Hypotonic stimulation induced Ca^{2+} release from IP_3-sensitive internal stores in a Green monkey kidney cell line

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1. Hypotonic stimulation (180 ± 5 mosmol l^{-1}) increased $[Ca^{2+}]_i$ in fura-2-loaded Green monkey kidney cells (COS-7 cells) and depolarized the membrane.

2. COS-7 cells were depolarized up to $-3{\cdot}5 \pm 4{\cdot}4$ mV from a resting membrane potential of $-35{\cdot}2 \pm 2{\cdot}3$ mV in response to hypotonic stimulation, when the patch electrode was filled with a 160 mM KCl–0·5 mM EGTA-based intracellular medium.

3. The increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation was divided into two phases. One was transient and oscillatory, and observed in Ca^{2+}-free medium; the other was persistent, blocked by 100 μM La^{3+}, and observed only in Ca^{2+}-containing medium.

4. The increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium was blocked by pretreatment with 10 μM thapsigargin. The increase in $[Ca^{2+}]_i$ induced by 10 μM thapsigargin was reduced after hypotonic stimulation which induced an increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium.

5. The increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium was not affected by treatment with 5 mM caffeine or 1–10 μM ryanodine. Neither caffeine nor ryanodine induced an increase in $[Ca^{2+}]_i$.

6. Adenosine 5′-*O*-2-thiodiphosphate (ADP-β-S; a P_{2Y} receptor agonist) induced an increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium and caused phosphoinositide breakdown in COS-7 cells. Exposure to 10 μM ADP-β-S blocked the increase in $[Ca^{2+}]_i$ induced in the Ca^{2+}-free medium by hypotonic stimulation. The results of summary points 4, 5, and 6 suggest that the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation is due to Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive internal stores.

7. The hypotonic stimulation-activated hydrolysis of phosphoinositides was decreased by pertussis toxin (PTX) in a dose-dependent manner.

8. These observations strongly suggest that hypotonic stimulation induced an increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium through activation of cascades using PTX-sensitive guanine nucleotide binding protein (G protein) and IP_3.

A number of cell species have stretch-activated ion channels which are gated by expansion or distortion of the cell membrane (Sachs, 1987). In hair cells and endothelial cells, their functions have been studied in detail (Ohmori, 1985; Lansman, Hallam & Rink, 1987). In several species of cells, Ca^{2+} influx through stretch-activated channels is observed under negative pressure in the patch pipette or when the cell is bathed in a hypotonic solution. It has been shown that an increase in $[Ca^{2+}]_i$ which is induced by cell swelling may be involved in the regulatory volume decrease (RVD) process in certain cells, such as the cultured human epithelial cell line Intestine 407 (Hazama & Okada, 1988) and renal proximal tubule cells (Suzuki *et al.* 1990).

Recent studies using Ca^{2+} indicators demonstrated that Ca^{2+} was released from intracellular Ca^{2+} stores under hypotonic stimulation in cells derived from a proximal tubule of rabbit kidney (McCarty & O'Neil, 1991), human umbilical vein (Oike, Droogmans & Nilius, 1994), rat epididymal cells (Leung & Wong, 1993), rabbit gastric gland (Negulescu, Munck & Machen, 1992), and human colon cancer (Nitschke, Leipziger & Greger, 1993). It was shown that Ca^{2+} mobilization was related to pertussis toxin (PTX)-sensitive G protein (Suzuki *et al.* 1990) or arachidonic acid (Oike *et al.* 1994). Hypotonic stimulation activated PTX-sensitive G protein and phospholipase A_2 in human platelets (Margalit, Livne, Funder & Granot, 1993).

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Physical stretch also induced inositol 1,4,5-trisphosphate (IP_3) and tetrakisphosphate accumulation through PTX-sensitive G protein in cardiomyocytes (Dassouli, Sulpice, Roux & Crozatier, 1993). There are, however, further mechanisms which remain to be elucidated.

ATP receptors known as P_2 purinoceptors are entirely distinct from the adenosine receptors (or P_1 purinoceptors). The P_2 purinoceptor category has been further divided into P_{2X} and P_{2Y} receptors (Barnard, Burnstock & Webb, 1994). P_{2Y} receptors show slower responses to agonist through second messenger systems. It has been shown that ADP-β-S stimulates phosphoinositide breakdown and increases intracellular Ca^{2+} concentration in several tissues, acting as a P_{2Y} receptor agonist (Boyer, Downes & Harden, 1989; Keppens, Vandekerckhove & Wulf, 1993).

In the present study, we found that $[Ca^{2+}]_i$ was elevated under hypotonic stimulation in the COS-7 cell line, a cell line which is derived from Green monkey kidney and is widely used for expression cloning of a variety of genes in molecular biology. We also found that COS-7 cells had endogenous P_{2Y} receptors. P_{2Y} receptors are coupled with G protein, phospholipase C and IP_3, and bearing this in mind we investigated the mechanism of the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation. We concluded that the increase in $[Ca^{2+}]_i$ is divided into Ca^{2+} influx from an extracellular medium and Ca^{2+} release from intracellular Ca^{2+} stores. The latter Ca^{2+}-release mechanism was investigated in detail and was found to be a consequence of activation of PTX-sensitive G protein and phosphoinositide breakdown.

METHODS

Cells and solutions

COS-7 cells derived from Green monkey kidney were purchased from RIKEN (Wako, Japan) and were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Flow Labs Inc., USA) (330 ± 5 mosmol l^{-1}) on plastic dishes. Uncloned Intestine 407 cells derived from human embryonic intestine were purchased from Flow Labs Inc. and cultured in Fischer's medium (Gibco) with 10% newborn calf serum (Flow Labs Inc.). Experiments for patch clamp recording and fluorescence measurements were started in isotonic media and the tonicity of the solution was changed in the course of experiments. Compositions of solutions used in the experiments were as follows (mM). Normal medium: NaCl, 155; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 17; buffered to pH 7·4 by 10 mM K-Hepes. Ca^{2+}-free isotonic medium: NaCl, 155; $MgCl_2$, 3·5; glucose, 17; EGTA, 1; buffered to pH 7·4 by 10 mM K-Hepes. 50% NaCl medium: NaCl, 77·5; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 17; buffered to pH 7·4 by 10 mM K-Hepes. Isotonic 50% NaCl–mannitol medium: NaCl, 77·5; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 17; mannitol, 155; buffered to pH 7·4 by 10 mM K-Hepes. Ca^{2+}-free X% NaCl medium: NaCl concentration was reduced to X% of the normal medium while concentrations of other chemicals were maintained. Experiments for phosphoinositide hydrolysis were performed in isotonic Li medium (containing (mM): LiCl, 10; NaCl, 145; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 17; buffered to pH 7·4 by 10 mM K-Hepes) or in X% NaCl–Li medium. In the X% NaCl–Li medium, the concentration of NaCl was reduced by an amount corresponding to (100 − X)% of the normal medium while maintaining other concentrations; for example 50% NaCl–Li medium contained (mM): NaCl, 67·5; LiCl, 10; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 17; buffered to pH 7·4 by 10 mM K-Hepes. Depending on the osmolarity required, NaCl concentration was varied between 100 and 50%. The osmolarity of these cultures and experimental media was measured by an osmometer (Advanced Instruments Inc., Model 3L, USA) and values were as follows: Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 330 ± 5 mosmol l^{-1}; normal medium, 330 ± 5 mosmol l^{-1}; 50% NaCl medium, 180 ± 5 mosmol l^{-1}.

