0026-895X/90/060823-07$03.00/0

MOLECULAR PHARMACOLOGY, 38:823-828

Phosphorylation-Independent Effects of Second Messenger System Modulators on γ-Aminobutyric Acid$_A$ Receptor Complex Function

NANCY J. LEIDENHEIMER, MICHAEL D. BROWNING, THOMAS V. DUNWIDDIE, LISA D. HAHNER, and R. ADRON HARRIS

Denver Veterans Administration Medical Center and Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received July 9, 1990; Accepted September 10, 1990

SUMMARY

Recent studies investigating the functional significance of γ-aminobutyric acid$_A$ (GABA$_A$) receptor complex phosphorylation have employed membrane-permeant compounds to manipulate second messenger systems. Although these compounds affect GABA$_A$ receptor function, the dependence of these effects on phosphorylation has not been established. Here we report that several second messenger system modulations can decrease GABA$_A$ receptor function independently of their effects on protein phosphorylation. Brain membrane vesicles were lysed and resealed in the presence of EDTA to chelate internal Mg^{2+}. Under these conditions, phosphorylation of vesicle proteins was almost completely inhibited, as determined by incorporation of ^{32}P into phosphoproteins. In these lysed/resealed vesicles, an inhibition of muscimol-stimulated $^{36}Cl^-$ uptake was observed with the cAMP analogs 8-(4-chlorophenylthio)-cAMP, N_6,O_2'-dibutyryl-cAMP, and 8-bromo-cAMP, the protein kinase inhibitor H7, and the adenylate cyclase activator forskolin. In both intact and EDTA-treated lysed/resealed microsacs, cAMP analogs and H7 inhibited binding of the GABA$_A$ receptor ligand [^{3}H]SR 95531 at concentrations shown to inhibit muscimol-stimulated $^{36}Cl^-$ uptake. Forskolin was observed to inhibit the binding of *t*-butylbicyclophosphoro-[^{35}S]thionate, a ligand that binds to a site on the chloride channel. These results demonstrate that compounds commonly used to alter second messenger systems affect the receptor sites and function of the GABA$_A$ receptor chloride channel by mechanisms that do not involve protein phosphorylation. In light of these findings, results obtained with these compounds should be interpreted with caution.

The GABA$_A$ receptor complex is a ligand-gated chloride channel located on synaptic membranes throughout the brain (1). Activation of the complex by GABA increases chloride conductance, resulting in membrane hyperpolarization and neuronal inhibition. GABA$_A$ receptor-mediated inhibition represents a major inhibitory mechanism in the central nervous system and its modulation by drugs such benzodiazepines, barbiturates, and ethanol underscore its clinical importance.

The recent cloning and sequencing of the GABA$_A$ receptor complex has revealed the presence of a consensus sequence for phosphorylation on the intracellular loop of the complex, suggesting that the GABA$_A$ receptor may be regulated by phosphorylation (2). It has since been reported that the GABA$_A$ receptor complex is phosphorylated by PKA (3, 4), Ca^{2+}-phospholipid-dependent protein kinase (4), and an unidentified endogenous kinase (5).

The physiological significance of GABA$_A$ receptor phosphorylation remains unclear, because there is indirect evidence that phosphorylation both maintains (6, 7) and decreases (8–11) GABA$_A$ receptor function. GABA$_A$ receptor "run down" is prevented by Mg^{2+}-ATP and ATP-γ-S, but not by a nonhydrolyzable ATP analog, suggesting that protein phosphorylation may be necessary to maintain GABA$_A$ receptor activity (6, 7). In contrast, GABA receptor function is decreased by activators of protein kinases, indicating that phosphorylation may inhibit the GABA$_A$ receptor. Phorbol esters that are activators of Ca^{2+}-phospholipid-dependent protein kinase inhibit GABA$_A$ receptor-gated currents, whereas phorbol esters that are inactive toward this kinase are without effect (8). Analogs of cAMP that stimulate PKA inhibit GABA-mediated Cl^- currents in hippocampal neurons (9), inhibit muscimol-stimulated $^{36}Cl^-$ transport in synaptoneurosomes (10), and enhance GABA receptor desensitization in chick cortical neurons (11). Similarly,

This work was supported by the Veterans Administration and ADAMHA Grants AA06399 and AA03527.

ABBREVIATIONS: GABA, γ-aminobutyric acid; PKA, cAMP-dependent protein kinase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CPTcAMP, 8-(4-chlorophenylthio)-cAMP; 8BrcAMP, 8-bromo-cAMP; Bt$_2$cAMP, N_6,O_2'-dibutyryl-cAMP; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H8, *N*-[2-(methylamino)-ethyl]-5-isoquinoline-sulfonamide; TBPS, *t*-butylbicyclophosphorothionate; SDS, sodium dodecyl sulfate.

forskolin, a compound that increases intracellular cAMP levels through activation of adenylate cyclase, increases $GABA_A$ receptor desensitization rate (11) and reduces muscimol-stimulated $^{36}Cl^-$ uptake (10).

The effects of these compounds on $GABA_A$ receptor function have not, however, been conclusively demonstrated to be dependent on phosphorylation. It is possible that cAMP analogs may affect the $GABA_A$ receptor complex independently of protein kinase activation, because extracellular, but not intracellular, application of cAMP and its analogs inhibits GABA-mediated Cl^- currents (12). Furthermore, the inhibitory effects of forskolin on muscimol-stimulated $^{36}Cl^-$ transport do not correspond well to increases in intracellular cAMP levels, and a forskolin analog that is inactive toward adenylate cyclase has been noted to decrease $GABA_A$ receptor function (10). In light of this, it is possible that compounds employed to manipulate phosphorylation pathways may affect the $GABA_A$ receptor complex independently of their effects on second messenger systems. Here we report that a variety of these compounds can affect the $GABA_A$ receptor complex in a phosphorylation-independent manner.

