British Journal of Pharmacology (2005) 145, 916–925
www.nature.com/bjp

Structural features of phenol derivatives determining potency for activation of chloride currents *via* α_1 homomeric and $\alpha_1\beta$ heteromeric glycine receptors

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1 Phenol derivatives constitute a family of neuroactive compounds. The aim of our study was to identify structural features that determine their modulatory effects at glycine receptors.

2 We investigated the effects of four methylated phenol derivatives and two halogenated analogues on chloride inward currents *via* rat α_1 and $\alpha_1\beta$ glycine receptors, heterologously expressed in HEK 293.

3 All compounds potentiated the effect of a submaximal glycine concentration in both α_1 homomeric and $\alpha_1\beta$ glycine receptors. While the degree of maximum potentiation of the glycine 10 μM effect in $\alpha_1\beta$ receptors was not different between the compounds, the halogenated compounds achieved half-maximum potentiating effects in the low μM range – at more than 20-fold lower concentrations compared with their nonhalogenated analogues ($P<0.0001$). The coactivating effect was over-ridden by inhibitory effects at concentrations $>300\,\mu$M in the halogenated compounds. Neither the number nor the position of the methyl groups significantly affected the EC_{50} for coactivation.

4 Only the bimethylated compounds 2,6 and 3,5 dimethylphenol (at concentrations $>1000\,\mu$M) directly activated both α_1 and $\alpha_1\beta$ receptors up to 30% of the maximum response evoked by 1000 μM glycine.

5 These results show that halogenation in the para position is a crucial structural feature for the potency of a phenolic compound to positively modulate glycine receptor function, while direct activation is only seen with high concentrations of compounds that carry at least two methyl groups. The presence of the β subunit is not required for both effects.
British Journal of Pharmacology (2005) **145,** 916–925. doi:10.1038/sj.bjp.0706254;
published online 23 May 2005

Keywords: Glycine receptors; inhibitory synaptic transmission; phenol derivatives

Abbreviations: α_1 glycine receptor, homomeric glycine receptor consisting only of α_1 subunits; $\alpha_1\beta$ glycine receptor, heteromeric glycine receptor consisting of α_1 and β subunits; EC_{50}, half-maximum effect concentration; $GABA_A$ receptor, γ-aminobutyric acid A receptor; HEK 293, human embryonic kidney cell, expression system; n_H, Hill coefficient

Introduction

While phenol derivatives constitute a family of potentially neuromodulatory drugs (James & Glen, 1980; Krasowski *et al*., 2001), the only compound in clinical use at the present time is the anaesthetic propofol (2,6 di-isopropylphenol). Several studies have addressed the minimal structural features that determine either the activating effects at γ-aminobutyric acid ($GABA_A$) receptors (Trapani *et al*., 1998; Krasowski *et al*., 2001; Mohammadi *et al*., 2001) that best mirror the *in vivo* anaesthetic effect of a phenolic compound (Krasowski *et al*., 2001), or the sodium channel blocking properties (Haeseler *et al*., 2001; 2002) as molecular basis for a potential local anaesthetic-like action. It is unknown at the present time whether the activating and coactivating effects at glycine receptors that have been described for propofol (Hales & Lambert, 1991; Pistis *et al*., 1997; Dong & Xu, 2002) are elicited by other phenol derivatives. Glycine receptors, like $GABA_A$ receptors, inhibit neuronal firing by opening chloride channels following agonist binding. Glycine receptors are mainly found in lower areas of the central nervous system and are involved in the control of motor rhythm generation, the coordination of spinal nociceptive reflex responses and the processing of sensory signals (Laube *et al*., 2002). Their role in modulating ascending nociceptive pathways and pain processing (Sherman *et al*., 1997a, b) makes them a potentially interesting target site for analgesic and spasmolytic agents.

$GABA_A$ and glycine receptors belong to the ligand-gated ion channel superfamily, which have a common structure in which five subunits form an ion channel (Jentsch *et al*., 2002). α and β subunits assemble into a pentameric receptor with a proposed *in vivo* stochiometry of $3\alpha:2\beta$ (Langosch *et al*., 1988). The glycine receptor α_1 subunit shares primary sequence homology with transmembrane segments of α, β and γ subunits of the

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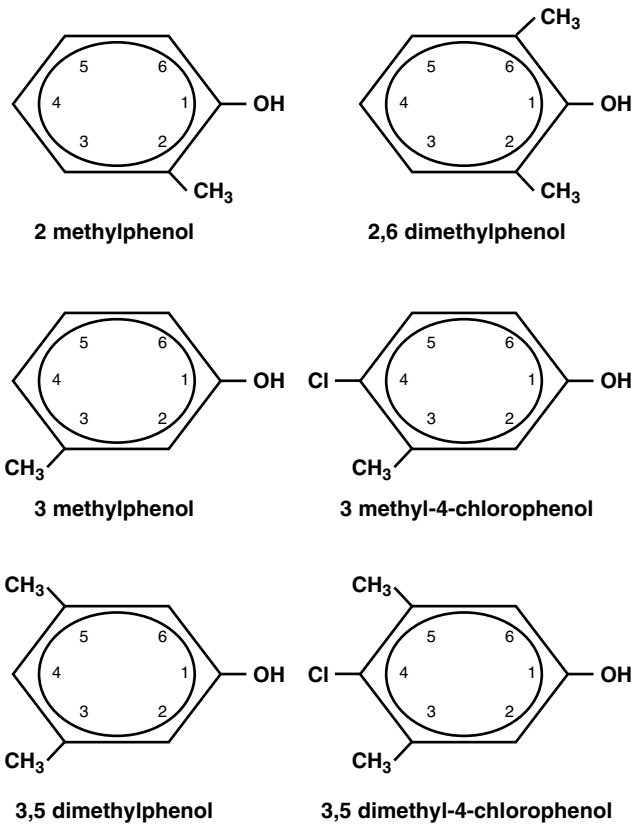

Figure 1 Structures of the phenol derivatives included in this study. From top to bottom: compounds with the methyl groups in ortho position to the phenolic hydroxyl group, the compound with a single methyl group in meso position and its halogenated analogue, and the compound with two methyl groups in meso position and its halogenated analogue.

