0026-895X/90/040578-05$02.00/0

MOLECULAR PHARMACOLOGY, 37:578-582

Electrophysiological Study of *tert*-Butylbicyclophosphorothionate-Induced Block of Spontaneous Chloride Channels

MARTINE HAMANN, MICHEL DESARMENIEN, PATRICK VANDERHEYDEN, PASCALE PIGUET, and PAUL FELTZ

Laboratoire de Neuroendocrinologie Comparée (URA 98 CNRS), 67084 Strasbourg Cedex, France

Received November 30, 1988; Accepted January 22, 1990

SUMMARY

The action of TBPS (*tert*-butylbicyclophosphorothionate) on spontaneous chloride channels recorded from porcine pars intermediate lobe cells in primary culture has been studied. This compound, which binds specifically to the γ-aminobutyric acid$_A$ (GABA$_A$) receptor complex, is known as a channel-gating (noncompetitive) GABA antagonist. The present results show that TBPS reduces spontaneous chloride channel activity in a dose-dependent manner, with an IC_{50} equal to 55 nM, which is a value comparable to its affinity for the GABA$_A$ binding sites. Single-channel analysis revealed that TBPS affects neither the amplitude nor the open time of these spontaneous channels but prolongs the longer closed times, resulting in a dramatic decrease in opening probability.

Neuroendocrine cells from the porcine IL display a chloride channel activity in the absence of any added pharmacological compound. As described by Taleb *et al.* (1), this channel shares a number of functional properties with the GABA-activated chloride channels (conductance levels, sequence of permeabilities to halides, sensitivity to internal calcium). Whereas the GABA-activated channels frequently occur in bursts with complex kinetics of activation and inactivation, the openings of the spontaneous chloride channels occurred regularly and no bursting activity was observed. The regularly occuring openings of the sponataneous channels, indeed, made this chloride channel activity suitable as a simplified model for studying the conformational changes of chloride channels independently from ligand binding kinetics.

Previous studies (1) showed that the competitive GABA$_A$ antagonists SR42641 and bicuculline do not alter the spontaneous activity at concentrations at which they block GABA-evoked channels. The aim of the present work was to improve this pharmacological characterization by testing the effects of TBPS. TBPS is a ligand with high affinity for GABA-activated chloride channels (2–4). Biochemical and electrophysiological studies showed that this compound acts as a noncompetitive GABA$_A$ antagonist by interaction with chloride channels (5–8).

We also tested picrotoxinin, the active component of picrotoxin, which is a well known inhibitor of GABA-mediated inhibition (9, 10) with noncompetitive properties (11, 12).

Our results, obtained from the recording of chloride channel activity in outside-out patches of IL cell membranes, indicate that both compounds decrease the activity of spontaneous chloride channels.

Materials and Methods

Preparation and recording. The porcine IL cell culture methods were described previously (13). Cells were kept in culture for 4–10 days in Dulbecco's modified Eagle medium (70%) containing Ham's F12 medium (25%) and fetal calf serum (5%). Gigaseals were established on isolated firmly attached cells with electrodes of 5–10 MΩ. All recordings were performed in the outside-out configuration. Care was taken to optimize the bandwidth of the system; soft glass (hematocrit) pipettes were coated with a silicone elastomer (RTV 141; Rhône Poulenc) and root mean square noise level was below 190 fA (bandwidth, direct current to 10 KHz) once the electrode was fixed on the head stage. All experiments were carried out at room temperature (18–20°).

Data were recorded from the patch-clamp amplifier (LIST EPC7) on a FM tape recorder (Racal Store 4) with a bandwidth of 5 or 20 kHz. Single-current traces were plotted on a HP 7470A plotter and, for long runs, on a Gould brush 280 pen recorder.

