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U-93631 Causes Rapid Decay of γ-Aminobutyric Acid-Induced Chloride Currents in Recombinant Rat γ-Aminobutyric Acid Type A Receptors

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SUMMARY

We discovered the ability of U-93631 (4-dimethyl-3-*t*-butylcarboxyl-4,5-dihydro[1,5-*a*]imidazoquinoxaline) to accelerate decay of γ-aminobutyric acid (GABA)-induced currents, and we explored its mechanism in human embryonic kidney cells (HEK-293) stably expressing the $\alpha1\beta2\gamma2$ subtype of $GABA_A$ receptors. Inward currents (Cl^- efflux) induced by 5 μM GABA at the holding potential of −60 mV (under a symmetrical Cl^- gradient) decayed with an exponential time course with a mean time constant (τ) of 222 ± 25 sec, as examined with the whole-cell configuration of the patch-clamp technique. The monoexponential decay was greatly accelerated in the presence of U-93631 at 5 μM, with the mean τ value being 5.2 ± 0.5 sec. The τ values were dependent on the concentration of U-93631, with an estimated K_d of approximately 2 μM. Outward currents at the holding potential of +60 mV decayed with a similar τ value in the presence of the drug, suggesting the voltage independence of the drug action. The initial amplitude of the GABA (5 μM)-induced Cl^- current was not affected by preincubation with U-93631 (5 μM) or GABA (200 nM) alone but was reduced by preincubation with the combination of the two. In the presence of U-93631 at 5 μM, the peak amplitude decreased as a function of GABA concentration, with the half-maximal inhibitory concentration being approximately 100 nM, which is close to the K_d for the high affinity GABA site (85 nM). It appears that the drug interacts with GABA-bound receptors (at least monoliganded) and accelerates receptor desensitization, rather than acting as an open channel blocker. The binding site for U-93631 on $GABA_A$ receptors seems not to overlap with GABA, barbiturate, or benzodiazepine sites, because the drug effect persisted in the presence of excess ligands for those sites. With cloned $GABA_A$ receptors composed of only $\alpha1\beta2$, $\beta2\gamma2$, or $\alpha1\gamma2$ subunits, U-93631 also accelerated the decay rate. This lack of subtype selectivity raises the possibility that the compound interacts with a region common among the three subunits, probably a novel modulatory site, which can possibly be exploited as a novel therapeutic target.

The neurotransmitter GABA is the predominant inhibitory neurotransmitter in the vertebrate central nervous system and interacts with $GABA_A$ receptors, which are receptor-chloride channel complexes of multimeric subunits (1–4). Several families of subunits, designated α, β, γ, δ, and ρ, with multiple isoforms (designated numerically) for each family, exist for $GABA_A$ receptors in the brain (3–5). Among various combinations of $GABA_A$ receptor subunits, the $\alpha1\beta2\gamma2$ subtype, when expressed in human embryonic kidney cells, accommodates all the $GABA_A$ receptor ligands known to date and responds to positive and negative allosteric modulators in the same way as do neuronal receptors (6).

Recently, we have developed various templates for benzodiazepine site ligands and examined their actions in the $\alpha1\beta2\gamma2$ subtype of $GABA_A$ receptors. One of the new templates was imidazoquinoxaline, the derivatives of which were antagonists or agonists at the benzodiazepine site of the cloned $GABA_A$ receptor (7). On the other hand, its reduced congener, the dihydroimidazoquinoxaline template, produced a unique response, in that the reduced derivatives accelerated decay of GABA-induced Cl^- currents without producing noticeable changes in the initial amplitude of the currents. Here we examined the interaction of a prototypical dihydroimiadzoquinoxaline, U-93631 (4-dimethyl-3-*t*-butylcarboxyl-4,5-dihydro[1,5-*a*]imidazoquinoxaline), with the $\alpha1\beta2\gamma2$ subtype of cloned $GABA_A$ receptors. It appears that the drug reversibly desensitizes $GABA_A$ receptors when GABA sites are occupied.

Materials and Methods

Cloned $GABA_A$ receptors. Combinations of rat α1, β2, and γ2 subunits of the $GABA_A$ receptor were expressed in human embryonic kidney cells (HEK-293) as described previously (6). Briefly, the cells were transfected with plasmids containing cDNA and a plasmid encoding G418 resistance. After 2 weeks of selection in the presence of 1 mg/

ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

ml G418, resistant cells were assayed, by Northern blotting, for the ability to synthesize GABA$_A$ receptor mRNAs. Positive cells for appropriate subunits were used for electrophysiology. The majority of studies were conducted with cells expressing the $\alpha1\beta2\gamma2$ receptor configuration, but other receptor configurations (as noted below) were also studied.

