oxytocin was about 700 nM using the cell-free Ca_i^{2+} calibration method (Fig. 5D). The increase in Ca_i^{2+} was transient and returned to the basal level even in the presence of oxytocin. The normalized F_{360}, the ratio of Fcenter/Fwhole (see MATERIALS AND METHODS), increased during cell contraction and decreased during relaxation. As described in MATERIALS AND METHODS, I adopted the changes of the normalized F_{360} as the indicator of contraction. The normalized F_{360} increased behind the increase in Ca_i^{2+}, and the recovery of the normalized F_{360} after the contraction was usually slow and not perfect even after the washout of oxytocin. The Ca_i^{2+} response to oxytocin (0.1-1000 nM) increased dose-dependently in normal Ringer's solution (Fig. 6). Maximum responses were obtained at 100 nM oxytocin, and thus I used this dose in the following experiments. The Ca_i^{2+} response induced by oxytocin was strongly suppressed in cells loaded with 100 μM BAPTA-AM, and the cells did not contract (Fig. 7) indicating necessity of Ca_i^{2+} increase for contraction.

To clarify the effects of extracellular Ca^{2+} removal on oxytocin-induced increase in Ca_i^{2+} and contraction, oxytocin was applied 3 min after perfusion with the Ca^{2+}-free solution. Oxytocin increased Ca_i^{2+} and induced contraction even in the Ca^{2+}-free solution (Fig. 8). The increase in Ca_i^{2+} in the Ca^{2+}-free solution was nearly identical to that in the normal Ringer solution. The peak value of F_{340}/F_{360} ratio induced by oxytocin was slightly decreased (0.95±0.03 in normal Ringer's solution; 0.89±0.03 in the Ca^{2+}-free solution, Table 1, n=26), and a half decay time from the peak ($t_{1/2}$) was slightly shortened from

1.48±0.20 min to 1.03±0.12 min (Table 2, n=12). A long time perfusion (30 min) with the Ca^{2+}-free solution failed to abolish the response to oxytocin, but the increase in Ca_i^{2+} (F_{340}/F_{360} ratio) was further decayed (0.49±0.04 in the normal Ringer's solution; 0.37±0.03 in the Ca^{2+}-free solution, $P<0.001$ paired t-test, n=26). These results suggest that oxytocin increases Ca_i^{2+} by releasing Ca^{2+} from intracellular stores and then induces contraction.

In order to determine whether L-type Ca^{2+} channels were involved in oxytocin-induced Ca_i^{2+} increase and contraction, oxytocin was applied 3 min after perfusion with normal Ringer's solution containing 10 μM nifedipine. Nifedipine did not affect oxytocin-induced increase in Ca_i^{2+} and contraction (Fig. 9). The peak value of F_{340}/F_{360} ratio induced by oxytocin was unchanged (0.66±0.03 in normal Ringer's solution; 0.64±0.02 in the presence of nifedipine, n=24). A half decay time from the peak ($t_{1/2}$) was same as that of control values (1.18±0.13 min in normal Ringer's solution; 1.18±0.17 min in the presence of nifedipine, Table 2, n=24). The results indicate that L-type Ca^{2+} channels do not contribute to the increase in Ca_i^{2+} induced by oxytocin.

The whole-cell patch clamp experiments were performed to assess electrical excitability of myoepithelial cells. The average resting potential was -31±3 mV (±SE, n=34). Application of oxytocin (10 nM) depolarized membrane potential slightly, but sometimes it induced hyperpolarization (data not shown). Hyperpolarizing and depolarizing step pulses (-100 - +90 mV with 10 mV step, 90 ms duration) from holding potential at -70 mV did not induce any clear

voltage-dependent currents (data not shown). These results suggest that myoepithelial cells are electrically non-excitable.

Thapsigargin, a plant alkaloid that selectively inhibits endoplasmic reticulum Ca^{2+}-ATPase in many tissues (Thastrup et al., 1990), was used to determine the contribution of the release of Ca^{2+} from intracellular stores induced by oxytocin. After application of 1 μM thapsigargin, Ca_i^{2+} increased and subsequently decreased to a plateau level (Fig. 10). After that, 100 nM oxytocin failed to induce the Ca_i^{2+} increase (Fig. 10). The result suggests that oxytocin increases Ca_i^{2+} by releasing Ca^{2+} from thapsigargin-sensitive stores.

The effects of caffeine and ryanodine, modifiers of Ca^{2+}-induced Ca^{2+} release (CICR) (Endo, 1977; Lai et al., 1988), on Ca_i^{2+} in myoepithelial cells were examined. Caffeine (1 or 10 mM) did not induce the increase in Ca_i^{2+} (Fig. 11A). Furthermore ryanodine (10 μM) did not affect the oxytocin-induced increase in Ca_i^{2+} (Fig. 11B). These results suggest that CICR is not involved in myoepithelial contraction.

3. Ca_i^{2+} responses induced by various substances

Various substances other than oxytocin were examined whether they induced the Ca_i^{2+} increase and contraction. Bradykinin (10-1000 nM), acetylcholine (10-1000 μM), arg-vasopressin (1-100 nM) and ATP (10-100 μM) increased Ca_i^{2+} (Fig. 12) and induced contraction. However, noradrenaline (100 μM), histamine (100 μM), endothelin-1 (100 nM), endothelin-3 (10 nM), substance P (100 nM) and angiotensin-II (10 μM) did not have any effects on Ca_i^{2+} (data not

shown). Bradykinin induced a full Ca_i^{2+} response at the dose of 10 nM in some cells. Other cells did not respond to bradykinin even at the dose of 1000 nM. So the dose-response curve had a large standard error (Fig. 13A). The Ca_i^{2+} response to acetylcholine was dose-dependently increased and maximal at 100 μM (Fig. 13B). Atropine (1 μM), muscarinic receptor antagonist, suppressed the Ca_i^{2+} response induced by acetylcholine (100 μM) (0.63±0.02 in control; 0.03±0.01 in the presence of atropine, $p<0.01$ paired t-test, n=17). Arg-vasopressin increased Ca_i^{2+} dose-dependently, and the potency to increase Ca_i^{2+} was one order lower than that of oxytocin (Fig. 13C). ATP (10-100 μM) also increased Ca_i^{2+} dose-dependently (Fig. 13D). The Ca_i^{2+} responses to bradykinin, acetylcholine, arg-vasopressin and ATP were not affected in the Ca^{2+}-free solution (Table 1). These results indicate that various substances act on myoepithelial cells and increase Ca_i^{2+} by releasing Ca^{2+} from intracellular stores.

4. Extracellular nucleotide-induced the Ca_i^{2+} responses and contraction

I investigated effects of nucleotides on myoepithelial cells in more detail, because extracellular nucleotides released from the mechanically stimulated secretory epithelial cells play a key role in propagating the intercellular Ca^{2+} wave in these cells (Enomoto et al., 1992, 1994, 1996; Furuya et al., 1993a,b). ATP (10 μM) induced an increase in Ca_i^{2+} and contraction in the myoepithelial cells even in the Ca^{2+}-free solution (Fig. 14). The peak value of F_{340}/F_{360} ratio in response to ATP (10 μM) was unchanged (0.74±0.03 in normal Ringer's

solution; 0.70±0.04 in the Ca^{2+}-free solution, Table 1, n=25), and $t_{1/2}$ did not alter (Table 2, n=8). When the Ca^{2+}-free solution was changed to normal Ringer's solution, the Ca_i^{2+} slightly increased in some cells that responded to ATP (Fig. 14) or oxytocin (data not shown). The increase in Ca_i^{2+} induced by ATP was transient, and $t_{1/2}$ for ATP (0.58±0.12 min) was shorter than that for oxytocin (1.15±0.10 min, Table 2, n=8). The increase in Ca_i^{2+} and the contraction induced by ATP were suppressed by 100 μM suramin, an antagonist of ATP receptors (Fig. 15). The peak value of F_{340}/F_{360} ratio induced by ATP was decreased from 0.66±0.04 to 0.16±0.03 ($p<0.01$, Student's paired t-test, n=21). Suramin (100 μM) did not affect the Ca_i^{2+} increase induced by 100 nM oxytocin (0.55±0.02 in control; 0.55±0.02 in the presence of suramin, n=26).

About 80% of the myoepithelial cells responded to ATP (10 μM) and ADP (10 μM), less than half responded to UTP (10 μM), and a few responded to UDP (10 μM) (Fig. 16). The dose-response curve (Fig. 17) showed that the Ca_i^{2+} responses to various nucleotides were dose-dependently increased in the following order of potency: ATP = ADP > UTP > UDP. The Ca_i^{2+} responses to ATP and ADP were sharply increased between 1 and 10 μM.

5. Synergistic effects of ATP and oxytocin on the Ca_i^{2+} responses

Application of 1 μM ATP plus 0.1 nM oxytocin induced the sudden but not gradual increase in Ca_i^{2+} and contraction, although each of the stimulants did not have any effects on Ca_i^{2+} at the doses (Fig. 18A). The peak value of F_{340}/F_{360}

ratio induced by ATP plus oxytocin (0.32±0.03, n=26) was significantly increased as compared with that induced by ATP (0.05±0.01, n=26) or oxytocin alone (0.14±0.04, n=19, Table 3). Dose-response curves for oxytocin (0.01-10 nM) were shifted to the left by addition of 1 μM ATP. However, when ATP (10 μM) plus oxytocin (0.1 or 10 nM) was applied to the cells, the increase in Ca_i^{2+} was not significantly different from that induced by ATP (10 μM) or oxytocin (10 nM) alone (Fig. 18B).

1. Morphological characteristics of mammary myoepithelial cells

I have developed a new method for the isolation and culture of myoepithelial cells from the mouse lactating mammary gland. In previous work myoepithelial cells from the mammary glands in rat and cow have been isolated using collagenase (Soloff et al., 1980; Olins and Bremel, 1982; Zavizion et al., 1992). The myoepithelial cells, however, were isolated from the involuting mammary gland (Olins and Bremel, 1982). Even when the myoepithelial cells were isolated from the lactating gland, they did not show good responsiveness to oxytocin (Zavizion et al., 1992). Moreover I could not apply their procedures using only collagenase to the mouse, since mouse myoepithelial cells were vulnerable to a long time treatment of collagenase. The strategy for isolation of myoepithelial cells in this study was based on morphological property of myoepithelial cells which are located at the basal of alveoli wrapped in connective tissues (Warburton et al., 1982). I used Dispase first and then applied collagenase in short time. Dispase did not damage myoepithelial cells after a long time incubation and digested only the connective tissues around alveoli rendering the subsequent collagenase digestion time minimal. A necessary incubation time of collagenase was critical (13-15 min). Long time incubation with collagenase (>20 min) damaged myoepithelial cells, while short time (<12 min) was not enough to dissociate myo- and secretory epithelial cells by pipetting. Separation of myo- and

secretory epithelial cells was achieved by Percoll density gradient centrifugation, since the former had higher buoyant density than the latter which have fat droplets in the cytoplasm. Isolated myoepithelial cells were cultured in the fetal bovine serum (FBS)-free defined medium (see MATERIALS AND METHODS) that was reported to maintain the contractility of cultured smooth muscle cells (Bowers and Dahm, 1993). The medium was also useful to remove contamination of secretory epithelial cells and fibroblasts, since they required FBS for culture and usually detached from collagen-coated coverslips without FBS. These isolation and culture methods of mammary myoepithelial cells could be applied to myoepithelial cells in other exocrine glands. It may facilitate elucidating the cell physiology of myoepithelial cells.

