Pergamon

Biochemical Pharmacology, Vol. 50, No. 10, pp. 1665–1671, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02067-M

REGULATION OF NICOTINIC ACETYLCHOLINE RECEPTORS ON HUMAN NEUROBLASTOMA CELLS DURING DIFFERENTIATION

STANLEY W. HALVORSEN,* NING JIANG and RENAE MALEK

Department of Biochemical Pharmacology, State University of New York at Buffalo, Buffalo, NY 14260, U.S.A.

(*Received* 24 *January* 1995; *accepted* 14 *June* 1995)

Abstract—Neuronal nicotinic acetylcholine receptors are expressed on a variety of cells in the nervous system where they play key roles in synaptic transmission and information transfer. Little is known, however, about the molecular mechanisms that control their expression, distribution, and function during nervous system development. We have investigated the control of expression during differentiation of one class of acetylcholine receptors that bind α-bungarotoxin of human neuroblastoma cells. We report that induction of differentiation of SH-SY5Y, SK-n-SH or IMR-32 cells by the phorbol ester 12-*O*-tetradecanoyl phorbol 13-myristate (10 nM, TPA) or by retinoic acid resulted in as much as a 70% decline in α-bungarotoxin receptors on the cells. The response to the phorbol ester was blocked by the protein kinase C inhibitors staurosporine and bisindolylmaleimide. The decrease in receptors induced by 10 μM retinoic acid was not affected by either agent. However, responses to lower (10 nM) concentrations of retinoic acid were blocked by staurosporine but not bisindolylmaleimide, suggesting a dual mechanism of action for retinoic acid in regulating acetylcholine receptors. It appears that acetylcholine receptors on neuroblastoma cells are regulated during differentiation by both protein kinase C-dependent and -independent mechanisms.

Key words: nicotinic receptor; neuronal acetylcholine receptor; phorbol ester; bungarotoxin; retinoic acid; protein kinase C

Neurons have the potential to express a variety of nicotinic ACh† receptor subtypes. It is generally believed that neuronal nicotinic receptors have the same basic structure as muscle nicotinic receptors and are formed as hetero-pentameric complexes with at least two ACh-binding alpha subunits [1]. In the case of muscle-type receptors, the alpha subunit is designated α1, and the remaining subunits β1, γ (or ε), and δ. Neuronal ACh receptors are capable of forming from a much larger group of gene products; thus, when expressed in *Xenopus* oocytes, various combinations of one type of alpha subunit with a single type of non-alpha subunit form a functional ACh-gated ion channel [1]. In embryonic chick ciliary ganglia, at least four distinct alpha subunits and two different non-alpha subunit genes are active [2]. However, recent results from studies using subunit specific antibodies provide evidence that a population of receptors in ciliary ganglia are composed of at least three different subunits, α3, α5 and β4, that participate in rapid signaling events at the synapse [3–5]. In addition, these neurons express a second type of receptor that binds αBgt and contains the α7 subunit but not any of the other known ACh receptor subunits [4]. Activation of the α7 containing receptors results in an increase in intracellular Ca^{2+} and regulates neurite outgrowth [6, 7]. A key question emerges as to how cells control the genes for different subunits and how, when multiple genes are active, cells direct particular combinations of subunits to assemble and form functional receptors. Thus, the effects of electrical activity, neurotransmitter-generated activity, and exposure to environmental cues, such as hormones and neurokines, on ACh receptor expression are being examined in detail on nerve and muscle cells [8–16].

The human neuroblastoma cell lines SH-SY5Y, SK-n-SH, and IMR-32 express neuronal-type nicotinic ACh receptors similar to those expressed in autonomic ganglia and in the central nervous system [17–19]. SH-SY5Y cells are known to contain ACh receptors that bind αBgt and contain the α7 gene product [18, 19]. Neuroblastoma cell lines are being utilized increasingly as model systems to examine the properties of neurons undergoing certain phases of development and differentiation. These cells are sympathetic ganglia-like precursor cells that undergo limited differentiation when exposed to phorbol esters, cyclic AMP derivatives, or retinoic acid [20–23]. For example, SH-SY5Y cells, when treated with TPA, a phorbol ester, slow their rate of division, extend neurites, undergo a series of morphological changes, and enhance their adrenergic properties by increasing tyrosine hydroxylase activity and ACh-stimulated norepinephrine release [21].

We report here that treatment with TPA and retinoic acid, but not cyclic AMP (cAMP), leading to the differentiation of the human neuroblastoma cells SH-SY5Y, SK-n-SH and IMR-32 resulted in a large reduction in the numbers of αBgt-binding ACh receptors on the cells. The reduction is consistent with involvement of mechanisms, both inclusive and exclusive, of protein kinase C activation.

MATERIALS AND METHODS

Materials

αBgt was purified from *Bungarus multicinctus* venom as described previously [24] and radioiodinated to a spe-

* Corresponding author: Dr. Stanley W. Halvorsen, Department of Biochemical Pharmacology, School of Pharmacy, 448 Hochstetter Hall, SUNY at Buffalo, Buffalo, NY 14260. Tel. (716) 645-3936; FAX (716) 645-3850.

