

## Receptor Autoradiographic Analysis of Muscarinic Receptors in the Rat Atrioventricular Node

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**ABSTRACT:** We carried out investigations on muscarinic acetylcholine receptors (m-AChR) in the rat heart, including the atrioventricular (AV) node. The related tissue sections were incubated with  $^3\text{H}$ -quinuclidinyl benzilate ( $^3\text{H}$ -QNB), then autoradiography and an image analysis coupled with computer-assisted microdensitometry were done. A single type of specific and high affinity binding sites of  $^3\text{H}$ -QNB was found to be highly concentrated in the AV node, the maximum binding capacity (Bmax) being 1.4 pmol/mg protein and with a dissociation constant (Kd) of 0.5 nM. The density and affinity of the binding to the AV node were the highest, when compared with findings in the atrium (interatrial septum) and ventricle (interventricular septum). The binding was competitively displaced by AF-DX 116, a selective antagonist for the  $M_2$  AChR subtype, with a high affinity, whereas pirenzepine, an antagonist for the  $M_1$  AChR subtype was much less potent in displacing the binding. Therefore, vagal-cholinergic stimulation presumably plays a significant role in functions of the rat AV node, probably by interacting with the specific, high affinity  $M_2$  AChR subtype.

### INTRODUCTION

Vagal-efferent neurons are widely distributed in cardiac tissues and densely innervate the sinoatrial node and atrioventricular (AV) node, a specialized cardiac conduction system.<sup>9)</sup> Vagal-cholinergic stimulation exerts negative chronotropic and inotropic actions, through muscarinic acetylcholine receptors (m-AChR).<sup>9)</sup> Specific acetylcholine-activated single channel  $\text{K}^+$  currents in isolated pacemaker cells were recorded using patch-clamp methods and the findings differed from events seen in atrial and ventricular cells.<sup>14) 16)</sup> Although there is evidence that the  $M_2$  AChR subtype in the mammalian

heart is more abundant than the  $M_1$  subtype,<sup>5) 6) 7)</sup> little is known of characteristics of m-AChR in conduction systems in the heart. Distribution and characterization of the  $\beta$ -adrenergic receptors in the rat and guinea pig heart areas have been extensively studied. It was found that the AV node, an area richly innervated by sympathetic nerves, has highly dense binding sites for  $^{125}\text{I}$ -cyanopindolol, a radiolabeled ligand for  $\beta$ -adrenergic receptors.<sup>12) 15)</sup> Of particular interest is the observation made by Molenaar *et al.*<sup>12)</sup> of a higher proportion of  $\beta$ -1 to  $\beta$ -2 adrenergic receptor present in the conduction system, as compared with that of the surrounding myocardium. Using the quantitative receptor autoradiographic method<sup>17) 23)</sup>

with  $^3\text{H}$ -quinuclidinyl benzilate ( $^3\text{H}$ -QNB), a radiolabeled ligand for m-AChR,<sup>22)</sup> we characterized the m-AChR in the rat heart, including the AV node.

## MATERIALS AND METHODS

### Animals and sample preparations

Twenty five male Wistar rats weighing 250–300g were fed standard chow (F-2, Funabashi Farm Co., Japan), and water *ad libitum*, and housed at 24°C with lights on 06:00 to 18:00. These rats were decapitated between 16:00 and 18:00, the heart was rapidly removed and placed in 3 mM HEPES buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1.0 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub> and 11 mM glucose for 20 sec at 24°C to remove the blood. The tissues containing the AV node were then immediately dissected under the orientation for the tricuspid valves, coronary sinus and fossa ovalis, using the method of Saito *et al.*,<sup>15)23)</sup> frozen in isopentane at -30°C, and stored at -80°C. Within 24 hr, frozen, 16  $\mu\text{m}$ -thick sections were cut in a cryostat at -16°C, and thaw-mounted into gelatin-coated glass slides. Sections adjacent to related tissue sections for the binding studies were treated using Karnovsky's acetylcholinesterase-staining method<sup>10)</sup>, for orientation of localization of the AV node. Alternate, unstained sections were stored overnight, under vacuum at 4°C.