Patch clamp recording

COS-7 cells were seeded on coverglasses in 35 mm dishes at a density of 3×10^4 cells per dish and cultured for 3–5 days at 37 °C in an atmosphere of 95% O_2–5% CO_2. Conventional patch pipette recording techniques were used in these experiments (Hamill, Marty, Neher, Sakman & Sigworth, 1981). Pipettes were fabricated from a low-melting-temperature soda glass, coated with Sylgard 184 (Dow Corning, Japan) up to about 50–100 μm from the tip and were heat polished before experiments. They were backfilled with a KCl–EGTA-based intracellular medium containing 160 mM KCl–0·5 mM EGTA and buffered to pH 7·4 by 10 mM K-Hepes. Patch electrode resistances were between 5 and 10 MΩ. Series resistances were calculated as 11 ± 5·2 MΩ (mean ± S.D., $n = 5$ cells) from the capacitative current transients generated in response to voltage pulses of 5 or 10 mV under the whole-cell recording condition. The efficiency of cell dialysis through the patch pipette was evaluated from the rate of fluorescence intensity increase when 200 μM fura-2 pentapotassium salt was introduced into the cell through the patch pipette. Fluorescence intensity attained the 90% of saturated level within at least 5 min of achievement of the whole-cell recording configuration (10 cells).

The recording chamber was placed on the stage of an inverted microscope (Olympus, IMT-2, Tokyo, Japan) with Nomarski differential interference contrast optics. The head stage of the patch amplifier (EPC-17, List Electronics) was fixed to a three-dimensional Huxley-type micromanipulator (Shoshin-EM, Okazaki, Japan), which was fixed on a side stage firmly attached to the inverted microscope.

The bathing solution in the recording chamber was circulated continuously (3–5 ml min^{-1}) by a peristaltic pump (P-3, Pharmacia, Sweden). The volume of the recording chamber was approximately 500 μl and 99% of the medium was exchanged in 50 s. This was confirmed by measuring fluorescence intensity changes from various points within the chamber. After placing 5 μM fura-2 pentapotassium salt dissolved in normal medium within the chamber, we measured the time course of fluorescence disappearance by circulating the fura-2-free normal medium. When 50% NaCl hypotonic medium was perfused extracellularly, the expected change of liquid junction potential relative to the normal medium was −3·7 mV. The reference electrode of Ag wire coated with AgCl was immersed in the agarose gel prepared in the normal medium, and the measured liquid junction potential was −3 mV. Most experiments were carried out at room temperature (22–25 °C). In several cases, we examined the effect of physiological temperature on the hypotonicity-induced membrane depolarization.

Loading cells with fura-2 and fluorescence measurement

COS-7 cells and Intestine 407 cells were seeded on coverglasses as described above. The acetoxymethyl ester form of the Ca^{2+}-sensitive fluorescence dye fura-2 (fura-2 AM, Molecular Probes Inc.) was used to load the cell. Fura-2 AM was dissolved in DMSO (1 mM). Cells were incubated with 2 μM final concentration of fura-2 AM in normal bathing medium for approximately 30 min at room temperature (Fig. 1*A*). Fluorescence intensity was measured through an image intensifier-coupled video microscope system (AVEC/VIM system, Hamamatsu Photonics, Hamamatsu, Japan). Detailed methods of the fura-2 fluorescence imaging and the on-line monitoring of $[Ca^{2+}]_i$ changes have been described previously (Ohmori, 1988; Ashmore & Ohmori, 1990; Shigemoto & Ohmori, 1990). Briefly, by drawing windows on fluorescence images of cells displayed on a video screen, and accumulating fluorescences from these window regions by alternating the excitation wavelength between 340 and 380 nm, the ratio of fluorescences excited at 340 nm (F_{340}) and at 380 nm (F_{380}) was calculated every 2 s. The size of the recording window was 10 μm^2 and covered only the central region of the COS-7 cell. The cell swelling induced by hypotonic medium was reflected in the gradual decrease of fluorescence intensities at both F_{340} and F_{380}. Figure 1*B* demonstrates an approximately 25% decrease of fluorescence intensities. These changes in fluorescences common to F_{340} and F_{380} were cancelled by taking ratios (Fig. 1*B*). The hypotonicity (50% NaCl medium)-induced expansion of cell volume did not affect the cell's viability as long as the stimulus was limited to less than 10 min, since more than 99% of cells were not stained by Trypan Blue (0·4% in phosphate-buffered saline: Chroma-Gesellschaft). The Trypan Blue exclusion test was performed on more than 200 cells, and results were counted in triplicate.

The $[Ca^{2+}]_i$ was estimated from the ratio (R) of F_{340}/F_{380}. $[Ca^{2+}]_i$ was calibrated using solutions of standard EGTA-buffered Ca^{2+} medium in the experimental chamber. The estimate of $[Ca^{2+}]_i$ was given by the following relationship, using a bilinear hyperbolic fit between the R value and $[Ca^{2+}]$ of a series of calibration media,

$$[Ca^{2+}]_i = \beta K_d(R - 0{\cdot}65)/(5{\cdot}85 - R),$$

where K_d is the dissociation constant of fura-2–Ca binding and β is the fluorescence ratio of fura-2 to fura-2–Ca at 380 nm. For the best fit, βK_d was 1·50 μM. β was 12·0 implying that K_d was 125 nM (Grynkiewicz, Poenie & Tsien, 1985).

Measurements of inositol phosphate formation

Inositol phosphate formation was measured as described previously (Yokohama, Tanaka, Ito, Negishi, Hayashi & Hayaishi, 1988; Nakajima, Tsuchida, Negishi, Ito & Nakanishi, 1992). COS-7 cells were seeded in six-well plates at a density of 1×10^5 cells per well and cultured for 2 days. Cells were labelled with [^{3}H]inositol (1 μCi ml^{-1}) for 22–25 h in inositol-free Dulbecco's modified Eagle's medium with 10% dialysed fetal bovine serum. Cells were washed once with phosphate-buffered saline (PBS) and incubated with the same solution for 20 min and then with PBS containing 10 mM LiCl for 20 min at 37 °C. The reaction was started by replacing the PBS with 1 ml of the isotonic Li medium, the isotonic Li medium containing ADP-β-S, or the hypotonic Li medium and was stopped by replacing the medium with 10% (w/v) trichloroacetic acid solution after a given time. Extraction and separation of ^{3}H-labelled inositol phosphates were carried out as described previously (Nakajima *et al.* 1992). The radioactivity in elutes was determined by a liquid scintillation spectrometer. All experiments were carried out in triplicate.

Statistical method

Differences between means were statistically evaluated using Student's unpaired t test. Pearson's correlation coefficient was adopted for correlation analysis between increases of total inositol phosphates and increases in $[Ca^{2+}]_i$ induced by the hypotonic medium or by ADP-β-S. Differences and correlations were considered statistically significant at $P < 0{\cdot}05$. Results are given as means ± S.D. unless otherwise stated with the number of cells (n) given in parentheses.

RESULTS

Hypotonic stimulation induces an increase in $[Ca^{2+}]_i$

When the hypotonic medium (180 ± 5 mosmol l^{-1}) was applied to COS-7 cells, fura-2 fluorescence was modified (Fig. 1*B*). The hypotonic medium was made by reducing sodium chloride levels in the normal medium by 50% (50% NaCl medium). The fluorescence excited at 340 nm wavelength (F_{340}) was increased after a transient decrease, and the fluorescence excited at 380 nm wavelength (F_{380}) was decreased in two phases after the introduction of hypotonic medium. The first slow phase corresponded to the gradual decrease of F_{340} and could be due to the cell swelling induced by hypotonic stimulation. Later changes in F_{340} and F_{380} fluorescence were symmetrical and were probably reflecting changes in $[Ca^{2+}]_i$. The fluorescence ratio (F_{340}/F_{380}) was calculated at each time interval. The grand average of the resting fluorescence ratio and the estimated $[Ca^{2+}]_i$ were 1·08 ± 0·08 and 310 ± 50 nM, respectively ($n = 10$). The hypotonic stimulation increased the fluorescence ratio to 2·61 ± 0·40 ($n = 10$) in 100 s and this corresponds to 950 ± 80 nM $[Ca^{2+}]_i$. The timing of hypotonic stimulation is indicated below the traces (Fig. 1*B*). Since fura-2 fluorescence reflects the Ca^{2+} concentration with relatively high specificity (Grynkiewicz *et al.* 1985), we hereafter describe these ratio changes as changes in $[Ca^{2+}]_i$.

