British Journal of Pharmacology (1997) 122, 821–824

PNU-107484A with α isoform-dependent functional changes in αxβ2γ2 subtypes of rat recombinant $GABA_A$ receptors

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1 We discovered a novel γ-aminobutyric $acid_A$ ($GABA_A$) receptor ligand displaying seemingly opposite functionalities, depending on the α isoform of the αxβ2γ2 subtypes. PNU-107484A enhanced GABA-induced Cl^- currents in the α1β2γ2 subtype, but inhibited the currents in the α3β2γ2 and α6β2γ2 subtypes, and its half-maximal concentrations in the subtypes were 3.1 ± 0.5, 4.2 ± 1, and 3.5 ± 0.2 μM, respectively, without showing much dependency on α isoforms.

2 In the α1β2 subtype, the drug at concentrations up to 40 μM showed no effect on GABA-induced Cl^- currents, suggesting the requirement of the γ subunit for its action.

3 PNU-107484A behaved like a positive allosteric modulator of the α1β2γ2 subtype with its binding site distinct from those for benzodiazepines, barbiturates and neurosteroids. With the α3β2γ2 subtype, the drug behaved like a non-competitive inhibitor of GABA, thus blocking Cl^- currents by GABA alone or in the presence of pentobarbitone and neurosteroids.

4 It appears that PNU-107484A is a unique $GABA_A$ receptor ligand with α isoform-dependent functionalities, which may provide a basis for development of α isoform-selective ligands, and it could be useful as a probe to investigate the physiological roles of the various α isoform subtypes.

Keywords: $GABA_A$; GABA-induced Cl^- currents; PNU-107484A; α-isoform-selective ligand

Introduction

γ-Aminobutyric $acid_A$ ($GABA_A$) receptors are responsible for inhibitory neurotransmission in mammalian brains, and various allosteric modulators of the receptors are clinically useful as anxiolytics, hypnotics, anticonvulsants and anaesthetics (Haefly *et al*., 1985; Sieghart, 1992). The receptor/Cl^- channel complexes are composed of multiple subunits, each subunit consisting of several isoforms (Bateson *et al*., 1991; Wisden & Seeburg, 1992; Barnard *et al*., 1993; Harvey *et al*., 1993). Among the numerous possible subtypes, the αxβ2γ2 subtypes share many functional characteristics with native neuronal receptors, and interact with all the known ligands for $GABA_A$ receptors (Pritchett *et al*., 1989a,b; Draguhn *et al*., 1990; Verdoorn *et al*., 1990; Puia *et al*., 1991). This class of $GABA_A$ receptor subtypes also displays α isoform-dependent receptor pharmacology; the best known example is the benzodiazepine site with three types (Type I (α1β2γ2), Type II (α2, 3 or 5β2γ2), and the third type unique to cerebellar granule cells (α6β2γ2)) (Pritchett *et al*., 1989a,b; Pritchett & Seeburg, 1990; Lüddens *et al*., 1990). This and the differential localization of α isoforms in the brain (Wisden *et al*., 1992) have produced considerable interest in their physiological roles and their utility as therapeutic targets. Further progress depends on the development of ligands selective for individual α-isoform subtypes. Among known ligands, several selected benzodiazepine site ligands (e.g., zolpidem and Cl 218872), as noted above, show differential affinities for the α-isoform subtypes (Pritchett *et al*., 1989b; Pritchett & Seeburg, 1990), but other known allosteric modulators, such as barbiturates and neurosteroids, seem not to differentiate them at all. Here we describe PNU-107484A (9-methyl-2,4-di-1-pyrrolidinyl-9H-pyrido[3′,4′:4,5]pyrrolo[2,3-d]pyrimidine hydrochloride) (Figure 1) which functionally differentiates the α-isoform subtypes. The drug enhanced GABA currents in the α1β2γ2 subtype, but inhibited the currents in the α3β2γ2 and α6β2γ2 subtypes with little change in their affinity.

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Methods

Cloned $GABA_A$ receptors

The stable cell lines expressing the indicated combinations of α1, α3, α6, β2 and γ2 subunits of $GABA_A$ receptors were derived by transfection of plasmids containing cDNA and a plasmid encoding G418 resistance into human embryonic kidney cells (HEK 293 cell) (Hamilton *et al*., 1993). After two weeks of selection in 1 mg ml^{-1} G418, resistant cells were assayed for the ability to synthesize all three $GABA_A$ receptors mRNA by Northern blotting. Positive cells were used for electrophysiology to measure GABA-induced Cl^- currents.

