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Differential Drug Responses on Native $GABA_A$ Receptors Revealing Heterogeneity in Extrasynaptic Populations in Cultured Hippocampal Neurons

Victoria A. L. Seymour · Andrea B. Everitt · M. Louise Tierney

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Abstract Hippocampal pyramidal neurons potentially express multiple subtypes of $GABA_A$ receptors at extrasynaptic locations that could therefore respond to different drugs. We activated extrasynaptic $GABA_A$ receptors in cultured rat hippocampal pyramidal neurons and measured single-channel currents in order to compare the actions of two drugs that potentially target different $GABA_A$ receptor subtypes. Despite the possible difference in receptor targets of etomidate and diazepam, the two drugs were similar in their actions on native extrasynaptic $GABA_A$ receptors. Each drug produced three distinct responses that differed significantly in current magnitude, implying heterogeneous $GABA_A$ receptor populations. In the majority of patches, drug application increased both the single-channel conductance (>40 pS) and the open probability of the channels. By contrast, in the minority of patches, drug application caused an increase in open probability only. In the third group high-conductance channels were observed upon GABA activation and drug application increased their open probability only. The currents potentiated by etomidate or diazepam were substantially larger in patches displaying high-conductance GABA channels compared to those displaying only low-conductance channels. Factors contributing to the large magnitude of these currents were the long mean open time of high-conductance channels and the presence of multiple channels in these patches. In conclusion, we suggest that the local density of extrasynaptic $GABA_A$ receptors may influence their single-channel properties and may be an additional regulating factor for tonic inhibition and, importantly, differential drug modulation.

Keywords $GABA_A$ receptor · Single-channel conductance · Etomidate · Diazepam

This work is dedicated to the memory of Professor P. W. Gage.

V. A. L. Seymour · A. B. Everitt · M. L. Tierney (✉)
Division of Molecular Bioscience, The John Curtin School of Medical Research, The Australian National University, Building 54, Garran Road, Canberra, ACT 0200, Australia
e-mail: Louise.Tierney@anu.edu.au

Introduction

γ-Amino-butyric acid (GABA) activates an array of $GABA_A$ receptors, distinct in their subunit composition, subcellular location and brain region distribution (Farrant and Nusser 2005; Fritschy and Brünig 2003; Mody 2005). As a result, the brain is provided with a considerable ability to diversify its inhibitory response. Classically, $GABA_A$ receptor-mediated inhibition has been considered the realm of fast synaptic (phasic) neurotransmission. However, more recently the identification of nonsynaptic (extrasynaptic) GABA-mediated currents has defined a distinct form of neurotransmission, referred to as "tonic" inhibition (Farrant and Nusser 2005; Mody et al. 1994). It has been suggested that it is primarily the response of these extrasynaptic $GABA_A$ receptors to endogenous effectors (neurosteroids) and the array of prescription (general anesthetics, antidepressants, sleeping pills, antiepileptics) and recreational drugs (alcohol) known to modulate $GABA_A$ receptors that produce their physiological effects in vivo (Birnir et al. 1994; Chandra et al. 2006; Mody et al. 1994).

Tonic inhibition results from the persistent activation of extrasynaptic $GABA_A$ receptors and, consequently, the inhibitory current contributes to signal integration in the brain by setting the threshold for action potential

generation (Farrant and Nusser 2005). Variation in the subunit composition of extrasynaptic $GABA_A$ receptors would be expected to affect the tonic current. Indeed, extrasynaptic $GABA_A$ receptors, located in a variety of principal cells and interneurons, appear to be heterogeneous in their composition as determined from their response to a wide array of drugs. For example, in addition to the benzodiazepine-sensitive $\alpha\beta\gamma$ receptors at extrasynaptic locations, the high GABA sensitivity and the demonstrated potentiation of currents by drugs such as etomidate and neurosteroids have led to the suggestion that $\alpha\beta\delta$ receptors also exist here (Bianchi and Macdonald 2003; Mortensen and Smart 2006; Stell et al. 2003). Most recently, inhibition of the tonic current by zinc ions implies the presence of $\alpha\beta$ receptors also (Mortensen and Smart 2006).

Immunocytochemical imaging reveals localization of both γ- and δ-containing extrasynaptic $GABA_A$ receptors, with both receptor subtypes at times showing a clustered appearance on the cell body (Christie et al. 2002; Petrini et al. 2004). The influence of clustering extrasynaptic $GABA_A$ receptors on tonic current remains unclear, although disruption of the cytoskeleton with nocodozol results in a dispersion of receptors in the membrane accompanied by a reduction in the amplitude of the tonic current (Petrini et al. 2003).

The general anesthetic etomidate preferentially activates recombinant δ-containing $GABA_A$ receptors (Brown et al. 2002), and potentiation by etomidate has been reported to increase the open probability and mean open time of single native GABA channels (Yang and Uchida 1996). In contrast, diazepam is specific for γ-containing $GABA_A$ receptors (Sigel and Baur 1988) and potentiates the GABA current by either increasing open probability without altering the channel kinetics of native channels (Rogers et al. 1994) or increasing both open probability and conductance (Eghbali et al. 1997). We examined the action of these two drugs on extrasynaptic $GABA_A$ receptors in cultured hippocampal pyramidal neurons using single-channel patch clamping in order to determine whether such drugs were targeting different extrasynaptic $GABA_A$ receptor subtypes, as might be expected from their drug profiles in recombinant systems. From the kinetic parameters measured, etomidate and diazepam produced similar changes in single-channel properties, which included the classical response of an increase in the open probability of the channel but in some, although not all, patches an increase in the single-channel conductance to levels above 40 pS was also observed. While this ability to increase single-channel conductance is a novel finding for the action of etomidate, it is well documented for diazepam (Eghbali et al. 1997). That both drugs produced a differential response suggests heterogeneity in extrasynaptic $GABA_A$ receptor populations, which is unlikely to be solely related to receptor compositional differences. The source of this heterogeneity was further investigated.

Methods

Hippocampal Cultures

Hippocampal neurons from newborn Wistar rats were cultured as described previously (Curmi et al. 1993). Briefly, a newborn rat was killed according to a protocol approved by The Australian National University's Animal Ethics Committee. The isolated hippocampus was triturated using a glass pipette and the dispersed neurons were grown on poly-L-lysine-coated glass coverslips. Cultures were maintained in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with glucose (360 mg/100 ml), fetal bovine serum (10%, Invitrogen), penicillin–streptomycin (1%) and serum extender (0.001%). Cultures were maintained at 37°C in a controlled atmosphere (5% CO_2) and used in patch-clamp studies 7–21 days postculture.

