Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2919–2923, March 1995
Neurobiology

Effects of serotonergic agents on neuronal nicotinic acetylcholine receptors

(5-hydroxytryptamine/*Xenopus* oocytes/neurotransmitter interactions)

J. GARCÍA-COLUNGA AND R. MILEDI*

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA 92717-4550

Contributed by R. Miledi, January 3, 1995

ABSTRACT **In *Xenopus* oocytes expressing neuronal nicotinic acetylcholine receptors (nAcChoRs), made up of $\alpha 2$ and $\beta 4$ subunits, acetylcholine (AcCho) elicited ionic membrane currents (AcCho currents) that were modulated by serotonergic agents. Both agonists and antagonists specific for various serotonin (5-hydroxytryptamine, 5HT) receptor subtypes interacted directly with $\alpha 2\beta 4$ nAcChoRs: 5HT, (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin, methysergide, spiperone, and ketanserin reversibly reduced the amplitude of AcCho currents and accelerated their decay. The AcCho-current time course decayed with two exponential functions. In the presence of 5HT, the fast time constant of current decay (τ_f) was not greatly modified, but the slow time constant (τ_s) was reduced. With AcCho and 5HT both at 100 μM, τ_s was reduced from 140 s to 85 s. The order of potency for inhibition of AcCho current amplitudes was (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin > methysergide > spiperone > ketanserin > 5HT. The inhibition was voltage-dependent but the magnitude of the voltage dependence for the different blockers did not correspond to their blocking potency: e.g., the block with spiperone was stronger than with 5HT, but it was less voltage-dependent. Our results suggest that serotonergic agents block neuronal nAcChoRs in a noncompetitive manner, similar to the block of muscle nAcChoR by curare and other substances. These results show that neuronal nAcChoR channels that have been activated by their specific neurotransmitter may be modulated by nonspecific neurotransmitters and their antagonists. These effects may help to better understand brain functions as well as the mode of action of the many serotonergic agents that are used in medical practice.**

The nicotinic acetylcholine receptor (nAcChoR) is a ligand-gated ion channel that opens after it binds the neurotransmitter acetylcholine (AcCho) and that mediates synaptic transmission between nerve and muscle as well as between neurons in the peripheral and central nervous systems. The muscle nAcChoR is a pentameric structure composed of four homologous subunits (α, β, γ or ε, and δ) with a stoichiometry of $\alpha_2\beta\gamma\delta$, whereas nAcChoRs expressed in the peripheral and central nervous systems appear to be composed of two different subunits, one α-like and the other β-like, with a probable stoichiometry of $\alpha_2\beta_3$ (1, 2).

The function of nAcChoRs can be modified not only by their specific neurotransmitter and related agonists and antagonists, but also by other neurotransmitters such as serotonin (5-hydroxytryptamine, 5HT) (3, 4) and substance P (5, 6). However, the mechanisms involved in the modulation of nAcChoRs by 5HT are still not well understood. In this paper, we report that neuronal nAcChoRs are affected by serotonergic agents in much the same way that 5HT acts on muscle and *Torpedo* nAcChoRs (4). These agents act on nAcChoRs in a noncompetitive and voltage-dependent way, probably blocking the open nAcChoR channel complex, as has been proposed for the actions of curare (7, 8), substance P (6), nicotinic agonists (9), quinacrine (10), and local anesthetics (11).

The actions of heterologous neurotransmitters and antagonists on the responses of neuronal nAcChoRs to their specific transmitter are important for the processes of synaptic cross-regulation that are postulated to occur in the central nervous system.

MATERIALS AND METHODS

cRNAs and Oocyte Injection. Plasmids containing cDNA clones encoding $\alpha 2$ and $\beta 4$ rat neuronal nAcChoR subunits were linearized with *Hin*dIII ($\alpha 2$) or *Xho* I ($\beta 4$) and transcribed *in vitro* with SP6 ($\alpha 2$) or T3 ($\beta 4$) RNA polymerase. The RNA was extracted with phenol/chloroform, precipitated with ethanol, and suspended at 1 μg/ml in RNase-free water. A mixture of equal volumes of $\alpha 2$ and $\beta 4$ cRNAs was made and stored at −80°C until used for injecting oocytes.