† Abbreviations: ACh, acetylcholine; TPA, 12-*O*-tetradecanoyl phorbol 13-myristate; αBgt, α-bungarotoxin; and BIM, bisindolylmaleimide.

cific activity of 300–400 cpm/fmol using a modified chloramine T method [10, 25]. $Na^{125}I$ was purchased from Amersham (Arlington Heights, IL). Staurosporine and BIM were from Calbiochem (La Jolla, CA), cell culture reagents were from GIBCO (Grand Island, NY), and other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO).

Cell culture

The human neuroblastoma cell line SH-SY5Y was obtained from Dr. June Biedler of the Sloan Kettering Institute for Cancer Research [26]. SK-n-SH (the parent line of SH-SY5Y) and IMR-32 [27] cells were obtained from the American Type Culture Collection. Cultures of SH-SY5Y and SK-n-SH were grown in a 1:1 mixture of Ham's F12 and Eagle's minimal essential medium (MEM) containing 1% nonessential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum in a humidified incubator with 95% air/5% CO_2 atmosphere at 37°. IMR-32 cells were cultured in MEM supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum. Cultures were grown to confluence in 75 cm^2 tissue culture flasks; before each experiment cells were replated into 35-mm wells of 6-well trays in the same medium without penicillin/streptomycin. After 24 hr, the culture medium was supplemented as indicated with TPA, retinoic acid, or the indicated reagent.

Binding assays

Binding assays for ACh receptors on cells in culture were initiated by replacing the culture medium in duplicate or triplicate wells with 1.0 mL fresh medium containing 3×10^{-9} M ^{125}I-αBgt as previously described [10]. After 60 min at 37°, the reaction was terminated by removing the medium and rinsing the cells three times with 2-mL aliquots of rinse buffer (MEM with 10 mM HEPES, pH 7.2). The cells were solubilized in 1 mL of 0.1 N NaOH and measured for radioactivity in a gamma counter. Specific binding was defined as the difference between total binding and nonspecific binding, which occurred in the presence of 2.5 mM *d*-tubocurarine. Binding data were normalized for growth and plating differences between wells following a Lowry protein determination, using BSA as a standard. Statistical analyses of data were performed using a one-tailed Student's *t*-test.

RESULTS

Regulation of αBgt binding to cells

Treatment of neuroblastoma cells with retinoic acid or phorbol ester resulted in a time-dependent decrease of ^{125}I-αBgt binding to cells (Fig. 1). Treatment of SH-SY5Y cells with either retinoic acid (10 μM) or TPA (10 nM) elicited a significant response within 1 day and a maximal response after 2 days of exposure, resulting in a 60–70% decline in surface ^{125}I-αBgt binding (Fig. 1). During this time period, the cells showed a decrease in growth rate and most extended processes and developed morphological changes consistent with previously published results [20, 21]. The response to TPA was relatively stable for up to an additional 4 days of treatment, showing only a modest recovery of binding activity. In contrast, the response to retinoic acid was more transient. Upon reaching a maximum decline of about 40%

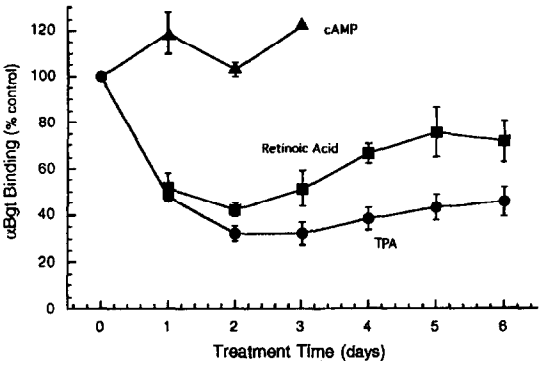

Fig. 1. Time course of the effects of TPA and retinoic acid on ^{125}I-αBgt binding. SH-SY5Y cells were planted into 35 mm dishes as described in Materials and Methods. After 24 hr, all cells were grown for an additional 6 days. Prior to assay for ^{125}I-αBgt, binding cells were incubated in the presence of 10 nM TPA (●), 1–2 mM 8-borom-cAMP (▲), or 10 μM retinoic acid (■) for the indicated number of days. All cultures were assayed at the same time and normalized to binding levels from untreated cells [100% (no treatment control) was 32 ± 5 fmol/mg]. Results are the means ± SEM from 3 independent experiments for retinoic acid and TPA and the means ± range from 2 experiments for 8-bromo-cAMP.

of control at 2 days, a gradual and partial recovery was observed until day 5 of continuous treatment, reaching a new steady-state level of about 70–75% of control. Treatment of cells for as long as 3 days with 1–2 mM 8-bromo-cAMP did not cause a reduction in αBgt binding (Fig. 1). These results suggested that induction of cell differentiation by itself was not sufficient to cause down-regulation of αBgt receptors.