Electrophysiology. The whole-cell configuration of the patch-clamp technique (8) was used to study GABA-induced Cl^- currents. Patch pipettes were constructed from borosilicate glass (Kimax-51; Kimble Products, Toledo, OH) that was pulled (Flaming/Brown, P-80/PC; Sutter Instrument Co., Novato, CA) and fire-polished to a tip impedance of 0.5–2 MΩ when filled with the following pipette solution (in mM): CsCl, 140; EGTA, 4; HEPES, 10; $MgCl_2$, 0.4; pH 7.2. Coverslips containing the cultured cells were transferred to a small chamber (1 ml) on the stage of an inverted light microscope (Nikon) and superfused continuously (2 ml/min) with the following external solution (in mM): NaCl, 125; KCl, 5.5; $CaCl_2$, 3.0; $MgCl_2$, 0.8; Na-HEPES, 20; dextrose, 25; pH 7.3.

Whole-cell currents were recorded with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage. A bath headstage (BH-1) was used to compensate for bath potentials. GABA-induced Cl^- currents were monitored on an oscilloscope and stored on a computer using commercially available software (pCLAMP; Axon Instruments). All studies, except those examining voltage dependence, were conducted with the cells voltage-clamped at −60 mV.

Experimental protocol. GABA was dissolved in the external solution (described above) and applied to the target cell through a U-tube positioned within 100 μm of the cell. In experiments in which preincubation with drugs was required, the cells were bathed for at least 5 min in external solution containing the drugs (GABA, U-93631, pentobarbital, diazepam, and/or Ro 15–1788) at the indicated concentrations. Typically, to monitor GABA response, a GABA pulse (5 μM, unless noted otherwise) was applied before, during, and after preincubation. Note that the GABA pulse during preincubation was carried out with a mixture of GABA and preincubated drugs, so as to not disturb the concentration of the preincubated drugs in the vicinity of the cell during the pulse.

Data analysis. The time constant for desensitization (τ) was obtained by fitting a one-exponential function to time course-current profiles with the aid of one of three software programs (Clampfit from Axon Instruments, Sigmaplot from Jandel Scientific, or Origin from Microcal Software). All programs produced similar τ values.

Results

GABA, at the concentration of 5 μM, induced an inward current in the $\alpha1\beta2\gamma2$ subtype at the holding potential of −60 mV under a symmetrical Cl^- gradient (Fig. 1A). The current decayed very little during a 15-sec GABA application, and the decay phase could be fitted with a one-exponential function with a mean time constant (τ) of 222 ± 25 sec. In the presence of U-93631 at 5 μM, the decay was still monoexponential but with a faster τ value (5.2 ± 0.5 sec; Fig. 1A, *middle*). The drug effect was reversible (Fig. 1A, *right*) and appeared to be independent of the GABA concentration, a major determinant of channel opening probability. For example, τ was still 4.1 ± 1.0 sec as the concentration of GABA was raised from 5 to 10 μM, which increased the current amplitude >2-fold. With GABA (10 μM) alone, the τ value was 157.1 ± 13.1 sec.

Dose dependence. Fig. 2 shows typical tracings for Cl^- currents displaying different decay rates as a function of the concentration of U-93631, in the $\alpha1\beta2\gamma2$ subtype of GABA$_A$ receptors. The decay phase of each tracing could be fitted well with a one-exponential function. The time constant decreased as a function of the concentration of U-93631. The τ values were 17.7 ± 3.0, 10.8 ± 1.0, 8.1 ± 1.0, and 5.2 ± 0.5 sec in the

