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ADRENERGIC CONTROL OF LIPOLYSIS
IN
PORCINE ADIPOCYTES

By

Luiz Lehmann Coutinho

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ABSTRACT**ADRENERGIC CONTROL OF LIPOLYSIS IN PORCINE ADIPOCYTES**

By

Luiz Lehmann Coutinho

In this study an isolated porcine adipocyte system was characterized and used to investigate the adrenergic control of lipolysis.

Studies on the mode of action of ractopamine (Rac) and clenbuterol (Cle) have indicated a lipolytic effect of these compounds on porcine adipocytes. The action of Rac was investigated further and demonstrated to be dependent on removal of adenosine from the media.

Lipolysis studies revealed a difference in ED 50 and maximal lipolytic rate for Iso, Rac and Cle. Receptor binding studies indicated that the affinity of these compounds for the β AR could not explain the difference in lipolytic rates. This observation suggests a different ability among the compounds studied to promote the coupling of the ligand-receptor complex to the stimulatory G protein.

Receptor binding studies also revealed the

presence of two β AR subtypes in porcine adipocytes. They were tentatively classified as β 1 and β 2AR and determined to be present in the proportion of 45% β 1 and 55% β 2.

Studies on the negative regulatory axis of the adrenergic system demonstrated the presence of α 2AR in porcine adipocytes. The expression of an α 2AR mediated inhibitory action on lipolysis was dependent on the androgenic status of the animals and on adipocyte size and/or age. Approximately 50% inhibition of theophylline stimulated lipolysis by α 2AR stimulation was observed in intact heavy weight (161 ± 19 kg and cell diameter of 97 ± 8 μ m) male pigs. No antilipolytic activity was observed in near market weight intact (104 ± 4 kg) and castrated male (92 ± 0.5 kg) pigs or heavy weight (225 ± 8 kg) castrated male pigs.

The absence of α 2AR inhibitory axis in near market weight pigs suggests that another component of the adrenergic system is playing a negative regulatory function. A candidate for this role is the adenosine receptor. In this study, a decrease in lipolytic rate was observed in adipocytes of near market weight pigs upon stimulation of adenosine receptors. Furthermore, an inhibitory G protein was shown to mediate the adenosine inhibitory action.

The studies reported here should help enhance the

understanding on adrenergic control of lipolysis in
porcine adipocytes.

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Introduction

Meat is an important component of the human diet due to its excellent quality protein, mineral and vitamin content. Despite these important nutritional characteristics, excess fat imposes a negative health image to this product. High levels of dietary fat have been associated with coronary heart disease, cancer, obesity and reduction of life expectancy in humans [Caster, 1976; Young, 1978; Watkin, 1979; Committee on Diet, Nutrition and Cancer, 1982; Creasy, 1985; Guyton, 1986]. Excess fat deposition also represents a problem for the meat industry. In 1987, fat trimmed from red meat and poultry amounted to 6.7 billion kg [American Meat Institute, 1989]. The excessive deposition of fat not only decreases the value of meat products, but also represents an inefficient use of nutrients present in diets of farm animals. These observations clearly indicate the need for a better understanding of the control of fat deposition in farm animals.

Fat deposition is the result of lipid accumulation, primarily triacylglycerol, in adipocytes [Allen et al., 1976]. This accumulation is dependent upon cellular mechanisms regulating the pathways of de novo fatty acid synthesis and/or esterification into triacylglycerol and the catabolic pathway for triacylglycerol hydrolysis and release of fatty acids and glycerol (lipolysis) from fat

cells [Allen et al., 1976, Vernon, 1980]. The amount of fat deposited is the net result of the difference between triacylglycerol synthesis and lipolysis. An increase in lipolysis and/or a decrease in lipogenesis will reduce fat deposition. Obviously then, manipulation of the mechanisms that modulate these activities are important to decrease fat deposition in food producing animals.

Both lipolysis and lipogenesis are controlled by cAMP, which in turn is regulated by the activity of the adenylate cyclase (AC) system [Garcia-Sainz & Fain, 1982, Brownsy, 1979, 1980]. The AC system is activated/inactivated through hormone receptor interaction [Stryer & Bourne, 1986]. Three receptors known to modulate the activity of the AC system are the β -adrenergic receptors (β AR), adenosine receptors (AdR) and the α 2-adrenergic receptors (α 2AR). Activation of the β AR promotes stimulation of the AC system, resulting in an increase in lipolysis and a decrease in lipogenesis. Activation of AdR (subtype A1) and/or α 2AR promotes inhibition of the AC, resulting in decrease in lipolysis and increase in lipogenesis.

Control of the AC system in adipocytes has been characterized in several species, but as will be discussed in the following section, limited information is available for porcine adipocytes. This observation became evident when studies to understand the mode of action of some phenethanolamines were conducted. These compounds can decrease fat deposition and in short-term studies promote

an increase in plasma free fatty acids and glycerol in pigs [Anderson, personal communication; Mersmann, 1987]. Studies with adipose tissue slices, however, could not demonstrate a direct effect of these compounds [Anderson, personal communication; Mersmann, 1987], raising questions about the adequacy of the in vitro systems employed.

Knowledge is also limited with respect to the inhibitory axis of the AC system in porcine adipocytes. Several studies have demonstrated involvement of the β AR in activation of the AC system [Hu et al., 1987], but studies to demonstrate the presence of the α 2AR have been unsuccessful [Mersmann, 1984c]. With respect to the AdR, at the initiation date of these studies, there were no reports in the literature on the presence of AdR in porcine adipocytes.

Thus, the objective of this dissertation was to provide a better understanding of the adrenergic control of lipolysis in porcine adipocytes. To achieve this objective an in vitro incubation system for porcine adipocytes was characterized; the action of the phenethanolamines isoproterenol (Iso), ractopamine (Rac) and clenbuterol (Cle) was demonstrated and characterized; the presence of two β AR subtypes was demonstrated; the affinities of Iso, Rac and Cle for the β AR receptors were determined and a developmental and androgenic status dependent presence of the α 2AR in porcine adipocytes was demonstrated.

Literature Review

Adenylate cyclase system:

The adrenergic receptor coupled adenylate cyclase system has been one of the most extensively studied signal transduction mechanisms. The basic components (receptors, G protein and catalytic unit) have been isolated, characterized in considerable detail and their interaction in the signal transduction mechanism is under intensive investigation [Lefkowitz et al., 1983; Casey & Gilman, 1988; Sibley & Lefkowitz, 1985]. The current understanding is that hormone (H) receptor (R) interaction initiates signal transduction by activating a G protein. In the activated state, the G protein exists as a heterotrimer (α , β , γ) with GDP bound to the α subunit (α -GDP). The interaction of the H-R complex promotes a conformational change in G protein that favors exchange of GDP for GTP in the α subunit. The activated G protein (α -GTP), dissociates into free $\beta\gamma$ subunits and activated α -subunit (α -GTP). The α -GTP interacts with the catalytic unit of AC and alters the rate of cAMP synthesis. An intrinsic GTPase activity of the α -subunit promotes GTP hydrolysis and dissociation from the catalytic unit. The deactivated α -GDP subunit then reassociates with the $\beta\gamma$ subunit and becomes available for a new cycle [Stryer & Bourne, 1986; Gilman, 1987].

G proteins:

As in many other biological regulatory systems the catalytic unit of AC is under both positive and negative control. Binding of Iso (β_1 , β_2 -adrenergic agonist) or epinephrine (Epi, α and β adrenergic agonist) to the β adrenergic receptor promotes the exchange of GDP for GTP in G_s (stimulatory G protein). This event promotes dissociation of the activated α_s which interacts with the catalytic unit and increases the rate of cAMP production. Alternatively, binding of Epi to the α_2AR or adenosine to the AdR (A1 subtype) promotes G_i (inhibitory G protein) mediated signal transduction. Activation of G_i promotes the dissociation of the heterotrimer (α , β , γ) generating α_i and free $\beta\gamma$ subunits and a decrease in cAMP synthesis [Naer & Clampham, 1988]. The mechanism by which activation of G_i inhibits adenylate cyclase is unclear [Naer & Clampham, 1988; Milligan, 1988]. There currently are four theories. One is that excess free $\beta\gamma$ subunit generated by G_i dissociation can decrease the α_s available by promoting α_s and $\beta\gamma$ association. This so called mass action inhibition is independent of the direct action of α_i on the catalytic unit [Gilman, 1987]. Another theory is that α_i -GTP, generated by G_i activation, interacts with the catalytic unit of the AC and decreases cAMP synthesis [Stryer & Bourne, 1986]. A third theory is the direct inhibition of the catalytic unit by the $\beta\gamma$ subunit [Stryer

& Bourne, 1986]. The fourth one is direct inhibition of the G_s -catalytic subunit complex by G_i [Casey & Gilman, 1988; Allende, 1988]. Despite the different mechanisms proposed, in all four theories the final event in the G_i activation cascade is decreased cAMP production [Geirschik et al., 1988].

Involvement of the G proteins in the AC signal transduction mechanism has been demonstrated by several observations. Initial indication of such involvement is the requirement of GTP for hormonal activation of AC and the alteration of agonist (but not antagonist) receptor affinity in the presence of GTP analogues. Later, it was demonstrated that agonist receptor interaction promotes the exchange of GTP for GDP followed by subsequent GTP hydrolysis [Gilman, 1987]. These experimental observations provided early indications of some enzymatic activity (GTPase) or binding of guanine nucleotides to some proteins [Milligan, 1988].

Exploration of G proteins was greatly enhanced by the discovery that certain bacterial toxins interact with these proteins. Hence, these toxins have become valuable tools in characterization of the G proteins. The toxin isolated from Vibrio cholerae (CTX) and Bordetella pertussis (PTX) promotes transfer of an ADP-ribose moiety from NAD to the α subunit of the G_s and G_i , respectively. The ADP ribosylation of G_s , by CTX promotes permanent activation of α_s by inhibiting GTP hydrolysis which then enhances cAMP

synthesis. The covalent modification of G_i by PTX appears to involve uncoupling of the G_i from the receptor, thus preventing receptor induced depression of the AC [Milligan, 1988]. Because of the specificity exhibited by these toxins, activation of a receptor mediated response by CTX and blocking of the normal G_i response by PTX were used to demonstrate the involvement of G_s and G_i , respectively, in α and β adrenergic regulation of lipolysis. Based on earlier work it was believed that PTX was a specific probe for the G_i involved in α_2AR signal transduction. Recent studies, however, have shown that PTX may also interact with and modify the functionality of G proteins which are not part of the adrenergic signal transduction system [Milligan, 1988]. This finding clearly negates exclusive use of these bacterial toxins to identify specific G proteins coupled to adrenergic receptors. Even so, there is no question that CTX and PTX are valuable tools to demonstrate involvement of G proteins in signal transduction.

There is no reason to doubt that both G_s and G_i are present in porcine adipocytes. However, to date there have been no studies to determine their presence or function in pig adipose tissue.

β -Adrenergic Receptors:

The βAR can be further classified into β_1 and β_2

subtypes [Lefkowitz & Caron, 1987]. In mammals $\beta 1$ and $\beta 2AR$ have molecular weights of 60,000 - 65,000 and cannot be distinguished based solely on their molecular weight [Graziano et al., 1985]. Both receptors have been cloned and sequenced [Frielle et al., 1987; Dixon et al., 1986]. Hydropathicity studies of the $\beta 1$ and $\beta 2AR$ amino acid sequences reveals the presence of seven transmembrane domains [Lefkowitz & Caron, 1988]. Despite their similarities, $\beta 1$ and $\beta 2AR$ are products of two distinct genes. Both receptors are coupled to G_s and stimulate cAMP synthesis [Levitzki, 1988]. Several ligands with $\beta 1$ and $\beta 2$ selectivity have been developed and used in adipocytes for receptor subtype characterization [Mersmann, 1986; Cabelli & Malbon, 1979; Rothwell et al., 1985].

In an extensive series of in vivo and in vitro (adipose tissue slices) studies, using several different agonists and antagonists, Mersmann [1984a,b and 1986] concluded that the βAR present in porcine adipose tissue could not be classified as $\beta 1$ or $\beta 2AR$ subtypes. Similar observations have been made on adipose tissue of other species, in particular human adipocytes [Bojanic & Nahorski, 1983].

The existence of at least another βAR subtype has recently been documented. Emorine et al. [1989] have cloned a $\beta 3AR$ from a human genomic library. This gene codes for a protein with 402 amino acids and has an apparent molecular mass of 65,000 daltons. Expression of

this gene in Chinese hamster ovary cells resulted increased cAMP accumulation when the cells were challenged with β AR agonists. The amino acid sequence suggests, as for the β 1 and β 2AR subtypes, the presence of seven transmembrane regions. Comparison of the β 3AR with the β 1 and β 2 receptors reveals 50.7 and 45 % conservation of amino acids, respectively. Hybridization studies with mRNA isolated from mouse white adipose tissue suggest expression of this gene in adipocytes. The existence of a β 3AR subtype supports the previous observations, of atypical receptors, obtained with lipolytic studies.

α -Adrenergic Receptors:

Similar to β AR, there also are two α adrenergic receptor subtypes (α 1 and α 2). The α 1 is associated with phosphatidylinositol breakdown and does not regulate activity of the AC system. The α 2AR is directly involved with the AC system and its activation inhibits the catalytic unit. The α 2AR is a glycoprotein with a molecular weight of about 64,000 [Geirschik & Jakobs, 1988] and it has been cloned and sequenced [Kobilka et al., 1987]. The amino acid sequence indicates seven transmembrane domains and several regions of homology to the β receptors. In a recent report, this cDNA clone (from human platelets) was used to clone the receptor from a human kidney cDNA library [Regan et al., 1988]. The results demonstrate that

platelets and kidney α 2AR originate from two different genes. Recently, Guyer et al. [1990] described the cloning of a porcine α 2AR. The derived amino acid sequence yields a protein with 450 residues. Analysis of the amino acid sequence reveals greater than 93% similarity to the α 2AR cloned from human platelets. As for the β receptors, several α 2AR agonists and antagonists are available for lipolysis studies [Geirschik & Jakobs, 1988].

The α 2AR has been demonstrated in adipocytes of hamsters [Hittelman and Butcher, 1973, Giudicelli et al., 1977 and Garcia-Sainz et al., 1980], rabbits [Lafontan, 1981], humans [Lafontan and Berlan, 1980, Burns et al., 1981], dogs [Berlan et al., 1982] and, despite some controversy in the past, rats [Rebourcet et al., 1988]. These studies relied on the ability of specific α 2AR agonists and antagonists to interact with the receptor and either inhibit or stimulate lipolytic activity, respectively. While such an approach is useful, the inability of agonists or antagonists to modify the lipolytic rate should not be interpreted as absence of the receptor. Negative observations may simply indicate lack of specificity of the agonist/antagonist for the receptor. The importance of this concept became evident in 1988, when Rebourcet and others clarified the controversy involving the presence of α 2AR in rat adipocytes. These researchers were able to show the presence of α 2AR by employing a new, more potent and specific agonist called UK 14,304.

In porcine adipose tissue, despite careful investigation by Mersmann, the $\alpha 2AR$ could not be detected [Mersmann, 1984c]. Utilizing tissue slices, Mersmann compared the lipolytic activity of epinephrine (both $\alpha 2AR$ and βAR agonist) and isoproterenol (pure βAR agonist), in either young, mature or genetically obese pigs. No difference in maximal lipolytic rate was observed. He used several $\alpha 2AR$ agonists and antagonists and also was unable to indicate receptor associated activity.

Despite the above observations it cannot be concluded that porcine adipocytes do not have an $\alpha 2AR$. It is difficult to conceive that pigs are different, in regard to the $\alpha 2AR$, from all the other species investigated so far.

Recent studies have indicated cell size to be an important factor to demonstrate $\alpha 2AR$ activity in hamsters, dogs and rats [Rebourcet et al., 1988; Carpene et al., 1983; Taouis et al., 1987]. No $\alpha 2AR$ activity could be detected in small adipocytes from young animals, but activity was observed in larger adipocytes from adult animals [Carpene et al., 1983; Taouis et al., 1987]. Receptor sites were also observed to be more numerous in larger adipocytes of adult dogs [Taouis et al., 1987]. Carpene et al [1983] showed that exposure of adult hamsters to cold temperature (6 °C) reduced adipocyte size to that of 4-5 week old animals. Reduction in size of the adipocyte resulted in complete disappearance of $\alpha 2AR$

binding sites and $\alpha 2AR$ responsiveness, which was demonstrated before cold exposure. These observations indicate that adipocyte size is an important factor in development of $\alpha 2AR$ activity.

Sex steroids play an important role in the expression of $\alpha 2AR$ activity in adipocytes [Pecquery et al., 1988]. In male hamsters, castration almost completely suppressed $\alpha 2AR$ mediated inhibition of lipolysis. Testosterone replacement therapy re-established the $\alpha 2AR$ response. These are very important observations, since in most of the studies conducted with pigs, castrated animals have been utilized.

Adenosine receptors:

The adenosine receptor, similar to α and β receptors, has been classified into two subtypes. The subtype R_a (A_2) has been associated with activation of AC [Schwabe, 1984]. The subtype R_i (A_1) interacts with G_i and depresses AC activity [Schwabe, 1984]. The A_2 type has not been shown to be present in fat cells [Daly, 1984]. The A_1 receptor is a glycoprotein with an apparent molecular weight, determined by photoaffinity labeling, of approximately 35,000 [Lohse et al., 1988]. Similar to α and β receptors, several agonists and antagonist are available for receptor characterization [Lohse et al., 1988]. Unfortunately, the adenosine receptor has not been cloned and limited

information is available about its molecular structure.

Little is known about the effect of adenosine in porcine adipose tissue metabolism. Nevertheless, since the start of this project, Coutinho et al. [1989] and Liu et al. [1989] have realized and demonstrated the importance of accounting for adenosine in porcine adipocyte studies. Our findings on this aspect will be discussed in Chapter II.

Overall control of fat deposition:

As described above, the α_2 AR, β AR and AdR are coupled to the AC system. Upon stimulatory hormonal activation, the catalytic unit of AC increases the rate of cAMP production. cAMP in turn activates protein kinase A, which phosphorylates intracellular enzymes. Phosphorylation converts hormone sensitive lipase into an active form resulting in triacylglyceride hydrolysis [Brownsy et al., 1979]. cAMP dependent protein kinase can also phosphorylate acetyl CoA carboxylase, reducing its activity and decreasing lipogenesis [Brownsy & Hardie, 1980; Garcia-Sainz & Fain, 1982].

In porcine adipose tissue slices, Hu et al. [1987] demonstrated that upon stimulation of lipolysis by isoproterenol, cAMP accumulation can be observed. Furthermore, cAMP response and lipolysis could be blocked by the β AR antagonist propranolol. These observations indicate that, as expected, the β AR in porcine adipose

tissue are coupled to the adenylate cyclase system and to production of cAMP. Dose titration studies with isoproterenol stimulation of AC indicated less than 50 % increase in maximal cAMP accumulation over basal. Free fatty acid accumulation responded with a 13-fold increase over basal. The higher amplification in free fatty acids is due to the amplification resulting from the cAMP cascade system. More importantly, these results indicate that determination of lipolytic response can be more sensitive than cAMP response, to evaluate the activity of the AC system.

Mode of action of ractopamine and clenbuterol in porcine adipose tissue:

Rac and Cle are analogs of the naturally occurring hormone Epi. Like other phenethanolamines, Rac and Cle feeding decrease fat deposition in farm animals [see Mersmann, 1989 and Williams, 1987 for review].

Studies with ractopamine feeding to finishing swine have resulted in reduced fat deposition [Anderson et al, 1987; Hancock et al., 1987, Prince et al., 1987, Merkel et al., 1987]. Similar results have also been observed in swine fed clenbuterol [Van Weerdon, 1987].

In vivo studies with Rac [Anderson, personal communication] and Cle [Mersmann, 1987] have shown that on short term infusion experiments, both compounds increase

plasma free fatty acids and glycerol. In studies conducted with adipose tissue of finishing swine fed Rac, Merkel and coworkers [1987] have observed increased basal lipolytic activity, reduction in lipogenesis and the lipolytic activity of lipogenic enzymes.

A lipolytic effect of Rac in isolated rat adipocytes has been demonstrated by Hausmann et al. [1989]. Direct lipolytic effect of Cle in human adipocytes has been demonstrated by Mauriege et al. [1988]. Furthermore, Dickerson [1990] demonstrated, in the adipogenic cell line TA1, that Rac stimulates lipolysis and inhibits activity of the lipogenic enzyme fatty acid synthase. A reduction in the activity of malic enzyme and glycerol-3-phosphate dehydrogenase was also observed. The decrease in the lipogenic enzyme activity appears to be modulated at a pretranslational level.

In vitro studies with porcine adipose tissue slices, however, could not detect a direct lipolytic effect of Rac [Anderson, personal communication] or Cle [Mersmann, 1987]. As will be discussed later, Coutinho et al. [1989] have demonstrated that upon imposing strict experimental conditions, lipolytic activities of Rac and Cle can be demonstrated in porcine adipocytes. Our observations were confirmed by Liu et al. [1989]

CHAPTER I

Characterization of a porcine adipocyte lipolysis system

INTRODUCTION

Previous investigators have used adipose tissue slices to study porcine adipose tissue metabolism. We, however, beleived it was important to work with isolated adipocytes to minimize contamination from other cell types present in adipose tissue. Unfortunately, limited information on experimental conditions for porcine adipocyte isolation and lipolysis assay was available.

Previous studies conducted with adipose tissue slices and adipocytes have indicated that a number of experimental conditions for cell digestion and cell incubation need to be determined [Mersmann et al., 1975; Mersmann and Hu, 1987; Mersmann, 1989 and Etherton and Chung, 1981]. Among these are: 1) collagenase concentration and source, 2) cell digestion time, 3) bovine serum albumin (BSA) source and concentration, 4) preincubation time and 5) linearity of response in relation to time of incubation and cell concentration.

Another important consideration, as will be discussed in more detail in another section, is the presence of adenosine (Ad) in the incubation media. Studies with human fat cells have demonstrated that during the procedure of cell isolation and (or) incubation, lysis of adipocytes can lead to accumulation of Ad in the media [Kather, 1986]. Interaction of Ad with the adenosine receptors (Ri) present in adipocytes promotes inhibition of the adenylate cyclase

system [Londons et al., 1980].

MATERIALS AND METHODS

A biopsy of subcutaneous adipose tissue was obtained from the middle layer of the dorsal neck region of anesthetized crossbred castrated male pigs (60 - 85 kg) using aseptic techniques. The sample was placed in Basal Media Eagle (BME) maintained at 38 °C and transported to the laboratory within 15 min. A modification of the procedure developed by Rodbell [1964a] for isolation of rat adipocytes was adopted. Using sterile techniques, the tissue was finely minced and digested with collagenase in BME medium containing 1% BSA. The digestion was conducted for 50 min at 38 °C under an atmosphere of 95% O₂ and 5% CO₂ with gentle shaking. Digestion was terminated by washing the cells with collagenase-free buffer. Cells were isolated from undigested tissue by filtration through a 500 um mesh polypropylene screen. Several washes were then employed to separate (by flotation) the adipocytes from cell debris. Lipolysis was assessed by determining glycerol released in the incubation media using an enzymatic assay kit.

Lipolysis assays were conducted at least in duplicate. Cells isolated from an individual pig were considered as the experimental unit. The SAS program was used for the statistical analyses of the lipolysis studies, in which

each pig was considered a block. Treatment means were compared as described in figure legends.

Glycerol assay kit, collagenase and adenosine deaminase were obtained from Boehringer Mannheim (Indianapolis, IN). Bovine serum albumin (CRG-7) was from Armour Pharmaceutical (Kankakee, IL). Incubation media were from Gibco Laboratories (Grand Island, NY). Isoproterenol, BSA fraction V and fatty acid free were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

The most critical factor in the digestion procedure was the selection of a collagenase source that generated lipolytic responsive adipocytes. The criteria used for selection of collagenase and other conditions discussed below was the lipolytic response to isoproterenol stimulation (Iso 10^{-4}M). Of six collagenases tested, two were equally effective and allowed a 30-fold increase in lipolysis over basal, two generated adipocytes with low lipolytic response (15-fold over basal) and two resulted in unresponsive adipocytes when the cells were treated with Iso (10^{-4}M ; data not shown).

Several incubation media (Krebs-Ringer bicarbonate buffer with one half the indicated Ca^{2+} , DMEM, and BME with either 5 or 25 mM glucose) were compared for incubation of isolated cells (data not shown). BME (5 mM glucose) was

selected for all the other studies. Three different sources of BSA were tested (CRG-7 from Armour, Sigma fraction V and Sigma fraction V fatty acid free). The use of fatty acid free BSA resulted in lower lipolytic rate than observed with the other BSA sources (Fig. 1). CRG-7 was selected for all the other studies. Preincubation of isolated adipocytes did not affect cell responsiveness and no preincubation was used thereafter (data not shown).