Hypotonic stimulation depolarizes COS-7 cells

When the hypotonic solution was applied to COS-7 cells (at 22–25 °C), the membrane potential was depolarized immediately from the resting level of −35·2 ± 2·3 mV ($n = 8$) to a steady-state value of −3·5 ± 4·4 mV under current clamp conditions (Fig. 1*C*). After hypotonic stimulation, the membrane potential slowly repolarized. Similar results were obtained when the experiment was performed at 35–37 °C. As a result of the hypotonic stimulation, the membrane was depolarized to 0·2 ± 2·3 mV from a resting potential of −32·4 ± 6·7 mV ($n = 6$). When the isotonic 50% NaCl–mannitol medium (for compositions see Methods) was applied, the change in membrane potential was less than 3 mV. When the Cl^- concentration of the patch pipette medium was reduced

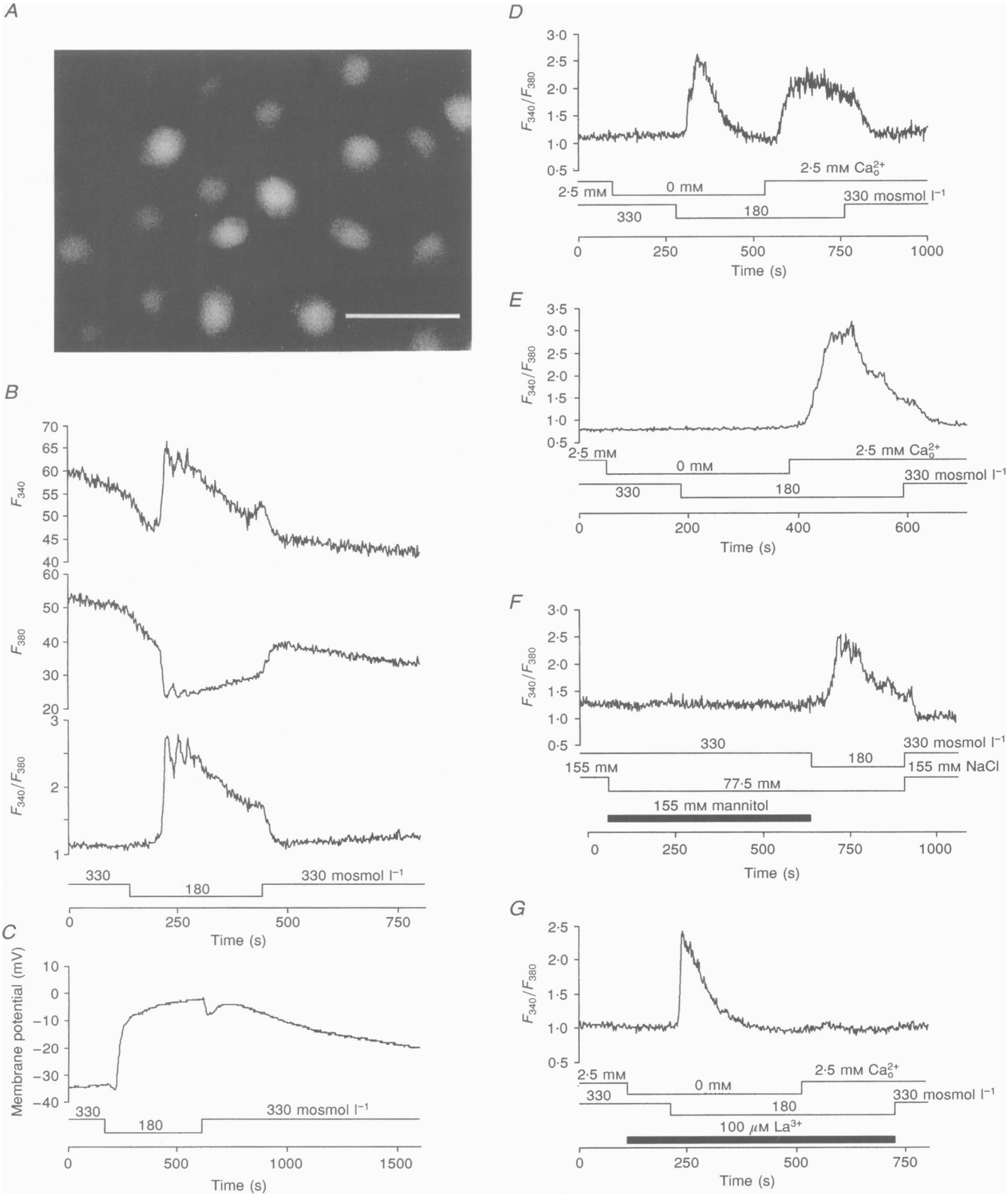

Figure 1. For legend see facing page.

from 160 to 20 mM by using a reduced Cl^- intracellular medium (composition (mM): potassium gluconate, 130; CsCl, 20; EGTA, 0·5; buffered to pH 7·4 by 10 mM K-Hepes), the level of depolarization was reduced to a membrane potential of $-18 \pm 1{\cdot}1$ mV ($n = 6$). The Cl^- equilibrium potentials were +16 mV in a KCl–EGTA-based intracellular medium and −36 mV in a reduced Cl^- intracellular medium when 50% NaCl medium was applied. Replacement of intracellular KCl with CsCl did not change the level of hypotonicity-induced depolarization ($+4{\cdot}5 \pm 4{\cdot}8$ mV, $n = 6$). These results indicate that the change in membrane potential is at least partly due to an increase in Cl^- conductance.

Hypotonic stimulation induced an increase in $[Ca^{2+}]_i$ in Ca^{2+}-free extracellular medium

The increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in COS-7 cells had two phases. The first phase was the increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium (Ca^{2+}-free 50% NaCl medium) and the second phase was the increase in $[Ca^{2+}]_i$ in Ca^{2+}-containing medium (50% NaCl medium) (Fig. 1*D*). The former was transient but oscillatory, lasted about 150 s, and was observed in twenty-eight out of thirty cells. When isotonicity was maintained by replacing NaCl with mannitol (isotonic 50% NaCl–mannitol medium), the reduction of NaCl to 50% (77·5 mM) in the extracellular medium did not induce $[Ca^{2+}]_i$ change in any out of fifteen cells (Fig. 1*F*). It is concluded that the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation is due not to the decrease in $[NaCl]_o$ but to the reduced osmolarity. The induction of $[Ca^{2+}]_i$ increase by hypotonicity in Ca^{2+}-free medium is not a ubiquitous phenomenon, since Intestine 407 cells did not show an increase in $[Ca^{2+}]_i$ with hypotonic stimulation in Ca^{2+}-free medium, as described elsewhere (Hazama & Okada, 1988) (Fig. 1*E*).

In COS-7 cells, the increase in $[Ca^{2+}]_i$ observed in the second phase in Ca^{2+}-containing medium (50% NaCl medium) was persistent and was blocked by 100 μM La^{3+} (Fig. 1*G*) and 1 mM Gd^{3+}. La^{3+} and Gd^{3+} are well known blockers for stretch channels (Yang & Sachs, 1989) and for Ca^{2+} channels (Nachshen, 1984). La^{3+} and Gd^{3+}, however, did not affect the rise of $[Ca^{2+}]_i$ in the first phase in the Ca^{2+}-free medium (Fig. 1*G*). These results indicate that the increase in $[Ca^{2+}]_i$ in the second phase in the Ca^{2+}-containing medium is probably due to the influx of extracellular Ca^{2+}.

The increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium was due to release from intracellular Ca^{2+} stores

Thapsigargin is a blocker of Ca-ATPase in endoplasmic reticulum and depletes Ca^{2+} from the intracellular Ca^{2+} stores (Thastrup, Cullen, Drøbak, Hanley & Dawson, 1990). The increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium (Ca^{2+}-free 50% NaCl medium) was blocked after 10 μM thapsigargin was applied (Fig. 2*A*). Reciprocally, when the increase in $[Ca^{2+}]_i$ was induced by hypotonic stimulation, subsequent thapsigargin-induced Ca^{2+} release was reduced to $58 \pm 5\%$ ($n = 10$ cells) (Fig. 2*B*) of the control when Ca^{2+} release was first induced by thapsigargin (Fig. 2*A*). These results show that the increase in $[Ca^{2+}]_i$ in the Ca^{2+}-free medium is due to Ca^{2+} release from intracellular stores that are sensitive to thapsigargin.

The increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium was unlikely to be due to Ca^{2+}-induced Ca^{2+} release

We examined the effects of 5 mM caffeine and 1 μM ryanodine on the increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium. Caffeine is known to release Ca^{2+} through the ryanodine receptors from intracellular Ca^{2+} stores (Endo, 1975). Ryanodine is known to block the ryanodine receptors at doses over about 1 μM or to keep the ryanodine receptors in open states at doses below about 1 μM (Meissner, 1986). When 5 mM caffeine was applied, hypotonic stimulation still induced an increase in $[Ca^{2+}]_i$ in eighteen out of twenty cells in Ca^{2+}-free medium and in all twenty cells in the Ca^{2+}-containing medium (Fig. 3*A*). When 1 μM ryanodine was applied, hypotonic stimulation increased $[Ca^{2+}]_i$ in twenty-seven out of thirty cells in Ca^{2+}-free medium and in all thirty cells in Ca^{2+}-containing medium (Fig. 3*B*). We

Figure 1. Fluorescence changes generated by hypotonic stimulation

A, fura-2 fluorescence image of COS-7 cells. Scale bar, 50 μm. *B*, upper two traces, fluorescence intensity changes were measured by exciting the cell at wavelengths of 340 nm (F_{340}) and 380 nm (F_{380}); the ordinate is in arbitrary units; lower trace, the ratio intensity F_{340}/F_{380} at each time interval was calculated from measurements in the upper two traces. Note that hypotonic stimulation with 50% NaCl medium induced the increase in $[Ca^{2+}]_i$ in about 100 s. *C*, hypotonic stimulation depolarized the membrane potential under current clamp conditions. *D*, fluorescence changes induced by hypotonic stimulation in the absence and presence of extracellular Ca^{2+} (2·5 mM). *E*, fluorescence changes in Intestine 407 cell. Note that hypotonic stimulation did not induce the Ca^{2+} response in the absence of extracellular Ca^{2+}. All the other panels are records from COS-7 cells. *F*, substitution of 77·5 mM NaCl for 155 mM mannitol did not induce the increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (2·5 mM). Subsequent hypotonic stimulation induced the increase in $[Ca^{2+}]_i$. *G*, the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-containing medium was blocked by 100 μM La^{3+}. However, the increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium was not affected. In this and subsequent figures, the timing for changes of ion concentration or osmolarity is indicated below each ratio measurement.

could not block the Ca^{2+} release with 10 μM ryanodine in any of ten cells measured. We also measured the onsets, peak ratios, and durations of the Ca^{2+} increase as indicated in Fig. 3*C* in order to examine the effect of caffeine or ryanodine on the hypotonicity-induced Ca^{2+} increase. The onsets were 50 ± 21, 55 ± 20 and 55 ± 36 s (n = 30, 20 and 27 cells) for the control, in the presence of 1 μM ryanodine, and in the presence of 5 mM caffeine, respectively. The peak ratios were 3·29 ± 0·50 and 3·26 ± 0·40, and the durations were 52 ± 21 s and 57 ± 20 s in the absence and presence of 1 μM ryanodine, respectively. We could not find any statistical significance in these differences. Therefore, 5 mM caffeine or 1–10 μM ryanodine has no gross effects on the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation. The hypotonic stimulation-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium appears to be unrelated to the Ca^{2+}-induced Ca^{2+} release mechanism.

The increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium was due to Ca^{2+} release from IP_3-sensitive internal stores

In COS-7 cells, $[Ca^{2+}]_i$ increased in response to 1 μM ATP but not to 100 μM adenosine, which indicates that COS-7 cells have P_2 purinoceptors. We found that ADP-β-S induced an increase in $[Ca^{2+}]_i$ in these cells. It has been shown that ADP-β-S is an agonist specific to P_{2Y} receptors that is involved in Ca^{2+} release from internal stores coupled with G protein and IP_3 (Keppens *et al.* 1993; Castro, Tomé, Miras-Portugal & Rosário, 1994). Immediately after Ca^{2+} was released by 10 μM ADP-β-S, hypotonic stimulation did not increase $[Ca^{2+}]_i$ in the Ca^{2+}-free 50% NaCl medium in any of ten cells investigated (Fig. 4*A*). When a hypotonic stimulation-induced increase in $[Ca^{2+}]_i$ preceded application of ADP-β-S, stimulation by ADP-β-S failed to induce Ca^{2+} release in all ten cells investigated (Fig. 4*B*). Moreover, ryanodine at 1 μM has no gross effect on IP_3-induced Ca^{2+} release triggered by 10 μM ADP-β-S. We have measured, as in Fig. 3*C*, the onsets, peak ratios, and durations of the Ca^{2+} increase induced by 10 μM ADP-β-S and compared the results in control conditions and in the presence of 1 μM ryanodine. The onset values were 14·8 ± 9·4 and 11·3 ± 2·0 s, the peak ratios were 4·12 ± 0·74 and 4·56 ± 1·02, and the duration values were 21·3 ± 7·8 and 21·9 ± 6·7 s, in the control and in the presence of 1 μM ryanodine, respectively (n = 29 and 20 cells). There were no statistically significant differences between them. Taken together,

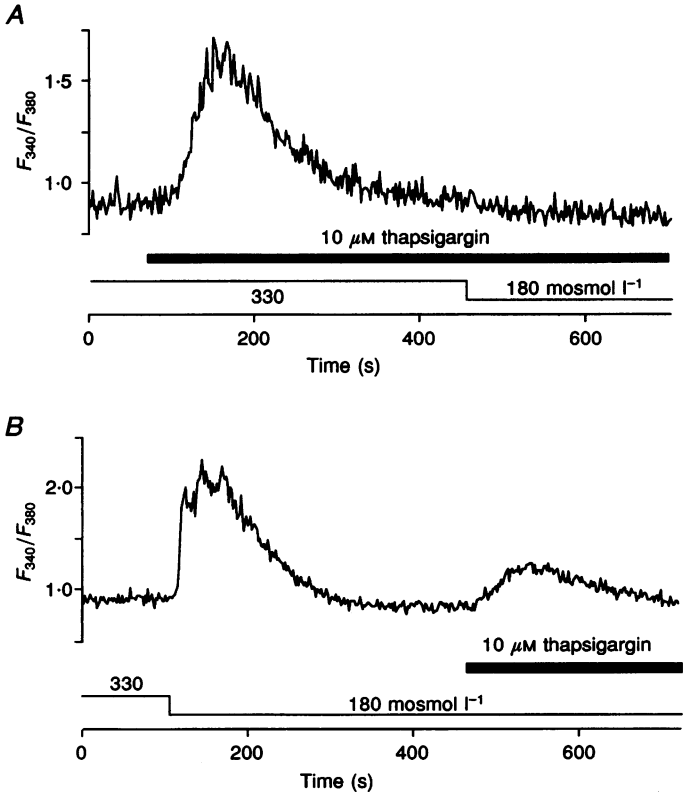

Figure 2. Effects of thapsigargin on the rise in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium

A, thapsigargin (10 μM) induced Ca^{2+} release and blocked the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium. *B*, the Ca^{2+} release induced by thapsigargin (10 μM) was reduced by hypotonic stimulation which induced Ca^{2+} release in the Ca^{2+}-free medium.

it is conceivable that the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium is due to Ca^{2+} release from IP_3-sensitive internal stores.

