Conductance of Recombinant GABA<sub>A</sub> Channels Is Increased in Cells Co-expressing GABA<sub>A</sub> Receptor-associated Protein*  

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High conductance γ-aminobutyric acid type A (GABA<sub>A</sub>) channels (>40 picosiemens (pS)) have been reported in some studies on GABA<sub>A</sub> channels in situ but not in others, whereas recombinant GABA<sub>A</sub> channels do not appear to display conductances above 40 pS. Furthermore, the conductance of some native GABA<sub>A</sub> channels can be increased by diazepam or pentobarbital, which are effects not reported for expressed GABA<sub>A</sub> channels. GABARAP, a protein associated with native GABA<sub>A</sub> channels, has been reported to cause clustering of GABA<sub>A</sub> receptors and changes in channel kinetics. We have recorded single channel currents activated by GABA in L929 cells expressing α<sub>1</sub>, β<sub>1</sub>, and γ<sub>2S</sub> subunits of human GABA<sub>A</sub> receptors. Channel conductance was never higher than 40 pS and was not significantly increased by diazepam or pentobarbital, although open probability was increased. In contrast, in cells expressing the same three subunits together with GABARAP, channel conductance could be significantly higher than 40 pS, and channel conductance was increased by diazepam and pentobarbital. GABARAP caused clustering of receptors in L929 cells, and we suggest that there may be interactions between subunits of clustered GABA<sub>A</sub> receptors that make them open co-operatively to give high conductance “channels.” Recombinant channels may require the influence of GABARAP and perhaps other intracellular proteins to adopt a fuller repertoire of properties of native channels.

Some, but not all, native γ-aminobutyric acid type A (GABA<sub>A</sub>) channels have been reported to have maximum conductances above 40 picosiemens (pS) and many subconductance states (1–3), whereas recombinant GABA<sub>A</sub> channels formed by a wide range of subunits do not have such high conductances. Furthermore, the conductance of some native channels is increased by drugs such as diazepam, pentobarbital, and propofol (4–6), which are effects not reported for expressed GABA<sub>A</sub> receptors. A possible explanation for these differences is that native receptors are not identical to expressed receptors perhaps because of specific interactions that occur in native but not heterologous cells.

A GABA<sub>A</sub> receptor-associated protein, GABARAP, is an intracellular protein that can interact with the γ<sub>2</sub> subunit of GABA<sub>A</sub> receptors (7). When co-expressed with GABA<sub>A</sub> subunits in QT-6 quail fibroblasts, GABARAP causes clustering of GABA<sub>A</sub> receptors accompanied by changes in whole cell current kinetics (8). Although this protein was not found in close proximity to mature native GABA<sub>A</sub> receptors in the plasmalemma of cortical neurons in a study using fluorescent antibodies to GABARAP (9), there is compelling evidence that co-expression of GABARAP with subunits of GABA<sub>A</sub> receptors does result in increased clustering of receptors in many cells (8). These observations suggest that GABARAP may be involved at an early stage in the clustering of GABA<sub>A</sub> receptors even though it appears not to be present as part of the anchoring mechanism for the receptors.

Because it has been suggested that the clustering of GABA<sub>A</sub> receptors may allow co-operative opening of channels and hence a higher apparent single channel conductance (see Ref. 10 for review), we have co-expressed GABARAP with subunits of GABA<sub>A</sub> receptors in an attempt to cause clustering of the receptors. We report here that co-expression of GABARAP with subunits of GABA<sub>A</sub> receptors causes the clustering of receptors in L929 fibroblasts and also causes the appearance of high conductance channels that are only rarely seen in control cells transfected in the same way with the same GABA<sub>A</sub> subunits but without GABARAP.

