α_2-Adrenergic receptor binding in canine Purkinje fibers

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Abstract Autoradiograms were developed from free running strands of Purkinje fibers and from left ventricular myocardium of dog hearts exposed to [7-*methoxy*-^{3}H]prazosin or [*O-methyl*-^{3}H]yohimbine in the presence or absence of excessive non-radioactive ligands. Both Purkinje fiber and ventricular myocardium showed high density, tissue-specific binding to [^{3}H]prazosin. In contrast, high density, tissue-specific binding to [^{3}H]yohimbine was present in Purkinje fibers but not in ventricular myocardium. Membrane fractions showed high affinity, saturable, and displaceable binding with [^{3}H]yohimbine in preparations from canine cardiac Purkinje fibers but not those from canine cardiac ventricular myocardium. Scatchard analysis of the canine Purkinje membrane α_2-adrenergic receptor binding showed a B_{max} of 54.9 fmol/mg protein with a K_d of 6.25 nM. These results confirm the electrophysiological findings that post-junctional α_2-adrenergic receptors are present in Purkinje fibers.

Key words: Purkinje fibers; α_2-Adrenergic receptors; α_1-Adrenergic receptors; Yohimbine; Prazosin; Autoradiography; Receptor binding

1. Introduction

α-Adrenergic receptors are important in the regulation of cardiac functions [1,2]. Based on physiological and radioligand binding studies, the postjunctional α-adrenergic receptors within the heart are believed to be exclusively of the α_1 subtype [3–5]. However, the existence of α_2-adrenergic receptors in the His-Purkinje system has not been thoroughly examined. Recently, we have demonstrated that α_2-adrenergic stimulation results in prolongation of action potential duration in isolated canine Purkinje fibers and such effects are mediated through a pertussis toxin-sensitive G protein [6]. Such α_2-mediated electrophysiological effects were not identified in the ventricular myocardium adjacent to the Purkinje fibers. In this study, we used autoradiographic and radioligand binding techniques, to confirm the presence of α_2-adrenergic receptors in isolated canine cardiac Purkinje fibers and its absence in canine ventricular myocardium.

2. Materials and methods

2.1. Animals

Adult Mongrel dogs of either sex, weighing 18–24 kg each, were anesthetized with 100 mg/kg chloralose i.v. The hearts were excised and placed in phosphate buffered saline (PBS) that contained (in mM): NaCl 136, KCl 2.7, KH_2PO_4 1.4, NaH_2PO_4 15.2, $CaCl_2$ 1.0, $MgCl_2$ 0.8, pH 7.4 and equilibrated with 100% O_2. Free running strands of Purkinje fibers were removed from the right and left ventricles and used for the studies. Samples of myocardium from the left ventricles were also studied and compared with the results from Purkinje fibers.

2.2. Autoradiographic studies

Binding of radioligands to isolated Purkinje fibers and ventricular myocardium was performed at 4°C using a previously described method with modification [7]. Freshly isolated samples of Purkinje fibers and ventricular myocardium were placed in 5 ml of PBS containing either 9.2 nM [7-*methoxy*-^{3}H]prazosin or 8.0 nM [*O-methyl*-^{3}H]yohimbine (1.54×10^6 dpm/ml for both). The concentrations of radioligands used should saturate the α receptors [7–9]. To determine non-specific binding, separate samples of Purkinje and ventricle were placed in 5 ml of PBS containing 9.2 nM [^{3}H]prazosin with 100 μM prazosin, or 8.0 nM [^{3}H]yohimbine with 100 μM yohimbine. Specificity of radioligand binding was assessed by the radioactivity displaced by non-radioactive ligands. Samples of Purkinje and ventricle in PBS without radioligands served as controls.

All samples were incubated for 90 min at 4°C followed by six rinses with 10 ml aliquots of PBS containing 1% bovine serum albumin. The samples were then fixed with 2.5% glutaraldehyde and 0.1 M sodium cacodylate solution at 4°C. Serial sections were cut (5 μm thickness) on a microtome and mounted on microscope slides. The slides were then dipped in nuclear emulsion (Kodak NTB2) and placed in light-tight boxes stored at 4°C for six to fifteen weeks. After the designated period of exposure, autoradiographic images were developed in D19 solution (Kodak, diluted 1:2 in distilled water) and fixed in 30% sodium thiosulfate.