Recording solutions. The composition of the various solutions was chosen so as to record Cl^- currents in isolation. For this purpose, Na^+ was replaced extracellularly by the impermeant cation choline; movements of K^+ ions were reduced by the combined intracellular blocking effects of Cs^+ and TEA. The standard extracellular solution contained, in mM: choline chloride, 128; $MgCl_2$, 10; $CaCl_2$, 0.5; HEPES/Tris, 10; and glucose, 10; pH 7.4. The standard intracellular solution contained: CsCl, 120; $MgCl_2$, 2; $CaCl_2$, 0.9; TEA·Cl, 10; and HEPES/CsOH, 9; pH 7.2. A Ca-buffer, EGTA at 10 mM, was used to control the concentration of free Ca_i. All measurements have been performed at an internal

This work was supported by INSERM (CRE 87-6011), by Direction de Recherche et d'Etudes Techniques (89-34036), and by a Fondation pour la Recherche Médicale research fellowship awarded to P.V. (CNRS-URA 309).

ABBREVIATIONS: IL, intermediate lobe; GABA, γ-aminobutyric acid; TBPS, *tert*-butylbicyclophosphorothionate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEA, tetraethylammonium; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid).

calcium concentration of 10^{-8} M, which was optimum for the recording of spontaneous chloride channel activities (1).

TBPS (0.45 mM in ethanol) from New England Nuclear Corp. was kept at 5° and used within 6 months. Picrotoxinin from Sigma, at a concentration of 1 mM, was dissolved in 0.175 N NaOH. These stock solutions were diluted in extracellular medium immediately before use. Media were exchanged by perfusing the Petri dish (volume reduced to <0.25 ml) for 5 min at a rate of 2.5 ml/min.

Data analysis. In a first analysis, data were digitized at 5 kHz (Bessel filter at 3 kHz), which allowed the vizualisation of a large increase in closed time in the presence of TBPS (see Results). Thereafter, data were digitized at 16 kHz (Bessel filter at 4 kHz) for detailed analysis of openings, closures, and conductances.

The system used to analyze the opening and closing time distributions was composed of a microcomputer (Olivetti M28), a data acquisition card (41 points/pA; Labmaster; Scientific Solutions, Solon, OH), and PCLAMP software (version 4.05; Axon Instruments, Burlingame, CA). Analysis of current amplitudes was performed on a Plessey 6220 DEC-compatible computer (LSI 11/73).

For kinetic measurement, one channel opening was considered to occur when the current trace deviated from the baseline by at least 1.4 pA (at holding potential −70 mV) and a channel closure when the current went back under this limit. Neither subconductance levels nor nachschlag phenomena were considered in the present study. Patches in which double openings were observed were not considered in this study.

After tabulating the successive openings and closures of channels, open and closed time interval histograms were plotted as a frequency histogram and a nonlinear least squares method (Levenberg-Marquardt algorithm) was used to compute the corresponding time constants of either mono- or biexponential distributions (5). All fittings were also performed separately in case of multiexponential distributions. In the case of multiexponential distributions, a given exponential was considered valid only if it exceeded 15% of the total area. The threshold of the open and closed time duration for resolvable events was obtained by using a value equal to 3 to 4 times the sampling interval.

Measurements of the amplitudes were determined using an interactive program (14) that allows the selection of windows. Histograms of the amplitudes of the resolved events were established, taking an average value fitted by computer for the current level of the individual events. Data could be described by a Gaussian curve, for which the position of the peak and the value of the standard deviation could be individually adjusted.

Statistics. All results are expressed as means ± standard errors. When appropriate, comparison of groups was performed using paired Student's *t* tests.

Results

Spontaneous inward current deflections similar to those described by Taleb *et al.* (1) were observed in most of the outside/out patches successfully excised. They could be recorded and displayed a regular activity for periods up to 0.5 hr. Their reversal potential, measured in some occasions, was similar to the Cl^- equilibrium potential (−3 mV). These spontaneous Cl^- activities are illustrated in Fig. 1.

Current amplitude. At −70 mV, the most frequently observed substate of the spontaneous Cl^- channels had an amplitude of 1.95 ± 0.28 pA (mean ± SE). The conductance of these channels thus equaled 28 pS. In the presence of 1 μM TBPS, no detectable difference was observed in the amplitude of these Cl^- channels. Fig. 2 illustrates the cumulative amplitude histograms summarizing results from five patches in control and TBPS conditions, showing that the amplitude of this conductance was not affected by TBPS (27 pS).