Materials and Methods

Drugs

All test drugs were obtained from Sigma Chemical Co. (St. Louis, MO) except forskolin, H7, and H8, which were purchased from Calbiochem (San Diego, CA).

$^{36}Cl^-$ Uptake Experiments

ICR mice between 2 and 3 months of age (Institute of Cancer Research, Harlan, IN) were decapitated, and microsacs were prepared as described previously (13). To prepare intact microsacs, whole brains were homogenized with 16 strokes in 4.5 ml/brain of ice-cold microsac buffer containing 145 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5, using a glass-Teflon homogenizer (Thomas, size C). Membranes were centrifuged at 900 × *g* for 15 min and the pellet was resuspended in 8.0 ml of buffer/brain. Membranes were again centrifuged and the pellet was resuspended as before in ice-cold buffer, to give a final protein concentration of 4 mg/ml. Microsacs were placed on ice, preincubated with drugs for 10 min, aliquoted (200 μl/tube), incubated for 5 min at 22°, and assayed for muscimol-stimulated $^{36}Cl^-$ uptake.

Lysed and resealed microsacs were prepared as above until the second resuspension, at which point the pellet was resuspended in lysing buffer (6 mM tricine, 3 mM EDTA, pH 7.5), incubated at 34° for 10 min, and resealed with 10 volumes of calcium-free buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 3 mM EGTA, pH 7.5). The resealing buffer was gradually added over 5 min while membranes remained at 34°. After the final addition of resealing buffer, membranes were kept at 34° for 5 min and then placed on ice for 5 min. Resealed membranes were centrifuged at 17,300 × *g* for 5 minutes, the supernatant was decanted, and the pellet was resuspended in 4 ml of resealing buffer/whole brain.

Muscimol-stimulated $^{36}Cl^-$ uptake was measured by a modification of the method of Allan and Harris (13). Microsacs were incubated at 22° for 5 min before measurement of $^{36}Cl^-$ uptake. To initiate $^{36}Cl^-$ transport, 200 μl of buffer containing $^{36}Cl^-$ (2.0 μCi/ml of buffer), with or without muscimol, were pipetted into 200 μl of rapidly mixing microsacs. $^{36}Cl^-$ transport was stopped after 3 sec with 4 ml of ice-cold microsac buffer containing picrotoxin (100 μM), and the microsacs were vacuum filtered over glass microfiber filters, using a Hoefer manifold (6.0 inches of Hg). Filters were rapidly rinsed twice with 6 ml/rinse of microsac buffer containing picrotoxin (100 μM), and radioactivity was determined by liquid scintillation counting.

All experiments were performed in triplicate. Muscimol-stimulated $^{36}Cl^-$ uptake is defined as the amount of $^{36}Cl^-$ uptake in the presence of muscimol minus the amount of $^{36}Cl^-$ uptake in the absence of muscimol. Muscimol-stimulated $^{36}Cl^-$ uptake is expressed in nmol of Cl^- uptake/mg of protein/3 sec. Protein content was determined by the method of Lowry *et al.* (14).

Receptor Binding Experiments

[^{3}H]SR 95531 binding. Intact microsacs were prepared as described for the $^{36}Cl^-$ uptake experiments, except that the final pellet was resuspended in calcium-free buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 3 mM EGTA, pH 7.5), to give a final protein concentration of 3 mg/ml. Lysed/resealed microsacs were prepared as described for the $^{36}Cl^-$ uptake experiments. The final pellet was resuspended in calcium-free buffer, to give a final protein concentration of 3 mg/ml. Microsacs were placed on ice, treated with drugs for 10 min, aliquoted (100 μl; 0.3 mg of protein) into microcentrifuge tubes, and incubated at 22° for 5 min. Following this incubation GABA (100 μM) (to define nonspecific binding) and [^{3}H]SR 95531 (3 nM) were added, and samples were incubated at 22° for 30 min. Samples were then centrifuged for 1.5 min at 13,000 × *g*, the supernatant was aspirated, and the surface of the pellet was washed with 750 μl of cold buffer. The buffer was aspirated and the pellet was air dried, digested overnight in 0.1 N NaOH, and neutralized the following day with HCl. Radioactivity was determined by liquid scintillation counting. All experiments were performed in triplicate.

[^{35}S]TBPS binding. Microsacs were prepared in microsac buffer as for $^{36}Cl^-$ uptake experiments, except that the final resuspension was in calcium- and magnesium-free buffer (145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 3 mM EDTA, pH 7.5). Membranes (100 μl, 0.3 mg of protein) were incubated with test drugs, picrotoxin (100 μM) (to define nonspecific binding), and [^{35}S]TBPS (3 nM) for 2 hr on ice. Membranes were diluted with 5 ml of ice-cold calcium- and magnesium-free buffer and immediately filtered either over glass microfiber filters on a Hoefer filter box or over Schleicher and Schuell no. 32 glass microfiber filter sheets on a Brandell harvester. Filters were washed with 5.0 ml of calcium- and magnesium-free buffer and radioactivity was determined by liquid scintillation counting. All binding experiments were performed on triplicate.

