0026-895X/93/050807-06$03.00/0

MOLECULAR PHARMACOLOGY, 43:807-812

Modulation by Albumin of Neuronal Cholinergic Sensitivity

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Received December 8, 1992; Accepted February 20, 1993

SUMMARY

Bovine serum albumin greatly enhanced the cholinergic response mediated by neuronal nicotinic acetylcholine receptors in chick ciliary ganglion neurons. The enhancement exceeded 5-fold in some experiments (mean ± standard error, 3.26 ± 0.43-fold) and was rapid, was dose dependent, and occurred without changes in the unitary conductance or the mean open time of the acetylcholine receptor channel. This lack of detectable change in permeation or kinetic properties suggests that bovine serum albumin might increase acetylcholine responses by increasing the number of functional receptors. The enhancement appears to be specific to the albumin molecule, because activity could not be removed by detergent extraction, gel filtration, or dialysis. Acetylcholine responses in these cells are known to be enhanced by a cAMP-dependent mechanism that converts existing acetylcholine receptors from a nonfunctional to a functional state. We found that the enhancement by bovine serum albumin occurred without an increase in cAMP and that pretreatment with membrane-permeable cAMP analogs prevented any additional enhancement of the cholinergic response by bovine serum albumin. These observations are consistent with a cAMP-dependent modulation of the enhancement produced by bovine serum albumin or a convergence of the two enhancement mechanisms onto a single pathway.

nnAChRs belong to the superfamily of multisubunit, ligand-gated, ion channels that includes glutamate, glycine, $GABA_A$, and muscle nicotinic ACh receptors (1–3). Different subtypes of nnAChRs composed of various subunits are found in the brain, in the spinal cord, and in autonomic ganglia, where they mediate excitatory neurotransmission (4, 5). Studies on the receptors in this superfamily suggest that their phenotypic expression, distribution, maturation, turnover, and degradation are subject to regulation by neurotrophic factors during development and after maturation of the nervous system (6–9). The extent and diversity of these regulatory mechanisms for nnAChRs are not yet known, but the loss of such regulation accompanies aging and dementias such as Huntington's disease and Alzheimer's disease (10), in which nicotinic transmission is impaired (11, 12).

In primary cultures of chick ciliary ganglion neurons, nnAChRs respond to their neurotransmitter and to regulatory stimuli that have been localized within the developing ganglion (6, 13, 14). Substance P, VIP, and a 50-kDa protein extracted from eye tissue modulate the properties of nnAChRs in ciliary and dorsal root ganglion neurons. The eye extract protein enhances embryonic nnAChR responses after several days of treatment in culture (6, 8). A more rapid effect is seen with the peptides. Activation of substance P receptors accelerates desensitization of the nnAChRs (15, 16), probably via an inositol trisphosphate-dependent pathway, whereas VIP appears to reactivate quiescent nnAChRs through a cAMP-dependent pathway (17). Given the variety of regulatory effects on nnAChRs that are known to occur, it is likely that additional neuromodulators remain unidentified.

Here we report that BSA can specifically, rapidly, and potently enhance the cholinergic sensitivity of parasympathetic neurons from chick ciliary ganglia, via a cAMP-independent mechanism, with no effect on receptor desensitization or single-channel properties. BSA is a 67-kDa globular protein found in plasma and interstitial fluid. It serves as a carrier and transporter of insoluble ligands [reviewed by Peters (18)] and is recognized by specific membrane receptor proteins (19, 20–22). Although BSA is unlikely to be an endogenous ligand in chicks, the characteristics of its effect suggest that similar serum proteins may nevertheless have important regulatory roles in the nervous system.

This work was supported by National Institutes of Health Grants NS24417 (J.F.M.), NS27177 (A.T.H.), and DC00256 and NS15344 (V.E.D.), by the Howard Hughes Medical Institute (A.T.H.), by a predoctoral fellowship from the Pharmaceutical Manufacturer's Association (D.G.), and by a Basil O'Connor Starter Scholar Research Award from the March of Dimes Birth Defects Foundation (J.F.M.).

ABBREVIATIONS: nnAChR, neuronal nicotinic acetylcholine receptor; GABA, γ-aminobutyric acid; BSA, bovine serum albumin; 8Br-cAMP, 8-bromo-cAMP; IBMX, 3-isobutyl-l-methylxanthine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); ACh, acetylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; VIP, vasoactive intestinal peptide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FlCRhR, fluorescein/rhodamine tagged protein kinase A.

