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Surface receptors for pancreatic hormones in dog and rat hepatocytes: Qualitative and quantitative differences in hormone–target cell interactions

(insulin/glucagon/pancreatic polypeptide/hepatocyte isolation/hormone binding and dissociation)

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ABSTRACT **In order to evaluate potential differences in the kinetics of peptide hormone–receptor interactions in hepatocytes of different species, we developed a simple procedure for the isolation of canine hepatocytes. Cells (obtained by collagenase perfusion of an extirpated dog liver lobe) were isolated with uniform high viability and yield. In addition, isolated dog hepatocytes tolerated incubation for at least 4 hr in defined medium with only a slight decrease in viability and with no change in the kinetics of [^{125}I]iodoinsulin or [^{125}I]iodoglucagon binding to cell-surface receptors. Comparisons of peptide hormone interactions with isolated dog and rat hepatocytes showed that (*i*) [^{125}I]iodoglucagon associated with specific membrane receptors more rapidly than did [^{125}I]iodoinsulin, for both rat and dog hepatocytes and at both 30°C and 37°C; (*ii*) the steady-state binding of [^{125}I]iodoglucagon at 30°C was greater than that of [^{125}I]iodoinsulin in dog hepatocytes, but the reverse relationship held in rat hepatocytes; (*iii*) the rate of dissociation of [^{125}I]iodoinsulin from hepatocytes of both species was enhanced by the presence of the unlabeled hormone, whereas the rate of dissociation of receptor-bound [^{125}I]iodoglucagon was enhanced by the presence of unlabeled glucagon only in hepatocytes derived from the dog; and (*iv*) [^{125}I]iodopancreatic polypeptide bound to neither rat nor dog hepatocytes, although the [^{125}I]iodotyrosylated peptide bound to rat hepatocytes with an unusually high apparent dissociation constant. While confirming essential findings of pancreatic hormone binding to isolated hepatocytes, this comparison suggests that both qualitative and quantitative aspects of hormone–target cell interactions can show interspecies variability.**

Many investigations have demonstrated that formation of a hormone–receptor complex at the cell plasma membrane represents the initial event in the mechanism of peptide hormone action (1–3). Although different cell types such as human leukocytes (4, 5), fibroblasts (6, 7), and adipocytes (8) have proved useful in examining peptide hormone–receptor interactions, most studies on peptide hormone receptors have relied on isolated cells from the rat [including both adipocytes (9–12) and hepatocytes (13–15)] and their derived cell membranes. Methods for the isolation of hepatocytes from the sheep (16) and the pig (17) have also been described, but these preparations have not been examined in detail for hormone binding. In order both to examine the interspecies generality of peptide hormone–receptor interactions and to approach an understanding of peptide hormone physiology in the dog, we have developed a method for the isolation of canine hepatocytes. The simple procedure, which results in cells having high and prolonged viability, has permitted a direct comparison of insulin, glucagon, and pancreatic polypeptide (PP) interactions with dog and rat hepatocytes.

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MATERIALS AND METHODS

Isolation of Canine Hepatocytes. Mongrel dogs were anesthetized with sodium pentobarbital and the left medial liver lobe was exposed by a midline abdominal incision; the vascular supply was cut, and the lobe was removed and placed on ice. Within 5 min, an angiographic catheter (kept in place by hand), was inserted into the hepatic vein (the largest vessel) and the liver lobe was perfused [at 38°C (13), using a peristaltic pump and a flow rate of 250 ml/min] with 1 liter of calcium-free Hanks' balanced salt solution containing 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and 5 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine). The solution was equilibrated with 95% O_2/5% CO_2 and was adjusted to pH 7.4 prior to use. The perfusion solution flowed out passively through the portal vein, which was compressed intermittently to increase vascular pressure. The topography of an extirpated liver lobe showing the site for catheter insertion is shown in Fig. 1.