Effects of hypotonic stimulation on phosphoinositide metabolism

We have investigated the possibility that the Ca^{2+} release induced by hypotonic stimulation is coupled with phosphoinositide metabolism and have examined whether hypotonic stimulation activated hydrolysis of phosphoinositides. For the evaluation of phosphoinositide metabolism, [^{3}H]inositol-labelled COS-7 cells were treated with X% NaCl–Li medium as described in Methods. Figure 5*A* shows time courses of [^{3}H]inositol phosphate ([^{3}H]IP) levels following hypotonic stimulation (50% NaCl–Li medium). [^{3}H]IP_3 formation slightly increased at 3 and 10 min. However, [^{3}H]IP_3 formation decreased to the control level at 20 and 30 min. An almost linear increase in [^{3}H]inositol bisphos-

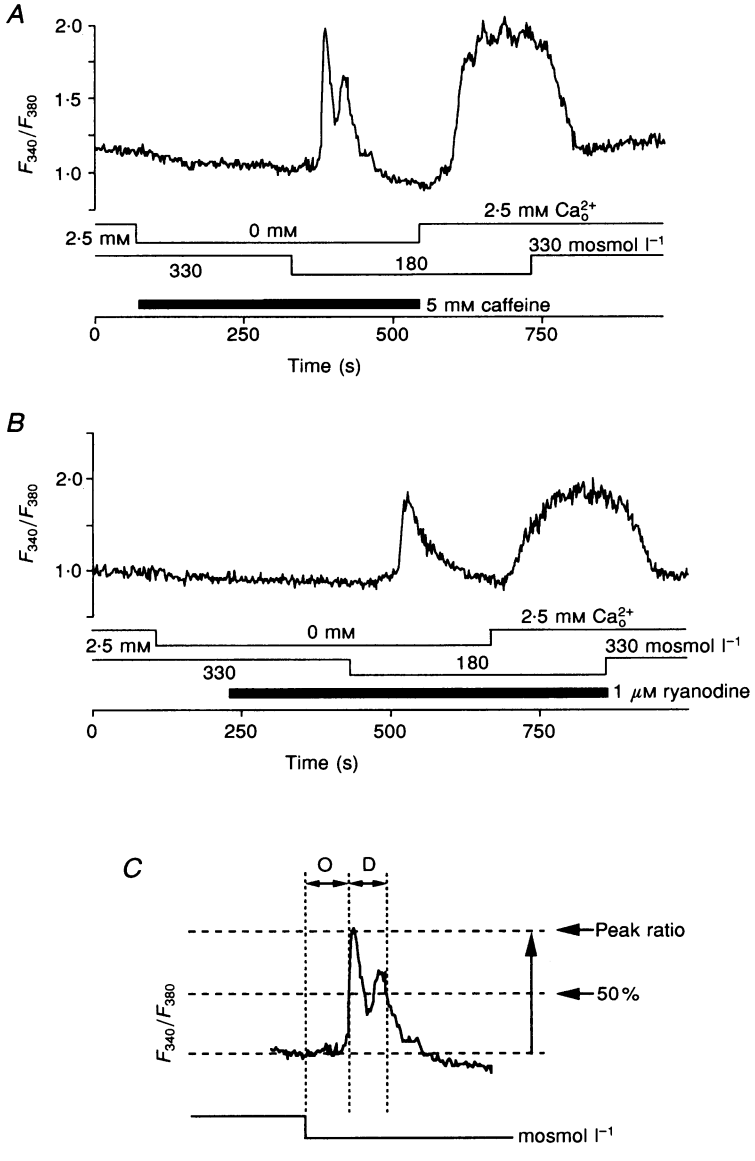

Figure 3. Effects of caffeine and ryanodine on the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium

The increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation was not affected by 5 mM caffeine (*A*) or 1 μM ryanodine (*B*). *C*, peak ratio, onset, and duration were evaluated as indicated. Onset (O) is defined as time from the application of the hypotonic medium to the half-rising level of $[Ca^{2+}]_i$. Duration (D) is defined as time from the half-rising level to the half-falling level of $[Ca^{2+}]_i$. When $[Ca^{2+}]_i$ demonstrated oscillation as in the case illustrated, the total duration of time until the $[Ca^{2+}]_i$ signal finally crossed the 50% level was measured as the duration of responses.

phate (IP_2) formation was observed, reaching a maximum value of 220% of the initial IP_2 level at 30 min. [^{3}H]inositol monophosphate (IP_1) formation was also observed but in lesser amounts than [^{3}H]IP_2. [^{3}H]IP_1 formation increased continuously after 10 min. Figure 5*B* shows the effect of various concentrations of NaCl in the *X*% NaCl–Li medium on total [^{3}H]IP levels measured at 10 min. Formation of total [^{3}H]IP was dose dependent over extracellular NaCl concentrations from 100 to 50% (Fig. 5*B*). When the 50% NaCl medium was adjusted to isotonicity by the addition of 155 mM mannitol, total IP accumulation stayed at the control level (open circle at 50% NaCl in Fig. 5*B*). This result excluded the possibility that total IP accumulation induced by hypotonic stimulation was simply due to a decrease in $[NaCl]_o$. Total [^{3}H]IP formation was similar in the absence and presence of 100 μM La^{3+} (data not shown). La^{3+} blocked Ca^{2+} influx induced by hypotonic stimulation from an extracellular medium (Fig. 1*G*). This result shows that the increase in total [^{3}H]IP is not due to Ca^{2+} influx.

As phosphoinositide metabolism has been shown to be followed by Ca^{2+} mobilization from the endoplasmic reticulum via IP_3 (Berridge & Irvine, 1984), we measured $[Ca^{2+}]_i$ and calculated the percentage of cells showing an increase in $[Ca^{2+}]_i$ at each concentration of NaCl in the bathing medium (Fig. 5*C*). Since the increase in $[Ca^{2+}]_i$ occurred in an all-or-none manner in individual cells, we used the percentage of cells showing an increase in $[Ca^{2+}]_i$ for our analysis rather than the exact value of the increase in $[Ca^{2+}]_i$. Probabilities were calculated from observations of more than twenty-nine cells for each measurement by counting the number of cells which demonstrated fluorescence ratio increases to a level higher than 1·5 within 300 s. When $[NaCl]_o$ was decreased to less than 70% of the [NaCl] of normal medium, both total IP accumulation (Fig. 5*B*) and the percentage of cells showing an increase in $[Ca^{2+}]_i$ (Fig. 5*C*) were elevated above the control level.