Myoepithelial cells differ from secretory epithelial cells in their ability to contract in response to oxytocin. The cultured myoepithelial cells in this study contracted well in response to oxytocin. The responsiveness to oxytocin was retained for at least 3 days in the FBS-free defined medium. When cells were cultured in the medium containing 10% FBS, the cells became flattened and lost the responsiveness to oxytocin within 2 days. Myoepithelial cells express a number of contractile proteins such as α-smooth muscle actin and smooth muscle myosin, and immunocytochemical reactions for these proteins are used as a cell marker for myoepithelial cells (Warburton et al., 1982; Gugliotta et al., 1988). In this study, F-actin stained with NBD-phallacidin easily identified myoepithelial cells on a quantitative basis, since the large amount of F-actin bundles contrasted with the relatively low content of F-actin in the secretory

epithelial cells. The stain has been utilized to visualize the three-dimensional arrangement of myoepithelial cells in whole tissue (Emerman and Vogl, 1986; Moore et al. 1987).

The ultrastructural study of cultured myoepithelial cells indicated that they were well organized as a contractile cell. Abundant bundles of microfilaments were prominent in the cytoplasm. Both thin and thick filaments existed. In contrast to skeletal and cardiac muscles, however, the filaments were not arranged in regular arrays and the striations were not observed in myoepithelial cells. At the basal surface of the cell, caveolae were arranged in rows that lay parallel to the long axis of the cell and associated with smooth endoplasmic reticulum. Myoepithelial cells resembled smooth muscle cells in these respects. Caveolae are small, flask-shaped invagination of the plasma membrane and closely associated with sarcoplasmic reticulum (SR) in smooth muscles (Gabella, 1971). Caveolae have been discussed as functional equivalents with terminal ends of transverse tubules (T tubules) in skeletal and cardiac muscles, although function of caveolae remains obscure (Forbes et al., 1979). Since recent studies revealed that caveolae contained heterometric G proteins, IP_3 receptor-like proteins (Fujimoto et al., 1992) and Ca^{2+}-ATPases (Fujimoto, 1993), there is a possibility that they are involved in the process of Ca_i^{2+} homeostasis.

Using the new method, I could isolate and culture the myoepithelial cells, the morphology of which showed contractile features like a smooth muscle cell.

2. Contractile characteristics of myoepithelial cells

Oxytocin-induced contraction of myoepithelial cells is critical for milk ejection. In oxytocin-deficient female mice, maternal behavior was normal, but all offspring dead shortly after birth because of the inability to nurse. Postpartum injections of oxytocin to oxytocin-deficient mothers restore milk ejection and rescue the offspring (Nishimori et al., 1996).

In myoepithelial cells, the increase in Ca_i^{2+} always preceded contraction, and contraction never occurred in the cells loaded with Ca^{2+} chelators. These facts indicate the necessity of the increase in Ca_i^{2+} for contraction. In skeletal and cardiac muscles, Ca^{2+} regulates contractile processes through acting on the troponin-tropomyosin system (Ebashi, 1972). In smooth muscle, muscle contraction occurs via activation of Ca^{2+}-calmodulin-regulated myosin light chain kinase (Somlyo and Somlyo, 1994). Olins and Bremel (1982) reported that oxytocin stimulates the phosphorylation of 20,000 mol wt myosin light chain in rat mammary myoepithelial cells. Olins and Bremel (1984) reported that the phosphorylation was inhibited by trifluoperazine, a inhibitor of calmodulin. Above reports and morphological data in this study suggested that the contractile system of myoepithelial cells are very much like that of smooth muscle. The oxytocin-induced increase in Ca_i^{2+} was transient without following a sustained phase, but contraction was persistent. It may be due to an absence of tension to stretch the contracted cell on the collagen-coated coverslip, because the contraction may have induced a partial detachment of cell processes from the coverslip. It may be also explained by "latch bridge" state that

dephosphorylated myosin cross-bridges remain attached to actin for several time after the Ca_i^{2+} falls (Murphy, 1994).

The amplitude and the time course of the Ca_i^{2+} increase induced by oxytocin were not markedly affected in the Ca^{2+}-free solution. The results suggest that oxytocin increased Ca_i^{2+} by releasing Ca^{2+} from intracellular stores and induced contraction. The dependence of mammary gland tissue contraction on extracellular Ca^{2+} was reported previously. Moore et al. (1987) reported that the myoepithelial cells in the tissue of mammary gland failed to contract with oxytocin after a 30 min incubation in a Ca^{2+}-free solution at 37°C. Polácek et al. (1967) reported that mammary strips contracted in response to oxytocin in a Ca^{2+}-free solution, but the response gradually decreased and disappeared upon further incubation in the same solution. Incubation in a Ca^{2+}-free solution causes depletion of Ca^{2+} from intracellular stores at a faster rate at higher temperature (Somlyo et al., 1971). In this study, oxytocin induced the contraction even 30 min after perfusion with the Ca^{2+}-free solution at 24°C, although the amplitude of the Ca_i^{2+} increase was reduced. The data imply that the incubation in the Ca^{2+}-free solution might cause the gradual depletion of Ca^{2+} in intracellular stores and reduce the contractility.

Thapsigargin inhibited the oxytocin-induced Ca_i^{2+} increase by inhibiting endoplasmic reticulum Ca^{2+}-ATPase. Thapsigargin application led to depletion of the intracellular stores due to the intrinsic leak of Ca^{2+} from the endoplasmic reticulum and the inability to resequenster Ca^{2+} because of Ca^{2+}-ATPase

inhibition. The result was consistent with intracellular stores being the primary source of Ca^{2+} for oxytocin-induced Ca_i^{2+} increase.

The results with nifedipine (Fig. 9) suggest that the Ca^{2+} influx through L-type Ca^{2+} channels dose not contribute to the contraction of myoepithelial cells, while activation of L-type Ca^{2+} channels is important to induce contraction in most muscle cells. In skeletal muscle, depolarization activates L-type Ca^{2+} channels that serve as the voltage sensor and triggers the release of Ca^{2+} from the SR. In cardiac muscle, the Ca^{2+} influx through L-type Ca^{2+} channels triggers the release of Ca^{2+} from the SR (Ca^{2+}-induced Ca^{2+} release, CICR). In smooth muscle, high K^+- or agonists-induced depolarization activates L-type Ca^{2+} channels, leading to the increase in Ca_i^{2+} and contraction. Agonists, however, also increase Ca_i^{2+} by releasing Ca^{2+} from the sarcoplasmic reticulum without a necessary change of membrane potential in contrast to skeletal and cardiac muscles (Somlyo and Somlyo, 1994). In highly excitable smooth muscles such as vas deferens, agonist-induced contraction largely depends on the activity of L-type Ca^{2+} channels, whereas in poorly excitable ones such as spleen and airway smooth muscles, agonist-induced contraction is resistant to the nifedipine treatment (Han et al., 1987; Murray and Kotlikoff, 1991). The increase in Ca_i^{2+} was found to largely depend on Ca^{2+} release from the SR and/or Ca^{2+} influx through receptor-operated Ca^{2+} channels (Murray and Kotlikoff, 1991). In myoepithelial cells, oxytocin-induced Ca_i^{2+} increases were resistant to both nifedipine treatment and extracellular Ca^{2+} removal. The electrophysiological data implied that myoepithelial cells would be electrically

non-excitable. These results suggest that myoepithelial cells belong to the poorly excitable type of smooth muscle where the increase in Ca_i^{2+} mainly depends on the release of Ca^{2+} from intracellular stores.

Generally Ca^{2+} release from intracellular stores proceeds through inositol 1,4,5-trisphosphate (IP_3)-regulated channels, or by CICR via ryanodine-sensitive channels (Pozzan et al. 1994). The intracellular signalling for oxytocin-induced increase in Ca_i^{2+} might involve the inositol phosphate pathway, since CICR was not active in myoepithelial cells. In uterine myometrium, oxytocin activates phospholipase C (PLC) through its receptor-coupled G protein, which promotes the production of the second messenger IP_3, thereby releasing Ca^{2+} from intracellular stores (Arnaudeau et al., 1994; Phaneuf et al., 1993). Further study is needed to clarify the intracellular signalling in the myoepithelial cells.

In secretory epithelial cells of the mammary gland, Ca_i^{2+} responses and oscillation occurred through Ca^{2+} release from intracellular stores (Furuya et al., 1993a). There seems to be a similar mechanism for the regulation of Ca_i^{2+} in myo- and secretory epithelial cells. This similarity may be relevant to a fact that they are derived from common stem cells (Radnor, 1972a).

3. Effects of neuroactive and vasoactive substances on myoepithelial cells

Acetylecholine increased Ca_i^{2+} and induced contraction in myoepithelial cells. The acetylecholine-induced Ca_i^{2+} increase was mediated by activation of muscarinic receptors, since the response was blocked by atropine. The results

in this study are consistent with the previous works performed in the mammary gland tissues or isolated myoepithelial cells. Linzell (1955) observed that an application of acetylcholine to the mammary gland tissue produced alveolar contraction, and then milk drained into the ducts. Olins and Bremel (1984) reported that acetylcholine stimulated phosphorylation of myosin light chain in myoepithelial cells. Physiological significance of the acetylecholine act on myoepithelial cells remains unknown, since acetylcholine is released from parasympathetic nerve endings, but the alveoli in the mammary gland are not innervated (Linzell, 1952). In the salivary glands, parasympathetic nerves are innervated, and acetylcholine has a important roles on fluid secretion by directly acting on secretory epithelial cells, or probably producing myoepithelial contraction (Garrett and Emmelin, 1979). Noradrenaline did not affect changes in Ca_i^{2+}. In the mammary gland tissue, noradrenaline exerted no action on the alveoli but produced intense vasoconstriction. It led to inhibition of milk ejection by preventing adequate access of oxytocin to the myoepithelial cells (Linzell, 1955).

Bradykinin increased Ca_i^{2+} by releasing Ca^{2+} from intracellular stores and induced contraction in myoepithelial cells. Bradykinin-induced increases in Ca_i^{2+} were observed in mammary cancerous cell lines but not in normal secretory epithelial cells (Furuya et al., 1993a). In mammary cancerous cell lines bradykinin activated pertussis- and cholera-toxin-insensitive G protein-coupled receptor, and then produced IP_3 leading to the release of Ca^{2+} from intracellular stores (Enomoto et al., 1996). The role of bradykinin in the

mammary gland remains unknown. In the salivary glands, kallikrien, a protease which converts kininogens to kinins, was localized on the apical membrane of striated duct cells (Simson et al., 1983). Bradykinin is a highly potent vasodilator and so probably responsible for the increase in blood flow during actively secreting fluid. It may also acts on myoepithelial cells and raise the intraluminal pressure of ducts to prevent back-flow of fluid (Emmelin et al., 1970).

Arg-vasopressin increased Ca_i^{2+} at low concentrations. The result is not surprising since arg-vasopressin binds to oxytocin receptors as well as to vasopressin receptors with high affinity (Zingg, 1996). So the arg-vasopressin-induced Ca_i^{2+} response is considered to be mediated by oxytocin receptors. However one can not rule out the possibility that a part of the Ca_i^{2+} response may be mediated by vasopressin receptors (Soloff et al., 1989). It seems questionable that vasopressin is involved in the milk ejection, since electrical activities of vasopressin-secreting neuron are never correlated with milk ejection during suckling, although vasopressin- and oxytocin-secreting neurons are located at the same hypothalamic nuclei (Crowley and Armstrong, 1992). Plasma vasopressin concentrations are not significantly increased and not correlated with oxytocin concentrations during suckling (Kasting, 1988).