At the close of the perfusion, the medium was replaced with 1 liter of collagenase solution (0.2 mg/ml, Worthington type II, prepared in Hanks' balanced salt solution containing 4.7 mM $CaCl_2$) and the perfusion was continued for 4 min. The tissue was gently teased apart and the crude cell suspension (with any remaining enzyme solution) was divided between two 250-ml Erlenmeyer flasks; the flasks were then reequilibrated with 95% O_2/5% CO_2 and were incubated with gentle shaking at 30°C for 15 min. The resulting suspension was washed through a double nylon mesh (60-μm pores) with cold Hanks' solution to obtain a final volume of 200 ml. Cells were washed four to six times with 100-ml volumes of Hanks' buffer by centrifugation (200 × *g*, 4°C, 45 sec) in silane-treated 50-ml tubes. Dead cells layered above darker brown, viable cells during centrifugation and were easily removed by aspiration. A second incubation of the cells (at a concentration of about 3×10^7 cells per ml) for 30 min at 30°C in minimal essential medium (GIBCO), followed by several additional washings, resulted in the further removal of dead cells. The yield of cells prepared from single liver lobes was $4.2 \pm 1.7 \times 10^8$ (mean ± SD, $n = 16$). Cell viability, determined by trypan blue exclusion, was always greater than 99%.

Isolation of Rat Hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats (175–250 g) according to published procedures (13, 18); the viability of the cells accepted for study was always greater than 97%. In order to determine the

Abbreviation: PP, pancreatic polypeptide.

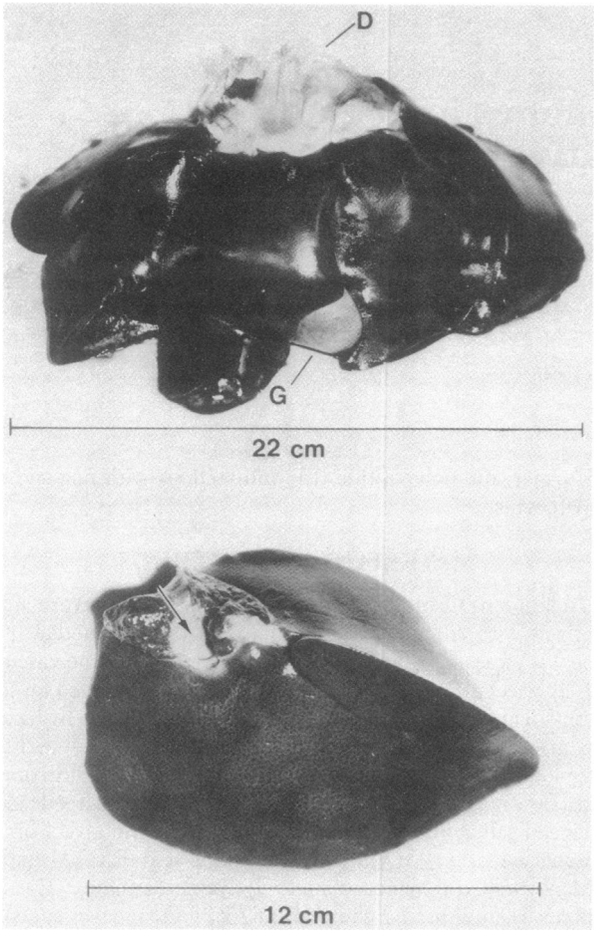

FIG. 1. Frontal view of an extirpated dog liver (*Upper*), showing the four main lobes and their relationships to the diaphragm (D) and the gall bladder (G), and dorsal view of the left medial lobe selected for hepatocyte isolation (*Lower*). The liver lobe is the third from the left in *Upper*. The arrow indicates the hepatic vein into which the perfusion catheter is inserted.

distribution of Kupffer cells in cell preparations from the rat and dog, suspensions of both types of hepatocytes were analyzed on a discontinuous Percoll (Pharmacia) gradient. Kupffer cells banded at the interface between 7.3% and 17% Percoll during centrifugation for 10 min at 1,088 × *g*. These experiments showed that the percentage of phagocytosing cells (as assessed by the uptake of latex beads) were the same in the two species (10–20%).

