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Role of Cell–Cell Adhesion in the Regulation of Prolactin Gene Expression by Extracellular $CaCl_2$

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We have investigated a role for calcium-dependent cell–cell adhesion in the regulation of prolactin gene expression in rat pituitary GH_3 cells. Cells cultured in a calcium-free, serum-free medium (SFM) express low levels of prolactin and growth hormone mRNA. As expected, addition of 0.5 m*M* $CaCl_2$ to GH_3 cells in SFM produced a specific, severalfold increase in prolactin mRNA levels. $CaCl_2$ also promoted intercellular adhesion, during which cells assembled end-to-end into cords. Prolactin mRNA increased after a delay of several hours. This latency period ranged from 4–12 h among different experiments, but always occurred after the onset of cell–cell adhesion. The voltage-sensitive calcium channel (VSCC) blocker, nitrendipine, inhibited the $CaCl_2$-induced increase in prolactin mRNA without affecting cord formation. However, the VSCC agonist, BAY K-8644, was unable to induce prolactin gene expression prior to the onset of intercellular adhesion at 8 h, even though it produced a cellular response (tyrosine phosphorylation of a ca. 130-kDa protein) within 30 min. Blocking cell–cell adhesion inhibited the calcium-dependent induction of prolactin gene expression. Low levels (0.0025–0.02%) of trypsin blocked cell–cell adhesion and the prolactin mRNA induction by $CaCl_2$ without affecting the levels of other mRNAs or cell–matrix adhesion. Heparin also specifically blocked the induction of both cell–cell adhesion and prolactin gene expression. Based on these data, we propose a role for both VSCCs and calcium-dependent cell–cell adhesion in the induction of prolactin gene expression by extracellular $CaCl_2$.

Key Words: Calcium; adhesion; prolactin.

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Introduction

The rat pituitary tumor GH_3 cell line represents a bipotential somatolactotrope that produces both prolactin (PRL) and growth hormone (GH) (Bancroft, 1981). GH_3 and related cell lines have been invaluable in the study of the regulation of PRL and GH gene expression by multiple hormones and growth factors (e.g., Rosenfeld et al., 1987), of the tissue-specific regulation of PRL and GH gene expression (e.g., Ingraham et al., 1990), and of pituitary cell differentiation (Frawley and Boockfor, 1991; Billis et al., 1992; Missale et al., 1994; Felix et al., 1995). One potentially important component in the regulation of pituitary cell function and differentiation that has largely been ignored is cell adhesion, either to other cells or to the extracellular matrix. It has been shown that lactotropes adhere to gonadotropes in vivo and in primary cultures (Nakane, 1970; Nogami and Yoshimura, 1982; Tougard and Tixier-Vidal, 1994), and that culturing GH_3 cells on an extracellular matrix modestly enhanced PRL production (Elias et al., 1990). Other than the finding that the pituitary and GH_3 cells express high levels of the cell-adhesion molecule, N-CAM (Lahr et al., 1993), there is little information about which cell-adhesion proteins are expressed in the anterior pituitary.

The culture of GH_3 cells in a chemically defined, serum-free medium (SFM) with no added calcium (Ca^{2+}) produces a significant decline in both PRL and GH gene expression. Addition of 0.5 m*M* $CaCl_2$ to these cultures results in a significant and specific increase in PRL mRNA levels and PRL synthesis (White et al., 1981, 1989). A notable characteristic of the $CaCl_2$-induced increase in PRL mRNA levels is the slowness with which it occurs. Typically, there is a delay of several hours before a measurable increase in PRL mRNA levels occurs. Thereafter, PRL mRNA levels increase gradually over a period of several hours or days. Experiments in cell suspension have shown that this induction is not dependent on cell–substrate adhesion (White et al., 1981). GH_3 cells cultured in SFM alone appear as single cells, whereas those cultured with $CaCl_2$ eventually form tightly adherent cords (White, 1985). Interestingly, there exists a temporal correlation between

the $CaCl_2$-induced stimulation of PRL gene expression and Ca^{2+}-dependent cell–cell adhesion.

Given the evidence for a role of cell adhesion in the regulation of phenotypic gene expression in other cell types (e.g., Saadat and Thoenen, 1986; Xanthopoulos et al., 1989; Alcivar et al., 1990; Doherty et al., 1991; Kobayashi et al., 1992; Hodivala and Watt, 1994; Reisfeld and Vardimon, 1994), we examined the temporal relationship between Ca^{2+}-induced cell–cell adhesion and PRL gene expression in GH_3 cells more closely. We present both correlative and causal evidence for the requirement of cell–cell contact for the induction of PRL gene expression by $CaCl_2$ and the Ca^{2+} channel agonist, BAY K-8644.

Results

$CaCl_2$-Induced Cell–Cell Adhesion Temporally Correlates with the Ca^{2+}-Dependent Induction of PRL Gene Expression

GH_3 cells cultured in a Ca^{2+}-free, SFM appeared as randomly dispersed single cells, which expressed low levels of PRL mRNA (Fig. 1A, SFM). Addition of 0.5 m*M* $CaCl_2$ to these cells induced end-to-end cell–cell adhesion, which ultimately resulted in the formation of cords containing as many as 10–20 cells (Fig. 1A, $CaCl_2$). As cord formation progressed, cell boundaries became obscured, and the cells assumed a more compacted, rectangular shape (Fig. 1B). $CaCl_2$ addition also induced a typical fivefold increase in PRL mRNA levels (Fig. 1A, $CaCl_2$). Transmission electron microscopy of GH_3 cell cords revealed the presence of small adherens-like contacts and closely apposed cell membranes on the sides of the cells that form contact (Fig. 1B–D).

