British Journal of Pharmacology (1999) 126, 1230–1236
http://www.stockton-press.co.uk/bjp

Actions of 3-[2′-phosphonomethyl[1,1′-biphenyl]-3-yl]alanine (PMBA) on cloned glycine receptors

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1 PMBA is a novel antagonist of strychnine-sensitive glycine receptors in the rat spinal cord, however, its mode of action is unknown. The actions of PMBA on rat glycine receptor α1 and α2 homomers in *Xenopus* oocytes were studied under two-electrode voltage-clamp.

2 Co-application of PMBA and glycine to both α1 and α2 homomers yielded inward currents which decayed to a steady-state. Responses rose slowly to the same steady-state amplitude following a 2 min pre-incubation in PMBA. Strychnine, but not picrotoxinin, showed similar antagonism to PMBA. The potency of PMBA was independent of membrane potential between −100 and 0 mV.

3 When tested against EC_{50} concentrations of glycine, PMBA was almost equally potent on α1 (IC_{50}, 406±41 nM: Hill coefficient, 1.5±0.2) and α2 (IC_{50}, 539±56 nM; Hill coefficient, 1.4±0.2) homomers.

4 PMBA (1–10 μM) and strychnine (200 nM) reduced the potency of glycine and the amplitude of the maximal agonist response of α1 and α2 homomers. In 10 μM PMBA, two distinct classes of glycine response were observed on α2, only a single class of responses were observed on α1.

5 There are similarities in PMBA and strychnine antagonism, although these compounds are structurally distinct. The possibility that PMBA interacts at two binding sites which differ in α1 and α2 subunits is discussed. PMBA may provide a lead structure for novel antagonists with which to investigate structural differences in glycine receptor at α1 and α2 subunits.

Keywords: Glycine receptor antagonist; PMBA; strychnine; picrotoxinin

Abbreviations: CTP, cyanotriphenylborate; GABA, γ-aminobutyric acid; nACh, nicotinic acetylcholine; NMDA, N-methyl-D-aspartate; PMBA, 3-[2′-phosphonomethyl[1,1′-biphenyl]-3-yl]alanine; PTXN, picrotoxinin; strych, strychnine

Introduction

Strychnine-sensitive glycine receptors mediate inhibitory neurotransmission in the central nervous system of vertebrates, particularly in the spinal cord (Wermann *et al*., 1967; Aprison, 1990). They are members of the cys-loop receptor super family (Karlin & Akabas, 1995) that includes nicotinic acetylcholine (nACh), γ-aminobutyric acid (GABA) and serotonin type-3 receptors, and are oligomeric proteins formed by the assembly of five subunits around a central transmitter-gated ion-channel (Langosch *et al*., 1988). Native glycine receptors are formed by the co-assembly of two subunit types, termed α and β (Grenningloh *et al*., 1987; 1990; Langosch *et al*., 1988). In the rat, three isoforms of the α subunit are known (α1–3) (Kuhse *et al*., 1995). Although the α subunit isoforms exhibit very high identity in their primary amino acid structure, their expression is closely regulated in the developing nervous system. Thus, α2 is predominant in the embryonic nervous system (Akagi *et al*., 1991a), but is replaced by α1 and α3 isoforms within 3 weeks of parturition (Becker *et al*., 1988; Akagi & Miledi, 1988; Akagi *et al*., 1991b; Kuhse *et al*., 1990a,b; Malosio *et al*., 1991). There is evidence that in the developing spinal cord, the widespread substitution of α1 subunits for α2 underlies changes in the kinetics of glycine receptor mediated post synaptic currents (Takahashi *et al*., 1992) and that the α3 isoform confers reduced agonist sensitivity relative to α1 and α2 (Kuhse *et al*., 1990a).

In the course of studies aimed at developing new antagonists for the glycine binding site of N-methyl-D-aspartate (NMDA) receptors, 3-[2′-phosphonomethyl[1,1′-biphenyl]-3-yl]alanine (PMBA) (Patent No. JP 4-275265) (Figure 1) was found to antagonize strychnine-sensitive glycine responses in the spinal cord of the new-born rat (Saitoh *et al*., 1994). PMBA is a derivative of phenylbenzene amino acids, containing both a glycine moiety and an ω-phosphono group and has a high specificity for glycine receptors; as it shows low potency as an antagonist of $GABA_A$ receptors and has no effect on NMDA receptors (Saitoh *et al*., 1994). In studies on the new-born rat spinal cord, PMBA was approximately 60 fold less potent than strychnine, however, its mode of action and potency on different isoforms of the glycine receptor has yet to be determined. In the present study, the actions of PMBA on α1 and α2 subunit homomers expressed in *Xenopus* oocytes are compared. PMBA is equally potent on both receptors at EC_{50} glycine and its mode of action bears similarities to that of strychnine. However PMBA has an unusual effect on the glycine dose-response relationship of α2 but not that of α1 homomers and may give insight into differences in the structures of these subunits.