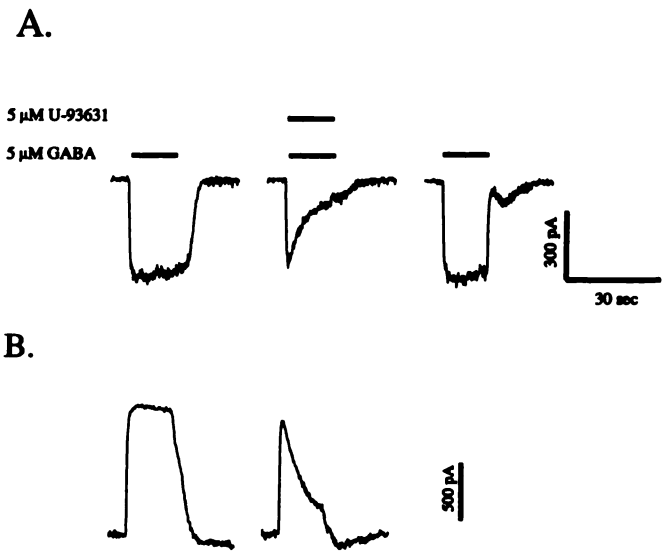

Fig. 1. Acceleration of GABA-induced Cl^- current decay by U-93631. A, Human embryonic kidney cells expressing the $\alpha1\beta2\gamma2$ subtype of GABA$_A$ receptors were voltage clamped at −60 mV under a symmetrical Cl^- gradient. GABA (5 μM) induced inward currents, as measured using the whole-cell configuration of the patch-clamp technique. The Cl^- current showed only a modest decay during a 15-sec period of GABA application, but its decay was reversibly accelerated in the presence of U-93631 at 5 μM (pre-equilibrated in the bathing medium). B, Voltage independence of the effect of U-93631. GABA (5 μM) induced an outward Cl^- current in the cells at the holding potential of +60 mV, and U-93631 (5 μM) accelerated the current decay. Fitting of the decay phase with an exponential function yielded τ values of 5.2 and 6.8 sec for inward (*top*) and outward (*bottom*) currents, respectively.

presence of U-93631 at 0.2, 0.8, 2, and 5 μM, respectively. It is noteworthy that the initial amplitude of Cl^- currents was not changed at all concentrations of U-93631. In the case of U-93631 at 5 μM, the peak current appeared to be reduced because of a rapid rate of current decay but could be extrapolated to the initial amplitude of the control using a one-exponential function with a τ of 5.2 sec. This distinguishes U-93631 from some other ligands, such as barbiturates (9) and benzodiazepines (10, 11), that accelerate the current decay after a large increase in the initial amplitude of Cl^- currents.

The lack of considerable decay of Cl^- current in the presence of GABA (5 μM) alone (mean τ of 222 sec) suggests that the channel opening/closing is in equilibrium and does not contribute much to the current decay measurements under our experimental conditions. Changes in τ in the presence of U-93631, therefore, arose solely from its interaction with the receptors, particularly with GABA-bound receptors, because pre-equilibration of the receptors with the drug did not change the initial peak amplitude of GABA-induced Cl^- currents (see below). A minimal model for current decay is

$$R^* \underset{k_{-1}}{\overset{k_{+1}[D]}{\rightleftharpoons}} R^*D$$

where R^* is the GABA-bound receptor, D is U-93631, R^*D is the drug-bound, nonconducting receptor, and k_{+1} and k_{-1} are the association and dissociation rates, respectively, for U-93631 with GABA-bound receptors. Using this model, the τ for the decay phase equals $1/(k_{+1}[\text{drug}] + k_{-1})$. From a plot of $1/\tau$