A closer study of cell–cell adhesion in GH_3 cells was prompted by the observations that a lag of several hours occurred before PRL mRNA increased, and that this increase correlated temporally with the onset of cell–cell adhesion. An example of this correlation between PRL gene expression and intercellular adhesion is shown in Fig. 2. PRL mRNA levels were compared to cord formation as measured by counting the number of cords containing three or more cells within a field of vision. As previously described, PRL mRNA levels were low in GH_3 cells cultured in SFM for 1 d (time 0 h, Fig. 2). PRL mRNA levels were slightly elevated at 2 and 4 h (two- to threefold) after addition of 0.5 m*M* $CaCl_2$, and increased significantly (sixfold) by 8 h. Cell–cell adhesion showed a similar time-course. Evidence of some cell–cell adhesion was noted as early as 2 h after $CaCl_2$ addition, in that cells were lined up next to one another and had begun the process of compacting into cords. There was a significant increase in cord formation by 4 h, prior to the increase in PRL mRNA. The number of cords with three or more cells appeared to decrease between 12 and 48 h. However, this was owing to formation of networks between individual cords rather than an actual decrease in cord formation (*see* Fig. 1A, $CaCl_2$). In actuality, there was an approximate threefold increase in cords with six or more cells between 8 and 24 h (data not shown).

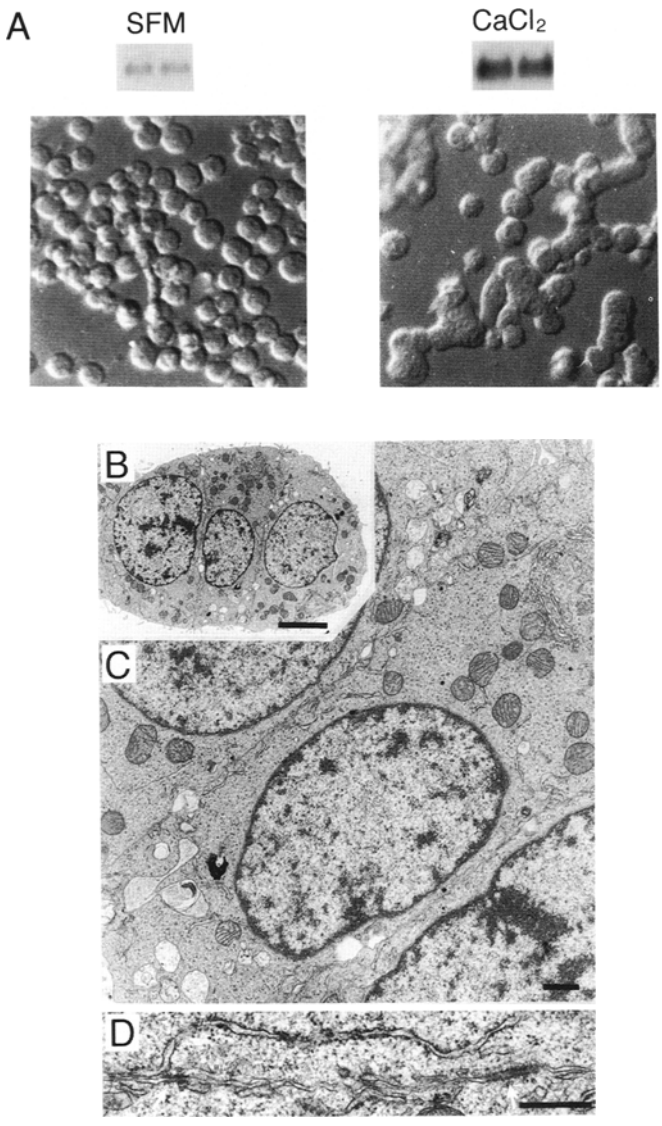

Fig. 1. Ca^{2+} induction of PRL mRNA and cord formation. **(A)** GH_3 cells were cultured for 24 h in either SFM or SFM plus 0.5 m*M* $CaCl_2$, then photographed at 400× magnification using Hoffman Modulator optics, and processed for cytoplasmic RNA. PRL mRNA was measured in duplicate samples by Northern blot analysis for SFM or $CaCl_2$ treatment. **(B, C, D)** TEM of GH_3 cells treated with SFM plus $CaCl_2$ for 24 h. Adjoining cell membranes are closely apposed (B) Bar, 6 μm (C) bar, 1 μm (D) white arrows, adherens-like junctional contacts are found in GH_3 cells; Bar, 0.5 μm.

It is striking that although the latency period from the time of addition of $CaCl_2$ to the onset of an increase in PRL mRNA levels and cord formation varied by several hours among experiments, these two events always displayed a close temporal correlation. For example, in the time-course shown in Fig. 3A, cord formation commenced relatively quickly, so that both the onset of cell–cell adhesion and an