Methods

The cloning of cDNA encoding α1 and α2 subunits and the synthesis of their respective cRNAs has been described previously (Akagi *et al*., 1991a,b). Stage V and VI oocytes were removed from the ovaries of mature *Xenopus laevis* and defolliculated manually after a 40 min incubation with

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collagenase type IA (2 mg ml^{-1}) in a low-calcium version of oocyte saline, standard saline composition (in mM): NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.6. Each oocyte was injected with 5 ng of cRNA in 25–50 nl and incubated at 17–18°C in saline supplemented with penicillin (100 u ml^{-1}), streptomycin (100 μg ml^{-1}), gentamycin (50 μg ml^{-1}) and 2.5 mM sodium pyruvate. Electrophysiological examination was performed 18–72 h after injection.

Oocytes were secured in a perspex chamber (volume approximately 200 μl) and continually perfused with oocyte saline at a rate of 5 ml min^{-1}. Oocytes were voltage-clamped at −70 mV using an Axoclamp 2A amplifier, and membrane currents monitored on an oscilloscope (Nihonkoden) and recorded with chart and DAT recorders (NF Electronic Instruments). All drugs were readily soluble in saline and were applied dissolved in the perfusate. The pH of drug stock solutions was tested before dilution, and adjusted to 7.6 where necessary. All measurements were made at room temperature (18–22°C).

Only oocytes which yielded stable responses to at least three applications of EC_{50} or EC_{100} glycine were used. Unless otherwise stated oocytes were preincubated in saline containing the given concentration of antagonist for 2 min before co-application of glycine and the antagonist. The process was repeated with increasing concentrations of agonist or antagonist, as appropriate. Antagonist dose-response relationships were determined at EC_{50} glycine.

The amplitudes of peak responses to glycine were measured and normalized to the maximal control response. Microcal Origin 4.10 (Microcal Software, MA, U.S.A.) was used to fit the appropriate logistic equation to the averaged, normalized data:

$$\text{Activation} \qquad 1 = \frac{I_{max}}{1 + (EC_{50}/[X])^{n}} \qquad (1)$$

$$\text{Inhibition} \qquad 1 = \frac{I_{max}}{1 + ([X]/IC_{50})^{n}} \qquad (2)$$

where I is the current induced by a given concentration of drug ([X]) expressed as a percentage of the maximal response (I_{max}), EC_{50} or IC_{50} is the concentration for half maximal agonism or antagonism respectively and n is the Hill coefficient. All data is presented as the means ± standard error of the mean (s.e.mean) of *n* observations.

The voltage-dependence of PMBA block was assayed at glycine concentrations that elicited currents displaying little or no desensitisation (i.e. EC_{50}). The holding potential was stepped briefly (1–3 s) from −70 mV to values ranging from −100 to 0 mV. The leakage current recorded in saline or PMBA alone was subtracted from that recorded in the presence of glycine or glycine and PMBA to yield the current due to receptor activation.

Sources of reagents

All reagents were obtained from Nacalai Tesque (Japan), except for collagenase type IA (Sigma, Japan) and PMBA, which was a generous gift from Nippon Chemiphar.

Results

Glycine induced dose-dependent inward currents in *Xenopus* oocytes injected with α1 or α2 cRNA and voltage clamped at −70 mV. The EC_{50} for α1 homomers was estimated to be 242 ± 16 μM ($n = 7$), and that of α2 homomers was 387 ± 11 μM ($n = 11$), with Hill coefficients respectively of 2.2 ± 0.2 and 3.3 ± 0.2, close to values reported in previous studies (Schmieden *et al*., 1989, 1992; Kuhse *et al*., 1990b).

PMBA (100 nM–10 μM) reduced the glycine response of both α1 and α2 homomers. When glycine and PMBA were co-applied the amplitude of the initial inward current was reduced relative to control, and this was followed by a rapid decay in current amplitude to a steady plateau (Figure 1a and b). This time-dependent decay is superficially similar to the use-dependent antagonism associated with open-channel blockers (e.g. Rundström *et al*., 1994) and could reflect PMBA binding to a site which is only accessible when the receptor is activated. However the decay in glycine-induced current was not observed when oocytes were preincubated in PMBA for 2 min prior to co-application of glycine and the antagonist. Rather, the response to glycine and PMBA developed slowly, but reached the same steady state amplitude as seen without pre-incubation (Figure 1a and b). PMBA antagonism was rapidly reversible, with a full agonist response being seen within 5 min of the removal of PMBA (data not shown). These data suggest that the slow rate at which PMBA block reaches equilibrium reflects a slow rate of association with the receptor and that binding of PMBA to the receptor is not dependent on receptor activation. Thus PMBA antagonism is not truly use-dependent.

