BETA-ADRENERGIC RECEPTOR SUBTYPES IN MYOCYTES, ARTERIES, AND ARTERIOLES OF THE PORCINE HEART IDENTIFIED WITH AUTORADIOGRAPHIC PROCEDURES

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Date

To the Dean of Graduate Studies and Research:

I am submitting herewith a thesis written by Doug B. Boliver entitled "BETA-ADRENERGIC RECEPTOR SUBTYPES IN MYOCYTES, ARTERIES, AND ARTERIOLES OF THE PORCINE HEART IDENTIFIED WITH AUTORADIOGRAPHIC PROCEDURES." I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Molecular Biology.

Lynda Uphouse
Major Professors Lynda Uphouse
and Kathy Muntz (deceased)

We have read this thesis and
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I dedicate my research in memory of Dr. Kathy Muntz for both her love of science and teaching.

ABSTRACT

BETA-ADRENERGIC RECEPTOR SUBTYPES IN MYOCYTES, ARTERIES, AND ARTERIOLES OF THE PORCINE HEART IDENTIFIED WITH AUTORADIOGRAPHIC PROCEDURES

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Beta (β) adrenergic receptors are localized in several tissue compartments of the heart, but it has been unclear as to the relative numbers contained in each compartment. The goal of the current study was to use receptor autoradiography to analyze β receptor agonist binding characteristics in different tissue compartments of pig heart including cardiac myocytes (predominately β_1), coronary arteries (predominately β_1) and coronary arterioles (predominately β_2). In addition, the relative numbers of β_1- and β_2-adrenergic receptors in these tissue compartments were estimated. Blocks of porcine anterior interventricular sulcus were frozen and tissue sections cut. Sections were incubated in [^{125}I]-(-)iodopindolol (^{125}I-IPIN) in the presence of 10^{-5} M of the β agonist, DL-propanolol HCl, or 5×10^{-7} M of the β_1 selective antagonist, CGP 20712A. For the validation experiments radioactivity was determined in the gamma counter and the sections demonstrated rapid binding, saturability and stereoselectivity. For autoradiography, emulsion-coated coverslips were

attached to the slides. After exposure, the slides were developed and stained, and grain density quantified. There was a greater density of β-adrenergic receptors, identified by autoradiographic grains, over arterioles as compared to the left anterior descending (LAD) artery. The LAD artery had only one sixth the density of β-adrenergic receptors seen in the arterioles, while the myocytes were equivalent to the arterioles.

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CHAPTER I

INTRODUCTION

Several neurotransmitters modulate the cardiovascular system in mammals. Of these, the adrenergic system has received particular emphasis and interest. The catecholamines, norepinephrine and epinephrine, activate a various responses by stimulating multiple adrenoceptors. The subclassification of adrenoceptors into alpha (α-) and beta (β-) and later into α_1-, α_2-, β_1- and β_2-subtypes was originally made on the basis of the rank orders of potency of structurally related catecholamines (Jones *et al.*, 1989). For cardiovascular modulation, β- adrenergic receptors appear to be the most important. β_1-adrenergic receptors are known to exist on cardiac myocytes and their activation of the cardiovascular system includes an increase in contractility (force), heart rate, the rate of conduction and blood flow (Jones *et al.*, 1989). β_2-adrenergic receptors, located on smooth muscle of blood vessels, intestines, lungs and bronchi, produce vasodilation (Jones *et al.*, 1989). The role of the β_2 subtype in cardiac myocytes has not been clearly defined, and it is not known if they play any role in contractility and heart rate.

The β-adrenergic receptors in cardiac myocytes have been reported to be predominately of the β_1 subtype, although the β_2 subtypes may be present in

smaller numbers depending upon the species. For instance, nearly one third to one quarter of the β-adrenergic receptors in rodents (Muntz *et al.*, 1994; Cui *et al.*, 1996) and canines (Muntz, 1992) are of the β_2 subtype. Similar results have been reported in humans (Minnemann *et al.*, 1979; Buxton *et al.*, 1985; Hedberg *et al.*, 1980; Stiles *et al.*, 1983; Brodde *et al.*,1983; Golf *et al.*,1985), whereas a 50:50 distribution in the β_1/β_2 ratio in humans has been reported by others (Heitz *et al.*, 1983; Robberecht *et al.*, 1983; Buxton *et al.*, 1987; Jones *et al.*, 1989; Summers *et al.*, 1989;Steinfath *et al.*, 1992). It has been reported that non-human primates (baboon's) have a β-adrenergic receptor subtype distribution where the β_1/β_2 ratio is 59/41 (Cui *et al., 1996)*, similar to that reported in human myocytes. The characterization of β-adrenergic receptors on cardiovascular tissues other than cardiac myocytes has not been investigated except in canine coronary arteries where a predominately β_2-adrenergic population (75-90%) was identified (Muntz, 1992).

Considerable cardiovascular research is performed in the porcine species because of the similarity it shares to the cardiovascular system of humans; yet, there are limited data on the β-adrenergic receptors of the porcine myocardium. It has been reported that of all the β receptors in porcine myocardium, a majority (85%) are of the β_1 subtype. The presence of β_2 receptors was not addressed (Stiles *et al.*,1983). In another study on homogenates from porcine myocardium, it was claimed that only β_1 receptors are present (Bjornerheim *et al.*, 1989). It is

therefore surprising that the β-receptor subtypes have not been characterized in the separate tissue types for the porcine myocardium.

In the following experiments, β-adrenergic receptor subtypes in myocytes, arteries, and arterioles of the pig heart were identified with autoradiographic procedures. Autoradiography offers advantages over homogenate binding because it offers better anatomical localization, and it allows assessment of receptors in several tissue types on the same slide. Since receptors in the different tissue compartments have been exposed to exactly the same incubation conditions, data from the different tissues are directly comparable. In addition, this technique avoids the problems of tissue homogenization, in which several cell types are mixed together. Most studies done on porcine myocardium have used the homogenization technique.