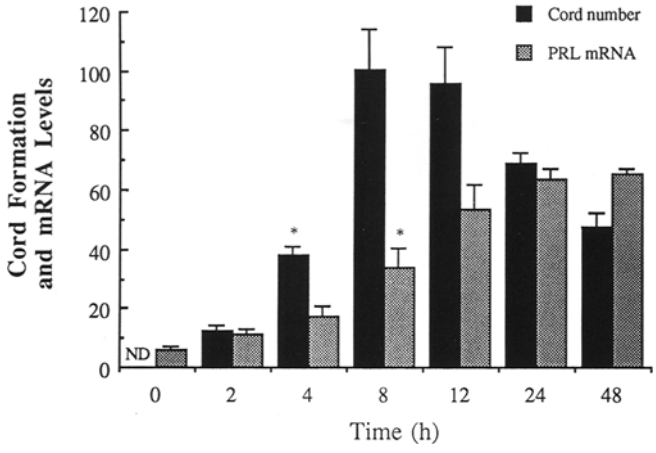

Fig. 2. Temporal correlation between the Ca^{2+} induction of PRL mRNA and cell–cell adhesion. GH_3 cells were cultured overnight in SFM. $CaCl_2$ was added at 0 h, then cord formation quantified, and cytoplasmic RNA isolated at the indicated times (0, 2, 4, 8, 12, 24, 48 h) after $CaCl_2$ treatment. Cord formation values represent the number of compacted cords containing three or more cells in a field of view and are given as the mean ± SE of five fields of view. PRL mRNA levels were analyzed by Northern blot hybridization and are given as the mean ± 0.5 range of densitometry values for duplicate samples. (*) Indicates a significant ($p \leq 0.05$) increase in cord formation or PRL mRNA levels over the previous time-points. ND, cord formation was not detectable at the 0 h time-point.

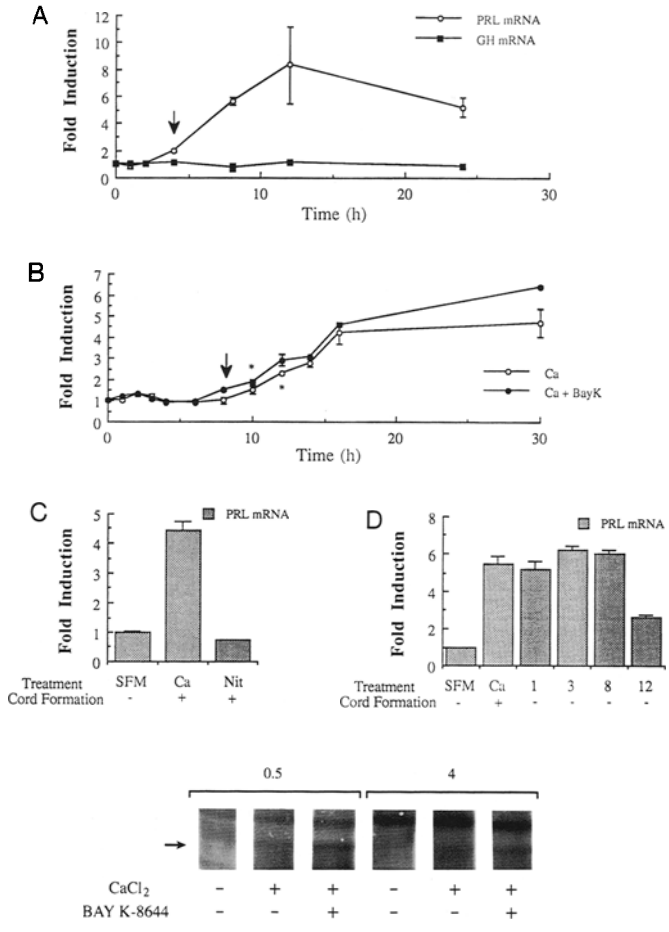

Fig. 3. (A) Time-course of the Ca^{2+} induction of PRL mRNA and cord formation. GH_3 cells were treated as in Fig. 2, and then cytoplasmic RNA isolated at the indicated time-points (0, 1, 2, 4, 8, 12, 24 h) after $CaCl_2$ treatment. Arrow indicates onset of cord formation at 4 h. **(B)** Time-course for induction of PRL mRNA by $CaCl_2$ or $CaCl_2$ plus the VSCC agonist, BAY K-8644. GH_3 cells were treated for the indicated times (1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 30) with $CaCl_2$ alone or with $CaCl_2$ plus 500 n*M* BAY K-8644. Optimal dosage of BAY K-8644 for PRL mRNA induction in GH_3 cells was previously determined. Arrow indicates onset of cord formation at 8 h. (*) Indicates a significant ($p \leq 0.05$) increase in PRL mRNA levels over the previous time-points. **(C)** The VSCC blocker, nitrendipine, blocks the Ca^{2+} induction of PRL mRNA without affecting cord formation. GH_3 cells were treated with SFM, or $CaCl_2$, or $CaCl_2$ plus 500 n*M* nitrendipine for 24 h prior to RNA isolation. Presence (+) or absence (–) of cord formation is indicated. **(D)** Time-course of the deinduction of PRL mRNA and loss of cell–cell adhesion after $CaCl_2$ removal. GH_3 cells were cultured for 16 h in SFM plus $CaCl_2$. The medium was then changed to SFM without $CaCl_2$ at 0 h. Cell–cell adhesion was monitored and PRL mRNA analyzed at the indicated time-points after removal of $CaCl_2$. SFM and $CaCl_2$ indicate levels of PRL mRNA after a 16-h incubation under these conditions. Presence (+) or absence (–) of cord formation is indicated. For (A, B, C, D), PRL and/or GH mRNA levels were analyzed by Northern blot hybridization and are given as the mean ± 0.5 range of fold induction values for duplicate samples. **(E)** Both $CaCl_2$ and BAY K-8644 treatment induce tyrosine phosphorylation of a ca. 120–130 kDa protein band (arrow). GH_3 cells were treated for the indicated times (0.5 or 4 h) with SFM, $CaCl_2$, or $CaCl_2$ plus 500 n*M* BAY K-8644 prior to isolation of protein lysates.

increase in PRL mRNA were apparent by 4 h. As previously shown (White et al., 1981) and illustrated in this experiment, the effect of $CaCl_2$ is specific to PRL gene expression, since GH mRNA levels are unaffected. In contrast, in the experiment shown in Fig. 3B (open circles), cell–cell adhesion was not apparent until 8 h, and PRL mRNA levels did not rise until 10–12 h.

