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Effect of SB-205384 on the decay of GABA-activated chloride currents in granule cells cultured from rat cerebellum

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1 4-Amino-7-hydroxy-2-methyl-5,6,7,8,-tetrahydrobenzo[b]thieno[2,3-b]pyridine-3-carboxylic acid, but-2-ynyl ester (SB-205384) and other γ-aminobutyric acid$_A$ ($GABA_A$) receptor modulators were tested for their effects on GABA-activated chloride currents in rat cerebellar granule cells by use of the whole-cell patch clamp technique.

2 The major effect of SB-205384 on $GABA_A$-activated current was an increase in the half-life of decay of the response once the agonist had been removed. This is in contrast to many $GABA_A$ receptor modulators that have previously been shown to potentiate GABA-activated currents.

3 This profile could be explained if SB-205384 stabilizes the channel in open and desensitized states so that channel closing is dramatically slowed. Such a modulatory profile may produce a novel behavioural profile *in vivo*.

Keywords: $GABA_A$ modulators; benzodiazepine; pentobarbitone; SB-205384; chloride channel

Introduction

Ligands that modulate the function of γ-aminobutyric acid$_A$ ($GABA_A$) receptor gated chloride channels have important therapeutic properties. Agents that increase chloride current flow have sedative, anaesthetic, hypnotic, anxiolytic and anticonvulsant actions. These modulatory effects are produced by interactions at a range of distinct allosteric binding sites on the $GABA_A$ channel complex. For two of the major classes, the benzodiazepines and barbiturates, the molecular mechanism of action of the modulators has been described at the single channel level (Study & Barker, 1981). Benzodiazepines, such as diazepam, increase the affinity of the receptor for GABA causing a leftward shift in the GABA dose-response curve and increasing the rate of channel opening at any submaximal GABA concentrations. Barbiturates, such as pentobarbitone, act by increasing the mean open time of the channel, resulting in an increase in maximal current evoked by GABA (Twyman *et al*., 1989). Steroid modulators exemplified by 5α-pregnane-3α,21-diol-20-one (THDOC) share some similarities with benzodiazepines. THDOC increases the frequency of channel opening at the single channel level but these compounds to not interact with the benzodiazepine binding site (Puia *et al*., 1990). At the macroscopic current level, the main effect of all three classes of modulator is to increase the peak amplitude of $GABA_A$ channel currents evoked by submaximal doses of GABA.

In the course of work aimed at identifying novel types of GABA channel modulator, we examined the properties of 4-amino-7-hydroxy-2-methyl-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-b]pyridine-3-carboxylic acid, but-2-ynyl ester (SB-205384) (Figure 1) (Benham *et al*., 1994), one of a group of related benzothiophene compounds which have a non benzodiazepine modulatory site (Benham *et al*., 1995). We compared the modulatory actions of this compound with diazepam, pentobarbitone and THDOC representing the classes described above and tracazolate (Goldberg *et al*., 1983), a non benzodiazepine modulator whose actions on $GABA_A$ currents have not been directly examined. Electrophysiological studies on cultured cerebellar granule cells revealed that SB-205384 had a novel mechanism of action, dramatically prolonging the decay of GABA-activated current on removal of agonist. Effect on peak GABA-activated current was in contrast much less marked. Since an important function of neurones is to integrate a variety of synaptic inputs over time, this temporal modulatory effect on GABA-gated currents may result in a novel behavioural profile *in vivo*.

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Methods

Cell culture

Rat cerebellar granule cells were obtained from 8 day old rats as described by Levi *et al*. (1989). The cells were plated on poly-ornithine coated coverslips and were maintained in culture 8–10 days before recording.

Electrophysiology

Cells were placed on the stage of an inverted microscope and continuously superfused (4–6 ml min^{-1}) with a solution containing (mM): NaCl 140, KCl 2.5, $CaCl_2$ 1.5, $MgCl_2$ 1.2, HEPES 10 and glucose 10 (pH 7.4 with NaOH). The patch pipette solution contained in mM: CsCl 140, $MgCl_2$ 4, HEPES 10, EGTA 10 and NaATP 2 and was adjusted to pH 7.2 with CsOH. SB-205384 and THDOC were made up as 10 mM stock solutions in dimethyl sulphoxide, pentobarbitone as a 10 mM stock solution in deionized water, and diazepam and tracazolate as 1 mM stock solutions in slightly acidified deionized water (3 drops 5 M HCl in 20 ml H_2O) before being diluted to their final concentrations in external solution.

