Muscarinic receptor activities potentiated by desensitization of nicotinic receptors in rat superior cervical ganglia

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KEY WORDS nicotinic receptors; muscarinic receptors; desensitization; nicotine; oxotremorine; superior cervical ganglion; patch-clamp techniques

ABSTRACT

AIM: The influences of desensitized nicotinic acetylcholine receptors (nAChR) on the activities of muscarinic acetylcholine receptors (mAChR) were investigated in single cultured rat superior cervical ganglion. **METHODS:** Whole-cell patch-clamp techniques were used. **RESULTS:** An inward current was induced by nicotine 80 μmol/L in the sympathetic neurons and desensitized rapidly after the prolonged exposure to nicotine. An outward current was induced by oxotremorine 100 μmol/L or pilocarpine 100 μmol/L and it showed no desensitization after exposure to its agonists. After nAChR desensitized completely, the current evoked by oxotremorine was increased significantly compared with its control. There were (42±38) % (n=8, P<0.05) and (165±66) % (n=5, P<0.01) increases induced by 100 and 500 μmol/L oxotremorine, respectively. Similar results were also obtained from pilocarpine and the current evoked by 100 μmol/L pilocarpine increased by (66±33) % (n=6, P<0.05) after nAChR desensitization. Once nicotine was removed, nAChR recovered from desensitization gradually and the enhanced mAChR activity also subsided along with it. Furthermore, the facilitatory effect of desensitized nAChR on mAChR activity could be prevented by mecamylamine. **CONCLUSION:** The activities of mAChR to its agonists were potentiated by the desensitization of nAChR in rat sympathetic neurons.

INTRODUCTION

Nicotinic and muscarinic acetylcholine receptors are distinctively different and belong to ion channel-gated and GTP binding protein-coupled receptor superfamily respectively. But they have the same endogenous agonist acetylcholine (ACh) and mediate all physiological functions of cholinergic nervous system together[1,2]. It is reasonable to assume that there must be a close relationship between the two types of receptors.

The series of research works in our laboratory have indicated that the sensitivity of brain muscarinic receptor to its agonist was increased in nicotine tolerant animals, which were developed by acutely or chronically repeated administrations of nicotine[3,4]. Similar results were obtained in peripheral nervous system and other laboratory also had the parallel findings[5,6]. Now many papers have been published about the regulatory effects of nicotine on muscarinic receptors, and the novel hypothesis was proposed that muscarinic receptor activity could be modulated by the desensitized nicotinic receptors.

Sympathetic neuron in primary cell culture bears both nicotinic and muscarinic receptors, their currents are relatively simple and readily identified, so it seems like a good model for detailed studies in the possible

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modulating effects between nicotinic and muscarinic receptors. In this paper we tried to elucidate the influences of desensitized nicotinic receptors on the activities of muscarinic receptors in single rat superior cervical ganglion by whole-cell patch-clamp techniques.

MATERIALS AND METHODS

Cell culture Neonatal Wistar rats were sterilized by alcohol and decapitated[7]. Superior cervical ganglia were carefully isolated and cut into small pieces, which were then incubated in L-15 Leibovitz Medium containing 0.25 % trypsin at 36 °C. After digestion for 30-35 min, the tissue suspensions were centrifuged at 500×*g* for 2 min. The pellet was gently suspended in DMEM containing 10 % horse serum and triturated with a fire-polished Pasteur pipette. The dissociated neurons were plated onto 35-mm culture dishes coated with poly-*L*-lysine (Sigma, 25 mg/L). Each dish was added 50 μL nerve growth factor. They were grown in a 95 % air-5 % CO_2 incubator at 37 °C. In the present experiment, the neurons were cultured for 5 to 8 d before electrophysiological recording.

Current recording Currents were recorded with standard whole-cell patch-clamp technique[8]. The extracellular bathing solution contained (in mmol/L): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 3, HEPES 10, glucose 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mmol/L): KCl 140, $MgCl_2$ 2, HEPES 10, egtazic acid 10, K_2ATP 5, adjusted to pH 7.3 with KOH. Before the experiment, the culture medium was replaced with the extracellular solution. Under the inverted microscope, the microelectrode was moved to the membrane surface of a neuron with a hydraulic micromanipulator. When the giga seal was formed between the tip of the microelectrode and the membrane, a swift pulse of suction was applied to the microelectrode interior to rupture the membrane and a whole-cell recording configuration was established. Data were collected by Axopatch 200B amplifier and analyzed by pCLAMP7.0 (Axon Instruments, Foster City, CA, USA). All experiments were performed at room temperature (20-25 °C).

