Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8155–8159, November 1987
Neurobiology

Cyclic AMP regulates the proportion of functional acetylcholine receptors on chicken ciliary ganglion neurons

(nicotinic/acetylcholine sensitivity/patch clamp/monoclonal antibodies)

JOSEPH F. MARGIOTTA†, DARWIN K. BERG‡, AND VINCENT E. DIONNE†

Departments of †Pharmacology (M-036) and ‡Biology (B-022), University of California at San Diego, La Jolla, CA 92093

Communicated by Dan L. Lindsley, July 13, 1987

ABSTRACT **Previous studies have shown that the number of functional acetylcholine receptors (AcChoRs) on chicken ciliary ganglion neurons in culture is considerably smaller than the total number of AcChoRs detected on the neurons by labeled receptor probes. Here we use patch-clamp recording to show that a cAMP-dependent process enhances the AcCho response of the neurons by a mechanism likely to involve an increase in the number of functional AcChoRs. The increase occurs without requiring protein synthesis and without involving a detectable increase in the total number of AcChoRs on the cell surface measured with a labeled receptor probe. The results imply that the neurons have functional and nonfunctional pools of AcChoRs and that functional receptors can be recruited from intracellular receptors or from nonfunctional receptors on the cell surface by a cAMP-dependent process. A cAMP-dependent regulation of the number of functional neurotransmitter receptors would provide a reversible mechanism by which cell–cell interactions could modulate synaptic transmission in the nervous system.**

cAMP has been shown to regulate the number and gating properties of ion channels (1, 2). Examples include a decrease in the number of available serotonin-sensitive K^+ channels in *Aplysia* sensory neurons (3), an increase in the opening probability and average number of voltage-sensitive Ca^{2+} channels on cardiac myocytes (4–6), and an increase in the rate of desensitization of nicotinic acetylcholine receptor (AcChoR) channels in skeletal muscle (7, 8) and in *Torpedo* electric tissue (9). Recent studies with mammalian GH_3 pituitary tumor cells suggest that cAMP maintains a class of Ca^{2+} channels in a functionally available state (10). In each of these cases, the regulation is thought to result from phosphorylation of the ion channel or related membrane proteins by a cAMP-dependent protein kinase (9–12).

The role of cAMP-dependent phosphorylation in regulating neuronal AcChoRs has not been examined. Neuronal AcChoRs are similar to muscle and electric organ AcChoRs in permeation and kinetic properties (13, 14) and in certain types of modulation (15, 16). They differ, however, in aspects of pharmacology (references in ref. 14), recognition by antibodies (17, 18), and subunit structure (19, 20). AcChoRs from neuron and muscle also appear to be regulated differently. We have shown that the number of functional AcChoRs on chicken ciliary ganglion neurons can be reduced by a factor of 2–3 by chronic exposure to elevated K^+, without altering single-channel properties and without changing the total number of AcChoRs on the neuron surface (14, 21, 22). Moreover, functional receptors appear to represent at most 10% of the total AcChoRs on the neuron surface (14). These findings raise the possibility that neurons, unlike muscle, maintain functional and nonfunctional AcChoRs in the plasma membrane and that the ratio of the two is subject to regulation.

In this report we examine the effects of cAMP on the operation and number of AcChoR channels on chicken ciliary ganglion neurons in cell culture. A change in receptor operation would suggest modulation of a receptor property such as desensitization rate, agonist affinity, single-channel conductance, or opening probability. A change in the number of functional receptors might indicate a mechanism for interconverting receptors between functional and nonfunctional forms. The results support the latter possibility by showing that cAMP elevation causes a 2- to 3-fold increase in the AcCho response without appreciably changing receptor properties or the total number of AcChoRs on the neuron surface.