Several concentrations of BSA were tested (Fig. 2). The lipolytic response increased and then plateaued as BSA concentration was increased from 0.5 to 4%. Three percent BSA was then selected for the next studies. To determine the concentration of adenosine deaminase (ADA) needed to remove the inhibitory effect of Ad, 0, 2 and 4 ug of ADA per ml of incubation media were tested (Fig 3). ADA at 2 ug/ml caused a 75% increase in stimulated lipolysis over no ADA ($p < 0.05$). The increase of ADA (4 ug/ml) over 2 ug/ml resulted in no additional stimulation.

With the optimum conditions set, linearity of the system in respect to incubation time (Fig. 4) and cell number (Fig. 5) were demonstrated. Finally a dose titration with Iso was conducted to demonstrate the sensitivity of the system to β AR agonist (Fig. 6).

DISCUSSION

Two factors were found indispensable for the

development of a sensitive porcine adipocyte system. The first was the selection of collagenase. Out of six collagenases tested, two generated adipocytes unresponsive to Iso stimulation. It was even more intriguing that one of the collagenases, that generated unresponsive porcine adipocytes, resulted in responsive cells if the source of tissue was rat epididimal fat pads (data not shown).

In summary, the lipolysis assay system was optimum at 2 ug/ml 3% BSA (CRG-7) in BME, 90 min incubation time and 0.4 to 1.2×10^5 cells in 1 ml of buffer. Under these conditions, basal release was 35 ± 20 and maximal lipolysis (ISO $10^{-5}M$) was 1016 ± 110 n mol glycerol $\times 10^6$ cells $\times$ hour $^{-1}$.

Figure 1: Lipolytic activity of isolated adipocytes incubated in the presence of three sources of BSA. Isolated adipocytes were obtained and incubated as described in materials and methods. Lipolytic activity was stimulated by Iso 10^{-4} M. Values represent mean $\pm$ standard error of treatment mean of three independent studies. Different letters denote significant difference at $P < 0.05$ determined with a Tukeys's test.

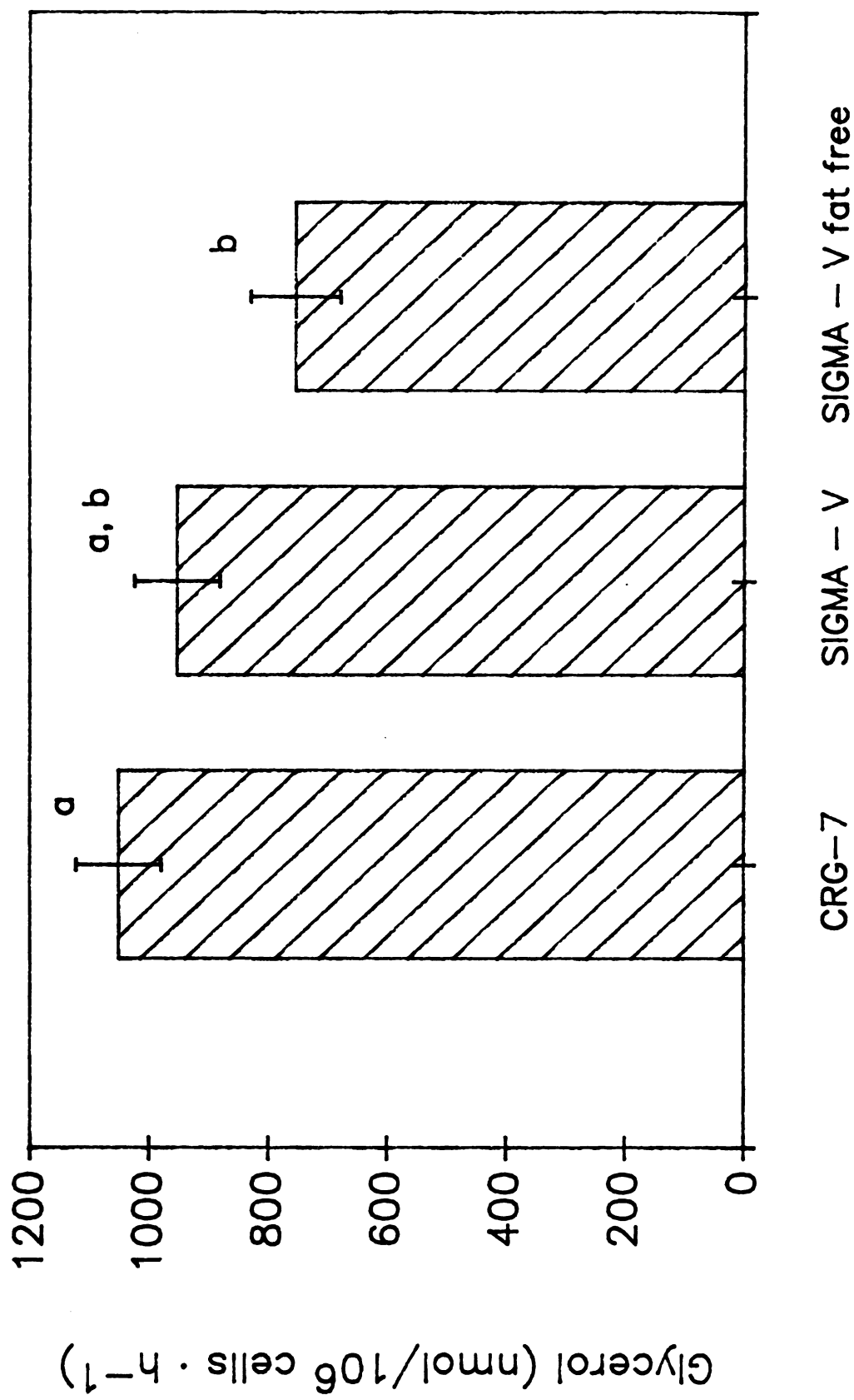


Figure 2: Lipolytic response of isolated adipocytes in function of concentration of BSA in the media. Isolated adipocytes were obtained and incubated as described in materials and methods. Lipolytic activity was stimulated by Iso 10^{-4} M. Values represent mean $\pm$ standard error of treatment mean of six independent studies. Treatment means were compared using student t test.

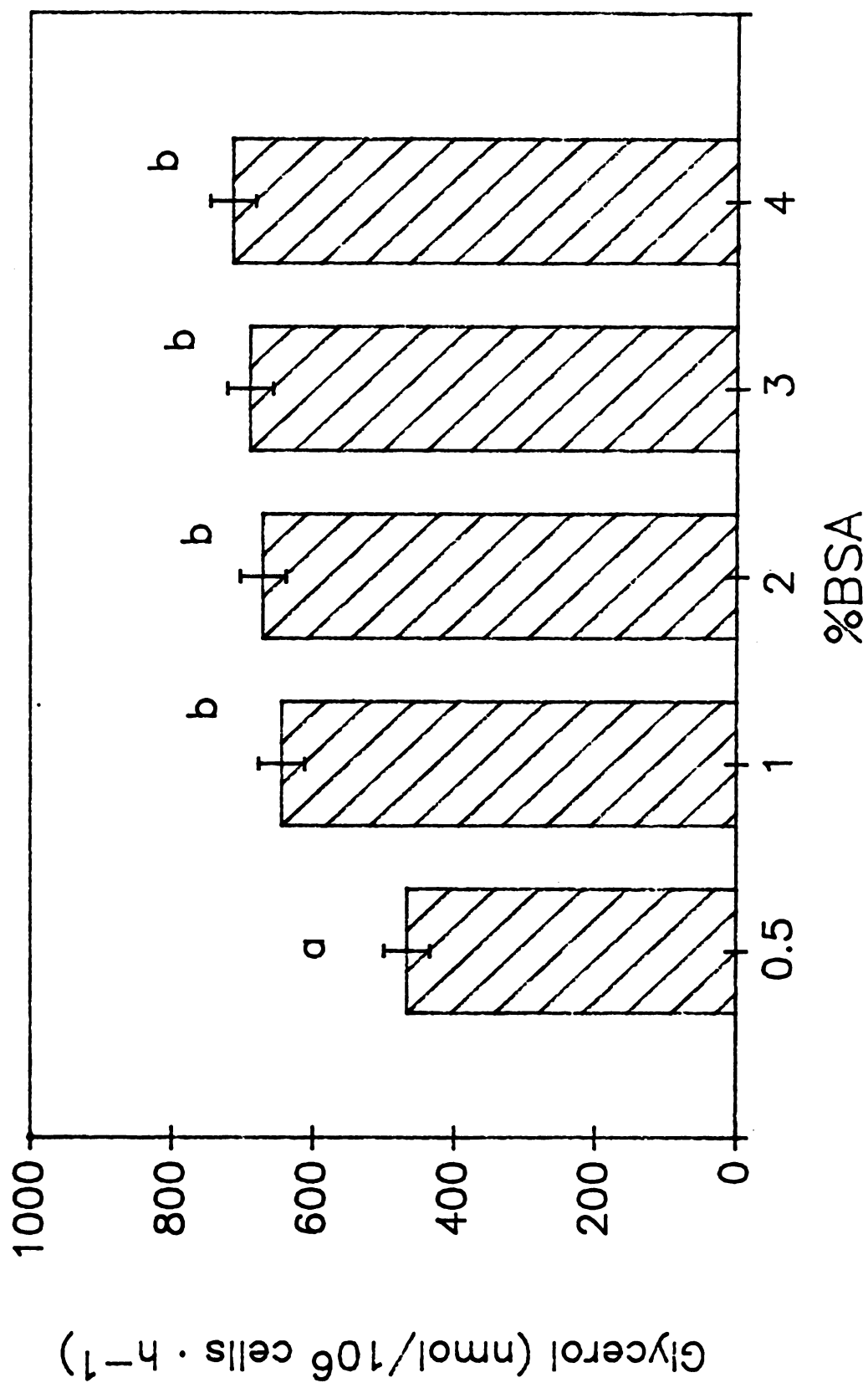


Figure 3: Lipolytic activity of isolated adipocytes incubated at different concentrations of ADA. Isolated adipocytes were obtained and incubated as described in materials and methods. Lipolytic activity was stimulated by Iso 10^{-4} M. Values represent mean $\pm$ standard error of treatment mean of six independent studies. Different letters denote significant difference at $P < 0.05$ determined using Bonferroni t test.

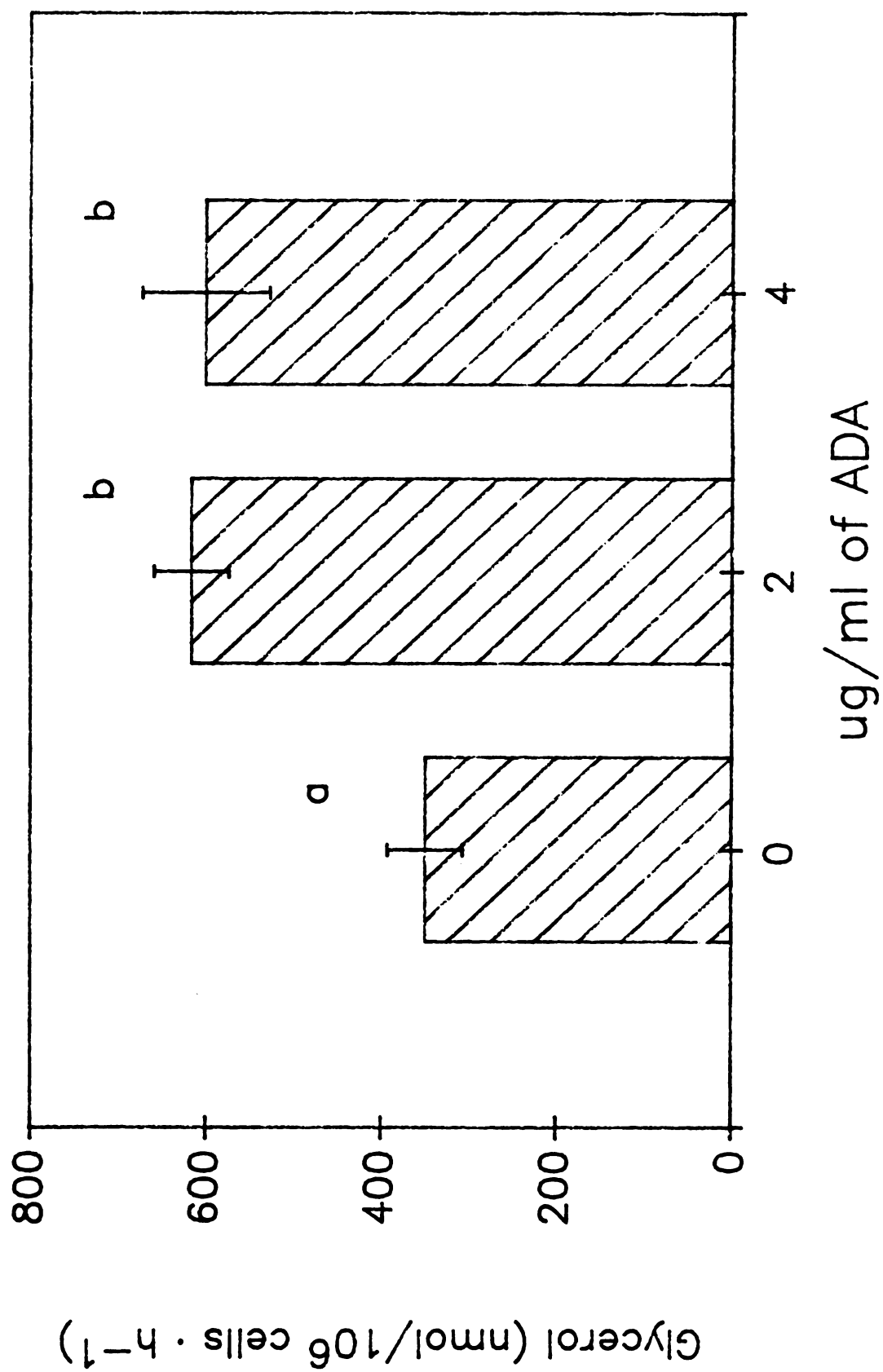


Figure 4: Lipolytic activity of isolated adipocytes at different incubation times. Isolated adipocytes were obtained and incubated as described in materials and methods. Lipolytic activity was stimulated by Iso 10^{-4} M. Values represent mean six independent studies. r^2 for linear regression was 0.999.

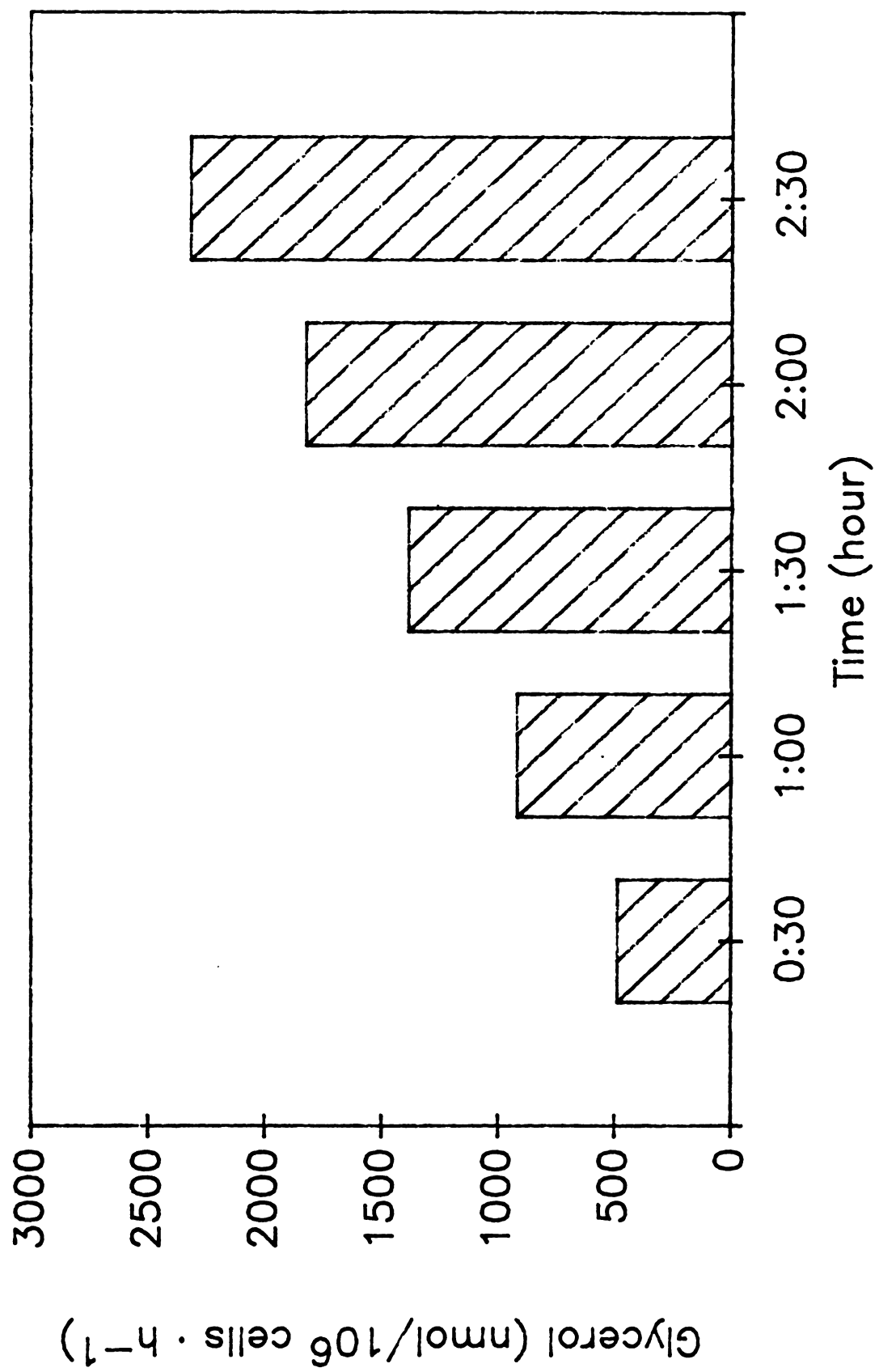


Figure 5: Lipolytic activity of isolated adipocytes as a function of cell number. Isolated adipocytes were obtained and incubated as described in materials and methods. Lipolytic activity was stimulated by Iso 10^{-4} M. Values represent mean of six independent studies. r^2 for linear regression was 0.996.

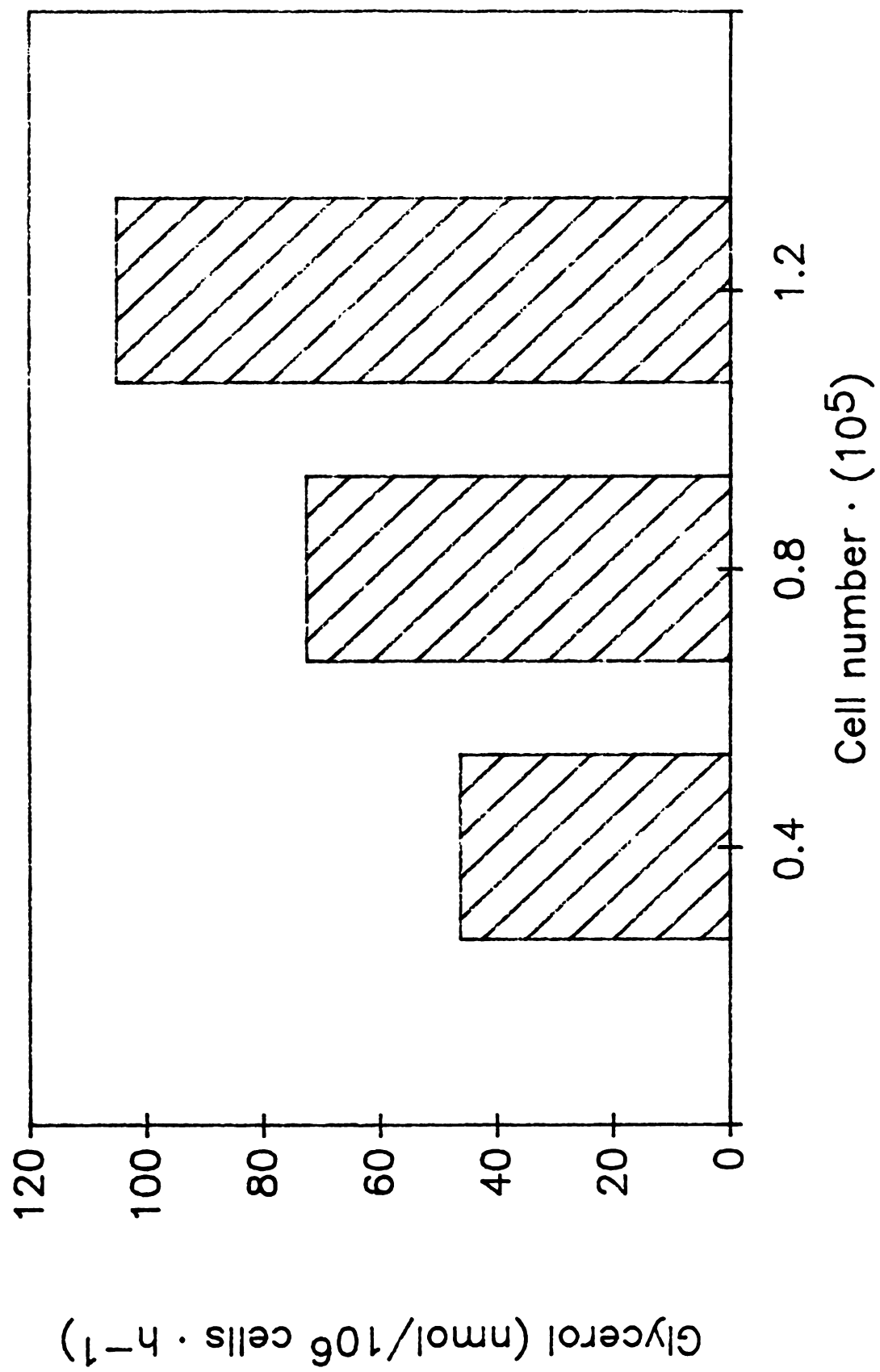
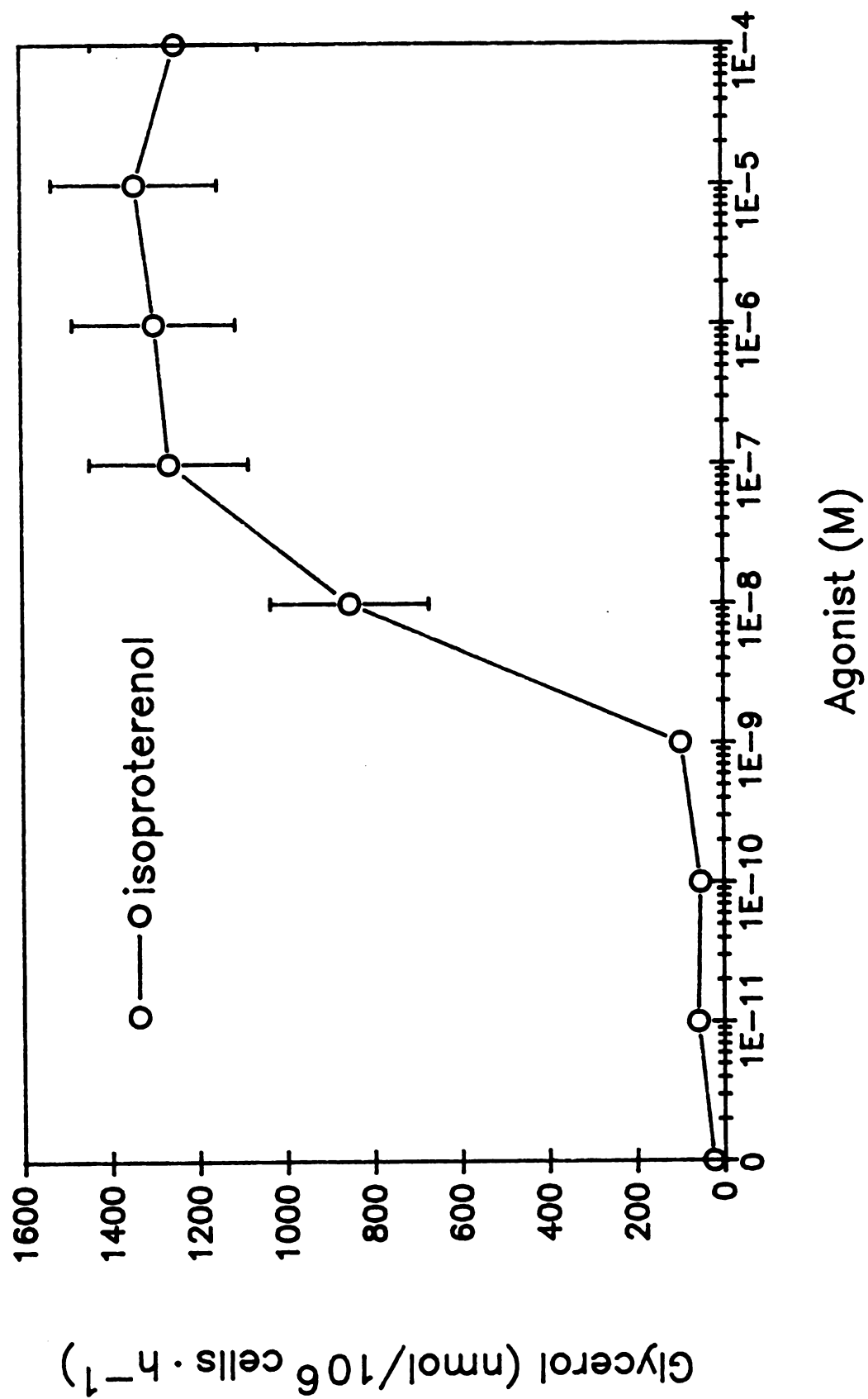


Figure 6: Dose titration of the lipolytic response of isolated adipocytes to isoproterenol stimulation. Isolated adipocytes were obtained and incubated as described in materials and methods. Values represent mean of six independent studies.