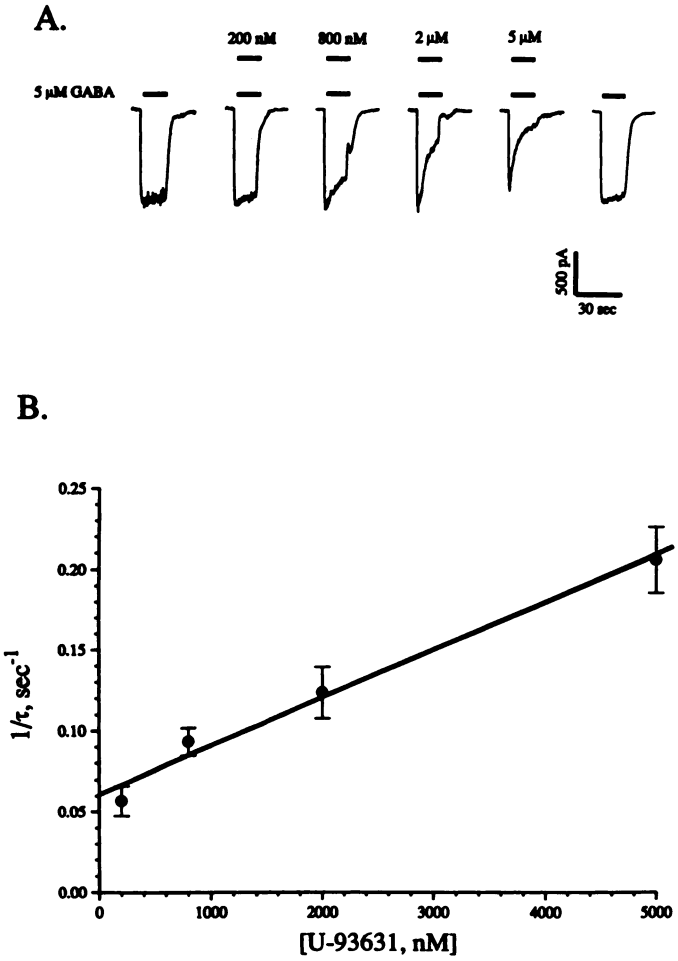

Fig. 2. Dose-dependent acceleration of Cl^- current decay by U-93631. A, Decay of GABA (5 μM)-induced Cl^- currents was accelerated in the kidney cells expressing the $\alpha1\beta2\gamma2$ subtype as the concentration of U-93631 was raised. The amplitude of the peak current was not noticeably affected by the drug. B, The decay phase of Cl^- currents was fitted with an exponential function and the values of $1/\tau$ were plotted as a function of the concentrations of U-93631. *Solid line*, best fit to the equation $1/\tau = k_{+1}[\text{drug}] + k_{-1}$, with a value of 0.06 sec^{-1} for k_{-1} from the *y*-intercept and a value of 3.3×10^4 M^{-1} sec^{-1} for k_{+1} from the slope. The dissociation constant for U-93631 obtained from the rate constants ($K_d = k_{-1}/k_{+1}$) was 1.8 μM.

versus the concentration of U-93631, we obtained a value of 0.06 sec^{-1} for k_{-1} from the *y*-intercept and a value of 3.3×10^4 M^{-1} sec^{-1} for k_{+1} from the slope (Fig. 2). The dissociation constant for U-93631 obtained from the rate constants ($K_d = k_{-1}/k_{+1}$) was 1.8 μM.

Voltage dependence. We examined whether U-93631 also accelerates the decay of GABA-induced Cl^- current in the opposite direction, by changing the holding potential from −60 to +60 mV (Fig. 1B). Outward current was observed and the decay phase could be fitted with a one-exponential function with a τ of 169.3 ± 33.8 sec, during a 15-sec period of GABA (5 μM) application. Again, U-93631 accelerated the current decay with an exponential time course with a τ of 6.8 ± 1.8 sec, similar to that (5.2 ± 0.5 sec) seen at the holding potential of −60 mV. We concluded that the acceleration of the decay rate by U-93631 is not voltage dependent.

Involvement of high affinity GABA sites in the action of U-93631. The finding that U-93631 did not produce changes in the peak amplitude of Cl^- currents indicates its interaction with only GABA-bound receptors. Two types of GABA binding sites, high and low affinity sites, are known to exist on $GABA_A$ receptors, based on binding studies and measurements of Hill coefficients of ≥2 in Cl^- current studies; with the $\alpha1\beta2\gamma2$ subtype the high affinity site has a K_d of 83 nM and the low affinity site appears to have a K_d value in the 10–20 μM range (12). To examine whether the high affinity GABA site is involved in the action of U-93631, we preincubated the cells for at least 5 min in the presence of GABA, at concentrations ranging from 50 to 200 nM. Preincubation with low concentrations of GABA alone did not affect Cl^- currents induced by GABA at 5 μM, but preincubation with both GABA (200 nM) and U-93631 (5 μM) reduced the initial amplitude of Cl^- currents (Fig. 3A). With a fixed concentration of U-93631 (5 μM), the initial amplitude (after extrapolation using an exponential function) was reduced by 4 ± 2, 43 ± 5, and 52 ± 5% at GABA concentrations of 50, 100, and 200 nM, respectively (Fig. 3B). It should be noted that varying the preincubation time from 5 to 20 min in the presence of GABA at 200