METHODS

Cell Culture. Cultures of dissociated ciliary ganglion neurons were prepared from 8-day chicken embryos and grown on glass coverslips for electrophysiological experiments or in 16-mm wells for antibody-binding studies as described (14, 21, 23). After 5–7 days, some cultures received culture medium for 3–48 hr supplemented with 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate, or 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) at 0.02–2.00 mM or with 3-isobutyl-1-methylxanthine (IBMX) at 1 mM either alone or in combination with 8-Br-cAMP. Culture media containing these reagents were passed through 0.22-μm filters (Millipore) before use. All reagents were obtained from Sigma, except for 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate, which was obtained from Boehringer Mannheim.

Electrophysiology. Coverslip cultures were bathed in a recording solution containing (in mM) 145.0 NaCl, 5.4 $CaCl_2$, 5.3 KCl, 0.8 $MgSO_4$, 5.6 glucose, 5.0 Hepes (pH 7.4), and 0.15% bovine serum albumin and examined with patch-clamp recording techniques as described (14). In some cases, 20–100 μM cAMP or cGMP (±2 mM MgATP) or 100 μM AMP was added to the standard patch pipette solution, which contained (in mM) 145.6 CsCl, 1.2 $CaCl_2$, 2.0 EGTA, 15.4 glucose, and 5.0 Na Hepes (pH 7.3). Neuronal AcCho sensitivity was assessed from the whole-cell current induced by rapid microperfusion with AcCho (5 μM–1 mM) dissolved in recording solution (14). In separate experiments, power density spectra (24) were obtained from the variance of whole-cell current fluctuations using a fast Fourier-transform method (14). Single-channel currents from AcChoRs were

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Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine; CH, cycloheximide; mAb, monoclonal antibody; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate.

recorded from outside-out patches and analyzed using methods similar to those described (14, 25). All numerical results are expressed as the mean ± SEM. Significance was determined using Student's *t* test.

Monoclonal Antibody (mAb) 35 Binding. To measure the total number of surface AcChoRs on ciliary ganglion neurons in culture, mAb 35 (26) was radioiodinated to a specific activity of 2–3 × 10^{18} cpm/mol and used in binding studies as described (21, 22). Nonspecific binding constituted 15–25% of total.

RESULTS

Incubation with cAMP Analogues. Ciliary ganglion neurons incubated with the membrane-permeant analogue 8-Br-cAMP together with the phosphodiesterase inhibitor IBMX displayed 2- to 3-fold larger peak currents in response to 100 μM AcCho than untreated controls (Fig. 1, Table 1). Little difference was observed in the time course of desensitization between treated and control neurons; as in muscle (27–29), the desensitization was well described by the sum of fast and slow exponential components. In most treated neurons, the slow component decayed somewhat faster (Table 1), whereas the amplitude of both components was enhanced to about the same degree. The same 2- to 3-fold difference in peak response was obtained when the AcCho-induced currents were corrected for rapid desensitization by extrapolating the current record back to the level at zero time, as described (14).

