Recombinant $GABA_A$ receptor desensitization: the role of the $\gamma 2$ subunit and its physiological significance

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1. The purpose of these investigations was to examine the role that the $\gamma 2$ subunit plays in human $GABA_A$ receptor desensitization. Two different recombinant $GABA_A$ receptors ($\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$) were compared by measuring the relaxation of whole-cell currents during the application of GABA, isoguvacine or taurine.

2. At concentrations which trigger a maximum response (100–500 μM GABA) the current relaxation usually fitted the sum of two exponentials. For $\alpha 1\beta 3$ subunit receptors these values were $\tau_1 = 145 \pm 12$ ms and $\tau_2 = 6{\cdot}3 \pm 2{\cdot}1$ s (means ± S.E.M.). Receptors consisting of $\alpha 1\beta 3\gamma 2$ subunits desensitized faster: $\tau_1 = 41{\cdot}6 \pm 8{\cdot}3$ ms and $\tau_2 = 2{\cdot}4 \pm 0{\cdot}6$ s.

3. The Hill slope, determined for each receptor subunit combination, was the same and greater than 1·0, implying two binding steps in the activation of both receptor subunit combinations.

4. For $\alpha 1\beta 3$ subunit receptors the fast desensitization rates were unaltered by reducing the GABA concentration from the EC_{100} (100 μM) to the approximate EC_{50} values (10–20 μM), whereas for $\alpha 1\beta 3\gamma 2$ subunit receptors a significant slowing was observed. The fast desensitization disappeared at agonist concentrations below the EC_{50} for both subunit combinations. In contrast, the slow desensitization appeared at agonist concentrations near the EC_{20}. This rate was dependent on agonist concentration reaching a maximum near the EC_{60} value of GABA.

5. The fast desensitization rates were unaltered by changing the holding potential of the cell during agonist application. However, for $\alpha 1\beta 3\gamma 2$ subunit receptors the slow desensitization rate increased by approximately 15- to 20-fold over the range of voltages of −60 to +40 mV. This indicates that the $\gamma 2$ subunit makes $GABA_A$ receptor desensitization voltage dependent.

6. Recovery from desensitization was also biphasic. The first recovery phase was faster for $\alpha 1\beta 3\gamma 2$ than for $\alpha 1\beta 3$ subunit receptors (0·13 *vs.* 0·03 s^{-1}, respectively). The second phase of recovery for the two receptors were the same (~0·003 s^{-1}).

7. There was only a poor correlation between agonist potency and the degree or time course of desensitization. Isoguvacine ($EC_{50} \approx 10$ μM) induced biphasic relaxation for both $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ subunit receptors ($\tau_1 = 288{\cdot}6 \pm 43{\cdot}3$ and 167 ± 15 ms, and $\tau_2 = 8{\cdot}0 \pm 1{\cdot}9$ and $4{\cdot}4 \pm 0{\cdot}4$ s, respectively, for each subunit combination). Taurine ($EC_{50} \approx 7$ mM) usually induced monophasic relaxation for both subunit combinations ($\tau_2 = 7{\cdot}1 \pm 1{\cdot}6$ and $23{\cdot}0 \pm 6{\cdot}6$ s, respectively).

8. A computer model was developed to examine the effect of the $\gamma 2$ subunit on the time course of a synaptic potential. It was found that the $\gamma 2$ subunit theoretically prolongs the time course of a synaptic potential by inducing desensitization more rapidly. The subsequent relaxation of the desensitized receptors through the open state increases P_{open} (the probability that the $GABA_A$ receptor is in an open conducting state) altering the time course of the modelled potential. $\alpha 1\beta 3$ subunit receptors do not desensitize sufficiently rapidly to induce this desensitized state and, therefore, are shorter in time course. These data imply that the physiological role of the $\gamma 2$ subunit is to increase synaptic efficacy by prolonging P_{open}.

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In recent years molecular cloning studies in many species have identified a family of proteins which form the $GABA_A$ receptor complex. These have been classified according to their amino acid homology as belonging to one of four different subunit groups, α, β, γ and δ. In the vertebrate central nervous system (CNS) all these subunits, except the δ subunit, exist as subtypes. There are six α subunits (not including two splice variants of $\alpha 6$), four β subunits and three γ subunits (not including two splice variants of $\gamma 2$; see Burt & Kamatchi, 1991 for review). The expression of these subunits in host cell systems, forming recombinant $GABA_A$ receptors, has shown that it is likely that the majority of native $GABA_A$ receptors are heteroligomer complexes composed of at least an $\alpha\beta$ combination along with a γ and/or δ subunit. The exact stoichiometry remains controversial (see Burt & Kamatchi, 1991 for review).

A great deal of work has been done to learn how this molecular diversity defines the pharmacological properties of the $GABA_A$ receptor. Many studies have shown that the pharmacological properties of recombinant $GABA_A$ receptors are changed by varying subunit expression in host cells (Sigel, Baur, Gerhard, Möhler & Malherbe, 1990; Verdoorn, Draughn, Ymer, Seeburg & Sakmann, 1990; Burt & Kamatchi, 1991). For example, the benzodiazepine sensitivity varies greatly when the α subunit expression is altered (Pritchett, Luddens & Seeburg, 1990). The physiological significance of this differential expression of subunits in the CNS has recently begun to be addressed (Verdoorn, 1994; Gingrich, Roberts & Kass, 1995). For example Gingrich *et al.* (1995) have shown that the substitution of an $\alpha 3$ subunit for an $\alpha 1$ subunit slows the gating kinetics and desensitization of the recombinant receptors. This change was examined in a computer model and theoretically these attributes altered the time course of a modelled synaptic potential. Therefore, it seems reasonable to suppose that the differential expression of $GABA_A$ receptor subunits is important in governing the time course of a synaptic potential. Three different functional attributes would be important: (1) the rate at which the receptor activates after the increase in the GABA concentration in the synaptic cleft; (2) the inactivation of the receptor as a result of the decline in the GABA concentration; and (3) the rate of receptor desensitization. This final attribute, desensitization, has been found to be very important for limiting the time course of fast glutamatergic synaptic transmission (Trussel & Fischbach, 1989). In contrast, and perhaps paradoxically, desensitization has been shown to be important in prolonging the time course of GABAergic synaptic transmission (Jones & Westbrook, 1995). In this study the authors have provided evidence that desensitization produces a population of receptors which can reopen from a temporary desensitized state thus prolonging the probability that the channels can be open.

A great deal of attention has been focused on the functional importance of the γ subunits of the GABA receptor primarily from a pharmacological point of view. This is because γ subunits confer sensitivity to the benzodiazepine (BDZ) family of compounds (Pritchett *et al.* 1989). However, it has also been shown that γ subunits have profound affects on the biophysical behaviour of GABA receptors and therefore these subunits appear to have a physiological importance as well. Angelotti & Macdonald (1993) have shown that $\alpha 1\beta 1\gamma 2$ GABA receptors have a higher affinity for GABA, longer channel mean open times and a larger unitary channel conductance in comparison with receptors composed of $\alpha 1\beta 1$ subunits. In addition, Verdoorn *et al.* (1990) have provided evidence that the $\gamma 2$ subunit regulates desensitization. However, the subunit combination used in this study is likely to be rare *in vivo* because it lacked a β subunit. Therefore, the exact contribution of the γ subunits, particularly $\gamma 2$, to the desensitization properties of the native receptor remains unexplored. For this reason and because the $\gamma 2$ subunit is by far the most abundant γ subunit expressed in the CNS (Wisden, Laurie, Monyer & Seeburg, 1992), we chose to investigate its contribution to the desensitization kinetics of the $GABA_A$ receptor.

To do this we used recombinant $GABA_A$ receptors expressed in the human embryonic kidney cell line 293 (HEK-293). We have compared the desensitization kinetics of whole-cell currents produced by $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ subunit receptors. Since we were working in a relatively non-physiological context we developed a computer model to extrapolate our results to the potential physiological importance of the desensitization kinetics and the role of the $\gamma 2$ subunit. In this way, we were able to examine how the desensitization process alters the time course of a synaptic potential and how the kinetics of the $\alpha\beta\gamma$ receptor combination are important. Results of the study have been reported in a preliminary form (Poulter, Dominguez-Perrot & Feltz, 1996).

