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γ-Aminobutyric Acid$_A$ Receptor Desensitization in Mice Spinal Cord Cultured Neurons: Lack of Involvement of Protein Kinases A and C

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SUMMARY

Desensitization of the γ-aminobutyric acid$_A$ (GABA$_A$) receptor was studied in cultured mammalian spinal cord neurons, using a GABA-induced $^{36}Cl^-$ influx assay. GABA$_A$ receptor agonists such as GABA and muscimol produced desensitization of GABA$_A$ receptor-gated Cl^- channels. The ability of GABA to induce desensitization was time and concentration dependent and reversible. Involvement of protein kinase A in the desensitization phenomenon was studied by using activators of adenylate cyclase (forskolin analogs) and membrane-permeant analogs of cyclic AMP (8-bromo-cAMP and dibutyryl-cAMP). Both active forskolin and the inactive forskolin analog 1,9-dideoxyforskolin decreased GABA-induced $^{36}Cl^-$ influx alone, as well as when preincubated in conjunction with GABA. The effect of forskolin analogs appears to be nonspecific and unrelated to generation of cyclic AMP. GABA-induced $^{36}Cl^-$ influx was also inhibited directly by 8-bromo-cAMP, dibutyryl-cAMP, and cAMP. Furthermore, the protein kinase A inhibitor H-8 did not reverse the effect of cAMP analogs on the inhibition of GABA-induced $^{36}Cl^-$ influx. Taken together, these results suggest that cAMP analogs inhibit GABA-induced $^{36}Cl^-$ influx by acting via an extracellular site. The inability of the active phorbol ester to modify GABA-induced desensitization rules out the involvement of protein kinase C in the GABA receptor desensitization. These results suggest that protein kinases A and C are not involved in GABA$_A$ receptor desensitization in mouse spinal cord cultured neurons.

Prolonged exposure of neurotransmitter receptors to their agonists results in a decreased response to subsequent challenge by the agonist. This phenomenon, termed "desensitization," has been described in a variety of systems including the nicotinic ACh receptor (1), a ligand-gated ion channel. Although the exact mechanism involved in the desensitization is not known, phosphorylation of the ACh receptor by PKA and PKC has been suggested to be involved in this process (2–4). In contrast, little is known about regulation of the GABA-gated Cl^- channel (also a ligand-gated ion channel) by second messenger systems and protein phosphorylation. However, recent studies have suggested that an ATP-dependent process, possibly phosphorylation, may be involved in the maintenance of GABA$_A$ receptor function (5, 6). Electrophysiological (7, 8) and biochemical (9, 10) studies have also revealed the desensitization of GABA$_A$ receptor-coupled chloride channels during sustained exposure to GABA. Furthermore, it has also been reported that cAMP analogs decrease the functional activity of the GABA$_A$ receptor-gated Cl^- channels in rat brain synaptoneurosomes (10), chick cortical neurons (8), and rat hippocampal neurons (11, 12). Because the GABA$_A$ receptor is highly homologous to nicotinic ACh receptors (13), it is possible that similar mechanisms (i.e., phosphorylation by PKA and PKC) may be involved in the desensitization of GABA$_A$ receptor. A consensus sequence for cyclic AMP-dependent phosphorylation in the β-subunit of the GABA$_A$ receptor (13) and phosphorylation of the β-subunit of the GABA$_A$ receptor by PKA has also been reported (14). A recent study has shown that two β-subunits could be phosphorylated by distinct kinases (15). These investigators have shown that PKA and PKC phosphorylate two distinct β subunits, with molecular weights of 58,000 and 56,000.

In our laboratory, a biochemical model using primary mammalian cultured spinal cord neurons has been developed for studying GABA-benzodiazepine receptor-ionophore complex pharmacology and regulation (16–20). Hence, we have investigated the desensitization of GABA$_A$ receptor-gated chloride channels using a biochemical technique of $^{36}Cl^-$ influx in a primary culture of mouse spinal cord cultured neurons. Additionally, we have examined the effect of modulators of PKA and PKC on GABA$_A$ receptor desensitization.

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ABBREVIATIONS: ACh, acetylcholine; GABA, γ-aminobutyric acid; PKC, protein kinase C; PKA, protein kinase A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimum essential medium.