Solutions and Drugs

During recording the cells were bathed at room temperature (20–22°C) in a solution containing (in mM) 135 NaCl, 3 KCl, 5 $MgCl_2$, 2 $CaCl_2$ and 10 5,*N*-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid (TES), pH 7.3. For inside–out patch recording the pipette solution (extracellular solution) contained (in mM) 10 TES, 138 choline chloride, 0.3 KCl and 7 $MgCl_2$, pH 7.3 (adjusted with 6.12 mM NaOH). Using these solutions, the reversal potential for chloride ions was estimated to be -0.05 m*V*. For outside–out patch recording the pipette solution (intracellular solution) contained (in mM) 135 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 5 EGTA and 10 TES, pH 7.3. During application of drugs the reversal potential was monitored by checking the current activity at 0 m*V*; lack of current activity at 0 m*V* for the inside–out patch experiments was indicative of the current being carried by Cl^- ions. We saw no change in the reversal potential throughout these experiments. The drugs used, diazepam (Hoffman La Roche, Dee Why, Australia) and etomidate (Tocris Cookson, Avonmouth, UK), were added through fine tubes positioned very close to the patch (within $\sim$100 μm) or via the bath solution. We find that such lipophilic drugs may be added to either side of the patch membrane to be effective; hence, patch configuration does not influence drug effects (Eghbali et al. 1997). On the day of the experiment stocks of these drugs dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) were

diluted in bath solution to a final concentration of 1 μM. The final concentration of DMSO in solution was <0.01%. GABA was dissolved in pipette solution at a concentration of either 0.5 or 1 μM.

Recording Procedures

Standard voltage-clamp techniques were performed by obtaining gigaohm seals (Hamill et al. 1981) on inside–out and outside–out patches excised from the cell soma. Junction potentials (~2.5 m*V*) were not corrected for.

Patch pipettes were made from thick-walled borosilicate glass (Harvard Apparatus, Dover, MA) and coated with Sylgard resin (Dow Corning, Midland, MI). After fire polishing, the pipettes had resistances of 10–20 MΩ when filled with pipette solution.

Data Acquisition and Analysis

GABA-activated single-channel currents were recorded using an Axopatch 200A or 200B amplifier (Axon Instruments, Burlingame, CA) filtered at 5 kHz and digitized at 10 kHz. The in-house software Channel 2 (written by P. W. Gage and M. Smith) was used for current analysis. Single-channel current amplitudes were accepted only if their open duration was at least 0.3 ms (i.e., three times the sampling rate), thereby avoiding extraneous electrical noise in the analysis (Luu et al. 2006). Using these settings, a typical, single, independent channel opening was defined as a direct transition from the baseline current level within 200 μs (two data points) (Luu et al. 2006). Statistical analyses were performed using Prism (Graphpad, San Diego, CA) or Excel (Microsoft, Redmond, WA). Mean values were compared using a paired or unpaired, two-tailed Student's *t*-test, and values were considered significantly different at $P < 0.05$.

Single-Channel Currents in Inside–Out Patches

Single-channel currents were analyzed to determine the distribution of current amplitudes. Amplitude histograms were constructed using more than 500 openings in which currents were collected into bins 0.07 pA in width (Horn 1987). The mean amplitude levels of single-channel currents were then determined by fitting gaussian components to these histograms using least-squares minimization (Luu et al. 2006). The open times were measured as the average of the open times of those amplitudes that were between 2 standard deviations either side of the mean of that gaussian component. The mean open time of each conductance level was an average of at least three or more independent experiments. Mean current (I_m) was measured as the total current above zero (the middle of the baseline peak) over a 30-s period. Channel burst durations were measured by constructing burst duration histograms from current recordings. A burst was defined as an opening or group of openings separated by relatively long closed periods (tau critical, τ_c). The τ_c value was determined for each recording by deriving the intersection of the two exponential lines for the two shortest closed time components (Colquhoun and Sigworth 1983). Typical values for τ_c were 0.48–1.77 ms; all closings briefer than τ_c were considered to occur within a burst. Burst durations were allocated into frequency histograms using logarithmic binning. The square roots of the frequency histograms were fitted with the sums of two or three exponential components (Sigworth and Sine 1987). Data are expressed as mean ± SEM from *n* independent experiments.

In order to determine whether an increase in mean current in patches displaying just a single channel opening was due only to the increase in conductance or whether the open probability of the channel had also increased, the ratio of the average conductance (mean current divided by the driving force on the ion, $-Vp - E_{Cl}$) in the presence of GABA and GABA plus etomidate was compared to the ratio of the maximum conductance under the same two conditions (Eghbali et al. 1997). If the ratio of the average conductance is larger than the ratio of the maximum conductance, then the increase in mean current is due to both an increase in the conductance and open probability of the channel.

Results

Differential Effects of Etomidate Potentiation on $GABA_A$ Channels

Low concentrations of GABA (0.5–1.0 μM) are able to activate extrasynaptic channels that display a wide variation in single-channel conductance in cultured hippocampal neurons (Birnir et al. 2001; Eghbali et al. 1997). The ability of etomidate (1 μM) to potentiate the activity of such GABA-activated channels was examined in inside–out patches from cultured hippocampal neurons whose GABA-activated conductance varied between ~10 and ~80 pS ($n = 42$, $-Vp = +60$ m*V*). Current responses could be divided into three groups according to the effect of etomidate on the single-channel conductance.