4. Roles of ATP in the mammary gland

I found that extracellular ATP increased the Ca_i^{2+}, and then induced contraction in myoepithelial cells. Extracellular Ca^{2+} independency of ATP-induced Ca_i^{2+}

increase indicates that ATP increases Ca_i^{2+} by releasing Ca^{2+} from intracellular stores.

The pharmacological classification of ATP receptors is primarily based on selectivity for various nucleotides (Dubyak and El-Moatassim, 1993; Abbracchio and Burnstock, 1994), since neither specific agonist nor antagonist exists. The ATP receptors are classified into the intrinsic ion channel (P_{2X}, P_{2Z}, P_{2T}) and the G-protein coupled receptor (P_{2D}, P_{2U}, P_{2Y}). The molecular structure of several ATP receptors has recently been determined by gene cloning, and revealed that the former has two transmembrane domains (Brake et al., 1994), whereas the latter has seven transmembrane domains typical of G protein-coupled receptors (Chang et al., 1995). The ATP receptor in the myoepithelial cells may be of the P_{2Y} type, since (1) the increase in Ca_i^{2+} was due to release of Ca^{2+} from intracellular stores, (2) the response was suppressed by suramin, and (3) UTP and UDP were less potent than ATP and ADP. The ATP receptor in myoepithelial cells was distinct from that in secretory epithelial cells. In secretory epithelial cells, UTP preferentially increases Ca_i^{2+} via activation of P_{2U}-type ATP receptors (Enomoto et al., 1996). Enomoto recently detected mRNA of P_{2Y} and P_{2U}-type ATP receptors using RT-PCR in myo- and secretory epithelial cells, respectively (Enomoto, K., private communication). P_{2Y}-type ATP receptors are widely distributed in various types of cells such as endothelial cells (Carter et al., 1988), astrocytes (Pearce et al., 1989), osteoblasts (Reimer and Dixon, 1992) and hepatocytes (Charest et al., 1985).

Extracellular ATP is rapidly catabolized by extracellular ATPase and nucleotidase. These "ectoATPase" and "ectonucleotidase" activities are variably expressed on the cell surface of various tissues (Zimmermann, 1992). If ATP acts on myoepithelial cells in the mammary gland tissue, it may be released from nerve terminals or cells proximal to myoepithelial cells. As a source of ATP, however, the possibility of a co-transmitter in the synaptic vesicles of sympathetic or parasympathetic nerve terminals (Westfall et al., 1990) can be excluded, since the myoepithelial cells are not innervated (Linzell, 1952). Secretory epithelial cells release nucleotides such as ATP, UTP and UDP in response to mechanical stimulation, and these nucleotides induce intercellular Ca^{2+} waves via activation of P_{2U}-type ATP receptors (Enomoto et al., 1994). Secretory epithelial cells are located in proximity to the myoepithelial cells in the mammary gland and are mechanically stressed by the contraction of the latter during milk ejection (Pitelka et al., 1973). The pressure of milk accumulated in the alveoli may also gives mechanical stress to secretory epithelial cells. Mechanically stimulated secretory epithelial cells may thus provide nucleotides for the myoepithelial cells in a paracrine fashion.

The finding that ATP induces contraction of myoepithelial cells gave a new insight into the process of milk ejection. The contraction of myoepithelial cells induced by oxytocin mechanically stimulates secretory epithelial cells and may release nucleotides such as ATP, UTP and UDP from the latter cells to extracellular space. Released ATP may act on myoepithelial cells and induce contraction in turn. The contraction may give mechanical stimulation to

secretory epithelial cells again. Thus, milk ejection may be potenciated in a positive feedback manner. Actually, the intercellular Ca^{2+} wave in secretory epithelial cells was found to be triggered by the contraction of myoepithelial cells in a co-culture system (Furuya et al., 1996). This finding implies that extracellular nucleotides may play a role as an extracellular messenger between myo- and secretory epithelial cells and potentiate milk ejection.

Myoepithelial cells in this study rarely responded to 100 pM oxytocin, while the physiological concentration of oxytocin in blood was around 50 pM upon milk ejection (Higuchi et al., 1985). The discrepancy may not be just due to artifacts in *in vitro* experiments but have more positive meanings. It has been shown that different alveoli produce milk at different times (Saake and Heald, 1974). If the changes of plasma oxytocin level are the only means of regulation of myoepithelial cell contraction, all cells would contract at once, even if all the alveoli can not produce significant quantities of milk. To avoid this unnecessary expense of energy, other factors may regulate myoepithelial cell contraction so that only myoepithelial cells embracing alveoli with so much milk will contract. In this study ATP ensured the Ca_i^{2+} response and contraction induced by oxytocin at the physiological concentration (Fig. 18). Exogenous injections of milk into the ducts potentiated oxytocin sensitivity of the tissue upon milk ejection by bulging alveoli with milk (Denuccio and Grosvenor, 1971). ATP may be released from secretory epithelial cells in the alveoli with so much milk and act on myoepithelial cells to allow oxytocin-induced milk ejection.

In the lactating mammary gland, secretory epithelial cells suffer dual mechanical stress from the pressure of milk accumulated in the alveoli during milk secretion and contraction of myoepithelial cells upon milk ejection. Mechanically stressed secretory epithelial cells may provide extracellular nucleotides to myoepithelial cells and allow to initiate and potentiate oxytocin-induced milk ejection. Thus, in addition to the hormonal action of oxytocin, extracellular ATP may work as a paracrine mediator to enhance or regulate the milk ejection process.

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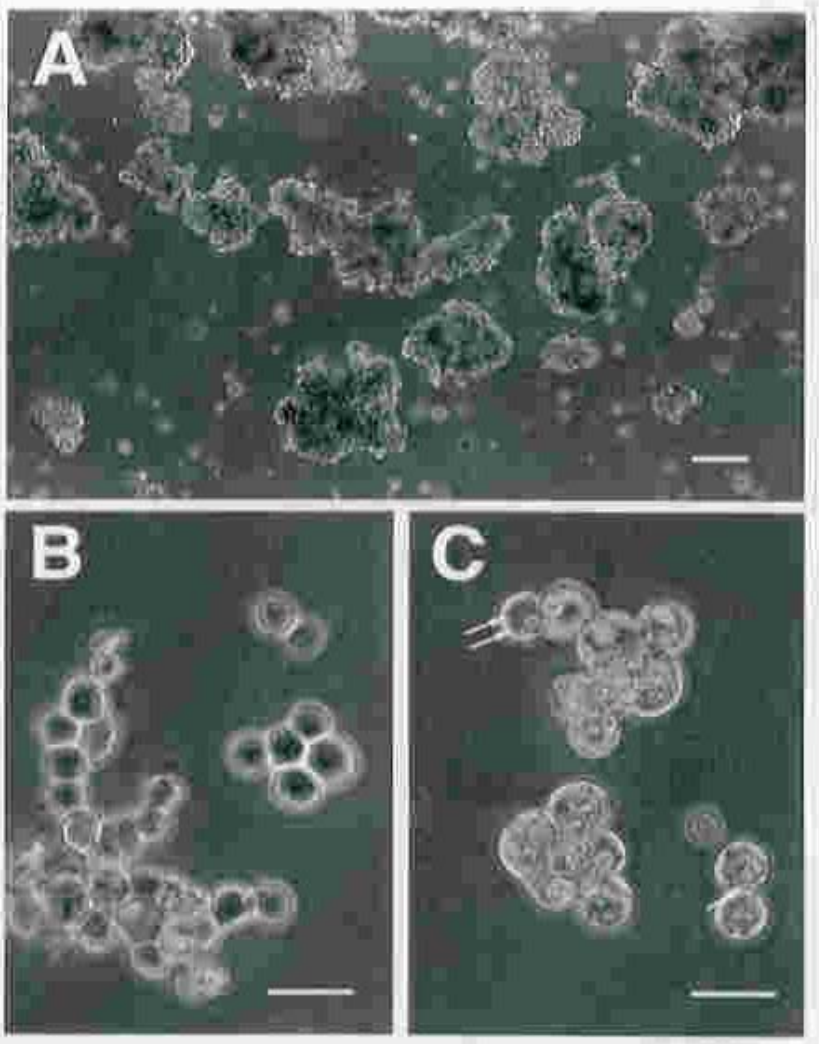

Figure 1. Phase-contrast images of alveoli, myoepithelial cells and secretory epithelial cells.

Cluster of alveoli after digestion with Dispase and collagenase (A). Isolated myoepithelial cells (B) and secretory epithelial cells (C). Note fat droplets were seen in the cytoplasm of secretory epithelial cells (arrows). Scale bars indicate 80 μm (A) and 20 μm (B,C).

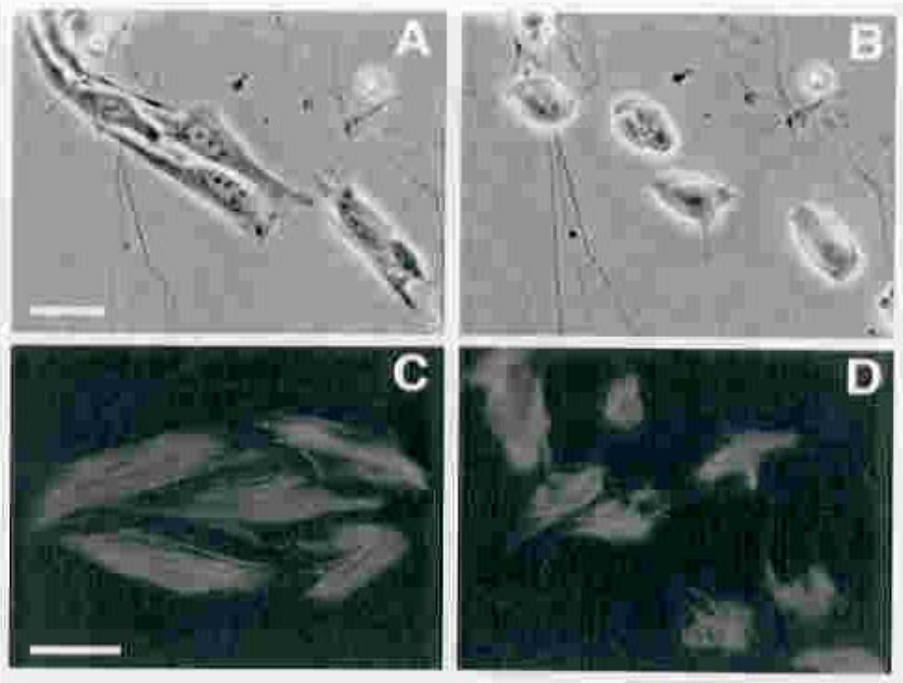

Figure 2. Isolated mammary myoepithelial cells in culture.

A and B are phase-contrast images before (A) and after (B) the application of oxytocin (100 nM) in the same field. C and D are NBD-phallacidin fluorescence images before (C) and after (D) the application of oxytocin (100 nM). Scale bars indicate 20 μm.