Experimental Procedures

Materials. Female and male C57BL/6J mice (8–10 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). $^{36}Cl^-$ (HCl) was purchased from ICN Radiochemicals (Irvine, CA). ACh chloride, cAMP sodium salt, 8-bromo-cAMP sodium salt, N^6-2′-*O*-dibutyryl-cAMP sodium salt, forskolin, GABA, muscimol, (+)-bicuculline, (±)-nipecotic acid, phorbol-12,13-dibutyrate, poly-L-lysine hydrobromide (molecular weight > 300,000), and 5-fluoro-2′-deoxyuridine were purchased from Sigma (St. Louis, MO), whereas uridine was purchased from Calbiochem-Boehring (La Jolla, CA). 1,9-Dideoxyforskolin was purchased from Calbiochem (San Diego, CA). (−)-Baclofen HCl was a gift from Ciba-Geigy (Basel, Switzerland). H-8 hydrochloride was purchased from Research Biochemicals (Natick, MA). Bicinchoninic acid protein assay reagents were purchased from Pierce Chemical (Rockford, IL). MEM and serum were obtained from GIBCO (Santa Clara, CA). All other chemicals were purchased from commercial sources. Drugs that were insoluble in water were dissolved in dimethyl sulfoxide and used at a final dimethyl sulfoxide concentration of ≤0.1%. Control experiments showed that dimethyl sulfoxide at up to 0.1% had no effect on GABA-induced $^{36}Cl^-$ influx.

Preparation of cell cultures. Spinal cords were dissected from 13- to 14-day-old C57BL/6J mouse embryos. The embryos, in their sacs, were removed and placed in a 60-mm culture dish containing ice-cold aerated (95% O_2/5% CO_2) Puck's buffer, pH 7.4 (100 ml of 10× Puck's saline (in mM: 0.054 KCl, 0.011 KH_2PO_4, 0.73 NaCl, 0.011 $Na_2HPO_4 \cdot 7\ H_2O$), 10 ml of 1 M HEPES, and 50 ml of 12% glucose/30% sucrose solution, <320–330 mOsm), and spinal cords were dissected out under a microscope fitted with a light source. The spinal cords were then minced with iridectomy scissors in an empty sterile 60-mm Petri dish. Tissue was then taken up in 1.5 ml of nutrient medium, pH 7.4 (MEM 10/10), which contained 80% Eagle's MEM, 33.3 mM glucose, 26.2 mM $NaHCO_3$, 10% heat-inactivated horse serum (56° for 30 min), and 10% fetal bovine serum, and was transferred to a sterile 15-ml centrifuge tube. Tissue fragments were subjected to dissociation by trituration. This cycle of resuspension in MEM 10/10, followed by trituration, was repeated until a supernatant volume of 0.75 ml/spinal cord was attained. Dissociated cells were plated on poly-L-lysine-coated sterile 25-mm round coverslips, by addition of 0.5 ml of suspension to dishes containing 1 ml of MEM 10/10 that had been preincubated with 95% air/5% CO_2 for at least 1 hr at 37°. The coverslips were prepared by placing 10 sterile plastic coverslips, with a tab bent on the edge for handling with forceps, in 100-mm polystyrene dishes to which a sterile solution of 0.1 M boric acid (pH 8.4 with NaOH) and poly-L-lysine HBr (1 mg/100 ml) was added. The coverslips were soaked in this solution overnight and then rinsed with MEM and placed in 35-mm tissue culture dishes containing 1 ml of MEM 10/10.

The plated cultures were incubated for 24 hr. After this time, 1 ml of growth medium was replaced with 1 ml of medium containing 10% heat-inactivated horse serum only (MEM 10), and a mixture of sterile 5-fluoro-2′-deoxyuridine plus uridine (2 mg/ml 5-fluoro-2′-deoxyuridine and 5 mg/dl uridine) at a final concentration of 10 mg/ml was added. A portion (1 ml) of the medium was replaced with MEM 10 after 3 days, and 500 μl of the medium were replaced again 24 hr before the experiment.

