0026-895X/96/010001-06$3.00/0

MOLECULAR PHARMACOLOGY, 49:1–6 (1996).

ACCELERATED COMMUNICATION

Agonist Responses of Neuronal Nicotinic Acetylcholine Receptors Are Potentiated by a Novel Class of Allosterically Acting Ligands

ANDRE SCHRATTENHOLZ, EDNA F. R. PEREIRA, ULRICH ROTH, KARL-HEINZ WEBER, EDSON X. ALBUQUERQUE, and ALFRED MAELICKE

Laboratory of Molecular Neurobiology, Institute of Physiological Chemistry and Pathobiochemistry, Johannes-Gutenberg University Medical School, Duesbergweg 6, D-55099 Mainz, Germany (A.S., U.R., K.-H.W., A.M.), Department of Pharmacology and Experimental Therapeutics, University of Maryland Medical School, Baltimore, Maryland 21201 (E.F.R.P., E.X.A.), and Laboratory of Molecular Pharmacology II, Federal University of Rio de Janeiro, Brazil (E.F.R.P, E.X.A.)

Received August 14, 1995; Accepted September 21, 1995

SUMMARY

Similar to the γ-aminobutyric acid$_A$ receptor and the *N*-methyl-D-aspartate subtype of glutamate receptor, neuronal nicotinic acetylcholine receptors are subject to positive modulatory control by allosterically acting ligands. Exogenous ligands such as galanthamine and the neurotransmitter 5-hydroxytryptamine, when applied in submicromolar concentrations with nicotinic agonists, significantly increase the frequency of opening of nicotinic receptor channels and potentiate agonist-activated currents. Because these effects have been shown to be blocked by the monoclonal antibody FK1, they are mediated by binding sites that are located on α subunits of nicotinic receptors and distinct from those for acetylcholine and acetylcholine-competitive ligands. At higher concentrations, the potentiating effect of these ligands decreases and is eventually overcome by an inhibition of the agonist-induced response. The sensitizing actions of galanthamine, 5-hydroxytryptamine, and related compounds, at submicromolar concentrations, may reflect the existence of cross-talk between adjacent neuroreceptors and synapses in the central nervous system and thus suggests the formation of transiently active chemical networks in the vertebrate brain.

The activity of many ionotropic neuroreceptors of the vertebrate brain is subject to modulation by ligands other than the natural transmitter. For instance, benzodiazepines increase the probability of GABA$_A$ receptor channel opening in response to GABA (1), and glycine must be bound to NMDA receptors to enable full activation by glutamate (2, 3). Additional modulatory ligands affect the amplitude and kinetics of response of these receptors (4–7), suggesting that multiple modes of modulatory control are essential elements of neurotransmission in the brain. Supporting this notion, neuronal nicotinic acetylcholine receptors are also subject to positive modulatory control by allosterically acting ligands (8–11). We have recently demonstrated that physostigmine, Gal, codeine, and structurally related compounds are able to induce single-channel activity of nAChRs in PC12 pheochromocytoma cells and in cultured neurons (8–11). This action is exerted via a binding site on the nAChR α-polypeptide that is distinct from the site for acetylcholine (12, 13), and therefore the above compounds constitute a new class of nicotinic ligands, which we have called NCAs (8). We also demonstrated that the single-channel activity induced by an NCA does not summate to sizable macroscopic responses (8–10), suggesting that the primary action of NCAs is not channel activation but rather a possible allosteric modulation of the nAChR activity induced by the natural agonist (8, 14). This prompted us to test whether NCAs can modify the nicotinic responses induced by ACh and ACh-competitive agonists. For this purpose, we chose two cell culture systems: PC12 pheochromocytoma cells and primary cultures of embryonic hippocampal neurons. Both are suitable models for the study of nicotinic

This work was supported by German Science Foundation Grants Ma 599/17–2 and Ma 599/18–1 (A.M.), Fonds der Chemischen Industrie, Bayer AG (Leverkusen), the European Community, the NMFZ of the Johannes-Gutenberg University, National Institutes of Health Grants NS25296 and ES05730 (E.X.A.), and the U.S. Army Medical Research Institute of Chemical Defense Contract DAMD 17-95-C-5063.

ABBREVIATIONS: ACh, acetylcholine; AnTX, (+)-anatoxin *a*; Gal, galanthamine; meGal, 1-methyl-galanthamine; 5-HT, 5-hydroxytryptamine; nAChR, nicotinic acetylcholine receptor; NCA, noncompetitive agonist; GABA$_A$, γ-aminobutyric acid type A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

neurotransmission (15–19) as they express several neuronal nAChR isoforms (19, 20). As we report here for the exogenous NCA Gal and the putative endogenous NCA 5-HT, these compounds, when applied in submicromolar concentrations with agonist, potentiate the agonist response of neuronal nAChRs to a similar extent as has been reported for the action of benzodiazepines on the agonist response of $GABA_A$ receptors.

