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cAMP Analogs Inhibit γ-Aminobutyric Acid-Gated Chloride Flux and Activate Protein Kinase A in Brain Synaptoneurosomes

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SUMMARY

The effects of permeant cAMP analogs were studied on the function of the γ-aminobutyric acid$_A$ ($GABA_A$) receptor and on the activation of protein kinase A in brain synaptoneurosomes. Incubation of cerebral cortical synaptoneurosomes with permeant cAMP analogs decreased muscimol-induced $^{36}Cl^-$ uptake in a concentration-dependent manner. The order of potency was chlorophenylthio-cAMP (CPT-cAMP) > dibutyryl-cAMP > 8-bromo-cAMP. This order of potency was reflected by the ability of the analogs to gain access to the intravesicular compartment. cAMP, which failed to penetrate the membrane, had no effect. The half-maximal and maximal effects of the cAMP analogs were similar in the cerebral cortex, hippocampus, striatum, and cerebellum. To determine whether the cAMP analogs were acting through the activation of protein kinase A, protein kinase A activity was measured in lysed synaptoneurosomes, using kemptide as the substrate. In the lysed preparation, where the cAMP analogs have direct access to intracellular enzymes, the order of potencies of the cAMP analogs to activate protein kinase A (8-bromo-cAMP > CPT-cAMP > dibutyryl-cAMP) differed from the order of potencies to inhibit muscimol-induced $^{36}Cl^-$ uptake. In regional studies, the greatest effect of CPT-cAMP was observed in the cortex, whereas the smallest effect was observed in the hippocampus and cerebellum. To determine whether cAMP inhibition of GABA-gated ion flux was due to activation of protein kinase A, the time course for each response was measured. Inhibition of muscimol-induced $^{36}Cl^-$ uptake by cAMP analogs was nearly complete by 5 sec. Significant activation of protein kinase A by CPT-cAMP was also observed as early as 5 sec, but protein kinase A activation continued up to 10 min. The protein kinase inhibitor peptide inhibited protein kinase A activity in lysed synaptoneurosomes but had no effect on ion flux in intact synaptoneurosomes, as expected. However, a permeant kinase inhibitor, H-8, also failed to inhibit the effect of cAMP analogs on the muscimol response, yet it inhibited protein kinase A activity. The failure of H-8 to inhibit cAMP analog effects on $GABA_A$ receptor function was most likely due to the presence of ATP inside the synaptoneurosomes, because H-8 inhibition of protein kinase A was reduced in the presence of ATP. These results indicate that cAMP and cAMP analogs must penetrate the intravesicular compartment to inhibit $GABA_A$ receptor function. Although cAMP analogs decrease GABA-gated ion flux under conditions in which they activate protein kinase A, a causal relationship remains to be established.

Phosphorylation of proteins plays an important role in modulation of protein function. Phosphorylation events regulate many neurotransmitter receptors, including second messenger-associated receptors such as the β-adrenergic receptor and ligand-gated ion channels such as the nicotinic cholinergic receptor. Because the nicotinic receptor shares both structural and functional properties with the GABA receptor (1, 2), it has served as a model for regulation of ion channel function by extracellular and intracellular signals.

The $GABA_A$ receptor complex is a multimeric protein comprising α, β, and γ subunits (3). The subunits are arranged to form a chloride channel and, when GABA binds to its recognition site, there is an increase in chloride conductance, resulting in neuronal inhibition. Recent studies have indicated that there is a consensus sequence on the β and γ subunits for cAMP-dependent and tyrosine-dependent phosphorylation, respectively (1, 3). Two groups have confirmed the former finding by demonstrating that the β subunit is phosphorylated by cAMP-dependent protein kinase (protein kinase A) (4, 5). Browning *et al.* (5) have shown that the β subunit is also a substrate for protein kinase C (5), whereas Sweetnam *et al.* (6) have reported that the α subunit is phosphorylated by an endogenous kinase.

We have reported previously that cAMP phosphorylation

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ABBREVIATIONS: GABA, γ-aminobutyric acid; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bt_2-cAMP, N^6,2′-*O*-dibutyryl-cAMP; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; 8-Br-cAMP, 8-bromo-cAMP; PKI, protein kinase A inhibitor; IBMX, isobutylmethylXanthine; HPLC, high performance liquid chromatography.

conditions inhibit $GABA_A$ receptor function (7). Various cAMP analogs, as well as endogenous cAMP generated by either forskolin or phosphodiesterase inhibition, inhibited muscimol-induced $^{36}Cl^-$ uptake in rat cerebral cortical synaptoneurosomes. cAMP analogs have also been reported to inhibit $GABA_A$ receptor function, as shown using electrophysiological techniques (8–10). In cultured cerebral cortical neurons, cAMP analogs promoted $GABA_A$ receptor desensitization (8). However, in cultured spinal cord neurons, the authors concluded that cAMP analogs do not promote $GABA_A$ receptor desensitization (11). Various investigators have concluded that the effect of cAMP analogs on GABA responses in hippocampal or spinal cord neurons is not mediated by activation of protein kinase A (11, 12). This was based on the failure of H-8, a protein kinase A (and C) inhibitor, to inhibit the effect of cAMP analogs on GABA responses (11, 12). However, in more direct studies, Porter *et al.* (13) have shown that the catalytic subunit of protein kinase A decreases the GABA-mediated chloride conductance in patch-clamped spinal cord neurons.

