Br. J. Pharmac. (1983), **79**, 623–628

Glycine-induced changes in acetylcholine release from guinea-pig brain slices

Lorenzo Beani, Clementina Bianchi, Anna Siniscalchi & Sergio Tanganelli

Department of Pharmacology, University of Ferrera, Via Fossato di Mortara, 64/B, 44100 Ferrara, Italy

1 The effect of glycine (Gly) on acetylcholine (ACh) release from superfused, resting or electrically-stimulated slices of guinea-pig caudate nucleus (CN), brain stem (BS) and cerebral cortex (CC) was studied.

2 The amino acid $1 \times 10^{-4} - 6 \times 10^{-3}$ mol l^{-1} reduced the electrically-induced release and increased the spontaneous and KCl-evoked transmitter outflow, mostly in CN but also in BS, whereas it was ineffective in CC. Taurine, chosen as a structurally related compound, moderately affected only the spontaneous release in CN.

3 Strychnine 2×10^{-7} mol l^{-1} was *per se* ineffective, but prevented most Gly effects. The Gly-induced increase of ACh outflow in resting CN slices, however, could be completely antagonized only by administering strychnine and picrotoxin together.

4 These findings suggest that: (i) the overall pattern of Gly influence on cholinergic function is similar to that previously described for γ-aminobutyric acid (GABA); (ii) specific receptors seem to be present in BS and, above all, in CN; (iii) a positive cooperation between endogenous GABA and Gly is evident in resting CN slices; (iv) the absence of any apparent endogenous glycinergic control on the cholinergic neurones casts doubt on but does not exclude the existence of glycinergic neurones in CN.

Introduction

A growing amount of evidence indicates that glycine (Gly) may be considered a major inhibitory transmitter not only in the spinal cord (Krnjevic, 1974; Johnston, 1978) but also at supraspinal sites, especially in the substantia nigra (Dray & Straughan, 1976; Pycock & Kerwin, 1981). High Gly concentrations have been found in the ventral grey matter, labelled amino acid is taken up by spinal cord and brain stem slices (Muller & Snyder, 1978) and it is released by KCl through a calcium-dependent process (Mulder & Snyder, 1974). Moreover, binding studies suggest that Gly recognition sites are present in various parts of the brain, such as the midbrain, and that strychnine binds to a specific component of the Gly-receptor-complex (Young & Snyder, 1973; 1974; Zarbin, Wamsley & Kuhar, 1981).

While electrophysiological investigations have clearly shown that Gly interacts with its postsynaptic receptors and causes hyperpolarization by enhancing chloride permeability, very few biochemical data exist concerning the direct Gly effect on neurotransmitter release. Cheramy, Nieoullon & Glowinsky (1978) reported that Gly, applied in the substantia nigra, reduced [^{3}H]-dopamine release from ipsilateral caudate nuclei in cats. Similarly, Scherber, Staib & Oelszner (1975) found that Gly, administered i.c.v., inhibited oxotremorine tremor in rats and reduced ACh release into the ventricles. However, these effects could depend on firing rate reduction of the dopaminergic and cholinergic neurones respectively and not on primary inhibition of the release processes. Only Kerwin & Pycock (1979), Giorguieff-Chesselet, Kemel, Wandscheer & Glowinski (1979) and, more recently, Martin & Mitchell (1980) and Mitchell (1982) offered direct evidence that Gly specifically stimulated [^{3}H]-dopamine release from slices of rat substantia nigra and striatum.

The following paper describes attempts to determine whether Gly affects the cholinergic structures present in brain slices surviving *in vitro*. A preliminary account of these results has already been presented (7th International Congress of Pharmacology, Paris, 1978).

Methods

Experiments were performed on guinea-pig cerebral cortex (CC), caudate nucleus (CN) and brain stem (BS) slices (0.4 mm thick) as previously described (Beani, Bianchi, Giacomelli & Tamberi, 1978; Bianchi, Tanganelli, Marzola & Beani, 1982). The tissue was allowed to equilibrate for 30 min at room temperature; 2–3 slices were then placed into two superfusion chambers of 0.9 ml and superfused at a constant rate of 0.5 ml min^{-1} with Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 10, $NaHCO_3$ 25, choline 0.02 and physostigmine 0.03) maintained at 37°C and gassed with a mixture of 95% O_2 plus 5% CO_2.