Materials and Methods

PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 150 IU/ml penicillin, 150 mg/ml streptomycin, 2 mM L-glutamine, and 15% heat-inactivated fetal calf serum at 37° in a humidified atmosphere of 5% CO_2/95% air. Before electrophysiological measurements, the cells were plated onto glass coverslips in

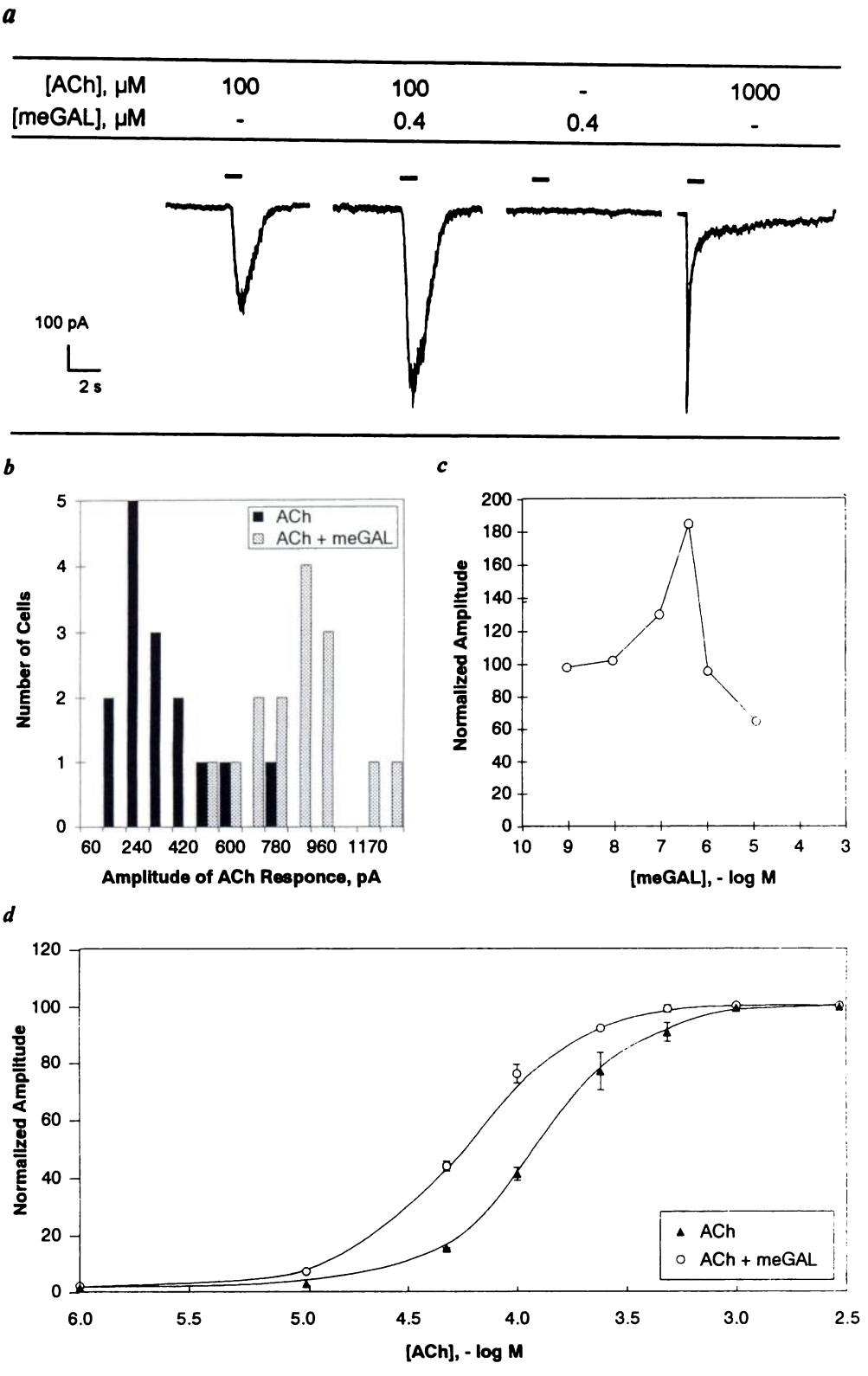

Fig. 1. Potentiation by meGal of ACh-elicited responses of cultured PC12 cells. a, Responses recorded from a single PC12 cell of bipolar morphology from a 3-day-old culture. The response to 100 µM ACh, in the absence of meGal (*first trace*), was nearly doubled in peak amplitude (*second trace*) when the same concentration of ACh was applied simultaneously with 0.4 µM meGal. At the same concentration, meGal alone did not produce a significant whole-cell response (*third trace*). The augmented response resembled in maximal amplitude, but not in the kinetics, the response to 1 mM ACh in the absence of meGal (*fourth trace*). b, Amplitude distribution of whole-cell responses to 100 µM ACh from 15 PC12 cells of the same culture in the absence (*filled bars*) and presence (*open bars*) of 0.4 µM meGal. The presence of meGal induced a shift in the average response amplitude from 375 to 815 pA. c, Modulation of peak amplitude of ACh (100 µM)-elicited currents versus the concentration of meGal applied. The average amplitudes of whole-cell recordings from three cells of the same culture dish were plotted versus the NCA concentration applied. The potentiating effect of meGal was limited to concentrations of <1 µM. d, Effect of meGal (0.4 µM) on the dose-response relationship for ACh. The averaged amplitudes of whole-cell currents recorded from three cells in one culture dish were plotted versus each concentration of ACh in the absence (▲) and presence (○) of meGal. The maximal amplitudes measured were ~1 nA; they were normalized to 100.

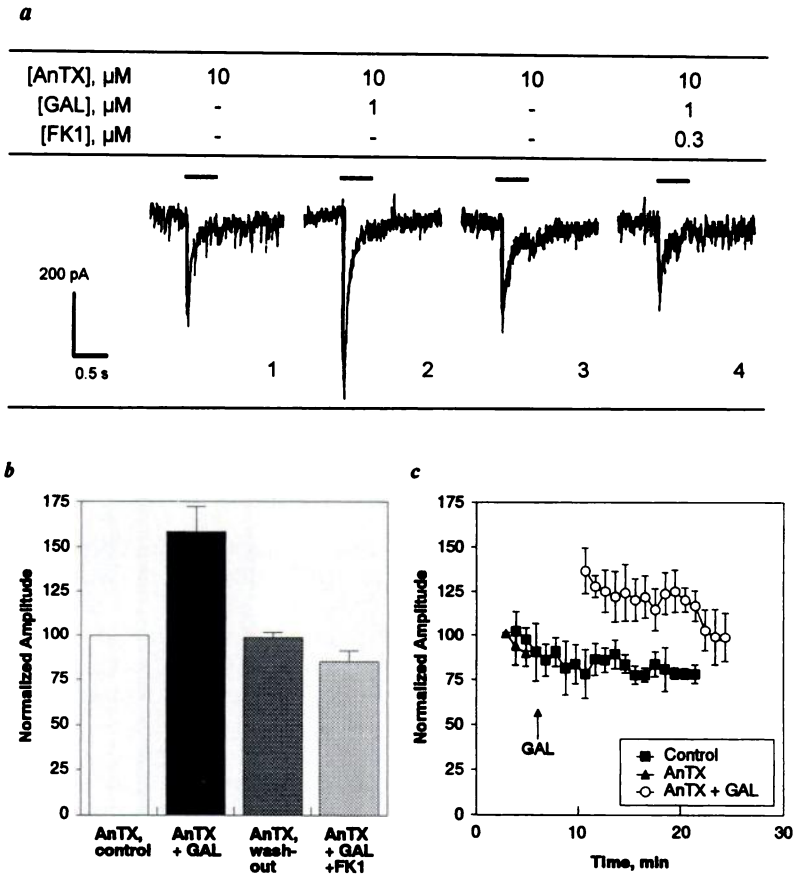

Fig. 2. Potentiation by Gal of AnTX-evoked α-bungarotoxin-sensitive nicotinic currents in cultured hippocampal neurons. a, Effects of Gal (1 μM) on α-bungarotoxin-sensitive currents evoked by a 500-msec pulse application of AnTX (10 μM) via the 250-μm aperture at the apex of a U-shaped tube onto a cultured hippocampal neuron. The first response of the cells to AnTX (10 μM) was recorded ~15 min after the patch was obtained, at which time the rate of rundown of the peak current amplitude is negligible. Immediately after the control record was obtained (*first trace*), the cell was superfused with Gal (1 μM)-containing external solution, the latter being applied directly to the neuron via a straight tube