Changes in $^3$H-Quinuclidinyl Benzilate Binding and Protein Synthesis in the Striatum Following Chronic Administrations of Muscarinic Agonist

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ABSTRACT Injection of the muscarinic agonist oxotremorine into rat striatum for seven consecutive days resulted in a 44% reduction in $^3$H-quinuclidinyl benzilate ($^3$H-QNB) binding, and it caused decreases in binding affinity to agonists. Furthermore, it eliminated the shift from the high affinity site to the low affinity site that occurs in the presence of $5'$-guanylyl imidodiphosphate (Gpp(NH)p). In addition, oxotremorine caused a 1.5-fold increase in the incorporation of $^3$H-leucine into the striatum, suggesting that it increased the synthesis of proteins other than the muscarinic receptor protein. The present results show that chronic treatment of the striatum with oxotremorine causes alterations in not only the quantity, but also the sensitivity of muscarinic receptors to guanine nucleotide.

Keywords: Muscarinic receptor, Down regulation, Striatum

On repeated exposure to an agonist, desensitization is generally observed as a reduction of the response to agonist-induced stimulation. Desensitization of receptors can be classified into two major types (1): One type is called the agonist-specific type or homologous desensitization, and it is thought to be due to phosphorylation, sequestration or down-regulation of receptors. The other type of desensitization is called the agonist-nonspecific type or heterologous desensitization.

Chronic administrations of the muscarinic agonist oxotremorine to mice is reported to result in the development of tolerance in the behavioral responses to muscarinic acetylcholine receptors (mAChR), because the mAChR binding sites in several brain regions decrease (2, 3). In addition, chronic inhibition of acetylcholinesterase by diisopropylfluorophosphate (DFP) leads to an increase in acetylcholine content and reduces the number of mAChR binding sites in the brain (4, 5). However, details of the molecular mechanisms of desensitization of mAChR are unknown, and there have been few studies on in vivo desensitization of mAChR in the brain.

In the present work, to obtain information about the mechanism of desensitization of mAChR, we studied the effects of long-term injections of oxotremorine into the striatum of rats on $^3$H-quinuclidinyl benzilate ($^3$H-QNB) binding and $^3$H-leucine incorporation as a marker of protein synthesis in the striatum. In this study, we used the striatum because this area is the richest in muscarinic receptors that connect functionally with other neurons (6).

Male Wistar rats (Kyudo, Kumamoto) weighing 200±20 g were used for all experiments. They were housed in a room at 20–25°C with a 12-hr light-dark cycle (light on 7:00 a.m.). Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. A guide cannula (20-gauge stainless steel) was inserted into the striatum bilaterally (A, 8.0 mm; L, 2.6 mm; V, −1.5 mm) according to the König and Klippel rat forebrain atlas (7). Seven days after the operation, 5 μl of oxotremorine (50 μg; Sigma, St. Louis, MO, USA) or saline (control) was injected bilaterally into the striatum once a day for one week. The rats were sacrificed 24 hr after the last injection and their brains were removed.

$^3$H-QNB binding in the striatum was assayed as described previously (6). The striatum was homogenized in 10 volumes of ice-cold 0.32 M sucrose and then centrifuged at 1,000 × g for 10 min. The supernatant was centrifuged at 17,000 × g for 60 min. The pellet was then resuspended in ice-cold distilled water for 10 min and centrifuged at 100,000 × g for 40 min. A 50-μl sample of crude synaptic membranes (approximately 50–100 μg protein) was incubated at 37°C in a reaction mixture (final volume of 560 μl) of the following composition: 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl$_2$, 0.8 mM CaCl$_2$, 10 mM glucose and 20 mM Tris-HCl, pH 7.4, containing $^3$H-QNB (33.2 Ci/mmol; New England Nuclear, Boston,
MA, USA) at a concentration of 0.01–5.0 nM. After incubating for 30 min, the reaction was terminated by adding ice-cold 50 mM Tris-HCl buffer (pH 7.4), and the mixture was filtered rapidly under vacuum through a Whatman GF/B glass filter, because $^3$H-QNB binding reached equilibrium after a 15-min incubation (6). The radioactivity on the filter was counted in a liquid scintillation spectrometer (Packard Tricarb 2660, Zurich, Switzerland). Specific binding was calculated as the difference between the radioactivities bound in the presence and absence of 1 μM atropine. Protein was measured by the method of Lowry et al. (8). After intrastriatal treatment with oxotremorine for one week or saline, rats received an intraperitoneal injection of 200 μl of $^3$H-L-leucine (20 μCi; Amersham, Buckinghamshire, UK) and then were decapitated 15, 30, 60 or 120 min later. The brains were rapidly removed, and the striatum and hippocampus were dissected out. These tissues were then homogenized in 10 volumes of 5% TCA in a glass-Teflon homogenizer, and the homogenate was boiled for 10 min and centrifuged at $1,000 \times g$ for 10 min. The pellet was delipidized with ice-cold acetone and weighed. The delipidized pellet was ashed by the "flame combustion-in-a-train method" (Oxidizer Model 305, Packard Instruction Co.), and the radioactivity was measured in a liquid scintillation counter. As $^3$H-leucine was incorporated time-dependently into the striatum for 60 min (data not shown), we determined its incorporation at 30 min. Data were analyzed statistically by Student's t-test.

