eminence is selectively increased (*21*). The existence of specific binding sites in the external median eminence for other polypeptide hormones, namely, lactogen (*10*), angiotensin II (*11*), and calcitonin (*22*), indicates that nerve terminals in this region have receptors for a variety of polypeptide hormones in addition to insulin. This broad hormone-recognition capacity suggests that the median eminence plays an important but unappreciated receptive role in hormone-to-brain communication.

Axonal or synaptic insulin receptors in the arcuate nucleus, on the other hand, seem ideally situated to influence synaptic transmission, and thereby to alter hypothalamic electrical activity, such as that following either systemic insulin administration (*23*) or microinjection of insulin into the ventral hypothalamus (*3*). In view of this possibility, a direct action of insulin on insulin-receptive axons and synaptic terminals in the arcuate nucleus may underlie a fundamental mechanism whereby fluctuations in blood-borne insulin can rapidly modulate the activities of hypothalamic circuits programming feeding behavior, body weight, and glucose homeostasis.

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16. Thin sections (pale gold interference color) were covered with a monolayer of emulsion (Ilford L-4) [B. M. Kopriwa, *Histochemie* **37**, 1 (1973)] and exposed for 4 months before development in fresh Kodak D-19B [B. M. Kopriwa, *ibid.* **44**, 201 (1975)]. Radioautographs were then stained in uranyl acetate and lead citrate and examined in an electron microscope (Hitachi HS-7S). Grains were photographed wherever they occurred (×8000) and enlarged (×22,000). The relative content of label in neuronal and glial structures was analyzed by allocating and tabulating grains according to the underlying structure (direct scoring method). An additional 205 grains were analyzed by both direct scoring and the statistical method of N. J. Nadler [*J. Cell Biol.* **49**, 877 (1971)] through the use of two different resolution circles (50 percent and 95 percent probability of enclosing the source of radioactivity). Computerized analysis of these results (N. J. Nadler and G. Levine, *Biol. Cellulaire*, in press) showed no statistical difference in the distribution obtained by direct scoring compared with resolution circles. This exercise demonstrates in our study that the simplified technique of direct scoring is as accurate in localizing the structural source of bound radioactivity as the more complicated methods available for quantitative fine structural radioautography, and that locating silver grains by direct scoring can be taken with maximum confidence to designate the location of [^{125}I]insulin binding sites.
17. In rats injected with [^{125}I]insulin alone, the average reaction per 6959 μm^2 of external median eminence was 145 ± 9.5 grains [mean ± standard error of the mean (S.E.M.)], and 119 ± 7.2 grains over the arcuate nucleus. In coinjected rats, the average reaction was 33 ± 2.6 grains and 37 ± 3.1 grains, respectively. Background fog was 7.2 ± 0.8 grains per 6959 μm^2. [The reaction reductions obtained from our analysis of radioautographs of sections (1 μm thick) of Epon-embedded hypothalamus fixed with aldehydes are in close agreement with our previous results obtained from radioautographs of sections (4 μm thick) of paraffin-embedded brain fixed with Bouin's solution (*7*), in which competitive binding sites for insulin in the ventral hypothalamus had the recognition specificity properties of biologically important insulin receptors.]
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24. The arbitrarily chosen number of grains analyzed in fine structural radioautographs from each region does not reflect the striking differences in grain density between injection groups (Fig. 1). Thus, in order to score large numbers of silver grains in fine structual radioautographs, from coinjected rats (Fig. 1B), many more sections were examined than were required for rats injected with [^{125}I]insulin alone (Fig. 1A).
25. Many unidentified structures resembled the "growth-cone-like" processes previously described in this region [M. van Houten and J. R. Brawer, *J. Comp. Neurol.* **179**, 719 (1978)].
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Local Effect of the Blastocyst on Estrogen and Progesterone Receptors in the Rat Endometrium

Abstract. *Nuclear receptors for both estradiol and progesterone were present in twofold higher concentrations in implantation sites than in nonimplantation regions of the endometrium of 6-day pregnant rats. Decidualization in the absence of an embryo was not accompanied by a similar increase in the concentration of nuclear receptors. Moreover, this difference in receptor distribution between the implantation and nonimplantation areas persisted when a major part of the maternal supply of sex steroids was suppressed by ovariectomy on day 5 of pregnancy. These results support the hypothesis that steroids originating from the embryo affect the endometrial implantation site.*