that was coassembled with the U-shaped tube. Under these conditions, the potentiating effect of Gal on the response to AnTX was observed within 1 min after the start of the perfusion (*second trace*). When the neuron was then superfused for 2 min with Gal-free external solution, the amplitude of the AnTX-evoked current returned to the previous level (*third trace*). The neuron then was superfused for 14 min with FK1 (dilution 1:100)-containing external solution, followed by superfusion for 1 min with external solution containing FK1 (dilution 1:100) and Gal (1 μM), after which the current evoked by an admixture of AnTX (10 μM), FK1 (dilution 1:100), and Gal (1 μM) was recorded from the cell (*fourth trace*). Notice that preincubation of the cell with FK1 prevented the potentiating effect of Gal. b, Quantification of the effects of Gal on AnTX-elicited currents in the absence or presence of FK1. The peak amplitude of the nicotinic currents elicited by the first application of AnTX to the cells was considered 100%, and the peak amplitudes of the currents elicited thereafter were normalized to this control. Perfusion of the cells (four experiments) with Gal-containing external solution for 1 min increased the peak amplitude of the currents elicited by the admixture of AnTX (10 μM) and Gal (1 μM) by 59 ± 13% over the control responses. Washing of the cells with Gal-free external solution for 2 min reversed the effect of Gal on type IA currents. After a 14-min perfusion of the cells with FK1-containing external solution, followed by a 1-min perfusion of the cells with external solution containing FK1 and Gal, application of the admixture of Gal and AnTX to the cells elicited currents whose peak amplitudes were approximately the same as those obtained under control condition. c, Time-dependent decrease of the peak amplitude of AnTX-evoked currents in cultured hippocampal neurons. ■, The rundown profile of the responses evoked by 500-msec pulses of AnTX (10 μM) applied to seven neurons (each value and error bar represent mean ± standard error, respectively). ▲, Average rundown profile of the AnTX-evoked currents recorded from four different cells before exposure to Gal. ○, Time-dependent changes in the effect of Gal (1 μM) on the AnTX-evoked currents recorded from those four cells (each value and error bar represent mean ± standard error, respectively). In these experiments, Gal was applied to the cells via the background perfusion. After beginning the exchange of the bath solution with Gal-containing external solution begins, it takes ~5 min for the potentiating effect of Gal to become maximal, and this effect disappears with time.