Electrophysiology

The whole cell patch clamp technique (Hamill *et al*., 1981) was used to record the GABA-mediated Cl^- currents in HEK 293 cells expressing various combinations of $GABA_A$ receptor subunits as described earlier (Draguhn *et al*., 1990). Briefly, patch pipettes were prepared from borosilicate glass tubes and were fire-polished to a tip resistance of 0.5–2 MΩ when filled with a solution containing (in mM): CsCl 140, EGTA 11, $MgCl_2$ 4, ATP 2 and HEPES 10, pH 7.3. Cells were bathed in an external solution containing (in mM): NaCl 135, KCl 5 $MgCl_2$ 1, $CaCl_2$ 1.8 and HEPES 5; pH 7.2. GABA was dissolved in the external solution to a final concentration ranging from 0.5 to 5 μM and was applied with or without indicated drugs through a U-tube placed within 100 μm of the target cell. A submaximal concentration of GABA was chosen for individual receptor subtypes at which the actions of allosteric modulators are optimally measurable. The current was recorded with an Axopatch 1D amplifier and a CV-4 headstage (Axon Instrument Co.). A Bh-1 bath headstage was used to compensate for changes in bath potentials. GABA currents were measured at a holding potential of −60 mV at room temperature (21–24°C).

Results

PNU-107484A was examined for its effect on GABA-induced Cl^- currents in the HEK 293 cells expressing the α1β2γ2,

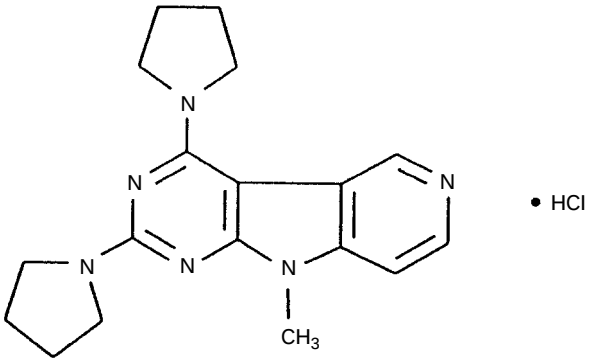

Figure 1 Chemical structure of PNU-107484A.

$\alpha3\beta2\gamma2$, $\alpha6\beta2\gamma2$, or $\alpha1\beta2$ subtypes of $GABA_A$ receptors, by use of the whole cell patch clamp technique (Figures 2 and 3). The current changes were analysed with a logistic equation $E = E_{max} \times [drug]^n/([drug]^n + [a\ half\ maximal\ drug\ concentration]^n)$. In the $\alpha1\beta2\gamma2$ subtype, the drug dose-dependently enhanced GABA (5 μM)-induced Cl^- currents (Figure 2) with a half-maximal concentration (EC_{50}) of 3.1 ± 0.5 μM, a net maximal current increase (E_{max}) of $127 \pm 54\%$ as normalized to the control GABA response, and a slope factor (n) of 1.3 ± 0.3. In the $\alpha3\beta2\gamma2$ subtype, the drug inhibited GABA (5 μM)-induced currents with a half-maximal inhibitory concentration (IC_{50}) of 4.2 ± 1 μM, a net maximal current decrease (I_{max}) of $100 \pm 9\%$ and a slope factor of 1. For inhibition, we modified the above equation to make the Y-axis equal to 100% – the degree of inhibition by the drug. In the $\alpha6\beta2\gamma2$ subtype, the