All experiments were performed at room temperature (20–25°C). Recordings were made by use of the whole-cell patch clamp technique (Hamill *et al*., 1981) with a List EPC7 patch clamp amplifier. Cells were held at a membrane potential of −60 mV. Currents were evoked in response to a pulse of GABA or GABA plus the test compound applied through the perfusion system for approximately 4 s. Current responses were evoked at approximately 60 s intervals and were stored on the computer for later analysis by voltage and patch clamp software (Cambridge Electronic Design). The perfusion system operated by gravity and used large bore tubing placed directly in line with the cell. Solutions were separated by an air bubble that was removed just before the solutions reached the cell via a small hole in the top of the tubing. This system allowed rapid solution exchange at the cell surface with exchange possible within approximately 200 ms. This was tested by changing the external solution between distilled water and 2 M KCl and

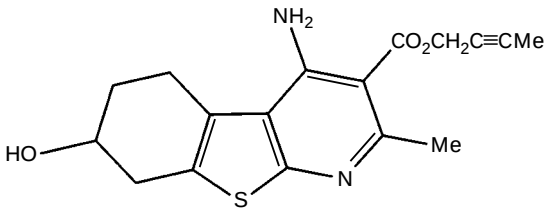

Figure 1 The structure of SB-205384. The Δ logP was measured as +3.91 using the procedure defined by Young *et al.* (1988).

measuring the rate of change of current. Thus, half times for decay of current of less than about 250 ms would not be distinguishable from solution exchange times. From dose-response curves to GABA, a concentration of GABA that gave approximately 20% of the maximum response was chosen for investigating the action of the GABA modulators. Half times for decay of current ($t_{1/2}$) were measured by calculating the time taken for the plateau current measured before GABA removal to decay to half its amplitude. All values are given as mean ± s.e.mean.

Materials

The following compounds were used: γ-amino-n-butyric acid (GABA; Sigma), diazepam (Fabbrica Italiano Sintetica S.p.A.), pentobarbitone sodium (Sigma), muscimol (Sigma), 5α-pregnane-3α,21-diol-20-one (THDOC; Sigma), 4-amino-7-hydroxy-2-methyl-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-b]pyridine-3-carboxylic acid, but-2-ynyl ester (SB-205384; Dept. of Medicinal Chemistry, SB, Harlow), tracazolate (ICI Pharmaceuticals), *cis* amino crotonic acid (CACA; Tocris Cookson).

Results

GABA application (0.1 to 1000 μM) to cerebellar granule cells after 8 days in culture consistently evoked rapidly rising inward currents at −60 mV holding potential (Figure 2a) with EC_{50} 3.4 μM (Figure 2b). Maximal currents of 2.4 nA were recorded under these conditions. These responses were consistent with chloride current flow through $GABA_A$ channels.

Many GABA channel modulators induce current flow in the absence of GABA (Cottrell *et al.*, 1987), so we first applied SB-205384 in the absence of GABA to assess any direct agonist effect and contribution of this mechanism to responses upon coapplication. Application of 10 μM SB-205384 alone gave a current after 4 s of application (i.e. the GABA application time used in the modulator experiments) that was 2.7 ± 0.9% ($n = 9$) of the peak current observed to an application of 100 μM GABA. Application of either 1 μM or 30 μM SB-205384 alone failed to produce any direct current ($n = 4$). Thus maximal contribution of a direct agonist effect of SB-205384 was about 10% of the EC_{20} GABA response. This direct effect was too small to account for the slowly deactivating current on washout of coapplied GABA and SB-205384 described subsequently. Simple addition of a GABA-evoked agonist current and an SB-205384-evoked agonist current does not explain the data.