Concentration-dependence of receptor regulation

To determine the effective concentration range, we treated SH-SY5Y cells with increasing concentrations of TPA from 0.1 to 100 nM. The cells showed decreasing levels of ^{125}I-αBgt binding for TPA concentrations up to 10 nM, which are concentrations expected to increase protein kinase C activity in these cells (Fig. 2A). TPA concentrations of 100 nM produced a less than maximal response (Fig. 2A). The primary effect that we would expect on these cells from TPA at 10 nM is the activation of protein kinase C, and this effect is specific for the biologically active 4-β phorbol esters [28]. SH-SY5Y cells treated with the non-tumor promoting 4α-phorbol ester derivative (4α-phorbol 12,13-didecanoate, 10 nM) had ^{125}I-αBgt binding levels similar to control (109 ± 7% of control), which were different from those of 10 nM TPA-treated cells (40 ± 4% of control, N = 6). The possibility that even at the relatively low 10 nM concentration TPA was down-regulating, and thus inhibiting, protein kinase C was examined by testing the effects of the protein kinase C inhibitors staurosporine and BIM. Staurosporine at 10 nM was shown previously to be effective at blocking PKC-mediated events in SH-SY5Y cells [22, 29]. Staurosporine (21 nM) was without effect on ^{125}I-αBgt binding when used alone on the cells, but blocked the effects of TPA (Table 1A). We also tested the activity of a newer, more selective PKC inhibitor, BIM, on the TPA response [30]. Partial inhibition of TPA-mediated biochemical and morphological effects are seen at 0.5 to 1.0 μM, and complete block is ob-

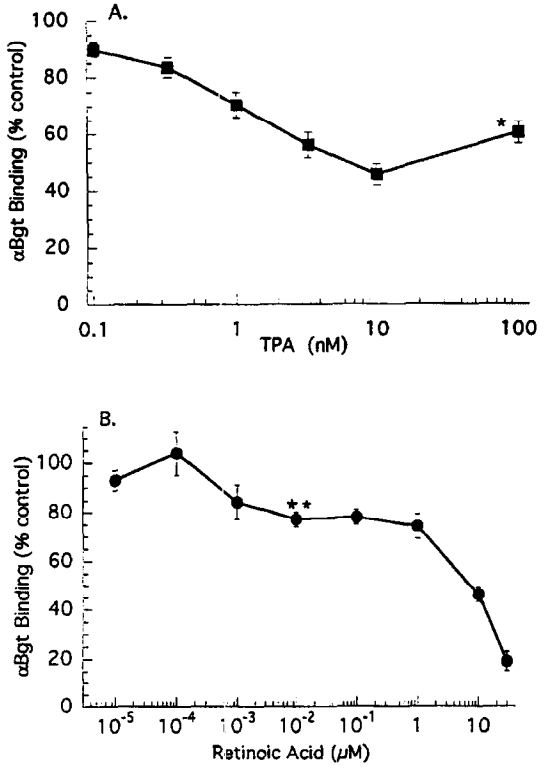

Fig. 2. Concentration dependence of TPA and retinoic acid on regulation of ^{125}I-αBgt binding. SH-SY5Y cells were planted into 35 mm dishes as described in Materials and Methods. After 24 hr, all cells were grown for an additional 2 days in the presence of the indicated concentration of TPA (A) or retinoic acid (B). Cells were assayed for ^{125}I-αBgt binding and normalized to binding levels from untreated cells (100% control); result are the means ± SEM from 13 independent experiments for TPA and 3–6 experiments for retinoic acid. The mean 100% control binding value was 35 ± 2 fmol/mg (TPA) and 28 ± 3 (retinoic acid). Key: (*) in panel A, $P < 0.05$ vs TPA at 10 nM, using Student's *t*-test; and (**) in panel B, $P < 0.025$ vs control, using Student's *t*-test.

served at 2–4 μM [31]. BIM at the submaximal concentration of 1 μM blocked approximately 45% of the TPA-induced down-regulation of αBgt binding (Table 1C). These results are consistent with the actions of TPA being mediated through activation of protein kinase C.

The response to increasing concentrations of retinoic acid on αBgt binding appeared biphasic. A decrease in ^{125}I-αBgt binding was seen following exposure to a concentration of 1 nM and appeared to plateau at about 80% of control between 10 nM and 1 μM (Fig. 2B). A further decrease was seen at concentrations greater than 1 μM (Fig. 2B). Activity over this concentration range of retinoic acid is consistent with previous studies showing that morphological differentiation of SH-SY5Y cells is observed at retinoic acid levels as low as 1 nM, and changes are optimal between 10^{-5} and 10^{-7} M [20]. Therefore, SH-SY5Y cells were responsive to retinoic acid over an extended concentration range, suggesting a complex mechanism(s) of action.

Distribution of response by cell type

Previous results have indicated that bovine chromaffin cells respond to TPA by increasing the numbers of ^{125}I-αBgt receptors [32]. Therefore, we examined cell types other than SH-SY5Y for their response to retinoic acid and TPA. The regulatory response was also measured in an unrelated neuroblastoma cell line by treating IMR-32 cells and a related cell line, SK-n-SH, and then compared with the results obtained from SH-SY5Y cells. All cell types responded to TPA and retinoic acid by slowing their proliferation, showing more extensive process outgrowth and through changes in morphology. αBgt binding was affected to varying degrees depending on the cell line (Table 2). SK-n-SH cells responded to TPA (10 nM) with a smaller reduction in binding than did SH-SY5Y cells, but increasing the concentration of TPA to 100 nM increased the response of SK-n-SH cells while decreasing the response of SH-SY5Y cells. The response of IMR-32 cells was intermediate between the SH-cell types. Differences in the degree of regulation of αBgt receptors among these different cell lines in response to retinoic acid was not as apparent. SK-n-SH cells were most sensitive and SH-SY5Y were the least responsive when assayed following exposure to 10 μM retinoic acid (Table 2). IMR-32 cells were intermediate between the other cell lines in the level of decrease of αBgt binding in response to retinoic acid.

