Biochemical Pharmacology, Vol. 38, No. 16, pp. 2587–2591, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

$^{36}Cl^-$ FLUX MEASUREMENTS ON $GABA_A$ RECEPTOR-ACTIVATED CHLORIDE EXCHANGE

MULTIPLE MECHANISMS OF THE CHLORIDE CHANNEL INACTIVATION

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(*Received* 1 *August* 1988; *accepted* 14 *February* 1989)

Abstract—The technique of radiotracer $^{36}Cl^-$ influx in primary culture of rat cerebellar granule cells was applied to study the mechanism of inactivation of the $GABA_A$ receptor-activated chloride channel. During sustained application of GABA, muscimol and THIP the specific bicuculline-sensitive $^{36}Cl^-$ influx tends to decline with time. The sequence in decay half-time is GABA < muscimol < THIP. Diazepam accelerates the rate of decay of the peak response to GABA. (−)-Baclofen enhances the rate of decline of the response to muscimol in a dose-dependent manner. Treatment of the cells with pertussis toxin antagonized the effect of (−)-baclofen. It is concluded that rat neonatal cerebellar neurons maintained in tissue culture exhibit complex inactivation of the $GABA_A$ channel, indicating some interaction with the $GABA_B$ receptor system.

The structure of the γ-aminobutyric acid/benzodiazepine ($GABA_A$) receptor [1] has many implications for the mechanisms by which binding of GABA to recognition site and opening of the integral chloride channel occurs. Both the electrophysiological [2–4] and the $^{36}Cl^-$ flux measurements [5–7] indicate that during sustained application of GABA the number of open $GAGA_A$ channels decreases with time (desensitization of the $GABA_A$ receptor). $^{36}Cl^-$ flux measurements performed after preincubation of membrane vesicles from the rat cerebral cortex with GABA demonstrated that the desensitization and chloride exchange processes occurred in the same ranges of time and GABA concentration. Evidence was obtained that channel opening and desensitization are independent processes each mediated by two GABA binding sites. Some other evidence obtained from $^{36}Cl^-$ flux measurements is also discussed in terms of desensitization of the $GABA_A$ channel [8–12].

We have used radiotracer $^{36}Cl^-$ influx in a primary culture from rat cerebellum [11, 12] to investigate the inactivation of $GABA_A$ channels on neurons. The cerebellar primary culture is extremely useful in this respect because of the large proportion (90%) of small and uniform granule cells receiving innervation from GAD antibody-positive GABAergic interneurons [13].

MATERIALS AND METHODS

Tissue culture. A primary culture of rat cerebellar neurons was obtained as previously described [14]. Cells from cerebella of 8-day-old rats were dissociated with trypsin and DNAse and plated at 5×10^6 cells/dish on poly-L-lysine (Sigma Chemical Co., St Louis, MO) coated dishes in Eagle's basal medium (Sigma Chemical Co.), 10% bovine serum (Gibco), 25 mm KCl, 2 mM glutamine and 100 μg/ml gentamycin. Cytosine arabinofuranoside (10 μM) was added to the culture after 18–24 hr *in vitro* to inhibit the replication of non-neuronal cells. Cultures were used after 6 days *in vitro*. Viability of cells was 98% as determined by the method of Jones and Senft [15].

$^{36}Cl^-$ *influx measurements.* We used the procedure previously described [11, 12] with slight modifications as follows. The growth medium was aspirated and immediately replaced with 1 ml HEPES buffer (15 mM, pH 7.4 [16]) containing 2 μM bicuculline methiodide [12]. Then the cells were allowed to cool slowly to 4° (30 min). The viability of the cultures did not change during the above procedures. The $^{36}Cl^-$ measurements, performed at 4°, were initiated by replacing the above solution with an identical one containing in addition 2.5 μCi/ml $^{36}Cl^-$. After a prescribed time interval (7–28 sec) the incubation solution was aspirated and the cells were rinsed rapidly three times (less than 3 sec) with 10 ml of the above buffer containing 1 mM furosemide and picrotoxin. The cultures were dried and solubilized in 1 ml of 0.1 N NaOH. After 2 hr at room temperature the radioactivity of a 0.9 ml sample was counted using a standard scintillation technique. A sample (0.1 ml) was taken for estimation of the protein content [17]. All data were normalized to a standard protein content (1 mg protein).

