

# GABA Increases both the Conductance and Mean Open Time of Recombinant GABA<sub>A</sub> Channels Co-expressed with GABARAP\*

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The single channel properties of recombinant  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>)  $\alpha\beta\gamma$  receptors co-expressed with the trafficking protein GABARAP were investigated using membrane patches in the outside-out patch clamp configuration from transiently transfected L929 cells. In control cells expressing  $\alpha\beta\gamma$  receptors alone, GABA activated single channels whose main conductance was 30 pS with a subconductance state of 20 pS, and increasing the GABA concentration did not alter their conductance. In contrast, when GABA<sub>A</sub> receptors were co-expressed with GABARAP, the GABA-activated single channels displayed multiple, high conductances ( $\geq 40$  pS), and GABA ( $\geq 10 \mu\text{M}$ ) was able to increase their conductance, up to a maximum of 60 pS. The mean open time of GABA-activated channels in control cells expressing  $\alpha\beta\gamma$  receptors alone was  $2.3 \pm 0.1$  ms for the main 30-pS channel and shorter for the subconductance state (20 pS,  $0.8 \pm 0.1$  ms). Similar values were measured for the 30- and 20-pS channels active in patches from cells co-expressing GABARAP. However higher conductance channels ( $\geq 40$  pS) remained open longer, irrespective of whether GABA or GABA plus diazepam activated them. Plotting mean open times against mean conductances revealed a linear relationship between these two parameters. Since high GABA concentrations increase both the maximum single channel conductance and mean open time of GABA<sub>A</sub> channels co-expressed with GABARAP, trafficking processes must influence ion channel properties. This suggests that the organization of extrasynaptic GABA<sub>A</sub> receptors may provide a range of distinct inhibitory currents in the brain and, further, provide differential drug responses.

Inhibitory signals in human brains are mediated primarily by  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>)<sup>2</sup> receptors. These ligand-gated ion channels are composed of multimembrane-spanning subunits that assemble into pentamers and function by gating a pore selective for chloride ions. The targeting and organization of GABA<sub>A</sub> receptors at specific membrane locations are critical

for their normal function. For example, GABA<sub>A</sub> receptors are clustered at inhibitory synapses but are also found both clustered and nonclustered at other sites on the neuronal cell surface (1, 2). These synaptic and nonsynaptic (extrasynaptic) sites reflect GABA<sub>A</sub> receptor involvement in both phasic and tonic signaling, respectively. The functional behavior of native GABA<sub>A</sub> receptors is complex. Much of the receptor's functional complexity has been attributed to its extensive structural heterogeneity as indicated by the 19 different genes identified to date ( $\alpha 1-6$ ,  $\beta 1-3$ ,  $\gamma 1-3$ ,  $\delta$ ,  $\rho 1-3$ ,  $\epsilon$ ,  $\theta$ , and  $\pi$ ).

Recombinant GABA<sub>A</sub> receptors are different from native GABA<sub>A</sub> receptors in that they never display single channel conductances greater than 40 pS, nor do drugs modulate their conductance, properties we and others have described for native non-synaptic (extrasynaptic) GABA<sub>A</sub> receptors (3–8). We have, however, been able to mimic the behavior exhibited by neuronal extrasynaptic GABA<sub>A</sub> receptors in a recombinant system and change the dispersion of receptors in the membrane simply by co-expressing the trafficking protein GABARAP with GABA<sub>A</sub> receptors (9). GABARAP (GABA<sub>A</sub> receptor-associated protein) was originally identified because of its physical association with GABA<sub>A</sub> receptors following their isolation by immunoprecipitation from solubilized rat brain (10). Subsequent immunolocalization data and the biochemical identification of the GABARAP interaction partners has led to the suggestion that it participates in trafficking and membrane fusion events underlying organizational processes at GABAergic synapses but does not remain associated with receptors once they are inserted at the synapse (11). In heterologous expression systems, recombinant GABARAP has been shown to promote clustering of  $\gamma$  subunit-containing GABA<sub>A</sub> receptors in the plasma membrane (9, 12). As a consequence of this ordered packing arrangement, the recombinant GABA<sub>A</sub> receptors function differently from receptors expressed in the absence of GABARAP. At the macroscopic level, changes in the rates of receptor desensitization and deactivation have been reported in addition to an increase in the GABA EC<sub>50</sub> (concentration required to elicit a half-maximal response) (12). At the single channel level we have shown that increased single channel conductances ( $\geq 40$  pS) are associated with GABA<sub>A</sub> receptors co-expressed with GABARAP and that drugs are able to increase the conductance of these channels (9). The aim of the present study was to analyze the single channel properties of GABA<sub>A</sub> receptors co-expressed with GABARAP and to see if these newly acquired properties correlated with these receptors being organized at a higher density (*i.e.* clustered) in the membrane. By analyzing single

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We dedicate this paper to the memory of Professor Peter Gage.

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<sup>2</sup> The abbreviations used are: GABA<sub>A</sub>,  $\gamma$ -aminobutyric acid type A; pS, picosiemens; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

channel currents activated by a range of GABA concentrations, we determined single channel conductances and mean open times and identified a correlation between these two parameters. Furthermore, at saturating GABA concentrations, our data indicate that co-expression with GABARAP does indeed increase the number of receptors in a patch.

## EXPERIMENTAL PROCEDURES

### Recombinant Expression System

Mouse L929 fibroblasts (American Type Culture Collection, Manassas, VA) were grown in minimum essential medium containing 200 IU ml<sup>-1</sup> penicillin and 200 µg ml<sup>-1</sup> streptomycin and supplemented with 10% heat-inactivated fetal bovine serum (Trace Biosciences) and incubated at 37 °C in 5% CO<sub>2</sub>, 95% air. Cells were transfected using a lipid-mediated reagent (Lipofectin; Sigma) as described in detail previously (13). For the identification of successfully transfected cells, the plasmid encoding enhanced green fluorescent protein (EGFP-N1; Clontech) was used in every transfection. Human α1, β1, γ2S, GABARAP, and enhanced green fluorescent protein plasmids were combined and added to the cells in equal ratios of 1:1:1:1 (α/β/γ/green fluorescent protein) or 1:1:1:1:1 (α/β/γ/GABARAP/enhanced green fluorescent protein) using 5 µg of each DNA. Cells that had bright green fluorescence were used for electrophysiological recordings between 24 and 72 h later.

### Electrophysiology

**Drugs**—GABA, (–)-bicuculline methiodide (both purchased from Sigma), and diazepam (gift from Hoffmann-La Roche) were diluted with bath solution to the final concentration on the day of the experiment. Concentrated stocks of drugs were made up in water, except for diazepam. Diazepam (2.8 mg) was first dissolved in Me<sub>2</sub>SO (50 µl) and then diluted to 1 mM with water. In the final diazepam solution, there was less than 0.001% Me<sub>2</sub>SO. It has been published that Me<sub>2</sub>SO, at concentrations as high as 0.1%, does not activate or modulate GABA<sub>A</sub> receptors (14, 15), and similarly, we observe no effect of the diluent on our patch clamp recordings.

**Recording Solutions and Techniques**—Standard outside-out patching techniques (16) were used to record currents. External recording medium (bath solution) contained the following 135 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM glucose, and 10 mM 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid (pH 7.4). The intrapipette solution contained 50 mM NaCl, 80 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 5 mM EGTA, and 10 mM TES (pH 7.3). This combination of bath and intrapipette solution produced a chloride equilibrium potential (17) of approximately –1.8 mV, whereas the equilibrium potentials for sodium and potassium were +19 and –70 mV, respectively. Patch pipettes were made using borosilicate glass capillaries (1.5-mm OD × 0.86-mm ID) fabricated on a pipette puller (Sutter Instruments Co.) and subsequently fire-polished and coated with Sylgard (Dow Corning) before use. Patch pipette resistances ranged from 10 to 20 MΩ for single channel recordings when filled with the pipette solution and immersed in bath solution. Drugs were applied via gravity-fed flow tubes and placed directly in front of the patch by lateral movement of the flow tube using a hydraulic micromanipulator. Data from two or

more separate transfections were pooled for analysis. All recordings were carried out at room temperature (20–22 °C). Currents were monitored with an Axopatch 200A amplifier, filtered at 5 kHz (3 dB, 4-pole Bessel; Axon Instruments) and directly recorded onto the computer (Axoscope7; ADC: digidata board 1200; Axon Instruments) sampling at either 10 or 20 kHz.