The induction of cell–cell adhesion and PRL gene expression required continuous exposure to $CaCl_2$. GH_3 cells treated with 0.5 m*M* $CaCl_2$ for 3 h, then changed to SFM alone prior to the onset of cord formation, showed no increase in either cell contacts or in PRL mRNA levels after a 24-h incubation (data not shown).

The effects of $CaCl_2$ on cell–cell adhesion and PRL gene expression were reversible, but showed very different time-courses (Fig. 3D). Cells treated with $CaCl_2$ for 24 h, then changed to Ca^{2+}-free SFM showed a complete dissociation of cords by 1 h after the medium change. In contrast, PRL mRNA levels remained elevated for at least 8 h, before declining to low levels at 12 h.

It is worth noting that cord formation is not required for all stimulators of PRL gene expression under these culture conditions. For example, fibroblast growth factor (FGF) increased PRL mRNA levels by severalfold in SFM in the absence of cord formation (unpublished observations). Thus, the requirement for cell–cell adhesion in the stimulation of PRL gene expression is not a universal one.

Both Cell–Cell Adhesion and Voltage-Sensitive Ca^{2+} Channels (VSCCs) Are Required for the Ability of $CaCl_2$ to Induce PRL Gene Expression

In cerebellar neurons, cell–cell adhesion has been linked to the activation of VSCCs (Doherty et al., 1991). Activation of L-type VSCCs has been shown to induce PRL gene expression in GH_3 cells (Hinkle et al., 1988; Jackson and Bancroft, 1988; Day and Maurer, 1990). If cell–cell adhesion activates VSCCs in GH_3 cells, we reasoned that: (1) A VSCC blocker, nitrendipine, would inhibit the $CaCl_2$ induction of PRL gene expression without affecting cord formation, and (2) a VSCC activator, BAY K-8644, would induce PRL gene expression prior to cord formation. Nitrendipine blocked the Ca^{2+} induction of PRL gene expression without perturbing cord formation (Fig. 3C). In two separate experiments, BAY K-8644 plus 0.5 m*M* $CaCl_2$ increased PRL mRNA levels approx 1.5- to 2-fold over that induced by $CaCl_2$ alone after 24–30 h (Fig. 3B). However, in both experiments, BAY K-8644 plus 0.5 m*M* $CaCl_2$ did not increase PRL gene expression prior to cord formation, which occurred 8 h after their addition.

Since BAY K-8644 did not induce PRL gene expression prior to cord formation, we examined whether $CaCl_2$ and/or BAY K-8644 was able to induce an earlier intracellular response in GH_3 cells. Since a prior study indicated that protein tyrosine kinase(s) (PTKs) may be required for the Ca^{2+} induction of PRL gene expression (Billis and White, submitted), we examined whether $CaCl_2$ and BAY K-8644 induced early changes in tyrosine phosphorylation. In the same experiment shown in Fig. 3B, tyrosine phosphorylation was examined by antiphosphotyrosine Western blot analysis on GH_3 cell extracts prepared 30 min and 4 h after the addition of $CaCl_2$ or $CaCl_2$ plus BAY K-8644. As shown in Fig. 3E, a diffuse faint band was observed in the mol-wt range of about 120–130 kDa. $CaCl_2$ alone induced the formation of a sharper band at the lower border of this diffuse band, and the intensity of this sharper band was clearly increased by $CaCl_2$ plus BAY K-8644 at both time-points. Thus, $CaCl_2$ and BAY K-8644 induced a cellular response (which may or may not be related to PRL gene expression) as early as 30 min after treatment, but PRL mRNA levels did not increase significantly until 10 h after treatment, i.e., after cord formation occurred.

Blockade of Cell-Cell Adhesion Specifically Inhibits the Ca^{2+}-Dependent Induction of PRL Gene Expression

The ability of GH_3 cells to form contacts was experimentally manipulated by several methods. One method used by other investigators to disrupt cell–cell interactions is the trypsinization of cell-surface proteins (Alcivar et al., 1990; Kobayashi et al, 1992; Reisfeld and Vardimon, 1994). We included low levels of trypsin at the time $CaCl_2$ was added to the SFM. In the range of 0.0025–0.02%, trypsin inhibited the ability of 0.5 m*M* $CaCl_2$ to induce both PRL gene expression and cord formation (Fig. 4). These concentrations of trypsin did not affect the ability of cells to adhere to tissue culture plastic, nor did they alter mRNA levels for GH (Fig. 4) or glucose-regulated protein 78 (GRP78) (data not shown). Low levels of trypsin also blocked the ability of $CaCl_2$ plus BAY K-8644 to induce PRL mRNA (data not shown).

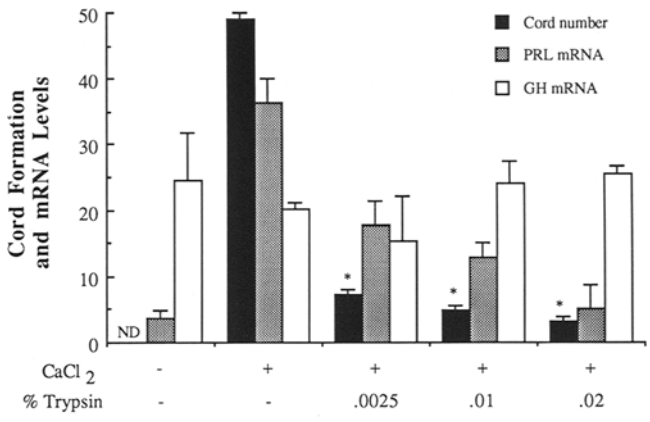

Fig. 4. Effect of trypsin treatment on cell–cell adhesion and PRL gene expression. GH_3 cells were cultured for 16 h in SFM alone, or plus $CaCl_2$, or plus $CaCl_2$ and the indicated amounts of trypsin. PRL and GH mRNA levels were analyzed by Northern blot hybridization and are given as the mean ± 0.5 range of densitometry values for duplicate samples. Cord formation represents the number of compacted cords containing three or more cells in a field of view and are given as the mean ± SE of five fields of view. (*) Indicates a significant ($p \leq 0.05$) decrease in cord formation from $CaCl_2$-treated cultures. ND, cord formation was not detectable at the 0 h time-point.