### Quantitative receptor autoradiography

The unstained sections were labeled with  $^3\text{H}$ -QNB (specific activity 30 Ci/mmol, New England Nuclear, U. S. A) in the incubation medium of 100 mM sodium phosphate buffer, pH 7.4, at 24°C, 30 min.<sup>3)</sup> Saturation experiments at equilibrium were performed in the presence of increasing amounts of  $^3\text{H}$ -QNB, ranging from 123 pM to 8.0 nM, without or with 5.0  $\mu\text{M}$  atropine for non-specific binding. In the kinetic studies to distinguish and characterize  $^3\text{H}$ -QNB binding to the heart tissues, we used three antagonists, atropine, AF-DX 116,<sup>5)7)</sup> and pirenzepine.<sup>6)</sup> After incubation, the slides were washed twice (2 sec each) in the same buffer, then rinsed quickly in distilled water. Tissue sections were dried under a cold stream of air, and exposed for 30 to 90 days with calibrated

$^3\text{H}$ -standards, prepared as described,<sup>17)</sup> to  $^3\text{H}$ -Ultrofilm (LKB Industries, U. S. A). The films were developed with a D19 Kodak developer, and optical densities were measured by computerized microdensitometry (UHG-101, Unique Medical Co, Japan). We measured the optical densities at four spots in each area of the autoradiograms, using a computerized microdensitometer. The computer automatically averaged the value. The data obtained from one rat was calculated from triplicate tissues sections. The optical densities observed were related to concentration of the radioactivity present, as deduced from a comparison with the standard curves.<sup>17)</sup>

### Data analysis

Data obtained from the radioligand saturation binding studies were analyzed using a LIGAND computer program.<sup>13)</sup> The inhibition constant (IC<sub>50</sub>) values from displacement curves were calculated, using the logit-log plot. Results are expressed as means  $\pm$  SEM. Differences were analyzed by one-way analysis of variance (ANOVA) using the F-test, followed by a modified t-test.