In the majority of patches the initial GABA-activated conductance ranged between 8 and 30 pS ($n = 32$), and 1 μM etomidate significantly increased both the single-channel conductance and mean current in 26/32 patches (HC_{ET}, Table 1). Following potentiation by etomidate, the channels displayed all the hallmarks of $GABA_A$ receptor-mediated, high-conductance channels; the current reversed

Table 1 Etomidate and diazepam each produce three distinct effects on native $GABA_A$ receptors

Drug treatment	Group	γ_{GABA} (pS)	γ_{Drug} (pS)	$\gamma_{Drug}/\gamma_{GABA}$	I_{mGABA} (pA)	I_{mDrug} (pA)	I_{mDrug}/I_{mGABA}	n
Etomidate	LC_{ET}	14.5 ± 1.3	24.3 ± 3.7*	1.7 ± 0.2	0.06 ± 0.02	0.44 ± 0.14*	13.1 ± 7.8	6
	HC_{ET}	16.8 ± 1.1	63.5 ± 2.5*	4.2 ± 0.3	0.12 ± 0.03	3.02 ± 0.51*	85 ± 17.8	26
	$HC_{GABA(ET)}$	64.0 ± 3.4	65.9 ± 1.7	1.0 ± 0.04	1.98 ± 0.44	4.56 ± 0.77	4.3 ± 1.6	10
Diazepam	LC_{DZ}	16 ± 2.1	21 ± 2.1*	1.3 ± 0.09	0.17 ± 0.1	1.67 ± 2.4*	6.5 ± 2.2	3
	HC_{DZ}	15.0 ± 1.0	60.2 ± 1.9*	4.3 ± 0.3	0.08 ± 0.05	2.16 ± 0.34*	125.3 ± 27.7	18
	$HC_{GABA(DZ)}$	59.5 ± 6.1	65.0 ± 3.9	1.2 ± 0.1	3.81 ± 1.28	4.70 ± 1.14	2.2 ± 0.6	8

Values are mean ± SEM from n patches recorded at $-Vp = +60$ mV

* Significant difference upon drug (1.0 μM) application compared to current activity in GABA (0.5–1.0 μM) (Student's t-test, $P < 0.05$)

I_m, mean current; γ, single-channel conductance; LC, low conductance (<40 pS); HC, high conductance (>40 pS)

close to 0 mV in equimolar chloride solutions, and the current–voltage relationship was outwardly rectifying (Fig. 1a–c). An example of the current activity in response to potentiation of the GABA response by etomidate from one inside–out patch is shown in Fig. 1a. As the membrane potential was stepped from −80 to +80 mV in 20-mV steps, both the single-channel conductance and the open probability of the channel were seen to increase. For example, at a pipette potential of +60 mV ($-Vp = -60$ mV) the GABA-activated channel had a conductance of 46 pS and an open probability of 0.27, but when the pipette potential was stepped to −60 mV ($-Vp = +60$ mV), the same channel's maximum conductance was now 75 pS and open probability was significantly higher at 0.39 (Fig. 1a). The influence of membrane potential on current amplitude was recorded over a range of potentials where the initial GABA response was potentiated by 1 μM etomidate, and the average I–V curves ($n = 6$) are shown in Fig. 1b. The increase in current amplitude was not caused by a change in the reversal potential because currents still reversed close to the Cl^- equilibrium potential (−0.05 mV) (Fig. 1a–c). Furthermore, bicuculline (100 μM) blocked high-conductance channels generated in outside–out patches following etomidate potentiation of the GABA response ($n = 2$, data not shown). Hence, etomidate was able to increase the single-channel conductance of native extrasynaptic $GABA_A$ receptors. The mean current generated upon etomidate potentiation of the GABA response was measured over a range of potentials in five inside–out patches, and average measurements are depicted in Fig. 1c. The current shows pronounced outward rectification consistent with the greater open probability and larger single-channel conductance of the channels.

Etomidate application did not always increase the amplitude of GABA currents. In some patches ($n = 6$) etomidate application increased the open probability but the single-channel conductance never exceeded 40 pS, as illustrated in Fig. 2a (LC_{ET} group). In the presence of GABA, channels exhibited a conductance of 12 pS; and upon etomidate application, the open probability of the channel increased, as indicated in the all-points histogram, while the conductance increased only slightly to 17 pS.

A third group consisted of patches in which the initial current amplitude in GABA was high, ranging 48–80 pS ($-Vp = +60$ mV, $n = 10$), and etomidate application did not significantly alter the conductance but channel open probability was increased ($HC_{GABA(ET)}$ group). A typical example is shown in Fig. 2c, where the conductance of the channel in the presence of GABA was 77 pS and upon etomidate application it was similar, 72 pS. The increase in open probability after etomidate application is illustrated by comparing the corresponding all-points histogram alongside each recording before and after drug in Fig. 2c. Average values for conductance and mean current according to the three groupings defined above are displayed in Table 1.

A plot of the maximum conductance upon etomidate application against the initial conductance in GABA is shown in Fig. 3a and illustrates the division of the responses into the three groups. The greater the distance a point is above the dissecting line, the greater the increase in single-channel conductance upon etomidate application. The majority of such values coalesce where the initial conductance in response to GABA is <40 pS and correspond to patches from the HC_{ET} group (solid triangles, $n = 26$).

Although etomidate increased the mean current (I_m) in all three groups, the magnitude of the current response varied greatly between them (Table 1, Fig. 3b). For example, in the group whose channels displayed a conductance <40 pS in response to GABA plus etomidate (LC_{ET}), I_m did increase but it was consistently small (mean = 0.44 ± 0.14 pA, range 0.11–1.04 pA; Fig. 3b, open circles). The relatively low channel open probability in these patches was reflected by the occurrence of only a single active channel throughout these recordings (Fig. 3c). In contrast, in those patches where the single-channel conductance was substantially increased by etomidate