a microtiter plate (density: 0.7×10^4 cells/ml) and cultured for 3 days. For whole-cell and single-channel recordings, the external bath solution (pH 7.4, 275 mOsM) consisted of 124 mM NaCl, 3.25 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM D-glucose, and 10 mM HEPES, and the internal pipette solution (pH 7.2, 280 mOsM) consisted of 120 mM CsF, 10 mM CsCl, 10 mM Cs-EGTA, and 10 mM HEPES. For whole-cell recordings, nAChR ligands were dissolved in external solution and applied via a U-shaped tube (21) positioned ~100 μm from the cell. For single-channel recordings, the ligands were dissolved in the external solution and applied to the patches via a perfusion system consisting of a double-barrel glass θ tube that was pulled and bent to the appropriate shape (22). The patch-pipette resistance was 5–7 MΩ. After a high resistance seal was formed on a PC12 cell, capacitance transients were minimized using the C-fast facility of the EPC-7 patch-clamp system. All experiments were performed at room temperature and at a holding potential of −70 mV. To prevent accumulation of the test compounds in the bath, the cells were superfused with the external bath solution at a rate of 1.5–3.0 ml/min. Data were analyzed using the pClamp 6.0 software package (version 6.0, Axon Instruments, Foster City, CA).

Neurons dissociated from the hippocampi of fetal rats were cultured as described previously (23). The fetuses were removed from pregnant rats (16–18 gestation days) and killed by cervical dislocation under CO_2 narcosis. For electrophysiological experiments, the

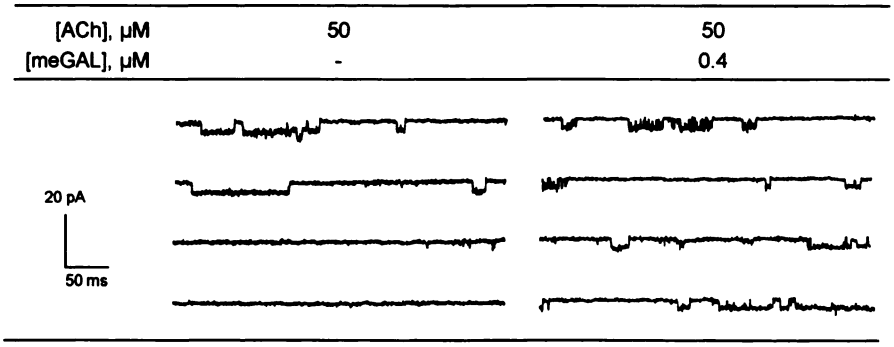

Fig. 3. Sample recordings of single-channel currents activated by ACh in the absence and presence of meGal. Recordings are from excised patches of PC12 cells that were cultured for 3 days on glass coverslips (see Fig. 1 legend). Data shown were filtered at 2.15 kHz. The populations of current amplitudes activated by 50 μM ACh (*left*) and by the concerted action of 50 μM ACh and 0.4 μM meGal (*right*) were of the same amplitude (2.1 ± 0.1 pA). At this concentration of ACh, the initial level of single-channel activity decreases with time due to desensitization. The major effects of meGal were an increase in the frequency of single-channel events evoked by ACh and a decrease in the rate of desensitization. Notice also the presence of noncompetitive nAChR-channel blockade (*flickering*) by meGal in the latter recordings.