EXPERIMENTAL PROCEDURES

Plasmid cDNAs and Antibodies—Full-length cDNAs for the human GABA<sub>A</sub> subtypes α<sub>1</sub>, β<sub>1</sub>, and γ<sub>2S</sub> were subcloned individually into the pcDNA3.1+ (Invitrogen) mammalian expression vector. Green fluorescent protein was expressed off of the pEGFP-N1 vector (Clontech, Palo Alto, CA). Full-length human GABARAP cDNA, cloned into pcDNA3 (Invitrogen), was a gift from Dr. R. W. Olsen (UCLA). The monoclonal antibody bd24 was purchased from Chemicon International, and fluorescein isothiocyanate conjugated rabbit anti-mouse IgG was purchased from Amersham.

Cell Culture—Mouse L929 fibroblasts (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco’s modified Eagle’s Medium plus 10% heat-inactivated fetal bovine serum (CSL), 10 mM HEPES, and 4 mM t-glutamine. Cells for passaging were lifted using a cell scraper or following a 2-min incubation in phosphate-buffered saline (pH 7.3) containing 0.5% trypsin and 0.2% EDTA.

Transfection—Cells were transfected with plasmids encoding GABA<sub>A</sub> subunits α<sub>1</sub>, β<sub>1</sub>, γ<sub>2S</sub> and green fluorescent protein, with or without GABARAP. Cells were plated onto glass coverslips in multwell
dishes at a density of 1.0–1.5 × 10⁵ cells/ml. Twenty-four hours later cells were washed with OptiMEM (Invitrogen) and then transfected with a total of 20 μg of plasmid DNA (in equal amounts) plus 10 μl of Lipofectin reagent (Invitrogen) in accordance with manufacturer’s instructions. After a 5-h incubation at 37 °C the mixture was replaced by fresh medium, and 48–72 h later the cells were used for electrophysiological experiments.

Western Blotting—Lysates from untransfected and transfected cells were run on 4–12% gradient gels (Invitrogen) in MES buffer. Proteins were transferred to nitrocellulose using a semidyne buffer system. Following blocking in milk powder the blot was incubated in the anti-GABARAP polyclonal antibody (7) (1/3000) at 4 °C overnight. After thorough washing the blot was incubated in secondary antibody (anti-rabbit horseradish peroxidase) for 2 h at room temperature and then visualized by ECL (West-Pico, Pierce). All blots included purified GABARAP as a positive control.

Immunofluorescent Labeling—Cells were transfected (as described above) with plasmids encoding GABA_A subunits α₁, β₁, and γ₂S with or without GABARAP. Staining and fixation were carried out at 4 °C 48 h post-transfection. Cells on coverslips were washed with phosphate-buffered saline containing 1% bovine serum albumin then incubated for 1 h with the anti-α₁, monoclonal antibody bd24 (1:50). Following fixation with 1% paraformaldehyde, cells were washed with phosphate-buffered saline, incubated for 1 h with fluorescein isothiocyanate-conjugated secondary antibody (1:50), and then washed again prior to mounting onto slides in Slowfade (Molecular Probes). Cells were imaged on a Radiance 2000 confocal microscope with a 60 × oil-immersion objective. Non-transfected cells treated as described above did not show any immunofluorescence.

Electrophysiology—Electrophysiological experiments were performed using conventional patch clamp techniques in cell-attached or outside-out patches. Cells were viewed using a light fluorescent attachment (Olympus BH2-RFL) so that only those cells successfully transfected with green fluorescent protein were selected for patching. Electrodes made from borosilicate glass (Clark Electromedical) and coated with Sylgard (Dow Corning) had resistances between 10 and 15 MΩ.

Cell-attached Patches—The bath solution contained (in mM): 142 NaCl, 4 KCl, 6 MgCl₂, 2 CaCl₂, and 10 TES, pH 7.4. Patch electrodes contained (in mM): 140 choline chloride, 0.3 KCl, 1 CaCl₂, 5 MgCl₂, and 10 TES, pH 7.4, in addition to the indicated concentration of GABA (3–10 μM, Sigma).