Experimental Procedures

Neuron isolation. Ciliary ganglion neurons were prepared from embryonic day 13–14 chick embryos (23). At this developmental stage, the cell yield per ganglion is higher than at later stages, the cholinergic responses are large, and the cAMP-dependent enhancement of these responses is clearly evident. Neurons were dissociated by incubating dissected ganglia for 30 min at 37° with collagenase A (0.4–0.5 mg/ml; Boehringer-Mannhein, Indianapolis, IN) in buffered avian saline that was nominally Ca^{2+}-free and contained the following (in mM): NaCl, 145.0; KCl, 5.3; $MgCl_2$, 1.0; glucose, 5.6; and HEPES, 5.0, pH 7.4. Enzyme-treated ganglia were washed four times with buffered saline containing 5.4 mM Ca^{2+} and 10% (v/v) heat-inactivated horse serum (supplemented saline). After the last wash, cells were dissociated by mechanical trituration in 1–2 ml of supplemented saline and were plated on glass coverslips coated with poly-D-lysine. The neurons were plated at a density of two ganglion equivalents (about 8000 cells)/coverslip, in 200-μl aliquots. After attachment (20 min), cells were incubated in fresh supplemented saline for 2–4 hr before the electrophysiological assays. In some experiments the cells were incubated for 6 hr after plating in supplemented saline containing 2 mM 8Br-cAMP and 1 mM IBMX. Unless noted otherwise, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Electrophysiology. Neurons plated on glass coverslips were examined at room temperature (21–24°) using Nomarski optics at 500× magnification. Whole-cell and single-channel currents were recorded using patch-clamp methods, as described previously (23, 24). The recording solution contained the following (in mM): NaCl, 145.0; KCl, 5.3; $CaCl_2$, 5.4; $MgCl_2$, 0.8; glucose, 5.6; and HEPES, 5.0, pH 7.4. According to the particular experimental design, BSA (1.5–15 mg/ml, fraction V; Scientific Protein Lab or Boehringer Mannheim), ovalbumin (10 mg/ml; Calbiochem, La Jolla, CA), bovine γ-globulin (10 mg/ml; Sigma), or sheep, rat, or chick serum albumin (10 mg/ml; Sigma) was added to the recording solution. The pipette solution contained the following (in mM): CsCl, 145.6; $CaCl_2$, 1.2; EGTA, 2.0; glucose, 15.4; and Na-HEPES, 5, pH 7.3 (free $[Ca^{2+}]$ = 130 nM). Currents were induced by pressure microperfusion from a pipette containing the agonist being tested dissolved in recording solution, i.e., ACh (1 μM to 2 mM) or GABA (5 μM).

Whole-cell and single-channel currents were recorded using an Axopatch 1C amplifier (Axon Instruments, Burlingame, CA) and an Indec laboratory computer system (Indec Systems, Inc., Sunnyvale, CA). Whole-cell currents were recorded at −70 mV, filtered at 5 kHz (four-pole Bessel filter), and digitized at 250-μsec intervals. Single-channel currents were recorded from outside-out patches at holding potentials of −80 or −120 mV, filtered at 8.5 kHz with an eight-pole Bessel filter, and digitized at 60–100-μsec intervals.

Data analysis. The whole-cell conductance (G_t) was calculated from the current records using the equation $G_t = I_t/(V_p - I_tR_s - V_r)$, where I_t is the current at time t, V_p is the pipette potential (−70 mV), R_s is the series resistance, and V_r is the nnAChR current reversal potential (−11 mV) determined previously (23). Neglecting to compensate for series resistance errors and expressing ACh sensitivity as current rather than conductance would not alter the conclusions presented here but would reduce the size of the largest responses by about 10%. Series resistance and membrane capacitance were measured directly during leak compensation by using the Axopatch amplifier. Membrane capacitance, assumed to be proportional to cell membrane surface area, was also estimated as the ratio of whole-cell capacitive charge to the applied voltage. Capacitative charge was calculated from the whole-cell capacitive current transient. Because nnAChRs desensitize during prolonged agonist application, ACh sensitivity was quantified from the maximal peak conductance at time $t = 0$ (G_0). For each cell, G_0 was obtained by extrapolation after fitting of the transformed conductance versus time records with exponential functions describing the fast and slow rates of desensitization, as described previously (23). The individual maximal peak conductance values were normalized for differences in cell membrane surface area by dividing the values by the membrane capacitance ($G_N = G_0/C_m$). G_N values therefore provide a normalized measure of the peak ACh response (in nS/pF). Measurements were obtained from neuron populations and are expressed as mean ± standard error. In some cases, ACh sensitivity was expressed as percentage (mean ± standard error) of the control G_N for each experiment. Statistical significance of differences between treatment groups was determined by an unpaired t test or by analysis of variance, using the INSTAT computer program (GraphPad, San Diego, CA).