composition of the external bath solution was 165 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, and 10 mM dextrose (pH 7.3, osmolarity 340 mOsM). The composition of the internal solution was 60 mMCsCl, 60 mM CsF, 10 mMEGTA, and 10 mM HEPES (pH 7.3, osmolarity 330 mOsM), and to prevent to a great extent the rundown of the nicotinic currents, 20 mM phosphocreatine, 50 units/ml creatine phosphokinase, and 5 mM ATP were added to the solution. Whole-cell, patch-clamp experiments were performed with hippocampal neurons cultured for 21–29 days. The patch pipettes were pulled from borosilicate capillary glass and had tip diameters ranging from 2 to 4 μm. The resistance of the patch pipettes, when filled with internal solution, was between 3 and 5 MΩ. Whole-cell currents were induced by fast application of AnTX (10 μM) to the neurons using a glass U-shaped tube positioned ~100 μm from the cell and recorded by an LM-EPC-7 patch system (List Electronics, Darmstadt, Germany). The signals were filtered at 3 kHz and directly sampled and analyzed by a microcomputer with the pClamp program.

Results and Discussion

The electrophysiological studies described below were performed with PC12 cells of bipolar morphology, which at the culture conditions applied is the predominant cell type (8). In Fig. 1a, the whole-cell responses of a single cultured PC12 cell to 100 μM ACh are shown in the absence and presence of 0.4 μM meGal. In the presence of meGal, a ~2-fold increase in response to ACh was observed (increase in amplitude from 380 to 740 pA), with the resulting response resembling in amplitude the response evoked by 1 mM ACh in the absence of meGal. Although NCAs are capable of activating the nAChR channel in the absence of ACh or ACh-competitive agonist (8–11), it is unlikely that the increase in peak amplitude of ACh-evoked nicotinic currents could be due to a mere summation of nAChR channel activity induced by ACh and NCA because at the concentrations of NCA at which the potentiation of ACh responses was observed, NCAs do not induce substantial single-channel activity or whole-cell currents (Fig. 1a) (8–10). Due to the very low probability of the NCA-activated channels being in the open state, they generally do not summate to give rise to substantial macroscopic responses, even at higher concentrations of NCA (8, 9). Fig. 1b shows the amplitude distributions of whole-cell responses from 15 randomly chosen PC12 cells studied under the same conditions as described above. The average potentiation by meGal of the ACh-induced response again was ~2-fold. The potentiating effect was limited to rather low concentrations of meGal (Fig. 1c). Above 0.4 μM meGal, the potentiating effect tended to decrease and was eventually overcome by an inhibition of the ACh-induced response. For a given concentration of meGal (e.g., 0.4 μM; Fig. 1d), potentiation of ACh-induced response was most pronounced at the low and intermediate range of the dose-response curve, and it gradually decreased as the maximal response was approached. The potentiating effect of meGal on the ACh response is likely to be due to an allosteric sensitization of the nAChR. Because the anti-nAChR antibody FK1 blocked the potentiating effect of meGal (not shown) without affecting the currents elicited by ACh (8), meGal probably exerted its action via the previously identified NCA binding site (12, 13).

Fig. 2 illustrates the ability of Gal to potentiate α-bungarotoxin-sensitive nicotinic currents in cultured hippocampal neurons and antagonism of this effect by the anti-nAChR antibody FK1 (9, 13). To limit the present study to only one of the three or more distinct types of whole-cell currents induced by nicotinic agonists in hippocampal neurons (18, 19), the alkaloid dihydro-β-erythroidine (0.1 μM) was added to the bathing solution (18, 23). Under these conditions, rapid application of the agonist AnTX (10 μM) to cultured hippocampal neurons consistently elicited fast desensitizing whole-cell currents (Fig. 2a), which are typical type IA responses (18). When the neurons were perfused with Gal (1 μM)-containing extracellular solution, the response of the cells to fast application of an admixture of AnTX (10 μM) and Gal (1 μM) was ~60% greater than the response of the cells to AnTX before their exposure to Gal (Fig. 2, a and b). In addition, exposure of the neurons to Gal prolonged the decay time constant of the AnTX-evoked currents from 23.6 ± 1.9 to 30.2 ± 2.1 msec (mean ± standard error from four experiments). Because the decay phase of type IA currents reflects mostly desensitization (19, 24), the present finding suggests that Gal might alter the rate of desensitization of α7-bearing neuronal nAChRs that give rise to these currents. After a 15-min incubation of the neurons with saturating concentrations of the antibody FK1, Gal was unable to potentiate the nicotinic currents evoked by AnTX (Fig. 2, a and b). As shown in Fig. 2c, the effect of Gal decreased with the time of exposure of the neurons to this agent. The potentiating effect peaked ~5 min after application of Gal-containing external solution and progressively decreased during the following 15 min. This slow

onset of the sensitizing action of Gal, which is not illustrated in the figure, requires further elucidation. However, the blockade by FK1 of Gal-induced sensitization suggests that it is not an indirect effect, as has been reported for the enhancement by dopamine of kainate-activated currents in cultured embryonic chick motoneurons (25) and for the enhancement by vasoactive intestinal peptide of ACh-activated nicotinic currents in chick ciliary ganglion neurons (26). Eventually, the amplitude of the response to admixtures of Gal and AnTX approached the typical amplitude of AnTX-induced responses, considering their normal rundown in cultured hippocampal neurons. This finding is in agreement with a previous report that Gal could desensitize its action on the nAChR (9). Taken altogether, these findings suggest that Gal can allosterically modulate nicotinic responses of hippocampal neurons in a manner similar to that shown above for PC12 pheochromocytoma cells.

