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Slow phases of $GABA_A$ receptor desensitization: structural determinants and possible relevance for synaptic function

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$GABA_A$ receptor fast desensitization is thought to shape the time course of individual IPSCs. Although $GABA_A$ receptors also exhibit slower phases of desensitization, the possible role of slow desensitization in modifying synaptic function is poorly understood. In transiently transfected human embryonic kidney (HEK293T) cells, rat $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ $GABA_A$ receptors showed distinct desensitization patterns during long (28 s) concentration jumps using a saturating (1 mM) GABA concentration. $\alpha1\beta3\gamma2L$ receptors desensitized extensively (~90 %), with four phases (τ_1 ~20 ms, τ_2 ~400 ms, τ_3 ~2 s, τ_4 ~10 s), while $\alpha1\beta3\delta$ receptors desensitized slowly and less extensively (~35 %), with one or two slow phases with time constants similar to τ_3 and τ_4 of $\alpha1\beta3\gamma2L$ receptors. To determine the structural basis of subunit-specific desensitization, δ-γ2L chimera subunits were expressed with α1 and β3 subunits. Replacing the entire N-terminus of the γ2L subunit with δ subunit sequence did not alter the number of phases or the extent of desensitization. Although extension of δ subunit sequence into transmembrane domain 1 (TM1) abolished the fast and intermediate components of desensitization, the two slow phases still accounted for substantial current loss (~65 %). However, when δ subunit sequence was extended through TM2, the extent of desensitization was significantly decreased and indistinguishable from that of $\alpha1\beta3\delta$ receptors. The importance of TM2 sequence was confirmed by introducing γ2 subunit TM2 residues into the δ subunit, which significantly increased the extent of desensitization, without introducing either the fast or intermediate desensitization phases. However, introducing δ subunit TM2 sequence into the γ2L subunit had minimal effect on the rates or extent of desensitization. The results suggest that distinct δ subunit structures are responsible for its unique desensitization properties: lack of fast and intermediate desensitization and small contribution of the slow phases of desensitization. Finally, to investigate the possible role of slow desensitization in synaptic function, we used a pulse train protocol. We observed inhibition of peak current amplitude that depended on the frequency and duration of GABA pulses for receptors exhibiting extensive desensitization, whether fast phases were present or not. The minimally desensitizing $\alpha1\beta3\delta$ receptor exhibited negligible inhibition during pulse trains. Because receptors that desensitized without the fast and intermediate phases showed pulse train inhibition, we concluded that receptors can accumulate in slowly equilibrating desensitized states during repetitive receptor activation. These results may indicate a previously unrecognized role for the slow phases of desensitization for synaptic function under conditions of repeated $GABA_A$ receptor activation.

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Agonist-induced desensitization has been observed for many types of ligand-gated ion channels and may subserve diverse functions *in vivo* (Jones & Westbrook, 1996). $GABA_A$ receptor desensitization has been shown to be multiphasic, suggesting that multiple desensitized conformations are possible (Celentano & Wong, 1994; Haas & Macdonald, 1999). We previously investigated the desensitization patterns of $\alpha\beta\delta$ and $\alpha\beta\gamma$ $GABA_A$ receptors (Haas & Macdonald, 1999), thought to comprise the majority of $GABA_A$ receptor isoforms in the brain (McKernan & Whiting, 1996). Both the rate and extent of desensitization were clearly dependent on subunit composition. For example, $\alpha1\beta3\gamma2L$ receptors showed extensive desensitization that was described by a fast phase (~10 ms), an intermediate phase (~150 ms) and a slow phase (~1500 ms). In contrast, $\alpha1\beta3\delta$ receptors desensitized less extensively, with a single slow phase (~1500 ms) that resembled the slow phase of $\alpha1\beta3\gamma2L$ receptor currents. Subunit-dependent desensitization, among other properties, may provide a molecular mechanism for regulating $GABA_A$ response efficacy during prolonged or repetitive activation.

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The subcellular compartmentalization of $GABA_A$ receptors is also related to subunit composition. $GABA_A$ receptors containing the δ subunit have been shown to reside in extrasynaptic membrane locations in the cerebellum (Nusser *et al.* 1998). Extrasynaptic receptors may sense ambient extracellular levels of GABA that fluctuate over a much slower time scale than synaptic transients. The slow desensitization kinetics of $\alpha\beta\delta$ isoforms are well suited for continuous response to sustained, low level GABA concentrations that may occur extracellularly (Lerma *et al.* 1986). In contrast, ternary receptors containing the γ subunits have been suggested to be predominantly localized to subsynaptic membranes (Nusser *et al.* 1998; Brickley *et al.* 1999). During individual synaptic events, fast desensitization is thought to shape the post synaptic current in part by prolonging its duration (a process known as receptor deactivation) by delaying the unbinding of GABA, although in principle all desensitized states share the property of trapping GABA on the receptor (Jones & Westbrook, 1995; Bianchi & Macdonald, 2001*a*). The rapid activation and desensitization kinetics of $\alpha\beta\gamma$ isoforms, thus, are well suited for responding to brief GABA transients during inhibitory synaptic transmission (Haas & Macdonald, 1999).

The role of intermediate and slow phases of desensitization for synaptic $GABA_A$ receptors is less clear. Slow desensitized states may not contribute to the shaping of individual IPSCs because equilibration occurs over a long time scale relative to the synaptic transient. However, persistent levels of GABA in the synaptic cleft are possible under certain conditions, such as spillover of GABA during high levels of activity (Isaacson *et al.* 1993; Rossi & Hamann, 1998). GABA spillover that reaches neighbouring inhibitory synapses is the equivalent of pre-incubation in low concentration GABA, which has been shown to desensitize $GABA_A$ receptors to subsequent pulses of saturating GABA (Overstreet *et al.* 2000). This prolonged exposure to GABA may promote entry into the slow phases of desensitization.

Slow phases of desensitization may also be involved in $GABA_A$ receptor responsiveness under conditions of repetitive receptor activation. Such conditions may occur during pathological states such as epilepsy, or during normal rhythmic network firing patterns. With exogenous application of GABA, even brief pulses can drive $GABA_A$ receptors into desensitized states, such that subsequent GABA applications evoke smaller amplitude currents. When repeated IPSCs are evoked, the decrement in synaptic current is often attributed to pre-synaptic mechanisms (Davies & Collingridge, 1993; Stevens & Wang, 1995). However, it is difficult to quantify post-synaptic receptor desensitization; if $GABA_A$ receptors can accumulate in slowly equilibrating desensitized states during repetitive stimulation, this may also constrain synaptic efficacy. Although dissecting the impact of post-synaptic receptor desensitization is complicated by the known presynaptic contributions to synaptic depression, recombinant systems employing exogenous GABA application allow direct testing of the hypothesis that slow phases of desensitization can regulate $GABA_A$ receptor availability during repeated applications of GABA.

In this study we used a chimera strategy to identify structures that conferred the distinct desensitization patterns of $GABA_A$ receptors containing δ or γ2L subunits. Interestingly, two distinct domains of the δ subunit were shown to be responsible for its unique desensitization pattern. The absence of the fast and intermediate phases of desensitization was dependent upon N-terminal and TM1 sequence, while TM2 sequence regulated the contribution of the slower phases of desensitization. Additionally, we took advantage of chimeric constructs that showed slow but extensive desensitization to explore the possible role of slow desensitization in synaptic function. Progressive inhibition of peak current during pulse trains suggested that accumulation of receptors in slowly equilibrating desensitized states might contribute to decreased synaptic efficacy during repeated receptor activation.

METHODS

Expression of recombinant $GABA_A$ receptors

The cDNAs encoding rat α1, β3, δ and γ2L, $GABA_A$R subunit subtypes and chimera subunits were individually subcloned into the plasmid expression vector pCMVNeo. See Bianchi *et al.* (2001) for construction of chimeras and splice site locations. All constructs have been confirmed by DNA sequencing (Sequencing Core, University of Michigan, MI, USA). Human embryonic kidney cells (HEK293T; a gift from P. Connely, COR Therapeutics, San Francisco, CA, USA) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10 % fetal bovine serum, at 37 °C in 5 % CO_2–95 % air. Cells were transfected with 4 μg of each subunit plasmid along with 1–2 μg of pHOOK (Invitrogen, Carlsbad, CA, USA) for immunomagnetic bead separation (Greenfield *et al.* 1997), using a modified calcium phosphate co-precipitation technique as previously described (Angelotti *et al.* 1993). The next day, cells were replated and recordings were made 18–30 h later.

Electrophysiology

Patch-clamp recordings were performed on transfected fibroblasts bathed in an external solution consisting of (mM): NaCl 142; KCl 8; $MgCl_2$ 6; $CaCl_2$ 1; Hepes 10; glucose 10 (pH 7.4, 325 mosmol l^{-1}). Electrodes were formed from thin-walled borosilicate glass (World Precision Instruments, Pittsburgh, PA, USA) with a Flaming Brown electrode puller (Sutter Instrument Co., San Rafael, CA, USA), fire-polished to resistances of 0.8–1.5 MΩ when filled with an internal solution consisting of (mM): KCl 153; $MgCl_2$ 1; MgATP 2; Hepes 10; EGTA 5 (pH 7.3, 300 mosmol l^{-1}). This combination of internal and external solutions produced a chloride equilibrium potential near 0 mV. Cells were voltage clamped at −10 to −60 mV using an Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA). No voltage dependence of desensitization was observed in this range. For isoforms exhibiting 'fast' desensitization, cells were gently

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lifted from the recording dish to reduce the solution exchange time. For isoforms without fast desensitization, we observed insignificant differences in fitting the desensitization whether cells were lifted or not (despite faster current rise times upon lifting), and thus data from the two conditions were pooled. GABA was applied (via gravity) to whole cells using a rapid perfusion system consisting of multi-barrel square glass connected to a Warner Perfusion Fast-Step (Warner Instrument Corp., Hamden, CT, USA). The glass was pulled to a final barrel size of approximately 250 μm. The solution exchange time was estimated routinely by stepping a dilute external solution across the open electrode tip to measure a liquid junction current. The 10–90 % rise times for solution exchange were consistently 1–2 ms or less, although the exchange around cells was probably slower.