PMBA antagonism was compared to that of two known glycine receptor antagonists, picrotoxinin (PTXN) and strychnine. PTXN antagonized both α1 and α2 homomers but showed little or no time-dependent decay (Figure 1c and d), as previously observed on α1 homomers expressed in human embryonic kidney cells (Lynch *et al*., 1995). Strychnine antagonism of both α1 and α2 homomers was however qualitatively very similar to that effected by PMBA. When strychnine and glcyine were co-applied, the amplitude of the initial response was reduced, and during prolonged application, decayed to a steady state (Figure 1e and f). As with PMBA, the decay was abolished by a 2 min pre-incubation in strychnine and the glycine-induced current developed slowly to the same steady-state amplitude seen without pre-incubation.

To compare the potency of PMBA on α1 and α2 homomers, the dose-dependency of PMBA antagonism was determined at approximately EC_{50} concentrations of glycine (250 μM glycine for α1, and 400 μM glycine for α2) following a 2 min pre-incubation in the antagonist. PMBA was almost equipotent as an antagonist of α1 and α2 homomers, with estimated IC_{50} values of 406 ± 41 nM ($n = 5$) and 539 ± 56 nM ($n = 4$) respectively (Figure 2a). Similarly, the slope coefficients for PMBA antagonism of α1 and α2 homomers were respectively 1.5 ± 0.2 ($n = 5$) and 1.4 ± 0.2 ($n = 4$), suggesting some cooperativity in PMBA action. Under the same conditions, strychnine has been found to be equipotent as an antagonist of α1 and α2 homomers with estimated IC_{50}s of between 9 and 50 nM (Schmieden *et al*., 1989; 1992; Langosch *et al*., 1994). In the present study, the potency of strychnine on α1 homomers was 18 ± 2 nM (slope coefficient = 1.1 ± 0.1, $n = 3$) (Figure 2a). In contrast to PMBA, PTXN showed subunit selectivity, being a more potent antagonist of α2 homomers (IC_{50} 141 ± 19 nM, $n = 3$) than homomers composed of α1 (IC_{50} 1.89 ± 0.16 μM, $n = 3$). The potency of PMBA on α1 and α2 was independent of membrane potential between −100 and 0 mV (Figure 2b). PMBA had no effect on the reversal potential of the currents mediated by either homomer (data not shown).

The effects of PMBA on the glycine dose-response relationship were assessed. Following a 2 min pre-incubation in 1 μM PMBA, the glycine dose-response curve of α1 homomers was shifted rightward (EC_{50} = 478 ± 30 μM), but