It is highly unlikely that the NCA-induced potentiation of nicotinic responses was related to inhibition of acetylcholinesterase activity by the NCA because (i) the expression levels of acetylcholinesterase in PC12 cells are very low (5–8 nmol/min/mg protein; Ref. 27), and the potentiating effect of meGal remained the same when it was applied after complete inhibition of acetylcholinesterase activity by phenylmethyl-sulfonylfluoride (data not shown); (ii) the experiments with hippocampal neurons (Fig. 2) were performed with the cholinesterase-insensitive nonester agonist AnTX; and (iii) physostigmine, a cholinesterase inhibitor more potent than meGal (8), is much less potent than meGal in potentiating nicotinic responses in the PC12 cells (data not shown).

On the single-channel level, the potentiation by NCA of the responses to ACh was observed as an increase in the frequency of channel opening, including a higher probability of simultaneous opening of several channels (Fig. 3). After application of 50 μM of ACh to excised patches of PC12 cells, there was an immediate burst of single-channel activity that tended to decrease as a result of desensitization (Fig. 3, *left*). In the presence of meGal (0.4 μM), we observed a significant increase in the frequency of single-channel events, the rate of desensitization was decreased, and there was evidence of a noncompetitive blocking action of the NCA (Fig. 3, *right*). The latter findings agree with the previous reports that NCAs could reduce desensitization of nAChR channels (9, 10) and that they directly block the agonist-activated channels at elevated concentrations (8, 9).

These data indicate that the action of NCAs on agonist responses of PC12 cells and hippocampal neurons resembled, in essence, the actions of benzodiazepines on $GABA_A$ receptors. NCAs potentiated the agonist responses (figs. 1–3), probably by allosterically enhancing the affinity of transmitter binding (Fig. 1d), and they slowed agonist-induced channel desensitization (figs. 1a, 2c, and 3). Although these actions of exogenous NCAs are interesting in their own right, we have also begun to investigate whether endogenous ligands with the properties of NCAs might exist. As a working hypothesis, neurotransmitters or neurohormones of other receptors could act as endogenous NCAs of nicotinic receptors, thereby permitting response integration and cross-talk between adjacent receptors and synapses. As Fig. 4 demonstrates, when 5-HT was applied simultaneously with the natural agonist, it produced an NCA-like potentiation of the ACh-evoked whole-cell response of PC12 cells. The response of the PC12 cells to ACh (50 μM) was increased by ~60% when the ACh-containing external solution was supplemented with 5-HT (0.1 μM). At this concentration, 5-HT alone was unable to evoke significant whole-cell response (Fig. 4; see also Ref. 28), and the potentiating effect of 5-HT was largely eliminated in the presence of high concentrations of the antibody FK1 (data not shown). In agreement with previous studies (29, 30), the concentration range at which 5-HT acted to potentiate ACh responses was well below that at which fast noncompetitive blocking of the nAChR channels was observed (Fig. 4). Although a potentiating action of submicromolar concentrations of 5-HT on nicotinic agonist-evoked responses was also observed with cultured hippocampal neurons with AnTX used as agonist, the variable size and slow onset of the effect require further elucidation. Taken together, our findings are consistent with a dual action of 5-HT on neuronal nicotinic receptors: an allosteric enhancement of the sensitivity to agonists at submicromolar concentrations and a noncompetitive blocking action of the nAChR channel at higher concentrations.

Conclusions

When applied at submicromolar concentrations with agonist, Gal, meGal, and the neurotransmitter 5-HT potentiated the response to agonist of neuronal nAChRs (Figs. 1–4), whereas at higher concentrations the same ligands acted as noncompetitive inhibitors of the agonist response. The potentiating action was inhibited by the anti-nAChR antibody FK1, which is known to interact with a site in the extracellular region of the nAChR α subunit that is distinct from the