The hormonal mechanism that triggers implantation of the ovum is poorly understood. Administration of estrogen or progesterone, or both, depending on the species, is necessary for implantation to occur in ovariectomized females (*1*). It has been proposed that steroids synthesized or accumulated by the blastocyst play an important role in implantation through a local action on the adjacent endometrium (*2*). Various histological and biological modifications have been shown to occur at the implantation sites, and the presence and metabolism of steroids in the blastocyst have been demonstrated. Thus the hypothesis according to which these local changes are brought about by hormones originating from the

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embryo remains in question (*3*). Steroid hormones provoke the translocation of their receptors from the cytosol to the nucleus (*4*). Thus local delivery of steroids by the blastocyst to the endometrium should be accompanied by an increased concentration of nuclear steroid-receptor complexes at the implantation sites. The present experiments were designed to test this prediction.

Six-day pregnant rats were injected with trypan blue to delineate the implantation sites (*1*). Endometrium was prepared from these regions and from nonimplantation regions. Estradiol and progesterone receptors were measured in the cytosol and in the nuclei. As shown in Table 1, the concentration of both estradiol and progesterone receptors in the nuclei displayed an approximately twofold difference between the implantation areas and the remaining areas of the endometrium. Cytosolic receptors (expressed on the basis of DNA content) were only slightly higher in the regions outside the implantation sites. We also compared the qualitative characteristics of estradiol and progesterone receptors from implantation and nonimplantation areas. No differences were found in the affinity of the cytosolic receptors for the hormones, as analyzed according to Scatchard (*5*), or in their sedimentation properties, as studied by density gradient ultracentrifugation.

The increased concentration of nuclear receptors for estradiol and progesterone at implantation sites could have been due either to a direct effect of the blastocyst or to a secondary effect of the vascular and cellular modifications that accompany decidualization (see, for example, in Table 1 the increased protein to DNA ratio at implantation sites). To examine this possibility, we used pseudopregnant rats, and on day 5 of pseudopregnancy we induced decidualization in one uterine horn by traumatization (Table 2). On day 6 the animals were killed and receptors measured in decidualized and nondecidualized endometrium. As shown in Table 2, the concentration of receptors (per milligram of DNA) was the same for both progesterone and estradiol and in both the nuclear and cytosolic fractions. Therefore, the increase in nuclear receptors that was observed at implantation sites in the pregnant animals could not be attributed merely to increased vascular permeability and to a better supply of maternal hormones at these sites. A direct effect of the blastocyst appeared more likely, since the phenomenon could be observed only when the embryo was present (*6*).

Because implantation still occurs in rats ovariectomized on the morning of day 5 of pregnancy (*7*), we designed another experiment in which most of the maternal supply of estradiol and progesterone would be suppressed (only adrenal hormones would be maintained). If differences in receptor distribution were really due to a local supply of hormones by the blastocyst, they should persist on day 6. We found that the concentration of estradiol receptors in the nuclei on day 6 was 5.49 ± 0.45 pmole per milligram of DNA (mean ± standard error; four determinations) at the implantation sites and 2.42 ± 0.33 in the intermediary regions. Progesterone nuclear receptors were present in the same regions at concentrations of 1.11 ± 0.02 and 0.67 ± 0.05 pmole per milligram of DNA, respectively. The twofold difference in nuclear receptors for both progesterone and estradiol was therefore conserved after ovariectomy (*8*).

Our finding that the concentrations of nuclear receptors for both estradiol and progesterone are increased twofold at implantation sites is compatible with the hypothesis that the blastocyst participates in the local delivery of these steroids to the endometrium. This conclusion is strengthened by the absence of differences in the amounts of receptors in decidualized and nondecidualized endometrium of pseudopregnant rats. Ovariectomy, which suppresses most of