Analysis of currents

Currents were low-pass filtered at 2–5 kHz, digitized at 10 kHz, and analysed using the pCLAMP8 software suite (Axon Instruments). The desensitization time courses of GABA$_A$R currents were fit using the Levenberg-Marquardt least squares method with one, two, three or four component exponential functions of the form $\Sigma a_n e^{(-t/\tau_n)}$, where n is the best number of exponential components, a is the relative amplitude of the component, t is time and τ is the time constant. Additional components were accepted only if they significantly improved the fit, as determined by an F test automatically performed by the analysis software on the sum of squared residuals. Five component fits were not considered. Numerical data were expressed as mean ± S.E.M. Statistical significance, using Student's t test (unpaired) was taken as $P < 0.05$.

Simulations

Simulated macroscopic currents were generated using the Berkeley Madonna 8.0 software package (www.berkeleymadonna.com) that numerically solves differential equations (4th order Runge-Kutta algorithm, 100 μs step size for all simulations shown).

RESULTS

Desensitization of $\alpha1\beta3\gamma2$L and $\alpha1\beta3\delta$ GABA$_A$ receptors differed in both rate and extent

There was a clear subunit dependence of GABA$_A$ receptor desensitization (Fig. 1). Lifted cells expressing $\alpha1\beta3\gamma2$L (Fig. 1*A*) or $\alpha1\beta3\delta$ (Fig. 1*C*) GABA$_A$ receptors were activated by GABA (1 mM) for 28 s using the concentration jump technique. $\alpha1\beta3\gamma2$L receptor currents desensitized rapidly and extensively (91.1 ± 1.1 %), with a time course that was best described by four exponential functions that we will refer to as fast, intermediate, slow and ultraslow phases of desensitization (Fig. 1*B*). In contrast to $\alpha1\beta3\gamma2$L receptors, $\alpha1\beta3\delta$ receptor desensitization was slow, accounted for only 38.1 ± 4.4 % current loss over 28 s and was well described by one (or sometimes two) exponential functions (Fig. 1*D* and *E*). The measured time constants for $\alpha1\beta3\delta$ receptors were similar to the slow and ultraslow phases of desensitization of $\alpha1\beta3\gamma2$L receptor currents. Longer duration pulses would be required to determine whether additional, slower phases of desensitization exist. Figure 1*E* showed the accuracy of multi-exponential fitting for the rapidly desensitizing $\alpha1\beta3\gamma2$L current. The exponential functions are superimposed on the trace, and the residual current (actual current − fitted curve) is shown above the trace. Minimal deviations of the residual current were observed, except for the first 30 ms of the trace, which may have contributed to the variation of the fast time constant (Fig. 1*E2*).

While excised patches have proven optimal for studying fast desensitization (Haas & Macdonald, 1999; Bianchi *et al.* 2001), whole cells offered two major advantages over patches for the study of slow desensitization. First, we found that the whole cell configuration provided considerably greater stability during the long duration GABA applications required to resolve slow phases of desensitization. Second, whole cell currents were typically much larger than patch currents, allowing accurate fitting of slow phases even after substantial current loss during many seconds of GABA exposure. The small current amplitudes typically remaining after even a few seconds of GABA application to patches may compromise fitting accuracy due to low signal to noise ratio. Nevertheless, two important considerations warranted further investigation to ensure that the whole cell configuration was appropriate for our study of slow desensitization. In the following sections we evaluated the potential contribution of chloride ion shifts to macroscopic desensitization and compared desensitization in whole cell and patch configurations.

Chloride ion redistribution did not account for the time course of desensitization

One difficulty in studying slow phases of desensitization is the requirement for unusually long agonist applications. It has been suggested that chloride ion shifts accounted in part for the fading of current during prolonged (on the scale of minutes) agonist applications (Akaike *et al.* 1987). For example, at hyperpolarized holding potentials, chloride ion efflux during the GABA application might drive the equilibrium potential for chloride (E_{Cl}) to negative potentials (it is normally near 0 mV using our solutions; see Methods), such that the driving force would decrease with time. This would lead to spurious overestimations of apparent desensitization. Despite the use of low resistance electrodes (that more easily dialyse internal solution into the cell cytoplasm to buffer chloride ion efflux), the possibility of chloride ion shifts needed to be addressed directly. Although the expression level (as reflected in current amplitude) of transfected GABA$_A$ receptors differed widely among individual cells, it was not uncommon to measure current amplitudes in the 5–15 nA range, particularly for the $\alpha1\beta3\gamma2$L receptors. Therefore we conducted control experiments using $\alpha1\beta3\gamma2$L receptors to determine whether potential redistribution of chloride ion could be affecting our measurements of desensitization. If significant chloride ion shifts were occurring over the course of GABA application, then the E_{Cl} would change as the time of GABA application increased. We repeatedly applied GABA (1 mM) for 10 s at various holding

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potentials and measured the current amplitude at the peak, middle (5 s) and end (10 s) of the response (Fig. 2*A*). We used shorter application durations for these experiments because most of the desensitization observed in 28 s pulses occurred by ~10 s, and cell stability was more robust for the required repeated GABA applications. Three cells that showed large current amplitudes (5–15 nA at −50 mV holding potential) were chosen for analysis. This protocol provided three *I–V* curves for each cell. The data from a representative *I–V* experiment (Fig. 2*A*) was plotted in Fig. 2*B*. Although this particular cell showed slight outward rectification at +50 mV, other cells showed no, or slight inward, rectification. Any change in the relative chloride ion concentration over the course of the application would be revealed as a change in the reversal potential. Chloride ion redistribution would also result in non-linearities of the *I–V* relations measured at different points during the 10 s application, which is theoretically ohmic (and thus linear), assuming no voltage-dependent gating. In excised patches (where chloride ion shifts were not relevant because of the small currents and large chloride ion reservoirs on both sides of the membrane), desensitization of $\alpha1\beta3\gamma2L$ receptor currents showed minimal apparent voltage dependence between −75 and +50 mV (data not shown). We did not observe any non-linearity of the relation (Fig. 2*B*), and there was no significant change in reversal potential (Fig. 2*C*). To rule out the possibility that series resistance errors (that were not compensated in this study) affected the *I–V* experiment, we measured the reversal potential in three

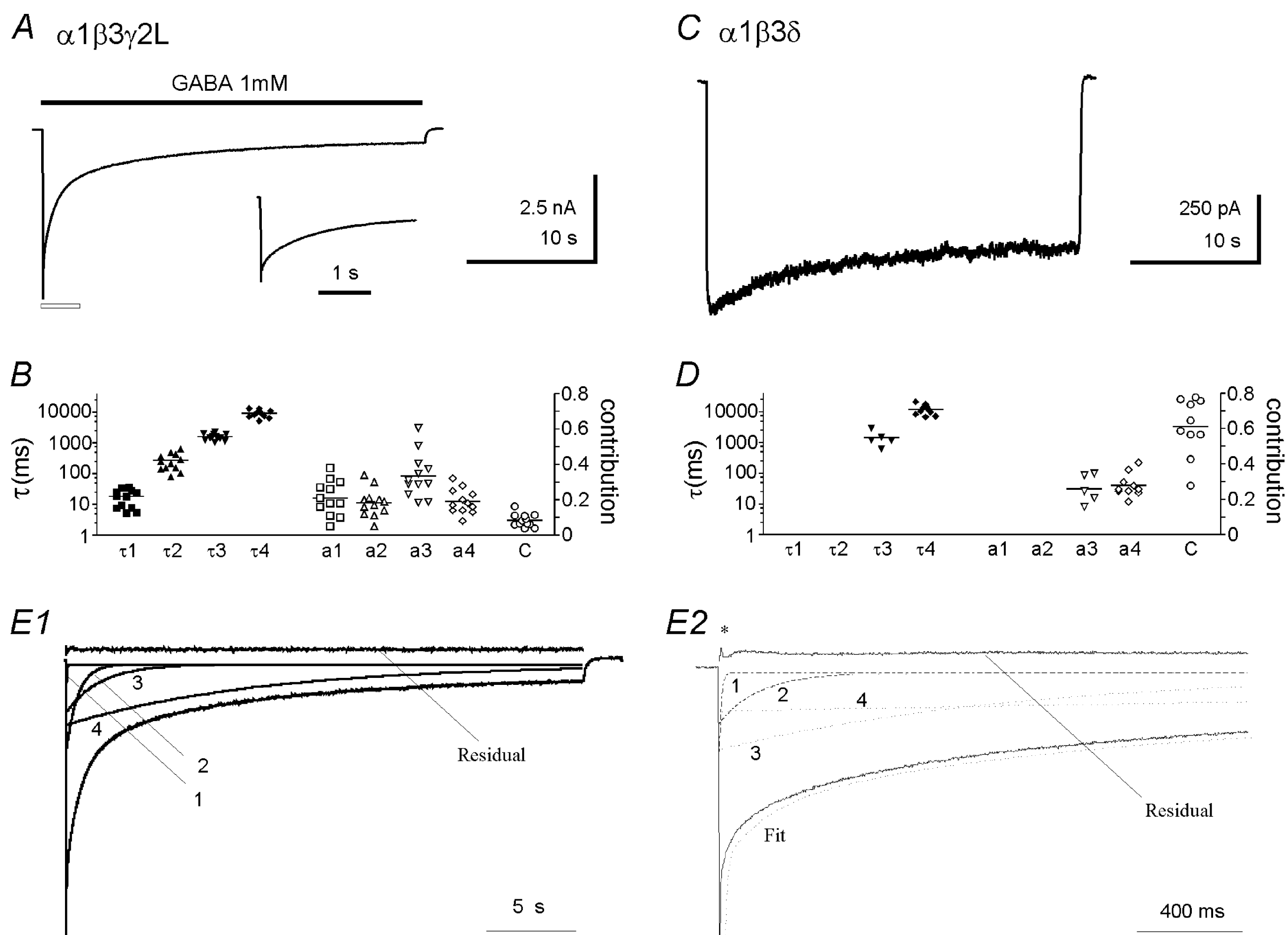

Figure 1. $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ desensitization differed in both rate and extent