Xenopus laevis (Xenopus I or Nasco) oocytes were isolated manually from the ovary and maintained at ≈16°C in Barth's solution [88 mM NaCl/1 mM KCl/0.33 mM $Ca(NO_3)_2$/0.41 mM $CaCl_2$/0.82 mM $MgSO_4$/2.4 mM $NaHCO_3$/5 mM Hepes, adjusted to pH 7.4 with NaOH and complemented with gentamicin sulfate at 0.1 mg/ml]. One day later the oocytes were injected with 0.5–50 ng of the cRNA mixture. After 2 days, oocytes were defolliculated by collagenase treatment (140 units/ml, Sigma, type I) for 0.5–1 hr, followed by manual dissection when necessary, and kept in Barth's solution until recording.

Electrophysiology Recording. Membrane currents were recorded 3–9 days after injection, using a voltage-clamp technique with two microelectrodes, both filled with 3 M KCl (12). The oocytes were placed in a recording chamber (volume, ≈0.1 ml) perfused continuously with normal frog Ringer's solution (115 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/5 mM Hepes/ adjusted to pH 7.0 with NaOH) at room temperature (19–23°C). The membrane potential was routinely held at −60 mV. AcCho and serotonergic substances were diluted in Ringer's solution and applied by superfusing the oocytes at a flow rate of 7–15 ml/min. When various drugs were applied to the same oocyte, the control response was allowed to recover 80–100% before each drug application. The current records were stored on discs for subsequent analysis. The apparent rate of desensitization was fitted by the sum of two exponential components and one steady-state component:

$$I = I_\infty + A_s \cdot \exp(-t/\tau_s) + A_f \cdot \exp(-t/\tau_f),$$

where I_∞ is the steady-state component; A_s and A_f are the amplitudes of the slow and fast components, extrapolated to

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: 5HT, 5-hydroxytryptamine; AcCho, acetylcholine; nAcChoR, nicotinic acetylcholine receptor; DPAT, (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin.

*To whom reprint requests should be addressed.

the time when the current was half-maximal; and τ_s and τ_f are the time constants for the slow and fast components, respectively.

RESULTS

Expression of $\alpha 2\beta 4$ Neuronal nAcChoRs. cRNA-injected oocytes exposed to AcCho responded with an inward current whose amplitude and time course depended on agonist concentration. The AcCho dose–response curve (Fig. 1), fitted with the Hill relation, gave an apparent dissociation constant of 8.3 ± 0.67 μM (n = 3–10) and a Hill coefficient of 1.08. At concentrations lower than 2 μM the AcCho current did not decrease much during 2- to 7-min exposures. With higher concentrations, the AcCho current showed a desensitization that became progressively faster as the concentration was increased (Fig. 1 *Inset*). In the range 1–10 μM AcCho, the current decay followed mainly one exponential function, while at higher AcCho concentrations it followed two exponentials. For example, in 15 oocytes exposed to 100 μM AcCho, the fast and the slow decay time constants (τ_s and τ_f) were on average about 4 and 140 s, respectively, and the steady-state component was close to 10% of the peak current. The two components (A_s and A_f) participated equally, being ≈45% of the total amplitude. The peak amplitude of the AcCho current was well maintained during repeated applications. Moreover, with 100 μM AcCho the time to full recovery was short (<5 min). After removal of the AcCho the currents returned rapidly to the base line; however, with high AcCho concentrations there was a transient increase of the inward current at the start of the wash, probably as a consequence of channel block by AcCho itself (Fig. 1 *Inset*).

Effects of 5HT on Neuronal nAcChoRs. In oocytes expressing nAcChoRs, 5HT alone (1–1000 μM) did not elicit significant ionic currents. However, when 5HT was applied simultaneously with AcCho the current evoked by AcCho was reduced in amplitude and its decay was accelerated (Fig. 2), presumably because of an increased desensitization. These effects were rapidly reversible. Coapplication of AcCho (100 μM) and 5HT (100 μM) reduced the peak current amplitude but, at this 5HT concentration, the fast time constant of current decay (τ_f) was not greatly affected. In contrast, the slow