nnAChR single-channel current amplitude and open durations were detected and measured off-line using a computer-assisted search routine. Open-duration histograms were fitted with exponential functions using the method of maximum likelihood (25). The appearance of single-channel opening events decayed at variable rates during these recordings, presumably due to a combination of desensitization and "run-down" of the patches. Open-duration lifetimes and unit current amplitude appeared to be independent of nnAChR desensitization (26).

Fractionation and purification of BSA. Dialysis, gel filtration, and detergent extraction were used to remove any contaminants that might be present in commercial BSA preparations and that could potentially be responsible for its ability to potentiate cholinergic sensitivity. Recording solution containing BSA (1.5–3 mg/ml, pH 7.4) was dialyzed overnight, with three bath changes, against recording solution alone. The dialysis membranes had molecular mass cut-offs of 3–14 kDa. ACh responses obtained in the presence of dialyzed or undialyzed BSA solutions were then tested in the electrophysiological assays described above and were compared with responses obtained in the absence of BSA.

BSA (10–20 mg/ml) was also subjected to gel filtration chromatography using Sephadex G-150 (Pharmacia, Piscataway, NJ), with recording solution or 3 N acetic acid as the mobile phase. The fractions obtained from the chromatographic runs displayed one protein peak, as determined by absorbance at 280 nm. This peak contained BSA, which was eluted with one third of the column volume and was diluted to a concentration of about 1.5 mg/ml during passage through the column. The BSA that was eluted with recording solution was applied directly to cells, whereas BSA eluted with acetic acid was dialyzed against recording solution and then applied to cells. Control BSA solutions with concentrations similar to that of the BSA peak were dissolved in 3 N acetic acid, dialyzed against recording solution, and used in parallel.

Commercial preparations of BSA were re-purified by detergent extraction using CHAPS. CHAPS (10 mM) was added to BSA solutions (10 mg/ml) for 2 hr at room temperature and then removed by dialysis against recording solution; the BSA was then tested for activity as described above.

Fluorescent detection of cAMP. Changes in free intracellular cAMP were monitored with a fluorescently labeled enzyme (FlCRhR) that was injected into cells (27). The reporter enzyme was prepared by covalently labeling the catalytic (C) and regulatory (R) subunits of mammalian protein kinase A with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate, respectively. In the absence of cAMP the subunits of protein kinase A form an R_2C_2 complex that holds the dye moieties sufficiently close together that excitation of the fluorescein donor results in emission from the rhodamine acceptor by resonance energy transfer. Increased levels of cAMP cause the subunits to dissociate, preventing energy transfer and resulting in an increase in the fluorescein/rhodamine emission ratio, measured at 500–530 nm for fluorescein and 570 nm for rhodamine.

Ciliary ganglion neurons from chick embryos were isolated as described above and plated in 35-mm culture dishes fitted with glass bottoms that had been precoated with poly-D-lysine. FlCRhR was injected into individual neurons at an estimated final concentration of 0.2–2 μM (in 25 mM K_3PO_4, 1 mM Na-EDTA, 0.5 mM β-mercaptoethanol, 2.5% glycerol, pH 7.3). Injected neurons were allowed to recover for 30–60 min in supplemented saline before examination. A base line for the fluorescence emission ratio was determined for each cell by repeated measurements for 10 min in recording solution. Recording solution containing the test agent was then applied and the measurements were continued for another 5–30 min. At the end of each

experiment, 1 mM IBMX, a phosphodiesterase inhibitor, was added to verify that the cells tested were capable of elevating cAMP.