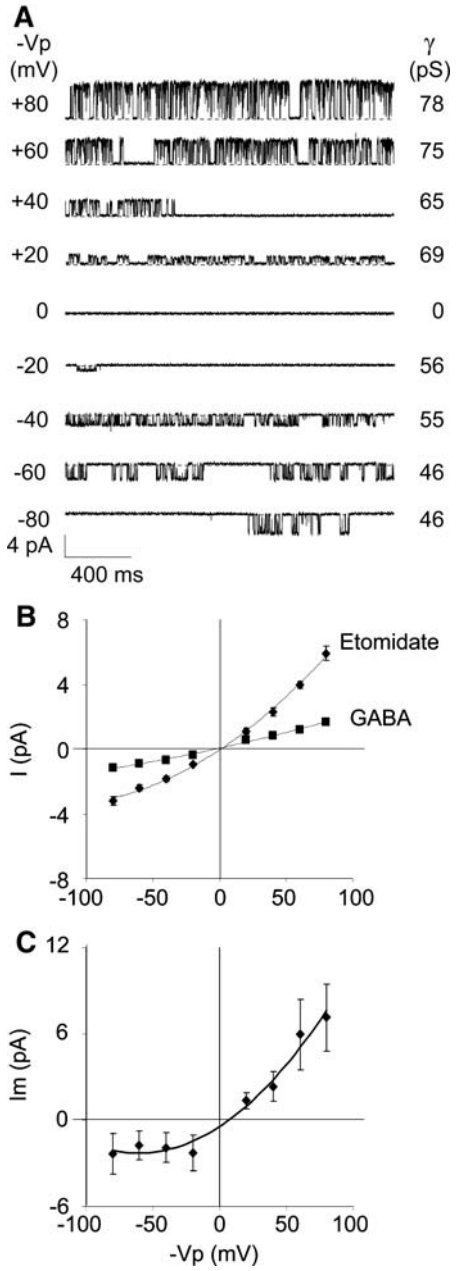

Fig. 1 Example of the effect of membrane potential on GABA-activated low-conductance channels potentiated by etomidate in cultured hippocampal pyramidal neurons. Except where indicated in **b**, etomidate was in all other cases applied to the neurons in conjunction with GABA. **a** Single-channel currents were recorded from inside–out patches at potentials from a –Vp of −80 to +80 m*V* in 20-m*V* steps. **b** Plot of single-channel current amplitude (*I*, pA) against membrane potential (–Vp, m*V*) ($n = 6$). **c** Plot of mean current (I_m) against membrane potential ($n = 5$). Both the conductance and mean current show pronounced outward rectification in response to etomidate potentiation, the latter due to increases in both conductance and open probability. All inside–out patches had either 0.5 or 1.0 μM GABA in the pipette and 1.0 μM etomidate added through a flow tube close to the cell

application (HC_{ET}; Fig. 3b, solid triangles), the mean current increased on average 85-fold (mean 3.02 ± 0.51 pA, range 0.27–10.52 pA) and was significantly larger than that in the LC_{ET} group ($P < 0.05$). In virtually all patches in this group (25/26) the ratio of the average conductance (I_m/–Vp) was larger than that of the maximum conductance ($\gamma_{drug}/\gamma_{GABA}$) and, hence, etomidate potentiation involved an increase in both open probability and single-channel conductance. The increase in open probability was reflected in the presence of more active channels following etomidate application in 16/26 patches in this group (two to five channels, Fig. 3c). In the third group, $HC_{GABA(ET)}$, while the mean current increased only about 4.3-fold on average upon etomidate application (compared to 85-fold on average for the HC_{ET} group), the magnitude of the mean current after etomidate was not significantly different from that attained in the HC_{ET} group (mean 4.56 ± 0.77 pA, range 1.54–9.94 pA; Table 1, Fig. 3b, dashes). Nine out of 10 patches in this group showed an increase in mean current upon etomidate application, and this increase in open probability was once again reflected in the presence of more active channels in the recordings of eight of these (two to five channels, Fig. 3c).

Differential Effects of Diazepam Potentiation on $GABA_A$ Channels

Etomidate is not unique in producing a differential drug response through extrasynaptic $GABA_A$ receptors. Examination of GABA currents potentiated by diazepam in these same neuronal cultures revealed a similar demarcation in responses. In the majority of patches GABA-activated currents displayed small amplitudes, ranging 10–23 pS (–Vp = +60 m*V*); but exposure to 1 μM diazepam caused an increase in the single-channel conductance to levels above 40 pS in 18/21 of these patches (HC_{DZ}, Table 1). In all 21 patches, however, the mean current was increased by diazepam, identifying the currents as being mediated by $GABA_A$ channels. In a third group in which channels with conductances ranging 46–98 pS were initially observed in the presence of GABA (0.5–1.0 μM), diazepam increased the open probability but did not affect the conductance of the channel ($HC_{GABA(DZ)}$, $n = 8$).

Diazepam increased channel open probability, with the range in the magnitude of the mean currents measured following the same trend as was seen upon etomidate application (Table 1). For example, those patches in which the conductance of the channel was >40 pS, either upon GABA application or following diazepam application