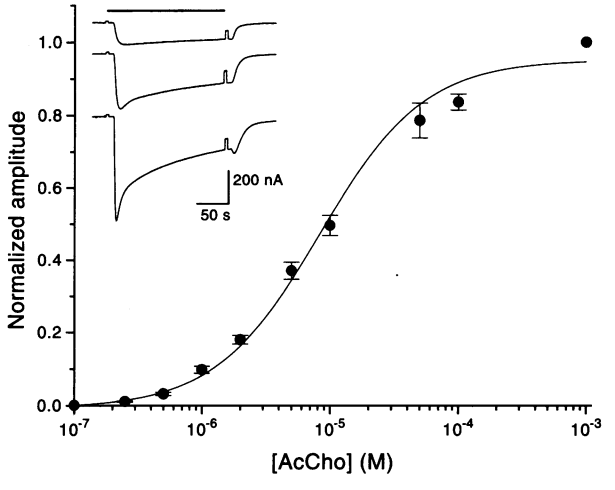

FIG. 1. Dose–response relation for the peak current evoked by AcCho acting on $\alpha 2\beta 4$ neuronal nAcChoRs. Responses were normalized to the current at 1000 μM AcCho and fitted with the Hill relation (solid line). Each point shows the mean ± SE for 3–10 oocytes. The average AcCho current at 100 μM was 594 ± 97 nA (n = 7). (*Inset*) Sample AcCho currents elicited by 2, 10, and 1000 μM AcCho. For this and subsequent figures the membrane was voltage-clamped at −60 mV and the drugs were applied during the time indicated by the bars at the top of the records.

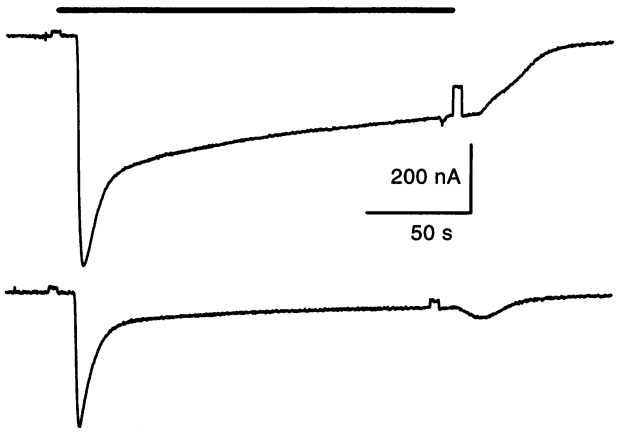

FIG. 2. Blockage of AcCho current by 5HT. Typical responses to 100 μM AcCho alone (upper record) and together with 300 μM 5HT (lower record) are shown.

time constant (τ_s) changed from 140 s to 85 s, while the steady-state component was reduced to 10% of the peak blocked response—i.e., about 6% of the control current.

Effects of Other Serotonergic Agents on Neuronal nAcChoRs. Serotonergic agents tested for their effects on AcCho currents included (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin (DPAT), a selective $5HT_{1A}$ agonist; spiperone, a $5HT_{1A}$ and $5HT_2$ antagonist; and methysergide and ketanserin, which are $5HT_{1c}$ and $5HT_2$ antagonists. For comparative purposes, all were applied at the same concentration (100 μM) together with 100 μM AcCho. Similar results were obtained when the drugs were tested in separate oocytes or applied sequentially to the same oocytes. In general, the effects were again a decrease in the current amplitude and an acceleration of its decay. DPAT was the most potent blocker of AcCho currents, and it reduced the peak current to 0.08 ± 0.01 (n = 5) of the control value whereas 5HT reduced it to 0.68 ± 0.03 (n = 15). The effects of the other serotonergic agents were intermediate between those of DPAT and 5HT. Their potency for blocking the AcCho current had the following sequence: DPAT > spiperone > methysergide > ketanserin > 5HT (Fig. 3).

The time course of the AcCho currents was also greatly altered by the presence of serotonergic agents. The current reached its peak earlier in the blocked responses than in the control currents (Fig. 4). The magnitude of this effect was estimated by measuring the time at which the current reached half of its peak value ($t_{1/2}$). For instance, when the AcCho current was blocked with methysergide, $t_{1/2}$ was 1.16 s compared with 2.52 s in the control response (Fig. 4). However, it should be noted that the maximal rate of change of the current was greater for the control response (284 nA/s) than for the blocked current (72 nA/s).

Dose Dependence of AcCho Current Block. The blockage of AcCho currents by all the serotonergic agents tested was enhanced when their concentration was increased. To examine the dose dependence in more detail, we focused on 5HT and spiperone. The blockage of the AcCho current was more potent with spiperone than with 5HT: the IC_{50} for spiperone was 30 ± 1.51 μM (n = 3) with a Hill coefficient of 0.85, whereas the IC_{50} for 5HT was 269 ± 27 μM (n = 3) with a Hill coefficient of 0.48. Even with 5 mM 5HT the current elicited by 100 μM AcCho was reduced only to 22%. In contrast, 500 μM spiperone blocked the AcCho current almost completely (Fig. 5).