Application of drugs All drugs were applied by a puff pipette that was connected to a pneumatic pressure ejector (Picospritzer II, Parker Hannifin Co) and N_2 pressure was adjusted to about 50-60 kPa. The puff pipette consisted of 3 microtubes and usually 2 of them were filled with different drugs. The diameter of a microtube was about 8-10 μm and the distance between the pipette and the recording neuron was 20-30 μm. Unless specifically indicated, the interval time between every application was 3 min and we detected that 3-min interval was long enough for nicotinic receptors recovering from the desensitization. After each application, the drug pipette must be moved out of the extracellular solution at once in order to avoid a possible diffusion of the drugs. In all experiments the cell membrane potential was held at -70 mV. Nicotine, mecamylamine, oxotremorine, pilocarpine, and atropine were all purchased from Sigma Chemical Corporation.

Statistical analysis The all evoked currents were measured by a computer program of pCLAMP7.0. The software of Origin 5.0 was conducted for statistical analysis and graphic display. One-way ANOVA and paired *t* test were performed for significant difference test. All data were expressed as mean±SD and *n* represented number of neurons.

RESULTS

Nicotinic and muscarinic current in superior cervical ganglia An inward current was elicited rapidly by application of nicotine 80 μmol/L to a neuron for 1 s (Fig 1 Part Ia). When the application time was prolonged to 30 s, the evoked current reached the peak amplitude instantaneously and then declined gradually, despite the continued presence of nicotine (Fig 1 Part Ib). It could not maintain in the apex during the whole period of drug application and after it disappeared completely, the subsequent 1-s nicotine stimulation failed to produce any detectable current, indicating that the nicotinic receptors were desensitized.

When a mixture of 80 μmol/L nicotine and different concentrations of mecamylamine was puffed to the neuron, the evoked nicotinic currents were depressed obviously. They were depressed by (36±4) %, (52±7) %, and (88±4) % by mecamylamine 0.1, 1, and 10 μmol/L, respectively. The differences between the inhibitory rates were significant and showed that mecamylamine blocked nicotinic receptors in a concentration-dependent manner in sympathetic neurons.

An outward current was elicited by application of muscarinic receptor agonist oxotremorine 100 μmol/L to a neuron for 1 s (Fig 1 Part IIa). When oxotremorine was continuously applied for 10 s or 20 s, the current was sustained with a little increase resulting from the rising local drug concentration and no obvious desensitization was observed (Fig 1 Part IIb, c). The evoked current was completely blocked in the pres-

ence of atropine 1 μmol/L. Likewise, another muscarinic receptor agonist pilocarpine 100 μmol/L evoked the similar response during prolonged application.

Response of mAChR after nAChR desensitization A puff pipette with 2 microtubes was used to drug application. One was filled with 80 μmol/L nicotine and the other filled with 100 or 500 μmol/L oxotremorine. Nicotine was puffed to a neuron for 30 s continuously resulting in the rapid desensitization of nicotinic receptors, and then oxotremorine was applied to the same neuron and the responses of mAChR were observed. It was found that the current elicited by 100 μmol/L oxotremorine was increased by (42±38) % (n=8, P<0.05) after nAChR desensitization compared with the evoked current on normal conditions (Fig 2 Part Ia, b). This effect was more prominent when 500 μmol/L oxotremorine was applied and there were (165±66) % (n=5, P<0.01) increases in contrast to its corresponding control (Fig 2 Part II). Similar results were also obtained from another muscarinic receptor agonist pilocarpine and the current induced by 100 μmol/L pilocarpine was enhanced by (66±33) % (n=6, P<0.05) compared with its control. These results indicated that the activity of muscarinic receptors to its agonists was potentiated after the desensitization of nicotinic receptors.

