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Direct neurotrophic action of glycyl-L-glutamine in the maintenance of acetylcholinesterase and butyrylcholinesterase in the preganglionically denervated superior cervical ganglion of the cat

(sympathetic nervous system)

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ABSTRACT **Intracarotid infusion of glycyl-L-glutamine (Gly-Gln) was shown previously to oppose the fall in the acetylcholinesterase and butyrylcholinesterase contents of the cat superior cervical ganglion (SCG) that otherwise follows preganglionic denervation. However, its effect was demonstrable only on the vasculary remote left SCG but not on the directly infused right SCG. Accordingly, it was concluded that a metabolite of Gly-Gln, formed in the blood, is an active neurotrophic factor. Glycyl-L-glutamic acid and L-glutamic acid were subsequently found to have a similar but less marked effect on both SCG. In the present study an alternative explanation has been tested: that Gly-Gln must combine slowly with some component of plasma to enable it to penetrate the ganglion cells and exert its neurotrophic effect. Findings are consistent with the latter proposal.**

The fall in the acetylcholinesterase (AcChoEase, EC 3.1.1.7) and butyrylcholinesterase (BtChoEase, EC 3.1.1.8) contents of the cat superior cervical ganglion (SCG) that follows preganglionic denervation (1, 2) is opposed by the intracarotid infusion of an aqueous extract of cat brain, spinal cord, and sciatic nerves (3) or its dialysate (M_r cutoff, 1000) (4). Following a report that glycyl-L-glutamine (Gly-Gln) showed a similar effect on cultured rat and chicken skeletal muscle (5), this compound was tested in the cat preparation. It was found to be ineffective at the directly infused right SCG but highly active in maintaining AcChoEase and BtChoEase at the circulatory remote left SCG (6). It was therefore concluded that a metabolite of Gly-Gln, formed in the blood, is a direct neurotrophic factor for the production of this effect. Of the possible candidates tested, glycine and L-glutamine were found to be inactive, but glycyl-L-glutamic acid (Gly-Glu) was moderately active at both the right and left SCG (6). It was shown subsequently that L-glutamic acid has a similar effect, whereas pyroglutamic acid, aspartic acid, and γ-aminobutyric acid were inactive (7). However, neither Gly-Glu nor L-glutamic acid, over a wide range of concentrations, was as effective in preventing the fall in AcChoEase at the right or left SCG as was Gly-Gln at the left SCG following infusion into the right common carotid artery.

Here we have tested an alternative explanation for the restriction of the neurotrophic action of Gly-Gln to the left SCG under the above conditions: that Gly-Gln combines relatively slowly with a component of plasma to permit its penetration to the cytoplasm of the neurons of the SCG. In contrast with Gly-Glu and L-glutamic acid, which are relatively lipid soluble, Gly-Gln is a highly polar compound and hence its permeation of the cell membrane should be limited. The results of the present study are consistent with this proposal and indicate that Gly-Gln has a potent, direct neurotrophic action.

METHODS

Anesthetic and surgical procedures, the methods for homogenization of ganglia and for determination of their AcChoEase, BtChoEase, and protein contents, and the methods for calculation of statistical significance of mean differences were identical with those reported (3). Under sodium pentobarbital anesthesia (35 mg/kg, intraperitoneally) 1 cm was resected from both cervical sympathetic trunks; the wound was sutured and Combiotic (penicillin/dihydrostreptomycin, 0.5 ml intramuscularly; Pfizer) was given. The following day, cats were again anesthetized and treated with atropine (1.0 mg/kg, intraperitoneally); artificial respiration was administered through a tracheal catheter attached to a Palmer pump, and a slow intravenous infusion of 5% glucose/0.45% NaCl was started. Heparin (50 units/kg, intravenously) was given just prior to bilateral ligation of the external carotid and lingual arteries and was repeated every 8 hr. Infusion of the solution under test was begun ≈24 hr after denervation and continued until the time of sacrifice, with a total of ≈400 ml. Solutions were infused by a 27-gauge hypodermic needle inserted into the right common carotid artery and attached by Tygon tubing to a reservoir enclosed in an ice-water bath. A Harvard peristaltic pump and a mercury manometer were interposed. Deep anesthesia was maintained by repeated intravenous doses of sodium pentobarbital as required; atropine or mephentermine was administered whenever wheezing respiration or persistent hypotension occurred. Exactly 48 hr postdenervation, the SCG and stellate ganglia were excised, weighed, and frozen until homogenization a few minutes later. AcChoEase and BtChoEase were assayed by a modification (8) of the method of Ellman *et al.* (9), and protein was determined according to Lowry *et al.* (10).