Table 1. Effects of protein kinase C inhibitors on response to TPA and retinoic acid

Treatment	αBgt binding (% of control) Control	+Staurosporine	+BIM
Section A			
–TPA	100	105 ± 6	
+TPA	33 ± 11	85 ± 6*	
Section B			
–Retinoic acid	100	99 ± 2	
+Retinoic acid (10 nM)	80 ± 4†	95 ± 7‡	
+Retinoic acid (10 μM)	31 ± 1	30 ± 3	
Section C			
–TPA and retinoic acid	100		93 ± 3
+TPA	25 ± 5		58 ± 4§
+Retinoic acid (10 nM)	72 ± 6		67 ± 4
+Retinoic acid (10 μM)	41 ± 2		34 ± 1

SH-SY5Y cells were plated into 35 mm dishes as described in Materials and Methods. After 24 hr all cells were grown for an additional 2 days in the presence or absence of (A) TPA (10 nM) and/or staurosporine (21 nM), (B) retinoic acid and/or staurosporine, or (C) either TPA or retinoic acid and/or BIM (1 μM), as indicated. Cells were assayed for ^{125}I-αBgt binding/mg protein, and the results were normalized to binding levels from untreated cells (100% control). Results are the means ± SEM of the percent of control determined from N = 3 for sections A and C and N = 4 for section B. Control values are: section A, 13 ± 1 fmol/mg; section B, 14 ± 1 fmol/mg; section C, 18 ± 1 fmol/mg.

* $P < 0.005$ compared with +TPA control (A).
† $P < 0.05$ compared with –retinoic acid control (B).
‡ $P < 0.05$ compared with +retinoic acid (10 nM) control (B).
§ $P < 0.005$ compared with +TPA control (C).

Differential mechanism of regulation

The reduction in ^{125}I-αBgt binding could have been due to either a decrease in receptor affinity and/or a decrease in receptor number. SH-SY5Y cells treated for 2 days with either TPA (10 nM) or retinoic acid (10 μM)

Table 2. Regulation of α-bungarotoxin receptors on different neuroblastoma cells

Cell type	αBgt binding (% of control) TPA 10 nM)	TPA (100 nM)	Retinoic acid (10 μM)
IMR-32	60 ± 5 (N = 9)	ND*	41 ± 4 (N = 9)
SH-SY5Y	46 ± 4 (N = 13)	61 ± 4 (N = 13)	50 ± 3 (N = 6)
SK-n-SH	71 ± 6 (N = 6)	30 ± 3 (N = 6)	32 ± 6 (N = 6)

The indicated cell types were plated into 35 mm dishes as described in Materials and Methods. After 24 hr all cells were grown for an additional 2 days in the presence of TPA or retinoic acid. Cells were assayed for ^{125}I-αBgt binding/mg protein, and the results were normalized to binding levels from untreated cells (100% control). Results are the means ± SEM of the percent of control determined from the number of independent experiments (N) indicated. Control binding levels were: IMR-32 cells, 21 ± 2 fmol/mg; SH-SY5Y cells, 36 ± 2 (TPA), 33 ± 2 (retinoic acid); and SK-n-SH cells, 16 ± 2.

* ND = not determined.

had no change in affinity, whereas the density of αBgt receptors declined by 49 and 51%, respectively, compared with control cells based on Scatchard analysis of equilibrium ^{125}I-αBgt binding (Fig. 3). Treatment of cells for 2 days at the lower retinoic acid concentration, 10 nM, followed by Scatchard analysis of ^{125}I-αBgt binding produced a similar result. Control cells had a K_d of 1.9 ± 0.1 nM and a B_{max} of 31 ± 2 fmol/mg, whereas cells treated with 10 nM retinoic acid had a K_d of 2.1 ± 0.2 nM and a B_{max} of 24 ± 2 fmol/mg (79 ± 7% of control, N = 3). These results indicated that the affinity of the receptor for ^{125}I-αBgt had not changed following treatment with TPA or either concentration of retinoic acid, and that the total density of receptors on the cells was decreased following each treatment.

Since both TPA and retinoic acid induce differentiation of SH-SY5Y cells and since the maximal decrease and the time-course for the decrease were qualitatively similar, we explored the possibility that these agents were utilizing a common mechanism to elicit the decrease in αBgt receptors. We performed a series of mixing experiments by co-administering retinoic acid and TPA to the cells to determine if the regulatory response was additive. Addition of retinoic acid at the lower concentration of 10 nM to cells incubated with increasing concentrations of TPA resulted in a gradual diminution of the response to retinoic acid such that no additional effect of retinoic acid was seen at a maximal concentration of TPA (3 nM in this set of experiments; Fig. 4). However, treatment of cells with a higher concentration of retinoic acid (10 μM) resulted in a significant decrease compared with cells treated only with 10 nM TPA (22 ± 4 vs 40 ± 6%, respectively, N = 9, P < 0.025).