Open time duration. Fig. 1 illustrates the spontaneous activity recorded from excised patches in control conditions and in the presence of TBPS. Within the recordings of each patch, there were neither declines nor periods of enhanced activity. Open time frequency histograms could be fitted by a single-exponential curve. No noticeable differences (paired *t* test; $p < 0.001$) were detected between control (0.54 ± 0.04 msec, n = 19) and TBPS (0.1–1 μM)-containing media (0.42 ± 0.04 msec, $n = 17$), as illustrated in Fig. 3. No significant difference was observed between data digitized at 5 and 16 kHz, although the determination was more precise in the latter case.

Closed time duration. Under control conditions, three different closing times were observed. In 17 cases, closed time histograms were fitted by two exponentials, a fast one (T_1 = 0.66 ± 0.09 msec and an additional intermediate (T_2 = 20 ± 4 msec; $n = 10$) or slow exponential (T_3 = 117 ± 15 msec; $n = 7$). An example is illustrated in Fig. 4, in which control curves represent the sum of two exponentials with a fast and a slow time constant of 1 and 18.2 msec, respectively. Furthermore, in 3 cases, histograms were fitted by the sum of three exponentials, T_1 = 0.5 ± 0.1 msec; T_2 = 14.8 ± 3 msec; and T_3 = 88 ± 9 msec.

In the presence of TBPS (0.1–1 μM), in 11 cases, closed time histograms were fitted by two exponentials, a fast one (T_1' = 0.67 ± 0.06 msec), which is unchanged as compared with the fast constant in control conditions (T_1), and an additional intermediate (T_2' of 78 ± 8 msec, $n = 5$) or slow (T_3' 474 ± 119 msec; $n = 6$) one. The long lasting closures (T_2' and T_3') were significantly prolonged, as compared with the corresponding constant in control conditions ($p < 0.001$). In 4 cases, the distribution could be fitted by three exponentials (T_1' = 0.7 ± 0.1 msec; T_2' = 86 ± 7 msec; T_3' = 352 ± 60 msec). Fig. 4 shows the effect of 1 μM TBPS; the closed time distribution was fitted by three exponentials of 0.98, 103, and 410 msec.

Because of the low activity of spontaneous channels in the presence of TBPS, the period of recording in the presence of the compound had to be as long as possible, in order to get enough data for histogram fittings. For this reason, a single dose of TBPS was always applied on each patch recorded. The variability in the rate of activity between patches under control conditions did not allow a determination of the dose dependency of closed times on TBPS. This dose dependency was, thus, evaluated by comparing, in a single patch, the overall activity under control conditions (standardized to 100%) and in the presence of TBPS. The activity of the spontaneous chloride channels was, thus, expressed by the ratio T_o/T_f (total time passed in the open state versus total time passed in a closed state) as a function of increasing concentrations of TBPS. This ratio (0.042 ± 0.032; $n = 20$, control conditions) is a measure of the opening probability. We expressed the ratio T_o/T_f in the presence of TBPS relative to its value in control conditions. Fig. 5 illustrates a plot of these ratios versus log [TBPS] concentrations, showing a decrease in the concentration range between 10^{-8} and 10^{-4} M. A sigmoid curve has been fitted to the data, with an IC_{50} value of 55 nM and a Hill coefficient of −0.95.

In the presence of picrotoxinin, the progressive decline of channel activity hampered a single-channel analysis similar to that performed for TBPS. Indeed, at concentrations of at least 1 μM, we observed in 11 patches that this compound was able to inhibit spontaneous chloride channels (data not shown).