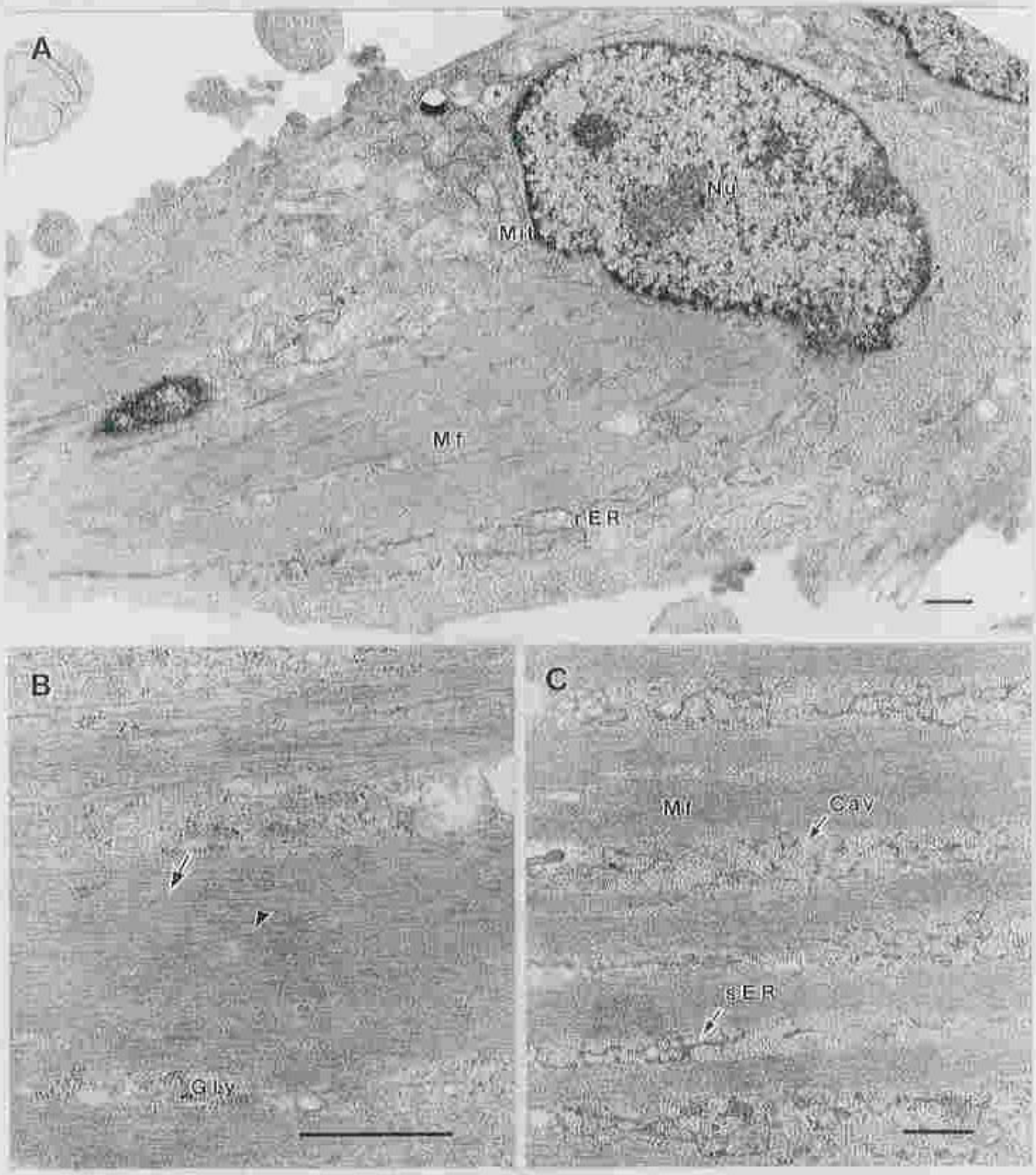
A
Nu
Mit
Mf
rER
B
Gly
C
Mit
Cav
sER

Figure 3. Electron micrograghs of cultured myoepithelial cells.

A: An overview of a myoepithelial cell abundant in microfilaments (Mf). Mitochondria (Mit) and elongated rough endoplasmic reticulum (rER) are present in the cytoplasm. Polyribosomes are evident around the nucleus (Nu) (x 8550). B: High magnification of the bundle of microfilaments. Thin (arrow) and thick filaments (arrowhead) are noted. Glycogen granules (Gly) are distributed over microfilaments (x 28000). C: Section close to the basal surface of a myoepithelial cell. Caveolae (Cav) are arranged in rows parallel to bundles of the microfilaments and are associated with cisternae of smooth endoplasmic reticulum (sER) (x 12000). Scale bars show 1 μm.

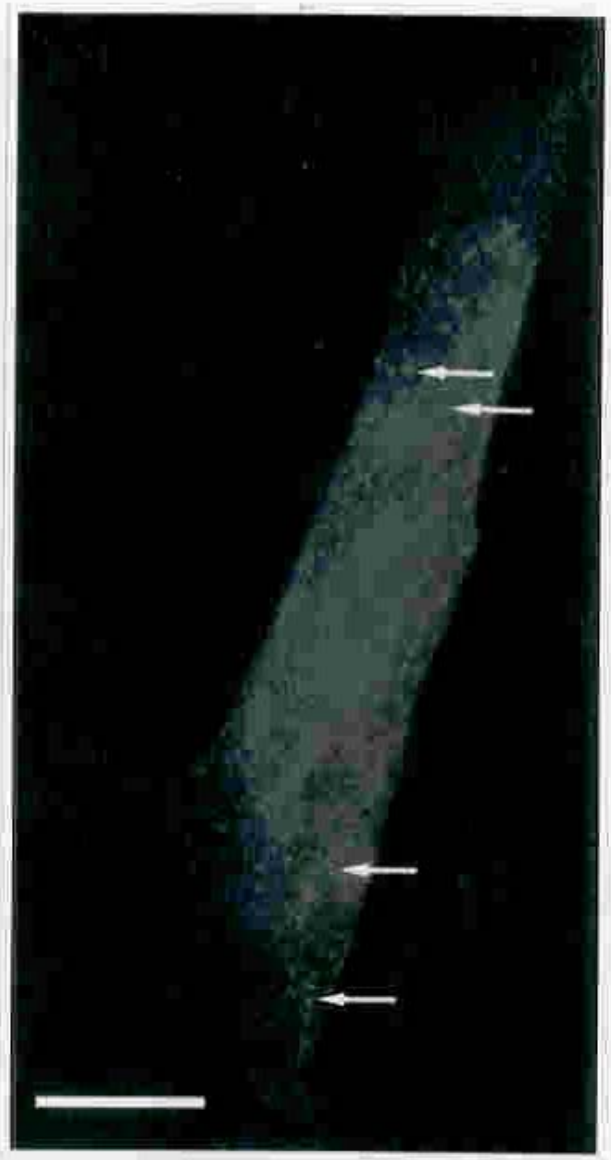

Figure 4. Immunofluorescence localization of caveolin-1. myoepithelial cells were incubated with polyclonal anti-caveolin-1 antibody visualized with biotin-labeled anti-rabbit IgG followed by streptavidin Texas-red. Note caveolin-1 immunoreactivity was seen as dots (arrows). Scale bars indicate 10 μm.

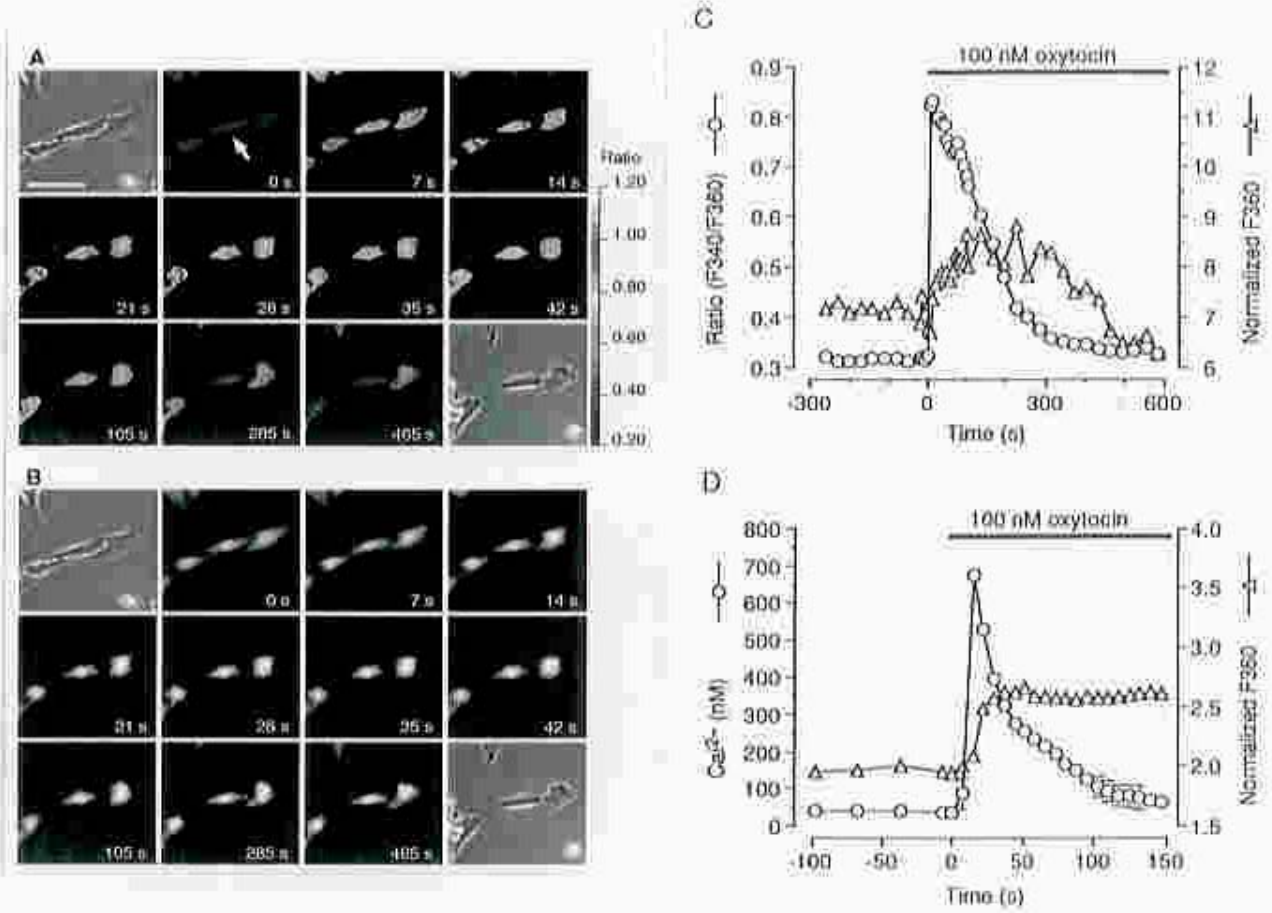

Figure 5. Oxytocin-induced Ca_i^{2+} responses and contraction.

A, B: Fura-2 fluorescence ratio (F_{340}/F_{360}) images (A) and F_{360} images (B) taken at 7, 63 or 180 s intervals. Oxytocin (100 nM) was applied just after the first image (at 0 s) was taken. The upper left and lower right images are phase-contrast images before and after the application of oxytocin, respectively. Scale bar is 50 μm. C: Changes in fura-2 fluorescence ratio (circles) and normalized F_{360} (triangles) in a cell (indicated by arrow in A). D: Ca_i^{2+} calibration was performed in the separate experiment. The values of Ca_i^{2+} concentration (circles) and normalized F_{360} (triangles) are averages of 4 cells. Error bars indicate S.E.M.

