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Complement action on secretory cells identified by the reverse hemolytic plaque assay: modified assay eliminates exposure of secretory cells to complement

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The application of a hemolytic plaque assay to antigen-secreting endocrine cells has brought about great advances in the study of regulation of hormone secretion. The reverse hemolytic plaque assay (RHPA) has enabled quantitation of secretion at the single cell level with simultaneous analysis of the population response. Moreover it has allowed unambiguous identification of specific cell types in mixed cell populations while maintaining the viability of the cells for further physiological experiments. Concern has arisen, however, regarding potential complement attack on those cells of interest, causing sublytic permeabilization leading to altered physiological function. To test this possibility, prolactin release from dispersed anterior pituitary cells was quantitated in two protocols of the RHPA. Cells were exposed to complement either subsequent to the termination of antiserum incubation or simultaneously with antiserum incubation, during which time hormone release is being detected. The presence of complement during antiserum incubation resulted in significant increases in mean plaque area as compared to the separate incubation procedure (13 709 ± 698 *vs* 9251 ± 547 μm^2). Analysis of the population profile of plaques indicated that the increased mean plaque area reflected a rightward shift in the frequency distribution of plaque size. The general increase in hormone release in the antibody/complement group is consistent with a predicted permeabilizing action of the complement on the secretory cells. To avoid this potentially damaging effect of complement on secretory cells to be used in subsequent physiological experiments, we have developed a modification of the RHPA in which the secretory cells are unequivocally identified without being exposed to complement.

Keywords: reverse hemolytic plaque assay; complement; secretion; pituitary; prolactin

Introduction

The mammalian anterior pituitary gland contains several secretory cell types, and single cell experiments, including electrophysiology and Ca^{2+} measurements, are rendered ambiguous in the heterogeneous populations of primary dissociated cell cultures. Only enriched (not pure) preparations of some of the cell types in the normal anterior pituitary gland are obtainable either in naturally-occurring sources (Gross *et al.*, 1984) or through separation techniques (Hymer and Hatfield, 1983). In recent years the modification and application of an immunological technique, the reverse hemolytic plaque assay (RHPA), has enabled the identification of cell types in mixed cell cultures while preserving the viability of the cells (Neill & Frawley, 1983). The procedure uses immunologically triggered complement-induced red blood cell lysis around individual pituitary cells to identify those secreting a particular hormone. The successful application of this technique established the means for distinguishing functional cell types in a heterogenous population which could not be distinguished on morphological criteria. This brought about the beginning of physiological studies on known PRL-secreting cells obtained from normal pituitary tissue (Lingle *et al.*, 1986).

A major concern regarding the use of the RHPA to identify cells for subsequent use in physiological experiments is the potential action of complement on the cell of interest. Although more resistant to lysis than erythrocytes, nucleated cells may still be adversely affected by complement attack. Indication that this sometimes occurs with cells identified by the RHPA is mostly anecdotal. Following an initial publication of physiological studies on plaque-identified cells, several investigators have returned to less equivocal means of identifying cells for later work. Leong (1989) reported that 'preplaquing' ACTH cells prior to calcium measurements produced 'highly variable results' which led that investigator to routinely identify the cells retroactively (complement added *after* the physiological studies were completed). In this paper, our aim was two-fold: firstly, we present quantitative evidence for a subtle action of complement on secretory cells assayed in the RHPA; and secondly, we describe a modified plaque assay which we developed to unequivocally identify secretory cell types without exposing them to complement.

Results

The effect of guinea pig serum (complement) on the release of PRL is illustrated in Figures 1 and 2. Using the standard RHPA procedure of incubating the cells first with antiserum alone and then, after rinsing, infusing guinea pig complement to elicit the hemolytic reaction, plaques of varying sizes surrounding single cells were formed (Figure 1A; Figure 2, filled circles). This heterogeneity of plaque size in the absence of PRL regulators and its non-normal distribution has been reported by several laboratories. In this assay, the division between 'small' and 'large' plaques was chosen to be $4.3 \times 10^2 \mu m^2$ based on the curve from the control group (Figure 2, filled circles). Although the profile was not a clearcut bimodal distribution, as was reported by Luque and colleagues (1986), there was a slight dip in the center of the distribution and the data did fall into an approximately even distribution of small and large plaques. The presence of a maximally effective dose of DA (500 nM) inhibited the formation of large plaques and most of the plaques were very small (Figure 1B, arrows). This shifted the frequency histogram to the left, toward a unimodal distribution (Figure 2, open circles), consistent with the original report by Luque and colleagues (1986) and subsequent reports.