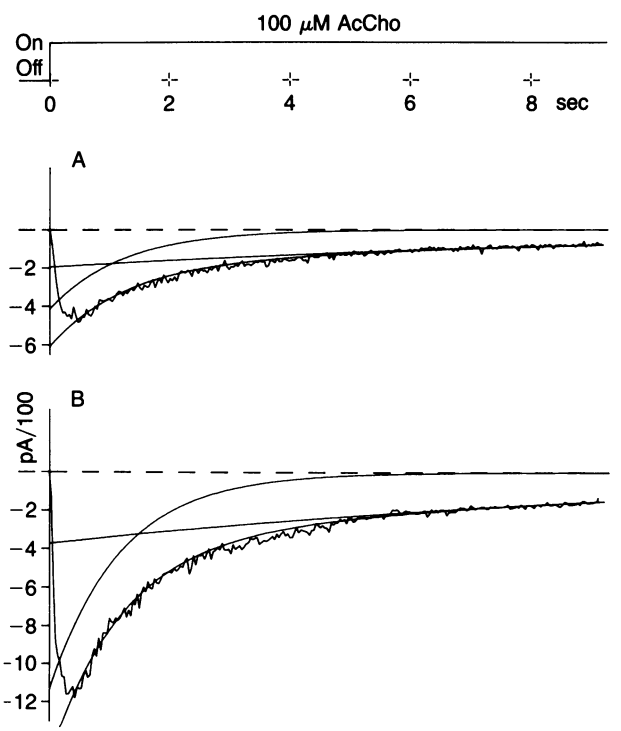

FIG. 1. Enhancement of the neuronal AcCho response by 8-Br-cAMP and IBMX. Records show whole-cell currents induced by 100 μM AcCho for a control neuron (*A*) and a neuron from the same plating pretreated with 2 mM 8-Br-cAMP and 1 mM IBMX at 37°C for 6 hr (*B*). The neurons were each voltage clamped at −70 mV and exposed to AcCho by microperfusion as indicated by the top template. Extrapolated peak whole-cell currents were −610 pA in *A* and −1550 pA in *B*. The fast and slow decaying exponential components of the currents (curves above records) and their sums (curves fit to records) were increased in the treated neuron, while the respective time constants (1.2 sec and 9.2 sec in *A* and 1.2 sec and 10.0 sec in *B*) were unchanged. Currents were filtered at 5 kHz and digitized at 500 μsec.

Table 1. 8-Br-cAMP + IBMX enhances whole-cell AcChoR currents and the slow rate of AcChoR desensitization

Culture medium supplement	I, pA	τ_f, sec	τ_s, sec	γ_a, pS	τ_b, msec
None	562	1.2	11.9	22	1.8
	± 50	± 0.1	± 0.1	± 4	± 0.2
	(25)	(25)	(25)	(13)	(13)
8-Br-cAMP + IBMX	1497	1.3	9.0	16	1.8
	± 108	± 0.1	± 0.4	± 3	± 0.2
	(24)	(24)	(24)	(15)	(15)

The extrapolated peak whole-cell currents (I) and the time constants of the fast (τ_f) and slow (τ_s) decaying components were obtained from neurons held at −70 mV using 100 μM AcCho. γ_a and τ_b were estimated from whole-cell current noise spectra obtained with 5 μM AcCho. Treated neurons were maintained in 2 mM 8-Br-cAMP + 1 mM IBMX for 6–48 hr. n values are given in parentheses. The differences in peak current and slow time constant of desensitization between treated and control neurons were significant ($P < 0.001$).

A near-maximal increase in the peak AcCho response was caused by 2 mM 8-Br-cAMP and 1 mM IBMX within 6 hr (net increase of 160 ± 16%, n = 17) and was not significantly changed by prolonging the treatment to 48 hr (net increase of 205 ± 33%, n = 11). Omitting IBMX slowed the enhancement, requiring incubations of 24 hr or longer in 8-Br-cAMP (0.2–2.0 mM) to achieve a 2-fold elevation of the AcCho response. The enhanced AcCho response was not preferentially abolished by internal perfusion of the cell. Even when large-bore recording pipettes (0.5–1.0 MΩ) were used to accelerate internal dialysis, the AcCho response declined by 30–50% during a 15-min recording period both in control neurons and in neurons pretreated with 8-Br-cAMP and IBMX.

Other membrane-permeant cAMP analogues, as well as IBMX alone, caused a similar increase in the AcCho response (Fig. 2*A*). The effect was specific for cAMP since it could not be produced by 8-Br-cGMP and was not accompanied by changes in resting membrane conductance, capacitance, or voltage-activated Na^+ currents.

