Journal of Cell Science 111, 1217-1225 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS7201

Roles of Ca^{2+}, inositol trisphosphate and cyclic ADP-ribose in mediating intercellular Ca^{2+} signaling in sheep lens cells

Grant C. Churchill[1] and Charles F. Louis[1,2,*]

Departments of [1]Biochemistry and [2]Veterinary PathoBiology, University of Minnesota, St Paul, MN 55108, USA
*Author for correspondence at Department of Veterinary PathoBiology, University of Minnesota, 1988 Fitch Avenue, Room 295, St Paul, MN 55108, USA (e-mail: louis003@maroon.tc.umn.edu)

Accepted 17 February; published on WWW 20 April 1998

SUMMARY

To further characterize how gap junction-dependent Ca^{2+} waves propagate between sheep lens cells, we examined the possible roles of inositol 1,4,5-trisphosphate (IP_3), Ca^{2+} and cyclic ADP-ribose (cADPR) in mediating intercellular Ca^{2+} waves. Second messengers were microinjected into a single cell in a monolayer of sheep lens cells while monitoring cytosolic Ca^{2+} with fura-2 and fluorescence microscopy. All three compounds initiated intercellular Ca^{2+} waves, but more cells responded following the injection of either IP_3 or cADPR than responded following the injection of Ca^{2+}. When either IP_3 or cADPR was co-injected with the Ca^{2+} chelator EGTA, cytosolic Ca^{2+} in the injected cell decreased but cytosolic Ca^{2+} in the adjacent cells increased, indicating that the intercellular messenger was IP_3 or cADPR, rather than Ca^{2+}. The phospholipase C inhibitor U73122 eliminated mechanically initiated intercellular Ca^{2+} waves, indicating that mechanical initiation probably requires IP_3 production. In U73122-treated cells, injected IP_3 initiated an intercellular Ca^{2+} wave in which the number of cells responding increased as the amount of IP_3 injected increased, indicating that the distance traveled by the Ca^{2+} wave was dependent on cell-to-cell diffusion of IP_3. In contrast, the ability of cADPR both to increase cytosolic Ca^{2+} in the injected cell and to initiate intercellular Ca^{2+} waves was greatly attenuated by U73122. In conclusion, Ca^{2+}, IP_3 and cADPR can all mediate intercellular Ca^{2+} waves by passing through gap junction channels, but both IP_3 and cADPR are more effective intercellular messengers than Ca^{2+}.

Key words: Cytosolic calcium, Calcium wave, Intercellular communication, Gap junction, Lens, Epithelium, Second messenger

INTRODUCTION

Many extracellular signals are relayed into the cell by an increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$; Berridge, 1993). In individual cells, Ca^{2+} increases can be organized over time to produce Ca^{2+} oscillations or organized over space to produce Ca^{2+} waves (Berridge, 1993; Tsien and Tsien, 1990; Meyer and Stryer, 1991; Clapham, 1995). In groups of cells connected via gap junctions, Ca^{2+} waves can propagate between cells (Sanderson et al., 1994; Sanderson, 1995). These intercellular Ca^{2+} waves are thought to coordinate multicellular processes including cilary beating in tracheal epithelial cells (Sanderson et al., 1990), bile expulsion in the liver (Robb-Gaspers and Thomas, 1995), hormone secretion in the pancreas (Loessberg-Stauffer et al., 1993; Meda, 1996; Yule et al., 1996) and information processing in neural cells (Cornell-Bell et al., 1990).

The identity of the messenger(s) that propagates intracellular or intercellular Ca^{2+} waves has been the subject of many experimental and theoretical studies (Allbriton et al., 1992; Boitano et al., 1992; Berridge, 1993; Jaffe, 1993; Meyer and Stryer, 1991; Sanderson et al., 1990, 1994; Sanderson, 1995; Clapham, 1995). The original candidates for the intercellular messenger were Ca^{2+} and IP_3 (Sanderson et al., 1990; Cornell-Bell et al., 1990), as both molecules are small enough to pass through gap junctional channels (<1 kDa; Goodenough et al., 1996) and both can release Ca^{2+} from intracellular Ca^{2+} stores (Berridge, 1993; Clapham, 1995; Meissner, 1994). In mechanically stimulated airway epithelial cells, the intercellular messenger is probably IP_3, because inhibiting either IP_3 synthesis (Hansen et al., 1995; Sanderson et al., 1994; Sanderson, 1995) or the binding of IP_3 to its receptor (Boitano et al., 1992; Sanderson et al., 1994) eliminates intercellular Ca^{2+} waves. However, studies with pancreatic acinar cells demonstrated that intercellular Ca^{2+} waves could be initiated by microinjecting either IP_3 or Ca^{2+}, but Ca^{2+} required stimulation of the cells with a low concentration of agonist (Yule et al., 1996). Presumably, the agonist increased the basal concentration of IP_3 sufficiently to enable Ca^{2+} activation of the IP_3 receptor (Yule et al., 1996), for which IP_3 and Ca^{2+} are co-agonists (Bezprozvanny et al., 1991). Therefore, the messenger that passes through gap junction channels to propagate the Ca^{2+} wave may depend both on the cell type and the concentration of other modulators of intracellular Ca^{2+} release channels. One possibility that has not been addressed to date is that cyclic ADP-ribose (cADPR) could function as an intercellular messenger. cADPR is an attractive candidate for propagating Ca^{2+} waves between cells as it is small enough to

traverse gap junctions (541 Da), stimulates Ca^{2+} release in a variety of cell types (Lee et al., 1994), mediates an intracellular Ca^{2+} wave when injected into sea urchin eggs (Lee et al., 1994), and acts as a global messenger within single cells (Kasai and Petersen, 1994; Petersen et al., 1995).

The ocular lens is a transparent tissue containing only two cell types: epithelial cells, which contain organelles and form a single layer on the anterior surface, and fiber cells, which lack organelles and comprise the bulk of its mass (Paterson and Delamere, 1992). In the lens, the loss of calcium homeostasis is implicated in cataract formation (Duncan et al., 1994), so it is important to better define the mechanisms by which Ca^{2+} is regulated in the lens. To date, only IP_3 has been shown to release Ca^{2+} in lens cells (Duncan et al., 1993). Although cADPR has been shown to mediate the release of Ca^{2+} from intracellular stores in sea urchin eggs and certain mammalian cells (Lee et al., 1994), a potential role for cADPR in lens cell Ca^{2+} regulation has not been investigated.

To further characterize the mechanism of intercellular Ca^{2+} wave propagation in sheep lens cells, we examined the relative abilities of IP_3, Ca^{2+} and cADPR to mediate intercellular Ca^{2+} waves. Sheep lens cells were studied because they are well-coupled via gap junctions (TenBroek et al., 1994) and exhibit intercellular Ca^{2+} waves in response to mechanical stimulation (Churchill et al., 1996). We now report that microinjection of either IP_3, Ca^{2+} or cADPR increased $[Ca^{2+}]_i$ in the impaled cell and initiated an intercellular Ca^{2+} wave, but more cells participated in the intercellular Ca^{2+} wave following the injection of IP_3 or cADPR than following the injection of Ca^{2+}. This is the first report demonstrating that cADPR can mediate intercellular Ca^{2+} waves. The phospholipase C inhibitor U73122 blocked intercellular Ca^{2+} waves initiated either by mechanical stimulation or by injection of cADPR, but not those initiated by injection of IP_3, indicating that IP_3, but not cADPR, was necessary and sufficient to propagate intercellular Ca^{2+} waves.

MATERIALS AND METHODS

Materials

Cyclic ADP-ribose was a generous gift from Dr Timothy Walseth (University of Minnesota). Sheep eyes were obtained from John Morrell and Co. (Sioux Falls, SD). Fetal calf serum was obtained from Hyclone (Logan, UT). Fura-2 and fura-2 acetoxymethyl ester were obtained from Molecular Probes (Eugene, OR). Thapsigargin, ionomycin, D-IP_3 and L-IP_3 were obtained from LC Laboratories (Woburn, MA). U73122 and U73144 were obtained from BIOMOL (Plymouth Meeting, PA). Medium 199, Hanks' balanced salt solution (HBSS), ADPR, EGTA and all other chemicals were obtained from Sigma Chemical Company (St Louis, MO).