Incorporation of ^{32}P into Microsac Phosphoproteins

Lysed and resealed microsacs were prepared as described for the $^{36}Cl^-$ uptake experiments, except that they were lysed in the presence of 100 μCi/ml [^{32}P]ATP, 100 μM ATP, and either 1) 6 mM tricine and 3 mM EDTA or 2) 6 mM tricine and 3 mM magnesium acetate. Following lysing/resealing, microsacs were placed on ice for 10 min and then 0.5-ml aliquots were treated with either CPTcAMP or H7 for an additional 10 min, on ice. Microsacs were incubated at 22° for 5 min and then 250 μl of SDS-polyacrylamide gel electrophoresis stop solution [final concentrations of 2.3% (w/v) SDS, 62.5 mM Tris·HCl, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, pH 6.8] were added to terminate phosphorylation. Proteins were heat denatured and resolved by SDS-polyacrylamide gel electrophoresis (15). Gels were stained for protein using Coomassie blue stain, and ^{32}P incorporation was assessed by autoradiography.

Electrophysiology Experiments

Coronal sections (400 μm) were taken from the middle portion of the rat hippocampi, as described previously (16–18), and incubated in artificial cerebrospinal fluid at 33 ± 1°. Electrophysiological recordings were made with 2–3-MΩ glass microelectrodes placed in the pyramidal cell layer, and synaptic responses were evoked by stimulation of the Schaffer and commissural inputs to the CA1 region in stratum radiatum. After a stable baseline was obtained, H7 and H8 dissolved in physiological buffer were added directly to the recording chamber to achieve the final concentrations.

Statistics

Paired *t* tests were performed on $^{36}Cl^-$ uptake and receptor binding data in which a single comparison was made for a given control. For data involving multiple comparisons, a repeated measures one-way analysis of variance was performed, followed by a *post hoc* Scheffé test.

Results

As previously reported by Heuschneider and Schwartz (10), cAMP analogs decrease muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes. Thus, to replicate these findings, our first series of experiments examined the effects of cAMP and its analogs on muscimol-stimulated $^{36}Cl^-$ uptake in intact (nonlysed) brain microsacs (Table 1). Treatment of intact microsacs with low millimolar concentrations of cAMP, 8BrcAMP, or CPTcAMP inhibited muscimol-stimulated $^{36}Cl^-$ uptake. cAMP (2 mM) and 8BrcAMP (3 mM) each decreased muscimol-stimulated $^{36}Cl^-$ uptake by approximately 15%. CPTcAMP inhibited muscimol-stimulated $^{36}Cl^-$ uptake in a concentration-dependent manner, with 2 mM CPTcAMP reducing $^{36}Cl^-$ uptake by 75%. To further examine the CPTcAMP effect, muscimol concentration-response experiments were performed following treatment of the microsacs with CPTcAMP (Fig. 1). CPTcAMP (2 mM) decreased the maximal muscimol response without affecting the muscimol EC_{50}. Thus, increasing concentrations of muscimol failed to overcome the inhibition produced by 2.0 mM CPTcAMP, suggesting that CPTcAMP decreases the muscimol response through a noncompetitive mechanism. Similarly, the inhibition of muscimol-stimulated $^{36}Cl^-$ flux in synaptoneurosomes by Bt_2cAMP was not overcome by increasing muscimol concentrations (10).

To investigate whether the effects of CPTcAMP on muscimol-stimulated $^{36}Cl^-$ uptake were due to stimulation of PKA, intact microsacs were treated with the protein kinase inhibitor H8 before CPTcAMP treatment. H8, by itself, had no effect and also failed to antagonize the effect of CPTcAMP on muscimol-stimulated $^{36}Cl^-$ uptake (Table 1). The protein kinase inhibitor H7 (300 μM) inhibited muscimol-stimulated $^{36}Cl^-$ uptake by 50% and, thus, could not be used to characterize the CPTcAMP effect.

TABLE 1

Effect of second messenger system modulators on muscimol-stimulated $^{36}Cl^-$ uptake in intact microsacs

Treatment	Muscimol-stimulated $^{36}Cl^-$ uptake[a]
	nmol/mg of protein/3 sec
Control	28.1 ± 1.9
cAMP, 2.5 mM	24.3 ± 2.2[b]
Control	30.4 ± 1.5
8BrcAMP, 3.0 mM	26.7 ± 1.8[c]
Control	22.0 ± 1.3
CPTcAMP	
0.1 mM	20.0 ± 0.6
0.5 mM	16.8 ± 0.3
1.0 mM	11.7 ± 0.8[c]
2.0 mM	5.4 ± 1.0[d]
Control	20.4 ± 3.4
H7	
10 μM	24.2 ± 1.8
30 μM	17.6 ± 0.2
300 μM	10.7 ± 1.6[b]
Control	18.8 ± 3.4
H8	
100 μM	20.3 ± 1.2
300 μM	17.2 ± 1.3
CPTcAMP, 2 mM plus H8	8.3 ± 1.4
30 μM	7.0 ± 0.8
100 μM	7.1 ± 0.5
300 μM	6.6 ± 1.2

[a] Muscimol-stimulated $^{36}Cl^-$ uptake is expressed as nmol of $^{36}Cl^-$/mg of protein/3 sec (mean ± standard error of three to six experiments, with each experiment performed in triplicate).
[b] Significantly different from control, $p < 0.05$.
[c] Significantly different from control, $p < 0.01$.
[d] Significantly different from control, $p < 0.001$.