A, current response of transiently expressed $\alpha1\beta3\gamma2L$ receptors to a 28 s concentration jump using 1 mM GABA (filled bar). The inset shows the first 3 s (open bar) on an expanded time scale. *C*, current response of $\alpha1\beta3\delta$ receptors to the same protocol as in *A*. The parameters used to fit $\alpha1\beta3\gamma2L$ and $\alpha1\beta3\delta$ currents are shown as scatter plots in *B* and *D*, respectively. The left ordinate indicates the time constants ($\tau1$–$\tau4$; note the logarithmic scale), and the right ordinate indicates the relative contribution of the corresponding time constants (a1–a4), as well as the constant term to account for incomplete desensitization. For each parameter, a horizontal line is drawn through the mean. Exponential fitting of $\alpha1\beta3\gamma2L$ receptor currents is shown for the entire 28 s application (*E1*) as well as the first ~2 s expanded (*E2*). The time constants (1–4) and residual current (actual – fitted) are labelled in both panels. The asterisk in *E2* indicates the slight deviation between the fitted curve and the actual current, as indicated by a non-zero residual.

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cells (that had large conductance changes in response to GABA) in which the series resistance was compensated by 85–90 % (Fig. 2*C*). E_{Cl} was not different when measured at the peak or 5 s later and was not different from that calculated without compensation. In Fig. 2*D* the relative charge transfer occurring over the course of a 28 s application from five randomly chosen $\alpha1\beta3\gamma2$L receptor current traces was plotted. The cumulative charge carried at 10 s was over 700 times greater than that carried by the time the peak was reached (< 5 ms). Despite this large difference in chloride flux, no evidence was found for significant redistribution of chloride ions in our

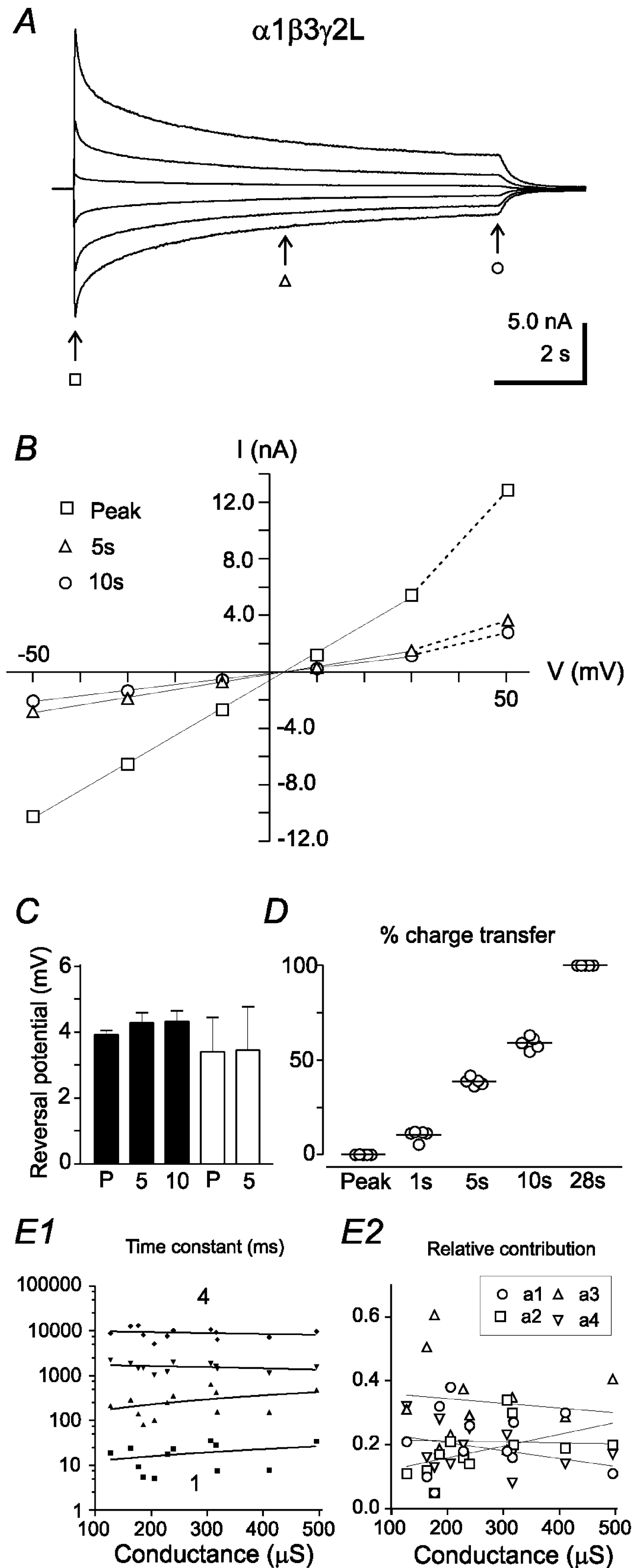

Figure 2. Chloride shifts were not responsible for the fading of current during prolonged GABA application

A, currents were evoked by 10 s applications of 1 mM GABA to $\alpha1\beta3\gamma2$L receptors at several command voltages (50, 30, 10, −10, −30, −50 mV, from the top trace to the bottom trace). The open symbols with arrows indicate the current measurements made at the peak (□), 5 s (△) and 10 s (○) for the plot in *B*. *B*, current–voltage relation plots were derived from current measurements at three different time points of each GABA application from the cell shown in *A*. Similar plots were obtained in three other cells. *C*, calculated chloride reversal potentials were measured as the voltage corresponding to zero current from fitting the *I–V* relations with a straight line from −50 to +30 for each cell. Values were not significantly different among the three measurement time points (filled bars). E_{Cl} was also calculated from 3–4 GABA applications from −20 to +10 mV in cells where 85–90 % series resistance compensation was used (open bars). *D*, the cumulative charge transfer is shown for five randomly chosen $\alpha1\beta3\gamma2$L receptor currents to demonstrate the typical relative magnitude of chloride flux occurring at various times throughout long (28 s) GABA applications. The time constants of desensitization (*E1*) and their relative contributions (*E2*) are plotted *vs.* conductance for each $\alpha1\beta3\gamma2$L receptor current (from Fig. 1*B*). Linear regression lines are shown for each parameter; none of the eight regression lines had slopes that differed from zero. Note the log scale used in *E2*, where the time constants are shown with the fastest (1) on the bottom, followed by the intermediate and slow time constants, with the ultraslow (4) on the top.

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experiments. Although series resistance errors may alter both the extent and the kinetics of desensitization (for example, the larger voltage error at the peak current would decrease the 'true' peak value and thus lead to underestimation of desensitization extent), we did not investigate these effects. However, neither the time constants of desensitization nor their relative contributions to the decay were correlated with conductance for the rapidly desensitizing $\alpha1\beta3\gamma2$L receptor currents (Fig. 2*E1* and *E2*).

Comparison of GABA$_A$ receptor desensitization in excised patches and in whole cells using various perfusion techniques

The concentration jump technique has been used to demonstrate rapid, multiphasic desensitization in excised patches containing native (Celentano & Wong, 1994) and recombinant (Haas & Macdonald, 1999) GABA$_A$ receptors, as well as nucleated patches from cerebellum (Tia *et al.* 1996). However, desensitization was usually slower when observed at the whole cell level, even with relatively rapid perfusion (for example, see Dominguez-Perrot *et al.* 1997). One concern with comparisons of desensitization between the whole cell and excised patch configurations was that channel behaviour might be altered if patch excision disrupts interactions with cytoplasmic factors. For example, phosphorylation and interactions with clustering proteins are known to affect GABA$_A$ receptor function (Jones & Westbrook, 1997; Chen *et al.* 2000). However, it was also possible that the differences were related to the technical limitations in perfusing whole cells on a time scale sufficiently rapid to resolve fast processes. To test this possibility, we quantified the time constant and relative contribution of 'fast' desensitization, as well as the current rise time, for rapidly desensitizing $\alpha1\beta3\gamma2$L receptor currents evoked by GABA (1 mM) under various conditions of perfusion efficiency (Fig. 3). The slowest perfusion technique we investigated was a commonly used Y-tube apparatus (Fig. 3*A*), with exchange times of 70–150 ms measured at an open electrode tip (Greenfield & Macdonald 1996). Currents required ~100 ms to reach peak, and subsequent desensitization was fitted with time constants in the range of 1000 ms (not shown). A motor driven 'stepper' that switches solutions by rapidly translating parallel flow pipes across a cell (Warner Instruments, see methods) provided much faster exchange times, with open tip measurements in the range of 1–2 ms (with faster exchange possible using increased solution flow rates). When GABA was applied using this technique, current rise times (10–90 %) were 7.3 ± 0.8 ms (Fig. 3*B* and *E*). Also, a fast component of desensitization could be resolved with relatively small amplitude and time constant of 31.8 ± 2.0 ms (Fig. 3*F* and *G*). The efficiency and speed of solution exchange around whole cells could be further improved by gently lifting the cell from the culture dish (Bianchi & Macdonald, 2001*a*). In this configuration (Fig. 3*C*), the current rise time was decreased to 1.7 ± 0.1 ms, and the first component of desensitization was both faster and of greater proportion (Fig. 3*C* and *F*). (Note that the current rise time was not a measure of solution exchange time because activation reflects agonist binding and channel gating, and desensitization curtailed the peak current.) Finally, using excised patches and optimized solution exchange times of 0.2–0.4 ms, we observed rise times of 0.55 ± 0.04 ms (Fig. 3*D*), along with a fast desensitization time constant of 6.0 ± 0.7 ms, which had a greater relative amplitude (Fig. 3*D* and *G*). The extent of apparent desensitization was also sensitive to application method (compare Fig. 3*A*–*D*), with greater apparent desensitization observed with faster application methods. This was probably accounted for by failure to reach the 'true' peak (from which the extent of desensitization is measured) with slower applications. Although 'back-extrapolation' has been used to correct for this failure to reach peak current, such manipulations cannot account for missed currents that decay with time constants that are near to or less than the time of current activation. The extrapolation process assumes that the portion of the time course available for measurement is an accurate representation of the process in question (i.e. fast desensitization). In other words, if the fast time constant is blunted by slow GABA application, the extrapolation will underestimate the 'true' peak. Figure 3*H* demonstrates the effects of back extrapolating a typical excised patch current using various fitting windows.

