Quantitative Autoradiographic Localization of the M1 and M2 Subtypes of Muscarinic Acetylcholine Receptors in the Monkey Brain

Rie MIYOSHI, Shozo KITO and Masanori SHIMOYAMA
Third Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

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Abstract—The distribution of muscarinic acetylcholine receptors (mAChR) was investigated in the monkey brain by means of quantitative in vitro autoradiography. \(^{3}\text{H}-\text{QNB}, \(^{3}\text{H}-\text{pirenzepine (PZ)}\) and \(^{3}\text{H}-\text{AF-DX 116}\) were used for labelling total mAChR, M1 and M2 receptors, respectively. \(^{3}\text{H}-\text{PZ}\) and \(^{3}\text{H}-\text{AF-DX 116}\) showed specificity to each receptor subtype in the monkey brain. On sections containing the putamen and globus pallidus, the sum of \(B_{\text{max}}\) values of \(^{3}\text{H}-\text{PZ}\) and \(^{3}\text{H}-\text{AF-DX 116}\) binding sites was almost close to that of \(^{3}\text{H}-\text{QNB}\) binding sites. Autoradiographic distributions of muscarinic subtype receptors in the monkey brain were similar to those reported in the rat brain; that is, M1 receptors were dominant in most areas of the telencephalon, while M2 receptors were richly distributed in the brainstem and cerebellum. However, some nuclei of the brainstem such as the central gray matter, superior colliculus, substantia nigra, nucleus of the oculomotor nerve, pontine nucleus and inferior olivary nucleus, had relatively high ratios of M1 receptors in the monkey brain. In addition, the cortical lammar distribution of M2 receptors noticed in the rat was not observed in the monkey brain. Knowledge about the localizations of M1 and M2 receptors in various brain regions in the monkey brain will increase our understanding of the functions of the brain cholinergic system in the primate.

Heterogeneity of muscarinic acetylcholine receptors (mAChR) has been shown in various mammalian tissues by using a radioreceptor assay. Quinuclidinyl benzilate (QNB), a classical muscarinic antagonist, binds to all mAChR with an equal affinity (1, 2). On the other hand, pirenzepine (PZ), a muscarinic antagonist, has contributed, together with substantial pharmacological and clinical evidence, to the classification of mAChR into M1 (high affinity for PZ) and M2 (low affinity for PZ) subtypes (3-6). M1 and M2 receptors are considered to be independent and to represent distinct gene products (7, 8). In addition, competitive binding experiments with AF-DX 116, a M2 cardio-selective antagonist (9, 10), have provided evidence that M2 receptors do not represent a single subtype, but rather a heterogeneous group of receptors (11-13). Among the low affinity binding sites for PZ, AF-DX 116 has about 10-25 times greater affinity for mAChR in the isolated heart than in smooth muscle preparations (11, 13). In the rat brain, receptors with multiple affinities for AF-DX 116 have been also found; that is, brain mAChR consist of M1 (high affinity for PZ, low affinity for AF-DX 116), cardiac M2 (low affinity for PZ, high affinity for AF-DX 116) and glandular M2 (low affinity for both PZ and AF-DX 116) (10, 12).

Autoradiographic studies of M1 receptors in the brain using \(^{3}\text{H}-\text{PZ}\) have been well-described (14-18), and they have revealed that M1 receptors have the highest density in the cerebral cortex (layers I-II), striatum, nucleus accumbens, hippocampus, dentate gyrus and amygdala, while they are sparse in the cerebellum, nucleus of the solitary tract and facial nerve nucleus. As for autoradio-
graphic studies of M2 receptors, direct binding of 3H-oxotremorine-M (17) and 3H-N-methyl-scopolamine binding displaced by unlabelled carbachol (18-20) have been performed. Regenold et al. (21) first reported direct visualization of M2 receptors within the rat brain using 3H-AF-DX 116 and demonstrated that the binding sites were highly concentrated in the septal nuclei, certain thalamic nuclei, superior colliculus and motor nuclei of the lower brainstem.

In the present study, the authors performed quantitative in vitro autoradiography of 3H-QNB, 3H-PZ and 3H-AF-DX 116 in the monkey brain and compared binding sites among them.

**Materials and Methods**

Japanese monkeys, 6 to 8 years old, were used in these experiments (the average life span of the Japanese monkey is 20 years), and they consisted of two males and one female. Animals were injected intramuscularly with ketamine at 10 mg/kg, and 20 mg/kg of pentobarbital was administered intravenously to deepen the anesthesia. Brains were rapidly removed, frozen over liquid nitrogen and stored at -80°C until sectioned using a cryostat. Ten-μm-thick sections were taken serially and mounted onto chrome alum/gelatin-coated slides. Slides were stored in microscope slide boxes at -20°C until use.