Results

BSA enhancement of cholinergic sensitivity. Neurons were tested for responses to ACh in the presence and absence of BSA. Exposure for 3–60 min to 10 mg/ml BSA at room temperature increased by severalfold the whole-cell conductance elicited by 1 mM ACh (Fig. 1A). The mean peak G_N for BSA-treated neurons was 309 ± 29% ($p < 0.001$) of the value for untreated neurons (Fig. 1B). This BSA-induced enhancement developed rapidly, it was maximal after a 3-min exposure to BSA (the shortest exposure that could be tested), and the effects after 3- or 60-min exposures were the same. Our experimental design did not allow tests of BSA exposures of <3 min, and individual neurons could be tested with ACh only once because desensitization caused a long-lasting reduction in the response magnitude. BSA did not alter the kinetics of ACh-induced desensitization or the relative contributions of the two components of desensitization. For both control and BSA-treated neurons, the fast and slow desensitization components of currents induced with 1 mM ACh had time constants of 0.2–0.6 sec and 2.3–5.9 sec, respectively; the amplitude of the fast component relative to G_0 was 0.66–0.70. The enhancement of the responses was not accompanied by a change in cell size, inasmuch as the mean cell capacitance of the control and BSA-treated cells was similar (22.4 ± 0.72 and 21.5 ± 0.71 pF for control and BSA-treated cells, respectively). The ability of BSA to increase neuronal ACh sensitivity occurred over the full range of ACh concentrations to which the cells responded (Fig. 1C). BSA increased the mean ACh-induced conductance at all ACh concentrations tested but otherwise did not affect the ACh dose-response curve (Table 1).

BSA did not alter the unitary conductance or the mean open time of single nnAChR channels. Single-channel currents were recorded from outside-out patches excised from the somatic membrane of neurons. As reported previously (23), two classes of unitary currents, with conductances of 25 and 40 pS, are present in these neurons. Currents from the 25-pS conductance class represented <10% of the total number of events; although BSA treatment did not appear to alter the kinetic properties of the 25-pS events or their relative contribution to the single-channel records, these events were too infrequent to be analyzed in detail. A summary of data for the predominant 40-pS channel is given in Table 2. Neither the single-channel conductance nor the distribution of open times was affected by BSA. The opening probability of the nnAChR channels could not be determined because of more pronounced "run-down" of the single-channel currents in outside-out patches than was seen previously. As an internal control, voltage-gated Na^+ and Ca^{2+} currents were measured in every cell studied. BSA had no effect on the magnitude or time course of these currents (data not shown).

The target selectivity of the BSA effect was further evaluated by testing the response to GABA. Ciliary ganglion neurons possess $GABA_A$ receptors (28), which are members of the same receptor family as nnAChRs (2). Responses to 5 μM GABA were significantly enhanced by BSA (9 mg/ml), but they were enhanced to a smaller degree than were responses to 500 μM ACh measured in the same experiments (Table 3). BSA increased the GABA-induced G_0 to 160 ± 14% of control ($p < 0.0001$), and in the same experiments BSA increased the response to 500 μM ACh by >3-fold ($p < 0.0001$). The GABA responses were induced with a submaximal dose (5 μM) because the whole-cell currents produced by higher concentrations of GABA saturated the recording system. Because the effect of