When the assay was performed by incubating the cells with antiserum and complement simultaneously, a dramatic increase in the size of the plaques was observed, both under basal condition (Figure 1C) and in the presence of DA (Figure 1D). The general increase in the size of the plaques as well as the paucity of very small plaques formed under

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these assay conditions was readily apparent from the photomicrographs (Figure 1C *vs* 1A and Figure 1D *vs* 1B). Figure 2 illustrates that the inclusion of complement during the antiserum incubation resulted in rightward shifts in the distributions of plaque area (triangles *vs* circles) both in the absence of PRL regulators (Figure 2, filled symbols and 'control' inset), and in the presence of DA (Figure 2, open symbols and 'dopamine' inset). In fact, when antiserum and complement were incubated simultaneously, the inhibition of plaque area by a very high dose of DA (500 nM) was similar to that observed in response to submaximal doses of DA (see Luque *et al.*, 1986).

A summary of the plaque data are presented in Table 1. When antiserum and complement were incubated separately, the distribution of plaque size between 'small' and 'large' categories was 48% and 52%, respectively. Dopamine (500 nM) shifted this distribution such that small plaques comprised 85% of the population and the mean plaque area was reduced by two-thirds. This dose of DA also reduced the percentage of total pituitary cells that formed detectable plaques, from 42% to 15%. The presence of complement during the antibody incubation augmented PRL release as indicated by all these parameters. First, the distributions of plaque size were shifted to the right, and large plaques comprised a greater proportion of the plaque-forming population as compared to the groups in which antiserum and complement were incubated separately. This occurred both in the presence and absence of DA. The mean plaque areas were significantly greater when antiserum and complement were incubated simultaneously. In addition, although under basal conditions (43.9% *vs* 42%), complement had no effect on the percentage of total pituitary cells that formed detectable plaques, it doubled the percentage of pituitary cells that formed plaques in the presence of 500 nM DA (30% *vs* 15%). These increases in secretory parameters did not occur when heat-inactivated (56°C, 30 min) guinea pig serum was used (1/50) during the antibody incubation (data not shown). Heat inactivated serum also did not support hemolysis.

These findings demonstrated that complement (guinea pig serum) could alter secretory cell function. Therefore, we sought to avoid exposure of the secretory cells to complement altogether. To do this, we developed a modification of the RHPA which is described under 'methods'. Figures 3 and 4 illustrate the identification of PRL-secreting cells using this 'split chamber RHPA'. The photomicrographs show the erythrocytes (Figures 3A, C and 4A) and rat anterior pituitary cells (Figures 3B, D and 4B) attached to what had been apposing surfaces of the chambers. Following incubation with the antiserum to rat PRL, the two coverslips of

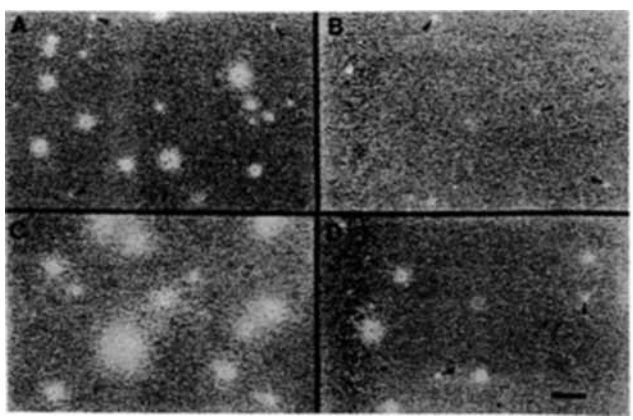

Figure 1 Photomicrographs illustrating the effect of complement action on PRL plaque area (PRL release per lactotrope). PRL release was assayed in the absence (**A** and **C**) or presence (**B** and **D**) of 500 nM dopamine. (**A, B**) Cells were incubated for 60 min with anti-PRL (1/200; ± DA). After rinsing the chambers, plaques were developed by incubation with guinea pig complement (1/80) for 90 min and then fixed. (**C, D**) Cells were incubated for 60 min in the presence of anti-rPRL (1/200) and complement (1/80) simultaneously (±DA). After rinsing the chambers, plaques were further developed for 30 min with complement (1/80) before fixation. Calibration bar = 300 μm