Pertussis toxin treatment. Pertussis toxin treatment was performed as described by Xu *et al.* [16]. The cells were treated with 1 μg/ml pertussis toxin (List Biochemicals, Lot No. 39) for 14 hr before the $^{36}Cl^-$ experiment. The effectiveness of the pertussis toxin treatment was checked in an experiment (kindly performed by Dr W. J. Wojcik) which utilized [^{33}P]NAD for ribosylation. The autoradiogram demonstrated that labelling of the 39–41 kD protein

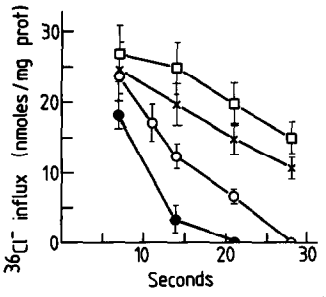

Fig. 1. Time course of the agonist-induced $^{36}Cl^-$ influx in cerebellar granule cells. (□) 30 μM THIP; (×) 3 μM muscimol; (○) 3 μM GABA; (●) 30 μM GABA. Five independent runs with five parallels each. Data are given as mean ± SE.

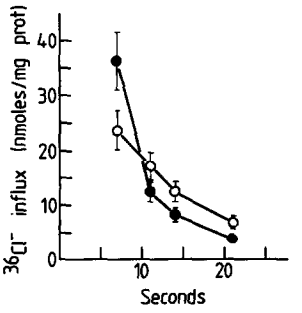

Fig. 2. Time course of the effect of diazepam on GABA-induced $^{36}Cl^-$ influx in cerebellar granule cells. (○) 3 μM GABA; (●) 3 μM GABA + 1 μM diazepam. Three independent runs with five parallels each. Data are given as mean ± SE.

occurred almost exclusively in non-treated cells, indicating that the pertussis toxin treatment was effective (>90% ribosylation).

Materials. THIP (RBI), (−)-baclofen (Ciba-Geigy, Basel, Switzerland), GABA, BMI, muscimol (Sigma), pertussis toxin (List Biochemials, Campbell, CA) and $^{36}Cl^-$ (NaCl solution, ICN) were used. Diazepam (Hoffman-LaRoche, Nutley, NJ) was dissolved in dimethylsulphoxide (DMSO). The presence of 0.005% DMSO did not influence the $^{36}Cl^-$ influx measurements.

RESULTS

$^{36}Cl^-$ *influx experiments*

Preliminary studies have shown that the maximal response to agonists was obtained with 3–5 μM GABA [12], 1–3 μM muscimol and 25–30 μM THIP in 7 sec. After 7 sec the peak responses start to decline with different time courses characteristic of the different agonists (Fig. 1). The sequence of the fading half-times with the different agonists was GABA < muscimol < THIP. Application of a 10-fold higher concentration of GABA gave a faster rate of fading of the peak response (Fig. 1).

In a separate experiment cells were preincubated with 3 μM GABA for 20 sec. Then the solution was aspirated and the cells were tested for GABA and muscimol-induced (3 μM each) $^{36}Cl^-$ influx. Table 1 shows that this preincubation resulted in a complete disapppearance of these responses to GABA and muscimol.

Diazepam (1 μM) not only potentiated the response to GABA but also accelerated the initial rate of its fading. After the initial phase (10 sec) the fading of the response in the presence of diazepam paralleled that with GABA alone (Fig. 2).

In the presence of (−)-baclofen (300 μM) the fading rate of the peak response to muscimol was changed (Fig. 3A). The response to 3 μM muscimol (measured after 14 sec) was decreased by (−)-baclofen in a dose-dependent manner (Fig. 3B). Statistical analysis indicated no significant deviation of the value measured for $^{36}Cl^-$ influx in the presence of 3 μM muscimol and 300 μM (−)-baclofen from the basal level of $^{36}Cl^-$ influx. Similar results were obtained with THIP (data not shown). Preincubation of the cells with 300 μM (−)-baclofen (15 min, 4°) did not influence the response to muscimol in a subsequent incubation. When the cells were preincubated with both muscimol (3 μM) and (−)-baclofen (300 μM) no response to muscimol was seen in the subsequent influx experiment after 7 sec. There was no change in basal chloride exchange activity of the cells or the responses to agonists after pertussis toxin treatment. However, no effect of (−)-baclofen on the responses to muscimol and THIP (at 14 sec) was seen in cells pretreated with pertussis toxin (Fig. 4B). Viability tests indicated no change in the proportion of viable cells after preincubation with 300 μM (−)-baclofen and pertussis toxin treatment.