METHODS

Cell culture and transfection

Human embryonic kidney-293 cells (HEK-293; American Type Culture Collection, CRL 1573, Rockville, MD, USA) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum (HS; Gibco BRL, France) and split every 4–5 days upon reaching confluency. For transfection, dishes were treated with 100 $\mu g\ ml^{-1}$ of poly-L-ornithine (PO; Sigma) solution 24 h before plating. Cells were plated at approximately 30% of confluence in the PO-treated 35 mm plastic culture dishes. After allowing another 24 h for the cells to adhere and begin exponential growth they were then ready for transfection. Rat GABA receptor subunit cDNAs coding for the $\alpha 1$, $\beta 3$ and $\gamma 2L$ (hereafter simply referred to as $\gamma 2$) subunits were cloned into the expression vector pGW1 (obtained from Dr. Steven Moss, University College London, UK; cf. Moss *et al.* 1991). A total of 3–4 $\mu g\ ml^{-1}$ GABA receptor subunit DNA was precipitated using a standard $Ca_3(PO_4)_2$ precipitation protocol (Sambrook, Fritsch & Maniatis, 1989) and was used to incubate the HEK-293 cells for 20–24 h, after which time the cells were washed twice with phosphate-buffered saline and finally medium (DMEM and/or 10% horse serum) was added. The cells were left for 24–72 h during which time they were used for recordings. Transfection efficiency

was approximately 30%. Finally, we checked for the identity of the expressed receptors by testing for BDZ sensitivity in cells co-transfected with $\alpha1\beta3\gamma2$ subunits. Invariably, if a cell responded to GABA, the response was potentiated by diazepam. This is similar to the observations of Angelotti & Macdonald (1993) who found that conductance states corresponding to the $\alpha\beta$ subunit receptor were never found in cells transfected with an $\alpha\beta\gamma$ combination. This indicates that the transfection process is an all-or-nothing phenomenon and that $\alpha\beta\gamma$ subunits tend to associate in preference to $\alpha\beta$ subunit receptors. Thus we were confident that the receptor populations were homogeneous.

Solutions

During experiments the extracellular medium contained (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; and Hepes, 10; (titrated to pH 7·2 with NaOH). The patch pipette solution contained (mM): CsCl, 140; NaCl, 18; MgATP, 2; EGTA, 1·1; glucose, 10; and Hepes, 10; (titrated to pH 7·2 with NaOH). All drug solutions were prepared from concentrated stocks diluted with the appropriate amount of extracellular medium. GABA and taurine were obtained from Sigma. Isoguvacine was obtained from Peninsula Laboratories GmbH, Heidelberg, Germany.

Perfusion technique and drug application

Drugs used in this study were applied by a modified U-tube apparatus similar to that described by Krishtal & Pidoplichko (1980). Here we greatly increased the diameter of the hole in the U-tube to approximately ~200–300 μm creating a flow rate of 1–2 ml min^{-1} during the application of the drug. To initiate flow from the tube we clamped the out-flow tube, either manually or with a solenoid valve. We calibrated this application in a manner similar to that of Vyklicky, Benveniste & Mayer (1990) and found that the rise time of the liquid-junction potential created at the tip of the open electrode was ~1–5 ms (mean 3·2 ± 1·6 ms). Using the highest concentrations of GABA, the rise time of whole cell currents was usually around 10 ms (but it could be as short as 5 ms). These activation rates were similar to those reported for other rapid perfusion techniques on whole cells (Vyklicky *et al.* 1990). Where possible the duration of the drug application was always 3–5 times longer than the time constant of the longest decay phase. Dishes were constantly perfused during recordings with extracellular medium at a rate equal to the uptake of the medium by the U-tube. Several reservoirs attached to the in flow of the U-tube allowed us to apply numerous drug solutions at varying concentrations.

Electrophysiological recordings

Recordings from transfected cells (usually 20–35 μm in diameter) were made at room temperature (18–22 °C). The dish was placed in a holder on an inverted modulation-contrast microscope. Whole-cell patch-clamp recordings were made using a List EPC-7 patch-clamp amplifier. The patch-pipette electrodes had resistances between 1·5 and 4 MΩ. Seals between the electrode and the cell membrane were between 1·5 and 3·5 GΩ. Access resistances were between 2·5 and 10 MΩ. Recordings with access resistances greater than 10 MΩ were discarded. Series resistance and whole-cell capacitance were compensated using the feedback circuit of the EPC-7. Compensation was usually 80% but never less than 60%. All data were obtained from isolated cells because it is known that these cells form gap junctions when they come into contact which gives rise to voltage-clamping errors and very large capacitance transients.

Acquisition and analysis

All data were stored on a pulse code modulation (PCM)-based video tape A/D system. The 10 kHz output channel of the EPC-7 was filtered at 5 kHz before being digitized at a rate of 22 kHz by the PCM (Sony model PCM-701ES). Off-line analysis was done using pCLAMP Fetchex software (Axon Instruments) in the gap-free acquisition mode. Acquisition was done at a sampling rate of 10 kHz after filtering at 2·5 kHz. Files were then analysed using a Macintosh microcomputer using AxoGraph software (Axon Instruments). The rise times of responses were calculated automatically at between 10 and 90% of their maximum. Exponential fits of the current decay were obtained using a least-squares protocol. All fits were also checked by the operator for accuracy by eye. All means are given ±standard error of the mean. Error bars in graphs represent the standard error of the means. Statistical significance was analysed by evaluating the data using Student's t test or one way ANOVA for comparison of multiple means (paired and unpaired where appropriate).

Dose–response curve data were fitted using the software GrafPad. Points were fitted to the equation:

$$y = 1/1 + (EC_{50}/[\text{agonist}])^{n_H},$$

where y is the agonist response as a percentage of the maximum response, n_H is the apparent Hill slope and EC_{50} is the agonist concentration which produces 50% of maximum response.

Computer simulations

The kinetic analysis of the activation and desensitization of the $GABA_A$ channel was modelled using the SCoP software package (Simulation Sources Inc., Berrien Springs, MI, USA). Association and dissociation rates were approximated from data reported by Maconochie, Zempel & Steinbach (1994). The desensitization rate constants were determined empirically. This modelling package allows control of both the rate of agonist application and its decay. $GABA_A$ receptor activity is represented by following the P_{open} (the probability that the $GABA_A$ receptor is in an open conducting state). Only one open state was modelled since it has been shown that for the subunit combinations used in this study, one state represents more than 90% of the openings (Angelotti & Macdonald, 1993).

RESULTS

The results described below are from more than 200 recordings from HEK-293 cells transfected with either $\alpha1\beta3$ or $\alpha1\beta3\gamma2$ GABA receptor subunits. All the data reported below are taken from recordings where the rise time of a response to a supramaximal GABA concentration was less than 25 ms for $\alpha1\beta3$ subunit receptors and 20 ms for $\alpha1\beta3\gamma2$ subunit receptors. Pulses of drug solution were applied to cells at regular intervals in all experiments for a duration of 2–30 s, depending on the information needed. Usually, the pulses were separated by 2 min, but this was often increased when the maximum amplitude was not reproducible. No doubt there was some 'tonic' desensitization (especially in light of the data shown in Fig. 7) but this was difficult to assess and so regular pulses of agonist were applied as the best way to eliminate this variability.

As shown in Fig. 1 the current relaxation was mostly biphasic for both receptor types. However, sometimes a third phase of desensitization could also be detected. But owing to its length (usually slower than $\tau = 50$ s) it could not be accurately defined from the responses lasting 10–30 s. Longer application times were not practically possible, owing to the flow rate from the U-tube which

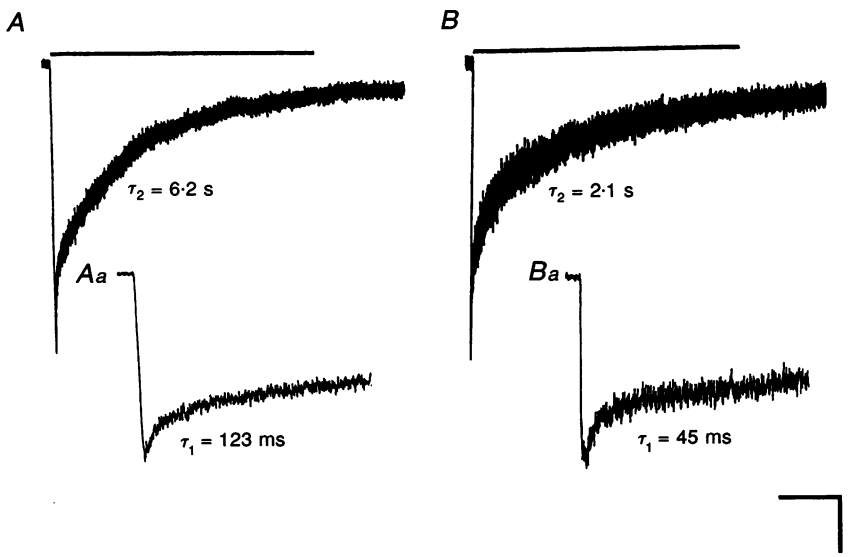

Figure 1. Desensitization of the two different GABA receptor subunit combinations
Responses to 100 μM GABA in both *A* and *B*. Receptors composed of $\alpha1\beta3$ subunits (*A*) desensitized more slowly than receptors composed of $\alpha1\beta3\gamma2$ subunit receptors (*B*). *Aa* and *Ba* show in detail the first phase of desensitization. Calibration: in *A* and *B*, time, 3 s; amplitude, 150 pA and 300 pA, respectively. In *Aa* and *Ba*, time, 250 ms; amplitude, 200 and 400 pA, respectively.