The purpose of this study was to determine whether the activation of protein kinase A mediates the effects of cAMP analogs on GABA responses in synaptoneurosomes. This question was addressed by comparing the ability of cAMP analogs to activate protein kinase A activity in lysed synaptoneurosomes with their ability to inhibit GABA-gated $^{36}Cl^-$ uptake. In addition, we addressed the inability of H-8 to inhibit protein kinase A in intact cell preparations.

Experimental Procedures

Materials. Experiments were performed on adult male Sprague-Dawley rats (150–225 g) obtained from Zivic-Miller Laboratories, Inc. (Zelienople, PA). Chlorine-36 (20–26 MBq/mmol) and [γ-^{32}P]ATP (0.96–1.44 TBq/mmol) were obtained from New England Nuclear (Boston, MA). Kemptide and PKI 5–24 were obtained from Peninsula Laboratories (Belmont, CA). H-8 was obtained from Seikagaku America, Inc. (St. Petersburg, FL). Bt_2-cAMP, CPT-cAMP, 8-Br-cAMP, cAMP, ATP, IBMX, dithiothreitol, EDTA, EGTA, aprotinin, leupeptin, Nonidet P-40, and other drugs and biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). P81 phosphocellulose filter paper was obtained from Whatman, Inc. (Maidstone, England).

Preparation of synaptoneurosomes. Synaptoneurosomes were prepared from adult male Sprague-Dawley rats as previously described by Schwartz *et al.* (14), with minor modifications. Brains were removed rapidly after decapitation and placed immediately in ice-cold buffer (pH 7.4) containing 10 mM glucose, 20 mM HEPES, 9 mM Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, and 1.0 mM $CaCl_2$. Various brain regions were dissected free of white matter and homogenized by hand (five strokes) in 7 volumes (w/v) of buffer, using a glass-glass homogenizer. The homogenate was diluted with 30 ml of buffer/g of tissue and filtered by gravity through three layers of nylon mesh (160 μm; Tetko Inc., Elmsford, NY). The filtrate was then gently filtered through a 10-μm Mitex filter (Millipore, Bedford, MA) and centrifuged at 1000 × *g* for 15 min. The supernatant was discarded and the pellet was gently resuspended and recentrifuged (1000 × *g*, 15 min). The final pellet was resuspended to a protein concentration of 10 mg/ml. The filtration steps described above effectively remove large cellular debris, oligodendrocytes, and intact neurons, resulting in a relatively homogeneous population of both pre- and postsynaptic membrane vesicles ["synaptoneurosomes"; for details see Hollingsworth *et al.* (15)].

Measurement of $^{36}Cl^-$ uptake. $^{36}Cl^-$ uptake was measured as previously described by Schwartz and co-workers (2, 14). Synaptoneurosomes (0.5–1.0 mg of protein) from various brain regions were preincubated for 10 min, at 30°, before the simultaneous addition of 0.5 μCi of $^{36}Cl^-$ and 50 μM muscimol. Basal uptake of $^{36}Cl^-$ was determined in the absence of muscimol. $^{36}Cl^-$ uptake was terminated after 5 sec by dilution of the assay mixture with 5 ml of ice-cold buffer containing 100 μM picrotoxin and filtration by vacuum through a glass fiber filter (no. 30; Schleicher & Schuell, Keene, NH). The filters were washed two times with 5 ml of cold buffer (containing picrotoxin) and counted by liquid scintillation spectrometry for retained radioactivity.

Measurement of protein kinase A activity. The procedure for assay of cAMP-dependent protein kinase activity described by Nestler and Tallman (16) was modified for use in synaptoneurosomes. Rats were decapitated and the brains were removed and dissected on ice. Synaptoneurosomes were prepared as described above. The final pellet was lysed by resuspension and homogenization (10 strokes by hand) in the following buffer: 20 mM Tris, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 50 kallikrein units/ml aprotinin, and 10 μg/ml leupeptin. The lysed vesicles (5 μg of protein/assay tube) were kept on ice for 10 min before assay for protein kinase A activity. To obtain particulate and soluble fractions of the tissue specimens, the final homogenate was subjected to ultracentrifugation (120,000 × *g*, 10 min at 4°) in a Beckman Airfuge.