Response of mAChR in course of recovery of nAChR from desensitization Nicotinic receptor desensitization was evoked by successive application of nicotine for 30 s, and then the further brief pulses of nicotine (1 s) were followed at various intervals (5, 30, 60, or 90 s) to determine the recovery of nicotinic receptors from desensitization (Fig 3). It was found that when nicotine was just removed, the desensitized nicotinic receptors failed to respond to the subsequent nicotine stimulation (Fig 3a). But as time going on, they recovered from desensitization gradually and accordingly, the evoked currents were increased step by step (Fig 3 b-d). Similar results were consistently observed from 3 cells. These phenomena indicated that once agonist was removed, the sensitivity of desensitized nicotinic receptors would recover in a time- dependent manner. As we observed, an interval about 3 min between each application of nicotine seemed to be enough for full recovery of all nicotinic receptors.

The responses of muscarinic receptors were observed during the period of recovery of nicotinic receptors. Under normal conditions, because muscarinic receptors showed no desensitization, the currents elicited by repetitive application of oxotremorine within a short time were almost unchanged (Fig 4 Part Ia). But after nicotinic receptor desensitization, oxotremorine-induced outward current, which had been elevated before, was decreased gradually and the supersensitivity of muscarinic receptors also subsided along with the recovery of nicotinic receptors (Fig 4 Part Ib). The

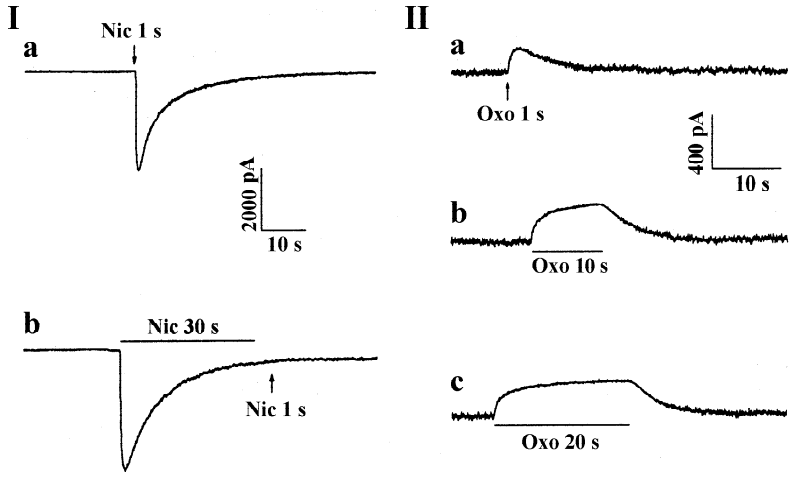

Fig 1. Current evoked by nicotine and oxotremorine in cultured rat superior cervical ganglia. The cell membrane potentials were held at –70 mV. Part I: (a) An inward current induced by application of nicotine 80 μmol/L for 1 s indicated by the arrow; (b) Nicotine was continuously applied for 30 s during the period indicated by horizontal bar and the evoked current declined rapidly despite the continued presence of nicotine. One second pulse of nicotine was then followed and failed to evoke any current, suggesting that the nicotinic receptors were completely desensitized at this moment. Part II: Typical outward currents induced by oxotremorine in the same sympathetic ganglion. Oxotremorine 100 μmol/L was continuously applied for 1 s (a), 10 s (b), and 20 s (c), respectively and no obvious desensitization was observed.

