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γ-Aminobutyric Acid Receptors Modulate Cation Binding Sites Coupled to Independent Benzodiazepine, Picrotoxin, and Anion Binding Sites

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SUMMARY

Benzodiazepine (BZ) receptors in rat brain membranes, dialyzed first against 1 mM EDTA, then water, were protected against heat inactivation (30 min, 60°) by a combination of $CaCl_2$ (5 mM) plus γ-aminobutyric acid (GABA) (500 μM), but by neither substance alone. In the presence of 500 μM GABA, $CaCl_2$ provided 50% of its maximal protective effect near 600 μM. Similar protection was provided by Mg^{2+}, Ba^{2+}, Sr^{2+}, and Mn^{2+} in the presence of GABA, but eight other divalent cations were inhibitory. In the presence of 5 mM $CaCl_2$, GABA and 10 GABA-A receptor agonists provided significant protection, with EC_{50} values in the 5–200 μM range. The GABA-B receptor agonist, baclofen, provided no protection alone or together with $CaCl_2$. Alone, NaCl provides almost complete protection with an EC_{50} value near 250 mM and this value is reduced to 30 mM by 500 μM GABA. Protection by NaCl seems to require simultaneous occupation of anion and cation recognition sites. Picrotoxin and several related convulsants (≤100 μM) alone provided no protection but enhanced the protective effect of $CaCl_2$ and high (≥100 mM), but not low (≤50 mM), NaCl. The protective effect of picrotoxin plus $CaCl_2$ was synergistically potentiated by 50 mM NaCl. Picrotoxin and related convulsants provided protection, with the lowest EC_{50} values (2–20 μM) in 200 mM NaCl. Time courses of heat inactivation (at 60°) in the presence of saturating concentrations of GABA mimetics plus 5 mM $CaCl_2$ are polyphasic and suggest the existence of at least three BZ receptor complexes in rat brain. There are probably independent but interacting recognition sites for anions, cations, picrotoxin (and related substances), GABA, and BZs in the receptor complexes. The cation site may reflect a membrane channel for both Na^+ and Ca^{2+}.

INTRODUCTION

The existence of multiple BZ[1]/ion/GABA receptor complexes is now generally accepted (1–9). There is good biochemical (1–4, 9–13) and electrophysiological (14–18) evidence for the presence of independent anion (chloride) recognition sites ("receptors[2]") in BZ/GABA receptor complexes. There is also increasing evidence for the presence of independent cation recognition sites in these complexes, in addition to anion sites. Chlordiazepoxide was reported to enhance the inhibitory action of GABA on spontaneous spike discharges in guinea pig cerebellar slices through an increase in K^+, but not Cl^- ion, permeability (19). GABA was found to depolarize the dorsal roots of the frog spinal cord, and to reduce dorsal root potentials in a bicuculline- or picrotoxin-reversible way (20). This depolarizing response to GABA remained in the absence of external chloride, but was abolished by removing Na^+ (20). The reduction in dorsal root potentials, which reflects presynaptic inhibition, is potentiated by diazepam (21). It has been found that GABA, applied to the dendrites of hippocampal CA1 pyramidal cells, causes partial depolarization of the cells, in contrast to the usual hyperpolarizing response to GABA when applied to the cell bodies (17, 18, 22). Both actions of GABA result in inhibition of neuronal firing, and increase chloride ion conductance across the neuronal membrane. Both the hyperpolarizing and the depolarizing responses to GABA are potentiated by BZs (17). In addition to the increase in chloride conductance, the application of GABA to the dendrites of hippocampal pyramidal cells also increases the membrane conductance of a still-unidentified cation (18). It is not yet known whether this dendrite response to GABA is mediated by two separate GABA receptors—one opening chloride channels, the

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[1] The abbreviations used are: BZ, benzodiazepine; GABA, γ-aminobutyric acid; FLU, flunitrazepam; BGPA, β-guanidino propionate; ImAA, imidazole acetate; THIP, 4,5,6,7-tetrahydroisoxazolo-4,5c-piperidine-3-ol; P4S, piperidine-4-sulfonate; APSA, 3-aminopropane sulfonate; IGV, isoguvacine.

[2] In this paper "receptor" is equivalent to "binding site" or "recognition site."

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other cation channels—or by a single GABA receptor which regulates both chloride and cation channels simultaneously (18). GABA and diazepam individually block the Ca^{2+}-dependent K^+-evoked release of [^{3}H]serotonin from rat hippocampal synaptosomes, and diazepam enhances the inhibitory effect of GABA on K^+-stimulated release (23). The Ca^{2+}-dependent K^+-stimulated release of [^{3}H]glutamate from 0.1-mm striatal prisms was facilitated by muscimol (50 μM) in a way potentiated by diazepam (24). Binding of [^{3}H]baclofen and [^{3}H]GABA to GABA-B receptors, which are insensitive to bicuculline and most classical GABA-A receptor agonists, was reported to be Ca^{2+} or Mg^{2+} ion-dependent (25), and GABA, acting on a GABA-B-like receptor (26), was found to decrease Ca^{2+} spikes in tetrodotoxin-blocked neurons from chick dorsal root ganglia grown in tissue culture (26). Although BZs do not interact significantly with GABA-B receptors, interaction of the latter with Ca^{2+} or Mg^{2+} raises the possibility that coupling to divalent cation sites may be a common feature of several types of GABA receptors, including those coupled to BZ receptors. Taken together, these findings suggest that brain-specific BZ receptors could be coupled to both anion and cation recognition sites.

We now present evidence for independent, but interacting, cation and anion recognition sites in BZ/GABA/picrotoxin receptor complexes.