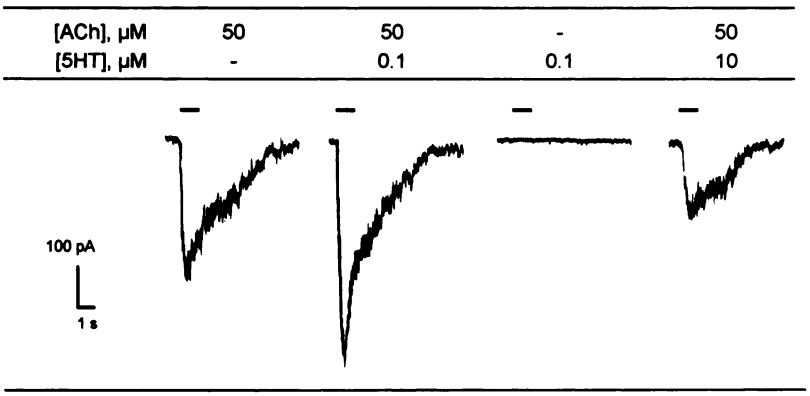

Fig. 4. Potentiation by the neurotransmitter 5-HT of the ACh responses of PC12 cells. Application of the same experimental protocol as described for Fig. 1a, with ACh at a concentration of 50 μM and 5-HT (0.1 μM) instead of meGal, the response to ACh in the absence of 5-HT (*first trace*) was increased by 67% (*second trace*) when 5-HT (0.1 μM) was applied simultaneous with ACh. At the same concentration, 5-HT alone did not produce a significant whole-cell response (*third trace*). At higher concentrations, 5-HT produced fast noncompetitive blocking action of the nAChR channels (*fourth trace*).

site of of ACh (13). 5-HT may not be the only endogenous ligand that is capable of allosterically enhancing (or decreasing or both) nicotinic agonist responses, and such modes of regulatory control may be a common feature of ligand-gated ion channels of the vertebrate brain. The multiplicity of regulatory ligands and binding sites identified for the ionotropic receptors of ACh, glutamate, and GABA suggest the existence of chemical networks that overlay and communicate with the neuronal network formed by synaptic contacts. In support of this notion, the potentiating action of nicotinic NCAs was observed at relatively low concentrations (submicromolar range) that are typical for many messenger molecules in the brain. NCA-mediated potentiation of ACh-evoked responses, therefore, may be the nicotinic counterpart of a regulatory mechanism that is a general characteristic of ionotropic neuroreceptors. Exogenous ligands with the capability of potentiating nicotinic responses may be useful in the treatment of nicotinic cholinergic deficits, as have been reported to be associated with several degenerative brain diseases (14).