flooded the dish with agonist forcing very long wash times. Furthermore, we considered that the length of this third relaxation process was so slow that it is probably physiologically irrelevant. At agonist concentrations equal to or exceeding the EC_{100}, the fast relaxation (τ_1) for $\alpha1\beta3$ subunit receptors had a time course of $145 \pm 12{\cdot}6$ ms; $n = 7$) whereas in $\alpha1\beta3\gamma2$ subunit receptors it was $41{\cdot}6 \pm 8{\cdot}3$ ms ($n = 8$). The second time constant of the relaxation was in the order of seconds. Similar to the results found for the fast time constant, the $\alpha\beta$ subunit receptor currents relaxed more slowly than $\alpha1\beta3\gamma2$ subunit receptor currents ($6{\cdot}3 \pm 2{\cdot}1$ s, $n = 7$ *vs.* $2{\cdot}4 \pm 0{\cdot}6$ s, $n = 8$, respectively). The fast component represented $14 \pm 1{\cdot}6\%$ of the total relaxation in $\alpha\beta$ subunit receptors in contrast to

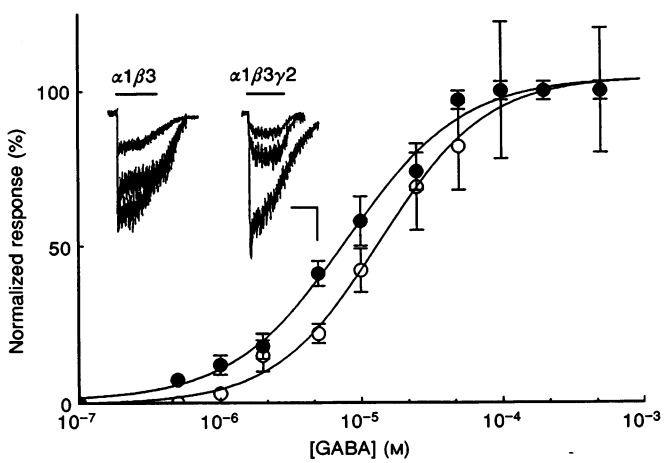

Figure 2. Normalized dose–response relationships for both receptor types
Responses are to 2, 25 and 100 μM GABA for $\alpha1\beta3$ subunit receptors and 2, 5 and 100 μM GABA for $\alpha1\beta3\gamma2$ subunit receptors. Both curves had Hill coefficients greater than 1, indicating that activation of each receptor is a bimolecular process. ○, $\alpha1\beta3$, $EC_{50} = 13 \pm 0{\cdot}1$ μM; ●, $\alpha1\beta3\gamma2$, $EC_{50} = 7 \pm 0{\cdot}1$ μM. Calibration: time, 2 s; amplitude, 50 and 100 pA for $\alpha1\beta3$ and $\alpha1\beta3\gamma2$, respectively.

it being 33 ± 1·3% of the desensitization in $\alpha1\beta3\gamma2$ subunit receptors. The steady state : peak ratios at the pseudo-steady state (5–30 s after the start of the pulse) was 0·20 ± 0·1 for $\alpha1\beta3$ receptors and 0·15 ± 0·1 for $\alpha1\beta3\gamma2$ receptors. The maximum rise time of the whole-cell current response from the two receptor types were different as well. Using agonist concentrations at or above EC_{100}, mean rise times were 18·7 ± 2·9 and 9·7 ± 1·4 ms for $\alpha1\beta3$ ($n = 7$) and $\alpha1\beta3\gamma2$ ($n = 20$; $P < 0·03$) subunit receptors, respectively. This implies that $\alpha1\beta3$ subunit receptors activate at least twice as slowly as $\alpha1\beta3\gamma2$ subunit receptors.

In these initial experiments, the maximum current elicited from these transfected cells was 416 ± 59 *versus* 1190 ± 160 pA, for $\alpha1\beta3$ and $\alpha1\beta3\gamma2$ transfected cells, respectively. The wide variability in the maximum currents probably reflects the variability of the success of the transfection and/or expression efficiency. It also reflects the time in culture before the cells were used. Cells expressing $GABA_A$ receptors after 24 h in culture tended to have smaller currents than cells that had spent longer times in culture after transfection. No correlation was found between the time constants of the relaxation and current amplitudes. Therefore, if a shift in the equilibrium potential for GABA (E_{GABA}) is occurring in cells having a large current flow, the redistribution of the chloride-ion gradient does not correlate with the current relaxation. This observation is in agreement with that of Frosch, Lipton & Dichter (1992) who showed that altering the chloride-ion concentration had little effect on $GABA_A$ current relaxation.

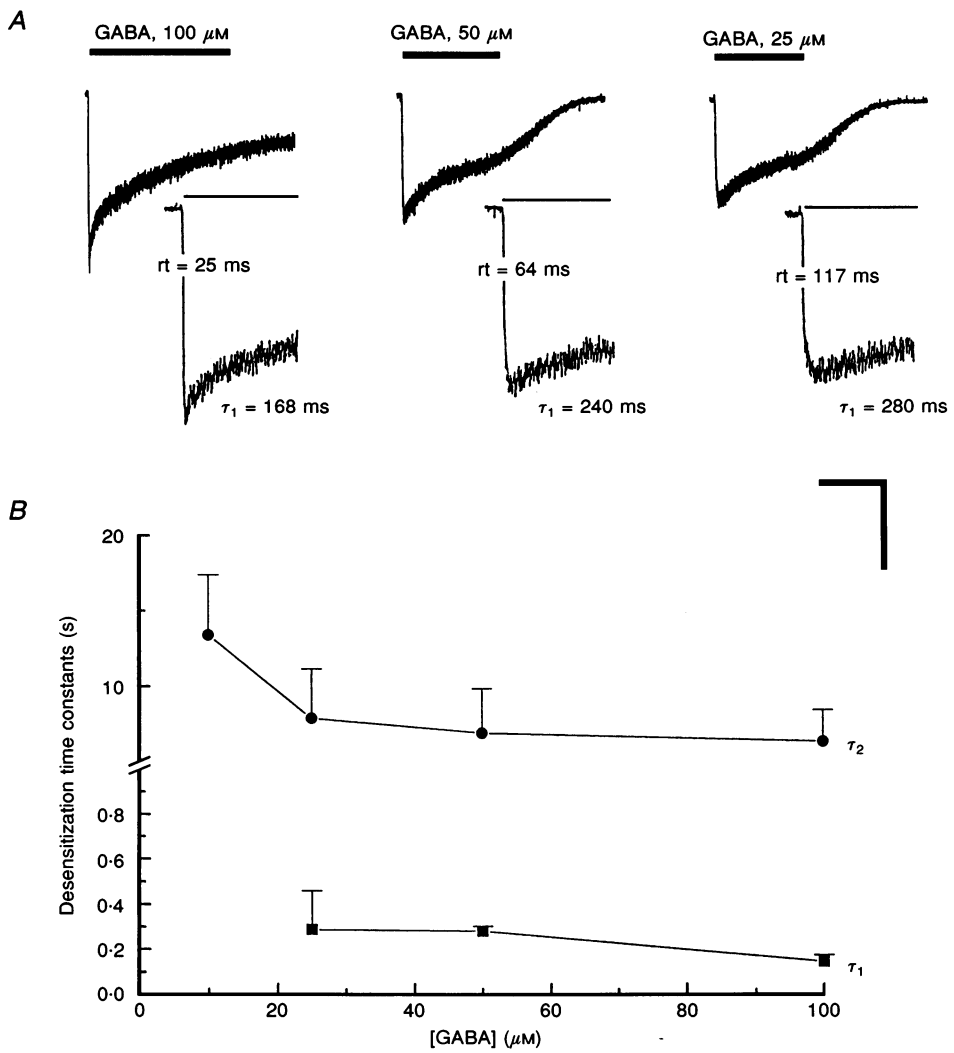

Figure 3. Concentration dependence of desensitization for $\alpha1\beta3$ subunit receptors

A, the fast rates of desensitization change little over the concentration range of 25–100 μM GABA. Insets show the same currents with a faster time scale (rt = rise time between 10 and 90% of maximum). *B*, graph showing the relationship between GABA concentration and the magnitude of the desensitization time constants. Calibration for *A* and insets, respectively: time, 1·5 s and 200 ms; amplitude, 70 pA and 100 pA.

Dose–response relationship

The dose–response relationship for the two receptor types was constructed in order to compare the kinetic properties of the activation of the receptor types under our experimental conditions. Most studies using recombinant $GABA_A$ receptors have indicated that the Hill slopes of the $\alpha\beta$ subunit receptors are close to 1·0 (Burt & Kamatchi, 1991), which implies a single binding step before activation. We hypothesized that these slopes reflect the slow application rates used in these studies. At high agonist concentrations significant desensitization was produced. This 'flattens' the dose–response relationship and the Hill slope. This appears to be the case since with our relatively fast application procedure we found that the Hill slope for both $\alpha1\beta3$ and $\alpha1\beta3\gamma2$ subunit receptors were the same and greater than 1·0 (1·3 ± 0·1 and 1·4 ± 0·1, respectively). This implies that there is more than one binding step before activation for both receptor types. Our dose–response curves fitted best with an EC_{50} of 13 ± 0·1 and 7 ± 0·1 μM for $\alpha1\beta3$ and $\alpha1\beta3\gamma2$ subunit receptors, respectively (Fig. 2; $n = 7–12$ cells for each curve).