**Analysis of Currents**—All single channel currents were analyzed using in-house software, CHANNEL 2 (written by M. Smith and P. W. Gage). Statistical analyses were performed using Excel (Microsoft). To compare sample means, paired or unpaired, a two-tailed *t* test, not assuming equal variance, was used. A critical value of *p* < 0.05 was used to define statistical significance.

**Single Channel Analysis**—All single channel recordings were performed at a holding potential of –60 mV using the outside-out patch clamp technique. Before analysis, the recordings were filtered at 2 kHz using the program CHANNEL 2, unless specified (e.g. mean open times). Single channel current amplitudes were measured directly and were only accepted as valid events if their open duration was at least 0.3 ms (*i.e.* 3 times the sampling rate). Amplitude histograms were then constructed using more than 500 openings in which the bin widths were 0.06 pA, and these were subsequently fitted to the sum of Gaussian components (Equation 1) using least-squares minimization. The number of Gaussian components required to fit the histogram was determined by the criteria set out by Horn (18). The Gaussian function (*g(I)*) used to fit an amplitude frequency histogram was as follows.

$$g(I) = A_1 e^{-0.5 \left( \frac{I - m_1}{s_1} \right)^2} + A_2 e^{-0.5 \left( \frac{I - m_2}{s_2} \right)^2} + \dots + A_n e^{-0.5 \left( \frac{I - m_n}{s_n} \right)^2} \quad (\text{Eq. 1})$$

**Single Channel Peak Current**—In outside-out patches, the rapid application of high GABA concentrations ([GABA]) invariably induced overlapping single channel openings whose maximum peak current was reached within 20 ms of the application. The single channel peak current for each [GABA] (1, 10, 100, and 1000 µM) was therefore determined as the peak amplitude of the current induced within the first 20 ms. The mean peak current was obtained by pooling data from three or more experiments.

**Single Channel Mean Open Time**—Unfiltered segments of typical single channel data were used for analysis only if simultaneous openings were rare or accounted for less than 1% of the total number of channel openings sampled. Using a program available in CHANNEL 2, the single channel amplitudes and their corresponding open duration can be measured automatically, after an “open” and “closed” threshold has been set. To determine the open time of single channel events that were ≤40 pS, the open and closed thresholds were set at half the amplitude of the smallest single channel conductance, typically 20 pS; hence, the threshold was set at 10 pS. At a holding potential of –60 mV, this threshold was set at 0.6 pA. Using the same criteria to determine the open time of single channel events that had conductances greater than 40 pS, the open and closed thresholds were set at 1.2 pA. In single channel recordings that exhibited both high and low single channel conductance events, the open times of these channel events were sampled twice. The first round was to collect the open times of the low conducting channels (≤40 pS), and the second was for the high conducting

channels ( $>40$  pS). This procedure was done because it was found that a number of the high conducting channels had brief closures (it was considered a closed event when the channel closed more than half the amplitude of that channel opening). These closures, however, did not cross the "lower" closed threshold set at 0.6 pA and was therefore deemed to be a single open event by the analysis program. Under these conditions, the mean open time of the higher conducting channels was biased toward longer times.

For each record, the single channel amplitude data set generated was used to construct an amplitude frequency histogram, and this was fitted with sum of Gaussians to determine the most frequently occurring single channel conductances. The resolved conductances determined from the histogram were confirmed by checking their presence in the single channel recordings. Subsequently, the open times were determined by taking the average of the open times of those amplitudes that were between two S.D. values below and above the mean of that Gaussian component. This was performed for each of the conductance levels that were resolved. The mean open time of each conductance state was derived from pooling three or more experiments that exhibited a particular conductance state.

**Probability of Simultaneous, Independent Channel Openings**—To determine whether high conductance channels could be due to the random simultaneous openings of independent channels, the probability of observing rapid transitions between the closed level and  $I_{max}$  (maximum single channel current) were calculated. We calculated whether the 60-pS conductances were due to the simultaneous openings of two 30-pS independent channel openings, because the 30-pS channels account for more than 50% of all opening events (Table 1). Furthermore, the low frequencies of the 20- and 40-pS channels make their random participation in generating a 60-pS channel less likely.

The probability of  $n$  independent channels opening simultaneously can be examined by a binomial distribution (Equation 2). Such methodology has been described by Sakmann and Neher (19).

$$\Pr(s) = \frac{n!}{s!(n-s)!} p_o^s (1-p_o)^{n-s} \quad (\text{Eq. 2})$$

The probability that two channels open simultaneously ( $s = 2$ ) thus becomes the following.

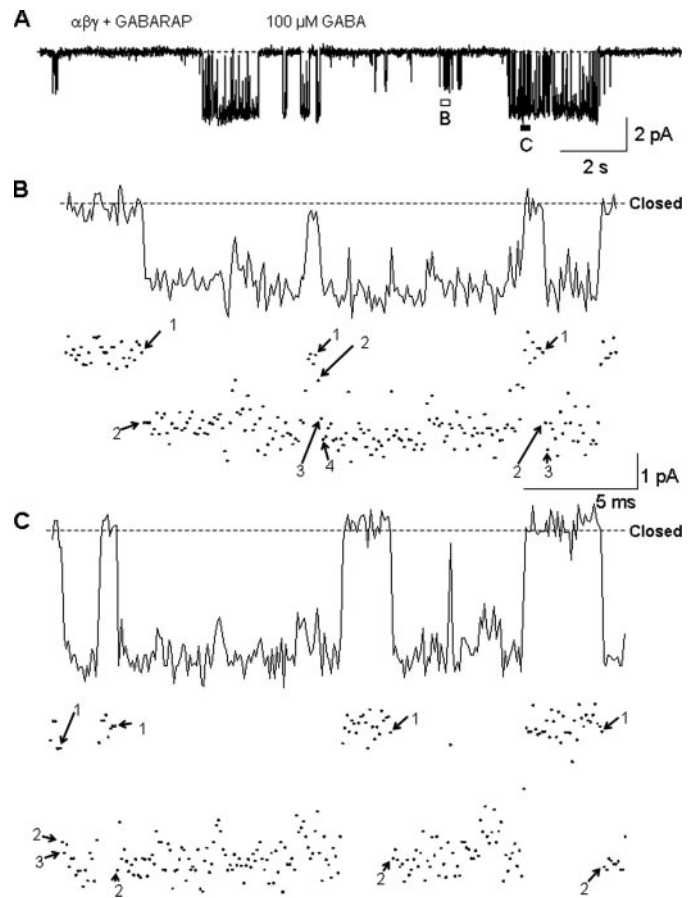
$$\begin{aligned} \Pr(s=2) &= \frac{n!}{2!(n-2)!} p_o^2 (1-p_o)^{n-2} \\ &= \frac{n(n-1)}{2} p_o^2 (1-p_o)^{n-2} \quad (\text{Eq. 3}) \end{aligned}$$

Since  $(1-p_o)^{n-2} < 1$ , then

$$\Pr(s=2) < \frac{n(n-1)}{2} p_o^2 \quad (\text{Eq. 4})$$

Since  $n^2 - n < n^2$ , then the following is true

$$\Pr(s=2) < \frac{(np_o)^2}{2} \quad (\text{Eq. 5})$$



**FIGURE 1. Single channel opening transitions.** These single channel traces are from a patch (P2) co-expressing  $\alpha\beta\gamma$  receptors and GABARAP that displayed 60-pS channel openings more than 50% of the time. A, 30 s of channel activity from an outside-out patch activated by 100  $\mu\text{M}$  GABA. On an expanded time scale, transitions to the 30- and 60-pS open states are depicted in B and C, respectively, illustrating that the fast transitions to each conductance occur within 400  $\mu\text{s}$ . Recordings were filtered at 5 kHz and sampled at a frequency of 10 kHz (hence, each data point represents 100  $\mu\text{s}$ ). Membrane potential was  $-60$  mV.

From our single channel recordings,  $np_o$  can be calculated as follows.

$$nPo = \frac{x_1 + 2x_2}{Tr} \quad (\text{Eq. 6})$$

where  $x_1$  is the number of 30-pS channel openings,  $x_2$  is the number of 60-pS channel openings, and  $Tr$  is the number of rapid transition periods in that particular recording.