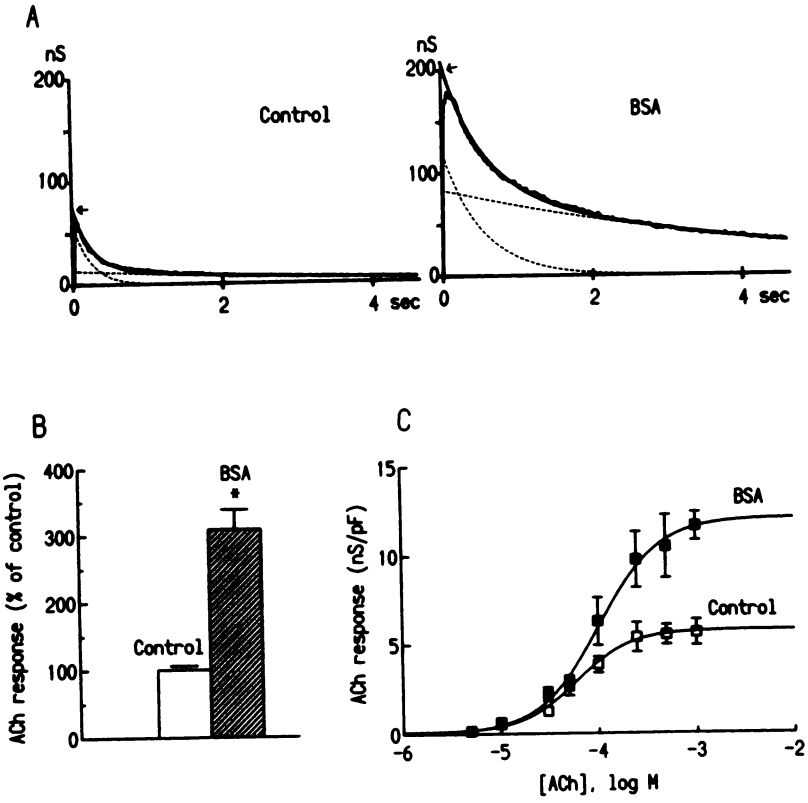

Fig. 1. Cholinergic responses in chick ciliary neurons are strongly enhanced after exposure to BSA. A, ACh-induced whole-cell conductance (computed from currents) in the absence (*left*) and presence (*right*) of 10 mg/ml BSA. BSA was applied ~10 min before the record was made. ACh (1 mM) was applied by pressure microperfusion throughout the recording, starting at $t = 0$. The ACh-induced conductance decayed due to receptor desensitization. The two *dashed curves* in each record are the fast and slow exponential components of desensitization; their sum is the *solid curve* that has been fitted to the response. For the control cell, $G_0 = 79$ nS (*arrow*), $C_m = 28.9$ pF, $G_N = 2.73$ nS/pF, $\tau_{fast} = 0.22$ sec, and $\tau_{slow} = 3.97$ sec. For the BSA-treated cell, $G_0 = 208$ nS (*arrow*), $C_m = 24.8$ pF, $G_N = 8.4$ nS/pF, $\tau_{fast} = 0.55$ sec, and $\tau_{slow} = 5.38$ sec. B, Mean ± standard error of the peak response elicited by 1 mM ACh after 10–60-min exposure to 10 mg/ml BSA, as a percentage of control. Control, 100 ± 5% ($n = 65$); BSA-treated, 309 ± 29% ($n = 55$). *, $p < 0.001$, Student's t test. C, BSA potentiated the ACh response at all doses. Peak whole-cell conductances (G_N) in the absence (□) and presence (■) of 10 mg/ml BSA were plotted for ACh concentrations ranging from 5×10^{-6} M to 10^{-3} M and were fitted with sigmoidal curves by nonlinear regression. The estimated EC_{50} values are 60 μM for the control data and 103 μM for the BSA-treated data. Each *point* represents the mean value from four cells, and for each *curve* all points are from a single experiment. Similar results were obtained for two control and three BSA experiments.

TABLE 1

ACh dose-response parameters

The measurements are mean ± standard deviation of n experiments.

	Control ($n = 2$)	BSA-treated ($n = 3$)
EC_{50} (μM)	56 ± 6	97 ± 14*
Hill coefficient	1.26 ± 0.41	1.35 ± 0.50

*$p < 0.03$.

TABLE 2

Unitary properties of the 40-pS nnAchR channel

The open-duration distributions were fitted by the sum of two exponential components with fast (τ_{fast}) and slow (τ_{slow}) time constants, corresponding to brief and long-lived openings. The fraction of total open time occupied by the component of brief openings is given by F_{brief}.

	Control	BSA-treated
Conductance (pS)	36.6 ± 0.4	37.2 ± 0.4
τ_{fast} (msec)	0.47 ± 0.04	0.49 ± 0.09
τ_{slow} (msec)	4.27 ± 0.40	4.49 ± 0.40
F_{brief}	0.47 ± 0.04	0.54 ± 0.04

TABLE 3

BSA enhanced the conductance elicited by 5 μM GABA and by 500 μM ACh

	Control	BSA-treated
GABA conductance (nS/pF)	2.22 ± 0.20 ($n = 33$)	3.37 ± 0.26* ($n = 32$)
ACh conductance (nS/pF)	3.26 ± 0.43 ($n = 12$)	10.78 ± 0.98* ($n = 16$)

*$p < 0.0001$.

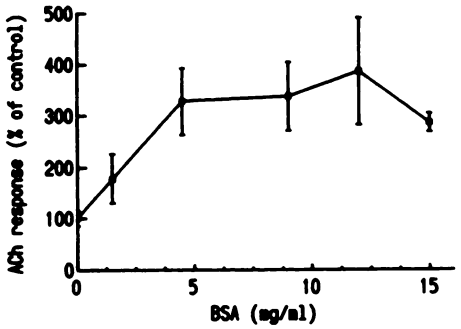

Fig. 2. Dose dependence of the BSA-induced enhancement of cholinergic sensitivity. Peak conductances induced by 500 μM ACh are plotted as a percentage of control for cells tested with different concentrations of BSA. Each *point* shows the mean ± standard error of four cells. Similar results were obtained in four other experiments.