Concentration dependence

For both receptor subunit combinations in the range of GABA concentrations corresponding to the EC_{50} and the EC_{100} there were two phases of desensitization which were

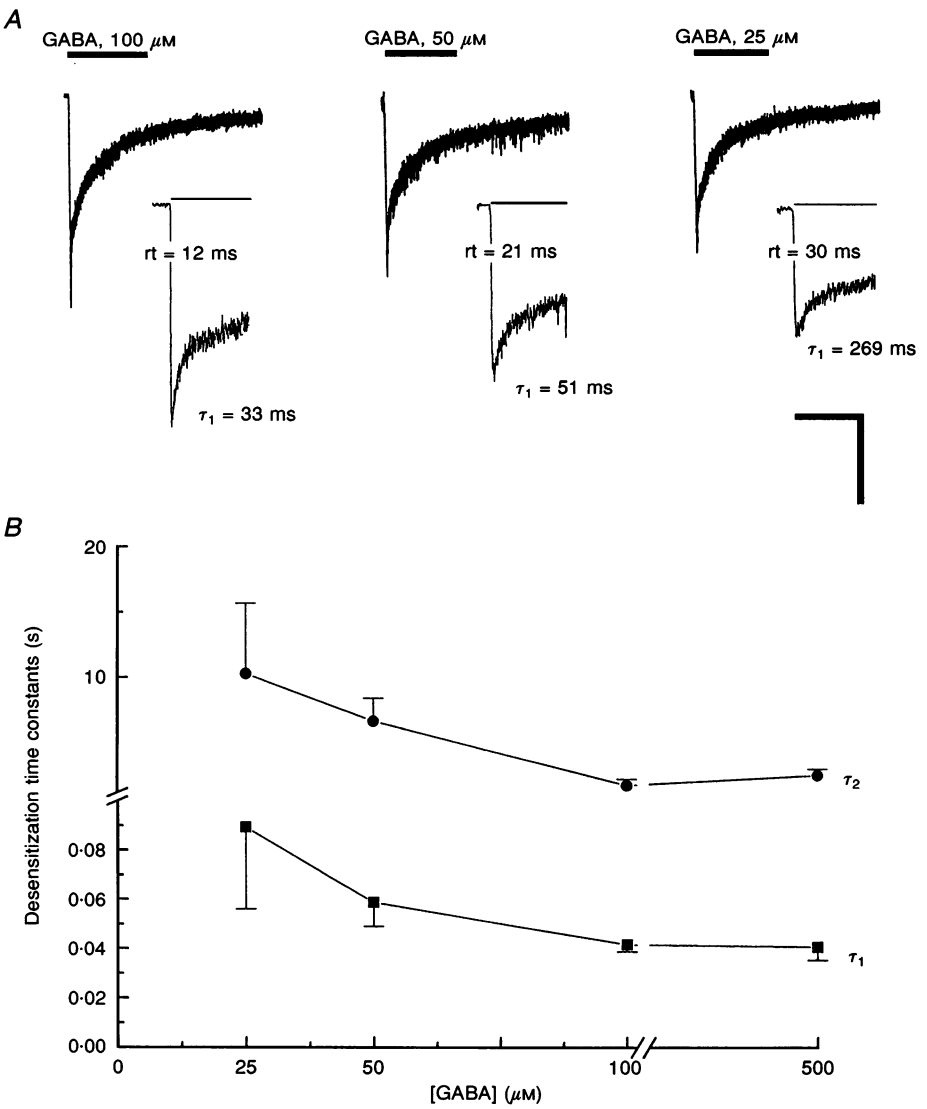

Figure 4. Concentration dependence of desensitization for $\alpha1\beta3\gamma2$ subunit receptors

A, the fast rate of desensitization slows with decreasing agonist concentration. Insets show detail of the same currents with a faster time scale. Calibration for *A* and insets, respectively: time, 5 s and 250 ms; amplitude, 200 and 250 pA. rt, rise time between 10 and 90% of maximum. *B*, graph showing the relationship between GABA concentration and the magnitude of the desensitization time constants.

immediately discernible during the 2–5 s pulses used in these experiments. Also evident over this concentration range was the decrease in the rise time as might be expected (cf. Maconochie *et al.* 1994). Figure 3*A* shows three responses to different concentrations of GABA applied to an $\alpha1\beta3$ subunit receptor-expressing cell. For the first two applications (100 and 50 μM) there was no statistically significant change in the fast rate of desensitization although the fast rate was less pronounced for the 50 μM application (146·0 ± 31 *versus* 224·6 ± 27 ms; $n = 7$; see inset traces in Fig. 3*A*). There was a decrease in the fast rate of desensitization for 25 μM GABA-induced responses in some cases. However, this phase was very minor and it was not possible to fit it accurately. This fact is reflected by the wide variability in the mean fitted value (228·8 ± 93 ms). None of these changes reached statistical significance in comparison with one another. A plot of the fitted time constants *versus* the GABA concentration is shown in Fig. 3*B* ($n = 7$ cells). In contrast, the fast desensitization of receptors made from $\alpha1\beta3\gamma2$ subunits appeared to be more clearly dependent on GABA concentration (Fig. 4*A* and its insets). For 100, 50 and 25 μM GABA the fast rate of desensitization was 41·6 ± 2·8, 59·2 ± 4·7 and 89·4 ± 33·3 ms, respectively. All these values are significantly different from one another ($P < 0·02$). Figure 4*B* shows a plot of these results obtained from eight or nine cells. However, as noted above, there was also a pronounced slowing of the activation rates for this receptor as well. This effect may account for the apparent concentration dependence of the fast phase for the $\alpha1\beta3\gamma2$ subunit receptor since the reduced activation rate might obscure a constant fast desensitization rate. For both receptors at concentrations less than the EC_{50} the desensitization was usually monophasic with only a slow, seconds-long rate of desensitization evident. For both results the slow rates of desensitization also appeared to be sensitive to agonist concentration. Very slow rates ($\tau_2 >$ than 100 s, not graphed) of desensitization were evident at concentrations near the EC_{20}. These rates became more rapid with increasing agonist concentration but this effect plateaued at concentrations near the EC_{60} (Fig. 3*B* and 4*B*).

Voltage dependence and recovery

The voltage dependence on the desensitization rates was examined by varying the holding potential between −100 and +40 mV in 20 mV increments and applying GABA for 10–30 s. For $\alpha1\beta3$ subunit receptors there was no apparent voltage dependence for either component (Fig. 5). This was also true for the fast component of responses triggered in cells expressing $\alpha1\beta3\gamma2$ subunits. However, for the slow component the time constant increased significantly 15–20 times on average (51·2 ± 29·6 s; $P < 0·01$ by paired t test) over the voltage range −40 to +20 mV (Fig. 6). However, it should be noted that when the desensitization became

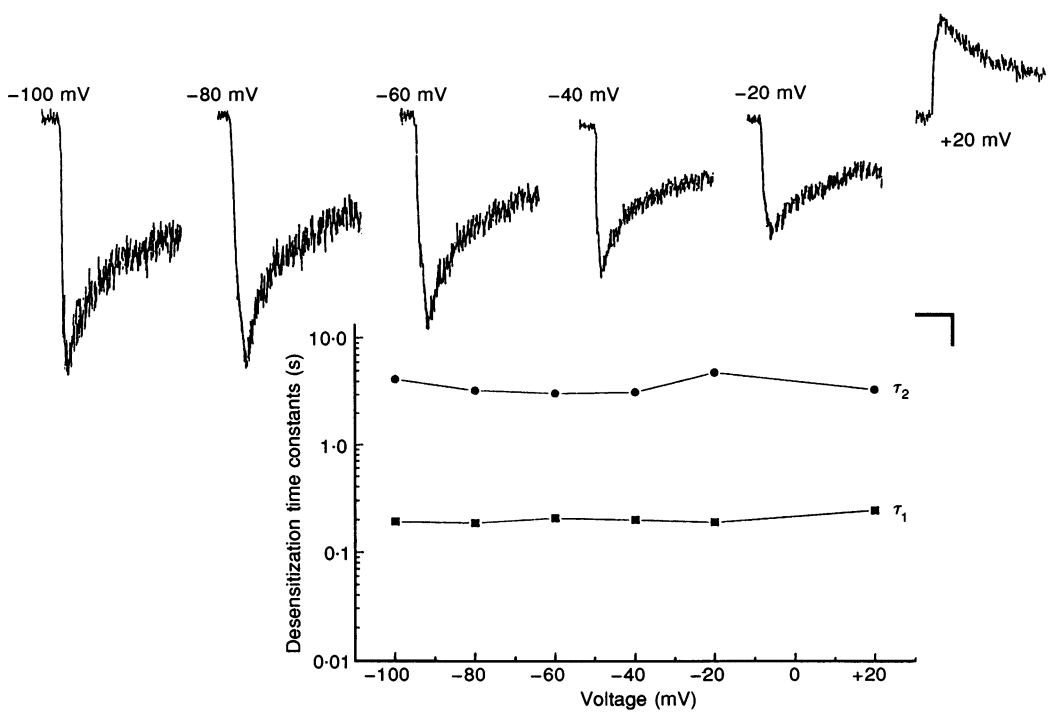

Figure 5. $\alpha1\beta3$ subunit receptor desensitization is independent of voltage

Whole-cell current responses to GABA applied to a cell clamped at different holding potentials. Graph shown is from the same cell, representative of six cells tested. Calibration: time, 200 ms; amplitude, 50 pA.

slower at depolarized potentials, the estimates of the time constants were inaccurate and the variability of the fitted values large (29–150). For this reason, only a single representative cell is shown in Fig. 6. These results indicate that the γ subunit is responsible for the voltage dependence observed in native receptors.