CHAPTER II

Lipolytic Activity of Phenethanolamines in Isolated Porcine Adipocytes

SUMMARY

Beta-adrenergic receptor (β -AR) mediated lipolytic activities of isoproterenol (ISO), ractopamine (RAC) and clenbuterol (CLE) were investigated in isolated adipocytes from subcutaneous adipose tissue of castrated male crossbred pigs (60 - 85 kg). Dose dependent lipolytic responses were demonstrated for the three compounds. Maximal lipolytic rates were 1264 ± 181 , 902 ± 167 and 121 ± 32 nmol/ 10^6 cells $\times$ h $^{-1}$ and estimated ED 50 were 5×10^{-9} M, 4×10^{-8} M and 1×10^{-8} M for ISO, RAC and CLE, respectively. Competition studies indicated that the lipolytic activity of ISO and RAC could be blocked by (-) propranolol. Adenosine proved to be an important factor in the evaluation of the lipolytic activities of phenethanolamines. In the absence of adenosine deaminase (ADA) the lipolytic activity of ISO was reduced, while the activity of RAC was abolished. Use of the adenosine analog, R-PIA, demonstrated that the lipolytic response of ISO was reduced to 50% while that of RAC was totally blocked by stimulation of the adenosine receptor. Receptor binding studies revealed that ISO, RAC and CLE had similar affinities for both porcine β -AR subtypes. The rank order for affinity to the β -AR was CLE > RAC > ISO. The rank order for lipolytic activity, however was ISO > RAC >> CLE. This apparent contradiction may be a result of different capacities of these compounds to promote the coupling of the ligand-receptor complex to the stimulatory G proteins.

INTRODUCTION

Work with livestock species in the last decade has demonstrated that phenethanolamines, in particular clenbuterol (CLE) and ractopamine (RAC), decrease fat and increase muscle accumulation in pigs [Mersmann, 1989, Hanrahan et al., 1986 and Anderson et al., 1988]. Infusion of CLE [Mersmann, 1987] and RAC [Anderson, personal communication] increased plasma free fatty acids and glycerol in pigs. Studies with adipose tissue slices to determine a direct effect of phenethanolamines in porcine adipose tissue could not demonstrate a lipolytic effect of CLE [Hu et al., 1987 and Mersmann, 1987] or RAC [Anderson, personal communication]. Liu et al. [1989], however, showed that both CLE and RAC induced lipolysis in porcine adipocytes when adenosine deaminase (ADA) or theophylline (THE) was included in the culture media.

Studies with human fat cells have demonstrated that during the procedure of cell isolation and (or) incubation, lysis of adipocytes can lead to accumulation of adenosine (Ad) in the media [Kather, 1988]. Interaction of Ad with the adenosine receptors (Ri) present in adipocytes promotes inhibition of the adenylate cyclase system [Londons et al., 1980]. The inhibitory process is mediated via an inhibitory G protein (Gi) which inhibits the catalytic unit of the adenylate cyclase system [Wolf et

al., 1981 and Gilman, 1987]. The presence of G_i can be demonstrated by the action of pertussis toxin (PTX). PTX promotes the ADP ribosylation of the G_i and blocks its inhibitory action on adenylate cyclase [Milligan, 1988].

The observation by Liu et al. [1989] that, unlike epinephrine, RAC and CLE could only stimulate lipolysis in the presence of THE or ADA suggests that these compounds either have low affinity for the β -adrenergic receptors or that they are partial agonists. The interaction of CLE with β -AR has been demonstrated [Cohen et al., 1982], but this observation has not been made for RAC.

In this study we report (1) the lipolytic activity of RAC and CLE in porcine adipocytes, (2) the presence, in porcine adipocytes, of an Ad inhibitory axis of the adenylate cyclase system via G_i and its effect on the lipolytic response of phenethanolamines, (3) the β -AR mediated activity of RAC, (4) the affinity of ISO, RAC and CLE for the porcine β -AR and (5) that the differences in lipolytic activity observed for the phenethanolamines studied are not due to their affinity for the porcine β -AR and may be due primarily to their ability to promote the coupling of the receptor to the stimulatory G protein.

MATERIALS AND METHODS

A modification of the procedure developed by Rodbell [1964a] for the isolation of rat adipocytes was used. The

middle layer of subcutaneous adipose tissue from the dorsal neck region of anesthetized crossbred castrated male pigs (60 - 85 kg) was obtained using aseptic techniques. The sample was placed in Basal Media Eagle (BME) maintained at 38 °C and transported to the laboratory within 15 min. Using sterile techniques, the tissue was finely minced and digested with collagenase in BME medium containing 1% BSA. The digestion was conducted for 50 min at 38 °C under an atmosphere of 95% O₂ and 5% CO₂ with gentle shaking. One lot of collagenase was selected (as discussed below) and used for all studies. Digestion was terminated by washing the cells with collagenase-free buffer and the adipocytes isolated by filtering the cells through a 500 um mesh polypropylene screen.

Isolated cells were incubated for 90 min at 38 °C in 1 ml of BME containing 3% BSA and 2 ug/ml of adenosine deaminase (ADA) at cell concentrations of 0.5 to 1 x 10⁵ cells/ml, unless otherwise stated. Lipolysis was assessed by determining glycerol released in the incubation media using an enzymatic assay kit.

Crude membranes were prepared from fat cell ghosts by a modification of the procedure developed by Rodbell [1964b]. Briefly, isolated cells were resuspended in a hypotonic lysing buffer (2.5 mM MgCl, 1 mM KHCO₃, 2 mM Tris.HCl, 5 ug/ml leupeptin, 3 mM EGTA and 100 μM phenylmethyl-sulfonyl fluoride (PMSF), pH 7.5) and centrifuged at 400 x g for 1 min at room temperature. The

infranatant and the pellet were transferred to a chilled tube and spun at 40,000 x g for 10 min at 4 °C. Crude plasma membranes were then obtained by resuspending the cell ghosts with a Polytron tissue homogenizer (Brinkmann; at setting 5 for 10 sec) in assay buffer (25 mM Tris.HCl, 120 mM NaCl, 10 mM MgCl and 1.1 mM ascorbic acid at pH 7.5). Aliquots of membranes were stored frozen at -80 °C until use (maximal of 4 wk with no loss of binding activity during this period). Protein concentration was determined by the Bio-Rad protein assay [Bradford, 1976] using bovine serum albumin as standard.

Receptor binding assays were conducted as described by Coutinho et al. [1990]. Briefly, crude plasma membranes were resuspended in assay buffer containing 100 µM GTP with the aid of a glass tissue grinder (Wheaton). The membranes (15 to 25 ug of protein) were incubated in glass tubes (Fisher) in a final volume of 200 ul. The samples were incubated at 38 °C with gentle shaking for 30 min. The radioligand used was ¹²⁵I-iodopindolol (IPIN). Nonspecific binding was determined in the presence of 0.1 µM (-) propranolol. Incubation was terminated by the addition of 6 ml of ice cold assay buffer. Samples were filtered through a glass microfiber filter (GF/B, Whatman) and washed with an additional 6 ml of ice cold assay buffer. Radioactivity was determined by a Micromedic gamma counter at 82% efficiency.

Lipolysis assays and competition studies were

conducted at least in duplicate. Cells or membranes isolated from an individual pig were considered as the experimental unit. The SAS program was used for the statistical analyses of the lipolysis studies, in which each pig was considered a block. The weighted least squares nonlinear regression analysis computer program Ligand [Munson & Rodbard, 1980] was used to estimate the binding parameters and the best fit model.

Glycerol kit, collagenase, adenosine deaminase, GTP, EGTA and leupeptin were obtained from Boehringer Mannheim (Indianapolis, IN). Bovine serum albumin (CRG-7) was from Armour Pharmaceutical (Kankakee, IL). BME was from Gibco Laboratories (Grand Island, NY). IPIN was from New England Nuclear (Boston, MA). Isoproterenol, propranolol, pertussis toxin and PMSF were obtained from Sigma Chemical Co. (St. Louis, MO). Ractopamine and clenbuterol were kindly donated by Lilly Research Laboratories and Boehringer Ingelheim Animal Health, Inc., respectively. All reagents were used without further purification unless stated otherwise.

RESULTS

Characterization of a porcine adipocyte lipolysis system.

A critical factor for the lipolysis system was the selection of a collagenase that allowed isolation of

adipocytes responsive to β -adrenergic agonist stimulation. Of six collagenases tested, two were equally effective and allowed a 30-fold increase in lipolysis over basal, two generated adipocytes with low lipolytic response (15-fold over basal) and two resulted in unresponsive adipocytes when the cells were treated with isoproterenol (ISO, 10^{-6} M; data not shown). Incubation conditions were optimized, based on the ability of adipocytes to respond to ISO stimulation, for: 1) incubation media (Krebs-Ringer bicarbonate buffer with one half the indicated Ca^{2+} , DMEM, and BME with either 5 or 25 mM glucose), 2) bovine serum albumin (BSA) source (CRG-7 from Armour, Sigma fraction V and Sigma fraction V fatty acid free) and concentration (0.5, 1, 2, 3 and 4%), 3) cell number (0.4 , 0.8 and 1.2×10^5 cells/ml) and 4) incubation time (30, 60, 90, 120 and 180 min) ($n = 6$). Because adenosine has antilipolytic activity and can be released into the media, the effect of ADA (0, 2 and 4 ug/ml) was tested. The system was optimum at 2 ug/ml of ADA, 3% BSA (CRG-7) in BME, 90 min incubation time and 0.4 to 1.2×10^5 cells in 1 ml of buffer (data not shown). Under these conditions, basal release was 35 ± 20 and maximal lipolysis (ISO 10^{-5} M) was 1016 ± 110 nmol glycerol / 10^6 cells $\times$ hour $^{-1}$.

Lipolytic activity of phenethanolamines

Dose titration of the lipolytic responses of porcine

adipocytes to ISO, RAC and CLE is presented in Fig. 1. Basal glycerol release was 25 ± 13 nmol/ 10^6 cells $\times$ h⁻¹. Upon stimulation by ISO a typical sigmoidal dose response curve was observed. Maximal rate of lipolysis was obtained at 10^{-7} M ISO (1264 ± 181 nmol/ 10^6 cells $\times$ h⁻¹). The estimated dose for half maximal lipolysis (ED 50) for ISO was 5×10^{-9} M. The maximal rate of lipolysis obtained with ISO was greater ($P < 0.05$) than the response obtained with RAC. Maximal lipolytic rate with RAC was obtained at 10^{-6} M (902 ± 167 nmol/ 10^6 cells $\times$ h⁻¹). The lipolytic response at 10^{-4} M was reduced, indicating a possible negative effect of RAC at this high concentration. The ED 50 for RAC was 4×10^{-8} M. Maximal lipolytic rate for RAC was also greater than the response obtained with CLE ($P < 0.05$). The dose response curve observed with CLE did not appear to be sigmoidal, possibly because the values of glycerol measured were in the lower end of the detection range of the assay. Nevertheless, the maximal lipolytic response, obtained at 10^{-4} M CLE (121 ± 32 nmol/ 10^6 cells $\times$ h⁻¹), was greater than the basal release ($P < 0.05$). The ED 50 for CLE was 10^{-8} M.

Lipolytic activity of phenethanolamines is mediated via β -adrenergic receptors.

Competition studies with (-)propranolol were conducted to determine the involvement of β -adrenergic receptors in

the lipolytic activity of ISO and RAC in porcine adipocytes. As shown in Fig. 2 (-) propranolol inhibited the lipolytic response of ISO and RAC in a dose dependent manner. Complete inhibition of ISO and RAC stimulated lipolysis (10^{-6}M) was obtained at (-) propranolol concentrations of 10^{-5}M and 10^{-6}M , respectively. Competition studies were not conducted with CLE because this compound presented only minor lipolytic activity.

Adenosine mediated inhibition of phenethanolamine response.

The role of adenosine (Ad) on the lipolytic activity of phenethanolamines in porcine adipocytes was investigated by dose titration studies with ISO and RAC in the presence or absence of ADA (2 ug/ml) in the incubation media. This concentration of ADA maximizes the lipolytic response to ISO (data not shown). As can be seen in Fig. 3., without ADA in the incubation media ISO stimulated lipolysis in a dose dependent manner. RAC, however, did not stimulate lipolysis in the absence of ADA even when RAC was included in the media at 10^{-5}M . The presence of ADA in the media resulted in greater lipolytic activities of ISO and RAC.

The inhibitory action of Ad was further characterized by dose titration studies conducted with (-)-N⁶-(R-Phenyl-isopropyl)-adenosine (R-PIA) in the presence of ADA (to remove endogenous Ad). R-PIA is an adenosine analog which is neither a substrate nor an inhibitor of ADA. The

inhibitory effect of R-PIA on the lipolytic activities of ISO and RAC at 10^{-6}M is presented in Fig. 4. A significant ($P < 0.05$) antilipolytic activity of R-PIA was observed at 10^{-8}M and 10^{-9}M or greater concentrations of R-PIA for ISO and RAC, respectively. Maximal inhibitory responses were obtained at 10^{-6}M R-PIA for ISO (50% inhibition) and at 10^{-7}M R-PIA for RAC (100% inhibition).

To further characterize the antilipolytic activity of Ad in porcine adipocytes, studies were conducted with pertussis toxin (PTX). Preliminary studies demonstrated that PTX blocks the inhibitory action of R-PIA on ISO stimulated lipolysis in a dose and time of preincubation dependent manner (data not shown). As shown in Fig. 5, the presence of R-PIA (10^{-6}M) promoted close to 50% inhibition of the lipolytic activity obtained with ISO (10^{-6}M). The presence of PTX (1,000 ng/ml) completely blocked the inhibitory action of the adenosine analog.

Affinities of phenethanolamines to β -adrenergic receptors

Receptor binding studies were conducted to investigate whether the differences in lipolytic response induced by ISO, RAC and CLE were due to their ability to bind to β -adrenergic receptors subtypes. Displacement curves were obtained at 220 nM IPIN and 15 to 17 concentrations (from 2.5 nM to 10 μM) of ISO, RAC or CLE. Combined nonlinear regression analysis obtained from 3 different animals was

used to determine the best fit model of the data. Typical displacement curves for ISO, RAC and CLE are presented in Fig. 6. Binding data were best fit by a one site model. Equilibrium dissociation constants are presented in Table 1.

The relationship between receptor occupancy and lipolytic response was investigated by plotting the lipolytic dose response curve and the percent receptor occupancy (Fig. 7). As can be seen in this figure, maximal lipolysis was obtained at 30%, 80% and 100% receptor occupancy for ISO, RAC and CLE, respectively.

DISCUSSION

In this report we demonstrate dose dependent lipolytic activities of ISO, RAC and CLE in isolated porcine adipocytes. Two factors were found indispensable for the development of a sensitive porcine adipocyte system. The first was the selection of collagenase. Out of six collagenases used, two generated adipocytes unresponsive to Iso ($10^{-5}M$) stimulation. It was even more intriguing to us that one of the collagenases that generated unresponsive porcine adipocytes resulted in responsive cells if the source of tissue was rat epididimal fat pads (data not shown).

The second important factor was the use of ADA. The present study not only indicates the presence of adenosine

receptors and Gi in porcine adipocytes, but also the importance in considering their inhibitory action when investigating the lipolytic activity of β -adrenergic receptor agonists. We have reported that if Ad is not removed from the culture media, the lipolytic activity of RAC cannot be measured, and the action of ISO is reduced by 50%. Our studies with the Ad analog, R-PIA, further demonstrate that the inhibitory action of adenosine receptor stimulation results in total blockage of a RAC response, and the ISO response is reduced to 50%. The action of PTX, which promotes inactivation of Gi, removed the inhibitory action of R-PIA on ISO-stimulated lipolysis providing evidence for the presence of Gi and the importance of this inhibitory axis of the adenylate cyclase system in porcine adipocytes.

In our competition studies we demonstrated total inhibition of RAC and ISO stimulated lipolysis by the non-selective β -adrenergic antagonist, (-)propranolol. These observations were expected for ISO, a known β -adrenergic agonist. In the case of RAC, a novel compound, it is the first time that a dose dependent β -adrenergic antagonist inhibition of its lipolytic activity has been reported in porcine adipocytes.

We have previously demonstrated the presence of two β -adrenergic receptor subtypes in porcine adipocytes [Coutinho et al., 1990]. The displacement studies with ISO, RAC and CLE were, however, best fit by a one site

model indicating that these compounds have equal affinities for both of the β -AR subtypes present in porcine adipocytes. These observations were expected for ISO, a nonselective β -agonist. The nonselectivity of CLE was unexpected, since CLE has been classified as a selective β_2 -AR agonist [Cohen et al., 1982]. RAC also presented nonselectivity for the β -AR presented in porcine adipocytes. RAC has been reported to be selective for the β_1 -AR when studied in C6- glioma cells [Smith & Coutinho, 1990]. It appears that the β -AR present in porcine adipocytes are somehow atypical. This statement is supported by the following observations: (1) Several β -adrenergic agonists have been reported to be lipolytic in rat adipose tissue, while having very low lipolytic activity in porcine adipose tissue [Mersmann, 1984b], (2) in our previous study on the characterization of the porcine β -AR subtypes, we reported that a selective β_1 -AR antagonist (ICI 89,406) could clearly separate the two β -AR populations, while a selective β_2 -AR antagonist (ICI 118,551) could not [Coutinho, et al., 1990]. In the same study, the integrity of ICI 118,551 was demonstrated by its selectivity in a C6-glioma cell line.

The calculated percentage of receptor occupancy for maximal lipolysis for ISO stimulation was 30%. This observation indicates the presence of spare β -adrenergic receptors in porcine adipocytes.

The rank order of potency for lipolytic response

observed in this study was ISO > RAC >> CLE. The rank order for receptor affinity, however, was CLE > RAC > ISO. The concentrations of R-PIA and propranolol necessary to promote half maximum inhibition (IC 50) of lipolytic response were greater for ISO than for RAC. Furthermore, maximal lipolytic response was obtained at 30%, 80% and 100% receptor occupancy for ISO, RAC and CLE. Taken together, these observations indicate that RAC and CLE are partial agonists and that the differences observed in lipolytic responses may result from differences in capacity of these compounds to promote the coupling of the β -adrenergic receptor to the G stimulatory protein (Gs).

The present study demonstrates β -adrenergic receptor-mediated lipolytic response of phenethanolamines in swine. These in vitro results indicate that modulation of adipose tissue metabolism via the β -adrenergic receptors may contribute to the decrease in fat deposition observed in in vivo studies [Mersmann, 1989, Hanrahan et al., 1986 and Anderson et al., 1988].

Table 1. Equilibrium dissociation constants (K_i) of phenethanolamines to β -adrenergic receptors from porcine adipocytes.

Inhibition of the binding of IPIN by phenethanolamines was measured as described in the materials and methods section. Data represent mean and the percent coefficient of variation (C.V.) of the equilibrium dissociation constant obtained by indirect binding (K_i), as determined by the computer program Ligand.

Phenethanolamine	K_i	CV(%)
(-) Isoproterenol	261 nM	15
Ractopamine	141 nM	7
Clenbuterol	28 nM	12

Fig. 1. Dose titration of the lipolytic activity of porcine adipocytes to (-) isoproterenol, ractopamine and clenbuterol.

Adipocytes were incubated with ADA (2 ug/ml) in the presence of the different phenethanolamines under the conditions described in the materials and methods section. The results are mean $\pm$ standard error of six independent experiments. Maximal lipolysis and ED 50 values were compared using Bonferroni t test.

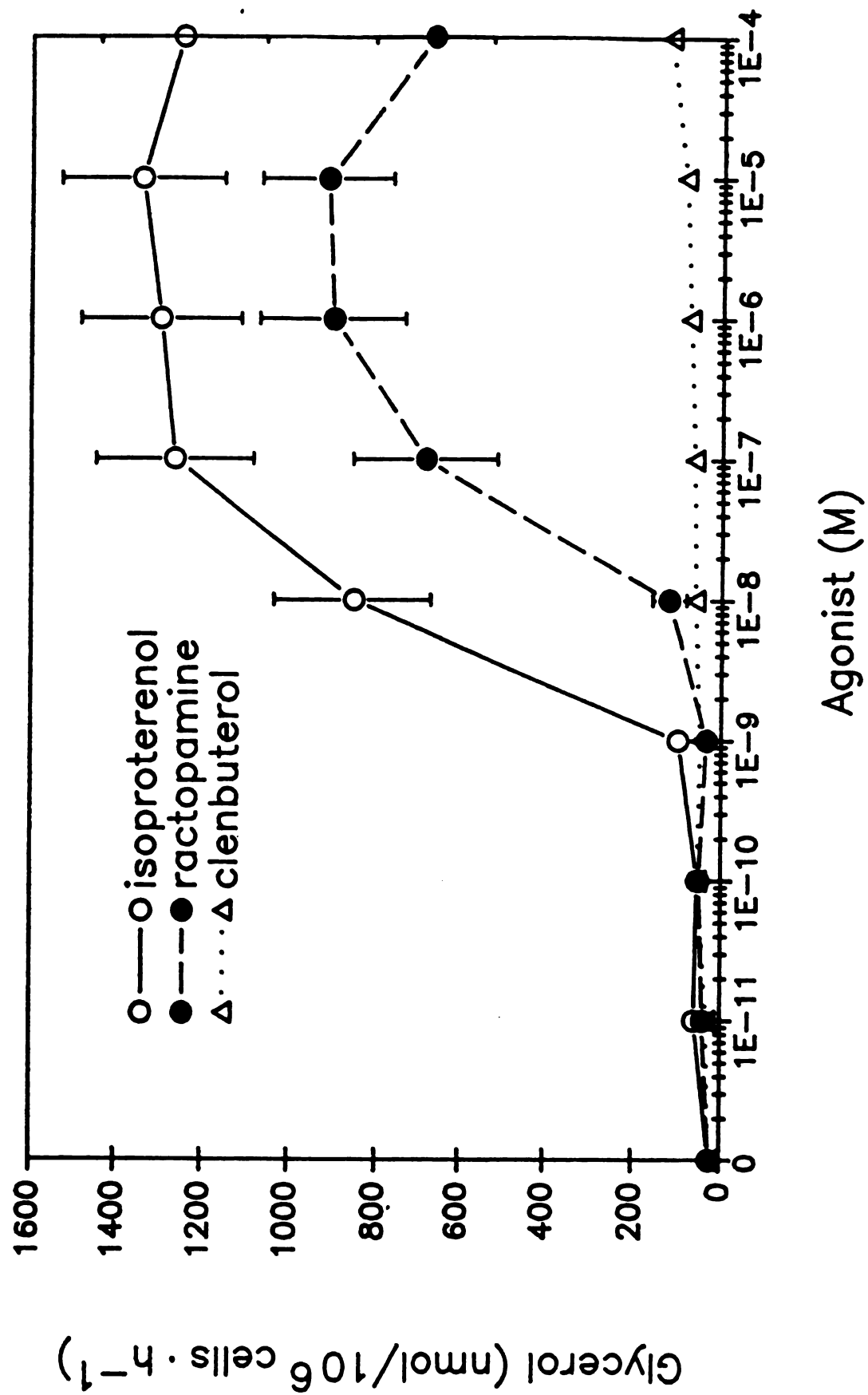


Fig. 2. Inhibition of the lipolytic response of isoproterenol and ractopamine by (-) propranolol.

Adipocytes were incubated in the presence of (-) isoproterenol at 10^{-6} M or ractopamine at 10^{-6} M and increasing concentrations of (-) propranolol. The results are mean $\pm$ standard error of three independent experiments.