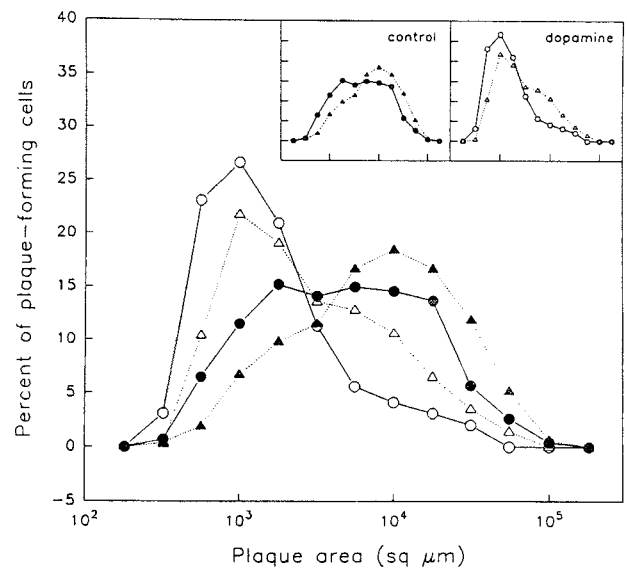

Figure 2 Frequency distribution profiles of PRL plaque area: effect of complement. PRL release was assayed in the presence (▲, ●) or absence (△, ○) of DA (500 nM). Cells were exposed to guinea pig complement either subsequent to (circles, solid lines) or simultaneously with (triangles, dashed lines) the antiserum. Inserts: comparison of separate *v.* simultaneous incubation of antiserum and complement for the two treatment groups. Due to large interassay variation, data shown are from a single plaque assay. Experiment was repeated in two additional assays which produced the same qualitative results

Table 1 Summary of RHPA results comparing the separate incubations of antiserum followed by complement and the simultaneous incubation of antiserum and complement together

			Plaque area (μm^2)			*Population distribution*[c]	
	N[a]	*%*[b]	*Mean ± SEM*	*Minimum*	*Maximum*	*Small*	*Large*
Separate incubations							
Control[d]	540	42.0	9251 ± 547	313	132 897	48%	52%
Dopamine (500 nM)	195	15.0	3014 ± 385†	279	39 085	85%	15%
Simultaneous incubation							
Control[d]	479	43.9	13 709 ± 698‡	373	109 129	30%	70%
Dopamine (500 nM)	367	30.0	5768 ± 465†‡	393	62 435	65%	35%

[a]Number of plaque-forming cells and number of plaque areas measured. [b]Percentage of total pituitary cells forming detectable plaques. [c]The division between 'small' and 'large' was chosen to be 4.3×10^3 μm^2 based on the frequency distribution plots in Figure 2. [d]Controls were run with or without 0.5 mM ascorbic acid, which was used as an antioxidant in the dopamine solution. Ascorbic acid alone had no effect. †$P < 0.001$ *vs* corresponding control group. ‡$P < 0.0001$ *vs* corresponding group in the separate incubations protocol.

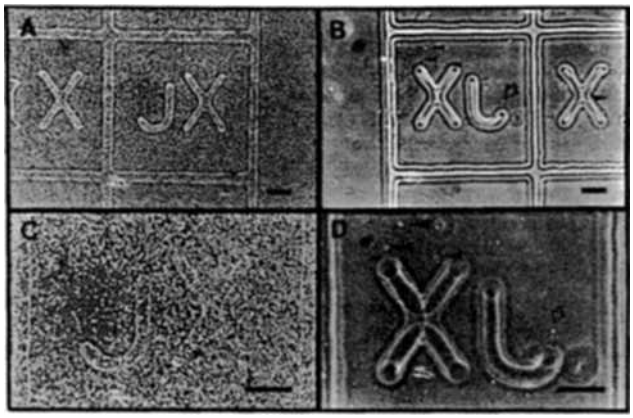

Figure 3 Identification of PRL-secreting cells using the 'split chamber' modification of the RHPA. Sheep erythrocytes (**A, C**) and rat anterior pituitary cells (**B, D**) are attached to what had been apposing surfaces of the chamber. Erythrocytes (3.5%) were plated onto the 'floor' of the chamber, which was the photoetched surface of the bottom coverslip. Anterior pituitary cells (3.5×10^5/ml) were plated onto the 'ceiling' of the chamber. This was the underside of the top coverslip, therefore the photoetched grid is in mirror-image and not in the same plane of focus as the adherent cells. Following incubation with antiserum, chambers were rinsed and then dismantled while submerged in prewarmed media. Chamber 'ceilings' with pituitary cells attached were placed in media while the chamber 'floor' with oRBCs attached were incubated with complement (1/80) for 30 min. Both components were later fixed and stained, as described in Methods and photographed under phase contrast optics. The only PRL secreting cell in this field is located at the tip of the 'J' (open arrows) as indicated by the corresponding plaque. Other cells (solid arrows) did not form plaques. Calibration bars = 100 μm