Single channels go from the closed state to an open state in a very short space of time. With our current sampling resolution, one point per 100  $\mu\text{s}$  (10 kHz), we have found that channels take less than four points to go from a closed state to an open state. Thus, this rapid transition from channel closed to channel open takes less than 400  $\mu\text{s}$  and therefore is defined as our rapid transition period. In a 30-s recording, there are 75,000 rapid transition periods ( $Tr = 30 \text{ s}/400 \mu\text{s}$ ). A typical single channel recording is depicted in Fig. 1 from a cell co-expressing GABA $_A$  receptors and GABARAP. The figure illustrates the time resolution of these rapid transitions from closed to open for both the 30-pS channels (Fig. 1B) and the 60-pS channels (Fig. 1C), where each data point represents 100  $\mu\text{s}$ .

**A**  $\alpha\beta\gamma$ 

No spontaneous channel activity

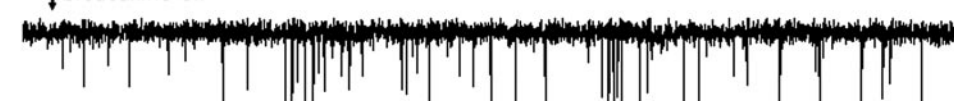
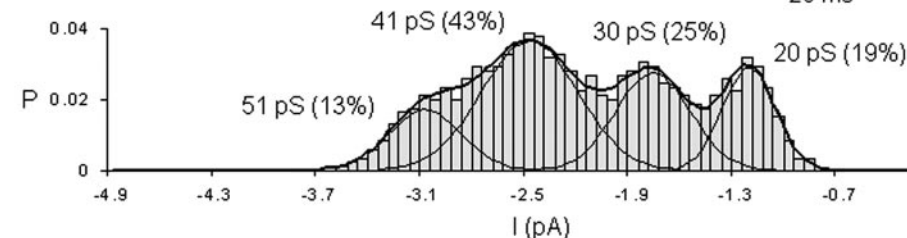
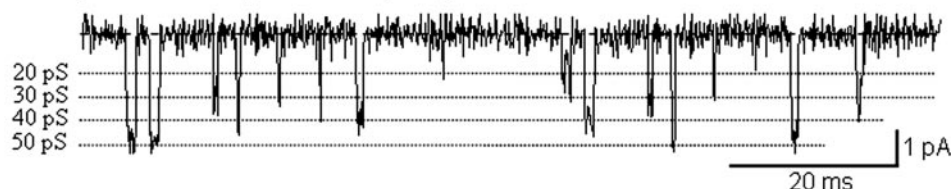
**B**  $\alpha\beta\gamma$  + GABARAP

Spontaneous channel activity

↓ bicuculline on



↓ bicuculline off

1 pA  
2 s**C** Spontaneous single-channel openings

**FIGURE 2. GABA<sub>A</sub> receptors show spontaneous channel activity when co-expressed with GABARAP.** L929 cells transfected with just  $\alpha\beta\gamma$  receptors typically showed no spontaneous channel activity, as shown in A. By comparison, spontaneous channel activity was invariably observed in cells co-transfected with  $\alpha\beta\gamma$  receptors and GABARAP. B shows the persistent nature of the spontaneous channel activity and its inhibition by bicuculline (100  $\mu$ M). From the same experiment as depicted in B, C shows, on an expanded time scale, the spontaneous channel events and their different single channel amplitudes. The amplitude frequency histogram constructed for this experiment is shown below and was best fitted with the sum of four Gaussians, indicating four predominant single channel conductances were present. All experiments were performed at  $-60$  mV, using the outside-out patch configuration.

From our single channel analysis, we can now find the following.

$$\Pr(s = 2) < \frac{(np_o)^2}{2} \approx \left( \frac{(x_1 + 2x_2)}{Tr\sqrt{2}} \right)^2 = \hat{\mu}$$

= sample mean probability for  $\Pr(s = 2)$  (Eq. 7)

$$\sigma = \sqrt{V(p_2)} = \sqrt{\frac{p_2(1 - p_2)}{Tr}}$$

$$\approx \sqrt{\frac{p_2}{Tr}} \approx \sqrt{\frac{(np_o)^2}{2Tr}} = \sqrt{\frac{\mu}{Tr}} = \hat{\sigma} = \text{sample S.D.} \quad (\text{Eq. 8})$$

Since  $p_2$  follows approximately the normal distribution, the  $Z$  distribution can be used here as follows,

$$Z = \frac{p_2 - \hat{\mu}}{\hat{\sigma}} \quad (\text{Eq. 9})$$

where  $Z \sim N(0,1)$ , and  $p_2$  is the observed probability of the 60-pS channels, measured from the raw data by  $x_2/Tr$ .

This is likely to overestimate the  $p$  value, so the final expression should be an inequality, as follows,

$$p < \frac{1}{2} \text{erfc} \left( \frac{p_2 - \hat{\mu}}{\hat{\sigma}\sqrt{2}} \right) \quad (\text{Eq. 10})$$

$$p < \frac{1}{2} \text{erfc} \left( \frac{Z}{\sqrt{2}} \right) \quad (\text{Eq. 11})$$

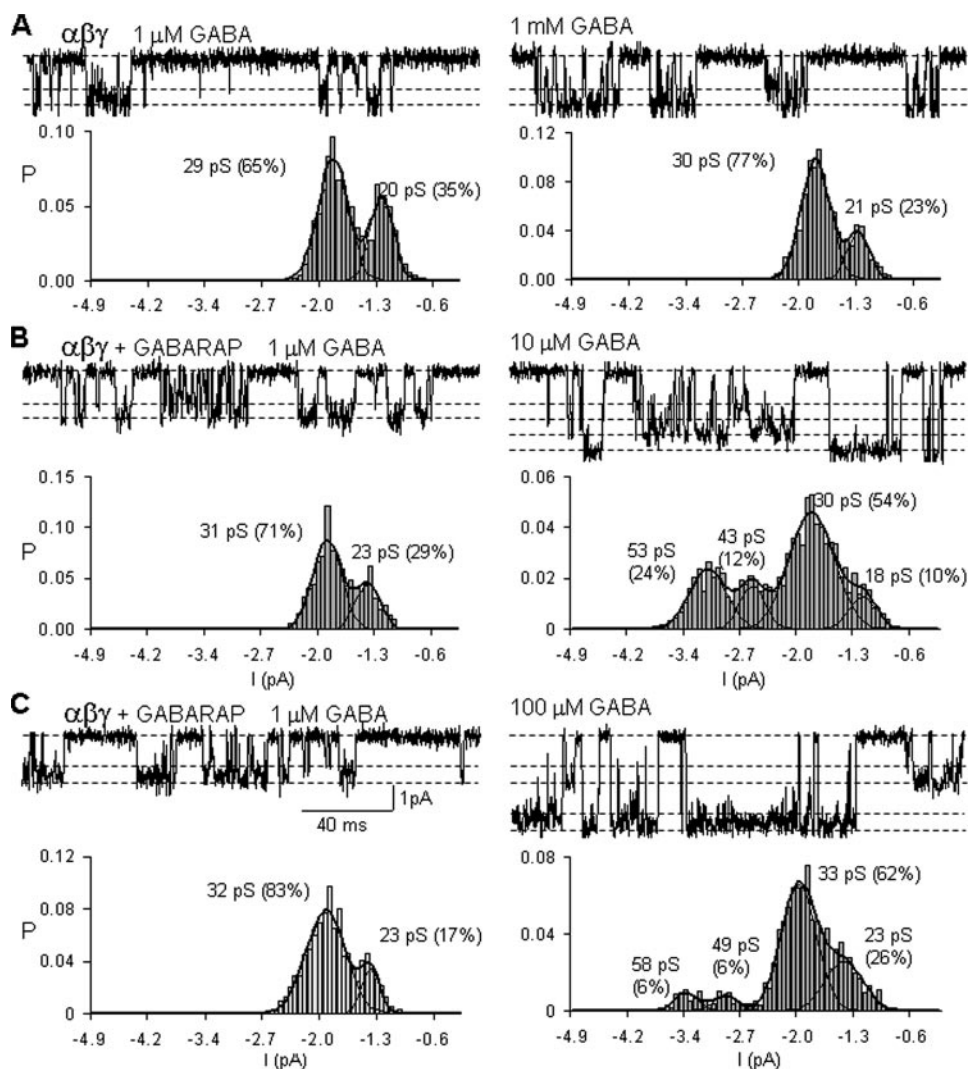
where  $p$  represents the statistical probability of observing  $p_2$  from our data. A  $p$  value of  $<0.01$ , for example, indicates that there is less than a 1% chance that our null hypothesis is true, which means there is less than a 1% chance of observing as many 60-pS single channel events as found in the data (*i.e.*  $p_2$ ) if they are indeed due to two independent 30-pS channels opening simultaneously. When the  $p$  value is close to zero, we can reject this null hypothesis.