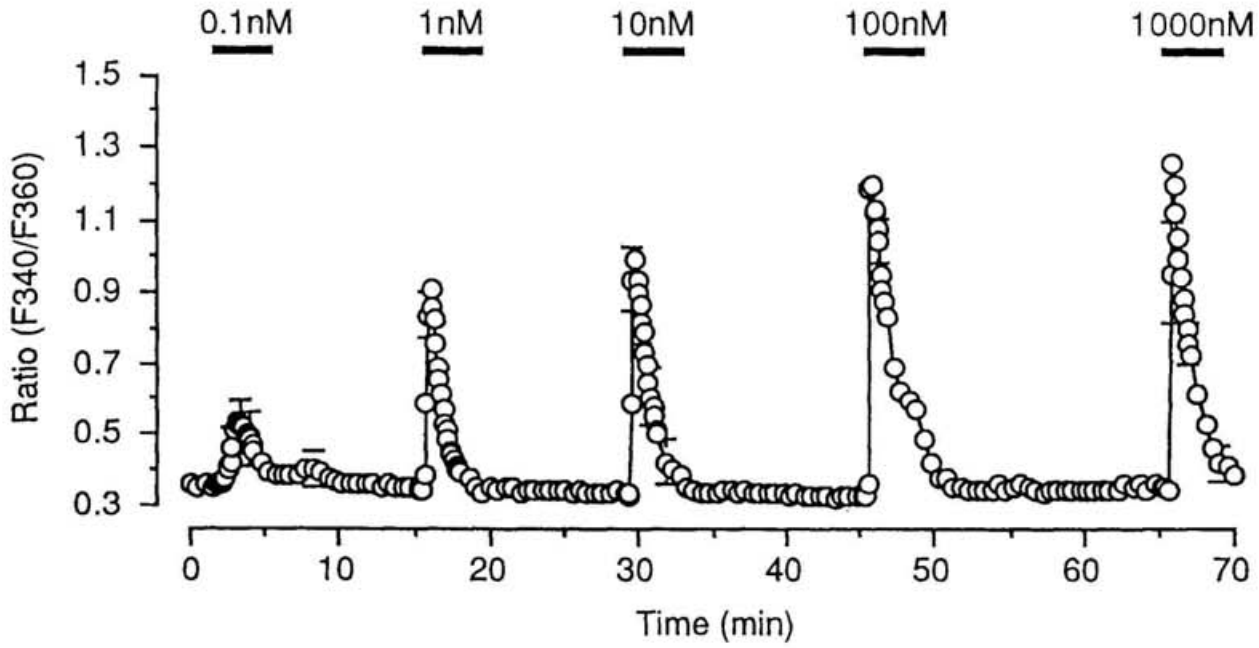

Figure 6. Ca_i^{2+} responses to various concentrations (0.1-1000 nM) of oxytocin. Average F_{340}/F_{360} ratios of 5 cells are shown.

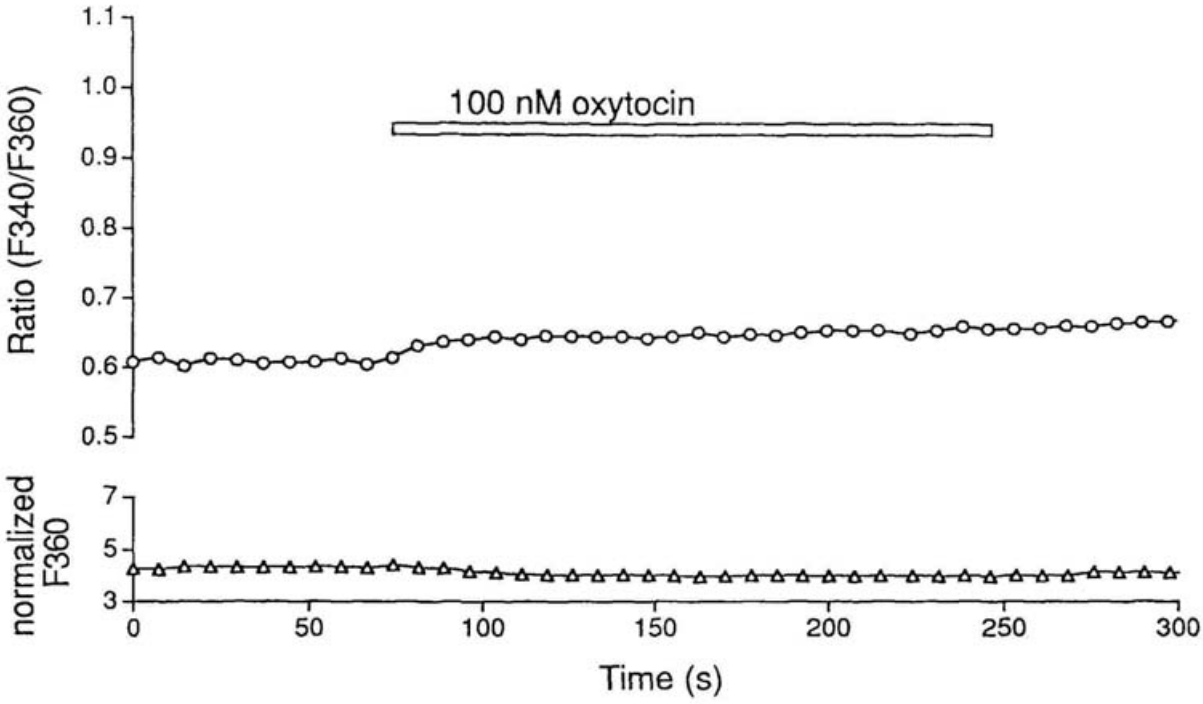

Figure 7. The effects of BAPTA-AM on the Ca_i^{2+} response and contraction induced by oxytocin.

Oxytocin (100 nM) was applied to the cells loaded with BAPTA-AM (100 μM) for 10 min at 37°C. The values of F_{340}/F_{360} ratio (circles) and normalized F_{360} (triangles) are averages of 4 cells. The same result was obtained from 2 separate experiments.

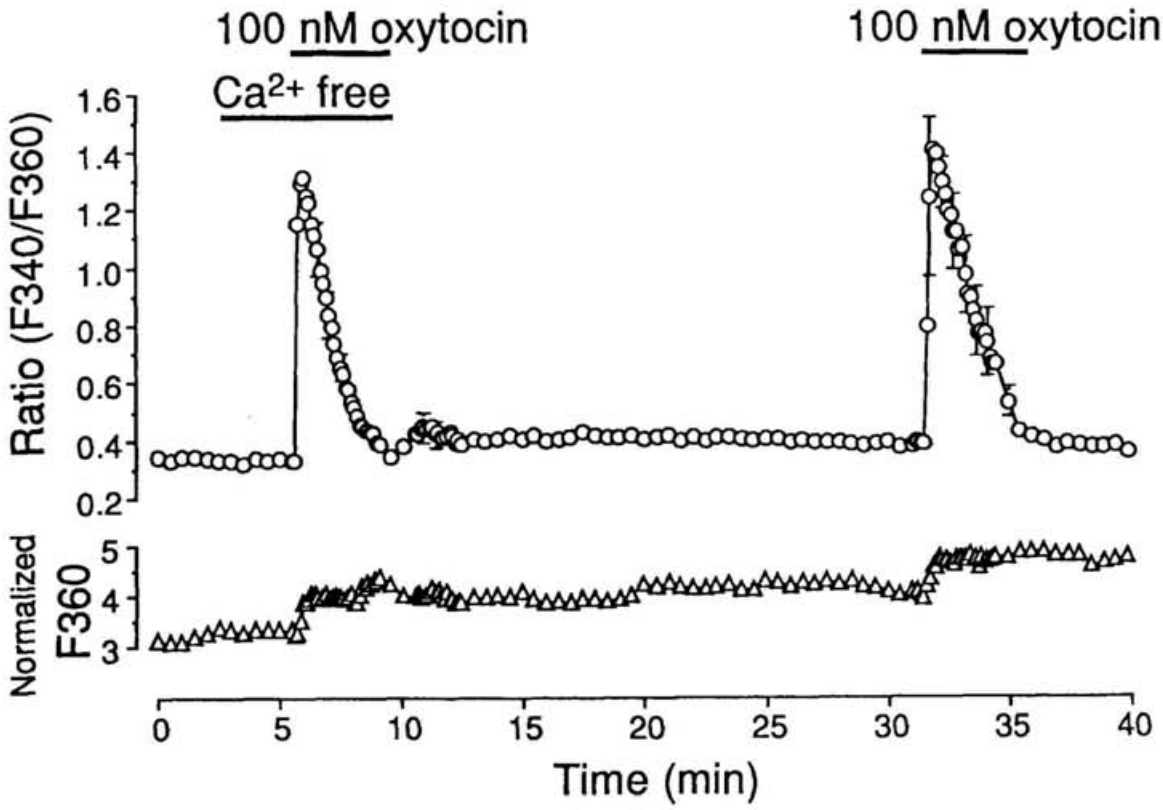

Figure 8. The effects of Ca^{2+} removal on the Ca_i^{2+} response and contraction induced by oxytocin.

Oxytocin (100 nM) was applied 3 min after perfusion of the Ca^{2+}-free solution (0.5 mM EGTA). The second application of oxytocin was carried out in normal Ringer's solution (Ca^{2+} 1.8 mM). The values of F_{340}/F_{360} ratio (circles) and normalized F_{360} (triangles) are averages of 4 cells. The same result was obtained from 5 separate experiments.

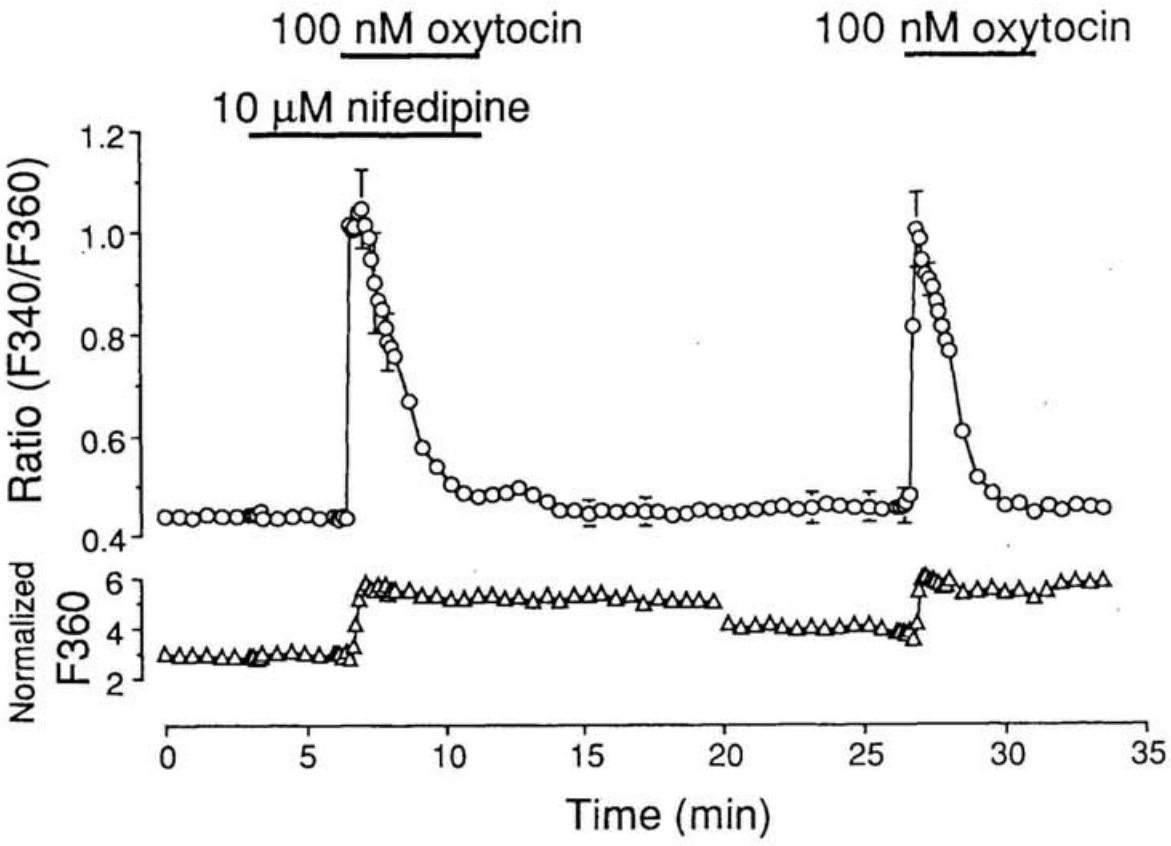

Figure 9. The effects of nifedipine on the Ca_i^{2+} response and contraction induced by oxytocin.