The results of the experiments described above combining TPA and retinoic acid suggested an overlapping pathway for TPA and 10 nM retinoic acid but not 10 μM retinoic acid (Fig. 4). Therefore, we tested if the effect of 10 nM retinoic acid on αBgt receptor levels was influenced by protein kinase C inhibitors. A concentration of staurosporine, 21 nM, that inhibited TPA effects (Table 1A) and effectively blocks TPA-induced differentiation of SH-SY5Y cells [22, 29] nearly abolished the response of SH-SY5Y cells to 10 nM retinoic acid, but was without effect on the response to 10 μM retinoic acid (Table 1B). The more selective PKC blocker, BIM, was without effect on the response to retinoic acid at either 10 nM or 10 μM (Table 1C). Thus, the mechanism of retinoic acid-induced regulation of αBgt receptors on SH-SY5Y cells at the lower 10 nM concentration is apparently not mediated via protein kinase C, but does require activation of a protein kinase that is inhibited by staurosporine

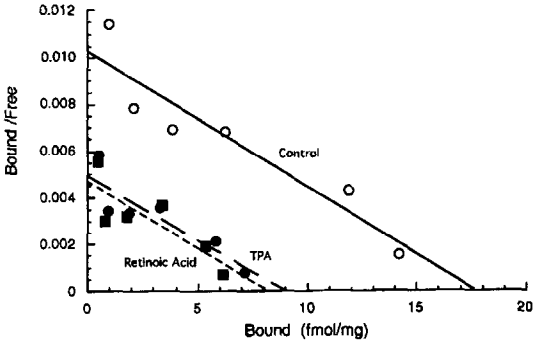

Fig. 3. Scatchard analysis of ^{135}I-αBgt binding to TPA- and retinoic acid-treated cells. SH-SY5Y cells were plated into 35 mm dishes as described in Materials and Methods. After 24 hr, all cells were grown for an additional 2 days in the presence of control medium (○,——), 10 nM TPA (■,— —) or 10 μM retinoic acid (●,– – – –). Cells were assayed for ^{125}I-αBgt binding in the presence of increasing concentrations of ^{125}I-αBgt and normalized per mg protein. The data were then analyzed by the method of Scatchard to determine the apparent K_d and B_{max} for binding. Results are from a single experiment, but similar results were obtained in a second experiment. The calculated K_d and B_{max} values were: control = 1.72 nM and 17.5 fmol/mg protein; TPA-treated = 1.83 nM and 9.0 fmol/mg; and retinoic acid-treated = 1.75 nM and 8.5 fmol/mg, respectively.

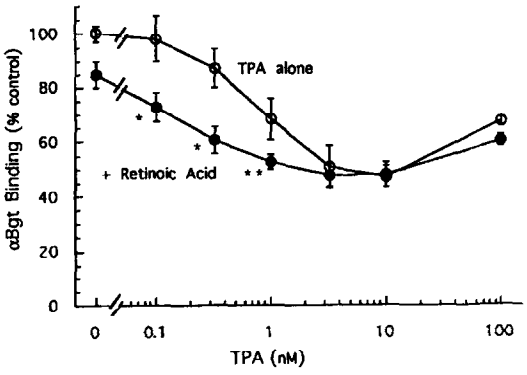

Fig. 4. Additive effects of TPA and retinoic acid on ^{125}I-αBgt binding to SH-SY5Y cells. SH-SY5Y cells were plated into 35 mm dishes as described in Materials and Methods. After 24 hr, all cells were grown for an additional 2 days in the presence of the indicated concentration of TPA alone (○), or TPA with 10 nM retionic acid (●). Cells were assayed for ^{125}I-αBgt binding and normalized to binding levels from untreated cells (32 ± 1 fmol/mg). Results are the means ± SEM of 3 independent experiments. Key: (*) P < 0.025 vs TPA at 0.1 and 0.3 nM, using Student's *t*-test; and (**) P < 0.1 vs TPA at 1.0 nM, using Student's *t*-test.

but not by BIM, whereas the effect of retinoic acid at 10 μM is not dependent on this protein kinase(s).

Reversal of down-regulation by TPA and retinoic acid

Removal of TPA and retinoic acid from the culture medium revealed an additional difference in their cellular responses. Recovery from the effects of TPA and retinoic acid exposure followed a similar time-course, but with different final results. Following removal of TPA (10 nM) from SH-SY5Y cells, ^{125}I-αBgt binding began to recover after 12–16 hr, and approached control values by 24 hr (Fig. 5). We tested the recovery of binding to cells following exposure to either a lower retinoic acid concentration (100 nM) or to the higher level (10 μM). After 2 days of exposure, removal of retinoic acid from the medium also resulted in a lag of 6–12 hr after which ^{125}I-αBgt binding began to recover; full recovery was achieved by 16 hr and rebounded to beyond 100% of control levels by 24 hr post-removal. The rebounded binding levels were greater following treatment with 10 μM retinoic acid (140%), and the lag was longer than that seen following the 100 nM treatment (120%) (Fig. 5). In either case, recovery from the down-regulation induced by retinoic acid appeared more rapid and more complete than recovery from TPA. During the recovery period, the cells retained their morphologically differentiated appearance, and the final difference in binding levels between the two agents did not simply reflect a delayed removal or reversal of TPA since at 24 hr the values had reached steady state and no further increases were observed over the next 24 hr (Fig. 5). Thus, the cells recovered from exposure to either TPA or retinoic acid by a relatively slow replacement of αBgt receptor to the cell surface, but following retinoic acid treatment and removal an overexpression of the receptor was observed.