The recovery from desensitization was examined by giving a 1–2 s application of GABA followed by a second test pulse at various time intervals between 5 and 120 s after the return to baseline (usually within 1–2 s). Each point is from a different cell usually at a different test time. We measured the recovery by plotting the test time in seconds *versus* the negative natural logarithm of the ratio of the two amplitudes (amplitude at time t/amplitude at time 0). In both cases a clear biphasic recovery was evident (Fig. 7*A* and *B*). These two phases, which were identifiable by eye, were fitted by a linear regression. The second slow phase was in approximately the same order of magnitude for both receptors and was very slow (~0·007 s^{-1} and 0·001 s^{-1}, for $\alpha1\beta3$ and $\alpha1\beta3\gamma2$ subunit receptors, respectively). However, the first fast phase of recovery was about three times slower for $\alpha1\beta3$ receptors than $\alpha1\beta3\gamma2$ receptors. The first component for $\alpha1\beta3$ receptors was 0·03 s^{-1} ($n = 17$) *vs.* 0·13 s^{-1} for $\alpha1\beta3\gamma2$ receptors ($n = 14$).

Agonist dependence

Finally, we determined whether different $GABA_A$ receptor agonists desensitize the two kinds of receptor differently and how their potency may be related to the rate and magnitude of desensitization. We did this because we wanted to determine whether the potency is related to desensitization as is the case for AMPA/kainate receptors (Patneau, Mayer, Jane & Watkins, 1992) and how the $\gamma2$ subunit may play a role in this potential relationship. To do this, we constructed dose–response curves for two different GABA receptor agonists selected for their relative high and low potency, respectively, isoguvacine and taurine. For $\alpha1\beta3$ subunit receptors GABA and isoguvacine were roughly equipotent agonists (13 *versus* 20 μM, respectively; $n = 5–7$ cells). As expected taurine was a poor agonist having an EC_{50} of 7 mM ($n = 7$ cells). Both isoguvacine and taurine were full agonists. The maximum current amplitude for isoguvacine and taurine was 421 ± 68 and 426 ± 32 pA, respectively ($n = 5$). Figure 8*A* shows the desensitization induced by these agonists (using EC_{100} concentrations) on

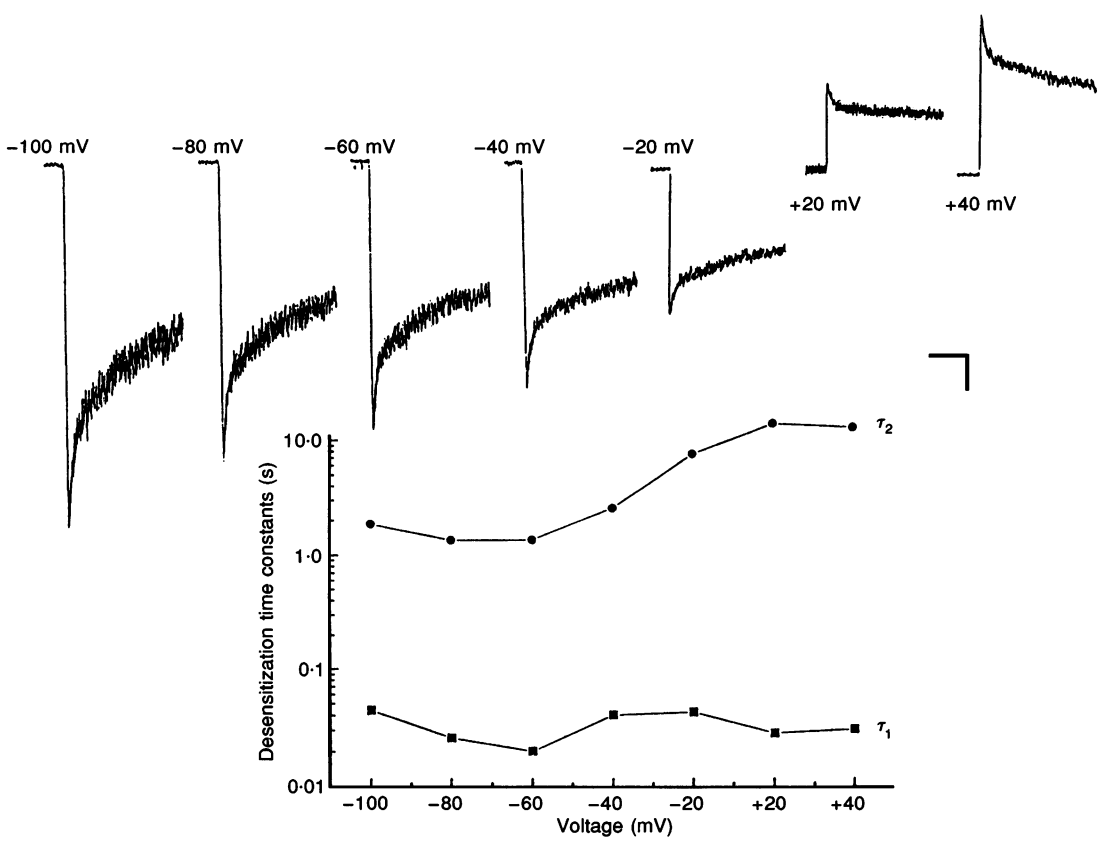

Figure 6. The slow phase of $\alpha1\beta3\gamma2$ subunit receptor desensitization is dependent on the membrane potential

Current responses to GABA application at different holding potentials. Note the slowing in the second decay phase with little change in the fast phase. Graph shown is from one cell representative of five cells. Calibration: time, 200 ms; amplitude, 90 pA.

$\alpha1\beta3$ subunit receptors. Taurine also activated the receptors slowly (352 ± 19·2 ms). All responses reached approximately the same levels of desensitization, independent of their potency (20–30% of the maximum; data not shown). Both isoguvacine and taurine induced substantially different current relaxation, both from each other and from GABA. The most striking difference was that in contrast to GABA and isoguvacine, taurine usually induced monophasic relaxation (6 out of 7 times). The time course of this slow relaxation was the same as the second phase of the desensitization of the GABA- and isoguvacine-induced relaxation (6·7 ± 2·8, 8·0 ± 1·9 and 7·1 ± 1·6 s for GABA, isoguvacine and taurine, respectively; $P < 0·2$; $n = 5–7$). In contrast, the fast phase of the isoguvacine-induced desensitization was about two times slower than the GABA-induced relaxation (288·6 ± 43·3 ms as compared with 126 ± 23·5 ms).

Similar results were obtained when these agonists were used to activate $\alpha1\beta3\gamma2$ subunit receptors. The EC_{50} values of isoguvacine and taurine were 16 μM and 9 mM, respectively. Again taurine activated the receptors slowly (in comparison with isoguvacine and GABA) but in this case taurine was not a full agonist (maximum current 886·9 ± 120 pA; $n = 9$). This was also true for isoguvacine. It could only induce responses that were 90% of the maximum GABA responses (1512 ± 136 *vs.* 1732 ± 160 pA; $P < 0·05$ by paired t test; $n = 9$). Thus the $\gamma2$ subunit altered the efficacy of taurine and isoguvacine. It also altered the time course of the second phase of desensitization for each agonist. Again, taurine had only a single slow phase of desensitization; however, it was much slower than the isoguvacine- or GABA-induced desensitization (23·0 ± 6·6, 4·4 ± 0·4 and 1·6 ± 0·2 s, respectively; $P < 0·02$; $n = 9$). The isoguvacine-induced fast phase of desensitization was faster than the rates observed in $\alpha1\beta3$ subunit receptors (167 ± 15 ms, $n = 9$; $P < 0·03$; Fig. 8*B*). However, it was slower than the GABA-induced fast phase (43 ± 4·2 ms). Also, for both receptor subtypes no apparent concentration dependence was evident for the time course of the fast phase of desensitization for isoguvacine-activated receptors (data not shown) and they all desensitized the receptor to similar levels at steady state.