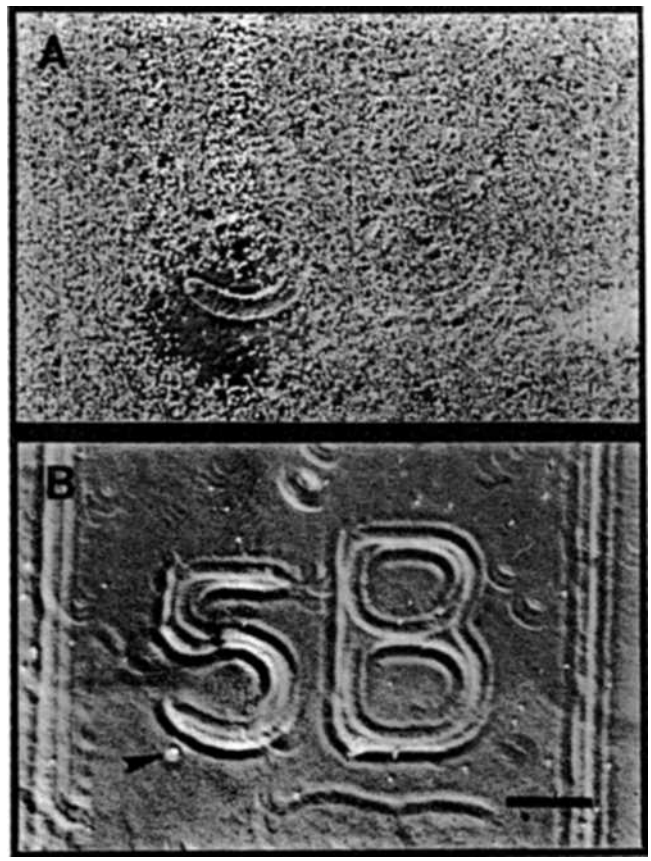

Figure 4 Viable PRL-secreting cell (**B**, arrow) identified using the 'split chamber RHPA'. This assay was performed for cells to be used in subsequent electrophysiological experiments. The cell was identified by the corresponding area of lysed erythrocytes (**A**) on the apposing coverslip of the assay chamber. The photomicrograph negative of the anterior pituitary cell was inverted for printing in order to facilitate identification of the locations of the cell and the plaque. Assay procedure was as described in the legend to Figure 4 except that the chamber 'ceilings' with pituitary cells attached were not fixed but were maintained in culture and the chamber 'floors' with oRBCs attached were exposed to complement at a dilution of 1/50 (30 min). Calibration bar = 100 μm

each chamber were separated and the oRBCs exposed separately to complement. PRL-secreting cells were identified by the presence of a hemolytic plaque in the same area of the photoetched grid on the apposing coverslip. One PRL-secreting cell can be identified in Figure 3 (B and D, open arrow) based on the location of a hemolytic plaque (Figure 3A and C) while several other pituitary cells in the same field have no corresponding plaque (solid arrows). For illustrative purposes, the anterior pituitary cells in this assay were plated at a higher density ($\sim 3.5 \times 10^5$ cells/ml) than used for identifying cells for physiological studies. Since the 'ceiling' of the chamber onto which the pituitary cells were plated was the underside of the top coverslip, the photoetched grid appears in mirror-image (Figures 2A *vs* 3B and 3C *vs* 3D) and not in the same plane of focus as the adherent cells. Figure 4 also shows a PRL-secreting cell identified with the 'split chamber' assay but the photomicrograph negative of the anterior pituitary cell was inverted for printing in order to facilitate identification of the locations of the cells and the plaque. Note that while the grids are no longer mirror images (Figure 4A *vs* 4B), the photoetched grid in Figure 4B is still not in the same plane of focus as the pituitary cell which is adhered to the opposite surface of the coverslip. The pituitary cell shown in Figure 4B was viable and identified for subsequent electrophysiological study. Anterior pituitary cells in this assay were plated at a lower density than that illustrated in Figure 3 (approximately one cell per photoetched square; original suspension = 1.5×10^5 cells/ml) to make identification of plaque-forming cells unequivocal.

Discussion

The application of the RHPA to antigen-secreting endocrine cells has brought about great advances in the study of regulation of hormone secretion. Concern arises, however, regarding potential complement attack on those cells of interest, causing sublytic permeabilization leading to altered physiological function. The mechanisms by which this would occur are established. Some secretory product is bound to the outside of the secretor itself. Antibodies will bind to this antigen, which is the basis for cell identification in fluorescence-activated cell sorting (St. John *et al.*, 1986). Antibodies on the external surface of the secreting cell can, in turn, bind and fix complement. In fact, complement-induced lysis has been exploited as verification of antibody binding to the surface of secretory synaptosomes (Jones *et al.*, 1981). Sublytic or transient permeabilization of secretory cells by complement may alter their function. The present data demonstrate that complement indeed can alter the function of secretory cells in the RHPA as evidenced by a generalized increase in hormone release.