The experiments were composed of two cycles of 40 min. Each cycle included two 5 min stimulation periods at 1 Hz and 5 Hz, each preceded by 5 min of rest and followed by 10 min of washout.

The cerebral tissue was stimulated with rectangular pulses of alternate polarity, intensity 30 mA per cm^2, duration 5 ms. The electrically-evoked release was calculated as net extra-release (Beani *et al.*, 1978). Potassium stimulation was performed with Krebs solution containing 25 mM KCl, no correction being made for osmolarity. Unless otherwise stated, in order to compare the ACh release between treated and untreated tissue of the same animal, the drugs were added to the perfusion medium of only one chamber 15–20 min before starting the second cycle. The first cycle served to check whether the release from the slices randomly set up in the two chambers was of the same order.

Acetylcholine bioassay

The ACh content of the perfusate was bioassayed against suitable standards on the guinea-pig ileum, pretreated for 1 h with tetrodotoxin 3×10^{-5} mol l^{-1}. The details of this technique have already been described (Beani *et al.*, 1978). The ACh release was expressed as ACh ng g^{-1} min^{-1} of fresh tissue.

Materials

Freshly prepared solutions of the following drugs

Table 1 Effect of glycine and taurine on acetylcholine release from slices of cerebral cortex, brain stem and caudate nucleus, at rest and in response to electrical stimulation for 5 min at 1 Hz and 5 Hz

	ACh release (ng g^{-1} min^{-1})		
	No stimulation (rest)	*1 Hz*	*5 Hz*
Cerebral cortex			
Controls	7.8 ± 0.3	39.0 ± 6.2	159.0 ± 19.0
Glycine 6×10^{-3} mol l^{-1} (5)	7.8 ± 0.5	43.0 ± 9.8	136.0 ± 12.0
	(100%)	(110%)	(86%)
Brain stem			
Controls	12.1 ± 0.67	14.4 ± 2.6	80.6 ± 10.0
Glycine 1×10^{-3} mol l^{-1} (5)	11.2 ± 1.09	12.0 ± 3.3	70.6 ± 3.2
	(92%)	(83%)	(88%)
Controls	11.0 ± 1.6	14.0 ± 1.4	97.0 ± 12.6
Glycine 6×10^{-3} mol l^{-1} (5)	13.0 ± 1.4	10.0 ± 1.0*	51.0 ± 5.7*
	(118%)	(71%)	(53%)
Caudate nucleus			
Controls	173.0 ± 21.0	322.0 ± 64.0	990.0 ± 68.0
Glycine 1×10^{-4} mol l^{-1} (6)	208.0 ± 17.0	289.0 ± 30.0	762.0 ± 80.0*
	(120%)	(90%)	(77%)
Controls	153.0 ± 17.0	361.0 ± 34.0	1030.0 ± 68.0
Glycine 3×10^{-4} mol l^{-1} (5)	293.0 ± 20.0**	168.0 ± 29.0**	679.0 ± 67.0**
	(192%)	(47%)	(66%)
Controls	170.0 ± 17.0	321.0 ± 30.0	999.0 ± 100.0
Glycine 3×10^{-3} mol l^{-1} (5)	314.0 ± 30.0**	159.0 ± 16.0**	530.0 ± 50.0**
	(185%)	(50%)	(53%)
Controls	137.0 ± 13.0	322.0 ± 51.0	902.0 ± 69.0
Taurine 3×10^{-4} mol l^{-1} (7)	187.0 ± 15.5**	363.0 ± 40.0	852.0 ± 106.0
	[136%)	(113%)	(92%)

The values given are ± s.e.mean.
The drugs were added to only one perfusion chamber in the interval between the 1st and 2nd cycle. The number in parentheses represents the number of experiments. The % changes with respect to the controls are given. The difference between treated and control slices of the same animal in the 2nd cycle is statistically significant, $*P < 0.05$; $**P < 0.01$, Student's *t* test for non-paired data.