To investigate the relationship between total IP accumulation and the percentage of COS-7 cells showing an increase in $[Ca^{2+}]_i$, P_{2Y} receptors in COS-7 cells were activated by ADP-β-S. Figure 5*D* shows the effect of various concentrations of ADP-β-S on total [^{3}H]IP levels after a 10 min exposure to ADP-β-S. Figure 5*E* shows the

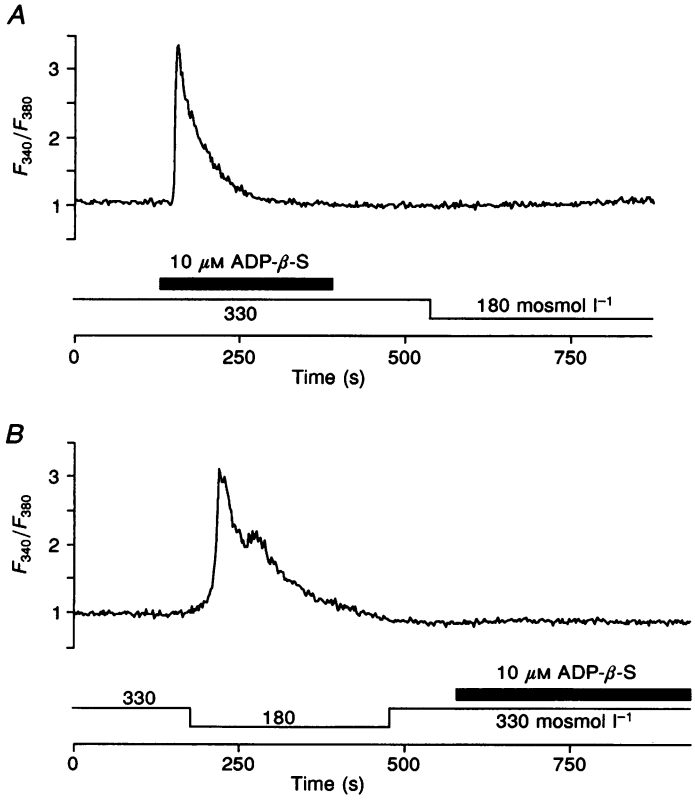

Figure 4. Effects of ADP-β-S on the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium

A, ADP-β-S (10 μM) induced Ca^{2+} release from IP_3-sensitive internal stores and blocked the following increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium. *B*, the Ca^{2+} release induced by ADP-β-S (10 μM) was suppressed by the preceding increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium.

percentage of cells showing an increase in $[Ca^{2+}]_i$. When at least 1 μM ADP-β-S was applied to COS-7 cells, it was observed that both total IP and the percentage of cells showing an increase in $[Ca^{2+}]_i$ were elevated above the control level (Fig. 5*D* and *E*) as was the case for hypotonic stimulation (Fig. 5*B* and *C*). The increase in total IP and the increase in $[Ca^{2+}]_i$ are correlated in both cases. The correlation coefficient for Fig. 5*B* and *C* was 0·93 and for Fig. 5*D* and *E* it was 0·91. Correlations were significant for both cases at $P < 0{\cdot}05$. Stimulation by 1 μM ADP-β-S increased the total [^{3}H]IP accumulation 1·2-fold, and increased the $[Ca^{2+}]_i$. This level of total IP accumulation was also induced by hypotonic stimulation. Taken together, it is likely that hypotonic stimulation increases $[Ca^{2+}]_i$ via IP_3.

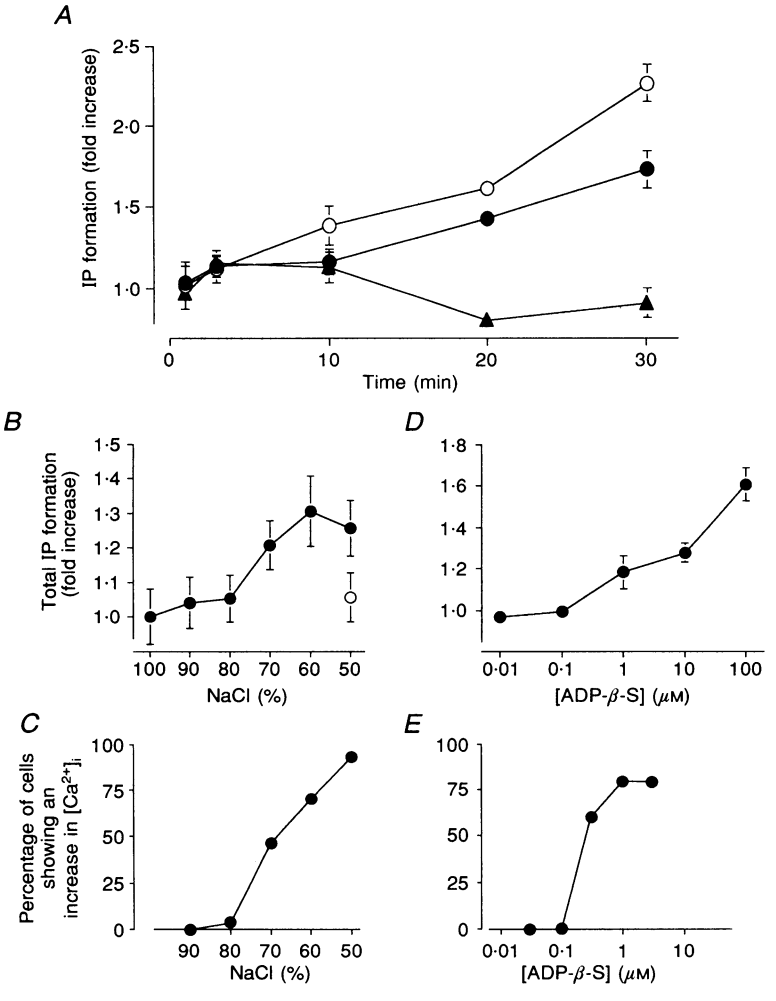

Figure 5. [^{3}H]Inositol phosphate accumulation and the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation and ADP-β-S

A, time courses of [^{3}H]inositol phosphate ([^{3}H]IP) accumulation induced by hypotonic stimulation (50% NaCl–Li medium). COS-7 cells were exposed to 50% NaCl–Li medium for the time indicated. The values (mean ± S.D., $n = 6$ measurements) of IP_1 (●), IP_2 (○), and IP_3 (▲) are expressed as multiples of the control at each exposure time. *B*, an osmolarity–response curve for total [^{3}H]IP. Total [^{3}H]IP formation in COS-7 cells after 10 min exposure to various concentrations of NaCl was measured. Each point represents the mean ± S.D. ($n = 6$ measurements). *C*, osmolarity–response curve for percentages of cells which showed an increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium. Percentages of cells showing an increase in $[Ca^{2+}]_i$ were determined from a population of more than 29 cells exposed to various concentrations of NaCl in the bathing medium. *D*, a dose–response curve of ADP-β-S for the increase in total [^{3}H]IP. Total [^{3}H]IP formation in COS-7 cells was measured after 10 min exposure to various concentrations of ADP-β-S. Each point represents the mean ± S.D. ($n = 3$ measurements). *E*, a dose–response curve for ADP-β-S for percentages of cells which showed the increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium. Percentages of cells showing an increase in $[Ca^{2+}]_i$ induced by stimulation with various concentrations of ADP-β-S were determined from a population of more than 30 cells.