$GABA_A$ receptor, which harbours amino-acid residues crucial for the binding of alcohols, volatile anaesthetics and propofol (Mihic *et al*., 1997; Carlson *et al*., 2000). Potentiating as well as directly activating effects of propofol have been seen in heterologously expressed α_1 and $\alpha_1\beta$ glycine receptors (Pistis *et al*., 1997). The focus of this *in vitro* investigation was to study simple propofol analogues with well-defined structural features (a halogen in the para position to the hydroxyl group and one or two methyl groups in the ortho or meso position, respectively) that have previously been shown to have a strong impact on the effects of phenol derivatives on other ion channels and receptors (see Figure 1) and to determine if these effects require coexpression of the β subunit.

Methods

Cell culture and transfection

Rat α_1 and $\alpha_1\beta$ glycine receptor subunits were transiently transfected into transformed human embryonic kidney cells (HEK 293). α_1 glycine receptor subunits efficiently form homomeric receptors in heterologous expression systems. β subunits do not form homomeric receptors but affect the function of heteromeric receptors, that is, decreasing the sensitivity to the agonistic effect of glycine and to the blocking effects of picrotoxin analogues (Shan *et al*., 2003). When cotransfecting the glycine receptor α and β subunits, their respective cDNAs were combined in a ratio of 1 : 10, since expression of the β polypeptide is less efficient than that of the α subunits (Pribilla *et al*., 1992). Reduced sensitivity to 1000 μM picrotoxin in $\alpha_1\beta$ heteromeric receptors was used as an assay of the efficacy of β subunit expression (Pribilla *et al*., 1992). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany), supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C in a 5% CO_2/air incubator. For transfection, cells were suspended in a buffer containing 50 mM K_2HPO_4 and 20 mM K-acetate, pH 7.35. For cotransfection of rat α_1 and β glycine receptor subunits, the corresponding cDNA, each subcloned in the pCIS2 expression vector (Invitrogen, San Diego, CA, U.S.A.) was added to the suspension. To visualize transfected cells, they were cotransfected with cDNA of green fluorescent protein (GFP, 10 μg ml^{-1}). For transfection, we used an electroporation device by EquiBio (Kent, U.K.). Transfected cells were replated on glasscoverslips and incubated for 15–24 h before recording.

All chemicals were from Sigma Chemicals (Deisenhofen, Germany), unless otherwise noted.

Solutions

The phenol derivatives under investigation were prepared as 1 M stock solution in ethanol, light-protected and stored in glass vessels at −20°C. Concentrations were calculated from the amount injected into the glass vials. Drug-containing vials were vigorously vortexed for 60 min. Glycine and picrotoxin were dissolved directly in bath solution.

Patch electrodes contained (mM) KCl 140, $MgCl_2$ 2, EGTA 11, HEPES 10, glucose 10; the bath solution contained (mM) NaCl 162, KCl 5.3, $NaHPO_4$ 0.6, KH_2PO_4 0.22, HEPES 15, glucose 5.6.

Experimental set-up

Standard whole-cell experiments (Hamill *et al*., 1981) were performed at –30 mV membrane potential. A tight electrical seal of several GΩ formed between the cell membrane and a patch-clamp electrode allows inward currents due to agonist-induced channel activation to resolve in the pA range. Electrical resistance of the pipettes was around 5 MΩ, corresponding to a total access resistance in the whole-cell configuration of about 10 MΩ. An ultrafast liquid filament switch technique (Franke *et al*., 1987) was used for the application of the agonist in pulses of 2 s duration. The agonist and/or the drug under investigation were applied to the cells *via* a smooth liquid filament achieved with a single outflow (glass tubing 0.15 mm inner diameter) connected to a piezo crystal. The cells were placed at the interface between this filament and the continuously flowing background solution. When a voltage pulse was applied to the piezo, the tube was moved up and down onto or away from the cell under investigation. Correct positioning of the cell with respect to the liquid filament was ensured by applying a saturating (1000 μM) glycine pulse before and after each test experiment. Care was taken to ensure that the amplitude and the shape of the glycine-activated current had stabilized before proceeding with

the experiment. Test solution and glycine (1000 μM) were applied *via* the same glass-polytetrafluoroethylen perfusion system, but from separate reservoirs. The contents of these reservoirs were mixed at a junction immediately before entering the superfusion chamber.

Drugs were applied either alone, in order to determine their direct agonistic effects, in combination with a subsaturating glycine concentration (10 μM), in order to determine their coactivating effects, or together with a saturating (1000 μM) concentration of glycine in order to detect open channel block. A new cell was used for each drug and each protocol, and at least three different experiments were performed for each setting. The amount of the diluent ethanol corresponding to the highest drug concentration used was 34,000 μM. We have previously shown that the ethanol itself has no effect at this concentration- neither on glycine receptor coactivation nor on direct activation (Ahrens *et al*., 2004). The lack of effect of 30,000 μM ethanol on glycine receptors has also been demonstrated elsewhere (Sebe *et al*., 2003).

Current recording and analysis

For data acquisition and further analysis, we used the Axopatch 200B amplifier in combination with pClamp6 software (Axon Instruments, Union City, CA, U.S.A.). Currents were filtered at 2 kHz. Fitting procedures were performed using a non-linear least-squares Marquardt–Levenberg algorithm. Details are provided in the appropriate figure legends or in the Results section.

The maximum current response induced by a compound acting directly as an agonist was expressed as percentage of the maximum response to 1000 μM glycine in the absence of drug immediately following the respective test experiment. The coactivating effect was expressed as percentage of the current elicited by 10 μM glycine according to $E\ (\%) = 100[(I-I_0)/I_0]$, where I_0 is the current response to 10 μM glycine. Activated or coactivated currents were normalized to their own maximum response. For the non-halogenated compounds, the dose–response curves did not always reach a plateau response, because phenol derivatives in concentrations larger than 3000 μM lead to a decline in seal resistance and thus did not yield reliable results. In these cases, the maximum response was the response at the highest concentration of the test compound for which a reliable response could be recorded. The dose–response curves were fitted according to $I_{\mathrm{norm}} = [1 + (\mathrm{EC}_{50}/[\mathrm{C}])^{n_{\mathrm{H}}}]^{-1}$, where I_{norm} is the current induced either directly by the respective concentration [C] of the agonist, or coactivated ($I-I_0$) by the agonist–glycine (10 μM) mixture, normalized to the maximum inward current or maximum coactivated current ($I_{\mathrm{max}}-I_0$), EC_{50} is the concentration required to evoke a response amounting to 50% of their own maximal response and n_{H} is the Hill coefficient.