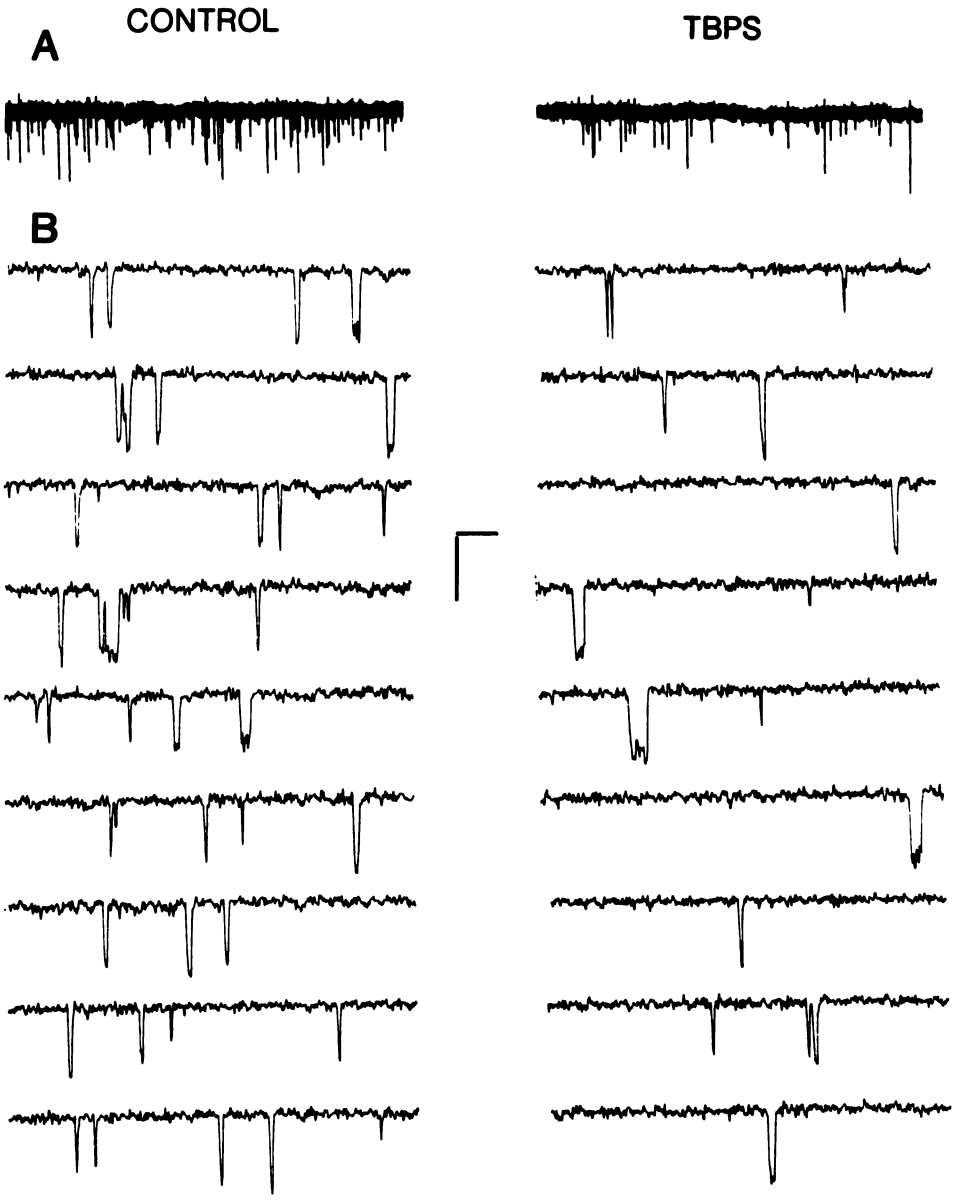

Fig. 1. Spontaneous chloride channel activities recorded from an outside-out patch in control conditions (*left*) and in the presence of 0.1 μM TBPS (*right*). Holding potential was −70 mV. Data are filtered at 3 kHz and digitized at 5 kHz. Inward currents appear as downward deflections. *Calibration bars*, 2 pA, 10 sec (A), and 10 msec (B).

