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ACCELERATED COMMUNICATION

Molecular Targets for the Myorelaxant Action of Diazepam

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ABSTRACT

Diazepam is used clinically for its myorelaxant, anxiolytic, sedative, and anticonvulsant properties. Although the anxiolytic action is mediated by α2 γ-aminobutyric acid A ($GABA_A$) receptors, the sedative action and in part the anticonvulsant action are mediated by α1 $GABA_A$ receptors. To identify the $GABA_A$ receptor subtypes mediating the action of diazepam on muscle tone, we have assessed the myorelaxant properties of diazepam in α2(H101R) and α3(H126R) knock-in mice harboring diazepam-insensitive α2 or α3 $GABA_A$ receptors, respectively. Whereas in α2(H101R) mice the myorelaxant action of diazepam was almost completely abolished at doses up to 10 mg/kg, the same dose induced myorelaxation in both wild-type and α3(H126R) mice. It was only at a very high dose (30 mg/kg diazepam) that α2(H101R) mice showed partial myorelaxation and α3(H126R) mice were partially protected from myorelaxation compared with wild-type mice. Thus, the myorelaxant activity of diazepam seems to be mediated primarily by α2 $GABA_A$ receptors and at high concentrations also by α3 $GABA_A$ receptors.

Classical benzodiazepines are in wide clinical use as hypnotics, tranquilizers, muscle relaxants, and anticonvulsants. These effects are caused exclusively by their interaction with the benzodiazepine site of $GABA_A$ receptors. Based on the presence of more than a dozen subunit genes, the central nervous system contains a plethora of structurally diverse $GABA_A$ receptors (Fritschy and Mohler, 1995; McKernan and Whiting, 1995; Barnard et al., 1998). The vast majority of $GABA_A$ receptors are benzodiazepine-sensitive and can be grouped into 4 classes characterized by the type of α subunit being either α1, α2, α3 or α5. Diazepam and related classical benzodiazepines interact with equal affinity with all benzodiazepine-sensitive $GABA_A$ receptors (Benke et al., 1996; Costa and Guidotti, 1996).

Recently, a promising strategy was developed to assign particular pharmacological effects of benzodiazepines to a specific $GABA_A$ receptor subtype. This approach is based on a mutation-induced molecular switch by which the respective $GABA_A$ receptor is rendered benzodiazepine-insensitive, as originally shown on recombinant receptors. When a conserved histidine residue in the benzodiazepine binding site of the respective α subunit is replaced by an arginine residue [α1(H101R); α2(H101R); α3(H126R); α5(H105R)], the respective receptor is insensitive to diazepam but remains responsive to GABA (Wieland et al., 1992; Kleingoor et al., 1993; Benson et al., 1998). This molecular switch has recently been introduced into $GABA_A$ receptors in vivo. A mutant mouse line was generated with a knock-in point mutation [α1(H101R)] in which those benzodiazepine effects mediated via α1 $GABA_A$ receptors were expected to be blunted (Rudolph et al., 1999). The behavioral analysis of this mutant mouse line demonstrated that the sedative, amnesic, and part of the anticonvulsant effects of diazepam are mediated by α1 $GABA_A$ receptors. In contrast, the anxiolytic and myorelaxant effects of diazepam were unaltered in the α1 (H101R) mice compared with wild-type mice, suggesting that these effects are mediated by other $GABA_A$ receptor subtypes (Rudolph et al., 1999).

To assign the contribution of α2 and α3 $GABA_A$ receptors to the pharmacological spectrum of benzodiazepines, two further mouse lines were recently generated that contain the α2(H101R) and α3 (H126R) point mutations, respectively (Löw et al., 2000). The α2 $GABA_A$ receptor is mainly ex-

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ABBREVIATIONS: GABA, γ-aminobutyric acid.

pressed in the limbic system, whereas α3 $GABA_A$ receptors are prominent in neurons of the reticular activating system of the brainstem. A detailed biochemical, autoradiographical, and immunohistochemical analysis demonstrated that the distribution and cellular location of the point-mutated receptors correspond to those of wild-type mice. However, their affinity for diazepam was reduced by a factor of at least 1000. An initial pharmacological analysis showed that the anxiolytic-like effect of diazepam is specifically mediated via α2 $GABA_A$ receptors but not by α3 $GABA_A$ receptors (Löw et al., 2000).