Cell culture

Primary cultures of cells isolated from the equatorial region of fresh ovine lens were prepared as described previously (TenBroek et al., 1994). Briefly, eyeballs were removed from freshly slaughtered lambs and maintained on ice until removal of the lenses 3-7 hours later. 6 lenses were placed in 6 ml of Hanks' balanced salt solution without added Ca^{2+} and Mg^{2+} (HBSS-CMF) and digested with 2.5 mg/ml trypsin for 15 minutes. 40 ml of ice-cold HBSS-CMF were added to slow the reaction, and the solution was triturated 20-30 times. Cells were centrifuged (230 ***g*** for 4 minutes) and resuspended at a density of 5×10^5 cells/ml in Medium 199, which included 10% fetal calf serum, 100 i.u./ml penicillin and 100 i.u./ml streptomycin. 2 ml of this cell suspension was placed into a 35-mm plastic Petri plate containing a 25-mm diameter glass coverslip coated with poly-D,L-ornithine (100 μg/ml). Cells were grown in medium 199 at 37°C in a humidified atmosphere containing 5% CO_2. The ovine lens epithelial cells used in this study were grown in culture for 5-28 days. Although some cells differentiated into fiber cells (TenBroek et al., 1994), only regions of epithelial-like cells were utilized for the Ca^{2+} imaging studies.

$[Ca^{2+}]_i$ determination and image analysis

Ca^{2+} imaging was performed as described previously (Churchill et al., 1996). Briefly, cells were loaded with fura-2 by incubation in 1 μM fura-2 acetoxymethyl ester in Medium 199 with fetal calf serum at 22°C in the dark for 20-40 minutes, and then rinsed three times with HBSS-H (HBSS supplemented with 10 mM Hepes, pH 7.2). The glass coverslip with attached cells formed the bottom of a microincubation culture chamber (MS 200D, Medical Systems Corporation, NY), which was maintained at 22°C. The chamber was mounted on the stage of an inverted epifluorescence microscope (IM 35, Zeiss) supported on a vibration-isolated table (Technical Manufacturing Corp., Peabody, MA). Cells were viewed through a 40×, 1.3 numerical aperture, oil-immersion objective lens (Fluor 40, Nikon).

$[Ca^{2+}]_i$ was determined by the ratio method and based on an in vitro calibration (Grynkiewicz et al., 1985). Fura-2 was excited alternately at 340 and 380 nm and fluorescence at 510 nm was detected with a SIT camera (VE-1000, Dage-MTI Inc, IN). Images were stored digitally with an optical memory disk recorder (TQ-3031F, Panasonic, Secauucus, NJ) and processed with the software Image-1/Fluorescence (Universal Imaging Corp., PA). A background subtraction and a shading correction were applied before calculating the ratio image.

Microinjection

As cells in this culture system show $[Ca^{2+}]_i$ increases in response to mechanical stimulation (Churchill et al., 1996), several measures were taken to minimize the possibility of micropipette movement. First, current was used rather than pressure for injection of the compounds. Second, the filter wheel was physically isolated from the microscope. Third, the micropipette was positioned with a low-drift hydraulic micromanipulator (MW-3, Narishige, Greenvale, NY).

Micropipettes were pulled from 2-mm outside diameter borosilicate capillary tubing (WPI, Sarasota, FL) on a Flaming-Brown-type pipette puller (P-87, Sutter Instruments, Novato, CA). Micropipettes had tip diameters of less than 1 μm and resistances of approx. 100–200 MΩ when filled with injection solutions. Compounds were dissolved in distilled deionized water and pH was adjusted to approx. 7.5 with either KOH (10 M) or Tris-base (10 M) to ensure that the compounds possessed a net charge. Micropipettes were loaded by backfilling with 10–30 μl of injection solution using a 1-ml syringe and a 28-gauge, plastic-coated glass needle (WPI, Sarasota, FL). Current was delivered through a chlorodized silver wire in contact with the micropipette solution, which contained 10 mM KCl to provide chloride for the Ag/AgCl half-cell.

In preparation for an injection, a micropipette was lowered 1-3 μm beyond apparent contact with the cell's plasma membrane, which either impaled or dimpled the cell. Then the 'tickle' button was depressed, or a 5-20 millisecond pulse of –25 to –100 nA, or both, were applied to ensure the micropipette had impaled the cell. Current was generated with an electrometer (Intra 767, WPI, Sarasota, FL). Current duration, magnitude and polarity were controlled with a pulse generator (A310 Accupulser, WPI, Sarasota, FL). Compounds were injected 3-6 minutes after impalement to allow $[Ca^{2+}]_i$ to recover from the increase in $[Ca^{2+}]_i$ in the impaled and surrounding cells that resulted from the mechanical stimulation accompanying impalement. Current passage through the micropipette was monitored with an oscilloscope and verified that it was the expected magnitude during each injection.

Data analysis

The results from single experiments that represent the typical response

obtained in five or more independent experiments are presented. Statistical analyses were performed with the program StatView (Abaccus Concepts, Berkeley, CA). Data was excluded from analysis if during injection the maximum voltage deliverable by the electrometer was exceeded, or current application resulted in the loss of fura-2 from the impaled cell, as these typically resulted in mechanical stimulation and release of an extracellular messenger, respectively (Churchill et al., 1996). Where appropriate, data are presented as a mean ± s.e.m., and were tested for significance by analysis of variance with a subsequent separation of the means performed with Fisher's Least Significant Difference test.

RESULTS

Microinjected IP_3 and cADPR are more effective than Ca^{2+} in initiating intercellular Ca^{2+} waves

To determine whether IP_3 or cADPR could initiate intercellular Ca^{2+} waves, IP_3 and cADPR were microinjected into lens cells while monitoring $[Ca^{2+}]_i$ with fura-2 and Ca^{2+} imaging. Impaling a single cell resulted in an increase in $[Ca^{2+}]_i$ in the impaled cell that spread to the surrounding cells (Fig. 1A,B; open arrow), as reported previously for mechanically stimulated lens cells (Churchill et al., 1996). After several minutes to allow for recovery from the impalement-induced intercellular Ca^{2+} wave in the cells surrounding the imapaled cell, microinjection of either IP_3 (Fig. 1A,B) or cADPR (Fig. 1C,D) initiated an increase in $[Ca^{2+}]_i$ in the impaled cell and an intercellular Ca^{2+} wave. Although the $[Ca^{2+}]_i$ in the impaled cell recovered from its peak $[Ca^{2+}]_i$, the impaled cell never recovered to its pre-impaled resting $[Ca^{2+}]_i$ in any of these microinjection experiments. In contrast, microinjection of Ca^{2+} resulted in a $[Ca^{2+}]_i$ increase in the injected cell that spread to only some first-tier cells, and a Ca^{2+} gradient was evident across these cells (Fig. 1E,F). The results from several microinjections of IP_3, cADPR and Ca^{2+} are compared in the graphs shown in Fig. 1G.