Outside-out Patches—The bath solution contained (in mM): 135 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 TES, pH 7.4. Patch electrodes were filled with a solution containing (in mM): 50 NaCl, 80 KCl, 2 CaCl₂, 2 MgCl₂, 5 EGTA, and 10 TES, pH 7.3–7.4. With these solutions the predicted chloride equilibrium potential was close to 0 mV. Drugs were made up to final concentration in bath solution (as described above) and were applied to outside-out patches directly via gravity-fed flow tubes. The flow tubes had a diameter of 300 μm and were placed directly in front of the outside-out patch. The presence of GABA_A receptors in outside-out membrane patches was confirmed by an initial application of GABA (1 mM) applied via the flow tube.

Testing Drugs—Where indicated, pentobarbital (Sigma) was dissolved in bath solution. Diazepam (Hoffman-La Roche) was initially solved in dimethyl sulfoxide (Me₂SO, Sigma) before being dissolved in bath solution. The final concentration of Me₂SO in the bath solution was 0.01%. Both drugs were applied by gravity-fed perfusion through the bath. For application of bicuculline (Sigma) to a cell-attached patch, a sudden bolus of drug was ejected from the end of a fine polyethylene tube inserted in the electrode to within 0.5–1.0 mm of the tip. Because the increases in channel conductance caused by diazepam and pentobarbital were more prominent the lower the predrug (control) conductance (4, 6) was, patches containing low conductance (<30 pS) channels were normally used for testing the effects of these drugs on channel conductance.

Analysis of Currents—Currents were recorded using an Axopatch 200A current-to-voltage converter (Axon Instruments), filtered at 5 or 10 kHz, digitized at 44 kHz using a pulse-code modulator (Sony PCM 501), and stored on videotape for analysis. In some experiments, currents were digitized at 10 kHz with a Digidata 1200 A–D converter and stored on computer disk. Currents were played back and digitized at a frequency of 10 kHz for analysis using a computer program. Channel conductance histograms or from the directly measured amplitudes of individual currents. Where single channel currents displayed subconductance states, the single channel current amplitude was taken as the most frequently occurring and highest state. Channel conductance was calculated by dividing current amplitude by the difference between the pipette potential and the reversal potential, the latter being close to 0 mV for the solutions used (see Fig. 2, B and D). “Mean currents” were measured over periods of 10 s or more by integrating all data points, normally sampled at 10 kHz, to obtain total current and then dividing by the number of data points. The base-line current level was set during periods when there was no channel activity, and current variance was set at a minimum. The base-line level was monitored throughout a record to ensure that no shift had occurred. This measure is a rapid and observer-free method of monitoring changes in channel open probability when channel conductance is not changing. Average results are expressed as mean ± S.E., and the probability of significant differences between means was calculated using the Student’s t test.

RESULTS

Effects of GABARAP on GABA_A Receptor Clustering

In L929 cells transfected with GABA_A α₁, β₁, and γ₂S cDNAs, the fluorescently labeled antibody bd24 that labels the α₁ subunit was diffusely distributed over the surface of the plasma membrane (Fig. 1A). In contrast, when L929 cells were cotransfected with cDNAs for the same three subunits plus GABARAP a punctate distribution of fluorescent staining, as illustrated in Fig. 1B, was observed in 58–69% of cells. This punctuate appearance was visible in cells taken from five different sets of cells cotransfected with GABARAP but was not seen in “control” cells that had been transfected with the GABA_A subunits but not GABARAP. These results are similar to those obtained in QT-6 fibroblasts (8) and indicate that GABARAP causes clustering of GABA_A receptors in L929 fibroblasts also.