Although the results did not exclude the importance of intracellular factors or address their intactness following patch excision, they suggested that the differences in desensitization between receptors measured in whole cells and excised patches may have been accounted for in large part by the physical limitations in solution exchange times relative to the rapid channel kinetics of this type of GABA$_A$ receptor. Further support for this idea came from our observation that the pattern of desensitization of slowly desensitizing isoforms (such as for $\alpha1\beta3\delta$ receptors) was similar between intact, lifted and excised patch techniques, despite clear differences in current activation time (not shown). Despite our observation that fast processes, such as current activation and fast desensitization, were somewhat compromised by solution exchange limitations in whole cells (compared to patches), we concluded that the lifted cell mode was nevertheless appropriate for investigation of processes, such as the slower phases of desensitization, that equilibrated with slower time constants and were therefore less sensitive to solution exchange efficiency.

Structural determinants of multiphasic desensitization explored with δ-$\gamma2$L chimeras

To investigate the subunit structural domains responsible for the distinct desensitization of $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$L

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receptor currents, we tested a series of chimeras that contained δ subunit sequence in the N-terminal extracellular domain spliced at various points within the first two transmembrane domains to γ2L subunit sequence. Each chimera was expressed with α1 and β3 subunits and responses were recorded during 28 s applications of GABA (1 mM) (Fig. 4). Wild-type responses from Fig. 1 were shown again in Fig. 4*A* ($\alpha1\beta3\gamma$2L) and Fig. 4*F* ($\alpha1\beta3\delta$) for comparison. The first chimera, containing δ subunit sequence only in the N-terminus (M1e; Fig. 4*B1*), did not alter the pattern of desensitization, which still occurred with four phases of similar time constants (Fig. 4*B2*), and reached a mean current loss of 87.2 ± 1.9 % in the 28 s GABA application. The next chimera, M1pre-iso, contained two additional δ subunit residues moving the splice junction into transmembrane domain 1 (TM1) (Fig. 4*C1*). Using 400 ms pulses of GABA (1 mM) to excised patches, we previously showed that this chimera differed from M1e in that it blocked the fast phase of desensitization (Bianchi *et al.* 2001). Indeed, lifted cells expressing this chimera lacked the fast phase of desensitization. However, only two slow time constants of desensitization were resolved, with similar time constants to τ_3 and τ_4 observed in $\alpha1\beta3\gamma$2L, indicating that this chimera in fact lacked both the fast and intermediate phases of desensitization (Fig. 4*C2*). Despite the absence of these two desensitization phases, the currents nevertheless desensitized extensively (70.8 ± 2.7 %). Increasing the contribution of δ subunit sequence to include all of TM1 (M1i chimera) did not change desensitization; the extent of desensitization and its biphasic pattern was not different

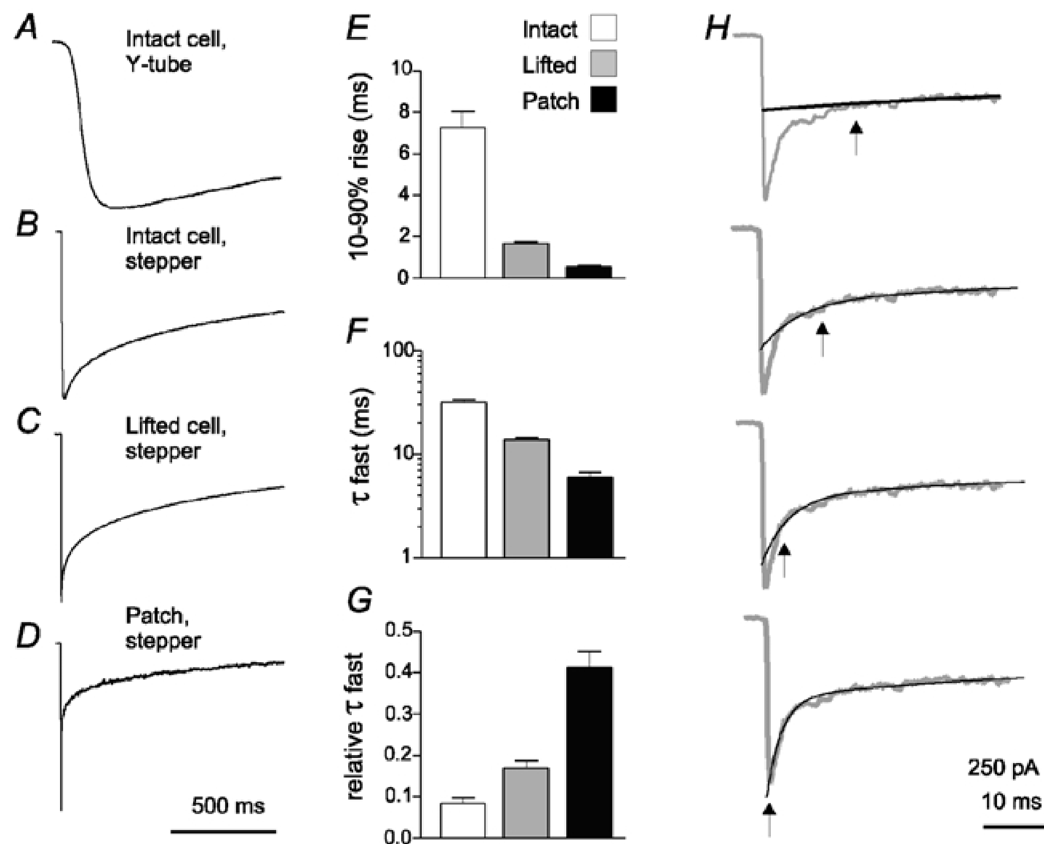

Figure 3. Comparison of activation and fast desensitization among various perfusion techniques

Representative currents were obtained from $\alpha1\beta3\gamma$2L receptors using a modified Y-tube (*A*), a stepper system applied to an intact cell (*B*) or a lifted cell (*C*), or an excised patch (*D*). Each current trace was obtained from a different cell, and normalized to peak amplitude for comparison. The scale bar in *D* applies to all four traces. *E*, current rise time, as indicated by the time elapsed between 10 and 90 % of the peak current, is shown for applications made with the stepper system using intact cells (open bars; $n = 34$), lifted cells (grey bars; $n = 38$) or excised patches (filled bars; $n = 13$) expressing $\alpha1\beta3\gamma$2L receptors. Bar colouration applies to panels *F* and *G* as well. *F*, the fastest fitted time constant of desensitization. Note the logarithmic ordinate. *G*, the relative contribution of the fastest desensitization component is shown. *H*, a typical current obtained from an excised patch (grey traces) is shown with an overlaid fitted curve (dark line) generated by extrapolating the fit to the time of current onset. The fits were generated between the 100 ms time point (not shown in the figure) and the time point indicated by the arrow. For the top trace, the best fit was a single exponential function, while the other three traces were fitted best by a two exponential function.

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from that observed for the M1pre-iso chimera (62.0 ± 2.8 %; Fig. 4*D1* and *D2*). However, extending δ subunit sequence to include TM2 clearly altered the extent of desensitization (41.3 ± 3.0 %; Fig. 4*E1*). Desensitization of the M2e chimera was indistinguishable from wild-type $\alpha 1\beta 3\delta$ receptor desensitization, except that it was always monoexponential (whereas 5 out of 10 $\alpha 1\beta 3\delta$ currents decayed biphasically), with a time constant resembling τ_4 (Fig. 4*D2*). These observations strongly implicated TM2 in the regulation of slow desensitization.

