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Characterization of Strychnine-sensitive Glycine Receptor in the Intact Frog Retina: Modulation by Protein Kinases

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We studied ^{3}H-glycine and ^{3}H-strychnine specific binding to glycine receptor (GlyR) in intact isolated frog retinas. To avoid glycine binding to glycine uptake sites, experiments were performed at low ligand concentrations in a sodium-free medium. The binding of both radiolabeled ligands was saturated. Scatchard analysis of bound glycine and strychnine revealed a K_D of 2.5 and 2.0 μM, respectively. Specific binding of glycine was displaced by β-alanine, sarcosine, and strychnine. Strychnine binding was displaced 50% by glycine, and sarcosine. Properties of the strychnine-binding site in the GlyR were modified by sarcosine. Binding of both radioligands was considerably reduced by compounds that inhibit or activate adenylate cyclase and increased cAMP levels. A phorbol ester activator of PKC remarkably decreased glycine and strychnine binding. These results suggest modulation of GlyR in response to endogenous activation of protein kinases A and C, as well as protein phosphorylation modulating GlyR function in retina.

KEY WORDS: Glycine receptor; binding; PKA; PKC; retina.

INTRODUCTION

The postsynaptic glycine receptor (GlyR) is a ligand-gated chloride channel composed of ligand-binding α-subunits and structural β-subunits, which form pentameric complexes. Molecular cloning has revealed four genes which encode the α-subunits (α1, α2, α3, α4) and only one gene encoding the β-subunit (1,2).

The strychnine-sensitive GlyR-channel complex has been reported to possess intracellular sites that can be phosphorylated (3), leading to the possible modulation of GlyR-mediated actions by intracellular protein kinases. Indeed, functional studies of the phosphorylation of GlyR show that protein kinase A (PKA) regulates the glycine-elicited Cl^- currents in cultured spinal trigeminal neurons (4); this kinase decreases the GlyR mediated responses in substantia nigra (5) and ventromedial hypothalamic neurons (6). Similarly, protein kinase C (PKC) inhibits the function of GlyR in the oocytes injected with brain mRNA (7) and also potentiates the glycine-induced currents in cultured spinal trigeminal neurons (8).

Several studies support the role of glycine as an inhibitory neurotransmitter in the vertebrate retina (9). The distribution of GlyR subunits has been confirmed by immunohistochemical studies in mammalian (10–12) and non-mammalian retina (13,14).

We report herein that GlyR in the intact isolated frog retina is modulated in response to the presence

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of activators of PKA and PKC. These results suggest that protein phosphorylation would modulate GlyR function in retina.

MATERIALS AND METHODS

Experimental Animals. Adult frogs (*Rana pipiens*) from a local commercial supply were housed under standard laboratory conditions (25 ± 1°C, 12 h light/dark cycle) and fed once a weak with Tenebrium worms. Dark-adapted frogs were pitted and decapitated, and the eyes were removed. The retina was isolated in Krebs medium containing in mM; choline chloride, 118; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.19; $KHCO_3$, 25; glucose, 5.6, pH 7.4. All experiments were carried out at room temperature (25°C) under dim red light.

Materials. Forskolin, isobutyl methyl xantine (IBMX), staurosporine and phorbol 12-myristate 13-acetate (PMA) were solubilized in DMSO and then diluted in medium. DMSO final concentration was lower than 0.05% and the same as in the respective controls.

Binding Assay. Retinas were incubated at 25 °C for 10 min in Krebs medium containing ^{3}H-glycine (40 Ci/mmol) or ^{3}H-strychnine (21 Ci/mmol. New England Nuclear, Boston, M.A.). After incubation tissues were washed with cold medium, weighed and dissolved in 0.5 ml of 1% (w/v) sodium dodecyl sulfate. Radioactivity in the solubilized tissue was determined by liquid scintillation counting. Specific binding was defined as the binding displaced by 1 mM or 100 μM (unlabeled glycine or strychnine, respectively. The kinetic analysis of data was performed by computational methods using Inplot 4 (version 4.03, Graph Pad Software). Statistical analysis was carried out using Student's *t*-test.

Cyclic nucleotides and protein determination. Retinas were incubated under the same conditions as those for the binding assays. After incubation, retinas were homogenized in 0.5 ml of ice-cold 0.4 M perchloric acid. Protein was removed by centrifugation, the pellet solubilized with NaOH and the protein content determined by a microplate reader spectrophotometer, using a commercial assay (Bio-Rad DC).

Perchloric supernatants were neutralized with $KOH–K_2CO_3$; adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) content was determined by radio immunoassay, using a commercial kit from Amersham (Biosciences, UK).