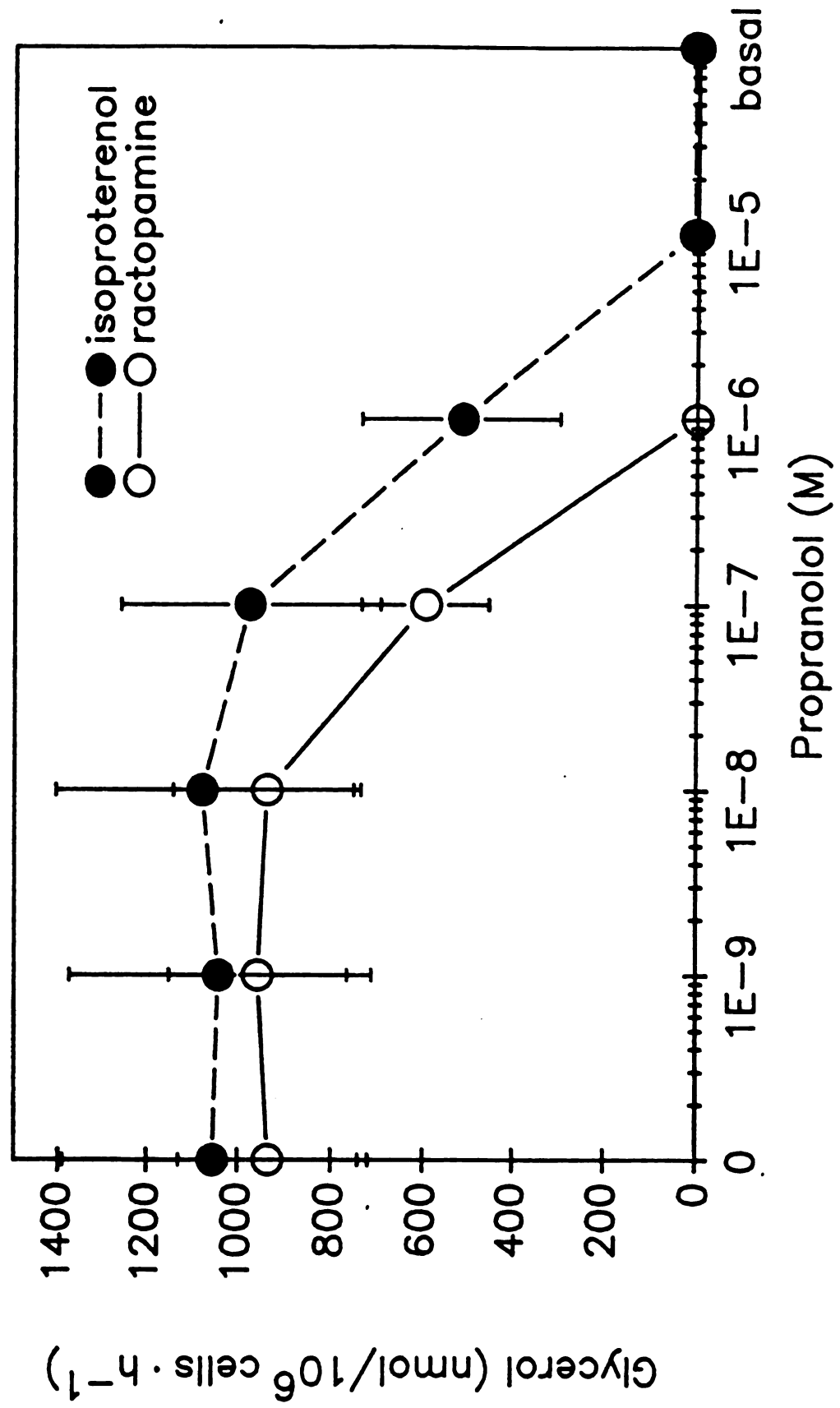


Fig. 3. Dose titration of the lipolytic response of porcine adipocytes to (-) isoproterenol and ractopamine in the presence or absence of adenosine deaminase.

Adipocytes were incubated in the presence or absence of ADA (2 ug/ml) and increasing concentrations of (-) isoproterenol and ractopamine. The results are mean $\pm$ standard error of four independent experiments. Treatment means were compared using Scheffe t test.

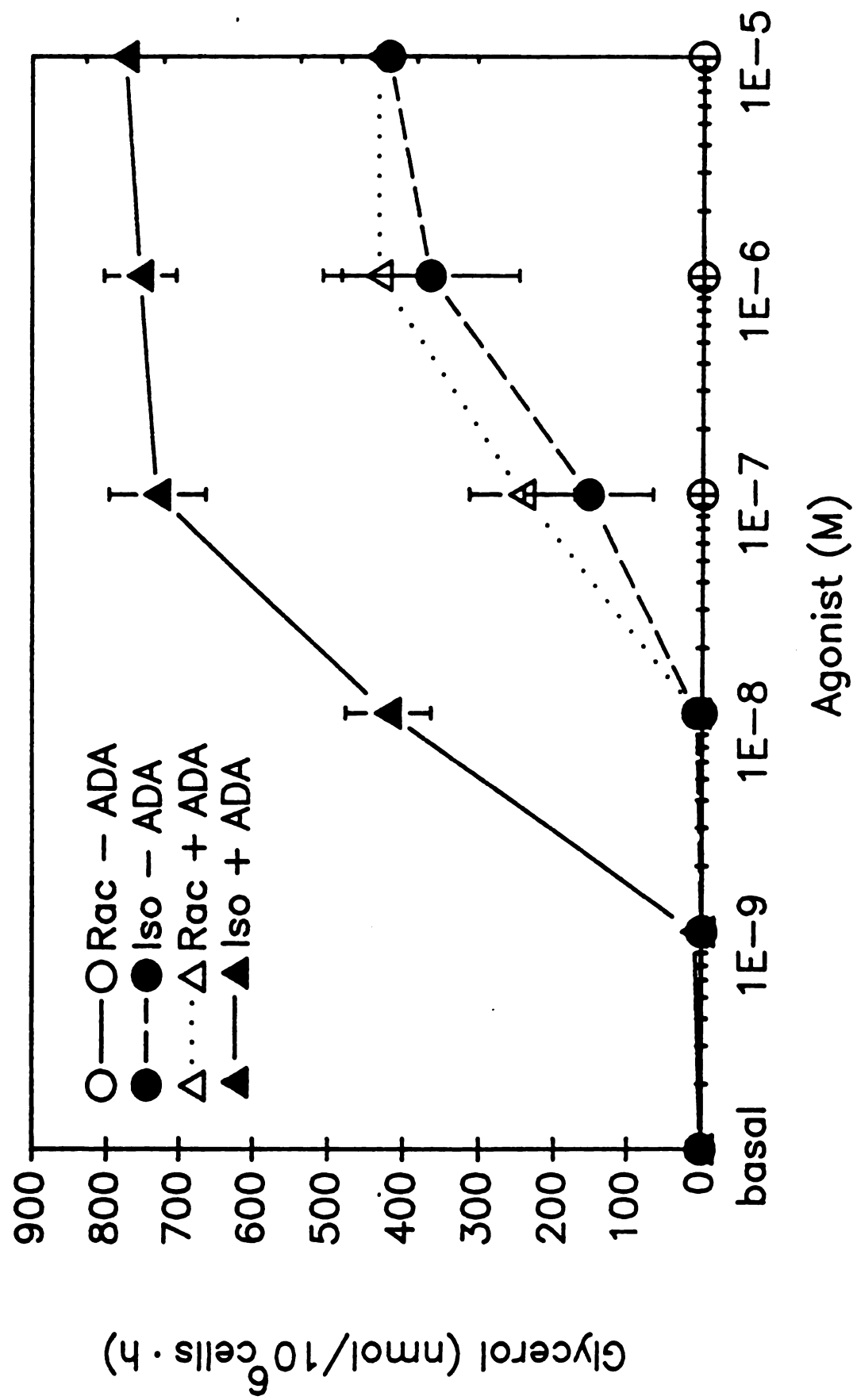


Fig. 4. Dose titration of the inhibitory action of the adenosine analog R-PIA on (-) isoproterenol and ractopamine stimulated lipolysis.

Adipocytes were incubated in the presence of ADA (2 ug/ml) to remove endogenous adenosine. The results are mean $\pm$ standard error of three independent experiments. Treatment means were compared using Scheffe t test.

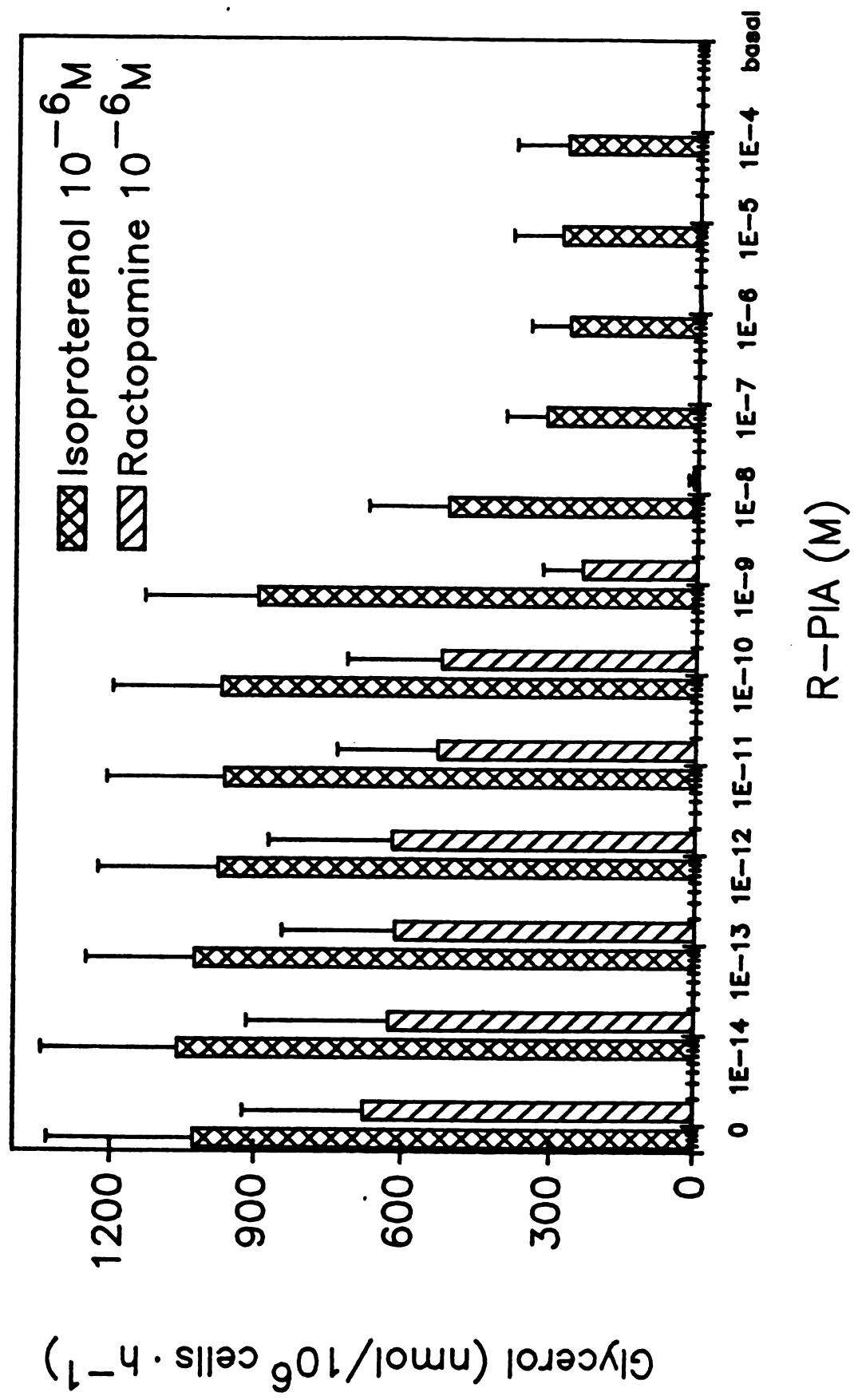


Fig. 5. Determination of blockage of the R-PIA inhibitory action on lipolysis by pertussis toxin.

Adipocytes were incubated in the presence of (-) isoproterenol (Iso, 10^{-6} M), pertussis toxin (PTX, 1,000 ng/ml) and R-PIA, 10^{-6} M) as indicated in the figure. The adipocytes in the PTX treatment were preincubated with the toxin for 2 h. The other treatments were preincubated in control media. The results are mean $\pm$ standard error of four independent experiments.

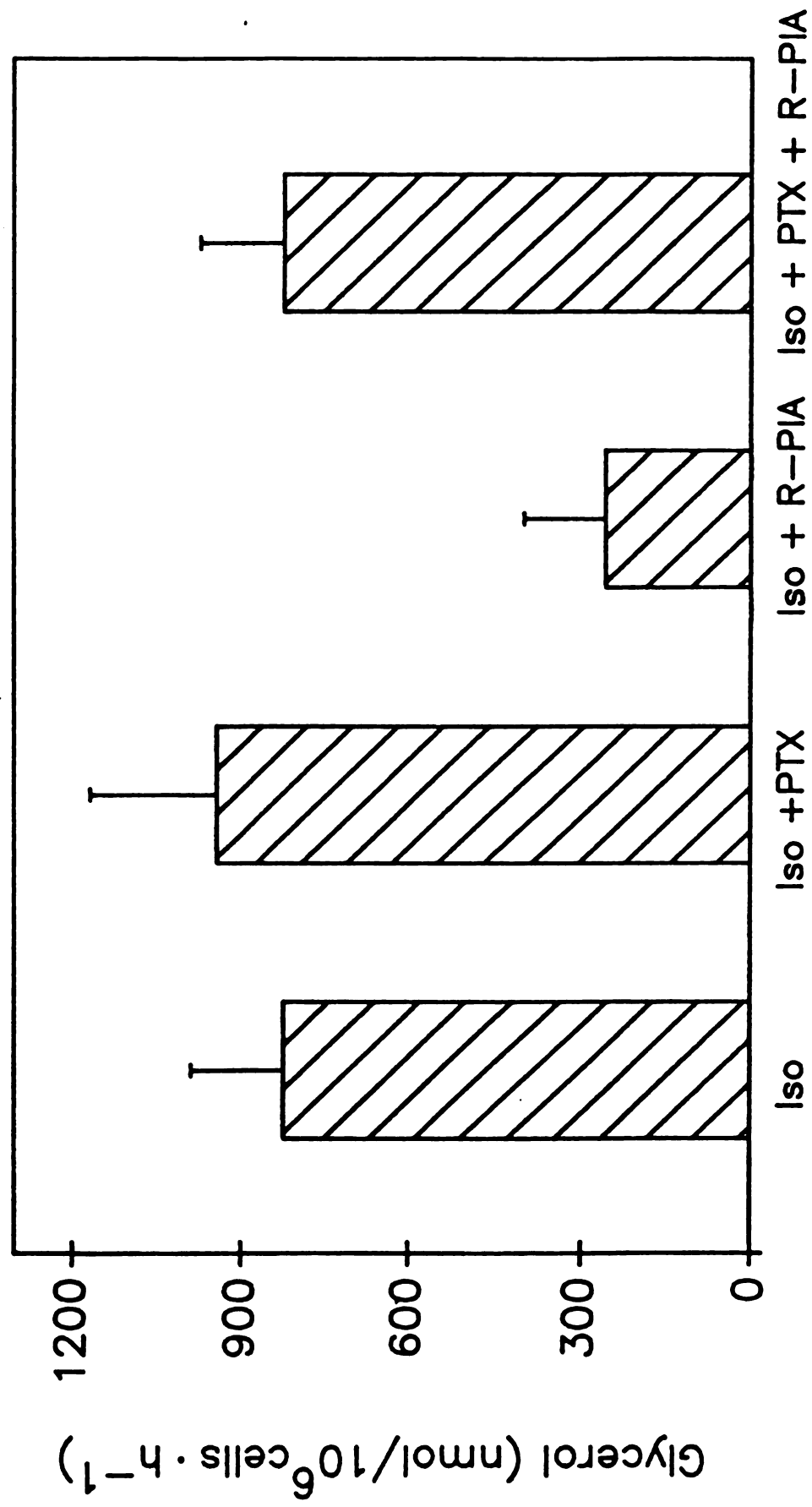


Fig. 6. Inhibition of the specific binding of IPIN to porcine adipocyte membranes by (-) isoproterenol, ractopamine and clenbuterol.

Data from a representative experiment done in duplicate are presented. Binding was studied at 38 °C after 30 min incubation in the presence of increasing concentrations of the displacing drug as described in the materials and methods. Results are expressed as percentage of IPIN specifically bound in the absence of displacing drug.

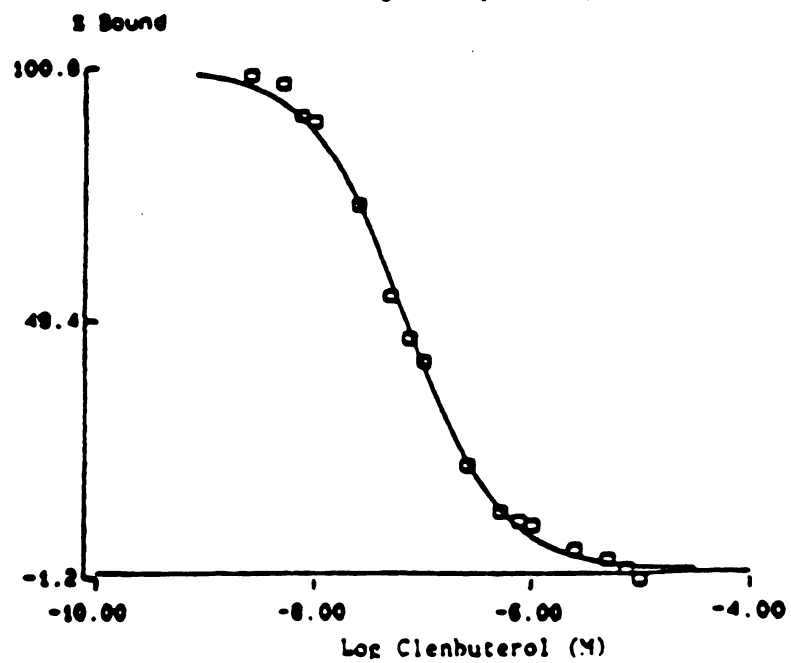
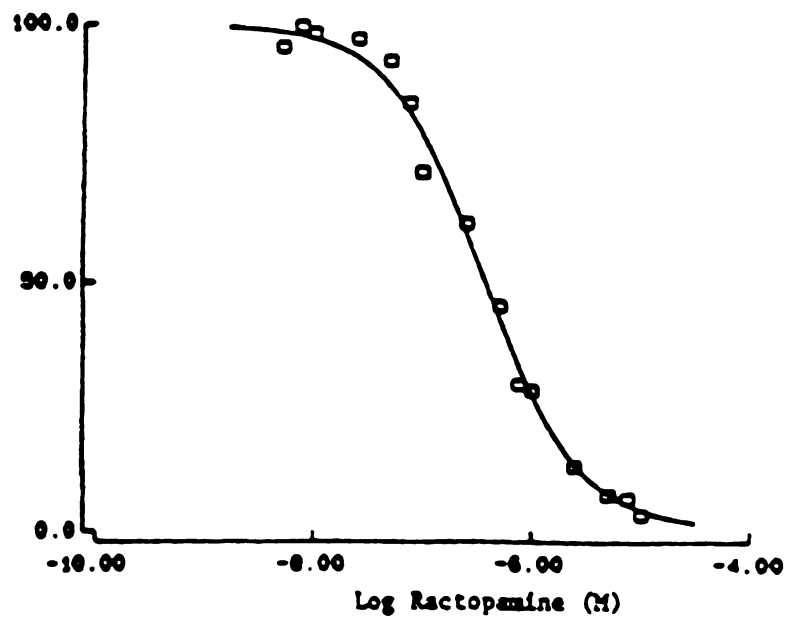
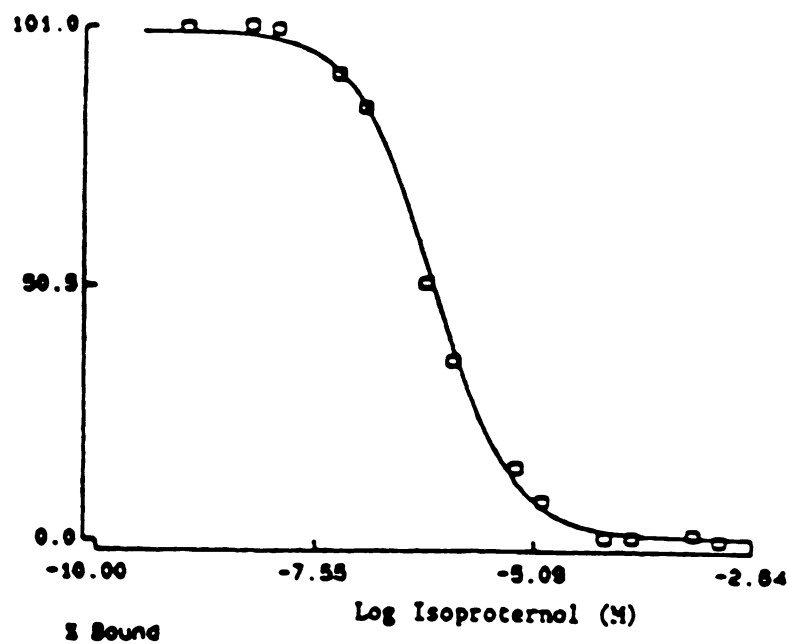
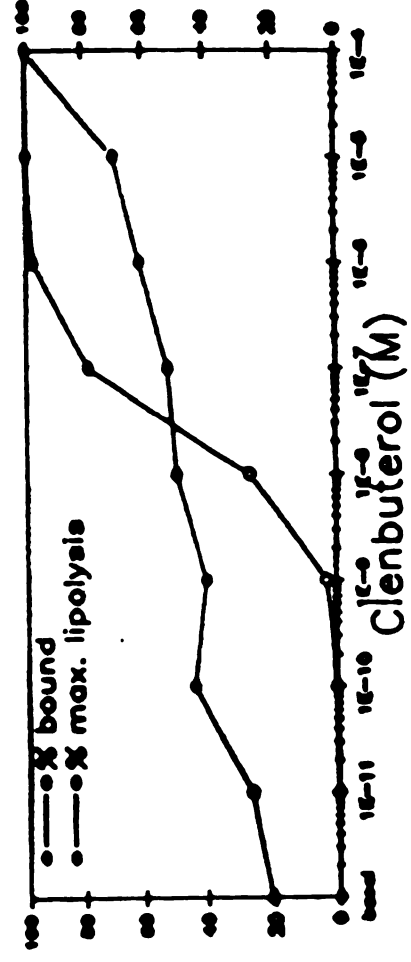
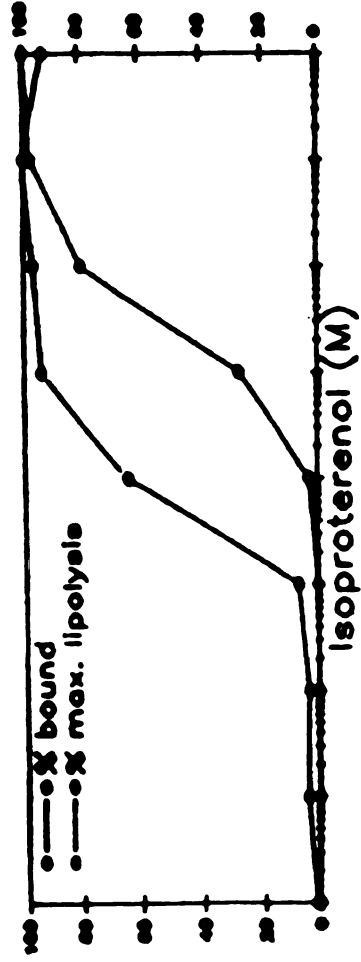
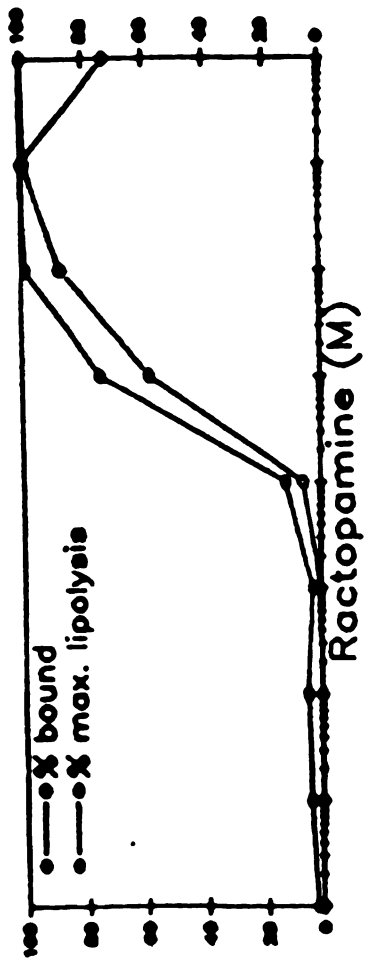


Fig. 7. Plots of the dose response curve of the lipolytic response and calculated receptor occupancy.

Receptor occupancy was calculated from the formula $B/B_{\text{max}} = L/(L + K_d)$, where B represents amount of ligand bound at a specific ligand (L) concentration and B_{max} represents the maximum binding capacity. K_d represents the affinity of the ligand for the receptors. The $K_i(s)$ reported in Table 1 were utilized for the calculation of receptor occupancy for each of the phenethanolamines.