Aliquots (10 μl) of lysed synaptoneurosomes and/or particulate or soluble fractions were incubated for 5 min (unless otherwise noted) at 30°, in a final volume of 75 μl, in phosphorylation assay buffer (final concentrations: 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.05% Nonidet P-40, 10 mM dithiothreitol). Kemptide (5 μg) was included as the substrate for phosphorylation with 50 μM [^{32}P] ATP (1 μCi/tube). Protein kinase A was activated by cAMP analogs (0.01 μM to 3 mM), and PKI (5 μg/tube) was added to some tubes to define basal protein kinase A activity. Following the incubation period, protein kinase A activity was terminated by addition of 15 μl of ice-cold "stop" solution, containing 250 mM EDTA and 20 mM ATP, pH 7.0. Aliquots (50 μl) of the phosphorylation mixture were blotted on 2- × 2-cm squares of P81 phosphocellulose filter paper. After drying, the filter paper squares were washed three times with 0.5% orthophosphoric acid (approximately 15 ml/square) followed by acetone. The ^{32}P retained by the filter paper squares was quantitated by counting for Cerenkov emission. Under the assay conditions used, protein kinase A activity was linear up to 7 min.

HPLC assay of cAMP analogs. Synaptoneurosomes were incubated with cAMP analogs (1 mM) for 10 min at 30°, as described for measurement of $^{36}Cl^-$ uptake. The incubation was terminated by centrifugation (29,000 × *g*, 20 min at 0–4°), and the supernatant containing extravesicular cAMP analog was removed. Intravesicular cAMP analog was released by lysis of the pellet in water, with sonication. Internal standards (8-Br-cAMP for cAMP samples and controls, cAMP for 8-Br-cAMP samples, Bt_2-cAMP for CPT-cAMP samples, and CPT-cAMP for Bt_2-cAMP samples) were added to each sample to control for extraction loss. Proteins and noncyclic nucleotides were precipitated from resuspended pellet and supernatant fractions by the addition of equal volumes of 0.03 N $Ba(OH)_2$ and $ZnSO_4$. Separate samples containing known concentrations of cAMP analogs were extracted in an identical manner, to calculate the absolute recovery (approximately 25%). After centrifugation (10,000 × *g*, 5 min at 0–4°), the respective supernatants were removed and assayed for cAMP analogs. cAMP analogs were chromatographed using ion pair, reverse phase HPLC, with UV (254 nm) detection.[2] For detection of cAMP and 8-Br-cAMP, the mobile phase included 2 mM tetrabutylammonium chloride, 0.05 M NaH_2PO_4 (pH 5.0), and 12% acetonitrile. For detection of CPT-cAMP and Bt_2-cAMP, the mobile phase consisted of 0.5 mM tetrabutylammonium chloride, 0.05 M NaH_2PO_4 (pH 5.0), and 20% acetonitrile. Peak heights for each analog were measured and corrected for appropriate dilution factors. Data are presented as the ratios of the cAMP analog peak heights in the respective fractions (intravesicular/extravesicular).

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* (17), using bovine serum albumin as standard.

Data analysis. The calculation of IC_{50} and EC_{50} values was based on curve-fitting programs, and Student's *t* tests were used for statistical

[2] C. D. Kilts, personal communication.

comparisons of two groups of means. A probability level (p value) of <0.05 was considered to be statistically significant.

Results

Incubation of cerebral cortical synaptoneurosomes with permeant analogs of cAMP decreased muscimol-induced $^{36}Cl^-$ uptake, in a concentration-dependent manner (Fig. 1). CPT-cAMP was the most potent (IC_{50} = 0.9 mM). At higher concentrations, Bt_2-cAMP produced a biphasic inhibition of the muscimol response. 8-Br-cAMP was the least potent derivative, inhibiting the muscimol response by 80% at 10 mM. High concentrations of cAMP (10 mM), which does not penetrate the membrane (see below), produced only 10% inhibition of muscimol-induced $^{36}Cl^-$ uptake. The order of potency for inhibition of $^{36}Cl^-$ uptake by the cAMP analogs (CPT-cAMP > Bt_2-cAMP > 8-Br-cAMP > cAMP) corresponded to their ability to penetrate synaptoneurosomal membranes, as determined by their relative distributions into intravesicular and extravesicular fractions (Table 1). After incubation of synaptoneurosomes with the most permeable analog, CPT-cAMP (1 mM), 2.67% (17 nmol/mg of protein) of the total CPT-cAMP was found in the intravesicular fraction. Bt_2-cAMP (1.6%) and 8-Br-cAMP (0.7%) were found to a lesser extent in the intravesicular fraction. In the presence of IBMX (1 mM), only 0.2% of cAMP was found in the intravesicular fraction (Table 1).