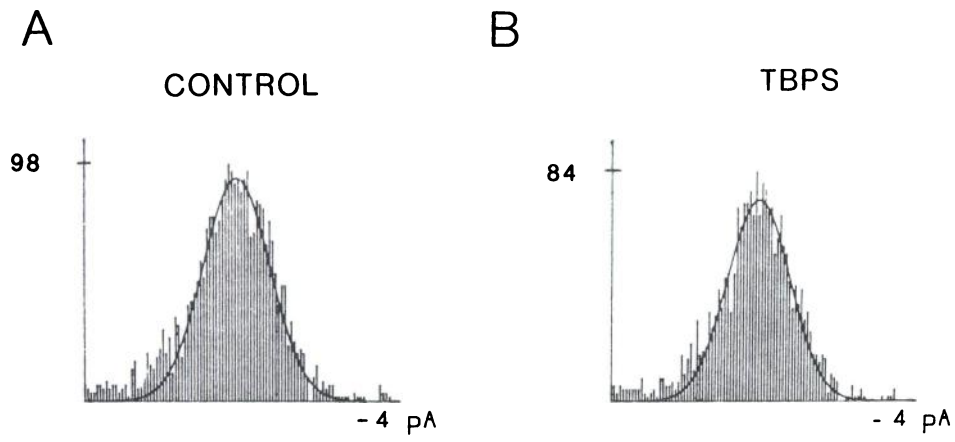

Fig. 2. Cumulative histogram (from five patches) of spontaneous channel amplitude in control conditions (A) and in the presence of 0.1 μM TBPS (B). The number of events at the peak is indicated on the *ordinate*. Gaussian curves have been superimposed on the data, the average amplitude values being −1.94 ± 0.28 pA (A) and −1.92 ± 0.24 pA (B).

Discussion

TBPS and picrotoxinin bind to a site that is allosterically linked to GABA, benzodiazepine, and chloride ion recognition sites in the $GABA_A$ receptor complex (3, 15). The chloride channel block hypothesis came originally from the observation that the picrotoxinin effect on crustacean muscle was reduced at high chloride concentrations, suggesting a competition between the permeant ion and the blocker inside the channel (10). The first radioligand studies to characterize the convul-

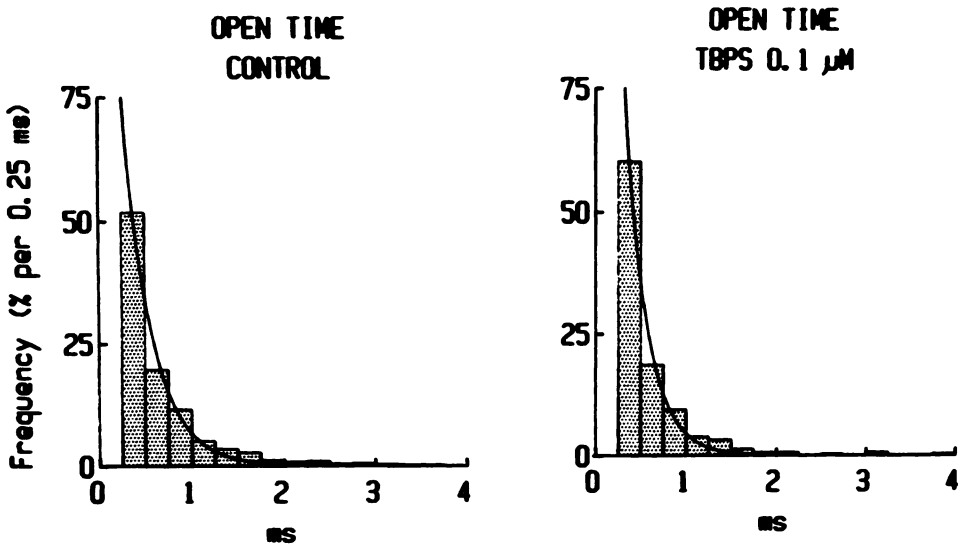

Fig. 3. Open time histograms fitted by single exponentials, with corresponding time constants of 0.48 msec (control) and 0.40 msec (0.1 μM TBPS). Distributions were plotted as probability density functions from a total of 2052 (control) and 1821 (TBPS) events.

