Br. J. Pharmac. (1987), **90**, 287–298

Electrophysiological study of SR 42641, a novel aminopyridazine derivative of GABA: antagonist properties and receptor selectivity of $GABA_A$ versus $GABA_B$ responses

M. Desarmenien, E. Desaulles[1], P. Feltz & M. Hamann

Laboratoire de Physiologie Générale (ULP) and Laboratoire de Neuroendocrinologie comparée (UA 309 CNRS), 21 rue René Descartes, 67084 Strasbourg Cedex, France

1 A new arylamino-pyridazine γ-aminobutyric acid (GABA) derivative, SR 42641, has been tested for its ability to antagonize the actions of GABA on mammalian sensory neurones.

2 SR 42641 and bicuculline reversibly decreased $GABA_A$-induced depolarizations and currents recorded intracellularly from dorsal root ganglion neurones (DRG). Dose-response curves were shifted to the right in a parallel fashion. K_B values (determined under voltage clamp conditions) were respectively 0.12 ± 0.05 and $0.38 \pm 0.08\,\mu M$. Similar values were obtained with current clamp recording conditions.

3 The study of the GABA-induced Cl^- current under voltage-clamp conditions did not show any voltage-dependency of the antagonist effect of SR 42641.

4 In nodose ganglion neurones, SR 42641 ($0.4–4.5\,\mu M$) did not alter the (−)-baclofen-induced shortening of the calcium component of action potentials. At concentrations higher than $10\,\mu M$, SR 42641 itself prolonged calcium-dependent action potentials.

5 Patch-clamp recordings from DRG cultured neurones indicated that SR 42641 did not affect the calcium current responsible for sustained calcium entry into cells.

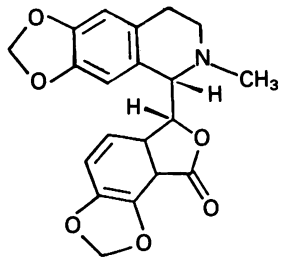
6 We conclude that SR 42641 is a potent competitive GABA antagonist, specific for the $GABA_A$ receptor. It does not act at the level of the chloride ionophore.

Introduction

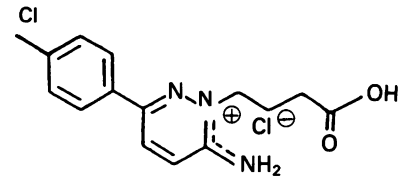
γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. At least two types of receptors are involved: $GABA_A$, activation of which opens chloride channels and $GABA_B$, activation of which may diminish the entry of calcium into cells (see Bowery, 1982). Both of these receptors could therefore be involved in inhibiting spike propagation, soma electrical activity or synaptic transmission. Classically two $GABA_A$ receptor antagonists are known: bicuculline and picrotoxin. Bicuculline is known to be a competitive antagonist, whilst picrotoxin is thought to act either on some specific picrotoxin binding site (Barker *et al.*, 1983; Ticku *et al.*, 1978), and/or by blocking some chloride conductance subclasses triggered by $GABA_A$ receptor activation (Yasui *et al.*, 1985). However, since neither of these compounds is entirely specific for the $GABA_A$ receptor site (Biscoe *et al.*, 1972; Goldinger & Muller, 1980; Simonds & DeGroat, 1980; Michaud *et al.*, 1986), there is a need for new GABA antagonists. This would also be useful to characterize structure-activity relationships, to differentiate binding site subclasses and to provide information about the molecular shape of the GABA binding sites.

We have recently described (Chambon *et al.*, 1985) a new GABA receptor antagonist: SR 95103. SR 95103 belongs to a family of chemicals which have incorporated a GABA molecule by its N-terminal group into an amino-phenyl pyridazine heterocycle. Binding studies suggested that this antagonist had a 20 fold greater affinity for the $GABA_A$ receptors than bicuculline; whereas electrophysiological studies indicated that this compound is slightly less potent than bicuculline in inhibiting GABA-induced responses. Later structure-activity relationship studies (Wermuth, 1985) led to the compound SR 42641 (Figure 1).

[1] Author for correspondence.



Bicuculline



SR 42641

Figure 1 Structure of SR 42641: (2-(carboxy-3'-propyl)-3-amino-6-*para*chloro-phenylpyridazinium chloride) and of bicuculline.

In binding studies, this compound has an approximate affinity for the $GABA_A$ receptor ten fold greater than SR 95103. *In vivo* studies of neuronal firing of cuneate cells (Michaud *et al.*, 1986) indicate that SR 42641 is more specific than SR 95103 when tested against glycine-induced inhibition and is a more potent GABA antagonist than bicuculline. In this latter study, drugs were applied iontophoretically so potencies could not be compared in terms of concentration.

The purpose of the present study was to evaluate, by use of electrophysiological techniques, the potency of SR 42641 relative to bicuculline. Dose-response curves were obtained and K_B value (dissociation constant) calculated for both products. As $GABA_A$ receptor activation triggers the opening of chloride channels of which the elementary conductance and open-time are voltage-sensitive (Hamill *et al.*, 1983; Segal & Barker, 1984; Gray & Johnston, 1985), the voltage-dependency of SR 42641 antagonism was tested in order to detect an eventual action at the chloride ionophore level. Furthermore, the specificity versus $GABA_B$ receptor recognition site was evaluated. Since calcium conductance decrease may be the result of $GABA_B$ receptor activation (Deisz & Lux, 1985), the action of SR 42641 alone was investigated. A preliminary report of some parts of this work has been published previously (Desarmenien *et al.*, 1986).

Methods

Effect on $GABA_A$ receptor

Relative potencies of SR 42641 and bicuculline The isolated dorsal root ganglion preparation of the rat (L_4–L_5) was used (Desarmenien *et al.*, 1984). Intracellular recordings were performed in large neurones, selected for their well known large response to $GABA_A$ receptor activation. The preparation was continuously perfused with saline solution referred to as 'control Ringer', the composition of which was (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26 and glucose 10; warmed to 30–35°C and bubbled with O_2/CO_2 (95/5%; pH 7.4). Glass microelectrodes (20–100 MΩ tip resistance), filled with K-acetate (4 M) were used. Responses were recorded either under current clamp or voltage clamp. Under current clamp, membrane potential was recorded via a d.c. amplifier. A bridge circuit was used to allow the injection of current through the recording electrode, thus a whole experiment could be performed from a constant resting membrane potential. Membrane input resistance was measured by means of 150 ms duration hyperpolarizing current steps. For the experiments performed under voltage-clamp, the membrane potential was measured via a WPI amplifier, which was controlled by a sample-and-hold circuit (derived from the technique described by Wilson & Goldner, 1975) allowing single electrode voltage-clamp to be performed (switching frequency 1 kHz with a 50% duty cycle; gain of the comparison amplifier set at 2200). We selected electrodes with a low capacity and limited rectification. The time constant of the electrode-amplifier assembly was fast enough (approx. 10 ms) to allow confident measurement of the slow GABA-induced currents and of the steady current induced by voltage steps of 150 ms, used for membrane conductance measurements. GABA was applied by releasing 5 μl drops of a concentrated drug solution (50 mM to 10 μM in Ringer) upstream from the neurone. The effective dilution of this drop in the superfusion Ringer could be estimated for each cell in the following way. The plateau depolarization resulting from the addition of 9 mM KCl to the superfusion medium was compared to transient depolarizations evoked by drops of various more concentrated (250 to 50 mM) solutions of KCl. The relative concentrations eliciting similar amplitude depolarizations provided a dilution factor which, in these experiments ranged from 10 to 100,

depending on the rate of superfusion and of the position at which the drop was placed in the chamber. The constancy of responses administrated in this way was good in most experiments ($< \pm 10\%$ variation from mean response). A critical appraisal of this method has been given in Desarmenien *et al.* (1981) and Chambon *et al.* (1985). In the experiments designed to evaluate full dose-response relationships, each value is the mean of at least three determinations for any given concentration. The dose-response curves were obtained in the absence and at least 15 min after onset of perfusion of the antagonist, thus allowing the dissociation constant K_B to be calculated (Furchgott, 1972). Curve fitting and calculations were performed using a microcomputer program (Barlow, 1983).