BSA on saturating concentrations of GABA was not determined, it is possible that neuronal sensitivities to both GABA and ACh are enhanced to a similar extent.

Dose dependence of BSA effect. BSA was applied to neurons at 1.5–10 mg/ml (20–150 μM) to determine the dose dependence of its effect on ACh sensitivity (Fig. 2). The EC_{50} for potentiation by BSA was 1.0–1.5 mg/ml (15–20 μM), and its effect was maximal with concentrations of ≥5 mg/ml. The enhanced cholinergic sensitivity produced by BSA was slowly reversible, requiring washes longer than 1 hr after 15-min applications of BSA at 1.5 mg/ml. The effect was more sustained when BSA was removed after a prolonged (>1-hr) incubation with a high concentration (10 mg/ml).

Specificity for BSA. We evaluated whether the enhancement of cholinergic sensitivity was specific for BSA by testing several other globular proteins. Neither bovine γ-globulin, bovine ovalbumin, sheep serum albumin, rat serum albumin, nor chick serum albumin caused a statistically significant increase or decrease in the cholinergic sensitivity (data not shown).

Purification of BSA. BSA is a soluble carrier protein that can bind to a wide variety of ligands. For this reason, we considered the possibility that the effect of BSA on cholinergic sensitivity might be due to an unidentified factor that copurified with the BSA. BSA from different commercial preparations showed similar abilities to enhance cholinergic sensitivity. To test whether the activity resided in the 67-kDa BSA molecule itself or in an associated impurity, several different techniques were used to repurify the BSA. Equilibrium dialysis at pH 7.4 using 3.5-, 6–8-, and 12–14-kDa dialysis membranes did not remove the activity. Gel filtration to remove small molecules larger than 14 kDa that could not be removed by equilibrium dialysis had no effect. Chromatographically purified BSA (seven experiments) at 1.5 mg/ml produced a 174 ± 12.4% enhancement ($p < 0.001$) of ACh responses, which was comparable to the enhancement elicited by untreated BSA. BSA was the only detectable protein eluted from the gel filtration column, and fractions without detectable protein had no effect on ACh responses. Gel filtration in the presence of 3 N acetic acid was performed to weaken interactions between the BSA molecule and a putative molecule strongly attached to it. However, the BSA fraction obtained in the presence of 3 N acetic acid and then dialyzed against recording solution also produced enhancement of ACh responses (165 ± 21%, $p < 0.03$, six cells/experimental group). Extraction with the detergent CHAPS to remove potential hydrophobic molecules that might be strongly associated with BSA was ineffective; the extracted and dialyzed BSA solution from four experiments enhanced the cholinergic response 4-fold, compared with control dialyzed detergent solution (11–19 cells/experimental group). Furthermore, the activity of BSA was heat stable as long as the BSA remained in solution.

Evidence that BSA does not elevate intracellular cAMP. To test whether the action of BSA is mediated by an elevation of intracellular cAMP, we imaged cells that had been injected with the fluorescent indicator FlCRhR (see Experimental Procedures). Exposure to BSA did not alter intracellular cAMP levels in any of the neurons tested ($n = 14$), whereas the same cells did show cAMP elevation upon subsequent addition of 1 mM IBMX, a phosphodiesterase inhibitor (Fig. 3A). Cells were also pretreated with a submaximal dose of IBMX (0.1 mM) to prevent degradation of cAMP. As with the untreated cells, BSA had no effect on the intracellular cAMP levels after pretreatment (Fig. 3B).

Evidence that pretreatment with 8Br-cAMP prevents cholinergic potentiation by BSA. Pretreatment of neurons with 8Br-cAMP prevented the enhancement by BSA of cholinergic sensitivity. Neurons were incubated with 2 mM 8Br-cAMP plus 1 mM IBMX for 6 hr before their sensitivity to ACh was tested. The cells were then tested in the absence or presence of 10 mg/ml BSA to determine whether the responses were additive. The measurements were performed with a submaximal concentration (100 μM) of ACh to eliminate the possibility of saturation of the transduction pathways. Individually, both BSA and 8Br-cAMP/IBMX produced the expected enhancement of ACh sensitivity; however, no additive effect was observed when the cells were treated with BSA after 8Br-cAMP/IBMX (Fig. 3C).