Initiation of intercellular Ca^{2+} waves is specific for D-IP_3 and cADPR

Several compounds with a similar structure to D-IP_3 and

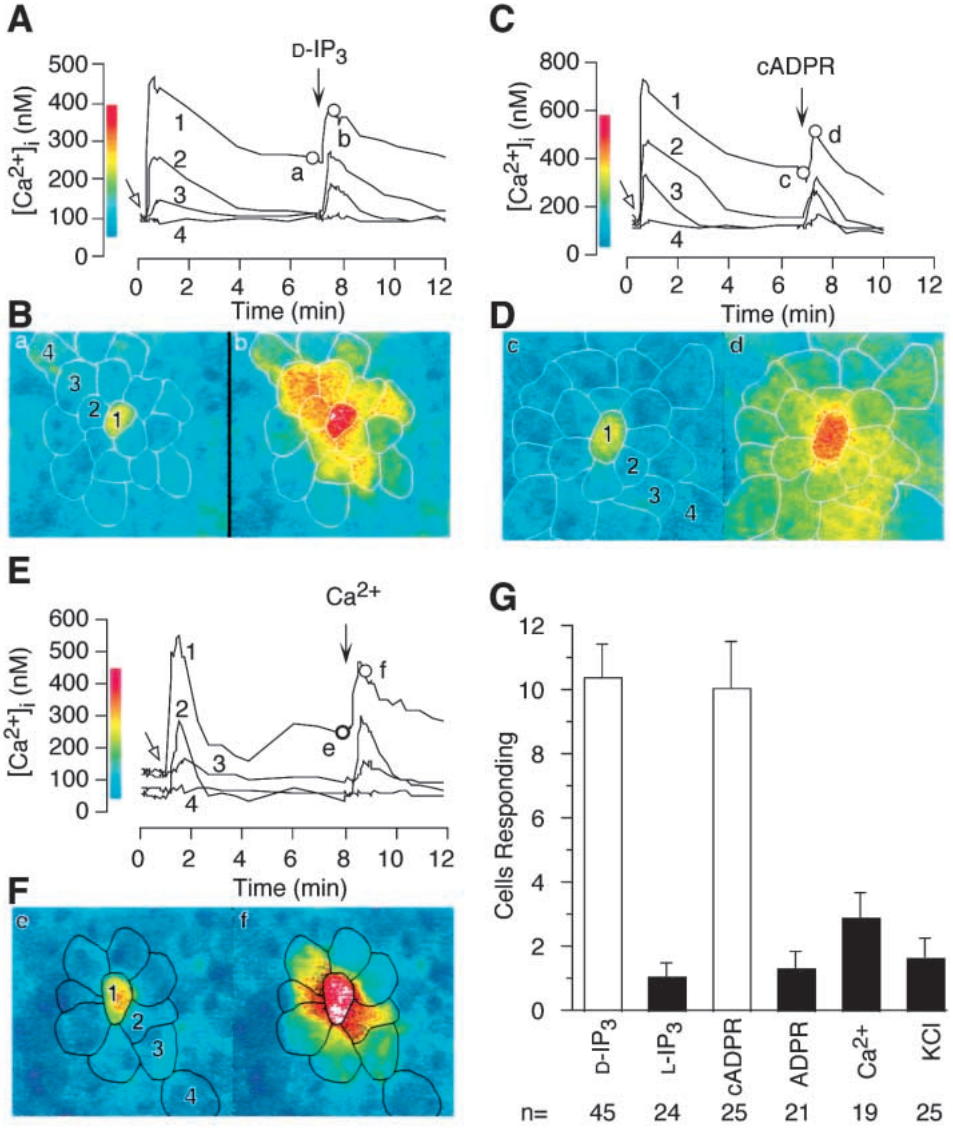

Fig. 1. Initiation and spread of intercellular Ca^{2+} waves following the injection of Ca^{2+}-releasing second messengers and related compounds. Results from the microinjection of IP_3 (A,B), cADPR (C,D) and Ca^{2+} (E,F) are presented as both traces of $[Ca^{2+}]_i$ over time in the impaled and surrounding cells (A,C,E) and as pseudocolor images of these same cells (B,D,F). The cell labeled 1 was impaled with the micropipette (open arrow), and following recovery of $[Ca^{2+}]_i$ in the surrounding cells (approx. 5 minutes), current was applied (filled arrow) to inject the indicated compound into the cell. Compounds were injected by application of a current of −50 nA for 10 milliseconds at 100-millisecond intervals for 10 seconds. Pseudocolor corresponds to $[Ca^{2+}]_i$ as indicated by the calibration scale. The approximate borders of the cells are outlined, and the numbered cells correspond to the numbered traces. The images in B, D and F are from the time points on the traces above marked with the circles and labeled with lower-case letters. (G) Summary showing the number of cells participating in the Ca^{2+} wave following microinjection of the indicated compounds. The number of experiments (*n*) is shown below each compound label. Cells that increased $[Ca^{2+}]_i$ ≥50 nM were designated as responding. Bars with the same fill-pattern are not significantly different based on a one-way analysis of variance, followed by means separation with Fisher's Least Significant Difference ($P \leq 0.05$). The microinjection solution was 10 mM KCl supplemented with the following concentrations of the test compounds (in mM): cADPR (0.5), ADPR (0.5), D-IP_3 (1.6) and L-IP_3 (1.6), KCl (10).

cADPR were injected as negative controls to ensure that the cells were responding to the microinjected chemical per se and not to a change in membrane potential or the injection of any small molecule. Fig. 1G shows that, in contrast to the intercellular Ca^{2+} waves initiated following the injection of either D-IP_3 or cADPR, intercellular Ca^{2+} waves were not consistently initiated following the injection of KCl, L-IP_3, the less active IP_3 enantiomer (Potter, 1990), and ADPR, a metabolite resulting from the hydrolysis of cADPR (Lee et al., 1994). The instances where injection of the related compounds did initiate an intercellular Ca^{2+} wave (Fig. 1G) may have resulted from mechanical stimulation due to voltage-induced cell damage. Although the response to the microinjection of any given compound was variable (Fig. 1G), the frequency of eliciting a response and the number of cells responding was markedly different for D-IP_3 and cADPR compared to the other related compounds. Thus, it appears that in lens cells the initiation of intercellular Ca^{2+} waves is specific for D-IP_3 and cADPR.

Both IP_3 and cADPR can pass through gap junctions

Attempts to directly demonstrate the requirement for functional gap junctions in propagating intercellular Ca^{2+} waves by uncoupling gap junctions proved equivocal because the treatment either attenuated agonist-mediated increases in $[Ca^{2+}]_i$ (both CO_2-mediated acidification and octanol) or failed to completely uncouple gap junctional communication (18α-glycyrrhetinic acid). The potential problems with agents that not only uncouple gap junctions but also interfere with Ca^{2+} signaling have been investigated and described in detail previously (Deutsch et al., 1995). Although participation of an extracellular messenger cannot be completely ruled out, such a mechanism seems unlikely given that sheep lens cells do not release an extracellular messenger unless a cell is ruptured (Churchill et al., 1996). Thus, it appears reasonable to conclude that second messenger-initiated intercellular Ca^{2+} waves were propagated via gap junctions.

Two approaches were used to determine whether the messenger molecule passing cell-to-cell through gap junction