Characteristics of Recombinant α₁ β₁ γ₂S Channels

Conductance—Typical single channel currents recorded in cell-attached patches on cells transfected with the three GABA_A subunits are shown in Fig. 2A (GABA concentration in the pipette was 3 μM). The currents reversed at a pipette potential (V_p) close to 0 mV, as expected for chloride currents with the pipette solutions used, and showed no rectification (Fig. 2B). The maximum conductance (γ_max) of the channels shown in Fig. 2, A and B was 21 pS. The average maximum conductance level of channels recorded in the 15 cell-attached patches was 22.3 ± 1.2 pS (mean ± 1 S.E.). In none of the 15 cell-attached patches was γ_max above 35 pS. The application of bicuculline (100 μM) to the outer membrane surface (facing the pipette solution) in three of the patches blocked channel activity.
Single channel currents were also recorded in outside-out patches. Typical currents activated by 1 μM GABA in one of these patches can be seen in Fig. 2C (V_p = +60 mV top trace, −60 mV bottom trace). The currents reversed at 0 mV and were non-rectifying (Fig. 2D). In the experiment illustrated in Fig. 2, C and D, channels had an average conductance of 29.2 ± 0.008 pS (n = 934 openings). In 33 outside-out patches, similar non-rectifying currents had an average maximum conductance of 30.5 ± 0.41 pS.

**Effects of Diazepam**—In five of the six patches tested, diazepam caused no significant increase in conductance of recombinant channels formed by the three GABA_A subunits alone as illustrated in Fig. 3, A and B. Channels were activated in a cell-attached patch with 3 μM GABA (Fig. 3A, V_p = −60 mV). The channels had a conductance of 17–23 pS and mean current was 0.17 pA. Within 2–3 min of switching to a bath solution containing 10 μM diazepam (Fig. 3B), channel activity had clearly increased (mean current 0.75 pA) and channel conductance was 22–26 pS. Similar results (an increase in open probability but no significant increase in channel conductance) were obtained in five cell-attached patches where the mean conductance of channels activated by 3 μM GABA was 16.6 ± 3.60 pS before exposure to 1 or 10 μM diazepam and 26.4 ± 3.40 pS after exposure to diazepam. In one of six patches channel conductance increased from 16 to 56 pS following the application of 10 μM diazepam. In eight outside-out patches, channels activated by 1 μM GABA had an average conductance of 29.8 ± 0.74 pS before exposure to 1 μM diazepam and a conductance of 29.6 ± 1.0 pS in the presence of 1 μM GABA plus 1 μM diazepam.

**Effects of Pentobarbital**—Pentobarbital also increased channel activity but there was no increase in channel conductance, as illustrated in Fig. 3, C and D. Channels in a cell-attached patch (V_p = −60 mV) activated by 3 μM GABA (Fig. 3C) had a maximum conductance of 25 pS (mean current 0.09 pA). Within 1 min of exposing the cell to 100 μM pentobarbital (Fig. 3D) channel activity had increased, and currents from two channels were superimposing. Mean current had doubled (0.18 pA), but there was no significant change in channel conductance. Similar results were obtained in three cell-attached patches exposed to 100 μM pentobarbital. Average channel conductance before exposure to pentobarbital was 21.7 ± 5.0 and 30.0 ± 1.0 pS after exposure to the pentobarbital.

**Characteristics of Recombinant α_2 β_3 γ_2s Channels in Cells Co-expressing GABARAP**

**Cell-attached Patches**

**Conductance**—Single channel chloride currents were recorded in cells expressing the three GABA_A subunits together with GABARAP. Typical examples of these currents in a cell-attached patch are shown in Fig. 4. Current traces shown in...
average channel conductance recorded in the 11 cell-attached and open probability during the exposure to diazepam. The 

each trace, show a progressive increase in channel conductance 

pam, and the associated all-points histograms to the right of 

patch on a cell expressing 

10

2.5, 26, and 80 s after the exposure of the cell to 10

ing to a conductance of 33 pS. The traces in Fig. 5

B

A

higher maximum conductance than channels recorded in the 

patches, channels activated by GABA had a significantly 

4

not co-expressing GABARAP. The current-voltage curve in Fig. 5

GABAA subunits and GABARAP. An increase in 

increase the open probability (11) and conductance (4, 5) of native 

channels blocked channel activity.

mum current amplitude was 6.5 pA when 

another prominent peak at 

pS). The average channel conductance in the nine patches not 

60.7

60 mV) was 

m

6.30 pS (p < 0.05) after exposure to diazepam. Similar results were obtained from four inside-out patches in which 

increased from 52.3 ± 8.93 to 84.3 ± 6.30 pS (p < 0.05) after exposure to diazepam.