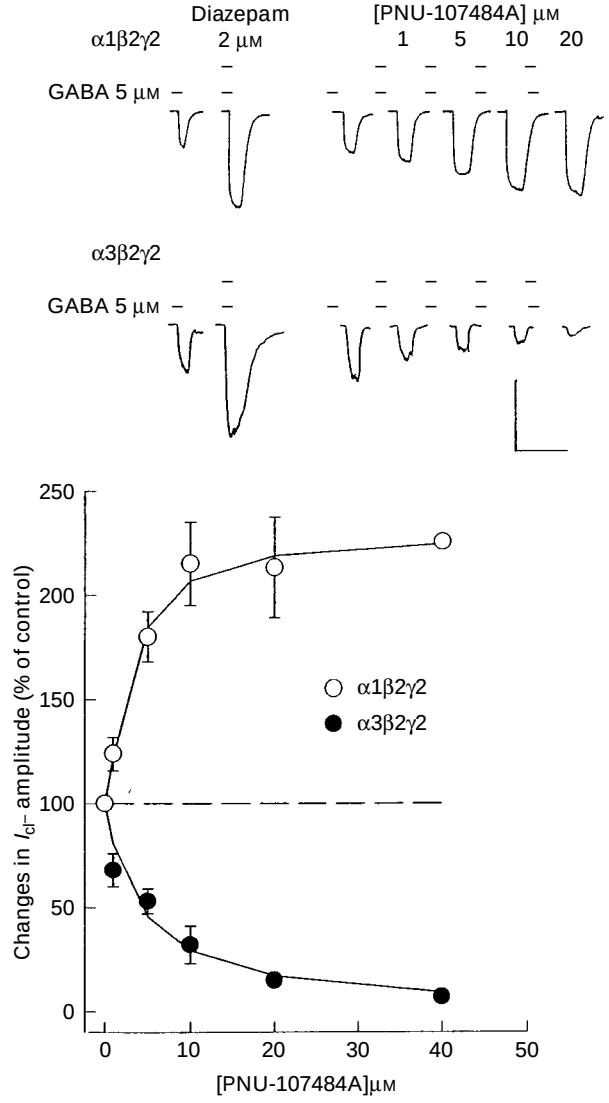

Figure 2 Current traces and plots comparing the effects of PNU-107484A on GABA-induced Cl^- currents in the $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ subtypes of $GABA_A$ receptors. GABA (5 μM)-induced Cl^- currents in HEK 293 cells expressing the $\alpha1\beta2\gamma2$ or $\alpha3\beta2\gamma2$ subtypes were measured in the presence of PNU-107484A at the indicated concentrations, by means of the whole cell patch clamp technique. The current amplitude was normalized to that observed with GABA (5 μM) alone. The data represent the mean from three or more measurements; vertical lines show s.e.mean. The solid lines represent the data fit to the logistic equation (see text) for the $\alpha1\beta2\gamma2$ subtype and for the $\alpha3\beta2\gamma2$ subtype. The vertical calibration, 500 pA; the horizontal bar, 30 s.