In the present investigation, an attempt is made to attribute the myorelaxant action of diazepam to α2 or α3 $GABA_A$ receptors by comparing the diazepam-induced changes in muscle tone in the α2 and α3 mutant mouse strains compared with wild-type. The muscle tone was assessed in the horizontal wire test, in which the ability of the animals to grasp and hang on to a wire is measured. As a control that is independent of the benzodiazepine site, the myorelaxant activity of the $GABA_B$ agonist baclofen was tested. The myorelaxant activity was differentiated from the diazepam-induced sedation by including measurements of the spontaneous locomotor activity of the mutant and wild-type mice.

Materials and Methods

Animals. Wild-type, α2(H101R), and α3(H126R) (five to six backcrosses to the 129/SvJ background) were generated as described previously (Löw et al., 2000). Female mice were raised in group-housed cages (8 to 10 mice per cage) under reversed light-dark cycle conditions (light on from 8:00 pm to 8:00 am) in the test room. Food and water were provided ad libitum. At the time of testing, body weight was 18 to 22 g.

Behavioral Procedures. The horizontal wire test was used to assess the drug effect on muscle tone (Bonetti et al., 1982). The number of mice unable to grasp the wire with both front paws and at least one hind paw within three trials was noted 30 min after oral administration of vehicle, diazepam (3–30 mg/kg), or baclofen (3–30 mg/kg). Another measure of muscle tone was obtained in the inverted screen test (Gasior et al., 1999). Mice were brought on a 22- $\times$ 9.5-cm wire mesh screen (0.9 cm screen mesh) placed 48 cm above the ground. The screen was inverted slowly by 180 degrees. Wild-type and α2(H101R) mice were able to move to the upper side of the screen three times. Thirty minutes after treatment with diazepam (20 mg/kg), the screen with the mice on the upper side was inverted and the latency to fall off the screen was noted (120-s observation period).

Spontaneous locomotor activity was assessed for 1 h as the mean number of crossings in an automated two-chamber apparatus (Imetronic, Pessac, France) 30 min after oral administration of vehicle or diazepam (3–30 mg/kg) during the early dark phase of the day-night cycle.

Drugs. Diazepam (gift from F. Hoffmann-LaRoche, Basel, Switzerland) was suspended in a 0.3% Tween 80/saline solution. Baclofen (Sigma, Buchs, Switzerland) was dissolved in saline. The drugs were administered in a volume of 5 ml/kg by mouth.

Data Analysis. Continuous random variables were analyzed using two-way analysis of variance (ANOVA) followed by Dunnett's test, Newman-Keuls' test, or unpaired or paired t tests for post hoc mean comparisons when appropriate. χ^2 Analysis and Fisher's exact tests were used for dichotomous variables (Conover, 1999). In addition, ANOVAs were performed on dichotomous variables after angular transformation.