Cell Incubations. Hepatocytes from either dog or rat were diluted to a density of 2×10^6 cells per ml, using minimal essential medium (13) and were incubated with shaking in glass vials containing 1 ml of suspension with added supplements of ^{125}I-labeled (0.2–0.4 pmol) and unlabeled hormones. Aliquots of the cell suspensions (0.5 ml) were layered on 4 ml of ice-cold Hanks' solution containing 1% bovine serum albumin (fraction V, Miles), and cells were collected by centrifugation at 850 × *g* at 4°C for 1 min; the supernatant fluid was removed and the radioactivity in the pellet was measured. The amount of radiolabeled peptide bound to cell receptors was determined as the percent of total radioactivity appearing in the cell pellet.

For studies of glycogen synthesis, hepatocytes were incubated with 10 mM glucose and 2 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{14}C]fructose (uniformly labeled, 230 mCi/mmol, Amersham) in the presence of glucagon (10^{-12}–10^{-7} M) for 60 min at 37°C. Cell pellets were dissolved in 1 ml of 30% (wt/vol) KOH containing glycogen at 3 mg/ml during a 30-min incubation in a boiling water bath; glycogen was precipitated at 4°C overnight by the addition of 4 ml of 30% KOH and 10 ml of ethanol. The centrifuged pellet was dissolved in 4 ml of water and the glycogen was again precipitated with 10 ml of ethanol during 4 hr at 4°C. The final pellet was dissolved in 1 ml of water and the radioactivity of the solution was measured.

Preparation of Radioiodinated Hormones. Porcine insulin, porcine glucagon, and bovine PP were gifts from R. Chance, Lilly Research Laboratory. [^{125}I]Iodoinsulin (^{125}I-insulin) was prepared as described (19, 20) except that the amounts of the reactants were: insulin, 3 nmol; carrier-free Na^{125}I, 1 nmol; and freshly prepared chloramine-T, 2 nmol. The reaction was terminated after 10 min by adding *N*-acetyl-L-tyrosine (1 μmol), and the peptide (which contained about 0.1 mol of ^{125}I per mol) was purified by cellulose chromatography (20, 21). [^{125}I]-Iodoglucagon (^{125}I-glucagon) was prepared by a similar procedure, using 1.4 nmol of glucagon, 1 nmol of Na^{125}I, and 1.5 nmol of chloramine-T. The reaction was terminated by adding 3 nmol of $Na_2S_2O_5$ and the labeled peptide (containing about 0.1 mol of ^{125}I per mol) was purified by chromatography on QAE-Sephadex A-25 (Pharmacia) (22), a method which yields mono-iodoglucagon. No differences in the receptor binding of ^{125}I-glucagon were noted with material obtained by reducing the amount of chloramine-T to 0.3 nmol during the iodination. [^{125}I]Iodo-PP (^{125}I-PP) was prepared by direct chloramine-T-based oxidation as described above, whereas [^{125}I]iodotyrosyl-PP (^{125}I-tyrosyl-PP) was prepared by use of *tertiary*-butyloxycarbonyl-L-[^{125}I]iodotyrosine *N*-hydroxysuccinimide ester (23); both peptides (which contained about 0.1 mol of ^{125}I per mol) were purified by cellulose chromatography (20, 21).

RESULTS

As shown in Fig. 2, maximal binding of ^{125}I-glucagon to hepatocytes from both the rat and the dog occurred within the first 5–10 min of incubation at 37°C, whereas binding of ^{125}I-insulin proceeded more slowly. Nevertheless, whether considering initial rates of binding or the appearance of the transient maxi-