2.3. Analysis of autoradiograms

Analysis of autoradiograms was performed by using a laser-scanning confocal image analysis system (Bio-Rad MRC-600) in the reflected light mode [10]. Hard copies of high fidelity photomicrographs were generated using a high resolution color video printer (Sony UP-5000) linked directly to the microscope so that loss of definition was minimal. Autoradiographic grains were distinguished on the basis of color (white grains against a black background) and quantified based on the number of grains per unit area (100 μm × 100 μm). For each autoradiogram, the background signal was defined as the density of autoradiographic grains in areas without tissue, and this number was subtracted from the density of autoradiographic grains in areas with tissue to obtain the tissue-associated signal. Total radioligand binding was defined as the tissue-associated density of autoradiograms labeled with [^{3}H]prazosin or [^{3}H]yohimbine. Non-specific binding was defined as the density of tissue-associated grains on autoradiograms labeled with [^{3}H]prazosin plus 100 μM prazosin or [^{3}H]yohimbine plus 100 μM yohimbine. Specific binding values were then calculated by subtracting non-specific binding values from total binding values.

2.4. Radioligand binding studies

Free running cardiac Purkinje fibers from 20 dogs were collected over a 20 week period and were frozen immediately under liquid nitrogen and stored at −70°C. Canine ventricular myocardium from the mid-lateral free wall of the left ventricle was also collected in similar manner. The Purkinje fibers and ventricular myocardium were homogenized and the membrane fractions were prepared by differential centrifugation as previously described [11]. Membrane protein assays were performed as described by Bradford [12].

α_2-Adrenergic receptor binding in the Purkinje and ventricular myocardial membrane fractions was performed using modifications of previously published methods [13,14]. In brief, 70 μg of membrane proteins were incubated with [^{3}H]yohimbine (88.7 Ci/mmol) at 0, 0.25, 0.5, 1.0, 2.5, and 10.0 nM in the presence or absence of 20 μM of nonradioactive yohimbine in a total volume of 140 μl with a buffer containing 50 mM

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Tris, 1 mM EDTA, 1 mM DTT, pH 7.5 at 23 °C for 90 min. Free and bound ligands were separated by passing through Whatman GF/C filters followed by three washings with 10 ml aliquots of ice-cold buffer containing 50 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.5 under a constant vacuum. The filters were dried, then placed in a vial containing 10 ml of scintillation fluid and the radioactivity counted in a scintillation counter. Specific α_2-adrenergic receptor binding was measured by the amount of membrane-associated radioactivity displaced by competition with excessive amounts of unlabeled yohimbine. Analysis of α_2-adrenergic receptor bindings was performed using Scatchard plot [15].

2.5. Materials

Prazosin and yohimbine were obtained from Sigma Chemical Co., St. Louis, MO. [7-*methoxy*-^{3}H]prazosin (specific activity 76.2 Ci/mmol) and [*O-methyl*-^{3}H]yohimbine (specific activity 88.7 Ci/mmol) were purchased from New England Nuclear, Boston, MA.

2.6. Statistical analysis

Autoradiographic grain densities were analyzed in four different preparations. Data were expressed as mean ± S.E.M. and significance was determined using Student's *t*-test at $P < 0.05$. Results for the radioligand binding studies were expressed as the mean of duplicates.

3. Results

3.1. Autoradiographic studies

Autoradiograms were developed after 6 to 15 weeks of radioligand exposure to nuclear emulsion. Examination of sections after serial developments revealed that optimal images were obtained after 11 weeks of exposure. Fig. 1 represents the results of a typical experiment in Purkinje fibers that showed the presence of specific α_2-adrenergic receptor binding. Unlabeled controls demonstrated low density of autoradiographic grains scattered indiscriminately throughout areas with or without tissue (Fig. 1B). Exposure to [^{3}H]yohimbine produced high density of tissue-associated grains (Fig. 1C). The tissue-associated radioligand binding was displaced by non-radioactive yohimbine, suggesting that the binding manifested by autoradiographic grains was α_2-adrenergic receptor specific (Fig. 1D).

Binding of the α_1-adrenergic specific radioligand, [^{3}H]prazosin, to Purkinje fibers was similar to that of [^{3}H]yohimbine (Fig. 2). Again, control autoradiographic images showed scarce and scattered grains (Fig. 2B). Incubation with [^{3}H]prazosin was associated with high tissue-specific grain densities that were displaced by unlabeled prazosin (Fig. 2C and D).