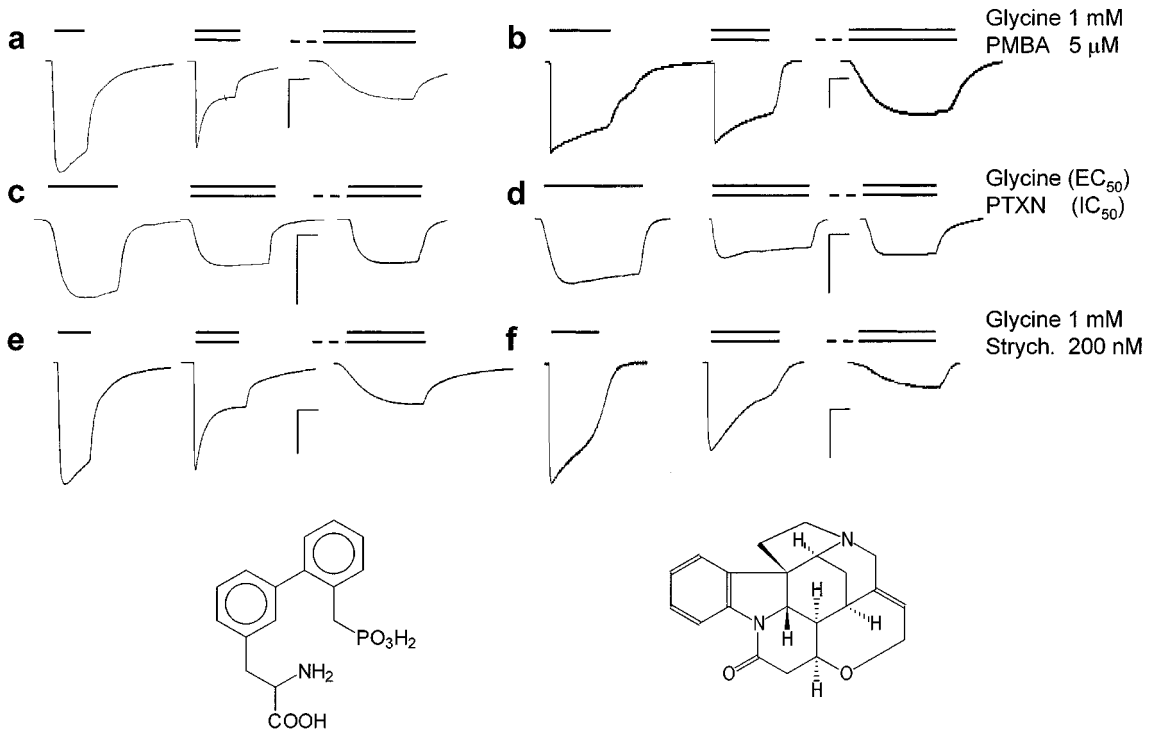

Figure 1 Actions of PMBA, PTXN and strychnine on α1 and α2 homomers expressed in *Xenopus* oocytes, voltage clamped at −70 mV. Co-application of PMBA (5 μM) and 1 mM glycine to α1 (a) and α2 (b) homomers yielded inward currents which rose rapidly before decaying to a steady-state. Responses rose slowly to the same steady-state amplitude following a 2 min pre-incubation in the antagonist (right hand traces in a and b). No time-dependent decay was observed with PTXN on the glycine responses of α1 (c; 1 μM PTXN, 250 μM glycine) and α2 (d; 100 nM PTXN, 400 μM glycine) but strychnine (strych.) had similar effects to PMBA on α1 (e) and α2 (f) homomers. This PMBA- and strychnine-induced decay was observed at all agonist concentrations tested; traces show antagonism of responses to EC_{100} glycine, those with PTXN show the responses to EC_{50} glycine. Traces are typical of responses from at least three oocytes. The horizontal and vertical scale bars represent 5 s and 500 nA respectively. The structures of 3-[2′-phosphonomethyl[1,1′-biphenyl]-3-yl]alanine (PMBA, left) and strychnine (right) are shown.

there was little change in the cooperativity of activation (antagonized Hill coefficient = 1.9 ± 0.2, $n = 5$). This rightward shift was concomitant with a slight decrease in the amplitude of the maximal agonist response (Figure 3a). Thus, in the presence of 1 μM PMBA, the mean amplitude of currents induced by 10 and 30 mM glycine were respectively 89 ± 3% ($n = 5$) and 91 ± 3% ($n = 3$) of the maximal control response (elicited by ⩾10 mM glycine). At higher concentrations of PMBA the rightward shift and decrease in the amplitude of the maximal response were more pronounced; in the presence of 10 μM PMBA the glycine EC_{50} for α1 homomers was 1883 ± 59 μM, while the current induced by 50 mM glcyine was 83 ± 2% of the maximum control response ($n = 3$) (Figure 3a). Again there was little change in the Hill coefficient (2.4 ± 0.1, $n = 3$).

There was a clear qualitative difference in the glycine response of α1 and α2 homomers following a 2 min pre-incubation in high concentrations of the antagonist. As on α1 homomers, 10 μM PMBA reduced both the potency of glycine and the amplitude of the maximum agonist response of the α2 homomers. However, two classes of antagonized response were observed on α2 homomers, which had distinctly different rates of onset (no attempt has been made to fit time constants to these changes as we do not consider our *Xenopus* oocyte preparation suitable for detailed kinetic analysis). Following pre-incubation in 10 μM PMBA, the responses of α2 homomers to relatively low concentrations of glycine (<5 mM) arose very slowly, taking approximately 20 s to reach a steady state whereas higher concentrations of glycine (10 mM and above) elicited more rapid responses which peaked within 5 s and showed desensitization (Figure 4a). Surprisingly, the peak responses of α2 homomers to 5 and 10 mM glycine had the same amplitude in the presence of 10 μM PMBA; thus 5 mM glycine elicited responses 51 ± 2% of the maximum ($n = 8$) while responses to 10 mM glycine were 54 ± 3% of the maximum ($n = 6$). Increasing the glycine concentration above 10 mM elicited larger responses; e.g. 50 mM glycine elicited currents 86 ± 2% of the maximal control response ($n = 6$). Thus a plateau appears in the middle of the antagonized glycine dose-response curve. However the concentration range spanned by this plateau was so small that it was not possible to accurately fit the data to a model for two binding sites (Figure 4b). Using equation (1), the estimated EC_{50} for the glycine dose-response relationship of α2 homomers in 10 μM PMBA was 5619 ± 1590 μM, Hill coefficient = 1.1 ± 0.4 ($n = 6$). A further difference in PMBA antagonism of α1 and α2 homomers was apparent following the application of 10 mM glycine and 10 μM PMBA. When PMBA (10 μM) and glycine (10 mM) were washed from the bath following the attainment of a steady-state blockade of α2 homomers, there was a transient increase in the whole-cell current before it returned to the basal level (arrow in Figure 4a). This apparent reactivation of the receptors was consistently observed on antagonized α2 homomers with 10 mM glycine and occasionally at higher agonist concentrations (i.e. only with the fast rising population of responses) but never with α1 homomers. A similar reactivation at the onset of wash has been observed with penicillin-G (Fujimoto *et al.*, 1995) and pentobarbitone (Wooltorton *et al.*, 1997) on $GABA_A$ receptors and may reflect the blocking drug vacating