Table 2 Effect of strychnine 2×10^{-7} mol l^{-1} on acetylcholine (ACh) release from slices of brain stem and caudate nucleus at rest and in response to electrical stimulation for 5 min at 1 Hz and 5 Hz

	ACh release (ng g^{-1} min^1)		
	No stimulation (rest)	*1 Hz*	*5 Hz*
Brain stem			
Controls (3)	13.2 ± 2.7	20.2 ± 3.0	78.7 ± 2.0
Strychnine (a)	12.2 ± 0.25	19.2 ± 1.2	69.7 ± 9.0
	(92%)	(95%)	(88%)
Strychnine (b)	12.5 ± 0.75	13.0 ± 0.93	69.0 ± 4.4
Strychnine plus glycine 6×10^{-3} mol l^{-1} (5)	10.8 ± 0.93	15.5 ± 4.0	66.8 ± 7.8
	(87%)	(119%)	(97%)
Caudate nucleus			
Controls (3)	148.0 ± 18.0	257.0 ± 43.0	803.0 ± 90.0
Strychnine (a)	138.0 ± 23.0	306.0 ± 60.0	825.0 ± 95.0
	(93%)	(119%)	(102%)
Strychnine (b)	150.0 ± 7.8	328.0 ± 35.0	953.0 ± 114.0
Strychnine plus glycine 3×10^{-4} mol l^{-1} (6)	220.0 ± 23*	308.0 ± 37.0	1006.0 ± 114.0
	(146%)	(94%)	(106%)
Strychnine (b)	103.0 ± 5.8	318.0 ± 28.0	788.0 ± 28.0
Strychnine plus taurine 3×10^{-4} mol l^{-1} (7)	117.3 ± 9.0	350.0 ± 43.0	819.0 ± 48.0
	(114%)	(110%)	(104%)

(a) Strychnine was added in the interval between the 1st and 2nd cycle.
(b) Strychnine was added to both perfusion chambers from the beginning of the experiment, while Gly and taurine were added in the interval between the 1st and 2nd cycle in only one chamber.
The values given are ± s.e.mean.
In parentheses the number of experiments. The % changes caused by glycine or taurine, with respect to slices treated with strychnine alone, are given.
* The difference from slices treated with the antagonist alone is statistically significant, $P < 0.05$, Student's *t* test for non-paired data.

were used: glycine, taurine, picrotoxin, strychnine sulphate (Sigma Chemical Company, Ltd., U.S.A.), tetrodotoxin (TTX, from Sankyo, Japan), spiroperidol, solubilized as tartrate.

Statistical analysis of the results

Differences in ACh released from slices superfused with or without drugs were analysed using Student's *t* test for non-paired data. values of $P < 0.05$ were considered of statistical significance.

Results

Effect of glycine on acetylcholine release

As shown in Table 1, Gly up to 6×10^{-3} mol l^{-1} was inactive in CC, moderately inhibitory in BS and extremely active in CN. In this last brain area, the amino acid at $1 \times 10^{-4} - 3 \times 10^{-4}$ mol l^{-1} increased the ACh resting release and reduced the electrically-evoked output. The effect of Gly was maximum at 3×10^{-4} mol l^{-1}.

In another set of experiments the influence of Gly on KCl-evoked ACh release in CN was investigated: the amino acid at 3×10^{-4} mol l^{-1} not only increased the resting release but significantly ($P < 0.05$) enhanced the ACh outflow induced by KCl 25 mM. In other words, KCl depolarization and the Gly effect were additive (180 ± 17 ng g^{-1} min^{-1} in controls, 249 ± 33 in the presence of Gly 3×10^{-4} mol l^{-1}, 316 ± 33 in KCl-depolarized tissue, 420 ± 57 in KCl-depolarized tissue and in the presence of Gly 3×10^{-4} mol l^{-1}; means of 5 experiments).