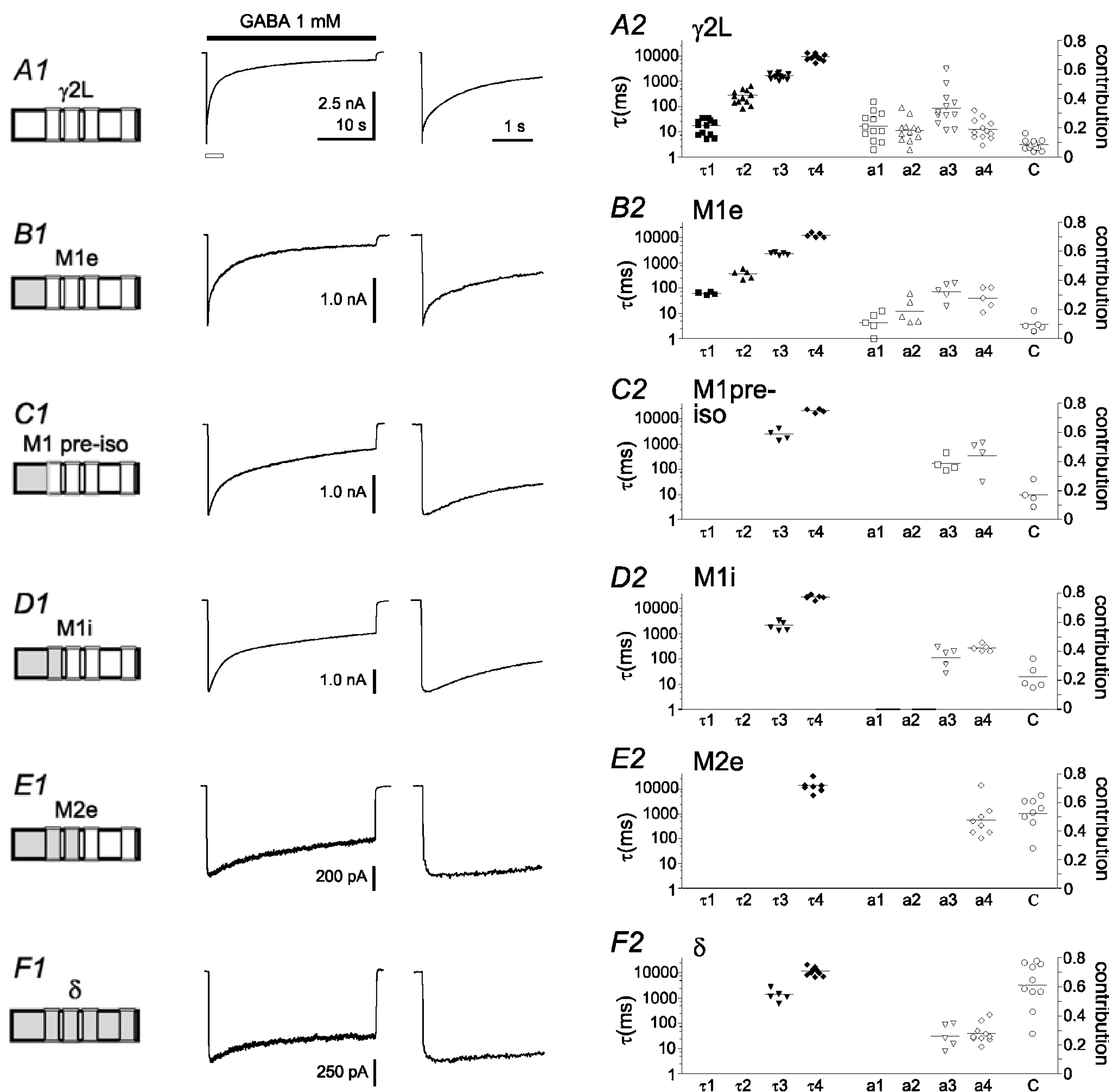

Figure 4. Structural determinants of desensitization explored through δ-γ2L chimeras

A1–F1, the subunit construct is shown in schematic form (left) with N-terminus to the left, and transmembrane domains represented by boxes. Open portions of the schematics indicate γ2L subunit sequence, while grey portions indicate δ subunit sequence. Current responses to 28 s GABA applications (filled bar in *A*1) for each construct (expressed with α1 and β3 subunits) are shown (middle), with the first 3 s (see open bar under trace in *A*1) expanded for comparison of initial phases of desensitization (right). *A2–F2*, scatter plots of all measured parameters obtained from fitting the desensitization time courses are shown (see methods). The left ordinate indicates the time constant of each component (left half of each plot), and the right ordinate indicates the relative contribution of the corresponding time constants, as well as the constant term to account for incomplete desensitization (right half of each plot). Wild-type traces and plots (*A* and *F*) are from Fig. 1.

We subsequently examined the desensitization of GABA$_A$ receptors containing δ or γ2L subunits in which the four divergent TM2 residues were replaced by γ2L or δ subunit sequence, respectively (Fig. 5). Replacing the δ TM2 with γ2L sequence clearly increased the extent of desensitization to 68.3 ± 1.3 %, significantly greater than $\alpha1\beta3\delta$ desensitization (38.1 ± 4.4 %) (Fig. 5*A*). The rate and extent of desensitization of δ(M2S) (Fig. 5*B*) was indistinguishable from M1pre-iso. This result suggested that the γ2L sequence in TM2 accounted for all of the observed desensitization in that chimera, which contained γ2L sequence in all of the transmembrane domains, except for the first two residues of TM1. Mutating subsets of the δ subunit TM2 to γ2L sequence did not significantly change desensitization compared to wild-type δ (not shown). Having shown that δ sequence in TM2 was necessary to decrease the extent of slow desensitization, we then tested whether it was sufficient. The reverse swap, with δ sequence introduced into TM2 of the γ2L subunit, had minimal impact on the pattern of desensitization (Fig. 5*C* and *D*). The extent of desensitization was slightly, but significantly, less for γ2L(M2S) (86.0 ± 1.7 compared to 91.1 ± 1.1 %). A summary of the desensitization extent is shown in Fig. 6 for comparison among the isoforms tested.

Possible role of slow desensitization for inhibitory synaptic transmission

Previous studies have suggested that GABA$_A$ receptor deactivation following brief agonist pulses (to mimic IPSC time course) was selectively shaped by fast desensitization; simulations suggested that slower phases had little or no effect on deactivation (Jones & Westbrook, 1995; Haas & Macdonald, 1999). Few studies have investigated the role of intermediate or slow phases of desensitization on channel function. Overstreet *et al.* (2000) demonstrated that persistent low concentrations of GABA decreased IPSC amplitude, and suggested that persistent low GABA concentrations favoured slow desensitization (which limited synaptic receptor availability). The model proposed by Haas & Macdonald (1999) also predicted inhibition of peak currents by pre-incubation with a similar IC$_{50}$ to that reported by Overstreet *et al.* (2000) (~2 μM; not shown), although occupancy of all three desensitized states (fast, intermediate and slow), contributed significantly to the inhibition. Our model supposes that all three proposed desensitized states are accessible only to the di-liganded receptor, in contrast to the model of Jones & Westbrook (1995). Multiphasic desensitization during the continued presence of saturating GABA has been demonstrated for both native and recombinant GABA$_A$ receptors (Celentano & Wong, 1994; Dominguez-Perot *et al.* 1997; Tia *et al.* 1996; Mellor & Randall, 1998; Haas & Macdonald, 1999; this study), indicating that slow desensitized states must be available to the fully liganded receptor. Therefore, to begin investigating the possible roles of specific desensitized states in GABA$_A$ receptor function, we conducted simulations using our comprehensive kinetic model that accounted for both single channel and macroscopic behaviour of $\alpha1\beta3\gamma$2L receptor currents across a range of GABA

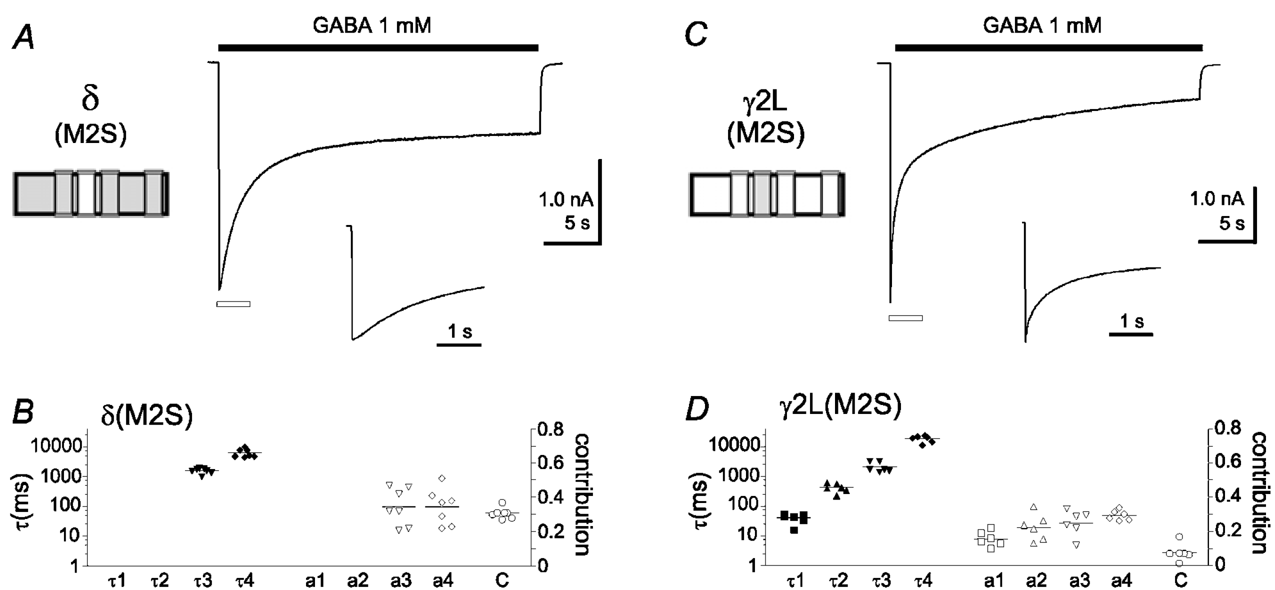

Figure 5. δ subunit sequence in TM2 is necessary but not sufficient to block desensitization

A, current response of $\alpha1\beta3\delta$(M2S) receptors to 28 s application of GABA (1 mM, filled bar) is shown. The four residues in the δ subunit that were exchanged for the corresponding residues in the γ2L subunit were: V264T, M278S, V279T, S280I (numbered according to the δ subunit mature peptide). The first 3 s (open bar) is expanded in the inset. *B*, scatter plot of fitted desensitization parameters is shown. *C*, current response of $\alpha1\beta3\gamma$2L(M2S) receptors to the same protocol and the fitted desensitization parameters (*D*) are shown.