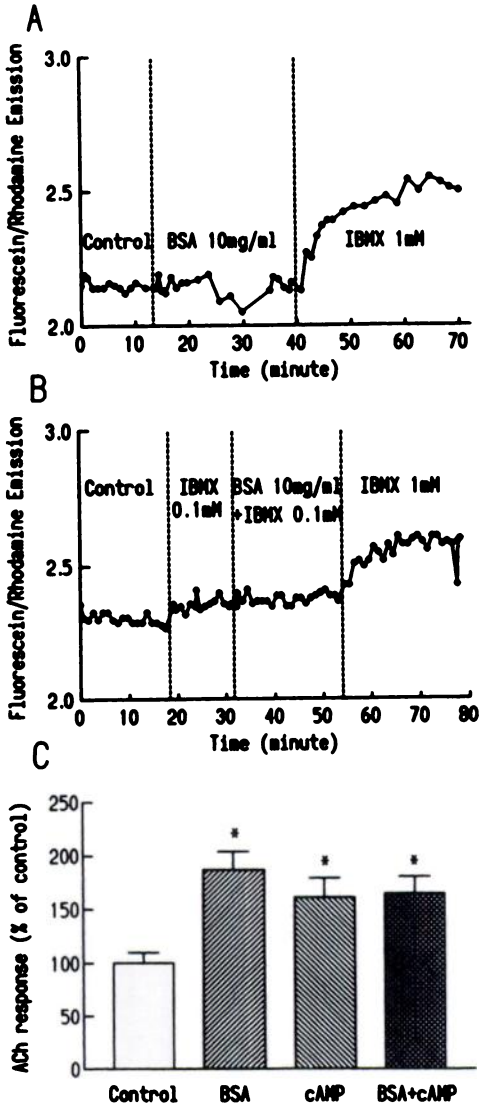

Fig. 3. BSA treatment does not change the intracellular cAMP levels. Individual neurons were injected with FICRhR and their fluorescence emission ratio (fluorescein/rhodamine) was measured periodically, at 500–530 nm and >570 nm, for 70–80 min. A, BSA did not influence intracellular cAMP concentrations. After a control period, 10 mg/ml BSA was added to the bath; there was no change in the emission ratio, indicating that the intracellular cAMP level did not change. The subsequent addition of 1 mM IBMX to the bath caused a rapid and sustained increase in the signal. B, After a control period, the cell was sensitized with 0.1 mM IBMX, resulting in a small increase in fluorescence. The cell was then exposed to 10 mg/ml BSA with 0.1 mM IBMX; there was no change in the fluorescence signal, indicating that the intracellular cAMP level did not change. As in A, the subsequent addition of IBMX to the bath caused a rapid and sustained increase in the signal. C, Preincubation with 8Br-cAMP prevented the response to BSA. Neurons were incubated with 8Br-cAMP (2 mM) plus IBMX (1 mM) for 6 hr, tested for response to a submaximal dose of ACh (100 μM) in the presence or absence of 10 mg/ml BSA, and compared with neurons that were tested with BSA alone and control neurons that were tested in parallel (n = 24–28/experimental cell group). The enhancement of ACh responses was 187 ± 17% (of control) for BSA-treated cells, 161 ± 19% for 8Br-cAMP/IBMX-treated cells, and 164 ± 16% for the combined treatments. *, $p < 0.05$, by analysis of variance.

Discussion

These results indicate that BSA or possibly a structure within the BSA molecule activates a process to enhance the nicotinic cholinergic sensitivity of chick ciliary ganglion neurons. The enhancement can be as much as 3-fold, making BSA the most efficacious rapidly acting neuromodulator of nnAChRs known. The ability of BSA or a similar endogenous molecule to modulate AChR function could help maintain normal neurotransmitter sensitivity as part of a sustained homeostatic process or could serve to regulate sensitivity in response to synaptic activity. Because the response was not shared by other albumins or globular proteins, it cannot be attributed to the presence of protein in the test media or to removal of a soluble inhibitor that derepresses the cholinergic response. Based upon the dose dependence and specificity for BSA, the effect appears to be mediated by a receptor-based mechanism. The EC_{50} for BSA was approximately 15–20 μM, which is similar to the binding affinity of a membrane receptor for albumin that was described previously (20). Although BSA did not elicit an increase in voltage-activated conductances, it did enhance neuronal GABA sensitivity. Furthermore, BSA has been shown to promote glutamate toxicity in cerebellar granule cells (29). Taken together, these results suggest that the mechanism of BSA enhancement may selectively affect the superfamily of ligand-gated ion channels.