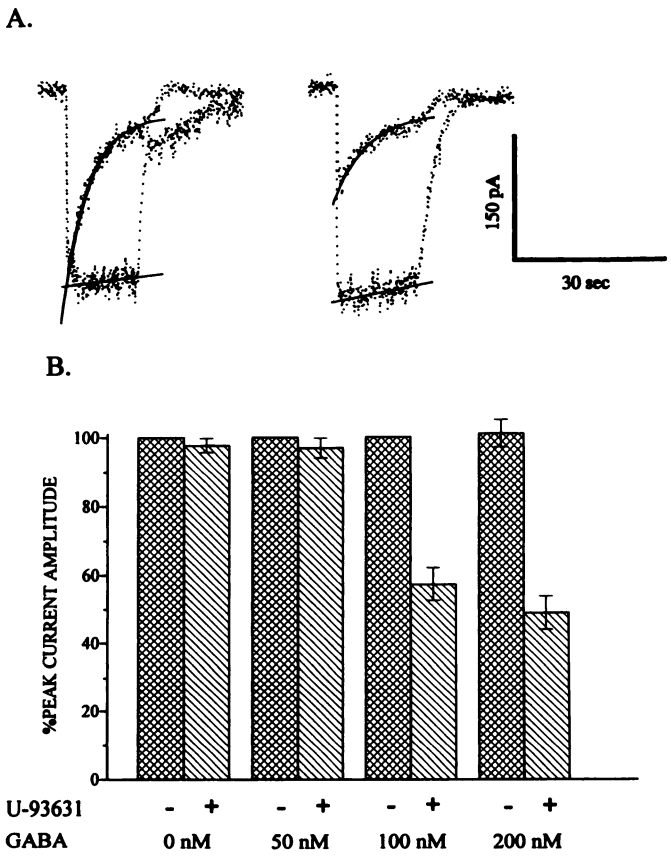

Fig. 3. Effect of preincubation of the $\alpha1\beta2\gamma2$ receptor with low GABA concentrations, in the presence of U-93631, on test GABA (5 μM) currents. The kidney cells expressing the $\alpha1\beta2\gamma2$ subtype were preincubated for 5 min with GABA concentrations ranging from 50 to 200 nM, in the presence or absence of U-93631 (5 μM), and then a test pulse of GABA (5 μM) was applied under the whole-cell configuration of the patch-clamp technique. A, *Solid lines*, fitting of the decay phase of Cl^- currents with an exponential function. In the presence of U-93631 the amplitude of Cl^- currents decreased as a function of GABA concentration during the preincubation period with 50 nM GABA (*left*) or 200 nM GABA (*right*). B, Decreases in the amplitude of the peak current as a function of GABA concentration during preincubation in the presence of U-93631 at 5 μM. The amplitude was decreased by 4 ± 2, 43 ± 5, and 52 ± 5% with GABA at 50, 100, and 200 nM, respectively. The data represent means ± standard errors from three experiments.

nM did not change the degree of reduction in the initial amplitude of Cl^- currents. Also, the residual currents decayed at the same rate as that observed without preincubation, suggesting one mechanism for Cl^- current decay in the presence of U-93631.

Acceleration of Cl^- current decay by high GABA concentrations (5–500 μM), on the other hand, was not noticeably altered by preincubation with low GABA concentrations (200 nM) in the $\alpha1\beta2\gamma2$ subtype. Fig. 4 shows the plots of τ versus GABA concentrations, with or without preincubation with 200 nM GABA, and representative current tracings. The slope was not considerably changed by preincubation with 200 nM GABA. In both cases, the τ value progressively decreased as the concentration of GABA increased, i.e., from >200 to 3 sec as the concentration of GABA was changed from 5 to 500 μM. It appears that the exponential decay of Cl^- currents in the presence of GABA alone is solely dependent on the magnitude of the evoked current.

Lack of interaction of U-93631 with known ligand binding sites on GABA$_A$ receptors. Benzodiazepines and barbiturates are well known allosteric modulators interacting with GABA$_A$ receptors via independent sites (3, 4, 9–11, 13–17). Diazepam (2 μM) markedly potentiated Cl^- currents induced by GABA at 5 μM and displayed a tendency to increase the current decay rate, reducing the τ from 222 to 67 ± 9 sec (Fig. 5A). U-93631, even in the presence of excess diazepam or Ro 15–1788 (a classical benzodiazepine antagonist) further accelerated Cl^- current decay, changing the τ values to 5.8 ± 1.0 and 4.3 ± 0.5 sec, respectively (Fig. 5B). This indicates that there is no interaction of U-93631 with the benzodiazepine site. Similarly, U-93631 accelerated the Cl^- current decay in the presence of 2 μM pentobarbital (data not shown).

Lack of subunit specificity. All of the experiments described above were conducted with cells expressing the $\alpha1\beta2\gamma2$ subtype of GABA$_A$ receptors. In an attempt to determine whether any one subunit is required for U-93631 action, we examined the effect of the drug on GABA-induced Cl^- currents in receptors consisting of only $\alpha1\beta2$, $\beta2\gamma2$, or $\alpha1\gamma2$ subunits (Fig. 6). In all cases, U-93631 markedly accelerated Cl^- current decay, whereas peak Cl^- current was not affected significantly.