The objective of the current study was to investigate whether β-adrenergic receptors are localized in cardiac myocytes, coronary arteries and coronary arterioles. In addition, the relative numbers of β_1- and β_2-adrenergic receptors in these tissue compartments were estimated. It was hypothesized that the porcine myocardium would be similar to that of other mammalian species and contain the β_2 subtype in one quarter of the overall β receptors and that coronary arteries would contain predominately the β_2 subtype.

CHAPTER II

MATERIALS AND METHODS

Materials

Porcine hearts were obtained from Owens Country Sausage slaughterhouse (Richardson, Texas). All pigs used for this study were females and weighed at least 500 pounds. For binding, the β-adrenergic receptor antagonist [^{125}I]-(-) iodopindoldol (^{125}I-IPIN) (Dupont/New England Nuclear, Boston, Mass.) was used. The competitors that were utilized are the antagonist propranolol (Sigma Chemical Co., St. Louis, Mo.) and CGP 20712A (Sigma Chemical Co., St. Louis, Mo.). All other supplies were obtained from Fisher Scientific Supply Company (Houston, Texas).

Methods

Porcine hearts were transported as quickly as possible (approximately 1.0 hr) after the animal was killed. The dissected hearts were transported on ice to Texas Woman's University where a 6 x 1.5 cm block of the anterior interventricular sulcus (AIV) was removed. The AIV was separated into five blocks approximately 10 x 14 mm in length and 3 mm thick. Each block was mounted on a piece of cork and frozen in isopentane, cooled with liquid nitrogen. The blocks were stored frozen at -80° C until the day of sectioning. Sections

(10 μm) were cut at a temperature of -20° C, thaw-mounted on gelatin-coated slides, and stored frozen at -80° C.

For the validation experiments, sections were allowed to warm to room temperature prior to binding of the appropriate radioligand. Sections were preincubated in binding buffer [50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1mM EDTA, and 10^{-4} M ascorbic acid] for 10 min at room temperature to remove endogenous ligand. Sections were then incubated at room temperature in the binding buffer with ^{125}I-IPIN plus or minus the appropriate competitors. For initial validation, each section was scraped off the slide and placed, along with the end of the swab, in an appropriately labeled test tube and radioactivity was determined in the gamma counter for one min. All experiments were done in quadruplicate.

When autoradiographic procedures were used, sections were incubated in buffer for 75 min at room temperature with 150 pM ^{125}I-IPIN to obtain total binding. Incubation of ^{125}I-IPIN, in the presence of 10^{-5} M DL-propranolol HCl or 5×10^{-7} M of the β_1 selective antagonist CGP 20712A, were used to determine total β- adrenergic receptors and β_1-adrenergic receptors, respectively. This concentration of CGP 20712A blocks 98% of β_1-adrenergic receptors and only 1% of β_2-adrenergic receptors (Cooke *et al.*, 1993). After incubation, sections were washed for three 20 min periods in chilled buffer and then quickly dipped in ice-cold distilled water. Sections were dried with a stream of cold air and stored at 4° C in sealed boxes containing Drierite.

Binding sites on the sections were localized autoradiographically with the emulsion-coated method as described previously (Muntz *et al.*, 1984). Acid-washed coverslips were coated by dipping into the Kodak NTB2 nuclear emulsion (Eastman Kodak Co., Rochester, NY) (diluted 1:1 with distilled water) at 43° C and dried at room temperature for three hrs in the darkroom. A coverslip was glued (Superglue, Loctite) to one end of each slide opposite the radiolabeled section. The coverslips were attached to the slide using a binder clip and a plastic spacer to protect the coverslip from breakage. The slides were exposed in boxes with Drierite for four days; the binder clip was removed, and the coverslip was gently lifted; an alligator clip was placed between the coverslip and the slide. All slides were developed simultaneously in Kodak D19 (Eastman Kodak Co., Rochester, NY) (diluted 1:1 with distilled water) for 4 min at 19° C, fixed for 5 min, and rinsed in water for 20 min. After photographic processing, the sections were stained with hematoxylin and eosin, and mounted with Permount.

The tissues and overlying developed grains in the emulsion layer were examined by light microscopy. Autoradiographic grains were quantified on a computer-based image analysis system according to previously described methods (Muntz, 1992). Cardiac myocytes, coronary arterioles and the smooth muscle of the left anterior descending artery (LAD) were quantified. Ten different areas were quantified in each tissue compartment. Only arterioles that

were less than 100 μm in diameter and in which a lumen could be visualized were analyzed. Otherwise, the areas within each compartment were chosen randomly. For arterioles that did not fill the video screen, the computer allowed an outline of the arteriole to be analyzed to determine the area of the arteriole, as well as the number of grains over the arteriole. After quantification, the grains were normalized to a standard area for comparison with cardiac myocytes and LAD arteries. Two duplicate sections were analyzed in a similar manner, and the results were averaged. Total β-adrenergic receptors were defined as the binding of ^{125}I-IPIN minus the binding of ^{125}I-IPIN in the presence of 10^{-5} M DL- propranolol HCl. The number of β_2-adrenergic receptors in each compartment were calculated by determining the relative optical density in sections incubated with ^{125}I-IPIN minus those present when 5×10^{-7} M CGP 20712A (to block β_1-adrenergic receptors) (see above) was included in the incubation. The number of β_1-adrenergic receptors were calculated by subtracting the number of β_2-adrenergic receptors from the total β-adrenergic receptors.

CHAPTER III

RESULTS

The evaluation of specific binding for ^{125}I-ICYP and ^{125}I-IPIN under different washing conditions revealed that ^{125}I-ICYP was low (58% and 64%), while ^{125}I-IPIN was much higher (86% and 90%) (figure 1). The reduced binding of ^{125}I-ICYP may result from non-β-adrenergic binding as has been described by Bjornerheim *et al.* (1991). For neither ligand was there a difference between specific binding under the various washing conditions. Therefore, ^{125}I-IPIN was selected as the radiolabeled ligand for the following experiments and three 20 min washes were used.