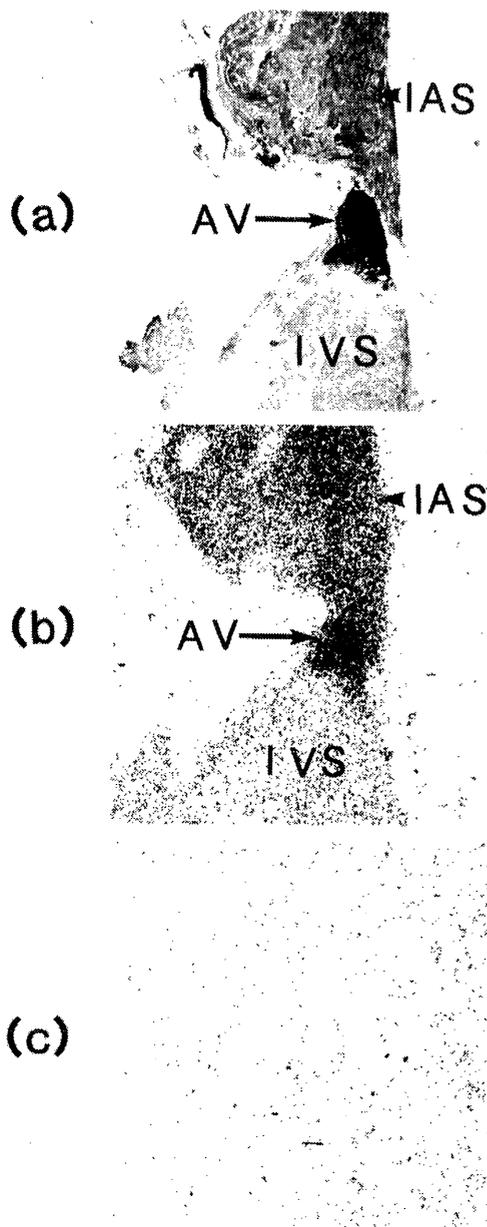
### Drugs

AF-DX 116 and pirenzepine dihydrochloride were donated by Dr. Karl Thomae GmbH, Federal Republic of Germany, and Nippon Boehringer Ingelheim Co., Japan, respectively. (-)-Propranolol was a gift from ICI Pharma Co., Japan. Atropine sulfate and reagents used were purchased from Sigma Chemical Co., U. S. A.

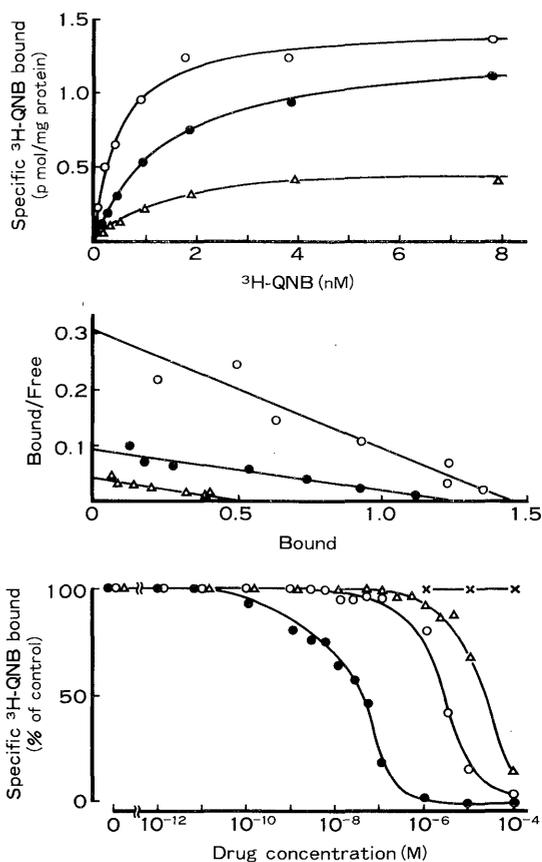
## RESULTS

In the initial experiment, we found that specific binding of  $^3\text{H}$ -QNB, obtained at the amount of 8.0 nM to the related sections reached the maximum at 5 min of incubation time, and maintained a plateau for up to 90 min at 24°C.

The AV node in the Wistar rat was localized in the area between the interatrial septum and the interventricular septum and was heavily stained for acetylcholinesterase (Figure 1-a). As expected, there was a high density of  $^3\text{H}$ -QNB binding to the AV node, whereas a relatively low density of the binding was detected in the



**Fig 1.** Receptor autoradiographic evidence of  $^3\text{H}$ -QNB binding sites in the atrioventricular (AV) node, interatrial septum (IAS) and interventricular septum (IVS). Sixteen- $\mu\text{m}$  thick sections were incubated with 8.0 nM  $^3\text{H}$ -QNB in the absence (b, total binding) and presence (c, non-specific binding) of 5.0  $\mu\text{M}$  atropine. After incubation, dried sections were exposed to  $^3\text{H}$ -Ultrafilm for 30 days. A picture of (a) adjacent to (b) is a Karnovsky's acetylcholinesterase-stained section.



**Fig 2.** Typical saturation experiments at equilibrium of specific  $^3\text{H}$ -QNB binding to the atrioventricular (AV) node (O), interatrial septum (●) and interventricular septum (Δ)(upper panel). Scatchard plots (middle panel) were obtained using the program LIGAND, based on data from saturation curves. Displacement curves (lower panel) of specific  $^3\text{H}$ -QNB binding to the AV node were obtained from the incubation with 8.7 nM of the radiolabeled ligand in the presence of increasing concentrations of atropine (●), AF-DX 116 (○), pirenzepine (Δ) and (-)-propranolol (×).

interatrial and interventricular septa (Figure 1-b). Non-specific binding was less than 2% of the total binding (Figure 1-c).