Internal Perfusion with cAMP. The peak response to 100 μM AcCho was also enhanced above control levels in cases in which the recording pipette contained cAMP (Fig. 2*B*). In these acute exposures, the time elapsed between establishing the whole-cell configuration and the AcCho trial was 2–5 min, suggesting a short latency for the cAMP-dependent change from normal to high AcCho sensitivity. The amplitudes of fast and slow decaying components of the current induced by AcCho were enhanced by the treatment to about the same extent, and, again, the slow component decayed somewhat faster. No change in the AcCho response was seen when the intracellular solution contained AMP or cGMP instead of cAMP (Fig. 2*B*).

AcChoR Function. The enhanced AcCho response caused by incubation in cAMP analogues does not involve a change in AcChoR affinity, single-channel permeation, or kinetic parameters. The dose–response curves for control and treated neurons indicated similar apparent K_d values and Hill coefficients while showing a 2- to 3-fold difference in maximal response over the AcCho concentration range tested (Fig. 3). Whole-cell current fluctuations evoked by 5 μM AcCho were analyzed for information about possible cAMP-dependent changes in the apparent single AcChoR channel conductance and burst duration (14). The noise spectra obtained from control neurons and neurons pretreated with 8-Br-cAMP and IBMX for 6–48 hr were well-fit by single Lorentzian functions, which predicted nearly identical values for single AcChoR channel burst duration (τ_b) and apparent conductance (γ_a) (Table 1).

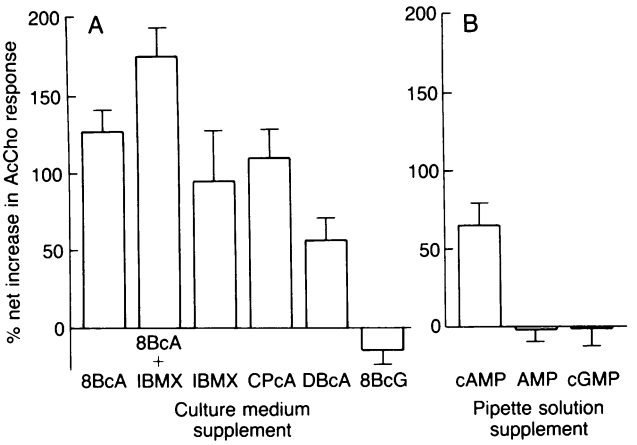

FIG. 2. Relative effectiveness of drugs applied externally (*A*) and internally (*B*) in causing increased AcCho sensitivity. (*A*) Neurons were incubated in the indicated drugs at the following concentrations: 8-Br-cAMP (8BcA), 2 mM; IBMX, 1 mM; 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPcA), 0.5 mM; N^6,2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate (DBcA), 1 mM. Values represent the mean net % change (±SEM; n = 6–34 neurons from two to eight separate experiments) in the extrapolated peak current response to 100 μM AcCho over an equivalent number of untreated controls from the same set of cultures; all increases were significant ($P < 0.02$). Incubation in 0.2 mM 8-Br-cGMP (8BcG) for 24 hr had no effect, whereas 0.2 mM 8-Br-cAMP enhanced the AcCho response 2-fold (not shown). (*B*) Whole-cell responses to 100 μM AcCho were obtained sequentially from neurons in two or three cultures using patch pipettes containing either normal filling solution (n = 32) or the same solution supplemented with 20–100 μM cAMP (n = 13), 50 μM AMP (n = 7), or 50 μM cGMP (n = 10). Results are expressed as the net % stimulation over the extrapolated peak AcCho current from neurons in the same culture obtained with normal filling solution. The AcCho response was enhanced by 66% ($P < 0.001$) when the pipettes contained cAMP, without a change in τ_f. τ_s was reduced from 12.5 ± 0.8 sec to 8.5 ± 0.8 sec ($P < 0.01$).