Both total and specific binding of ^{125}I-IPIN increased with increasing incubation time (respectively $F_{5,21} = 45.2$ and 34.6, $p \leq .0001$) (figure 2). Binding increased significantly during the first 30 min of incubation. By 30 min, there was a significant increase in binding relative to the zero time point (Tukey's test, $q_{21,6} = 11.8$, $p \leq .001$); there were no significant changes in binding from 30 min to 90 min (Tukey's test, all $q < 4.37$, $p > .05$). In the following experiments, incubations were carried out for 75 min, where a plateau in specific binding had clearly occurred.

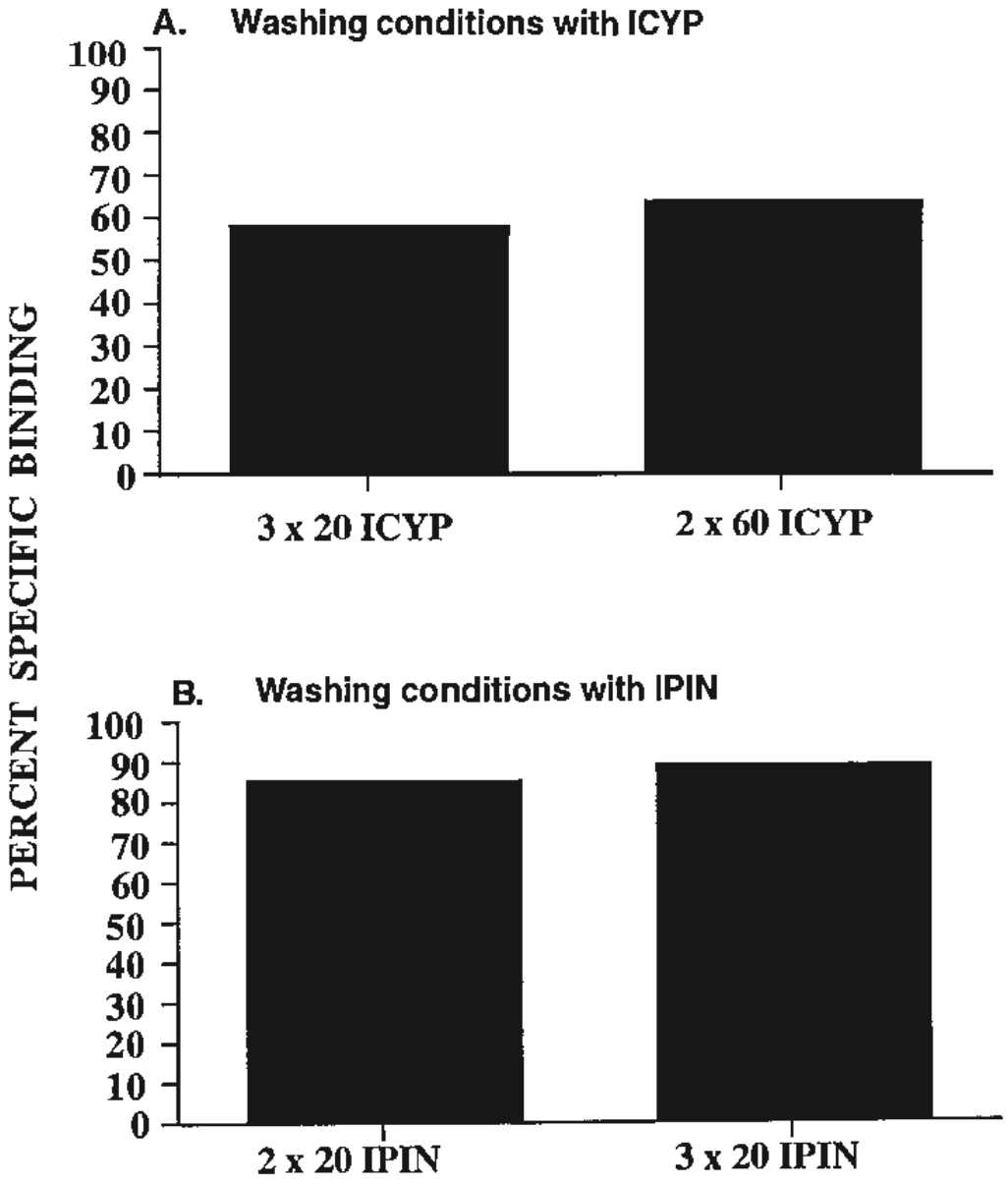
A. Washing conditions with ICYP
100
90
80
70
60
50
40
30
20
10
0
3 x 20 ICYP
2 x 60 ICYP
PERCENT SPECIFIC BINDING
B. Washing conditions with IPIN
100
90
80
70
60
50
40
30
20
10
0
2 x 20 IPIN
3 x 20 IPIN

FIGURE 1: Percent specific binding under different washing conditions.

Sections from porcine heart were incubated with either ^{125}I-pindolol (IPIN) or ^{125}I-cyanopindolol (ICYP) as described in the methods. After incubation, tissue was placed for 2 or 3 washes in washing buffer (20 min for IPIN and 20 and 60 min for ICYP). Data are percent specific binding under the different washing conditions. For ICYP, one experiment under each condition is represented. For IPIN, data for 3 x 20 min represents one experiment while the 2 x 20 min data are the average of 2 independent experiments.