Oxytocin (100 nM) was applied in the presence or absence of nifedipine (10 μM). The values of F_{340}/F_{360} ratio (circles) and normalized F_{360} (triangles) are averages of 3 cells. The same result was obtained from 4 separate experiments.

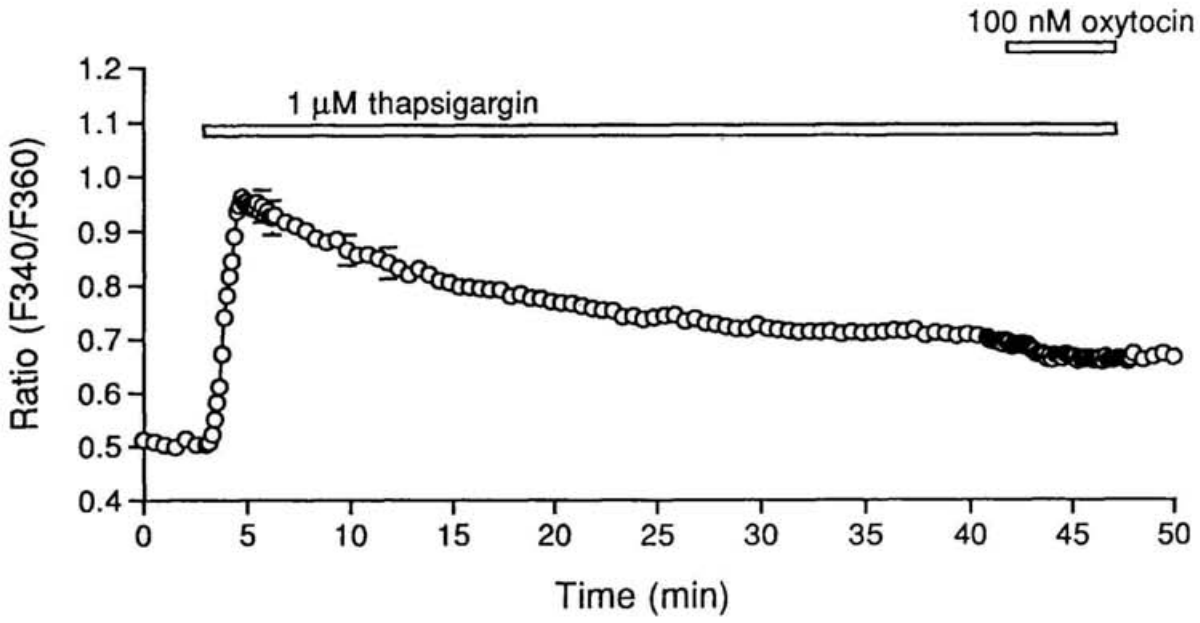

Figure 10. The effects of thapsigargin on the Ca_i^{2+} responses induced by oxytocin. Cells were exposed to thapsigargin (1 μM) in normal Ringer's solution prior to the subsequent application of oxytocin (100 nM). The values of F_{340}/F_{360} ratio (circles) are averages of 4 cells. The same result was obtained from 3 separate experiments.

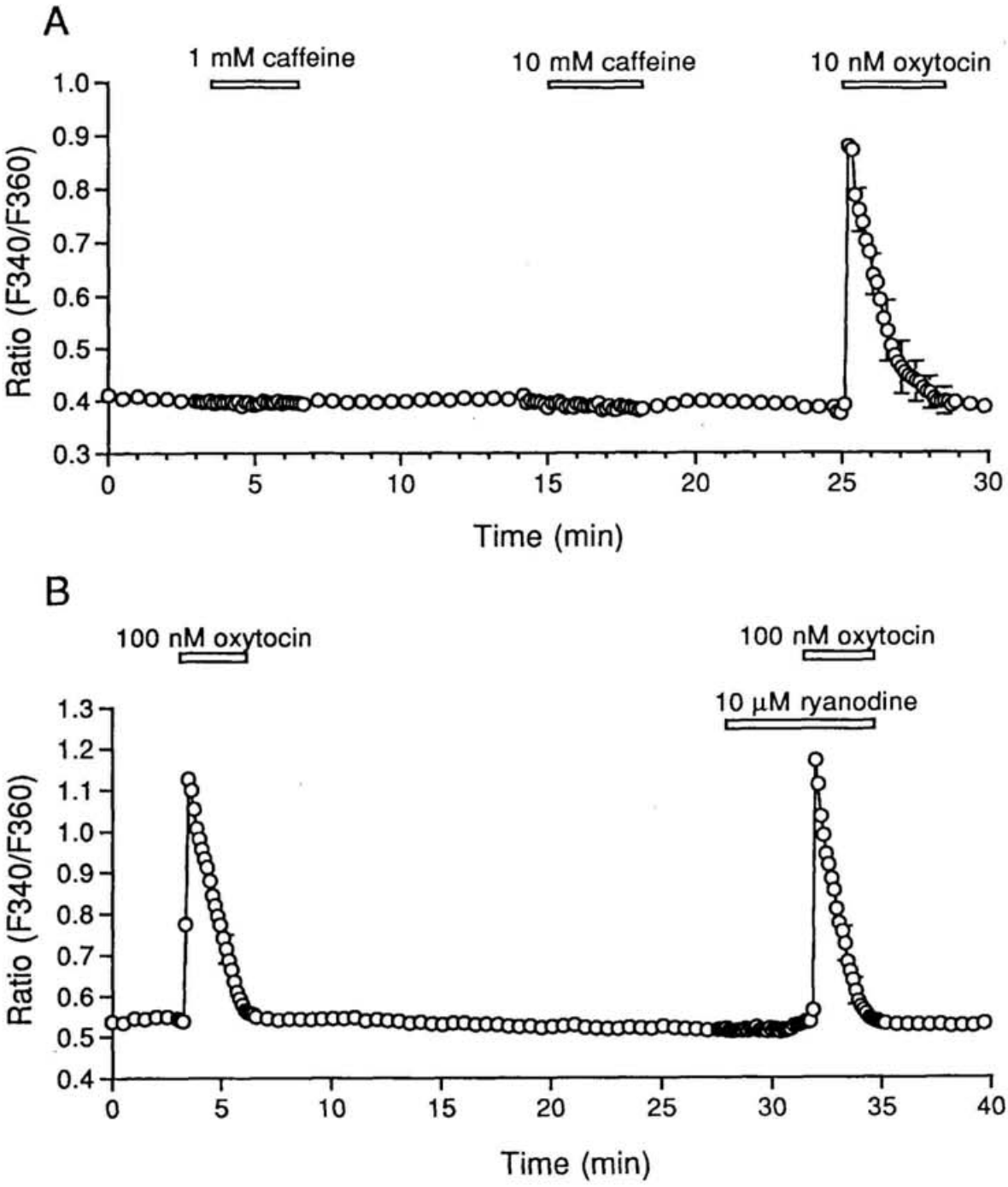

Figure 11. The effects of caffeine and ryanodine on Ca_i^{2+}.

A: Caffeine (1 or 10 mM) was applied to cells. B: Oxytocin (100 nM) was applied in the presence or absence of ryanodine (10 μM). The values of F_{340}/F_{360} ratio (circles) are averages of 5 cells (A) or 7 cells (B).

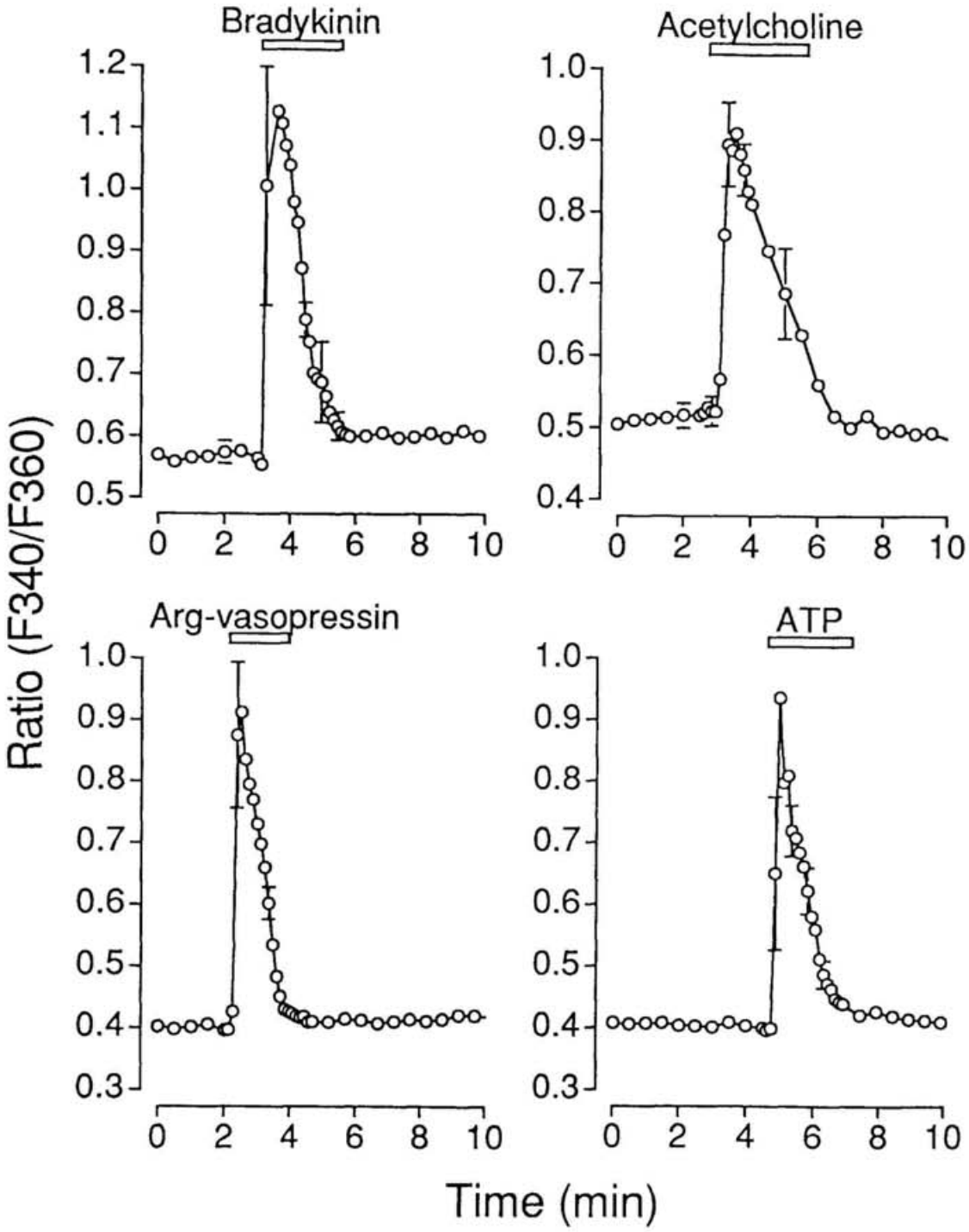

Figure 12. Ca_i^{2+} responses induced by bradykinin, acetylcholine, arg-vasopressin or ATP.