Demonstrating whether complement could affect secretory cell function in the plaque assay itself was important in light of anecdotal information regarding high variability of physiological responses by cells identified with this technique. In addition, some controversies exist in the literature. For example, investigators using fluorescence-activated cell sorting to identify normal lactotropes have reported spontaneous firing of action potentials in these cells (Lewis *et al.*, 1988), while others (Lingle *et al.*, 1986) have reported intrinsic activity in negligible numbers (1 or 2 out of >60) of

normal PRL secretors identified by the plaque assay. The difference in methodologies used to identify the primary lactotropes has been cited as a possible reason for this discrepency (Lewis *et al.*, 1988).

Numerous studies have demonstrated that sublytic doses of complement elicit efflux of preloaded ^{86}Rb from cells (Burakoff *et al.*, 1975; Boyle *et al.*, 1976), while direct measurements of membrane potential in nucleated cells demonstrate decreased resting membrane potential and membrane resistance a few minutes after adding lytic or sublytic doses of complement (Stephens and Henkart, 1979). Taken together, these responses indicate that complement induces a non-selective increase in ionic permeability in nucleated cells. Such actions would be expected to result in the general increase in PRL release observed when complement is included in the antiserum incubation of the plaque assay (Figures 1 and 2). Complement action can also explain the greater percentage of plaque-forming cells observed in the presence of DA. Increased membrane permeability would counteract, at least partially, dopaminergic inhibition resulting in a greater number of lactotropes secreting within the detectable range of the plaque assay. The lack of increase in the percentage of plaque-forming cells in the absence of DA is not surprising. The 42–44% value is in the range of lactotrope composition in the female pituitary as determined by immunohistochemistry and even non-specific stimulation of the cells would not be expected to increase the number of cells secreting PRL. Indeed, in separate studies, we have found no increase in the percentage of plaque-forming cells in the presence of 10^{-7} M TRH, although this peptide dramatically increases mean plaque area.

In validating the use of the RHPA to identify cells for physiological studies, we have previously compared the electrical properties of untreated GH_3 cells and plaque-assayed GH_3 cells and detected no adverse effects in a variety of electrophysiological measurements (Gregerson and Oxford, 1987). Average resting potential and the properties and pharmacology of Ca^{2+}, Na^+ and K^+ currents were not different between assayed and non-assayed GH_3 cells. The assay also did not interfere with spontaneous electrical activity in the GH_3 cells. These findings indicate that the RHPA need not cause long-term alterations of the membrane and/or electrical properties of secretory cells being identified. It must be emphasized that extremely rigorous conditions for the RHPA were used for those studies. The conditions included a very low density of the erythrocyte lawn (ie, decreased substrate) which enabled substantial reduction in the concentration of complement needed for hemolysis (1/100 to 1/120 dilution). Exposure of the secretory cells to complement was further reduced by removal of free complement at the first sign of hemolysis. Experimental evidence indicates that only one functional complement channel is sufficient to lyse a single erythrocyte (the 'one-hit theory', Mayer, 1961) and complement channels are relatively stable in the plasma membrane of erythrocytes with a half-life of several days at 37°C (Ramm & Mayer, 1980; Ramm *et al.*, 1983). Moreover, erythrocytes cannot survive even limited complement attack unless osmotically protected. Thus, hemolysis and plaque formation will continue for some time after free complement has been rinsed from the chambers.

In contrast to erythrocytes, lysis of nucleated cells requires multiple functional complement channels (Koski *et al.*, 1983) and these channels are quite unstable in the plasma membrane. Once free complement is rinsed away, complement channels are removed from the membrane of nucleated cells in a temperature-dependent manner with a half-life of 1 to 3.5 min at 37°C (Ramm *et al.*, 1983; Morgan *et al.*, 1984). Nucleated cells can also survive limited complement attack even in the absence of osmotic protection. The complement-induced changes in cell membrane permeability described above are reversible when sublytic doses of complement are used (Stephens & Henkart, 1979). Full recovery from limited complement attack may occur, but this undoubtedly will depend upon the assay conditions and the type of cells being identified. Different cell types have different susceptibility to complement. Long-term alterations in the cells' physiological or metabolic state are still a potential risk and may be difficult to distinguish from naturally-occurring biological variation.