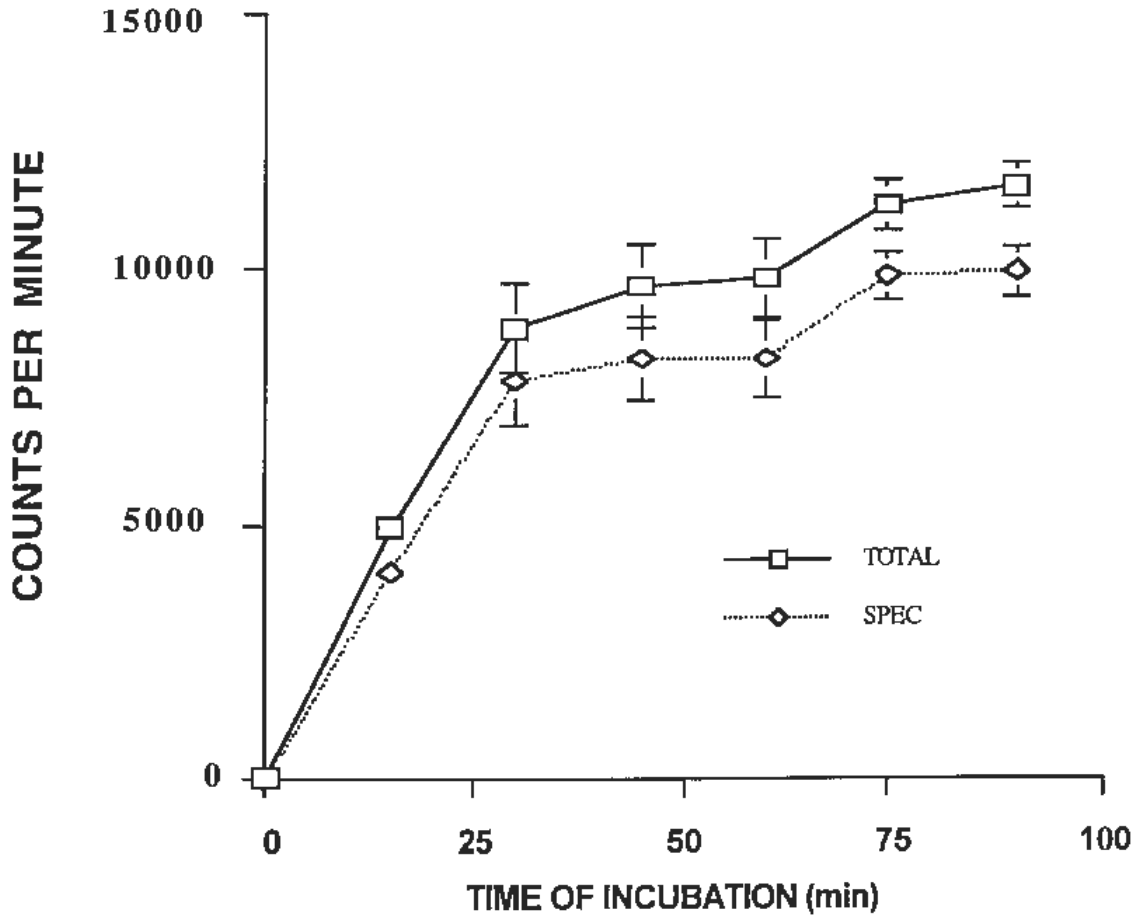

FIGURE 2: Duration of incubation and ^{125}I-pindolol binding.

Sections from porcine heart were incubated with ^{125}I-pindolol with (SPEC) or without (TOTAL) propranolol as described in the methods. Incubation continued up to 90 min. Data are the mean ±S.E. for 3-5 experiments at each incubation time except for 15 min where 2 replications were performed.

There was more competition for ^{125}I-IPIN binding with increasing concentration of either D- or L-isoproterenol (see figure 3). Stereoselectivity was evidenced by the greater potency of L-isoproterenol as a competitor relative to the D-isoproterenol.

Histology of three cardiac tissues (myocytes, arterioles and artery) are shown on the left; grains over the same tissue sample are shown on the right (see figure 4). Autoradiographic grains appear to be distributed uniformly over cardiac myocytes. Grains appeared to be more concentrated in arterioles than in myocytes and they appeared to be the least concentrated over the LAD artery.

There were significant tissue differences in total (β_1 and β_2) receptors ($F_{2,14} = 32.49$, $p \leq .0001$) as well as in the density of β_1 ($F_{2,14} = 11.84$, $p \leq .001$) and β_2 ($F_{2,14} = 104.73$, $p \leq .0001$) receptors (figure 5). Arterioles had significantly more total β adrenergic receptors than either myocytes or arteries ($q_{14,3} \geq 3.70$, $p \leq .05$), while myocytes had significantly more than arteries ($q_{14,3} \geq 3.70$, $p \leq .05$).

Arterioles contained significantly more β_2 receptors than either myocytes or arteries ($q_{14,3} \geq 3.70$, $p \leq .05$), while myocytes and arteries did not differ. In contrast, β_1 receptors were highest in myocytes (relative to arteries and arterioles; $q_{14,3} \geq 3.70$, $p \leq .05$), while arteries and arterioles were not different.

Therefore, not only the density of individual subtypes, but the distribution of subtypes within tissue compartments, characterized the three tissues examined. For the percent of $\beta_1 + \beta_2$, respectively, there were significant tissue differences ($F_{2,14} = 25.37$ and 25.37, $p \leq .0001$) (figure 6). Myocytes and arteries contained predominately the β_1 subtype (respectively 78% and 84%) while arterioles contained primarily the β_2 subtype (87%). The percent of β_1 in arteries and myocytes was significantly greater than in arterioles ($q_{14,3} \geq 3.70$, $p \leq .05$). The percent β_2, in contrast, was greatest in arterioles (significantly different from both arteries and myocytes, $q_{14,3} \geq 3.70$, $p \leq .05$) while arteries did not differ from myocytes ($p \geq .05$).