Analysis of the voltage-dependency of SR 42641 antagonism Experiments were performed on dorsal root ganglion neurones. Responses to GABA were evoked at various membrane potentials in order to determine whether the blocking effect of SR 42641 may result from an interaction of this drug with the receptor-linked chloride channels. Indeed, depolarization induces an increase in GABA-evoked macroscopic conductance reflecting voltage sensitivity of the rate of channel opening and closing (Segal & Barker, 1984). If SR 42641 were to act as a channel blocker, its effect should depend on membrane potential. These experiments were performed under single electrode voltage-clamp conditions (as described previously) by combining voltage jumps and iontophoretic GABA applications (1 M, pH = 4). Iontophoresis allowed the delivery of small amounts of GABA (dose adjusted to trigger half maximal responses), thus response amplitude remained constant and receptor desensitization progressing with time was avoided even at relatively high rates of repetitive application (usually 1 application every 20 s). Furthermore, GABA applications could be synchronized with voltage jumps (Figure 4). To take into account small variations in the delivery of GABA, test responses (T) during the voltage jump were normalized against the control response (C), recorded at the holding potential just before the voltage jump and T/C (A) was plotted versus amplitude of the voltage jump in the absence and presence of antagonist. In order to avoid contamination of the chloride response by other conductances, GABA was applied 2–3 s after the beginning of each voltage jump, at a time when sodium conductance was inactivated. Calcium conductances were blocked with $CdCl_2$ (0.01 mM). KH_2PO_4 was replaced by KCl to avoid cadmium precipitation. Potassium conductances were blocked by adding TEA-Br (7.5 mM) and NaCl was lowered to 116.5 mM to maintain osmolarity. In some experiments, potassium conductance blockade was improved by filling the micropipette (40–60 MΩ tip resistance) with either CsCl (2 M) or Cs_2SO_4 (2 M).

Microiontophoretic constant currents were generated by an operational amplifier based technique (Bionic Instruments – Phymep, Paris) with an optical isolated trigger system. The total return current was isolated from the recording ground (feedback through a silver wire included in the recording chamber) and a backing current of 20–25 nA was applied continuously to the GABA-containing pipette (always placed downstream to the cell of interest). This current was automatically interrupted during application of the calibrated pulses of current used for drug delivery.

Effect on $GABA_B$ receptor

$GABA_B$ receptor activation was monitored (Desarmenien *et al.*, 1984) as a shortening of the calcium component of action potential elicited by the $GABA_B$ receptor agonist, (−)-baclofen. Experiments were mainly performed on nodose ganglia (prepared as described for dorsal root ganglia) because they have a high proportion of C cells which possess $GABA_B$ receptors and whose action potential displays a marked calcium component. Some experiments were also performed on A delta cells of dorsal root ganglia, in which such a calcium component, although smaller, does exist. This calcium component was enhanced with tetraethylammonium bromide (TEA-Br, 10 mM) in the 'control Ringer' solution, NaCl being lowered to 114 mM. Intracellular recordings were made with micropipettes (80–100 MΩ tip resistance) filled with K-acetate (4 M). Action potentials were elicited by orthodromic stimulation of the vagus or sciatic nerve, or by injection of depolarizing current through the recording electrode. Frequency of stimulation was once every 20 or 30 s to avoid progressive fading of the spike calcium component. The duration of the action potentials was measured as the time required for the maximal amplitude value to decay by half (time to half decay: t.h.d.). Baclofen and SR 42641 were applied by perfusion.

Effect on calcium current

Experiments were carried out on new-born (4 day-old) rat dorsal root ganglion neurones maintained in short term (3–10 h) primary culture, as described by Bossu *et al.* (1985). Voltage-clamp recordings were performed in the whole cell configuration of the patch-clamp method (Hamill *et al.*, 1981). Ionic conditions were chosen to minimize contamination by other currents. Thus Na^+ and K^+ were omitted from the external medium, K^+ channels were blocked by Cs^+ in the patch-electrode and by TEA both in the patch electrode and in the external solution. This latter contained (in mM): choline chloride 125, $CaCl_2$ 10, TEA 7.5, Hepes/Tris 10, pH 7.4. The patch electrode

contained: choline chloride 70.5, $CaCl_2$ 3, $MgCl_2$ 2, TEA 20, EGTA/CsOH 33, Hepes/Tris 10 and ATP 2, pH 7.2. The concentration of $CaCl_2$ and EGTA/CsOH were chosen so as to give low Ca^{2+} activities in the intracellular compartment (approximately pCa 8) thus minimizing Ca^{2+}-dependent inactivation of Ca^{2+} currents (Lee *et al.*, 1985; Bossu & Feltz, 1986). Experiments were carried out at room temperature. Data were stored on a tape recorder and measurements were made on a storage oscilloscope.

Drugs

The following were used: GABA: γ-aminobutyric acid (NBC); bicuculline methiodide (Sigma); SR 42641; (2-(2-(carboxy-3' propyl)-3-amino-6-*para*chloro-phenyl-pyridazinium chloride) Sanofi Recherche Montpellier); (−)-baclofen; (−)-β-(*p*-chlorophenyl)-GABA (kindly provided by Ciba-Geigy).

Statistics

Results are expressed as mean ± s.e.mean. Statistical differences were evaluated using non parametric Mann-Whitney U test for independent samples and Wilcoxon matched pairs signed-ranks test for related samples (Siegel, 1956). Slope differences between control and test curves were evaluated individually using a *t* test for slopes (Schwartz, 1963).

Results

$GABA_A$ receptor antagonism: comparison with bicuculline

In a first series of experiments carried out on 25 cells (resting potential, − 64 ± 3 mV; input resistance 28 ± 2 MΩ), we evaluated the antagonistic effects of