% MAXIMAL LIPOLYSIS



% BOUND

CHAPTER III

Quantitative characterization of the β -adrenergic receptor subtypes in porcine adipocytes

SUMMARY

Two populations of beta-adrenergic receptor (β AR) subtypes and their proportions were characterized in adipocytes isolated from subcutaneous adipose tissue of castrated male crossbred pigs (60 - 85 kg). Specific binding of the radioligand ^{125}I -Iodopindolol (IPIN) to crude plasma membrane (70 to 90% of total binding) reached equilibrium conditions in 30 min (38 °C), was tissue concentration dependent, stereospecific and saturable ($B_{\text{max}} = 168 \pm 5.8$ fmol/mg protein). The presence and proportion of β AR subtypes were investigated by simultaneous regression analysis of multiple inhibition curves with ICI 89,406 and ICI 118,551 (β 1AR and β 2AR antagonists, respectively). Data were analyzed by weighted least squares nonlinear regression. Displacement curves with ICI 118,551 were best fit by a one site model, indicating nonselectivity of this antagonist for the β AR subtypes present in porcine adipocyte membranes. Displacement curves by ICI 89,406 were best fit by a two site model ($p < 0.01$) that indicated the presence of two receptor populations and selectivity of IPIN for the β 2AR subtype. Forty-five percent of the receptors had a high affinity for ICI 89,406, $K_i = 2.27 \pm 0.68$ nM and were classified as β 1AR. The K_i for the low affinity binding site, β 2AR, was 57.6 ± 4.6 nM. IPIN was 3.7-fold selective for the β 2AR subtypes ($K_d \beta$ 2AR = 98 ± 12 pM and $K_d \beta$ 1AR = 368 ± 180 pM).

INTRODUCTION

Recently, a group of phenethanolamines have been reported to decrease fat and increase muscle accumulation in livestock species [see Mersmann, 1989 and Hanrahan et al., 1986 for review]. Studies in our laboratories have demonstrated that several phenethanolamines, including isoproterenol, clenbuterol and ractopamine increase lipolysis in porcine adipocytes in a dose dependent manner [Coutinho et al., 1989]. The action of these compounds has been shown, in porcine adipose tissue slices [Hu, et al, 1987], and C-6 glioma cells [Smith and Coutinho, 1990], to involve the beta-adrenergic-receptor (β AR) coupled adenylate cyclase system.

Our laboratory is presently involved in understanding the mode of action of these compounds in adipose tissue of pigs. A prerequisite for these studies is a characterization of the β AR present in porcine adipocytes. The presence of the β AR in swine adipose tissue has been demonstrated by Mersmann et al. [1974] through measurements of lipolytic response. This observation was confirmed by Bocklen et al. [1986] with the use of a radioligand binding assay. Studies to determine the β AR subtype were conducted by Mersmann [1984 a and b]. The approach used was to determine the lipolytic response of adipose tissue slices to several selective β AR agonists and antagonists. The results were inconclusive.

Therefore, the objective of this study was to employ a receptor binding assay to characterize the β AR subtypes present in isolated porcine adipocytes. With this approach we were able to demonstrate the presence of two β AR populations and quantitate the proportion of the receptor subtypes present.

MATERIALS AND METHODS

A modification of the procedure developed by Rodbell [1964 a] for the isolation of rat adipocytes was used. The middle layer of subcutaneous adipose tissue from the dorsal neck region of anesthetized crossbred castrated male pigs (60 - 85 kg) was obtained using aseptic techniques. The sample was placed in Basal Media Eagle (BME) kept at 38 °C and transferred to the laboratory within 15 min. Using sterile techniques, the tissue was finely minced and digested with collagenase in a BME media containing 1% BSA. The digestion was conducted for 50 min at 38 °C under an atmosphere of 95% O₂ and 5% CO₂ with gentle shaking. The same batch of collagenase was used for all studies. Digestion was terminated by washing the cells with collagenase free buffer and the adipocytes were isolated by filtering the cells through a 500 um mesh polypropylene screen.

Crude membranes were prepared from fat cell ghosts by a modification of the procedure developed by Rodbell

[1964b]. Briefly, isolated cells were resuspended in a hypotonic lysing buffer (2.5 mM MgCl, 1 mM KHCO₃, 2 mM Tris.HCl, 5 µg/ml leupeptin, 3 mM EGTA and 100 µM phenylmethyl-sulfonyl fluoride (PMSF), pH 7.5) and centrifuged at 400 x g for 1 min at room temperature. The infranatant and the pellet were transferred to a chilled tube and spun at 40,000 x g for 10 min at 4 °C. Crude plasma membranes were then obtained by resuspending the cell ghosts with a Polytron tissue homogenizer (Brinkmann; at setting 5 for 10 sec) in assay buffer (25 mM Tris.HCl, 120 mM NaCl, 10 mM MgCl and 1.1 mM ascorbic acid at pH 7.5). Membranes were aliquoted and stored frozen at -80 °C until used (maximal of 4 wk with no lost of binding activity during this period). Protein concentration was determined by the Bio-Rad protein assay (Bradford [1976]) using bovine serum albumin as standard.

Crude plasma membranes were resuspended in assay buffer containing 100 µM GTP with the aid of a glass tissue grinder (Wheaton). The membranes (15 to 25 µg of protein) were incubated in glass tubes (Fisher) in a final volume of 200 µl. Except where stated, the samples were incubated at 38 °C with gentle shaking for 30 min. The radioligand used was ¹²⁵I-iodopindolol (IPIN). Nonspecific binding was determined in the presence of 0.1 µM (-) propranolol. Incubation was terminated by the addition of 6 ml of ice cold assay buffer. Samples were filtered through a glass microfiber filter (GF/B, Whatman) and washed with an

additional 6 ml of ice cold assay buffer. Radioactivity was determined on a Micromedic gamma counter at 82% efficiency.

Saturation assays were run in triplicate and competitive binding studies were conducted in duplicate. The membranes isolated from an individual pig were considered the experimental unit. The weighted least squares nonlinear regression analysis computer program Ligand [Munson & Rodbard, 1980] was used to estimate the binding parameters and the best fit model.

Collagenase, GTP, EGTA and leupeptin were obtained from Boehringer Mannheim (Indianapolis, IN). Bovine serum albumin (CRG-7) was from Armour Pharmaceutical (Kankakee, Ill). BME was from Gibco Laboratories (Grand Island, NY). ICI 89,406 and ICI 118,551 were kindly provided by Dr. P.B. Molinoff (Dept. of Pharmacology, Univ. of Pennsylvania). IPIN was from New England Nuclear (Boston, MA). Isoproterenol, propranolol and PMSF were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents were used without further purification unless stated otherwise.

RESULTS

Characterization of the binding of IPIN to porcine adipocyte membranes.

The concentration of (-) propranolol used to estimate

nonspecific binding was determined based on displacement studies with IPIN (Fig. 1). The equilibrium dissociation constant obtained by indirect binding assay (K_i) for (-) propranolol was calculated to be 717 ± 11 pM. Since 100 times the K_i should, theoretically displace up to 99% of the binding of the radioligand [McGonile & Molinoff, 1989], 0.1 μ M was chosen to estimate nonspecific binding in all subsequent studies. As can be seen in Fig. 1, this concentration of (-) propranolol completely displaced IPIN.

Specific binding of IPIN (determined at 25 pM) reached equilibrium conditions by 30 min at 38 °C and was stable for at least 90 min (Fig. 2). Nonspecific binding did not change from 10 to 90 min. Specific binding increased linearly ($r^2 = 0.995$) from 2.5 to 40 μ g of crude membrane protein (Fig. 3). Saturation binding analysis showed that specific binding of IPIN was saturable, while nonspecific binding increased linearly from 25 to 400 pM (Fig. 4). The specific binding ranged from 70 to 90% of total binding. The Scatchard transformation of a typical saturation study is presented in Fig. 5. The Hill coefficient was near unity (0.98 ± 0.004), indicating absence of positive or negative cooperativity or selectivity of IPIN for the receptor subtypes [Cornish-Bowden & Koshland, 1975]. The equilibrium dissociation constant (K_d) of IPIN for the receptor populations, obtained from nonlinear regression analysis of 4 experiments was 0.14 ± 0.006 nM. The total concentration of receptors (B_{max}) was 168 ± 5.8 fmol/mg

protein. Stereospecificity was demonstrated by displacement studies with (+) and (-) isoproterenol. The results of three experiments indicated a K_i for (+) isoproterenol of $6.91 \pm 0.83 \mu\text{M}$ and for (-) isoproterenol of $0.261 \pm 0.04 \mu\text{M}$ (Fig. 6).

Displacement studies with β AR selective antagonists.

Displacement curves were obtained with concentrations of IPIN of 60, 86, 137, 220 and 300 pM and 15 to 17 concentrations of the β 1AR selective antagonist ICI 89,406 (from 0.1 nM to 10 μM). Combined nonlinear regression analysis obtained from three different animals (15 displacement curves) was used to determine the best fit model of the data. Binding of IPIN to a two site model, with no parameter constraints was superior ($P < 0.01$) to a model representing binding to one site. The two site model with no constraint, was also superior ($P < 0.01$) to a two sites model where, the binding affinity of IPIN for the two receptor sites was held equal and constant. Receptor binding parameters are shown in Table 1. The results indicate a 3.7-fold selectivity of IPIN for the receptor population with low affinity for ICI 89,406. The antagonist ICI 89,406 had a 25-fold selectivity for the high affinity binding sites and recognized 45% of the binding sites with high affinity and 55% with low affinity. A typical displacement curve is shown in Fig. 7. The

displacement curves with the β 2AR selective antagonist ICI 118,551, at concentrations of IPIN of 86, 136 and 220 pM indicated no selectivity of this antagonist for the two binding sites (linear Hofstee plot, data not shown). Nonlinear analyses of the displacement curves were best fit by a one site model, with no improvement of the fit with a two site model. The estimated K_i for ICI 118,551 was 110 ± 8 nM.

DISCUSSION

We have previously demonstrated that our cell isolation procedure generates adipocytes that, when challenged with (-) isoproterenol, produce a greater increase in basal lipolysis than adipose tissue slices [Coutinho et al., 1989]. This observation indicates that our collagenase digestion did not degrade the receptors to any significant extent. We have now validated a β AR binding assay by demonstrating, at equilibrium conditions, linearity of binding with tissue concentration, saturability and stereospecificity. IPIN was chosen as the radioligand because of its high specific activity (2200 Ci/mmol) and low nonspecific binding.

Saturation studies indicated the presence of 168 ± 5.8 fmol of β AR per mg of protein in crude membrane preparations of porcine adipocytes. This value corresponds closely to that previously reported for crude membranes of

rat [Williams et al., 1976], human [Muriege et al., 1988] and dog adipocytes [Touis et al, 1987]. A previous report by Bocklen et al. [1986] indicated the presence of 596 fmol of β AR per mg of protein in purified membranes of porcine adipocytes. It is, however, difficult to compare the results, since Bocklen et al. [1986] used purified membranes, while we worked with crude preparations.

An important consideration for a quantitative determination of receptor subtype is the selectivity of the radioligand for the receptor subtypes being studied. As reported by McGonigle et al. [1986], inaccurate estimates of receptor subtype proportion can be obtained even if the radioligand is only 3-fold selective. The use of a slightly selective radioligand, below saturation conditions, will lead to overestimation of the high affinity binding sites and underestimation of the low affinity binding sites [McGonigle et al, 1986]. Alternatively, the use of the radioligand at saturation conditions will lead to a higher level of nonspecific binding and a reduction in the accuracy of the estimates [McGonigle et al, 1986].

Several methods to investigate the selectivity of radioligands have been reported [McGonigle et al, 1986 and Burgisser, 1983]. The Scatchard transformation is inappropriate, since a linear plot can be obtained even if the sites differ in their affinities by less than 5- to 7-fold [Burgisser, 1983]. The most sensitive method appears

to be the use of simultaneous regression analysis of multiple displacement curves (at several concentrations of the radioligand) [McGonigle et al, 1986]. As reported by Neve et al. [1986] several of the radioligands most commonly used for β AR binding assays, including IPIN, present some degree of selectivity. In our studies, simultaneous regression analysis of multiple inhibition curves obtained with the β 1AR selective antagonist ICI 89,406 and concentrations of IPIN of 60, 86, 137, 220 and 300 pM, revealed a 3.7-fold selectivity of IPIN. The displacement curves with ICI 89,406 also indicated the presence of two receptor subtypes. The high affinity binding sites represented 45% of the total and were tentatively classified as β 1AR. The low affinity binding sites represented 55% of the total and were tentatively classified as β 2AR. The presence of two β AR subtypes has also been determined in human adipocytes [Mauriege et al, 1988]. Using the radioligand ^{125}I -labeled cyanopindolol (ICYP) and the β AR selective antagonist ICI 89,406, Mauriege et al. reported the proportion of the β 2AR sites to be 58% and of the β 1AR sites 42% in human adipocytes [Mauriege et al, 1988]. The two receptor subtypes appear to be involved in lipolysis and the β 2AR has been speculated to act as a "hormonal" receptor responding to epinephrine while the β 1AR could be associated with responses to norepinephrine [Mauriege et al, 1988 and Ariens & Simonis, 1983].

The displacement curves with the β 2AR selective antagonist ICI 118,551 generated complete displacement of the IPIN and were best fit by a one site model, even though several radioligand concentrations were utilized. These results indicate that this antagonist is recognizing the porcine β AR subtypes with similar affinity. The lack of selectivity of the ICI 118,551 could be caused by an unknown degradation or modification of the compound. This, however, was not the case since we were able to demonstrate in our laboratories, with the same batch of compound, a 40-fold selectivity of ICI 118,551 for the β 2AR subtype in C-6 glioma cells [Smith and Coutinho, 1990]. The difference in selectivity in the two cell types might be explained by differences in protein [Heron et al, 1980] and lipid composition of the plasma membranes [Seeman et al, 1984]. Alternatively, this observation might indicate a difference in the β 2AR subtype present in porcine adipocytes.

To our knowledge, this is the first report to utilize simultaneous regression analysis of multiple displacement curves to obtain quantitative determination of receptor subtypes in adipocytes. It is also the first time the presence of two receptor populations of β AR has been demonstrated in porcine adipocytes. The characterization of IPIN binding to porcine adipocytes and the determination of β AR receptor subtypes will now allow us to evaluate effects of development, genetic lineage and nutritional

manipulation on the β AR population in adipocytes. We can now also determine the affinity of several of the phenethanolamines for the β AR subtypes. These studies will enhance our understanding of the adrenergic regulation of adipose tissue metabolism and the mode of action of phenethanolamines.

Table 1. Parameters obtained by simultaneous regression analysis of multiple displacement curves obtained with ICI 89,406.

Inhibition of the binding of IPIN (60, 86, 137, 220 and 320 pM) by the β 1AR selective antagonist ICI 89,406 (15 to 17 concentrations) was measured as described in the materials and methods section. Data represent the mean and the percent coefficient of variation (C.V.) for the parameters of a two site model as determined by the computer program Ligand.

	mean	% C.V.
Kd (β 1AR) IPIN (pM)	367	49
Kd (β 2AR) IPIN (pM)	98	12
Ki (β 1AR) ICI 89,406 (nM)	2.27	30
Ki (β 2AR) ICI 89,406 (nM)	57	8
% β 1AR	45	34
% β 2AR	55	7

Fig. 1. Inhibition of specific binding of IPIN to porcine adipocyte membranes by the β AR antagonist (-) propranolol. Data from a representative experiment done in duplicate are presented. Binding was studied at 38 °C after 30 min incubation in the presence of increasing concentrations of the displacement drug as described in materials and methods. Results are expressed as percentage of IPIN specifically bound in the absence of displacement drug.

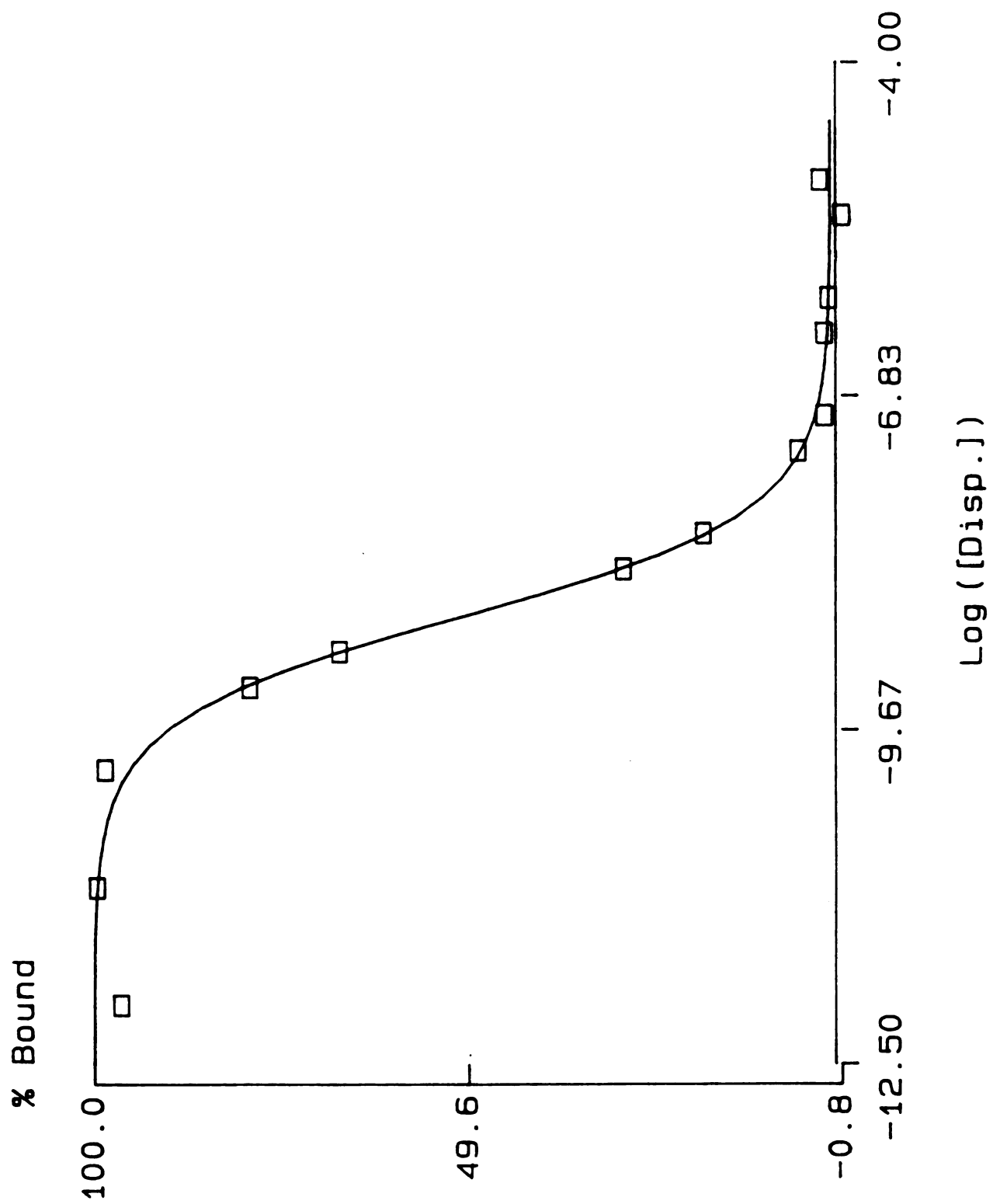


Fig. 2. Binding of IPIN to porcine adipocyte membranes at different times of incubation.

Binding was studied at 38 °C as described in materials and methods. Results are expressed as percentage of maximum IPIN bound. Data represent means $\pm$ SEM of three experiments done in triplicate.

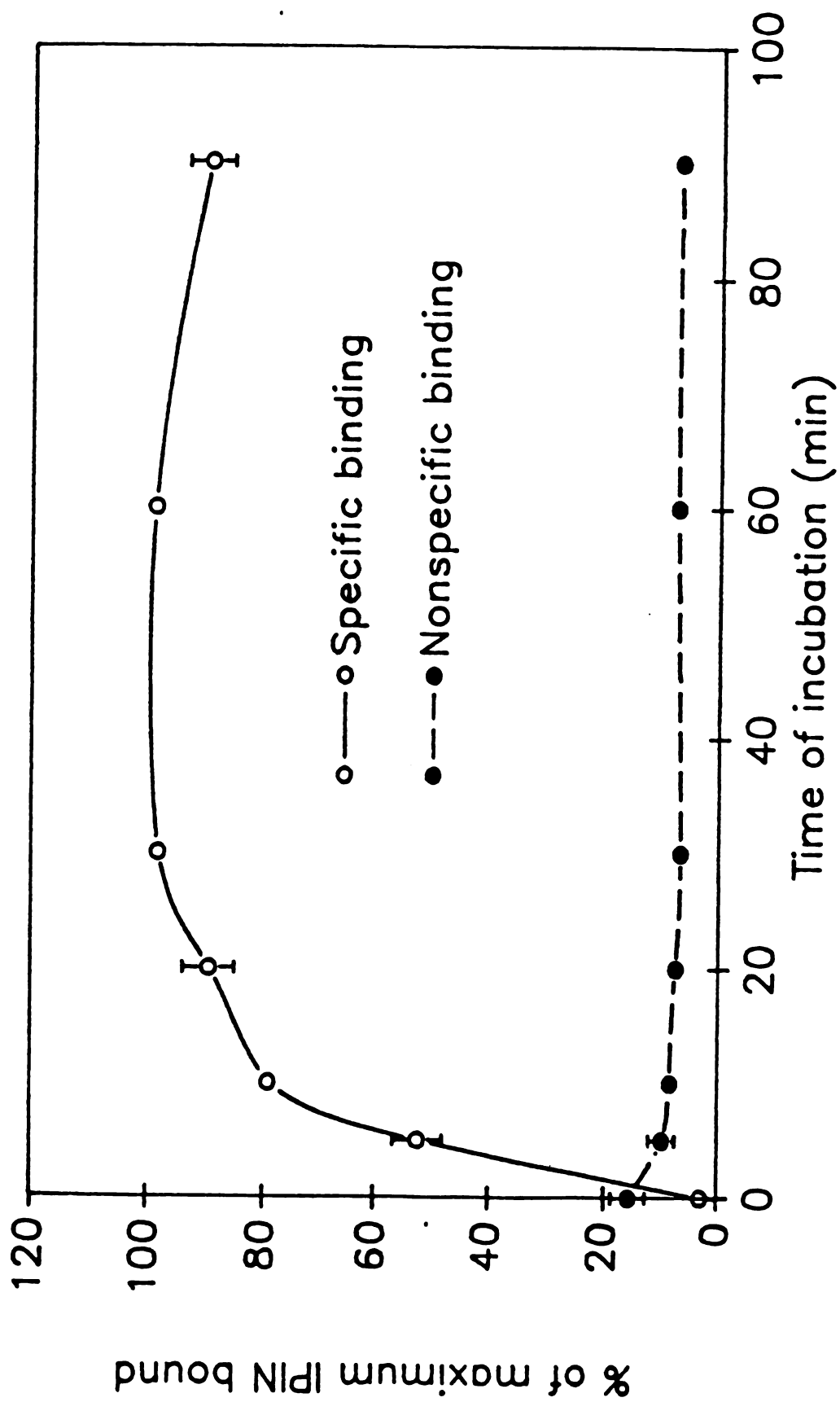


Fig. 3. Specific binding of IPIN to porcine adipocytes at different concentrations of protein.

Protein concentration was determined in "crude" membranes as described in materials and methods. The results are expressed as percentage of IPIN specifically bound. Binding at 40 μ g was considered 100%. The data represent means $\pm$ SEM of two experiments done in triplicate.

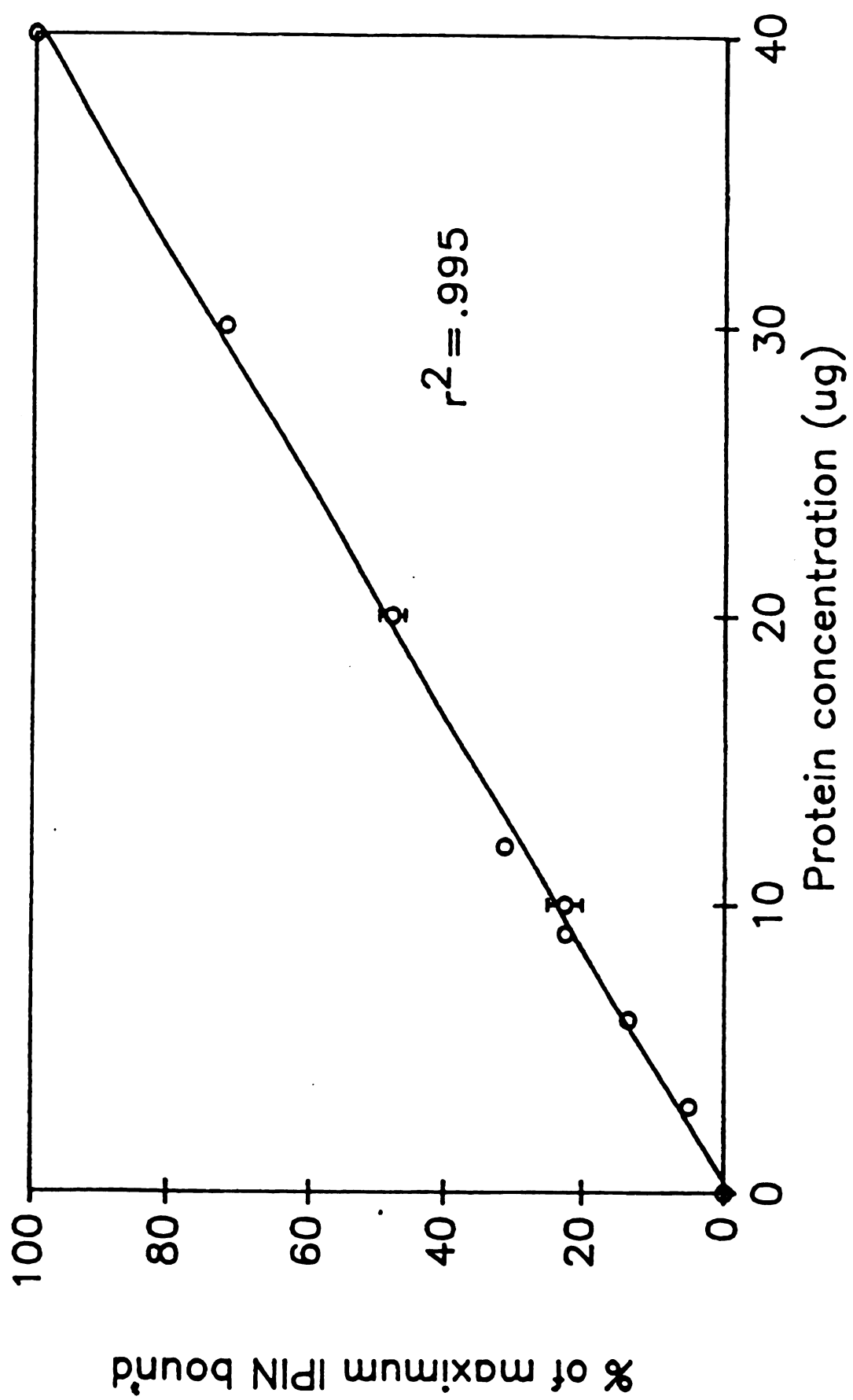


Fig. 4. Binding of IPIN to porcine adipocyte membranes.