The effects of trypsin treatment were reversible. In the experiment illustrated in Fig. 5, all cells were treated overnight with 0.5 m*M* $CaCl_2$ and 0.01% trypsin. Again, this treatment resulted in very low levels of PRL mRNA. After the overnight treatment, the medium was changed to serum-containing growth medium for 1 h in order to neutralize the trypsin and allow repair of the cell surface. The medium was then changed back to SFM, plus or minus 0.5 m*M* $CaCl_2$. Cell–cell adhesion was monitored by visual inspection, and PRL and histone 3 (H3) mRNA levels were measured at 9 h. PRL mRNA was increased threefold over SFM controls after 9 h and was accompanied by cord formation. These results show that the effects of trypsin on both adhesion and PRL gene expression were reversible, and that cord formation accompanied the increase in PRL gene expression. The ability of the cells to reverse the effects of trypsin was dependent on ongoing protein synthesis since cycloheximide blocked the ability of $CaCl_2$ to induce cell–cell adhesion and PRL gene expression (Fig. 5).

Several adhesion molecules, some of which mediate Ca^{2+}-dependent cell adhesion, contain heparin binding domains (Cole and Glaser, 1986; Nelson et al., 1993; Rapraeger, 1993; Elenius and Jalkanen, 1994). Cell–cell adhesion mediated by these molecules is disrupted on treat-

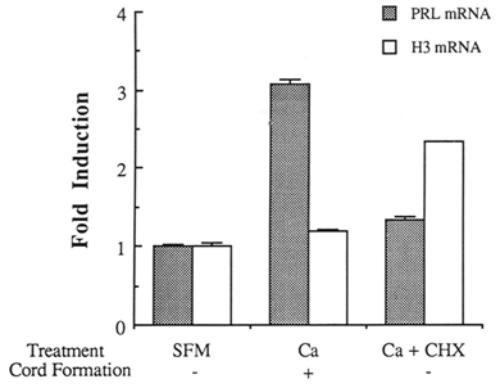

Fig. 5. Effect of $CaCl_2$ on PRL mRNA levels and cell–cell adhesion after trypsin removal in the absence and presence of ongoing protein synthesis. GH_3 cells were treated overnight in SFM plus $CaCl_2$ and 0.01% trypsin. Culture medium was changed to serum-containing growth medium for 1 h. Medium was then changed to SFM alone (SFM), or plus $CaCl_2$ (Ca), or plus $CaCl_2$ and 2.5 μM cycloheximide (CHX). Cell–cell adhesion was monitored and RNA isolated 9 h after the last medium change. PRL and histone 3 (H3) mRNA levels were measured by RNA dot-blot analysis and are given as the mean ± 0.5 range of fold induction values for duplicate samples. Presence (+) or absence (–) of cord formation is indicated. Induction of H3 mRNA served as a positive control for an effect of cycloheximide (Harris et al., 1991).

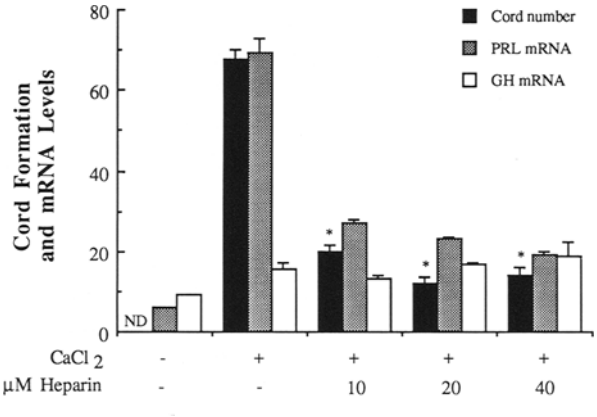

Fig. 6. Effect of heparin on Ca^{2+}-dependent cell–cell adhesion and the Ca^{2+}-dependent induction of PRL gene expression. GH_3 cells were cultured for 16 h in SFM alone, or plus $CaCl_2$, or plus $CaCl_2$ and 10, 20 or 40 μM heparin. PRL and GH mRNA levels were analyzed by Northern blot hybridization and are given as the mean ± 0.5 range of densitometry values for duplicate samples. Cord formation represents the number of compacted cords containing three or more cells in a field of view and are given as the mean ± SE of five fields of view. (*) Indicates a significant ($p \leq 0.05$) decrease in cord formation from $CaCl_2$-treated cultures. ND, cord formation was not detectable at the 0 h time-point.

ment with extracellular heparin or heparan sulfate. In a representative experiment in Fig. 6, heparin treatment in the 10–40 μM range significantly inhibited Ca^{2+}-dependent cord formation without affecting the ability of cells to adhere to tissue-culture plastic. Heparin also blocked the ability of $CaCl_2$ to induce PRL gene expression. As is sometimes observed, $CaCl_2$ induced a small (i.e., less than two-fold) increase in GH mRNA in this experiment. Heparin had no effect on these slightly elevated GH mRNA levels (Fig. 6), or on GRP78 and H3 mRNA levels (data not shown). Exogenous dermatan sulfate and chondroitin sulfate C had no effect on either Ca^{2+}-dependent cell–cell adhesion or PRL gene expression (data not shown).