Before autoradiographic procedures were performed, initial biochemical experiments were conducted for each ligand to determine optimal conditions for binding using serial sections containing the putamen and globus pallidus in duplicate. For labelling with 3H-QNB (New England Nuclear, 42.9 Ci/mmol), slide-mounted tissue sections were incubated with 3H-QNB (0.05-1.6 nM) for 60 min at room temperature in 0.1 M sodium-potassium phosphate buffer, pH 7.4. Following the incubation period, sections were rinsed in the buffer twice for 5 min at 4°C. For 3H-PZ (New England Nuclear, 84.2 Ci/mmol), sections were incubated in 0.1 M sodium-potassium phosphate buffer, pH 7.4, containing 3H-PZ (1.25-40 nM) for 90 min at room temperature. After that, slides were given a five-minute rinse in fresh buffer and then a one-minute one in distilled water at 4°C. For 3H-AF-DX 116 (New England Nuclear, 58.6 Ci/mmol), sections were incubated with 3H-AF-DX 116 (1.25-40 nM) at 25°C for 60 min in 50 mM sodium-potassium phosphate buffer, pH 7.4, fixed by glutaraldehyde vapor for 10 min and then washed in the buffer twice for 3 min at 4°C. The specific binding was determined as the difference between the binding in the presence and absence of 1 μM atropine for both 3H-QNB and 3H-PZ, and 100 μM atropine for 3H-AF-DX 116. Then, the labelled sections were wiped from the slides with filter paper, and the radioactivity was counted with a liquid scintillation spectrophotometry.

For autoradiography, cryostat sections were incubated with 2 nM 3H-QNB, 15 nM 3H-PZ or 25 nM 3H-AF-DX 116 under the above-described conditions. After labelling, slides were rapidly air-dried and apposed to a sheet of tritium-sensitive film (Amersham). In order to quantify the receptor density, 10 μm sections of the autoradiographic 3H-microscale of the Amersham Company were coexposed as a standard. Autoradiograms were exposed for 4 weeks for 3H-QNB and 3H-PZ or 8 weeks for 3H-AF-DX 116 at 4°C. The films then were developed for 13 min at 20°C in Kodak Microdol X, fixed for 10 min, washed in running water and dried in air. Obtained films were placed in a photographic illumination apparatus and optical densities of various brain regions and the 3H-microscale were calculated using LBAS II from Zeiss. The molar quantities of bound ligands were determined from standard curves representing the relationship between optical density and the level of radioactivity (22). The authors confirmed that values of specific binding sites obtained from both radioreceptor assay and in vitro autoradiography were almost totally consistent with each other (23).

**Results**

Figure 1 shows saturation curves and Scatchard plots of 3H-QNB, 3H-PZ and 3H-AF-DX 116 binding sites on sections containing the putamen and globus pallidus. Scatchard analysis revealed that each of these three ligands had a high affinity single binding site. The Kd values were 0.39 nM, 27.4 nM and 30.4 nM for 3H-QNB, 3H-PZ and 3H-AF-DX 116, respectively. The sum of the Bmax
The values of $^3$H-PZ and $^3$H-AF-DX 116 binding sites was remarkably close to that of the $^3$H-QNB binding sites.

The summary to compare the numbers of binding sites of these three ligands is shown in Table 1. In the case of $^3$H-QNB, non-

**Fig. 1.** Saturation curves and Scatchard plots of $^3$H-QNB (a), $^3$H-PZ (b) and $^3$H-AF-DX 116 (c) binding sites on cryostat sections containing the putamen and globus pallidus of the monkey brain. These values are the means in three repeated experiments.
specific binding was not visually apparent. For $^3$H-PZ and $^3$H-AF-DX 116, the specific binding was determined by the difference between the bindings in the absence and presence of atropine. $^3$H-PZ binding sites composed a large portion of the $^3$H-QNB binding sites in most of the telencephalic regions, whereas their proportions were less in the thalamic and hypothalamic nuclei than in other telencephalic areas. In contrast, $^3$H-AF-DX 116 binding sites were higher in the thalamus and hypothalamus than in other telencephalic areas. In the brainstem, $^3$H-AF-DX 116 binding sites were dominant, especially in the nuclei of the motor trigeminal, facial and hypoglossal nerves. In addition, $M_2$ receptors were also dominant in the cerebellar cortex. On the other hand, the ratio of $^3$H-PZ binding sites was relatively high in the central gray matter, superior colliculus, substantia nigra, nucleus of the oculomotor nerve, pontine nucleus and inferior olivary nucleus.