Results

Myorelaxant Action of Diazepam in α2(H101R) and α3(H126R) Mice. To assess the potential involvement of the α2 and α3 $GABA_A$ receptors in the muscle relaxant action of diazepam, α2(H101R) and α3(H126R) mice carrying diazepam-insensitive α2 and α3 receptors, respectively, were subjected to the horizontal wire test. Diazepam produced a dose-dependent impairment of the grasping reflex in wild-type mice ($\chi^2 = 47.11$; $P < 0.001$). The percentage of mice that were unable to grasp the horizontal wire was significantly increased in response to 10 and 30 mg/kg of diazepam compared with vehicle ($P < 0.001$, Fisher's exact test) (Fig. 1 A). In contrast, diazepam up to 10 mg/kg did not affect the grasping reflex in α2(H101R) mice carrying a diazepam-insensitive α2 $GABA_A$ receptor. Only at the highest dose (30 mg/kg) was the grasping reflex impaired in 33.3% of α2(H101R) mice ($P < 0.01$ versus vehicle) ($\chi^2 = 17.89$, $P < 0.001$) (Fig. 1A). ANOVA revealed a significant genotype X treatment interaction [$F(3,72) = 3.45$, $P < 0.05$]. An independent measure of the muscle relaxant activity of diazepam in wild-type and α2(H101R) mice was obtained using the inverted screen test. The administration of 20 mg/kg diazepam was associated with a decreased latency to fall off the grid in wild-type mice (73.75 ± 15.20 s, $n = 8$; $P < 0.05$, paired t test) but not in α2(H101R) mice (111.07 ± 6.72; $P < 0.05$ versus wild-type). Repeated-measures ANOVA on the same subjects revealed a significant genotype X treatment interaction [$F(1,20) = 6.70$, $P < 0.05$]. In contrast, the $GABA_B$ receptor agonist baclofen impaired the grasping reflex in the horizontal wire test dose-dependently to the same extent in wild-type ($\chi^2 = 19.29$, $P < 0.001$) and α2(H101R) mice ($\chi^2 = 20.14$, $P < 0.001$) (Fig. 1B), indicating that the α2(H101R) mice are responsive to other myorelaxants. Diazepam produced a similar impairment of grasping reflex in wild-type ($\chi^2 = 67.88$, $P < 0.001$) and α3(H126R) mice ($\chi^2 = 43.77$, $P < 0.001$) at doses up to 10 mg/kg. At the highest dose tested (30 mg/kg), a genotype difference was observed in that a significantly lower percentage of α3(H126R) mice (61.8%) showed impaired grasping reflex compared with wild-type mice (100%) ($P < 0.001$, Newman-Keuls' test). ANOVA revealed a significant genotype X treatment interaction [$F(3,237) = 3.23$, $P < 0.05$] (Fig. 1C).

Effect of Diazepam on Spontaneous Locomotor Activity in α2(H101R) and α3(H126R) Mice. To exclude the possibility that the differences in the myorelaxant action of diazepam observed in wild-type and mutant mice are caused by a differential sensitivity for the sedative action of the drug, the spontaneous locomotor activity was assessed in a familiar environment. Diazepam produced a dose-dependent decrease in locomotor activity similarly in wild-type and α2(H101R) mice [$F(3,71) = 16.94$, $P < 0.001$] (Fig. 2A). This depressant drug effect was significant from the dose of 3 mg/kg ($P < 0.01$ versus vehicle, Dunnett's post hoc mean comparisons) in the two genotypes. Similarly, diazepam at all doses tested depressed spontaneous locomotor activity in both wild-type and α3(H126R) mice [$F(3,64) = 14.39$, $P < 0.001$] (Fig. 2B).

Discussion

Apart from baclofen, classical benzodiazepines represent the main group of drugs that are widely used to reduce the

heightened muscle tone that accompanies various neurological diseases and injuries of the brain or spinal cord as well as states of anxiety. However, their clinical use as myorelaxants is limited by the lack of selectivity. The reduction in muscle stiffness is frequently associated with drowsiness and sedation. Until recently, it was not possible to dissociate, on the molecular level, the effects of diazepam on motor control systems from its various other actions. However, in a recent study using α1(H101R) mice , we were able to attribute the sedative effect of diazepam to the α1 $GABA_A$ receptors, whereas the reduction in muscle tone was associated with other $GABA_A$ receptor subtypes (Rudolph et al., 1999). To identify the molecular substrate of the myorelaxant property of diazepam, we have recently generated two novel lines of mice that contain the point mutations α2(H101R) and α3(H126R) (Löw et al., 2000). These two novel animal models are exquisite tools to investigate the specific role of the α2 or α3 $GABA_A$ receptor subtypes in mediating the myorelaxant effect of diazepam.

The reduction in muscle tone produced by diazepam was found to be almost exclusively mediated by α2 $GABA_A$ receptors, at least up to the dose of 10 mg/kg by mouth. This is demonstrated by the failure of diazepam to impair the grasping reflex in α2(H101R) mice (Fig. 1A) but not in α3(H126R) mice (Fig. 1C).