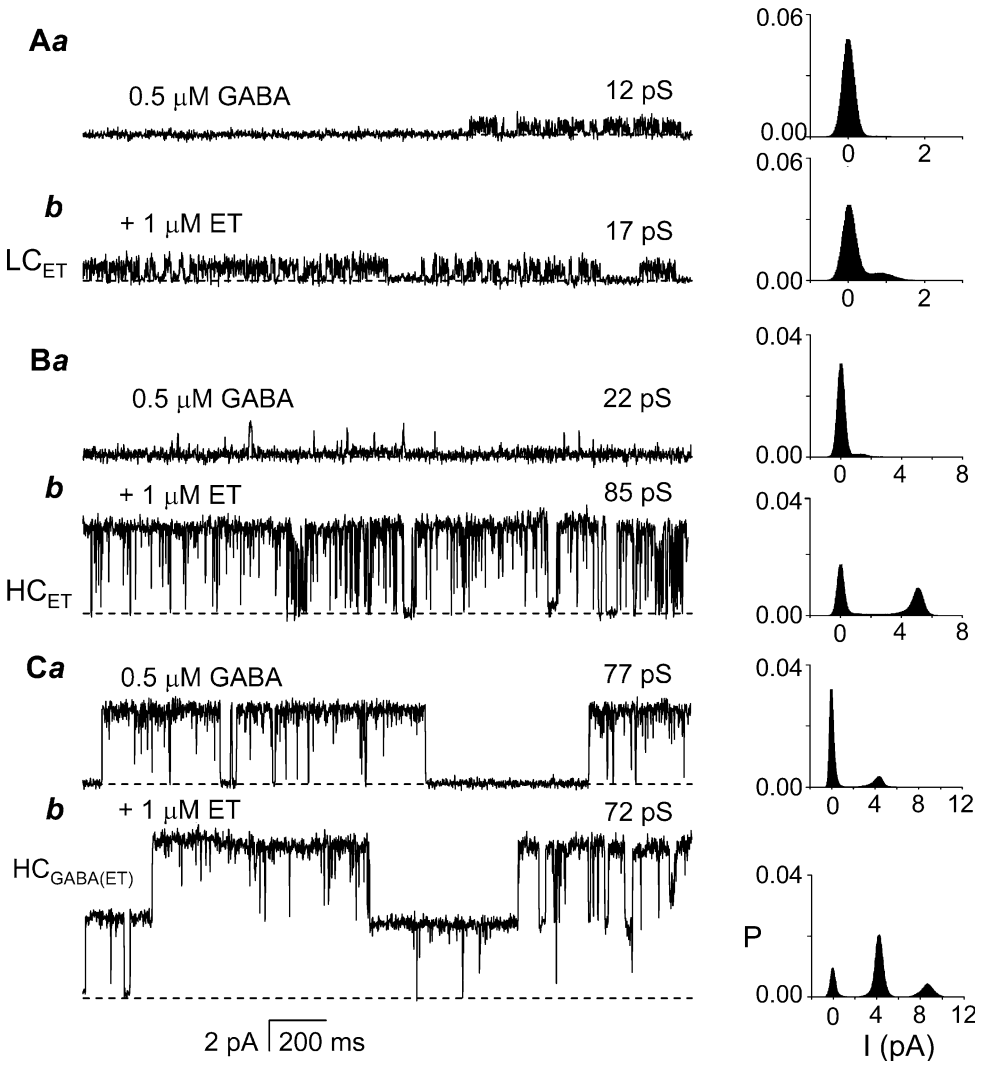

Fig. 2 Etomidate potentiation produces three distinct responses from extrasynaptic $GABA_A$ receptors in cultured hippocampal pyramidal neurons. **a** *Upper* In a minority of patches ($n = 6/42$) GABA-activated channels that displayed a low conductance (12 pS) and etomidate mainly increased the open probability with only a slight increase in conductance (*lower*) (LC_{ET}). **b** *Upper* In the majority of patches (26/42) low-conductance channels activated by GABA (22 pS) responded to etomidate application by greatly increasing both their conductance (88 pS) and open probability (*lower*) (HC_{ET}). **c** *Upper* Low concentrations of GABA occasionally produced high-conductance channels (10/42, 77 pS) and etomidate increased their open probability only (*lower*) ($HC_{GABA(ET)}$). Corresponding all-points histograms represent 30 s of current recording. All recordings were at $-Vp = +60$ m*V*. All inside–out patches had 1.0 μM GABA in the pipette and 1.0 μM etomidate (ET) added through a flow tube close to the cell

(HC_{DZ}, $HC_{GABA(DZ)}$), had the largest mean currents, while those in which the single-channel conductance remained <40 pS upon diazepam application generated relatively smaller mean currents (LC_{DZ}, Table 1).

Relationship Between Channel Kinetics and Conductance

We investigated the correlation between single-channel conductance and mean open time in native GABA-activated channels potentiated by either etomidate or diazepam. Figure 4a shows a typical example of currents activated by GABA (0.5 μM) where channels with conductances of 18 ± 1.8 and 23.7 ± 3.4 pS ($-Vp = +60$ m*V*) had brief open times (0.6 ± 0.5 and 1.2 ± 1.3 ms, respectively, mean ± SD), as depicted in the corresponding open time distribution plot alongside the trace. By contrast, channels whose conductance had been increased, as depicted in Fig. 4b (58.6 ± 2.9 pS, mean ± SD, $-Vp = +60$ m*V*), showed a much longer mean open time of 9.7 ± 8.9 ms. Similar data were obtained from all patches in the HC_{ET} group amenable to this analysis ($n = 8$), revealing a significant difference in the mean open time of such high-conductance channels generated by etomidate application compared to the low-conductance channels activated by GABA alone ($P < 0.05$, Table 2).

The high-conductance channels generated either by GABA alone or by diazepam potentiation of the GABA response also displayed significantly longer mean open times than the low-conductance channels activated by GABA ($P < 0.05$, Table 2). An example of high-conductance activity in the presence of 0.5 μM GABA is shown in Fig. 4c, where channels with a conductance of 80 pS had a mean open time of 4.3 ms. Figure 4d shows the current activity observed upon diazepam application where the single-channel conductance has been increased to 55 pS and such channels have a mean open time of 16 ms.

Paired data are displayed in Fig. 4e, f, where single-channel conductance is plotted against the mean open time of the low-conductance channels initially activated by