The effect of 5HT on the current decay was studied in more detail. Since the time course of the AcCho current was clearly influenced by the speed of perfusion, we analyzed the responses under comparable conditions. Increasing the 5HT concentration from 10 μM to 5 mM did not alter the fast time

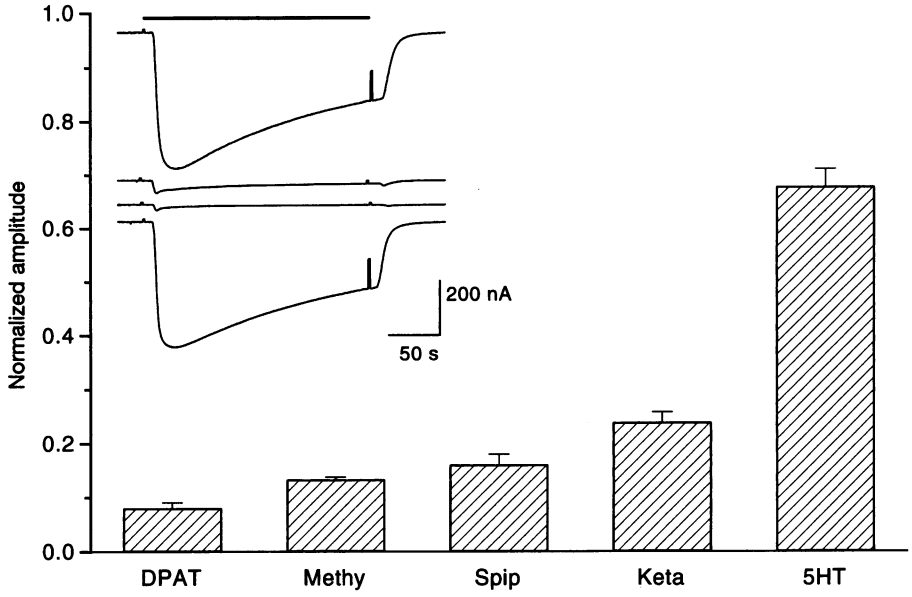

FIG. 3. Blockage of AcCho currents by serotonergic agents. Columns show the peak currents evoked by 100 μM AcCho in the presence of 100 μM DPAT, methysergide (Methy), spiperone (Spip), ketanserin (Keta), and 5HT, normalized to the currents obtained with AcCho alone. Each column represents the mean + SE of 4–15 oocytes. (*Inset*) AcCho current evoked by AcCho alone (top and bottom records) or together with methysergide (second record from top) or DPAT (third record from top). The currents illustrated were all recorded from the same oocyte and in the sequence shown.

constant of decay (τ_f), while the slow time constant (τ_s) decreased from about 140 s to 40 s. The amplitude ratio, A_s/A_f, decreased with increasing 5HT concentrations and A_f remained higher than A_s. Thus, the slow component of the AcCho current was affected more, in both time course and amplitude, than the fast component. Furthermore, the steady-state component decreased with increasing 5HT concentration and was reduced to 80% of the peak response by 10 μM 5HT and to 0% by 5 mM.

Voltage Dependence of Serotonergic Block. The AcCho current–voltage relation showed strong rectification at positive potentials. Between −10 and +20 mV the current was approximately zero and there was no clear reversal (Fig. 6*A*), due mainly to the rectifying characteristics of the channel itself (13, 14). When the AcCho currents were modulated by 5HT, the responses were reduced at all membrane potentials (Fig. 6*A*), and the current–voltage relation in the presence of 5HT or spiperone showed the same type of rectification and null current point as that of the control responses. These features suggest that the serotonergic agents did not alter the selectivity of the AcCho-gated channel.

Although spiperone blocked the AcCho currents more potently than 5HT, its block was less voltage-dependent. Between −130 and −60 mV the cord conductances, taken as a measure of the voltage-dependent block, were 2.5 for 5HT and 1.4 for spiperone (Fig. 6*B*). Moreover, the time course of the AcCho current decay was accelerated more by 5HT than by spiperone. At low AcCho concentrations, the decay was sometimes drastically accelerated by 5HT and clearly decayed with two time contants, whereas the control current decayed with only one exponential time course.