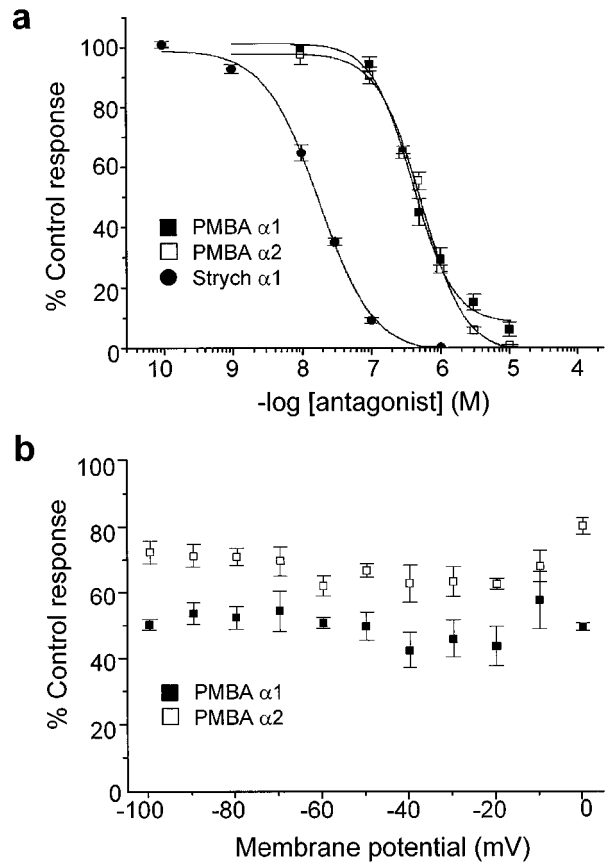

Figure 2 Relative potency and voltage dependence of PMBA on glycine receptor α1 and α2 homomers expressed in *Xenopus* oocytes. Currents induced by EC_{50} concentrations of glycine (250 μM for α1; 400 μM for α2) were blocked dose-dependently by PMBA. Oocytes were incubated in PMBA for 2 min prior to the co-application of PMBA and glycine. Data were normalized to the responses elicited by EC_{50} doses of glycine alone and each point represents the means ± s.e.mean of data from five oocytes for α1 and four oocytes for α2 homomers. PMBA was equipotent as antagonist of the response to EC_{50} glycine of α1 and α2 homomers, voltage clamped at −70 mV. The dose-inhibition curve for strychnine (strych., $n=3$) on α1 homomers is shown for comparison. (b) PMBA (400 nM) antagonism of the EC_{50} glycine response of α1 ($n=5$) and α2 ($n=5$) homomers was unaffected by membrane potential between −100 mV and 0 mV.