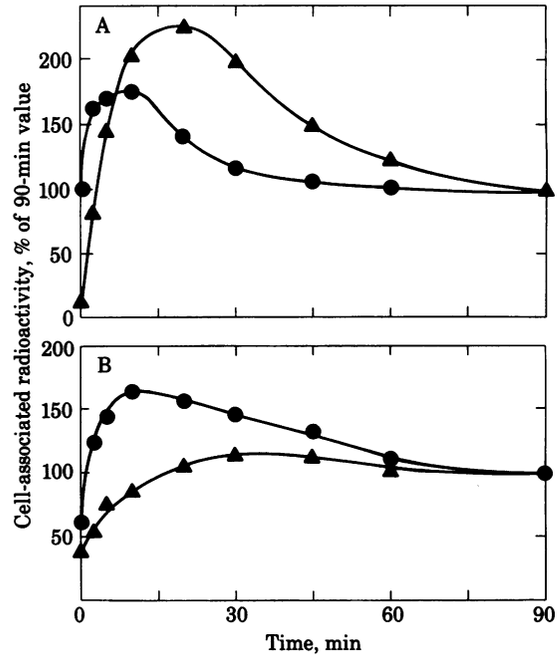

FIG. 2. Time courses of association at 37°C of ^{125}I-insulin (▲) and ^{125}I-glucagon (●) to rat (*A*) and dog (*B*) hepatocytes. The data have been normalized by considering the amount of radiolabeled hormone bound at 90 min (in the absence of any competitor) to represent 100%.

Table 1. Degradation of radioiodinated pancreatic hormones by isolated dog and rat hepatocytes

Species	Incubation period, min	Trichloroacetic acid-precipitable radioactivity, % of total		
		Insulin	Glucagon	PP
Dog	30	91 ± 3 (14)	87 ± 5 (14)	89 ± 9 (5)
Dog	60	89 ± 4 (10)	74 ± 7 (6)	68 ± 8 (3)
Rat	30	86 ± 11 (4)	86 ± 10 (6)	88 ± 6 (5)
Rat	60	57 ± 30 (3)	62 ± 30 (11)	52 (2)

Radiolabeled peptide hormones (0.2–0.4 pmol) were incubated with dog or rat hepatocytes for the indicated periods at 30°C. Aliquots (1 ml) of the well-mixed supernatants from the cell pellet were added to equal volumes of 16% (wt/vol) trichloroacetic acid; the solutions were mixed and the radioactivity of the pellet obtained after centrifugation was measured. Values represent percent of total radioactivity present in the cell supernatant that was precipitated by trichloroacetic acid ± SD; the number of experiments is shown in parentheses. The radiolabeled PP used for these experiments was ^{125}I-tyrosyl-PP.

mum, cell association of the two hormones in these complex profiles was more rapid for hepatocytes derived from the rat than it was for those derived from the dog (a result confirmed in four separate experiments). At 30°C, the rates of hormone binding to rat and dog hepatocytes were decreased and binding approached a simple steady state, with the initial rate of binding of ^{125}I-glucagon again exceeding that of ^{125}I-insulin for hepatocytes from both species (data not shown). However, the extent of steady-state ^{125}I-glucagon and ^{125}I-insulin binding was not equivalent for hepatocytes from the two sources: after 30 min of incubation at 30°C, rat hepatocytes bound 5.8 ± 2.5% (n = 15) of the ^{125}I-glucagon and 7.7 ± 1.6% (n = 5) of the ^{125}I-insulin; for dog hepatocytes, the binding of ^{125}I-glucagon averaged 13.7 ± 4.0% (n = 12), whereas the binding of ^{125}I-insulin averaged only 4.0 ± 1.0% (n = 10). Importantly, degradation of these labeled hormones was slow and uniform for hepatocytes from both species (Table 1). Separate experiments showed for both cell types that the binding of a peptide hormone (insulin, glucagon, or PP) was specific for its receptor. For example, no inhibition of ^{125}I-insulin binding was seen in the presence of glucagon or PP.