**RESULTS**

The outside-out patch configuration was used to examine the effects of GABA concentration on the single channel properties of recombinant GABA<sub>A</sub> receptors co-expressed with GABARAP and to

compare these properties with GABA<sub>A</sub> receptors expressed alone in L929 cells.

**GABA<sub>A</sub> Receptors Co-expressed with Recombinant GABARAP Display Spontaneous Channel Openings**—Outside-out patches excised from cells co-expressing recombinant GABARAP and  $\alpha 1\beta 1\gamma 2S$  receptors exhibited spontaneous single channel activity with openings being very brief in duration. These spontaneous single channel currents originated from GABA<sub>A</sub> receptors, because in all cases tested, bicuculline (100  $\mu$ M,  $n = 7$ ) reversibly inhibited their activity (Fig. 2A), and currents reversed close to the chloride equilibrium potential ( $\sim 0$  mV), consistent with the current being carried by  $\text{Cl}^-$  ions. What was distinct about these spontaneous single channels was



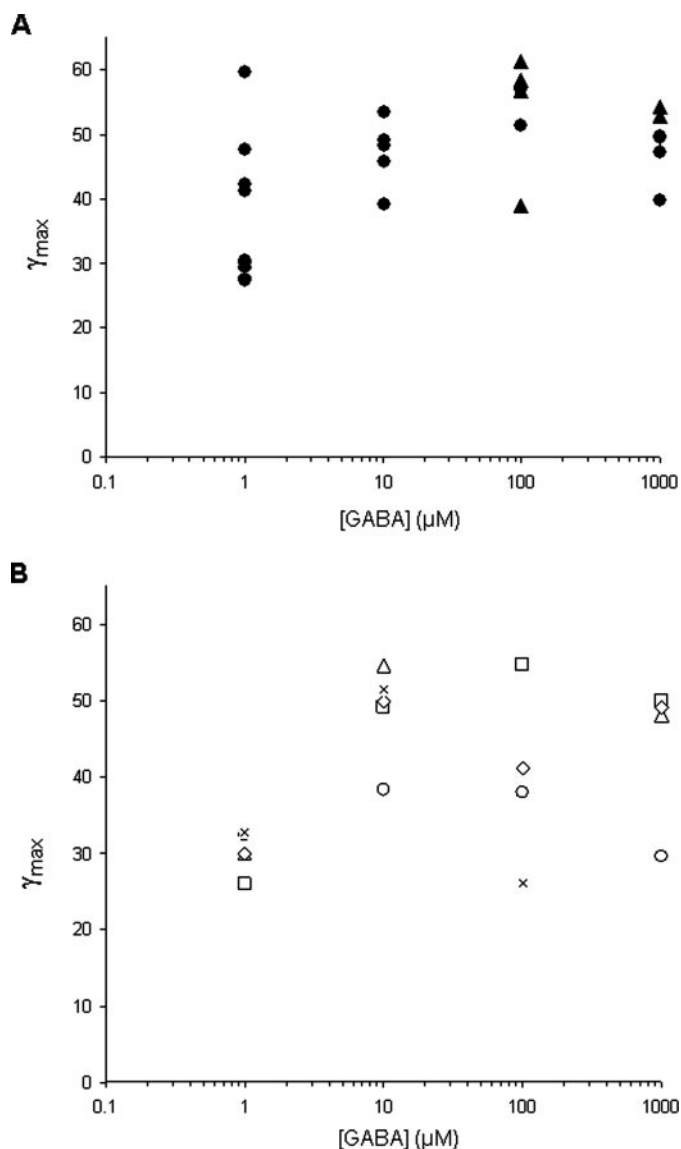
**FIGURE 3. GABA increases the single channel conductance of  $\alpha\beta\gamma$  receptors co-expressed with GABARAP.** *A*, single channel responses from  $\alpha\beta\gamma$  receptors in a patch that was exposed to 1  $\mu\text{M}$  GABA and then to 1 mM GABA. The maximum single channel conductance did not change with the increase in [GABA]. In cells co-expressing  $\alpha\beta\gamma$  receptors and GABARAP, single channel conductance was increased by [GABA] as depicted in *B* and *C*. *B* shows an example of a 10  $\mu\text{M}$  GABA application that increased the maximum single channel conductance to 53 pS from 31 pS activated by 1  $\mu\text{M}$  GABA. *C* shows another experiment in which the patch was exposed to 1  $\mu\text{M}$  GABA and then to 100  $\mu\text{M}$  GABA. In this patch, the maximum single channel conductance increased from 32 to 58 pS. An amplitude frequency histogram was constructed for each GABA application and shows the predominant single channel conductances during the exposure to GABA. All data shown were recorded in the outside-out patch configuration and at a holding potential of  $-60$  mV.

that they were often high ( $\geq 40$  pS) and they displayed a range of conductances (2–5 states), characteristics never seen in control cells expressing GABA<sub>A</sub> receptors alone (13). To illustrate the variation in the single channel conductances of the spontaneous currents and the frequency of the amplitudes within a single patch, a typical example is shown in Fig. 2*B*. Here the spontaneous channels displayed conductances as high as  $\sim 50$  pS and as low as  $\sim 20$  pS with conductance states in between in integrals of  $\sim 10$  pS. In general, the maximum conductance of spontaneous channels ranged between 30 and 60 pS ( $n = 25$ ), and the frequency of particular conductance states varied between patches but all patches expressing GABA<sub>A</sub> receptors and GABARAP displayed spontaneous GABA<sub>A</sub> channel activity, unlike those expressing just  $\alpha\beta\gamma$  receptors. These later patches occasionally displayed spontaneous channel activity ( $n =$

10/33), but current activity was short lived and always dissipated within 1 min.

**GABA Increases the Maximum Single Channel Conductance When Recombinant GABARAP Is Co-expressed**—The effect of GABA concentration ([GABA]) on single channel conductance was examined by exposing outside-out patches to a range of GABA concentrations (1, 10, 100, and 1000  $\mu\text{M}$ ). In our hands and in all other published reports, GABA *per se* does not alter the single channel conductance of recombinant GABA<sub>A</sub> receptors (Fig. 3*A*). By contrast, GABA could increase the maximum single channel conductance in patches pulled from cells co-expressing  $\alpha\beta\gamma$  and GABARAP. For example, in 16 patches, the application of 1  $\mu\text{M}$  GABA generated low conductance channels, indistinguishable from control cells ( $\sim 30$  and 20 pS). However, subsequent application of a higher [GABA], either 10, 100, or 1000  $\mu\text{M}$ , always resulted in an increase in the maximum conductance when GABARAP was co-expressed. Fig. 3 illustrates the effect of [GABA] on single channel conductance. In the *left-hand trace* in *A–C*, 1  $\mu\text{M}$  GABA activated channels with a maximum conductance of  $\sim 30$  pS. However, when a higher [GABA] was subsequently applied to the same patch, the maximum conductance increased but only in patches co-expressing GABA<sub>A</sub> receptors and GABARAP (*B* and *C*). The ampli-

tude histograms *below* each *trace* show the distribution and frequency of conductances displayed in the patch over a longer time period. These amplitude histograms illustrate the quantal distribution of the mean conductance levels observed in this and in all patches in this group. In a limited subset of patches, an additional application of an even higher [GABA] (100 or 1000  $\mu\text{M}$ ) did not significantly further increase the maximum conductance of these high conductance channels ( $n = 5/18$ ) (Fig. 4*B*, *open symbols*). In one of these experiments, the conductance actually decreased (Fig. 4*B*, *open circle*). The agonist GABA produced a range of maximum conductances between 40 and 60 pS that increased in elementary steps of  $\sim 10$  pS and appeared to be independent of concentrations  $\geq 10$   $\mu\text{M}$ . Fig. 4*A* shows a scatter plot of these conductance values from 25 experi-



**FIGURE 4. GABA modulation of conductance in outside-out patches co-expressing  $\alpha\beta\gamma$  and GABARAP.** The data represent individual experiments where the maximum single channel conductance is plotted against GABA concentration. GABA, at concentrations  $\geq 10 \mu\text{M}$ , increased the conductance of low conductance channels activated by  $1 \mu\text{M}$ . **A** illustrates this result with filled circles representing patches exposed to  $1 \mu\text{M}$  GABA followed by a second, higher GABA concentration ( $n = 13$ ). Application of a single high ( $100 \mu\text{M}$ ) or saturating dose of GABA ( $1 \text{ mM}$ ) opened channels to the highest conductances (filled triangles) ( $n = 7$ ). **B** shows five experiments in which the patch was exposed to three or four GABA concentrations (unfilled symbols), illustrating that  $[\text{GABA}] \geq 10 \mu\text{M}$  did not significantly increase channel conductance. All patches were held at  $-60 \text{ mV}$ .