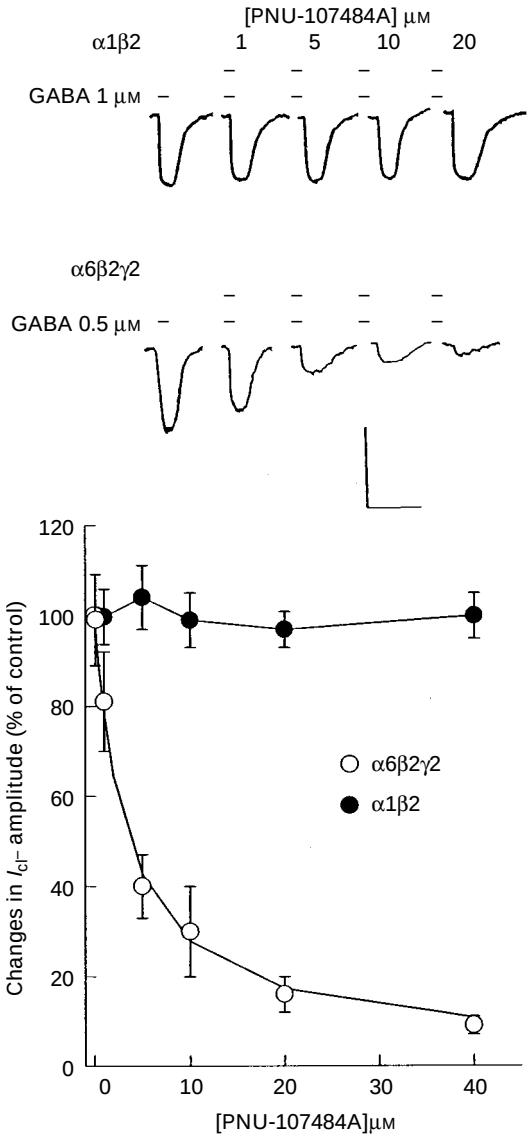

Figure 3 Effects of PNU-107484A on GABA-induced Cl^- currents observed with the $\alpha1\beta2$ and $\alpha6\beta2\gamma2$ subtypes of $GABA_A$ receptors. GABA-induced Cl^- currents in HEK 293 cells expressing the $\alpha1\beta2$ or $\alpha6\beta2\gamma2$ subtypes were measured in the presence of PNU-107484A at the indicated concentrations, by means of the whole cell patch clamp technique. In the case of the $\alpha6\beta2\gamma2$ subtype, the drug action did not level off within a drug application period of 10 s, and therefore, the cells were preincubated with the drug through bath application for at least 30 s. The GABA concentration was 1 μM for the $\alpha1\beta2$ subtype and 0.5 μM for the $\alpha6\beta2\gamma2$ subtype. The current amplitude was normalized to that observed with GABA alone. The solid lines represent the data fit to the logistic equation (see text) for the $\alpha6\beta2\gamma2$ subtype. The vertical calibration, 500 pA; the horizontal bar, 30 s.

drug also inhibited GABA (0.5 μM)-induced currents with an IC_{50} of 3.5 ± 0.2 μM, I_{max} of $100 \pm 7\%$, and a slope factor of 1 (Figure 3). In the $\alpha 1\beta 2$ subtype, the drug at concentrations up to 40 μM had no effect on GABA (1 μM)-induced currents. It should be pointed out that in the absence of GABA, PNU-107484A produced no appreciable effects in all the subtypes we examined here (data not shown). This shows that the drug is not a GABA mimetic, but rather an allosteric modulator.

To explore the identity of its binding site, functional competition experiments were carried out with PNU-107484A at saturating concentrations and several known allosteric modulators (Figure 4). In the $\alpha 1\beta 2\gamma 2$ subtype, we observed a GABA current enhancement of $111 \pm 9\%$ with PNU-107484A 15 μM (saturating concentration), $179 \pm 25\%$ with pentobarbitone 20 μM, and $210 \pm 32\%$ with 5α-THDOC 50 nM. With the co-application of the drug and pentobarbitone, the level increased to $287 \pm 46\%$, and with the drug plus 3α,21-dihydroxy-5α-pregnan-20-one (5α-THDOC), to $337 \pm 51\%$. This shows that the effect of PNU-107484A is additive with that of pentobarbitone or the neurosteroid. Also, the current enhancement by PNU-107484A was not blocked by ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a][1,4]benzodiazepine-3-carboxylate (Ro 15-1788) (10 μM) a typical antagonist of the benzodiazepine site (Figure 4). These results indicate that PNU-107484A shares no common binding site with barbiturates, neurosteroids or benzodiazepines, the three major allosteric modulators. In the $\alpha 3\beta 2\gamma 2$ subtype, PNU-107484A 20 μM reduced GABA-induced currents by 78%, whereas pentobarbitone (20 μM) and 5α-THDOC (50 nM) enhanced the currents by 112 and 188%, respectively. When the drug was co-applied with pentobarbitone or with 5α-THDOC it still reduced the currents by nearly 80%, showing the prevalence of its inhibitory action over the positive allosteric actions of pentobarbitone or 5α-THDOC. Also, Ro 15-1788 (10 μM) failed to reverse the inhibitory action of PNU-107484A.

We also examined how GABA at different concentrations affected the action of PNU-107484A (Figure 5). In the $\alpha 1\beta 2\gamma 2$