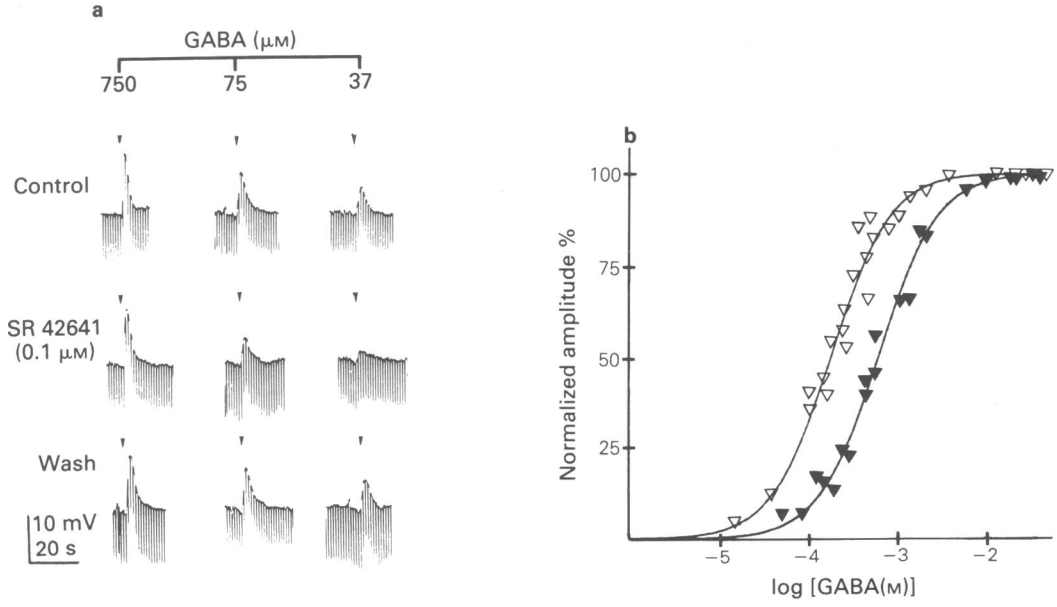


Figure 2 SR 42641 antagonizes $GABA_A$ depolarizations in large dorsal root ganglion neurones. (a) Penwriter recordings showing depolarizations and decreases of membrane resistance (monitored by hyperpolarizing current pulses: 1 nA; 150 ms) induced by various concentrations of GABA (numbers indicate the effective concentration of GABA in the Ringer solution). Note the diminution of GABA responses during perfusion of SR 42641 (middle row) and the recovery after washout (bottom row). (Resting membrane potential, − 62 mV; input resistance 25 MΩ). (b) GABA log-dose response curve obtained from a sample of 6 cells (mean resting potential, − 65 ± 5 mV; mean input resistance 33 ± 5 MΩ) before and during perfusion of a given concentration of SR 42641 (0.1 μM): (△) control; (▲) in presence of SR 42641. The amplitudes of GABA-induced depolarizations were expressed as % of the maximal control response = 100. Experimental points could be fitted (coefficient of multiple correlation: 0.925) with a 'two parallel sigmoid curves' microcomputer programme. SR 42641 shifted the dose-response curve to the right in a parallel manner. EC_{50}s calculated in the presence of SR 42641 were significantly different from those obtained in the control medium (0.57 ± 0.15 and 0.24 ± 0.06 mM; $P < 0.001$).

low concentrations of SR 42641 (0.1 to 1 μM) on short membrane depolarizations evoked by GABA. Figure 2 shows that continuous perfusion of the preparation with 0.1 μM SR 42641 reversibly decreased the dose-dependent responses caused by brief applications of GABA. Similar effects but with slower delay of onset were observed with bicuculline (0.7 to 7 μM). Neither of the two antagonists affected resting membrane potential or input resistance. However, the delay of drug washout for full recovery of the GABA-induced responses was always slower after application of bicuculline. With both antagonists, dose-response curves to GABA displayed a parallel shift to the right (as illustrated for SR 42641 in Figure 2) which we have examined further in voltage-clamp experiments. In keeping with the data obtained in current-clamp experiments, the mean maximal amplitude of GABA-induced responses (10.4 ± 0.8 mV) was not significantly affected by SR 42641 although the individual maxima were slightly decreased in some instances (up to 20% in 4 out of 16 cells). These data provided a first evaluation of the apparent dissociation constant: $K_B = 0.10 \pm 0.02\,\mu$M (14 determinations from 9 cells) for SR 42641 and 0.36 ± 0.06 μM (16 determinations from 16 cells) for bicuculline. The difference between these values was statistically significant ($P < 0.001$).

To assess further the competitive nature of this antagonist, voltage-clamp experiments were carried out on 16 cells (mean resting potential, − 65 ± 3 mV; mean input resistance 26 ± 6 MΩ). The dose-dependent inward currents evoked by GABA and their reversible inhibition by SR 42641 are shown in Figure 3a. As already noticed in current-clamp experiments, SR 42641 (0.2–0.4 μM) shifted the log-dose response curve to the right in a parallel manner (Figure 3c). In the presence of SR 42641, the EC_{50} values were always

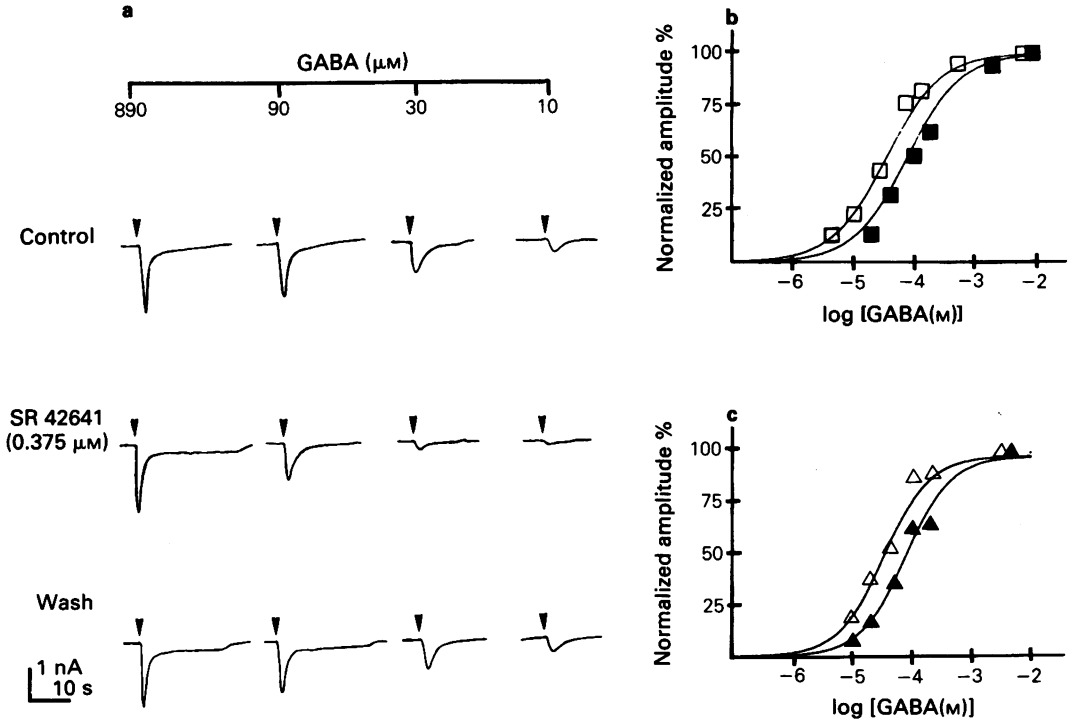


Figure 3 Analysis, under voltage-clamp conditions, of the GABA$_A$ antagonist properties of SR 42641 and bicuculline. (a) Same comments as in Figure 2a except that inward currents were recorded instead of depolarizations (Holding potential = Resting membrane potential = − 68 mV; input resistance = 10 MΩ). (b) GABA log-dose response curves obtained in the presence (■) and absence (□) of bicuculline 0.475 μM. The amplitudes of GABA-induced inward currents were expressed as % of the maximal control response = 100. Bicuculline shifted the dose-response curve to the right in a parallel manner, without affecting the maximal response. Experimental points were fitted with a 'two parallel sigmoid curves' microcomputer programme (coefficient of multiple correlation : 0.86). (Holding potential = Resting membrane potential = − 80 mV; input resistance = 10 MΩ.) (c) GABA dose-response curves obtained in the same cell as in (a) in the presence (▲) and absence (△) of SR 42641 (0.375 μM). Same comments as in (b). Coefficient of multiple correlation: 0.936.

greater than those of control curves (68 ± 24 μM) and the difference was statistically significant when paired measurements ($P < 0.005$) were compared. Moreover, the slope of the dose-response curves obtained in the absence and presence of SR 42641 never differed significantly from each other when tested within their linear portion. In addition, no statistical difference was found between maximal current amplitudes (2.6 ± 0.7 nA), although this maximum sometimes decreased up to 20% (2 out of 10 cells). Finally, the calculated K_B of SR 42641 was 0.12 ± 0.05 μM (10 cells), which is close to the value determined from current-clamp experiments. A similar shift to the right was obtained for dose-response curves established with bicuculline (Figure 3b). The same remarks as for SR 42641 concerning maximal amplitudes and slopes apply to this antagonist. The mean of the calculated K_B values for bicuculline was 0.38 ± 0.08 μM (6 cells) which was statistically different from that of SR 42641 ($P < 0.001$).