Autoradiographic distributions of $^3$H-QNB, $^3$H-PZ and $^3$H-AF-DX 116 binding sites on

| Table 1. Specific binding sites of $^3$H-QNB, $^3$H-PZ and $^3$H-AF-DX 116 in various areas of the monkey brain |
|---------------------------------------------------------------|
|                  | $^3$H-QNB (fmol/mg tissue) | $^3$H-PZ (fmol/mg tissue) | $^3$H-AF-DX 116 (fmol/mg tissue) | $^3$H-AF-DX 116 |
| Frontal cortex    | 405.7±21.2                  | 250.2±24.7                  | 70.2±2.5                        | 3.6              |
| Caudate nucleus   | 616.0±38.9                  | 363.2±19.0                  | 69.5±10.7                       | 5.2              |
| Putamen           | 449.6±15.2                  | 269.2±12.9                  | 57.9±8.4                        | 4.6              |
| Globus pallidus, externa | 44.4±2.3                | 27.1±3.3                    | 4.6±1.2                         | 5.9              |
| Globus pallidus, interna | 105.8±20.8             | 65.7±8.6                    | 11.0±0.6                        | 6.0              |
| Claustrum         | 288.7±18.9                  | 175.6±18.1                  | 49.5±7.3                        | 3.5              |
| Amygdala          | 280.1±31.0                  | 194.8±23.4                  | 52.4±9.7                        | 3.7              |
| Thalamus, anteroventralis | 257.2±32.1             | 67.9±16.3                   | 76.4±13.9                       | 0.88             |
| Thalamus, posterolateralis | 102.5±11.5            | 31.0±11.8                   | 41.5±3.6                        | 0.75             |
| Thalamus, dorsomedialis | 219.5±46.5              | 55.5±12.0                   | 61.7±8.5                        | 0.90             |
| Mammillary body   | 274.6±28.9                  | 110.0±15.3                  | 80.8±3.3                        | 1.4              |
| Parietal cortex   | 287.9±15.1                  | 227.8±12.2                  | 68.1±9.4                        | 3.3              |
| Insular cortex    | 300.7±22.7                  | 206.4±12.6                  | 60.1±10.7                       | 3.4              |
| Temporal cortex   | 334.8±29.2                  | 228.8±18.2                  | 73.3±13.2                       | 3.1              |
| Hippocampus, Ammon's horn | 402.5±21.5          | 266.0±4.1                   | 80.0±3.9                        | 3.3              |
| Hippocampus, dentate gyrus | 373.7±17.8          | 261.7±11.7                  | 77.2±15.1                       | 3.4              |
| Hippocampus, parahippocampal gyrus | 406.4±42.1        | 268.0±6.5                   | 68.1±4.4                        | 3.9              |
| Occipital cortex  | 407.9±36.2                  | 211.4±7.6                   | 84.6±7.3                        | 2.5              |
| Central gray matter | 195.7±28.1               | 58.6±9.0                    | 30.8±6.9                        | 1.9              |
| Superior colliculus | 193.9±12.0             | 68.9±7.1                    | 26.3±3.8                        | 2.6              |
| Substantia nigra  | 127.8±13.0                  | 59.3±5.1                    | 24.6±4.5                        | 2.4              |
| Ventral tegmental area | 144.1±12.4            | 32.0±6.8                    | 19.9±1.9                        | 1.6              |
| Oculomotor nucleus | 144.0±12.3             | 45.0±8.2                    | 22.7±4.0                        | 2.0              |
| Interpeduncular nucleus | 102.6±12.4            | 22.0±6.2                    | 17.6±6.0                        | 1.3              |
| Pontine nucleus   | 159.6±18.7                  | 31.5±5.1                    | 17.4±2.6                        | 1.8              |
| Motor trigeminal nucleus | 322.6±36.7           | 51.2±8.8                    | 47.0±6.0                        | 1.1              |
| Spinal trigeminal nucleus | 143.5±18.3          | 38.4±7.1                    | 28.6±4.6                        | 1.3              |
| Facial nucleus    | 232.2±23.5                  | 26.6±3.9                    | 51.5±9.3                        | 0.5              |
| Hypoglossal nucleus | 196.9±9.6              | 34.6±5.3                    | 37.5±3.3                        | 0.9              |
| Inferior olive    | 59.4±5.5                    | 34.4±2.6                    | 21.7±4.0                        | 1.6              |
| Cerbellar cortex  | 36.0±4.4                    | 17.3±4.9                    | 18.6±1.7                        | 0.93             |

The numbers express the mean±S.D. (n=5-13). These values represent molar quantities of bound ligands at one particular concentration of $^3$H-ligands. About 65% of the maximal $^3$H-QNB binding and about 50% of the maximal $^3$H-PZ and $^3$H-AF-DX 116 binding were occupied under our experimental conditions.
serial sections are shown in Figs. 2 to 6. $M_1$ and $M_2$ receptors were often found in the same telencephalic structures. In the frontal.