Discussion

In this study we have shown that U-93631, a dihydroimidazoquinoxaline derivative, accelerates decay of GABA-induced Cl^- currents in a dose-dependent, voltage-independent manner in the $\alpha1\beta2\gamma2$ subtype of cloned GABA$_A$ receptors expressed in human embryonic kidney cells. Although GABA$_A$ receptor function is known to be modulated in various ways (including positive, neutral, and negative modes) by allosteric ligands (4, 6, 9–11, 13–18), the acceleration of Cl^- current decay by U-93631 is unique, in that the initial amplitude of the current was not altered even with preincubation with the drug. This suggests that the action of the drug arises from its interaction with GABA-bound receptors.

High and low affinity binding sites for GABA exist on GABA$_A$ receptors (17), and we found that occupancy of high affinity GABA sites is sufficient to trigger the action of U-93631. For example, preincubation of the receptors with GABA

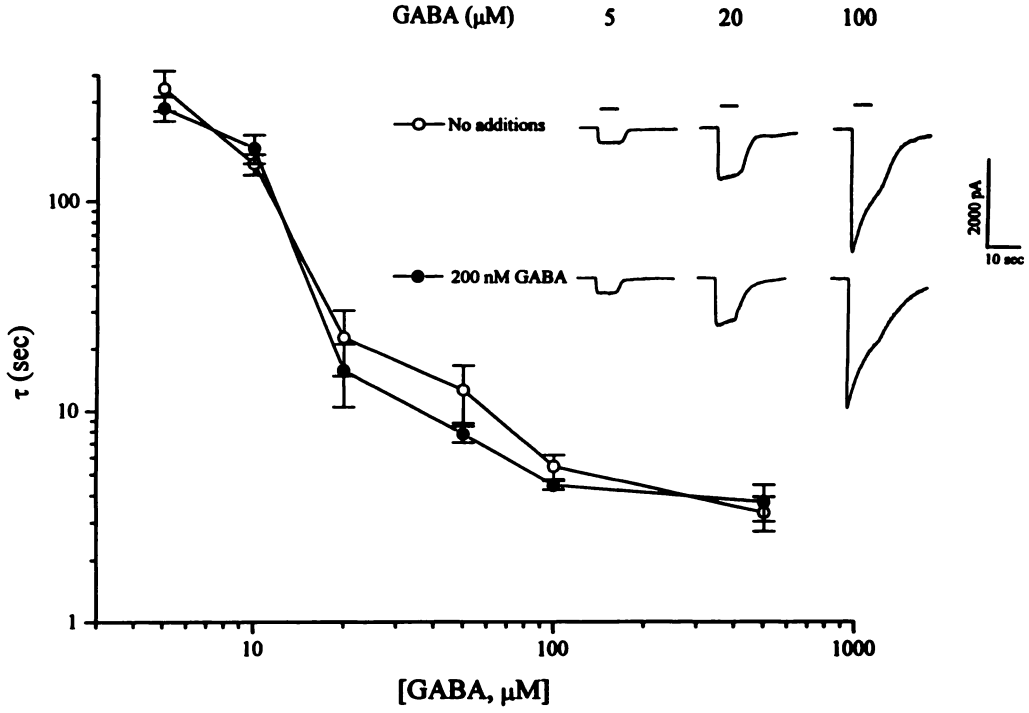

Fig. 4. Effect of low GABA concentrations on high GABA concentration-induced desensitization of Cl^- currents. The kidney cells expressing the $\alpha1\beta2\gamma2$ subtype of GABA$_A$ receptors were preincubated for at least 5 min in the absence or presence of GABA at 200 nM, and then a test pulse of GABA ranging from 5 to 500 μM was applied for 5 sec (*inset*, representative *tracings*). The decay phase of Cl^- current observed during each test pulse was fit with an exponential function. The time constants (τ) were plotted as a function of the concentration of GABA for the test pulse. The τ value decreased as the concentration of GABA increased from 5 to 500 μM with the $\alpha1\beta2\gamma2$ subtype; it was >200 sec with 5 μM GABA and was reduced to 3 sec with 500 μM GABA. Note that preincubation with 200 nM GABA had no significant effect on the τ values at all test concentrations of GABA.