Statistics

Only results obtained with $\alpha_1\beta$ receptors were enrolled in the statistical tests. As a consequence of the higher glycine sensitivity in α_1 homomeric receptors, a maximum coactivating response (with respect to the effect of 1000 μM glycine) might occasionally be observed at low drug concentrations leading to an underestimation of the EC_{50} values derived from Hill fits in α_1 homomeric receptors. Statistical analysis was performed in order to reveal differences in the maximum effect, on the one hand, and in the concentrations required to achieve half-maximum effect (EC_{50}), on the other hand, between halogenated and nonhalogenated analogues, between compounds with one vs two methyl groups, or between compounds with ortho or meso position of the methyl groups with respect to the phenolic hydroxyl group. Curve fitting and parameter estimation of the Hill curves were performed using the program 'PROC NLMIXED' of SAS Release 8.02. In this model, the 'experiment' is treated as the subject variable and the parameter values (EC_{50} and n_{H}) are treated as normally distributed random factors. The mean differences of these parameters between two substances were entered into the common model as fixed shift parameters $\Delta\mathrm{EC}_{50}$ and Δn_{H}, activated for all data of the second data set. The corresponding (asymptotic) t-value was used to test the null hypothesis of no parameter difference against the two-sided alternative. The null hypothesis was rejected at $P<0.05$. All data are depicted as means $\pm$ s.d.

A two-sample t-test was applied to analyse significance of differences in the maximum potentiating effect between halogenated and nonhalogenated, mono- and bimethylated or ortho- vs meso-methylated structural analogues.

Results

A total of 94 cells were included in the study. Expression of rat α_1 homomeric and $\alpha_1\beta$ mRNA in HEK 293 cells generated glycine receptors that showed glycine-activated inward current with amplitudes of -1.0 ± 0.5 nA in α_1 and 1.3 ± 0.9 nA in $\alpha_1\beta$ receptors following saturating (1000 μM) concentrations of the natural agonist. Successful coexpression of the β subunit was verified with picrotoxin 1000 μM coapplied with 1000 μM glycine after each experiment. In this experimental setting, picrotoxin 1000 μM blocked α_1 homomeric receptors by $55\pm0.05\%$ while $\alpha_1\beta$ receptors were hardly affected by picrotoxin ($19\pm0.05\%$ block). When α and β cDNAs were used at a 1 : 10 ratio for cotransfection, successful coexpression of the β subunit verified with picrotoxin was 100%. The current transient showed a fast increase, followed by a monophasic decay. The time constant of desensitization was 958 ± 250 ms in α_1 homomeric and 1026 ± 212 ms in $\alpha_1\beta$ receptors. The respective steady-state current that did not desensitize in the presence of 1000 μM glycine was at 86 ± 6 and $84\pm8\%$ of the peak current amplitude.

When applied without glycine, only 2,6 dimethylphenol and 3,5 dimethylphenol directly activated receptor-mediated inward currents in a concentration-dependent manner. Currents reached $30\pm12\%$ (α_1, $n=3$) and $38\pm5\%$ ($\alpha_1\beta$, $n=3$), and $32\pm6\%$ (α_1, $n=3$) and $33\pm10\%$ ($\alpha_1\beta$, $n=3$) of the maximum glycine (1000 μM) response in the presence of high concentrations (3000 μM) of either 3,5 dimethylphenol or 2,6 dimethylphenol, respectively. The estimates for half-maximum concentrations (EC_{50}) were 1468 ± 208 and 1466 ± 83 μM for 3,5 dimethylphenol and 1410 ± 101 and 1549 ± 164 μM for 2,6 dimethylphenol in α_1 and $\alpha_1\beta$ receptors, respectively.

As illustrated by the tracings in Figure 2, currents induced by both compounds did not desensitize during the 2 s application.

Dose–response curves for glycine at α_1 and $\alpha_1\beta$ receptors are shown in Figure 3. The EC_{50} for glycine was 12.8 ± 2.3 μM at α_1