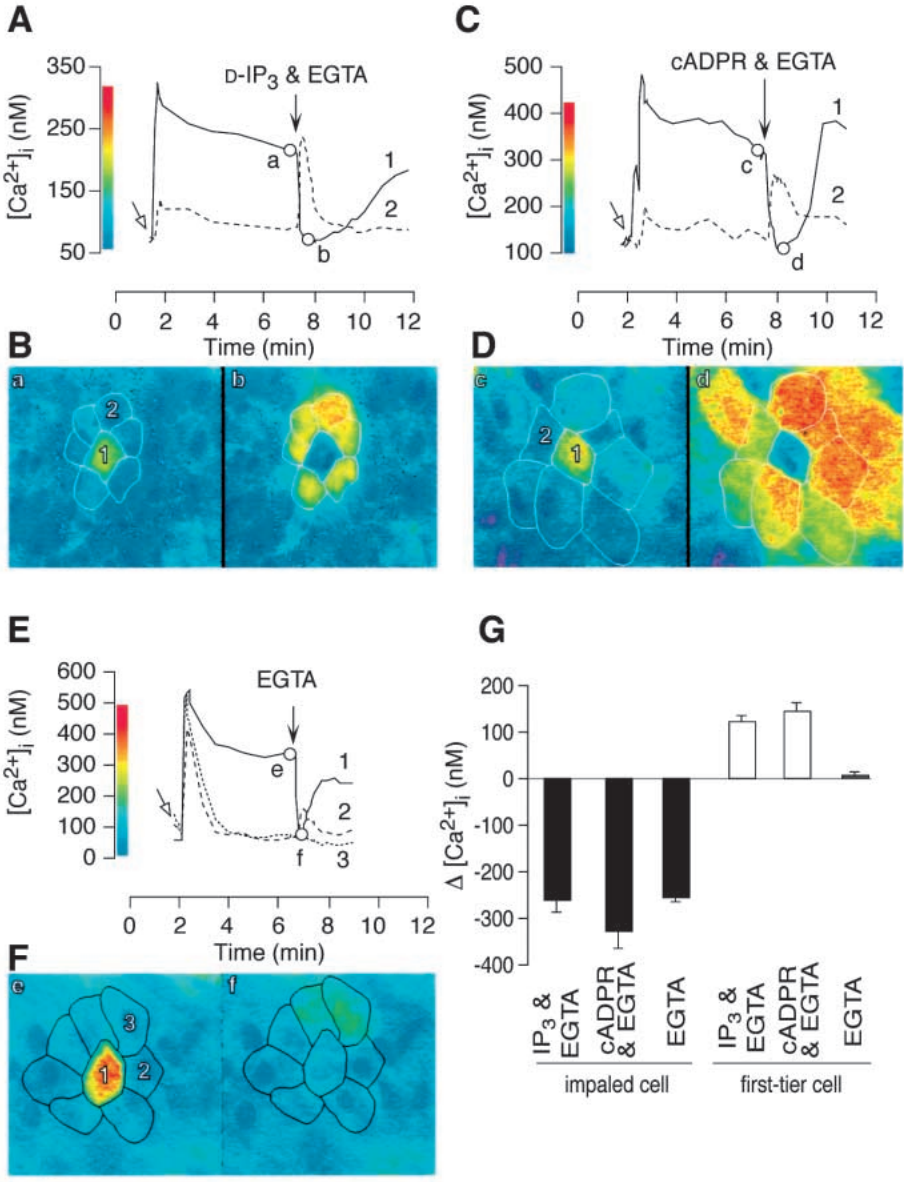

Fig. 2. IP_3 and cADPR pass through gap junctions to mediate intercellular Ca^{2+} waves. Effect of co-injecting IP_3 and EGTA (A,B), cADPR and EGTA (C,D) or EGTA alone (E,F) on $[Ca^{2+}]_i$ in the impaled and immediately adjacent cells. The cell labeled 1 was impaled with the micropipette (open arrow), and following recovery of $[Ca^{2+}]_i$ to approximately resting levels in all but the impaled cell (approx. 5 minutes), current was applied (filled arrow) to inject the compound(s) into the cell. Compounds were injected by application of a current of –50 nA for 10 milliseconds at 100-millisecond intervals for 10-15 seconds. Pseudocolor corresponds to $[Ca^{2+}]_i$ as indicated by the calibration scale. The approximate borders of the cells are outlined, and the numbered cells correspond to the numbered traces. The images in B, D and F are from the time points on the traces above marked with the circles and labeled with lower-case letters. The microinjection solution contained 10 mM KCl supplemented with the following concentrations of compounds (in mM): IP_3 (1.6) and EGTA (5); cADPR (5) and EGTA (5); and EGTA alone (5). (G) Summary of the changes in $[Ca^{2+}]_i$ in both the impaled and first-tier cells following the injection of the indicated compounds. Bars with the same fill-pattern are not significantly different, based on a one-way analysis of variance, followed by means separation with Fisher's Least Significant Difference ($P \leq 0.05$). Data are based on (A,B) 31 co-injections of IP_3 and EGTA (four cultures), (C,D) 27 co-injections of cADPR and EGTA (4 cultures) and (E,F) 55 injections of EGTA alone (eight cultures).

channels was the injected messenger (IP_3 or cADPR) or Ca^{2+}. In the first approach, IP_3 or cADPR were co-injected with EGTA, which resulted in a large and rapid *decrease* in $[Ca^{2+}]_i$ in the impaled cell, but an *increase* in $[Ca^{2+}]_i$ in most first-tier cells (Fig. 2A–D). In contrast, injecting EGTA alone resulted in a rapid and large *decrease* in the impaled cell, which was accompanied by a *decrease* in $[Ca^{2+}]_i$ in most first-tier cells (Fig. 2E,F). The percentage of first-tier cells in which $[Ca^{2+}]_i$ respectively increased, decreased or did not change, was: IP_3+EGTA: 76, 19 and 6 (n=21); cADPR+EGTA: 71, 16 and 12 (n=15); EGTA alone: 29, 44 and 27 (n=43). The changes in $[Ca^{2+}]_i$ from several injections are summarized in Fig. 2G.

These results raise the question as to why upon co-injection there is a decrease in $[Ca^{2+}]_i$ in the impaled cell but an increase in $[Ca^{2+}]_i$ in the surrounding cells. A possible explanation is that in the impaled cell EGTA lowers the $[Ca^{2+}]_i$ sufficiently to prevent IP_3 from activating the IP_3 receptor, which requires $[Ca^{2+}]_i$ to be between 100 and 300 nM for maximal release (Bezprozvanny et al., 1991). Such a mechanism would require EGTA to lower $[Ca^{2+}]_i$ before IP_3 could activate the IP_3 receptor to release Ca^{2+}. When EGTA is injected it is mostly complexed with Ca^{2+} because the $[Ca^{2+}]_i$ is elevated in the impaled cell before injection. Thus, in the impaled cell the predominant form of EGTA following its injection is the EGTA:Ca^{2+} complex, rather than free EGTA, so it is then both free IP_3 and EGTA:Ca^{2+} complex that diffuse through gap junction channels into the surrounding cells. The EGTA:Ca^{2+} complex cannot decrease $[Ca^{2+}]_i$, therefore, unlike in the impaled cell, the $[Ca^{2+}]_i$ remains sufficiently elevated so that the IP_3 that enters the surrounding cells can activate Ca^{2+} release through the IP_3 receptor.

In the second approach used to determine whether the injected messenger or Ca^{2+} passed through gap junction channels to mediate the intercellular Ca^{2+} wave, the amount of compound injected was varied and compared with the distance traveled by the Ca^{2+} wave. If the injected messenger was the diffusing messenger, then varying the amount of messenger injected into a cell should alter the number of cells participating in the intercellular Ca^{2+} wave independently of changes in $[Ca^{2+}]_i$ in the impaled cell. When the amount of IP_3 injected into a given cell was varied by changing the injection current amplitude, the number of cells responding with an increase in $[Ca^{2+}]_i$ increased proportionately to the amount of IP_3 injected (Fig. 3A,B). The same relationship was observed when an increasing amount of cADPR was injected (Fig. 3C,D). Note that the increase in $[Ca^{2+}]_i$ in a given cell was not necessarily greater as more messenger was injected; rather, more cells were recruited as more messenger was injected (Fig.

