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Hepatocyte Growth Factor Disrupts Cell Contact and Stimulates an Increase in Type 3 Inositol Triphosphate Receptor Expression, Intracellular Calcium Levels, and Apoptosis of Rat Ovarian Surface Epithelial Cells

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The present studies revealed that hepatocyte growth factor (HGF) disrupts cell contact, increases both type 3 IP_3 receptor and intracellular calcium ($[Ca^{2+}]_i$) levels and induces apoptosis of rat ovarian surface epithelial cells (ROSE-179 cells). Type 3 IP_3 receptor was only increased in cells that lost cell contact. Disrupting cell contact by depleting extracellular calcium (Ca^{2+}) also resulted in an increase in $[Ca^{2+}]_i$ levels and an increase in apoptosis. These responses were prevented by the addition of 0.7 m*M* Ca^{2+}. Actinomycin D and cycloheximide prevented apoptosis that resulted from Ca^{2+} removal. *In situ* hybridization studies revealed that type 3 IP_3 receptor was expressed at relatively low levels by ROSE-179 cells cultured with Ca^{2+} but at high levels in the absence of Ca^{2+}. ROSE-179 cells cultured in Ca^{2+}-free medium with type 3 IP_3 receptor antisense oligonucleotide lost cell contact but did not show an increase in either type 3 IP_3 receptor protein, $[Ca^{2+}]_i$, or apoptosis. The nonsense oligonucleotide did not alter these responses to Ca^{2+} removal. Thus, the disruption of cell contact by either HGF or Ca^{2+} depletion increases the expression of type 3 IP_3 receptor, which causes an increase in $[Ca^{2+}]_i$ and the apoptotic death of ROSE-179 cells.

Key Words: Rat; ovarian surface epithelial cells; intracellular calcium; IP_3 receptor; apoptosis; hepatocyte growth factor.

Introduction

It is well-known that numerous hormones and growth factors prevent cells from undergoing apoptosis *(1–3)*. Nonhormonal factors such as interactions with either extracellular matrix proteins or other cells also inhibit apoptosis *(4–8)*. For example, cell contact has been shown to inhibit granulosa cells *(9)*, ovarian surface epithelial cells *(10)*, bronchial epithelial cells *(11)*, and leukemic B-lymphocytes *(12)* from undergoing apoptosis. Although the signal transduction pathways associated with extracellular matrix interaction are beginning to be identified, very little is known about the mechanism by which cell contact prevents apoptosis *(13–15)*. Recent studies have shown that N-cadherin-mediated cell contact preserves the viability of rat granulosa cells *(9)* and cells derived from the rat ovarian surface epithelium (i.e., ROSE-179 cells) *(10)*. In these cells, it is likely that cell contact maintains viability by activating fibroblast growth factor receptors *(10)*.

Conversely, hepatocyte growth factor (HGF) triggers ROSE-179 cell apoptosis by decreasing cell contact *(16)*. This in turn leads to a prolonged increase in intracellular calcium levels ($[Ca^{2+}]_i$) *(16)*. Once $[Ca^{2+}]_i$ levels are elevated, ROSE-179 cells undergo apoptosis without any further requirement for RNA or protein synthesis *(16)*. Although HGF's ability to reduce cell contact and increase $[Ca^{2+}]_i$ is dependent on *de novo* RNA and protein synthesis *(16)*, it is not clear which genes are involved in mediating HGF's action.

Inositol triphosphate (IP_3) receptors may be involved in initiating the increase in $[Ca^{2+}]_i$ in ROSE-179 cells. The IP_3 receptors are a family of gene products that promote the release of Ca^{2+} from intracellular stores on binding IP_3 *(17,18)*. There is considerable homology among these receptors. For example, these receptors are approx 300 kDa and have 62 and 64% identity in their amino acid sequences *(17)*. However, the affinity of these three receptors varies with type 2 having the highest and type 3 the lowest affinity *(19)*.

Type 1, 2, and 3 IP_3 receptors have been detected within the ovary *(20–22)*. In the ovary, type 1 is the most readily detectable by Western blot analysis, whereas type 3 IP_3 receptor is expressed at extremely low levels *(20)*. Interestingly, the type 3 IP_3 receptor is likely to be involved in

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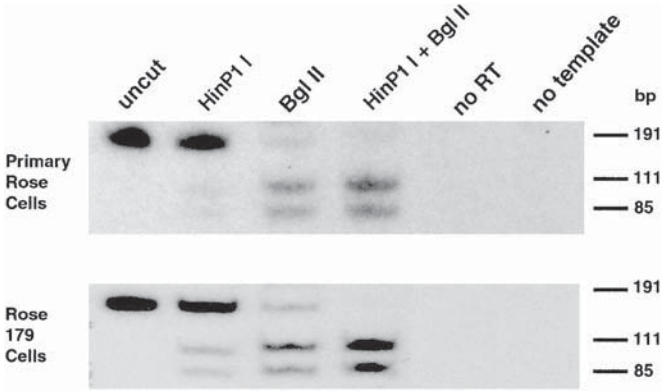

Fig. 1. Detection of type 1 and 3 IP_3 receptor mRNA in RNA isolated from primary ovarian surface epithelial cells **(top)** and ROSE-179 cells **(bottom)**. An RT-PCR reaction was run on each sample. An aliquot of each reaction was either not digested (uncut) or digested with *Hin*P1 I, *Bgl* II, or both. As negative controls, separate RT-PCRs were conducted in which either the reverse transcriptase (no RT) or mRNA template (no template) was omitted from the RT-PCR reaction. In this procedure, exposure to *Hin*P1 I cleaves the type 3 IP_3 receptor (fragments ≤ 111 bp), whereas *Bgl*II digests type 1 IP_3 receptor (fragments ≤ 111 bp). When RT-PCR product was subjected to digest with both enzymes, all the RT-PCR product was cleaved.

promoting a sustained increase in $[Ca^{2+}]_i$ in ROSE-179 cells, because it mediates a prolonged increase in $[Ca^{2+}]_i$ and plays an essential role in apoptosis of T- and B-lymphocytes *(23,24)*. Therefore, the present studies were designed to determine whereas if type 1 and 3 IP_3 receptors are selectively expressed by primary ovarian surface epithelial cells and ROSE-179 cells, and whether these IP_3 receptors are involved in regulating sustained increases in $[Ca^{2+}]_i$ and subsequent apoptosis of ROSE-179 cells.

Results

Using the reverse transcriptase polymerase chain reaction (RT-PCR) ratio method, mRNAs that encode type 1 and type 3 IP_3 receptors were detected in RNA isolated from primary ovarian surface epithelial cells. *Hin*P1 I, which cleaves type 3 IP_3 receptor mRNA into 106- and 80-bp fragments, did not digest much of the RT-PCR product. By contrast, *Bgl*II, which digests type 1 IP_3 receptor mRNA into 111- and 85-bp fragments, cleaved most of the RT-PCR product. Together, both enzymes completely digested RT-PCR product. These findings indicated that in primary ovarian surface epithelial cells more transcripts encode type 1 than type 3 IP_3 receptor (Fig. 1, top). A similar pattern of expression was observed in ROSE-179 cells, although ROSE-179 cells had a higher type 3: type 1 ratio than did primary ovarian surface epithelial cells (Fig. 1, bottom). In both primary ovarian surface epithelial cells and ROSE-179, approx 80% of the IP_3 receptor mRNA was type 1. Standard RT-PCR also detected type 1 and 3 IP_3 receptors in ROSE-179 cells (Fig. 2A). Analysis of these PCR products revealed sequences that were identical to the published sequences of rat type 1 and type 3 IP_3 receptors, respectively. Type 2 IP_3 receptor could not be detected by RT-PCR (data not shown). The presence of type 1 and 3 IP_3 receptors was also confirmed by Western blot analysis (Fig. 2B). However, usually more protein was required to be loaded on the gel to detect type 3 IP_3 receptor compared to type 1 IP_3 receptor. In addition, the SuperSignal ULTRA detection reagent (Piercer,Rockford, IL) had to be used at full strength to visualize the type 3 IP_3 receptor as opposed to a 1:5 dilution for the type 1 IP_3 receptor.