The present data also demonstrate that errors in the quantitation of hormone secretion may occur when antibody and complement are used simultaneously. This simultaneous incubation with antiserum and complement is the procedure utilized by some investigators routinely and is also necessary for the 'sequential plaque assay' in which the same set of secretory cells are assayed multiple times (Frawley *et al.*, 1985). Artifacts in the sequential plaque assay, unrelated to complement exposure, have also been reported by Neill and colleagues (1987). An alternative method for the sequential quantitation of hormone release from single cells is the sequential immunoblot assay ('SCIBA') described by Arita *et al.* (1991). This modification of Kendall and Hymer's immunoblot assay (1987) takes advantage of the protein binding properties of nitrocellulose membranes. In the SCIBA, a coverslip on which cells are plated, is gently placed on the transfer membrane with the cells in contact with a thin layer of medium on the water-repellent surface of the membrane. Secreted hormone binds to the membrane and later can be quantitated by immunostaining along with a series of blots of hormone standards.

The immunoblot assay may also be used for identification for subsequent physiological experiments. In our experience, however, locating the cells which correspond to the immunostained blots can be ambiguous, particularly when the cells of interest represent a small percentage of the total population. In contrast, the 'split chamber RHPA' which we have herein described, provides unequivocal and faster identification of secretory cells. Potential problems arising from complement actions on secretory cells can be completely avoided by the use of the split chamber RHPA. The cells of interest are never exposed to complement and they are easily and unequivocally identified based on their secretory product.

Materials and methods

Materials

Sprague-Dawley derived female rats were obtained from Charles River (Wilmington, MA) and maintained on a 14 h: 10 h light: dark cycle with food and water available *ad libitum*. Sources of reagents were as follows: collagenase (~125 U mg protein; Worthington Biochemical Corp., Freehold, NJ); culture media (Irvine Scientific, Santa Ana, CA); horse serum (HyClone Laboratories, Logan, UT); ovine erythrocytes (Waltz Farms, Smithburg, MD); and guinea pig complement (GIBCO BRL, Grand Island, NY). Rabbit anti-rat PRL for use in the plaque assay was generated in our laboratory and characterized as described below. Reagents for the radioimmunoassay of rat PRL were provided by the NIDDK through the National Pituitary Agency. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Generation of antiserum

We have generated our own supply of rabbit antiserum ('arPRL-86') against purified rat PRL for use in the RHPA. Briefly, antigen (NIDDK rPRL-I-3) was prepared by reconstitution in phosphate-buffered saline (PBS). Half of the preparation was denatured by boiling for 2 min to expose more antigenic sites. Rabbits were first inoculated with 100 μg of antigen in an emulsion with Freund's complete adjuvant (0.5 ml total volume, *i.m.*), then boosted 3 weeks later with 100 μg of antigen in Freund's incomplete adjuvant (0.5 ml, *i.m.*). Subsequent inoculations of 50 μg antigen in

PBS were administered every 4 weeks *i.v.* via the marginal ear vein. Blood collections for antiserum were taken from the ear artery between 7 and 10 days following each inoculation, when antibody titers were highest. This regimen produced high antiserum titers by the third month. Antiserum was screened in plaque assays in which only protein A-coated erythrocytes were plated in the chambers and a fixed concentration of purified rPRL-I-3 or I-5 (3-5 μg/ml) was present. The antiserum demonstrated no non-specific hemolytic activity in that hemolysis did not occur if either the purified rat PRL or the complement incubation was omitted. Also, preimmunized sera from the rabbits in place of antiserum did not support complement-induced hemolysis. Validation of this antiserum demonstrated that preabsorption of the antiserum with purified rat PRL (rPRL-I-3 or I-5, 0.5 μg/ml) abolished plaque formation while preabsorption of the antiserum with purified rat GH (rGH-I-5 or I-6; 50 μg/ml) did not diminish plaque formation. In radioimmunoassay, standard curves generated with purified rat PRL (NIDDK RP-3) and our antiserum (final dilution 1/600 000) or arPRL-S-9 antiserum (final dilution 1/13 750) were parallel (logit-log: -2.26 ± 0.12 and -2.09 ± 0.08, respectively). The relative potency of purified rat GH (GH-I-5) was 2.0×10^{-4} and 1.0×10^{-4} for our antiserum (arPRL-86) and arPRL-S-9, respectively.