In order to investigate the effects of GABA modulators on GABA-induced chloride currents it was important to first ascertain a concentration of GABA to enable the consistent detection of any change in the response. A concentration of GABA of 1 μM was chosen for use in subsequent experiments. This concentration gave approximately 20% of the maximum response to GABA (EC_{20} 0.6 μM, Figure 2b). This allowed a large window for potentiation whilst still having the potential to show up any inhibition. Control responses were obtained to 1 μM GABA which was then reapplied with increasing concentrations of the modulator compounds. Pretreatment with the modulator rather than coapplication had no significant effect on the peak responses measured, or the time course of the decay of current, so we used coapplication for all subsequent experiments.

Examples of original traces showing the effect of coapplying diazepam and then SB-205384 with GABA (1 μM) are shown in Figure 3. All compounds gave some degree of potentiation of inward current amplitude. However, in addition to this effect, tracazolate and SB-205384 slowed the decay of the inward current on GABA washout, such that the current now took many seconds to relax back to basal levels (Figure 3 lower trace).

Figure 4a summarizes the effect of the various modulators on the amplitude of the control GABA response. The control GABA response was taken as 100%. All the modulators tested show some degree of potentiation of the control current. SB-205384 was more potent than the barbiturate, pentobarbitone, and was virtually equipotent to tracazolate but less potent than the steroid, THDOC. Diazepam only gave a very small degree of potentiation at all concentrations tested (maximum potentiation only 44% above control).

Decay of GABA-activated currents in granule cells is multiexponential (e.g. Maconochie *et al.*, 1994). Such detailed analysis was inappropriate in these experiments due to the limitations of our solution exchange confounding the analysis of fast components. Values for $t_{1/2}$ were therefore measured to give a robust indication of modulator-induced changes in decay rate. Figure 4b shows the effect of the various modulators on the half-life of decay of the GABA-activated current on removal of agonist from the same experiments analysed in Figure 4a. SB-205384 and tracazolate both dramatically prolonged the half-life for decay of the GABA response in a concentration-dependent manner. At the highest concentration of SB-205384 used, the half-life of decay was increased about 10 fold compared to control responses. THDOC also caused a prolongation of the half-life of decay at 1 μM. This prolongation was of similar magnitude to that observed for SB-205384 at the same concentration. However, at 1 μM, THDOC potentiated GABA-induced currents to a far greater extent than SB-205384. The other standard compounds (diazepam, pentobarbitone) had very little effect on the decay time of the response, whilst still causing potentiation of the response. Thus the major action of SB-205384 and tracazolate was to prolong the duration of GABA-activated current rather than increase the amplitude of the response, as was seen with the other modulator compounds.

In order to investigate further the mechanism of action of SB-205384, the agonist dependence of SB-205384 modulation was examined. This might indicate if SB-205384 was acting by increasing the affinity of the receptor site for agonist, as is thought to be the case for diazepam, or acting on a mechanism discrete from agonist binding. The effect of SB-205384 on the half-life of decay for three agonists with different affinities for the $GABA_A$ receptor was investigated. The concentrations of agonists were chosen to give the same absolute responses in order to generate the same proportion of channels in the open state. CACA was approximately 200 fold less potent than GABA or muscimol, producing a similar response at 0.5 mM to that produced by GABA or muscimol at 1 μM. There was little difference in the half-life of decay that we observed for each agonist alone (CACA 0.23 ± 0.07 s ($n = 3$), GABA 0.18 ± 0.02 s ($n = 12$) and muscimol 0.43 ± 0.12 s ($n = 3$)). This was expected as the resolution of the recording system was insufficient to distinguish the faster decay rate predicted for CACA due to its low affinity. However, in the presence of 1 μM SB-205384 decay times were dramatically slowed and therefore able to be resolved above the time constant of the solution exchange. Interestingly the half time varied less than 4 fold between the responses to CACA (0.46 ± 0.09 s, $n = 3$), GABA (0.70 ± 0.08 s, $n = 12$) and muscimol (1.78 ± 0.42 s, $n = 3$) in the presence of SB-205384. This is in contrast to the 200 fold range in their potencies. This suggests that the modulation by SB-205384 is relatively independent of the agonist used.