Table 1. Estradiol and progesterone receptors at implantation sites in the rat endometrium. Wistar rats (3 to 4 months old) housed and treated as described (*9*) were taken on the morning of day 6 of gestation (day 1 being when spermatozoids were present in the vagina) and given an intravenous injection of 0.75 ml of 1 percent trypan blue in saline. Thirty minutes after the injection the animals were killed and the uteri were excised and slit longitudinally in the cold room. Implantation sites, colored in blue, were excised, and the endometrium was removed by gentle scraping. Portions of endometrium from the intermediary regions (outside implantation zones) were also prepared. The samples were kept frozen in liquid nitrogen up to 2 months (without any modification of receptor characteristics). Cytosolic and nuclear fractions were prepared, and the nuclear receptor for estradiol (*10*) and the cytosolic and nuclear receptors for progesterone (*11*) were measured by exchange techniques. Cytosolic receptor for estradiol was directly assayed by incubating 200 μl of cytosol in triplicate with (i) 10 n*M* [^{3}H]estradiol alone or (ii) 10 n*M* [^{3}H]estradiol plus 1 μ*M* unlabeled diethylstilbestrol. The cytosol was incubated for 2 hours at 0°C. Receptor concentration was calculated as the difference between the bound radioactivity [measured by charcoal adsorption (*12*)] in (i) and (ii). Since 85 to 90 percent of estradiol receptor complexes are located in the nuclei (*13*) it was not necessary to use an exchange assay for measuring estrogen receptors in the cytosol. The results given (*14*) are the means (± standard error) of five measurements for estradiol receptors and six for progesterone receptors; 30 animals were used for each measurement. N.S., not significant.

Fraction	Implantation site		Nonimplantation site		*P**
	pmole/mg DNA	pmole/mg protein	pmole/mg DNA	pmole/mg protein	
			Estradiol receptors		
Cytosol	11.33 ± 1.73	0.42 ± 0.03	12.11 ± 1.05	0.96 ± 0.06	N.S.
Nuclei	8.83 ± 0.34		4.62 ± 0.33		< .001
			Progesterone receptors		
Cytosol	1.41 ± 0.04	0.17 ± 0.03	1.74 ± 0.39	0.35 ± 0.10	N.S.
Nuclei	0.86 ± 0.07		0.45 ± 0.02		< .001

*Determined from concentrations expressed as picomoles per milligram of DNA.

Table 2. Estradiol and progesterone receptors in decidualized and nondecidualized endometrium of pseudopregnant rats. Oviduct was sectioned on day 1 of pregnancy. On the morning of day 5 a cotton thread (exceeding by 5 mm the length of the horn) was introduced into one of the uterine horns. Twenty-four hours later the thread was removed, the endometrium was prepared from each horn, and receptors measured (see Table 1). Results are given as the means (± standard error) of four determinations. Twenty rats were used for each determination. There were no statistically significant differences between the decidualized and the nondecidualized horns (with receptor concentrations expressed as picomoles per milligram of DNA).

Fraction	Decidualized horn		Nondecidualized horn	
	pmole/mg DNA	pmole/mg protein	pmole/mg DNA	pmole/mg protein
		Estradiol receptors		
Cytosol	1.23 ± 0.12	0.093 ± 0.001	1.33 ± 0.17	0.38 ± 0.07
Nuclei	1.43 ± 0.14		1.38 ± 0.16	
		Progesterone receptors		
Cytosol	2.25 ± 0.21	0.161 ± 0.016	2.27 ± 0.23	0.44 ± 0.06
Nuclei	0.94 ± 0.09		0.93 ± 0.05	

the steroids of maternal origin, did not modify receptor distribution in the endometrium of pregnant rats. We cannot completely exclude the possibility that the blastocyst secretes an unknown factor that enhances receptor translocation from cytosol to nucleus. Nevertheless, our observations are in agreement with the hypothesis that assigns an active role to the blastocyst in implantation.

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6. Another striking feature was the lower total cellular concentration of endometrial estrogen receptors in pseudopregnant as compared to pregnant rats. The presence of embryonic estrogen might explain this difference.
7. After ovariectomy on the morning of day 5, normal implantation occurs on the morning of day 6. In the absence of progesterone, however, implantation is not maintained thereafter (P. Sartor, in preparation).
8. Cytosolic receptors for estradiol were present in concentrations of 5.49 ± 0.38 and 2.42 ± 0.81 pmole per milligram of DNA (four determinations) at the implantation sites and in the intermediary regions, respectively. The values for cytosolic receptors for progesterone were 4.17 ± 0.09 and 2.23 ± 0.26, respectively, in the same regions. Thus ovariectomy caused an important increase in the total cellular concentration of progesterone receptors at the implantation sites.
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Calcium Transients During Excitation-Contraction Coupling in Mammalian Heart: Aequorin Signals of Canine Purkinje Fibers