The molecular target for the muscle relaxant effect is clearly distinct from that mediating sedation, because the α2(H101R) mice showed a marked decrease in spontaneous locomotor activity in response to the respective dose of diazepam (10 mg/kg) (Fig. 2A). The failure of α2(H101R) mice to display diazepam-induced myorelaxation is not attributable to a principal inability to respond in the horizontal wire test because the α2(H101R) mice were responsive to the muscle

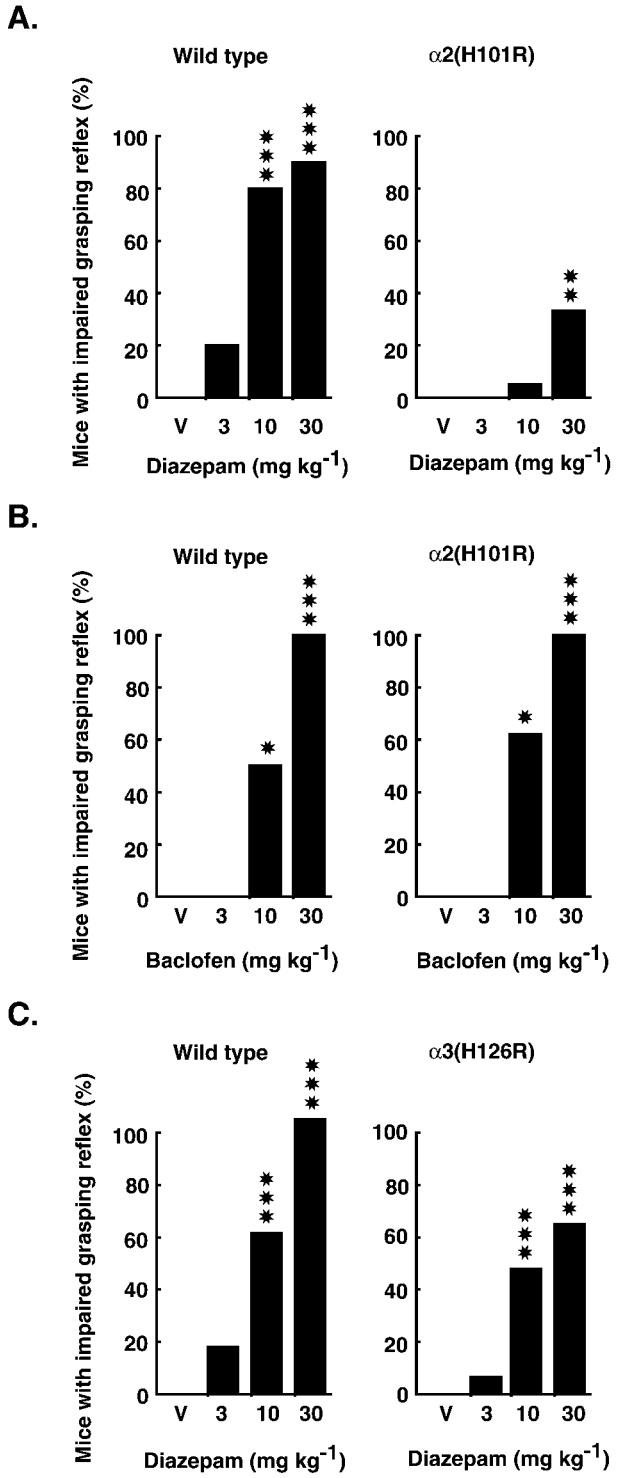

Fig. 1. Effects of diazepam (3–30 mg/kg by mouth) (A,C) and baclofen (3–30 mg/kg p.o.) (B) in the horizontal wire test in wild-type, α2(H101R) (A,B), or α3(H126R) mice (C). Results are expressed as percentage of mice with impaired grasping reflex. n = 6 to 21 mice per group. V, vehicle. ✱$P < 0.05$, ✱✱$P < 0.01$, and ✱✱✱$P < 0.001$, Fisher's exact tests.