In an equilibrium binding study done in the presence of increasing concentrations of the radiolabeled ligand, specific  $^3\text{H}$ -QNB binding to the AV node was saturable. Scatchard analysis of the data obtained from a LIGAND computer program<sup>13)</sup> gave a straight line, thereby indi-

**Table 1.** Dissociation constant (Kd) and maximum binding capacity (Bmax) of <sup>3</sup>H-QNB binding sites in the rat atrioventricular (AV) node, interatrial septum (IAS), and interventricular septum (IVS)

	Kd (nM)	Bmax (pmol/mg protein)
AV node	0.477 ± 0.013 a	1.422 ± 0.029 d
IAS	1.321 ± 0.299 b	1.245 ± 0.049 e
IVS	1.336 ± 0.198 c	0.503 ± 0.042 f

Results are means ± SEM of 3 individual rats.

One-way ANOVA,  $p < 0.01$  ( $f < d$ ,  $F = 329.16$ ;  $f < e$ ,  $F = 132.53$ ),  $p < 0.05$  ( $a < c$ ,  $F = 18.77$ ;  $a < b$ ,  $F = 7.97$ ;  $e < d$ ,  $F = 9.55$ )

**Table 2.** Inhibition ( $IC_{50}$ ,  $\mu M$ ) of <sup>3</sup>H-QNB binding to the a atrioventricular (AV) node, interatrial septum (IAS) and interventricular septum (IVS) by muscarinic antagonists

Antagonists	AV node	IAS	IVS
Atropine (4)	0.029 ± 0.009 a	0.091 ± 0.022 b	0.121 ± 0.036 e
AF-DX 116 (5)	1.729 ± 0.352 d	2.698 ± 1.025 e	3.663 ± 1.039 f
Pirenzepine (4)	15.018 ± 3.288 g	19.771 ± 0.858 h	19.430 ± 3.806 i
Propranolol (4)	100000 <	100000 <	100000 <

The  $IC_{50}$  is the concentration of antagonist displacing 50% of specifically bound <sup>3</sup>H-QNB obtained at the concentration of 8.7 nM. Results are means ± SEM of 4 to 5 individual rats.

One-way ANOVA,  $p < 0.01$  ( $a < d$ ,  $F = 1.16$ ;  $d < g$ ,  $F = 20.8$ ;  $e < h$ ,  $F = 151.59$ ;  $f < i$ ,  $F = 19.79$ )

$p < 0.05$  ( $a < d$ ,  $F = 6.89$ ;  $a < c$ ,  $F = 6.36$ ;  $c < f$ ,  $F = 9.19$ )

cating that <sup>3</sup>H-QNB binds to a single population of sites (Figure 2, upper and middle panels). A single population of saturable binding sites was noted in the interatrial and interventricular septa. As summarized in Table 1, the dissociation constant (Kd) of the binding to the AV node was the lowest, and the maximum binding capacity (Bmax) was the highest, among the areas studied. The Bmax of the binding in the interatrial septum was significantly higher than that in the interventricular septum.

Typical inhibition curves of <sup>3</sup>H-QNB binding to the AV node by atropine, AF-DX 116, pirenzepine, and (–)-propranolol are illustrated in the lower panel of Figure 2. Atropine and AF-DX 116 competitively displaced the binding, with a high affinity, and pirenzepine was much less potent. Atropine showed the highest potency and (–)-propranolol did not displace the binding as shown in  $IC_{50}$  values (Table 2).