The regional sensitivity of muscimol-induced $^{36}Cl^-$ uptake to CPT-cAMP was examined. Inhibition of muscimol-induced $^{36}Cl^-$ uptake by CPT-cAMP (3 mM) was nearly maximal in four brain regions, cerebral cortex, hippocampus, striatum, and cerebellum (Fig. 2). In addition, the IC_{50} values did not vary significantly among brain regions (1.4 ± 0.1, 1.6 ± 0.1, 0.9 ± 0.4, and 0.7 ± 0.2 mM, respectively).

One of the major actions of intracellular cAMP is the activation of protein kinase A. Thus, we determined the ability of these analogs to activate protein kinase A in synaptoneurosomes, using kemptide as the substrate. Cerebral cortical synaptoneurosomes were lysed, and three fractions (particulate, soluble, and total) were analyzed for activation of protein kinase A by cAMP analogs (Table 2). Although Bt_2-cAMP activated protein kinase A in both the particulate and soluble fractions,

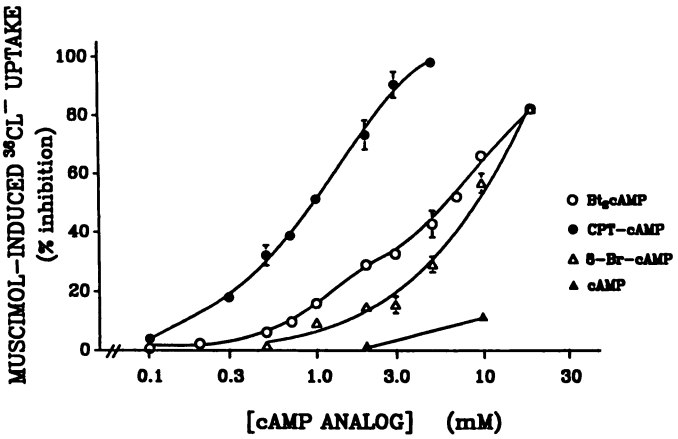

Fig. 1. Inhibition of muscimol-induced $^{36}Cl^-$ uptake by cAMP analogs. Synaptoneurosomes were incubated with cAMP or cAMP analogs for 10 min at 30° before the addition of muscimol and $^{36}Cl^-$, as described in Experimental Procedures. The amount of muscimol-induced $^{36}Cl^-$ uptake in the absence of inhibitors was 37.1 ± 1.1 nmol/mg of protein. The data are the mean ± standard error of three experiments performed in triplicate.

TABLE 1

Distribution of cAMP analogs into intravesicular and extravesicular fractions of synaptoneurosomes

Synaptoneurosomes were incubated for 10 min (30°) with cAMP analogs (1 mM), as for measurement of $^{36}Cl^-$ uptake. IBMX (1 mM) was included in samples in which cAMP was added. Cyclic AMP analogs were assayed by HPLC, as described in Experimental Procedures. Data are reported as the ratio of the corrected cAMP analog peak height in the intravesicular fraction to its peak height in the extravesicular fraction. Data are the mean ± standard error of three determinations, except where noted. Endogenous cAMP was not detectable in control samples.

cAMP analog	Intravesicular/extravesicular peak height
cAMP	0.002[a]
8-Br-cAMP	0.007 ± 0.002
Bt_2-cAMP	0.016 ± 0.002
CPT-cAMP	0.027 ± 0.004

[a] Average of two determinations.

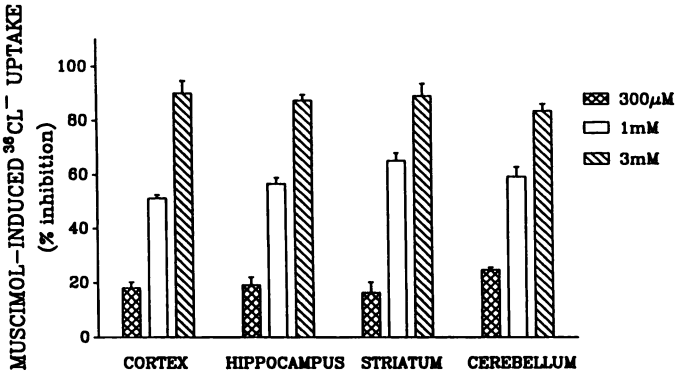

Fig. 2. Inhibition of muscimol-induced $^{36}Cl^-$ uptake in several brain regions by CPT-cAMP. Synaptoneurosomes from various brain regions were incubated with 300 μM to 3 mM CPT-cAMP, as described in Fig. 1. Data are the mean ± standard error of three to seven experiments performed in triplicate.