MATERIALS AND METHODS

Preparation of EDTA-dialyzed rat brain P_2 fraction. Rat forebrain (minus cerebellum and pons-medulla) was homogenized using a glass-Teflon homogenizer in 50 volumes of ice-cold 1 mM EDTA (pH 7.5), and a P_2 fraction was prepared by conventional differential centrifugation. The P_2 pellet was resuspended in 50 volumes of ice-cold 1 mM EDTA and dialyzed against three successive portions of ice-cold distilled water (1–2 hr for each portion). The volume of distilled water was at least 20 times the volume of P_2 suspension inside the dialysis bag. After dialysis, the P_2 membranes were centrifuged once more, resuspended in 5 times the original volume of brain tissue, and stored frozen at −20° in 1.0-ml aliquots for a maximum of 14 days. [^{3}H]FLU binding sites were more stable when the P_2 membranes were stored at −20° as firm pellets, after centrifugation, rather than as suspensions in water.

Standard binding assay. Thawed P_2 suspensions were dispersed in a glass-Teflon homogenizer and diluted, usually 30-fold, in double-distilled or double-ion exchanged water. One milliliter of this diluted P_2 suspension was used per assay in standard 125 × 13 mm Pyrex test tubes. An amount of P_2 membranes was used which bound no more than 15% of the total [^{3}H]FLU added. Appropriate buffers, salts, and test substances were added together with 100 μl of 4 nM [^{3}H]FLU (final concentration 0.2 nM) in a final volume of 2.0 ml. The tubes were then incubated for 15 min at 37° followed by an additional 60 min at 0° (ice bath)[3] before filtration through Whatman GF/A glass microfiber filter discs (2.4-cm diameter) on a Millipore 12-sample filtration manifold with slight vacuum. The filters were washed twice with 5.0-ml portions of ice-cold 10 mM Tris-HCl (pH 7.5) and transferred to disposable polypropylene scintillation vials (Walter Sarstedt, Inc.); 5 ml of liquid scintillation cocktail [Liquiscint, National Diagnostics, (Somerville, N. J.) containing 8% water] were added, and the samples were counted for tritium by conventional scintillation counting.

Heat inactivation experiments. An appropriately diluted rat brain P_2 suspension was combined with test substances in a volume of 1.0 ml [all containing 2 mM Tris-HCl (pH 7.5)] and the samples were placed for 30 min in a 60° thermostat-regulated, constant temperature water bath. All samples were then placed in an ice bath, and 900 μl of a mixture containing Tris-HCl (pH 7.5), NaCl, and GABA to give a final concentration of 50 mM, 200 mM, and 500 μM, respectively (to ensure maximal [^{3}H]FLU binding), plus 100 μl of 4 nM [^{3}H]FLU to give a final concentration of 0.2 nM, in a final volume of 2.0 ml, were added. Incubation, filtration, and counting were then performed as described above.

Time courses of heat inactivation. P_2 membranes, appropriately diluted and combined with salts and test substances, in a volume of 1.0 ml as described above, were placed in a thermostat-regulated water bath at 60°; they were removed at times ranging from 5 to 120 min and placed in an ice bath. At the end of the time course, the Tris-HCl/NaCl/GABA mixture and [^{3}H]FLU were added, and incubation, filtration, and counting were performed as described above.

Calculation of B_{max}, EC_{50}, and Hill numbers. Linear concentration-response curves (salt or GABA mimetic concentration versus specific [^{3}H]FLU binding) were drawn. Values for ΔB were obtained by subtracting [^{3}H]FLU binding in the absence of added ligand from binding in the presence of ligand to produce a function going from zero to a limiting value, which can be analyzed by the Michaelis-Menten equation (Langmuir isotherm) or one of its variants. A ΔB_{max} value was estimated with a double-reciprocal (Lineweaver-Burk) plot ($1/\Delta B$ versus $1/L$), using the three or four highest ligand concentrations. In cases where the double-reciprocal plots deviated drastically from linearity, the ΔB_{max} values were taken directly from the plateaus of linear plots. The ΔB_{max} value was then used to construct a Hill plot, log [($\Delta B_{max}/\Delta B$) − 1] versus log [ligand], from which an EC_{50} value (the concentration of ligand producing 50% of its maximal effect) and a Hill coefficient were obtained.

RESULTS

In preliminary experiments the protective effects of various ions and test substances were compared by using water-dialyzed and EDTA/water-dialyzed rat brain P_2 membrane preparations. The specific [^{3}H]FLU (BZ) binding sites in both preparations were more than 99% destroyed by heating for 30 min at 60° in 2 mM Tris-HCl (pH 7.5) alone. It was striking that 5 mM $CaCl_2$, 50 mM NaCl, or 50 mM $NaPO_4$ (pH 7.5) alone provided several-fold more protection in the ordinary water-dialyzed preparation than in the EDTA/water-dialyzed preparation (data not shown). A combination of GABA (500 μM) and

[3] Preincubation at 37° allows [^{3}H]FLU, at 0.2 nM, to reach equilibrium binding in about 60 min at 0°, and yields about twice as much specific binding as that obtained after incubation for 60 min at 0°, without the 37° preincubation (data not shown).

TABLE 1

Concentration-response analysis for five divalent cations in the presence of 500 μM GABA

EDTA/water-dialyzed P_2 membranes from rat forebrain were heated for 30 min at 60° with varying concentrations of divalent cations and 500 μM GABA. After heating, [^{3}H]FLU binding to specific receptors was measured as described in the text. ΔB_{max}, EC_{50}, and Hill numbers (α) were estimated by double-reciprocal plots and Hill analysis as described in the text. ΔB_{max} is the maximal increase in protective effects, expressed as percentage of unheated control [^{3}H]FLU binding. Binding in the absence of divalent cations (about 4% of control) is subtracted from total binding to obtain ΔB. The EC_{50} values, derived from Hill plots, represent the concentrations of cations required to produce 50% of their maximal protective effects. All values are means of n separate determinations ± standard deviation.