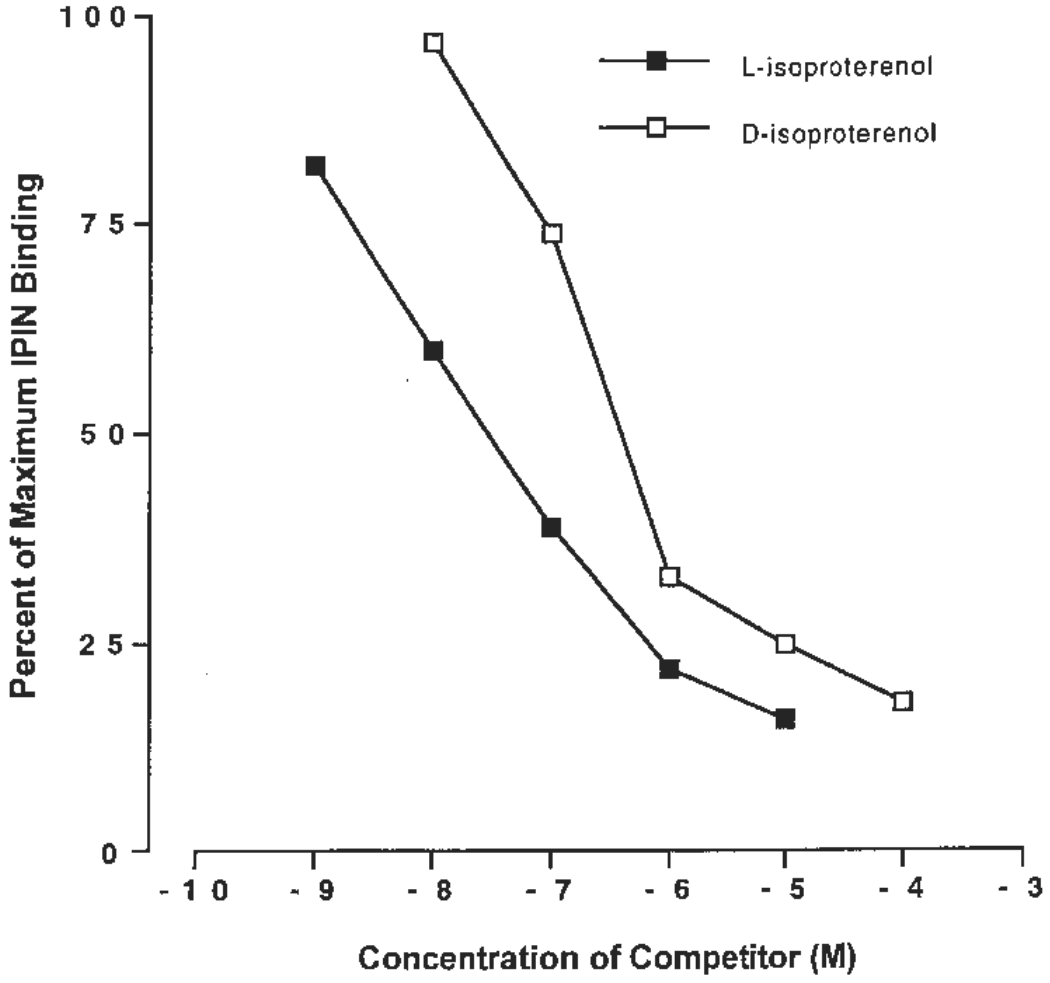

FIGURE 3: Comparison of D-and L-isoproterenol as competitors for ^{125}I-pindolol binding.

Sections from porcine heart were incubated with ^{125}I-pindolol as described in the methods. Separate sections were incubated with either D- or L-isoproterenol as competitor. Data are the percent of ^{125}I-pindolol bound without competitor (percent of maximum binding) that remained in the presence of competitor at the indicated concentrations.

