We examined living cultures by phase-contrast and interference-contrast microscopy. Representative replicate cultures were fixed (Bouin's fixative) at 8-hour intervals from 16 to 114 hours and then stained with toluidine blue or hematoxylin.

Development of gametocytes in vitro after a single asexual generation is significant because it demonstrates that control of the transition from schizogony to gametogony is a genetic trait, largely independent of host influence. These experiments demonstrated that abbreviation of the life cycle was responsible for the shortened prepatent period, rather than a more rapid development of each stage as was originally suspected. Further, we observed macrogametocytes and microgametocytes within isolated clusters of parasites (presumably the progeny of a single sporozoite), suggesting that sporozoites, although haploid in chromosome number, may be bisexual.

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Blockade of Prolactin Action by an Antiserum to Its Receptors

Abstract. *A guinea pig antiserum to prolactin receptors selectively inhibited the binding of [^{125}I]prolactin to its membrane receptors as well as prolactin-mediated incorporation of [^{3}H]leucine into casein and transport of [^{14}C]aminoisobutyric acid, but was without effect on the binding of [^{125}I]insulin and insulin-mediated events in explants of rabbit mammary glands maintained in culture. These findings provide direct evidence for an obligatory functional role of a membrane receptor in mediating the action of a polypeptide hormone.*

That plasma membrane receptors are involved in mediating the biological effects of polypeptide hormones is a widely accepted concept. However, the hypothesis is based principally on indirect evidence derived from studies correlating the extent of specific binding of hormones to plasma membranes or target cells with activation of adenylate cyclase or other cellular functions (*1*). A review of these data still leaves unanswered the question whether specific hormone binding sites or receptors are obligatory mediators of hormone action. To test the hypothesis more directly we have examined the question of whether antibodies to prolactin receptors would block the biological effect of the hormone. In the case of acetylcholine, Patrick *et al.* (*2*) have used a similar approach to establish the essential role of the receptor in mediating the action of this neurotransmitter. We have found that an antiserum to prolactin receptors which were purified from rabbit mammary glands not only specifically inhibited the binding of prolactin to its receptors but also selectively blocked prolactin-stimulated casein synthesis and amino acid transport in explants of rabbit mammary glands. The same antiserum, however, had no effect on insulin binding, on insulin-stimulated amino acid transport, or on glucose oxidation; nor did it bind prolactin. Our results provide evidence for an obligatory functional role of the prolactin receptor in mediating the action of this hormone.

Prolactin receptors were purified from extracts of mammary glands of pregnant rabbits by affinity chromatography (*3*) and were used to immunize guinea pigs (*4*). These antiserums to receptors have been characterized and described (*4*). One of these antiserums was used in our study to examine its effect on the binding of prolactin to membrane receptors and on prolactin-mediated biological actions in rabbit mammary glands. The isolation of rabbit mammary gland particles which contain receptors for prolactin and insulin has been described (*5*). The procedure described (*5*) for the determination of specific binding of ^{125}I-labeled ovine prolactin ([^{125}I]oPRL) and [^{125}I]insulin was also used in the study reported here. Figure 1 shows that the addition of 100 μl of diluted (1/100) guinea pig antiserum to receptor to the prolactin binding assay mixture resulted in approximately 90 percent inhibition of specific binding of [^{125}I]oPRL to membrane particles, whereas the same amount of a control guinea pig serum (CS; serum obtained from an animal which was injected with vehicle alone) had no effect. Not included in Fig. 2 is the observation (*4*) that even antiserums obtained from guinea pigs immunized with crude membrane particles isolated from either mammary gland or liver of rabbits did not inhibit the binding of [^{125}I]oPRL to membrane receptors. Moreover, the specific binding of [^{125}I]insulin to the same membranes was not affected by this antiserum to receptor (*4*). The same antiserum to the receptor not only inhibited the binding of [^{125}I]oPRL to crude particulate membrane receptors, but also to receptors solubilized by Triton X-100 and then purified by affinity chromatography (*3*, *4*). Specific binding of prolactin to membrane particles derived from several rat and rabbit tissues was also inhibited.