DISCUSSION

Our results show that serotonergic agents block the AcCho currents of neuronal nAcChoRs in a noncompetitive and

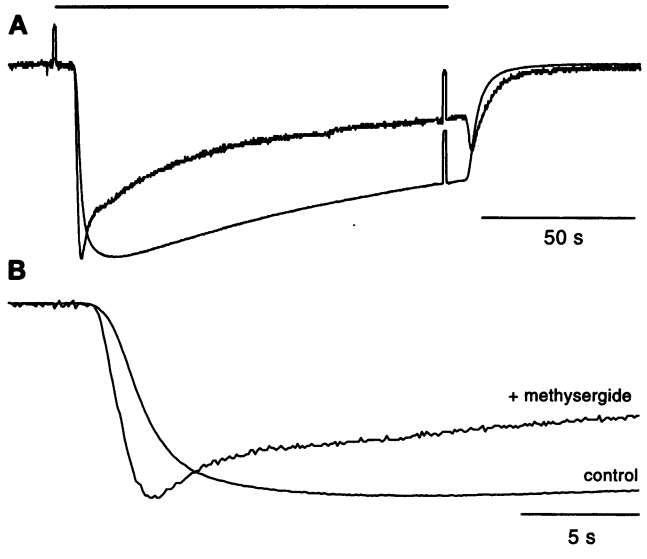

FIG. 4. Effect of methysergide on the time course of the AcCho current. AcCho and methysergide were both at 100 μM and the currents were scaled to the same peak amplitude. (*A*) Responses to AcCho alone and with methysergide. (*B*) Same records expanded.

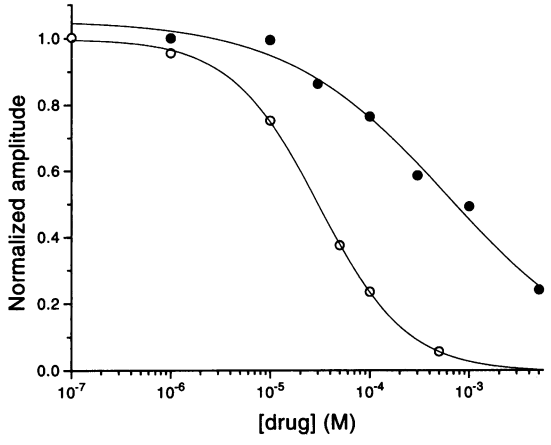

FIG. 5. Inhibition of AcCho currents by 5HT (●) and spiperone (○). The currents were normalized to the amplitude of the currents obtained with AcCho alone. Data were obtained from two different oocytes. The AcCho concentration was 100 μM for inhibition with 5HT and 10 μM for inhibition with spiperone.