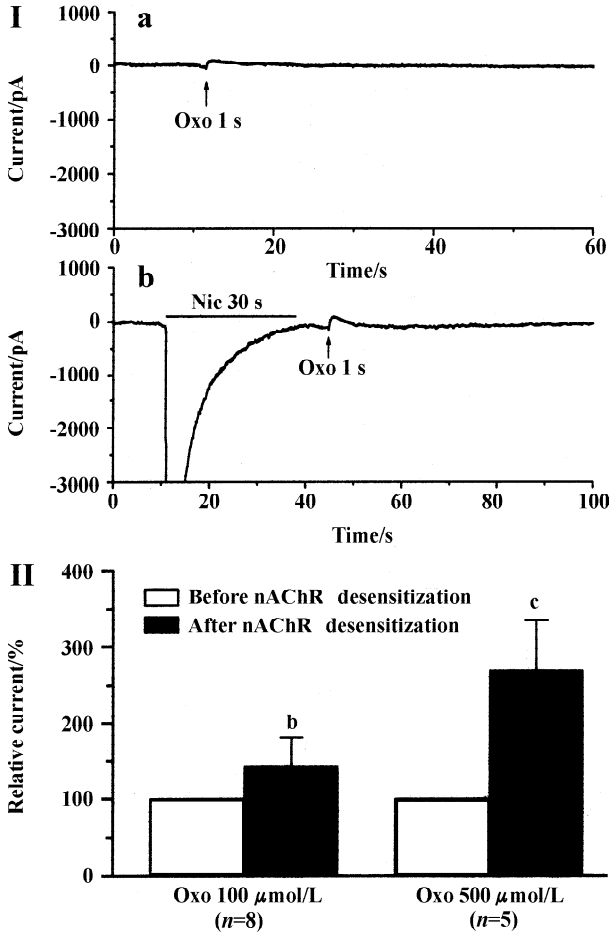

Fig 2. Currents induced by oxotremorine before and after the desensitization of nAChR. Part I: Representative traces of current recording. Traces in (a) and (b) were obtained from the same neuron. (a) Current evoked by application of oxotremorine 100 μmol/L for 1 s on normal conditions. (b) Current evoked by oxotremorine 100 μmol/L after the desensitization of nAChR. Nicotine 80 μmol/L was continuously applied for 30 s during the period indicated by horizontal bar and resulted in the rapid desensitization of nAChR. Oxotremorine added to the bath for 1 s evoked an outward current with larger amplitude. Part II: The current induced by oxotremorine 100 and 500 μmol/L respectively before and after the desensitization of nAChR. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

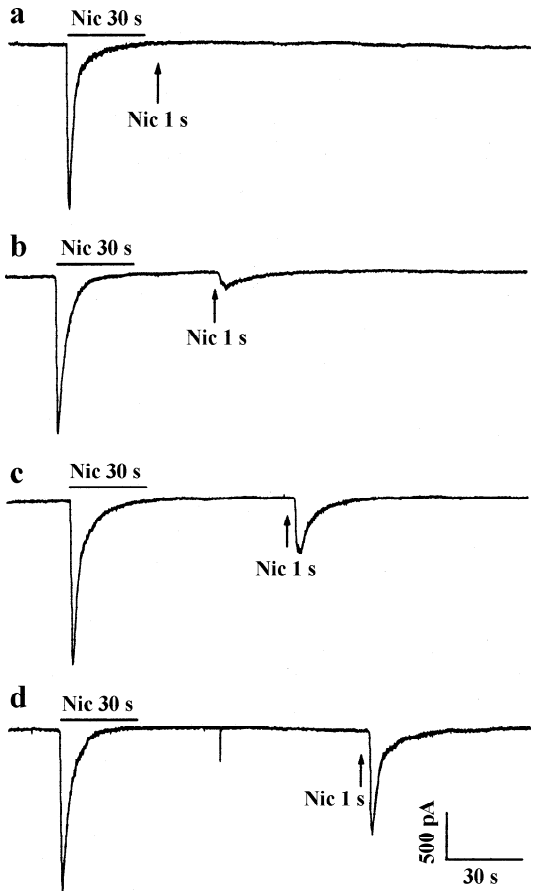

Fig 3. Time-dependent recovery of nicotinic receptors from desensitization revealed by repeated application of nicotine. All these graphs were obtained from the same neuron and intervals between different recordings were more than 3 min. Desensitization was evoked by application of nicotine 80 μmol/L for 30 s during the periods indicated by horizontal bars, and then further brief pulses of nicotine (1 s) were followed at various intervals (a: 5 s; b: 30 s; c: 60 s; d: 90 s) to detect the recovery of nicotinic receptors from desensitization. The holding potentials were all kept at -70 mV.

data obtained from six cells demonstrated that after nicotinic receptor desensitization, as time going on, the increased muscarinic receptor activity was reduced step by step and nearly restored to the normal level finally (Fig 4 Part II).

These results indicated that the desensitization of nicotinic receptors was the prerequisite of the hypersensitivity of muscarinic receptors. So once nicotinic receptors recovered from desensitization, the muscarinic receptor hypersensitivity also disappeared.