Divalent cation (n)	ΔB_{max} (% of control)	EC_{50} (μM)	α Hill no.
$CaCl_2$ (5)	61 ± 4.5	600 ± 160	0.99 ± 0.07
$MgCl_2$ (3)	61 ± 5.4	630 ± 360	0.86 ± 0.10
$BaCl_2$ (3)	63 ± 5.3	930 ± 420	0.83 ± 0.20
$SrCl_2$ (3)	64 ± 5.6	830 ± 260	0.83 ± 0.18
$MnCl_2$ (3)	39 ± 0.2	200 ± 86	0.95 ± 0.12

$CaCl_2$ (5 mM), but neither substance alone, provided about 50% protection of the receptor in both preparations. Similarly, GABA together with NaCl (50 mM) gave about equal protection in both preparations. These results suggested that the removal of divalent cations from the P_2 fraction by EDTA also facilitated the removal of GABA, which is not removed by dialysis against water alone. Preliminary GABA measurements indicated that EDTA dialysis removes about 90% of the GABA present in ordinary water-dialyzed P_2 membranes.[4]

All of the remaining experiments were performed with EDTA/water-dialyzed rat brain membranes. In the presence of 500 μM GABA, Ca^{2+}, Ba^{2+}, Mg^{2+}, and Sr^{2+} ions (all 5 mM) provided about the same protection (50–60%) after 30 min at 60° while Mn^{2+} provided somewhat less protection (40%) (Table 1; Fig. 1). Be^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+}, La^{3+}, and Cd^{2+} failed to protect BZ receptors in the presence of GABA (500 μM) and antagonized the protective effect of $CaCl_2$ plus GABA in noncompetitive ways (data not shown). Concentration-response curves for the active divalent cations revealed that Ca^{2+}, Mg^{2+}, Ba^{2+}, and Sr^{2+} were about equipotent, with EC_{50} values in the 500–900 μM range. Mn^{2+} seemed to have a higher affinity (EC_{50} near 200 μM) for the cation site (Table 1; Fig. 1).

In the presence of a saturating concentration of $CaCl_2$ (5 mM), GABA and 10 GABA-A receptor agonists yielded saturable concentration-response curves, with 50% of maximal effect at concentrations ranging from 5 μM for muscimol to 60 μM for BGPA and 130 μM for ImAA (Table 2; Fig. 2). The GABA-B receptor agonist baclofen provided no protection alone or together with 5 mM $CaCl_2$.

The concentration-response curves for GABA and muscimol yielded Hill numbers less than 1, possibly reflecting the existence of two or more BZ/GABA receptor complexes with different affinities for GABA (or muscimol). The Hill number for THIP, the GABA mimetic providing the least protection, was near 1.4, possibly reflecting positive cooperativity between interacting subunits within a receptor complex (Table 2).

[4] T. Hare and R. Squires, unpublished data.

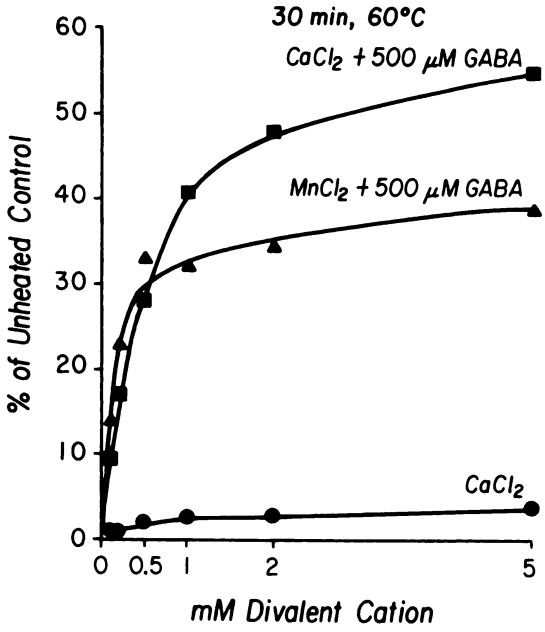

FIG. 1. *Concentration-response curves for $CaCl_2$ and $MnCl_2$ in the presence of 500 μM GABA and $CaCl_2$ alone*

EDTA/water-dialyzed rat brain P_2 membranes were heated for 30 min at 60° together with varying concentrations of $CaCl_2$ or $MnCl_2$ + 500 μM GABA. After heating, BZ receptors ([^{3}H]FLU binding) were assayed as described in the text.

Time courses of heat inactivation of BZ receptors in the presence of saturating concentrations of $CaCl_2$ (5 mM) and GABA mimetics (at least 10 times their EC_{50} values, listed in Table 2) were in all cases polyphasic, having "slow" components with half-lives ranging from 30 min with THIP to about 95 min with GABA and 146 min with GABA plus 50 mM NaCl (Fig. 3; Table 3).

Since the GABA mimetics were used at saturating concentrations, the large differences in the half-lives of the slow components probably reflect different efficacies of the GABA mimetics in inducing heat-resistant conformations of the BZ/GABA/ion receptor complexes. The

TABLE 2

Concentration-response analysis of GABA and seven GABA mimetics

EDTA/water-dialyzed rat brain P_2 membranes were heated for 30 min at 60° with varying concentrations of GABA mimetics and 5 mM $CaCl_2$. [^{3}H]FLU binding and estimation of ΔB_{max}, EC_{50}, and α values were as described in the text. All values are means of n separate determinations ± standard deviation.