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concentrations and application durations (Fig. 7*A*; Haas & Macdonald, 1999). Note that the longest application of GABA used to generate this model was 4 s, and thus the ultraslow phase of desensitization detected in the present study (t ~10 s) was not observed. A simulated current response to a prolonged GABA application was plotted in Fig. 7*B*, along with the probabilities of being in any of the three desensitized states. The multiphasic pattern of current loss reflected equilibration among the three desensitized states. The model response to a simulated 10 Hz train of 2 ms GABA pulses (1 mM) was plotted in Fig. 7*C1*, with the probabilities of occupying the three desensitized states given above the trace. Strong inhibition of subsequent GABA applications was observed. However, slower phases of desensitization were accumulating during the train, while the occupancy of the fast desensitization state (Df) was decreasing. Setting the entry rate constant into Df to zero altered the time course of inhibition during the train, but extensive current loss occurred nevertheless, as receptors accumulated in the slower desensitization states (Fig. 7*C2*). An additional prediction of fully liganded slow desensitization states was that for a given inter-pulse interval, the inhibition observed in the second response would be sensitive to the duration of the first pulse. In other words, when the first pulse was brief, occupancy of rapidly equilibrating states was favoured, such as Df. For a pair of 2 ms GABA pulses separated by 800 ms, the small amount of inhibition of the second pulse was accounted almost entirely by residual occupancy of the slower desensitized states (Fig. 7*D1*). As predicted, if the duration of the first pulse was increased to 200 ms, allowing additional time to equilibrate in the slower desensitized states, greater inhibition was observed in the second pulse (800 ms later) (Fig. 7*D2*). Again, this inhibition was accounted for by occupancy of the slow desensitized states, while Df occupancy was near zero. Finally, we tested the effect of smaller increases in the pulse duration of simulated pulse trains on occupancy of the three desensitized states. Increasing the pulse durations from 2 to 20 ms revealed a small increase in occupancy of the desensitized states (Fig. 7*E*). This simulation was important for the following experimental section, in which pulse trains were delivered to whole cells, requiring longer pulse durations due to perfusion limitations.

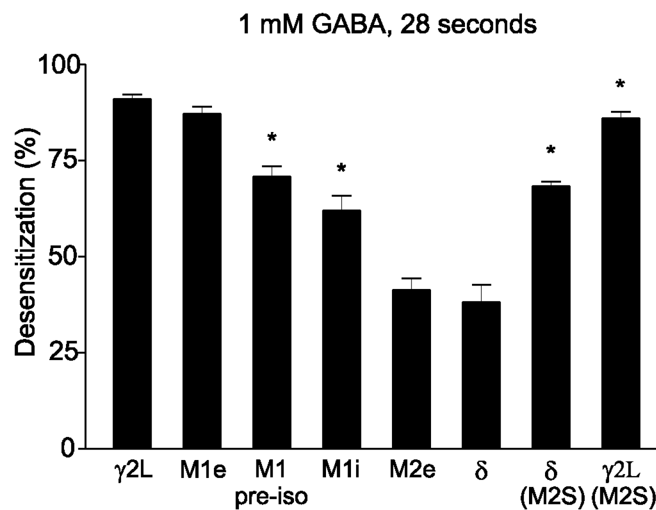

Figure 6. Summary of desensitization extent during 28 s applications of 1 mM GABA

Extent of desensitization was measured for each isoform as the following percentage: (peak current − current at offset of GABA)/peak current. * Significant difference compared to both $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors.

Although the models provided a theoretical framework for understanding the role of specific desensitized states in $GABA_A$ receptor function, we sought a model-independent test of the prediction that the slow phases of desensitization were relevant for $GABA_A$ receptor inhibition during repeated GABA applications. Several receptors with chimeric subunits were observed to desensitize extensively despite the absence of the fast and intermediate phases of desensitization (namely M1i, M1pre-iso and δ(M2S)). These receptors provided the functional equivalent of setting the entry rate constants into the fast and intermediate desensitized states to zero. We delivered trains of GABA (1 mM) applications that varied in duration and frequency. Although the individual pulses delivered to whole cells were, for technical reasons, longer than the likely duration of synaptic transients (~1 ms), they were a reasonable approximation and not likely to significantly increase entry into slower desensitized states compared to brief pulses (see simulation, Fig. 7*E*). The responses of $\alpha1\beta3\gamma2L$ receptors to trains of 10 ms pulses delivered at intervals of 100, 200, 500 or 2000 ms are presented in Fig. 8*A*. The extensive inhibition observed with 100 ms intervals (left trace) was gradually diminished as the interval was increased (next three traces). Intervals of 8 s or longer were required to completely eliminate depression (not shown). For the 2000 ms interval, we also tested 200 ms GABA pulse durations (right trace). Longer pulses favoured equilibration in slower phases of desensitization. Although little desensitization accumulated for brief GABA pulses when the interval was 2000 ms, when the duration of each GABA application was increased to 200 ms, inhibition was observed. Similar results were found in three other cells. Figure 8*B* shows current responses obtained from a cell expressing $\alpha1\beta3\delta$(M2S) using a similar train protocol. Inhibition was observed for 20 ms GABA pulses delivered every 100 ms (left trace), suggesting that fast and intermediate desensitized states were not solely responsible for the decreased current during repetitive stimulation. The extent of inhibition was decreased with increasing intervals (next three traces). Inhibition was then observed to increase for the 2000 ms interval when the GABA application duration was increased to 200 ms, similar to

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the results obtained with the rapidly desensitizing $\alpha 1\beta 3\gamma 2L$ receptors. Similar results were obtained from four other cells (expressing either M1pre-iso or δ(M2S)). Figure 8*C* shows the lack of inhibition observed with repetitive stimulation of $\alpha 1\beta 3\delta$ receptors, even with pulse durations of 1000 ms delivered at 1200 ms intervals (middle trace). This was expected based on the minimal extent of desensitization observed during a continuous 28 s GABA (1 mM) application for this cell (right trace).

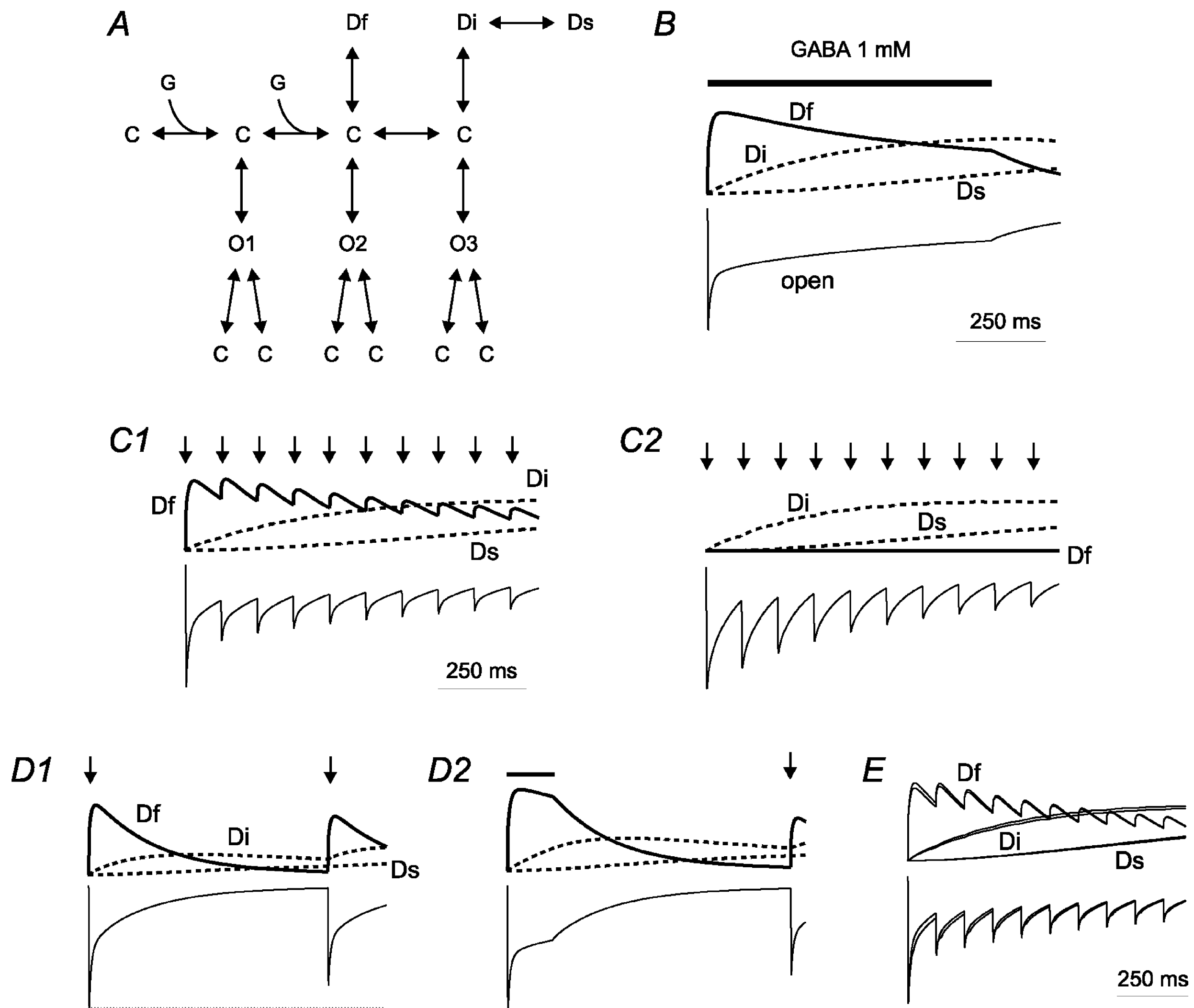

Figure 7. Simulations predict a role for slow phases of desensitization during repetitive stimulation