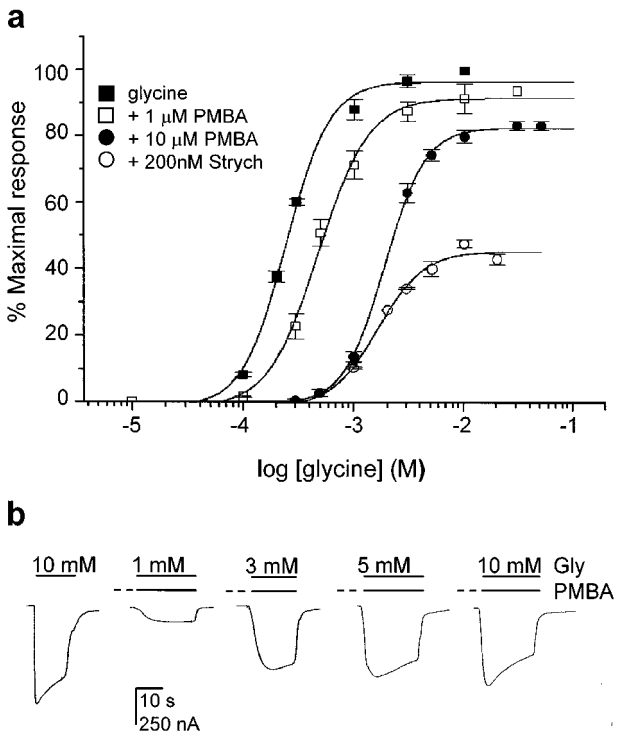

Figure 3 Glycine dose-response relationships of α1 homomers obtained in the absence and presence of PMBA and strychnine. Glycine was applied for 15–20 s at increasing concentrations, 5 min being allowed between each application to minimize the possible effects of desensitization. Oocytes were incubated in PMBA or strychnine for 2 min before co-application of glycine and the antagonist. Numerical data for each oocyte were normalized to the maximum response to glycine alone, and are shown as the means ± s.e.mean of n observations. (a) Glycine dose response relationship of α1 homomers. Relative to the control ($n=7$), higher concentrations of glycine were required to elicit a half maximal response, and the amplitude of the maximum response was reduced in the presence of 1 μM PMBA ($n=5$), 10 μM PMBA ($n=3$) and 200 nM strychnine ($n=3$). (b) Responses of α1 homomers to increasing concentrations of glycine in the absence and presence of 10 μM PMBA. The responses of the antagonized receptors developed slightly more slowly than in the control. The sample traces illustrate responses from one oocyte, and are typical of all five oocytes tested. Horizontal bars represent the duration of drug application.

the open channel, or the channel re-opening as the receptor leaves a desensitized state. As the currents induced by other concentrations of glycine in the absence or presence of PMBA terminated rapidly upon washing, we do not believe that this phenomenon results from poor solution exchange, however further experiments using more rapid solution exchanges on cells smaller than *Xenopus* oocytes would help clarify this.

At concentrations close to its IC_{50}, strychnine has previously been found to be a competitive antagonist of α1 and α2 homomers (Schmieden *et al.*, 1989). Given the similarities between PMBA and strychnine antagonism and that a reduction in the maximal agonist response is only seen at high concentrations of PMBA, the effects on the glycine dose-response curves of equally high concentrations of strychnine were determined. At concentrations 10 fold higher than the estimated IC_{50}, strychnine (200 nM) caused both a rightward shift and depression of the maximum in the glycine dose-response curves of both α1 and α2 homomers (EC_{50}s respectively; 1635 ± 197 μM, $n=3$; 2121 ± 236 μM, $n=3$). Strychnine elicited a near parallel shift in the α1 dose-response curve (Hill coefficient, 2.1 ± 0.4), but while there was no evidence for two populations of glycine response on strychnine-antagonized α2 homomers (Figure 4b), the Hill coefficient was markedly reduced relative to control (1.1 ± 0.2). Although 200 nM strychnine caused a similar reduction in the glycine EC_{50} of both α1 and α2 homomers, the reduction in the amplitude of the maximum response differed on these two receptors. Thus, the plateau of the α1 dose-response curve was 48 ± 3% ($n=3$) of the control, whereas the highest agonist concentration tested (50 mM) on α2 homomers elicited responses 74 ± 4% ($n=3$) of the maximum control response.

Discussion

PMBA is a novel antagonist of strychnine-sensitive glycine receptors, with a structure unlike that of other antagonists including strychnine, α and β-amino acids and piperidine carboxylic acids (Young & Snyder, 1974; Krogsgaard-Larsen *et al.*, 1982). PMBA is inactive at the glycine binding site of NMDA receptors (Saitoh *et al.*, 1994), in the same way that many antagonists of the glycine site of NMDA receptors have little or no affinity for strychnine-sensitive glycine receptors