RESULTS

^{3}H-Glycine Binding

In order to characterize glycine sites at GlyR in the intact retina, it was necessary to separate them from the glycine transport system as well as from the binding at the *N*-methyl-D-aspartate receptor (NMDAR). It is well known that the high affinity glycine transport system is a sodium-dependent, temperature sensitive mechanism (15,16). Therefore, we studied binding of ^{3}H-glycine in sodium-free medium. In order to determine equilibrium time, retinas were incubated in the presence of 10 nM of radioligand. The binding reaction was stopped at incubation times from 5 to 40 min. Specific binding increased up to 10 min remaining constant thereafter. All other experiments were performed at 10 min incubation. Under these conditions, specific binding of ^{3}H-glycine represents around 30 % of the total radioactivity bound, at all radioligand concentrations studied.

Varying ^{3}H-glycine concentration within the range of 2–4000 nM generated a saturation curve with a single slope by Scatchard analysis (Fig. 1). This suggested a single population of receptors with a K_D of 2.5 ± 0.37 μM, a B_{max} of 320 ± 48 pmol/g, and a Hill coefficient of 1.8 ± 0.24.

^{3}H-Strychnine Binding

Specific binding was found to be saturated at 10 min incubation. Specific ^{3}H-strychnine binding to intact retina accounts for 20 % of the total radiolabeled strychnine bound. Specific binding was studied in a concentration range between 2 and 1000 nM ^{3}H-strychnine (Fig. 2). Scatchard analysis of the data showed the presence of one binding site with K_D of 1 ± 0.1 μM and a B_{max} of 153 ± 15 pmol/g. The Hill coefficient for strychnine was close to the unity (1.3 ± 0.18).

Displacement of Glycine and Strychnine Binding

As shown in Table I glycine, strychnine, β-alanine and sarcosine displace ^{3}H-glycine to a similar extent. Conversely, glycine and sarcosine displace specific ^{3}H-strychnine binding only in approximately 50%. At 1mM, β-alanine alone, and sarcosine in combination with glycine displaced ^{3}H-strychnine to a greater extent than strychnine itself. However, sarcosine in combination with strychnine did not displace strychnine.

Effect of Protein Kinases Activity on Glycine and Strychnine Binding

In order to test the possible effect of protein kinases on glycine and strychnine binding to the GlyR, binding experiments were carried out in the presence of different drugs known to activate or inhibit the activity of PKA or PKC.

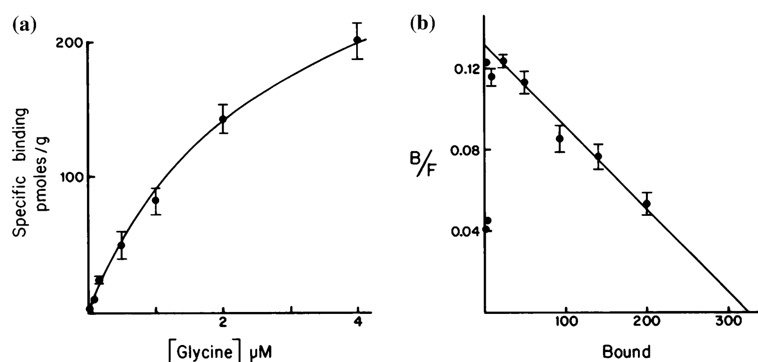

Fig. 1. Specific binding of ^{3}H-glycine to intact frog retina. Binding was assayed by incubating the retinas in the presence of increasing ^{3}H-glycine concentrations. (a) Specific binding was calculated with 1 mM glycine. (b) Scatchard plot from the data in (a). $r = 0.95$. Points are the mean of three independent experiments ± SEM.

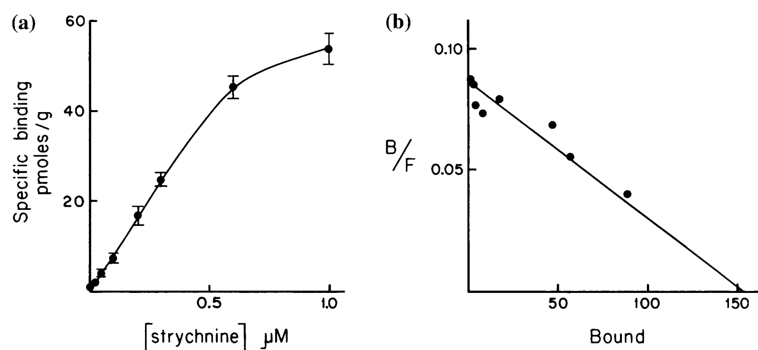

Fig. 2. Specific binding of ^{3}H-strychnine to intact frog retina. (a) Retinas were incubated in the presence of increasing concentrations of ^{3}H-strychnine. Specific binding was calculated with 100 μM strychnine as described in Methods. (b) Scatchard analysis from the data. $r = 0.99$. Points are the mean of three independent experiments ± SEM.