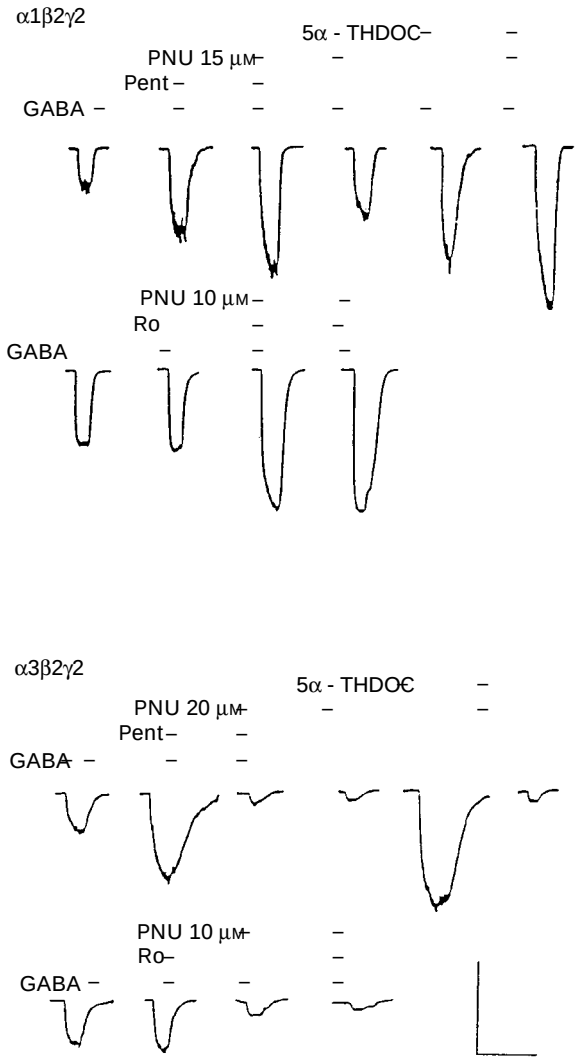

Figure 4 Representative current traces showing the effects of PNU-107484A (PNU), pentobarbitone (Pent), 5α-THDOC, Ro 15-1788 (Ro) or in combination as indicated on GABA currents in the $\alpha 1\beta 2\gamma 2$ and $\alpha 3\beta 2\gamma 2$ subtypes of $GABA_A$ receptors. GABA (5 μM)-induced Cl^- currents were measured in the HEK 293 cells expressing the $\alpha 1\beta 2\gamma 2$ or $\alpha 3\beta 2\gamma 2$ subtypes in the presence of Ro 15-1788 (10 μM), PNU-107484A (15 or 20 μM), pentobarbitone (20 μM), or 5α-THDOC (50 nM) and in combination as indicated. The vertical calibration, 500 pA; the horizontal bar, 30 s.

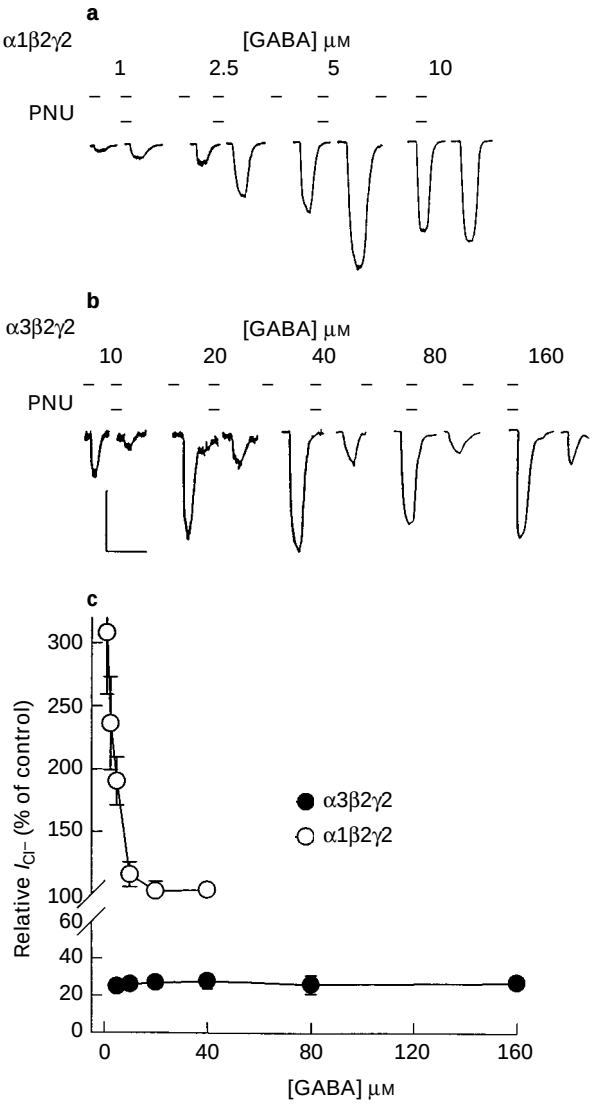

Figure 5 Representative current traces and plots showing how GABA at different concentrations affects the actions of PNU-107484A in the $\alpha 1\beta 2\gamma 2$ and $\alpha 3\beta 2\gamma 2$ subtypes. The concentration of GABA varied from 1 to 40 μM in the $\alpha 1\beta 2\gamma 2$ subtype with or without 10 μM PNU-107484A (PNU) and from 5 to 160 μM in the $\alpha 3\beta 2\gamma 2$ subtype. The currents were measured by means of the whole cell patch clamp technique. In (a): the vertical calibration represents 250 pA for the traces with GABA at 1, 2.5 and 5 μM, and 500 pA for the trace with GABA at 10 μM. In (b): 125 pA for the traces with GABA at 10 and 20 μM and then 250, 500, and 1000 pA for the traces with GABA at 40, 80 and 160 μM, respectively. The horizontal calibration bar represents 30 s. In (c), the amplitude of GABA currents observed in the presence of PNU-107484A were normalized to those observed with GABA alone.