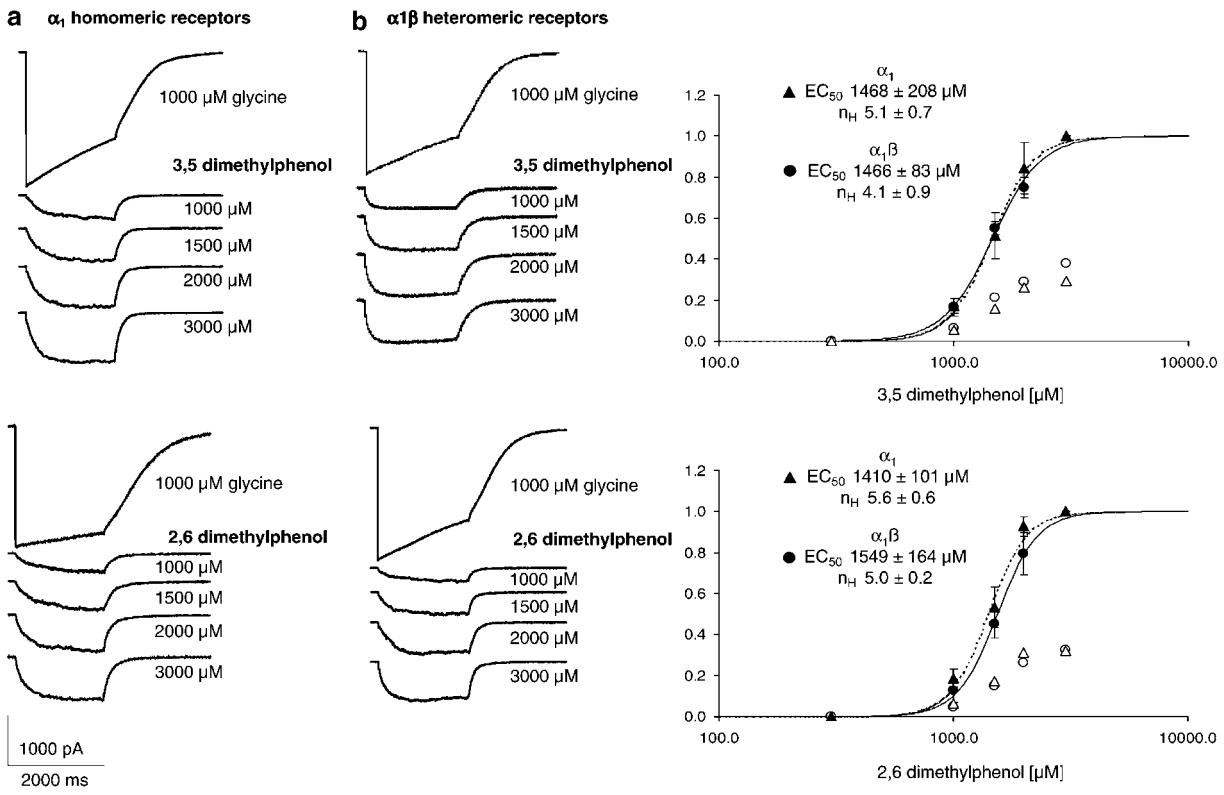

Figure 2 (a) Representative current traces elicited by 2 s application of 3,5 dimethylphenol or 2,6 dimethylphenol with respect to the current elicited by 1000 μM glycine in the same experiment (upper trace). Tracings were obtained from one HEK 293 cell each expressing either α_1 homomeric or $\alpha_1\beta$ glycine receptors. (b) Normalized Cl^- currents activated in the absence of glycine *via* α_1 homomeric (triangles) or $\alpha_1\beta$ (circles) glycine receptors (mean $\pm$ s.d.; $n=3$ each), plotted against the concentration of 3,5 dimethylphenol (upper diagram) or 2,6 dimethylphenol (lower diagram) on a logarithmic scale. Currents were normalized either to maximum value achieved by high concentrations (3000 μM) of the compound (filled symbols) or to maximum value achieved by 1000 μM glycine (empty symbols). Solid lines are Hill fits to the data with the indicated parameters. The concentration–response plots were almost superimposable for α_1 homomeric and $\alpha_1\beta$ glycine receptors, and no difference between the ortho- and meso-methylated compound could be detected.

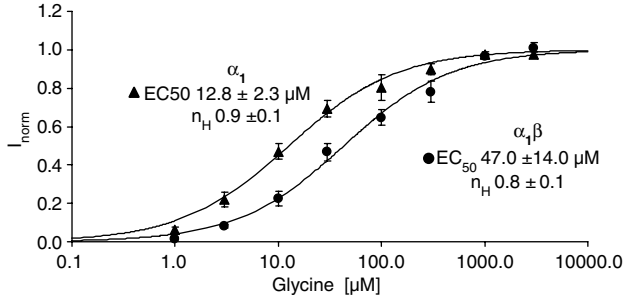

Figure 3 Normalized Cl^- currents activated by glycine *via* α_1 homomeric (triangles) or $\alpha_1\beta$ (circles) glycine receptors (mean $\pm$ s.d.; $n=3$ each), plotted against the concentration of glycine. Solid lines are Hill fits to the data with the indicated parameters.

and 47.0 $\pm$ 14.0 μM at $\alpha_1\beta$ receptors. Glycine 10 μM evoked a current response of 21 $\pm$ 7% ($n=34$) in $\alpha_1\beta$ and 46 $\pm$ 5% ($n=24$) of the response to 1000 μM glycine in α_1 receptors; this difference in glycine sensitivity was significant ($P<0.001$). Less than 10% of the current response to 10 μM glycine desensitized as long as glycine was present.

All phenol derivatives investigated potentiated the current response to glycine 10 μM in both α_1 and $\alpha_1\beta$ receptors, Figures 4 and 5 show representative current traces obtained with $\alpha_1\beta$ receptors, Figure 6 shows current traces obtained with α_1 homomeric receptors.

No significant differences between the compounds were detected with respect to the degree of maximum potentiation. Only the potentiating effect seen with 3 methylphenol was higher than with 2 methylphenol ($P=0.04$), which, however, might be a consequence of the lower response to glycine 10 μM in the experiments with 3 methylphenol with respect to the experiments with 2 methylphenol.

The halogenated compounds 3,5 dimethyl-4-chlorophenol and 3 methyl-4-chlorophenol achieved half-maximum potentiating effects at more than 20-fold lower concentrations compared with their nonhalogenated analogues; this difference was statistically significant ($P<0.0001$). The estimates for the EC_{50} values for the compounds with the methyl groups in the meso position (3 methylphenol and 3,5 dimethylphenol) in $\alpha_1\beta$ receptors were not significantly different from the EC_{50} values for their ortho-methylated structural analogues (2 methylphenol and 2,6 dimethylphenol). The bimethylated compounds were not significantly more potent than their structural analogues with only one methyl group in $\alpha_1\beta$ receptors. The concentration dependence of current potentiation in $\alpha_1\beta$ receptors derived from five to six experiments for each compound is depicted in Figure 7.