A, the kinetic model presented by Haas & Macdonald (1999) to account for single channel gating and macroscopic currents for $\alpha 1\beta 3\gamma 2L$ receptors is shown; rate constants were taken from that study. *B*, response of the model in *A* to an 800 ms pulse of GABA (1 mM; filled bar) is shown. The probability of Df (fast desensitization; continuous line), Di (intermediate desensitization; labelled dotted line) and Ds (slow desensitization; labelled dotted line) are shown above the current trace (downward dark line labelled as open). *C1*, the response of the model to repeated 2 ms GABA pulses (1 mM; arrows) every 100 ms is shown. The probability of each desensitized state is shown above the simulated current (continuous dark line). Occupancy of desensitized states is shown, as in *B*. *C2*, the same protocol as C*1* was used, except that the entry rate constant for Df is set to zero. *D1*, the response of the model to a pair of 2 ms GABA applications separated by 800 ms is shown. A horizontal dotted line is shown for visual comparison of the small inhibition of amplitude for the second peak current. *D2*, when the first GABA application is extended to 200 ms, the test pulse (2 ms) occurring 800 ms later shows greater inhibition. The model suggested that the greater inhibition was due to an increase in the probability of the slower phases of desensitization. *E*, same protocol as in *C1*, except that both 2 and 20 ms pulse durations were shown. The longer pulse duration resulted in a slight increase in the occupancy of all three open states, and a slight decrease in the simulated current amplitude.

DISCUSSION

We employed chimeras between the δ and γ2L subunits of the $GABA_A$ receptor to investigate the structural determinants of the slow components of multiphasic desensitization. Long duration GABA applications (28 s) permitted resolution of four phases of desensitization for $\alpha1\beta3\gamma$2L receptors (fast, intermediate, slow and ultraslow), whereas $\alpha1\beta3\delta$ receptor currents exhibited only two phases with time constants similar to the slow and ultraslow phases observed for $\alpha1\beta3\gamma$2L receptors. δ-γ2L chimeras indicated that distinct domains of the δ subunit were responsible for blocking the fast and intermediate phases of desensitization and regulating the contribution of the slow phases. Additionally, we demonstrated that slow phases of desensitization might play an important role in synaptic function under conditions of repetitive $GABA_A$ receptor activation.

Interpretation of macroscopic desensitization patterns

Using the concentration jump technique applied to excised patches, we previously showed that $\alpha1\beta3\gamma$2L receptors desensitized with three phases during a 4 s application of 1 mM GABA (τ_1 ~10 ms, τ_2 ~150 ms, τ_3 ~1500 ms), while $\alpha1\beta3\delta$ receptor desensitization was

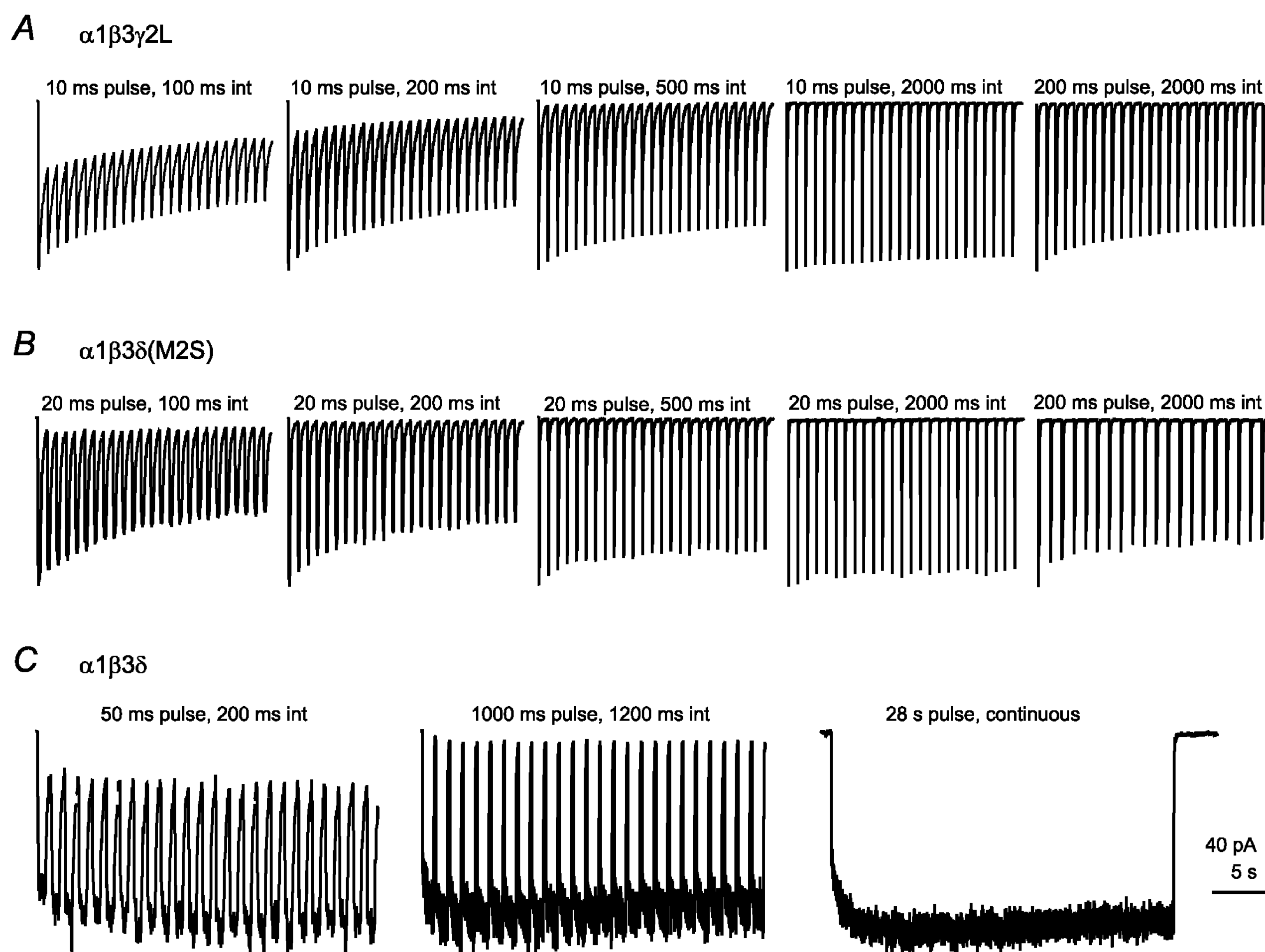

Figure 8. Fast desensitization is not required for inhibition during repetitive stimulation

A, current responses of $\alpha1\beta3\gamma$2L receptors to a series of 25 applications of GABA (10 ms; 1 mM) is shown. The interval between the start of each pulse is shown above the traces. The progressive inhibition of peak current amplitude decreased as the interval between pulses increased from left to right for the first four traces. The right trace shows the effect of increasing the duration of the GABA pulse to 200 ms for a 2000 ms interval (compare to the fourth trace). *B*, pulse train protocol was applied to $\alpha1\beta3\delta$(M2S) receptors, except that GABA was applied for 20 ms. Inhibition of peak currents during the repetitive stimulation was observed for this isoform, which lacks the two fast phases of desensitization. The right trace indicates the effect of increasing the GABA application duration to 200 ms (compare to fourth trace). *C*, $\alpha1\beta3\delta$ receptors show minimal inhibition during trains of GABA applications (left), even during 1000 ms applications separated by only 200 ms of wash (for a start–start interval of 1200 ms; middle). The current response to a continuous application of GABA (1 mM) is shown for comparison (right). The calibration applies only to the continuous current trace.

dominated by a slow phase of τ ~1500 ms (Haas & Macdonald, 1999). Using longer (28 s) applications to whole cells, we now report four phases of desensitization for $\alpha1\beta3\gamma2$L and two phases of desensitization for $\alpha1\beta3\delta$ receptors that corresponded to the slow and ultraslow phases (τ_3 and τ_4) of $\alpha1\beta3\gamma2$L receptor desensitization. The similar time constants τ_3 and τ_4 suggested that similar slow desensitized states were common to both isoforms, but that the overall desensitization extent was less in the $\alpha1\beta3\delta$ receptor. The δ subunit appeared to constrain the desensitization of GABA$_A$ receptors in two ways: by blocking entry into the fast and intermediate desensitized states (that account for τ_1 and τ_2), and by decreasing the overall extent of desensitization (accounted for by the remaining slow and ultraslow phases).