Data from a representative experiment done in triplicate are presented. Binding was studied at several concentrations of IPIN as described in materials and methods. Nonspecific binding was determined in the presence of 0.1 μ M of (-) propranolol. Specific binding was determined by the difference between total and nonspecific binding. Data represent means $\pm$ SEM.

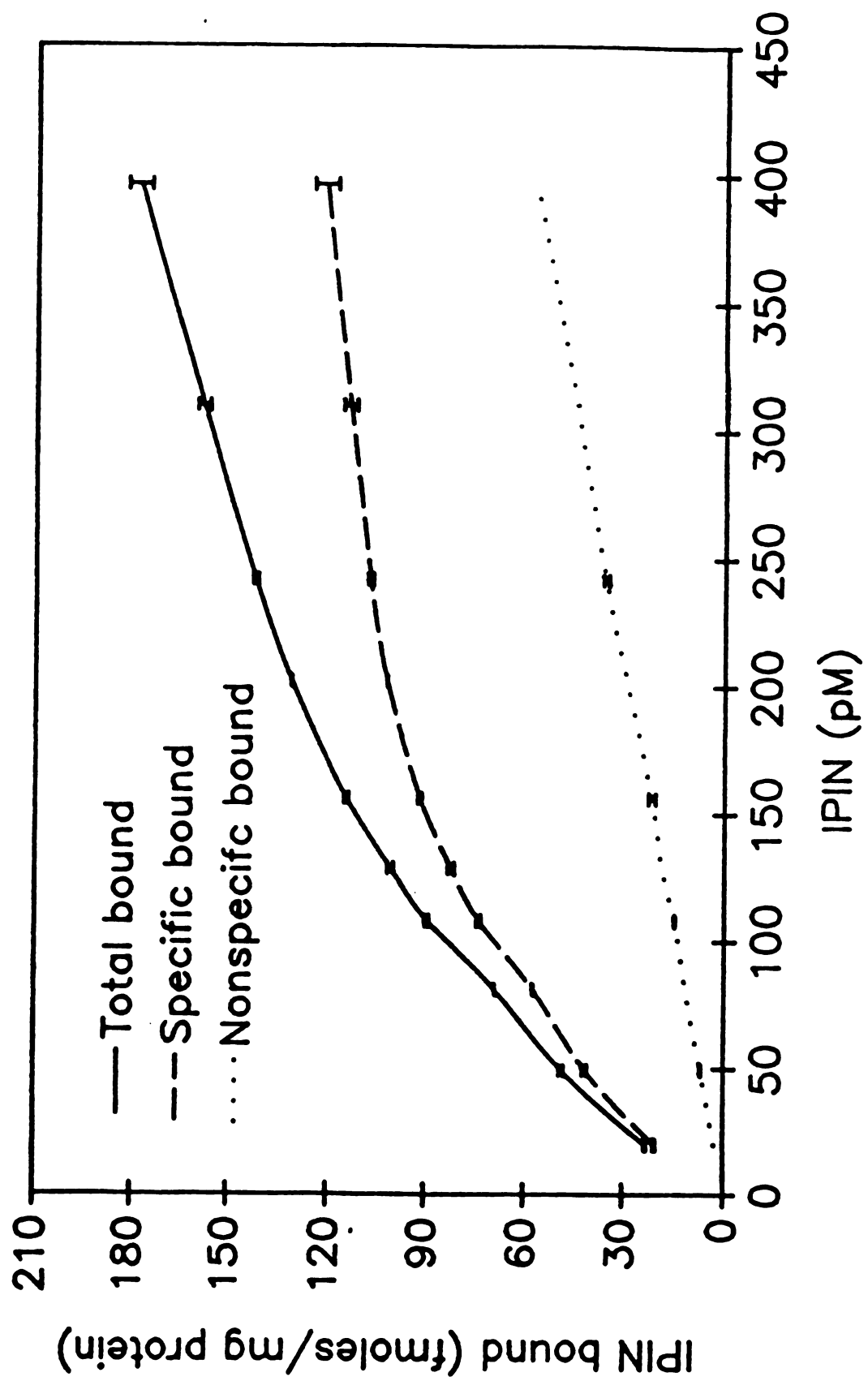


Fig. 5. Scatchard plot of specifically bound IPIN to porcine adipocyte membranes.

Data from a representative experiment conducted in triplicate.

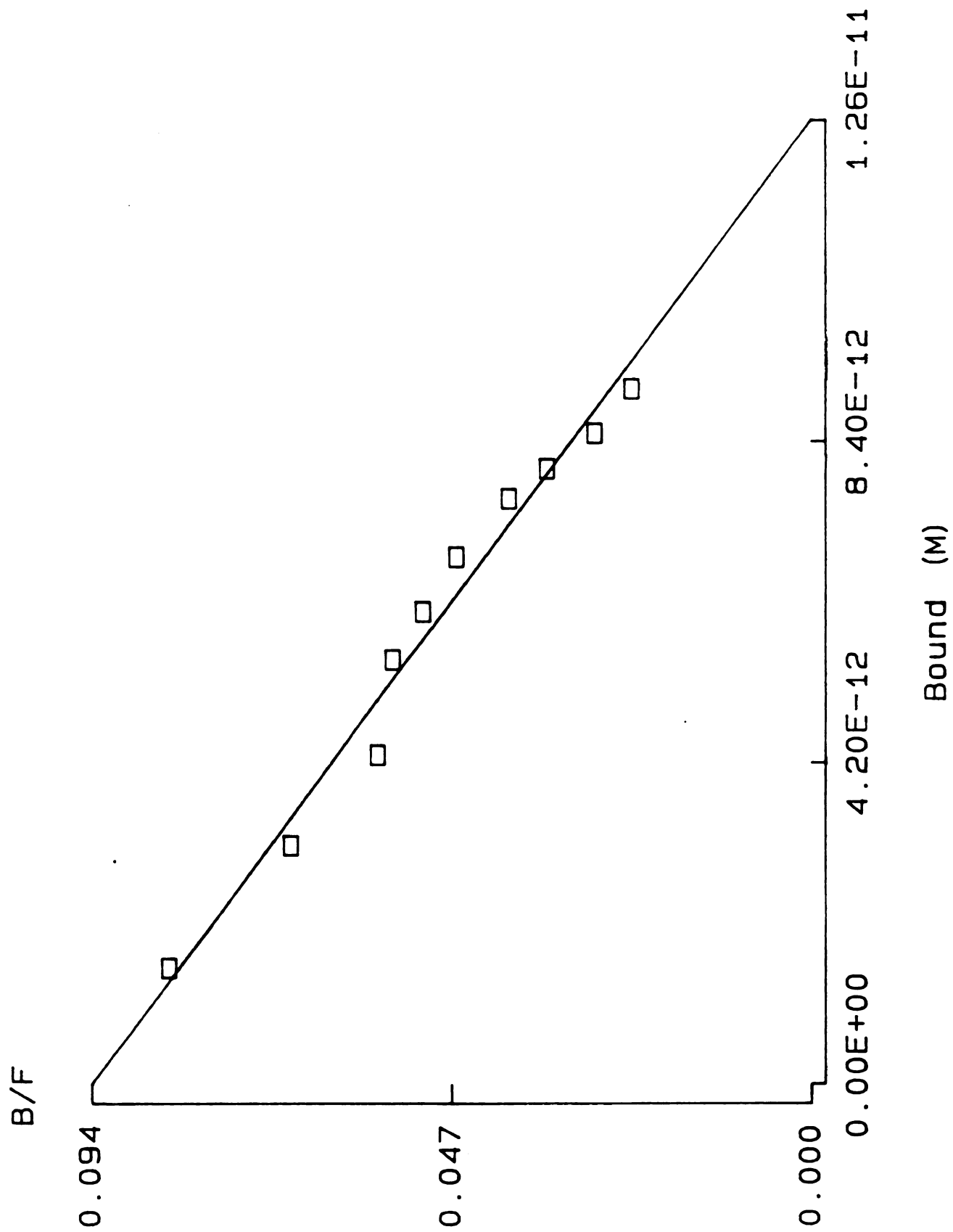


Fig. 6. Inhibition of IPIN specifically bound to porcine adipocyte membranes by (+) and (-) isoproterenol.

Binding was studied in the presence of increasing concentrations of the displacement drugs as described in materials and methods. Results are expressed as percentage of IPIN specifically bound in the absence of isoproterenol. Open squares represents (-) isoproterenol and open circles (+) isoproterenol.

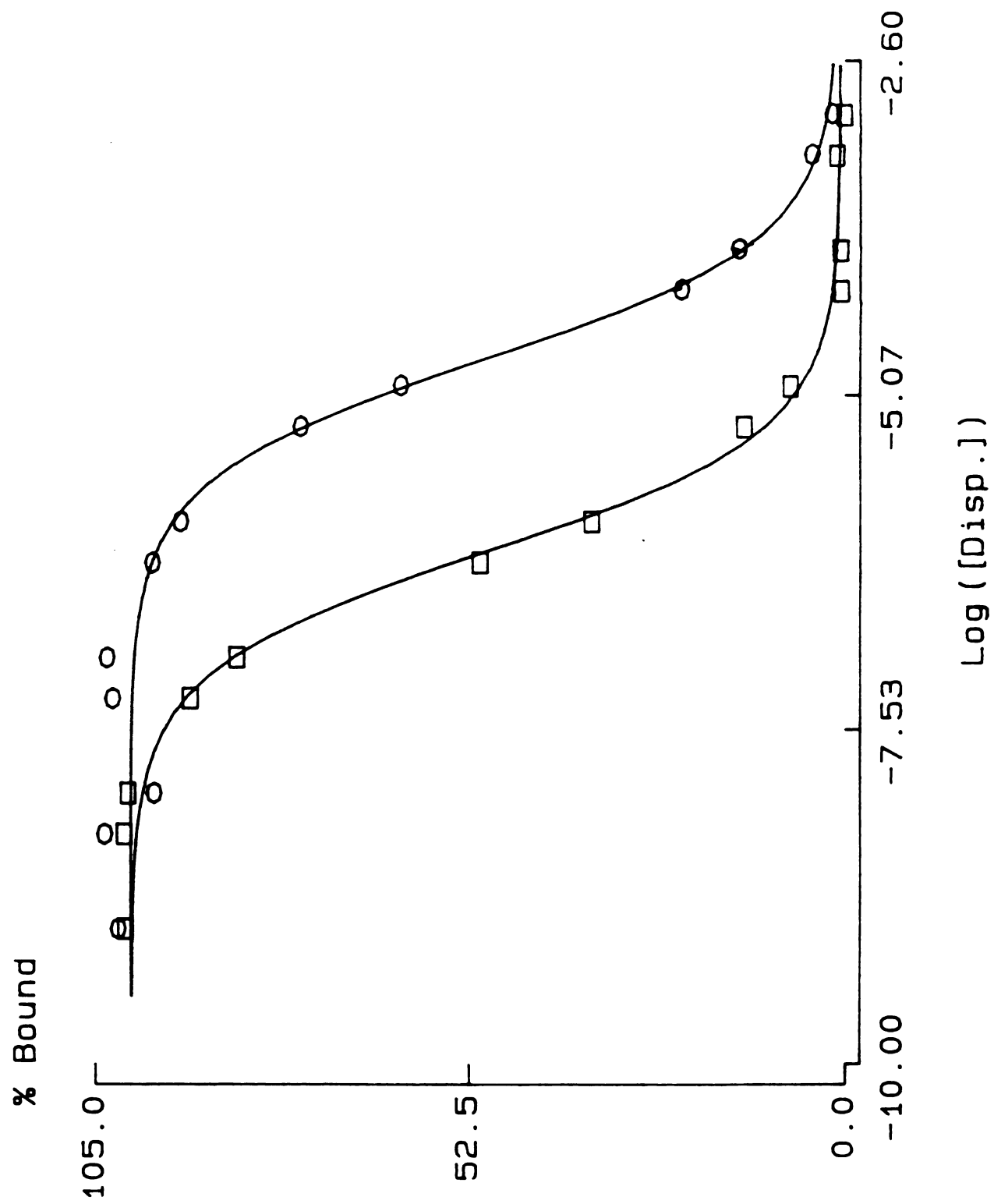
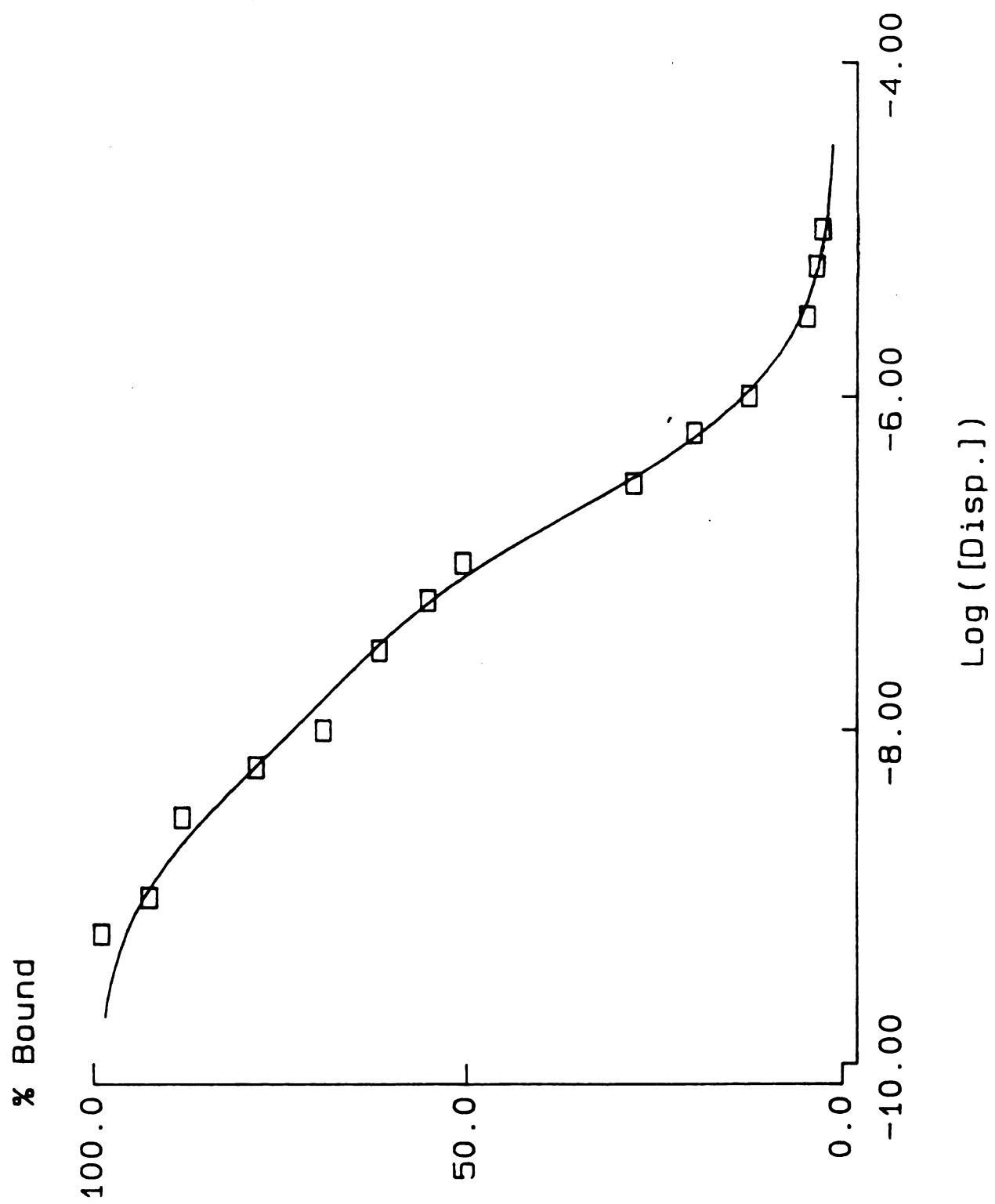


Fig. 7. Inhibition of specifically bound IPIN to porcine adipocyte membranes by the β 1AR selective antagonist ICI 89,406.

Data from a representative experiment done in duplicate are presented. Binding was studied at 300 pM of IPIN in the presence of increasing concentrations of ICI 89,406 as described in Materials and methods. Results are expressed as percentage of IPIN specifically bound in the absence of displacing drug. The best fit line is the result of a two site model as determined by the computer program Ligand.



CHAPTER IV

Androgenic status and developmental dependent α 2AR activity in porcine adipocytes

SUMMARY

Lipid metabolism in adipose tissue is under control of the adrenergic receptor coupled adenylate cyclase system (AC). Stimulation of β AR activates AC, increases cAMP and results in stimulation of lipolysis and inhibition of lipogenesis. Stimulation of α 2AR inhibits AC, decreases cAMP and results in inhibition of lipolysis and stimulation of lipogenesis. Presence of α 2AR has been demonstrated in adipocytes of all the species investigated thus far, but the porcine. Thus, the objective of this study was to re-investigate the presence of α 2AR in isolated porcine adipocytes. Cells were incubated with different agonists and antagonists and glycerol released in the media determined. Epinephrine (Epi, a β and α 2AR agonist) was used in combination with propranolol (β antagonist) so only the α 2AR would be stimulated by Epi. Theophylline was used to promote basal lipolysis, so an inhibitory action from α 2AR stimulation could be determined. In experiments conducted with near market weight castrated male pigs (92 ± 0.5 kg and mean adipocyte diameter 98 ± 3 μ m) no inhibition of basal lipolysis was observed. To evaluate the effect of cell size and/or age, castrated male pigs were fed ad libitum until 158 ± 8 kg and cell diameter of 119 ± 6 μ m. Again no inhibition of basal lipolysis by Epi was observed. To evaluate the effect of androgenic status, adipocytes from intact male pigs at near market weight (104 ± 4 kg and

cell size $84 \pm 4 \mu\text{m}$) and at greater weight (161 ± 17 and cell size of $97 \pm 8 \mu\text{m}$) were studied. No inhibition of lipolysis was observed in near market weight boars, but close to 50% inhibition of basal lipolysis was observed in the older heavier boars ($p < 0.01$). To further demonstrate the presence of $\alpha 2\text{AR}$, the inhibitory action of yohimbine ($\alpha 2\text{AR}$ antagonist) was tested and resulted in a dose dependent inhibition of the antilipolytic action of Epi. Finally to investigate if the effect of castration could be overcome by age, castrated male pigs were fed until $225 \pm 8 \text{ kg}$ (cell size was $122 \pm 4 \mu\text{m}$). No inhibition of basal lipolysis by Epi was observed. In conclusion the presence of $\alpha 2\text{AR}$ receptor has been demonstrated in adipocytes of pigs. Furthermore, the inhibitory action on lipolysis via the $\alpha 2\text{AR}$ is dependent on androgenic status, cell size and or/age.

INTRODUCTION

Both lipolysis and lipogenesis are controlled by cAMP, which in turn is regulated by the activity of the adenylate cyclase (AC) system [Garcia-Sainz & Fain, 1982, Brownsy, 1979, 1980]. The adenylate cyclase system is activated/deactivated through hormone receptor interaction [Stryer & Bourne, 1986]. Two receptors known to modulate

the activity of the AC system are the β -adrenergic receptors (β AR) and the α 2-adrenergic receptors (α 2AR). Activation of β AR promotes stimulation of AC, increasing lipolysis and decreasing lipogenesis. Activation of α 2AR promotes inhibition of AC, decreasing lipolysis and increasing lipogenesis.

The presence of α 2AR has been demonstrated in adipocytes of hamsters [Hittelman and Butcher, 1973, Giudicelli et al., 1977 and Garcia-Sainz et al., 1980], rabbits [Lafontan, 1981], humans [Lafontan and Berlan, 1980, Burns et al., 1981], dogs [Berlan et al., 1982] and, despite some controversy in the past, rats [Rebourcet et al., 1988]. These studies relied on the ability of specific α 2AR agonists and antagonists to interact with the receptor and either inhibit or stimulate lipolytic activity, respectively. While such an approach is accurate and reliable, the inability of agonists or antagonists to modify the lipolytic rate should not be interpreted as absence of the receptor. Negative observations may simply indicate lack of specificity of the agonist/antagonist for the receptor. The importance of this concept became evident in 1988, when Rebourcet and others clarified the controversy involving the presence of the α 2AR in rat adipocytes. These researchers were able to show the presence of the α 2AR by employing a new, more potent and specific agonist called UK 14,304.

In porcine adipose tissue, despite careful

investigation, the $\alpha 2AR$ could not be detected [Mersmann, 1984c]. Utilizing tissue slices, Mersmann compared the lipolytic activity of epinephrine (Epi, an agonist of both $\alpha 2AR$ and βAR) and isoproterenol (Iso, pure βAR agonist), in either young, mature or genetically obese female or castrated male pigs. No difference in maximal lipolytic rate was observed. The use of several $\alpha 2AR$ agonists and antagonists were also unable to indicate receptor associated activity.

Despite the above observations it cannot be concluded that porcine adipocytes do not possess an $\alpha 2AR$. It is difficult to conceive that pigs are different from other species investigated in regard to the $\alpha 2AR$. Recent studies have indicated that cell size is an important factor to demonstrate $\alpha 2AR$ activity in hamsters, dogs and rats [Rebourcet et al., 1988; Carpene et al., 1983; Taouis et al., 1987]. No $\alpha 2AR$ activity could be detected in small adipocytes from young animals, but activity was observed in larger adipocytes from adult animals [Carpene et al., 1983; Taouis et al., 1987]. Receptor sites were also observed to be more numerous in larger adipocytes of adult dogs [Taouis et al., 1987]. Carpene et al [1983] showed that exposure of adult hamsters to cold temperature (6 °C) reduced adipocyte size to that of a 4-5 week old animals. The reduction in size of the adipocytes resulted in complete disappearance of $\alpha 2AR$ binding sites and $\alpha 2AR$ responsiveness which had been demonstrated before cold exposure.

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It has also been demonstrated that sex steroids play an important role in the expression of α 2AR activity in adipocytes [Pecquery et al., 1988]. In male hamsters, castration resulted in an almost complete suppression of the α 2AR mediated inhibition of lipolysis. Testosterone replacement therapy re-established the α 2AR response. These are very important observations, since in most of the studies conducted with pig, castrated animals are utilized.

In this study we demonstrate the presence of the α 2AR in porcine adipocytes of intact male adult pigs. Furthermore we demonstrate that the inhibitory activity of the α 2AR on lipolysis is associated with cell size and/or age and androgenic status.

MATERIALS AND METHODS

For this study crossbred (York, Landrace and Duroc) pigs were housed in environmentally controlled facility and fed ad libitum.

A modification of the procedure developed by Rodbell [1964 α] for the isolation of rat adipocytes was used. The middle layer of subcutaneous adipose tissue from the dorsal neck region of anesthetized pigs was obtained using aseptic techniques. The sample was placed in Basal Media Eagle (BME) maintained at 38 °C and transported to the laboratory within 15 min. Using sterile techniques, the tissue was finely minced and digested with collagenase

in BME medium containing 1% BSA. The digestion was conducted for 50 min at 38 °C under an atmosphere of 95% O₂ and 5% CO₂ with gentle shaking. One lot of collagenase was used for all studies. Digestion was terminated by washing the cells with collagenase-free buffer and the adipocytes isolated by filtering the cells through a 500 um mesh polypropylene screen.

Lipolysis assay was conducted as described by Coutinho et al. [1989]. Briefly, isolated cells were incubated for 120 min at 38 °C in 1 ml of basal media Eagle (BME) containing 3% BSA and 2 ug/ml of adenosine deaminase (ADA) at cell concentrations of 0.5 to 1 x 10⁵ cells/ml, unless otherwise stated. Lipolysis was assessed by determining glycerol released in the incubation media using an enzymatic assay kit.