$^{36}Cl^-$ influx. $^{36}Cl^-$ influx was measured by a modification of the previously described method of Thampy and Barnes (21), as described earlier (16, 18–20). Briefly, coverslips were removed from tissue culture medium, rinsed quickly three times at room temperature in HEPES-buffered saline, pH 7.4 (in mM: 136 NaCl, 5.4 KCl, 1.4 $MgCl_2$, 1.2 $CaCl_2$, 1 NaH_2PO_4, 20 HEPES, adjusted to pH 7.4 with Tris base), and drained rapidly on tissue paper, followed by immediate transfer to 2 ml of HEPES-buffered saline containing ^{36}Cl (2 μCi/ml), in the absence and presence of various drugs. For desensitization experiments, coverslips with attached neurons were rinsed twice in HEPES-buffered saline, followed by transfer to 2 ml of HEPES-buffered saline containing the drug, and were incubated for 10 min, unless otherwise mentioned in Results. After this time, the coverslips were drained rapidly on tissue paper and $^{36}Cl^-$ influx measurement was carried out. For recovery from desensitization experiments, the coverslips were removed following the incubation with the drug left in HEPES-buffered saline for a specified period, as mentioned in Results, and drained rapidly on tissue paper. $^{36}Cl^-$ influx was carried out as described above. GABA-mediated $^{36}Cl^-$ influx was measured in the presence of the uptake blocker nipecotic acid (100 μM), and basal $^{36}Cl^-$ influx was measured in the presence of 10 μM bicuculline. This concentration of bicuculline did not affect the basal flux. Influx was terminated after 5 sec by rapid transfer of the coverslip to 1000 ml of ice-cold stop solution and then immersion for 7 sec in another beaker containing 1000 ml of ice-cold stop solution, which was being stirred continuously. The stop solution contained in mM: 150 NaCl, 5.4 KCl, 1.4 $MgCl_2$, 1.2 $CaCl_2$, 1 NaH_2PO_4, and 5 HEPES, adjusted to pH 7.4 with Tris base. After a 7-sec immersion in the stop solution, each coverslip was drained on tissue paper and transferred to a scintillation vial containing 1.5 ml of 0.2 N NaOH. After 1 hr, a 0.5-ml aliquot was removed for protein determination, and the balance was neutralized with 1 N HCl (200 μl), mixed with 10 ml of Hydrofluor, and counted by liquid scintillation. Protein was estimated by the bicinchoninic acid protein assay (22). All values for $^{36}Cl^-$ influx were expressed per mg of cellular protein.

Cyclic AMP measurements. For cyclic AMP measurements, the neurons were pooled in HEPES buffer containing 4 mM EDTA (pH 7.4) and frozen. On the day of the assay, they were centrifuged at 45,000 rpm, and the pellet was homogenized in HEPES buffer, sonicated, and heated for 8 min in a boiling water bath. Following centrifugation, the cAMP in the supernatnat was assayed, using an Amersham cAMP assay kit (23). The results were expressed as pmol/mg of protein.

Data analysis. Statistical analysis was performed using Student's *t* test and analysis of variance; $p < 0.05$ was considered statistically significant.

Results

$GABA_A$ agonist-induced desensitization. GABA-induced $^{36}Cl^-$ influx in spinal cord neurons is specific for $GABA_A$ agonists and is concentration dependent, with half-maximal and maximal responses to GABA occurring at 10^{-5} and 10^{-4} M, respectively (19, 20). Preincubation of cultured spinal cord neurons with GABA (10^{-4} M) for 10 min produced a decrease in the ability of subsequent applications of GABA to induce $^{36}Cl^-$ influx, relative to control neurons. This effect, termed desensitization, was specific for $GABA_A$ receptor ligands, because the decrease in response was observed with GABA and muscimol but not with non-$GABA_A$ receptor ligands such as baclofen and ACh (Fig. 1). Both GABA (10^{-4} M) and muscimol (10^{-5} M) produced a similar ~50% decrease in response to subsequent application of GABA. Fig. 2 shows that the desensitization with GABA was concentration dependent. Incubation of spinal cord neurons with 5×10^{-6} M GABA produced a decrease in subsequent responses to GABA (10^{-4} M) by ~17%, compared with control. At 2×10^{-5} M GABA, the response was decreased by ~33%, and maximal desensitization of ~50% of the response was obtained with 10^{-4} M GABA. Fig. 2 also shows that the desensitization produced by GABA was time dependent. With 10^{-4} M GABA, $t_{1/2}$ for desensitization was 4 sec, with maximal desensitization occurring at 10–15 sec. Fig. 3 shows that the desensitization induced by GABA was reversible.