Thus, the desensitization of both these GABA receptor subunit combinations have agonist-dependent characteristics. In particular, the time course of the fast phase was agonist dependent for both subunit combinations. Interestingly, the time course of the slow phase switched from being agonist independent ($\alpha1\beta3$) to agonist dependent ($\alpha1\beta3\gamma2$). Finally,

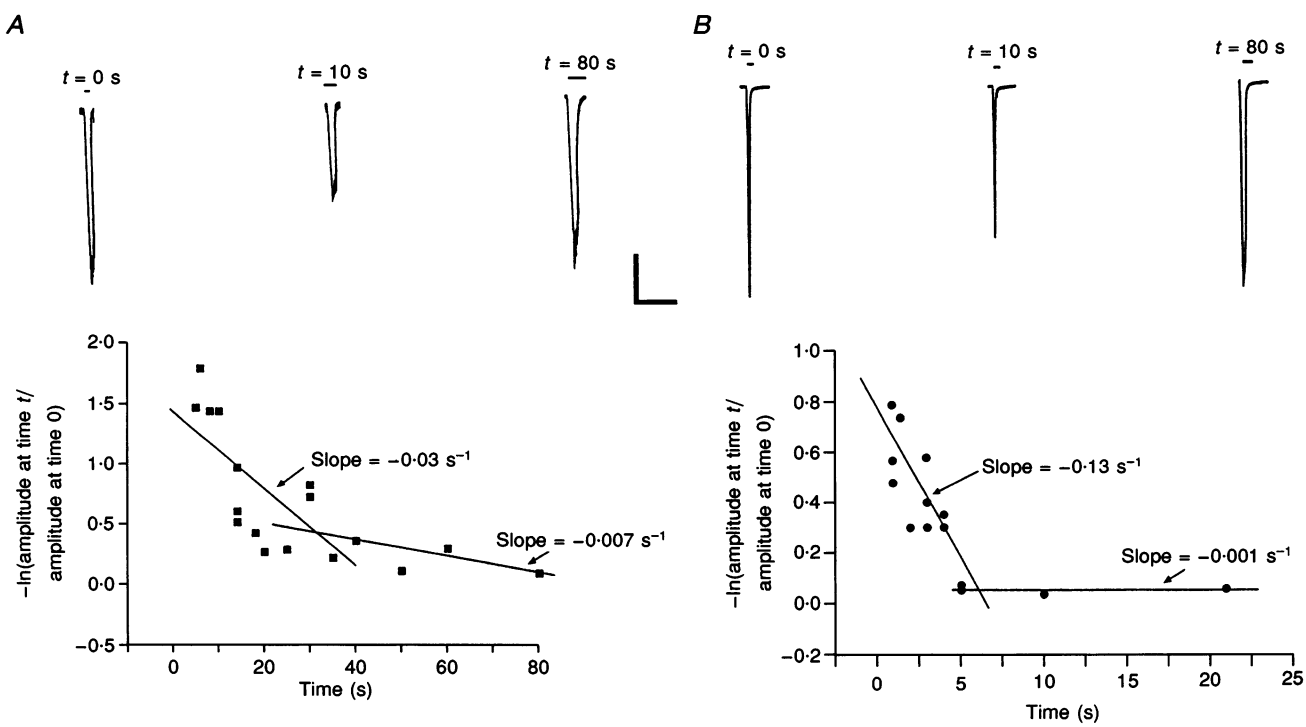

Figure 7. Recovery from desensitization for both receptor types is biphasic

A, $\alpha1\beta3$ receptor recovery; *B*, $\alpha1\beta3\gamma2$ receptor recovery. Note that the first phase of the recovery for $\alpha1\beta3$ receptors is much slower than for $\alpha1\beta3\gamma2$ receptors. Each point is from a different cell; $n = 14–17$ cells. The lines were fitted by linear regression In *A* the fast phase recovery r (goodness of fit) = −0·73, and in the second slow phase recovery $r = −0·5$. In *B* the fast phase recovery $r = −0·82$ and for the slow phase recovery $r = −0·53$. Calibration: time, 10 s; amplitude, 150 pA and 1 nA for *A* and *B*, respectively.

we should note that potency correlated poorly with both the desensitization rates and the level of desensitization. GABA and isoguvacine had EC_{50} values of approximately the same order of magnitude but the faster desensitization rates were quite different. The only correlation which could be made comes from comparing the EC_{50} values and the desensitization rates in $\alpha 1\beta 3\gamma 2$ subunit receptors. Taurine desensitized the receptor slowly and was a poor agonist in contrast to the more potent GABA and isoguvacine, which both desensitized the receptors more rapidly.

DISCUSSION

In this study we have characterized the desensitization kinetics of two different recombinant GABA receptors. The first subunit combination, $\alpha 1\beta 3$, is thought to occur only rarely in neurones. The other combination, $\alpha 1\beta 3\gamma 2$, is believed to represent a native receptor subunit combination ($\alpha x\beta y\gamma z$ where x, y and z are subunit-subtype designations). We have found, by comparing the current relaxation during agonist application, that both receptors desensitize in a predominately biphasic manner using GABA. Adding the $\gamma 2$ subunit to the 'basic' GABA receptor composed of an α and β subunit speeds the activation, desensitization and recovery processes. The $\gamma 2$ subunit also conferred voltage sensitivity on the slow desensitization phase. Using different agonists we have demonstrated that GABA-receptor desensitization has both agonist dependent and independent features. This implies that the desensitization phases must be considered as independent kinetic states. Furthermore, although we have found that the $\gamma 2$ subunit alters the efficacy of the receptors, it plays no role in the fundamental kinetics of $GABA_A$ receptor desensitization. It appears only to regulate the time course of the desensitization. Most of this discussion will concern itself with evaluating the physiological significance of the fast phase of desensitization since it is potentially the most important determinant of the time course of a synaptic potential.

Comparison with past work

The results of these experiments partly confirm the observations of Verdoorn *et al.* 1990 who found that GABA receptors composed of α and β subunits have two principal desensitization phases, one in the range of hundreds of milliseconds and the other in the range of seconds. Verdoorn *et al.* (1990) found that this first, very fast phase disappears

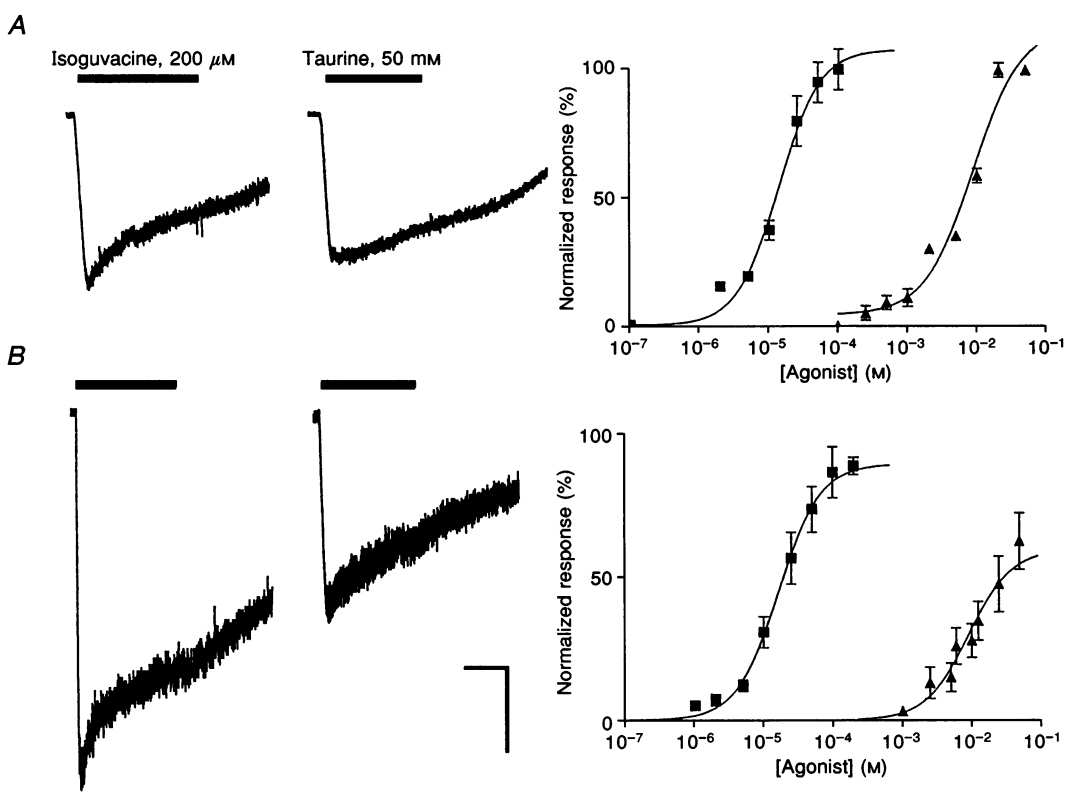

Figure 8. Agonist dependent desensitization rates and dose–response curves for isoguvacine and taurine

A, response of $\alpha 1\beta 3$ receptors to isoguvacine and taurine. EC_{50} values for isoguvacine and taurine were 20 μM and 7 mM, respectively. *B*, responses of $\alpha 1\beta 3\gamma 2$ receptors to the same agonists. EC_{50} values for isoguvacine were 16 μM and 9 mM, respectively. Calibration: time, 1 s; amplitude, 200 pA. Responses were normalized to 100 μM GABA response in same cell.

in a receptor expressing the $\gamma 2$ subunit. In contrast, we have found that this millisecond-long phase becomes faster. It may be possible that the β subunit is required for biphasic GABA receptor desensitization. Our study differs from most other reports of the apparent Hill slope of $\alpha\beta$ subunit receptors in comparison with $\alpha\beta\gamma$ subunit receptors. In most studies the Hill slope of $\alpha\beta$ receptors is 1 whereas $\alpha\beta\gamma$ subunit receptors usually have a greater Hill slope in the range of 1·3–2·0 (Burt & Kamatchi, 1991). Our results, obtained using relatively faster agonist applications, gave Hill slopes which were the same for the two different receptor types and they were greater than 1. These Hill slopes suggest that there are two binding sites and/or steps for both receptor subtypes, but the Hill slope is still much less than 2. These values suggest significant desensitization at high agonist concentrations or differences in the binding steps as suggested in Maconochie *et al.* (1994). However, regardless of the mechanism responsible for these values, the binding kinetics leading to the activation of the two receptors appear to be equivalent.