The patterns of α-adrenergic receptor subtype binding in ventricular myocardium were very different from those in Purkinje fibers (Fig. 3). While myocardial tissue demonstrated high density tissue-specific binding to the α_1-adrenergic radioligand (Fig. 3C), tissue-specific binding to the α_2-adrenergic radioligand was absent (Fig. 3D).

The results of quantitative analysis of tissue-specific autoradiographic grain densities from four different preparations are summarized in Table 1. The density of α_1-adrenergic receptors is comparable between Purkinje fiber and ventricular myocardium. The density of α_2-adrenergic receptors in Purkinje is also

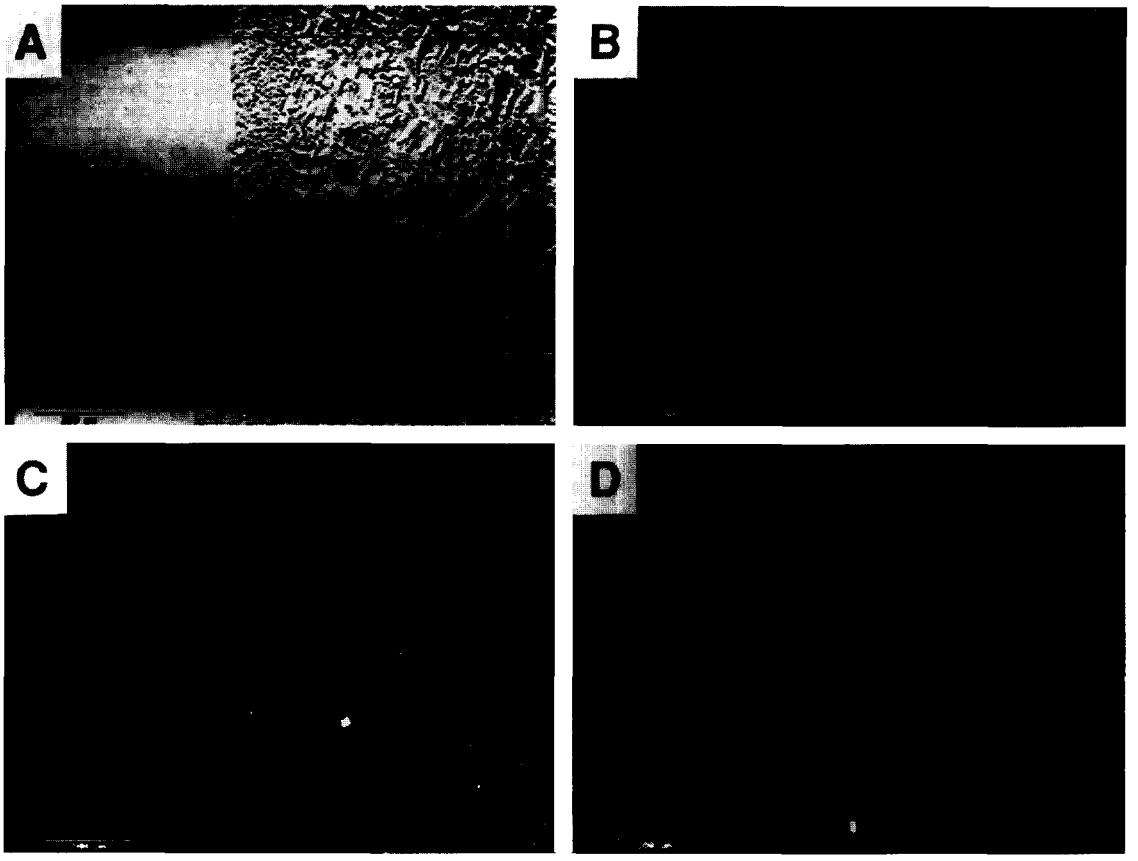

Fig. 1. Photomicrographs of canine Purkinje fibers after 11 weeks of exposure to [^{3}H]yohimbine demonstrating the presence of specific α_2-adrenergic receptor binding. Top left (A): Transmitted light mode showing section of tissue being analyzed. Top right (B): Reflected light mode of similar section without radiolabel. Bottom left (C): Reflected light mode of same section as in (A) labeled with [^{3}H]yohimbine. Bottom right (D): Reflected light mode of similar section labeled with [^{3}H]yohimbine + 100 μM yohimbine. Autoradiograms show the presence of specific α_2-adrenergic receptor binding in Purkinje fiber. Bar = 100 μm.