An assessment of the stability of dog hepatocytes to conditions of *in vitro* incubation showed that both the initial rates of ^{125}I-glucagon and ^{125}I-insulin binding and the periods required to reach steady-state binding were unaffected by preincubation of the cells at 30°C for 4 hr (Fig. 3). As shown in Fig. 4, the concentrations of insulin and glucagon required to inhibit by half the binding of the corresponding ^{125}I-labeled probes to dog hepatocytes [1.0 ± 0.2 nM (n = 7) and 5.7 ± 0.8 nM (n = 6), respectively] were similarly unaffected by the 4-hr preincubation. Scatchard plots of data reflecting insulin binding to dog hepatocytes revealed typical curvilinearity (see Fig. 4*A Inset*); the apparent dissociation constant for insulin was 0.90 ± 0.02 nM [for the linear component having the steeper slope ("high-affinity" receptors), n = 7] and the number of corresponding

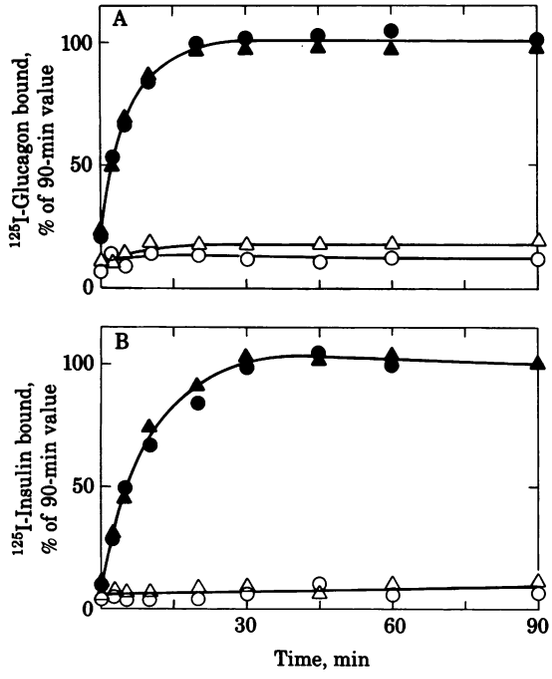

FIG. 3. Time courses of association at 30°C of ^{125}I-glucagon (*A*) and ^{125}I-insulin (*B*) to freshly isolated dog hepatocytes (▲) and to dog hepatocytes preincubated at 30°C for 4 hr (●). ^{125}I-Glucagon association in the presence of 140 μM glucagon (△, fresh cells; ○, preincubated cells; in *A*) and ^{125}I-insulin association in the presence of 160 μM insulin (△, fresh cells; ○, preincubated cells; in *B*) are also shown. Preincubation occurred at a cell density of 2 × 10^6 cells per ml in minimal essential medium; preincubated cells were not reisolated for this or other experiments. The data have been normalized by considering the amount of radiolabeled hormone bound at 90 min to represent 100%.