DISCUSSION

Rat cerebellar cells maintained in primary cultures

Table 1. The effect of preincubation (20 sec) with GABA (3 μM) on the muscimol- and GABA-induced $^{36}Cl^-$ influx in cerebellar granule cells

	$^{36}Cl^-$ influx (nmol/mg protein) at 7 sec*	
Agonists	No preincubation	Preincubation
3 μM GABA	22.7 ± 3.1	14.0 ± 2.1
3 μM muscimol	23.8 ± 3.6	12.6 ± 0.9

* Three independent runs with five parallels each. Data are given as mean ± SE. Basal value is 13.3 ± 0.7 nmol/mg protein.

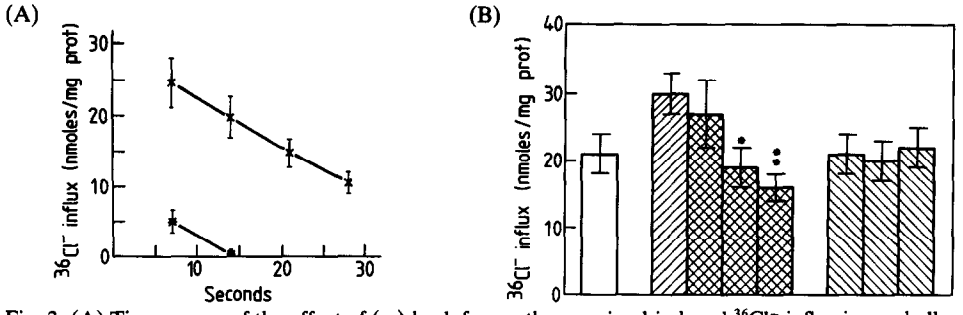

Fig. 3. (A) Time course of the effect of (−)-baclofen on the muscimol-induced $^{36}Cl^-$ influx in cerebellar granule cells. (×) 3 μM muscimol; (*) 3 μM muscimol + 300 μM (−)-baclofen. Five independent runs with five parallels each. Data are given as mean ± SE. (B) The effect of (−)-baclofen on the muscimol-induced $^{36}Cl^-$ influx at 14 sec in cerebellar granule cells. Empty bar: basal influx; right-hatched bar: muscimol 3 μM alone; criss-cross bars: 3 μM muscimol + 50, 100, 300 μM (−)-baclofen; left-hatched bars: 50, 100 and 300 μM (−)-baclofen alone. Data are given as mean ± SE. *$P < 0.01$, **$P < 0.05$.

show desensitization of the $GABA_A$ channel after preincubation with GABA. The concentration of GABA required for complete desensitization in 20 sec is in the micromolar concentration range. Similar conditions were observed on neurons in electrophysiological measurements (for a review see Ref. 4). Experiments performed on membrane vesicles [5–7] showed that the desensitization was determined by the time of exposure and the concentration of GABA. We have calculated their product (R_d = [GABA] × t) and found it to be constant over a large range of response (5×10^{-5} [Mxsec]). Interestingly the R_d value characterizing $GABA_A$ channels in cultured cerebellar neurons is found to be similar, $\sim 5 \times 10^{-5}$ Mxsec. The desensitization of the $GABA_A$ channel in these different models (e.g. cortical vesicles vs cerebellar neurons) is similar in this respect.