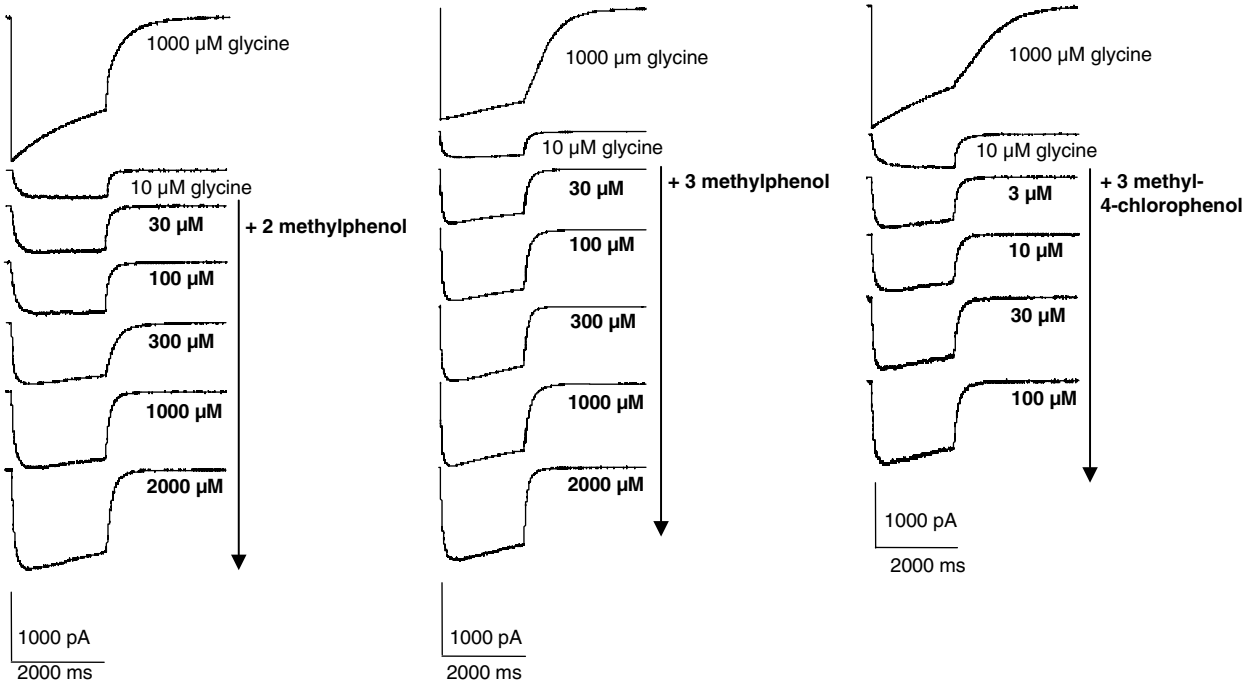

Figure 4 Representative current traces elicited by 2 s coapplication of 10 μM glycine and (from left to right) 2 methylphenol, 3 methylphenol and 3 methyl-4-chlorophenol with respect to the current elicited by 1000 μM glycine in the respective control experiment (upper trace) in $\alpha_1\beta$ heteromeric receptors. All compounds increased the amplitude of the response evoked by 10 μM glycine. In the halogenated compound (right row of traces), this effect was observed in the low μM concentration range.

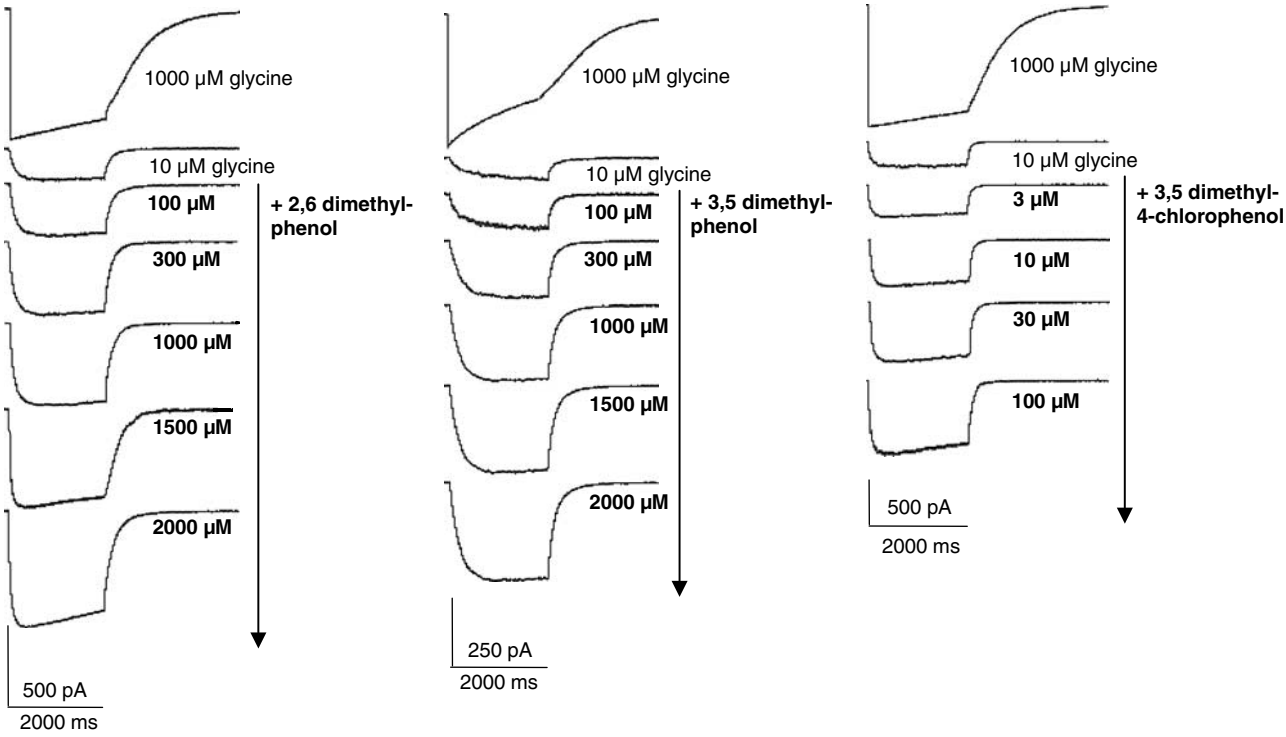

Figure 5 Representative current traces elicited by 2 s coapplication of 10 μM glycine and (from left to right) 2,6 dimethylphenol, 3,5 dimethylphenol and 3,5 dimethyl-4-chlorophenol with respect to the current elicited by 1000 μM glycine in the respective control experiment (upper trace) in $\alpha_1\beta$ heteromeric receptors. The halogenated compound (right row of traces) showed coactivating effects in the low μM concentration range.