Voltage-dependency of the antagonism

The voltage-dependency of GABA responses was determined in 7 cells (mean resting potential, − 60 ± 3 mV; mean input resistance 28 ± 6 MΩ). Figure 4 illustrates such an experiment in which A = T/C values were plotted versus membrane potential. Increasing depolarizations (holding potential, − 60 mV) elicited a decrease in the GABA response as the GABA reversal potential was approached. The curve cannot be described by a straight line between potential − 100 and 0 mV but rather as a part of a bell-shape curve. In no case did the curves obtained in the presence of SR 42641 (0.2–0.5 μM) differ from the control one. GABA reversal potentials (− 4 ± 6 mV, $n = 7$) were the same in the absence or in the presence of SR 42641, as determined by extrapolation or by direct measurement.

Specificity for $GABA_A$ versus $GABA_B$ receptors

Experiments ($n = 8$) were performed on two types of cells classified with respect to action potential duration measured as the time to half decay (t.h.d.) of spike amplitude. The recordings illustrated in Figures 5 and 6 were obtained respectively with group C neurones (nodose ganglia; mean resting potential, − 78 ± 9 mV: mean input resistance 109 ± 36 MΩ; mean control t.h.d. 150 ms; $n = 5$) and with A delta cells (dorsal root ganglia; mean resting potential, − 62 mV; mean input resistance 48 MΩ; mean control t.h.d. 1.75 ms; $n = 3$). These figures show firstly that the superfusion of (−)-baclofen (1 μM) produced a reversible decrease of the t.h.d. and secondly that SR 42641 (0.1–10 μM) did not modify the effect of baclofen. Higher concentrations of antagonist were not used because, at doses

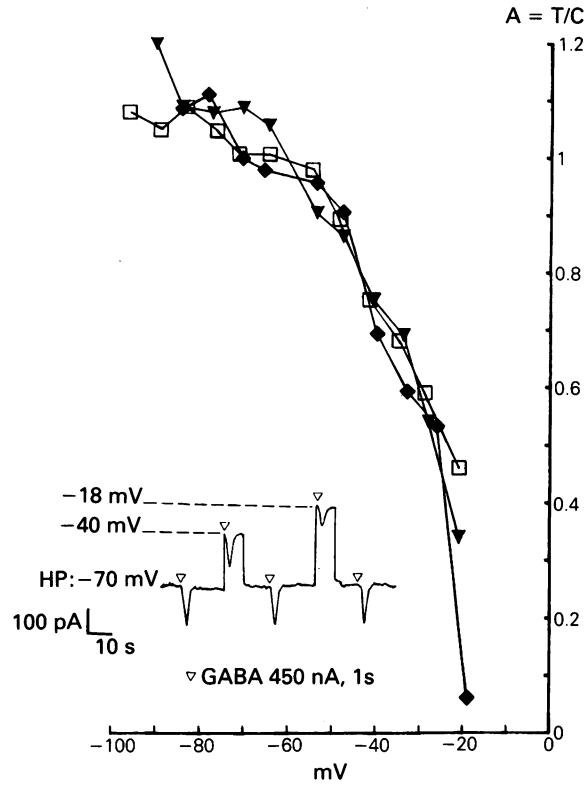


Figure 4 Absence of voltage-dependency of SR 42641 antagonism. Inset illustrates the procedure used. Experiments were performed under voltage-clamp conditions. Iontophoretic pulses (400 nA; 1s) of GABA (1 M, pH 4) elicited transient inward currents. The amplitude (T) of GABA responses evoked during voltage jumps (to the potential indicated on the abscissa scale) was normalized to the amplitude (C) of the control response recorded at the holding potential (− 60 mV) immediately before the voltage jump. I/V curves were obtained before (□), during (▼) and after (◆) perfusion of SR 42641 (0.2 μM). Notice that all three curves are superimposed, indicating that SR 42641 antagonism was not voltage-dependent.

greater than 10 μM, SR 42641 produced a significant increase of the t.h.d. value (Figure 7) reaching 79% at 50 μM. This latter effect was reversible during washout. In no case did SR 42641 affect the peak amplitude of the action potential.

Action on calcium current

By using the tight seal whole cell configuration for recording of dorsal root ganglion cells in culture (see methods), the I/V relationships of Ca^{2+} currents were