As the slowing of channel closing did not seem to be due to a direct effect on agonist binding, we next examined the effect

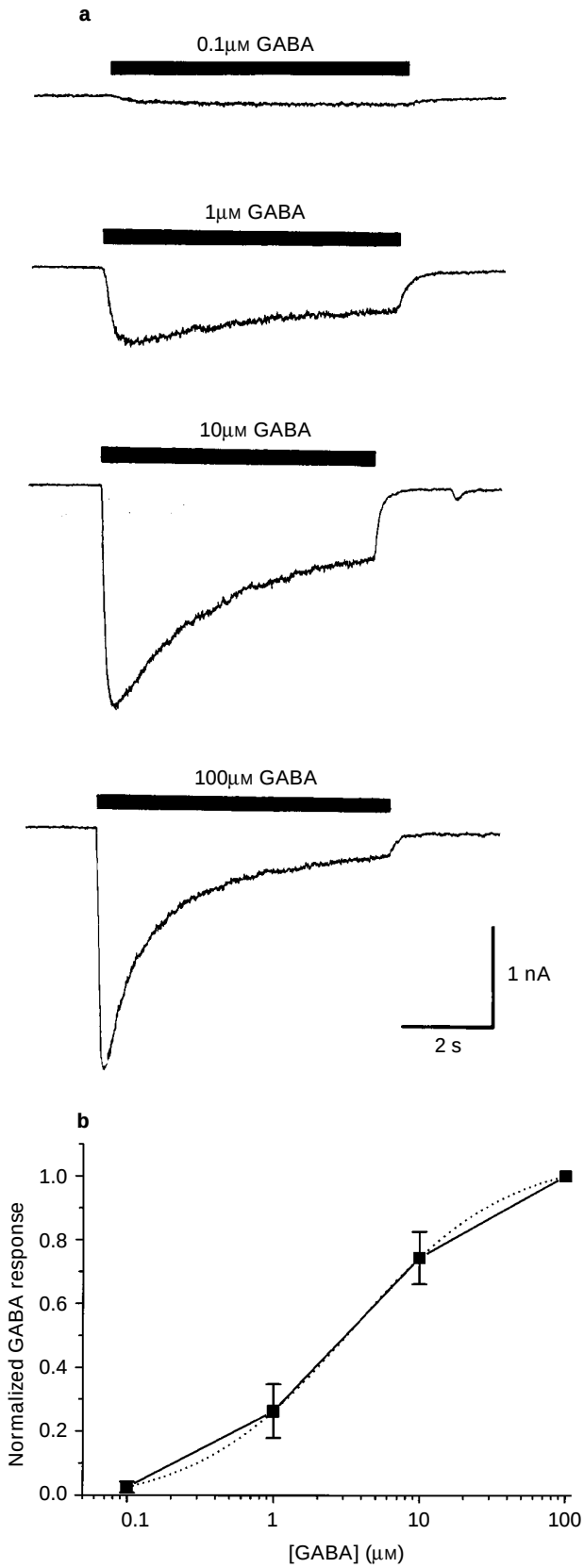

Figure 2 Concentration-response data for GABA-evoked currents recorded from rat cerebellar granule cells, holding potential −60 mV. (a) Typical current responses evoked by application of increasing concentrations of GABA. All data were obtained from one cell. (b) Concentration-response curve for GABA-evoked currents. Peak inward current amplitudes were normalized to currents evoked by 100 μM GABA. Each point represents the mean and vertical lines show s.e.mean of four cells. Concentration-response curve was fitted to the logistic equation by Origin software, EC_{50} 3.4 μM.