Figure IA shows Scatchard plots for specific $^3$H-QNB binding to rat striatum. Scatchard analysis of specific $^3$H-QNB binding revealed a single high affinity binding component with a $K_d$ of 142.0 ± 6.9 pM and a $B_{max}$ of 841.9 ± 20.9 pmoles/g protein. Long-term injections of oxotremorine into the striatum resulted in time-dependent decrease in $^3$H-QNB binding. The $B_{max}$ value was significantly reduced by 44% after treatments with oxotremorine for one week ($B_{max} = 475.8$ pmoles/g protein vs control value $B_{max} = 841.9$ pmoles/g protein), without any change in the $K_d$ value (Fig. 1A, Table 1). The $IC_{50}$ value of oxotremorine for the $^3$H-QNB binding to the control striatum was 3.4 μM, whereas that after treatment with oxotremorine for one week was 15.9 μM, indicating a decrease in the affinity for the muscarinic agonist (Fig. 1, B and C).

The Hill slopes of the displacement curve for oxotremorine in the control and oxotremorine-treated groups were less than 1.0 (Hill coefficient: control rats = 0.60, oxotremorine-treated = 0.67), suggesting the presence of heterogenous affinities for the binding of oxotremorine to mAChR in the striatum. Muscarinic agonists are known to bind to receptors of two major affinity states, high and low affinity states, which are known to be interconvertible.

![Fig. 1. Effects of chronic treatments with oxotremorine for 7 days on Scatchard plots of $^3$H-QNB binding and the displacement by oxotremorine in the striatum. A: Effects of chronic treatments with oxotremorine on Scatchard plots of $^3$H-QNB binding in the striatum. ○: control, ●: oxotremorine-treated. B, C: Effects of chronic treatments with oxotremorine on the displacement by oxotremorine of 0.5 nM $^3$H-QNB specific binding in the striatum. B, control; C, oxotremorine-treated. Values are means for 3 or 4 experiments. ○: -100 μM Gpp(NH)p, ●: +100 μM Gpp(NH)p.](image-url)
The heterogeneity of agonist binding sites is due, in part, to the association of the receptor coupled with G proteins; that is, the high affinity binding sites consist of receptor-G protein complexes, whereas the low affinity sites are not coupled with G protein (10). The high-affinity state of receptors coupled with G protein is important in various physiological responses (11). Moreover, 100 μM 5′-guanylyl imidodiphosphate (Gpp(NH)p), a non-hydrolyzable analogue of GTP, shifted the displacement curve of oxotremorine at low concentration to the right in control rats, but had no effect in the striatum of oxotremorine-treated rats (Fig. 1, B and C). This finding indicates that long-term treatment with oxotremorine causes uncoupling of muscarinic receptors from G proteins in rat striatum.

The mechanism of desensitization is in general thought to involve phosphorylation of receptors, which causes uncoupling of receptors from the G proteins and results in a decrease in their affinity to agonists (12). Muscarinic receptors are phosphorylated by protein kinase A, protein kinase C and muscarinic-receptor-specific kinase (13). However, the relationship between the decrease of binding sites and phosphorylation of receptors is unknown. Recently, chronic treatment with muscarinic agonists was reported to decrease the levels of mAChR-mRNA and -protein, but to cause an increase in the c-fos mRNA level associated with down-regulation of mAChR in cultured cerebellar granule cells (14). Moreover, chronic treatments with agonists were shown to change the transcription rate of mAChR-mRNA (14).

The incorporation of 3H-leucine into protein is associated with protein synthesis. Interestingly, in the present study we found that long-term treatment with oxotremorine increased the incorporation of 3H-leucine into the striatum 1.5-fold, but did not alter that in the hippocampus (Table 2). If the number of binding sites is associated with the level of receptor protein, the present finding of increased incorporation of 3H-leucine in the striatum suggests increases of protein other than the protein of mAChR.

Indeed, the development of refractoriness of catecholamine receptors in glioma cells is actually prevented by the addition of the protein synthesis inhibitor cycloheximide (15). Therefore, some protein other than mAChR protein may be involved in the chronic oxotremorine-induced desensitization of the striatum. Possibly this protein is the product of a proto-oncogene such as c-fos in vivo. Alternatively, the receptors may be lost or internalized by an endocytotic process of some unknown protein.

In conclusion, the present results show that chronic treatment of the striatum with a muscarinic agonist caused alteration in not only the quantity, but also the sensitivity of muscarinic receptors to guanine nucleotide.

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### Table 1. Effects of chronic treatment with oxotremorine on the B_max and K_d values of specific 3H-QNB binding to the striatum

| Treatment                  | B_max (pmoles/g protein) | K_d (pM) |
|----------------------------|--------------------------|----------|
| 5 days                     |                          |          |
| Control (n = 3)            | 150.8 ± 11.7             | 855.3 ± 30.6 |
| Oxotremorine-treated (n = 3)| 154.2 ± 27.8             | 706.1 ± 94.3 |
| 7 days                     |                          |          |
| Control (n = 4)            | 142.0 ± 6.9              | 841.9 ± 20.9 |
| Oxotremorine-treated (n = 3)| 136.4 ± 14.4             | 475.8 ± 32.5* |

Values are means ± S.E. *Significantly different from the control at P < 0.05.

### Table 2. Effects of chronic treatment with oxotremorine for 7 days on the incorporation of 3H-leucine

| Treatment                  | 3H-Leucine incorporation (dpm/mg weight/30 min) |
|----------------------------|-----------------------------------------------|
| Control                    | 403.3 ± 26.4 (n = 6)                        |
| Oxotremorine-treated       | 584.2 ± 37.5* (n = 6)                       |
| Hippocampus                | 351.6 ± 14.3 (n = 6)                        |
| Oxotremorine-treated       | 323.9 ± 12.2 (n = 6)                        |

Values are means ± S.E. *Significantly different from the control at P < 0.05.
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