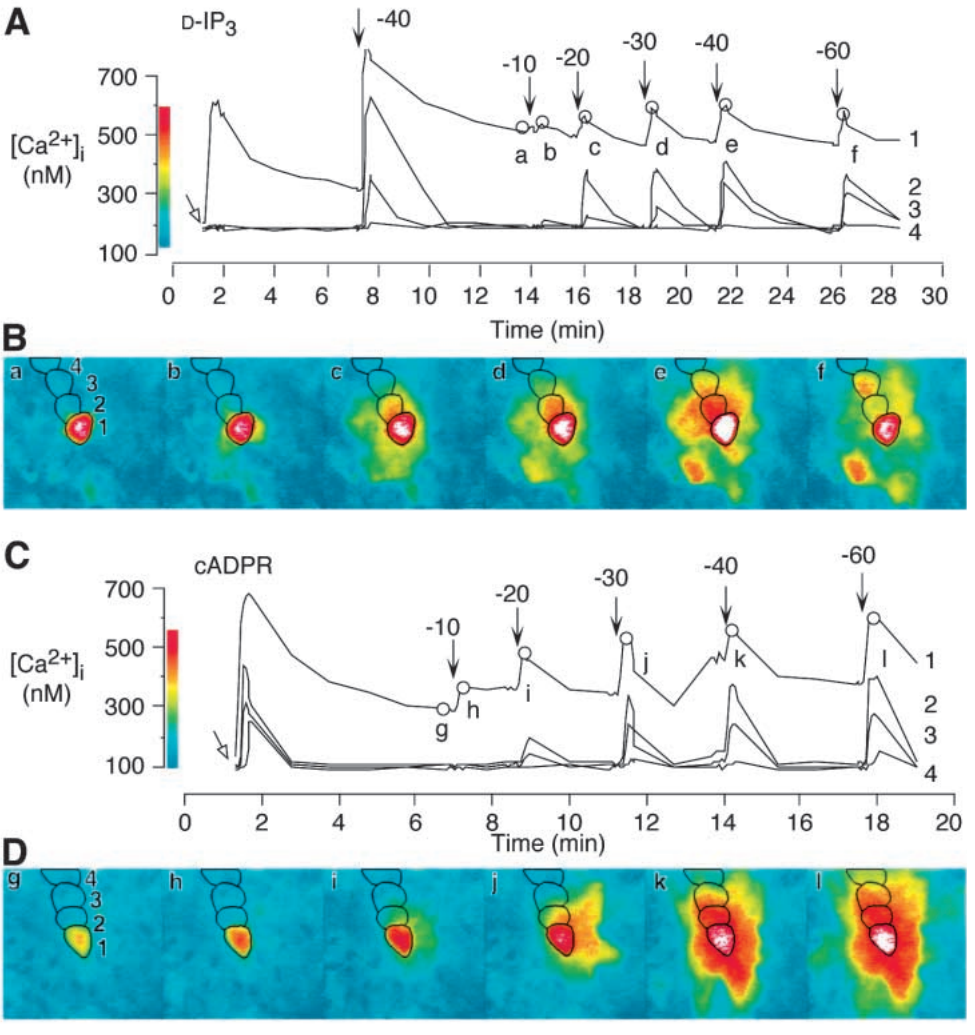

Fig. 3. Effect of the amount of IP_3 or cADPR injected on the spread of the intercellular Ca^{2+} wave. Results from the microinjection of IP_3 (A,B) and cADPR (C,D) are presented as both traces of $[Ca^{2+}]_i$ over time in the impaled and surrounding cells (A,C) and as pseudocolor images of these same cells (B,D). The cell labeled 1 was impaled with the micropipette (open arrow), and following recovery of $[Ca^{2+}]_i$ to approximately resting levels in all but the impaled cell (approx. 5 minutes), current was applied (filled arrow) to inject the compound into the cell. Compounds were injected by application of a current for 10 milliseconds at 100-millisecond intervals for 15 seconds. The amplitude of the current was used to vary the amount of compound injected; current amplitudes are indicated above the filled arrows (−10 to −60 nA). The images in B and D are from the time points on the traces above marked with the circles and labeled with lower-case letters. Pseudocolor corresponds to $[Ca^{2+}]_i$ as indicated by the calibration scale. The approximate borders of the cells are outlined, and the numbered cells correspond to the numbered traces. The data are representative of the response observed in 7/11 experiments using seven cultures (A,B) and 7/10 experiments using five cultures (C,D). In the other experiments the cells became insensitive to injection of the second messenger during the prolonged impalement.

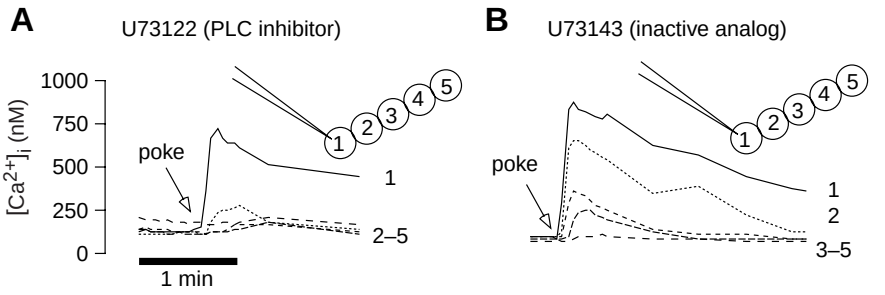

Fig. 4. Effect of the phospholipase C inhibitor U73122 on the initiation and spread of mechanically stimulated intercellular Ca^{2+} waves. Cells were exposed to either the phospholipase inhibitor U73122 (A) or its inactive analog U73143 (B) (both at 10 μM) for ≥30 minutes before the first data point of each set of traces. Each numbered trace corresponds to the numbered cell in the inset diagram. The cell marked 1 was stimulated with a micropipette at the open arrow. The data are representative of the response to mechanical stimulation observed in 18/21 experiments (five cultures) following incubation in U73122 (A), and 7/7 experiments (two cultures) following incubation in U73143 (B).

3). This correlation between the amount of messenger injected and the number of responding cells, and the lack of correlation between the magnitude of the increase in $[Ca^{2+}]_i$ in the injected cell and the number of responding cells, suggests that it is the injected compound and not Ca^{2+} that is passing through gap junction channels.

Effect of the phospholipase C inhibitor U73122 on mechanically activated intercellular Ca^{2+} waves

That IP_3 and cADPR can mediate increases in $[Ca^{2+}]_i$ by passing through gap junctions is consistent with a possible role for either compound mediating intercellular Ca^{2+} waves initiated by mechanical stimulation in lens cells. To determine the requirement for IP_3 synthesis in mediating intercellular Ca^{2+} waves, cells were mechanically stimulated after incubation with the phospholipase C inhibitor U73122 (Thompson et al., 1991). U73122 has been shown to inhibit mechanically activated intercellular Ca^{2+} waves in other cell types (Hansen et al., 1995; Dandrea and Vittur, 1997) and significantly attenuates agonist-stimulated $[Ca^{2+}]_i$ increases in these sheep lens cells (Churchill and Louis, 1997). After incubation with 10 μM U73122, mechanical stimulation of a single cell with a micropipette resulted in an increase in $[Ca^{2+}]_i$ in the stimulated cell, but the intercellular spread of the $[Ca^{2+}]_i$ increase was essentially eliminated (Fig. 4A). In contrast, incubation with 10 μM U73143, the inactive analog of U73122, had no effect on a mechanically activated intercellular Ca^{2+} wave (Fig. 4B). The effect of U73122 was not due to the inhibition of gap junctional communication, as 5 minutes after a single cell was injected with Lucifer yellow the number of cells receiving dye was 28.0±3.2 cells (n=4) in the absence and 26.8±1.7 cells (n=5) in the presence of 10 μM U73122, and these values were not significantly different (unpaired t-test, P=0.74).

Effect of U73122 on intercellular Ca^{2+} waves initiated by injecting IP_3 or cADPR

The ability of U73122 to essentially eliminate mechanically activated intercellular Ca^{2+} waves provided an opportunity to investigate the role of the injected messenger in propagating intercellular Ca^{2+} waves without the complication of a mechanically activated response. If IP_3 or cADPR was passing through gap junctions to propagate the Ca^{2+} wave between cells, then injected IP_3 or cADPR should trigger Ca^{2+} waves in U73122-treated cells. Similar to the experiment shown in Fig. 4A, when a cell incubated with 10 μM U73122 was