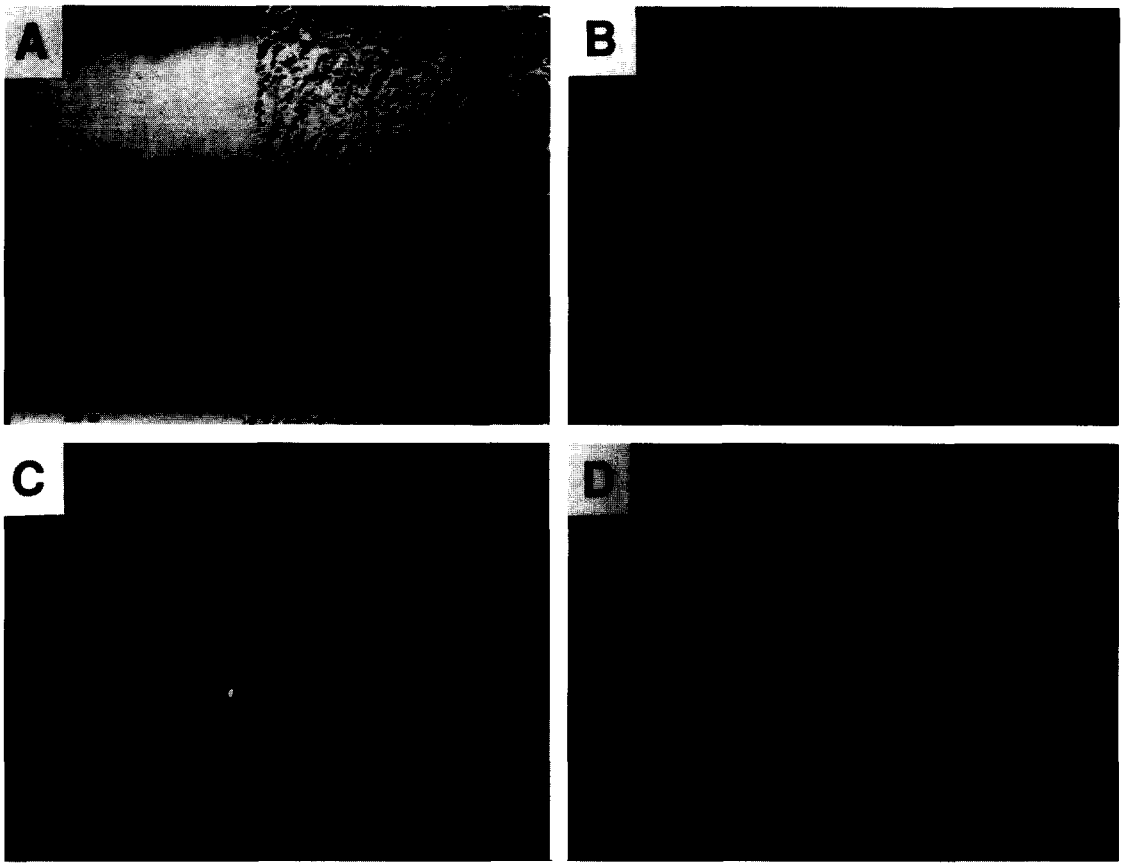

Fig. 2. Photomicrograph of canine Purkinje fiber after 11 weeks of exposure to [^{3}H]prazosin demonstrating the presence of specific α_1-adrenergic receptor binding. Top left (A): Transmitted light mode showing section of tissue being examined. Top right (B): Reflected light mode of similar section without radiolabel. Bottom left (C): Reflected light mode of the same section as in (A) labeled with [^{3}H]prazosin. Bottom right (D): Reflected light mode of similar section labeled with [^{3}H]prazosin + 100 μM prazosin. Autoradiograms show the presence of specific α_1-adrenergic receptor binding in Purkinje fiber. Bar = 100 μm.

not significantly different from those of α_1-adrenergic receptors in Purkinje or in myocardium. In contrast, the autoradiographic grain densities from α_2-adrenergic radioligand binding in ventricular myocardium were at least one-thousandfold less than that detected in Purkinje fibers and were not significantly different from background. These results are in good agreement with electrophysiological measurements that action potentials are modulated in both Purkinje and myocardium by α_1-adrenergic stimulation, but action potentials are modulated only in Purkinje fibers and not in myocardium by α_2-adrenergic stimulation [6].