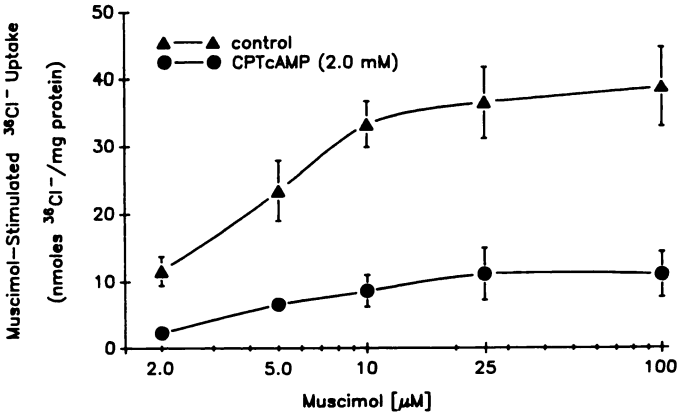

Fig. 1. CPTcAMP inhibition of muscimol-stimulated $^{36}Cl^-$ uptake in mouse brain microsacs. Values on the *ordinate* represent muscimol-stimulated $^{36}Cl^-$ uptake as nmol of Cl^-/mg of protein/3 sec. Muscimol concentration-response curves for control and CPTcAMP (2.0 mM)-treated microsacs were significantly different from each other, using an analysis of variance with repeated measures, $F(1)=18.8$, $p < 0.05$, three experiments.

To further examine whether cAMP analogs inhibited muscimol-stimulated $^{36}Cl^-$ uptake through a phosphorylation-dependent mechanism, we performed $^{36}Cl^-$ uptake experiments in a microsac preparation in which PKA-dependent phosphorylation was essentially inhibited. This preparation involved the lysing/resealing of the microsacs in the presence of EDTA to chelate internal Mg^{2+}. Because Mg^{2+} is required by PKA (and most other kinases) for phosphorylation, chelation of internal Mg^{2+} by EDTA should completely inhibit phosphorylation of most proteins. To demonstrate this inhibition, the incorporation of [^{32}P]ATP into phosphoproteins was examined in microsacs that had been lysed and resealed in the presence of either Mg^{2+} or EDTA. Microsacs lysed and resealed in the presence of Mg^{2+} retained their ability to phosphorylate proteins, as shown by the incorporation of ^{32}P into a variety of different molecular weight phosphoproteins (Fig. 2, *lane 1*). In contrast, microsacs lysed and resealed in the presence of EDTA failed to show incorporation of ^{32}P into microsac phosphoproteins, with the exception of a M_r 40,000 band and two lower molecular weight bands (Fig. 2, *lanes 2–4*). The incorporation of ^{32}P into these bands was unaffected by either CPTcAMP or H7 treatment (Fig. 2, *lanes 3* and *4*, respectively). Thus, PKA-dependent phosphorylation was essentially inhibited in EDTA-treated lysed/resealed microsacs.

EDTA-treated lysed/resealed microsacs were used to examine the phosphorylation dependence of second messenger system modulators on muscimol-stimulated $^{36}Cl^-$ uptake (Table 2). Under these conditions, in which phosphorylation has been precluded, second messenger system modulators would not be expected to affect muscimol-stimulated $^{36}Cl^-$ uptake, except by mechanisms that do not involve phosphorylation. CPTcAMP (2.5 mM) and Bt_2cAMP (2.5 mM) each inhibited muscimol-stimulated $^{36}Cl^-$ uptake by 30–50%. Similarly, H7 (300 μM) decreased muscimol-stimulated $^{36}Cl^-$ uptake by 30%, whereas H8 was without effect. Other compounds that inhibited muscimol-stimulated $^{36}Cl^-$ uptake in EDTA-treated lysed/resealed microsacs were 8BrcAMP and the adenylate cyclase activator forskolin. Thus, a variety of second messenger system modulators can influence GABA$_A$ receptor function through a phos-

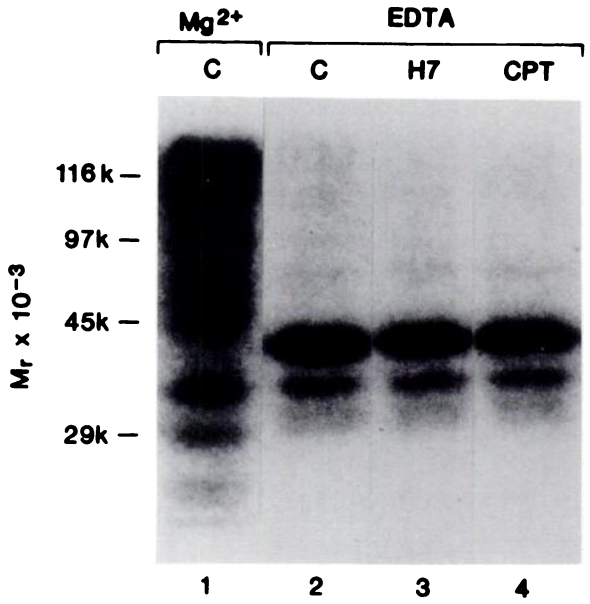

Fig. 2. Incorporation of [^{32}P]ATP into phosphoproteins in lysed/resealed microsacs. Microsacs were lysed/resealed in the presence of either 3 mM magnesium acetate (*lane 1*) or 3 mM EDTA (*lanes 2–4*). EDTA-treated microsacs were incubated with either 300 μM H7 (*lane 3*) or 2 mM CPTcAMP (*lane 4*) following the resealing procedure, as described in Materials and Methods. Mg^{2+}-dependent phosphorylation was essentially inhibited in EDTA-treated microsacs. Phosphorylation in EDTA-treated microsacs was unaffected by either H7 or CPTcAMP. Shown is a representative gel from three experiments, each performed in triplicate.