Pertussis toxin (PTX) has been shown to suppress the increase in IP_3 accumulation which is triggered by the activation of G protein-coupled receptors through the ribosylation of G proteins in certain cells (Fu, Okano & Nozawa, 1988). As shown in Fig. 6*A*, PTX pretreatment for 24 h caused inhibition of total IP accumulation in a dose-dependent manner. IP accumulation was induced by hypotonic stimulation for 10 min (50% NaCl–Li medium) in a dose-dependent manner. Pretreatment with 100 ng ml^{-1} PTX markedly inhibited the total IP accumulation. Pretreatment for 24 h with 100 ng ml^{-1} PTX also suppressed the increase in $[Ca^{2+}]_i$ which was induced by the Ca^{2+}-free 60% NaCl medium (Fig. 6*B*). PTX pretreatment did not suppress the increase in $[Ca^{2+}]_i$ induced by Ca^{2+}-free 50% NaCl medium. This is probably because PTX pretreatment blocks G protein coupling to phospholipase C activity only partially. The partial block of G protein coupling to phospholipase C by PTX was also observed in Chinese hamster ovary cells (Aramori & Nakanishi, 1992) and in NIH3T3 cells (Fu *et al.* 1988). Although there remains a possibility that treatment with PTX simply deteriorated cells in our experiments, it is more likely that hypotonic stimulation increased $[Ca^{2+}]_i$ through IP_3 and PTX-sensitive G proteins.

DISCUSSION

We have studied Ca^{2+} responses generated by hypotonic stimulation in COS-7 cells. During hypotonic stimulation, an increase in $[Ca^{2+}]_i$ was observed (Fig. 1*B*). In these experiments, COS-7 cells were loaded with the membrane-permeable ester form of fura-2, fura-2 AM, and fluorescence intensities were measured by exciting at two wavelengths reciprocally, 340 and 380 nm. Volume changes have been reported to induce artifactual increases in the F_{340}/F_{380} ratio, but the ratio changes reported were mostly due to a decrease of F_{380} with little or no change in F_{340} (Botchkin & Matthews, 1993). In our experiments, the fluorescence excited at the 340 nm wavelength was increased and the fluorescence excited at the 380 nm wavelength was decreased reciprocally when the fluorescence ratio increased. Therefore, it is likely that the changes in fluorescence ratio at F_{340}/F_{380} were not artifactual, due to volume changes, but reflected genuine changes in $[Ca^{2+}]_i$.

The depolarized membrane potential induced by hypotonic stimulation was at least partly due to the increase in Cl^- conductance. This shows the possibility of the existence of a stretch-activated Cl^- channel in COS-7 cells. In several other cells, it has already been reported that there are stretch-activated Cl^- channels (Ackerman, Wickman & Clapham, 1994). There are also contributions of stretch-activated cationic channels to the membrane potential in COS-7 cells, since the membrane potential (−3·5 mV) attained in the 50% NaCl medium deviated from the Cl^- equilibrium potential of +16 mV in a negative direction. If we assume that hypotonic stimulation induces a simultaneous activation of non-selective cationic channels, this conductance would make the depolarization level deviate in a negative direction from the Cl^- equilibrium potential, since the reversal potential for a non-selective cationic channel could be −16 mV in the 50% NaCl medium.

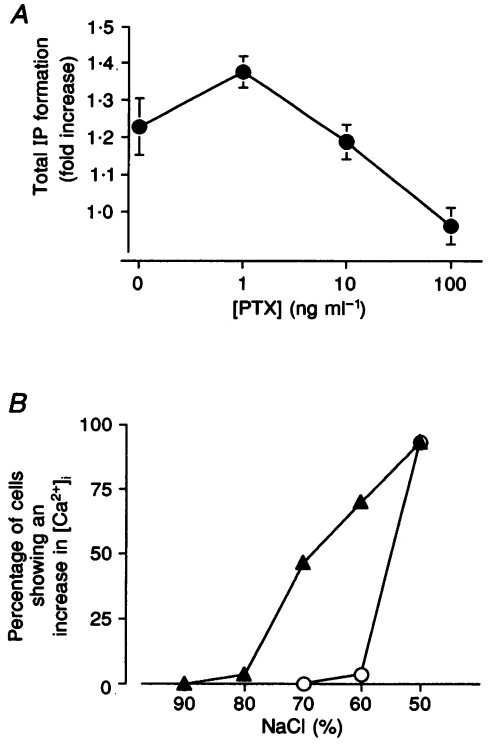

Figure 6. Effects of PTX on IP formation and hypotonicity-induced Ca^{2+} release

A, inhibition of hypotonicity-induced total IP formation by PTX pretreatment for 24 h. Total IP formation induced by 10 min exposure to 50% NaCl–Li medium (180 mosmol l^{-1}) was determined in COS-7 cells after incubation for 24 h with various concentrations of PTX. Each point represents the mean ± S.D. ($n = 3$). *B*, inhibition of hypotonicity-induced Ca^{2+} release by 100 ng ml^{-1} PTX pretreatment for 24 h. Percentages of cells showing an increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation at various concentrations of NaCl were determined for the control population (▲, 30 cells) and for a PTX-pretreated population (○, 30 cells) in Ca^{2+}-free medium.

Since an increase in $[Ca^{2+}]_i$ was not observed in 77·5 mM $[NaCl]_o$ medium when isotonicity was maintained by the addition of 155 mM mannitol (Fig. 1*F*), the $[Ca^{2+}]_i$ increase was not due to a decrease in $[NaCl]_o$ but could be due to the hypotonicity. Therefore, the Na^+–Ca^{2+} exchanger makes little contribution to the change in $[Ca^{2+}]_i$ in COS-7 cells. Similar conclusions were reached in renal proximal tubule cells (McCarty & O'Neil, 1991).

The increase in $[Ca^{2+}]_i$ was divided into two phases (Fig. 1*B* and *D*). Phase one was observed in the Ca^{2+}-free medium; phase two was observed only in the Ca^{2+}-containing medium. The latter was thought to be due to the influx of extracellular Ca^{2+} and was blocked by 100 μM La^{3+} (Fig. 1*G*) or 1 mM Gd^{3+}. Both La^{3+} and Gd^{3+} have been demonstrated to be blockers of stretch-activated ion channels (Yang & Sachs, 1989) and of voltage-gated Ca^{2+} channels (Nachshen, 1984). Hypotonic stimulation induced depolarization up to −3·5 mV under current clamp recording conditions in COS-7 cells (Fig. 1*C*) and 100 μM La^{3+} blocked this depolarization (data not shown). Therefore, the membrane depolarization seems closely associated with the influx phase of Ca^{2+}. However, further studies will be needed to evaluate the influx of extracellular Ca^{2+}, whether through stretch-gated ion channels or voltage-gated Ca^{2+} channels or both.

We focused our experiments on the elucidation of the mechanism of the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation in Ca^{2+}-free medium with 1 mM EGTA. The increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation has already been shown in Ca^{2+}-free medium in several other cells. However, the mechanism of the increase in $[Ca^{2+}]_i$ remains to be elucidated. The transient but oscillatory increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation is probably attributed to Ca^{2+} released from intracellular Ca^{2+} stores because (1) the increase in $[Ca^{2+}]_i$ was induced even in Ca^{2+}-free medium with 1 mM EGTA (Fig. 1) and (2) the depletion of intracellular Ca^{2+} stores by 10 μM thapsigargin blocked the increase in $[Ca^{2+}]_i$ induced by hypotonic stimulation (Fig. 2). We examined the effect of ryanodine or caffeine on Ca^{2+} release from intracellular Ca^{2+} stores (Fig. 3) and excluded the possibility of Ca^{2+} release being due to the Ca^{2+}-induced Ca^{2+} release mechanism. In human endothelial cells, hypotonic stimulation induced a rise in $[Ca^{2+}]_i$ through the activation of phospholipase A_2 and the production of arachidonic acid (Oike *et al.* 1994). Arachidonic acid induced Ca^{2+} release from IP_3-sensitive Ca^{2+} stores. In COS-7 cells, however, arachidonic acid did not increase $[Ca^{2+}]_i$ at all (data not shown).