Rat cerebellar cells tested in resting conditions ($E_{rev} = E_0 = -70$ mM) show a decay of the peak responses to different agonists and GABA with diazepam. A higher concentration of GABA gives a faster decay of the peak response indicating that the faster rate of disappearance of the peak response to GABA is not due to metabolism or uptake. The decrease of the peak response to 30 μM GABA with time is different from the decay of the peak response to 3 μM GABA with diazepam. We regard the differential decline of the peak responses to agonists and GABA with diazepam as a result of two processes, $GABA_A$ channel controlled $^{36}Cl^-$ efflux and desensitization of the $GABA_A$ channel. Supposing desensitization is the rate limiting process the different half-time obtained with different agonists and GABA with diazepam reflect their different desensitization properties. The estimated half-times characterizing the decay of the peak response to the different agonists in our $^{36}Cl^-$ exchange measurements at 4° (GABA 14 sec; muscimol 25 sec; THIP 35 sec) seems to correlate with the desensitization half-times obtained from electrophysiological measurements on hippocampal neurons at room temperature (GABA 1.1 ± 0.3 sec; muscimol 3.3 ± 2.3 sec; THIP 6 ± 3 sec [18]). Differences in the corresponding absolute values are consistent with a decrease in the conductance of $GABA_A$ channels at low temperature [19]. There is also electrophysiological evidence for the desensitization accelerated by diazepam [20, 21]. In addition, in agreement with our observations, the extent of desensitization was also found to be higher after GABA response was stimulated by chlordiazepoxide [21].

In contrast, the sequence of the decay half-times for the different agonists does not correlate with the sequence in the process of equilibrium binding to $GABA_A$ receptors (K_d values). Why does the $GABA_A$ channel-activated chloride exchange decay considerably faster in the presence of GABA than in the presence of muscimol? One possible answer is because the $GABA_A$ channel-activated chloride exchange and desensitization of the $GABA_A$ channel are independent processes [7]. In this work we addressed another possible answer. In the line of evidence that GABA is also an agonist at the $GABA_B$ receptor, we investigated the effect of the $GABA_B$ agonist, (−)-baclofen, on the response to muscimol. The response to muscimol disappeared completely in 14 sec when 300 μM (−)-baclofen was present and decreased considerably in 7 sec. This effect of (−)-baclofen needs $GABA_A$ channels to be activated, because (−)-baclofen alone does not influence the basal chloride influx. Preincubation with (−)-baclofen did not decrease the response to muscimol. However, when preincubation was performed with (−)-baclofen together with muscimol the response to muscimol was abolished. A viability test indicated no change in the number of viable cells after preincubation with 300 μM (−)-baclofen; thus, the above effects of (−)-baclofen are not related to some toxic effect of higher (−)-baclofen concentration. Preliminary electrophysiological measurements suggest that the effect of (−)-baclofen is not due to the release of GABA because bath application did not depolarize the cells to induce such a release [(−)-baclofen was not seen as an effective inactivator of the muscimol response in these trials, indicating a need for Ca^{2+} or some other second messenger being absent or washed out during the whole cell recording procedure].

To address this issue further we tried to antagonize the effect of (−)-baclofen. Much evidence suggests