Discussion

The three major findings of this article are as follows:

1. Induction of cell–cell adhesion and PRL mRNA are temporally correlated;
2. BAY K-8644 in the presence of extracellular $CaCl_2$ cannot induce PRL gene expression prior to induction of cell–cell adhesion; and
3. Two general inhibitors of cell–cell adhesion also inhibit the induction of PRL gene expression.

Based on these findings, we propose a role for cell–cell adhesion in the Ca^{2+}-dependent induction of PRL gene expression.

An increasingly large body of work supports a role for cell–cell adhesion, cell–matrix adhesion, and cell shape in the regulation of gene expression in a variety of systems, including phenotypic gene expression in differentiated cells. For example, the glucocorticoid-dependent induction of glutamine synthetase gene transcription in retinal tissue is dependent on the presence of cell–cell contacts between glia and neurons (Reisfeld and Vardimon, 1994). Also, cell–cell adhesion is required for induction of tyrosine hydroxylase in adrenal chomaffin cells (Saadat and Thoenen, 1986), of L1 adhesion molecule expression in PC12 cells (Kobayashi et al., 1992), and for maintenance of C/EBP gene transcription in hepatocytes (Xanthopoulos et al., 1989). Disruption of cell–cell contacts in hepatocytes results in a prolonged increase in junB gene transcription as well as the decline in C/EBP transcription (Xanthopoulos et al., 1989). Similarly, disruption of cell–cell contacts in testicular cells triggers an increase in junB and c-Jun mRNA (Alcivar et al., 1990). *N*-cadherin-mediated, Ca^{2+}-dependent cell–cell adhesion induces a neuronal phenotype in PC12 cells (Doherty et al., 1991). Another example is the Ca^{2+}-dependent regulation of keratinocyte differentiation in vitro. Ca^{2+}-induced keratinocyte differentiation is marked by regulation of tyrosine kinases (Filvaroff et al., 1990; Zhao et al., 1992, 1993; Calautti et al, 1995), and changes in cell–cell adhesion (Hennings et al., 1980; Hennings and Holbrook, 1983), as well as expression of specific gene markers (Yuspa et al., 1989).

We have now provided both correlative and causal evidence for a role of cell–cell adhesion in the stimulation of PRL gene expression by $CaCl_2$. The correlative evidence is based on the finding that PRL gene expression increases after a latency period of several hours following the addition of extracellular $CaCl_2$, after the time of onset of cord

formation. Over the past few years, we have found that although the time required for cells to form cords varies from experiment to experiment by as much as 8 h, the correlation was maintained between the onset of cord formation and the increase in PRL mRNA levels. The observation that the removal of $CaCl_2$ after several hours of treatment, but before the onset of cord formation, does not result in an increase in PRL mRNA further supports the requirement for cell adhesion.

The finding that nitrendipine blocks the ability of $CaCl_2$ to increase PRL mRNA confirms previous studies that have provided pharmacological evidence for a role of L-type VSCCs in the regulation of PRL gene expression (Hinkle et al., 1988; Jackson and Bancroft, 1988; Day and Maurer, 1990). Similar findings in neuronal cell–cell adhesion systems have led some investigators to propose a model in which cell–cell adhesion activates VSCCs, which in turn activate intracellular Ca^{2+}-dependent signal transduction pathways involved in producing a cellular response (in this case, the stimulation of neurite outgrowth; Doherty et al., 1991). Our finding that nitrendipine blocked the effect of $CaCl_2$ without perturbing cord formation is consistent with a similar model in GH_3 cells. However, if cord formation activated VSCCs in GH_3 cells, it should have been possible to bypass cord formation and accelerate the induction of PRL gene expression by addition of the VSCC activator, BAY K-8644, along with 0.5 m*M* $CaCl_2$. Although $CaCl_2$ plus BAY K-8644 increased PRL mRNA more so than did $CaCl_2$ alone, the VSCC agonist failed to induce PRL mRNA prior to cord formation. One possible explanation for this would be that VSCCs are repressed by some mechanism in the absence of intercellular adhesion, and are unresponsive to the drug. However, this possibility is not consistent with the finding that $CaCl_2$, and to a greater extent, $CaCl_2$ plus BAY K-8644, increased the tyrosine-specific phosphorylation of a ca. 130-kDa protein within 30 min, long before cord formation occurred. The data from the BAY K-8644 time-courses provide compelling evidence that cell–cell adhesion is a requirement for $CaCl_2$ induction of PRL gene expression, since BAY K-8644 plus $CaCl_2$ clearly affected GH_3 cells by 30 min, yet had no significant effect on PRL gene expression for 8–10 h, again after the time when cord formation was first noted. Although these observations do not support the hypothesis that cell–cell adhesion acts exclusively through the activation of calcium channels, they indicate that functional VSCCs are required for the ability of extracellular $CaCl_2$ to induce PRL gene expression.

Experimental blockage or disruption of cord formation by inclusion of trypsin or heparin provided more causal evidence for the requirement of cell–cell adhesion in the induction of PRL gene expression by $CaCl_2$. Trypsinization has been used successfully in several systems to examine the effects of the loss of intercellular adhesion on gene expression (Alcivar et al., 1990; Kobayashi et al, 1992; Reisfeld and Vardimon, 1994). In GH_3 cells, the inclusion of low levels of trypsin effectively inhibited cell–cell adhesion. This loss of adhesion was accompanied by a reduced ability of $CaCl_2$ to stimulate PRL gene expression. Significantly, this effect of trypsin was highly specific for cell–cell adhesion and PRL mRNA. The cells adhered well to the tissue-culture dish, PRL mRNA levels did not fall below those observed in basal SFM conditions, and GH and GRP78 mRNA levels did not significantly change. Also, the effects of trypsin were reversible. GH_3 cells exhibit a normal response to $CaCl_2$ after trypsin removal, both in terms of cord formation and PRL gene expression. As expected, new protein synthesis was required for the reversal of the trypsin-induced effects.