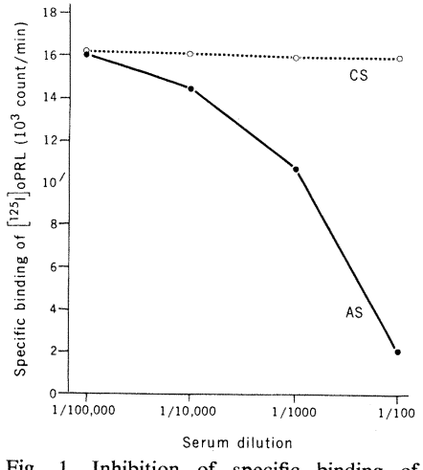

Fig. 1. Inhibition of specific binding of [^{125}I]ovine prolactin (oPRL) to membrane particles from pregnant rabbit mammary tissues by guinea pig antiserum to receptor. The preparation of crude membrane particles derived from pregnant rabbit mammary glands and the method for the determination of specific binding of [^{125}I]oPRL to membranes have been described (*5*). The incubation mixture (final volume, 0.5 ml) contained 230 μg of membrane proteins, 1.2×10^5 count/min (approximately 0.5 ng) of [^{125}I]oPRL and 100 μl of antiserum to receptor (AS) or control serum (CS) that had been previously diluted. Each point represents the mean of duplicate determinations.

To exclude the possibility that the antiserum to the receptor was binding [^{125}I]oPRL and thereby inhibiting the tissue binding of prolactin, additional experiments were performed with the use of a double antibody immunoprecipitation procedure. Trace amounts (~ 0.5 ng) of [^{125}I]oPRL were incubated with increasing concentrations of the antiserum to receptor for 3 days at 4°C, at which time excess rabbit antiserum to guinea pig γ-globulin was added to precipitate guinea pig antibodies. Virtually no [^{125}I]oPRL was precipitated even when the guinea pig antiserum to the receptor was used at a concentration of 10 percent by volume. When the same experiment was repeated with specific antiserum to oPRL in place of the antiserum to the receptor, 90 percent of the [^{125}I]oPRL was precipitated when the antiserum dilution was 1/1000.