TABLE 2

Activation and inhibition of protein kinase A in lysed cerebral cortical synaptoneurosomes

Synaptoneurosomes were separated by centrifugation into particulate and soluble fractions. Nonfractionated preparations are indicated as "total." Basal values for cerebral cortical synaptoneurosomal PKA activity in the absence and presence of 5 μg of PKI were 1.07 ± 0.01 and 0.27 ± 0.01 nmol/mg of protein/min, respectively. Basal values in the presence of 5 μg of PKI have been subtracted from those in the presence of 10 μM cAMP analog, to obtain net activation of kinase. Data are the mean ± standard error of four to six experiments, performed in triplicate.

Fraction	cAMP analog (10 μM)	^{32}P-Kemptide formed		
		No inhibitor	+5 μg of PKI	+30 μM H-8
		nmol/mg of protein/min		
Particulate	Bt_2-cAMP	1.18 ± 0.03		0.40 ± 0.02
Soluble	Bt_2-cAMP	0.73 ± 0.03		
Total	Bt_2-cAMP	1.12 ± 0.03	0.02 ± 0.001	
Total[a]	CPT-cAMP	7.92 ± 0.24	0.05 ± 0.001	
Total	8-Br-cAMP	7.75 ± 0.10	0.05 ± 0.002	

[a] In other experiments, 50 μM H-8 inhibited 10 μM CPT-cAMP by 90%.

the protein kinase A activity of the total synaptoneurosomal preparation was similar to that measured in the particulate fraction. Subsequent determinations of protein kinase A activity were performed in the total synaptoneurosomal fraction. CPT-cAMP and 8-Br-cAMP also activated protein kinase A in the total synaptoneurosomal fraction. When complete concentration-response curves were obtained (Fig. 3), 8-Br-cAMP and CPT-cAMP were the most potent activators (EC_{50} = 134 ± 28 and 219 ± 52 nM, respectively). Bt_2-cAMP was 1000 times less potent at activating protein kinase A in cerebral cortical synaptoneurosomes (EC_{50} = 309 ± 193 μM).

In regional studies, CPT-cAMP activated protein kinase A to different degrees (Fig. 4). Basal protein kinase A activity was not significantly different among the four brain regions. The greatest maximal effect of CPT-cAMP was measured in the cerebral cortex, whereas the smallest maximal effect occurred in the cerebellum. The potencies of CPT-cAMP did not differ significantly among the four brain regions studied (EC_{50} values ranged from 0.1 to 0.3 μM).

Experiments were performed to determine whether the cAMP inhibition of GABA-gated ion flux was due to activation of protein kinase A. First, the time courses for cAMP actions were compared. Inhibition of muscimol-induced $^{36}Cl^-$ uptake by CPT-cAMP, Bt_2-cAMP, and 8-Br-cAMP was rapid; a 5-sec preincubation of synaptoneurosomes with the cAMP analogs resulted in the same extent of inhibition as observed after a 10-min preincubation (Table 3). Significant activation of protein kinase A by CPT-cAMP (10 μM) occurred as early as 5 sec, whereas a slower phase of protein kinase A activation occurred over the next 10 min (Table 3 and Fig. 5). Second, the ability of protein kinase A inhibitors to inhibit cAMP-mediated events was tested in both protein kinase A assays and ion flux assays. In lysed synaptoneurosomes, both basal and cAMP-activated protein kinase A were inhibited by the protein kinase inhibitors PKI and H-8 (Table 2). Although 5 μg of PKI maximally inhibited basal and cAMP-activated protein kinase A, 0.5–1 μg was sufficient for maximal inhibition (data not shown). As expected, PKI preincubation had no effect on muscimol-induced $^{36}Cl^-$ uptake in intact synaptoneurosomes, because it cannot gain access to the vesicle interior (Table 4). However, H-8, which would be expected to permeate the vesicles (18), did not affect the ability of Bt_2-cAMP to alter GABA-gated ion flux (Table 4). Previous studies have indicated that H-8 is inhibited competitively by ATP (18). To test whether this could explain the lack of H-8 inhibition of the effect of cAMP analogs on muscimol-induced $^{36}Cl^-$ uptake, we incubated lysed synaptoneurosomes with increasing ATP concentrations and measured the ability of H-8 to inhibit protein kinase A activity. In the presence of 50 μM ATP (standard assay conditions), H-8 (50 μM) inhibited CPT-cAMP (10 μM)-stimulated protein kinase A activity by 90% (data not shown). The inhibition by H-8 was reduced to 60% when concentrations of up to 1 mM ATP were added. Technical considerations precluded the addition of