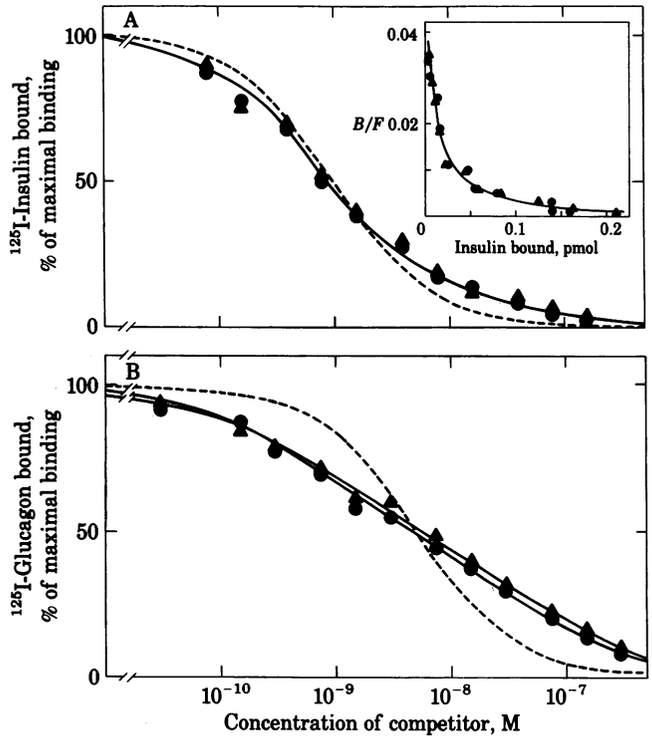

FIG. 4. Competition for ^{125}I-insulin binding by insulin (*A*) and for ^{125}I-glucagon binding by glucagon (*B*) to freshly isolated dog hepatocytes (▲) and to dog hepatocytes preincubated at 30°C for 4 hr (●). The temperature for the binding study was 30°C and the incubation period was 30 min. The data, expressed as percent of maximal binding (in the absence of any competitor), have been corrected for the amount of radiolabeled hormone remaining cell associated in the presence of competitor (160 μM insulin or 140 μM glucagon). Theoretical binding competition curves, assuming homogeneous receptor populations and reversible interactions, are shown for insulin (dissociation constant = 1 nM, *A*) and glucagon (dissociation constant = 5 nM, *B*) in broken lines. (*Inset*) Scatchard plot of the competition for ^{125}I-insulin binding by insulin to freshly isolated dog hepatocytes (▲) and to dog hepatocytes preincubated at 30°C for 4 hr (●). The data are plotted as (cell-bound insulin) ÷ (free insulin) vs. cell-bound insulin (assuming equivalency in the labeled and unlabeled forms) and have been corrected for the amount of radiolabeled hormone remaining cell associated in the presence of 160 μM insulin.

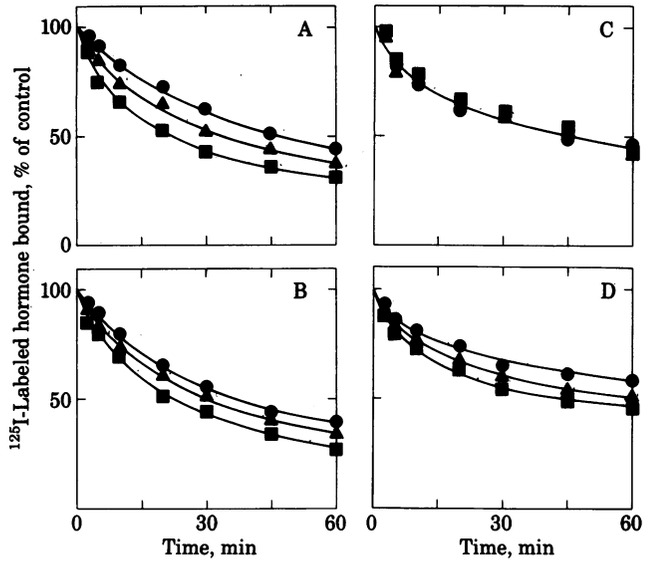

FIG. 5. Dissociation of ^{125}I-insulin and ^{125}I-glucagon from rat and dog hepatocytes. Isolated hepatocytes were incubated with radiolabeled hormone for 30 min at 30°C and were washed twice with cold medium to remove unbound tracer. The cells were diluted to 12 times their original volume and were reincubated at 30°C. Dissociation of ^{125}I-insulin from rat (*A*) and dog (*B*) hepatocytes: ●, in the absence of any supplement; ▲, in the presence of 1.6 nM insulin; ■, in the presence of 16 nM insulin. Dissociation of ^{125}I-glucagon from rat (*C*) and dog (*D*) hepatocytes: ●, in the absence of any supplement; ▲, in the presence of 2.9 nM glucagon; ■, in the presence of 29 nM glucagon. Data are reported as the percent of radiolabeled peptide bound to cells at the start of the dissociation (control) that remained cell associated after the indicated periods.