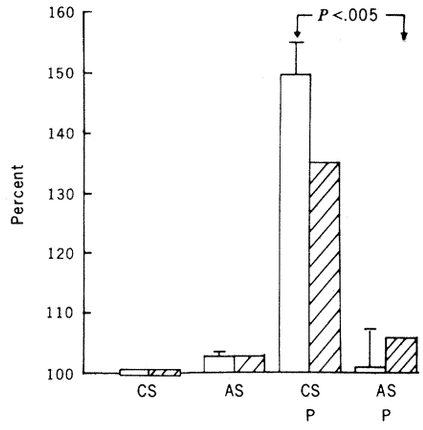

Fig. 2. Effect of antiserum to receptor on the prolactin-stimulated incorporation of [^{3}H]leucine into casein in rabbit mammary tissue explants. Rabbit mammary explants were maintained for 3 days in culture medium containing insulin and hydrocortisone; they were then transferred to dishes containing fresh medium. When ovine prolactin (NIH-P-S10, 26 I.U./mg) was present in the fresh medium, a concentration of 1 μg/ml was employed. Incubation in the presence or absence of prolactin was continued for another 3 days. The concentration of and time of exposure to prolactin were determined in an experiment designed to produce maximal stimulation of the incorporation of [^{3}H]leucine into casein in the mammary explants. Guinea pig antiserum to receptor or control guinea pig serum was sterilized by passage through a Millipore membrane and introduced into culture medium at a final concentration of 10 percent (by volume) 1 day before the explants were exposed to prolactin. γ-Globulin fractions were obtained by precipitation with 5*M* sodium phosphate with ammonium sulfate (33 percent saturation) (*4*) and reconstituted with 0.05*M* sodium phosphate buffer, *p*H 7.4, in a volume equal to that of the original serum. The γ-globulin content (by volume) was filtered through Millipore membranes and added at a final concentration of 10 percent (by volume). Serums or γ-globulin fractions were present in the cultures for the remaining 4 days. For each culture condition, dishes were set up in quadruplicate. At the end of the day 6, 5 μc of L-[4,5-^{3}H]leucine (43 c/mmole; New England Nuclear) in 0.1 ml of medium was added to each dish and the dishes were incubated for 4 hours; the explants were then weighed, and the amount of [^{3}H]casein synthesized was determined (*6*). Casein was precipitated with rennin and calcium. The washed casein pellet was solubilized in 0.5 ml of Protosol (New England Nuclear) and the radioactivity was counted in toluene-based scintillation fluid. Results are expressed as a percentage of [^{3}H]casein synthesis. The basal level (control serum or γ-globulin fractions derived from it) was considered equal to 100 percent in each experiment. Open bars, whole serum (mean ± standard error of the mean) for three experiments; the basal levels of [^{3}H]casein synthesis in these three experiments were 927 ± 50, 853 ± 44, and 1020 ± 29 (mean ± S.E.M., N = 4) count/min per milligram of tissue. The hatched bar, γ-globulin fraction (one experiment); the basal level of [^{3}H]casein synthesis being 984 ± 34 (N = 4) count/min per milligram of tissue. Statistical data were evaluated by Student's *t*-test. Abbreviations P, prolactin; AS, antiserum to receptor; and CS, control serum.

Since the antiserum to the receptor at a concentration of 10 percent did not bind even a trace amount of prolactin, it would have no effect on the prolactin (at a concentration of 1.2 μg/ml) used in the culture media. These observations demonstrate that the antiserum to receptor selectively blocks the binding of prolactin to its receptor site.

The next series of experiments were therefore designed to examine if the same antiserum would, by inhibiting the binding of prolactin to its receptor site, block the biological action of the hormone. The incorporation of [^{3}H]leucine into casein, an event that is specifically stimulated by prolactin in mammary tissues, was used to determine the effect of antiserum to receptor on the biological activity of prolactin. We used a mammary gland organ culture technique similar to that of Juergens *et al.* (*6*). Adult female rabbits were made pseudopregnant by a single intravenous injection of 100 international units (I.U.) of human chorionic gonadotropin (Ayerst). The animals were killed on day 12 of pseudopregnancy, and the mammary glands were removed under aseptic conditions. Explants (0.5 to 1 mg each) were prepared, and three to four explants were cultured on siliconized lens paper that was floated on 1 ml of culture medium contained in a plastic culture dish (Falcon Plastics and Corning Glass Works). The explants were cultured in medium 199 containing Hanks balanced salts (Grand Island Biological), penicillin G (50 μg/ml), streptomycin (50 unit/ml), Fungizone (2.5 μg/ml), porcine insulin (10 μg/ml), hydrocortisone (10 μg/ml), and HEPES [*N*-(2-hydroxy)ethylpiperazine-*N*'-2-ethanesulfonic acid] buffer (15 m*M*) (Sigma), with the *p*H adjusted to 7.4. Other details were similar to those described by Juergens *et al.* (*6*) unless otherwise noted.

The addition of 10 percent (by volume) guinea pig antiserum to receptors to the culture medium did not alter the baseline rate of [^{3}H]casein synthesis observed in the presence of the same concentration of control guinea pig serum (Fig. 2). Addition of prolactin in the presence of control serum resulted in a 50 percent