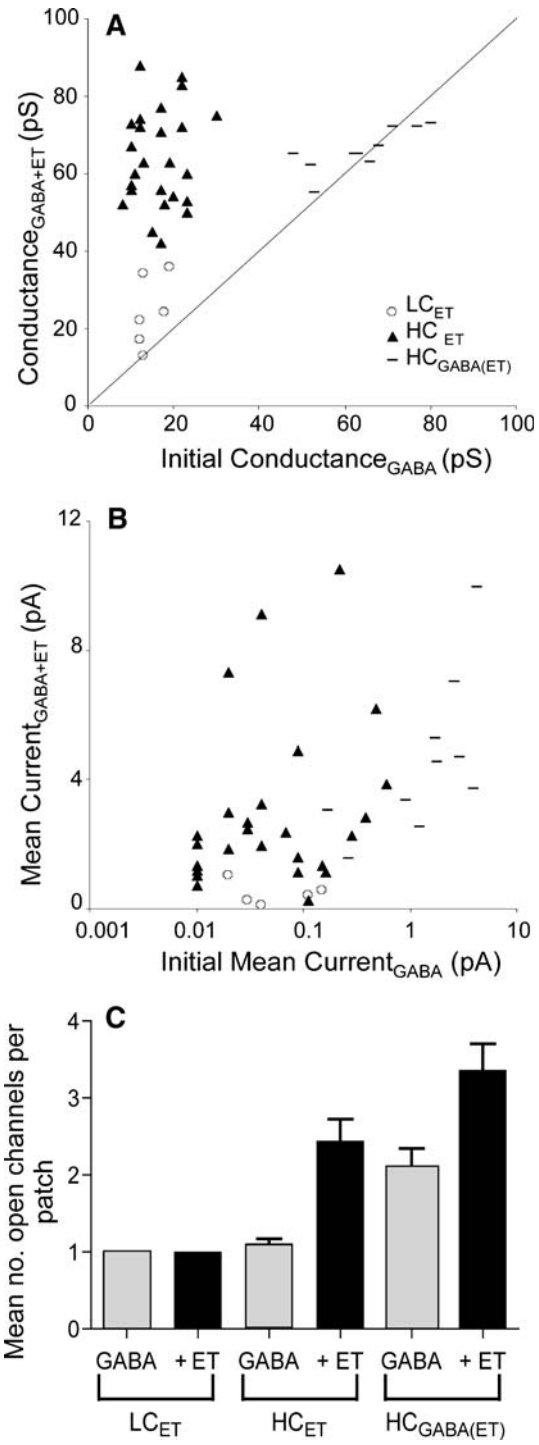

Fig. 3 Variation of responses following etomidate potentiation of the GABA response in cultured hippocampal neurons. **a** Plot of maximum conductance during GABA application (*x* axis) against maximum conductance upon subsequent etomidate application (*y* axis). Values above the diagonal line indicate an increase in single-channel conductance produced upon etomidate application, while values along the diagonal indicate no change upon etomidate application. Etomidate only increased the conductance of channels whose initial conductance in GABA was low (▲), although a small number of these were not significantly affected (o). **b** Plot of mean current (I_m) upon GABA + etomidate application (*y* axis) against initial mean current during GABA activation (*x* axis). The largest electrical signal in response to etomidate application was generated in patches displaying high-conductance GABA channels (▲, –). Mean current was measured over 30 s. **c** Plot of the average number of channels observed in a patch upon GABA activation (*gray bars*) and subsequent application of etomidate (*black bars*) for each of the three groups, LC_{ET} ($n = 6$), HC_{ET} ($n = 26$) and $HC_{GABA(ET)}$ ($n = 10$). Patches displaying high-conductance channels tended to have more active (overlapping) channels (HC_{ET} and $HC_{GABA(ET)}$ groups) than those displaying low-conductance channels (LC_{ET}). All recordings were at –Vp = +60 m*V*. ET, etomidate

GABA (<40 pS) and the subsequent high-conductance channels (>40 pS) potentiated by etomidate or diazepam, respectively. The data were best fit with a linear relationship (diazepam $R = 0.78$, $P < 0.05$; etomidate $R = 0.56$), showing that the conductance and open time of these channels are intricately linked.

One of the actions of drugs acting at $GABA_A$ receptors is to increase the open probability of the channel, leading to the appearance of bursts of channel openings and, hence, increasing the size of the electrical signal. The mean burst duration of high-conductance channels was measured and found to be similar irrespective of the drug used to potentiate the current. For example, the average burst lengths of high-conductance channels potentiated by etomidate and diazepam were 37.8 ± 10.3 and 37.7 ± 6.8 ms, respectively (Table 2). High-conductance channels activated by GABA alone also displayed long bursts of activity (19.6 ± 2.6 ms). The long mean burst associated with high-conductance channels reflects, at least to some extent, the long mean open time of the channels. Insufficient data prevented analysis of the burst behavior of low-conductance $GABA_A$ channels in these cultured neurons.

Conductance and its Correlation with Open Probability

We investigated native extrasynaptic $GABA_A$ receptor density indirectly by examining channel open probability, where the minimum number of channels in a patch was determined by simply counting the number of overlapping channels. We then compared this open probability with the occurrence of particular channel characteristics, namely, single-channel conductance.

Following etomidate application, multiple (overlapping) channels were observed in patches exhibiting high-conductance channels ($HC_{GABA(ET)}$ $n = 10/10$, HC_{ET} $n = 16/26$). In contrast, none of the patches whose conductance remained low (<40 pS) upon etomidate application contained multiple channels (LC_{ET} $n = 6$), even though there was a significant increase in channel open probability

Table 2 Comparison of the single-channel kinetic properties of native $GABA_A$ channels activated by GABA or potentiated by etomidate or diazepam

Drug treatment	Group	[Drug] (μM)	Conductance (pS)	Mean open time (ms)	Mean burst length (ms)
GABA	LC_{GABA}	0.5–1.0	15.4 ± 0.7 ($n = 50$)	0.9 ± 0.1 ($n = 8$)	nd
GABA	HC_{GABA}	0.5–1.0	62.0 ± 3.2 ($n = 18$)	7.7 ± 2.3* ($n = 6$)	19.6 ± 2.6 ($n = 3$)
GABA + etomidate	HC_{ET}	1.0	63.5 ± 2.5 ($n = 26$)	7.0 ± 1.4* ($n = 8$)	37.8 ± 10.3 ($n = 7$)
GABA + diazepam	HC_{DZ}	1.0	60.2 ± 1.9 ($n = 18$)	9.6 ± 1.9* ($n = 10$)	37.7 ± 6.8 ($n = 4$)

Mean ± SEM from n patches recorded at $-Vp = +60$ mV

* Significantly different from GABA (LC_{GABA}) (Student's t-test, $P < 0.05$). GABA 0.5–1 μM, etomidate 1 μM, diazepam 1 μM

(Table 1). Similarly, upon diazepam application, patches in which the conductance of GABA channels remained low only ever appeared to exhibit one active channel (LC_{DZ} $n = 3$) but those displaying high-conductance channels contained multiple channels (HC_{DZ} $n = 7/8$, $HC_{GABA(DZ)}$ $n = 9/18$). These data reveal a dependence between high-conductance $GABA_A$ channels and multiple, active $GABA_A$ channels (i.e., channel open probability) in patches (chi-squared test $HC_{GABA(ET)}$ $P < 6.3 \times 10^{-5}$, HC_{ET} $P = 6.5 \times 10^{-3}$, HC_{DZ}, $P < 3.5 \times 10^{-3}$, $HC_{GABA(DZ)}$, $P < 0.04$, $-Vp = +60$ mV).

Discussion

Analysis of single-channel recordings from cultured hippocampal neurons reveals that extrasynaptic $GABA_A$ receptors, activated with low concentrations of GABA and modulated by clinical concentrations of etomidate or diazepam, are differentially modulated. This diversity of responses implies the existence of heterogeneous populations of extrasynaptic $GABA_A$ receptors. Some of this functional heterogeneity is undoubtedly due to different receptor compositions (Brickley et al. 1999; Herd et al. 2008). However, some of the functional heterogeneity, we suggest, may arise due to differences in the local density of extrasynaptic $GABA_A$ receptors in the plasma membrane, as implied herein by the dependence observed between high-conductance GABA channels and multiple channels in the patch (Fig. 3c).