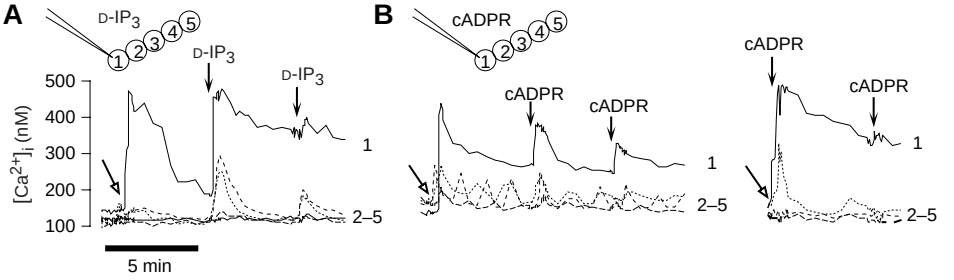

Fig. 5. Ability of microinjected IP_3 or cADPR to initiate intercellular Ca^{2+} waves in cells treated with the phospholipase C inhibitor U73122. Cells were incubated with the phospholipase C inhibitor U73122 (10 μM) for ≥20 minutes prior to impaling a single cell with a micropipette containing either (A) IP_3 or (B) cADPR. The relative positions of the cells are shown schematically. The cell labeled 1 was impaled with the micropipette (open arrow), and following recovery of $[Ca^{2+}]_i$ to approximately resting levels in the surrounding cells (approx. 5 minutes), current was applied (filled arrow) to inject the compound into the cell. Compounds were injected by application of a current of –50 nA for 10 milliseconds at 100-millisecond intervals for 10 seconds. The microinjection solution contained 10 mM KCl supplemented with either IP_3 (1.6 mM) or cADPR (5 mM). The data are representative of the response observed in 26/32 injections of IP_3 using 13 cultures (A), cADPR injections using seven cultures (B) 6/17 (left traces) and 11/17 (right traces).

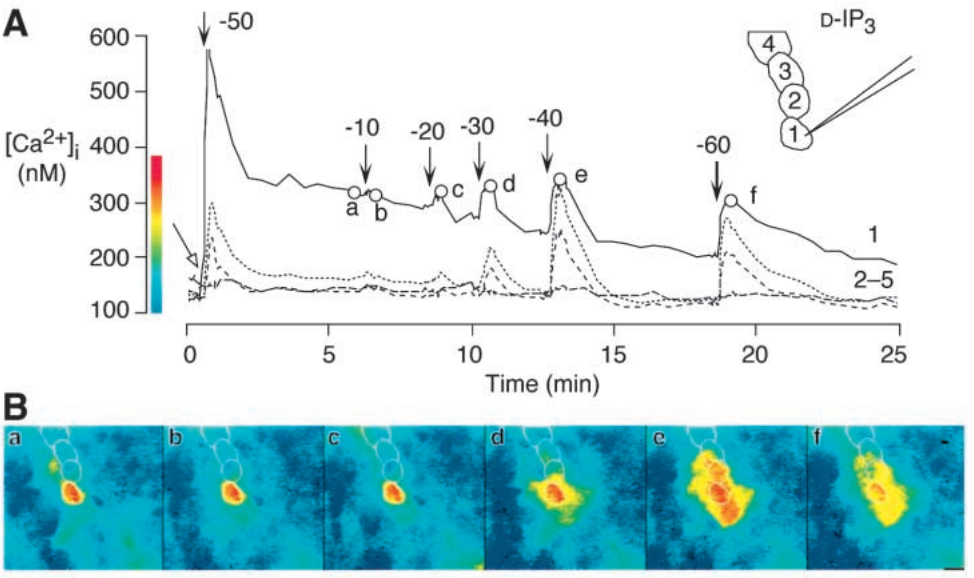

Fig. 6. Effect of the amount of IP_3 injected on the spread of intercellular Ca^{2+} waves in cells treated with the phospholipase C inhibitor U73122. Cells were incubated with the phospholipase C inhibitor U73122 (10 μM) for ≥20 minutes prior to impaling a single cell with a micropipette containing IP_3. Results are presented as both traces of $[Ca^{2+}]_i$ over time in the impaled and surrounding cells (A) and as pseudocolor images of these same cells (B). The cell labeled number 1 was impaled with the micropipette (open arrow), and following recovery of $[Ca^{2+}]_i$ to approximately resting levels in the surrounding cells (approx. 5 minutes), current was applied (filled arrow) to inject the compound into the cell. IP_3 was injected by application of a current for 10 milliseconds at 100-millisecond intervals for 15 seconds. The amplitude of the current was varied to control the amount of IP_3 injected; current amplitudes are given above the filled arrows (−10 to −60 nA). The images in B are from the time points on the traces marked with the circles and labeled with lower-case letters. Pseudocolor corresponds to $[Ca^{2+}]_i$ as indicated by the calibration scale. The approximate borders of the cells are outlined, and the numbered cells correspond to the numbered traces. The data are representative of the response observed in 8/14 IP_3 injections using eight cultures.

impaled, the $[Ca^{2+}]_i$ increase was mostly restricted to that cell (Fig. 5A); however, an intercellular Ca^{2+} wave was initiated following the injection of IP_3 (Fig. 5A). In contrast to IP_3, the ability of cADPR to initiate intercellular Ca^{2+} waves was essentially eliminated by U73122. In 11 out of 17 injections, injecting cADPR failed to increase $[Ca^{2+}]_i$ in the impaled cell (Fig. 5B, right traces), and in the remaining 6 out of 17 injections, cADPR increased $[Ca^{2+}]_i$ in the impaled cell but failed to elicit an intercellular Ca^{2+} wave (Fig. 5B, left traces). Moreover, in only 4 out of 17 of these injections did cADPR initiate an intercellular Ca^{2+} wave. These results provide evidence that IP_3 but not cADPR is necessary and sufficient for intercellular Ca^{2+} wave propagation.

The role of IP_3 diffusion in intercellular Ca^{2+} wave propagation

Although the experiment shown in Fig. 3 demonstrates that the number of cells participating in the intercellular Ca^{2+} wave was dependent on the amount of IP_3 injected into the impaled cell, it does not rule out the possibility that IP_3 regeneration is at least partially responsible for Ca^{2+} wave propagation. Phospholipase C can be activated by Ca^{2+} following the addition of ionophore in some cell types (Harootunian et al., 1991) but not others (Bird et al., 1997). These variable results may be explained by the isoform of phospholipase C present in a given cell type, as it now has been shown that phospholipase Cδ but not β or γ is activated by Ca^{2+} (Allen et al., 1997). Thus, in sheep lens cells, IP_3 regeneration stimulated by the increase in $[Ca^{2+}]_i$ may have played a role in propagating the intercellular Ca^{2+} wave, rather than just the diffusion of the injected IP_3. To distinguish between an IP_3-diffusion mechanism and an IP_3-regeneration mechanism for intercellular Ca^{2+} wave propagation, U73122-treated cells were injected with varying amounts of IP_3. The amount of IP_3 injected was correlated with the number of cells participating in the Ca^{2+} wave (Fig. 6), indicating that IP_3 diffusion alone could account for the spread of the Ca^{2+} wave to a limited number of cells. The number of cells responding to the injection of IP_3 in the presence of U73122 was 6.8±0.9 (n=28), which is significantly less than the 10.4±1 cells (n=45) responding in the absence of U73122 (unpaired t test, P=0.01).

DISCUSSION

In this report the mechanism by which intercellular Ca^{2+} waves are propagated was investigated in sheep lens cells in culture. It was demonstrated that IP_3 and cADPR were much more effective than Ca^{2+} both in initiating and in propagating intercellular Ca^{2+} waves. The ability of IP_3 to initiate intercellular Ca^{2+} waves has been reported previously for hepatocytes (Saez et al., 1989), tracheal epithelial cells (Sanderson et al., 1990; Sanderson, 1995) and pancreatic acinar cells (Yule et al., 1996). The limited ability of Ca^{2+} to induce both intracellular and intercellular Ca^{2+} waves in cells not stimulated by agonist is consistent with previous reports in other cell types (Rose and Loewenstein, 1975; Saez et al., 1989; DeLisle and Welsh, 1992; Christ et al., 1992; Yule et al., 1996). The importance of phospholipase C for initiating and propagating intercellular Ca^{2+} waves in sheep lens cells is also consistent with the previously reported results with other cell types (Hansen et al., 1995; Dandrea and Vittur, 1997). This is the first report to show that injecting IP_3 into U73122-treated cells resulted in intercellular Ca^{2+} waves, thus demonstrating that IP_3 was necessary and sufficient to mediate intercellular Ca^{2+} waves between sheep lens cells. Moreover, this is the first report for any cell type to show that injecting cADPR can initiate intercellular Ca^{2+} waves.