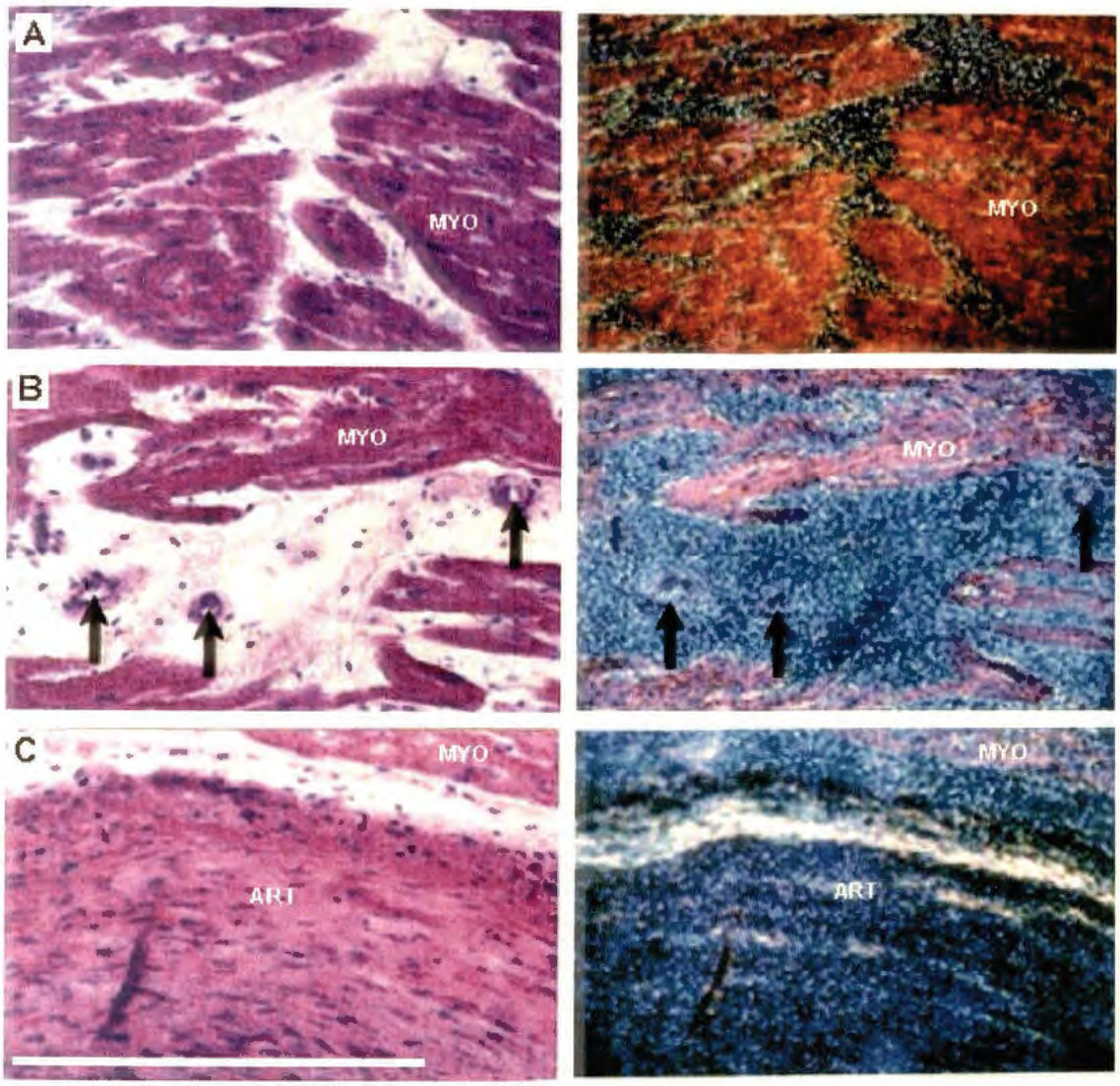

FIGURE 4: Representative photographs of tissue sections.

On the left portion of the figure, hematoxylin and eosin stained examples of cardiac myocytes (A) (MYO indicates myocytes), arterioles (B) (arrows indicate arterioles), and artery (C) (ART indicates artery) are shown. Autoradiographic grains from the same sections are shown to the right of each tissue photograph. Scale bar, 427 μm.

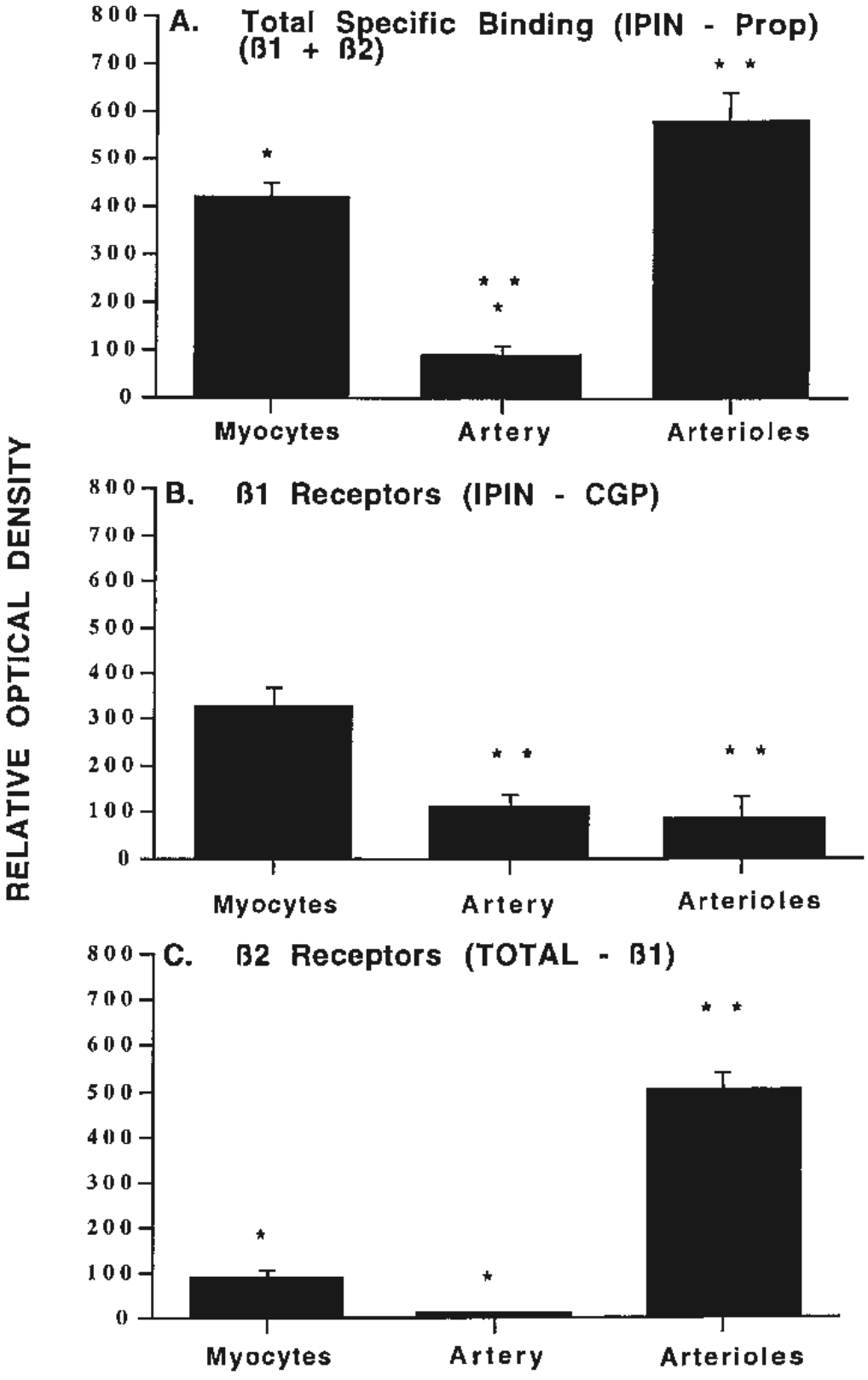
A. Total Specific Binding (IPIN - Prop) (ß1 + ß2)
B. ß1 Receptors (IPIN - CGP)
C. ß2 Receptors (TOTAL - ß1)
RELATIVE OPTICAL DENSITY
800
700
600
500
400
300
200
100
0
Myocytes
Artery
Arterioles

FIGURE 5: Relative optical density under different binding conditions.