GABA mimetic varied (n)	ΔB_{max} (% of control)	EC_{50} (μM)	α Hill no.
GABA (5)	52 ± 4.1	20 ± 3.9	0.84 ± 0.07
APSA (3)	48 ± 4.8	14 ± 2.3	0.86 ± 0.10
BGPA (3)	47 ± 9.2	72 ± 23	0.88 ± 0.10
ImAA (3)	40 ± 4.6	130 ± 50	0.96 ± 0.05
Muscimol (3)	47 ± 5.6	5 ± 0.47	0.82 ± 0.10
Dihydromuscimol (1)	45	10	0.79
IGV (3)	28 ± 5.9	49 ± 21	0.89 ± 0.16
P4S (3)	30 ± 3.9	26 ± 7.6	0.87 ± 0.08
THIP (3)	14 ± 2.4	45 ± 12.5	1.4 ± 0.06
(±)-β-Homoproline (1)	34	150	1.0
trans-4-Aminocrotonic acid (1)	48	53	0.90

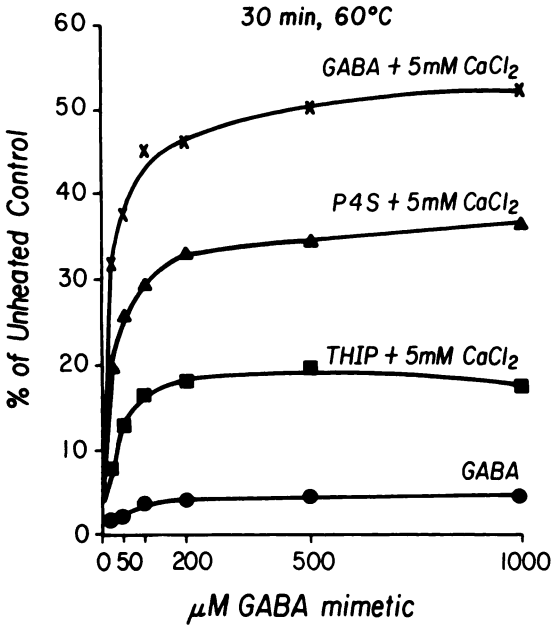

FIG. 2. *Concentration-response curves for GABA, P4S, and THIP in the presence of 5 mM $CaCl_2$ and GABA alone*

EDTA/water-dialyzed rat brain P_2 membranes were heated for 30 min at 60° together with varying concentrations of GABA, P4S, or THIP in the presence of 5 mM $CaCl_2$. After heating, BZ receptors were assayed as described in the text.

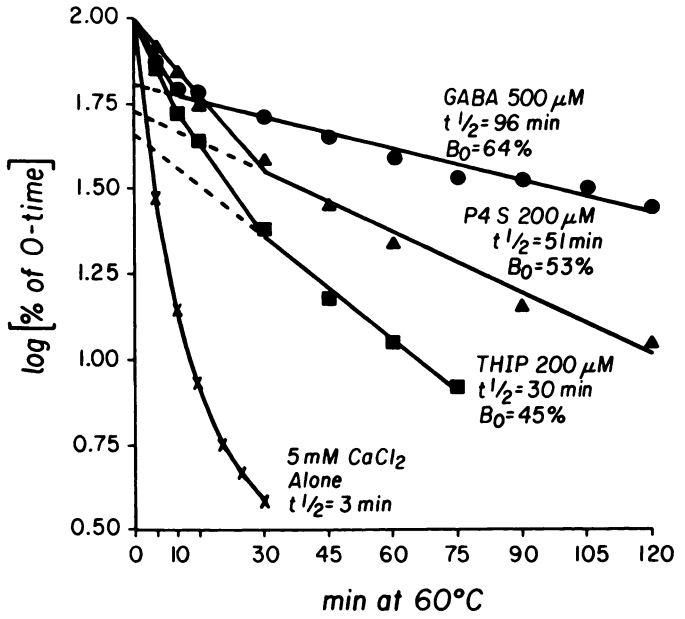

FIG. 3. *Time courses of BZ receptor inactivation at 60° in the presence of saturating concentrations of $CaCl_2$ (5 mM) and GABA mimetics*

Concentrations of GABA, P4S, and THIP are given in Table 3. EDTA/water-dialyzed rat brain P_2 membranes were used. Samples were withdrawn from the 60° bath at the indicated times (ranging from 5 to 120 min) and placed in an ice bath. At the end of the time course, BZ receptors were assayed as described in the text. The half-lives of the "slow" components of the time courses are presented. The B_0 values were calculated by extrapolating the slow components back to zero time, and are expressed as percentages of the unheated control values, which were not significantly different from each other [see zero times (% of control), Table 3].

B_0 values, presented in Table 3, represent the sizes of the slow components, extrapolated back to time zero and expressed as percentages of unheated control binding. It is striking that these B_0 values fall into four groups: one consisting of the values obtained using the piperidine-derived GABA mimetics THIP, IGV, and P4S (near 45% of the unheated controls); another consisting of ImAA, BGPA, and APSA (58%); a third of GABA and muscimol (68%); and a fourth of 1 mM APSA or GABA plus 50 mM NaCl (94%). It appears, therefore, that saturating concentrations of THIP, IGV, or P4S together with a saturating concentration of $CaCl_2$ (5 mM) selectively protect a subpopulation of [^{3}H]FLU binding sites constituting about 45% of all [^{3}H]FLU binding sites in rat forebrain. These B_0 values are different from the B_0 values for GABA (69%) at a high level of statistical significance ($p < 0.001$, using Student's t-test). Similarly, the B_0 value for GABA plus 50 mM NaCl is also significantly greater than the value for GABA alone ($p < 0.001$). Thus, the B_0 values seem to reflect the existence of at least three subpopulations of [^{3}H]FLU binding sites constituting 45%, 68%, and 95% of all [^{3}H]FLU binding sites.

The affinity of GABA for its receptor seemed to be regulated by both cations and anions. Alone, GABA increased the number of [^{3}H]FLU binding sites protected by only 3.4% with an apparent EC_{50} near 68 μM. In the presence of a saturating concentration of $CaCl_2$ or $MgCl_2$ (5 mM), the EC_{50} value for GABA was about 15–16 μM, and this value was not affected by picrotoxin. In the presence of 50 mM NaCl the EC_{50} for GABA was reduced to about 8 μM (Table 4).