DISCUSSION

In this report, we have shown that the induction of differentiation in several neuronal cell lines resulted in the down-regulation of nicotinic ACh receptors on the cells. The reduction occurred whether the cells were induced by the phorbol ester, TPA, or the hormone, retinoic acid. These are compounds considered to have distinct targets of action in the cells. The effects seen following TPA treatment were most consistent with a requirement for the activation of protein kinase C. TPA at 10 nM has been shown previously to produce a peak in protein kinase C activity within 6 hr on SH-SY5Y cells [28]. The blockade of the effects of TPA by the protein kinase C inhibitors, staurosporine and BIM (Table 1), provides further evidence for involvement of this kinase. Both staurosporine and BIM completely block the differentiation effects of TPA on SH-SY5Y cells [31]. Although staurosporine is not very selective among protein kinases, BIM shows good selectivity for inhibition of protein kinase C over protein kinase A, receptor tyrosine kinases, and Ca^{2+}/calmodulin-dependent kinases at the concentrations used here [30, 33]. However, blockade of responses by protein kinase C inhibitors should, in general, not be considered conclusive for indicating a protein kinase C-dependent mechanism. The reduced effect of TPA observed at concentrations above 10 nM (Fig. 2A, Table 2) may be a result of protein kinase C desensitization, which occurs frequently at higher concentrations and longer treatment times [28]. Another possibility for the reduced response at higher TPA concentrations is that the cells express multiple types of protein kinase C with different sensitivities to TPA. SH-SY5Y cells are known to contain α, β, δ and ε forms of the kinase that respond differently to activators and inhibitors of protein kinase C [29].

The effects seen following retinoic acid treatment suggest different mechanisms of action for lower (1–100 nM) compared with higher (10–30 μM) concentrations (Fig. 2B). The response to lower, but not higher, concentrations was inhibited effectively by staurosporine but not BIM, suggesting that a functional protein kinase other than protein kinase C is necessary for its effect (Table 1). Alternatively, retinoic acid at 10 nM may activate a protein kinase C subtype not sensitive to inhibition by BIM. The biphasic effects observed with retinoic acid of different concentrations is likely the result of actions at distinct retinoic acid receptors. SH-SY5Y cells express several types of retinoic acid receptors that are regulated differently during differentiation [34]. The continued exposure of cells to 10 μM retinoic acid resulted in a gradual recovery of αBgt receptor levels after several days (Fig. 5). This recovery may be the result of retinoic acid receptor desensitization or it could be due to the generation of a final cell phenotype with distinct αBgt receptor regulatory properties as compared with cells undergoing differentiation.

The extended time courses for down-regulation and recovery of αBgt receptors (Figs. 1 and 5) suggest that changes in protein turnover are involved rather than a more transient sequestration of receptors within cells. However, an additional factor to consider for the recovery phase is the length of time required for complete elimination of the lipophilic retinoic acid and TPA from the cells following their removal from the medium. An interesting feature of the recovery was the overshoot seen following removal of retinoic acid. This may be related to the cells developing different phenotypes. SH-SY5Y cells are known to develop distinct biochemical and morphological characteristics following TPA and

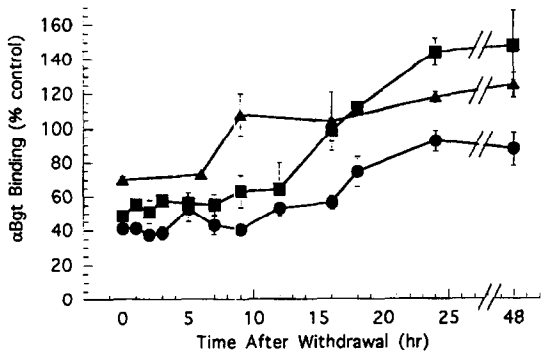

Fig. 5. Time course for recovery of ^{125}I-αBgt binding to SH-SY5Y cells following treatment with TPA or retinoic acid. SH-SY5Y cells were plated into 35 mm dishes as described in Materials and Methods. Following treatment for 2 days with either 10 nM TPA (●), 0.1 μM retinoic acid (▲), or 10 μM retinoic acid (■) or no treatment, the medium was removed, cells were rinsed thoroughly, and the medium was replaced with fresh control medium. At the indicated times following removal of the reagents, cells were assayed for ^{125}I-αBgt binding and normalized to binding levels from untreated cells (17 ± 2 fmol/mg) as previously described. Treatments were initiated at various times after replating to permit all cells to be assayed on the same day. Results are the means ± range from 2 experiments.

retinoic acid-induced differentiation [20]. Studies are in progress to discern the precise fate of the receptors undergoing regulation.