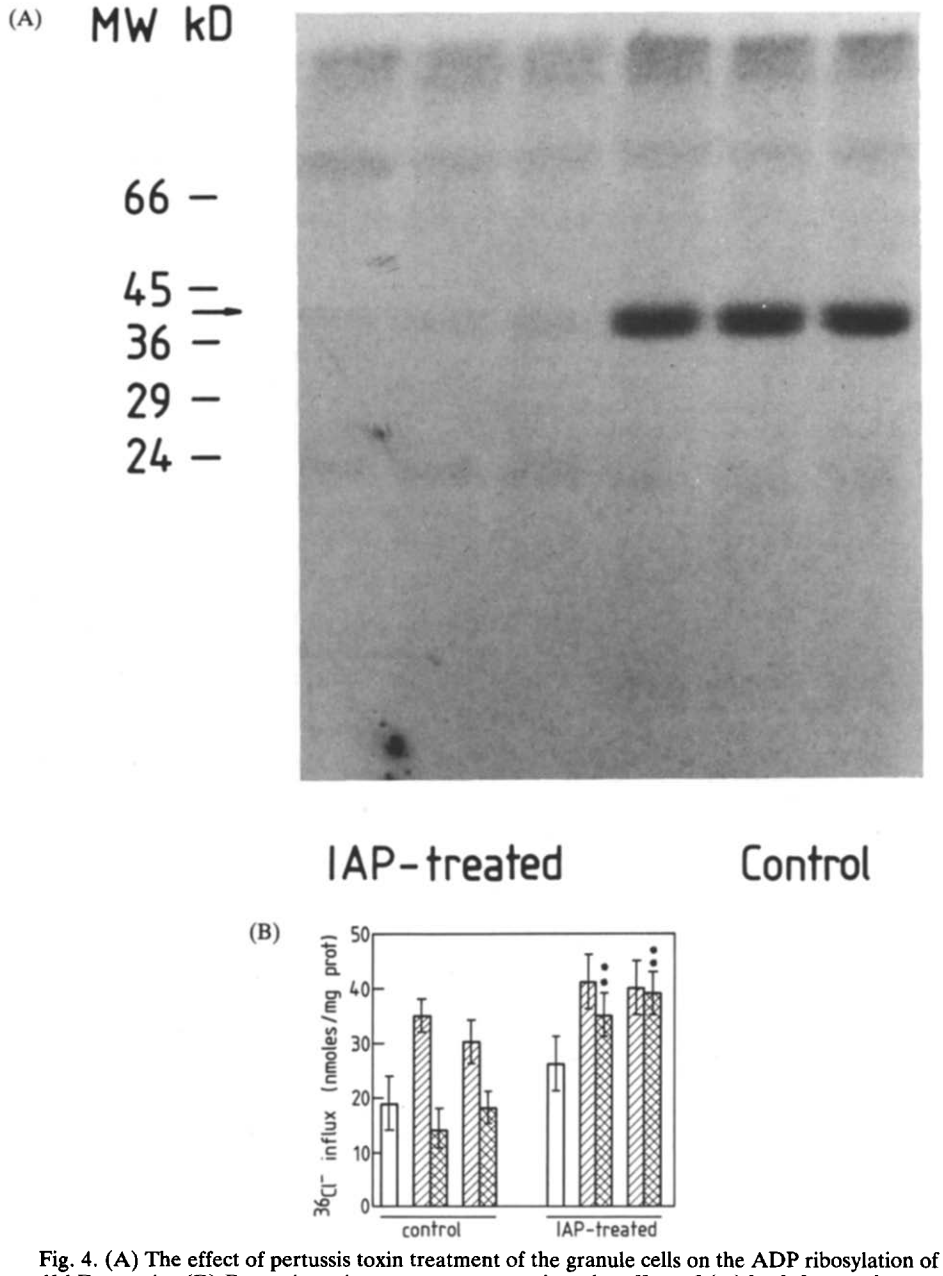

Fig. 4. (A) The effect of pertussis toxin treatment of the granule cells on the ADP ribosylation of a 39–41 kD protein. (B) Pertussis toxin treatment antagonizes the effect of (−)-baclofen on the muscimol induced $^{36}Cl^-$ influx at 14 sec in cerebellar granule cells. Empty bar: basal influx; right-hatched bars: 30 μM THIP and 3 μM muscimol, respectively; criss-cross bars: 30 μM THIP + 300 μM (−)-baclofen and 3 μM muscimol + 300 μM (−)-baclofen, respectively. Data are given as mean ± SE. $^{**}P < 0.05$.

that the $GABA_B$ receptor belongs to the superfamily of GTP binding protein coupled receptors [16, 22, 23]. Therefore we examined the effect of pertussis toxin, which is known to inactivate some G protein by ADP-ribosylation [16]. The effective treatment with pertussis toxin clearly abolished the effect of (−)-baclofen on the response to muscimol and THIP. The above results suggest that at least two mechanisms exist by which $GABA_A$ channels quieten down. One of them is the intrinsic desensitization of the $GABA_A$ channel, the other is one which proceeds via interaction with a G protein coupled to the $GABA_B$ receptor.

Acknowledgements—We thank E. Costa, A. Guidotti and W. Wojcik (Fidia—Georgetown Institute for the Neurosciences, Washington, DC) for working facilities and for helpful discussions.