Because the increase in AcCho response persists with extended dialysis, any differences in single AcChoR channel properties attributed to cAMP analogues should be apparent in recordings from excised outside-out membrane patches. Single AcChoR channel currents recorded from soma membrane patches from 11 control and 5 treated neurons (Fig. 4 *A* and *B*) had reversal potentials of −8.4 ± 1.9 mV and −6.8 ± 1.4 mV, respectively, and single-channel conductances of 40.1 ± 1.6 pS and 41.4 ± 1.9 pS, respectively, revealing no significant difference in either parameter (Fig. 4*D*). The currents represent AcChoR channel openings since they were present only in patches perfused with AcCho. In some patches a smaller single-channel event (20–30 pS), comprising <5% of the openings, was also present (data not shown). We do not know if the smaller currents represent a second species of AcChoR, but their occurrence was not influenced by pretreatment with 8-Br-cAMP and IBMX.

The kinetic behavior of the 40-pS conductance AcChoR channels on the neurons was examined from the stochastic properties of the time intervals between closing and opening transitions in the single-channel current records, as described (14, 25). According to a simple model for AcChoR activation (30), the transitions of the receptor between closed and open states will be governed by closing and opening rate constants α and β, where α is the inverse of the mean channel open time (τ), β is related to the closed time distribution, and the opening probability $p_o = \beta/(\beta + \alpha)$. Estimates of α and β did not depend on assumptions about the number of AcChoRs in the patch (25). Interval distributions between successive opening and closing transitions from control neurons and

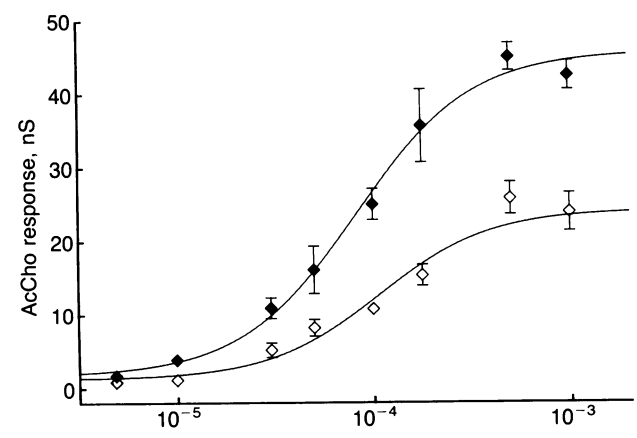

FIG. 3. The AcChoR dose–response relationship is not altered by cAMP analogues. Neurons were incubated either in standard culture medium (open symbols) or in culture medium containing 2 mM 8-Br-cAMP and 1 mM IBMX for 6–48 hr (filled symbols) and tested with 5 μM–1 mM AcCho. Values represent the mean ± SEM (n = 4–36 neurons from 2–16 separate culture sets) of the extrapolated maximum whole-cell conductances (G) given by $G = I/(V_h - V_r)$, where I is the extrapolated peak whole-cell current for each concentration, V_h is the holding potential, and V_r is the reversal potential (−8 mV). Curves were drawn by nonlinear, least squares regression and indicate similar apparent K_d values (85 and 100 μM) and Hill coefficients (1.5 and 1.6) for treated and control neurons, respectively.