The number of BZ receptors protected by $CaCl_2$, as

TABLE 3

Time courses of heat inactivation of BZ receptors at 60° in the presence of saturating concentrations of various GABA mimetics and $CaCl_2$ (5 mM)

The half-lives ($t_{1/2}$) of the slow components are presented. They are usually taken in the time range 30–120 min from semilog plots (log %B versus time). The B_0 values represent the amounts of the "slow" components, expressed as percentage of unheated control, obtained by extrapolating the linear portions of the semilog plot back to time zero. All values are means of n separate experiments ± standard deviation. The presence of the GABA mimetics used did not significantly affect unheated control binding [see 0-time (% of control)]. Assay of BZ receptors was as described in Table 1 and the text.

Substance added (n)	B_0 for slow components (% of 0 time)	Slow component $t_{1/2}$ (min)	Zero time (% of control)
None (3)	100[a]	2.8 ± 0[a]	107 ± 9
THIP, 200 μM (3)	42 ± 7[b]	28 ± 2[b]	93 ± 2
IGV, 1 mM (3)	46 ± 11[c]	52 ± 5[b]	98 ± 3
P4S, 200 μM (3)	48 ± 11[c]	46 ± 5[b]	98 ± 3
ImAA, 1 mM (3)	56 ± 10	58 ± 8[c]	91 ± 2
BGPA, 1 mM (3)	61 ± 4	83 ± 8	98 ± 4
APSA, 200 μM (2)	58 ± 5	76 ± 16	92 ± 6
GABA, 500 μM (8)	69 ± 7	95 ± 14	96 ± 7
Muscimol, 100 μM (3)	66 ± 3	70 ± 9	96 ± 6
Picrotoxin, 200 μM + NaCl, 50 mM (2)	68 ± 3	46 ± 6[c]	93 ± 3
APSA, 1 mM (2)	92 ± 4	81 ± 13	92 ± 2
GABA, 500 μM + NaCl, 50 mM (5)	95 ± 4[b]	150 ± 21[b]	95 ± 6

[a] Not resolved into fast and slow components.
[b] Significantly different from GABA, 500 μM ($p < 0.001$, Student's t-test).
[c] Significantly different from GABA, 500 μM ($p < 0.01$, Student's t-test).

TABLE 4

Concentration-response analysis for GABA alone or in the presence of $CaCl_2$ (5 mM), $MgCl_2$ (5 mM), NaCl (50 mM), or picrotoxin (100 μM) plus $CaCl_2$ (5 mM)

Brain membranes together with varying concentrations of GABA plus the substances indicated were heated at 60° for 30 min. Assays and calculations as described in Table 1 and in the text. All values are means of n separate determinations ± standard deviations.

Substance added (n)	ΔB_{max} (% of control)	EC_{50} (μM GABA)	α Hill no.
None (GABA alone) (1)	3.4	68	1.8
$CaCl_2$ 5 mM (5)	52 ± 4.1	20 ± 3.9	0.84 ± 0.07
$MgCl_2$ 5 mM (3)	49 ± 7.2	17 ± 4.2	0.86 ± 0.2
NaCl 50 mM (3)	50 ± 7.5	7.5 ± 2.2[a]	0.89 ± 0.2
Picrotoxin, 100 μM + $CaCl_2$, 5 mM (3)	47 ± 10	20 ± 6.5	0.92 ± 0.2

[a] Significantly different from $CaCl_2$, 5 mM ($p < 0.01$, Student's t-test).

well as the affinity of the receptor complex for Ca^{2+}, appeared to be modulated by GABA, NaCl, and picrotoxin acting on independent, but interacting, receptors. Alone, $CaCl_2$ increased the number of BZ receptors protected by 6% of the unheated control, with an EC_{50} near 3.3 mM, whereas in the presence of a saturating concentration of GABA (500 μM) the number of receptors protected was increased 10-fold (to 61% of the unheated control) and the EC_{50} for $CaCl_2$ was reduced to 0.60 mM (Table 5). Picrotoxin (100–200 μM) increased the number of BZ receptors protected by $CaCl_2$ but did not increase the affinity for $CaCl_2$. A combination of picrotoxin (200 μM) plus NaCl (50 mM) allowed $CaCl_2$ to increase the maximal number of receptors protected to about 70% of the unheated control but, again, without increasing the affinity of the receptor complex for $CaCl_2$ (Table 5).

Similarly, the affinity of the receptor complex for picrotoxin was modulated by $CaCl_2$ and NaCl in a synergistic way. Alone, or together with 50 mM NaCl, picrotoxin provided little protection. However, in the presence of saturating (5 mM) $CaCl_2$, picrotoxin increased the number of protected receptors by about 22%, with a EC_{50} near 150 μM. In the presence of 5 mM $CaCl_2$ plus 50 mM NaCl, picrotoxin increased the maximal protection by about 40% of the unheated control with an EC_{50} near 50 μM (Table 6; Fig. 4). Thus 50 mM NaCl, while providing

TABLE 5

Concentration-response analysis of $CaCl_2$ alone or in the presence of GABA, picrotoxin, or picrotoxin plus NaCl

Membranes containing varying concentrations of $CaCl_2$ were heated for 30 min at 60°. Assay of BZ receptors ([^{3}H]FLU binding) and calculations of ΔB_{max}, EC_{50}, and α values were as described in Table 1 and the text. All values are means of n separate determinations ± standard deviation.

Substance added (n)	ΔB_{max} (% of control)	EC_{50} (mM $CaCl_2$)	α Hill no.
None ($CaCl_2$ alone) (1)	6	3.3	0.9
GABA, 500 μM (5)	61 ± 4.5	0.6 ± 0.16	0.99 ± 0.07
Picrotoxin, 100 μM (2)	19 ± 1.8	4.2 ± 1.2[a]	0.86 ± 0.06
Picrotoxin, 200 μM (3)	23 ± 1.0	3.8 ± 1.0[a]	0.99 ± 0.07
Picrotoxin, 200 μM + NaCl, 50 mM (3)	70 ± 12	3.2 ± 1.0[b]	0.98 ± 0.03

[a] Significantly different from GABA 500 μM, $p < 0.001$.
[b] $P < 0.01$, Student's t-test.