The EC_{50} values and Hill coefficients ($\pm$s.d.) derived from fits of the Hill equation to the normalized response in α_1 and $\alpha_1\beta$ receptors are depicted in Table 1. As a consequence of the higher glycine sensitivity in α_1 homomeric receptors, a maximum coactivating response (with respect to the effect of 1000 μM glycine) might occasionally be observed at low drug

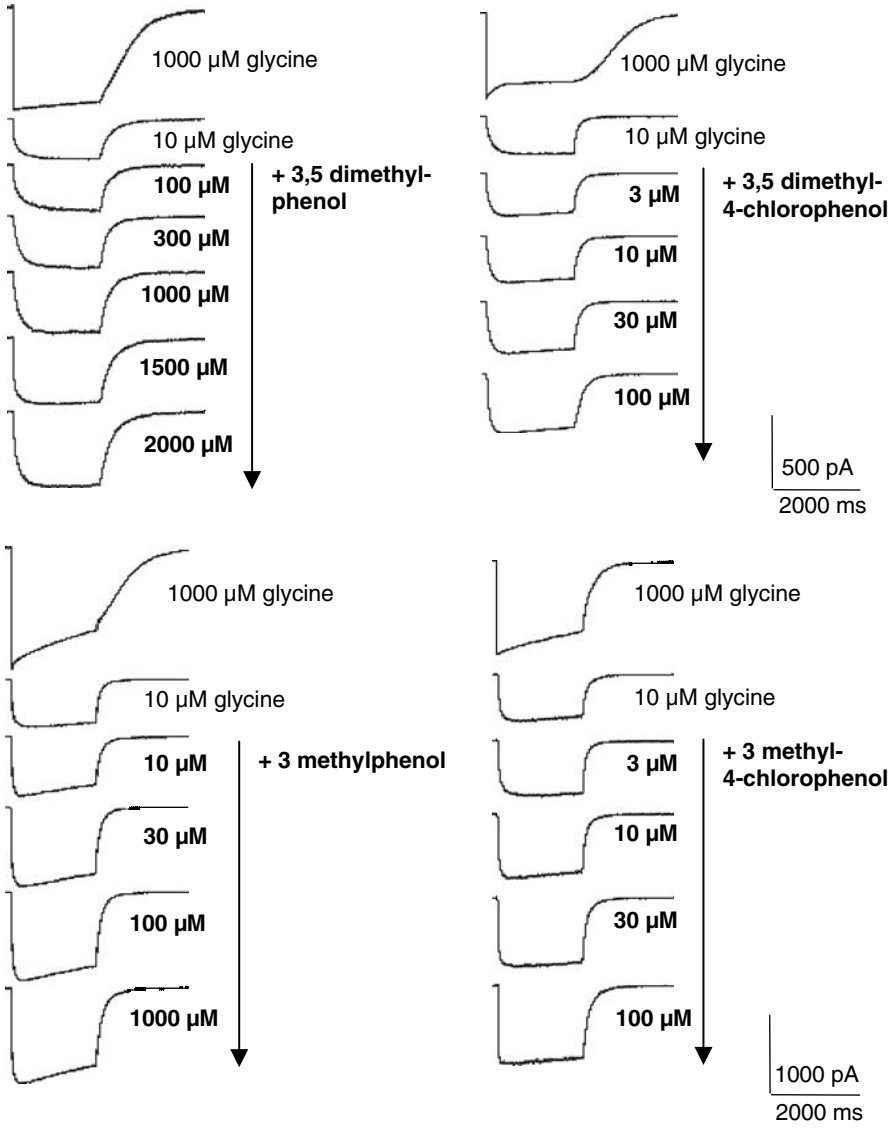

Figure 6 Representative current traces elicited *via* α_1 homomeric receptors by 2 s coapplication of 10 μM glycine with either 3,5 dimethylphenol (upper row of traces), 3 methylphenol (lower row of traces) or their respective halogenated analogue (right row of traces) with respect to the current elicited by 1000 μM glycine (upper trace). The effect elicited by 10 μM glycine is higher in α_1 homomeric receptors than in $\alpha_1\beta$ heteromeric receptors (compare with tracings in Figures 3 and 4). Coactivating effects of phenol derivatives in α_1 homomeric receptors are seen in a similar concentration range compared to $\alpha_1\beta$ heteromeric receptors.

concentrations, leading to an underestimation of the EC_{50} values derived from Hill fits in α_1 homomeric receptors. Thus, the parameters given for the α_1 homomeric receptors should not be used for potency determinations. However, as revealed by the current traces in Figures 4–6 and by the values given in Table 1, all phenol derivatives coactivate currents *via* α_1 homomeric receptors in a similar concentration range compared to $\alpha_1\beta$ receptors; thus, the expression of the β-subunit is not required for the coactivating effects.

The halogenated compounds 3,5 dimethyl-4-chlorophenol and 3 methyl-4-chlorophenol in concentrations larger than 300 and 600 μM, respectively, produced a reduction in the peak current amplitude when coapplied with 1000 μM glycine along with a large response rebound when coapplication was terminated. The current decay was accelerated during coapplication of the respective compound and glycine (1000 μM). A total of three experiments were performed for each compound to substantiate this effect. Figure 8 shows representative current traces.

Discussion

Our study shows that substituted phenol derivatives that carry a chloride in the para position to the phenolic hydroxyl group coactivate glycine receptors at low concentrations and thus may offer a potential for therapy of spasticity, muscle relaxation and pain relief. At much higher concentrations, only the bimethylated and nonhalogenated compounds directly activated the glycine receptor in the absence of the natural agonist. These results show that direct activation and coactivation of glycine receptors by phenol derivatives require