Extrasynaptic $GABA_A$ Receptor Populations

Different receptor composition may underlie some of the variation in drug responses. Hippocampal neurons contain both extrasynaptic γ- and δ-containing $GABA_A$ receptors, with the most likely combinations suggested to be $\alpha5\beta\gamma$ and $\alpha4\beta\delta$ receptors (Caraiscos et al. 2004; Glykys et al. 2008; Herd et al. 2008; Mangan et al. 2005). In addition, receptors including just α- and β-subunits may contribute to the extrasynaptic pool of $GABA_A$ receptors (Mortensen and Smart 2006). Etomidate has been shown to potentiate the whole-cell current response produced by all three of these receptor combinations in heterologous expression systems, although it is most efficacious at δ-containing $GABA_A$ receptors (Brown et al. 2002; Uchida et al. 1995). Some of the GABA-activated channels potentiated by etomidate displayed conductances that are within the range exhibited by recombinant $\alpha\beta$ receptors (10–15 pS, $n = 2$, Fig. 3a, LC_{ET} group) (Angelotti and Macdonald 1993; Luu et al. 2005). Other GABA-activated channels potentiated by etomidate ($n = 4$, LC_{ET}) and some potentiated by diazepam ($n = 3$, LC_{DZ}) exhibited single-channel conductances of 18–35 pS, consistent with recombinant $\alpha\beta\delta$- and $\alpha\beta\gamma$- containing receptors (Angelotti and Macdonald 1993; Fisher and Macdonald 1997; Luu et al. 2005). It is possible, therefore, that in these patches, where channel conductance did not change significantly upon drug application (remaining <40 pS), both γ- and δ-containing $GABA_A$ receptors exist. However, what they all have in common, we suggest, is that their local density in the membrane is low, consistent with their low open probability (mean current) and with the observation of only ever a single active channel throughout each recording (some >20 min in length). The increase in mean current only in response to either etomidate or diazepam application is similar to what is observed in recombinant expression systems expressing combinations of $\alpha\beta\gamma$- and $\alpha\beta\delta$-subunits (Brown et al. 2002; Fisher and Macdonald 1997). The low abundance of these receptor types at extrasynaptic locations in cultured hippocampal neurons together with their small electrical signal suggest that these particular channels would contribute relatively little to the drug-induced tonic inhibitory response of extrasynaptic $GABA_A$ receptors.

$GABA_A$ receptors in those patches that display high-conductance channels following either GABA activation or potentiation by either etomidate or diazepam must be different from those in which the conductance remained low (<40 pS). The biggest currents in response to drug application were measured in patches displaying high-conductance GABA-mediated channels (Table 1). Hence, it would be expected that modulation of these extrasynaptic receptors, which leads to increases in both single-channel conductance and open probability (mean open time and