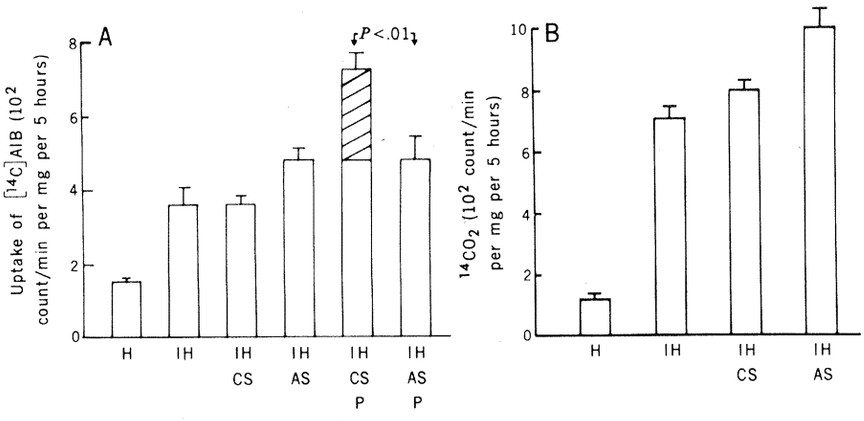

Fig. 3. (A) The effect of antiserum to the receptor on transport of [^{14}C]aminoisobutyric acid (AIB) into mammary explants. The experimental procedures were similar to those described in the legend to Fig. 1, except that serum was added 4 hours before the addition of insulin on day 1. The total exposure to serum was therefore 6 days. At the end of the day 6, 0.5 μc of 2-amino[1-^{14}C]isobutyric acid (60 mc/mmole; Amersham/Searle) in 0.1 ml of medium was added to each dish. After exposure to [^{14}C]AIB for 4 hours, the explants were weighed, washed twice with ice-cold medium, and digested in 0.5 ml of Protosol; radioactivity was then determined. Results are expressed as means ± the standard error of the mean for four observations. Abbreviations: H, hydrocortisone; I, insulin. The hatched area represents prolactin-stimulated uptake of [^{14}C]AIB. (B) the effect of antiserum to receptor on oxidation of [1-^{14}C]glucose to $^{14}CO_2$. The experimental procedures were identical to those described in the legend to Fig. 2. At the end of the culture period, mammary explants from each dish were weighed, quickly refloated on a fresh lens paper in the same culture medium which had been transferred to a 25-ml erlenmeyer flask. Then 0.5 μc of D-[1-^{14}C]glucose (660 mc/mmole; Amersham/Searle) was added in 0.1 ml of medium to each flask; the flasks were then closed with a rubber stopper to which a plastic well was attached (Kontes Glass). After incubation for 5 hours at 37°C with gentle shaking of the flasks, the mixtures were treated with 0.3 ml of Hyamine injected through the stopper into the plastic well, and 1 ml of 5*N* HCl was then injected into the medium. The flasks were allowed to stand overnight. The plastic well, which contained the Hyamine, was conveniently dropped into a counting vial by cutting the stem. The $^{14}CO_2$ trapped was counted. In this experiment, mammary explants that had been boiled for 20 minutes before addition of [1-^{14}C]glucose were used as the tissue blank. Results are expressed as mean ± the standard error of the mean for three observations.

increase in [^{3}H]casein synthesis, whereas in the presence of antiserum to receptor no increase was observed. Essentially the same results were obtained when γ-globulin fractions isolated either from control or antiserum to the receptor were used. These results suggest that the inhibitory factor in the serum was due to the presence of antibodies. However, direct studies indicated that the specific binding of [^{125}I]oPRL to explants was reduced by 85 to 90 percent in the presence of antiserum to the receptor (*7*). These findings also demonstrate that the antiserum did not bind or destroy prolactin. The above observations taken together indicate that the antibodies to the receptor block the biological action of prolactin by blocking receptor sites, rendering them inaccessible to the hormone.

To demonstrate that the inhibitory effect of the antiserum was specifically limited to prolactin-mediated effects, two additional experiments were performed (Fig. 3). When transport of [^{14}C]aminoisobutyric acid (AIB) was measured in mammary explants cultured in a manner similar to that used for the determination of [^{3}H]casein synthesis, it was observed that insulin alone stimulated the uptake of [^{14}C]AIB transport, a finding that was observed previously (*8*). Addition of either control guinea pig serum or antiserum to the receptor did not influence the effect of insulin. However, the addition of prolactin to the medium containing control serums caused a further 50 percent stimulation of the uptake of [^{14}C]AIB above that observed in the presence of insulin plus serum. The prolactin-dependent portion of the [^{14}C]AIB transport was completely abolished on the addition of antiserum to the receptor.