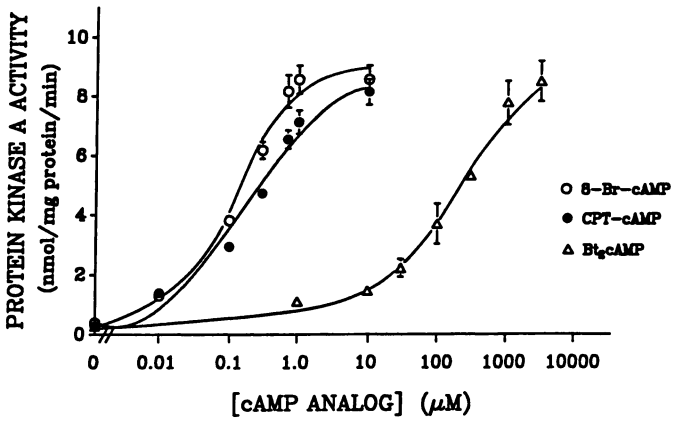

Fig. 3. Stimulation of protein kinase A in lysed cerebral cortical synaptoneurosomes by cAMP analogs. Synaptoneurosomes were prepared as for $^{36}Cl^-$ uptake assays (total fraction) but were lysed before incubation (5 min, 30°) with cAMP analogs and the phosphorylation substrate kemptide. Protein kinase A activity represents nmol of ^{32}P-kemptide formed/mg of protein/min. Data are the mean ± standard error of three experiments performed in duplicate.

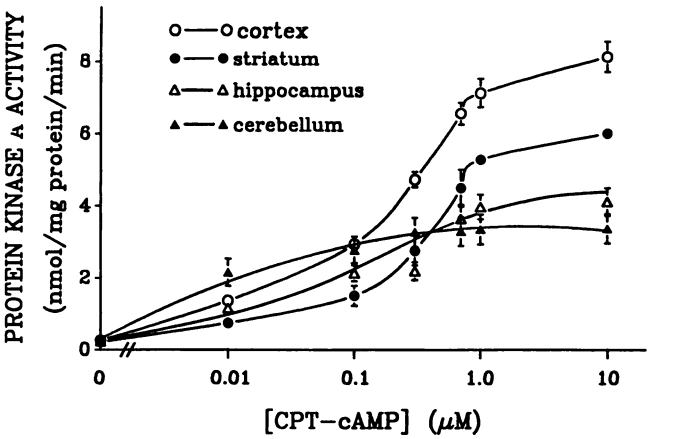

Fig. 4. Stimulation of protein kinase A in lysed synaptoneurosomes from several brain regions by CPT-cAMP. Synaptoneurosomes were prepared from various brain regions and lysed as described in Experimental Procedures. CPT-cAMP (0.01–10 μM) and kemptide were incubated with lysed synaptoneurosomes for 5 min. Protein kinase A activity is reported as described in Fig. 3. The basal values for protein kinase A activity were 0.27 ± 0.03, 0.21 ± 0.09, 0.23 ± 0.04, and 0.21 ± 0.06 nmol/mg/min for cortex, striatum, hippocampus, and cerebellum, respectively. Data are the mean ± standard error of three experiments performed in duplicate.

TABLE 3

Time course of cAMP analog inhibition of muscimol-induced $^{36}Cl^-$ uptake and activation of protein kinase A in lysed and intact synaptoneurosomes

In $^{36}Cl^-$ flux assays, the cAMP analog was added simultaneously with muscimol and $^{36}Cl^-$ for the 5-sec time point. The data are the mean ± standard error of three experiments, performed in triplicate.

cAMP analog	Muscimol-induced $^{36}Cl^-$ uptake		^{32}P-Kemptide formed	
	5 sec	10 min	5 sec	10 min
	% of inhibition		*nmol/mg of protein*	
CPT-cAMP[a]	50.0 ± 3.1	45.3 ± 0.9	12.1 ± 0.5	58.6 ± 2.1
Bt_2-cAMP[b]	27.7 ± 3.2	24.5 ± 1.2		
8-Br-cAMP[b]	24.5 ± 4.9	19.5 ± 2.1		

[a] 1 mM and 10 μM for $^{36}Cl^-$ uptake and protein kinase A assays, respectively.
[b] 2 mM for $^{36}Cl^-$ uptake.