Abstract. *Aequorin signals in mammalian heart muscle cells reveal the existence of two temporally distinct processes that increase cytoplasmic calcium ions after membrane excitation. The differential dependence of these processes on the pattern of stimulation suggests that the first process is, or is closely related to, calcium entry through the surface membrane and that the second is calcium release from intracellular storage sites.*

The ability to directly observe intracellular Ca^{2+} transients is central to the solution of problems in the investigation of excitation-contraction coupling (*1*). In this report I show that through the use of the Ca^{2+}-sensitive photoprotein aequorin, such direct observations of intracellular Ca^{2+} transients have now been made in mammalian heart muscle. These observations are relevant to several concepts about excitation-contraction coupling in mammalian heart cells: First, Ca^{2+} enters the cell during the plateau of the action potential (*2*). As part of this process the ion channels inactivate but later recover in a simple exponential fashion (*3*) during the repolarized interval between action potentials. Second, during the action potential Ca^{2+} is released into the cytoplasm from intracellular storage sites (stores) (*4*). The stores are replenished by the Ca^{2+} entering during the action potential, and by active sequestration of the previously released Ca^{2+}. The Ca^{2+} taken up by the storage mechanism is not again available for release until after a delay. As a consequence (of replenishment and delayed reavailability), the amount of Ca^{2+} released during an action potential has been thought to depend in a rather complex way on the pattern of stimulation (*4*). The aequorin signals in canine Purkinje fibers have features consistent with these concepts.

Strands of Purkinje tissue were excised from either ventricle of canine hearts and mounted in a small (1 ml), temperature controlled (35° ± 0.5°C), recording chamber. The superfusing solution flowed continuously (4 ml/min) and was equilibrated with 95 percent O_2 and 5 percent CO_2 and contained 123 m*M* NaCl, 5.4 m*M* KCl, 2.7 m*M* $CaCl_2$, 0.42 m*M* NaH_2PO_4, 23.8 m*M* $NaHCO_3$, 1.0 m*M* $MgCl_2$, and 5.5 m*M* glucose. The strand was electrically stimulated by 0.5-msec pulses of current flowing between a punctate cathode near one end of the strand and a distant anode. The force of contraction was measured with an AME semiconductor force transducer. Membrane potential was measured with the micropipettes used to pressure-inject aequorin. The extraction and purification of aequorin was as described in (*5*). Light from the aequorin-injected strand was detected with an EMI 9635 B photomultiplier tube (PMT) and recorded as anode current. Signals (light, force, membrane potential) were stored on magnetic tape for later analysis (signal averaging). In each muscle 10 to 20 intracellular microinjections of aequorin were made along a 3-mm length of the strand.

Figure 1A shows examples of the three kinds of signals routinely recorded in these experiments. Light and isometric force development were normally recorded simultaneously throughout the experiment. Membrane potential was recorded before and after microinjection, but not during light recording.

Light begins to rise to levels detectably above the photomultiplier tube noise about 10 msec after the upstroke of the action potential. It then continues to rise rapidly for approximately 18 msec. In a distinctly separate phase, the light rises more slowly over a period of about 55 msec and reaches a peak roughly 80 msec after the onset of excitation. In this signal light declined exponentially (τ = 31 msec) over a period of 80 msec beginning about 10 msec after peak light intensity.

The configuration of aequorin signals is highly dependent on the inotropic state of the muscle (*6*, *7*). In the present experiments variations in inotropic state and the aequorin signal were produced by varying the pattern of stimulation. Figure 1B illustrates the simplest situation—that of stimulation at regular intervals. In this protocol stimulation was begun at the longest interval and progressed through successively shorter intervals as a steady-state response was achieved at each interval. The resulting relation between stimulus interval and steady-state maximum isometric force qualitatively resembled that observed in mammalian atrium (*8*) and sheep Purkinje fibers (*3*) in that the contractions at long intervals were large. In Fig. 1B, for intervals of 5 seconds and less, the aequorin signal consists of the initial rapid rise followed either by a plateau or an additional inflection on the declining phase. The light