REFERENCES

1. Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriquez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg PH and

Barnard EA, Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature (Lond)* **328**: 221–227, 1987.
2. Krnjevic K and Phillips JW, Iontophoretic studies of neurons in the mammalian cerebral cortex. *J Physiol (Lond)* **165**: 274–304, 1963.
3. Nowak LM, Young AB and MacDonald RL, GABA and bicuculline action on mouse spinal cord and cortical neurons in cell culture. *Brain Res* **244**: 155–164, 1982.
4. Mathers DA, The $GABA_A$ receptor: new insights from single-channel recording. *Synapse* **1**: 96–101, 1987.
5. Cash DJ and Subbarao K, GABA mediated transmembrane chloride flux with membrane vesicles from rat brain measured by quench flow technique: kinetic homogeneity of ion flux and receptor desensitization. *Life Sci* **41**: 437–445, 1987.
6. Cash DJ and Subbarao K, Desensitization of GABA from rat brain: two distinguishable receptors on the same membrane. *Biochemistry* **26**: 7556–7562, 1987.
7. Cash DJ and Subbarao K, Channel opening of GABA receptor from rat brain: molecular mechanisms of the receptor responses. *Biochemistry* **26**: 7562–7570, 1987.
8. Allan AM and Harris RA, GABA agonists and antagonists alter chloride flux across brain membrane. *Molec Pharmacol* **29**: 497–505, 1986.
9. Schwartz RD, Suzdak PD and Paul SM, GABA- and barbiturate-mediated $^{36}Cl^-$ uptake in rat brain synaptoneurosomes: evidence for rapid desensitization of the GABA receptor-coupled chloride ion channel. *Molec Pharmacol* **30**: 419–426, 1986.
10. Yang JSJ and Olsen RW, GABA receptor-regulated $^{36}Cl^-$ flux in mouse cordical slices. *J Pharmacol Exp Ther* **241**: 677–685, 1987.
11. Kardos J and Maderspach K, $GABA_A$ receptor-controlled $^{36}Cl^-$ influx in cultured rat cerebellar granule cells. *Life Sci* **41**: 265–272, 1987.
12. Kardos J and Guidotti A, Desensitization of GABA-stimulated $^{36}Cl^-$ influx in cerebellar granule cells. In: *Chloride Channels and Their Modulation by Neurotransmitters and Drugs* (Eds. Biggio G and Costa E), pp. 73–85. Raven Press, New York, 1988.
13. Vaccarino FM, Alho H, Santi MR and Guidotti A, Coexistence of GABA receptors and GABA-modulin in primary cultures of rat cerebellar granular cells. *J Neurosci* **7**: 65–76, 1987.
14. Gallo V, Wise BC, Vaccarino F and Guidotti A, GABA- and benzodiazepine-induced modulation of [^{35}S]-t-butylbicyclophosphorothionate binding to cerebellar granules cells. *J Neurosci* **5**: 2432–2438, 1985.
15. Jones KH and Senft JA, An improved method to determine cell viability by simultaneous staining with fluorescein diacetate–propidium iodide. *J Histochem Cytochem* **33**: 77–79, 1985.
16. Xu J and Wojcik WJ, $GABA_B$ receptor-mediated inhibition of adenylate cyclase in cultured cerebellar granule cells: blockade by islet activating protein. *J Pharmacol Exp Ther* **239**: 568–573, 1986.
17. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analyt Biochem* **72**: 248–254, 1976.
18. Thallman RH and Hershkowitz N, Some factors that influence the decrement in the response to GABA during its continuous iontophoretic application to hippocampal neurons. *Brain Res* **342**: 219–233, 1985.
19. Mathers DA and Barker JL, GABA- and glycine-induced Cl^- channels in cultured mouse spinal neurons require the same energy to close. *Brain Res* **224**: 441–445, 1981.
20. Bormann J and Clapham DE, GABA receptor channels in adrenal chromaffin cells: a patchclamp study. *Proc Natn Acad Sci USA* **82**: 2168–2172, 1985.
21. Mierlak D and Farb DH, Modulation of neurotransmitter receptor desensitization: chlordiazepoxide stimulates fading of the GABA response. *J Neurosci* **8**: 814–820, 1988.
22. Andrade R, Malenka RC and Nicoll RA, A G protein couples sorotonin and $GABA_B$ receptors in the same channels in hippocampus. *Science* **234**: 1261–1265, 1986.
23. Asano T and Ogasawara M, Uncoupling of GABA B receptors from GTP-binding proteins by *N*-ethylmaleimide: effect of *N*-ethylmaleimide on purified GTP-binding proteins. *Molec Pharmacol* **29**: 244–249, 1986.