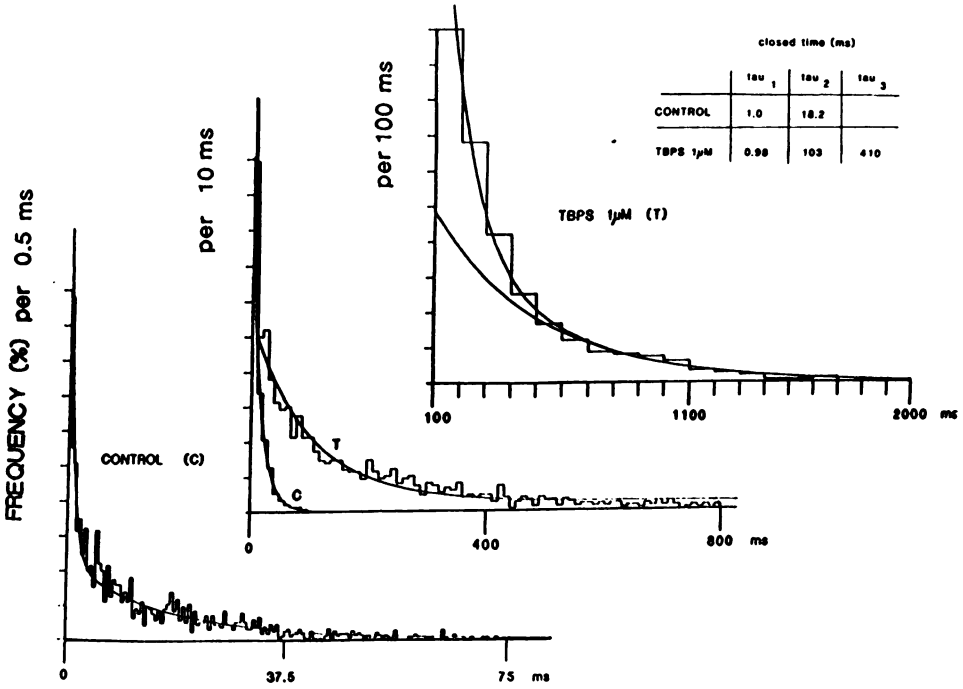

Fig. 4. Closed time distribution as the time intervals between channel openings, in control conditions (*C*) and in the presence of 1 μM TBPS (*T*). The histograms are given with three different time scales, with the corresponding fitted curves (*continuous lines*) superimposed on each of them. In the *left panel*, the number of events was 1473 (control); in the *middle panel*, 1524 (control) and 1879 (TBPS); and in the *right panel*, 1037 (TBPS). On the *ordinate*, each *mark* represents 10%. The corresponding closed time constants are indicated in the *inset*. The fast component obtained in the presence of TBPS is similar to the control one (see *middle panel* and *inset*) and, thus, is not illustrated in details. The *two lines* in the *right panel* indicate the intermediate and compound exponentials.

sant binding site were performed with the binding of [^{3}H] dihydropicrotoxinin (16). However, because of the relatively low affinity and high nonspecific binding of [^{3}H]dihydropicrotoxinin (17), [^{35}S]TBPS was introduced to label this binding site (18). Although TBPS is classically described for the characterization of chloride channels that are associated with the $GABA_A$ receptor, it is not known whether its binding sites are directly related to the functional state of the chloride channels.

In this study, the primary culture of IL cells of the pituitary offers the possibility to explore the presence of functional TBPS binding sites that are directly linked to the chloride channels and that are not activated by GABA. We recorded chloride activities in an endocrine preparation of IL cells of porcine hypophysis. These channels displayed a regular activity in the absence of any added ligand. To prevent possible activation of receptor-linked chloride channels by endogenous ligands, we continuously superfused our outside-out patches above the cell layer with the extracellular medium.

The chloride channels described here had an amplitude and kinetic properties comparable to those described by Taleb *et al.* (1) in the same preparation. A conductance level of 28 pS with a mean open time of 1 msec was most frequently observed. In order to improve the pharmacological characterization of these channels, we investigated the action of TBPS.

We showed that the spontaneous chloride channels, not activated by any pharmacological compound, are inhibited by TBPS. Concerning the mechanism of action of TBPS on these channels (spontaneous chloride channels), our data indicate that this compound affects neither the amplitude of the most frequently encountered conductance state nor the mean open time. Furthermore, in our recording conditions, we did not detect any noticeable effect on short closures. The predominant TBPS effect appears to be a prolongation of long lasting closures, resulting in a decrease in the occurences of the single-channel events. Its effect was dose dependent, with an IC_{50}