3.2. Radioligand binding studies

The binding of [^{3}H]yohimbine to canine cardiac Purkinje membranes showed high affinity, saturable binding that was displaceable by an excess of unlabeled yohimbine (Fig. 4A). In contrast, this specific α_2-adrenergic receptor binding was absent in membranes prepared from canine ventricular myocardium. Scatchard analysis showed a linear fit suggestive of a single binding site with a K_d of 6.25 nM and a B_{max} of 54.9 fmol/mg protein. These values are similar to those obtained in other cell types [2,7,13].

4. Discussion

This study demonstrated that α_2-adrenergic receptors are present in canine cardiac Purkinje fibers. The binding affinity and receptor density in the canine Purkinje fibers is similar to those in other cell types [2] such as prostate [7] and coronary arterial smooth muscle [13]. This high affinity α_2-adrenergic receptor specific binding is absent in ventricular myocardium. These results directly support our previous observation that activation of postjunctional α_2-adrenergic receptors results in lengthening of action potential durations and suppression of β-adrenergic induced afterdepolarizations in isolated canine cardiac Purkinje fibers but not in ventricular myocardium [6]. These results are also in agreement with the recent report that α_2-adrenergic stimulation prolongs the Purkinje relative refractory period but not ventricular refractory period in intact dog hearts [16]. Our results explain why previous studies using radioligand binding and functional approaches failed to detect the presence of post-junctional α_2-adrenergic receptors in the heart because the Purkinje fibers were not examined separately and carefully [2–5].