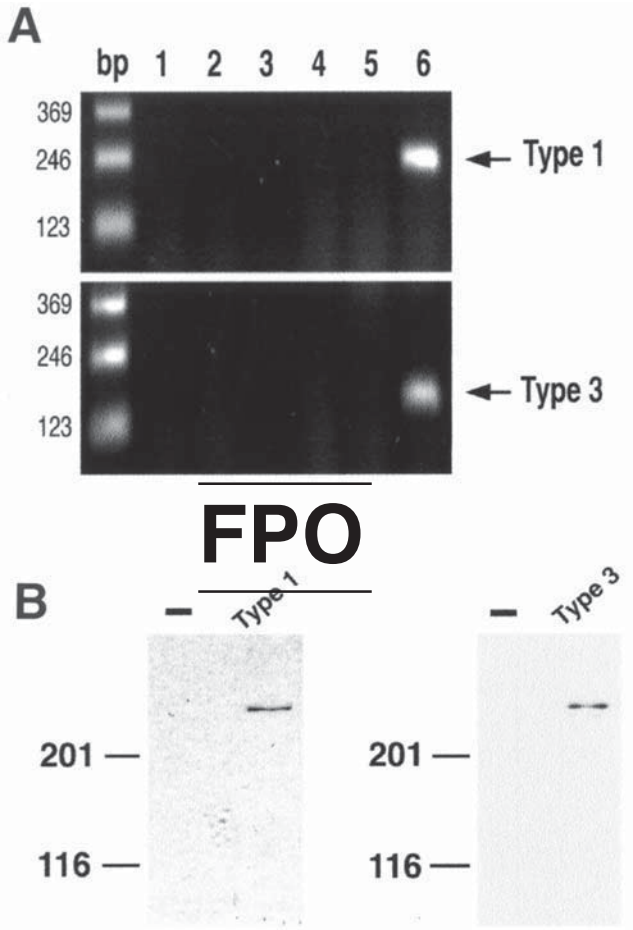

Fig. 2. Expression of type 1 and 3 IP_3 receptor mRNA **(A)** and protein **(B)** in ROSE-179 cells. In (A), ROSE-179 cell mRNA was detected by RT-PCR using specific primers for each isoform. Arrows indicate a product of the expected size. In lanes 1 and 2, RT-PCRs were conducted in the absence of the sense and antisense primer, respectively. In lane 3, the template was omitted, and in lanes 4 and 5 the RT was omitted. Lane 6 represents mRNA isolated from ROSE-179 cells. The Western blot analysis (B) shows that type 1 and 3 IP_3 receptor proteins are expressed in ROSE-179 cells. Western blots conducted without the primary antibody served as a negative control (–).

Having determined that both type 1 and 3 IP_3 receptors are expressed in ROSE-179 cells, we designed the next series of experiments to determine whether the ratio of type 1:type 3 receptor changes as ROSE-179 cells undergo

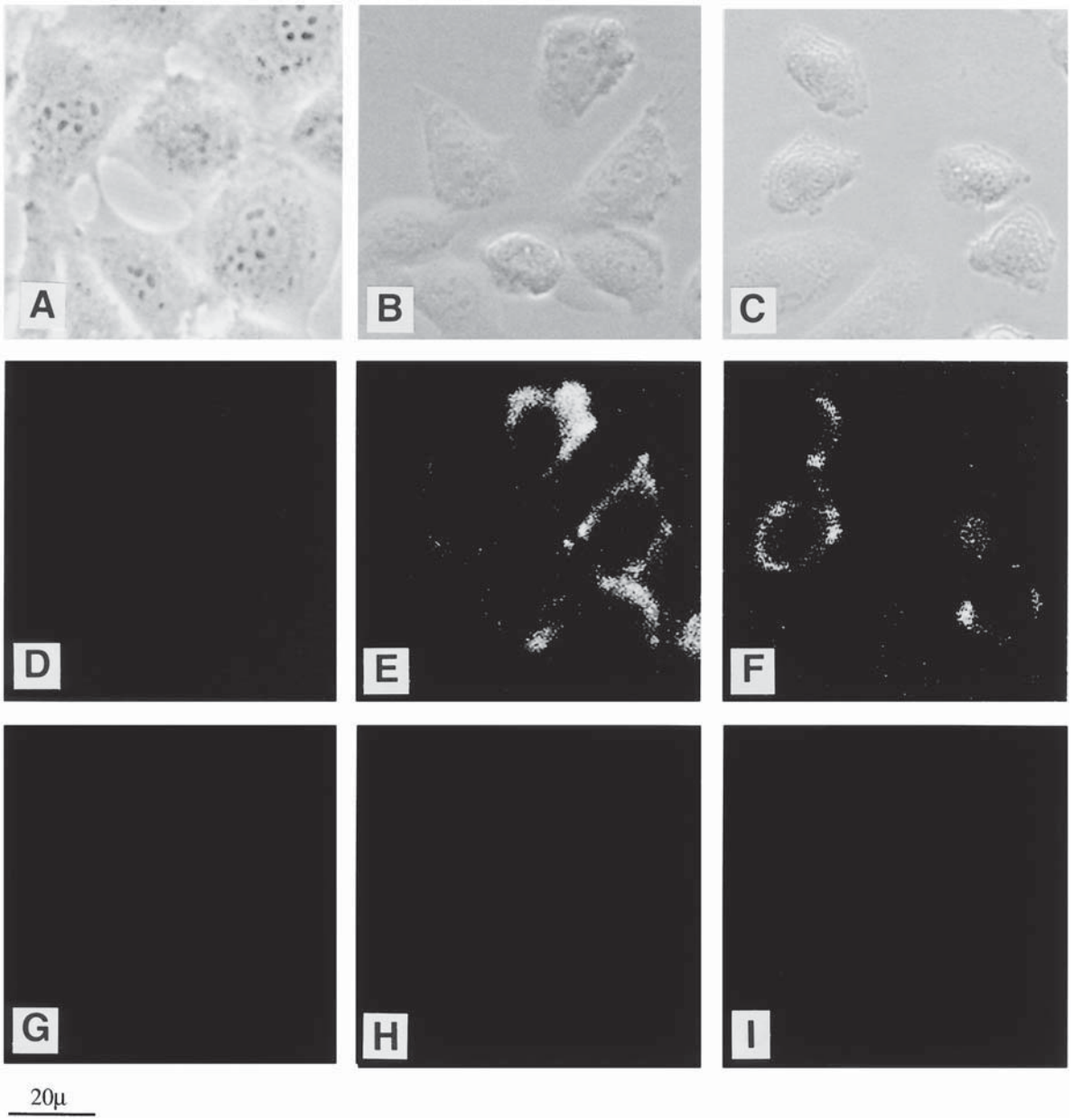

Fig. 3. The effect of HGF and calcium depletion on annexin V and propidium iodide staining. In this study, ROSE-179 cells were cultured for 3 h in either serum-free calcium-supplemented media in the absence (controls) **(A, D, G)** or presence of HGF **(B, E, H)**. Cells were also cultured for 3 h in serum-free calcium-free media **(C, F, I)**. Cells were shown under phase optics (A, B, C), or stained with annexin V (D, E, F) or propidium iodide (G, H, I).

apoptosis. Previously published work has demonstrated that HGF induced a decrease in cell contact within 1 h, a 2- to 3-fold increase in $[Ca^{2+}]_i$ by 3 h, and an increase in apoptotic nuclei by 6 h *(16)*. Although many cells had lost contact and $[Ca^{2+}]_i$ levels were elevated by 3 h, the cells did not possess apoptotic nuclei as judged by hydroethidine staining. To confirm that cells were undergoing apoptosis by 3 h after HGF, cells were costained with annexin V and propidium iodide. As a positive control, cells were fixed with 10% formalin and stained with propidium iodide. Fixation resulted in 100% of the cells staining with propidium iodide. In this assay, the ability to bind annexin V but not stain with propidium iodide indicated that the cells were in the early stages of apoptosis *(25)*. Compared to controls (e.g., cells cultured in serum-free calcium-supplemented media) (Fig. 3A,D,G), cells that showed a lost or reduced cell contact in response to HGF bound annexin V but did not stain with propidium iodide (Fig. 3B,E,H). Together with the previously published data, these observations indicate that 3 h after HGF is an optimal time to assess the expression of IP_3 receptors.