TABLE 2

Effect of second messenger system modulators on muscimol-stimulated $^{36}Cl^-$ uptake in EDTA-treated lysed/resealed microsacs

Treatment	Muscimol-stimulated $^{36}Cl^-$ uptake[a]
	nmol/mg of protein/3 sec
Control	17.9 ± 1.3
8BrCAMP, 2.5 mM	15.8 ± 0.6
Bt_2cAMP, 2.5 mM	11.7 ± 1.9[b]
CPTcAMP, 2.5 mM	8.1 ± 1.2[c]
H7, 300 μM	12.0 ± 1.2[b]
H8, 300 μM	16.2 ± 2.4
Control	16.5 ± 0.1
8BrcAMP, 4.0 mM	13.3 ± 0.6[d]
Control	19.5 ± 0.9
Forskolin, 100 μM	12.3 ± 2.7[d]

[a] Muscimol-stimulated $^{36}Cl^-$ uptake is expressed in nmol of Cl^-/mg of protein/3 sec (mean ± standard error of three experiments, with each experiment performed in triplicate).
[b] Significantly different from control, $p < 0.01$.
[c] Significantly different from control, $p < 0.001$.
[d] Significantly different from control, $p < 0.05$.

phorylation-independent mechanism.

To elucidate this mechanism, binding experiments were performed with the $GABA_A$ receptor antagonist [^{3}H]SR 95531. Cyclic AMP and its analogs inhibited the binding of [^{3}H]SR 95531 to the same extent in both intact and EDTA-treated lysed/resealed microsacs (Table 3). CPTcAMP (0.1–2.0 mM) produced a concentration-dependent inhibition of [^{3}H]SR 95531 binding, with 2 mM CPTcAMP inhibiting binding in intact and EDTA-treated lysed/resealed microsacs by 69 and 66%, respectively. A concentration-dependent inhibition of [^{3}H]SR 95531 binding was also observed with 8BrcAMP (0.1–3.0 mM), with the highest concentration of 8BrcAMP producing 58 and 57% inhibition in intact and EDTA-treated lysed/resealed microsacs, respectively. Cyclic AMP (2.5 mM) also

TABLE 3

Effect of second messenger system modulators on [^{3}H]SR 95531 binding in intact and EDTA-treated lysed/resealed microsacs

Treatment	[^{3}H]SR 95531 specific binding[a]	
	Intact	Lysed/resealed
	cpm	
Control	1298 ± 158	2066 ± 179
cAMP, 2.5 mM	916 ± 123[b]	1540 ± 81[c]
AMP, 2.5 mM	1136 ± 30	1757 ± 59
8BrcAMP		
0.1 mM	1316 ± 114	2052 ± 89
0.5 mM	1122 ± 157	1817 ± 102
1.0 mM	973 ± 144[c]	1633 ± 93[c]
2.0 mM	541 ± 62[d]	890 ± 71[d]
CPTcAMP		
0.1 mM	1298 ± 159	1958 ± 105
0.5 mM	885 ± 91[b]	1407 ± 75[d]
1.0 mM	658 ± 103[d]	1062 ± 63[d]
2.0 mM	402 ± 77[d]	694 ± 28[d]
Control	1730 ± 67	2458 ± 80
H7		
10 μM	1730 ± 87[c]	2024 ± 87[d]
100 μM	1003 ± 50[b]	1528 ± 109[d]
300 μM	59 ± 97[d]	351 ± 58[d]

[a] [^{3}H]SR 95531 specific binding is expressed as mean cpm ± standard error (three experiments, with each experiment performed in triplicate).
[b] Significantly different from control, $p < 0.01$.
[c] Significantly different from control, $p < 0.05$.
[d] Significantly different from control, $p < 0.001$.

inhibited [^{3}H]SR 95531 binding in both membrane preparations, whereas AMP did not significantly reduce binding. H7 produced a concentration-dependent decrease in [^{3}H]SR 95531 binding in both intact and EDTA-treated lysed/resealed microsacs. At 300 μM H7, [^{3}H]SR 95531 binding was inhibited by 97 and 86% in intact and lysed/resealed preparations, respectively.

The binding of [^{35}S]TBPS, a ligand that binds to a site on the chloride channel, was also examined. [^{35}S]TBPS binding was inhibited by CPTcAMP but not by cAMP or 8BrcAMP. The following compounds produced the indicated percentage of inhibition (three experiments, $p < 0.05$) of [^{35}S]TBPS binding: CPTcAMP (2.5 mM), 73 ± 5%; AMP (2.5 mM), 21 ± 4%; isobutylmethylxanthine (1 mM), 20 ± 4%; and forskolin (60 μM), 16 ± 3%.

In addition to functional studies in brain microsacs, the effects of H7 and H8 on hippocampal slice electrophysiology were examined (Fig. 3). Treatment of brain slices with 100 μM H7 markedly increased the amplitude of the evoked population spike response (mean increase of 389 ± 99%, four experiments) and elicited the appearance of secondary and tertiary spikes. One slice tested with 100 μM H8 showed a 13% increase in the population spike response, whereas two other slices tested with 200 μM H8 showed a mean response of 102% above controls. In neither case did any multiple spikes develop (Fig. 3B).