Verdoorn *et al.* (1990) have also shown that increasing concentrations of GABA increase the rate of desensitization for both phases. We have obtained similar results but they were the least equivocal for the slow phase of desensitization. Jones & Westbrook (1995) have ruled out any concentration affect on the millisecond-desensitization rate. In our case, the fast desensitization phase for $\alpha 1\beta 3\gamma 2$ subunit receptors seemed dependent on concentration. However, this may not be strictly true. It is possible that the slowing activation rate alters the apparent time course of the fast desensitization. That is, the slower desensitization rate is the result of a combination of two macroscopic exponential rates, the activation (variable) and the desensitization (constant). This interpretation is supported by the observation that the slower rate of fast desensitization of the $\alpha 1\beta 3$ subunit receptors was relatively insensitive to GABA concentration. We believe that only after the onset slows to about the same order of magnitude as the fastest desensitization rate does an apparent slowing in the desensitization become evident. This was particularly evident for $\alpha 1\beta 3\gamma 2$ receptors. The activation rates slow to a magnitude closer to the fast desensitization rate at higher GABA concentrations and therefore the 'concentration dependence' is more apparent. This conclusion is further supported by the isoguvacine data. The slower fast desensitization rates (in comparison with GABA) were completely insensitive to agonist concentrations over the same range. Based on these observations we conclude that in fact the fast desensitization rates are independent of the agonist concentration. Similar conclusions have been reached by the study of Calentano & Wong (1994). We have interpreted these results to indicate that the fast desensitization develops only from the open fully bound conducting state of the receptor, although other interpretations are possible (cf. Jones & Westbrook, 1995). In contrast, the slow desensitization, being more concentration dependent, develops from the closed but fully bound conducting state of the receptor (ignoring other states like singly bound and unbound desensitized states).

Numerous studies have examined the desensitization of native $GABA_A$ receptors in vertebrate neurones (Brown & Galvan, 1977; Ben-Ari, Krnjevic & Reinhardt, 1979; Desarmenien, Feltz & Headley, 1980; Hackman, Ausander, Grayson & Davidoff, 1982; Gallagher, Nakamura & Shinnick-Gallagher, 1983; Akaike, Inoue & Krishtal, 1986; Frosch *et al.* 1992; Puia, Costa & Vincini, 1994; Calentano & Wong, 1994; Maconochie *et al.* 1994; Jones & Westbrook, 1995; Zhang & Jackson, 1995). Most of these studies have identified single desensitization time constants in the range of seconds. However, a few have identified rates in the millisecond range (Puia *et al.* 1994; Calentano & Wong, 1994; Maconochie *et al.* 1994; Jones & Westbrook, 1995). For example, Puia *et al.* (1994) have reported two desensitization phases in the millisecond range in granule and Purkinje cells of rat cerebellum. The first had a time course of approximately 5 and 7 ms in these two cell types, respectively. The time course of the second phase was in the order of 100 ms (granule) and 50 ms (Purkinje cells); rates which are similar to the maximum desensitization rate of the $\alpha 1\beta 3\gamma 2$ combination. Similar rates of desensitization to ours (in the tens of milliseconds) were the fastest reported by Jones & Westbrook (1995). They have also shown that desensitization does account, in part, for the time course of a synaptic current.

We have also found that in cells expressing $\alpha 1\beta 3\gamma 2$ subunits the slow, but not the fast component of desensitization was dependent on the voltage at which the cell was clamped. It has been suggested that this voltage dependence is potentially important. Under some physiological or pathological stresses reducing the rate at which desensitization develops may be a mechanism augmenting the effectiveness of GABA. However, it seems unlikely that the slowly developing desensitized state would have an effect on the time course of a single synaptic potential. Nevertheless, this effect of membrane potential might manifest itself when a GABAergic synapse is under some kind of tetanus lasting for seconds.

The agonist dependent desensitization rates and the poor correlation between potency and 'desensitizing power' are surprising in view of the data reported by Patneau *et al.* (1992) from glutamate receptors. They found a good correlation between the potency of the Willardine derivatives employed as AMPA/kainate receptor agonists and the degree of desensitization. However, there was little difference in the desensitization rates between agonists. In contrast, we found no clear relationship between potency or efficacy and the degree of desensitization, but the rates varied. The simplest interpretation of our observations is that desensitization occurs from the open state of the channel after the equilibrium which reflects agonist potency.

Patneau *et al.* (1992) concluded that the desensitizing potency of the agonist is determined by the conformational rigidity of the molecule which sterically hinders the conformation change of the receptor. In our case a similar interpretation could be made. The agonist dependence on the fast desensitization rate reflects the physiochemical properties of the agonists. However, it is likely that it has little to do with conformational rigidity since taurine is able to rotate freely around its chiral centres and desensitizes slowly, whereas the more conformationally constrained isoguvacine desensitizes more quickly. In agreement, we have found that musimol, a conformationally constrained molecule, desensitizes GABA receptors at a rate similar to isoguvacine ($\tau_1 \approx 100$ ms).

Computer model

Based on our experimental data and the above interpretations we were interested in finding rate constants which quantitatively reproduced the data obtained from concentration dependence experiments. A number of criteria had to be met: (1) increased activation rates over the range of concentration used; (2) appropriate relative proportioning of the fast and slow components of desensitization; and (3) inactivation rates consistent with those reported by Maconochie *et al.* (1994; and therefore with two binding steps). As we were most interested in determining whether the fast desensitization affected by the $\gamma 2$ subunit could potentially be important during the time course of a synaptic potential, we focused our efforts on adequately modelling the fast desensitization. For this reason the voltage dependence was ignored for the slow desensitization. This allowed us to follow the time course of a modelled response as a function of the probability that the receptor is in the open conducting state (P_{open}). Therefore, the following scheme was used to develop rate constants that describe $GABA_A$ receptor activation, inactivation and desensitization. We have ignored schemes which include recovery states which do not relax through the binding and unbinding equilibria in the absence of more data.

$$\begin{array}{ccccccc} A+R & \underset{k_{-1}}{\overset{2k_1[GABA]}{\rightleftharpoons}} & A+AR & \underset{2k_{-2}}{\overset{k_2[GABA]}{\rightleftharpoons}} & ARA & \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} & ARA_{open} \\ & & & & \Big\| k_{-4} \\ & & & & ARA_{ds} & & \end{array} \qquad \begin{array}{c} \\ \\ \Big\| k_4 \\ \\ \end{array} \qquad \begin{array}{c} \\ \\ k_{-3} \Big\| k_3 \\ ARA_{df} \end{array}$$

Scheme 1

where A is the agonist and R is the receptor; AR and ARA are single and double-bound non-conducting states, respectively; ARA_{open} is the double-bound single open state and ARA_{df} and ARA_{ds} are the fast and slow desensitized states, respectively.

We should also add that although we believe it is critical that the fast desensitized state develops from the open-channel state, the equivalent argument could be made for the slowly developing desensitization state. However, we found that it makes no difference where the equilibrium is placed in modelling runs and we have chosen the closed fully bound state for the simulations presented here.

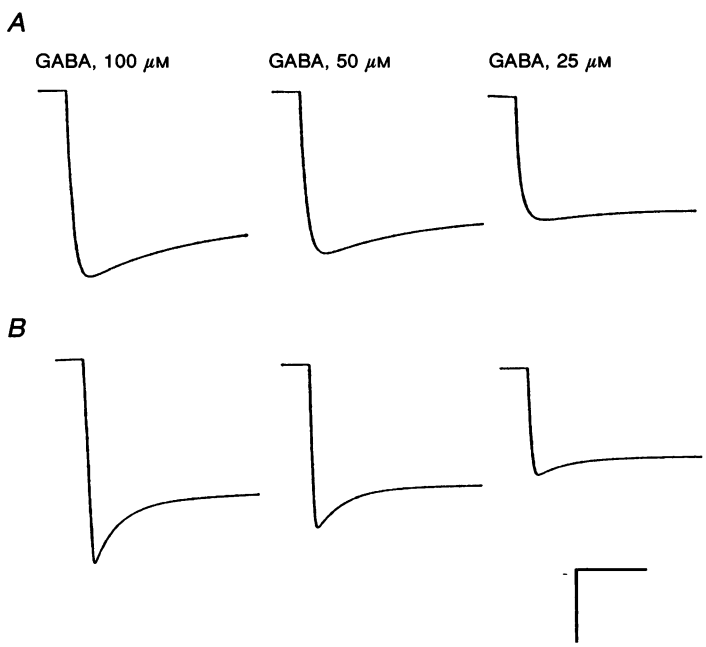

Figure 9. Computer simulations of the desensitization of the $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors

100, 50 and 25 μM agonist simulations were used to model the time course of desensitization of the $\alpha 1\beta 3$ (*A*) and $\alpha 1\beta 3\gamma 2$ (*B*) receptors. The results shown here model the essential characteristics of Figs 3*A* and 4*A* (see text for explanation). Calibration: time, 250 ms, and P_{open}, 0·25.