Heparin treatment of GH_3 cells had similar effects to those of trypsin. Heparin specifically blocked the Ca^{2+}-dependent induction of both PRL gene expression and cord formation, but had no significant effect on GH mRNA levels or cell–substrate adhesion. Heparin is known to affect the action of several types of adhesion molecules, including N-CAM (Cole and Glaser, 1986) and some members of the selectin family (Nelson et al., 1993). Additional characterization of the adhesion proteins that are expressed in GH_3 cells is required to understand the basis for this effect of heparin.

Analysis of cell–cell boundaries by electron microscopy revealed the presence of adherens-like junctions. Thus, members of the Ca^{2+}-dependent cadherin family may be involved in cord formation in GH_3 cells. We have observed a low level of expression of *N*-cadherin, R-cadherin, and possibly several other cadherins in GH3 cells (our unpublished observations). Thus, cell–cell adhesion may affect PRL gene expression though pathways involving cadherins, catenins, and associated cytoskeletal components. However, use of a blocking antibody to *N*-cadherin (Volk et al., 1990) does not consistently inhibit the induction of PRL gene expression by calcium or specifically inhibit cell–cell adhesion (our unpublished observations). Also, expression of an *N*-cadherin-dominant negative mutant (Fujimori and Takeichi, 1993) has no effect on either cell–cell adhesion or PRL gene expression (our unpublished observations). Other cell–cell adhesion molecules, such as N-CAM (Lahr et al., 1993), β1 integrin (our unpublished observations), and syndecan I (our unpublished observations), are expressed in GH_3 cells, and may contribute to cord formation and involve different signaling pathways. The electron micrograph also revealed the close apposition of cell membranes from adjacent cells, raising the possibility for juxtacrine and/or paracrine signaling between cells. Exogenous heparin interferes with a number of potential juxtacrine and paracrine signaling molecules, e.g., Wnt family members (Jue et al., 1992), syndecan/fibroblast growth factor interactions (Rapraeger, 1993; Elenius and Jalkanen, 1994), and FGF-receptor/ligand interactions (Guimond et al., 1993; Kan et al., 1993). Thus, cell–cell adhesion may play a permissive rather than direct role in the induction of PRL

mRNA. It is possible that induction of cell–cell adhesion and induction of PRL mRNA are separate events that correlate well, but are unrelated. However, the fact that latency periods vary as much as 8 h from experiment to experiment argues against this since the onset of cell–cell adhesion is always closely followed by the induction of PRL gene expression. Further characterization of the adhesion and cell membrane molecules in GH_3 cells will allow the design of experimental protocols, which more specifically address the role adhesion plays in the regulation of PRL gene expression.

It is likely that cell–cell adhesion plays a role in normal lactotrope function in the pituitary. Lactotropes are nonrandomly distributed in the rat pituitary (Nakane, 1970), have been described as clusters during pregnancy (Horvath and Kovacs, 1988), and are often found surrounding gonadotropes in vivo (Nogami and Yoshimura, 1982; Chronwell et al., 1996). In vitro studies have shown that lactotropes and gonadotropes are connected by gap junctions (Fletcher et al., 1975) and small adherent junctions (Horvath et al., 1977), and further, that association with gonadotropes affects PRL secretion in lactotropes through a paracrine mechanism (Denef, 1985). It is probable that expression of cell–cell adhesion molecules responsible for both the distribution of lactotropes and preferential association of lactotropes and gonadotropes in vivo contribute either permissively or specifically to regulation of PRL gene expression.

Materials and Methods

Cell Culture

GH_3 cells were obtained from American Type Culture Collection (Rockville, MD) free of mycoplasma contamination, and maintained in suspension culture as previously described (White and Bancroft, 1987). For experiments, cells were centrifuged out of serum-containing growth medium, washed once in SFM (White and Bancroft, 1987), and plated onto 60 × 15 or 100 × 20 mm Falcon tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) in SFM prior to treatment. Duplicate cultures were used for each experimental condition or treatment. The details for each experiment are described in the legend for the corresponding figure. $CaCl_2$ was always added to a final concentration of 0.5 m*M*. BAY K-8644 and nitrendipine were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Trypsin (2.5%) was obtained from Gibco BRL (Grand Island, NY). Cycloheximide, heparin, chondroitan sulfate C, and dermatan sulfate were obtained from Sigma (St. Louis, MO).

DNA Clones

The cDNA clones for PRL, histone 3 (H3), glucose-regulated protein 78 (GRP78), and GH were described previously (White et al., 1981; Preston et al., 1990; Delidow et al., 1991).