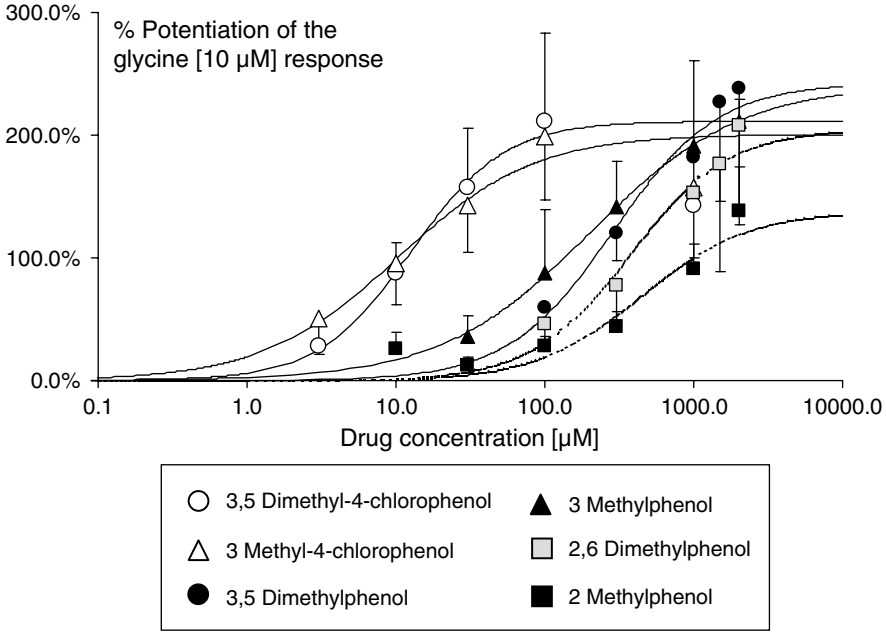

Figure 7 Potentiation (%) of the current elicited by 10 μM glycine (mean $\pm$ s.d. of 5–6 independent experiments) by each compound in $\alpha_1\beta$ heteromeric receptors, plotted against the concentration applied on a logarithmic scale. Solid lines are Hill fits to the data with the parameters indicated in Table 1. The concentrations required for a half-maximum coactivating response were significantly smaller in the halogenated compounds compared with their nonhalogenated structural analogues ($P<0.0001$). No significant differences between the compounds were detected with respect to the degree of maximum potentiation. Only the potentiating effect seen with 3 methylphenol was higher than with 2 methylphenol ($P=0.04$), which, however, might be a consequence of the lower response to glycine 10 μM in the experiments with 3 methylphenol with respect to the experiments with 2 methylphenol.

Table 1 EC_{50} values and Hill coefficients ($\pm$s.d.) derived from fits of the Hill equation to the normalized coactivating response (with respect to the effect of the highest concentration tested) in α_1 and $\alpha_1\beta$ receptors

	α_1 *homomer*		$\alpha_1\beta$ *heteromer*	
	EC_{50} (µM)	n_H	EC_{50} (µM)	n_H
3 methyl-4-chlorophenol	8±5	1.1±0.4	**4±1***	1.2±0.3
3 methylphenol	59±19	0.9±0.3	222±45	1.0±0.1
3,5 dimethyl-4-chlorophenol	13±4	1.4±2.9	**11±2***	1.3±0.1
3,5 dimethylphenol	254±139	1.7±0.9	308±46	1.3±0.2
2 methylphenol	70±29	0.7±0.1	448±89	1.2±0.2
2,6 dimethylphenol	226±104	1.5±0.4	373±51	1.2±0.1

The halogenated compounds 3 methyl-4-chlorophenol and 3,5 dimethyl-4-chlorophenol were significantly more potent than their nonhalogenated structural analogues (bold and *, $P<0.0001$). No significant differences were detected between compounds with one vs two methyl groups or between ortho- vs meso-methylated compounds in $\alpha_1\beta$ receptors.

distinct structural features. The presence of the β subunit is required neither for positive modulation nor for direct activation of glycine receptors by phenol derivatives.

$GABA_A$ and glycine receptors are the main receptors for inhibitory neurotransmission in the mammalian central nervous system (Laube *et al*., 2002). $GABA_A$ is the most important neurotransmitter in the brain, and glycine plays a major role in the spinal cord and lower brain stem. While $GABA_A$ receptors have been identified as a common target site for structurally diverse sedative-anaesthetic and anxiolytic drugs (Belelli *et al*., 1996; Banks & Pearce, 1999), clinically applicable compounds that specifically target glycine receptors have yet to be identified. Glycine receptors have been suggested as potential candidates for therapeutics that mediate antinociceptive and muscle relaxant effects (Laube *et al*., 2002).

All phenol derivatives investigated in this study were capable of positively modulating glycine receptor function to a certain extent. Apparently, one important structural feature that determines the potency of a phenol derivative to coactivate glycine receptors is halogenation in the para position to the phenolic hydroxyl group. Insertion of a second symmetrical methyl group did not further increase the potency of the single-methylated compound, and the position of the methyl group with respect to the phenolic hydroxyl group had no influence on the coactivating potency. At higher concentrations (>300 μM), the coactivating effect of the halogenated compounds was over-ridden by inhibitory effects revealed by a reduction in the peak current amplitude during coapplication with 1000 μM glycine. The large response rebound when coapplication was stopped simultaneously is consistent with the assumption of open

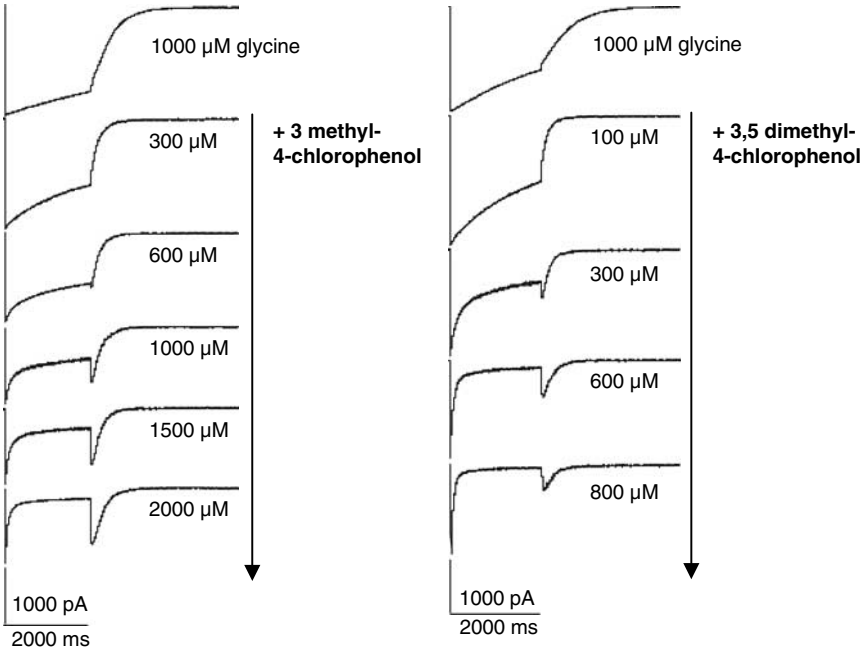

Figure 8 Inhibitory effects induced by 3 methyl-4-chlorophenol (left row of traces) and 3,5 dimethyl-4-chlorophenol (right row of traces) at concentrations $\geqslant$600 and 300 μM, respectively, as revealed by a reduction in the peak current amplitude during coapplication with glycine 1000 μM, a concentration-dependent acceleration of the current decay during application followed by channel reopening at the end of the application.