Effects of Pentobarbital—Barbiturates have also been re-

ported to increase GABA A channel-open probability (11) and conductance (6) in native channels. In patches in cells cotrans-

fected with the GABA A subunits but not with GABARAP, pen-

tobarbital did increase channel conductance in the eight patches containing lower conductance channels with an 
average conductance of 24.9 ± 3.40 pS, conductance increased 

to 45.4 ± 5.70 pS (p < 0.005) after exposure to diazepam.

Effects of Diazepam—Diazepam has been reported to in-
crease the open probability (11) and conductance (4, 5) of native 

GABA A channels. Diazepam produced both effects on channels in 11 cell-attached patches on cells cotransfected with the 

GABA A subunits and GABARAP. An increase in γm, caused by 

10 μM diazepam is illustrated in Fig. 5. Before exposure to 
diazepam, the amplitude of single conductance currents (Vp = −40 mV) activated by 3 μM GABA (Fig. 5A) was 1.32 pA corresponding 
to a conductance of 33 pS. The traces in Fig. 5B, recorded 2.5, 26, and 80 s after the exposure of the cell to 10 μM diaze-
pam, and the associated all-points histograms to the right of each trace, show a progressive increase in channel conductance and open probability during the exposure to diazepam. The average channel conductance recorded in the 11 cell-attached 

patches activated by 3 μM GABA was 32.3 ± 4.74 pS and after 
exposure to 10 μM diazepam was increased to 56.3 ± 7.74 pS 
(p < 0.01). Conductance after diazepam application increased 
regardless of the initial conductance seen in the patch. In 3 of 

the 11 patches in which the initial GABA-activated conductance 

was on average 52.0 ± 8.93 pS, conductance increased 

85.3 ± 14.7 pS (p = 0.05) after exposure to diazepam. In the 
eight patches containing lower conductance channels with an 
average conductance of 24.9 ± 3.40 pS, conductance increased 
to 45.4 ± 5.70 pS (p < 0.005) after exposure to diazepam.

Effects of Pentobarbital—Barbiturates have also been re-
ported to increase GABA A channel-open probability (11) and conductance (6) in native channels. In patches in cells cotrans-

fected with the GABA A subunits but not with GABARAP, pen-
tobarbital caused no increase in channel conductance as illustrated for a cell-attached patch in Fig. 3, C and D. In contrast, 
in 10 patches from cells expressing the GABA A subunits and GABARAP, pentobarbital did increase channel conductance from 41.9 ± 5.65 to 63.9 ± 5.2 pS (p < 0.01). The effect of pentobarbital on conductance was most marked in 5 of the 10 patches where the initial GABA-activated currents were small (corresponding to a γm < 40 pS) increasing from 28.4 ± 4.5 to 

59.8 ± 5.16 pS following application of 100 μM pentobarbital 
(p < 0.001). This effect is illustrated in Fig. 6. Before exposure to pentobarbital (Fig. 6A), single channel conductance was 25 pS (Vp = −40 mV), and the all-points histogram contained multiple peaks that corresponded to the superimposed activity of several channels in the patch. The separation of 1.0 pA between adjacent peaks reflects the single channel conductance of 25 pS. Following exposure of the patch to 100 μM pentobarbital, channel activity became greater and single channel current amplitude increased (Fig. 6B). There was still evidence of several channels in the patch, but the separation between
current levels in the all-points histogram was now 2.72 pA (Fig.
6B) corresponding to a conductance of 68 pS. The increase in the areas of the non-base-line peaks relative to the base-line peak at 0 pA indicates an increase in channel-open probability, and the increased separation between peaks indicates an increase in channel conductance.