As shown in Fig. 4, the total amount of IP_3 receptor RT-PCR product was increased by 3 h after HGF treatment (cf. the uncut control vs HGF lanes). HGF did not significantly

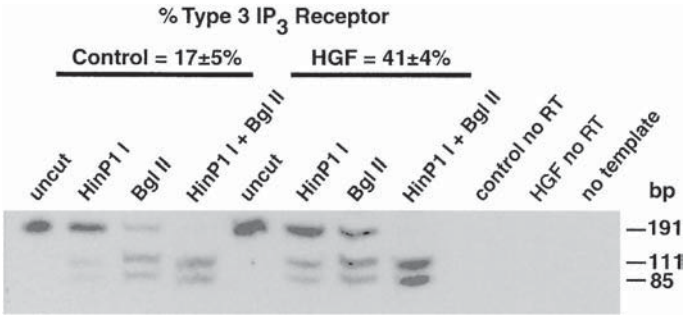

Fig. 4. The effect of HGF on the relative levels of type 1 and 3 IP_3 receptor mRNA. These mRNAs were detected using the RT-PCR ratio method. Representative RT-PCR reactions are shown. On the right half, uncut, *Hin*P1 I, *Bgl* II, and *Hin*P1 I + *Bgl*II digests are shown for ROSE-179 cells cultured for 3h in serum-free medium. In the middle are the same digest treatments for ROSE-179 cells cultured for 3h in serum-free medium with HGF. The appropriate controls for these RT-PCR reactions were conducted as described in Fig. 1. The relative amount of type 3 IP_3 receptor mRNA, as a percentage of all RT-PCR products, is shown for both control and HGF treatments. These values represent the mean ± SE of four control and eight HGF-treated samples. These samples were collected from two separate experiments in which the controls were run in duplicate and the HGF samples in quadruplicate.

change the relative level of type 1 IP_3 receptor mRNA (cf. the amount of the ≤111-bp fragments in the *Bgl*II lanes of the control and HGF). However, HGF increased the amount of mRNA that encodes the type 3 IP_3 receptor (cf. the amount of ≤111-bp fragments in the *Hin*P1 I lanes of the control to that of HGF). Although all the RT-PCR reactions for each experiment were run at the same time under the same conditions, there could be slight variations in each reaction tube. To account for this and other potential sources of error, quantitative comparisons were made between the bands that represent the type 1 (191 bp) and type 3 (≤111-bp) IP_3 receptor mRNA in the *Hin*P1 I digest. This comparison revealed that by 3 h after HGF, the percentage of type 3 IP_3 mRNA increased from 17 ± 5 to 41 ± 4% ($p < 0.05$; Fig. 4).

The cellular distribution of type 1 and 3 IP_3 receptor protein was assessed by immunocytochemistry. In control media, type 1 IP_3 receptor was observed within the perinuclear region of the cytoplasm and at the plasma membrane. Neither the cellular distribution nor the relative amount of type 1 IP_3 receptor protein was affected by HGF. Similarly, the level of expression and cellular localization of the type 1 IP_3 receptor were independent of cell contact, as clearly observed by confocal microscopy (Fig. 5). By contrast, the type 3 IP_3 receptor was detected exclusively within the perinuclear region of the cytoplasm. HGF did not influence the localization of the type 3 IP_3 receptor protein, although HGF increased the amount of this receptor within cells in which cell contact was either reduced or lost (Fig. 6).

Because HGF disrupts cell contact, experiments were conducted to determine whether simply disrupting cell contact mimics HGF's actions. In these studies, cell contact was disrupted by depleting extracellular Ca^{2+}. Depletion of Ca^{2+} resulted in cell contacts being broken by 1 h. Cells that lost contact bound annexin V but did not stain with propidium iodide (Fig. 3C,F,I). By contrast, cells cultured in serum-free medium supplemented with 0.7 m*M* $CaCl_2$ maintained cell contact, did not bind annexin V, and did not stain with propidium iodide (Fig. 3A,D,G).

Intracellular calcium levels were relatively constant in cells cultured in the presence of 0.7 m*M* $CaCl_2$. Removal of extracellular Ca^{2+} resulted in a two to three fold increase in $[Ca^{2+}]_i$ within 2 h (Fig. 7). These elevated levels were maintained from 2 to 6 h after Ca^{2+} removal compared to that of cells cultured with supplemental Ca^{2+} ($p < 0.05$). Apoptotic nuclei were frequently observed by 6 h after Ca^{2+} depletion (Fig. 8A). The number of apoptotic nuclei was decreased by Ca^{2+} supplementation (Fig. 8A; $p < 0.05$). Treatment with either actinomycin D (ACT-D) ($p < 0.05$) or cycloheximide (CHX) ($p < 0.05$) reduced the number of apoptotic nuclei associated with Ca^{2+} depletion (Fig. 8B).

In situ hybridization (ISH) studies revealed that type 3 IP_3 receptor mRNA was present in cells cultured in 0.7 m*M* $CaCl_2$, albeit at very low levels (Fig. 9A). An increase in type 3 IP_3 receptor mRNA was observed within ROSE-179 cells that had either a reduction or loss of cell contact 3 h after the removal of extracellular Ca^{2+} (Fig. 9B). Confocal microscopic analysis revealed relatively low levels of type 3 IP_3 protein within the cytoplasm of ROSE-179 cells that maintained cell contact (Fig. 10). Like mRNA levels, levels of type 3 IP_3 receptor protein increased in cells that lost contact in response to depletion of extracellular Ca^{2+} (Fig. 10). Regardless of whether the cells were aggregated or not, type 3 IP_3 receptor was always localized within the perinuclear region of the cytoplasm and not to the plasma membrane (Fig. 10).

These observations suggest a role for type 3 IP_3 receptors in inducing $[Ca^{2+}]_i$ and subsequent apoptosis. To test this hypothesis, ROSE-179 cells were cultured in Ca^{2+}-free medium with either nonsense or type 3 IP_3 receptor antisense oligonucleotide. In both nonsense and antisense oligonucleotide treatment groups, many cell contacts were disrupted in response to Ca^{2+} depletion. As seen in Fig. 11A, cells that lost contact in the presence of the nonsense oligonucleotide possessed relatively high levels of type 3 IP_3 receptor protein as assessed by intensity of the fluorescent staining (Fig. 11C). Conversely, antisense oligonucleotide treatment attenuated the Ca^{2+} depletion-induced increase in type 3 IP_3 receptor protein in cells that had lost contact (cf. Fig. 11C with D). Quantitative assessment of the fluorescent intensity associated with type 3 IP_3 receptor protein staining confirmed that type 3 IP_3 receptor antisense oligonucleotide treatment suppressed type 3 IP_3 receptor expression compared to treatment with nonsense oligonucleotide ($p < 0.05$; Fig. 12). Type 3 IP_3 receptor antisense oligonucleotide treatment also suppressed $[Ca^{2+}]_i$ levels