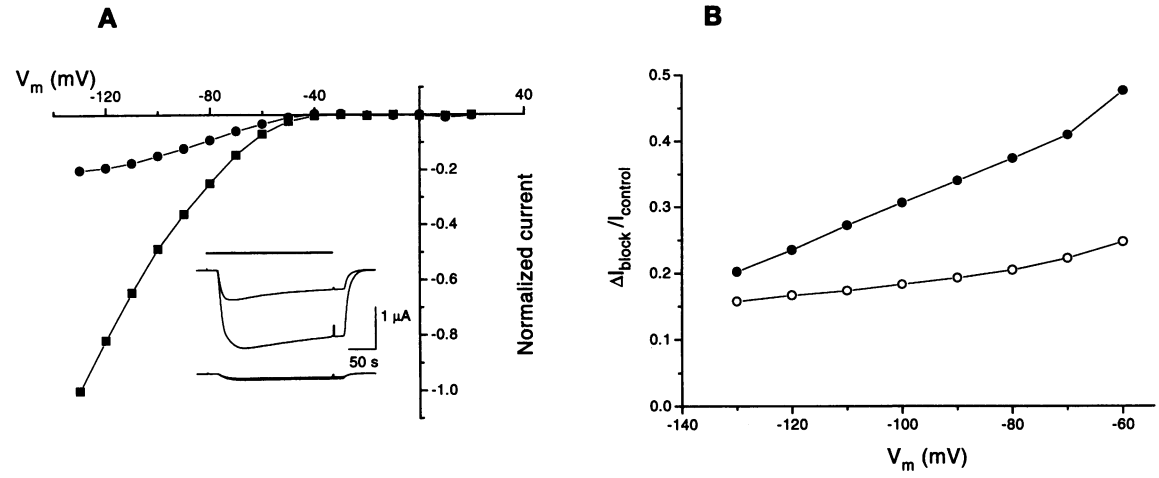

FIG. 6. (*A*) Current–voltage relation of the AcCho current evoked by 100 μM AcCho alone (■) or together with 100 μM 5HT (●). Each point represents the peak current elicited by AcCho when the potential was clamped at the potentials indicated. The responses were normalized to the current obtained with AcCho alone at −130 mV. (*Inset*) Current block by 5HT with the oocyte clamped at −120 mV (upper pair of records) and at −40 mV (lower pair). In this example, the current was elicited by 5 μM AcCho and blocked with 50 μM 5HT. (*B*) AcCho currents blocked by 5HT (●) or spiperone (○) at various holding potentials and normalized to their corresponding control response. The AcCho current was elicited by 5 μM AcCho and blocked by 100 μM 5HT or spiperone.

voltage-dependent manner. A common feature of the effect of 5HT on both muscle and neuronal cells is the reduction of their responses to AcCho (3, 4, 15–18), and various mechanisms have been proposed to explain the inhibitory action of 5HT on nAcChoRs. For example, it has been suggested that 5HT antagonizes the neuronal nAcChoRs in a competitive manner (16), that it acts through a cAMP-dependent mechanism (19), or that it interacts noncompetitively with AcCho on muscle and neuronal nAcChoRs (4, 18). Whatever is the mechanism involved, 5HT has similar potency for inhibiting AcCho currents in a wide variety of cells. The IC_{50} for blockage of AcCho responses by 5HT was ≈100 μM in chick ciliary ganglion neurons (17), C2 myotubes (4), muscle receptors expressed in oocytes (ref. 4 and unpublished results), in bovine chromaffin adrenal cells (18), and in α2β4 neuronal nAcChoRs.

The effect of serotonergic agents on neuronal nAcChoRs is clearly not mediated by 5HT receptors, because the oocyte membrane rarely has 5HT receptors (R. M., unpublished results) and because the application of up to 5 mM 5HT did not evoke appreciable ionic currents. Furthermore, agonists and antagonists of different 5HT receptor subtypes affected the AcCho currents in the same manner, and none of them produced the oscillatory currents characteristic of the activation of 5HT receptors expressed in oocytes (20). Thus, our results show that not only 5HT but also other serotonergic agents act directly on nAcChoRs, much in the same way that 5HT affects the nAcChoRs in other cells (4, 16, 18).

The effects of 5HT on nAcChoRs were consistent with a noncompetitive inhibition, because the block was relatively independent of the agonist concentration. Thus, serotonergic agents may bind to the same site with different affinities or may bind to multiple sites, but more work is required to discriminate between these possibilities. Nevertheless, it is interesting that neuronal and muscle nAcChoRs differ structurally in their subunit composition. The neuronal receptors are formed by one or two different types of subunits, whereas the muscle receptors are formed by four different types of subunits (for review, see ref. 21). Although the binding site(s) for serotonergic agents on nAcChoRs is still not identified, our results suggest that in neuronal nAcChoRs the site is located in the α or β subunits, or in the α/β interface, because of the molecular composition of the neuronal receptors. Therefore, to explain the inhibitory effect of 5HT on muscle nAcChoRs, the simplest possibility is that 5HT is binding to the same site as in neuronal receptors.

Our results also show that the serotonergic agents accelerated the decay of AcCho currents of neuronal nAcChoRs. For muscle nAcChoRs it was previously observed that the amplitude of the single-channel currents was not affected in the presence of 5HT, suggesting that the ionic permeability was not altered; but the frequency of channel openings, as well as the mean burst duration, was reduced (4). All this, together with the voltage-dependent block of AcCho currents by 5HT or spiperone, is consistent with a noncompetitive block and with a block of the open channel or an increase in the rate of desensitization (4, 6–8, 10, 11, 22, 23). The voltage dependence suggests that serotonergic agents interact with the nAcChoRs in the vestibule of the channel or within the channel itself. The different voltage dependences of the 5HT and spiperone blockages could be due to the existence of multiple binding sites for serotonergic agents. If that is the case, our results suggest that the binding site for 5HT is closer to the internal end of the channel than the binding site for spiperone.