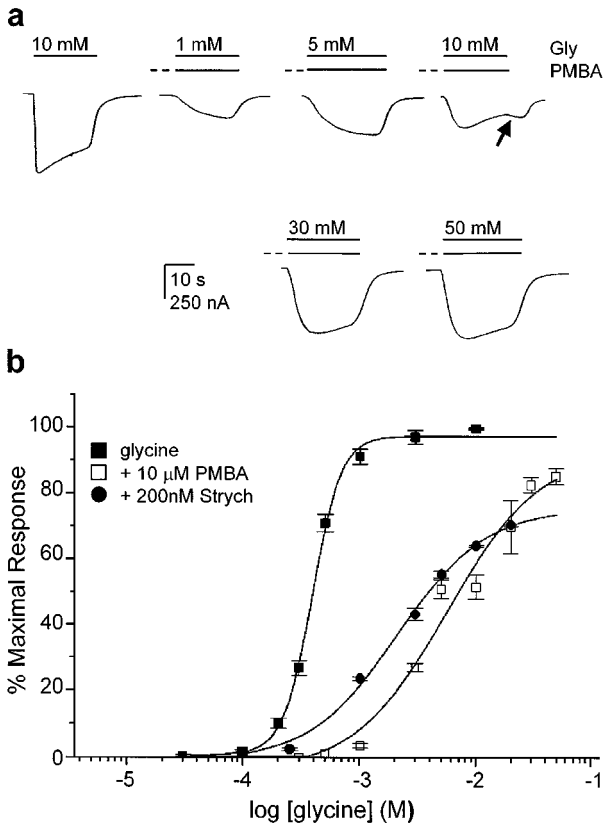

Figure 4 Effects of PMBA and strychnine on the glycine dose-response relationship of α2 homomers (a). Following 2 min pre-incubation in 10 μM PMBA, the response of α2 to low concentrations of glycine (≤5 mM) developed slowly. Higher concentrations of glycine (10–50 mM) elicited more rapid responses which showed desensitization. However, 5 and 10 mM glycine elicited responses of similar amplitudes. A transient increase in the holding current was consistently observed upon removal of 10 mM glycine and 10 μM PMBA (solid arrow) but only occasionally with higher concentrations of agonist. Traces are from one oocyte and are typical of recordings from eight oocytes. (b) These unusual responses of α2 homomers to glycine and PMBA lead to a flattening of the agonist dose-response curve in the presence of 10 μM PMBA ($n=5-8$) relative to control ($n=11$). Both the potency of glycine and the cooperativity of channel activation were reduced by 200 nM strychnine ($n=3$).

(Pullan & Powel, 1992). PMBA has a low potency on both vertebrate (Saitoh *et al*., 1994) and invertebrate GABA receptors (AMH, unpublished observations on recombinant *Drosophila* GABA receptors) and is therefore a relatively selective antagonist of strychnine-sensitive glycine receptors.

Although structurally distinct, PMBA and strychnine exhibited a number of similarities in their antagonism of recombinant glycine receptors. (1) Both antagonist show a slowly developing block when co-applied with glycine and this time-dependent phenomenon is abolished by pre-incubation. This suggests that the antagonists binding site(s) can be accessed when the channel is closed, albeit with a low rate of association; (2) PMBA and strychnine (Schmieden *et al*., 1992) show no subunit selectivity when tested at EC_{50} glycine distinguishing them for PTXN, which was approximately 10 fold more potent on α2 than on α1 homomers, and which does not show the time-dependent onset of blockade characteristic of PMBA and strychnine. PMBA and strychnine's lack of subunit-selectivity and PMBA's voltage-insensitivity also distinguishes them from cyanotriphenylborate (CTP), a non-competitive antagonist of recombinant glycine receptors (Rundström *et al*., 1994). CTP's actions are consistent with it binding to a site in the open channel and its subunit selectivity is dependent on the identity of a residue in the presumed channel lining; (3) At high concentrations, both PMBA and strychnine reduced the potency of glycine and the amplitude of the maximal agonist response of α1 and α2 homomers. This effect, which has elements of both competitive and non-competitive antagonism has been termed mixed antagonism (Constanti, 1978; Smart & Constanti, 1986). Previous electrophysiological studies of α1 and α2 homomers suggested that strychnine was a purely competitive antagonist (Schmieden *et al*., 1992). This contradiction with the present study most likely reflects the lower concentrations of strychnine used in previous studies (50 and 100 nM on α1 and α2 respectively) as the non-competitive aspects of strychnine and PMBA antagonism are only seen at concentrations well above their IC_{50}s; at concentrations equivalent to those of strychnine used by Schmieden *et al*. (1992), 2.5–5 times the IC_{50}, PMBA also appears to be a purely competitive antagonist. It is therefore possible that PMBA and strychnine bind to similar, and possibly overlapping, sites on glycine receptors, as suggested by previous radioligand binding studies (Saitoh *et al*., 1994).

Mixed antagonism could result from an antagonist binding to a single site and allosterically stabilizing closed conformations of the receptor (Smart & Constanti, 1986). However, mixed antagonism could also reflect the existence of two antagonist binding sites, one of which overlaps the agonist binding site (competitive antagonism) while binding to the second either blocks the open channel directly, or again stabilizes the receptor in a closed channel conformation. There is compelling evidence that glycine and strychnine compete for an overlapping site on α subunits, as residues which affect the binding and potency of strychnine are identical, or lie adjacent to those which alter the potency of glycine (Grenningloh *et al*., 1987; Schmieden *et al*., 1992; 1993; Vandenberg *et al*., 1992a,b; Rajendra *et al*., 1995). Indeed, theoretical studies have identified a glycine-like motif in strychnine (Aprison *et al*., 1995). PMBA bears a glycine moiety which is linked at its second (C_α) position to the biphenyl group, and as such it may be considered an α-amino acid. In a study of the actions of glycine analogues on rat neurones, Tokutomi *et al*. (1989) demonstrated that α-amino acids such as D-serine and L-cysteine which bear bulky functional groups on C_α (respectively, methanol and methanethiol groups) have little or no agonist efficacy at glycine receptors, whilst enantiomers of α-alanine, which have less bulky methyl groups, act as partial agonists. On α1 homomers, the agonist potency and efficacy of α-amino acids varies inversely with the steric bulkiness of the C_α functional group and α-amino acids with large C_α-atom substituents, such as D-serine, act as competitive antagonists of glycine (Schmieden & Betz, 1995). Thus PMBA, with its large C_α-atom substituent, could act as a competitive antagonist and occupy the agonist binding site without eliciting channel opening. However, at EC_{50} glycine, PMBA (IC_{50}, 406–539 nM) is approximately 10,000 fold more potent an antagonist than the α-amino acids assayed by Schmieden & Betz (1995) (IC_{50}s, 3–17 mM). This, the non-competitive element of PMBA's action and the cooperativity observed in the PMBA dose-inhibition relationship suggests that the antagonist does not act solely by competing with glycine for a common site, but may also block the channel either directly or allosterically.