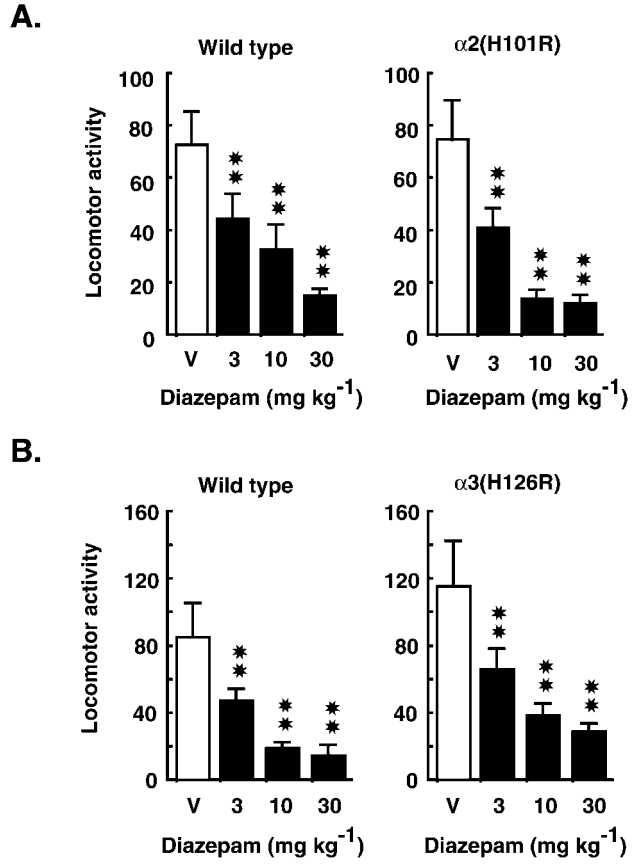

Fig. 2. Effects of diazepam (3–30 mg/kg by mouth) on spontaneous locomotor activity in wild-type, α2(H101R) mice (A), and α3(H126R) mice (B). Locomotor activity was recorded for 1 h. Results were expressed as mean number of crossings ± S.E.M. n = 8 to 10 mice per group. V, vehicle. ✱✱$P < 0.01$, Dunnett's post hoc tests.

relaxant baclofen, indicating that the polysynaptic spinal reflex transmission was not impaired in the α2(H101R) mice (Fig. 1B). The attribution of the myorelaxant effect of diazepam to α2 $GABA_A$ receptors is in line with the highly specific expression of the α2 $GABA_A$ receptor in the spinal cord, notably in the superficial layer of the dorsal horn and in motor neurons (Bohlhalter et al., 1996).

The anxiolytic effect of diazepam has recently been shown to be mediated via α2 $GABA_A$ receptors, which are located mainly in limbic areas. However, the two α2 $GABA_A$ receptor mediated effects are apparent at different dose ranges. Low doses (1–2 mg/kg) are sufficient for anxiolysis (Löw et al., 2000), whereas myorelaxation is evident in the horizontal wire test only at doses of ≥10 mg/kg (Fig. 1A).

The α3 $GABA_A$ receptors seem to contribute to the muscle relaxant effect at high doses of diazepam as shown by the significantly reduced percentage of α3(H126R) mice with impaired grasping reflex in response to 30 mg/kg of diazepam (Fig. 1C). This is consistent with the widespread expression of the α3 subunit in the spinal cord and notably its colocalization with the α2 subunit on primary afferent terminals, which are the targets of GABA-ergic presynaptic inhibition (Bohlhalter et al. 1996). Again, this partial loss of drug effect was restricted to the control of the muscle tone. The spontaneous locomotor activity of the α3(H126R) mice was similarly affected by diazepam as in wild-type mice (Fig. 2B). We did not use doses of diazepam higher than 30 mg/kg because they produce a significant degree of sedation, which would nonspecifically affect the performance in tests used to assess muscle tone.

It is noteworthy that in α2(H101R) mice, a residual myorelaxation was observed only at the highest dose of diazepam tested (30 mg/kg) (Fig. 1A). This effect may be attributable to α3 $GABA_A$ receptor-dependent impairment of the grasping reflex at high doses of diazepam (Fig. 1C).

The β-carboline abecarnil is an anxiolytic compound that is markedly less myorelaxant than diazepam, although both types of benzodiazepine actions are thought to be mediated via α2 $GABA_A$ receptors. This differential modulation might be explained by the partial agonistic activity of abecarnil at α2 $GABA_A$ receptors (Pribilla et al., 1993; Turner et al., 1993), which appears to be sufficient to induce anxiolytic but not muscle relaxant activity.

In summary, the present results demonstrate that the myorelaxant effect of diazepam is largely mediated via α2 $GABA_A$ receptors. The respective α2 $GABA_A$ receptors are presumably those expressed on motor neurons and in the superficial layer of the dorsal horn although supraspinal α2 $GABA_A$ receptors may also be involved. In response to high doses of diazepam, the α3 $GABA_A$ receptors may additionally contribute to the muscle relaxant action. These results are of relevance for the development of future selective myorelaxants acting at the benzodiazepine binding site.

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