Discussion

In the present study, cyclic AMP and its analogs CPTcAMP and 8BrcAMP inhibited muscimol-stimulated $^{36}Cl^-$ uptake in intact microsacs. These results are consistent with previous findings that these compounds inhibit GABA-dependent Cl^- currents in hippocampal neurons (9), reduce muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes (10), and enhance the desensitization rate of the $GABA_A$ receptor in chick cortical neurons (11). These effects on $GABA_A$ receptor function have not been shown to be correlated with concurrent changes in either $GABA_A$ receptor phosphorylation or PKA activity. Therefore, the phosphorylation dependence of these effects

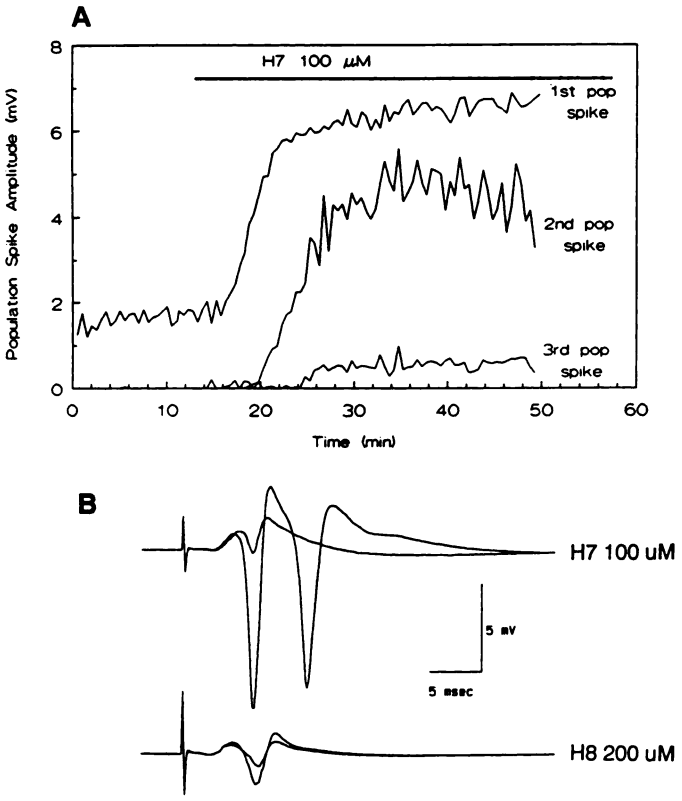

Fig. 3. Effects of H7 and H8 on hippocampal physiology. A, Population spike responses were recorded from rat hippocampal slices before and during treatment with 100 μM H7, as indicated by the *bar* along the top. The initial population spike was increased approximately 4-fold by H7, and a second and third population spike appeared as well. Shown is a representative recording from one of four experiments. B, Averaged evoked responses are shown before and during treatment with H7 and H8. The H7-treated slice showed an even larger increase than did the slice illustrated in A, along with a large amplitude secondary spike not seen in the control record. The effect of 200 μM H8 was statistically significant (95% confidence limits on the control and H8 waveforms did not overlap) but was quantitatively much smaller than the response to H7 and did not show secondary spiking.

remains to be established.

To investigate whether the effects of CPTcAMP on muscimol-stimulated $^{36}Cl^-$ uptake were mediated through PKA, microsacs were pretreated with the protein kinase inhibitor H8 to antagonize any PKA-mediated effects of CPTcAMP. H8 failed to antagonize the effect of CPTcAMP on muscimol-stimulated $^{36}Cl^-$ uptake, indicating that CPTcAMP may be altering GABA$_A$ receptor function independently of phosphorylation. It is unlikely that the ineffectiveness of H8 in antagonizing the CPTcAMP effect was due to the failure of H8 to reach PKA-inhibiting concentrations inside the microsacs, because H8 is known to cross membranes (19) and the K_i of H8 (1.3 μM) (20) is far below the concentration of H8 (300 μM) used in our experiments.

To further investigate the possibility that CPTcAMP may alter GABA$_A$ receptor function through phosphorylation-independent mechanisms, we developed a microsac preparation in which Mg^{2+}-dependent phosphorylation was inhibited. This procedure involves the hypoosmotic lysing and resealing of microsacs in the presence of EDTA to chelate internal Mg^{2+}, which, because PKA is a Mg^{2+}-dependent enzyme, should prevent PKA-dependent phosphorylation. Under these conditions, inhibition of phosphorylation was essentially complete, with the exception of three protein bands. One of these bands, a M_r 40,000 phosphoprotein, corresponds to pyruvate dehydrogenase, one of the few phosphoproteins that can undergo Mg^{2+}-independent phosphorylation (21). The lower molecular weight bands are believed to be breakdown products of pyruvate dehydrogenase. In contrast to the EDTA-treated lysed/resealed microsacs, microsacs that were lysed and resealed in the presence of Mg^{2+} retained their ability to phosphorylate a variety of phosphoproteins.

In EDTA-treated lysed/resealed microsacs, CPTcAMP markedly inhibited muscimol-stimulated $^{36}Cl^-$ uptake, despite the almost total absence of phosphorylation. Further, CPTcAMP did not stimulate phosphorylation of the M_r 40,000 band or of the two lower molecular weight bands. Other cAMP analogs that inhibited muscimol-stimulated $^{36}Cl^-$ uptake under these conditions include 8BrcAMP and Bt$_2$cAMP.

To determine the mechanism by which cAMP analogs inhibited GABA$_A$ receptor function, binding experiments were performed with [^{3}H]SR 95531, a ligand for the GABA$_A$ site (22). CPTcAMP, 8BrcAMP, and cAMP each inhibited [^{3}H]SR 95531 binding in both intact and EDTA-treated lysed/resealed microsac preparations. Although it is possible that phosphorylation may alter receptor binding, it is clear that phosphorylation is not involved in the inhibition of [^{3}H]SR 95531 binding by these compounds, because the percentage of inhibition of [^{3}H]SR 95531 binding produced by each compound was the same in both intact and EDTA-treated lysed/resealed microsacs.