Bradykinin (200 nM), acetylcholine (100 μM), arg-vasopressin (100 nM) or ATP (10 μM) was applied to cells. The values of F_{340}/F_{360} ratio (circles) are averages of 3-4 cells.

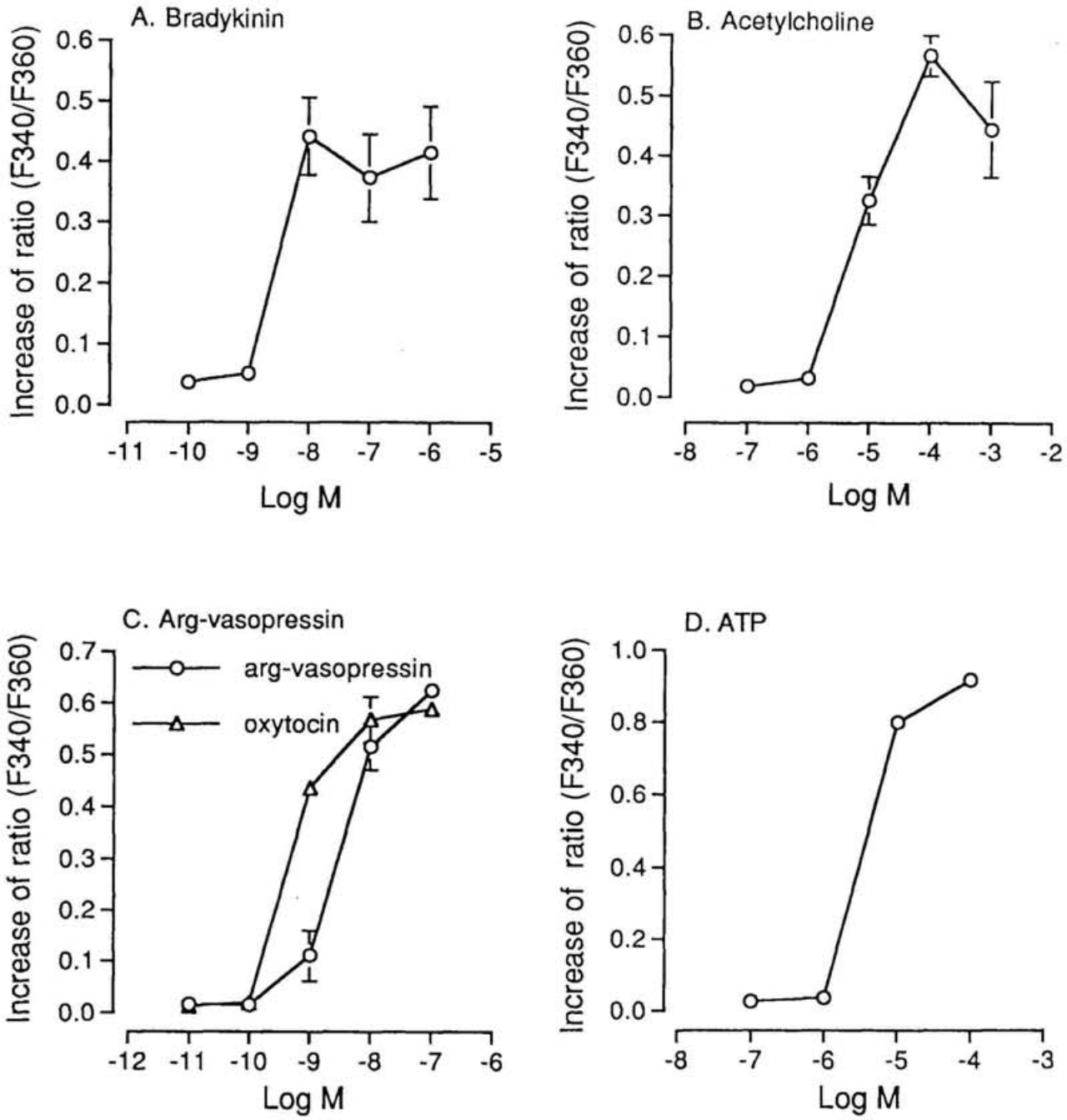

Figure 13. Dose-response curves for bradykinin, acetylcholine and arg-vasopressin and ATP.

The ordinate represents the peak increase in F_{340}/F_{360} ratio upon application of the stimulants (A: bradykinin, B: acetylcholine, C: arg-vasopressin, D: ATP). Each point is the average of 3-18 cells from 2 or 3 separate experiments. For comparison dose-response curve for oxytocin was set in C.

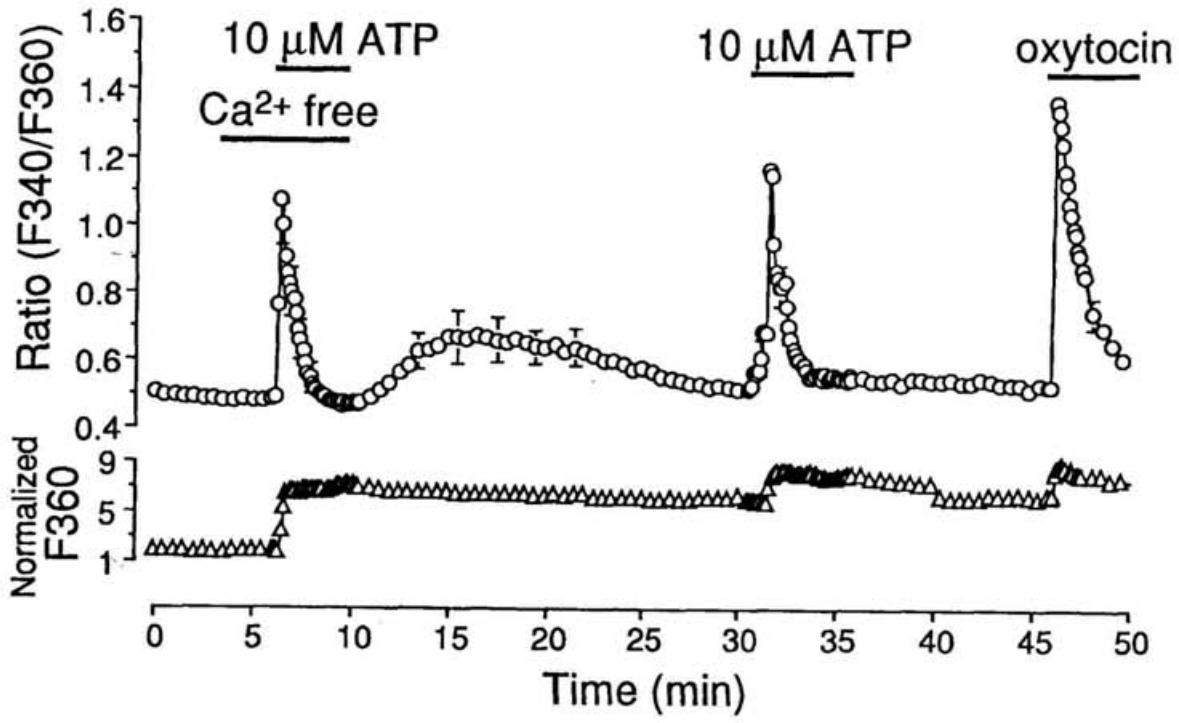

Figure 14. The effects of Ca^{2+} removal on the Ca_i^{2+} response and contraction induced by ATP.

ATP (10 μM) was applied 3 min after perfusion of the Ca^{2+}-free solution (0.5 mM EGTA). The second application of ATP was carried out in normal Ringer's solution (Ca^{2+} 1.8 mM). Oxytocin (100 nM) was applied at the end of the experiment. The values of F_{340}/F_{360} ratio (circles) and normalized F_{360} (triangles) are averages of 4 cells. The same result was obtained from 5 separate experiments.

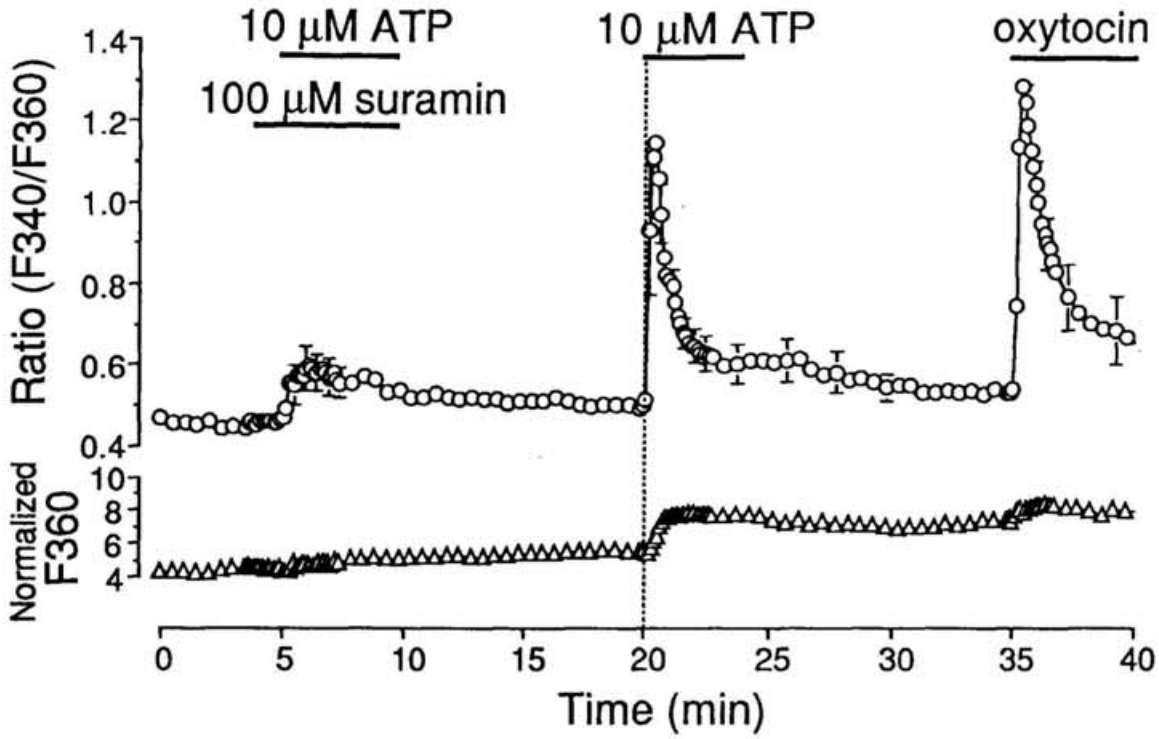

Figure 15. Effects of suramin on the response to ATP.

ATP (10 μM) was applied in the presence or absence of suramin (100 μM). Oxytocin (100 nM) was applied at the end of the experiment. The values of F_{340}/F_{360} ratio (circles) and normalized F_{360} (triangles) are averages of 4 cells. The same result was obtained from 5 separate experiments.