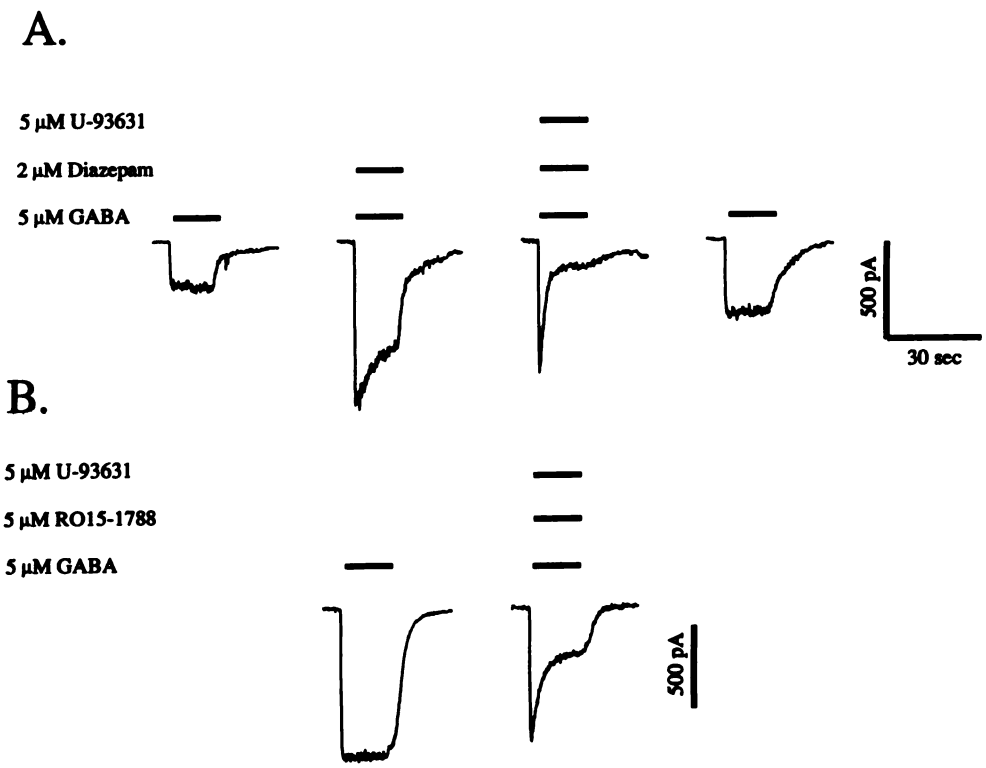

Fig. 5. Lack of interaction of U-93631 with the benzodiazepine site of $GABA_A$ receptors. U-93631 accelerated Cl^- current decay despite the presence of excess diazepam (2 μM) (A) or Ro 15–1788 (B). Diazepam alone potentiated the peak Cl^- current and mildly enhanced desensitization. Note that the GABA-induced current did not recover completely (A, *right*), due to the residual effect of diazepam.

at 100 nM and the drug at 5 μM (see Fig. 4) reduced the initial peak of GABA (5 μM) current by 43%. GABA at 100 nM may occupy about one half of the high affinity GABA sites (K_d of 83 nM for GABA in this subtype) (12).

Two interesting questions are the following: what type of conformational change is induced by occupancy of the high affinity GABA sites and where is the binding site for U-93631 in the monoliganded receptors? Information in the literature about the conformations of monoliganded $GABA_A$ receptors is scanty; indeed, it is not even known whether the monoliganded channels conduct Cl^- currents. Single-channel openings of short duration (2–3 msec) have been postulated to originate from monoliganded receptors (19). In preliminary cell-attached patch studies with the $\alpha1\beta2\gamma2$ subtype, however, we have not observed a single event of channel opening, in periods of up to 5 min, with 100 nM GABA in the pipette. With 5 μM GABA, we have observed robust channel openings. It appears, therefore, that the reduction of Cl^- currents by the drug during preincubation with 100 nM GABA is not likely due to open channel blocking by U-93631. Also, the voltage independence of the drug action indicates no possibility for penetration of the drug into the mouth of chloride channels to any depth, as is the case for a classical open channel blocker. Moreover, the physical property of the drug as a positively charged secondary amine compound makes it difficult to designate this drug as an open channel blocker acting deep within the pore of the anion-selective channel.