Specific binding (as indicated by relative optical density) of ^{125}I-pindolol to total, β_1 or β_2 receptors are shown in the figure. Total specific binding (A) reflects the binding of ^{125}I-pindolol in the presence of propranolol. β_1 receptors (B) represent binding in the presence of CPG. β_2 receptors (C) were determined by subtraction of β_1 from the total. Data are the mean ±S.E. for 5-6 experiments for each condition. Single asterisks indicate a significant difference from arterioles. Double asterisks indicate a significant difference from myocytes.

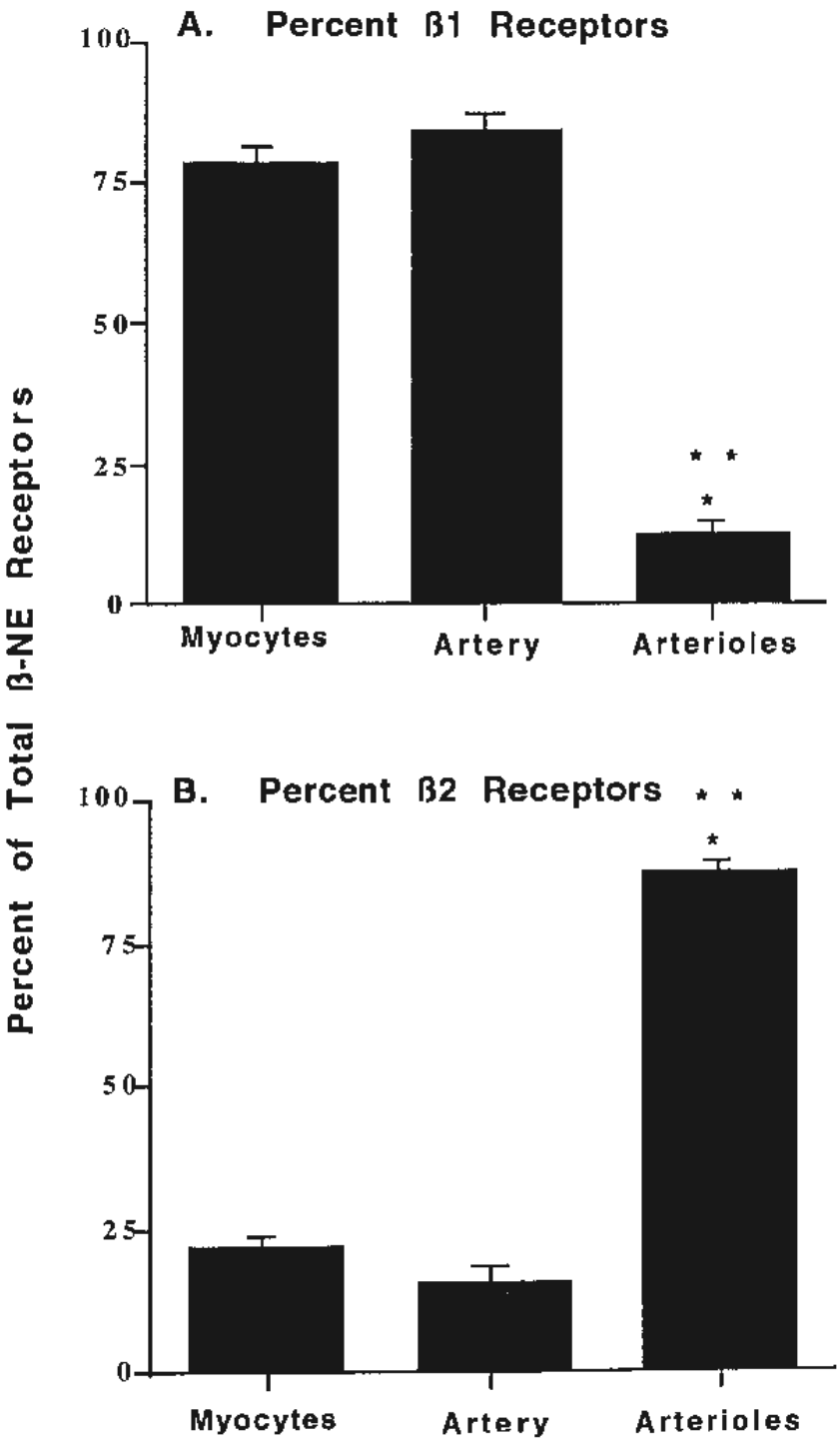
A. Percent ß1 Receptors
100
75
50
25
0
* *
*
Myocytes
Artery
Arterioles
Percent of Total ß-NE Receptors
B. Percent ß2 Receptors * *
*
100
75
50
25
0
Myocytes
Artery
Arterioles

FIGURE 6: Relative distribution of β_1 and β_2 receptors in cardiac tissue.

For each tissue, the specific binding of ^{125}I-pindolol in the presence of propranolol was taken as 100% of β-adrenergic receptors. β_1 and β_2 receptors, present in each of the cardiac tissues, were expressed as a percent of this total. The percent of β_1 receptors are shown in figure 6A; percent of β_2 receptors are shown in figure 6B. Single asterisks indicate a significant difference from artery. Double asterisks indicate a significant difference from myocytes.