Although all three messengers investigated here can pass through gap junction channels, there are at least four reasons for believing that the messenger passing through gap junctions is predominantly IP_3 or cADPR rather than Ca^{2+}. First, there was a large increase in $[Ca^{2+}]_i$ in the mechanically stimulated cell in the presence of U73122 that did not spread significantly to other cells, indicating that an increase in $[Ca^{2+}]_i$ alone was not sufficient for intercellular spread of the Ca^{2+} wave, which is consistent with the results reported previously in other cell types (Hansen et al., 1985; Dandrea and Vittur, 1997). Second, injecting Ca^{2+} resulted in intercellular Ca^{2+} waves that were communicated to fewer cells than the Ca^{2+} waves initiated by the injection of either IP_3 or cADPR. Again, this demonstrates that there can be an increase in $[Ca^{2+}]_i$ in a single cell without triggering an extensive intercellular Ca^{2+} wave. Third, the number of cells responding to either IP_3 or cADPR correlated more closely with the amount of compound injected than with the magnitude of the increase in $[Ca^{2+}]_i$ in the impaled cell. This direct relationship between the amount of compound injected and the extent of the Ca^{2+} wave suggests that diffusion of the injected messenger determines the distance traveled by the intercellular Ca^{2+} wave. Fourth, when either IP_3 or cADPR was co-injected with EGTA there was an increase in the adjacent cells even when the injected cell showed a decrease in $[Ca^{2+}]_i$, indicating that an increase in $[Ca^{2+}]_i$ in the injected cell was not required for spread of the Ca^{2+} wave to adjacent cells. The results from these co-injections are similar to the results obtained by mechanical stimulation of cells incubated in low extracellular Ca^{2+}, in which the stimulated cell shows a decrease in Ca^{2+} but the surrounding cells show an increase in Ca^{2+}, which propagates as an intercellular Ca^{2+} wave (Sanderson et al., 1990; Demer et al., 1993; Sanderson, 1995; Churchill et al., 1996). In contrast to initiating the response with mechanical stimulation, however, microinjecting either IP_3 or cADPR enables the response to be attributed to the injected compound.

The role of IP_3 diffusion in mediating the propagation of intercellular Ca^{2+} waves was investigated with the phospholipase C inhibitor U73122. In the presence of U73122, the number of cells responding to IP_3 injection was correlated with the amount of IP_3 injected, indicating that the number of responding cells was controlled by the amount of IP_3 diffusing from a single cell. Although the number of cells that participated in the intercellular Ca^{2+} waves initiated by IP_3 injection was reduced in the presence of U73122, this does not necessarily demonstrate a requirement for regeneration of IP_3 in propagating the intercellular Ca^{2+} wave because U73122 has been shown to have several non-specific effects (Willems et al., 1994)). Thus, the interpretation of these results is limited to the conclusion that IP_3 diffusion can explain propagation of the intercellular Ca^{2+} wave to a limited number of cells (a maximum of about 18).

The explanation for the U73122 inhibition of cADPR-mediated intercellular Ca^{2+} waves is unknown. However, it may be that either cADPR alone cannot release Ca^{2+} and requires phospholipase C to produce IP_3, or that U73122 inhibits some step required for cADPR-mediated Ca^{2+} release. It is unlikely that cADPR cannot release Ca^{2+} without requiring phospholipase C activation because cADPR releases Ca^{2+} from permeabilized lens cells (our unpublished observations). It is possible that cADPR releases only a small amount of Ca^{2+} and that this Ca^{2+} stimulates phospholipase C to synthesize IP_3, which then propagates the Ca^{2+} wave. This mechanism would require Ca^{2+} activation of phospholipase C, which has been reported in some cell types (Harootunian et al., 1991; Gromada et al., 1995) but not others (Bird et al., 1997). This mechanism is unlikely in lens cells because injecting Ca^{2+} alone does not trigger intercellular Ca^{2+} waves, as would be predicted for a Ca^{2+}-induced IP_3 regeneration mechanism. Alternatively, U73122 has been shown to have several effects on Ca^{2+} signaling other than just phospholipase C inhibition (Willems et al., 1994); thus, U73122 might either inhibit cADPR-mediated Ca^{2+} release directly or deplete cADPR-sensitive Ca^{2+} stores.

The U73122 inhibition of cADPR-mediated Ca^{2+} waves may reveal an important cooperativity between cADPR and IP_3 in mediating Ca^{2+} release and Ca^{2+} waves initiated by cADPR. It may be that the Ca^{2+} channels activated by IP_3 outnumber the Ca^{2+} channels activated by cADPR. The resting concentration of IP_3 should be higher than in cells treated with U73122, and because the IP_3 receptor requires IP_3 for Ca^{2+} activation (Bezprozvanny et al., 1991), the IP_3 receptors in U73122-treated cells could not be activated by a cADPR-mediated increase in $[Ca^{2+}]_i$. In contrast, in cells not treated with U73122, the IP_3 receptors could be activated by a small or localized increase in $[Ca^{2+}]_i$ because of the higher steady-state IP_3 concentration. Thus, in the absence of U73122, the cADPR-mediated increase in $[Ca^{2+}]_i$ could be amplified by IP_3 receptors and coordinated into a Ca^{2+} wave.

Although lens cells exhibit intercellular Ca^{2+} waves in response to the microinjection of either IP_3 or cADPR, the question arises as to whether these results have a physiological relevance. There is much evidence that IP_3 is a physiological mediator of Ca^{2+} increases in lens cells. For example, lens cells synthesize and degrade IP_3 in an agonist-dependent manner (Vivekanandan and Lou, 1989), permeabilized lens cells respond to IP_3 (Duncan et al., 1994), and agonists that operate through IP_3 in other cell types (Berridge, 1993) increase $[Ca^{2+}]_i$ in lens cells (Duncan et al., 1994; Churchill and Louis, 1997). In contrast to IP_3, this is the first report of cADPR increasing $[Ca^{2+}]_i$ in lens cells, and there is currently no information regarding whether agonists or metabolites modulate cADPR concentrations in the lens. Future studies will be required to determine whether cADPR may play a physiological role in mediating intercellular Ca^{2+} waves between lens cells or indeed any other cell type.

In summary, this study has demonstrated that both Ca^{2+} and IP_3 can mediate intercellular Ca^{2+} waves by passing through gap junction channels. It has also demonstrated for the first time that cADPR can mediate intercellular Ca^{2+} waves by passing through gap junction channels. The phospholipase C inhibitor U73122 blocked intercellular Ca^{2+} waves initiated by mechanical stimulation and injection of cADPR, but not those initiated by injection of IP_3. This indicates that IP_3 but not cADPR is necessary and sufficient to propagate intercellular Ca^{2+} waves between sheep lens cells.

This research was supported by the National Institutes of Health Grant EY-05684.

REFERENCES

Allbritton, N. L., Meyer, T. and Stryer, L. (1992). Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science* **258**, 1812-1815.

Allen, V., Swigart, P., Cheung, R., Cockcroft, S. and Katan, M. (1997). Regulation of inositol lipid-specific phospholipase Cδ by changes in Ca^{2+} ion concentrations. *Biochem. J.* **327**, 545-552.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315-325.

Bezprozvanny, I., Watras, J. and Ehrlich, B. E. (1991). Bell-shaped calcium response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* **351**, 751-754.

Bird, G. S. J., Obie, J. F. and Putney, J. W. (1997). Effect of cytoplasmic Ca^{2+} on (1,4,5)IP_3 formation in vasopressin-activated hepatocytes. *Cell Calcium* **21**, 253-256.

Boitano, S., Dirksen, E. R. and Sanderson, M. J. (1992). Intercellular propagation of calcium waves mediated by inositol trisphosphate. *Science* **258**, 292-295.