TABLE 6

Concentration-response analysis of picrotoxin in the presence of $CaCl_2$, $CaCl_2$ plus NaCl (50 mM) or NaCl (200 mM)

Membranes containing varying concentrations of picrotoxin were heated for 30 min at 60°. Assay of BZ receptors after heating, and calculation of ΔB_{max}, EC_{50}, and α values were as described in the text. All values are means of n separate determinations ± standard deviation.

Substance added (n)	ΔB_{max} (% of control)	EC_{50} (μM picrotoxin)	α Hill no.
$CaCl_2$ 5 mM (3)	22 ± 1.8	180 ± 50[a]	0.96 ± 0.16
$CaCl_2$ 5 mM + NaCl, 50 mM (3)	42 ± 5.4	57 ± 14	0.99 ± 0.06
NaCl, 200 mM (3)	39 ± 1.6	15 ± 2.7[b]	0.95 ± 0.25

[a] Significantly different from $CaCl_2$, 5 mM + NaCl, 50 mM ($p < 0.02$, Student's t-test).
[b] Significantly different from $CaCl_2$, 5 mM + NaCl, 50 mM ($p < 0.01$, Student's t-test).

little protective effect alone or in combination with picrotoxin, potentiated the protective effects of picrotoxin plus $CaCl_2$ and increased the affinity of the receptor complex for picrotoxin in the presence of $CaCl_2$.

High concentrations of NaCl (>500 mM) alone provided almost complete protection against heat inactivation, with an EC_{50} near 250 mM; this value was not changed much by the presence of 5 mM $CaCl_2$. GABA, on the other hand, reduced the EC_{50} for NaCl from 250 mM to 35 mM without significantly affecting the maximal number of protected receptors (Table 7; Fig. 5). Picrotoxin had a double effect on the concentration-response curve for NaCl. Like GABA, picrotoxin shifted the curve to the left (EC_{50} value reduced from 250 mM to 132 mM). Picrotoxin also increased the Hill number for NaCl from 2 to about 3 (Table 7; Fig. 5). The combination of picrotoxin plus $CaCl_2$ reduced the EC_{50} for NaCl to 64 mM, with a Hill number near 2 (Table 7).

In the presence of 200 mM NaCl, which alone protected about 34% of the [^{3}H]FLU binding sites, saturating concentrations of picrotoxin roughly doubled the number of sites protected (to about 70% of the unheated control). The EC_{50} for picrotoxin in the presence of 200 mM NaCl

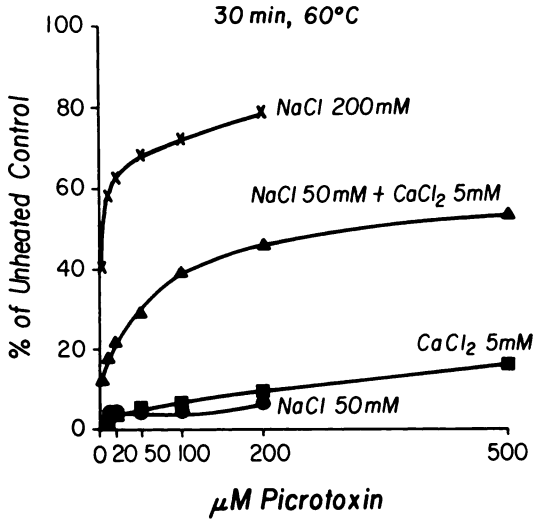

FIG. 4. *Picrotoxin concentration-response curves*

EDTA/water-dialyzed brain membranes were heated for 30 min at 60° together with varying concentrations of picrotoxin, alone or in the presence of 5 mM $CaCl_2$, or 5 mM $CaCl_2$ plus 50 mM NaCl. After heating, BZ receptors were assayed as described in the text.

TABLE 7

Concentration-response analysis of NaCl alone and in the presence of GABA, $CaCl_2$, picrotoxin, and picrotoxin plus $CaCl_2$

Concentrations of NaCl ranging from 5 mM to 500 mM and the substances indicated were heated for 30 min at 60°. Assay of BZ receptors after heating, and calculations of ΔB_{max}, EC_{50}, and α values were as described in the text. All values are means of n separate determinations ± standard deviations

Substance added (n)	ΔB_{max} (% of control)	EC_{50} (mM NaCl)	α Hill no.
None (NaCl alone) (6)	83 ± 4.8	260 ± 42	2.0 ± 0.20
GABA, 500 μM (4)	81 ± 8.0	35 ± 10[a]	1.6 ± 0.36
$CaCl_2$, 5 mM (4)	82 ± 10	210 ± 31	1.4 ± 0.26[b]
Picrotoxin, 100 μM (4)	83 ± 4.4	140 ± 17[a]	2.9 ± 0.30[a]
Picrotoxin + $CaCl_2$ (3)	68 ± 3.2	64 ± 7.1[a, c]	1.9 ± 0.17[d]

[a] Significantly different from NaCl alone (none) ($p < 0.001$).
[b] Significantly different from NaCl alone (none) ($p < 0.01$).
[c] Significantly different from both $CaCl_2$, 5 mM, and picrotoxin, 100 μM ($p < 0.001$).
[d] Significantly different from picrotoxin, 100 μM ($p < .01$).

was about 15 μM. Several other convulsants thought to act on picrotoxin receptors also potently increased the protective effect of 200 mM NaCl. These include picrotoxinin (EC_{50} = 25 μM), anisatin (3 μM), isopropyl bicyclophosphate (17 μM), *t*-butyl bicyclophosphate (3 μM), and isopropyl bicyclophosphothionate (3 μM). Picrotin was inactive at 100 μM.