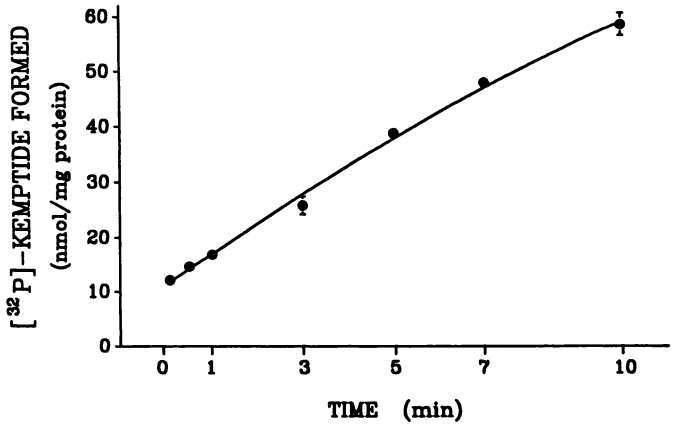

Fig. 5. Time course for protein kinase A activation by CPT-cAMP in lysed cerebral cortical synaptoneurosomes. Lysed synaptoneurosomes were incubated for various times with 10 μM CPT-cAMP and the substrate kemptide before the addition of the stop solution. Protein kinase A activity is indicated as nmol of ^{32}P-kemptide formed/mg of protein. Data are the mean ± standard error of three experiments performed in duplicate.

TABLE 4

Effect of protein kinase A inhibitors on Bt_2-cAMP inhibition of muscimol-induced $^{36}Cl^-$ uptake in intact cerebral cortical synaptoneurosomes

A maximally effective concentration of muscimol (50 μM) was used to determine $^{36}Cl^-$ uptake. Intact synaptoneurosomes were preincubated with PKI and H-8 for 5 min before the addition of Bt_2-cAMP. Data are the mean ± standard error of the number of experiments noted in parentheses.

cAMP analog	Muscimol-induced $^{36}Cl^-$ uptake
	% of inhibition
Bt_2-cAMP (1 mM)	16.0 ± 0.7 (22)
+ PKI (5 μg)	16.7 ± 1.3 (3)
Bt_2-cAMP (2 mM)	28.9 ± 1.9 (9)
+ PKI (5 μg)	30.0 ± 4.6 (3)
+ H-8 (50 μM)	28.0 ± 2.1 (3)

higher ATP concentrations that reflect typical intracellular levels [3–10 mM (19, 20)].

Discussion

Several investigators have begun to study the regulation of GABA receptor function by cAMP-dependent phosphorylation conditions. Various techniques have been used, and conflicting results have emerged. We reported earlier that Bt_2-cAMP inhibits GABA-gated $^{36}Cl^-$ flux in synaptoneurosomes; accumulation of endogenous cAMP levels with either forskolin to activate adenylate cyclase or IBMX to inhibit phosphodiesterase also decreased GABA-gated $^{36}Cl^-$ flux (7). However, the actions of forskolin were also independent of adenylate cyclase activation (7). In contrast, Tehrani *et al.* (8) have shown that the effect of forskolin on GABA-gated $^{36}Cl^-$ flux in cultured embryonic neurons was due to adenylate cyclase activation. In cultured hippocampal and spinal cord neurons, cAMP and cAMP analogs inhibited GABA responses, but this was attributed to a direct effect on the GABA-gated ion channel (10–12) (see below). These differences in the effects of cAMP or cAMP-dependent phosphorylation conditions on GABA responses may be due to different techniques or brain preparations. More studies will be needed to elucidate the nature of these conflicting results.