Given the qualitative differences between antagonism by PMBA and the channel blocker CTP, and PMBA's low potency on GABA receptors (Saitoh *et al*., 1994, A.M.H., unpublished observations), which have very similar channel linings to those of glycine α homomers (Grenningloh *et al*.,

1987; Kuhse *et al*., 1990a; Akagi *et al*., 1991a), it seems unlikely that PMBA's non-competitive actions reflect open channel block. In a previous study, alterations to the structure and location of the ω-phosphono group of PMBA derivatives profoundly affected their potency as glcyine receptor antagonists (Saitoh *et al*., 1994). Thus, the positioning of the phosphonic acid group relative to the biphenyl rings of PMBA is an important determinant of the molecules antagonist potency. It is therefore possible that part of the phosphonomethyl-biphenyl moiety ligands a second binding site from which it cannot be displaced by glycine and which allosterically inhibits channel opening. Whether this is in fact the case awaits the results of further studies. There is however evidence that strychnine may interact with residues outside the agonist binding site, and that these interactions may contribute to strychnine antagonism. Following pre-treatment with the protein-modifying reagents diazonium tetrazole or acetic anhydride, glycine, α- and β-amino acids and piperidine compounds fail to displace radiolabelled strychnine from rat synaptic membranes whereas displacement by unlabelled strychnine and related compounds is unaffected (Young & Snyder, 1974; Marvizon *et al*., 1986). Additionally, strychnine is a competitive antagonist of taurine on α1 homomers, but non-competitive on α2 homomers (Schmieden *et al*., 1992). These data suggest that strychnine antagonism may not arise solely from direct competition with the agonist for a common binding site, but also from allosteric inhibition of channel opening. The same may therefore be true of PMBA. Indeed, bicuculline, which has long been considered a competitive antagonist of $GABA_A$ receptors, was recently found to act allosterically (Uchida *et al*., 1996; Ueno *et al*., 1996).

Where the residues which mediate these non-competitive actions lie remains to be determined, but they could conceivably form an extension of the agonist binding site. As there are differences in the structure of the agonist binding sites on α1 and α2 subunits (Schmieden *et al*., 1992), differences in the relative locations of such competitive and non-competitive subsites on the two homomers may explain the subunit specific actions of PMBA and strychnine. Two classes of agonist response, defined by their rate of rise, were seen with PMBA antagonism of α2 homomers leading to a small plateau in the α2 dose-response curve, whereas only a single population of responses were observed on α1. While there was no evidence for two classes of responses in the presence of strychnine, the cooperativity of α2 homomer activation was greatly reduced by the antagonist (from 3.3 to 1.1), which is consistent with non-competitive actions. Furthermore there were differences in the degree of insurmountable depression effected by strychnine on α1 and α2. The molecular basis of these differences remains to be determined, but studies of mutant receptors and PMBA derivatives (Saitoh *et al*., 1994) will hopefully elucidate which residues mediate the competitive and non-competitive actions of PMBA and strychnine. PMBA is chiral at the alanine moiety and as the sample of PMBA used in the present study is racemic, it would also be worth investigating the relative potencies of PMBA enantiomers and the ω-phosphono group derivatives on glycine α homomers.

In conclusion, the present study demonstrates that like strychnine, PMBA targets the α subunit of rat glycine receptors and effects a mixed antagonist of α subunit homomers. PMBA may therefore act partly by competitive antagonism of agonist binding. However, PMBA also has non-competitive actions which may reflect an allosteric effect on channel gating. Unusual effects of PMBA were observed on the glycine dose-response relationship of α2 homomers. These data raise the possibility that the PMBA molecule recognizes two binding sites which differ on α1 and α2 subunits. PMBA and its derivatives may aid the development of potential non-competitive glycine receptor antagonists based on the phosphonomethyl-biphenyl motif and further studies of the structure-function relationships of glycine receptor subunits.

The authors would like to thank Drs S. Kawashima and T. Ono for use of cRNA synthesis facilities and to the referees for comments on the manuscript. A.M.H. gratefully acknowledges the Japanese Society for the Promotion of Science for a travelling fellowship. This work was supported in part by a grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to H.S. and M.I.

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(Received July 6, 1998
Revised November 15, 1998
Accepted December 3, 1998)