ADP-β-S is probably a selective agonist for P_{2Y} receptors, which belong to the P_2 purinoceptor group. ADP-β-S increases $[Ca^{2+}]_i$ through G protein, phospholipase C and IP_3 by activating P_{2Y} receptors in erythrocytes and liver cells (Boyer *et al.* 1989; Keppens *et al.* 1993). Actually, in COS-7 cells, we found that ADP-β-S increased $[Ca^{2+}]_i$ (Fig. 4*A* and 5*E*) and total IP accumulation (Fig. 5*D*). We found that at least 1·2-fold total IP accumulation lead to an increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium when the concentration of ADP-β-S was low, for example, 1 μM (Fig. 5*D* and *E*). We concluded that hypotonic stimulation induced enough phosphoinositide accumulation to increase $[Ca^{2+}]_i$ (Fig. 5*A*, *B* and *C*). Contrary to our observations, Suzuki *et al.* (1990) have reported that the level of phosphoinositide accumulation induced by hypotonic stimulation was not sufficient to lead to the increase in $[Ca^{2+}]_i$ in cultured renal proximal tubule cells.

We tried to introduce heparin into COS-7 cells by electroporation or by using the whole-cell patch recording technique in order to ensure that the increase in $[Ca^{2+}]_i$ is via the IP_3 receptor. However, electroporation killed COS-7 cells, and the frequency of the Ca^{2+} increase in the Ca^{2+}-free medium was markedly reduced to 12·5% (3 out of 24 cells; data not shown) in the whole-cell recording condition. Further trials of heparin introduction were, therefore, abandoned.

There might be other explanations for the Ca^{2+} release than the IP_3 receptor mechanism discussed above. For example, the voltage-dependent, IP_3-insensitive Ca^{2+} channels in the endoplasmic reticulum could be another likely candidate for Ca^{2+} release, as described in rat exocrine pancreas (Schmid, Dehlinger-Kremer, Schulz & Gögelein, 1990). The voltage-dependent Ca^{2+} channel in the endoplasmic reticulum has been activated by caffeine. However, since caffeine did not induce the increase in $[Ca^{2+}]_i$ in COS-7 cells (Fig. 3), there might be little contribution of this type of Ca^{2+} channel to the changes in $[Ca^{2+}]_i$.

Pertussis toxin (PTX) suppressed the total IP accumulation induced by hypotonic stimulation (Fig. 6). PTX has been shown to inactivate G protein by ADP ribosylation (Ui, 1984). Suzuki *et al.* (1990) have also shown that PTX suppressed phosphoinositide accumulation in cultured renal proximal tubule cells. These results led to the idea that the total IP accumulation induced by hypotonic stimulation is through the activation of PTX-sensitive G proteins. However, the exact mechanism of G protein activation by hypotonic stimulation is still unknown. Three possibilities may be considered in COS-7 cells (Fig. 7).

(1) ATP could be released into the extracellular medium by hypotonic stimulation, activate P_2 receptors and increase $[Ca^{2+}]_i$. Actually, 1 μM suramin, which is an antagonist for P_2 purinoceptors, blocked the hypotonicity-induced Ca^{2+} release (data not shown). However, suramin is not specific for P_2 purinoceptors and is known to have a number of effects on several kinds of receptors and enzymes, for example reverse transcriptase, DNA polymerase, protein kinase C, platelet derived growth factor receptors and so on (Voogd, Vansterkenburg, Wilting & Janssen, 1993). Therefore, antagonistic effects of suramin alone cannot

exclude the participation of P_2 purinoceptors. A specific P_{2Y} blocker Cibacron Blue (100 μM) (Burnstock & Warland, 1987) did not block the hypotonicity-induced Ca^{2+} release. However, 100 μM Cibacron Blue blocked the Ca^{2+} release induced by 10 μM ADP-β-S. Therefore, the activation of P_{2Y} receptors can be excluded as an explanation for hypotonicity-induced Ca^{2+} release. In COS-7 cells, 100 μM Cibacron Blue did not block the Ca^{2+} release induced by 10 μM ATP. The possibility therefore remains that hypotonic stimulation-induced Ca^{2+} release could be triggered via another type of P_2 purinoceptor.

(2) Neer & Clapham (1988) have proposed that the stability of subunits of G protein is maintained by cytoskeletal elements. Hypotonic stimulation generates local disruption of the cytoskeleton (Wilkerson, Dibona & Schafer, 1986) and probably induces binding of G protein to effector molecules as discussed by Suzuki *et al.* (1990). However, treatment with 50 μM cytochalacin D (an inhibitor for actin polymerization) for 20 min, 100 μM phalloidine for 1 h, or 10 μM taxol (a stabilizer for actin) for 22 h did not block the hypotonicity-induced Ca^{2+} release in COS-7 cells (data not shown). There might still be a possibility that other unidentified cytoskeletal molecules are related to the hypotonicity-induced Ca^{2+} release in COS-7 cells.

(3) Margalit *et al.* (1993) have shown that regulatory volume decrease following hypotonicity-induced swelling is mediated through PTX-sensitive G protein and Ca^{2+}-insensitive phospholipase A_2 in human platelets. They proposed that a G protein-coupled receptor acts as a mechanoreceptor and transforms the mechanical stimulus into the biochemical response. In COS-7 cells, it is also possible that a G protein-coupled receptor activates phospholipase C via G protein as a result of mechanical stimulation.

In Fig. 7, we summarize our findings and the possible mechanisms described above. Unfortunately in this paper, we were unable to determine the molecular mechanism which leads to the activation of G proteins following hypotonic stimulation. However, our findings in this cell line make future analysis and cloning of the related molecules much easier because large numbers of homogeneous cells and mRNA can readily be obtained from COS-7 cells.

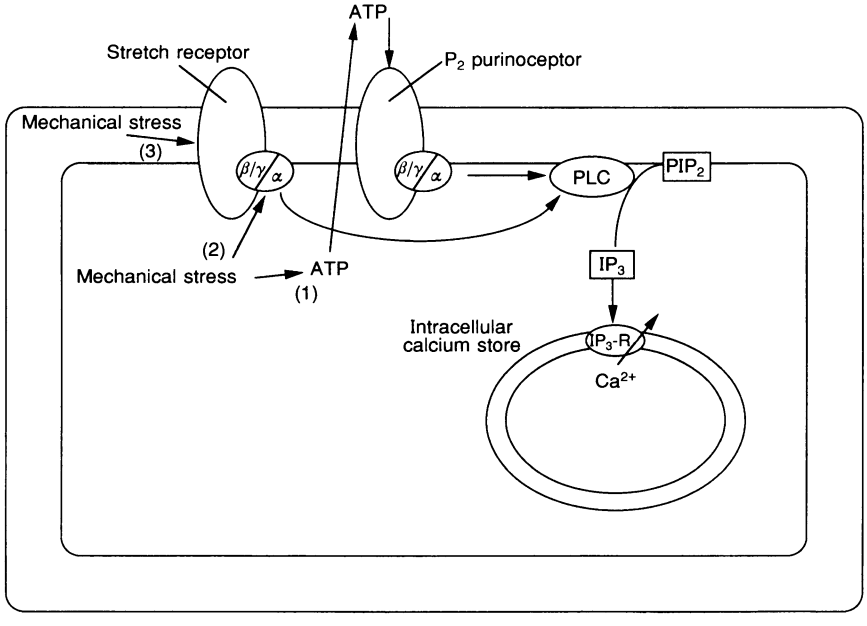

Figure 7. A model of hypotonicity-induced Ca^{2+} release from internal stores

Application of mechanical stress to the cell surface is assumed to induce activation of a G protein and the cascade of phospholipase C (PLC) and IP_3. IP_3 activates IP_3 receptors (IP_3-R) and induces Ca^{2+} release from internal stores. Several possibilities remain for the activation of a G protein. Numbers in parentheses correspond to numbered paragraphs in Discussion where full details are given. PIP_2, phosphatidylinositol 4,5-bisphosphate. α, β and γ indicate each subunit of G protein.

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