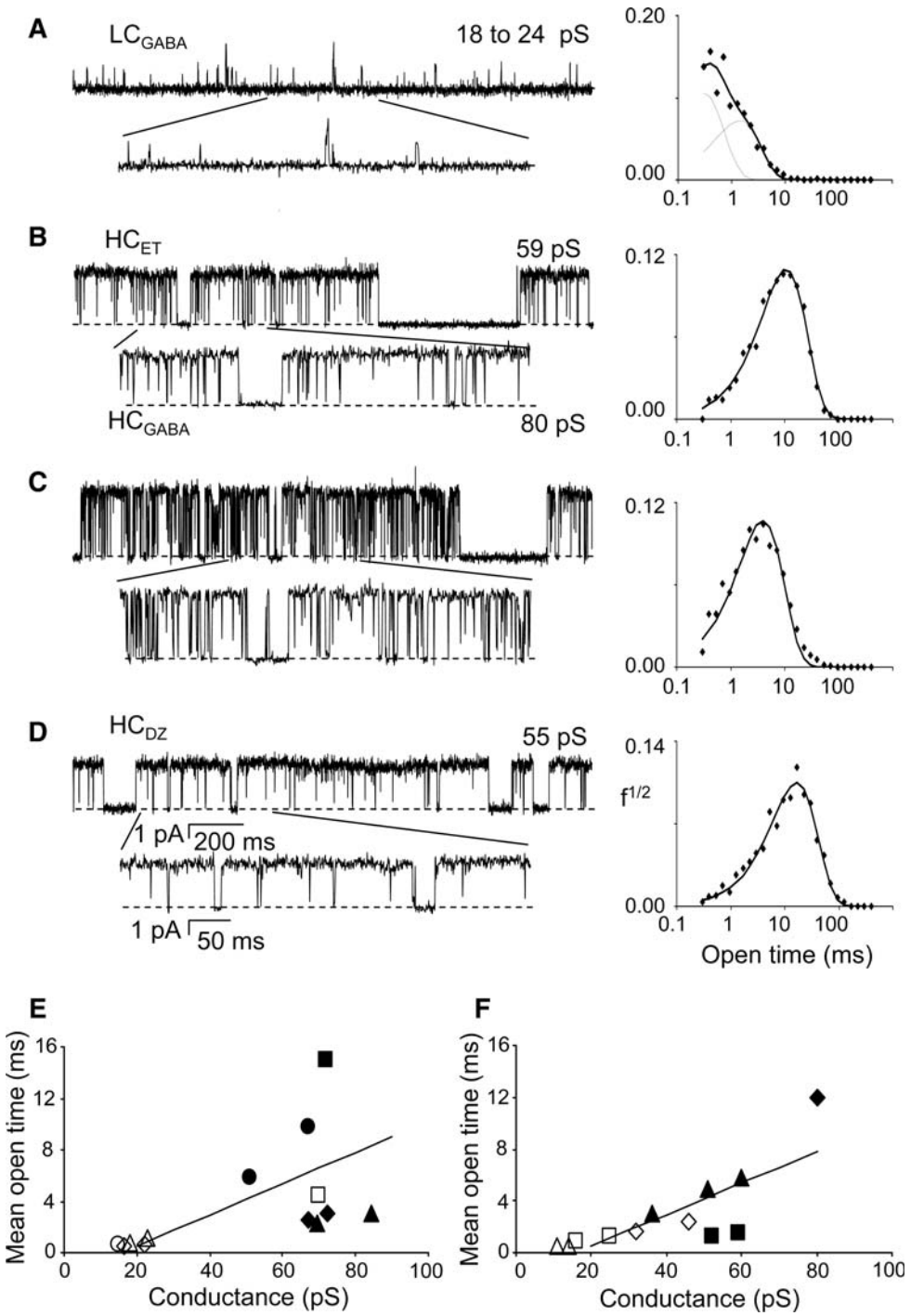

Fig. 4 Correlation between single-channel conductance and mean open time of extrasynaptic $GABA_A$ channels in cultured hippocampal pyramidal neurons. Each single-channel trace is accompanied by its corresponding open time distribution. **a** An example of the low-conductance channels activated by GABA in cultured pyramidal neurons (18 and 24 pS); such channels displayed brief openings (0.6 and 1.2 ms, respectively). **b** High-conductance channels (59 pS) generated by etomidate potentiation displayed long mean open times, 9.7 ms (HC_{ET}). **c** Low concentrations of GABA (0.5 μM) could also occasionally generate high-conductance channels, and in the example shown such channels had a conductance of 80 pS and a mean open time of 4.3 ms (HC_{GABA}). **d** Diazepam potentiation of the GABA response similarly generated high-conductance channels (55 pS) with relatively long mean open times, 16 ms (HC_{DZ}). **e**, **f** The plots show paired data from five to six patches where currents were activated with GABA and subsequently potentiated by either etomidate (**e**) or diazepam (**f**). Single-channel conductance (*x* axis) is plotted against mean open time (*y* axis). The same symbol represents measurements from the same patch, while unfilled symbols represent measurements in GABA and filled symbols represent subsequent measurements in etomidate. All recordings were at $-Vp = +60$ m*V* and all inside–out patches had either 0.5 or 1.0 μM GABA in the pipette. Etomidate or diazepam was added (1.0 μM) by flowing the drug through a tube placed close to the cell

number of active channels, i.e., nP_o), would contribute the greatest inhibitory effect on tonic current in response to drugs.

In trying to determine what may underlie a $GABA_A$ channel's ability to respond differently to a drug, we noted that many patches displaying high-conductance channels also displayed multiple channels. This was not the case in patches in which the single-channel conductance remained low, thus revealing a dependence between high-conductance channels and multiple channels in the patch (chi-squared test). Similar observations have been made in the recombinant system where patches pulled from cells coexpressing $\alpha\beta\gamma$ $GABA_A$ receptors with a trafficking protein, GABARAP, displayed high-conductance channels and contained significantly more receptors than those displaying low-conductance channels from patches expressing just $\alpha\beta\gamma$ receptors (Luu et al. 2006). Together these results suggest that the local density of both native and recombinant $GABA_A$ receptors influences their ion channel properties.

Correlation Between Single-Channel Conductance and Kinetics

The mean open time of $GABA_A$ channels showed a strong correlation with conductance, demonstrated by high-conductance channels displaying significantly longer mean open times than low-conductance channels activated by GABA. A similar relationship was observed whether the channels were activated by GABA or potentiated by either diazepam or etomidate, suggesting that these compounds act similarly to stabilize the open conformation(s) of these high-conductance channels. We have previously described a similar correlation for recombinant $GABA_A$ $\alpha\beta\gamma$ receptors coexpressed with GABARAP (Luu et al. 2006). Based on the similarity in the "superchannel" properties (i.e., high conductance and long mean open time) of native high-conductance $GABA_A$ channels with those reproduced in the recombinant system upon coexpression of GABARAP, we suggest that processes similar to (or indeed the same as) GABARAP-mediated trafficking regulate the expression of native extrasynaptic $GABA_A$ receptors in the plasma membrane and, as a consequence, $GABA_A$ ion channel properties are altered. This suggestion, based on functional measurements, is consistent with previous confocal microscopy studies showing that the expression of recombinant GABARAP in neurons leads to an increase in the cell surface number of γ-containing $GABA_A$ receptors (Leil et al. 2004).

Open Probability and Conductance

Etomidate and diazepam were able to increase the single-channel conductance of some, but not all, extrasynaptic $GABA_A$ channels; in some patches only the open probability of the channel was increased (mean current). This suggests that the actions of these drugs on conductance and open probability are independent and, indeed, that the ability of a drug to generate high-conductance channels is specific for a particular population of $GABA_A$ receptors.

By comparing the ratios of the mean current post- and pre-drug application, a number of details are revealed (Table 1). Firstly, the degree of modulation of native GABA-activated channels is quite variable for both etomidate and diazepam, but the two drugs show similar variation within their groups. Secondly, the greatest effect of drug potentiation is seen on GABA-activated channels that initially displayed conductances <40 pS but whose conductance could be increased upon drug application. This apparent similarity in the actions of diazepam and etomidate suggests that the equivalent response groups from the two drug treatments share common properties. One such property we have identified is the dependence between conductance and the local density of receptors in the membrane.

Differential Drug Effects

Since the kinetic parameters of GABA currents potentiated by either etomidate or diazepam were similar, we cannot be certain that these drugs were targeting the same or different receptor compositions in these neurons. However, from the specificity of the drugs used in this study, our results imply that γ-containing $GABA_A$ receptors are able to respond differentially to diazepam and that their single-channel properties are dependent upon their local extrasynaptic density. The single-channel properties of δ-containing receptors may also be dependent upon the receptor's local density, but a different approach is required other than kinetic measurements to confirm this. Synaptic (i.e., γ-containing) $GABA_A$ receptors are known to reside extrasynaptically for some of their lifetime. Both receptor insertion at synaptic sites and receptor exocytosis following lateral diffusion away from the synapse occur at extrasynaptic locations (Bogdanov et al. 2006). It is possible that the heterogeneity observed here upon diazepam application reflects γ-containing $GABA_A$ receptors awaiting different fates.

Conclusion

In conclusion, we have shown that etomidate and diazepam have a differential effect on extrasynaptic $GABA_A$ receptors and suggest that such responses are in part attributable to differences in the local density of receptors in the

membrane. This leads us to propose that regulating the density of native extrasynaptic $GABA_A$ receptors in the plasma membrane influences their response to drugs. A variation in the subunit composition of extrasynaptic $GABA_A$ receptors will affect the tonic current but so too will increased receptor density in the membrane that gives rise to high-conductance $GABA_A$ channels with enhanced kinetic properties. Both these variations will affect the neuronal response to endogenous and exogenous modulators, providing additional response factors for tonic inhibition.

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