**TABLE 1**

**Observed fast transitions and predicted probability of two independent simultaneous channel openings**

Values in parentheses represent the frequency (percentage) of that conductance  $Tr$ , number of transition segments (e.g.  $30 \text{ s}/400 \mu\text{s} = 75,000$ ).  $x_1$ , number of 30-pS channel openings observed.  $x_2$ , number of 60-pS channel openings observed. Observed probability of  $x_2 = x_2/Tr$ . Predicted probability of two simultaneous independent channel openings,  $\text{Pr}(s = 2) = ((x_1 + 2x_2)/Tr\sqrt{2})^2$ .  $Z$  = number of S.D. away from the predicted mean  $p_2$ , statistical probability derived from Equation 10 (see "Experimental Procedures").

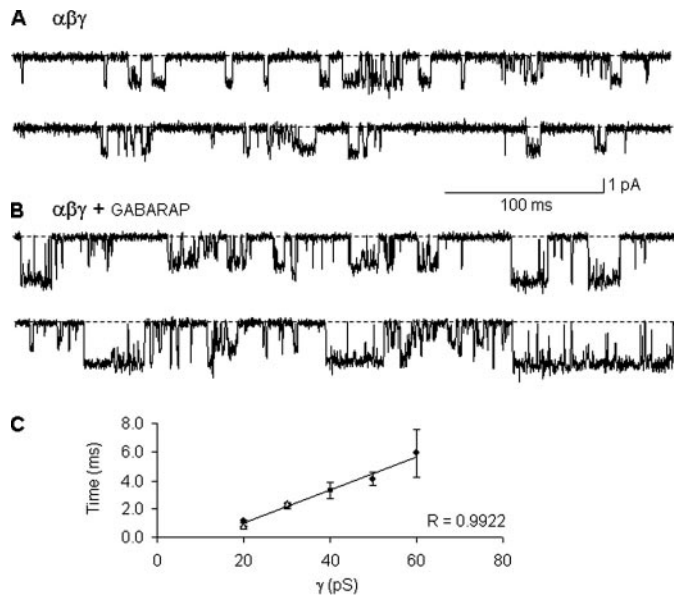
| Patch | Conductance |         |        |         |         | $Tr$   | $x_1$ | $x_2$ | Observed Pr of $x_2$ | Predicted Pr ( $s = 2$ ) | S.D.               | $Z$          | $p$                  |
|-------|-------------|---------|--------|---------|---------|--------|-------|-------|----------------------|--------------------------|--------------------|--------------|----------------------|
|       | pS          | pS      | pS     | pS      | pS      |        |       |       |                      |                          |                    |              |                      |
| P1    | 56 (16)     |         | 37 (8) | 31 (27) | 25 (50) | 75,000 | 452   | 135   | 0.0018               | $5 \times 10^{-5}$       | $2 \times 10^{-5}$ | 70.6         | $<6 \times 10^{-16}$ |
| P2    | 61 (22)     | 49 (12) |        | 33 (24) | 22 (6)  | 75,000 | 188   | 484   | 0.0064               | $1 \times 10^{-4}$       | $4 \times 10^{-5}$ | 159.2        | $<6 \times 10^{-16}$ |
| P3    | 57 (15)     | 48 (26) |        | 31 (59) |         | 75,000 | 1090  | 395   | 0.0052               | $3 \times 10^{-4}$       | $6 \times 10^{-5}$ | 76.5         | $<6 \times 10^{-16}$ |
| P4    | 58 (9)      | 51 (19) |        | 32 (50) | 23 (22) | 75,000 | 1711  | 421   | 0.0056               | $6 \times 10^{-4}$       | $9 \times 10^{-5}$ | 57.3         | $<6 \times 10^{-16}$ |
| P5    | 56 (6)      | 46 (2)  |        | 28 (68) | 19 (24) | 87,500 | 1518  | 109   | 0.0012               | $2 \times 10^{-4}$       | $5 \times 10^{-5}$ | 22.1         | $<6 \times 10^{-16}$ |
|       |             |         |        |         |         |        |       |       |                      |                          |                    | When $Z = 8$ | $6 \times 10^{-16}$  |

ments. At  $1 \mu\text{M}$  GABA, the maximum conductance centered around 30 pS ( $n = 18$ ), whereas higher conductance channels were more likely with higher  $[\text{GABA}]$  ( $10 \mu\text{M}$  ( $n = 11$  data points),  $100 \mu\text{M}$  ( $n = 10$ ), and  $1000 \mu\text{M}$  ( $n = 11$ )).

In order to determine the highest conductance obtainable by the agonist GABA, outside-out patches excised from cells co-expressing  $\alpha\beta\gamma$  receptors and GABARAP were initially exposed to a high  $[\text{GABA}]$  ( $100$  and  $1000 \mu\text{M}$ ), and single channel conductances were measured. Under these conditions, the maximum conductance, determined from amplitude histograms, could be as high as  $\sim 60$  pS and sometimes  $\sim 50$  pS ( $n = 7$ ) (Fig. 4A, filled triangles).

**Independent Versus Synchronized Gating Events**—To determine if the high conductance openings ( $\sim 60$  pS) could be due to random simultaneous opening of two independent 30-pS channels, we calculated the probability of rapid transition between the closed level and  $I_{\text{max}}$  ( $I_{\text{max}}$  = the maximum single channel current). If the observed number of rapid transitions from the closed level to  $2^*I_{\text{max}}$  is significantly greater than the predicted probability of two simultaneous independent channels opening, then this could not be attributed to random events (20). Table 1 presents the observed probability of the 60-pS channel in five single channel recordings from cells co-expressing GABA<sub>A</sub> receptors and GABARAP and, alongside these values, the associated mean predicted probability that these 60-pS channels arose from the simultaneous opening of two independent 30-pS channels. For example, in patch 1, the predicted probability of a single event in which two 30-pS channels open simultaneously is  $5 \times 10^{-5}$ , yet there are 135 such transitions. Hence, the actual number of fast transitions from closed to  $2^*I_{\text{max}}$  far exceeded the probability of two simultaneous random openings in this and in all patches co-expressing GABA<sub>A</sub> receptors and GABARAP. Given these data, we can now ask how confidently can we accept our null hypothesis, that the observed probability of the 60-pS channel events ( $p_2$ ) is due to two independent 30-pS channels opening simultaneously. These statistical probabilities ( $p$  values) are close to zero, indicating that the number of 60-pS channels observed is very unlikely to be due to random opening events of smaller conducting 30-pS channels. Hence, we can reject the null hypothesis.