Pituitary cell preparations

Pituitaries were dissected from random cycling female rats and enzymatically dispersed using sterile technique. A non-trypsin dissociation to isolate single, functional pituitary cells was developed based on the method of Weiner *et al.* (1983). In brief, after removal of the neurointermediate lobe, each anterior pituitary gland was placed in Hank's balanced salt solution (calcium- and magnesium-free; HBSS-CMF) containing antibiotics (100 U/ml penicillin G and 0.1 mg/ml streptomycin sulfate) and minced finely (<1 mm). The tissue fragments were incubated for 60 min in a shaking water bath (37°C) in HBSS-CMF containing 0.3 g% collagenase, 1 mg% DNase I and 0.1 mg% trypsin inhibitor. The cells were then mechanically dispersed by trituration (~12 times at 30 min and at 60 min) using a siliconized Pasteur pipet. Cells were pelleted by centrifugation (150 *g*, 10 min), the supernatant removed and the cells resuspended in fresh HBSS-CMF. The wash was repeated twice and then the cells were collected following filtration through sterile nylon mesh (20 μm pore). An aliquot of cell suspension was removed for cell counting with a standard hemacytometer and determination of cell viability using trypan blue exclusion technique. This method routinely yielded between 1.0 and 1.5 million cells per pituitary with cell viability always in excess of 95%. We initially chose to use a trypsin-free method because of concerns raised in the literature regarding damage to DA receptors by trypsin. It has been suggested, however, that collagenase produces very few single cells, which are absolutely required for the plaque assay (see Neill *et al.*, 1987). We have found, however, that our collagenase method yields a majority of single cells and that reclumping of cells is the major source of doublets, triplets, etc. Reclumping can be minimized by making all dilutions of the dispersed cells in HBSS-CMF. Then the re-exposure of the cells to divalent cations (which promotes cell adhesion) is upon their mixture with the oRBCs, which buffer the physical contact of the pituitary cells and minimize re-aggregation.

Reverse hemolytic plaque assay

Standard assay The standard RHPA was used as described by Smith *et al.* (1986). For the assay, ovine red blood cells (oRBCs) were coupled to Staphylococcus protein A in the presence of an aged solution of chromium chloride and suspended in DMEM (with 10 mM HEPES-HCl; without phenol red) containing 0.1% BSA (DMEM/BSA). Dispersed anterior pituitary cells (3×10^6/ml) were mixed with protein A-coated oRBCs (12%) and the suspension infused into modified Cunningham chambers. The chambers were placed in a 95% air-5% CO_2 atmosphere at 37°C, and the cells allowed to attach to the polylysined slide (45 min). After unattached cells were rinsed out with DMEM/BSA, detection of PRL secretion was initiated by infusion of antiserum (arPRL-86, final dilution 1/200) with or without dopamine (500 nM). To test for the effects of complement on PRL secretion, some chambers also included guinea pig complement (final dilution 1/80) in the antiserum incubation. Following a 60 min incubation, all chambers were rinsed with DMEM/BSA to remove unbound complement and/or antiserum. In chambers that had been exposed to the simultaneous mixture of antiserum and complement, plaques had begun to form but were not complete at the end of the 60 min. Therefore, complement alone (1/80) was infused into all chambers and allowed to incubate for 30 min to ensure complete plaque development for quantitation. All chambers which had *simultaneous* incubation of antiserum with complement and half of those which had been exposed to *separate* incubations of antiserum and complement were then rinsed with DMEM/BSA. The remaining chambers that had incubated with antiserum alone were subjected to a second, 60 min incubation with complement in order to equal the total complement exposure of the *simultaneous* antiserum/complement group. Subsequent analysis demonstrated that hemolysis was complete after 30 min of complement alone since there were no quantitive differences in plaque size between the two *separate* incubation groups (30 min *vs* 90 min complement: 9055 ± 467 *vs* 9251 ± 547 for spontaneous release; 2887 ± 393 *vs* 3014 ± 385 in the presence of 500 nM DA).

Following complement incubation and rinse with DMEM/BSA, the cells were fixed with 2% gluteraldehyde in DMEM/BSA for 10 min on ice. After a 10 min exposure to 0.1% Triton X-100, cells were stained with 2.5% phloxine followed by azure II and methylene blue (0.05% and 0.02%, respectively, in 0.05% Na-borate). The stains were differentiated in a rosin-ethanol solution. This produced bright blue nuclei and pale pink cytoplasm, making even non-plaque-forming pituitary cells readily identifiable with bright-field microscopy.

Plaque areas were quantitated by morphometric measurements using an image-analysis system with JAVA Imaging software (Jandel Scientific, Corta Madera, CA). The area of each plaque was calculated from two perpendicular measurements of diameter. The experiment was repeated on three different cell preparations. All the experiments produced the same results internally. The data presented in Figure 2 and Table 1 were calculated from measurements made from a single experiment, due to inter-assay variability. Statistical significance was determined by two-way analysis of variance followed by a t-test between independent means.