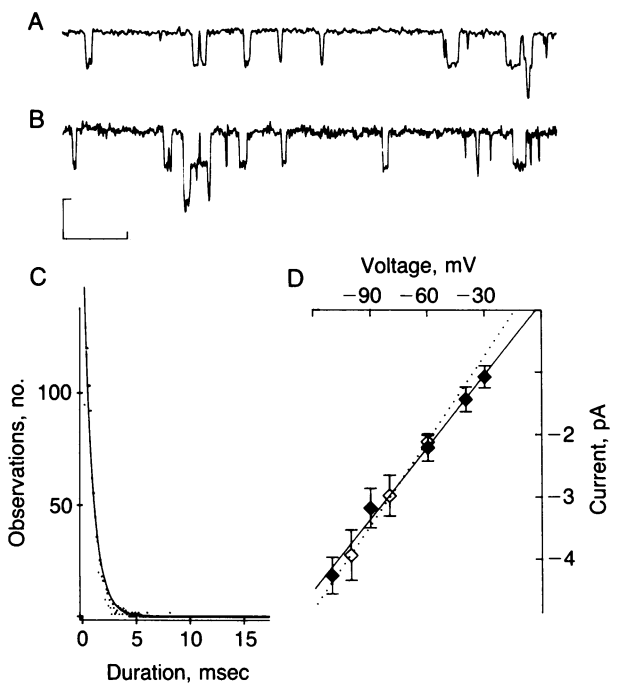

FIG. 4. Single AcChoR channel properties are not altered by treatment with cAMP analogues. Recordings were obtained from outside-out patches of soma membrane excised from control neurons (*A*) and neurons pretreated with 8-Br-cAMP and IBMX (*B*) during exposure to 5 μM AcCho. One major class of single AcChoR channel current is revealed in both cases. Currents were digitally sampled at 100 μsec and low-pass filtered at 2 kHz in *A* and 5 kHz in *B*. Holding potentials were −80 mV in *A* and −90 mV in *B*. Calibration: 4 pA, 10 msec. (*C*) Open duration distribution for the single-channel currents depicted in *B*. Bin width: 100 μsec. The distribution is fitted with an exponential function predicting a mean channel open time of 0.9 msec, identical to that expected for untreated neurons. (*D*) Current–voltage plots for the single-channel currents in *A* (open symbols) and *B* (filled symbols) are similar, revealing single-channel conductances of 45 and 39 pS and extrapolated reversal potentials −13 and −3 mV.

those pretreated with 8-Br-cAMP and IBMX were similar and fit the predictions of the model equally well. In excised patches from four control neurons and four treated neurons no differences were detected in τ (0.93 ± 0.12 msec and 0.82 ± 0.37 msec, e.g., Fig. 4*C*), β (519 ± 31 sec^{-1} and 580 ± 88 sec^{-1}), or p_o (0.320 ± 0.032 and 0.318 ± 0.049).

AcChoR Number. The enhanced AcCho response caused by cAMP analogues does not require protein synthesis or detectable increases in the total number of AcChoRs present on the cells. Thus, cycloheximide (CH) at 5 μg/ml, a concentration shown to block 95% of protein synthesis in the neurons within 10 min (31), did not block the increase in AcCho sensitivity caused by 8-Br-cAMP and IBMX (Fig. 5*A*). Assays of total AcChoRs on the neurons using a radiolabeled AcChoR-specific mAb (^{125}I-labeled mAb 35) as described (21, 22) detected no difference between control neurons and neurons exposed to 8-Br-cAMP and IBMX for 6 hr (Fig. 5*B*).

DISCUSSION

The conclusions drawn from the present study are that (*i*) a cAMP-dependent mechanism increases the AcCho response of chicken ciliary ganglion neurons, (*ii*) it does so by increasing the number of functional AcChoRs on the cells, and (*iii*) the increase in functional receptors apparently involves a conversion of preexisting nonfunctional receptors to a functional form. Evidence for the first conclusion is straightforward: exposure of the neurons to membrane-permeant cAMP analogues and a phosphodiesterase inhibitor specifically increases both components of the whole-cell current induced by AcCho. Moreover, internal perfusion of the neurons with cAMP from the recording pipette also increases the AcCho response, and the increase cannot be mimicked by internal perfusion with AMP or cGMP. The second conclusion is drawn by inference: cAMP treatments that cause an increase in the AcCho response do so without causing a significant change in AcChoR agonist affinity, single-channel conductance, reversal potential, τ, or p_o. A small increase is observed in the decay rate for the slow component of the whole-cell current, but the effect is opposite to that required to account for the enhanced AcCho response. We conclude that the enhanced AcCho response arises from an increased number of functional AcChoRs on the neuron surface. This does not exclude the possibility that the increased AcCho response reflects a cAMP-dependent block of some very rapid form of agonist-induced receptor inactivation.