Cellular metabolism, as reflected by the oxidation of [1-^{14}C]glucose in the cultured mammary explants, was not impaired by the addition of antiserum to the receptor (Fig. 3B). This observation shows that the antiserum selectively blocks the action of prolactin without any effect on insulin-mediated actions such as amino acid transport and oxidation of [1-^{14}C]glucose in the same tissue. The inhibition of prolactin action by the antiserum is thus specific and is not due to a general inhibition of membrane-mediated functions or due to a "toxic" effect of the antiserum on mammary cells.

Our results support the hypothesis that the membrane structures which bind prolactin are essential for mediating several actions of this hormone. Thus by definition, these structures are receptors for prolactin. The immunological approach used in our study may also be extended to elucidate receptor functions in intact animals as well as to locate putative target organs for prolactin.

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7. Mammary explants were cultured in medium containing only insulin and hydrocortisone in the presence of antiserum to receptor or control serum. About 5×10^5 count/min (0.5 ng) of [^{125}I]oPRL was added to each dish. For each determination of binding of [^{125}I]prolactin, two dishes contained only [^{125}I]prolactin and two dishes contained the same amount of [^{125}I]-prolactin plus 10 μg of unlabeled prolactin. After incubation for 10 hours, explants were weighed and washed twice with ice-cold medium, and the radioactivity was determined. Specific binding refers to the amount of [^{125}I]prolactin bound to explants in the absence of unlabeled prolactin minus that found in the presence of unlabeled hormone. Similar inhibition of binding of [^{125}I]prolactin to explants was observed 1 day and 4 days after the explants had been exposed to antiserums to receptors.
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Morphine-Induced Rotation in Naive, Nonlesioned Rats

Abstract. *In rats injected with morphine in the midbrain reticular formation, pronounced ipsilateral rotation behavior was elicited by mild auditory and visual stimuli. The frequency of occurrence and rate of rotation were dose-dependent. This effect was site specific and drug specific; other drugs (except heroin) failed to induce this behavior. Naloxone potentiated the morphine rotation. Pretreatment with drugs that either potentiated or attenuated the morphine rotation indicated involvement of the noradrenergic and cholinergic systems and excluded a role for the dopaminergic system. No analgesia was observed after morphine microinjection in this site; thus, the hyperresponsivity to mild auditory and visual stimuli and concurrent analgesia previously seen in animals with morphine microinjections in the periaqueductal gray matter appear to be dissociable effects of morphine, and site specific.*

Morphine, a potent analgesic opiate, has a wide spectrum of physiological and behavioral effects, including analgesia, hypothermia, respiratory depression, stimulation of locomotor activity, and euphoria; repeated administrations lead to development of tolerance and drug-seeking behavior. Cessation of morphine administration after repeated administrations leads to another behavioral syndrome, "abstinence," characterized by irritability, weight loss, increased intestinal motility, wet shakes, ear blanching, teeth chattering, and so forth. It seems probable that different sites in the central nervous system mediate these diverse effects of morphine and that different neurochemical systems underlie the different effects observed.

Rotation behavior has been reported (*1*) in morphine-dependent animals during naloxone-precipitated abstinence after various pretreatments involving alterations in the dopaminergic system. Typically, rotation behavior occurs after administration of dopamine agonists and antagonists in animals with lesions of the nigro-neostriatal pathway, and it is currently the focus of intensive investigations as an animal model for extrapyramidal malfunction (which occurs in such disorders as Parkinsonism). The results of these studies implicated primarily the dopaminergic system, although there were indications that serotoninergic and cholinergic mechanisms were also involved (*2*). Thus, the finding that rotation behavior was precipitated during morphine withdrawal in lesioned or pretreated rats suggested the involvement of dopaminergic mechanisms in the phenomenon of morphine dependence.

We report here the occurrence of pronounced rotation behavior following intracerebral microinjection of morphine in naive, nonlesioned rats. Following microinjection of morphine in the midbrain reticular formation (MRF), rats responded to previously neutral stimuli, such as auditory and visual ones, by a burst of "pivots" (rapid rotations) on the ipsilateral hind leg. The rate of rotation, often greater than 2 per second, was at least several times that reported to follow ad-