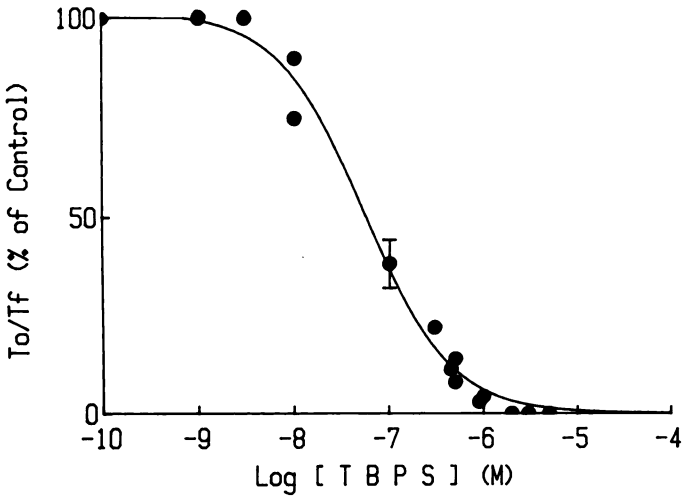

Fig. 5. Plot of T_o/T_f (see text) versus log concentration of TBPS, indicating that the efficacy of TBPS to block spontaneous chloride channels is dose dependent. The sigmoid curve fitted to the data indicates an IC_{50} of 55 nM and Hill coefficient of −0.95. The mean value of the T_o/T_f ratio at 0.1 μM TBPS, determined in five different patches, equaled 38.7 ± 6.8 nM.

value of 55 nM and a Hill number of −0.95. This IC_{50} value is in the same range as the affinity constant of [^{35}S]TBPS (K_D = 20 nM), as determined by direct binding on rat brain membranes (3).

As for TBPS, we found that the noncompetitive GABA antagonist picrotoxinin, at concentrations of at least 1 μM, was also able to inhibit spontaneous chloride channel activities. This picrotoxinin concentration is in the same range as that reported to block GABA receptor responses (19) and that obtained by direct binding studies in rat brain (16).

Although there is only indirect evidence that TBPS binding sites are located at or near the chloride channels, our study provides electrophysiological evidence that TBPS acts as a chloride channel inhibitor in the absence of any ligand. Indeed, specific [^{35}S]TBPS binding sites have been shown to be distinct from those linked to competitive GABA antagonists (3, 20). This is in agreement with TBPS inhibition of basal Cl^- uptake in rat cerebral neurons (21) and with previous electrophysiological studies on *Xenopus* oocytes (8), in which both the onset and offset of TBPS blocking effect occured at slow rates in the absence of any agonist. This, however, does not exclude a distinct agonist-dependent mechanism of TBPS and picrotoxinin (8, 19).

Taken together, these results suggest that TBPS can also exert its effect as GABA antagonist by direct blockade of chloride channels, independently of ligand activation.

Acknowledgments

The authors wish to thank Mrs. Roth for technical assistance, Dr. A. Feltz for helpful comments on the manuscript, and SANOFI-Recherche for the facilities placed at our disposal throughout the present study.