As previously described for GABA (Bianchi *et al.*, 1982), tetrodotoxin 5×10^{-7} mol l^{-1} abolished Gly facilitation on resting ACh release (data not given). To check for Gly selectivity, taurine, an amino acid structurally related to Gly, was tested. Taurine added at 3×10^{-4} mol l^{-1} to CN slices increased the ACh release at rest as did Gly, but was almost ineffective against electrically-evoked ACh efflux (Table 1).

Table 3 Effect of glycine 3×10^{-4} mol l^{-1} on acetylcholine (ACh) release from slices of caudate nucleus, pretreated with picrotoxin 8×10^{-5} mol l^{-1} or spiroperidol 2.5×10^{-7} mol l^{-1}, maintained at rest and stimulated for 5 min at 5 Hz

Experimental conditions	*ACh release* (ng g^{-1} min^{-1}) *No stimulation (rest)*	5 Hz
Controls	123.0 ± 17.0	824.0 ± 68.0
Glycine (5)	235.0 ± 20.0**	544.0 ± 54.0
Spiroperidol	147.0 ± 18.0	810.0 ± 100.0
Spiroperidol plus (3) glycine	268.0 ± 40.0** (182%)	322.0 ± 48.0** (48%)
Picrotoxin	144.0 ± 12.0	1030.0 ± 155.0
Picrotoxin plus (3) glycine	210.0 ± 26.0 (146%)	871.0 ± 95.0 (84%)
Picrotoxin and strychnine 2×10^{-7} mol l^{-1} (4)	142.0 ± 26.0	905.0 ± 90.0
Picrotoxin and strychnine plus glycine	119.0 ± 28.0 (83%)	1055.0 ± 54.0 (116%)

The values given are ± s.e.mean.
The antagonists were added to both perfusion chambers from the beginning of the experiment while glycine was added to one perfusion chamber between the 1st and 2nd cycle.
In parentheses the number of experiments. The % changes caused by glycine with respect to antagonist treated slices are given.
The differences between Gly treated and Gly untreated slices are statistically significant, $^{**}P < 0.01$. Student's *t* test for non-paired data.

Effect of receptor antagonists

In order to assess whether the effect of Gly depended on interaction with Gly receptors, strychnine was tested. The antagonist alone, added at 2×10^{-7} mol l^{-1}, did not change the resting or evoked ACh release in BS and CN slices (Table 2). At higher concentrations ($1 \times 10^{-6} - 1 \times 10^{-5}$ mol l^{-1}), it reduced both resting and electrically-evoked ACh efflux, probably due to unspecific effects. The addition of strychnine (2×10^{-7} mol l^{-1}) to BS slices reversed Gly facilitation of resting ACh outflow and prevented Gly inhibition of electrically-evoked ACh release (Table 2). Although in CN slices the antagonist cancelled Gly inhibition of electrically-evoked ACh release, it only reduced the facilitatory effect of the amino acid on spontaneous release (Table 2).

The increase of resting ACh release caused by taurine 3×10^{-4} mol l^{-1} was also prevented by strychnine (Table 2).

Other antagonists were then tested in an attempt to determine whether or not Gly acted indirectly on the caudate cholinergic neurones.

As shown in Table 3 spiroperidol 2.5×10^{-7} mol l^{-1}, added to CN slices did not modify the effect of Gly. On the contrary, picrotoxin 8×10^{-5} mol l^{-1}, partly antagonized the increase of ACh resting release (Table 3). Interestingly, only in the presence of both strychnine and picrotoxin was the increase in ACh efflux completely prevented (Table 3).

Discussion

These results show that exogenous Gly is able to modulate ACh release especially in the CN. The amino acid is 10–20 times less active in BS cholinergic structures, while its control does not involve the neocortex. This finding agrees with electrophysiological data excluding any Gly influence on the cortical neurones (Kelly & Krnjević, 1969).