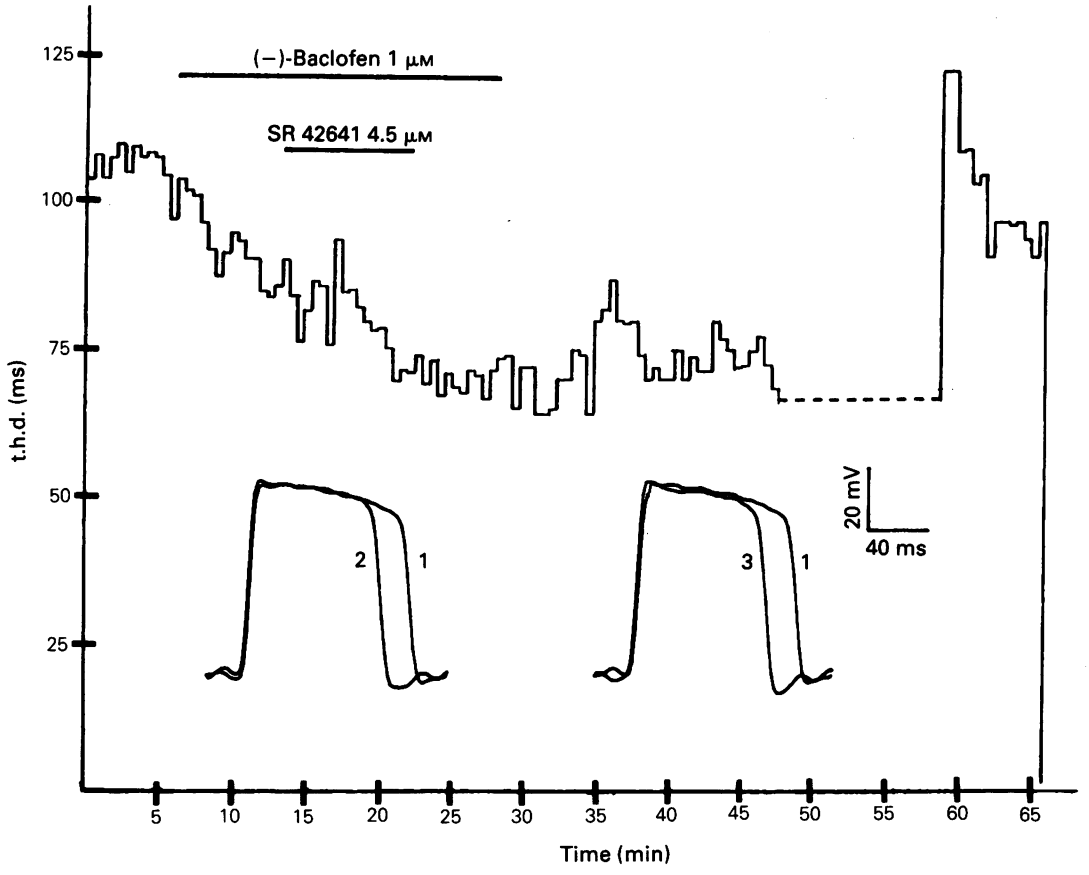


Figure 5 Lack of effect of SR 42641 against GABA$_B$ receptors tested on long-lasting calcium component of action potential. GABA$_B$ receptor activation was monitored as a (−)-baclofen-induced shortening of the calcium component of action potentials (evaluated as time to half decay: t.h.d.). Action potentials were elicited by somatic stimulation of a nodose C ganglion cell (resting membrane potential, − 100 mV, held at − 51 mV during the experiment; stimulation frequency: 0.032 Hz). The calcium component was enhanced by adding TEA in the perfusion solution. t.h.d. was plotted versus time; notice that the decrease of t.h.d. values induced by (−)-baclofen was unaffected by simultaneous perfusion of SR 42641. The dotted line indicates that no recordings were performed during this period of time. Inset illustrated this, showing action potentials recorded during control conditions (1), perfusion of (−)-baclofen (2) and perfusion of (−)-baclofen plus SR 42641 (3). Recordings were digitized and redrawn using a microcomputer.

examined while using two levels of holding potentials (− 80 or − 40 to − 30 mV; 12 cells). Using the criteria defined by Nowycky *et al.* (1985), three types of Ca^{2+} currents could be observed on these cells: S for sustained, T for transient and N for neither. The sustained Ca^{2+} current is more likely to display a sensitivity to GABA$_B$ agonists (Deisz & Lux, 1985; Dolphin & Scott, 1986; Holtz *et al.*, 1986) and, for the sake of clarity, we focused our attention mainly on this current. Accordingly, long lasting sustained Ca^{2+} currents were recorded as illustrated in Figure 8. The potential was stepped to 0 mV for 300 ms, after a 300 ms prepulse to − 40 mV; the holding potential being − 80 mV. Under these conditions, a sustained current could be recorded. Although this is likely to correspond to $ICa^{2+}{}_S$, it is perhaps not entirely free of contamination by N, because the conditioning depolarizing pulse used was only 300 ms long. SR 42641 (100 μM; 3 experiments) did not modify the amplitude or the shape of this calcium current (Figure 8).

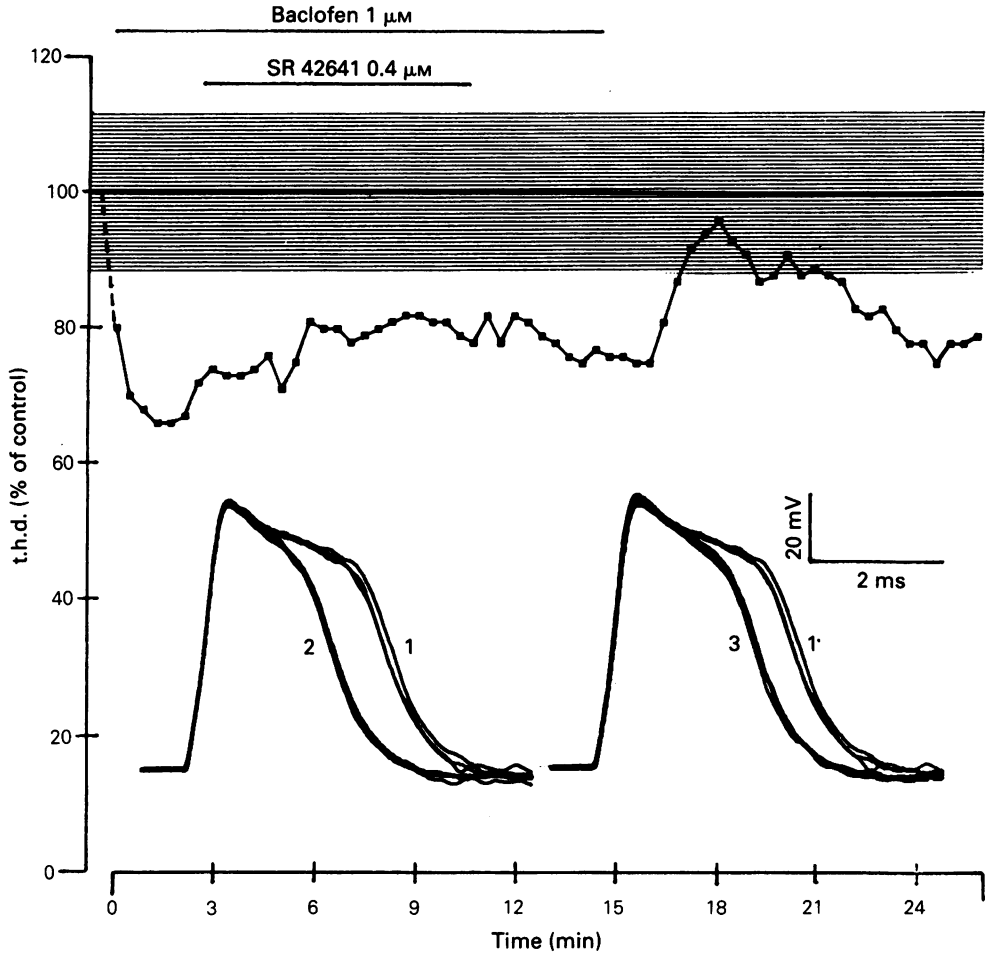


Figure 6 Lack of effect of SR 42641 against $GABA_B$ receptors, tested on short lasting calcium component. Same comments as in Figure 5 except that t.h.d. values were plotted as % of mean control value before application of baclofen (hatched area represents mean as 100% ± s.d.).

Discussion

Our results provide a number of electrophysiological arguments for considering SR 42641 as a competitive $GABA_A$ receptor antagonist. Statistical analysis of the data obtained under both current- and voltage-clamp recording conditions indicate that SR 42641 shifted GABA dose-response curves to the right in a parallel fashion, as did bicuculline. Furthermore, the maximum response was also well maintained from a statistical point of view. With respect to the antagonist potency of bicuculline, which served as a reference compound, it is worth noting that the present results are in agreement with those obtained by other authors using electrophysiological techniques (Simmonds, 1980; Kemp *et al.*, 1985). However, non-competitive antagonism could be suspected in some cases in our experiments, as maximal GABA responses were sometimes depressed by the antagonist. However, the real maximal GABA responses may not have been produced because of receptor desensitization or non steady-state equilibrium conditions due to the short application used (short applications had to be used precisely to try to avoid desensitization). Another factor could be the uptake of GABA by satellite glial cells. However Desarmenien *et al.* (1980) have shown that in the superficial neurones studied, this glial uptake does not affect the GABA response. Information concerning these possibilities could have been drawn from a Schild's plot analysis. However, such