CHAPTER IV

DISCUSSION

The objective of these studies was to identify the relative distribution of β_1 and β_2 receptors in porcine myocardium. This is the first experiment in which these receptor subtypes have been characterized in the separate tissue types of porcine myocardium. Evaluation of the individual tissues is important since the porcine heart is a primary animal model that is used to better understand the human heart and should facilitate our understanding of the role β-adrenergic receptor subtypes play in cardiac function. Autoradiographic studies were performed to characterize the individual tissues. With autoradiography, it was possible to identify grain density over cardiac myocytes, blood vessels, and connective tissue. The resolution of the system was not adequate for localization of receptor subtypes to individual cells, but it was possible to exclude connective tissue and large nerves so that myocytes could be analyzed separately.

The most notable result of this study was the distribution of β-adrenergic receptors over blood vessels in the porcine heart (see figure 5). There was a greater density of β-adrenergic receptors, identified by autoradiographic grains, over arterioles as compared to the LAD artery. Such a high density of receptors

may be related to the high concentration of adrenergic nerve innervation that is associated with coronary arterioles (Muntz *et al., 1984*). This would fit with the physiological role that resistance vessels have in controlling blood flow. The arterioles studied are considered to be resistance vessels since they are less than 90 microns in diameter (Cooke *et al.*, 1993) and because changes in their diameter affect the resistance to blood flow (Martini, 1995). The LAD artery had only one sixth the density of β-adrenergic receptors seen in the arterioles, while the myocytes were slightly less than arterioles. There was more variability in the data from the LAD artery, possibly because there are fewer receptors in this tissue. We can argue against a blood vessel-specific difference in β-adrenergic receptor binding because the binding pattern in the LAD artery was more like that in myocytes than that in arterioles, suggesting that the binding pattern observed in arterioles was not necessarily a general pattern for all blood vessels.

These studies are also significant in their demonstration of tissue differences in the relative proportion of β_1 and β_2 receptors. In the myocytes, there was a predominance of β_1 receptors (78%) (see figure 6). The majority of β-adrenergic receptors in arterioles were of the β_2 type (87%), while the LAD artery had a predominance of β_1 receptors (84%). β_1-adrenergic receptors activate the cardiovascular system by increasing contractility (force), heart rate, the rate of conduction and blood flow, while β_2 receptors produce vasodilation

(Jones *et al.*, 1989). These physiological responses are mediated by the β-adrenergic receptors activating adenylate cyclase via the stimulatory guanine nucleotide regulatory protein (Cooke *et al.*, 1993). Through this study we were able to deduce that the porcine myocardium does contain the β_2 subtype, but the physiological role of the β_2 subtype in the myocardium is not clearly defined. It is possible that the β_2 subtype is involved with contractility and heart rate, but that it mediates an increased heart rate through a different mechanism.

The technique of in vitro labeling of tissue sections offers advantages for studying receptors in the heart and other organs by allowing quantification of nonspecific and total binding in tissue sections while avoiding the problems of tissue homogenization. It allows us the ability to simultaneously study several systems in the same tissue, and it does not rely on diffusion of ligand or delivery via the circulatory system. The fact that there is no concern for diffusion of the ligand is particularly significant in analyzing relative differences in binding to different tissue components, such as blood vessels and cardiac myocytes.

Although ^{125}I-ICYP has been used by several investigators for characterization of β receptors in various tissues, ^{125}I-IPIN was found to be preferable for the current experiments. ^{125}I-IPIN had a lower nonspecific binding than ^{125}I-ICYP in porcine heart sections. Engel *et al.* (1981) and Brodde *et al.* (1981) were the first to describe the use of the β-adrenergic receptor antagonist ^{125}I-ICYP as a radioligand for β receptors. Since the introduction of ^{125}I-ICYP, it

has been regarded as a highly specific ligand for β receptors. However, previous investigators have suggested that non-β- adrenergic binding of radioligands may be explained by the lipid solubility of the competitor as indicated by Bjornerheim *et al.* (1991). In their results, there was an apparent lack of the ability of a hydrophilic agonist and antagonist to compete with ^{125}I-ICYP for non-β- adrenergic sites, while all the lipophilic antagonists were capable of such competition. They did have one exception to this finding which was the hydrophilic agonist terbutaline. Terbutaline was capable of complete competition with ^{125}I-ICYP for binding to non-β- adrenergic sites. They also found that the non-β-adrenergic sites were protease sensitive and showed thermostability similar to β-adrenergic receptors. Bjornerheim *et al.* (1991) concluded that ^{125}I-ICYP and several β receptor antagonists interact specifically with receptor-like proteins other than β receptors. They also found considerable interspecies differences in the levels of these myocardial receptor-like proteins. Of particular significance to the current project, pigs were noted as having an especially high density of these receptor-like proteins. According to their results, rat hearts had the least binding to non-β-adrenergic receptor-like proteins, followed by humans and finally pigs. A possible candidate for these receptor-like proteins to which ^{125}I-ICYP binds are members of the serotoninergic receptor families (Bjornerheim *et al.,* 1991). However, ^{125}I-IPIN is structurally similar to ^{125}I-ICYP, and both IPIN and propanolol have a relatively high affinity

for serotonin receptors (Pazos *et al.*, 1985), so it is unlikely that binding to serotoninergic receptors totally accounts for the difference in nonspecific binding of IPIN and ICYP.

In conclusion, by using an autoradiographic technique, we were able to determine the distribution of β-adrenergic receptor binding over porcine cardiac myocytes and myocardial blood vessels. Although the relative ratio of β_1- and β_2-adrenergic receptors in porcine myocardium was found to be similar to that of other mammals studied, some investigators studying porcine myocardium have found minimal or no β_2-adrenergic receptors (Bjornerheim *et al.*, 1989). These investigators were using the homogenization technique for their analysis and may have missed the β_2 receptors because of their relatively low numbers. These present observations reinforce the value of the porcine heart as a model to better understand the human heart and the role of β-adrenergic receptor subtypes in cardiac function.

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