The large Hill numbers for NaCl (Table 7; Fig. 5) are in sharp contrast to the Hill numbers near, or slightly less than, unity for the divalent cations (Table 1), and probably reflect positive cooperativity between interacting subunits in the BZ receptor complexes. Cooperative interactions between separate cation and anion receptors seem possible.

The protective effects produced by ions and GABA as reported here seem to be specific for BZ receptors, since they are also obtained with the use of [^{3}H]2-phenylpyrazolo[4,3-*c*]quinolin-3(5H)-one (a potent BZ receptor antagonist) but not [^{3}H]quinuclidinyl benzilate (a potent muscarinic receptor blocker) as ligands (data not shown).

DISCUSSION

The dialysis of rat brain membranes against EDTA followed by water yields a BZ receptor preparation which is significantly different from water-dialyzed preparations. Dialysis against EDTA then water apparently removes, in addition to divalent cations, significant amounts of GABA which cannot be removed by dialysis against water alone.[4] This finding is consistent with reports that the binding of [^{3}H]diazepam and [^{3}H]FLU to sites on brain membranes is more effectively enhanced by added GABA or muscimol when the membranes are prewashed a few times with Tris-citrate buffer (e.g., ref. 27), which also chelates divalent cations. Alone, divalent cations (Ca^{2+}, Mg^{2+}), NaCl (50 mM), or $NaPO_4$ provided significant protection of BZ receptors in water-dialyzed membranes but little protection using EDTA/water-dialyzed membranes unless exogenous GABA was added. Does GABA produce the protective effects described above by acting on physiologically relevant GABA receptors? Muscimol, one of the most potent GABA mimetics

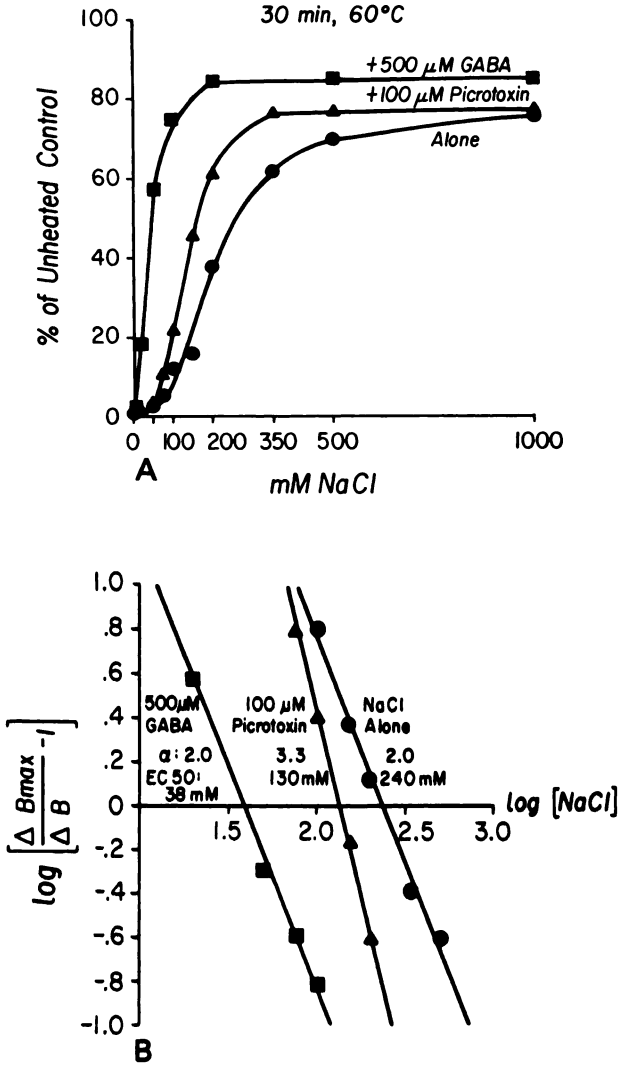

FIG. 5. *NaCl concentration-response curves*
EDTA/water-dialyzed membranes were heated at 60° for 30 min together with varying concentrations of NaCl, alone or in the presence of GABA (500 μM) or picrotoxin (100 μM). After heating, BZ receptors ([^{3}H]FLU binding) were assayed as described in the text. A, Linear graph of specific [^{3}H]FLU binding sites (as percentage of unheated control binding) versus NaCl concentration; B, Hill transformation of A.

known, is the most potent in protecting BZ receptors from heat inactivation in the presence of $CaCl_2$. The ability of 10 GABA-A receptor agonists with diverse chemical structures, but not baclofen (a GABA-B receptor agonist), to provide protective effects in the presence of 5 mM $CaCl_2$ strongly suggests that physiologically relevant GABA-A receptors are involved.

THIP is of special interest since it selectively protects the smallest fraction of BZ receptor complexes from heat inactivation (about 42% with a half-life of 28 min). THIP reduces the affinity of ions required for [^{3}H]FLU binding (13) and partially antagonizes the protective effects of GABA against heat inactivation.[5] Using an ordinary water-dialyzed P_2 preparation, THIP plus 50 mM NaCl failed to protect BZ receptors in rat brain membranes

[5] R. Squires, unpublished data.

against heat inactivation, in contrast to all other GABA mimetics tested (3). The failure of THIP to protect under those conditions (3) may be due to the generally weak protective effect of this GABA mimetic, together with the ability of THIP to antagonize a small protective effect of endogenous GABA in the ordinary water-dialyzed P_2 preparation. Gavish and Snyder (28) found that THIP protected BZ receptors from heat inactivation by using a well-washed bovine brain particulate preparation in the presence of 50 mM Tris-HCl (pH 7.4).