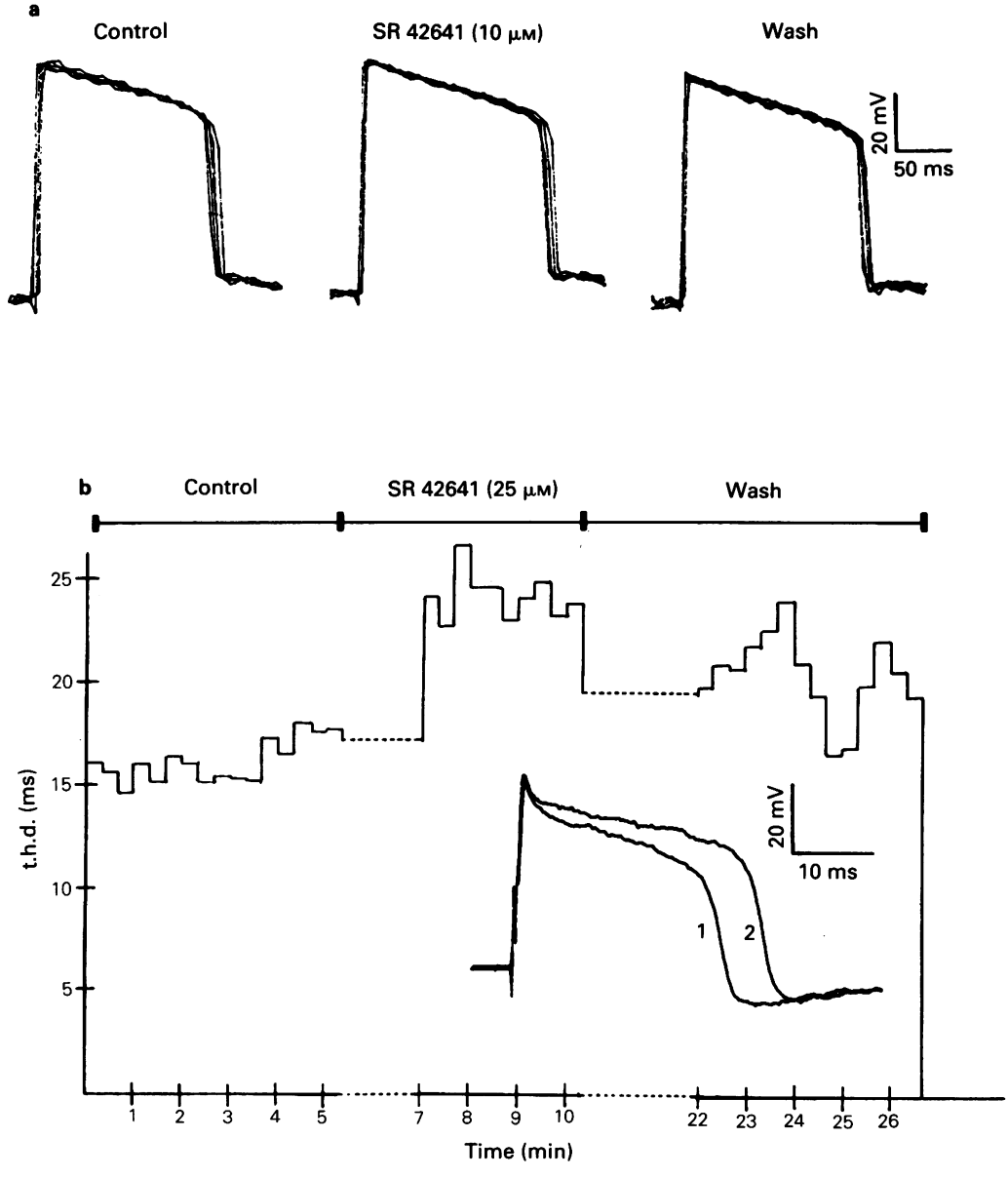


Figure 7 Effect of SR 42641 alone on the calcium component of action potentials. (a) At a concentration up to 10 μM, SR 42641 did not affect amplitude or shape of action potentials. Traces show superimposed action potentials elicited by orthodromic stimulation of a nodose C ganglion cell (resting membrane potential, − 64 mV). (b) At concentrations higher than 10 μM SR 42641 reversibly enhanced action potential duration. Similar protocol as in (a) except that action potentials were elicited by injection of brief depolarizing currents steps through the recording electrode; for dotted lines, see explanation in Figure 5. Time to half decay (t.h.d.) was plotted versus time (stimulation frequency 0.05 Hz). Inset illustrates this effect, by showing a control action potential (1) and an action potential recorded in the presence of SR 42641 (2). (Resting membrane potential, − 55 mV, held at − 40 during the experiment). Notice that the peak amplitude of action potentials was unaffected by SR 42641.

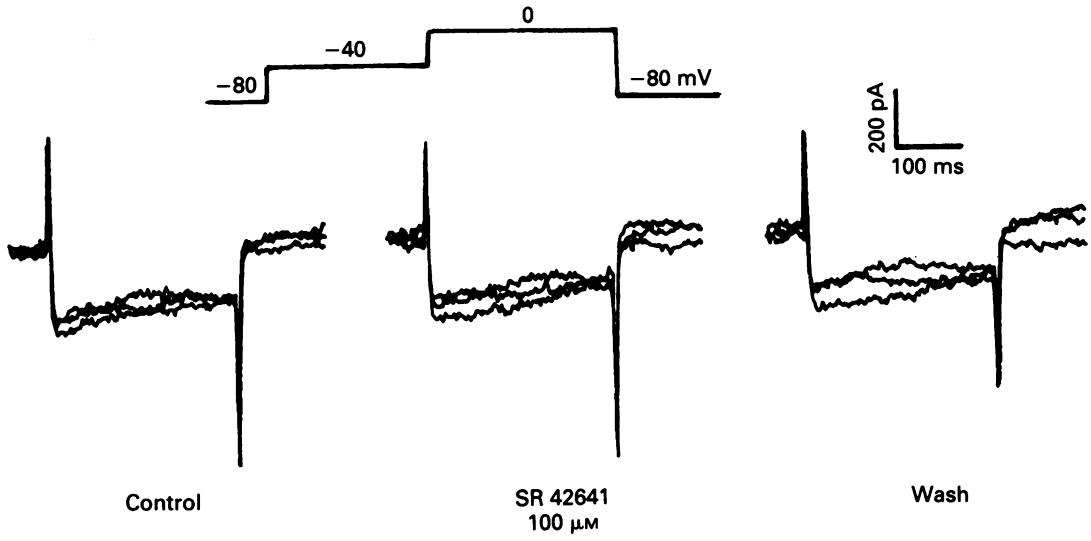


Figure 8 Whole cell recording (patch-clamp technique) of a sustained calcium current evoked in a cultured DRG cell. The potential was stepped to 0 mV after a prepulse at − 40 mV, the resting holding potential being − 80 mV. Three successive responses (digitized and redrawn) were superimposed. Notice that 2 min perfusion of SR 42641 did not significantly affect this Ca^{2+} current.

attempts providing further validation of our experimental conditions were not feasible, mainly because of the length of time required (several hours) for running such a protocol while maintaining intracellular recordings.

Competitive antagonism is strengthened by our data indicating that the antagonist does not seem to affect the chloride ionophore linked to the GABA receptor, as indicated by the non-voltage dependency of the antagonist effect of SR 42641. The observation that the curve cannot be fitted by a straight line but rather is part of a bell-shaped curve may reflect the greater probability of chloride channel openings at more depolarized and positive potentials (Segal & Barker, 1984; Gray & Johnston, 1985). The contribution of a Ca^{2+}-activated chloride conductance seems unlikely in our conditions since voltage-activated Ca^{2+} entries were blocked by Cd^{2+}. Thus, were SR 42641 to behave as a channel blocker, the effect would be more apparent at depolarized potentials. But in no case did we see any differences between the control curves and those obtained in the presence of SR 42641. In our experimental conditions, GABA reversal potentials occurred at depolarized values, so a decrease of chloride current as a decreased driving force may attenuate any differences between the two curves, so that a small channel blocker property may not have been seen. Another argument in line with our observations comes from the binding studies of Heaulme *et al.* (1986). These authors showed that SR 42641 does not interfere with the binding of [^{35}S]-*t*-butylbicyclophosphorothionate, a chloride-channel ligand (Squires *et al.*, 1984).