Autoradiographic techniques have been successfully em-

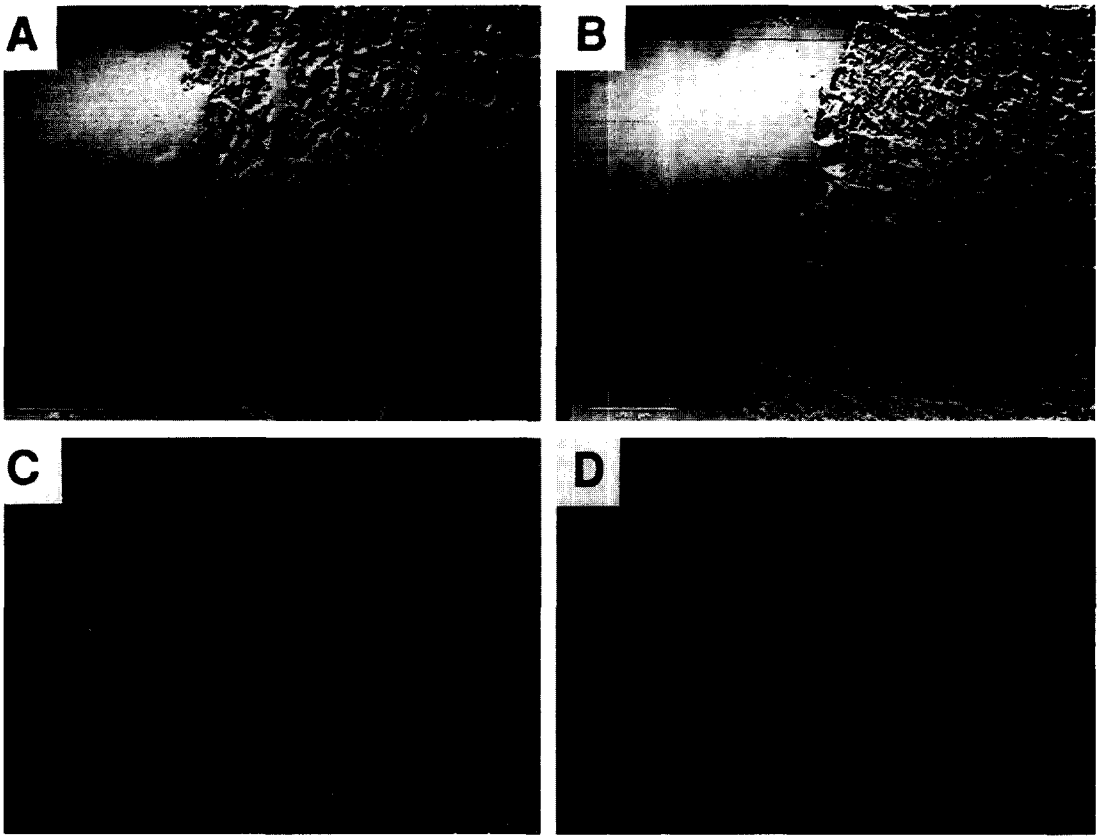

Fig. 3. Photomicrographs of canine left ventricular myocardium after 11 weeks of exposure to [^{3}H]prazosin and [^{3}H]yohimbine. Top left (A): Transmitted light mode of section in (C). Top right (B): Transmitted light mode of section in (D). Bottom left (C): Reflected light mode of section in (A) labeled with [^{3}H]prazosin. Bottom right (D): Reflected light mode of section in (B) labeled with [^{3}H]yohimbine. Autoradiograms show high density tissue-specific α_1- but no α_2-adrenergic receptor binding in ventricular myocardium. Bar = 100 μm.

ployed to examine the presence of β-adrenergic receptor subtypes in the cardiac conduction system [17]. Both β_1- and β_2-adrenergic receptor subtypes were found to be present in the canine sinoatrial and atrioventricular nodes. However, the relative density of the β_1 receptors was about four times greater than that of the β_2-receptors in those regions. Thus, the finding that two receptor subtypes co-exist in the conduction tissues of the heart is not unprecedented. Nevertheless, our findings suggest that the relative densities of the α_1 and α_2 receptor subtypes are similar in the Purkinje fibers.

It is unlikely that the radioligand binding in Purkinje fibers is from prejunctional α_2-adrenergic receptors. Anatomic studies have demonstrated that Purkinje cells in the 'terminal regions of the false tendons' show no innervation at all [18]. These 'false tendons' are the free running strands of Purkinje fibers that we used exclusively in this study. Histological examination of the sections analyzed showed no evidence of nerve endings or elements. The high density, tissue-specific autoradiographic grains are apparently associated with Purkinje cells. In contrast, [^{3}H]yohimbine binding in ventricular myocardium, which is known to be relatively richly innervated, would be expected to show higher cross-reactivity with prejunctional α_2 receptors. Our results to the contrary showed that ventricular tissue was virtually devoid of α_2 receptors and prejunctional α_2 receptors were hardly detectable at all. The finding that the density of α_2 receptors in Purkinje is at least one-thousandfold higher than that in myocardium represents, therefore, postjunctional α_2-adrenergic receptors in Purkinje cells.

The fact that postjunctional α_2-adrenergic receptors are present in Purkinje fibers but not in ventricular myocardium is intriguing. Purkinje fibers are thought to have the same embryonic origin as that of cardiac muscle fibers [19,20]. There is, however, recent evidence that the His-Purkinje system may be derived from neural crest cells [21]. Such a hypothesis of the neural crest origin of Purkinje fibers not only may explain the presence of α_2 receptors on Purkinje cells, but is also consistent with the primary function of the His-Purkinje system which is to conduct electrical impulses rather than to perform the actual work of cardiac contraction.

Table 1
Quantitative analysis of autoradiographic grain densities (grains/unit area of 100 μm × 100 μm) for [7-*methoxy*-^{3}H]prazosin binding and for [*O-methyl*-^{3}H]yohimbine binding in isolated canine Purkinje fibers and left ventricular myocardium

	Total binding (grains/unit area)	Specific binding (grains/unit area)
Purkinje + [^{3}H]prazosin	23.3 ± 5.4	22.7 ± 5.2
Purkinje + [^{3}H]yohimbine	24.6 ± 3.2	23.5 ± 3.1
Ventricle + [^{3}H]prazosin	19.8 ± 0.5	18.5 ± 1.0
Ventricle + [^{3}H]yohimbine	0.08 ± 0.04*	0.02 ± 0.02*

Data represent mean ± S.E.M., $n = 4$.
*Represents $P < 0.05$ compared with Purkinje + [^{3}H]yohimbine.