**Correlation between Mean Open Time and Single Channel Conductance**—It was noted in channel recordings that high conductance channels ( $\geq 40$  pS) opened for longer than low conductance channels ( $< 40$  pS). This is illustrated in Fig. 5 in recordings of typical GABA-activated single channel activity from



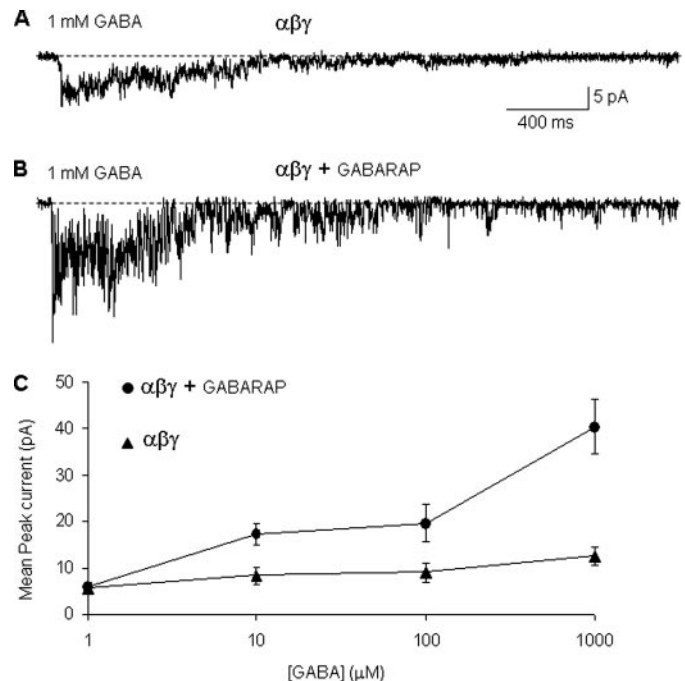
**FIGURE 5. The mean open time of recombinant  $\text{GABA}_A$  channels increases with increasing single channel conductance.** A and B show typical single channel traces recorded in the presence of 10  $\mu\text{M}$  GABA at  $-60$  mV from  $\alpha\beta\gamma$  receptors and  $\alpha\beta\gamma$  receptors + GABARAP, respectively. Note that the higher single channel conductances are typically open for longer periods than the smaller single channel openings. The graph in C shows the relationship between the mean open time and the single channel conductance. The high correlation co-efficient ( $R$  value) indicates that there is a very strong direct relationship between these two parameters. The linear relationship implies that as the single channel conductance increases the mean open time of the channel gets longer.

**TABLE 2**  
Relationship between single channel conductance and mean open time

Values represent mean  $\pm$  S. E.

| $\alpha\beta\gamma$ receptors + GABARAP |               |               |               |               |               |
|-----------------------------------------|---------------|---------------|---------------|---------------|---------------|
| GABA ( $n = 19$ )                       |               |               |               |               |               |
| Conductance                             | 60 pS         | 50 pS         | 40 pS         | 30 pS         | 20 pS         |
| Mean open time (ms)                     | $5.9 \pm 1.7$ | $4.1 \pm 0.5$ | $3.3 \pm 0.6$ | $2.2 \pm 0.2$ | $1.1 \pm 0.1$ |
| GABA + diazepam ( $n = 9$ )             |               |               |               |               |               |
| Mean open time (ms)                     | $6.8 \pm 1.0$ | $5.2 \pm 0.7$ | $3.8 \pm 0.2$ | $2.5 \pm 0.3$ | $0.9 \pm 0.1$ |
| $\alpha\beta\gamma$ receptors           |               |               |               |               |               |
| GABA ( $n = 24$ )                       |               |               |               |               |               |
| Mean open time (ms)                     |               |               |               | $2.3 \pm 0.1$ | $0.8 \pm 0.1$ |
| GABA + diazepam ( $n = 6$ )             |               |               |               |               |               |
| Mean open time (ms)                     |               |               |               | $2.5 \pm 0.3$ | $0.9 \pm 0.1$ |

patches expressing either  $\alpha\beta\gamma$  receptors (Fig. 5A) or co-expressing  $\alpha\beta\gamma$  receptors with GABARAP (Fig. 5B). In order to quantify these relative differences, we measured the open times of single channel events with different amplitudes. Amplitude frequency histograms were initially constructed, and the open times of the single channel events that fell within two S.D. values of Gaussian-fitted mean current amplitudes were then averaged. Open times derived in this way were then averaged from three or more experiments, and the mean open time was plotted against mean conductance (Fig. 5C). The high  $R$  value (0.9922) implies a strong correlation between these two parameters, indicating that as the conductance increases, so too does the mean open time of the channel when GABARAP is co-



**FIGURE 6. Cells co-expressing recombinant  $\text{GABA}_A$  receptors and GABARAP had larger peak currents in outside-out patches than those cells expressing just  $\alpha\beta\gamma$  receptors.** A and B show typical current responses to 1 mM GABA when recorded from  $\alpha\beta\gamma$  receptors and  $\alpha\beta\gamma$  receptors + GABARAP, respectively. The peak amplitude of the current recorded from  $\alpha\beta\gamma$  receptors was 11 pA, and channels had a maximum conductance of 30 pS. By contrast, the peak current recorded from  $\alpha\beta\gamma$  receptors + GABARAP was 30 pA, and channels had a maximum conductance of 47 pS. A summary of the peak current amplitudes recorded using different  $[\text{GABA}]$  is shown in C. The graph shows that the mean peak current amplitudes increase with increasing  $[\text{GABA}]$  both with and without GABARAP. However, patches expressing  $\alpha\beta\gamma$  receptors and GABARAP have a significantly higher mean peak amplitude when activated by  $[\text{GABA}] \geq 10 \mu\text{M}$ , compared with patches expressing just  $\alpha\beta\gamma$  receptors ( $p < 0.01$ ).

expressed with  $\text{GABA}_A$  receptors. The mean open times determined for single channels with different mean conductances from 19 patches are given in Table 2. The mean open times of comparable conductances are similar between the two expression regimes (*i.e.* with or without GABARAP). This is illustrated in Fig. 5C, where the mean open times for the  $\sim 30$ -pS main and 20-pS subconductance channels in control patches (no recombinant GABARAP) are overlaid on the graph for comparison. In this analysis, we did not find any correlation between the concentration of GABA used and the mean open times of channels, only a correlation between channel conductance and mean open time as shown.

**Outside-out Patches Co-expressing  $\text{GABA}_A$  Receptors plus GABARAP Contain More Receptors Than Those Expressing Just  $\alpha\beta\gamma$  Receptors**—We reasoned that if the changes in single channel properties that we observe result from the ability of GABARAP to cluster receptors, then one might expect to have more receptors (a larger  $N$  value) in these excised patches. In order to estimate the relative number of receptors in a patch, we applied a range of GABA concentrations to patches and measured the initial peak amplitude of the current. Under saturating GABA conditions, the size of this peak current is dependent only upon the number of receptors in the patch and their single channel conductance. Fig. 6 illustrates typical initial responses to a saturating GABA concentration (1 mM) obtained from out-

**TABLE 3**

Comparison of initial peak currents and estimation of  $N$ , number of receptors per patch, in outside-out patches expressing  $\text{GABA}_A \alpha\beta\gamma$  receptors with and without GABARAP

$I_i$ , initial peak current in response to saturating [GABA] (1.0 mM).  $i_{\max}$ , maximum single channel current.  $N$ , number of receptors per patch, is calculated by dividing  $I_i/i_{\max}$ , assuming that all receptors in the patch initially open to their maximum conductance. Values are mean  $\pm$  S.E.

| GABA <sub>A</sub> receptors + GABARAP |                              |                             | GABA <sub>A</sub> receptors |                |               |
|---------------------------------------|------------------------------|-----------------------------|-----------------------------|----------------|---------------|
| Peak $I$ (pA)                         | $i_{\max}$                   | $N$                         | Peak $I$ (pA)               | $i_{\max}$     | $N$           |
| 56.0                                  | 3.10                         | 18.1                        | 4.2                         | 1.67           | 2.5           |
| 30.0                                  | 2.67                         | 11.2                        | 7.5                         | 1.69           | 4.4           |
| 20.1                                  | 3.06                         | 6.6                         | 8.1                         | 1.5            | 5.4           |
| 71.0                                  | 2.75                         | 25.8                        | 23.0                        | 1.92           | 12            |
| 47.0                                  | 2.32                         | 20.3                        | 4.7                         | 1.66           | 2.8           |
| 26.5                                  | 3.03                         | 8.7                         | 4.4                         | 1.70           | 2.6           |
| 47.8                                  | 3.53                         | 13.5                        | 25.5                        | 2.10           | 12.1          |
| 44.3                                  | 3.26                         | 13.6                        | 22.1                        | 1.99           | 11.1          |
| 20.5                                  | 2.70                         | 7.6                         | 31.3                        | 2.05           | 15.2          |
|                                       |                              |                             | 16.2                        | 1.98           | 8.2           |
|                                       |                              |                             | 2.1                         | 1.54           | 1.4           |
|                                       |                              |                             | 11.3                        | 1.94           | 5.8           |
|                                       |                              |                             | 5.9                         | 1.57           | 3.7           |
|                                       |                              |                             | 18.8                        | 2.03           | 9.2           |
|                                       |                              |                             | 5.3                         | 1.55           | 3.4           |
|                                       |                              |                             | 16.2                        | 1.97           | 8.2           |
|                                       |                              |                             | 6.1                         | 1.60           | 3.8           |
|                                       |                              |                             | 10.5                        | 1.86           | 5.7           |
|                                       |                              |                             | 15.4                        | 2.01           | 7.7           |
| Mean                                  |                              |                             |                             |                |               |
| 40.4 $\pm$ 5.7 <sup>a</sup>           | 2.95 $\pm$ 0.12 <sup>a</sup> | 13.9 $\pm$ 2.1 <sup>a</sup> | 12.6 $\pm$ 1.9              | 1.8 $\pm$ 0.05 | 6.6 $\pm$ 0.9 |