We also examined the possibility that the desensitization may not be real but could be due to the inability of the cells to accumulate Cl^- against an altered osmotic gradient or electrical shift potential. To address this question, we preincubated the neurons with glycine (which also activates Cl^- channels) or GABA and then measured the response to these agonists. Table 1 shows that glycine pretreatment did not result in a reduced

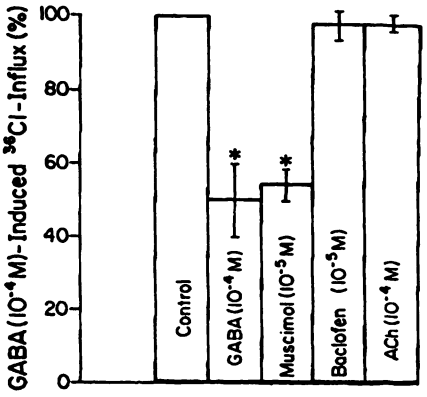

Fig. 1. Specificity of GABA$_A$ receptor-induced desensitization of GABA-gated Cl^- channels in mammalian spinal cord neurons. Spinal cord neurons were incubated in the absence or presence of various ligands for 10 min before measurement of GABA-induced $^{36}Cl^-$ influx for 5 sec, as described in the text. ACh produced similar effects in the absence or presence of an acetylcholinesterase inhibitor, physostigmine (10^{-5} M). The results are mean ± standard deviation of three experiments. * $p <$ 0.05, as compared with control.

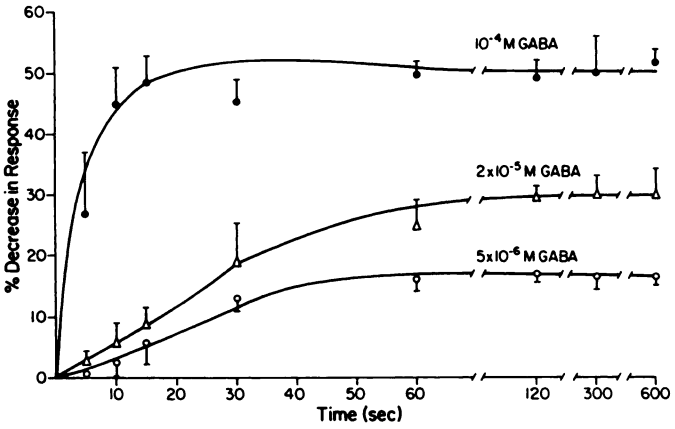

Fig. 2. Concentration- and time-dependent desensitization induced by GABA. Spinal cord neurons were incubated with various concentrations of GABA for various times before measurement of GABA (10^{-4} M)-induced $^{36}Cl^-$ influx. The results are mean ± standard deviation of three experiments, each done in triplicate.

response to GABA. Likewise, GABA pretreatment did not alter the response to glycine.

Effect of modulators of PKA on GABA receptor desensitization. Table 2 shows that an activator of adenylate cyclases, forskolin (10^{-5} M; 10-min pretreatment), *per se* did not affect the ability of GABA to induce $^{36}Cl^-$ influx or modify the response to GABA following desensitization induced by preincubation with GABA. Thus, GABA-induced desensitization was unaffected by 10^{-5} M forskolin. However, pretreatment with higher concentrations of forskolin (10^{-4} M) in the absence of GABA decreased the GABA response. Preincubation of the neurons with a combination of GABA (10^{-4} M) and forskolin (10^{-4} M) increased the reduction in response to GABA, as compared with GABA or forskolin preincubation alone (Table 2). Furthermore, use of a similar concentration of an inactive analog of forskolin such as 1,9-dideoxyforskolin (10^{-4} M) also produced a similar direct and additive effect with GABA (Table 2). Table 3 shows that, although forskolin increased cAMP production, 1,9-dideoxyforskolin did not significantly elevate