The concentrations of Gly required to modify ACh release were slightly higher than those found to be active against dopamine release in other species and under different experimental conditions (Kerwin & Pycock, 1979; Giorgieff *et al.*, 1979a; Mitchell, 1982), but they were lower than the GABA amounts needed to affect striatal cholinergic function (Bianchi *et al.*, 1982).

The most crucial finding was that Gly reduced stimulus-evoked ACh release and increased spontaneous or KCl-induced ACh outflow by acting upon specific receptors.

Although we cannot rule out the possibility that the inhibitory action is due to depression of neuronal excitability, the overall pattern of Gly-induced changes fits well with the theoretical scheme of a depolarizing effect on the cholinergic nerve endings, such as that of GABA (Bianchi *et al.*, 1982), and emphasizes the different significance of drug- (or transmitter-) induced changes in electrically- and KCl-evoked ACh release, respectively (Schoffel-

meer, Wemer & Mulder, 1981).

In our opinion, the electrical pulses mimic the physiological, phasic, nerve-conducted, sodium-dependent activity, triggering the calcium entry-secretion coupling in the nerve endings.

In contrast, KCl depolarization can, above all, enhance the spontaneous, voltage- and calcium-dependent neurosecretion, to be looked upon more as a 'synaptic noise' than as a 'synaptic signal'.

In this respect, at least in regard to cholinergic transmission, Gly is very similar to GABA (Bianchi *et al.*, 1982), since both amino acids reduce the 'signal to noise' ratio.

The specificity of the Gly effect is supported by the taurine and antagonist experiments. While the structural analogy of taurine may explain its limited Gly-like, strychnine-sensitive influence on CN resting ACh release (see also Martin & Mitchell, 1980), the full antagonism displayed by strychnine vs Gly in BS and in electrically-stimulated CN slices stressed the presence of specific receptors able to modulate not only dopaminergic (Mitchell, 1982), but also cholinergic function. On the other hand, the stimulatory effect exerted by the amino acid on spontaneous ACh release in CN was reduced by either strychnine 2×10^{-7} mol l^{-1} or by picrotoxin 8×10^{-5} mol l^{-1}, but it was completely blocked only when picrotoxin and strychnine were administered together. Thus, in this brain area Gly seems to act both through strychnine-sensitive receptors and through other mechanisms involving GABA release.

This conclusion agrees in part with the results obtained by Giorguieff-Chesselet, Besson, Cheramy & Glowinski (1979b) and Mitchell (1982), which indicate that picrotoxin antagonizes Gly stimulation of spontaneous [^{3}H]-dopamine release in rat striatal slices.

Since Gly facilitation of spontaneous ACh release was prevented by TTX, it may be that a sodium-dependent mechanism, i.e. the increased firing rate of GABAergic neurones, is involved; yet other hypotheses have also been suggested (Bianchi *et al.*, 1982).

In conclusion, these results show that exogenous Gly affects the cholinergic mechanisms in the BS and CN through specific receptors and modulates the functionally interconnected GABAergic cells in the CN.

On the other hand, the existence of an intrinsic glycinergic tone in the superfused slices can be excluded since strychnine alone was ineffective on normal ACh release. This conclusion is at variance with previous observations showing an endogenous GABAergic and noradrenergic control of ACh release in striatal and cortical slices (Beani *et al.*, 1978; Bianchi *et al.*, 1982). Gly action probably depends either on its interaction with certain recognition sites, devoid of any apparent physiological role, or on the fact that glycinergic neurones, if present, were not operative under these experimental conditions.

The expert technical assistance of Mr G. Marzola is gratefully acknowledged. This work was supported by a grant from the C.N.R. Rome (n. 81.00114.04).

References

BEANI, L., BIANCHI, C., GIACOMELLI, A. & TAMBERI, F. (1978). Noradrenaline inhibition of acetylcholine release from guinea-pig brain. *Eur. J. Pharmac.*, **48,** 179–193.

BIANCHI, C., TANGANELLI, S., MARZOLA, G. & BEANI, L. (1982). GABA-induced changes in acetylcholine release from slices of guinea-pig brain. *Naunyn-Schmiederbergs Arch. Pharmac.*, **318,** 253–258.