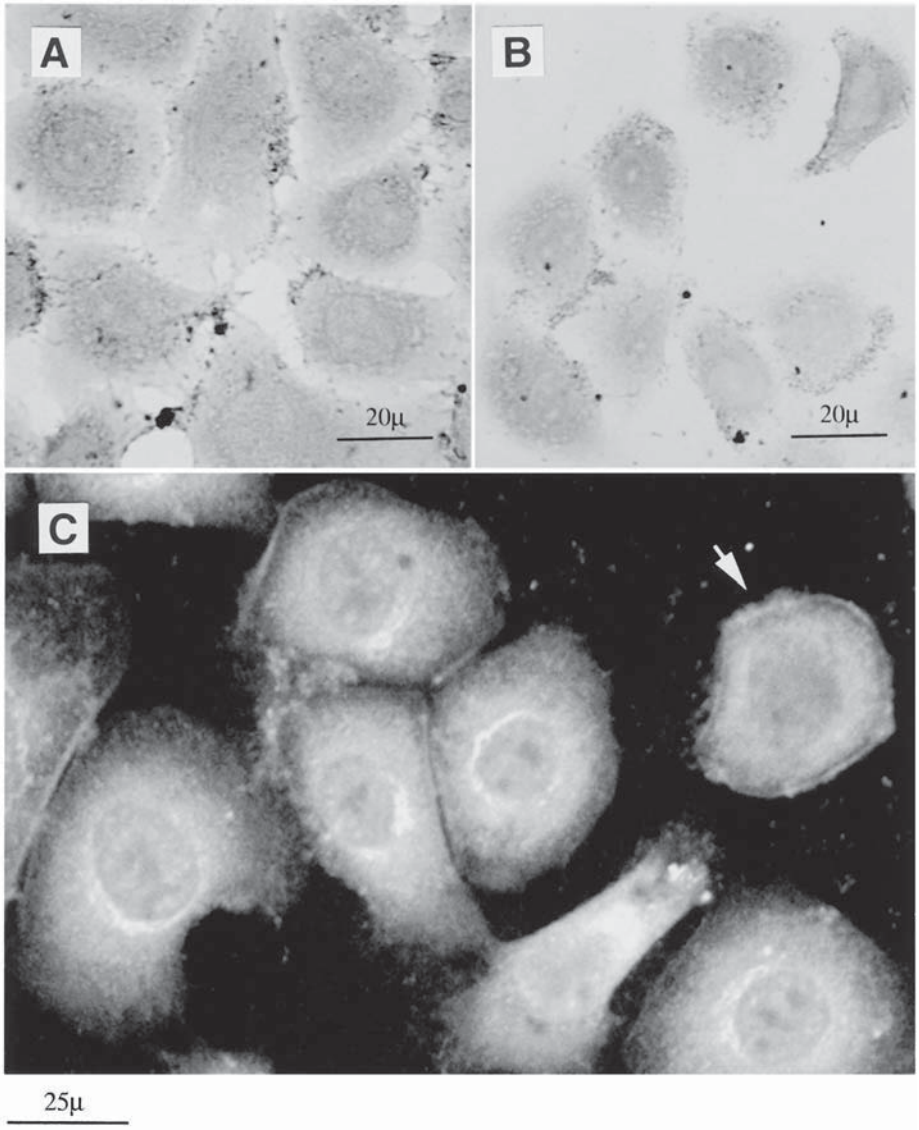

Fig. 5. The effect of HGF on the localization of type 1 IP_3 receptor protein. In control cultures **(A)**, type 1 IP_3 receptor protein was localized to both the perinuclear region of the cytoplasm and the plasma membrane. The most intense staining was observed at the plasma membrane. Often IP_3 receptor protein appeared to be concentrated at the point of cell-cell contact. HGF disrupted cell contact but did not alter the distribution pattern of IP_3 receptor protein **(B)**. The perinuclear and plasma membrane localization of IP_3 receptor protein could best be seen in cells prepared for confocal microscopic analysis **(C)**. Note that evens cells that had lost cell contact, IP_3 receptor protein can still be detected at the plasma membrane.

($p < 0.05$) and apoptosis ($p < 0.05$) compared to nonsense oligonucleotide treatment (Fig. 12).

Discussion

Although previous studies have demonstrated that type 1 and 3 IP_3 receptors are present within the ovary *(20–22)*, this is the first study to assess the expression of these receptors specifically within the ovarian surface epithelial cells. The present studies show that types 1 and 3 IP_3 receptors are expressed by primary rat ovarian surface epithelial cells and ROSE-179 cells. Furthermore, viable ovarian surface epithelial cells express more of the type 1 than type 3 IP_3 receptor. This conclusion is based on the detection of mRNAs by the RT-PCR ratio method. This method is well suited for this type of comparison because the same primers are used to detect both type 1 and 3 IP_3 receptors within the same sample. Unfortunately, it is not possible to use Western blot analysis to confirm that there is more type 1 and 3 IP_3 receptor expressed by ROSE-179 cells because antibodies that bind IP_3 receptors may have different binding affinities and are generated in different species. However, the observation that it is more difficult to detect type 3 than 1 IP_3 receptor by Western blot analysis is consistent with our RT-PCR findings.

Based on the data generated by the RT-PCR ratio method, it appears that ovarian surface epithelial cells and

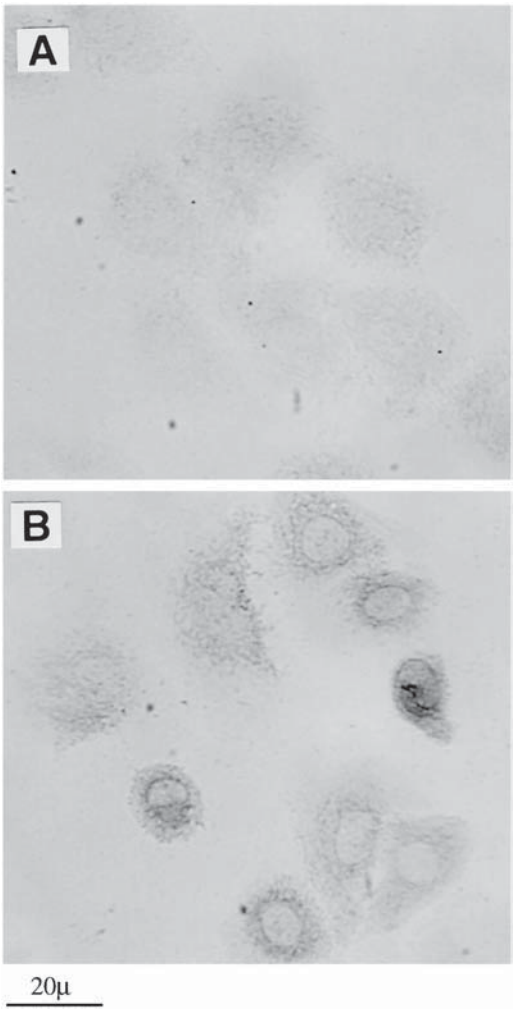

Fig. 6. The effect of HGF on the localization of type 3 IP_3 receptor protein. In control cultures **(A)**, type 3 IP_3 receptor protein was barely detectable. Unlike type 1 IP_3 receptor, type 3 was localized to the perinuclear region but not the plasma membrane. After HGF treatment **(B)**, the relative amount of type 3 IP_3 receptor protein staining increased in cells that had lost or were losing cell contact.