## DISCUSSION

The quantitative receptor autoradiographic method we used revealed that specific <sup>3</sup>H-QNB binding sites are highly localized in the AV node, and area richly innervated with vagal-

cholinergic neurons.<sup>9)</sup> Tissue quenching of radioisotope energy in quantitative receptor autoradiography with tritiated ligands is one factor linked to receptor-transmitter mismatch and to the failure to attain precise quantitations of receptors in myelin-rich areas such as brain white matter.<sup>4)8)</sup> The autoradiographic localization of specific <sup>3</sup>H-QNB binding sites we noted in areas of the heart corresponds to the histochemical distribution of acetylcholinesterase, a finding which differs from data on the central nervous system. As cardiac tissues are innervated by un-myelinated nerve fibers,<sup>2)</sup> quenching was not taken into consideration in the present study.

We firstly obtained data on difference in the affinity of specific <sup>3</sup>H-QNB binding between the AV node, atrial and ventricular tissues. Binding to the AV node had three times the affinity of binding to the interatrial and the interventricular septa. Although there are no documented, comparable data on the affinity of <sup>3</sup>H-QNB binding to the AV node, there is a difference between the Kd value of 1.3 nM in the atrial and ventricular septa and that of 0.6 nM noted in the rat cardiac membranes by Waelbroeck *et al.*,<sup>18)</sup> perhaps because of different experi-

mental conditions. AF-DX 116, an antagonist for the  $M_2$  AChR subtype<sup>5)7)</sup> potently competed for binding of  $^3\text{H-QNB}$  to the AV node, whereas pirenzepine, an antagonist for the  $M_1$  subtype<sup>16)</sup> was much less potent. ACh seems to modify pacemaking functions of the AV nodal cells by activating the muscarinic-gated potassium channel.<sup>14)16)</sup> Therefore, taken in conjunction with evidence of the anatomical distribution of vagal-cholinergic neurons,<sup>9)</sup> our data support the proposal that the functional significance of acetylcholine on the AV node predominantly involves the high affinity  $M_2$  AChR subtype.

The existence of multiple subtypes of mAChR has been suggested.<sup>1)</sup> The  $M_1$  subtype is mainly located in the cerebral cortex and hippocampus, whereas the  $M_2$  subtype with a low affinity for pirenzepine is in the heart, cerebellum and ileum.<sup>16)29)</sup> We characterized  $^3\text{H-QNB}$  binding sites in the heart areas, using atropine, AF-DX 116 and pirenzepine. When comparing the  $\text{IC}_{50}$  values obtained from the displacement study, atropine, a non-selective antagonist was the most potent competitor for the binding. Our observations of the potency in displacing  $^3\text{H-QNB}$  binding of three muscarinic antagonists is compatible with the findings of Watson *et al.*<sup>19)</sup> and of Yamada *et al.*<sup>21)</sup> Taking note of the recent finding that the *in vivo* potency in the chronotropic action of atropine was 10 to 100 times higher than that of AF-DX 116,<sup>7)11)</sup> the difference between the  $\text{IC}_{50}$  values of atropine and AF-DX 116 obtained here seems reasonable. As AF-DX 116 was a more potent competitor, as compared with pirenzepine, it seems likely that the  $M_2$  AChR subtype is predominantly distributed in heart areas. Although the affinity of  $^3\text{H-QNB}$  binding to the AV node was significantly higher than in the atrial and ventricular tissues, the potency in displacing of AF-DX 116 and pirenzepine was similar among the areas studied, the exception being the case of atropine. This may contradict the finding that AV nodal cells have a specific acetylcholine-activated  $\text{K}^+$  channel and differs from electrophysiological evidence obtained using atrial and ventricular cells.<sup>14)16)</sup> Whether or not  $M_2$  AChR in the AV node is distinct from the receptor in atrial and ventricular cells will probably be elucidated when more is known of

the molecular properties of  $^3\text{H-QNB}$  binding proteins.

In summary, we found specific  $^3\text{H-QNB}$  binding sites to be highly concentrated in the AV node and to be characteristically of the  $M_2$  AChR subtype. Therapeutic approaches to treatment of tachy- and bradyrhythmia with agents selectively affecting  $M_2$  AChR subtype may have to be designed.

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