Cell–Cell Adhesion Analysis

Cord formation was quantified by counting the number of cords formed in GH_3 cell cultures. A cord is defined as a cell cluster containing 3 or more cells tightly adhered to one another. Cord numbers are obtained by counting the number of cords within a microscopic field of view at 100× magnification. In Figs. 2, 4, and 6, cord formation is given as the mean ± SE of cord numbers from five fields of view. Fields were selected randomly from each of the four quandrants of a culture dish and from the center of the dish. In Figs. 3C, D, and 5, cell–cell adhesion is scored as either the presence (+) or absence (–) of cords. (–) Scored GH_3 cell cultures do not contain any cords and consist only of single cells. (+) Scored GH_3 cell cultures contain extensive cords typical of a culture incubated for 24 h in SFM plus $CaCl_2$, as shown in Fig. 1A. Note that the photographs in Fig. 1A represent a small selected area in a field of view taken at 400× magnification, whereas cell–cell adhesion analysis was done at 100X magnification. In Fig. 3A and B, cord formation onset is noted with an arrow. Cord formation onset is defined as the first appearance of tightly adhered cell clusters containing three or more cells in GH_3 cultures. Cell clusters containing two cells were excluded from quantification to eliminate the possibility of Ca^{2+}-independent associations.

Statistical Analysis

All experiments were performed a minimum of three times except for Figs. 3B, E and 5, which were performed twice. Cord formation data were shown to be normally distributed. PRL mRNA time-course data (Figs. 2, 3A, and B) and cord formation data (Figs. 2, 4, and 6) were analyzed by one-way analysis of variance followed by Student-Newman-Keuls multiple range test. Only $p \leq 0.05$ was considered significant.

RNA Isolation and Analysis

Preparation of cytoplasmic RNA for analysis by either Northern blot hybridization or RNA dot hybridization was performed according to the method of Greenberg and Ziff (1984), except that the spin step with vanadyl ribonucleoside complexes was omitted. For Northern blot and RNA dot hybridizations, cytoplasmic RNA samples (10 and 5 μg, respectively) were blotted onto nitrocellulose (MSI, Westboro, MA) and immobilized by UV crosslinking. Membranes were prehybridized in 50% formamide, 5X SSPE (1X SSPE = 0.18*M* NaCl, 0.01*M* NaH_2PO_4, 1 m*M* EDTA, pH 7.4) (Maniatis et al., 1982), 5X Denhardt's solution (Maniatis et al., 1982), 0.1% SDS, and 100 mg/mL denatured, sheared, sonicated salmon sperm DNA for 6–16 h at 42°C. DNA probes were labeled with [^{32}P]dCTP by nick translation (Amersham Corp., Arlington Heights, IL) (PRL, GH, H3, and GRP78) and purified by Sephadex G-50 chomatography. The radiolabeled DNA (10×10^6 cpm) was boiled for 5 min, cooled on ice, and added directly

to the prehybridization solution. Samples were hybridized for 36–48 h at 42°C, washed twice in 2X SSC (1X SSC = 0.15*M* NaCl, 0.015*M* sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min, then once in 0.1X SSC, 0.1% SDS at 55°C for 1 h, and exposed to Kodak XAR5 film (Eastman Kodak Co., Rochester, NY). Autoradiograms were quantified by scanning densitometry with a Gilford Response densitometer, or by computer scanning and analysis with IP Lab Spectrum software (Signal Analytics Corporation, Vienna, VA). mRNA levels in Figs. 2, 4, and 6 are represented by the mean of actual band density values ± 0.5 range. mRNA levels in Figs. 3 and 5 are represented as fold induction. The fold-induction value is the mean ± 0.5 range of densitometry values for duplicate samples divided by the mean densitometry value for SFM-treated samples, e.g., a fourfold PRL mRNA induction represents an increase of 400% over PRL mRNA levels in SFM-treated cultures. In some cases, the range or standard error was too small to show up on the graph. Ethidium bromide staining of ribosomal bands was examined to ensure equal loading.

Protein Analysis

For phosphotyrosine immunoblotting, samples were electrophoresed on 6% SDS-polyacrylamide gels. For analysis of whole cells, the medium was removed, and the cells (plated at equal densities for each treatment) were dissolved by scraping with a Teflon policeman in 250 µL of boiling Laemmli sample buffer (Laemmli, 1970). The samples were then boiled for 5 min, and the DNA was sheared by passing five times though a 26-gage needle. Insoluble material was removed by centrifuging the cell lysates for 5 min in a microcentrifuge at 12,000*g*. Prior to electrophoresis, cell extracts were diluted, and the protein concentration of each sample was determined (Bradford, 1976). Typically, 100 µg of protein were electrophoresed/lane. Nitrocellulose sheets were blocked with 10% bovine serum albumin (BSA, Boehringer Mannheim, Indianapolis, IN) in Tris-buffered saline (100 m*M* NaCl, 50 m*M* Tris-HCl, pH 7.6) containing 0.1% Tween-20 (Sigma) overnight with agitation at room temperature. After removal of blocking solution, antiphosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at a dilution of 1:1000 in TBS containing 3% BSA was added, and the blots were incubated at room temperature with agitation for 1–2 h. Horseradish peroxidase-conjugated sheep antimouse antibody (Kirkegaard and Perry, Inc., Gaithersburg, MD) at a dilution of 1:25,000 was used as a secondary antibody, and reactivity was determined by chemiluminescence (Kirkegaard and Perry, Inc., Gaithersburg, MD) according to manufacturer's instructions.

Electron Microscopy

GH_3 cells were cultured for 24 h in SFM plus 0.5 m*M* $CaCl_2$, then fixed for 1 h in 2.5% glutaraldehyde in 0.1*M* cacodylate (CAC) buffer, pH 7.4, rinsed in CAC buffer, and processed for transmission electron microscopy (TEM). Cells were postfixed in 1% osmium tetroxide with 0.8% potassium ferricyanide in 0.1*M* CAC buffer, rinsed, stained in block with 0.5% aqueous uranyl acetate, dehydrated in EtOH, and embedded in Polybed resin (Polysciences, Warrington, PA). Thin sections were cut with a diamond knife, examined after staining with aqueous uranyl acetate and lead citrate, and observed in a Philips CM-TEM.

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