Fig. 2. Autoradiographic distributions of $^3$H-QNB (a), $^3$H-PZ (b) and $^3$H-AF-DX 116 (c) binding sites in sections through the mamillary body of a monkey. AM, amygdala; GP, globus pallidus; HT, hypothalamus; I, insular cortex; NC, caudate nucleus; PU, putamen; TH, thalamus.

Fig. 3. Autoradiographic distributions of $^3$H-QNB (a), $^3$H-PZ (b) and $^3$H-AF-DX 116 (c) binding sites in sections containing the hippocampus of a monkey. D, dentate gyrus; H, hippocampal proper; PH, parahippocampal gyrus.
parietal, insular and temporal cortices, binding sites of these three ligands were diffusely observed throughout all the layers. In the hippocampal formation, M₁ receptors were the most numerous in the area CA1 and molecular layer of the dentate gyrus. M₂ receptors in the hippocampus were generally less in number, while densities in areas CA2 and CA3 were the highest. Figures 4 to 6 are typical autoradiograms of mAChR subtypes in the lower brainstem. Figure 4 shows the nucleus of the motor trigeminal nerve and pontine nucleus; Fig. 5, the nucleus of the facial nerve; and Fig. 6, the nucleus of the hypoglossal nerve and inferior olivary nucleus. It was clearly seen that M₂ receptors were dominant in the nuclei of the motor trigeminal and facial nerves, while M₁ receptors in the inferior olivary nucleus were apparently observed.

**Discussion**

The results of this study reveal localiza-
tions of $M_1$ and $M_2$ muscarinic receptor subtypes in the monkey brain through direct visualization of binding sites using selective antagonists for each receptor. High selectiveness of $^3$H-AF-DX 116 to $M_2$ receptors has been reported by Regenold et al. (21) in the rat brain. In our experiments, we were able to clearly discriminate between $M_1$ and $M_2$ receptors in the monkey brain by using $^3$H-PZ and $^3$H-AF-DX 116. It has been demonstrated that $K_d$ values for AF-DX 116 to cardiac $M_2$ and glandular $M_2$ are about 115 nM and 3200 nM, respectively (11, 12).

In this paper, the low affinity site for AF-DX 116 was not detected in the saturation experiment due to limitation of the concentration of the radioligand used. From the binding experiments, the sum of $B_{max}$ values of $^3$H-PZ and $^3$H-AF-DX 116. It has been demonstrated that $K_d$ values for AF-DX 116 to cardiac $M_2$ and glandular $M_2$ are about 115 nM and 3200 nM, respectively (11, 12). In this paper, the low affinity site for AF-DX 116 was not detected in the saturation experiment due to limitation of the concentration of the radioligand used. From the binding experiments, the sum of $B_{max}$ values of $^3$H-PZ and $^3$H-AF-DX 116 binding sites was remarkably close to that of the $^3$H-QNB binding sites on cryostat sections containing the putamen and globus pallidus. It was concluded that in this brain region, $M_1$ receptors were dominant, and the rest of the mAChR was largely cardiac $M_2$ receptors. At the concentration of $^3$H-AF-DX 116 used in this autoradiographic study, the radioligand is considered to label most of cardiac $M_2$ receptors.

Comparing our results with previous ones from the rat brain (14-17, 19, 21, 24), the similar data were obtained that $M_1$ receptors were dominant in most areas of the telencephalon, while $M_2$ receptors were richly observed in the brainstem and cerebellum. Some differences could be detected; i.e., in the rat cerebral cortex (for example the frontal and occipital cortices), the laminar distribution of $M_2$ receptors was observed, while $M_1$ receptors diffusely existed throughout all the layers (15, 24). In the monkey brain, however, no laminar distribution was found in any subtype receptor in the frontal, parietal, insular and temporal cortices. It is obscure what this difference between the rat and monkey means, since the functional significance of the laminar distribution of $M_2$ receptors has not been clarified. It has been reported that m3 mRNA displays the layered pattern in the rat cerebral cortex through in situ hybridization (25). Further analysis of gene expression of monkey and rat brains may postulate the physiological significance of the laminar distribution. In addition, $M_1$ receptors in some nuclei of the monkey brainstem were apparently observed, although the density was low, while those in the rat brainstem were not detectable. It was concluded that in the monkey, $M_1$ receptors were more involved in cholinergic mechanisms of the lower brainstem than in the rat.

These data will contribute to a better understanding of cholinergic functions in the primate.

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