Together, the parallel shift of dose-response curves and the absence of voltage-dependency indicate that SR 42641 is probably a competitive antagonist. This statement is confirmed by biochemical results showing the competitive displacement of [^{3}H]-GABA from its Na^+-independent binding site (Heaulme *et al.*, 1986). In these studies, SR 42641 appeared 200 times more potent than bicuculline; whereas, in our electrophysiological study, SR 42641 was only 3.6 times more potent than bicuculline. Such a discrepancy between binding and physiological studies may concern different subtypes of $GABA_A$ binding sites (Olsen *et al.*, 1981). On the other hand, binding and electrophysiological results are in agreement when comparing SR 42641 to its chemical parent, SR 95103. Such studies show a 10 fold greater potency for SR 42641, an interesting finding as regards potency and structure-activity relationship. SR 42641 does not affect resting membrane properties but may, at high doses, affect other conductances implicated in spike activity. Indeed, at concentrations above 10 μM, it prolongs the calcium component of action potentials. A similar result has been described for bicuculline (Heyer *et al.*, 1982). SR 42641 did not behave as an agonist for $GABA_B$ receptor sites as it did not shorten the calcium component of action potentials. Nor did it behave as a $GABA_B$ receptor antagonist since it did not affect baclofen action. Thus, SR 42641 may be a helpful tool in experiments in which $GABA_B$ receptor activation is studied with the natural ligand GABA, since it does not affect calcium current, which may be implicated in

some mechanism of action of baclofen.

Our evidence concerning the specificity of SR 42641 for the $GABA_A$ versus the $GABA_B$ receptors is based on spike duration measurements and recordings of ICa^{2+}_S. Changes in the ICa^{2+}_S component of inward currents are reflected in the timing of spikes, particularly in the presence of TEA (7.5 mM in our experiments). Indeed, there have been detailed studies showing a decrease of ICa^{2+}_S (Deisz & Lux, 1985; Dolphin & Scott, 1986; Taleb *et al.*, 1986; Holz *et al.*, 1986) which may account for shorter spikes during $GABA_B$ receptor activation. In our experiments, ICa^{2+}_S was not inhibited by SR 42641 and peak amplitudes and spike durations were not modified (doses up to 100 times the K_B). However, there are limitations on the analysis of spikes timing and of ICa^{2+}_S. For instance, in sensory ganglia, spike shape and duration in the presence of TEA may reflect the combined involvement of ICa^{2+}_S and a tetrodotoxin (TTX)-resistant Na current in a physiological medium with a normal Na^+/Ca^{2+} ratio (see Bossu & Feltz, 1984). Since SR 42641 also preserved the peak amplitude of spikes, one can speculate that this other inward current will also be unchanged as well. Similar kinetics of activation–inactivation were reported for ICa^{2+}_N, so it is for the same reason that we suspect that this current will also remain unaffected. In the case of ICa^{2+}_T, this third Ca^{2+} current is generally thought to play a major role in spike generation mechanisms rather than on its shape and/or timing (see discussion in Nowycky *et al.*, 1985; Bossu & Feltz, 1986). It should also be borne in mind that any significant decrease in spike duration by $GABA_B$ agonists on the rat ganglia also depends on K^+ currents (Desarmenien *et al.*, 1985; Dolphin & Scott, 1986) but biophysical experiments are needed to investigate whether the drug is acting directly or indirectly (affecting intracellular Ca^{2+}) on outward currents. This remains an open question, especially since higher doses of SR 42641 clearly increase spike duration.

Other potent antagonists for $GABA_A$ receptors have been described in the last few years. The more potent are R 5135 (Hunt & Clements-Jewery, 1981) and Pitrazepin (Gähwiler *et al.*, 1984). In electrophysiological experiments, R 5135 was 200 fold more potent than bicuculline but interfered greatly with the glycine receptor (Simmonds & Turner, 1985; Curtis & Malik, 1985). Pitrazepin, 3 fold more potent than bicuculline (Kemp *et al.*, 1985), interacts with the benzodiazepine and glycine receptors (Braestrup & Nielsen, 1985) but does not influence baclofen action. In binding studies, SR 42641 (Heaulme *et al.*, 1986) did not interact with either the benzodiazepine, glutamate, strychnine or $GABA_B$ binding sites. This latter result confirms our own observation concerning an absence of interaction with baclofen. In another type of electrophysiological study, using extracellular techniques, SR 42641 appeared to be a specific GABA antagonist in that it did not interact with the glycine receptor (Michaud *et al.*, 1986). Finally it is worth noting that this compound is easily soluble, chemically stable and its effects occur rapidly and are easily reversible, so it is a useful tool in investigating $GABA_A$ and $GABA_B$ receptors.

M.H. was supported by a fellowship in pharmacology from the Ministère de la Recherche et de la Technologie. We would like to thank Dr B.A. Demeneix and Dr N. Grant for correcting the English manuscript, and Dr J.C. Michaud (Sanofi Recherche) for helpful discussion all through this work.

This research was funded by CNRS, U.L.P. and Sanofi Recherche. The present study programme is sponsored by INSERM (CRL 846-09/10-1984).

References

BARKER, J.L., McBURNEY, R.N. & MATHERS, D.A. (1983). Convulsant-induced depression of amino acid responses in mouse culture spinal neurones studied under voltage clamp. *Br. J. Pharmac.*, **80**, 619–630.

BARLOW, R.B. (1983). *Biodata Handling with Microcomputers*. pp. 180–185. Amsterdam: Elsevier.

BISCOE, T.J., DUGGAN, A.W. & LODGE, D. (1972). Antagonism between bicuculline, strychnine and picrotoxin and depressant amino-acids in the rat nervous system. *Comp. J. gen. Pharmac.*, **3**, 423–433.

BOSSU, J.L., FELTZ, A., THOMANN, J.M. (1985). Depolarization elicits two distinct calcium currents in vertebrate sensory neurones. *Pflügers Arch.*, **403**, 360–367.

BOSSU, J.L. & FELTZ, A. (1984). Patch-clamp study of the tetrodotoxin-resistant sodium current in group C sensory neurones. *Neurosci. Lett.*, **51**, 241–246.

BOSSU, J.L. & FELTZ, A. (1986). Inactivation of the low-threshold transient calcium current in rat sensory neurones: Evidences for a dual process. *J. Physiol.*, **376**, 341–357.

BOWERY, N.G. (1982). Baclofen: 10 years on. *TIPS.*, **3**, 400–403.

BRAESTRUP, C. & NIELSEN, M. (1985). Interaction of pitrazepin with the GABA/benzodiazepine receptor complex and with glycine receptors. *Eur. J. Pharmac.*, **118**, 115–121.

CHAMBON, J.P., FELTZ, P., HEAULME, M., RESTLE, S., SCHLICHTER, R., BIZIERE, K. & WERMUTH, C.G. (1985). An arylaminopyridazine derivative of γ-aminobutyric acid (GABA) is a selective and competitive antagonist at the GABA-A receptor site. *Proc. natn. Acad. Sci., U.S.A.*, **82**, 1832–1836.