In addition to cAMP analogs, the protein kinase inhibitor H7 also affected GABA$_A$ receptor function. H7 inhibited muscimol-stimulated $^{36}Cl^-$ uptake in intact microsacs and in EDTA-treated lysed/resealed microsacs. Furthermore, in both intact and EDTA-treated lysed/resealed preparations, H7 produced a nearly complete inhibition of [^{3}H]SR 95531 binding at concentrations that inhibited muscimol-stimulated $^{36}Cl^-$ uptake. Thus, it appears that H7 can inhibit GABA$_A$ receptor function in a phosphorylation-independent manner, via a direct interaction with the binding sites within the GABA$_A$ receptor complex. In contrast to H7, H8 failed to significantly alter muscimol-stimulated $^{36}Cl^-$ uptake in either intact or EDTA-treated lysed/resealed microsacs; however, a slight decrease in muscimol-stimulated $^{36}Cl^-$ uptake was observed in two of three membrane preparations at the 300 μM concentration. At this concentration of H8, we have observed inhibition of [^{3}H]SR 95331 binding in a preparation similar to the lysed/resealed microsacs preparation presented here (data not shown).

The results of the electrophysiological experiments with H7 and H8 in hippocampal slices point to similar conclusions. As previously observed (23), H7 produced large increases in the amplitude of evoked population spike responses and the development of multiple spikes. These changes are identical to those produced by agents such as bicuculline (24) or picrotoxin, which block GABAergic transmission in the hippocampus. The observation by Muller *et al.* (25) that recurrent and/or feedforward inhibition in the hippocampus is reduced by H7 would also be consistent with an interaction with GABAergic systems. In comparison, H8 produced a small but significant increase in the evoked population spike response but did not elicit multiple spikes at the concentrations tested. Therefore, as in the $^{36}Cl^-$ uptake and [^{3}H]SR 95531 binding experiments, H7 was found

to produce robust effects in comparison with H8.

Another membrane-permeant compound that has been used to investigate the effect of phosphorylation on $GABA_A$ receptor function is the adenylate cyclase activator forskolin. Although forskolin inhibits $GABA_A$ receptor function, dideoxyforskolin, a forskolin analog that is inactive toward adenylate cyclase, also inhibits GABA receptor function (10). We have also observed inhibitory effects of both these compounds on muscimol-stimulated $^{36}Cl^-$ uptake in intact microsacs.[1] In the present experiments, forskolin inhibited the muscimol-stimulated $^{36}Cl^-$ uptake in EDTA-treated lysed/resealed microsacs, indicating that forskolin can alter $GABA_A$ receptor function independently of phosphorylation. In binding experiments with [^{35}S]TBPS, a ligand for a site on the $GABA_A$ receptor-gated Cl^- channel, forskolin inhibited [^{35}S]TBPS binding by 16%. This percentage of inhibition is similar to that observed in $^{36}Cl^-$ uptake studies (21%) using the same forskolin concentration (10). Therefore, it is likely that forskolin inhibition of $GABA_A$ receptor function is due to direct interference with the chloride channel. These data are consistent with previous findings that demonstrate that forskolin directly affects a variety of ion channels (26) including the ion channel associated with the acetylcholine receptor (27), a ligand-gated ion channel belonging to the same superfamily as the $GABA_A$ receptor (2).

The present results indicate that several commonly used second messenger system modulators can affect $GABA_A$ receptor function independently of Mg^{2+}-dependent protein phosphorylation. Our findings do not exclude the possibility that the effects of these compounds may be mediated through a cyclic nucleotide-dependent, Mg^{2+}-independent protein kinase; however, we are unaware of the existence of such a kinase and, therefore, believe this possibility is unlikely. Experiments involving the immunoprecipitation of the $GABA_A$ receptor would more directly answer this question, and the preparation of $GABA_A$ receptor-specific antibodies by one of us (M.D.B.) is currently underway.

In conclusion, second messenger system modulators appear to affect $GABA_A$ receptor function in a phosphorylation-independent manner and, therefore, results obtained with these agents should be interpreted with caution. In light of these findings, the use of protein kinases and phosphatases may provide a more specific approach to elucidating the functional significance of $GABA_A$ receptor phosphorylation. Preliminary results utilizing the catalytic subunit of PKA (28) and alkaline phosphatase (29) do indeed provide evidence that the $GABA_A$ receptor is regulated by protein phosphorylation.

Acknowledgments

The authors thank Marianne Taylor for her expertise in performing the electrophysiology experiments and Dr. Neil L. Harrison for suggesting the [^{35}S] TBPS binding experiments.