subtype, the drug (10 μM) enhanced 1 μM GABA-induced currents to 308 $\pm$ 49% of control. As the GABA concentration was raised to 2.5, 5 and 10 μM, the drug-induced enhancement of the currents progressively decreased; thus, the relative current level reached 236 $\pm$ 37%, 190 $\pm$ 19%, and 116 $\pm$ 10%, respectively, of those observed with GABA alone. At a GABA concentration of 20 μM or higher, the drug produced no appreciable effects on the currents. This shows that its mechanism of action is similar to that for other allosteric modulators in that it potentiates the GABA response more effectively at low GABA concentrations than at high GABA concentrations. In the $\alpha 3\beta 2\gamma 2$ subtype, the drug (10 μM) decreased 5 μM GABA-induced currents to 25% of control, but its inhibition level remained unaltered as the GABA concentration was progressively raised to 10, 20, 40, 80 and 160 μM (Figure 5). At all the GABA concentrations, the current level decreased to 26–27% of control (GABA alone). This shows that PNU-107484A is a non-competitive (allosteric) inhibitor of the effects of GABA. This could also explain the dominance of PNU-107484A effects over those of the three allosteric modulators in the $\alpha 3\beta 2\gamma 2$ subtype, as noted above. It is possible that PNU-107484A directly affects the action of GABA at a site upstream from those affected by the other allosteric modulators.

Discussion

In this study, we have shown that PNU-107484A, a novel $GABA_A$ receptor ligand, displays α isoform-dependent functionalities in cloned $GABA_A$- $\alpha x\beta 2\gamma 2$ subtypes, the most widely distributed receptors in mammalian brain (Wisden *et al*., 1992). The drug enhanced GABA currents in the $\alpha 1\beta 2\gamma 2$ as a positive allosteric modulator, with its binding site distinct from that for barbiturates, neurosteroids or benzodiazepines. In the $\alpha 3\beta 2\gamma 2$ (or $\alpha 6\beta 2\gamma 2$) subtype, the drug inhibited the currents and this inhibitory action prevailed over high concentrations of GABA or an allosteric action of the positive modulators. This supports the finding that PNU-107484A is a negative allosteric (non-competitive) modulator of GABA sites. Interestingly, this functional variation was not accompanied by changes in its half-maximal concentrations (ranging from 3 to 4.2 μM) to the subtypes, which are related to its affinity. Also its full dose-response profiles at a concentration range from 0.1 to 80 μM showed no signs of biphasic responses. These observations, along with high sequence homology among α isoforms (over 70%), suggest that binding pockets for PNU-107484A are similar among the above $\alpha x\beta 2\gamma 2$ subtypes.

Earlier studies with the benzodiazepine site have shown that ligand functionality could be altered with mutation of a single amino acid or a region in the α isoforms, e.g., the R100H mutation of $\alpha 6$ conferring on the $\alpha 6\beta 2\gamma 2$ subtype the agonistic response to diazepam (Wieland *et al*., 1992), and replacement of P161 to L187 of $\alpha 6$ with the corresponding $\alpha 1$ segment converting Ro 15-1788 from an agonist to an antagonist (Im *et al*., 1996). These facile functional changes, though not as dramatic as the one observed with PNU-107484A, point out the possibility that few divergent residues among the α isoforms could be responsible for the functionality changes in PNU-107484A, with little change in its affinity. Future mutational studies with the $\alpha x\beta 2\gamma 2$ subtypes may reveal the residue(s) responsible for the functionality of PNU-107484A. This line of information would be useful for delineating subtype-selective functional pharmacophore(s) for modulation of Cl^- channels as well as for developing high affinity ligands with α isoform-dependent functionality. Discovery of this type of ligand will aid in understanding the role of individual α isoforms and their potential as therapeutic targets.

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(Received March 21, 1997
Revised July 8, 1997
Accepted July 23, 1997)