Table I. Percent of Displacement of ^{3}H-Glycine and ^{3}H-Strychnine Specifically Bound to Intact Frog Retina

Displacer	Displacement (%)	
	^{3}H-Glycine	^{3}H-Strychnine
Glycine 1 mM	100	48 ± 4.8
Strychnine 100 μM	83 ± 5.3	100
β-Alanine 1 mM	109 ± 7.6	163 ± 11.0*
Sarcosine 1 mM	88 ± 5.4	54 ± 8.2
Sarcosine + glycine	124 ± 9.1	150 ± 11.9*
Sarcosine + strychnine	75 ± 5.4	127 ± 14.0

Displacement was assayed by incubating the tissue with either ^{3}H-glycine or ^{3}H-strychnine at 10 nM. Data are the mean ± SEM from between five and seven independent experiments. $*P < 0.05$.

Table II. Effect of Different Compounds on ^{3}H-Glycine and ^{3}H-Strychnine Specific Binding to the Frog Retina

	^{3}H-Glycine (pmol/g)	^{3}H-Strychnine (pmol/g)
None	1.30 ± 0.11	1.08 ± 0.10
Forskolin (10 μM)	0.86 ± 0.07*	0.2 ± 0.07*
IBMX (1 mM)	0.60 ± 0.08	0.01 ± 0.5
Staurosporine (10 nM)	1.36 ± 0.08	0.59 ± 0.04*
PMA (200 nM)	0.38 ± 0.12*	0.18 ± 0.013*

Retinas were incubated for 10 min in a sodium-free medium with 10 nM of ^{3}H-glycine or ^{3}H-strychnine in the absence or the presence of different compounds. Specific binding was calculated with 1 mM glycine or 100 μM strychnine as described in Methods. Data are the mean ± SEM of between four and seven independent experiments. $*P < 0.05$.

The presence in the incubation medium of 10 μM forskolin or 1 mM IBMX drugs, which increase the levels of cAMP by activating adenylate cyclase or by inhibiting phosphodiesterase, reduced between 30 and 90% the specific binding of ^{3}H-glycine and ^{3}H-strychnine (Table II). Also, 200 nM PMA, a tumor promoting agent known to stimulate PKC, reduced ^{3}H-glycine and ^{3}H-strychnine binding 70 and 80%, respectively. However, the PKC inhibitor staurosporine (10 nM) did not affect glycine binding but decreased strychnine binding by 45% (Table II).

Table III. Effect of Different Compounds on the Cyclic Nucleotides Content in the Frog Retina

Compound	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
None	32.2 ± 3.6	28.8 ± 4.0
Forskolin (10 μM)	50.0 ± 7.6*	21.0 ± 2.2
IBMX (1 mM)	123 ± 23*	23 ± 2.2
PMA (200 nM)	36.0 ± 7.0	22 ± 2.0

Retinas were incubated for 10 min in a sodium-free medium in the absence or presence of different compounds as described in the Experimental Procedures. Values are the mean ± SEM of between four and seven independent experiments. $^{*}P < 0.05$.

Cyclic Nucleotides Levels

Under binding incubation conditions, cAMP levels were increased 50% and 300% by forskolin and IBMX, respectively (Table III) but they were not affected by PMA. Although in all experimental conditions, the levels of cGMP appear lower than in the control, these differences were not statistically significant.

DISCUSSION

Although there is considerable evidence supporting the role of glycine as inhibitory neurotransmitter in the retina, little is known about the mechanisms of regulation.

Retina can be maintained *in vitro* in a nearly physiological state, which makes it an adequate model to study neural activity. Therefore, to understand the mechanism of glycine neurotransmission, in the present work we characterized the ligand binding properties of the GlyR and its possible modulation by protein kinases in the intact isolated frog retina in physiological *in vitro* conditions.