Christ, G. J., Moreno, A. P., Melman, A. and Spray, D. C. (1992). Gap junction-mediated intercellular diffusion of Ca^{2+} in cultured human corporal smooth muscle cells. *Am. J. Physiol.* **263**, C373-383.

Churchill, G. C., Atkinson, M. M. and Louis, C. F. (1996). Mechanical stimulation initiates cell-to-cell calcium signaling in ovine lens epithelial cells. *J. Cell Sci.* **109**, 355-365.

Churchill, G. C. and Louis, C. F. (1997). Stimulation of P_{2U} purinergic or α_{1A} adrenergic receptors mobilizes Ca^{2+} in lens cells. *Invest. Ophthalmol. Vis. Sci.* **38**, 855-865.

Clapham, D. E. (1995). Calcium signaling. *Cell* **80**, 259-268.

Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S. and Smith, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: Long range glial signalling. *Science* **247**, 470-473.

Dandrea, P. and Vittur, F. (1997). Propagation of intercellular Ca^{2+} waves in mechanically stimulated articular chondrocytes. *FEBS Lett.* **400**, 58-64.

DeLisle, S. and Welsh M. J. (1992). Inositol trisphosphate is required for the propagation of calcium waves in Xenopus oocytes. *J. Biol. Chem.* **267**, 7963-7966.

Demer, L. L., Wortham, C., Dirksen, E. R. and Sanderson, M. J. (1993). Mechanical stimulation induces intercellular calcium signaling in bovine aortic endothelial cells. *Am. J. Physiol.* **264**, H2094-H2102.

Deutsch, D. E., Williams, J. A. and Yule, D. I. (1995). Halothane and octanol block Ca^{2+} oscillations in pancreatic acini by multiple mechanisms. *Am. J. Physiol.* **269**, G779-788.

Duncan, G., Webb, S. F., Dawson, A. P., Bootman, M. D. and Elliott, A. J. (1993). Calcium regulation in tissue-cultured and human and bovine lens epithelial cells. *Invest. Ophthalmol. Visual Sci.* **34**, 2835-2842.

Duncan, G., Williams, M. R. and Riach, R. A. (1994). Calcium, cell signalling and cataract. *Prog. Retinal Eye Res.* **13**, 623-652.

Goodenough, D. A., Goliger, J. A. and Paul, D. L. (1996). Connexins, connexons, and intercellular communication. *Ann. Rev. Biochem.* **65**, 475-502.

Gromada, J., Jorgensen, T. D. and Dissing, S. (1995). Cyclic ADP-ribose and inositol 1,4,5-triphosphate mobilizes Ca^{2+} from distinct intracellular pools in permeabilized lacrimal acinar cells. *FEBS Lett.* **360**, 303-306.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.

Hansen, M., Boitano, S., Dirksen, E. R. and Sanderson, M. J. (1993). Intercellular calcium signaling induced by extracellular adenosine 5′-triphosphate and mechanical stimulation in airway epithelial cells. *J. Cell Sci.* **106**, 995-1004.

Hansen, M. S., Boitano, S., Dirksen, E. R. and Sanderson, M. J. (1995). A role for phospholipase C but not ryanodine receptors in the initiation and propagation of intercellular calcium waves. *J. Cell Sci.* **108**, 2583-2590.

Harootunian, A. T., Kao, J. P., Paranjape, S. and Tsien, R. Y. (1991). Generation of calcium oscillations in fibroblasts by positive feedback between calcium and IP_3. *Science* **251**, 75-78.

Jaffe, L. (1993). Classes and mechanisms of calcium waves. *Cell Calcium* **14**, 736-745.

Kasai, H. and Petersen, O. H. (1994). Spatial dynamics of second messengers: IP_3 and cAMP as long-range and associative messengers. *Trends Neurosci.* **17**, 95-101.

Lee, H. C., Galione, A. and Walseth, T. F. (1994). Cyclic ADP-ribose: metabolism and calcium mobilizing function. *Vitamins and Hormones* **48**, 199-257.

Loessberg-Stauffer, P., Zhao, H., Luby-Phelps, K., Moss, R. L., Star, R. A. and Muallem, S. (1993). Gap junction communication modulates $[Ca^{2+}]i$ oscillations and enzyme secretion in pancreatic acini. *J. Biol. Chem.* **268**, 19769-19775.

Meda, P. (1996). Gap junction involvement in secretion: the pancreas experience. *Clin. Exp. Pharm. Physiol.* **23**, 1053-1057.

Meissner, G. (1994). Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Ann. Rev. Physiol.* **56**, 485-508.

Meyer, T. and Stryer, L. (1991). Calcium spiking. *Ann. Rev. Biophys. Biophys. Chem.* **20**, 153-174.

Paterson, P. A. and Delamere, N. A. (1992). The lens. In *Adler's Physiology of the Eye* (ed. W. H. Hart, Jr.), pp. 348-390. Mosby Year Book, St. Louis.

Petersen, O. H. (1995). Inositol trisphosphate and cyclic ADP ribose as long range messengers generating local subcellular calcium signals. *J. Physiol.* **89**, 125-127.

Potter, B. V. L. (1990). Recent advances in the chemistry and biochemistry of inositol phosphates of biological interest. *Nat. Prod. Rep.* **7**, 1-24.

Robb-Gaspers, L. D. and Thomas, A. P. (1995). Coordination of Ca^{2+} signaling by intercellular propagation of Ca^{2+} waves in the intact liver. *J. Biol. Chem.* **270**, 8102-8107.

Rose, B. and Loewenstein, W. R. (1975). Calcium ion distribution in cytoplasm visualised by aequorin: diffusion in cytosol restricted by energized sequestering. *Science* **190**, 1204-1206.

Saez, J. C. Connor, J. A., Spray, D. C. and Bennet, M. V. L. (1989). Hepatocyte gap junctions are permeable to the second messenger inositol 1,4,5-trisphosphate and to calcium ions. *Proc. Nat. Acad. Sci. USA* **86**, 2708-2712.

Sanderson, M. J., Charles, A. C. and Dirksen, E. R. (1990). Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. *Cell Regul.* **1**, 585-596.

Sanderson, M. J., Charles, A. C., Boitano, S. and Dirksen, E. R. (1994). Mechanisms and function of intercellular calcium signaling. *Mol. Cell. Endocrinol.* **98**, 173-187

Sanderson, M. J. (1995). Intercellular calcium waves mediated by inositol trisphosphate. *Ciba Found. Symp.* **188**, 175-94.

Sneyd, J., Wetton, B. T., Charles, A. C. and Sanderson, M. J. (1995). Intercellular calcium waves mediated by diffusion of inositol (1,4,5)-trisphosphate: a two-dimensional model. *Am. J. Physiol.* **268**, C1357-C1545.

TenBroek, E. M., Johnson, R. G. and Louis, C. F. (1994). Cell-to-cell communication in a differentiating ovine lens culture system. *Invest. Ophthalmol. Vis. Sci.* **35**, 215-228.

Thompson, A. K., Mostafapour, S. P., Denlinger, L. C., Bleasdale, J. E. and Fisher, S. K. (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.* **266**, 23856-23862.

Tsien, R. W. and Tsien, R. Y. (1990). Calcium channels, stores, and oscillations. *Ann. Rev. Cell Biol.* **6**, 715-60.

Vivekanandan, S. and Lou, M. F. (1989). Evidence for the presence of phosphoinositide cycle and its involvement in signal transduction in the rabbit lens. *Curr. Eye Res.* **8**, 101-112.

Willems, P. H., Van de Put, F. H., Engbersen, R., Bosch, R. R., Van Hoof H. J. and de Pont, J. J. (1994). Induction of Ca^{2+} oscillations by selective, U73122-mediated, depletion of inositol-trisphosphate-sensitive Ca^{2+} stores in rabbit pancreatic acinar cells. *Eur. J. Physiol.* **427**, 233-243.

Yule, D. I., Stuenkel, E. and Williams, J. A. (1996). Intercellular calcium waves in rat pancreatic acini: mechanism of transmission. *Am. J. Physiol.* **271**, C1285-1294.