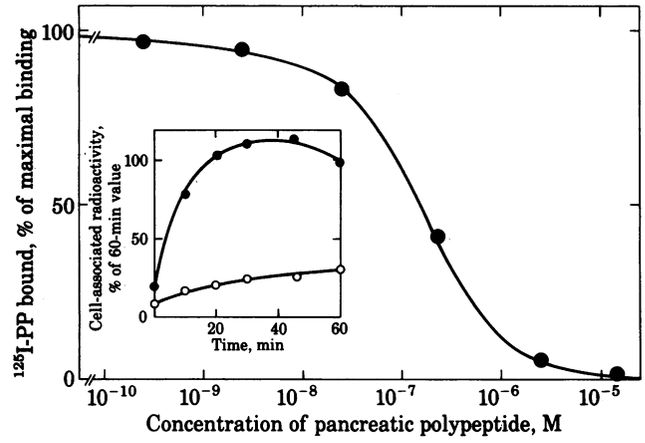

FIG. 6. Time course of association of ^{125}I-tyrosyl-PP (●, *Inset*) and competition for ^{125}I-tyrosyl-PP binding by PP to rat hepatocytes at 30°C. PP association in the presence of 1 μM PP (○) is also shown in the *Inset*; the data have been normalized by considering the amount of radiolabeled peptide bound at 60 min (in the absence of competitor) to represent 100%. The incubation period for the competition experiment was 30 min, and the data (expressed as percent of maximal binding in the absence of competitor) were corrected for the amount of radiolabeled peptide remaining cell associated in the presence of 23 μM PP.

receptors was 9,100 ± 2,600 per cell. These prolonged incubations elicited only a 1–2% decrease in dog hepatocyte viability, whereas similar incubations caused a 10–20% decrease in the viability of rat hepatocytes. In addition, Table 1 shows that the degradation of ^{125}I-insulin and ^{125}I-glucagon remained low for dog hepatocytes, but increased for rat hepatocytes, during 60-min incubations with the labeled hormones.

The deviation of the competition curves of Fig. 4 (as for those obtained with rat hepatocytes, data not shown) from those expected for a simple binding (broken lines, Fig. 4) was more severe for glucagon than it was for insulin. The span of glucagon concentrations required to decrease ^{125}I-glucagon binding from 90% to 10% of its original value, however, remained constant for glucagon iodinated to different degrees. Nevertheless, glucagon elicited its biological activity in dog and rat hepatocytes at concentrations much lower than those required for inhibition of ^{125}I-glucagon binding; 10–30 pM glucagon inhibited by 50% the basal rate of [^{14}C]fructose incorporation into glucagon in cells derived from both species.

Further studies examined the dissociation of receptor-bound ^{125}I-insulin and ^{125}I-glucagon from isolated hepatocytes. As shown in Fig. 5 *A* and *B*, respectively, the rates of dissociation of ^{125}I-insulin from rat and dog hepatocytes were similar, with 50% of the prebound material dissociating by 35 ± 7 min of incubation (n = 8). The addition of unlabeled insulin enhanced the rate of dissociation of labeled hormone within the shortest interval examined (2.5 min). Fig. 5 *C* and *D* shows that the rate of dissociation of ^{125}I-glucagon from rat hepatocytes (50% dissociation by 45 ± 13 min, n = 6) greatly exceeded that from dog hepatocytes. Although the addition of glucagon did not affect the dissociation of ^{125}I-glucagon from rat hepatocytes (Fig. 5*C*), increasing concentrations of the unlabeled hormone notably enhanced the rate of ^{125}I-glucagon dissociation from dog hepatocytes (Fig. 5*D*). Additional experiments showed that concentrations of glucagon as high as 0.3 μM did not alter the rate of ^{125}I-glucagon dissociation from rat hepatocytes and that increased incubation volumes during the dissociation (50 ml rather than 12 ml) did not diminish the differences between rat and dog hepatocytes (data not shown).