The regulation of nicotinic ACh receptors is an event commonly seen following treatments that induce differentiation. However, the extent and direction of the regulation are dependent on the type of receptor and the type of cell. In the case of rat PC12 cells, NGF-induced differentiation results in an increase in ACh-mediated channel activity and an increase in the levels of mRNA for several receptor subunits including α7 [15, 35]. Our results indicate that SH-SY5Y cells induced to differentiate with 8-bromo-cAMP show no decrease in levels of αBgt receptor after 3 days, and perhaps a small increase, consistent with results previously reported using IMR-32 cells. IMR-32 cells induced to differentiate with dibutyryl-cAMP for 13 days show a 3-fold increase in αBgt receptors compared with undifferentiated cells [23]. The apparent smaller response reported here could be due to either the extended treatment times previously utilized, the different cell types, or differences in the effects of dibutyryl- as compared with 8-bromo-cAMP. In any case, cells differentiated by retinoic acid and TPA express markedly fewer αBgt receptors than those differentiated following cAMP treatment. Chick skeletal muscle responds in a manner similar to SH-SY5Y cells, as electrical activity triggers an activation of protein kinase C and a decrease in synthesis of muscle-type ACh receptor [8]. The regulatory response of the adrenergic-like cell lines reported here to TPA was the opposite of those previously reported for primary adrenergic chromaffin cells in culture. Freshly isolated bovine adrenal cells respond to TPA by increasing as much as 10-fold their levels of αBgt receptors through an apparent protein kinase C mechanism [32]. The subunit structure of chromaffin nicotinic receptors is not known, but in culture they appear to express ganglia-type receptors that contain an α3 subunit [36] as well as relatively small numbers of αBgt receptors that do not contain either α1, α3 or α5 [37]. It will be interesting to learn the molecular basis for the different responses seen in chromaffin as compared with neuroblastoma cells.

Our results reported here indicate a potential role of protein kinase C in the regulation of neuronal ACh receptor assembly and/or turnover, and also suggest that additional pathways are utilized that require other protein kinases yet to be identified. Elucidation of the mechanisms controlling ACh receptor expression on differentiating neuroblastoma cells will enhance our understanding of the ways that cells regulate neurotransmitter receptors in developing nerve cells.

Acknowledgements—Grant support was provided by the National Science Foundation (IBN9210348 to S.W.H.), the Pharmaceutical Manufacturers Association, and the American Association of Colleges of Pharmacy. We thank Raquel Lima for expert technical assistance and Dr. June Biedler and Barbara Spengler for the SH-SY5Y cells.

REFERENCES

1. Sargent PB, The diversity of neuronal nicotinic acetylcholine receptors. In: *Annual Review of Neuroscience* (Eds. Cowan WM, Shooter EM, Stevens CF and Thompson RF), Vol. 16, pp. 403–444. Annual Reviews, Palo Alto, CA, 1993.
2. Corriveau R and Berg DK, Coexpression of multiple acetylcholine receptor genes in neurons: quantification of transcripts during development. *J Neurosci* **13:** 2662–2671, 1993.
3. Halvorsen SW and Berg DK, Subunit composition of neuronal nicotinic acetylcholine receptors from chick ciliary ganglia. *J Neurosci* **10:** 1711–1718, 1990.
4. Conroy W, Vernallis A and Berg DK, The α5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive receptor subtypes in brain. *Neuron* **9:** 679–691, 1992.
5. Jacob MJ, Lindstrom J and Berg DK, Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine receptor. *J Cell Biol* **103:** 205–214, 1986.
6. Vijayaraghavan S, Pugh P, Zhang Z, Rathous M and Berg DK, Nicotinic receptors that bind α-Bgt on neurons raise intracellular free Ca^{2+}. *Neuron* **8:** 353–362, 1992.
7. Pugh PC and Berg DK, Neuronal acetylcholine receptors that bind α-bungarotoxin mediate neurite retraction in a calcium-dependent manner. *J Neurosci* **14:** 889–896, 1994.
8. Huang C-F, Tong J and Schmidt J, Protein kinase C couples membrane excitation to acetylcholine receptor gene inactivation in chick skeletal muscle. *Neuron* **9:** 671–678, 1992.
9. Berg DK, Boyd RT, Halvorsen SW, Higgins LS, Jacob MH and Margiotta JF, Regulating the number and function of neuronal acetylcholine receptors. *Trends Neurosci* **12:** 16–21, 1989.
10. Halvorsen SW and Berg DK, Specific down-regulation of the α-bungarotoxin binding site on chick autonomic neurons by ciliary neuronotrophic factor. *J Neurosci* **9:** 3673–3680, 1989.
11. Halvorsen SW, McEachern AE, Schmid HA and Berg DK, Regulation of acetylcholine receptors on chick ciliary ganglion neurons by components from the synaptic target tissue. *J Neurosci* **11:** 2177–2186, 1991.
12. Corriveau R and Berg DK, Neurons in culture maintain acetylcholine receptor levels with far fewer transcripts than *in vivo*. *J Neurobiol* **25:** 1579–1592, 1995.
13. Lukas R, Effects of chronic nicotinic ligand exposure on functional activity of nicotinic acetylcholine receptors expressed by cells of the PC12 rat pheochromocytoma or the TE671/RD human clonal line. *J Neurochem* **56:** 1134–1145, 1991.
14. Simasko S, Durkin J and Weiland G, Effects of substance P on nicotinic acetylcholine receptor function in PC12 cells. *J Neurochem* **49:** 253–260, 1987.
15. Ifune C and Steinbach JH, Regulation of sodium currents and acetylcholine responses in PC12 cells. *Brain Res* **506:** 243–248, 1990.
16. Simasko S, Soares J and Weiland G, Two components of carbamylcholine-induced loss of nicotinic acetylcholine receptor function in neuronal cell line PC12. *Mol Pharmacol* **30:** 6–12, 1986.
17. Lukas R, Expression of ganglia-type nicotinic acetylcholine receptors and nicotinic ligand binding sites by cells of the IMR-32 human neuroblastoma clonal line. *J Pharmacol Exp Ther* **265:** 294–302, 1993.
18. Lukas R, Norman S and Lucero L, Characterization of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y human neuroblastoma clonal line. *Mol Cell Neurosci* **4:** 1–12, 1993.
19. Peng X, Katz M, Gerzanich V, Anand R and Lindstrom J, Human α7 acetylcholine receptor: Cloning of the α7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional α homomers expressed in *Xenopus* oocytes. *Molec Pharmacol* **45:** 546–554, 1994.
20. Påhlman S, Ruusala A-I, Abrahamsson L, Mattsson MEK and Esscher T, Retinoic acid-induced differentiation of cultured human neuroblastoma cells: A comparison with phorbol ester-induced differentiation. *Cell Differ* **14:** 135–144, 1984.
21. Påhlman S, Ruusala A-I, Abrahamsson L, Odelstad L and Nilsson K, Kinetics and concentration effects of TPA-in-