Figure 3 Effects of diazepam and SB-205384 on GABA-evoked inward currents in cerebellar granule cells. Holding potential −60 mV. Horizontal bars indicate the period of GABA or GABA plus modulator application. All data were obtained from one cell.

of a desensitizing GABA concentration on the action of SB-205384. We compared the actions of SB-205384 on responses elicited to 1 μM GABA, which showed little desensitization (Figures 2a and 3) and to 100 μM GABA, which showed rapid desensitization decaying to less than 10% of the peak response during 4 s application (Figure 2a). Amplitudes of peak and plateau current evoked by 1 μM GABA were altered to a similar degree by 10 μM SB-205384. The peak amplitude was increased to 252±48% of the control peak response ($n=7$), whilst the plateau was increased to 233±49% control plateau response ($n=7$). In contrast the currents induced by 100 μM GABA were inhibited by 10 μM SB-205384, with the plateau current showing a greater reduction to 67±5% of control plateau current, $n=4$, than the peak of the response (86±4% of control peak response, $n=4$). Responses to 100 μM GABA had a greater half-life of decay (0.45±0.05 s ($n=4$)) than responses to 1 μM GABA (0.15±0.03 s ($n=7$)). Coapplication of 10 μM SB-205384 still increased the half-life of decay of the response to 100 μM GABA to 3.24±0.41 s ($n=4$). For comparison the half-life of decay for coapplication with 1 μM GABA was 1.64±0.25 s ($n=7$). Thus SB-205384 did not increase the maximum current evoked by 100 μM GABA but the half-life decay was dramatically increased, as seen at the lower dose of 1 μM GABA.

Discussion

The present study shows that the main action of SB-205384 on GABA-gated chloride currents in cerebellar granule cells is to slow significantly the decay of current following agonist removal, in contrast to the dominant action of many existing GABA channel modulators of potentiating peak current.

In the absence of modulators GABA-gated currents show complex decay rates. Puia *et al.* (1994) used outside-out patches

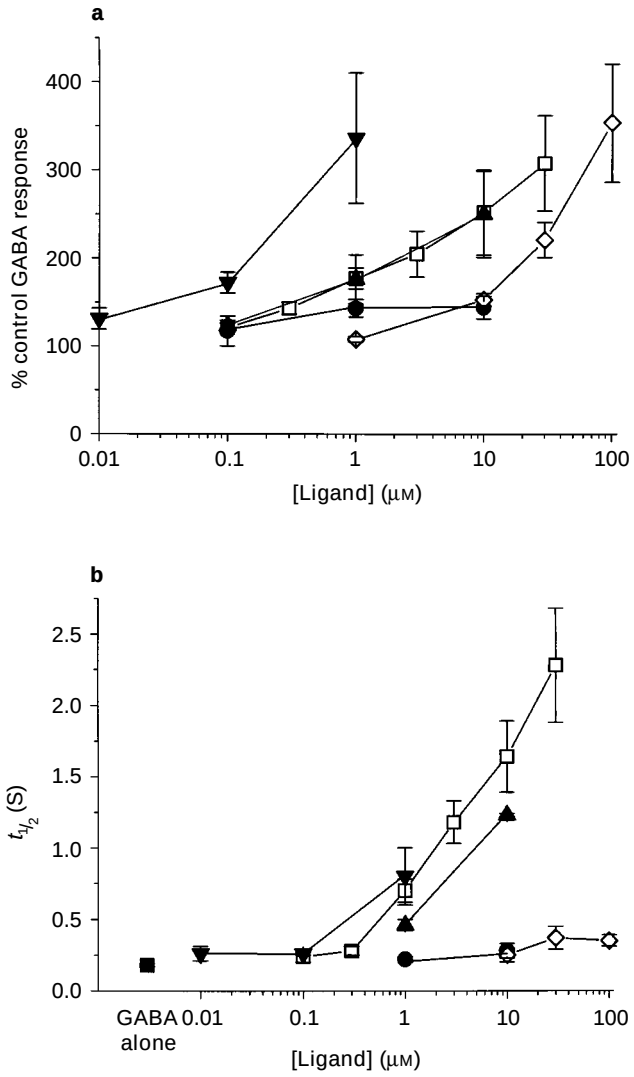

Figure 4 Effects of SB-205384 (□), diazepam (●), pentobarbitone (◇), THDOC (▼) and tracazolate (▲) on (a) peak current amplitude and (b) half-time of decay of GABA-activated inward currents. Values of peak amplitude were expressed as % control GABA response i.e. >100% indicates potentiation. Plotted values were the mean and vertical lines show s.e.mean of 3–33 repeats.