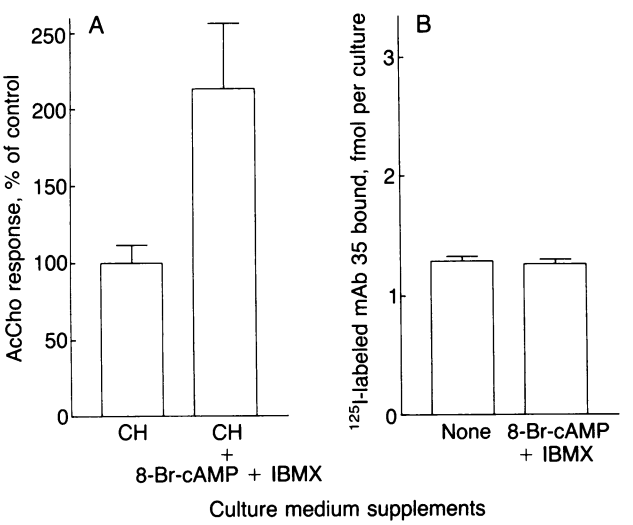

FIG. 5. The cAMP-dependent enhancement of AcCho sensitivity does not require protein synthesis or a detectable increase in the total number of surface AcChoRs on the neurons. (*A*) Six- to seven-day-old cultures were incubated first in medium containing 5 μg of CH per ml for 2 hr and then either continued for 6 hr in CH or supplemented with 2 mM 8-Br-cAMP and 1 mM IBMX (CH + 8-Br-cAMP + IBMX). Dishes were then rinsed with recording solution, and the neurons were tested for responses to 100 μM AcCho as in Fig. 1. Results are expressed as % of the control response in cultures treated with CH alone and represent the mean ± SEM of 18 control and 14 treated neurons in sister cultures from four platings. A 2-fold increase is observed ($P < 0.02$). (*B*) Surface AcChoRs were measured with ^{125}I-labeled mAb 35 by growing cultures in 16-mm wells (10^4 neurons per well) for 6 days and then incubating them for 6 hr either in fresh medium alone or in medium containing 2 mM 8-Br-cAMP and 1 mM IBMX. The cultures were then rinsed and assayed for specific ^{125}I-labeled mAb 35 binding per well ($n = 3$). Similar results were obtained in two additional experiments.

The third conclusion is most interesting. It follows from the observation that the cAMP-dependent increase in AcCho sensitivity does not require protein synthesis. The slow time course of 8-Br-cAMP and IBMX in producing the enhanced AcCho response presumably reflects time required for membrane permeation of the drugs. The rapid effect of cAMP applied intracellularly is consistent with a mechanism independent of protein synthesis.

If a cAMP-dependent mechanism increases the number of functional AcChoRs on the neurons without requiring *de novo* receptor synthesis, the source of preexisting receptors could be either intracellular AcChoRs or nonfunctional AcChoRs on the surface. Up to two-thirds of the AcChoRs associated with chicken ciliary ganglion neurons in culture, as revealed by ^{125}I-labeled mAb 35 binding, are intracellular, and only a small proportion of these appears destined for the cell surface (31). The role of the remaining intracellular AcChoRs is unknown. The possibility that many of the surface AcChoRs might be nonfunctional comes from the observation that the number of functional AcChoRs, even when increased by cAMP treatment, is far less than the total number of AcChoRs on the cell surface. All of the mAb 35 sites appear to be associated with AcChoRs (21, 22, 32). Assuming two mAb 35 binding sites per AcChoR (14, 22, 32), the amount of binding obtained here corresponds to about 5 × 10^4 surface AcChoRs for each neuron. Estimating the number of functional AcChoRs (N_f) from $N_f = G^*/\gamma p_o$, where G^* is the extrapolated maximum whole-cell conductance induced by a saturating concentration of AcCho (Fig. 3), γ is the single AcChoR channel conductance (40 pS), and $p_o = 0.32$, yields values of about 2000 and 4000 for control and for 8-Br-cAMP and IBMX treated neurons, respectively. The finding that functional AcChoRs can be changed in number, either by chronic exposure to elevated K^+ concentrations (14) or by a cAMP-dependent mechanism as reported here, without changing the total number of AcChoRs on the neuron surface implicates the surface pool as a likely source for recruiting functional AcChoRs through some form of conversion. The surface-labeling assays do not exclude the possibility, however, that the increase in functional AcChoRs arises through insertion of additional preexisting receptors into the plasma membrane. If only functional receptors were inserted under these conditions, the resulting increase in total surface AcChoRs would be too small to detect. Such a mechanism would still represent a "conversion" of nonfunctional AcChoRs to functional ones by cAMP, but the conversion would involve selective insertion of functional receptors into the plasma membrane rather than a physical alteration of receptors preexisting in the plasma membrane.