The action of 5HT on muscle and neuronal nAcChoRs seems to be a general phenomenon. 5HT exerts similar effects on mouse myotubes, human TE671/RD cells, and muscle and *Torpedo* nAcChoRs made up of α, β, γ or ε, and δ subunits (4). Also, 5HT modulates the responses to AcCho of sympathetic ganglion cells (17), bovine chromaffin cells (18), and α2β4 nAcChoRs. Thus we may conclude that 5HT, which is an endogenous molecule, acts as a neurotransmitter on specific receptors and may also have an important modulatory function at nAcChoRs in a wide variety of neurons. Moreover, the effects of serotonergic agents on neuronal nAcChoRs occur at such concentrations that they need to be considered when we try to understand the normal and pathological processes of synaptic transmission, as well as the mode of action of the many serotonergic agents presently used to alleviate brain malfunctions.

We are very grateful to Drs. J. Boulter and S. Heinemann for providing the neuronal nAcChoR subunit clones, Victor Ramirez for assistance during the preparation of RNAs, Rico Miledi for computer programming, and Drs F. Eusebi and R. O. Arellano for help with the manuscript. This work was supported by National Institutes of Health Grant NS23284.

1. Cooper, E., Couturier, S. & Ballivet, M. (1991) *Nature (London)* **350,** 235-238.
2. Anand, R., Conroy, W. G., Schoepfer, R., Whiting, P. & Lindstrom, J. (1991) *J. Biol. Chem.* **266,** 11192-11198.

3. Colomo, F., Rahamimoff, R. & Stefani, E. (1968) *Eur. J. Pharmacol.* **3,** 272-274.
4. Grassi, F., Polenzani, L., Mileo, A. M., Caratsch, C. G., Eusebi, F. & Miledi, R. (1993) *J. Neurosci. Res.* **34,** 562-579.
5. Livett, B. G., Kozousek, V., Mizobe, F. & Dean, R. M. (1979) *Nature (London)* **278,** 256-257.
6. Clapham, D. E. & Neher, E. (1984) *J. Physiol. (London)* **347,** 255-277.
7. Katz, B. & Miledi, R. (1978) *Proc. R. Soc. London B* **203,** 119-133.
8. Trautmann, A. (1982) *Nature (London)* **298,** 272-275.
9. Carter, A. A. & Oswald, R. E. (1993) *Biophys. J.* **65,** 840-851.
10. Adams, P. R. & Feltz, A. (1980) *J. Physiol. (London)* **306,** 261-281.
11. Charnet, P., Labarca, C., Leonard, R. J., Vogelaar, N. J., Czyzyk, L., Gouin, A., Davidson, N. & Lester, H. A. (1990) *Neuron* **2,** 87-95.
12. Miledi, R. (1982) *Proc. R. Soc. London B* **215,** 491-497.
13. Vernino, S., Amador, M., Luetje, C. W., Patrick, J. & Dani, J. (1992) *Neuron* **8,** 127-134.
14. Costa, A. C. S., Patrick, J. W. & Dani, J. A. (1994) *Biophys. J.* **67,** 395-401.
15. Magazanik, L. G., Illes, P. & Snetkov, V. A. (1976) *Dokl. Acad. Nauk. SSSR* **227,** 1968 (abstr.).
16. Akasu, T. & Koketsu, K. (1986) *J. Physiol. (London)* **380,** 93-109.
17. Schmid, H. A. & Vijayaraghavan, S. (1992) *Neurophysiology* **31,** 1001-1008.
18. Vijayaraghavan, S., Schmid, H. A. & Mapp, K. S. (1993) *J. Neurochem.* **61,** 324-331.
19. Turapev, T. M., Yurchenko, O. P. & Grigoriev, N. G. (1987) *Cell. Mol. Neurobiol.* **7,** 381-389.
20. Miledi, R., Parker, I. & Sumikawa, K. (1989) *Fidia Research Foundation Neuroscience Award Lectures:1987–1988* (Raven, New York), Vol. 3, pp. 57-90.
21. Sargent, P. B. (1993) *Annu. Rev. Neurosci.* **16,** 403-443.
22. Neher, E. & Steinbach, J. H. (1978) *J. Physiol. (London)* **277,** 153-176.
23. Stallcup, W. & Patrick, J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 634-638.