References

1. Taleb, O., J. Trouslard, B. A. Demeneix, P. Feltz, J. L. Bossu, and A. Feltz. Spontaneous and GABA-evoked chloride channels on pituitary intermediate lobe cells and their internal requirements. *Pflügers Arch.* **409**:620–631 (1987).
2. Lawrence, L. J., C. J. Palmer, K. W. Gee, X. Wang, H. I. Yamamura, and J. E. Casida. *t*-[^{3}H]Butylbicycloorthobenzoate: a new radioligand probe for the *gamma*-aminobutyric acid-regulated chloride ionophore. *J. Neurochem.* **45**:798–804 (1985).
3. Squires, R. F., J. E. Casida, M. Richardson, and E. Saederup. [^{35}S]-*t*-Butylbicyclophosporothionate binds with high affinity to brain specific sites coupled to γ-aminobutyric acid-A and ion recognition sites. *Mol. Pharmacol.* **13**:326–336 (1983).
4. Supavilai, P., and M. Karobath. [^{35}S]-Butylbicyclophophorothionate binding sites are constituents of the *gamma*-aminobutyric acid benzodiazepine receptor complex. *J. Neurosci.* **4**:1193–1200 (1984).
5. Colquhoun, D., and F. J. Sigworth. Fitting and statistical analysis of single channel records, in *Single Channel Recording* (B. Sakmann, and E. Neher, eds.). Plenum Press, New York, 191–263 (1983).
6. Obata, T., H. I. Yamamura, E. Malatynska, M. Ikeda, H. Laird, C. J. Palmer, and J. E. Casida. Modulation of *gamma*-aminobutyric acid stimulated chloride influx by bicycloorthocarboxylates, bicyclophosphorus esters, polychlorocycloalkanes and other cage convulsants. *J. Pharmacol. Exp. Ther.* **244**:802–806 (1988).
7. Olsen, R. W. GABA-benzodiazepine-barbiturate receptor interactions. *J. Neurochem.* **37**:1–13 (1981).
8. Van Renterghem, C., G. Bilbe, S. Moss, T. G. Smart, A. Constanti, D. A. Brown, and E. A. Barnard. GABA receptors induced in *Xenopus* oocytes by chick brain mRNA: evaluation of TBPS as a use-dependent channel blocker. *Mol. Brain Res.* **2**:21–31 (1987).
9. Mac Donald, R. L., and J. L. Barker. Specific antagonism of GABA-mediated postsynaptic inhibition in cultured mammalian spinal cord neurons: a common mode of convulsant action. *Neurology* **28**:325–330 (1978).
10 Takeuchi, A., and T. Takeuchi. A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. *J. Physiol. (Lond.)* **205**:377–391 (1969).
11. Desarmenien, M., P. Feltz, P. M. Headley, and F. Santangelo. SL 75 102 as a γ-aminobutyric acid agonist: experiments on dorsal root ganglion neurones *in vitro*. *Br. J. Pharmacol.* **72**:355–364 (1981).
12. Smart, T. G., and A. Constanti. Studies on the mechanism of action of picrotoxinin and other convulsants at the crustacean muscle GABA receptor. *Proc. R. Soc. London Biol.* **227**:191–216 (1986).
13. Demeneix, B. A., O. Taleb, J. P. Loeffler, and P. Feltz. $GABA_A$ and $GABA_B$ receptors in primary culture: functional role in modulating peptide release. *Neuroscience* **17**:1275–1285 (1986).
14. Sigworth, F. An example of analysis, in *Single Channel Recording* (B. Sakmann and E. Neher, eds.). Plenum Press, New York, 301–321 (1983).
15. Squires, R. F., and E. Saederup. $GABA_A$ receptor blockers reverse the inhibitory effect of GABA on brain-specific [^{35}S]TBPS binding. *Brain Res.* **414**:357–364 (1987).
16. Ticku, M. K., M. Ban, and R. W. Olsen. Binding of [^{3}H]-α-dihydropicrotoxinin, a γ-aminobutyric acid synaptic antagonist, to rat brain membranes. *Mol. Pharmacol.* **14**:391–402 (1978).
17. Ticku, M. K., and G. Maksay. Convulsant depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complexe. *Life Sci.* **33**:2363–2375 (1983).
18. Ramanjaneyulu, R., and M. K. Ticku. Binding characteristics and interactions of depressant drugs with [^{35}S]-*t*-butylbicyclophosphorothionate, a ligand that binds to the picrotoxinin site. *J. Neurochem.* **42**:221–229 (1984).
19. Twyman, R. E., C. J. Rogers, and R. L. Mac Donald. Pentobarbital and picrotoxin have reciprocal actions on single $GABA_A$ receptor. *Neurosci. Lett.* **96**:89–95 (1989).
20. Wong, D. T., P. G. Threlkeld, F. P. Bymaster, and R. F. Squires. Saturable binding of [^{35}S]-*t*--butylbicyclophosphothionate to the sites linked to the GABA receptors and the interaction with GABAergic agents. *Life Sci.* **34**:853–860 (1984).
21. Tehrani, M. H. J., R. Vaidyanathaswamy, and E. M. Barnes. Interaction of *t*-butylbicyclophosphorothionate with *gamma*-aminobutyric acid gated chloride channels in cultured cerebral neurons. *J. Neurochem.* **46**:1542–1548 (1986).

Send reprint requests to: Martine Hamann, Laboratoire de Neuroendocrinologie Comparée (URA 98 CNRS), 21 Rue R. Descartes, 67084 Strasbourg Cedex, France.