A more plausible mechanism for U-93631 action is that the drug interacts with the monoliganded receptors and transforms them into a conformation that is unlikely to open at higher concentrations of GABA. Similar interactions of the drug with multiliganded $GABA_A$ receptors in the open conformation cannot be ruled out, because application of high GABA concentrations (5 or 10 μM) in the presence of the drug also accelerated current decay. The lack of change in the initial peak amplitude of GABA-induced currents in this case indicates that the process of GABA-induced channel opening is much faster than drug binding. In any event, the drug-induced decay in Cl^- currents could, in a broad sense, be termed desensitization but appears to be distinct from desensitization induced by GABA or other agonists (9–11, 20–22). Indeed, our analysis of GABA-induced desensitization illustrates that it is unaffected by occupancy of the high affinity GABA site; similar results have been reported recently (22). The major determinant for desensitization by GABA appears to be the relative amplitude of induced current, i.e., an increased current amplitude leads to an increased rate of desensitization (see Fig. 4). This mechanism also appears to be primarily responsible for desensitization by diazepam and pentobarbital (8–10) and may distinguish these drugs from U-93631. In any event, single-channel studies may clarify the mode of interaction of U-93631 with $GABA_A$ receptors.

From the above discussion, the binding site for U-93631 appears not to be within the channel mouth. Also, it does not overlap with the sites for well known allosteric modulators such as benzodiazepines and barbiturates, because U-93631 was effective in the presence of diazepam, Ro 15–1788 (a classical benzodiazepine antagonist), and pentobarbital. Furthermore, the drug action was not sensitive to GABA concentration; the τ value in the presence of U-93631 did not change significantly as the concentration of GABA was increased from 5 to 10 μM. These results are consistent with the absence of direct interactions of U-93631 with GABA sites. The binding site for U-

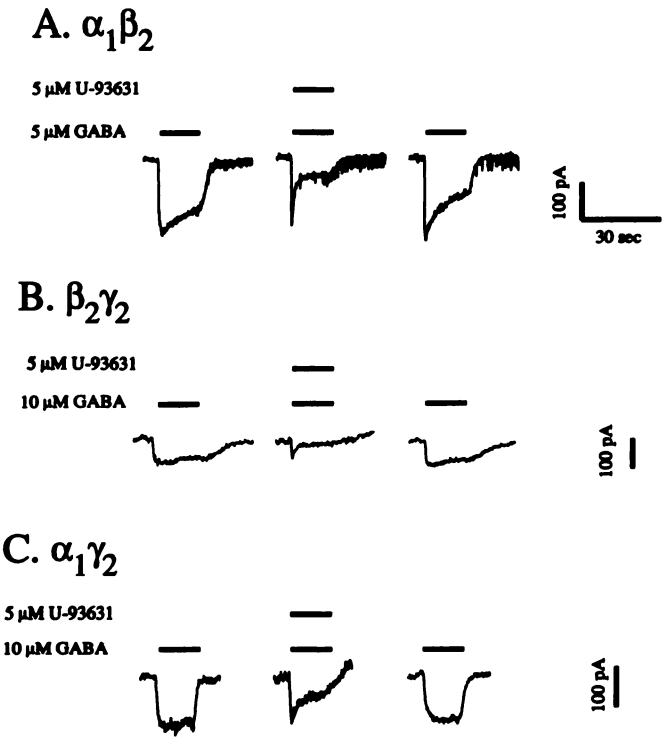

Fig. 6. Effect of U-93631 on GABA-induced Cl^- currents in the $\alpha1\beta2$, $\beta2\gamma2$, and $\alpha1\gamma2$ subtypes of $GABA_A$ receptors. The kidney cells were transfected with the indicated subunits of $GABA_A$ receptors. GABA induced Cl^- currents of varying magnitudes with the subtypes; the lowest was in the $\beta2\gamma2$ subtype (10 μM GABA), followed by the $\alpha1\gamma2$ (10 μM GABA) and $\alpha1\beta2$ subtype (5 μM GABA). This may reflect different levels of expression of stable receptor complexes. U-93631 reversibly accelerated decay of Cl^- currents in all the subtypes.

93631 thus appears to be novel. Our efforts to detect the association of the site with particular subunits were not successful; U-93631 was effective with receptors made of any combination of two subunits from $\alpha1$, $\beta2$, and $\gamma2$. Considering the homology in the primary sequences of the subunits (1, 2), it is possible that U-93631 could interact with a binding site arising from a common sequence among $\alpha1$, $\beta2$, and $\gamma2$.

In summary, we have characterized a novel compound, U-93631, that induces rapid, concentration-dependent desensitization of $GABA_A$ receptors when GABA sites are occupied. This drug action is not voltage dependent and is not dependent upon any one subunit of the GABA receptor. Our data are consistent with the presence of a novel modulatory site for U-93631 on $GABA_A$ receptors, which can possibly be exploited as a novel therapeutic target.

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