Cell diameter was determined with the use of a microscope equiped with a micrometer. Lipolysis studies were conducted at least in duplicate and cells isolated from an individual pig were considered as the experimental unit. The SAS program was used for the statistical analyses, in which each pig was considered a block. Each data set was tested for heterogeneous variance and treatment means tested as described in figure legends.

Glycerol kit, collagenase and adenosine deaminase were obtained from Boehringer Mannheim (Indianapolis, IN). Bovine serum albumin (CRG-7) was from Armour Pharmaceutical (Kankakee, IL). BME was from Gibco Laboratories (Grand

Island, NY). Isoproterenol, propranolol, epinephrine and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO). UK 14,304 was kindly donated by Pfizer (Sandwich, Kent.).

RESULTS AND DISCUSSION

The $\alpha 2$ AR associated inhibitory action on lipolysis was first investigated by comparing the maximal lipolytic rate of Iso and Epi. Iso is a β AR agonist with no $\alpha 2$ AR activity and epinephrine is both β AR and $\alpha 2$ AR agonist. Thus, a lower maximal lipolytic rate for Epi could be the result of its interaction with $\alpha 2$ AR.

Dose titration studies (Fig. 1) conducted with cells isolated from near market weight (96 ± 6 kg) castrated male pigs had a 14.6 % greater maximal lipolytic response for Iso (10^{-4} M) than for Epi (10^{-4} M), ($P < 0.01$), suggesting the presence of $\alpha 2$ AR. Alternatively, Epi could have a lower ability than Iso to promote the coupling of the ligand receptor complex to the Gs protein.

To further investigate the presence of the $\alpha 2$ AR, the ability of the $\alpha 2$ AR agonist UK 14,304 to inhibit Iso stimulated lipolysis (10^{-8} M, which provides half maximum lipolysis, Coutinho et al., [1989]) was investigated. Dose titration studies with UK 14,304 could not demonstrate any antilipolytic response of this agonist (Fig. 2).

Half maximum Iso stimulated lipolysis could be too

strong of a stimulation to be overcome by α 2AR activation. Since basal lipolytic rate of isolated porcine adipocytes is near zero, theophylline (Theo, 5 mM) was used to provide a basal lipolysis rate. Previous studies (data not shown) indicated that Theo at 5 mM stimulates lipolysis approximately 10% of the maximal rate of that observed with Iso. Dose titration studies with UK 14,304 under these conditions also did not inhibit lipolysis (data not shown).

The lack of response of an agonist or antagonist could simply mean that the compound does not recognize the receptor. To minimize this problem Epi, a naturally occurring hormone, was used as an α 2AR agonist. Because Epi also has β AR agonistic activity, studies were conducted to determine the doses of Epi and propranolol (Prop, β antagonist) that would maximize α 2AR activity, with no stimulation on lipolysis. Previous studies in our laboratory have indicated that Prop at concentrations higher than 10^{-4} M can be detrimental to adipocytes, so 10^{-5} M Prop was selected. Dose titration studies were then conducted with Epi in the presence of 5 mM Theo. As can be seen in Fig. 3, Epi did not inhibit lipolysis at any concentration tested. Epi at 10^{-5} M, however, was able to overcome the blocking effect of Prop and stimulated lipolysis ($P < 0.01$). To ensure that no lipolytic activity due to Epi stimulation of β AR would occur, Epi at 10^{-7} M and Prop at 10^{-5} were selected.

Epi in combination with Prop was then used to

investigate the presence of $\alpha 2AR$ in adipocytes isolated from near market weight castrated male pigs. It was expected that Prop would block the interaction of Epi with βAR . In that manner, Epi would act only as a $\alpha 2AR$ agonist and would decrease basal lipolysis, if $\alpha 2AR$ were present. As can be seen in the first column of Table 1, no antilipolytic effect of Epi was detected.

The results with UK 14,304 and with Epi plus Prop, unlike the comparison of Epi and Iso, do not suggest the presence of $\alpha 2AR$ in adipocytes of near market weight castrated male pigs.

As pointed out in the introduction the antilipolytic action of $\alpha 2AR$ has been shown to be dependent on cell size and/or age. To investigate these effects, castrated male pigs were kept on ad libitum feeding until reaching almost double the weight (158 ± 8 kg) of the pigs used in the previous studies (Table 1). As expected, these animals also had larger cells (Table 1, column 2). Treatment of adipocytes with Epi plus Prop, as shown for near market weight pigs, did not inhibit basal lipolysis.

As demonstrated by Pecquery et al. [1988], the presence of testosterone is necessary for the expression of $\alpha 2AR$ mediated antilipolytic action in adipocytes of hamsters. To evaluate if androgenic status was important the antilipolytic activity of Epi was tested in near market weight intact male pigs. As can be seen in Table 1, column 4, no antilipolytic activity was detected.

The next study was designed to test if the presence of $\alpha 2AR$ in porcine adipocytes was dependent on cell size and/or age and androgenic status. Adipocytes were isolated from intact male pigs weighing 161 ± 19 kg. As can be seen in Table 1, column 5, close to 50% inhibition of basal (Theo, 5 mM) stimulated lipolysis was detected ($P < 0.01$). To further demonstrate the presence of $\alpha 2AR$, an $\alpha 2AR$ antagonist (yohimbine) was used to demonstrate a dose dependent blockage of the antilipolytic effect of Epi (Fig. 4). The use of yohimbine on cells isolated from near market weight intact male had no effect on lipolysis (Fig. 5).

It thus appears that antilipolytic action of $\alpha 2AR$ in porcine adipocytes is dependent on androgenic status and either age and/or adipocyte size.

To investigate if the effect of castration on the expression of the $\alpha 2AR$ antilipolytic response could be overcome by age and/or cell size, adipocytes were isolated from castrate male pigs weighing 225 ± 8 kg. As can be seen in Table 1, column 3, cell size was not affected to any significant extent, even though body weight was greater than that of previous groups. The combination of Epi plus Prop caused no reduction in basal lipolysis (Table 1). Likewise the antagonist yohimbine also had no effect (Table 1).

The onset of puberty in pigs occurs at 5 - 8 months of age, which under the feeding regimen used in this study

would be less than 100 kg [Singleton et al., 1990]. This indicates that the near market weight intact male pigs used in this study had already entered puberty. These animals, however, did not express $\alpha 2AR$ mediated antilipolytic response. These observations indicate that presence of the sex steroids is not sufficient for expression of the $\alpha 2AR$ response. Cell size and/or age also appears to regulate expression of $\alpha 2AR$ mediated antilipolytic response.

Finally, it is important to observe that castrated pigs accumulate more fat and have larger adipocytes than intact male pigs. This appears as a contradiction, since the expression of a receptor that inhibits cAMP accumulation should result in lower rates of lipolysis and greater rates lipogenesis. It is important to consider, however, that the AC system in adipocytes presents several levels of regulation. It has been demonstrated, for instance, that intact male hamsters have greater AC activity [Pecquery et al, 1990], and this could result in higher rates of basal lipolysis in intact versus castrated animals. In our study castrated animals had a higher theophylline stimulated lipolysis rate than intact animals (Table 1). This effect was, however, confounded by adipocyte size. The comparison of castrated males versus intact male, at the same cell size, reveals basically the same rate of basal lipolysis (Table 1). It is thus difficult to explain why the expression of $\alpha 2AR$ response in porcine adipocytes is not associated with greater

accumulation of triacylglycerol in adipocytes. This, however, was not the objective of this study, but certainly deserves future investigation.

In this study we were able to demonstrate an antilipolytic action mediated via the α 2AR in porcine adipocytes. The α 2AR response in porcine adipocytes appears to be dependent on the androgenic status, cell size and/or age.

Table 1: Lipolytic response of isolated porcine adipocytes originated from castrated or intact males at different weights and cell size

sex:	male castrate	male castrate	male castrate	male intact	male intact
weight (Kg)	91.7±0.5	158.2±7.9	224.8±7.6	104.5±4.5	160.8±19.3
cell diameter (μ m)	97.8±2.8	119.4±6.5	122.1±4.5	84.2±4.3	97.1±8.3
number of animals	3	4	4	3	4
glycerol release (nmol/ 10^6 cells $\times$ h $^{-1}$)					
basal	177.0±6.1	330.7±37.4	1704.3±46.7	87.5±6.6	150.5±9.0
Epi + Prop ₅ (10^{-7}) (10^{-5})	183.9±6.1	305.7±37.4	1740.3±46.7	104.4±6.6	76.8±10.6

Lipolytic rates were determined in the presence of 5 mM theophylline. Weight and adipocyte diameter are means $\pm$ standard error and glycerol release are mean $\pm$ standard error of treatment means. ** represents significant difference at $P < 0.01$. Treatment effect was determined based on Dunnett t test.

Figure 1: Dose titration of the lipolytic response of Iso and Epi on adipocytes isolated from near market weight (96 ± 6 kg) castrated male pigs. Treatment comparison was made only at maximal lipolysis (10^{-4} M), since analysis of all data points resulted in heterogeneous variance. Error bars indicate standard error of treatment means. Values represent the average of four independent experiments. ** represents significant difference at $P < 0.01$. based on a F test.

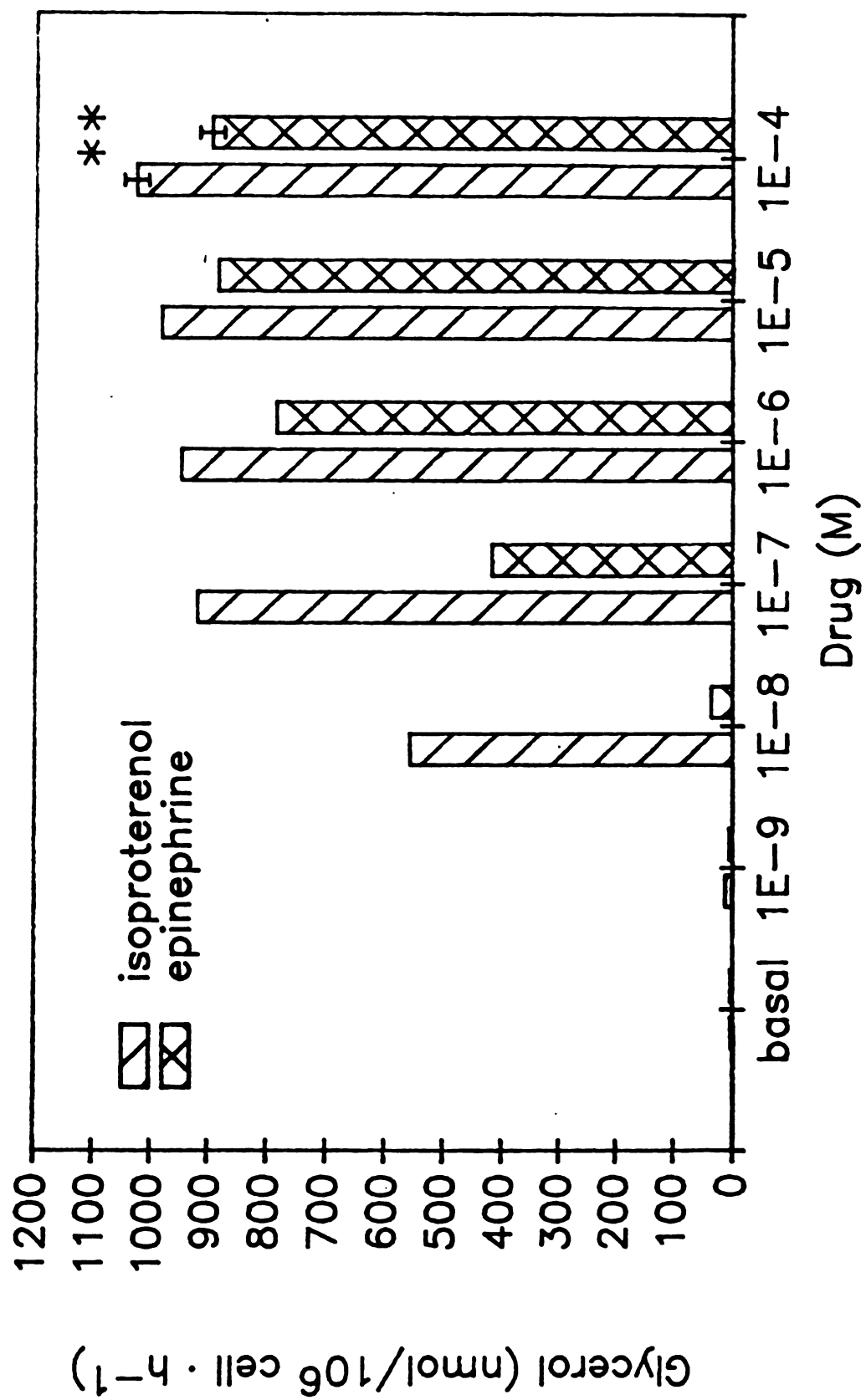


Figure 2: Dose titration of the lipolytic response of UK 14,304 on adipocytes stimulated with Iso (10^{-8} M). Error bars represent standard error of treatment means, and were calculated excluding basal values to avoid heterogeneous variance. Values represent the average of four independent experiments conducted with near market weight (96 ± 6 kg) castrated male pigs.

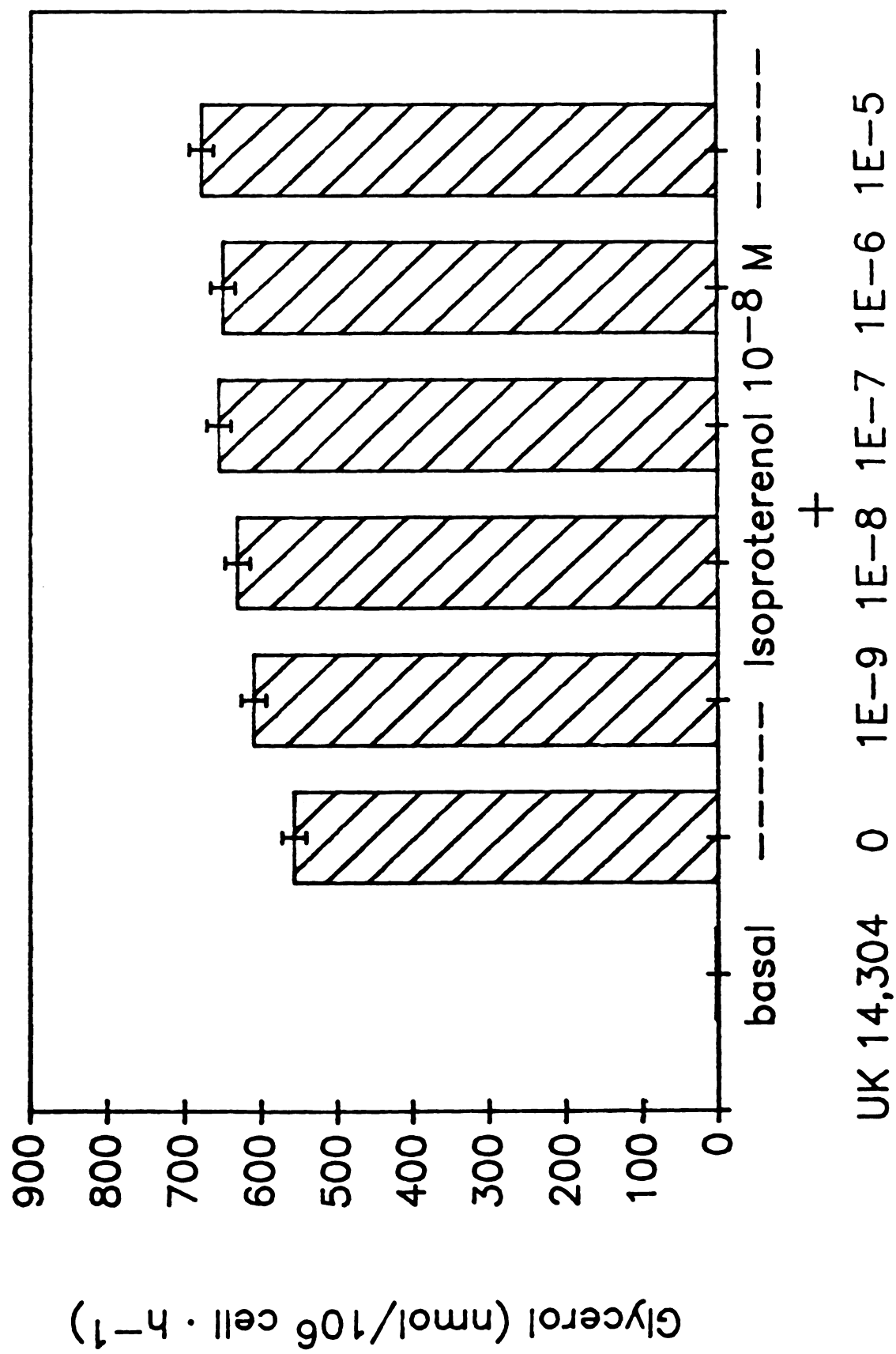


Figure 3: Dose titration of the lipolytic response of Epi on adipocytes incubated with Prop (10^{-5} M) and Theo (5 mM). Error bars indicate standard error of treatment means. Values represent the average of four independent experiments conducted with near market weight (92 ± 5) castrated male pigs. ** represents significant differences at $P < 0.01$. Treatment effect was determined based on Dunnet t test.

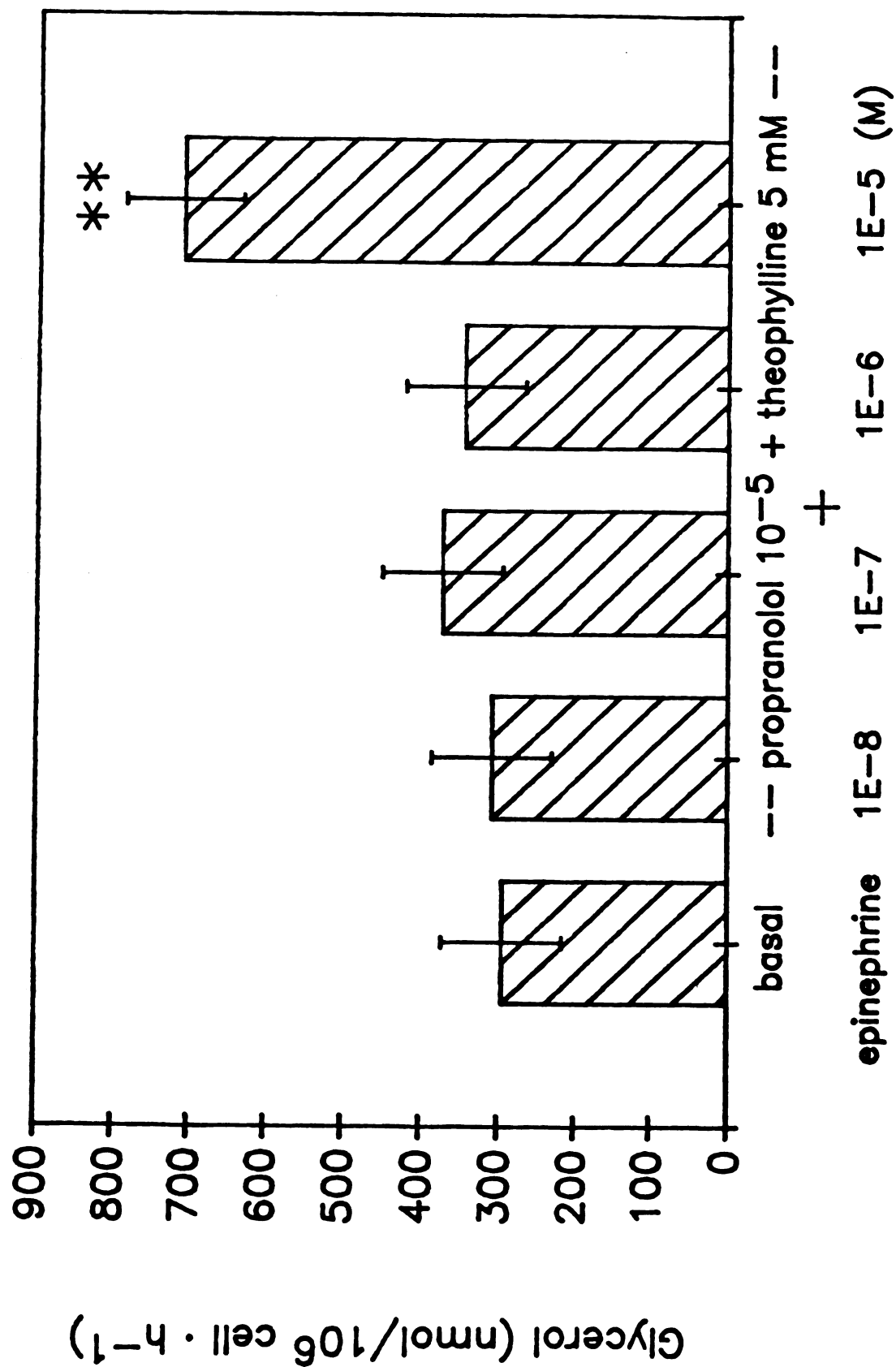


Figure 4:

yohimbine

kg) intact

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Figure 4: Dose titration of the lipolytic response of yohimbine on adipocytes from near market weight (104 ± 4 kg) intact male pigs. Adipocytes were incubated with Epi (10^{-7} M), Prop (10^{-5} M), Theo (5 mM) and several concentrations of yohimbine. Basal values were obtained in the absence of Epi. Error bars indicate standard error of treatment means. Values represent the average of three independent experiments.

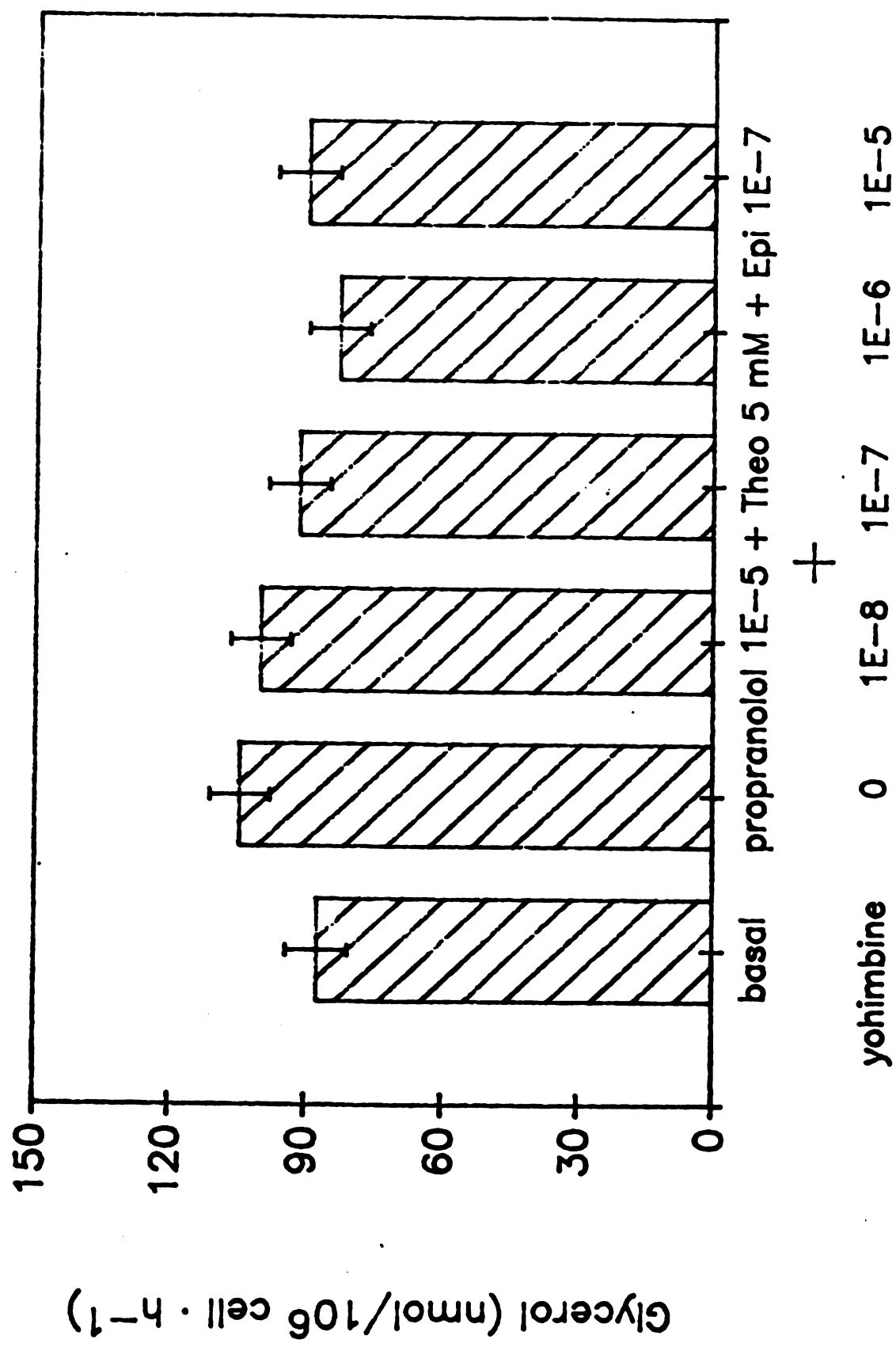


Figure 5: Dose titration of the lipolytic response of yohimbine on adipocytes from intact male pigs (161 ± 19 kg). Adipocytes were incubated with Epi (10^{-7}), Prop (10^{-5} M), Theo (5 mM) and several concentrations of yohimbine. Basal values were obtained in the absence of Epi. Error bars indicate standard error of treatment means. Values represent the average of four independent experiments. ** represents significant difference at $P < 0.01$. Treatment effect was determined based on Dunnet t test.