Response of mAChR after nAChR blockade

The effect of nicotinic receptor antagonist mecamylamine on the increased activity of muscarinic receptors were investigated. Firstly, the mixture of 80 μmol/L nicotine and 10 μmol/L mecamylamine was puffed to a neuron for 30 s and nearly no current was induced because of the blocking effect of mecamylamine on nicotinic receptors. And then 500 μmol/L oxotremorine was applied and it was found that the evoked current only increased (31±26) % (n=8, P<0.05) compared with its control, which was much lower than that seen after nAChR desensitization [(165±66) %, Fig 5]. This result implied that the facilitatory effects of nicotinic receptor desensitization on muscarinic receptor activ-

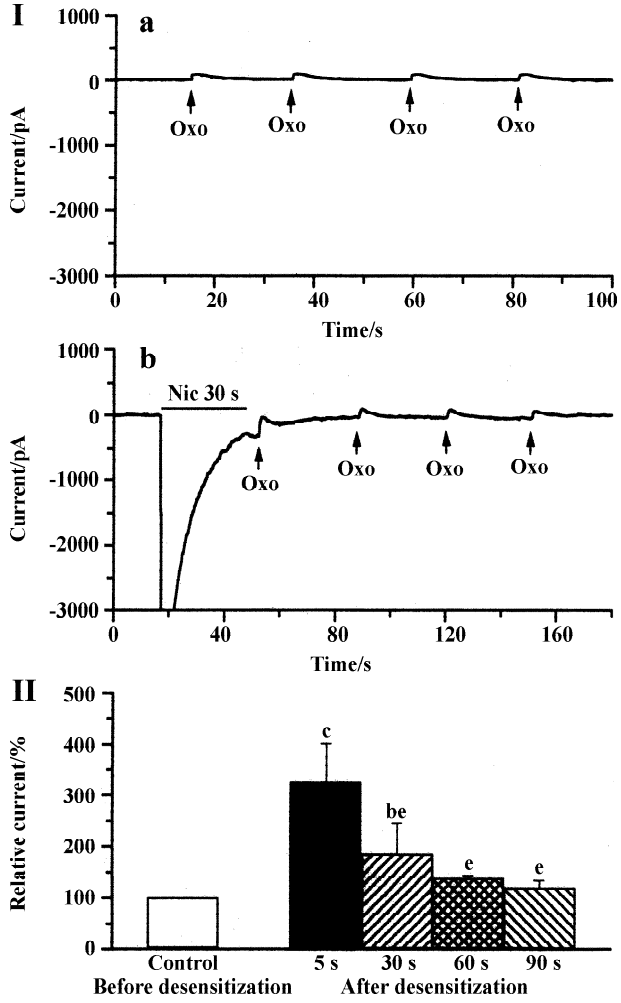

Fig 4. Currents induced by oxotremorine 500 μmol/L during repeated application before and after the desensitization of nicotinic receptors. Part I : (a) Oxotremorine-induced currents during 1-s repeated application within a short time on normal conditions; (b) The desensitization of nicotinic receptors was evoked by continuous application of nicotine 80 μmol/L for 30 s during the period indicated by horizontal bar, and then oxotremorine was repeatedly applied at different intervals. Part II: Time-dependent recovery of oxotremorine-induced current after the desensitization of nicotinic receptors. *n*=6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* the control. $^{e}P<0.05$ *vs* the group of 5 s. Tukey's test after one-way ANOVA.

ity could be prevented by mecamylamine. So it was reasonable to suggest that the desensitized nicotinic receptors were responsible for the hypersensitivity of muscarinic receptors in sympathetic neurons.

DISCUSSION

It is well known that nicotinic receptor is an allosteric protein which at least possesses three discrete and interconvertible conformations, including the resting