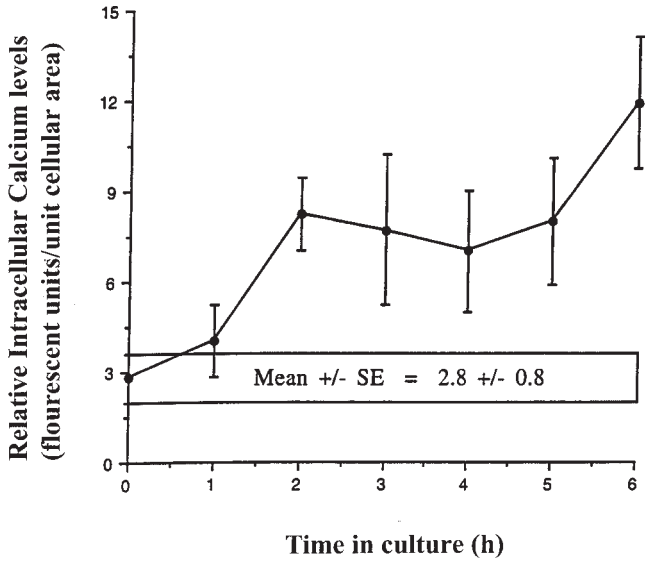

Fig. 7. Effect of Ca^{2+} removal on temporal changes in $(Ca^{2+})_i$ levels. $(Ca^{2+})_i$ levels were monitored at hourly intervals and expressed as a mean ± standard (SE). In cells treated with supplemental Ca^{2+}, $[Ca^{2+}]_i$ levels did not significantly change over the 6-h culture period. Therefore, these values were pooled and shown as a mean ± SE. After 2 h of culture, $[Ca^{2+}]_i$ levels for those cells cultured without supplemental Ca^{2+} were significantly increased ($p < 0.05$) compared to those cultured with Ca^{2+} supplementation.

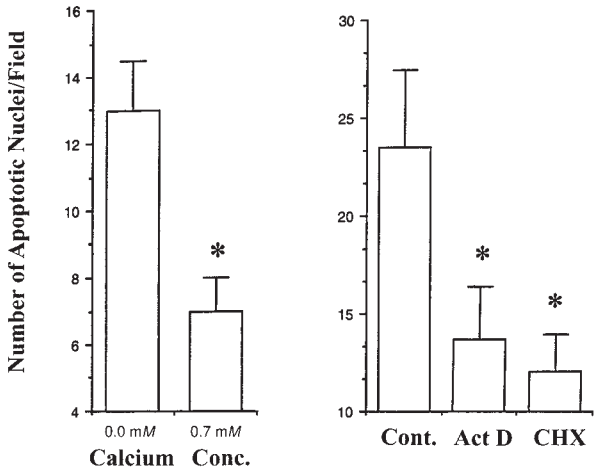

Fig. 8. Effect of Ca^{2+} removal **(A)**, ACT D and CHX **(B)** on ROSE-179 cell apoptosis. In (A), ROSE-179 cells were cultured for 6 h in serum-free medium with or without supplemental $CaCl_2$. In (B), cells were cultured for 6 h without Ca^{2+} (cont) and either ACT-D or CHX. After culture, cells from both experiments were stained with hydroethidine to identify apoptotic nuclei. Values in this and subsequent graphs represent means ± SE. * The value was significantly different from the control value ($p < 0.05$).

ROSE-179 cells express two of the three IP_3 receptors with the major isoform being the type 1 receptor. It is generally thought that genetics determine the type and amount of IP_3 receptor that is expressed by a specific cell type. By contrast, our study demonstrates that the expression pattern of these two IP_3 receptors dramatically changes as ROSE-179 cells undergo apoptosis. As such, the present study is one of just a few that describe changes in the level of IP_3 receptor expression. In other studies, an increase in type 3 IP_3 receptor expression was observed during the apoptosis of lymphocytes *(23,24)* and cardiomyocytes *(26)*. Taken together, these observations support the concept advanced by DeLisle et al. *(27)* that in response to physiological stimuli, cells can change the expression level of a given IP_3 receptor isoform or may even switch to another IP_3 receptor isoform.

In ROSE-179 cells, cell contact maintains Ca^{2+} homeostasis *(9,10)*. Conversely, HGF disrupts cell contact and promotes a sustained increase in $[Ca^{2+}]_i$ levels *(16)*. This sustained increase in $[Ca^{2+}]_i$ commits ROSE-179 cells to die by an apoptotic mechanism *(16)*. Studies utilizing ACT-D and CHX indicate that RNA and protein synthesis is required for HGF to increase $[Ca^{2+}]_i$ levels but not for the subsequent events

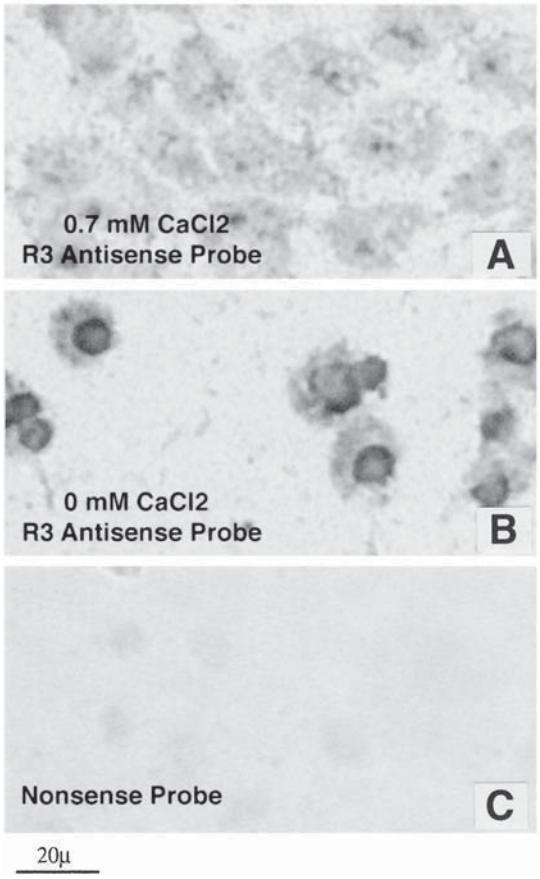

Fig. 9. Effect of Ca^{2+} removal on type 3 IP_3 receptor mRNA expression in ROSE-179 cells as assessed by ISH. ROSE-179 cells were incubated for 3 h in serum-free medium with **(A)** or without **(B)** supplemental $CaCl_2$. The cells were then processed for ISH. R3 antisense oligonucleotide was used to detect type 3 IP_3 receptor mRNA, and nonspecific staining was assessed using a nonsense oligonucleotide **(C)**. The photographs in (A and B) were taken under identical lighting conditions and photographic settings, thereby allowing a legitimate visual comparison. Contrast of the negative control image, shown in (C), was increased to reveal the presence of ROSE-179 cells.

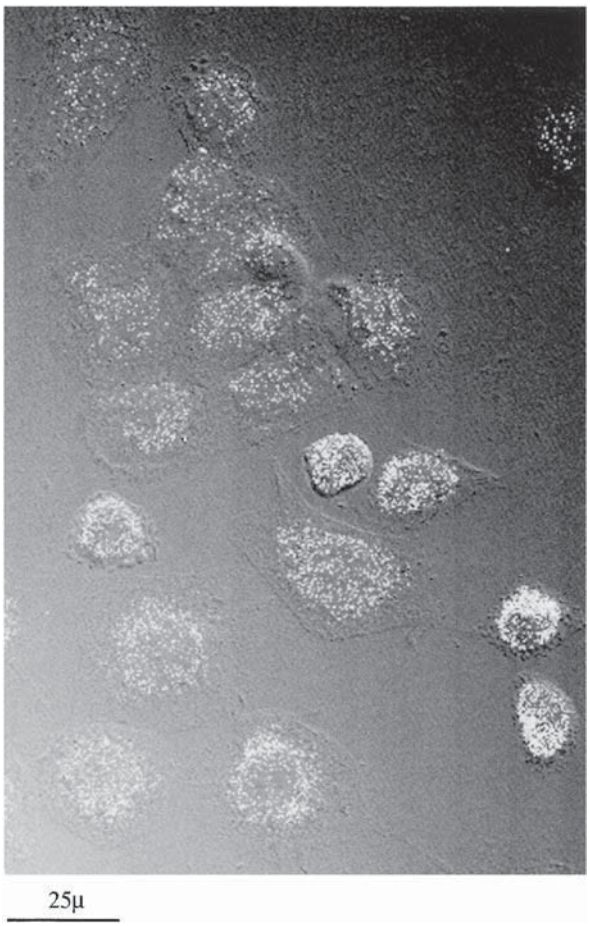

Fig. 10. A differential interference contrast micrograph merged with fluorescent image associated with specific type 3 IP_3 receptor staining. Both images were taken using a confocal microscope from the same focal plane. This merged image revealed that type 3 IP_3 receptor protein is localized to the perinuclear region of the cytoplasm and not to the plasma membrane. In addition, specific staining for type 3 IP_3 receptor in cells that had lost contact with other cells was considerably greater than that observed for cells within aggregates. Note that nonspecific fluorescence was subtracted from the fluorescent image associated with type 3 IP_3 receptor staining prior to being merged with the interference contrast image.