channel block as the underlying mechanism- a phenomenon that has previously been described for the modulation of glycine receptors by high concentrations of propofol (Dong & Xu, 2002; Ahrens *et al*., 2004) as well as for the modulation of $GABA_A$ receptors by inhalational agents (Banks & Pearce, 1999; Hapfelmeier *et al*., 2001). However, further studies should target voltage dependence of these effects in order to substantiate the hypothesis of open channel block. Alternatively, the reduction in peak current amplitude along with the acceleration of the current decay during coapplication with 1000 μM glycine might be explained by an allosteric mechanism of inhibition with high concentrations of halogenated phenol derivatives stabilizing the desensitized conformation of the receptor, analogous to a mechanism of block assumed for picrotoxin on ligand-gated chloride channels (Qian *et al*., 2005). None of the halogenated compounds directly activated the receptor in the absence of the natural agonist.

The structural features that determine the potency of a phenol derivative to activate or coactivate Cl^- inward currents *via* glycine receptors show similarities as well as differences with respect to the requirements that have previously been reported for activation of GABA-ergic receptors (Trapani *et al*., 1998; Krasowski *et al*., 2001; Mohammadi *et al*., 2001) or sodium channel blocking effects (Haeseler *et al*., 2001).

Qualitatively, the structure–activity relationship for coactivation of glycine receptors by phenol derivatives shows parallels with the structure–activity relationship to block voltage-operated sodium channels. In both cases, potency is strongly increased by the chloride in para position to the phenolic hydroxyl (Haeseler *et al*., 2001). Quantitatively, the half-maximum concentrations for glycine receptor coactivation in this study were about 10-fold (3,5 dimethyl-4-chlorophenol) and 100-fold (3 methyl-4-chlorophenol) lower than the concentrations required for half-maximum blockade of sodium channels by these compounds (Haeseler *et al*., 1999; Haeseler *et al*., 2001). While insertion of a chloride in para position led to a parallel increase in the potency to coactivate glycine receptors and to block of voltage-operated sodium channels, this is not the case for the insertion of a second methyl group. In contrast to the effect at glycine receptors, the potency for sodium channel blockade was increased when a second methyl group was attached in the meso position (Haeseler *et al*., 2001). As for the halogenated compound with one single methyl group in the meso position (3 methyl-4-chlorophenol), there is only little overlap in the concentration range where glycine receptor coactivation was observed in this study and the concentration range where sodium channel blockade was reported. For comparison, half-maximum effect at glycine receptors was achieved with 4 μM 3 methyl-4-chlorophenol, whereas half-maximum block of sodium channels in the resting state required 400 μM 3 methyl-4-chlorophenol. In the case of the nonhalogenated phenol derivatives with two methyl groups, coactivation of glycine receptors was detected in the same concentration range as sodium channel blockade. In this study, a half-maximum coactivating effect was observed with 370 μM 2,6 dimethylphenol compared to 187 μM for half-maximum blockade of voltage-operated neuronal sodium channels at a membrane potential close to the physiological resting potential (Haeseler *et al*., 2002). At much higher concentrations ($>$1000 μM), the bimethylated compounds directly activated the glycine receptor in the absence of the natural agonist. If these results can be generalized, halogenated phenol derivatives should show glycine receptor coactivation at low concentrations, with

increasing concentrations leading to blockade of voltage-operated sodium channels and open channel block of glycine receptors. Nonhalogenated bimethylated phenol derivatives should both coactivate glycine receptors and block sodium channels at intermediate concentrations and should directly activate glycine receptors at high concentrations. Halogenated phenol derivatives with one single methyl group would be expected to be sodium channel blockers, while having facilitating effects at glycine receptors at concentrations where sodium channel blockade would still be small.

In contrast to the glycinergic effects seen in this study, GABA-ergic effects were hardly affected by substitution in the para position (Trapani *et al*., 1998; Krasowski *et al*., 2001). GABA-ergic activity of phenolic compounds has been linked to the size and shape of alkyl groups in positions 2 and 6 of the aromatic ring relative to the phenolic hydroxyl group (Krasowski *et al*., 2001); however, the effect of 3,5 di-alkyl substitution has never been tested systematically. Direct activation of $GABA_A$ receptors was seen in the single-methylated compound only when the methyl group was in the ortho position (Mohammadi *et al*., 2001). Our study shows that, as far as direct activation of glycine receptors is concerned, at least two methyl groups are required for a detectable effect that is independent from their position with respect to the phenolic hydroxyl group.

In conclusion, our study suggests that it may be possible to find phenol derivatives that target preferably glycine receptors rather than voltage-operated sodium channels or $GABA_A$ receptors and thus might show a desirable pattern of anti-nociceptive, muscle relaxant and local anaesthetic/anticonvulsant effects.

We are indebted to Professor Betz, Frankfurt, for providing us with cloned subunits. We thank U. Jensen, Departement of Neurology, Hannover, for transfection and cell culture, J. Kilian and A. Niesel, Department of Neurology, Hannover, for technical support, and Dr B. Mohammadi, Department of Neurology, Hannover, for helpful discussions. This study was supported by a grant of the Deutsche Forschungsgemeinschaft (Bu 938/8-1) to Johannes Bufler.

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(Received November 25, 2004
Revised January 24, 2005
Accepted April 6, 2005
Published online 23 May 2005)