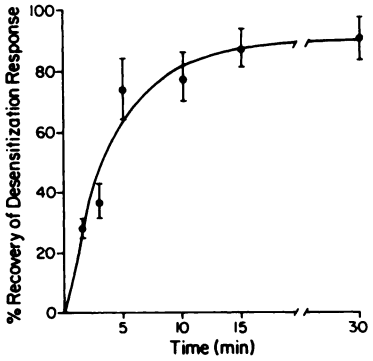

Fig. 3. Recovery of GABA (10^{-4} M)-induced desensitization. Spinal cord neurons were incubated with (±)-nipecotic acid (10^{-4} M) and GABA (10^{-4} M) for 10 min. After this time, each coverslip with attached neurons was left in contact with HEPES-buffered solution (2 ml) for various times, as shown in the figure. Following this, the coverslip was drained rapidly on tissue paper, passed quickly through another Petri dish containing 2 ml of HEPES-buffered saline, and drained rapidly before $^{36}Cl^-$ influx measurement was carried out. The results are mean ± standard deviation of three experiments, each done in triplicate.

TABLE 1

Lack of generalization of desensitization between GABA and glycine

Coverslips containing neurons were preincubated with glycine or GABA for 10 min before measurement of GABA/glycine-induced $^{36}Cl^-$ influx for 5 sec, as described in the text. The results are mean values ± standard deviation of individual experiments, each done in triplicate.

Treatment	Pretreatment (10 min)	Increase over basal
		%
GABA (10^{-4} M)	None	101 ± 3.1; 100 ± 2.2
GABA (10^{-4} M)	Glycine (10^{-4} M)	100 ± 7.2; 98 ± 3.5
Glycine (10^{-4} M)	None	96 ± 1.0; 90 ± 0.3
Glycine (10^{-4} M)	GABA (10^{-4} M)	97 ± 1.3; 91 ± 2.7

TABLE 2

Effect of pretreatment with forskolin (10^{-5} M, 10^{-4} M), 1,9-dideoxyforskolin (10^{-4} M), and GABA (10^{-4} M) on GABA-induced $^{36}Cl^-$ influx in mouse cultural spinal cord neurons

Coverslips containing neurons were preincubated with forskolin or GABA alone and in combination for 10 min before measurement of GABA (10^{-4} M)-induced $^{36}Cl^-$ influx for 5 sec. Experiments were performed as described in Experimental Procedures. Control refers to GABA (10^{-4} M)-induced $^{36}Cl^-$ influx in the absence of any pretreatment. Values are mean ± standard deviation of three experiments, each done in triplicate. Forskolin analogs did not affect the basal flux (2.66 ± 0.12 nmol/mg of protein).

Pretreatment	GABA (10^{-4} M)-induced $^{36}Cl^-$ influx	Increase over basal
	nmol/mg of protein	%
Control	5.26 ± 0.34	97.7
GABA (10^{-4} M)	3.75 ± 0.49[a]	40.9
Forskolin (10^{-5} M)	5.11 ± 0.35[b]	92.1
Forskolin (10^{-4} M)	4.70 ± 0.01[a]	76.7
1,9-Dideoxyforskolin (10^{-4} M)	4.81 ± 0.01[a]	80.9
Forskolin (10^{-5} M) + GABA (10^{-4} M)	3.73 ± 0.35[b]	40.1
Forskolin (10^{-4} M) + GABA (10^{-4} M)	3.33 ± 0.09[b]	25.0
1,9-Dideoxyforskolin (10^{-4} M) + GABA (10^{-4} M)	3.50 ± 0.47[b]	31.5

[a] $p < 0.05$, as compared with control.
[b] Not significant, as compared with respective control.

cAMP in the spinal cord neurons. Thus, the effects of forskolin analogs appear to be unrelated to the generation of cAMP.

Table 4 summarizes the effect of the membrane-permeant analogs of cAMP 8-bromo-cAMP and dibutyryl-cAMP on GABA receptor desensitization. At a concentration of 5 × 10^{-4}

TABLE 3

Effect of forskolins on cAMP levels in intact spinal cord neurons

Spinal cord neurons were incubated with forskolin for 10 min and cAMP accumulation was measured, as described in Experimental Procedures. The values are mean ± standard deviation of six coverslips.

	cAMP accumulation
	pmol/mg of protein
Control	3.92 ± 0.57
Forskolin (10^{-4} M)	13.23 ± 1.45[a]
1,9-Dideoxyforskolin (10^{-4} M)	5.72 ± 1.87

[a] $p < 0.05$, as compared with control.