Modified RHPA ('Split chamber RHPA')

The procedure for our modified RHPA is illustrated in Figure 5. Cunningham chambers were fabricated from two photoetched coverslips, both of which had been treated with poly-lysine. Using an inverted microscope (Nikon TMS) with phase-contrast optics to visualize the photoetched grids, the chambers were assembled such that the grids of the two coverslips were perfectly aligned. Initially, the top coverslip was attached directly to the pieces of double stick tape, making difficult any adjustments required to perfect grid alignment. Later, we began adding a thin strip of teflon tape before placement of the top coverslip which greatly facilitated manufacture of the chambers. With gentle pressure applied, the coverslips 'adhered' well to the teflon tape even when inverted, although they could be additionally anchored with a piece of standard tape.

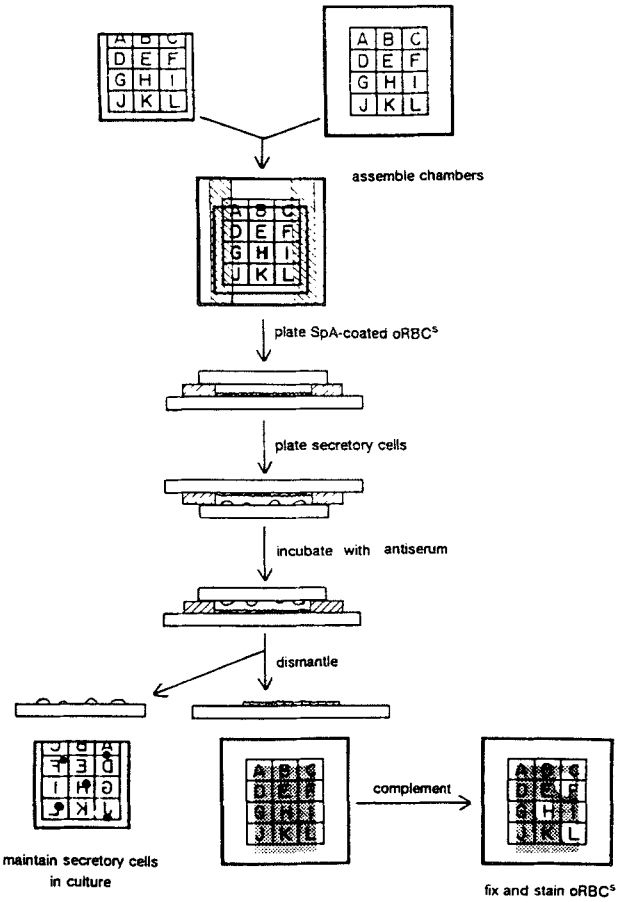

Figure 5 Protocol for the 'split chamber' modification of the RHPA. See text for explanation

A suspension (4–5%) of protein A-coated oRBCs was infused and allowed to plate on the 'floor' of the chamber (30 min, 37°C). Unattached erythrocytes were then rinsed out and a suspension of anterior pituitary cells ($1.5–2.0 \times 10^5$/ml) was infused, the chamber inverted, and the cells allowed to plate onto the 'ceiling' of the chamber (30 min, 37°C). In our experience, we have had greater success in achieving even oRBC 'lawns' when they are plated first. Again, unattached cells were rinsed from the chamber and the assay was initiated by the infusion of medium containing arPRL-86 (1/200). Following a 60 min incubation at 37°C, the chambers were rinsed and then dismantled while submerged in prewarmed, CO_2-equilibrated medium. Again, the use of the teflon tape enabled one to easily lift off the top coverslip with forceps. The 'ceilings' of the chambers, with anterior pituitary cells attached, were transferred to sterile petri dishes containing pre-equilibrated medium. The 'floors' of the chambers, with oRBCs attached, were incubated with complement (1/50 or 1/80) to rapidly develop plaques. PRL-secreting cells were identified by locating the pituitary cells which corresponded to the plaques. While this could be accomplished with photomicrographs as illustrated in Figures 3 and 4 (see Results), a much simpler method of identification was routinely used. A reproduction of the photoetched grid was xerographed onto transparency sheets. The location (and perimeter) of numerous plaques could be quickly drawn on one of these reproductions using a colored marker. The transparency sheet was then flipped over to produce the mirror image of the 'ceiling' coverslip and was used as a map to locate the plaque-forming cells for subsequent physiological experiments.

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