Picrotoxin did not modify the protection-enhancing effects of GABA in the presence of either $CaCl_2$ or NaCl, but potentiated the protective effects of $CaCl_2$ and NaCl, indicating that it binds to a separate site which interacts allosterically with independent sites for cations, anions, GABA, and BZs.

Time courses of BZ receptor heat inactivation in the presence of saturating concentrations of $CaCl_2$ and GABA mimetics are polyphasic, with "slow" components constituting about 42% of the unheated control binding in the presence of saturating THIP and about 69% in the presence of saturating GABA (a highly significant difference), suggesting the existence of at least three populations of BZ receptor complexes. Together with 5 mM $CaCl_2$, APSA at high concentrations (1 mM) differed from the other GABA mimetics tested by producing a slow component constituting about 92% of the unheated control. The combination of 5 mM $CaCl_2$/500 μM GABA/50 mM NaCl provided unusual protection, with time courses revealing a slow component ($t_{1/2}$ = 146 min) constituting 95% of the unheated control (Table 3). These results suggest that all BZ/GABA receptor complexes may be coupled to both anion and cation recognition sites (Fig. 6).

Are the cation recognition sites physiologically relevant? The submillimolar concentrations of Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, and Mn^{2+} (as chlorides) required to protect significantly [^{3}H]FLU binding sites against heat inactivation in the presence of saturating concentrations of GABA convincingly demonstrate a role for cations in the protective effects produced by these salts, since similar concentrations of NaCl or KCl are essentially inactive. However, the ability of NaCl at 50 mM, which alone gives little protection, to enhance synergistically the protective effect of $CaCl_2$ alone, or of $CaCl_2$ plus picrotoxin, indicates that anions are also involved in the protective effects, supporting earlier observations that NaCl, KCl, and CsCl produce one pattern of protection whereas sodium and potassium phosphate (pH 7.5) produce another (3). The concentration-response curves for NaCl also appear to support the view that both cations and anions are involved in the observed protective effects. The unusually high Hill numbers for NaCl, either alone or in the presence of picrotoxin or GABA, strongly suggest positive cooperativity between allosterically interacting binding sites, possibly for anions and cations, respectively. Thus, protection of [^{3}H]FLU binding sites by NaCl may require the *simultaneous* occupancy of a cation and an anion site by Na^+ and Cl^-, respectively. $CaCl_2$ at 5 mM alone slightly reduced the EC_{50} value and the Hill number for NaCl. Together with picrotoxin, $CaCl_2$ reduced the EC_{50} value for NaCl even more as compared with those obtained in the presence of picrotoxin alone (see Table 7). These results suggest that Ca^{2+} and Na^+ may bind to the same cation recognition site, with Ca^{2+} exhibiting a much higher affinity for the site than Na^+. Both Ca^{2+} and Na^+ have been implicated in GABA-regulated, BZ-potentiated processes on dendrites (17, 18) and on nerve terminals (20, 23, 24).

Recently, vitamin D-dependent calcium-binding proteins in several types of neurons in brain have been localized with the use of immunohistochemical techniques (e.g., ref. 29). It has been pointed out that three types of neurons containing the calcium-binding proteins, the Purkinje cells of the cerebellum, the neurons of the inferior olive, and CA_1 pyramidal cells of the guinea pig hippocampus are known to produce voltage-dependent calcium spikes within their dendritic trees (29). There is evidence for the presence of BZ receptors on the dendrites of CA_1 pyramidal cells (17) and cerebellar Purkinje cells (30). The BZ receptors on the dendrites of CA_1 pyramidal cells apparently potentiate the ability of GABA to open cation channels (17). The calcium-binding protein might act to "buffer" Ca^{2+} entering the pyramidal cells through these channels. GABA was also reported to inhibit calcium spikes in tetrodotoxin-blocked chick sensory neurons grown in tissue culture (26). It was suggested that this effect of GABA might reflect events associated with presynaptic inhibition (26). Thus, GABA might inhibit release of neurotransmitters by blocking influx of Ca^{2+} into nerve terminals (26). However, the reported action of GABA on calcium spikes is not potentiated by BZs.[6] The ionic mechanisms involved in both presynaptic inhibition and the partially depolarizing effects of GABA on CA_1 pyramidal cell dendrites remain unclear. In conclusion, the strong interactions of the BZ-coupled cation site with independent GABA and picrotoxin receptors suggest that it is physiologically relevant, although the cation involved remains unidentified. It

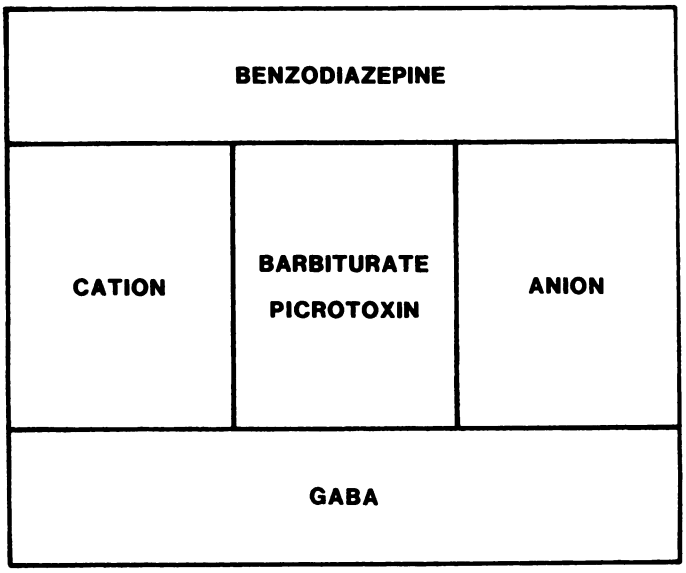

FIG. 6. *Schematic representation of a benzodiazepine/cation/anion/picrotoxin/GABA receptor complex*

[6] K. Dunlap, personal communication.

seems possible that both Na^+ and Ca^{2+} may use the same cation channel coupled to BZ receptors.

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