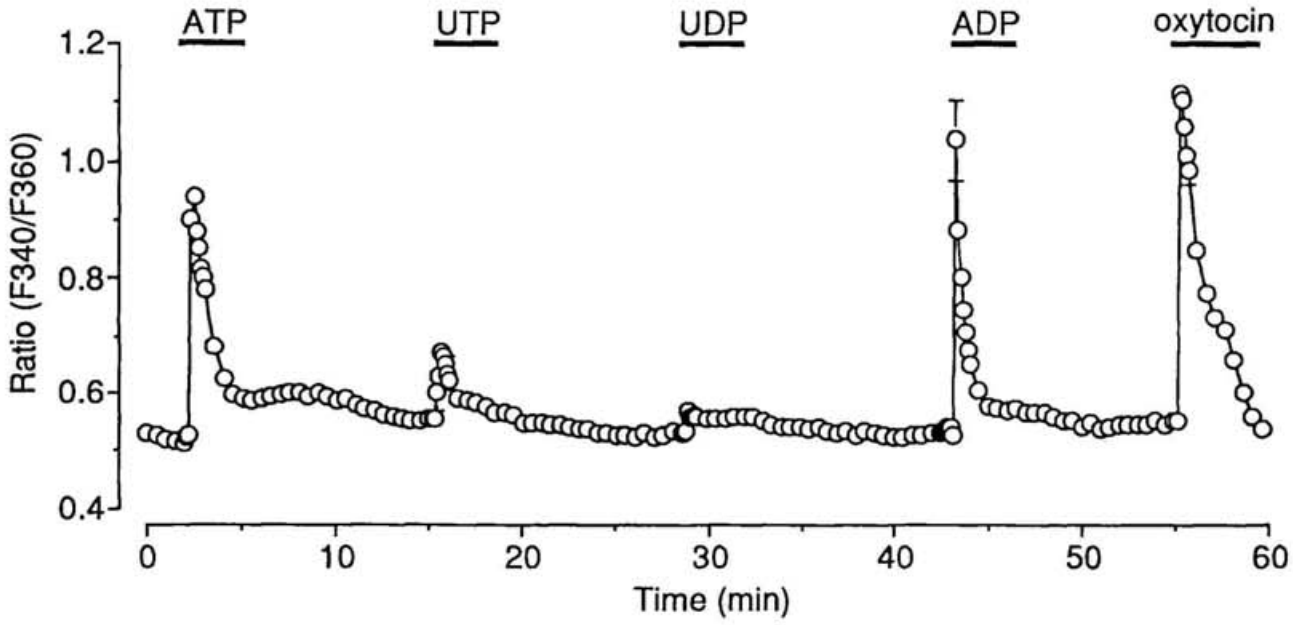

Figure 16. Differences in Ca_i^{2+} responses to ATP, UTP, ADP and UDP.

Of the cells that responded to oxytocin (100 nM), about 80% responded to ATP (10 μM) and ADP (10 μM). The response to UTP (10 μM) was less than that to ATP. Only a few responded to UDP (10 μM). The F_{340}/F_{360} ratio data are averages of 11 cells.

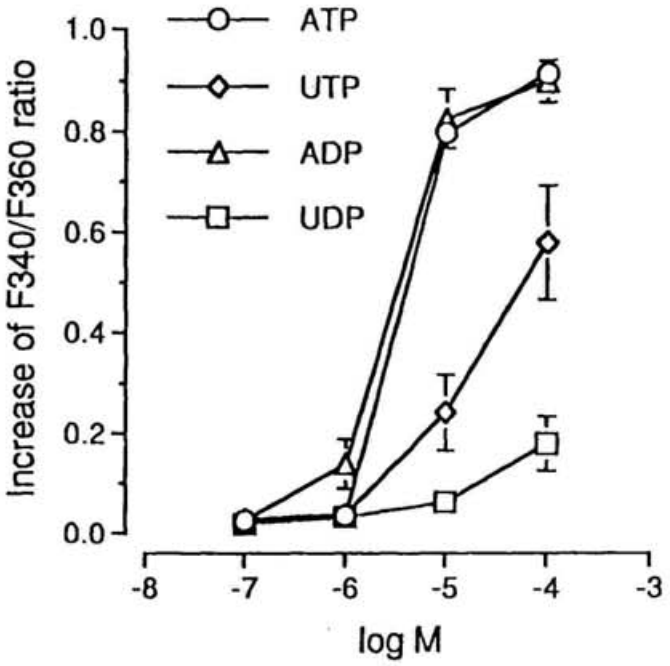

Figure 17. Dose-response curves for ATP, UTP, ADP and UDP.

The ordinate represents the peak increase in F_{340}/F_{360} ratio upon application of the stimulants. Each point is the average of 10-18 cells from 3 separate experiments.

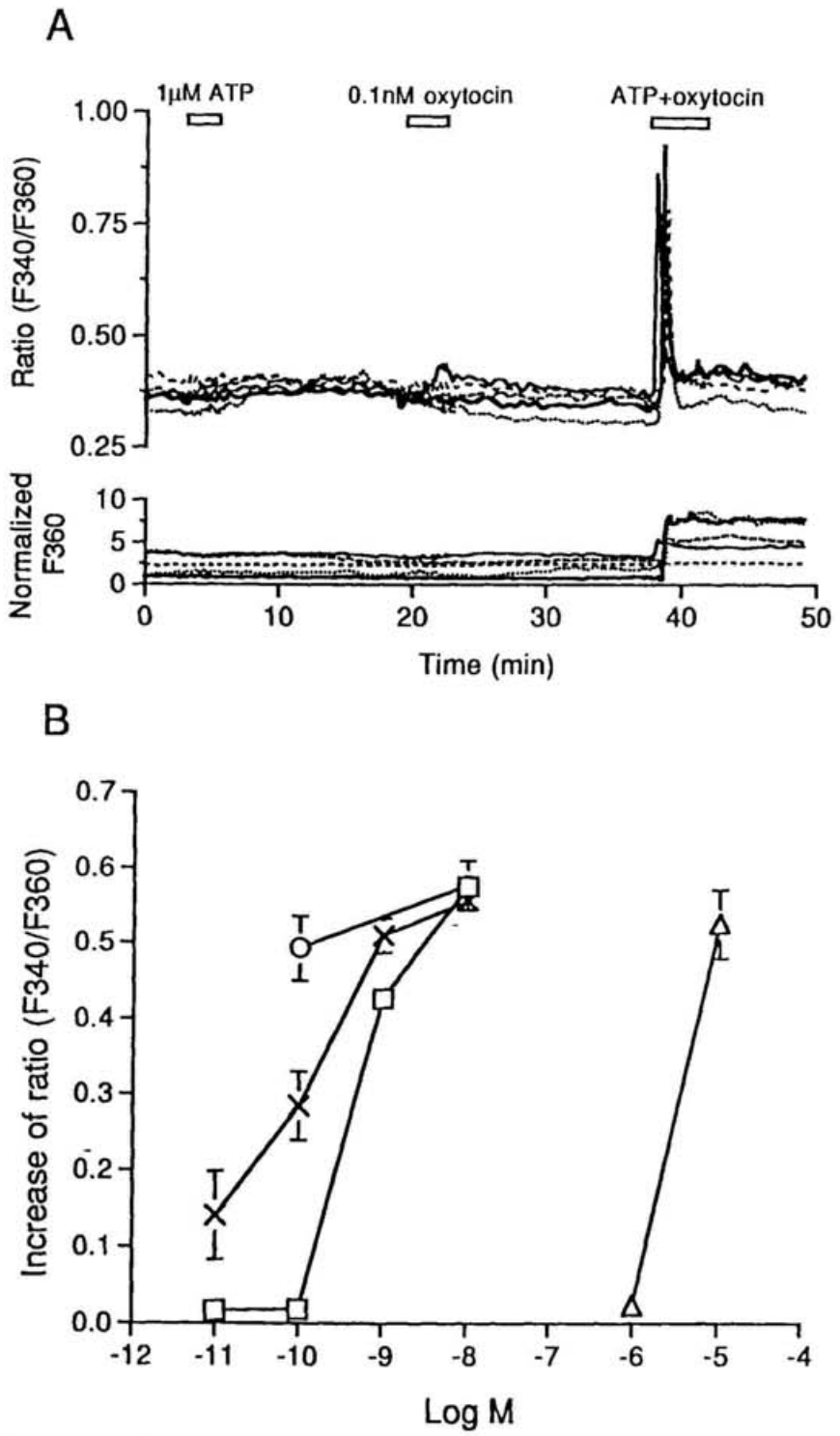

Figure 18. Effects of ATP on oxytocin-induced Ca_i^{2+} responses.

A: Synergistic effects of ATP (1 μM) and oxytocin (0.1 nM) on Ca_i^{2+}. The responses in 5 cells were drawn overlapped. B: The ordinate represents the peak increase in F_{340}/F_{360} ratio upon application of the stimulants. Oxytocin (square), ATP (triangle), oxytocin plus 1 μM ATP (cross) or oxytocin plus 10 μM ATP (circle) was applied. Each point is the average of 3-7 cells.

Table 1. Effects of extracellular Ca^{2+} removal on the peak Ca^{2+} responses induced by stimulants.

Stimulants	Control	Ca^{2+}-free (0.5 mM EGTA)	
oxytocin	0.95±0.03	0.89±0.03*	(n=26)
arg-vasopressin	0.57±0.02	0.56±0.01	(n=15)
acetylecholine	0.48±0.02	0.46±0.03	(n=10)
ATP	0.74±0.03	0.70±0.04	(n=25)
bradykinin	0.55±0.03	0.55±0.05	(n=10)

Oxytocin (100 nM), arg-vasopressin (100 nM), acetylecholine (100 μM), ATP (10 μM) or bradykinin (200 nM) was applied to cells. The values are peak increase of F_{340}/F_{360} ratio (average ± SE) induced by the stimulants. n is number of cells tested. *P < 0.05 compared with control group by Student's paired or unpaired t-test.

Table 2. Effects of Ca^{2+} removal and nifedipine (10 μM) on the half decay time from Ca_i^{2+} responses ($t_{1/2}$).

Stimulants	n	Control	Ca^{2+}-free	nifedipine	†oxytocin
oxytocin	12	1.48±0.20	1.03±0.12*		
oxytocin	24	1.18±0.13		1.18±0.17	
ATP	8	0.58±0.12	0.54±0.12		1.15±0.10*

Oxytocin and ATP were applied at 100 nM and 10 μM, respectively. Data represent the half decay time (min) from the peak value of F_{340}/F_{360} ratio induced by the stimulants. †For comparison, oxytocin was applied in normal Ringer's solution at the end of the experiment. The values are average ± S.E.M. n is number of cells tested. *P < 0.05 compared with control group by Student's paired t-test.

Table 3. Synergistic effects of ATP and oxytocin on the Ca_i^{2+} responses.

	ATP	oxytocin	ATP+oxytocin
Ratio (F_{340}/F_{360})	0.05 ± 0.01^{ac}	0.14 ± 0.04^{bc}	0.32 ± 0.03^{ab}
No. of cells	(n=26)	(n=19)	(n=26)

ATP (1 μM), oxytocin (0.1 nM) or ATP plus oxytocin was applied to cells. Data represent the peak value of F_{340}/F_{360} ratio induced by the stimulants. The values are average ± S.E.M. n is number of cells tested. The statistical significance was tested by ANOVA and Turkey-Kramer multiple comparisons test (a, b: $P < 0.001$. c: $P > 0.05$).