Outside-out Patches

Conductance—High conductance channels were seen in a lower percentage of outside-out patches than in cell-attached patches from cells transfected with the GABAA subunits plus GABARAP. GABAA channels with conductances above 40 pS were recorded in 6 of 20 outside-out patches activated with 1 μM GABA. Examples of these high conductance channels recorded in one of these outside-out patches are shown in Fig. 7A. The traces are shown on a fast time base to illustrate that there were immediate (within 100 ms) transitions between conductance levels, i.e. they were "single" channel openings and not superimposed non-synchronous openings. The average maximum conductance of the channels in the patch illustrated in Fig. 7A was 61.9 ± 0.011 pS (n = 475 openings). In the six patches containing the high conductance channels, the average γm was 53.7 ± 2.8 pS, which is significantly different from the γm in cells not expressing GABARAP described above (p < 0.0001). In the other 14 patches, channel conductance was 28.5 ± 0.85 pS, which is not significantly different from results obtained in cells not transfected with GABARAP.

Effects of Diazepam—In 5 of 13 outside-out patches tested with diazepam, channel conductance was increased by the drug. Two of these were exposed to 10 μM diazepam, the other three were exposed to 1 μM diazepam. Examples of this effect in two of the patches are shown in Fig. 7. In Fig. 7, B and C, single channel currents increased in amplitude after the patch was exposed to 10 μM diazepam (Vp = −60 mV). The channels in Fig. 7B had a maximum conductance of −30 pS (30.1 ± 0.012 pS, n = 193). After exposure to diazepam, γm increased by a factor of 2 to 60.0 ± 0.02 pS (n = 255). In the two patches showing an increase in channel conductance when exposed to 10 μM diazepam, γm increased from 27.7 to 42.3 pS and from 30.6 to 58.7 pS. The effect of 1 μM diazepam is illustrated in Fig. 7, D and E. The histograms show the distribution of channel conductances measured before (D) and after (E) exposure to 1 μM diazepam. There is a clear shift in channel conductance to higher values. The average channel conductance before diazepam was 25.9 ± 0.40 pS (n = 100) and after diazepam was 40.7 ± 0.52 pS (n = 133). In the three patches exposed to 1 μM diazepam, γm increased from 28.0 ± 0.71 to 48.8 ± 1.73 pS.

To test the possible link between channel clustering and conductance, cells were transfected with the three GABAA subunits plus a truncated GABARAP. A truncated form of GABARAP (deletion of the first 35 residues) loses the ability to bind to tubulin or microfilaments and to cause clustering (12). In contrast to the effects of full-length GABARAP, high conductance channels were not seen in cells expressing truncated GABARAP together with the three GABAA subunits. In seven cell-attached patches, γm was 28 ± 1.7 pS (Vp = −60 mV) (Table I), which is similar to the conductance of channels in the absence of GABARAP. In another series of experiments, we recorded GABAA single channel currents in cells expressing GABAA α1 and β1 subunits (no γm) together with full-length GABARAP. Because the GABARAP binding site is on the γ subunit, GABARAP would not bind to these receptors, and indeed there is no clustering of receptors under these conditions (8). In seven cell-attached patches on cells expressing α1, β1, and γm GABAA subunits plus GABARAP, A, examples of high conductance channels activated with 1 μM GABA in one of these outside-out patches. The currents were recorded at −60 mV, and the calibration bar shows 50 pS. B, conductance of channels recorded in another outside-out patch (1 μM GABA, Vp = −60 mV). C, conductance of channels recorded from the same patch after exposure to 1 μM GABA plus 1 μM diazepam. Vertical calibration bar shows 50 pS. D, channel conductance probability histogram from another outside-out patch exposed to 1 μM GABA. E, channel conductance probability histogram from the same outside-out patch exposed to 1 μM GABA plus 1 μM diazepam.
GABARAP and GABA<sub>A</sub> Channel Conductance

| Conductance | α1β1γ<sub>265</sub> + truncated GABARAP | α1β1 + GABARAP |
|-------------|----------------------------------------|----------------|
| pS          |                                        |                |
| 27<sup>a</sup> | 23                                      | 19<sup>a</sup> |
| 23          | 23                                      | 16             |
| 25          | 25                                      | 17             |
| 37          | 37                                      | 20             |
| 27          | 27                                      | 17             |
| 30          | 30                                      | 20             |
| 28 ± 1.7<sup>b</sup> | 18.9 ± 0.91<sup>b</sup> |               |