<sup>a</sup> $p < 0.05$ .

side-out patches. Current responses from  $\text{GABA}_A$  receptors expressed with (Fig. 6B, 30 pA) or without recombinant GABARAP (Fig. 6A, 11 pA) are shown to clearly illustrate the large, significant difference in the size of these currents ( $p < 0.01$ ). The maximum single channel conductance of the channels was 29 pS (Fig. 6A) and 47 pS (Fig. 6B, +GABARAP). Even taking into account the difference in maximum single channel current amplitudes between  $\text{GABA}_A$  receptors expressed with or without GABARAP, there remains a significant difference between the peak currents that can only be attributed to a difference in receptor number ( $N$ ). When  $N$  is calculated from a set of patches initially exposed to 1 mM GABA, on average patches from cells co-expressing  $\alpha\beta\gamma$  and GABARAP contained significantly more receptors ( $N \sim 14$ ) than those expressing just  $\alpha\beta\gamma$  receptors ( $N \sim 7$ ) (Table 3). Note, however, the wide range in the estimates of  $N$  in both data sets. These data support our hypothesis that this GABARAP-regulated packing arrangement alters the ion channel properties of the  $\text{GABA}_A$  receptor.

When initial peak current amplitudes in response to 1, 10, 100, and 1000  $\mu\text{M}$  GABA were averaged from a number of experiments ( $n = 8-35$ ) and plotted against [GABA], a significant difference in mean peak current amplitudes was observed at all GABA concentrations of  $\geq 10 \mu\text{M}$  (Fig. 6C). For example, the mean peak currents measured with increasing [GABA] for  $\alpha\beta\gamma$  receptors co-expressed with GABARAP were  $5.9 \pm 0.6$ ,  $17.3 \pm 2.2$ ,  $19.6 \pm 4.2$ , and  $40.4 \pm 5.8$  pA. These are compared with  $5.8 \pm 1.0$ ,  $8.3 \pm 1.9$ ,  $9.1 \pm 2.2$ , and  $12.6 \pm 1.9$  pA when  $\text{GABA}_A$  receptors were expressed alone.

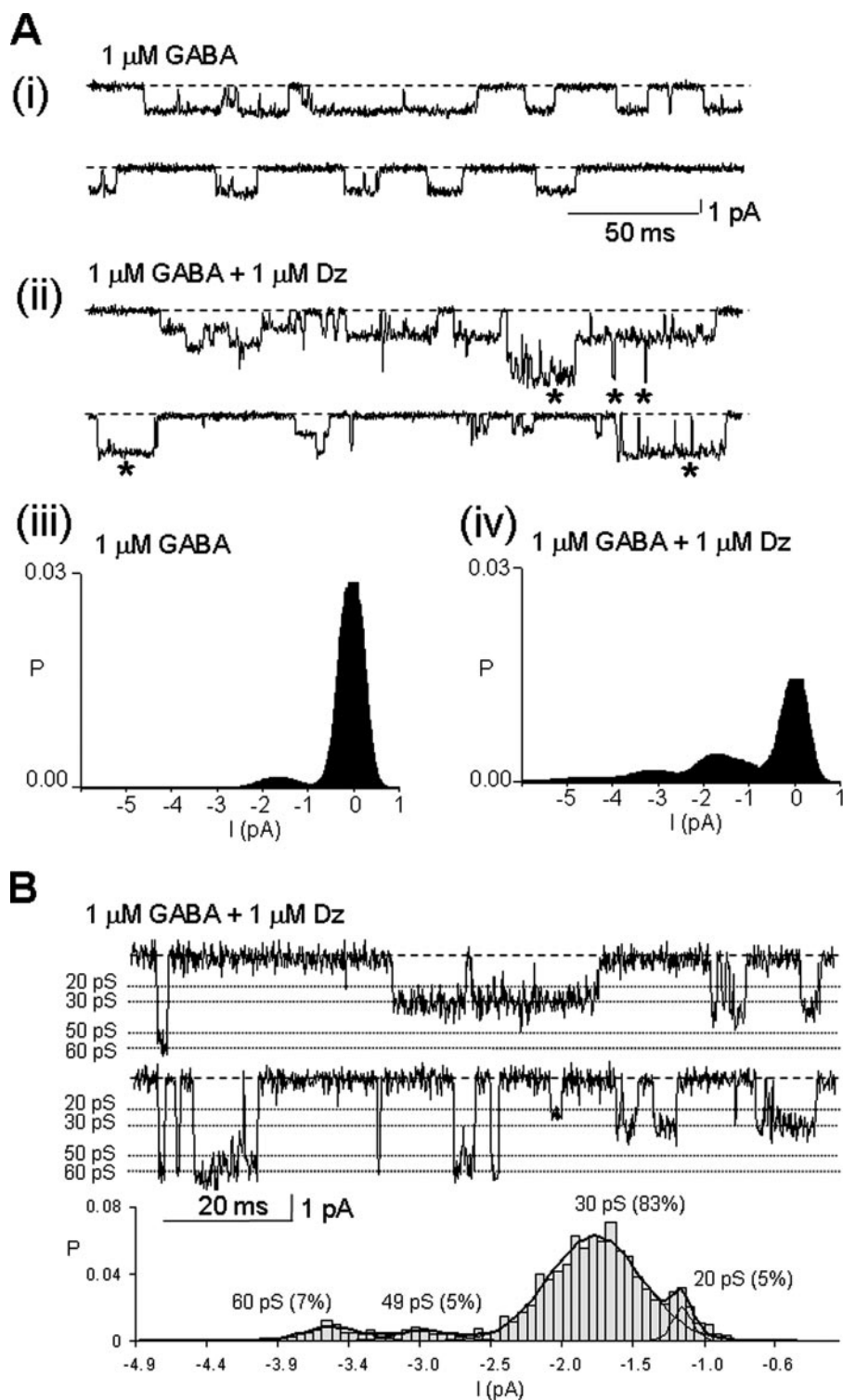
**Diazepam-modulated Channel Activity Shows a Correlation between Conductance and Mean Open Time**—Diazepam increases the open probability of recombinant  $\text{GABA}_A$  recep-

tors containing the  $\gamma$  subunit (21, 22). We have shown recently that diazepam is able to increase both the conductance and open probability (mean current) of recombinant  $\alpha\beta\gamma$  receptors co-expressed with GABARAP in cell-attached and outside-out patches (9). Here we have analyzed the effect of diazepam on the elementary conductance levels and mean open time of  $\text{GABA}_A$  channels for comparison with those modulated by GABA described above. A typical example of the modulation of the GABA response by diazepam is shown in Fig. 7. An increase in channel open probability upon diazepam application can be seen by comparing the all points histograms in Fig. 7, A (iii) (+ GABA) and A (iv) (GABA + diazepam). In addition,  $\text{GABA}_A$  receptors co-expressed with GABARAP show an increase in conductance, and this effect is illustrated in the single channel recordings before diazepam (Fig. 7A (i)) and during diazepam application (Fig. 7A (ii)). In the example shown, the single channel conductance increased from 30 pS to a maximum of 52 pS in the presence of diazepam. In Fig. 7B, another channel's response to diazepam is shown whose maximum conductance reaches  $\sim 60$  pS. The expanded time scale illustrates that the single channel amplitudes are single transitions from base line, and the corresponding amplitude frequency histogram depicts the distribution of current levels and their frequency in response to diazepam. By contrast, the maximum conductance did not change when GABA and diazepam were co-applied to control patches expressing  $\alpha\beta\gamma$  receptors alone (9); only their open probability increased. In general, diazepam increased conductance to a maximum of  $\sim 60$  pS, with all records displaying multiple single channel openings whose quantal distribution was similar to that produced by GABA. In effect, modulation by 1  $\mu\text{M}$  diazepam produced the same maximum conductance as a saturating GABA concentration (1 mM) in outside-out patches when receptors were co-expressed with GABARAP (Figs. 3 and 5).