TABLE 4

Effect of pretreatment with 8-bromo-cAMP and dibutyryl-cAMP on GABA-induced $^{36}Cl^-$ influx in cultured spinal cord neurons

Experiments were performed as described in Experimental Procedures. Values are mean ± standard deviation of five to eight experiments, each done in triplicate.

Pretreatment	Decrease in GABA (10^{-4} M)-stimulated $^{36}Cl^-$ influx
	%
8-Bromo-cAMP (5×10^{-4} M)	0.3 ± 8.2
8-Bromo-cAMP (10^{-3} M)	25.8 ± 4.6
Dibutyryl-cAMP (10^{-3} M)	30.6 ± 8.9
GABA (10^{-4} M)	49.6 ± 10.5
8-Bromo-cAMP (5×10^{-4} M) + GABA (10^{-4} M)	48.1 ± 4.6
8-Bromo-cAMP (10^{-3} M) + GABA (10^{-4} M)	68.1 ± 5.3
Dibutyryl-cAMP (10^{-3} M) + GABA (10^{-4} M)	80.2 ± 12.8

TABLE 5

Concentration-dependent effect of pretreatment (10 min) and 8-bromo-cAMP and dibutyryl-cAMP on GABA-induced $^{36}Cl^-$ influx in mouse cultured spinal cord neurons

Values are mean ± standard deviation of three to five experiments, each done in triplicate.

Pretreatment (M)	Decrease in GABA (10^{-4} M)-stimulated $^{36}Cl^-$ influx
	%
8-Bromo-cAMP	
0.5×10^{-3}	0.3 ± 8.2
1.0×10^{-3}	25.8 ± 4.6
2.0×10^{-3}	47.4 ± 4.0
5.0×10^{-3}	72.5 ± 5.1
Dibutyryl-cAMP	
1.0×10^{-3}	30.6 ± 8.9
2.0×10^{-3}	52.8 ± 3.1
5.0×10^{-3}	73.6 ± 3.7

TABLE 6

Direct effect of analogs of cAMP on GABA-induced $^{36}Cl^-$ influx

GABA-induced $^{36}Cl^-$ influx was measured for 5 sec in the absence and presence of test compounds, as described in Experimental Procedures. The results are mean ± standard deviation of three experiments, each done in triplicate.

	Increase over basal
	%
GABA (10^{-4} M)	104 ± 4
GABA (10^{-4} M) + 8-bromo-cAMP (10^{-3} M)	75 ± 6
GABA (10^{-4} M) + dibutyryl cAMP (10^{-3} M)	66 ± 4
GABA (10^{-4} M) + cAMP (10^{-3} M)	72 ± 3

M, 8-bromo-cAMP neither had any *per se* effect nor altered the desensitization induced by GABA. However, a higher concentration of 8-bromo-cAMP (10^{-3} M) alone decreased the effect of GABA by 26 ± 5%. This effect of 8-bromo-cAMP was additive with that of the GABA preincubation (Table 4). Likewise, dibutyryl-cAMP (10^{-3} M) alone decreased the effect of GABA by 31 ± 9%, and its effect was also additive with GABA preincubation (Table 4). Table 5 shows that both 8-bromo-cAMP and dibutyryl-cAMP pretreatment (10 min) alone produced a concentration-dependent decrease in GABA-induced $^{36}Cl^-$ influx. The maximal inhibition of GABA-induced $^{36}Cl^-$ influx with these two analogs was greater (~70%), relative to GABA-induced desensitization (~50%; Fig. 1). Based on this observation, we examined the possibility that the analogs of cAMP might affect GABA-gated Cl^- channels directly. Table 6 shows that 8-bromo-cAMP and dibutyryl-cAMP inhibited GABA-induced $^{36}Cl^-$ influx during a 5-sec measurement, i.e., without preincubation. Surprisingly, cAMP also directly inhibited GABA-induced $^{36}Cl^-$ influx. Table 7 shows the effects of an inhibitor of protein kinase A, H-8, and activators of adenylate cyclase on GABA receptor desensitization. H-8 had no effect on the direct inhibitory effects of 8-bromo-cAMP and dibutyryl-cAMP on GABA-induced $^{36}Cl^-$ influx (Table 7A). Furthermore, H-8 reversed neither the desensitization induced by GABA nor the inhibitory effect of 8-bromo-cAMP and dibutyryl-cAMP alone or in combination with GABA (Table 7B).