from granule cells in cerebellar slices to show two components to GABA currents of 3 and 70 ms (the 70 ms component representing less than 50% of decay). Time constants of 10 and 170 ms have been recorded from granule cells in culture (Maconochie *et al*., 1994). In both studies short millisecond applications of 1 mM GABA were used. A study with cultured hippocampal neurones has revealed a similar pattern of behaviour (Jones & Westbrook, 1995). These results suggest that the slow component of deactivation (time constant, 100–200 ms) is in fact a result of entry of channels into a desensitized state from which the channel can reopen before closing. Our experimental protocol used lower GABA concentrations at longer application times, a protocol that is also likely to produce a significant slow component to deactivation.

Existing GABA modulators, such as diazepam and pentobarbitone that have been studied in detail, either increase GABA affinity (diazepam) or stabilize channels in the open state increasing mean open time (pentobarbitone). Neither of these molecular actions results in a dramatic slowing of macroscopic channel closing.

We chose to measure the half-time of decay rather than multiexponential rate constants as we lacked very fast temporal resolution and the microscopic kinetics in a system where several types of GABA channel are expressed is very complex. This has the advantage of clearly representing the dominant time constant in one value, but the half-time of decay does not directly relate to multiple reaction rates in kinetic models. Hence, we have not tried to present a detailed kinetic description of the mode of action. However, the profound slowing of decay rate by SB-205384 suggests that the GABA-gated channels have been stabilized in a set of states oscillating between open and desensitized such that ultimate channel closing was very much slowed. If so, the slow current decay rate reflects the slow exit of channels from the drug bound desensitized state to the open state and then their rapid closing. In the presence of GABA the mean P_o of the channels was little changed by SB-205384 so that the macroscopic current showed little potentiation. SB-205384 had similar effects on current decay if most of the channels were driven into the desensitized state by application of high concentrations of GABA (100 μM). This suggests that the drug bound state can be entered readily from both open and desensitized states. The differential effects on peak current at low and high GABA concentrations appear complex but could be explained by a dual effect of small increases in channel mean open time increasing peak current at low GABA concentrations and a shift in the equilibrium to favour desensitization, which will predominate and tend to decrease current at higher GABA concentrations.

The observation that SB-205384 slows decay rates significantly when CACA was used as agonist supports a lack of effect on agonist affinity. The resolution of our recording system was not sufficiently fast to see the faster unmodulated decay rate that would be expected for CACA, which was 200 fold less potent than GABA and therefore will unbind faster. If SB-205384 was acting at this step it would be expected that the modulatory effect would be agonist-dependent, such that any slowing of CACA current decay would also be fast to detect in our system.

The main conclusion from our analysis is that it is possible to increase the duration of a transient GABA response with very little effect on peak amplitude – this occurred at low and high GABA concentrations. The implications on synaptic function of various modulatory mechanisms of GABA channel gating have been reviewed by Mody *et al*. (1994). As GABA channel kinetics rather than the duration of GABA in the synaptic cleft are most important for determining the time course of the synaptic current, direct modulation of GABA channel kinetics to increase the duration of inward current is likely to have a significant effect on GABA-activated inhibitory postsynaptic currents. Some indication of the behavioural effects of this form of modulation may be derived from *in vivo* studies on the effects of tracazolate which showed similar modulatory properties to SB-205384. Tracazolate has been shown to be anxiolytic and the main differences in its profile of activity as compared to diazepam is a less potent sedative action and weaker interaction with ethanol (Goldberg *et al*., 1983).

In addition to their unique kinetic properties, the overall CNS activity of any agent will also be dependent on the subunit specificity of the compound. $GABA_A$ channels exist as pentameric structures composed of a variety of subunit combinations with specific CNS localization (reviewed by Wisden & Seeburg, 1992). Investigating the activity of SB-205384 on $GABA_A$ channels in recombinant expression systems will provide data on subunit
specificity.

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