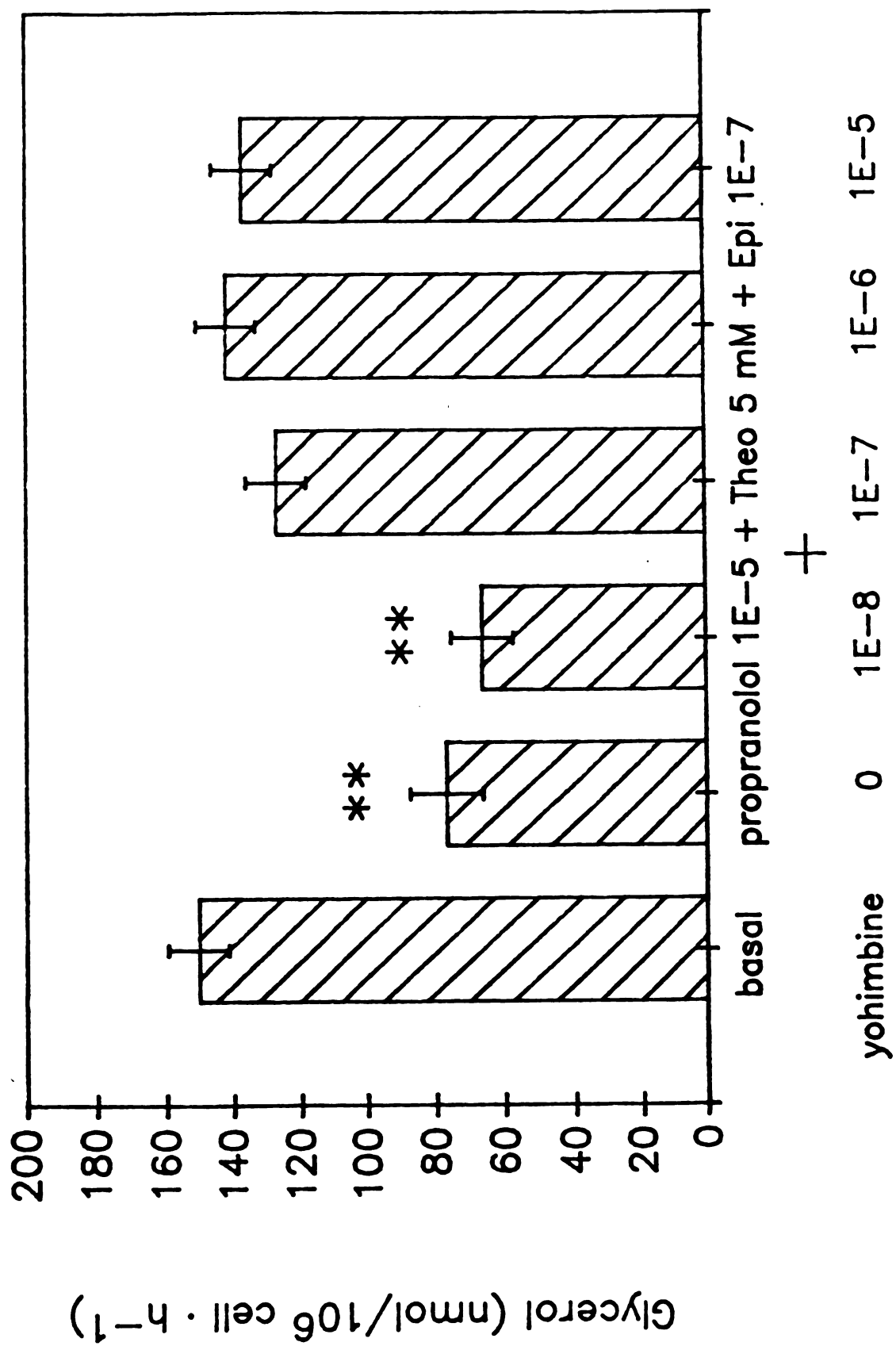
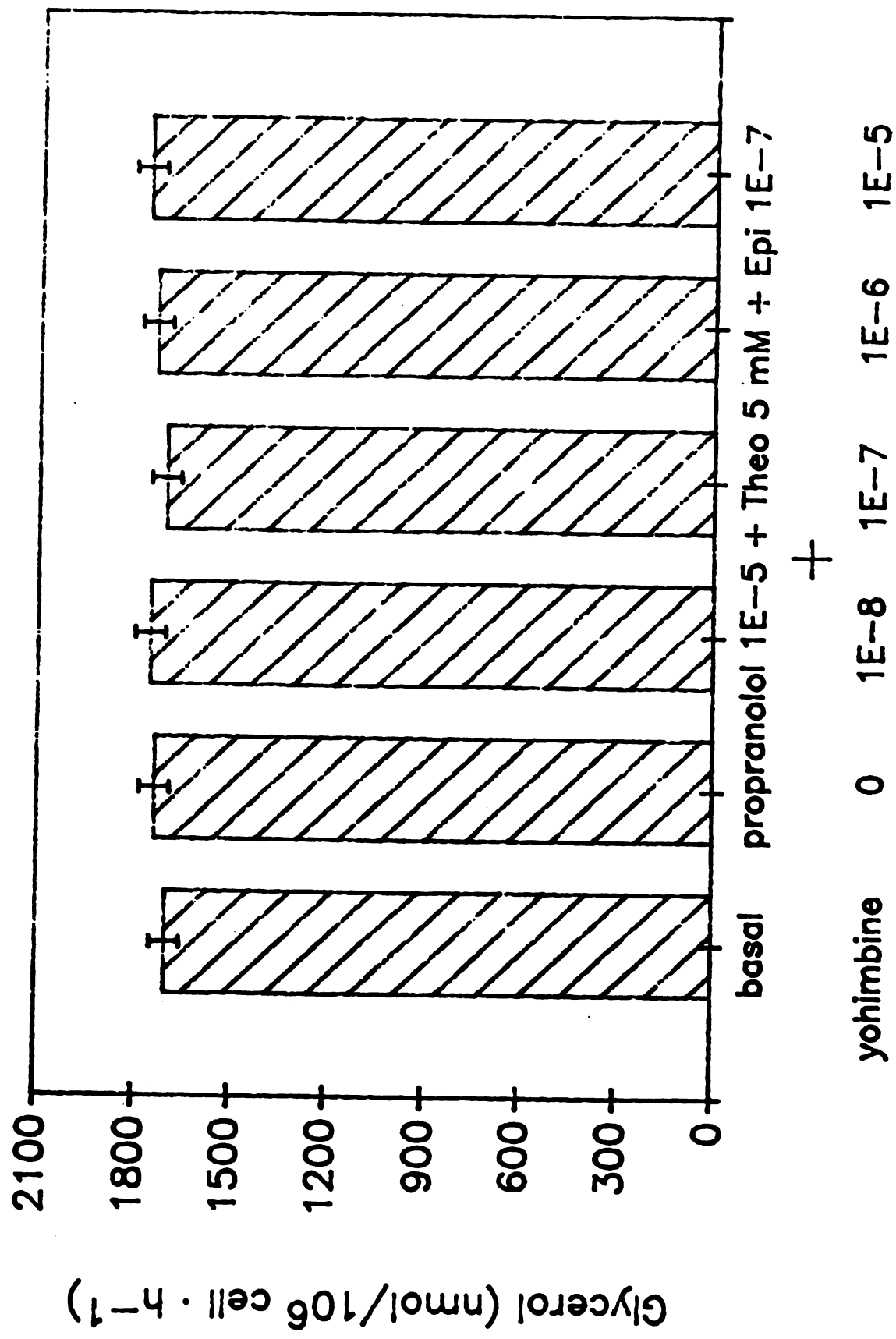


Figure 6: Dose titration of the lipolytic response of yohimbine on adipocytes from castrate male pigs (225 ± 8 kg). Adipocytes were incubated with Epi (10^{-7}), Prop (10^{-5} M), Theo (5 mM) and several concentrations of yohimbine. Basal values were obtained in the absence of Epi. Error bars indicate standard error of treatment means. Values represent the average of three independent experiments.



Conclusions

Studies conducted during the last decade have demonstrated that analogs of epinephrine, also called β -agonists, can reduce fat deposition in farm animals. This observation indicates that modulation of the adrenergic coupled adenylate cyclase system in adipose tissue can result in less fat deposition in pigs. To further improve the results obtained with these compounds an understanding of: 1) the adrenergic coupled adenylate cyclase system and its regulation of adipose tissue metabolism and 2) the mode of action of epinephrine analogs in pigs is needed.

Unfortunately, limited information was available on adrenergic control of lipolysis in porcine adipose tissue. Thus key questions in regard to the direct action of β -agonists on adipose tissue and presence of α_2 AR in porcine adipocytes were unanswered.

In this dissertation, a sensitive in vitro porcine adipocyte system was characterized and used to further improve the knowledge on adrenergic regulation of adipose tissue. A direct effect of Rac and Cle in porcine adipocytes was demonstrated. The lipolytic activity of ractopamine was then shown to be dependent on the removal of adenosine from the media. This could explain why, in previous studies, a direct effect of these compounds was not detected.

It was also observed that Rac, Cle and Iso had

different abilities (maximal rate and ED 50) to stimulate lipolysis, raising the question whether these compounds had different affinities for the β AR of porcine adipocyte membranes. A complete characterization of a receptor binding assay and receptor subtypes, however, had never been published for porcine adipocytes. Furthermore, Mersmann [personal communication] had been unsuccessful in developing a β AR binding assay. In Chapter II a complete characterization of a β AR binding assay, the presence of two β AR subtypes and a quantitative determination of their proportions is reported. This system was then used to determine the affinity of Iso, Rac and Cle for the β AR. The results indicated that the different lipolytic ability of these compounds could not be explained by their affinities for the receptors, suggesting a difference in the coupling of the ligand-receptor complex to the Gs. This observation adds another dimension to the action of β -agonists, indicating that the final response of a compound is dependent on its ability to bind to the receptor and also to promote the coupling to the G protein. Both events need to be taken into account during development or testing of new drugs.

Another aspect of adrenergic regulation of adipose tissue metabolism is that, as in any other important regulatory pathway, both positive and negative axis are present. Most studies conducted in farm animals have only explored the stimulatory side of the AC system. It is

important to consider that, at least theoretically, blockage of the inhibitory axis should result in greater activity of the catalytic unit of the AC system, and thus result in higher lipolysis, lower lipogenesis rates and less fat deposition. Previous studies conducted by Mersmann [1984c], however, could not demonstrate the presence of $\alpha 2AR$ in porcine adipose tissue. In chapter IV, the presence of $\alpha 2AR$ was demonstrated in adipocytes of pigs. Moreover, the presence of $\alpha 2AR$ antilipolytic mediated activity was shown to be dependent on both androgenic status and adipocyte size and/or age. For production purposes, however, $\alpha 2AR$ antagonists could not be explored in most commercial operations, since this receptor does not appear to modulate lipolysis in near market weight castrated pigs. It is possible that in such animals another negative regulatory mechanism is in place to control the AC system. An obvious candidate for this role is the adenosine receptor. Results from this study revealed the presence and coupling of the Ad receptor to G_i and inhibition of lipolysis with an Ad agonist. One could speculate that a new group of compounds, Ad antagonists, could be developed to modulate fat accretion in farm animals.

The methodology developed in this dissertation and results originated from it will allow new studies to be conducted.

The development of receptor binding assay for porcine

adipocytes will permit researchers to investigate the phenomenon of down regulation of receptors. To date, no direct information is available on receptor down regulation in adipose tissue of pigs fed β -agonists.

The observation made on the presence of a possible atypical β AR subtype is fascinating and could allow the development of β -agonists with minimal action outside the adipose tissue.

Finally, the androgenic status and adipocyte size or age-dependent expression of α 2AR activity poses several questions. Is the receptor uncoupled in castrate animals ? Is the expression of the α 2AR gene being regulated by sex steroids ? What other regulatory elements are operating in intact animals to reduce fat deposition, since one would expect the presence of α 2AR to be associated with greater fat deposition ?

In conclusion, this study provided new insights into the adrenergic regulatory mechanisms of lipolysis in porcine adipose tissue. Key questions in the field were answered while several others were raised.

REFERENCES

- Allen, C.E., Beitz, D.C. Cramer, D.A., & Kauffman, R.G. (1976) In: Biology of fat in meat animals, Publ. No. 234, Univ. of Wisconsin Press, Madison.
- Allende, J.E. (1988) FASEB J. 2:2356-2367.
- American Meat Institute (1989) Meat Facts, Washington, D.C.
- Anderson, D.B., Veenhuizen, E.L., Waitt, W.P., Paxton, R.E. & Mowrey, D.H. (1987) J. Anim. Sci. 65(Suppl. 1), 130 (Abstr.).
- Anderson, D.B., Veenhuizen, E.L., Waitt, W.P., Paxton, R.E. & Mowrey, D.H. (1988) J. Anim. Sci. 66(Suppl. 1), 279 (Abstr.).
- Ariens, E.J. & Simonis, A.M. (1983) Biochem. Pharmacol. 32, 1539-1545.
- Berlan, M., Carpenne, C., Lafontan, M. and Dang Tran, L. (1982) Horm. Metab. Res. 14:257-260.
- Berlan, M. & Lafontan, M. (1985) Eur. J. Clin. Invest. 15:341-348.
- Bocklen, E., Flad, S., Muller, E & vonFaber, H. (1986) Anim. Prod. 43, 335-340.
- Bojanic, D and S.R. Nahorski (1983) Eur. J. Pharmacol. 93:235-243.
- Bradford, M. (1976) Anal. Biochem. 72, 248-254.
- Brownsy, R.W., Hughes, W.A. and Denton, M. (1979) Biochem. J. 184:23-32.
- Brownsy, R.W. & Hardie, D.J. (1980) FEBS Lett. 20:67-70.
- Burgisser, E. (1983) J. Recept. Res. 3, 261-281.
- Burns, T.W., Langley, P.E., Terry, B.E., Bylund, D.B., Hoffman, B.B., Tharp, M.D., Lefkowitz, R.J., Garcia-Sainz, A. and Fain, J.N. (1981) J. Clin. Invest. 67:467-475.
- Cabelli, R.J. and Malbon, C.C. (1979) J. Biol. Chem. 254:8903-8908.
- Carpenne, C.M., Berlan, M. & Lafontan, M. (1983) J. Lipid Res. 24:766-774.
- Casey, P.J. and Gilman, A.G. (1988) J. Biol. Chem.

263:2577-2580.

-Caster, W.O. (1976) Nutrition, longevity and aging, M. Rockestein and M.L. Susasman (Eds.). New York: Academic Press Inc., 29-45.

-Cohen, M.L., Wiley, K.S. & Bemis, K.G. (1982) Arch. Pharmacol. 320, 145.

-Committee on diet, nutrition and cancer, Assembly of life sciences, National Research Council (1982) Diet, nutrition and cancer, Washington, D.C.: National Academy Press.

-Cornish-Bowden, A. & Koshland Jr., D.E. (1975) Mol. Biol. 95, 201-212.

-Coutinho, L.L., Cameron, J.K., Romsos, D.R., Merkel, R.A. and Bergen, W.G. (1989) J. Anim. Sci. 67 (Suppl. 2, Abstract).

-Coutinho, L.L., Bergen, W.G., Merkel, R.A. & Smith II, C.K. (1990) FASEB J. 4, A-378, Abstract # 650.

-Creasy, W.A. (1985) Diet and cancer, Lea and Febiger. Philadelphia, PA

-Daly, J.W. (1984) Adenosine: Receptors and Modulation of Cell Function. 625:31-46.

-Dickerson, P.S. (1990) PhD Dissertaion, Michigan State University, E. Lansing, MI.

-Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlmann, H.G., Freille, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Mumford, R.A., Slater, C.D. (1986) Nature (London) 321:75-79.

-Emorine, L.J., Marullo, S., Briend-Sutren, M., Patey, G., Tate, K., Delavier-Klutcho, C. and Strosberg, A.D. (1990) Science 245:1118-1121.

-Etherton, T.D. and Chung, C.S. (1981) J. Lipid. Res. 22:1053.

-Frielle, T., Collins, S., Daniel, K.W., Caron, M.G., Lefkowitz, R.J. and Kobilka, B.K. (1987) Proc. Natl, Acad. Sci. U.S.A. 84:7924-7924.

-Garcia-Sainz, A. & Fain, J.N. (1982) Trends Pharmacol. Sci. 21:201-203.

-Garcia-Sainz, A., Hoffman, B.B., Li, S.Y., Lefkowitz, R.J. and Fain, J.N. (1980) Life Sci. 27:953-969.

- Geirschik, P. and Jakobs, K.H. (1988) The alpha-2 adrenergic receptors, ed. L.E. Limbird, Chapt 3:75-105, Humana Press. Clifton, New Jersey.
- Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615-649.
- Giudicelli, Y., Agli, B., Brulle, D. and Nordmann, R. (1977) FEBS Lett. 83:225-230.
- Graziano, M.P., Mosham, C.P. and Malbon, C.C. (1985) J. Biol. Chem. 260:7665-7674.
- Guyer, C.A., Hortsman, D.A., Limbird, L.E. (1990) FASEB J. 4, A-808, Abstract # 3139.
- Guyton, A.C. (1986) Textbook of medical physiology, Saunders (Ed.), 7th edition, 826-827.
- Hancock, J.D., Peo, E.R., Lewis, A.J., Parrot, J.C. (1987) J. Anim. Sci. 65:(Suppl. 1):309.
- Hanrahan, J.P., Quirke, J.F., Bomann, W., Allen, P., McEwan, C., Fitzsimons, J.M., Kotzian, J. & Roche, J.F. (1986) In: Recent advances in animal nutrition, W. Haresign and D.J.A. Cole, Ed., Butterworths, London, pp. 125-138.
- Hausmann, D.B., Martin, R.J., Veenhuizen, E.L. and Anderson, D.B. (1989) J. Anim. Sci. 67:1455-1464.
- Heron, D.S., Shinitzky, Hershkowitz, M. & Samuel, D. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 7463-7467.
- Hittleman, K.J. and Butcher, R.W. (1973) Biochem. Biophys. Acta 316:403-410.
- Hu, C.Y., Novakofski, J. & Mersmann, H.J. (1987) J. Anim. Sci. 64, 1031-1037.
- Kather, H. (1988) J. Biol. Chem. 263, 8803-8809.
- Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, M.G., Lefkowitz, R.J. & Regan, J.W. (1987) Science 238:650-656.
- Lafontan, M. (1981) J. Lipid Res. 22:1084-1093.
- Lafontan, M. and Berlan, M. (1980) Eur. J. Pharmacol. 66:87-93.
- Lefkowitz, R.J., Stadel, J.M. and Caron, M.G. (1983) Ann. Rev. Biochem. 52:159-186.
- Lefkowitz, R.J. and Caron, M.G. (1987) Recent Prog. Horm.

Res. 43:469-497.

-Lefkowitz, R.J. and Caron, M.G. (1988) J. Biol. Chem. 263:4993-4996.

-Levitzki, A. (1988) Science 241:800-806.

-Liu, C.Y., Boyer, J.L. and Mills, S.E. (1989) J. Anim. Sci. 67, 2930-2936.

-Lohse, M.J., Klotz, K.N., Schwabe, U., Cristalli, G., Vittori, S. and Griftani, M. (1988) Rec. Adv. Rec. Chem. 107-121.

-Londos, C., Cooper, D.M.F. & Wolff, J. (1980) Proc. Natl. Acad. Sci. USA, 77, 2551-2554.

-McEwan, C., Fitzsimons, J.M., Kotzian, J. & Roche, J.F. (1986) In: Recent advances in animal nutrition, W. Haresign and D.J.A. Cole, Ed., Butterworths, London, pp. 125-138.

-McGonigle, P., Neve, K.A. & Molinoff, P. (1986) Mol. Pharmacol. 30, 329-337.

-McGonigle, P. & Molinoff, P.B. (1989) In: Basic Neurochemistry Molecular Cellular and Medical Aspects, 4th. edition, Ed. G.J. Siegel et al. Raven Press, Ltd. New York, pp. 183-201.

-Merkel, R.A., Burkett, R.L., Burnett, R.J., Dickerson, P.S., Johnson, S.E., Schroeder, A.L. and W.G. Bergen (1987) Mich. State Univ. Res. Rep. 487;143-149.

-Mersmann, H.J., Brown, L.J. Underwood, M.C. & Santon, H.C. (1974) Comp. Biochem. Physiol. 47, 263-270.

-Mersmann, H.J., Phinney, G., Brown, L.J. and Arakelian, M.C. (1975) Gen. Pharmac. 6:193-199.

-Mersmann, H.J. (1984a) Comp. Biochem. Physiol. 77C, 39-42.

-Mersmann, H.J. (1984b) Comp. Biochem. Physiol. 77C, 43-53.

-Mersmann, H.J. (1984c) Comp. Biochem. Physiol. 79C, 165-170.

-Mersamnn, H.J. (1986) Am. J. Physiol. 252:E85-96.

-Mersmann, H.J. (1987) Am. J. Physiol. 252, E85-E95.

-Mersmann, H.J. and Hu, C.Y. (1987) J. Anim. Sci. 64:148-164.

-Mersmann, H.J. (1989) In: Animal Growth Regulation, D.R. Campion, G.J. Hausmann and R.J. Martin, eds, Plenum Press, New York, pp. 337-357.

-Milligan, G. (1988) Biochem. J. 255, 1-13.

-Munson, P.J. & Rodbard, D. (1980) Anal. Biochem. 107, 220-239.

-Muriege, P., Pergola, G., Berlan, M. & Lafontan, M. (1988) J. Lipid. Res. 29, 587-601.

-Naer, E.J. & Clampham (1988) Nature 333:129-134.

-Neve, K.A., McGonigle, P. & Molinoff, P. (1986) J. Pharmacol. Exp. Ther. 238, 46-53.

-Pecquery, R., Leneveu, M-C and Giudicelli, Y. (1988), Endocrinology 122, 2590-2596.

-Pecquery, R., Dieudonne, M-N., Leneveu, M-C and Giudicelli, Y. (1990), Endocrinology 126, 241-245.

-Prince, T.J., Hoffman, D.L., Brown, P.M. and Gillespie, J.R. (1987) J. Anim. Sci. 65:309.

-Rebourcet, M.C., Carpene, C. and Lavau, M. (1988) Biochem J. 252:679-682.

-Regan, J.W., Kobilka, T.S., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J. and Kobilka, B.K. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:6301-6305.

-Rodbell, M. (1964a) J. Biol. Chem. 239, 375-380.

-Rodbell, M. (1964b) J. Biol. Chem. 242, 5744-5750.

-Rotwell, N.J., Stock, M.J. and Sudera, D.K. (1985) Am. J. Physiol. 248:E397-402.

-Schwabe, U. (1984) Adenosine: Receptors and Modulation of Cell Function. 625:15-28.

-Seeman, P., Ulpian, C., Wregget, K.A. & Wells, J.W. (1984) J. Neurochem. 43, 221-235.

-Sibley, D.R. & Lefkowitz, R.J. (1985) Nature 317:124-129

-Singelton, W.L., Shannon, J.W. & Day, B.N., 1990, Michigan State University Pork Industry Handbook, Fact Sheet 19.433.01.

-Smith, C.K. II & Coutinho, L.L. (1990) Quantitative characterization of the selectivity of Ractopamine for the

β -adrenergic receptor subtypes. Manuscript in preparation.

-Stryer, L. and Bourne, H.R. (1986) Ann. Rev. Biol. 2:391-419.

-Touis, M., Berlan, M., Montastruc, P. & Lafontan, M. (1987) J. Pharmacol. Exp. Ther. 242, 1041-1049.

-Van Weerdon, E.J. (1987) In: J.P. Hanrahan (Ed.) Beta-Agonists and Their Effects on Animal Growth and Carcass quality. pp 152-162. Elsevier Applied Science, London.

-Vernon, R.G. (1980) Prog. Lipid. Res. 19:23.

-Watkin, D.M. (1979) Nutrition and the world food problem, M. Rechcigl, Jr. (ed) New York: S. Karger, pp. 20-62.

-Williams, L.T., Jarret, L. & Lefkowitz, R.J. (1976) J. Biol. Chem. 251, 3096-3104.

-Williams, P.E.V. (1987) Nutr. Abstr. and Rev. (Series B) 57:453-464.

-Wolf, J., Londos, C. & Cooper, D.M.F. (1981) Adv. Cyclic Nucleotide Res. 14, 163.

-Young, V.R. (1978) Adv, Exptl. Med. Biol. 97:85.

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