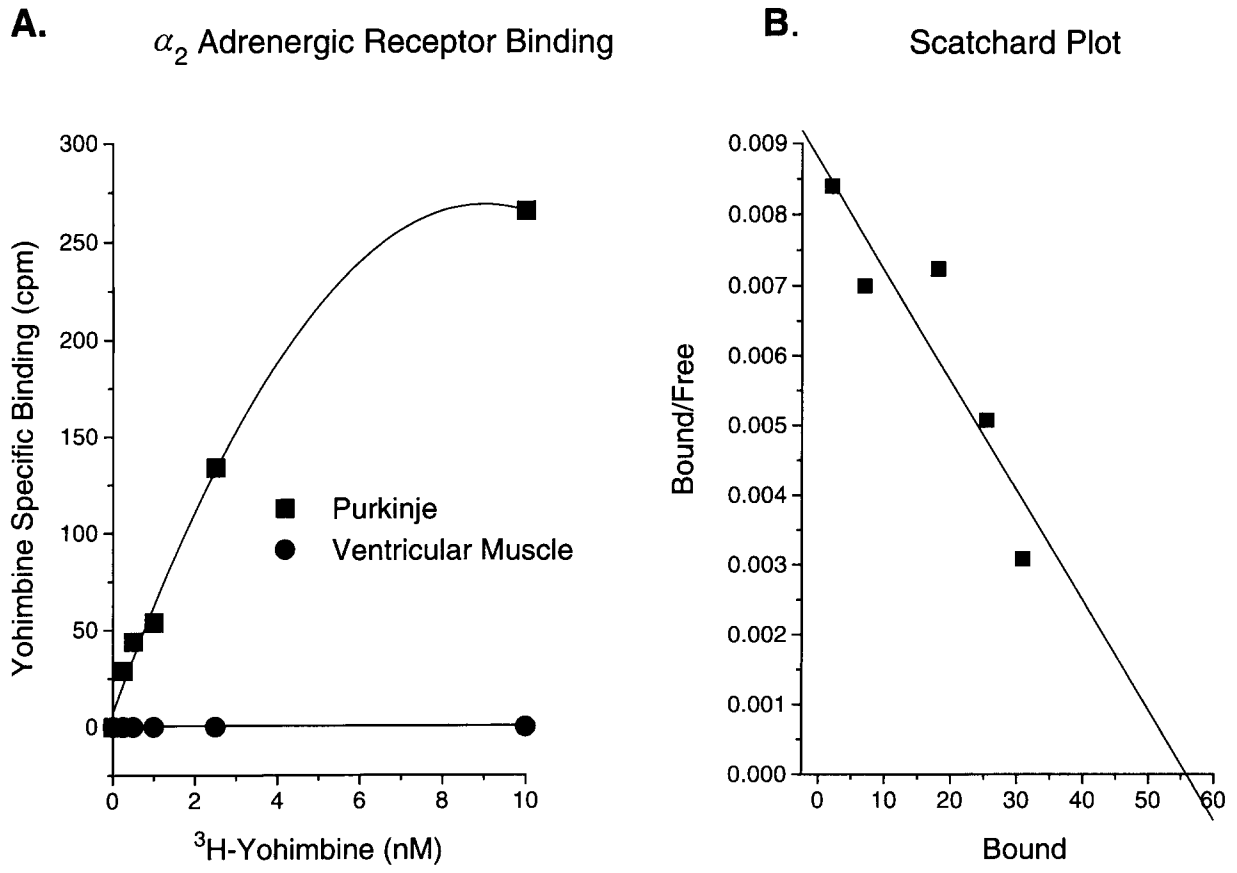

Fig. 4. Binding of [^{3}H]yohimbine to membrane fractions prepared from free-running strands of Purkinje fibers and from ventricular myocardium of canine hearts. Membrane fractions and α_2-adrenergic receptor specific binding were performed as described in Methods. Panel A: Purkinje membranes (■) show high affinity, saturable binding which is absent in myocardial membranes (●). Panel B: Scatchard analysis of the specific binding data. Line represents fit of data points by linear regression. B_{max} = 54.9 fmol/mg protein, K_d = 6.25 nM.

In conclusion, we have demonstrated that postjunctional α_2-adrenergic receptors are present in Purkinje fibers but not in ventricular myocardium. The relative density of the α_2 receptors is comparable to that of the α_1 receptors in Purkinje fibers. These results are consistent with our previous findings on the electrophysiological effects of α_2-adrenergic stimulation in canine cardiac Purkinje fibers.

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