in the apoptotic cascade *(16)*. In nonovarian cells, HGF disrupts cell contact by altering the adhesion proteins, β-catenin and cadherin. Specifically, HGF stimulates the phosphorylation of β-catenin *(28)* and promotes the degradation of cadherin *(29)*. These actions directly lead to the loss of cell contact *(30)*. The present study demonstrates that the disruption of calcium-dependent cell contact is sufficient to increase $[Ca^{2+}]_i$ and induce apoptosis. It is likely, then, that HGF's ability to disrupt cell contact accounts for most of its apoptotic action. It remains to be determined whether HGF disrupts contact by degrading cadherin and/or phosphorylating β-catenin.

Interestingly, RNA and protein synthesis is required for Ca^{2+} depletion to induce apoptosis. This indicates that the loss of cell contact, regardless of whether it is initiated by HGF or Ca^{2+} depletion, activates a genomic mechanism that eventually leads to the apoptotic death of these cells.

Type 3 IP_3 receptors appear to be one of the proteins synthesized in response to a loss of cell contact, because levels of type 3 IP_3 receptor mRNA and protein are elevated only in cells that have lost or are losing contact. This increase in the level of type 3 IP_3 receptor occurs relatively early in the apoptotic cascade. This is based on the observation that annexin V binds only to cells in the early stages of apoptosis *(25,31)*. In the present study, the binding of annexin V is observed only in cells that have lost contact. This is consistent with the previous work by Gatti et al. *(31)* that demonstrates that the binding of annexin V is not detected in cells that maintain cellular adhesion. Furthermore, the binding of annexin V occurs very early in the apoptotic pathway, well before major changes in membrane permeability, as assessed by propidium iodide

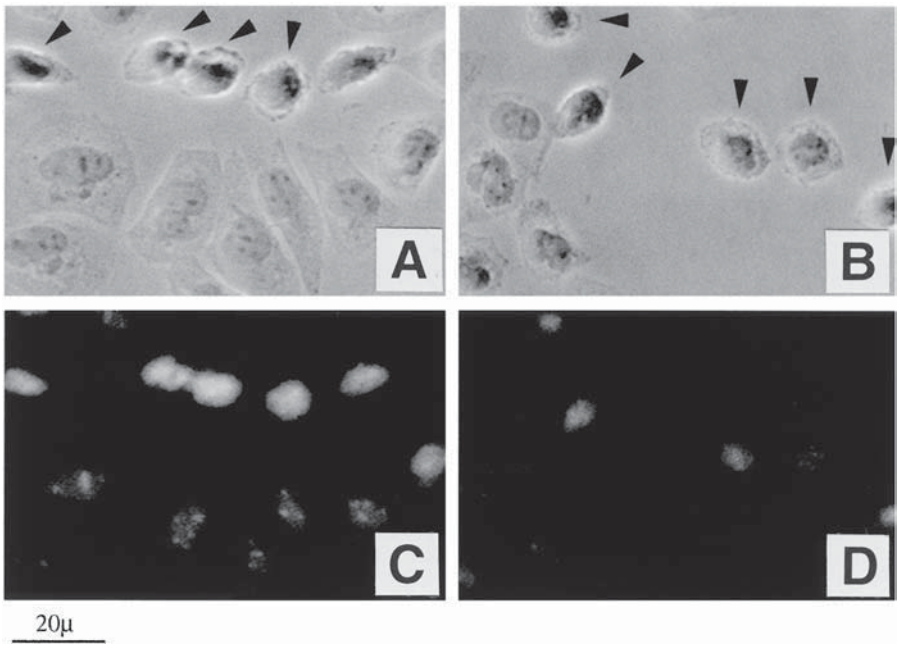

Fig. 11. Effect of type 3 IP_3 receptor nonsense **(A,C)** or antisense **(B,D)** oligonucleotide treatment on cell contact and type 3 IP_3 receptor protein levels. Type 3 IP_3 receptor levels were assessed by immunofluorescence after 3 h of culture. Cells that had lost contact in the presence of nonsense oligonucleotide (arrowheads in [A]) had relatively high amounts of type 3 IP_3 receptor as assessed by fluorescent intensity (C). However, in the presence of type 3 IP_3 receptor antisense oligonucleotide, cells that had lost contact (arrowheads in [B]) had relatively low levels of type 3 IP_3 receptor based on fluorescent intensity (D).

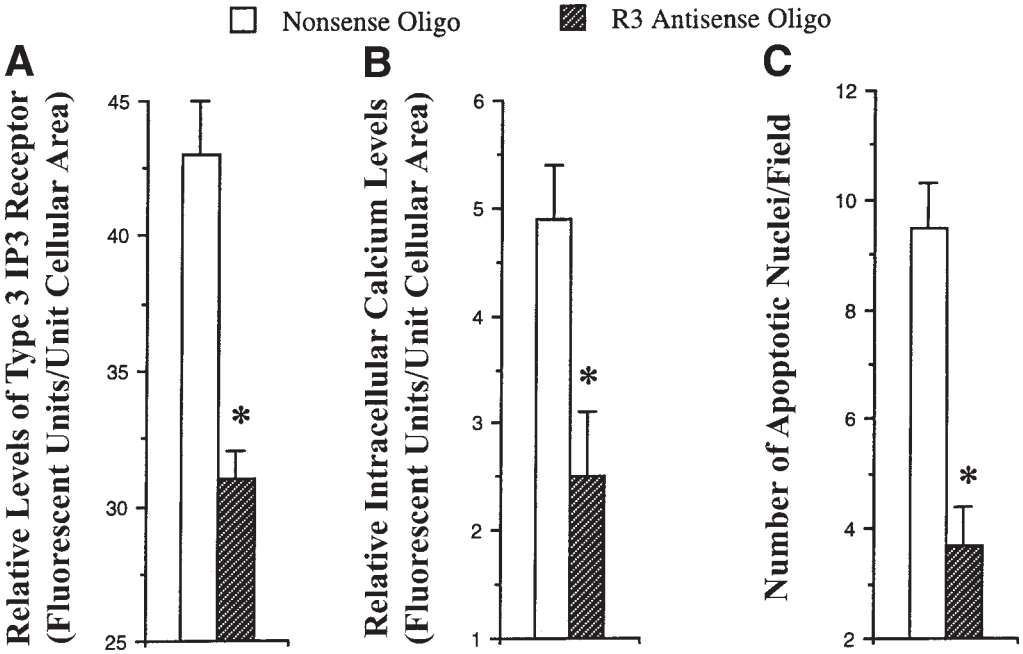

Fig. 12. Effect of type 3 IP_3 receptor antisense oligonucleotide on **(A)** type 3 IP_3 receptor protein levels, **(B)** $[Ca^{2+}]_i$, and **(C)** apoptosis in ROSE-179 cells. ROSE-179 cells were cultured as described in Fig. 11. Type 3 IP_3 receptor protein and $[Ca^{2+}]_i$ levels were assessed after 3 h of culture. Apoptosis was evaluated after 6 h of culture. * A significant difference was found between nonsense and type 3 IP_3 receptor antisense oligonucleotide treatments ($p < 0.05$).

staining or the formation of apoptotic bodies *(32)*. Thus, the enhanced expression of IP_3 receptors is exclusively associated with ROSE-179 cells that are in the early stages of apoptosis as judged by the binding of annexin V.