In the present study we have also demonstrated that cAMP analogs can activate protein kinase A in synaptoneurosomes from several brain regions. In order to determine whether cAMP analogs inhibit the muscimol response by activating protein kinase A, three experiments were performed. First, the order of potencies for cAMP analogs was compared with respect to inhibition of muscimol-induced $^{36}Cl^-$ uptake and activation of protein kinase A. Although both the absolute potencies and the order of potencies differed, this was probably due to the different degrees of membrane permeation of the cAMP analogs. This was supported by the data from the HPLC analysis that indicated the relative distribution of each analog in the intravesicular and extravesicular fractions. The inability of cAMP to inhibit the muscimol responses was reflected by its inability to penetrate the synaptoneurosome. The synaptoneurosomal preparation used in the protein kinase A assay, is identical to that used in the ion flux assay, except that it is lysed. Thus, the potencies of the analogs to stimulate protein kinase A are independent of their degree of membrane permeation. Second, the time courses for CPT-cAMP inhibition of muscimol-induced $^{36}Cl^-$ uptake and activation of protein kinase A were compared. Whereas the inhibition of the muscimol response by CPT-cAMP was maximal by 5 sec, the activation of protein kinase A followed a much slower time course. However, there was significant activation of protein kinase A by CPT-cAMP within 5 sec, so it is possible that partial activation of protein kinase A may be enough for full inhibition of the GABA-gated ion channel. Support for this hypothesis was provided by Porter *et al.* (13), who showed that the application of the protein kinase A catalytic subunit to patch-clamped spinal neurons produced an instantaneous inhibition of GABA-gated chloride conductance. Third, in the most direct experiment, we attempted to prevent the cAMP-induced inhibition of the muscimol response with inhibitors of protein kinase A activity. When PKI or H-8 was incubated with intact synaptoneurosomes before the addition of Bt_2-cAMP or CPT-cAMP, they had no effect on GABA responses, yet they both inhibited cAMP analog-activated protein kinase A activity in the lysed preparation. In the case of PKI, this result was expected, because the peptide is not membrane permeable and should not have access to the inside of the vesicle to inhibit protein kinase A. In contrast, H-8 is reported to be a permeant inhibitor of protein kinase A (18). Several investigators have concluded that the failure of H-8 to inhibit the effect of cAMP analogs on GABA responses indicates that cAMP analogs or cAMP do not affect GABA responses via stimulation of protein kinase A (11, 12). In studies using patch-clamped hippocampal membranes, H-8 failed to inhibit cAMP-induced decreases of GABA responses when placed on the inside of the membrane patch (12). The authors concluded that cAMP and cAMP analogs have a direct effect on the $GABA_A$ receptor, via an extracellular site. cAMP has been shown to have direct effects on other ion channels (21–23). In contrast, our findings suggest that the failure of H-8 to inhibit the response to cAMP analogs could be due to competitive inhibition of H-8 by ATP (18). We found that H-8 inhibition of protein kinase A in lysed synaptoneurosomes was reduced in the presence of added ATP. We did not completely eliminate the effect of H-8, because the highest concentration of ATP added was 1 mM. However, because intracellular ATP concentrations typically range from 3 to 10 mM (19, 20), it is likely that the ATP concentrations inside the intact synaptoneurosomes are high enough to completely inhibit the effects of H-8. In studies with patch-clamped neurons, 5 mM ATP is typically added to the internal perfusion medium to prevent "rundown" of the GABA response (12, 24). This could explain the failure of H-8 to inhibit cAMP effects on GABA responses in patch-clamped hippocampal membranes (12). Thus, H-8 may not be an appropriate tool to investigate inhibition of protein kinase A activity in intact cell systems or under conditions when high ATP concentrations are present.

The present studies do not distinguish between direct effects of cAMP analogs on the $GABA_A$ receptor and indirect effects on GABA responses via activation of protein kinase A. However, we cannot rule out the possibility that cAMP analogs inhibit $GABA_A$ receptor function via cAMP-dependent phosphorylation mechanisms. Recent studies have demonstrated cAMP-dependent phosphorylation sites on $GABA_A$ receptor subunits. Kirkness *et al.* (4) have shown that the β subunit is phosphorylated by protein kinase A. Browning *et al.* (5) have reported that the β subunit and, to a smaller degree, the α subunit are phosphorylated by both protein kinases A and C. Taken together with the studies of Porter *et al.* (13), in which the presence of the catalytic subunit of protein kinase A decreased GABA responses, it is highly likely that cAMP-dependent phosphorylation regulates GABAergic function.

At present, it is not known whether phosphorylation of the $GABA_A$ receptor alters its activity. Some studies have shown that the presence of "phosphorylation factors such as Mg^{2+}-ATP, and low Ca^{2+} maintains $GABA_A$ receptor function (24–26). In contrast, the catalytic subunit of protein kinase A reduces GABA-evoked single-cell currents by reducing channel opening frequency (13). Given the structural (1) and functional (2) similarities between the nicotinic and $GABA_A$ receptor, the phosphorylation state of the $GABA_A$ receptor could alter the rate of receptor desensitization or resensitization, similar to the nicotinic receptor (27, 28). Previously, we have shown $GABA_A$ receptor desensitization in the synaptoneurosome preparation (29) and an enhanced rate of $GABA_A$ receptor desensitization by noncompetitive nicotinic receptor blockers such as phencyclidine (2).

The phosphorylation state and desensitization of the $GABA_A$ receptor may have physiological significance. Prolonged or repeated exposure of the $GABA_A$ receptor to GABA can lead to desensitization and to increased neuronal excitation, as well as seizure activity (30, 31). Because permeant analogs of cAMP have been shown to increase neuronal excitability and induce seizures (Refs. 32–34; for review see Ref. 35), it could be hypothesized that cAMP-dependent phosphorylation of the $GABA_A$ receptor is involved. Alternatively, intracellular cAMP might interact directly with the $GABA_A$ receptor-gated chloride channel to increase neuronal excitability.

In summary, our results indicate that inhibition of $GABA_A$ receptor function by cAMP and cAMP analogs depends on their ability to reach the cytoplasmic compartment. It remains to be established whether inhibition of GABA responses is due to an interaction of cAMP analogs with the GABA-gated ion channel or to activation of protein kinase A. Ultimately, studies are necessary to show that cAMP-dependent phosphorylation of the $GABA_A$ receptor results in altered $GABA_A$ receptor function.

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