A comparison of the interactions of PP [the third major peptide of the pancreatic islet (24)] with dog and rat hepatocytes showed that hepatocytes from neither species bound ^{125}I-PP to an extent greater than 1%. However, rat hepatocytes, but not those of the dog, bound ^{125}I-tyrosyl-PP to a high and consistent degree (10–18% of added tracer, n = 5). As shown in Fig. 6 *Inset*, the binding of ^{125}I-tyrosyl-PP to rat hepatocytes reached a steady-state value by about 30 min at 30°C. Unlabeled PP competed for the binding of the labeled probe in a homogeneous manner (Fig. 6), but unlabeled insulin or glucagon had no effect (not shown). Nevertheless, the concentration of PP required to decrease by half the cellular binding of the tyrosylated derivative (200 nM) was 100 times greater than that usually seen for peptide hormone–receptor interactions (see Fig. 4). Importantly, the consistent degree of degradation of the labeled peptide (Table 1) and the coelectrophoresis of ^{125}I-tyrosyl-PP with material that had been incubated with hepatocytes for 30 min (on polyacrylamide gels at pH 8.7; data not shown) indicate no cause for ascribing the above findings to selective tracer degradation.

DISCUSSION

As illustrated in Fig. 3, ^{125}I-glucagon and ^{125}I-insulin both achieved simple steady-state binding to dog hepatocytes at 30°C; the initial rate for ^{125}I-glucagon binding to these cells, as for hepatocytes derived from the rat, exceeded that for ^{125}I-insulin at both 30°C and 37°C. Nevertheless, at 30°C the steady-state levels of ^{125}I-glucagon and ^{125}I-insulin bound to hepatocytes from the two species showed a reciprocal relationship: dog hepatocytes bound greater amounts of ^{125}I-glucagon, whereas rat hepatocytes bound greater amounts of ^{125}I-insulin. Although the higher level of ^{125}I-glucagon binding to dog hepatocytes may well reflect a greater number of glucagon receptors per cell, the complexity of the curves of Fig. 4*B* prevents a single graphical

approach to the determination of receptor number.

Indications of the effects of different iodination procedures were found for the cellular binding of PP: although ^{125}I-PP bound to hepatocytes from neither species, ^{125}I-tyrosyl-PP specifically associated with hepatocytes from the rat. Calculations based on both the apparent dissociation constant (200 nM) and the high binding of ^{125}I-tyrosyl-PP in the absence of any competitor would indicate a receptor number approaching 10^6 per cell. This very high number and the absence of ^{125}I-tyrosyl-PP binding to dog hepatocytes [despite the greater similarity of bovine and canine PP (R. E. Chance, personal communication)] suggest an unusual interaction of the iodotyrosylated peptide with rat hepatocytes rather than a physiological binding to plasma membrane receptors. Importantly, however, the binding of [B1-(^{125}I)iodotyrosine]insulin to rat hepatocytes does not differ from that of ^{125}I-insulin (20).

The enhanced rate of dissociation of ^{125}I-insulin in the presence of the unlabeled hormone in both rat and dog hepatocytes is consistent with the results of others (11, 25). However, the related phenomenon [which is often attributed to negatively cooperative interactions among filled receptors (ref. 25; cf. ref. 26)] was demonstrable for ^{125}I-glucagon and glucagon only in hepatocytes from the dog. Whether any causative conformational change is absent in rat hepatocytes, or whether the conformational change fails to affect the applicable rate constant to an observable degree, is not clear. Nevertheless, because both dog and rat hepatocytes are equivalently responsive to glucagon at concentrations between 10 and 100 pM, the phenomenon governing enhancement of the rate of ^{125}I-glucagon dissociation does not appear to play a major role in glucagon action.

Comparisons between peptide hormone–receptor interactions of isolated dog and rat hepatocytes have demonstrated differences in their relative and absolute abilities to bind ^{125}I-insulin, ^{125}I-glucagon, and ^{125}I-tyrosyl-PP at steady state and in the degree to which glucagon interactions with membrane receptors enhance the rate of dissociation of receptor-bound ^{125}I-glucagon. These differences document interspecies variability in ligand interactions with hepatocyte peptide hormone receptors and indicate the utility of species comparisons in assessing the generality of related findings. In addition, the tolerance of isolated dog hepatocytes to prolonged incubation suggests their applicability to further studies of peptide hormone binding, action, and metabolism.

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