<sup>a</sup> Holding membrane potential V<sub>m</sub> = −60 mV.
<sup>b</sup> Mean ± S.E.

and β<sub>1</sub> subunits plus GABARAP, no high conductance channels were seen (Table I). The average channel conductance was 18.9 ± 0.91 pS. These observations suggest that the effects of GABARAP on channel conductance require binding of GABARAP to the GABA<sub>A</sub> γ subunit at some stage of trafficking or assembling of GABA<sub>A</sub> receptors.

**DISCUSSION**

Our results show that co-expression of GABARAP with α<sub>1</sub>, β<sub>1</sub>, and γ<sub>265</sub> subunits of GABA<sub>A</sub> receptors can cause changes in the properties of GABA<sub>A</sub> channels. When compared with recombinant GABA<sub>A</sub> channels in cells expressing α<sub>1</sub>, β<sub>1</sub>, and γ<sub>265</sub> subunits, channels formed by these subunits in cells also expressing GABARAP often had a higher maximum conductance (above 40 pS), and their conductance could be increased by diazepam or pentobarbital. These properties are strikingly similar to those of GABA<sub>A</sub> channels in cultured hippocampal neurons from neonatal rats (3, 4, 6, 13).

GABARAP has been shown to cause the clustering of GABA<sub>A</sub> receptors (8). The cause of this clustering has not been established, and in particular whether GABARAP is closely associated with the receptors (14). Whether or not GABARAP anchors GABA<sub>A</sub> receptors to the cytoskeleton, it is clear that co-expression of GABARAP does produce aggregation of receptors in hot spots in Japanese quail QT-6 fibroblasts (8) perhaps by influencing their trafficking or insertion in the plasmalemma. We have confirmed that co-expression of GABARAP in the L929 fibroblasts we were using in the electrophysiological experiments caused the aggregation of receptors (Fig. 1). We did not detect endogenous GABARAP in L929 cells using Western blotting with the GABARAP antibody (7) (data not shown). Hence, if L929 cells do contain very low amounts of endogenous GABARAP below the sensitivity of Western blotting, it does not cause the same clustering as the co-expressed GABARAP.

We believe that it is the clustering of receptors produced by the co-expressed GABARAP that is responsible for the changes in "single channel" characteristics. It has been suggested previously that channels can open cooperatively (for reviews see Ref. 10). There is evidence for direct protein-protein interactions (cross-talk) between the intracellular domains of different channels in close proximity (15, 16). It is possible that GABARAP, by aggregating receptors, promotes interaction(s) between the intracellular domains of the closely packed receptors so that several pentameric channels open cooperatively. This hypothesis would predict that any method of aggregating receptors might induce cooperative opening and closing of channels. This remains to be tested.

Not all cell-attached patches and fewer outside-out patches from cells co-expressing GABARAP showed high conductance channels. If the high conductance channels depend on the dense packing of receptors, it would not be surprising if some patches did not contain clustered receptors and high conductance channels. Although receptors are presumably in their native state in cell-attached patches, in forming an outside-out patch it is necessary to drag both membrane and receptors into the patch, and it would not be surprising if interactions between receptors and receptor clusters were often disrupted during formation of the patch. This may explain why high conductance channels were seen less frequently in outside-out than in cell-attached patches from cells expressing GABARAP with the three GABA<sub>A</sub> subunits.

Although the absence of endogenous GABARAP in our L929 expression system is able to explain why high conductance recombinant GABA<sub>A</sub> channels are not observed here, it is not clear why there appears to be a discrepancy in some native systems. Perhaps there too, the presence or absence of proteins involved in the clustering mechanism is responsible. Also, our data indicate that patch configuration affects the probability of recording high conductance channels. Whatever the mechanism of the changes caused by GABARAP, our results suggest that recombinant channels may not always be ideal preparations for characterizing the properties, or the responses to drugs, of native channels.

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