Effect of modulator of PKC on GABA receptor desensitization. Table 8 shows the effect of an activator of PKC, such as phorbol-12,13-dibutyrate, on GABA receptor desensitization. This phorbol ester analog did not have any effect on GABA-induced desensitization of the $GABA_A$ receptor.

Discussion

In this study, we have presented data that indicate that GABA agonist pretreatment produces desensitization of $GABA_A$ receptors, as measured by GABA-gated $^{36}Cl^-$ influx, in mammalian spinal cord neurons. GABA-induced desensitization was both time and concentration dependent, with maximal desensitization (50% reduction in response) occurring at 10^{-4} M GABA. Furthermore, experiments with glycine rule out a generalized or heterologous desensitization of GABA-gated Cl^- channels in cultured spinal cord neurons.

$GABA_A$ receptors belong to a ligand-gated ion channel gene family, and involvement of PKA and PKC in the desensitization of nicotinic ACh receptors has been demonstrated (2–4). Furthermore, there are studies, both electrophysiological (7, 8, 24) and biochemical (9, 10), which suggest an involvement of these kinases in GABA receptor desensitization and "fading phenomenon" (5, 6). Based on these observations, we conducted studies to investigate the involvement of PKA and PKC in GABA-induced desensitization in well characterized mouse spinal cord cultured neurons (17–20).

Our studies with the activators of PKA rule out an involvement of cAMP-dependent PKA in the desensitization of $GABA_A$ receptors. This is based on the observations discussed below. Forskolin, at concentrations at which it produces a half-maximal enhancement of cAMP (10^{-5} M) (25), had no effect, either alone or in combination with GABA, on GABA-induced desensitization. Higher concentrations (10^{-4} M) of either forskolin or inactive 1,9-dideoxyforskolin alone (in the absence of

TABLE 7

Effect of inhibitor of PKA, H-8, on GABA receptor desensitization

Experiments were performed as described in the text. The results are mean ± standard deviation of three experiments, each done in triplicate.

	Pretreatment (10 min)	Increase over basal
		%
A. GABA (10^{-4} M)	None	108 ± 15
+8 Bromo-cAMP (10^{-3} M)	None	75 ± 6
+Dibutyryl-cAMP (10^{-3} M)	None	66 ± 4
+H-8 (5×10^{-5} M)	None	109 ± 12
+H-8 (5×10^{-5} M) + 8-Bromo-cAMP (10^{-3} M)	None	72 ± 7
+H-8 (5×10^{-5} M) + dibutyryl-cAMP (10^{-3} M)	None	69 ± 10
B. GABA (10^{-4} M)	GABA (10^{-4} M)	52 ± 4
GABA (10^{-4} M)	H-8 (5×10^{-5} M) + GABA (10^{-4} M)	54 ± 6
GABA (10^{-4} M)	8-Bromo-cAMP (10^{-3} M)	77 ± 5
GABA (10^{-4} M)	H-8 (5×10^{-5} M) + 8-Bromo-cAMP (10^{-3} M)	78 ± 6
GABA (10^{-4} M)	Dibutyryl-cAMP (10^{-3} M)	64 ± 8
GABA (10^{-4} M)	H-8 (5×10^{-5} M) + dibutyryl-cAMP (10^{-3} M)	63 ± 6
GABA (10^{-4} M)	GABA (10^{-4} M) + 8-Bromo-cAMP (10^{-3} M)	39 ± 10
GABA (10^{-4} M)	H-8 (5×10^{-5} M) + 8 Bromo-cAMP (10^{-3} M) + GABA (10^{-4} M)	36 ± 8
GABA (10^{-4} M)	GABA (10^{-4} M) + dibutyryl-cAMP (10^{-3} M)	35 ± 9
GABA (10^{-4} M)	H-8 (5×10^{-5} M) + GABA (10^{-4} M) + dibutyryl-cAMP (10^{-3} M)	35 ± 11

TABLE 8

Effect of pretreatment (10 min) with phorbol-12,13-dibutyrate (10^{-6} M) on GABA-induced $^{36}Cl^-$ influx in mouse cultured spinal cord neurons

Experiments were performed as described in Experimental Procedures. The results are mean ± standard deviation of a typical experiment, done in triplicate. Similar results were replicated two times (variation, ±10%).