Because the extent of desensitization is determined by the relative occupancy of open and desensitized states, it is possible that differences in open state stability might actually be responsible for the desensitization patterns observed in this study. Increasing open state occupancy, or decreasing desensitized state occupancy, will lead to a decreased rate and extent of desensitization. For example, we have previously demonstrated that increased gating efficacy accounted for the significantly decreased rate and extent of apparent desensitization of $\alpha1\beta3\gamma2$L(L9′S) receptors (Bianchi & Macdonald, 2001*b*). It is unlikely that such a phenomenon accounts for the distinct desensitization of $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$L isoforms because their desensitization is the opposite of that predicted based on differences in gating efficacy alone. $\alpha1\beta3\delta$ receptors have been shown to exhibit brief and infrequent openings, even under conditions of maximal activation by high GABA concentration (1 mM) (Fisher & Macdonald, 1997; Haas & Macdonald, 1999), and yet desensitization is minimal. In contrast, $\alpha1\beta3\gamma2$L receptors have much higher gating efficacy, yet their desensitization is rapid and extensive.

Several constructs exhibited desensitization patterns that were intermediate between those observed with $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$L receptors. For the reasons stated above, we concluded that the increased extent of desensitization relative to $\alpha1\beta3\delta$ receptors (through greater contributions of the two slow phases) observed for receptors containing constructs such as M1pre-iso, M1i and δ(M2S) (see Fig. 4) was indeed due to changes in the relative occupancy of slowly equilibrating desensitized states and not a secondary effect of decreasing open state stability. However, single channel analysis of these chimeras would be required to rule out the possibility that receptors containing these constructs had compromised gating efficacy compared to $\alpha1\beta3\delta$ receptors.

The association of high efficacy gating and rapid desensitization for $\alpha1\beta3\gamma2$L receptors (and the lack of either phenomena in $\alpha1\beta3\delta$ receptors) raised the possibility that the processes are structurally linked. Previously, Naranjo & Brehm (1993) demonstrated that subunit switches in the nAChR resulted in a concomitant alteration of gating and desensitization properties. Further structure–function analysis at both macroscopic and single channel levels should reveal additional information about the interrelatedness of open and desensitized states.

Structural determinants of desensitization

Although TM2 contains most of the channel lining residues (Xu & Akabas, 1996), the physical nature of the channel gate, and the manner in which desensitization occludes chloride ion conduction, remain poorly understood. Electron micrograph analysis of the Torpedo nAChR has led to the suggestion that the channel gate resides in the middle of TM2 (Unwin, 1995). However, the accessibility of engineered cysteines near the cytoplasmic end of TM2 to extracellularly applied sulfhydryl reagents suggested a deeper location of the gate (Wilson & Karlin, 1998). Structural insights into desensitized conformations are even less clear. A presumably desensitized state induced by a pulse of high concentration of acetylcholine just prior to freezing the membranes indicated structural changes in the transmembrane domains as well as the extracellular N-terminus (Unwin, 1995). There are also several mutation studies that suggested the importance of various TM1 and TM2 residues in desensitization of GABA$_A$ receptors and other related ligand-gated ion channels (Revah *et al.* 1991; Yakel *et al.* 1993; Im *et al.* 1995; Labarca *et al.* 1995; Lynch *et al.* 1997; Dalziel *et al.* 2000). Involvement of trans-membrane domains in desensitization may suggest a distinct mechanism from glutamate-gated AMPA receptors, in which desensitization appears to be under the control of extracellular ligand binding domains (Stern-Bach *et al.* 1998; Banke *et al.* 2001).

Using the same set of δ-$\gamma2$L chimeras shown in this study, we previously reported that the fast phase of desensitization was blocked by δ-subunit sequence in the N-terminus and adjacent TM1 residues (Bianchi *et al.* 2001). However, the short duration GABA pulses used in that study (400 ms) were insufficient to accurately resolve slower phases of desensitization. We now extend the role of δ subunit N-terminus and adjacent TM1 residues to include block of the intermediate phase of desensitization. However, slow phases still accounted for extensive desensitization in chimeras that contained δ subunit sequence in the N-terminus and TM1 (M1pre-iso, M1i). Only when δ subunit sequence was extended to include TM2 (M2e chimera) was desensitization similar to that observed for $\alpha1\beta3\delta$ receptor currents. Notably, the importance of δ subunit sequence in TM1 and TM2 for regulating desensitization patterns was dependent on δ subunit sequence in the N-terminus. Introducing δ subunit sequence into TM2 of the $\gamma2$L subunit had only minimal effects on desensitization (this study), and the TM1 residues identified by the M1pre-iso chimera were

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insufficient to block fast desensitization (Bianchi *et al.* 2001). In contrast, mutation of δ subunit residues in TM1 (Bianchi *et al.* 2001) or TM2 (this study) to γ2L residues increased desensitization. This structural 'asymmetry' may suggest that multiple δ subunit domains are required to constrain $GABA_A$ receptor desensitization. Mutation of a subset of δ domains can compromise the minimal desensitization phenotpye of $\alpha\beta\delta$ receptors, while introducing subsets of the δ subunit into the γ2L subunit was insufficient to alter desensitization. Additional chimeras will clarify domains of the δ-subunit N-terminus that are required (in combination with the transmembrane domains) for regulation of desensitization.

Our results indicated that separate δ subunit domains modulated the presence of fast and intermediate phases, and the contribution of slow and ultraslow phases of desensitization. This would be consistent with two distinct desensitization 'gates', one of which operates on a relatively fast time scale (tens to hundreds of milliseconds), and the other on a slower time scale (one to tens of seconds). However, it is also possible that the multiple desensitized states inferred from macroscopic current measurements reflect different conformations of a single structure that can be modulated separately through TM1 and TM2. In this regard, it is interesting that TM1 and TM2 may be interleaved at their extracellular ends, based on data obtained from cysteine scanning mutagenesis (Akabas *et al.* 1994; Akabas & Karlin, 1995). Either way, it remains unknown whether the collapse of the conduction pore during desensitization involves alternative conformations of the activation gate, or a physically separate structure. It is also possible that some forms of desensitization (such as the slow phases that depended on TM2 sequence) involve stable closed conformations of the channel gate, while others operate through distinct structures.

A possible role of slow desensitization for inhibitory synaptic function

The model proposed by Jones & Westbrook (1995) predicted that slow desensitization of $GABA_A$ receptors was accessible in the monoliganded state and therefore relevant only for slowly developing, low concentration GABA transients. Although we cannot rule out monoliganded, desensitized states, there is clear evidence for multiple phases of desensitization in native and recombinant $GABA_A$ receptors. With the assumption that there are minimal monoliganded receptors during application of 1 mM GABA, there must be at least four desensitized states accessible in the fully liganded receptor, one to account for each time constant of desensitization. We have previously developed a model that incorporated slow desensitized states that are accessible to the fully liganded receptor (Haas & Macdonald, 1999). Simulations using this model suggested that although Df is the most relevant desensitized state for brief transients (similar to the model of Jones & Westbrook), in fact the slower desensitized states (Di and Ds) accumulate during repetitive brief GABA pulses, accounting for the progressive loss of current. Repetitive applications of GABA resulted in pronounced inhibition of $GABA_A$ receptor currents that showed extensive desensitization, independent of the presence of fast and intermediate phases of desensitization (Fig. 8*A* and *B*). This strongly suggested that slow desensitized states could accumulate under conditions of repeated activation, even with brief pulses of GABA. Paired pulse protocols and trains of high frequency stimulation result in depression of IPSC amplitude (Davies *et al.* 1990; Galarreta & Hestrin, 1998; Jiang *et al.* 2000; Bartos *et al.* 2001). Presynaptic mechanisms related to vesicular release have been demonstrated (Davies & Collingridge, 1993; Stevens & Wang, 1995). Also, shifts in the postsynaptic chloride equilibrium potential have been detected during repetitive firing (McCarren & Alger, 1985; Thomson & Gahwiler, 1989). However, few studies of synaptic $GABA_A$ receptors have identified desensitization as a mechanism for depressed responses with repeated $GABA_A$ receptor activation (Alger, 1991). Our results indicated that accumulation in slow desensitized states might represent an additional post-synaptic mechanism. Note that the extension of any results obtained with recombinant receptors assumes that native receptors behave in a similar fashion. Although this has not been explicitly proven, recombinant $GABA_A$ receptors exhibit many pharmacological and kinetic properties (desensitization, deactivation, single channel kinetics) of native receptors (reviewed in Olsen & Macdonald, 2002).

It has been suggested that non-desensitizing receptors would be ideally suited for sustained responsiveness to extrasynaptic GABA (Saxena & Macdonald, 1994; Nusser *et al.* 1998; Haas & Macdonald, 1999). However, sustained responses are also possible from $\alpha1\beta3\gamma$2L receptors. Although desensitization was rapid and extensive, it was never complete on the time scale we examined (28 s). The currents were typically larger than those observed for $\alpha1\beta3\delta$ receptors, so that even after 90 % current loss over 28 s there was still significant current remaining (hundreds of picoamps). $\alpha1\beta3\delta$ receptors did not desensitize extensively during the long applications, but they had smaller peak currents on average, so that the current remaining was also in the hundreds of picoamps range. The basis for this large difference in current size may be related to differences in expression level, or gating efficacy, which is considerably greater for $\alpha1\beta3\gamma$2L receptors (Fisher & Macdonald, 1997; Haas & Macdonald, 1999). Whatever the basis may be, if the difference persisted in a neuronal environment, extrasynaptic $\alpha1\beta3\gamma$2L receptors might be able to contribute sustained membrane currents despite significant desensitization. There is evidence for $\alpha\beta\gamma$ isoforms in extrasynaptic membranes (Nusser *et al.* 1998; Brickley *et al.* 1999). Also, less apparent desensitization

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is observed in $\alpha1\beta3\gamma2$L receptors currents evoked by low agonist concentrations (Celentano & Wong, 1994; Haas & Macdonald, 1999). Thus, tonic inhibition resulting from prolonged GABA exposure may not be strictly limited to non-desensitizing isoforms.

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