CURTIS, D.R., & MALIK, R. (1985). Glycine antagonism by RU 5135. *Eur. J. Pharmac.*, **110**, 383–384.

DEISZ, R.A. & LUX, H.D. (1985). γ-aminobutyric acid-induced depression of calcium currents of chick sensory neurons. *Neurosci. Lett.*, **56**, 205–210.

DESARMENIEN, M., DESAULLES, E., FELTZ, P., HAMANN,

M., MICHAUD, J.C. & MIENVILLE, J.M. (1986). Electrophysiological study of pyridazine – GABA derivatives with GABA-A antagonist properties. *Br. J. Pharmac*, Proc. Suppl., **87**, 150P.

DESARMENIEN, M., FELTZ, P. & HEADLEY, P.M. (1980). Does glial uptake affect GABA responses? An intracellular study on rat dorsal root ganglion neurones in vitro. *J. Physiol.*, **307**, 163–182.

DESARMENIEN, M., FELTZ, P., HEADLEY, P.M. & SANTANGELO, F. (1981). SL 75102 as a GABA agonist: experiments on dorsal root ganglion neurones in situ. *Br. J. Pharmac.*, **72**, 355–364.

DESARMENIEN, M., FELTZ, P., OCCHIPINTI, G., SANTANGELO, F. & SCHLICHTER, R. (1984). Coexistence of $GABA_A$ and $GABA_B$ receptors on A delta and C primary afferents. *Br. J. Pharmac.*, **81**, 327–333.

DOLPHIN, A.C. & SCOTT, R.H. (1986). Inhibition of calcium currents in cultured rat dorsal root ganglion neurones by (−)-baclofen. *Br. J. Pharmac.*, **88**, 213–220.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Catecholamines, Handbook of Experimental Pharmacology*, vol 33. ed. Blaschko, H. & Muscholl, E. pp. 283–335. Berlin, Heidelberg, New York: Springer Verlag.

GÄHWILER, B.H., MAURER, R. & WÜTHRICH, H.J. (1984). Pitrazepin, a novel GABA-A antagonist. *Neurosci. Lett.*, **45**, 311–316.

GOLDINGER, A. & MÜLLER, W.E. (1980). Stereospecific interaction of bicuculline with specific (^{3}H) strychnine binding to rat spinal cord synaptosomal membranes. *Neurosci. Lett.*, **16**, 91–95.

GRAY, R. & JOHNSTON, D. (1985). Rectification of single GABA-gated chloride channels in adult hippocampal neurons. *J. Neurophysiol.*, **54**, 134–142.

HAMIL, O.P., BORMANN, J., SAKMANN, B. (1983). Activation of multiple-conductance state chloride channels in spinal neuron. *Nature*, **305**, 805–808.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.*, **391**, 85–100.

HEAULME, M., CHAMBON, J.P., LEYRIS, R., MOLIMARD, J. CH., WERMUTH, C.G. & BIZIERE, K. (1986). Biochemical characterization of the interaction of three pyridazinyl-GABA derivatives with the GABA-A receptor site. *Brain Res*, **384**, 224–231.

HEYER, E.J., NOWAK, L.M. & MACDONALD, R.L. (1982). Membrane depolarization and prolongation of calcium dependent action potentials of mouse neurons in cell culture by two convulsants: bicuculline and penicillin. *Brain Res.*, **232**, 41–56.

HOLTZ IV, G.G., RANE, S.G. & DUNLAP, K. (1986). GTP binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature*, **319**, 670–672.

HUNT, P. & CLEMENTS-JEWERY, S. (1981). A steroid derivative, R 5135, antagonizes the GABA/benzodiazepine receptor interaction. *Neuropharmacology*, **20**, 357–361.

KEMP, J.A., MARSHALL, G.R., WONG, E.H.F. & WOODRUFF, G.N. (1985). Pharmacological studies on pitrazepin, a GABA-A receptor antagonist. *Br. J. Pharmac.*, **85**, 237P.

LEE, K.S., MARBAN, E. & TSIEN, R.W. (1985). Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *J. Physiol.*, **364**, 395–411.

MICHAUD, J.C., MIENVILLE, J.M., CHAMBON, J.P. & BIZIERE, K. (1986). Interactions of three pyridazinyl-GABA derivatives with rat central GABA and glycine receptors, an in vivo microiontophoretic study. *Neuropharmacology*, (in press).

NOWYCKY, M.C., FOX, A.P. & TSIEN, R.W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature*, **316**, 440–443.

OLSEN, R.W., BERGMAN, M.O., VAN NESS, P.C., LUMMIS, S.C., WATKINS, A.E., NAPIAS, C. & GREENLEE, D.V. (1981). γ-aminobutyric acid receptor binding in mammalian brain. Heterogeneity of binding sites. *Mol. Pharmac.*, **19**, 217–227.

SCHWARTZ, D. (1963). La regression. In *Méthodes statistiques à l'Usage des Médecins et des Biologistes*. pp. 225–231. Paris: Flammarion Médecine Science.

SEGAL, M. & BARKER, J.L. (1984). Rat hyppocampal neurons in culture: properties of GABA-activated Cl ion conductance. *J. Neurophysiol.*, **51**, 500–515.

SIEGEL, S. (1956). *Non Parametric Statistics for the Behavioral Sciences*. New York: MacGraw Hill.

SIMMONDS, M.A. (1980). Evidence that bicuculline and picrotoxin act at separate sites to antagonize γ-aminobutyric acid in rat cuneate nucleus. *Neuropharmacology*, **19**, 39–45.

SIMMONDS, M.A. & TURNER, J.P. (1985). Antagonism of inhibitory amino acids by the steroid derivative RU 5135. *Br. J. Pharmac.*, **84**, 631–635.

SIMONDS, W.F. & DeGROAT, W.C. (1980). Antagonism by picrotoxin of 5-hydroxytryptamine-induced excitation of primary afferent neurons. *Brain Res.*, **192**, 592–597.

SQUIRES, A.F., CASIDA, J.E., RICHARDSON, M. & SAEDERUP, E. (1984). (^{35}S) t-butylbicyclophosphorothionate binds with high affinity to brain specific sites coupled to γ-aminobutyric acid –A and ion recognition sites. *Mol. Pharmac.*, **23**, 326–336.

TALEB, O., LOEFFLER, J.PH., TROUSLARD, J., DEMENEIX, B.A., KLEY, N., HOLLT, V. & FELTZ, P. (1986). Ionic conductances related to GABA action on secretory and biosynthetic activity of pars intermedia cells. *Brain Res. Bull.*, (in press).

TICKU, M.K., BAN, M. & OLSEN, R.W. (1978). Binding of ^{3}H-alpha-dihydropicrotoxinin, a gamma-aminobutyric acid synaptic antagonist, to rat brain membrane. *Mol. Pharmac.*, **14**, 391–402.

WERMUTH, C.G. (1985). Recherche en série pyridazinique – potentialité pharmacologique et thérapeutique des amino-3-pyridazines. *Actual. Chim. Ther.*, **12**, 3–35.

WILSON, W.A. & GOLDNER, M.M. (1975). Voltage clamping with a single microelectrode. *J. Neurobiol.*, **6**, 411–422.

YASUI, S., ISHIZUKA, S. & AKAIKE, N. (1985). GABA activates different types of chloride-conducting receptor-ionophore complexes in a dose-dependent manner. *Brain Res.*, **344**, 176–180.

(Received February 3, 1986.
Revised September 22, 1986.
Accepted October 3, 1986.)