That the increased expression of type 3 IP_3 receptor is important in regulating $[Ca^{2+}]_i$ and subsequently the apoptotic process is supported by three observations. First, HGF, an inducer of apoptosis, increases the expression of type 3 IP_3 receptor.

Second, the increase in type 3 IP_3 receptor is temporally associated with an increase in $[Ca^{2+}]_i$. Third, treatment with type 3 IP_3 receptor antisense but not nonsense oligonucleotide prevents Ca^{2+} depletion from increasing type 3 IP_3 receptor expression, $[Ca^{2+}]_i$, and apoptosis. Moreover, treatment with type 1 IP_3 antisense oligonucleotide does not prevent apoptosis (unpublished observations). Collectively, these findings suggest that type 3 IP_3 receptors play an essential role in inducing ROSE-179 cells to undergo apoptosis.

Although it is clear that the type 3 IP_3 receptor is involved in promoting an increase in $[Ca^{2+}]_i$, the mechanism through which it mediates this action is unknown. It appears that the type 3 IP_3 receptor–regulated increase in $[Ca^{2+}]_i$ was not due to Ca^{2+} influx, because $[Ca^{2+}]_i$ levels increase when the extracellular Ca^{2+} is depleted. Furthermore, type 3 IP_3 receptor is not localized to the plasma membrane of ROSE-179 cells. The inability to detect this receptor at the plasma membrane is consistent with the concept that membrane Ca^{2+} channels are not activated and do not contribute to the increase in $[Ca^{2+}]_i$ levels during ROSE-179 cell apoptosis. The sustained increase in $[Ca^{2+}]_i$ is more likely the result of the release of Ca^{2+} from its intracellular stores. The present study demonstrates that the type 3 IP_3 receptor is localized to the perinuclear region of the ROSE-179 cells. The detection with the perinuclear region is consistent with the well-known fact that IP_3 receptors are localized to the endoplasmic reticulum *(18)*. Because the endoplasmic reticulum is the major site of Ca^{2+} storage within nonmuscle cells *(33)*, an increase in type 3 IP_3 receptor levels could enhance the response to endogenous IP_3. This would lead to a release of Ca^{2+} from the endoplasmic reticulum and a subsequent increase in $[Ca^{2+}]_i$ *(17,18)*. Furthermore, the binding affinity of IP_3 for the type 3 IP_3 receptor has been shown to increase with slight increases in $[Ca^{2+}]_i$ *(17,18)*. In this manner, a slight increase in Ca^{2+} could act through a positive feedback mechanism to sustain elevated $[Ca^{2+}]_i$.

Finally, disrupting cell contact results in an increase in transcription that accounts in part for the increase in type 3 IP_3 expression. This statement is based on the marked increase in type 3 IP_3 receptor mRNA that was detected by ISH as well as those studies using ACT-D and CHX. In astrocytes, Ca^{2+} depletion stimulates the expression of a number of genes *(25,31)*. For example, mRNAs that encode the AP-1 transcription factors, c-*fos* and c-*jun*, are increased threefold within 1 h of Ca^{2+} depletion with a several fold increase in c-*myc* mRNA observed within 4 h of Ca^{2+} removal *(34)*. The mechanism by which simply disrupting cell contact leads to the expression of genes that ultimately induce an increase in $[Ca^{2+}]_i$ and apoptosis of ROSE-179 cells needs to be defined.

Materials and Methods

Isolation of Rat Primary Ovarian Surface Epithelial Cells

Rat primary ovarian surface epithelial cells were isolated by a dispase/collagenase treatment, as previously described by Hess et al. *(8)*. Two to three rats were used for each preparation. Two different preparations were assessed in these studies. This procedure resulted in the isolation of an approx 95% pure population of ovarian surface epithelial cells as judged by cytokeratin staining *(8)*. This protocol was approved by the animal care committee of the University of Connecticut Health Center.

ROSE-179 Cell Culture

ROSE-179 cells were generously provided by Dr. Robert Burghardt of Texas A&M University (College Station, TX) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum as previously described *(16)*. Cells were frozen in liquid N_2, thawed, and then used within 6 mo. For experimental procedures, ROSE-179 cells were plated in 35-mm Falcon tissue culture dishes (Becton Dickinson, Lincoln Park, NJ), 60-mm glass Petri dishes, or 8-chamber glass lab-tek slides (Nunc, Naperville, IL). After 24–72 h of culture in serum-supplemented medium, ROSE-179 cells were approx 80–90% confluent. The cells were then washed once with Joklik-modified MEM (minimal essential medium) supplemented with 15 m*M* HEPES (pH 7.0), Pen-Strep, and 9.2% defined serum substitute with lipids as described by White and Bancroft *(35)*. This medium contained approx 0.01 m*M* Ca^{2+} *(36)*. The cells were cultured for up to 6 h in one of the following supplemented Joklik-modified MEMs:

1. Serum-supplemented medium.
2. Serum-free, Ca^{2+}-free medium.
3. Serum-free medium with 0.7 m*M* Ca^{2+}.
4. Serum-free, Ca^{2+}-supplemented medium with HGF (4 ng/mL).

All tissue culture reagents were purchased from Sigma Chemical unless stated otherwise. Depending on the experimental design, Joklik-modified MEM was also supplemented with the following cell culture reagents at the indicated concentrations:

1. 11 μ*M* CHX.
2. 4 μ*M* ACT-D.
3. 50 μg/mL of type 3 IP_3 receptor–antisense oligonucleotide:5' TGGACATTTCATTCATGGCTTTGGC 3'.
4. 50 μg/mL of nonsense oligonucleotide: 5' TACACC TAGCCCAGAAAGTGAGAAT 3'.

Both oligonucleotides were purchased from Life Technologies (Gaithersburg, MD).

Assessment of Cell-Cell Contact, Intracellular Calcium Levels,and Apoptosis

Cell Contact

ROSE-179 cells were cultured for up to 6 h and cell contact was monitored by phase optics. To observe cell contact more precisely, studies were also conducted in which cells

were fixed in 10% buffered formalin. The cells were then stained with hematoxylin and eosin.

Intracellular Calcium Levels

Cells were cultured in serum-supplemented medium on glass lab-tek slides and then loaded with Fluo-3 (Molecular Probes, Eugene, OR). The relative level of $[Ca^{2+}]_i$ was estimated as previously described *(16)*. In each experiment, the specific Fluo-3 fluorescence (i.e., $[Ca^{2+}]_i$) for 40–100 cells per treatment was determined.

Apoptosis

After 6 h of incubation with various treatments, ROSE-179 cells were stained with hydroethidine (Polysciences, Warrington, PA) and assessed for apoptosis as previously described *(37)*. Because apoptotic bodies are not frequently detected until 6 h after exposure to an apoptotic stimulus, ROSE-179 cells were stained with annexin V and propidium iodide using the reagents and protocol provided by Oncogene (Cambridge, MA). The only deviation from the manufacturer's protocol was that the concentration of annexin V was doubled. Those cells that stained for annexin V but not propidium iodide were considered to be in the early stages of apoptosis *(25)*.