CHERAMY, A., NIEOULLON, A. & GLOWINSKI, J. (1978). Inhibition of dopamine release in the cat caudate nucleus by nigral application of glycine. *Eur. J. Pharmac.*, **47,** 141–147.

DRAY, A. & STRAUGHAN, D.W. (1976). Synaptic mechanisms in the substantia nigra. *J. Pharm. Pharmac.*, **28,** 400.

GIORGUIEFF-CHESSELET, M.F., KEMEL, M.L., WANDSCHEER, D. & GLOWINSKI, J. (1979a). Glycine stimulates the spontaneous release of newly synthetized [^{3}H]-dopamine in rat striatal slices. *Eur. J. Pharmac.*, **60,** 101–104.

GIORGUIEFF-CHESSELET, M.F., BESSON, M.J., CHERAMY, A. & GLOWINSKY, J. (1979b). Role of various amino acids on the modulation of dopamine release from nigro-striatal dopaminergic neurones. *J. Physiol.*, (Paris) **75,** 611–613.

JOHNSTON, G.A.R. (1978). Neuropharmacology of amino acid inhibitory transmitters. *A. Rev. Pharmac. Tox.*, **18,** 269–289.

KELLY, J.S. & KRNJEVIC, K. (1969). The action of glycine on cortical neurones. *Exp. Brain Res.*, **9,** 155–163.

KERWIN, R.W. & PYCOCK, C.J. (1979). Specific stimulating effect of glycine on ^{3}[H]-dopamine efflux from substantia nigra slices of the rat. *Eur. J. Pharmac.*, **54,** 93–98.

KRNJEVIC, K. (1974). Chemical nature of synaptic transmission in vertebrate. Physiol. Rev. **54,** 410–540.

MARTIN, I.L. & MITCHELL, P.R. (1980). Effects of some amino acids on K^+-induced release of ^{3}H-DA from rat striatal tissue. *Br. J. Pharmac.*, **68,** 162P–163P.

MITCHELL, R. (1982). Transmitter-like actions of L-proline and glycine in rat striatum. *Br. J. Pharmac.*, **77,** 505P.

MULDER, A.H. & SNYDER, S.H. (1974). Potassium-induced release of amino acids from cerebral cortex and spinal cord slices of the rat. *Brain Res.*, **76,** 297–308.

MULLER, W.E. & SNYDER, S.H. (1978). Glycine high affinity uptake and strychnine binding associated with glycine

receptors in the frog central nervous system. *Brain Res.* **143,** 487–498.

PYCOCK, C.J. & KERWIN, R.W. (1981). The status of glycine as a supraspinal neurotransmitter. *Life Sci.*, **28,** 2679–2686.

SCHERBER, A., STAIB, A.N. & OELSZNER, W. (1975). Wirkung verschiedener Amino-säuren und deren Antagonisten auf den cholinergen Tremor und die Azetylcholinfreisetzung im ZNS der Ratte. *Acta Biol. Med. Germ.*, **34,** 1517–1524.

SCHOFFELMEER, A.V.M., WEMER, J. & MULDER, A.H. (1981). Comparison between electrically-evoked and potassium-induced [^{3}H]-noradrenaline release from rat neocortex slices: role of calcium ions and transmitter pools. *Neurochem. Int.*, **3,** 129–136.

YOUNG, A.B. & SNYDER, S.H. (1973). Strychnine binding associated with glycine receptors of the central nervous system. *Proc. natn. Acad. Sci. U.S.A.*, **70,** 2832–2836.

YOUNG, A.B. & SNYDER, S.H. (1974). Strychnine binding in rat spinal cord membranes associated with the glycine receptor: cooperativity of glycine interactions. *Mol. Pharmac.*, **10,** 790–809.

ZARBIN, N.N., WAMSLEY, J.K. & KUHAR, M.J. (1981). Glycine receptor: light microscopic autoradiographic localization with [^{3}H]-strychnine. *J. Neurosci.*, **1,** 532–547.

(*Received January 24, 1983.*
Revised February 18, 1983.)