References

1. Kusama, T., C. E. Spivak, P. Whiting, V. L. Dawson, J. C. Schaeffer, and G. R. Uhl. Pharmacology of GABA receptors expressed in *Xenopus* oocytes and COS cells. *Br. J. Pharmacol.* **109:**200–206 (1993).
2. Johnston, J. W., and P. Ascher. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature (Lond.)* **325:**529–531 (1987).
3. Hollmann, M., and S. Heinemann. Cloned glutamate receptors. *Annu. Rev. Neurosci.* **17:**31–108 (1994).
4. Macdonald, R. L., and R. E. Twyman. Biophysical properties and regulation of $GABA_A$ receptor channels. *Semin. Neurosci.* **3:**219–230 (1991).
5. Olsen, R. W., D. M. Sapp, M. H. Bureau, D. M. Turner, and N. Kokka. Allosteric actions of central nervous system depressants including anesthetics on subtypes of the inhibitory γ-aminobutyric $acid_A$ receptor-chloride channel complex. *Ann. N. Y. Acad. Sci.* **625:**145–154 (1991).
6. Rock, D. M., and R. L. Macdonald. The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. *Mol. Pharmacol.* **41:**83–88 (1991).
7. Seeburg, P. H. The TINS/TIPS lecture. The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci.* **16:**359–365 (1993).
8. Storch, A., A. Schrattenholz, J. C. Cooper, E. M. Abdel Ghani, O. Gutbrod, K.-H. Weber, S. Reinhardt, C. Lobron, B. Hermsen, V. Soskic, E. F. R. Pereira, E. X. Albuquerque, C. Methfessel, and A. Maelicke. Physostigmine, galanthamine and codeine act as noncompetitive nicotinic agonists on clonal rat pheochromocytoma cells. *Eur. J. Pharmacol.* **290:**207–219 (1995).
9. Pereira, E. F. R., S. Reinhardt, A. Schrattenholz, A. Maelicke, and E. X. Albuquerque. Identification and functional characterization of a new agonist site on nicotinic acetylcholine receptors of cultured hippocampal neurons. *J. Pharmacol. Exp. Ther.* **265:**1474–1491 (1993).
10. Pereira, E. F. R., M. Alkondon, S. Reinhardt, A. Maelicke, X. Peng, J. Lindstrom, P. Whiting, and E. X. Albuquerque. Physostigmine and galanthamine characterize the presence of the novel binding site on the α4β2 subtype of neuronal nicotinic acetylcholine receptor stably expressed in fibroblasts cells. *J. Pharmacol. Exp. Ther.* **270:**768–778 (1994).
11. Maelicke, A., T. Coban, A. Schrattenholz, B. Schröder, S. Reinhardt, A. Storch, J. Godovac-Zimmermann, C. Methfessel, E. F. R. Pereira, and E. X. Albuquerque. Physostigmine and neuromuscular transmission. *Ann. N. Y. Acad. Sci.* **681:**140–154 (1993).
12. Schrattenholz, A., J. Godovac-Zimmermann, H.-J. Schäfer, and A. Maelicke. Photoaffinity labeling of *Torpedo* acetylcholine receptor by physostigmine. *Eur. J. Biochem.* **216:**671–677 (1993).
13. Schröder, B., S. Reinhardt, A. Schrattenholz, K. E. McLane, A. Kretschmer, B. M. Conti-Fine, and A. Maelicke. Monoclonal antibodies FK1 and WF6 define two neighboring ligand binding sites on *Torpedo* acetylcholine receptor α-polypeptide. *J. Biol. Chem.* **269:**10407–10416 (1994).
14. Maelicke, A., H. Schröder, and A. Schrattenholz. Modulatory control by noncompetitive agonists of nicotinic cholinergic neurotransmission in the central nervous system. *Semin. Neurosci.* **7:**103–114 (1995).
15. Ifune, C. K., and J. H. Steinbach. Rectification of acetylcholine-elicited currents in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* **87:**4794–4798 (1990).
16. Mathie, A., S. G. Cull-Candy, and D. Colquhoun. Conductance and kinetic properties of single nAChR channels in rat sympathetic neurons. *J. Physiol. (Lond.)* **439:**717–750 (1991).
17. Ifune, C. K., and J. H. Steinbach. Inward rectification of acetylcholine-elicited currents in rat pheochromocytoma cells. *J. Physiol. (Lond.)* **457:** 143–165 (1992).
18. Alkondon, M., and E. X. Albuquerque. Initial characterization of nAChRs in rat hippocampal neurons. *J. Receptor Res.* **11:**1001–1021 (1991).
19. Alkondon, M., S. Reinhardt, C. Lobron, B. Hermsen, A. Maelicke, and E. X. Albuquerque. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons: II. Run-down and inward rectification of agonist-elicited whole-cell currents and *in situ* hybridization studies. *J. Pharmacol. Exp. Ther.* **271:** 494–506 (1994).
20. Boulter, J., A. O'Shea-Greenfield, R. M. Duvoisin, J. G. Conolly, E. Wada, A. Jensen, P. D. Gardner, M. Ballivet, E. S. Deneris, D. McKinnon, S. Heinemann, and J. Patrick. α3, α5, and β4: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. *J. Biol. Chem.* **265:**4472–4482 (1990).
21. Spitzer, K., and J. H. B. Bridge. A simple device for rapidly exchanging solutions surrounding a single cardiac cell. *Am. J. Physiol.* **256:**C441–C447 (1989).
22. Fenwick, E. M., A. Marty, and E. Neher. A patch-clamp study of bovine chromaffin cells and their sensitivity to acetylcholine. *J. Physiol. (Lond.)* **331:**577–597 (1982).
23. Alkondon, M., and E. X. Albuquerque. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons: I. Pharmacological and functional evidence for distinct structural subtypes. *J. Pharmacol. Exp. Ther.* **265:** 1455–1473 (1993).
24. Castro, N. G., and E. X. Albuquerque. The α-bungarotoxin-sensitive hippocampal nicotinic receptor channel has a high calcium permeability. *Biophys. J.* **68:**516–524 (1995).
25. Smith, D. O., D. Lowe, R. Temkin, and H. Hatt. Dopamine enhances glutamate-activated currents in spinal motoneurons. *J. Neurosci.* **15:** 3905–3912 (1995).
26. Gurantz, D., A. T. Harootunian, R. Y. Tsien, V. E. Dionne, and J. F. Margiotta. VIP modulates neuronal nAChR function by a cAMP-dependent mechanism. *J. Neurosci.* **14:**3540–3547 (1994).
27. Greene, L. A., and A. Rukenstein. Regulation of AChE activity by NGF: role of transcription and dissociation on proliferation and neurite outgrowth. *J. Biol. Chem.* **256:**6363–6367 (1981).
28. Furukawa, K., N. Akaike, H. Onodera, and K. Kogure. Expression of 5-HT receptors in PC12 cells treated with NGF and 8Br-cAMP. *J. Neurophysiol.* **67:**812–819 (1992).
29. Grassi, F., A. M. Polenzani, C. G. Mileo, F. Caratsch, F. Eusebi, and R. Miledi. Blockade of nicotinic acetylcholine receptors by 5-hydroxytryptamine. *J. Neurosci. Res.* **34:**562–570 (1993).
30. Garcia-Colunga, J., and R. Miledi. Effects of serotonergic agents on neuronal nicotinic acetylcholine receptors. *Proc. Natl. Acad. Sci. USA* **92:** 2919–2923 (1995).

Send reprint request to: Alfred Maelicke, Phd.D., Laboratory of Molecular Neurobiology, Institute of Physiological Chemistry, Johannes-Gutenberg University Medical School, Duesbergweg 6, D-55099 Mainz, Germany.