Expression of IP_3 Receptors

IP_3 receptor mRNA levels were monitored by RT-PCR and ISH 3 h after exposure to an apoptotic stimulus. This timepoint was selected because it coincides with the increase in $[Ca^{2+}]_i$, which requires *de novo* RNA and protein synthesis *(16)*. In some experiments, the relative levels of type 1 and 3 IP_3 receptor mRNA were compared by an RT-PCR ratio method *(38)*. Type 1 and 3 IP_3 receptor proteins were detected by Western blot andimmunocytochemistry.

Reverse Transcriptase Polymerase Chain Reaction

ROSE-179 cell RNA was isolated using the Ultraspec RNA isolation system (BIOTECX, Houston, TX). For RT-PCR analysis, 2 μg of total RNA was converted to first-strand cDNA with Superscript II $RNaseH^-$ RT and oligo dT primer (Life Technologies). PCR was performed using 20% of the RT reaction product as template with primers specific for rat type 1 and 3 IP_3 receptors *(39)*. The following custom primers were used to detect the type 1 IP_3 receptor: 5'-TCC CTG GTC AGC AGT GAC TC-3' and 5'-CTC ATT TGC TTA GGC TGG CT-3' (Life Technologies). These primers correspond to nucleotides 8370–8389 and 8569–8588, respectively, of the rat type 1 IP_3 receptor gene sequence (accession no. J05510). The following custom primers were used to amplify the rat type 3 IP_3 receptor: 5''-GGT GAG CGG CGA GGG CGA GG-3' and 5'-GCA GTT CTG CAC GTC CAC GA-3' (Life Technologies). These primers correspond to nucleotides 7961–7980, and nucleotides 8116–8135, respectively, of the rat type 3 IP_3 receptor gene sequence (accession no. L06096). In addition to template, the PCR reaction also contained 1X PCR buffer (Life Technologies), 1.5 m*M* $MgCl_2$, 1 pmol/μL of each primer, 200 μ*M* of each deoxynucleotide (dATP, dCTP, dGTP, and dTTP) (Promega, Madison, WI), and 2.5 U of recombinant *Taq* polymerase (Life Technologies). Amplification of primer products by 40 cycles of PCR in a thermal cycler followed this profile: 95°C denaturation, 1 min; 72°C primer hybridization, 1 min; 72°C extension, 1 min; 72°C final extension, 10 min. After amplification, the type 1 IP_3 receptor PCR product was directly sequenced to confirm identity,and the type 3 IP_3 receptor PCR product was cloned with the TA Cloning System (Invitrogen, San Diego, CA) and sequenced to confirm identity.

RT-PCR Ratio Method to Detect Type 1 and 3 IP_3 Receptor mRNA

Type 1 and 3 IP_3 receptor mRNA were detected using the method described by De Smedt et al. *(38)*. Briefly, cDNA was prepared as described above. Twenty-five cycles of PCR were performed using 10% of the RT reaction as a template with primers that amplify a 191-bp sequence of high homology in both type 1 and 3 IP_3 receptor. The following template primers were used: 5'-GAC CTC CT(C/G) TTC TTC TTC AT-3' and 5'-TT(A/G) ATG TGC TCT TCA AAG G-3', which correspond to nucleotides 8034–8053 and 8206–8224, respectively, of rat type 1 IP_3 receptor (accession no. J05510), and to nucleotides 7620–7639 and 7792–7810, respectively, of rat type 3 IP_3 receptor (accession no. L06096). These primers were prepared by Life Technologies. The PCR reaction also contained 1X PCR buffer (Life Technologies), 2 m*M* $MgSO_4$, 1 pmol/μL of each primer, 200 μ*M* of each deoxynucleotide (dATP, dCTP, dGTP, dTTP; Promega), and 2.5 U of Platinum High Fidelity *Taq* DNA polymerase (Life Technologies). The primer products were amplified in a thermal cycler as follows: 94°C denaturation, 1 min; 56°C primer hybridization, 1 min; 68°C extension, 1 min. After 25 cycles, the PCR reaction was diluted by 20-fold using fresh PCR reaction mixture supplemented with 10–20 nCi/μL of (α-^{32}P)dCTP (Amersham, Arlington Heights, IL). An additional five cycles of PCR were carried out with a final extension of 10 min at 68°C. Unincorporated (α-^{32}P)dCTP was removed by the QIAquick PCR purification system (Qiagen, Valencia, CA). Type 1 and 3 IP_3 receptor mRNA was evaluated by digesting the PCR products with restriction enzymes, which selectively cut one of the IP_3 receptor isoforms. Specifically, *Bgl*II (Life Technologies) was used to cut type 1 IP_3 receptor, and *Hin*P1 I (New England BioLabs, Beverly, MA) was used to cut type 3 IP_3 receptor according to the procedure of De Smedt et al. *(38)*. Restriction digests were separated on an 8% polyacrylamide gel in 0.5X TBE. Radiographs were obtained with Kodak BioMax MS film. To make quantitative comparison, the films were scanned and the densities of each band associated with the *Hin*P1 I digest were determined using IP Lab Gel (Signal

Analytics, Vienna, VA). To calculate the percentage of type 3 IP_3 receptor mRNA, the density of each of the bands after *Hin*P1 l digest was determined. The density of the ≤111-bp bands was divided by the sum all the densities present after *Hin*P1 l digestion. This value was multiplied by 100.

In Situ Hybridization

ISH was carried out as previously described *(40)*. The following 5' digoxigenin-labeled probes were used:

1. Type 3 IP_3 receptor antisense oligonucleotide: 5' CCA TAA ATG TGG CAG CTC CGT G 3'.
2. Nonsense oligonucleotide: 5' TAC ACC TAG CCC TGA TAG TGA G 3' (Genosys Biotechnologies, Woodlands, TX).

The presence of digoxigenin-labeled mRNA was detected using a Genius 3 detection kit according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN).

Western Blot

ROSE-179 cells were cultured on 60-mm glass Petri dishes in serum-supplemented growth media until approx 95% confluent. Cell lysates were prepared as previously described *(9)*. Eighteen to thirty-six micrograms of protein per lane was electrophoresed on a 5% polyacrylamide gel at 100 V and then transferred to nitrocellulose. Type 1 and 3 IP_3 receptors were detected by Western blot analysis using a 1:1000 dilution of either a polyclonal antibody to type 1 IP_3 receptor (BIOMOL, Plymouth Meeting, PA) or a monoclonal antibody to type 3 IP_3 receptor (Transduction, Lexington, KY). Protein was detected by chemiluminescence using the SuperSignal ULTRA detection system (Pierce) at full strength for type 3 IP_3 receptor or at a 1:5 dilution for type 1 IP_3 receptor. Specific staining was assessed by omitting the primary antibody from the protocol.

Immunocytochemical Localization of IP_3 Receptors

IP_3 receptors were localized by immunocytochemistry using either a 1:50 dilution of the type 1 IP_3 receptor antibody (BIOMOL) or the type 3 IP_3 receptor antibody (Transduction). These proteins were detected using the Vectastain Elite ABC detection kit (Vector, Burlingame, CA) or a fluorescein isothiocyanate-labeled goat antimouse IgG. These studies were done on lab-tek slides. Because each treatment was present on the same lab-tek slide, the staining conditions were identical. This allowed for valid comparisons of staining intensity among treatment groups. Specific staining was assessed by omitting the primary antibody from the protocol.

Statistical Analysis

All experiments were repeated two to four times. Those experiments involving $[Ca^{2+}]_i$ or apoptosis were conducted in duplicate, with the results of each replicate being similar. Based on these experiments, the data were pooled and statistically analyzed. The results of each experiement were were analyzed by either a student's t test or a one-way analysis of variance followed by Student-Newman-Keuls multiple range test. Regardless of the statistical test, only p values ≤ 0.05 were considered to be significant. The values were presented as the means ± SEs.

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