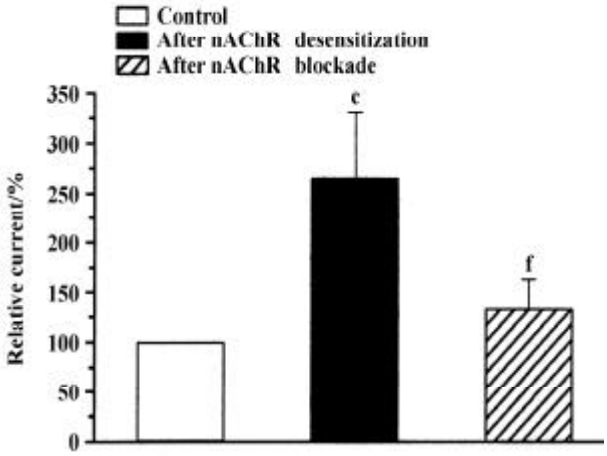

Fig 5. Current induced by oxotremorine 500 mmol/L after the desensitization or blockade of nAChR evoked by the continuous application of nicotine or mecamylamine respectively. *n*=5-8. Mean±SD. $^{c}P<0.01$ *vs* the control. $^{f}P<0.01$ *vs* the group after nAChR desensitization. Tukey's test after one-way ANOVA.

state, the active state, and the desensitized state. Desensitization is triggered by prolonged or repeated exposure to agonists and results in the inactivation of its receptor channel that does not allow for the passage of any ions. In this form it exhibits a higher affinity for agonists than the resting and active states, but the agonist cannot induce the receptor activation[9,10]. Desensitization is an essential intrinsic molecular property of nicotinic receptors and has been suggested to play an important role in nicotine dependence and withdrawal symptoms of smokers[11]. But the physiological significance of nicotinic receptor desensitization is not yet fully understood at present.

The phenomenon of desensitization is clearly seen in single rat superior cervical ganglion by use of whole-cell patch-clamp technique in our experiment. When nicotine was continuously applied, the evoked current disappeared gradually and failed to show any response to subsequent agonist stimulation, indicating that the nicotinic receptors were completely desensitized. But exactly during this period, the current evoked by oxotremorine or pilocarpine was increased markedly compared with the normal control, suggesting that the activities of muscarinic receptors to its agonists were enhanced. Furthermore, once the agonist was removed, desensitized nicotinic receptors recovered gradually and at the same time, the increased muscarinic receptor activity subsided along with it. These results firstly provided the direct evidences that it was the desensi-

tized nicotinic receptors to evoke the enhanced activities of muscarinic receptors, which implied that desensitization did not mean that nicotinic receptor was in a nonfunctional state and it might play some potential roles through increasing muscarinic receptor activity.

Our previous work found that preincubation of the membrane fraction derived from rat cerebral cortex with nicotine led to a decrease in the dissociation constant for muscarinic receptors binding to [^{3}H]oxotremorine-M and an increase for binding to its antagonist [^{3}H]QNB, while the maximal binding value was unchanged[12]. These results indicated that nicotine pretreatment increased the affinity of brain muscarinic receptors to its agonist, decreased the affinity to its antagonist, and had no effects on the amount of muscarinic receptors. This might imply that chronic nicotine treatment could change the conformation of muscarinic receptors, and by this way increased its sensitivity to agonists.

Moreover, we also found that the enhanced activity of muscarinic receptors after nicotinic receptor desensitization could be prevented by mecamylamine, which blocked nicotinic receptor activation in sympathetic neurons. It is likely that the desensitized state of nicotinic receptors was necessary for increased muscarinic receptor activity. Therefore, if the desensitization did not come into being, for example, it was blocked by mecamylamine, the muscarinic receptor activity would remain unchanged.

It is well known that chronic exposure to nicotine produces desensitization of nicotinic receptors and induces tolerance to many of the drug behavioral and physiological effects. This prolonged desensitization has been proposed as the potential mechanism of the tolerance to nicotine and nicotine tolerance may be the behavioral manifestations of nicotinic receptor desensitization[13]. Recently it was reported that mecamylamine could prevent the development of tolerance to nicotine in rats[14], which suggested that pharmacological blockade of receptor function was not equivalent to repeated agonist treatment in producing receptor desensitization and mecamylamine could block the desensitized state formation of nicotinic receptors. This was consistent with our present findings in some sense and it appeared that mecamylamine might just by this way antagonized the increased activity of muscarinic receptors.

The possible modulatory mechanisms of desensitized nicotinic receptors on muscarinic receptor activity maybe exist not only in receptor level, and nicotine may act upon muscarinic signal transduction at a site downstream of the receptor. Whether this regulation is through some intracellular factor, such as enzyme or Ca^{2+}, still requires for further detailed studies.

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