We have demonstrated that both ^{3}H-glycine and ^{3}H-strychnine binding sites can be detected in the intact retina. It is noteworthy that the specific binding values of ^{3}H-glycine are similar to those for ^{3}H-strychnine. Because strychnine has a negligible affinity for the glycine transporter (17), it is likely that in our Na^{+}-free conditions glycine binding involves only GlyR sites. Scatchard analysis of specific glycine and strychnine binding showed a K_D of 2.5 and 2 μM, which are one to two orders of magnitude higher than those found in spinal cord and other brain areas (18,19) as well as in synaptosomal membranes from frog retina (20). This low affinity may be explained by the low percentage of specific binding of radioligand observed, which would be explained to low diffusion through the tissue. Even so, specific ^{3}H-glycine binding observed in this study is approximately two orders of magnitude lower than the K_M for glycine uptake in retina (100 μM); (15,16).

The ability of GlyR agonists and antagonist to compete with ^{3}H-glycine and ^{3}H-strychnine binding were also determined. β-Alanine, the most potent agonist of GlyR, strychnine, and glycine analogue sarcosine, were effective in displacing ^{3}H-glycine as previously reported (20,21). Previous studies reported that glycine and strychnine do not share the same binding site on the GlyR both have two different binding sites that interact allosterically (19). In agreement with these results, we observed a Hill coefficient for glycine binding greater than the unity. In this regard, sarcosine partially displaced radiolabeled strychnine, but might affect allosteric interactions between glycine and strychnine sites, enhancing the accessibility to the strychnine site, as indicated by the higher displacement of ^{3}H-strychnine observed by coapplication of glycine and sarcosine. Instead, the slope of Hill plots from strychnine binding was consistently close to the unity. The reason for this is not clear, and requires further analyses. Since the specific binding of both radioligands represents a low percentage of the total binding, no further pharmacological characterization was possible. Even so these results altogether indicate that the binding we observed is, indeed, to the GlyR.

Evidence suggests that a variety of neurotransmitters that regulate intracellular second messengers may affect the efficacy of synaptic transmission by modulating phosphorylation of ion channels (22). The GlyR has been shown to be phosphorylated by intracellular second messenger-triggered protein kinases, including PKA and PKC. Both kinases alter the magnitudes of glycine-evoked currents; however, there are discrepancies regarding their precise effects. PKA and PKC enhance the glycine response in *Xenopus* oocytes injected with rat brain mRNA (7). PKA increases the channel opening probability in trigeminal neurons (4,23), but inhibits glycine responses in hypothalamic neurons (6). Similarly, PKC has been shown to inhibit glycine currents in hippocampal, trigeminal, and spinal neurons (8,23,24). In the salamander retinal ganglion cells, PKA down-regulated the glycine current slow component, while PKC up-regulated the glycine current fast component (25). These differences may be

ascribed to the heterogeneous combination of GlyR subunits as well as to different subtypes of PKs yielding to different responses.

Our results demonstrated that forskolin and IBMX, at concentrations reported to activate and inhibit adenylate cyclase and phosphodiesterase (26,27) respectively, decreased glycine and strychnine binding. IBMX and to a lesser extent forskolin increased cAMP levels, supporting the idea that PKA might phosphorylate the receptor. This, in turn, might lead to changes in affinity for its ligands and/or to phosphorylation and internalization of the receptor, as has been shown for other receptors (22,28).

Forskolin, IBMX, or PMA did not affect cGMP levels, suggesting that changes in binding produced by these compounds should unlikely be related to the NMDA receptor, since cGMP levels have been reported to increase during activation of NMDA receptors (29).

The production of intracellular second messengers through GTP binding proteins driven by membrane receptors are known to mediate coupled receptor cross-talk (22,28,30,31). It is, therefore, possible that the GlyR-mediated response is modulated not only by the amount of agonist acting on the GlyR extracellularly but also by the action of other receptors, such as norepinephrine and dopamine receptors, which are linked to the production of intracellular second-messengers and protein kinases activity. Indeed, activation of α2 adrenoreceptor coupled to a GTP binding protein, which decreases the intracellular cAMP and PKA activity, potentiates GlyR in rat substantia nigra neurons (32).

In addition, our results suggest that retinal GlyR are modulated upon activation of PKC, as indicated by the remarkable decrease in glycine and strychnine binding in the presence of PMA. However, the PKC inhibitor staurosporine partially inhibited strychnine but not glycine binding. These results may be ascribed to a direct interaction of staurosporine with the GlyR–strychnine site. Therefore, our results might suggest that PKs can mediate phosphorylation of the GlyR resulting in receptor internalization, leading to the decrease in the binding sites we observed.

In conclusion, we were able to identify binding properties of GlyR in the intact retina, which are modulated in response to the presence of activators of PKA and PKC. Further studies with this preparation may allow us to understand the role of GlyR in modulating synaptic transmission in retina.

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