The increased number of functional AcChoRs caused by cAMP could arise either from a direct effect of cAMP on membrane components or from a stimulation of cAMP-dependent protein kinase. Cyclic nucleotides do activate membrane conductances in other systems (33, 34). A direct

effect seems unlikely here, however, because cAMP does not alter the resting conductance of the neurons or change the single-channel properties of AcChoRs when applied to the cytoplasmic surface of the plasma membrane in excised patches (data not shown). The more likely mechanism is that the cAMP effect is mediated by protein phosphorylation, as has been suggested for a variety of ion channels (1, 2, 10, 35).

The cAMP-dependent regulation of neuronal AcChoRs seen here is quite different from that observed for AcChoRs from muscle and electric organ. Forskolin, known to raise cAMP levels by direct activation of adenylate cyclase (36), enhances the apparent rate of AcChoR desensitization in skeletal muscle fibers (7, 8). Preliminary results indicate that forskolin treatment of chicken ciliary ganglion neurons does cause a reduced response to AcCho, but the effect appears to be similar to that of a local anesthetic in producing direct receptor blockade (37), as reported for the pheochromocytoma cell line PC12 (38). Quench-flow experiments with lipid vesicles containing AcChoRs purified from *Torpedo* electric organ demonstrate directly that receptor phosphorylation by the catalytic subunit of cAMP-dependent protein kinase enhances a component of agonist-induced receptor desensitization (9). Intracellular application of cAMP in the present experiments causes a much smaller increase in the slow rate of desensitization; the major effect is to increase both components of the whole-cell AcCho current consistent with increased numbers of functional receptors.

Differences in cAMP-dependent regulatory effects on AcChoRs in ciliary ganglion neurons compared with muscle and electric tissue AcChoRs may reflect differences in their structures. The neuronal AcChoR α-subunit present in chicken ciliary ganglion neurons (39) is homologous to a rat neuronal AcChoR α-subunit whose deduced amino acid sequence reveals a serine moiety potentially available for cAMP-dependent phosphorylation (20). Because AcChoR α-subunits of muscle and *Torpedo* electric organ lack such a serine, and cAMP-dependent phosphorylation of *Torpedo* electric organ AcChoR occurs primarily on the γ- and δ-subunits (9, 40), cAMP-dependent phosphorylation may lead to different consequences for neuronal AcChoRs compared with AcChoRs in muscle and electric tissue. It is not clear whether neuronal AcChoRs have γ- or δ-subunit equivalents (41).

Regulating the conversion of transmitter receptors from nonfunctional to functional forms would provide a means of controlling the sensitivity of neurons to synaptic input. A mechanism of this type would be readily reversible and potentially responsive to a number of environmental influences. It could have general significance for modulating synaptic transmission in the nervous system.

We thank Drs. Stanley Halvorsen, Marthe Howard, and Michael White for helpful discussions and Dannielle Pellegrin for expert technical assistance. This work was supported by Grants NS 24417, NS 12601, and NS 15344 from the National Institutes of Health.

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