References

1. Olsen, R. W., and J. C. Venter. *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties.* Alan Liss, New York (1986).
2. Schofield, P. R., M. G. Darlison, N. Fugita, D. R. Burt, F. A. Stephenson, H. Rodriguez, L. M. Rhee, J. Ramachandran, V. Reale, T. Glencorse, P. H. Seeburg, and E. J. Barnard. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature* (*Lond.*) **328**:221–227 (1987).
3. Kirkness, E. F., C. F. Bovenkerk, T. Ueda, and A. J. Turner. Phosphorylation of *gamma*-aminobutyrate (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase. *Biochem. J.* **259**:613–616 (1989).
4. Browning, M. D., M. Bureau, E. M. Dudek, and R. W. Olsen. Protein kinase C and cAMP-dependent protein kinase phosphorylate the purified $GABA_A$ receptor-β subunit. *Proc. Natl. Acad. Sci. USA* **87**:1315–1318 (1990).
5. Sweetnam, P. S., J. Lloyd, P. Gallombardo, R. T. Malison, D. W. Gallager, J. F. Tallman, and E. J. Nestler. Phosphorylation of the $GABA_A$/benzodiazepine receptor α subunit by a receptor-associated protein kinase. *J. Neurochem.* **51**:1274–1284 (1988).
6. Stelzer, A., A. R. Kay, and R. K. S. Wong. $GABA_A$-receptor function in hippocampal cells is maintained by phosphorylation factors. *Science* (*Washington D. C.*) **241**:339–341 (1988).
7. Gyenes, M., M. Farrant, and D. H. Farb. "Run-down" of γ-aminobutyric $acid_A$ receptor function during whole-cell recording: a possible role for phosphorylation. *Mol. Pharmacol.* **34**:719–723 (1988).
8. Sigal, E., and R. Baur. Activation of protein kinase C differentially modulates neuronal Na^+, Ca^{2+}, and *gamma*-aminobutyrate type A channels. *Proc. Natl. Acad. Sci. U. S. A.* **85**:6192–6196 (1988).
9. Harrison, N. L., and N. A. Lambert. Modification of $GABA_A$ receptor function by an analog of cyclic AMP. *Neurosci. Lett.* **105**:137–142 (1989).
10. Heuschneider, G., and R. D. Schwartz. cAMP and forskolin decrease *gamma*-aminobutyric acid-gated chloride flux in rat brain synaptoneurosomes. *Proc. Natl. Acad. Sci. U. S. A.* **86**:2938–2942 (1989).
11. Tehrani, M. H. J., J. J. Hablitz, and E. M. Barnes, Jr. cAMP increases the rate of $GABA_A$ receptor desensitization in chick cortical neurons. *Synapse* **4**:126–131 (1989).
12. Lambert, N. A., and N. L. Harrison. Analogs of cyclic AMP decrease γ-aminobutyric $acid_A$ receptor-mediated chloride current in cultured rat hippocampal neurons via an extracellular site. *J. Pharmacol. Exp. Ther.* **255**:90–94 (1990).
13. Allan, A. M., and R. A. Harris. γ-Aminobutyric acid agonists and antagonists alter chloride flux across brain membranes. *Mol. Pharmacol.* **29**:497–505 (1986).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
15. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (*Lond.*) **227**:680–685 (1970).
16. Dunwiddie, T. V., and G. S. Lynch. Long-term potentiation and depression of synaptic responses in the rat hippocampus: localization and frequency dependency. *J. Physiol.* (*Lond.*) **276**:353–367 (1978).
17. Mueller, A. L., B. J. Hoffer, and T. V. Dunwiddie. Noradrenergic responses in rat hippocampus: evidence for mediation by *alpha* and *beta* receptors in the *in vitro* slice. *Brain Res.* **214**:113–126 (1981).
18. Lupica, C., W. A. Cass, N. R. Zahniser, and T. V. Dunwiddie. Effects of the selective adenosine A2 receptor agonist CGS 21680 on *in vitro* electrophysiology, cyclic AMP formation and dopamine release in the rat CNS. *J. Pharmacol. Exp. Ther.* **252**:1134–1141 (1990).
19. Hidaka, H., and M. Hagiwara. Pharmacology of the isoquinoline sulfonamide protein kinase C inhibitors. *Trends Pharmacol. Sci.* **8**:162–164 (1987).
20. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**:5036–5041 (1984).
21. Browning, M. B., W. F. Bennett, P. Kelly, and G. Lynch. Evidence that the 40,000 *M*, phosphoprotein influenced by high frequency synaptic stimulation is the *alpha* subunit of pyruvate dehydrogenase. *Brain Res.* **218**:255–266 (1981).
22. Heaulme, M., J.-P. Chambon, R. Leyris, J. C. Molimard, C. G. Wermuth, and K. Biziere. Characterization of the binding of [^{3}H]SR 95531, a $GABA_A$ antagonist, to rat brain membranes. *J. Neurochem.* **48**:1677–1686 (1987).
23. Corradetti, R., A. M. Pugliese, and N. Ropert. The protein kinase C inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) disinhibits CA1 pyramidal cells in rat hippocampal slices. *Br. J. Pharmacol.* **98**:1376–1382 (1989).
24. Dunwiddie, T. V., W. R. Proctor, and J. Tyma. Local anesthetic actions of cocaine: effects on excitatory and inhibitory synaptic responses in the hippocampus *in vitro*. *Br. J. Pharmacol.* **95**:1117–1124 (1988).
25. Muller, D., J. Turnbull, M. Baudry, and G. S. Lynch. Phorbol ester induced synaptic facilitation is different than long-term potentiation. *Proc. Natl. Acad. Sci. U. S. A.* **8597**:6997–7000 (1988).
26. Laurenza, A., E. M. Sutkowski, and K. Seamon. Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol. Sci.* **10**:442–447 (1989).
27. Middleton, P., F. Jaramillo, and S. M. Schuetze. Forskolin increases the rate of acetylcholine receptor desensitization at rat soleus endplates. *Proc. Natl. Acad. Sci. U. S. A.* **83**:4967–4971 (1986).
28. Porter, N. M., R. E. Twyman, M. D. Uhler, and R. L. Macdonald. Cyclic AMP dependent protein kinase decreases $GABA_A$ receptor current in mouse spinal neurons. *Soc. Neurosci. Abstr.* **15**:1150 (1989).
29. Chen, Q. X., and R. K. S. Wong. Ca^{2+} activated dephosphorylation destabilizes $GABA_A$ receptor function in hippocampal neurons. *Soc. Neurosci. Abstr.* **15**:1300 (1989).

[1] Unpublished observations.

Send reprint requests to: Dr. Nancy J. Leidenheimer, Department of Pharmacology, UCHSC, 4200 E. Ninth Ave., Denver, CO 80262.