The relationship between conductance and mean open time of channels activated by GABA plus diazepam was measured in the same way as described above for GABA-activated channels. Records or sections of records were chosen that did not contain overlapping channel events. The mean open time of the diazepam-modulated channels also showed a strong correlation with conductance ( $R = 0.9998$ ) and once again showed that as the conductance increased so too did the mean open time of the channel. For comparison, values of mean conductance and their corresponding mean open times are shown in Table 2 for channel events activated by GABA and those activated by GABA plus diazepam; these values are not statistically different. In control patches, the conductance and mean open times of  $\alpha\beta\gamma$  receptors expressed alone were the same either when GABA or GABA plus diazepam were applied (Table 2).

## DISCUSSION

Previously, we have shown that the co-expression of the trafficking protein GABARAP with  $\text{GABA}_A \alpha\beta\gamma$  receptors leads to the formation of channels whose conductance is increased by drugs and whose dispersion in the membrane is changed such that they form clusters (9). Here we have obtained single channel recordings that show for the first time that GABA itself at high concentrations increases the conductance of recombinant



**FIGURE 7. Diazepam increases the conductance of  $\alpha\beta\gamma$  receptors co-expressed with recombinant GABARAP.** Diazepam (1  $\mu\text{M}$ ) plus GABA (1  $\mu\text{M}$ ) were co-applied to outside-out patches excised from cells co-expressing  $\alpha\beta\gamma$  receptors and GABARAP. In the example shown in *A*, single channels activated by 1  $\mu\text{M}$  GABA had a maximum conductance of 30 pS recorded at  $-60$  mV as indicated in the corresponding all points histogram in *A* (iii). When GABA (1  $\mu\text{M}$ ) plus diazepam (1  $\mu\text{M}$ ) were subsequently co-applied to the same patch, the open probability of the channel increased as depicted in the trace in *A* (ii) and the corresponding all points histogram in *A* (iv). The single channel activity also showed the presence of additional, higher single channel conductance states, which in this example reached a maximum of 52 pS (\*). In another example, shown in *B*, the maximum conductance of single channels was increased from 31 pS in 1  $\mu\text{M}$  GABA to 60 pS when diazepam (1  $\mu\text{M}$ ) was subsequently co-applied. The time scale on the x axis has been expanded to show the single channel transitions to higher conductances, and the all points amplitude histogram below shows the distribution in current amplitudes and their relative frequencies. All traces were recorded at  $-60$  mV. The same scale bar is used for all traces.

$\alpha\beta\gamma$  receptors co-expressed with GABARAP (Figs. 3 and 5). This is significant, because a similar effect has been observed on extrasynaptic GABA<sub>A</sub> channels in neurons from the hippocampus (23) and dentate gyrus (24). The latter authors have suggested that the apparent ability of GABA to modulate the conductance of single channels may be due to the presence of different receptor subtypes in the patch. The data presented here show that this occurs with a single defined receptor composition, provided that it is co-expressed with GABARAP. Hence, it is probable that both receptor diversity and receptor organization play a role in plasticity of the GABA<sub>A</sub> response.

We have measured the mean open time of channels activated by GABA alone and those activated by GABA and modulated by diazepam and observed a strong correlation between higher conductances and longer open times (Fig. 5). In effect, the co-expression of GABARAP produces a form of GABA<sub>A</sub> "super-channel" in the recombinant system that is characterized by having a high conductance ( $>40$  pS) and a long open time. The control of neuronal excitability by tonic inhibition requires the generation of steady conductance that reduces the gain of neuronal input-output functions (25). Physiologically, it would be beneficial to have the potential to express a range of differently responding receptors from a given pool of subunits by modulating trafficking processes.

GABARAP is a trafficking protein. Its co-expression in the recombinant system could lead to higher receptor numbers in the membrane, clustering, different organizational modes affecting lateral movement in the membrane, or a combination of these. We have measured the number of receptors in patches from cells expressing  $\alpha\beta\gamma$  receptors with and without GABARAP. The co-expression of GABARAP does indeed lead, on average, to more receptors in the patch (Fig. 6 and Table 3). However, the presence of GABA<sub>A</sub> superchan-

nels is not strictly correlated with high receptor numbers in the patch. For example, patches containing the highest number of receptors expressed in the absence of GABARAP ( $n = 15$ ) did not display “superchannel” activity, whereas patches containing the lowest number of receptors ( $n = 7$ ) co-expressed with GABARAP did (Table 3). In other words, it is not just that GABARAP increases the number of receptors per patch but that there is an additional process occurring. Confocal immunofluorescent images from this laboratory (9) and others (12) showing punctate, GABARAP-mediated clusters of GABA<sub>A</sub> receptors in the plasma membrane lead us to infer that it is the clustering of recombinant GABA<sub>A</sub> receptors, initiated by GABARAP, that alters their ion channel properties. The trafficking of receptors by GABARAP must deliver receptors to sites where they are constrained; otherwise, receptors would simply diffuse apart. We therefore hypothesize a mechanism whereby receptor interactions within clusters promote coordinated channel openings, thereby explaining the presence of high conductance channels in cells co-expressing GABARAP and GABA<sub>A</sub> receptors.

The occurrence of high conductance channels in our patches co-expressing GABA<sub>A</sub> receptors and GABARAP far exceeds the predicted probability of their occurrence being due to the simultaneous opening of two independent 30-pS channels (Table 1); hence, these high conductance channels cannot be attributed to a random event. In addition, if the different conductances were attributable to double simultaneous opening events, then we might expect to observe such events in control patches expressing just GABA<sub>A</sub> receptors. However, we never observe this phenomenon. We suggest instead, that these high conductance openings arise through the coupled gating of clustered GABA<sub>A</sub> receptors.

In the L929 cell system that we use to express recombinant GABA<sub>A</sub>  $\alpha\beta\gamma$  receptors plus GABARAP, the maximum conductance of both the GABA-activated and the diazepam-modulated single channels was  $\sim 60$  pS using the outside-out configuration. This is lower than we observe in the cell-attached configuration in both recombinant (9) and native systems (4, 5, 23, 26, 27). The reason for this difference is not known. However, it is likely that in excising the patch, either there is disruption of some protein interaction(s) or, alternatively, there is a contribution from cytosolic components that is lacking.

Our discovery that GABA increases both single channel conductance and the mean open time of recombinant GABA<sub>A</sub> receptors co-expressed with GABARAP demonstrates that trafficking processes influence ion channel properties. In their totality, the correlation between channel conductance and open time and the quantal nature of the conductance levels, together with our data showing higher receptor numbers in excised patches from GABARAP-expressing cells, support a mechanism whereby GABARAP organizes GABA<sub>A</sub> receptors such that physical interactions between adjacent receptors enable cooperative channel openings. Whereas this proposed mechanism is novel for GABA<sub>A</sub> receptors, there is precedent.  $\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors are chaperoned to the plasma membrane by the trafficking protein Stargazin. Utilizing different domains, Stargazin modulates both the trafficking and the single channel conductance (gating) of the ion channel (28). A similar mechanism involving interaction partners for GABA<sub>A</sub> receptors, which

generates “superchannel” activity appears less plausible at present because biochemical and immunofluorescence data do not place GABARAP in the vicinity of GABAergic postsynaptic sites (11). It should be noted, however, that GABARAP co-localization with extrasynaptic GABA<sub>A</sub> has not been examined to date. Future experiments aimed at understanding GABA<sub>A</sub> superchannels will address such details as protein interaction partners that stabilize clustered receptors in the membrane and consequently alter their ion channel properties.

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