duced differentiation of cultured human neuroblastoma cells. *Cell Differ* **12:** 165–170, 1983.
22. Leli U, Cataldo A, Shea T, Nixon R and Hauser G, Distinct mechanisms of differentiation of SH-SY5Y neuroblastoma cells by protein kinase C activators and inhibitors. *J Neurochem* **58:** 1191–1198, 1992.
23. Gotti C, Sher E, Cabrini D, Bondiolotti G, Wanke E, Mancinelli E and Clementi F, Cholinergic receptors, ion channels, neurotransmitter synthesis and neurite outgrowth are independently regulated during the *in vitro* differentiation of a human neuroblastoma cell line. *Differentiation* **34:** 144–155, 1987.
24. Ravdin PM and Berg DK, Inhibition of neuronal acetylcholine sensitivity by α-toxins from *Bungarus multicinctus* venom. *Proc Natl Acad Sci USA* **76:** 2072–2076, 1979.
25. Lindstrom J, Einarson B and Tzartos S, Production and assay of antibodies to acetylcholine receptors. *Methods Enzymol* **74:** 432–459, 1981.
26. Biedler J, Helson L and Spengler B, Morphology and growth, tumorigenicity and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* **33:** 2643–2652, 1973.
27. Tumilowicz JJ, Nichols W, Chelan J and Green AE, Definition of a continuous human cell line derived from neuroblastoma. *Cancer Res* **30:** 2110–2218, 1970.
28. Heikkilä J, Akerlind G and Akerman K, Protein kinase C activation and down-regulation in relation to phorbol ester-induced differentiation of SH-SY5Y human neuroblastoma cells. *J Cell Physiol* **140:** 593–600, 1989.
29. Leli U, Shea T, Cataldo A, Hauser G, Grynspan F, Beerman M, Liepkalns V, Nixon R and Parker P, Differential expression and subcellular localization of protein kinase C α, β, δ, and ε isoforms in SH-SY5Y neuroblastoma cells: Modifications during differentiation. *J Neurochem* **60:** 289–298, 1993.
30. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D and Kirilovsky J, The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266:** 15771–15781, 1991.
31. Heikkilä J, Jalava A and Eriksson K, The selective protein kinase C inhibitor GF 109203X inhibits phorbol ester-induced morphological and functional differentiation of SH-SY5Y human neuroblastoma cells. *Biochem Biophys Res Commun* **197:** 1185–1193, 1993.
32. Geersten S, Afar R, Trifaró J-M and Quik M, Phorbol esters and *d*-tubocurarine up-regulate α-bungarotoxin sites in chromaffin cells in culture via distinct mechanisms. *Neuroscience* **34:** 441–450, 1990.
33. Muid R, Dale M, Davis P, Elliott L, Hill C, Kumar H, Lawton G, Twomey B, Wadsworth J, Wilkinson S and Nixon J, A novel conformationally restricted protein kinase inhibitor, Ro 31-8425, inhibits human neutrophil superoxide generation by soluble, particulate and post-receptor stimuli. *FEBS Lett* **293:** 169–172, 1991.
34. Lovat P, Pearson A, Malcolm A and Redfern CPF, Retinoic acid expression during the *in vitro* differentiation of human neuroblastoma. *Neurosci Lett* **162:** 109–113, 1993.
35. Henderson LP, Gdovin MJ, Liu CL, Gardner PD and Maue RA, Nerve growth factor increases nicotinic ACh receptor gene expression and current density in wild-type and protein kinase A-deficient PC12 cells. *J Neurosci* **14:** 1153–1163, 1994.
36. Criado M, Alamo L and Navarro A, Primary structure of an agonist binding subunit of the nicotinic acetylcholine receptor from bovine adrenal chromaffin cells. *Neurochem Res* **17:** 281–287, 1992.
37. Higgins LS and Berg DK, Immunological identification of a nicotinic acetylcholine receptor on bovine chromaffin cells. *J Neurosci* **7:** 1792–1798, 1987.