Although BSA is not identical to chick serum albumin, it may mimic the activity of an endogenous protein or peptide acting via an as yet unidentified cell surface receptor. Alternatively, structurally similar molecules have been found in commercial preparations of BSA and have been reported to have other biological activities (30). Their comparable biochemical and physical properties make them difficult to distinguish by isoelectric focusing, mobility on gel electrophoresis, or recognition by albumin antibodies (30, 31). BSA could also serve as a precursor to biologically active peptides (31). A recent preliminary study showed that one of three tryptic fragments of BSA can enhance the low-voltage Ca^{2+} current in dorsal root ganglion neurons (32), indicating that BSA contains a peptide sequence having a biological activity.

The actual change that BSA elicits in the nnAChR channels to enhance conductance remains uncertain. The most likely effect seems to be an increase in the number of functional channels, especially because there was no detectable change in the single-channel properties that could account for a 3-fold enhancement of whole-cell conductance. Previous work has shown that these cells contain nnAChR binding sites whose number far exceeds the number of activatable channels (33). Thus, recruitment of new functional channels from a pool of silent nnAChRs is a possible mechanism for potentiation. Such a mechanism appears to be activated by VIP in these cells through a pathway that involves elevation of intracellular cAMP (17). In contrast, BSA appears to mobilize nnAChRs by a different, cAMP-independent, pathway, because it enhances ACh responses without detectably increasing intracellular cAMP. Of course, BSA application could induce small increases in intracellular cAMP that go undetected in the imaging assay used here. It is unlikely, however, that such increases alone would be sufficient to produce the observed 3-fold enhancement of ACh responses, because agents such as IBMX, 8Br-cAMP, and VIP, when applied individually or in combination, have smaller effects on sensitivity than does BSA and yet cause readily detectable increases in cAMP (17, 33).[1] The failure of pretreatment with 8Br-cAMP to potentiate ACh sensitivity

[1] D. Gurantz, A. T. Harootunian, and J. F. Margiotta, unpublished observations.

beyond that seen with BSA alone does suggest, however, that the cAMP-dependent and -independent pathways involved in regulating ACh responses do interact. Given the submaximal doses of ACh used in the additivity experiments and the small relative size of the enhancement produced by 8Br-cAMP, it is unlikely that the lack of additivity represents a simple ceiling effect at the ACh level. Instead, it seems more likely that 8Br-cAMP may be stimulating an additional mechanism, such as one involving a kinase, to inhibit the BSA-mediated response or that the cAMP-mediated and BSA-mediated enhancements converge at some point onto a single pathway. The additivity of the BSA effect with that of VIP was not determined because the effect of VIP is much smaller than that of BSA and the variations in the electrophysiological measurements are such that additivity would be difficult to demonstrate. However, it is clear that BSA does not stimulate VIP receptors, because VIP causes increased levels of intracellular cAMP not seen with BSA.

In the central nervous system, neurological deficits in synaptic transmission occur with increasing frequency as individuals age. Certain of these changes occur in nicotinic cholinergic neurons and are associated with dementias such as Alzheimer's disease. Although changes in the levels of neuromodulators are not thought to be the primary causes of these diseases, the ability to enhance neuronal sensitivity to ACh could be a powerful therapeutic tool in relieving the symptoms of cholinergic neuron-related dementias, especially in early stages when deficits are only partial. Current therapeutic approaches rely on anticholinesterases to increase nonspecifically the duration of neurotransmitter action at all sites of cholinergic transmission, by blocking neurotransmitter degradation. Neuromodulator enhancement of cholinergic sensitivity, however, should avoid the serious side effects caused by esterase inhibition because it is potentially targetable; nnAChRs are differentially expressed and might be differentially modulated in different cell types (10). Although the present work did not evaluate whether nnAChRs in the central nervous system and in ciliary neurons are similarly regulated by BSA, the potential importance of this regulatory process warrants further study.

Acknowledgments

We thank Drs. Darwin K. Berg, Leslie Blair, and Nicholas C. Spitzer for helpful discussions and comments on the manuscript and Drs. Claudio Schteingart and Jan Schnitzer for advice on the characterization of the BSA activity.

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