For the binding reactions we have used values which reflect the activation and inactivation rates reported by Maconochie *et al.* (1994) as well as our own dose–response data concerning the $\alpha1\beta3$ subunit receptors. To do this we set the second binding step to be 10 times slower than the first and then adjusted the off rates accordingly. For $\alpha1\beta3$ receptor simulations the first forward binding rate (k_1) was set to 10 μM s^{-1} while the off rates (k_{-1} and k_{-2}) were set to 250 s^{-1}. The second binding step (k_2) was set to 1 μM s^{-1}. The opening rate of the channel (β) was set to 6000 s^{-1} (Maconochie *et al.* 1994) and α was set to 2500 s^{-1} to give a maximum P_{open} of 0·7. For $\alpha1\beta3\gamma2$ simulations everything was the same except k_{-1} was raised to 50 s^{-1} in order to account for the different EC_{50} values and the activation rates.

We next set the fast desensitization rates for these two simulations. For $\alpha1\beta3$ receptor simulation the k_3 and k_{-3} values were set so that the fast phase represented approximately 15% of the total desensitization, i.e. k_3 was set to 4 s^{-1} and k_{-3} to 3·5 s^{-1}. We used the fast recovery phase value of 0·03 s^{-1} for k_{-4}, and therefore k_4 was set to 0·12 s^{-1}. For simulations of the $\alpha1\beta3\gamma2$ subunit receptors, k_3 was set to 15 s^{-1} while k_{-3} was set to 10 s^{-1} so that the fast phase was approximately 30 % of the total desensitization. k_4 and k_{-4} were set to 0·21 s^{-1} and 0·10 s^{-1} (again the fast recovery phase), respectively. The application of GABA had a rise time of 1 ms and the concentrations used were 25, 50 and 100 μM GABA. Figure 9 shows the modelled responses for these two sets of values. In Fig. 9*A* the $\alpha1\beta3$ model reproduces the essential characteristics of the data shown in Fig. 3*A*. There is very little change in the desensitization rate over the range of concentrations used. In contrast, Fig. 9*B* shows a slight change in the rates of desensitization essentially reproducing the data shown in Fig. 4*A*. The fast phase of desensitization arising from the open state of the channel can be physically interpreted to mean that the channel can reopen directly from the desensitized state. This interpretation correlates with the work of Newland, Colquhoun & Cull-Candy (1991), who have suggested that the long closed times between bursts of openings represent the entry of the channel into a temporarily desensitized state.

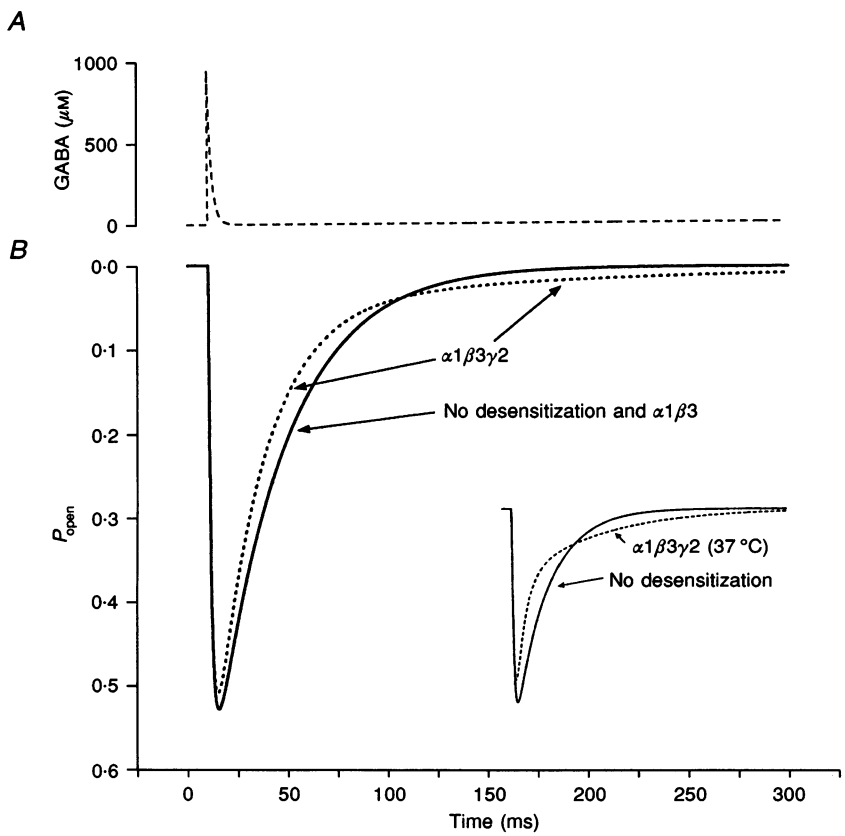

Figure 10. Computer simulations of an agonist stimulation

Agonist simulation having a 'synaptic time course' for $\alpha1\beta3$ or $\alpha1\beta3\gamma2$ receptors at 20 °C. Continuous and dotted lines in *B* represent the time course of P_{open} in response to a brief exponentially decaying pulse of GABA shown above in *A*. In *B* the continuous line represents the time course of P_{open} with either no desensitization or with the desensitization kinetics of an $\alpha1\beta3$ combination and these did not differ. The dotted line represents the time course of P_{open} of $\alpha1\beta3\gamma2$ receptors. Note the alteration in the time course and the prolongation of P_{open}. The inset trace represents the time course of P_{open} at 37 °C assuming a Q_{10} value of 2 for the desensitization kinetics.

Since the settings used above reproduced our experimental data, we next turned our attention to following the time course of P_{open} during a modelled 'synaptic application' of GABA. The release of neurotransmitter is believed to occur by the fusion of a vesicle with the terminal membrane resulting in the exocytosis of the neurotransmitter molecules into the synaptic cleft within a 100 μs period. Recent studies by Maconochie *et al.* (1994), as well as Jones & Westbrook (1995) indicate that the concentration of GABA must rise to between 500 μM and 1 mM within this 100 μs period. The subsequent time course of the fall in GABA concentration in the synaptic cleft is unknown. However, for excitatory synapses the neurotransmitter concentration has been estimated to decay with a time constant of approximately 1·5–2·0 ms (Clements, Lester, Gong, Jahr & Westbrook, 1992). Therefore, we have 'applied' in our model a 1 mM pulse of GABA peaking in 100 μs which declines with a time constant of 2·0 ms. Results of these simulations are shown in Fig. 10. Using the rate constants developed above, it is evident that the desensitization kinetics of the $\alpha1\beta3$ receptor gives a time course of P_{open} which is the same as when there is no desensitization present. In contrast, the time course of the $\alpha1\beta3\gamma2$ model is quite different. The desensitization changes the time course from being predominately monophasic to biphasic. Of particular interest is the long relaxation which greatly increases the length of the synaptic potential. These results are in close agreement with the recently published model of Jones & Westbrook (1995) who have shown that desensitization is responsible for this long relaxation. However, our model is different in that our desensitized state relaxes through the open state of the channel whereas the Jones & Westbrook (1995) model has the fast desensitization developing from the fully bound closed (but activatable) state. However, we could not reproduce our dose–response data using their constants and kinetic scheme, but we did confirm their conclusions using short pulses. Nevertheless, both models are simplifications of more complex processes and such discrepancies are to be expected. It is difficult to speculate further whether or not this prolongation is physiologically significant in either our case or theirs. However, our experiments were carried out at 18–22 °C and, therefore, our model reflects these temperatures. If one assumes a modest Q_{10} value of 2 for the desensitization process, then at physiological temperatures the rate of desensitization would increase by nearly 4-fold. The inset in Fig. 10 shows how this increase would be predicted to affect P_{open}. We should also indicate that integrating the curves to obtain the total areas showed that in all cases they are equivalent. Therefore, this model does not predict that there would be an increase in total charge transfer. What it does predict is that desensitization prolongs the time that the channels may be open and thus prolongs the time course of the synaptic potential. The important conclusions from both models (ours and that of Jones & Westbrook) is that desensitization drives the receptor into a 'reopenable' state increasing synaptic efficacy. What we have found in this study is that the $\gamma2$ subunit is important in creating the kinetics that ensures that this phenomena occurs more frequently.

In conclusion we have found that the $\gamma2$ subunit increases the rate at which the $GABA_A$ receptor desensitizes. The physiological importance of this behaviour is to prolong the time course of a synaptic current. This subunit also confers voltage dependence to the desensitization process although this is probably only relevant during times of stress and/or intense excitation of the synapse.

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