Pretreatment	Decrease in GABA (10^{-4} M)-stimulated $^{36}Cl^-$ influx
	%
Phorbol-12,13-dibutyrate (10^{-6} M)	1.4 ± 2.0
GABA (10^{-4} M)	49.6 ± 10.6
Phorbol-12,13-dibutyrate (10^{-6} M) + GABA (10^{-4} M)	53.3 ± 10.3

GABA) produced a decrease in GABA-induced $^{36}Cl^-$ influx, and this effect was additive with desensitization induced by pretreatment with GABA. Our results with forskolin analogs are similar to those reported by Heuschneider and Schwartz (10) but differ from the studies of Tehrani *et al.* (8). The latter investigators reported that forskolin, but not the inactive forskolin analog, accelerated GABA-induced desensitization in chick cortical neurons. Forskolin analogs have nonspecific effects on membranes, and they may also affect ion channel function, independent of cAMP systems (e.g., Refs. 26–28). Our results would tend to suggest that the effect of forskolin analogs observed is also independent of the cAMP system.

Likewise, 8-bromo-cAMP and dibutyryl-cAMP alone both inhibited GABA-induced $^{36}Cl^-$ influx, and their effect was also additive with GABA-induced desensitization. Furthermore, both these analogs produced a concentration-dependent inhibition of GABA-induced $^{36}Cl^-$ influx, and the maximal inhibition obtained with these compounds (~70%) was greater than that obtained with GABA$_A$ agonists (~50%). Because cyclic nucleotides have been reported to directly modulate ion channels (e.g., Refs. 29 and 30), we examined the direct effect of cAMP analogs on GABA-induced $^{36}Cl^-$ influx. We observed that 8-bromo-cAMP also inhibited GABA-induced $^{36}Cl^-$ influx, without prior incubation, thus suggesting a possible direct effect on the channel. Surprisingly, cAMP also directly (without preincubation) inhibited GABA-induced $^{36}Cl^-$ influx. Finally, the inability of H-8 [an inhibitor of PKA; $IC_{50} = 1.2 \times 10^{-6}$ M (31)] to reverse the inhibitory effect of 8-bromo-cAMP and dibutyryl-cAMP also rules out an involvement of PKA in the desensitization of GABA$_A$-gated Cl^- channels. It should be pointed out that H-8 is not a highly specific antagonist of PKA, becuase it also inhibits PKC ($IC_{50} = 1.5 \times 10^{-5}$ M) (31). However, the results obtained with H-8 are consistent with other observations described in this study, which rule out an involvement of PKA in the GABA receptor desensitization phenomenon. Our studies are in agreement with those of Lambert and Harrison (11, 12) but are in contrast to the studies of Tehrani *et al.* (8). Lambert and Harrison (12) have reported that analogs of cAMP, including cAMP, decreased GABA$_A$ receptor-gated Cl^- currents in cultured hippocampal neurons. These investigators concluded that these effects were mediated via an extracellular site. Our studies are consistent with the notion that analogs of cAMP may modulate GABA-gated Cl^- channels via an extracellular site.

In contrast to the PKA system, limited information is available regarding a role of PKC in GABA$_A$ receptor systems. Interestingly, PKC has been demonstrated to phosphorylate a β (β_{56}) subunit of the GABA$_A$ receptor, which can be photoaffinity labeled with [^{3}H]muscimol (18). Moreover, there is a report indicating that activators of PKC decreased GABA$_A$ responses in oocytes, which expressed RNA for GABA$_A$ receptors (23). Our studies with an activator of PKC do not suggest an involvement of this system in the regulation of GABA$_A$ receptor-gated Cl^- channels in spinal cord neurons. Taken together, these results suggest that PKA and PKC do not appear to be involved in GABA$_A$ receptor desensitization in mammalian spinal cord cultured neurons.

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