Heparinized blood was obtained from experimental cats by intracardiac puncture immediately following sacrifice; it was centrifuged on a Dynac centrifuge at 3500 rpm for 30 min at 5°C, and the plasma was removed and frozen until use. In the initial experimental series, freshly prepared Gly-Gln stock solution of the appropriate concentration was incubated at 5°C overnight with 40 ml of plasma. Immediately prior to infusion it was diluted to 400 ml with cold 0.9% NaCl solution. In the subsequent series, plasma was heated at 60°C for 60 min with mechanical stirring; it was then cooled, Gly-Gln stock solution was added, and the mixture was held at 5°C overnight prior to dilution and infusion as above. In the final series, 80-ml portions of heat-treated plasma were dialyzed overnight (Spectrapor membrane tubing, M_r cutoff,

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Abbreviations: AcChoEase, acetylcholinesterase; BtChoEase, butyrylcholinesterase; SCG, superior cervical ganglion (or ganglia).

10,000) against 80 ml of 0.9% NaCl solution at 5°C with constant shaking; to 40 ml of the retained portion or the dialysate was added Gly-Gln stock solution. The mixture was held overnight at 5°C and subsequently diluted to give a final concentration of 3 μM Gly-Gln and infused as previously.

Controls consisted of six SCG from three preganglionically denervated cats that were infused similarly for 24 hr with a 1:10 dilution of heat-treated plasma in 0.9% NaCl solution (see Table 2). The mean values for AcChoEase (206 ± 10 nmol of substrate hydrolyzed per mg of protein per min) and BtChoEase (409 ± 21) were somewhat lower and higher, respectively, than those of an earlier control series (254 ± 13 and 251 ± 33) that consisted of six SCG from similarly treated cats that were infused for 24 hr with 0.9% NaCl solution (6).

The possible anticholinesterase action of Gly-Gln was tested by incubating at room temperature for 60 min final concentrations of 1, 10, 100, and 1000 μM with a homogenate of cat stellate ganglion; the mixtures were then assayed by the standard procedure.

RESULTS

As in previous experiments of this series, values for AcChoEase were generally much more consistent than those for BtChoEase in both control and experimental groups.

When infused at concentrations of 1000 μM (Table 1, cat U) and 100 μM (cats I and K) following incubation at 5°C overnight with untreated plasma, Gly-Gln had a markedly inhibitory effect on the contents of AcChoEase and BtChoEase of the denervated SCG in comparison with controls (Table 2, cats W_2, G_2, and V). For AcChoEase, at 30 μM (Table 1, cats A and B) there was no difference from controls; a distinctly positive effect was obtained at 10 μM (cats W, Q, and W_1), which peaked at 3 μM (cats Y, Z, and A_1). Lower positive effects were noted at 1 μM (cats E and G) and at 0.3 μM (cats S and U). Results for BtChoEase showed great variability. Although positive results were obtained at concentrations from 30 to 0.3 μM, excepting at 1 μM, these were significant statistically only at 30 μM and 0.3 μM.

To eliminate the possibility that the forgoing results were due to plasma enzyme-conversion products of Gly-Gln, the following experiments were conducted. Prior to incubation at 5°C with Gly-Gln, cat plasma was kept at 60°C for 60 min as described under *Methods*. Results were nearly identical with those of the previous series. Under the latter conditions, infusion of 10 μM Gly-Gln had no apparent effect on ganglionic AcChoEase (Table 2, cats K_1 and M). At 3 μM a markedly positive effect was obtained (cats O–R). Lower positive effects occurred with 1 μM (cats H, I, J, and L) and 0.3 μM (cats S_1 and X). BtChoEase contents were significantly above controls at 10, 3, and 1 μM.

The dialysis experiments were performed in an attempt to obtain some information regarding the nature of the component of plasma with which Gly-Gln apparently combines to render it active as a neurotrophic factor. Results are presented in Table 3. Infusion of 3 μM Gly-Gln preincubated with either the dialysate or retentate of dialyzed heat-treated plasma resulted in significant elevation of the AcChoEase contents of denervated SCG above controls. The BtChoEase contents were also elevated, but because of the marked variation in individual values the mean was increased significantly only with the retentate.

In the *in vitro* tests described above, Gly-Gln had no effect on AcChoEase or BtChoEase activity at any concentration tested (1–1000 μM).

Table 1. Effects of intracarotid infusion of Gly-Gln preincubated with plasma on AcChoEase and BtChoEase contents of cat SCG 48 hr after preganglionic denervation

		Substrate hydrolyzed, nmol/mg of protein per min							
		AcChoEase				BtChoEase			
Cat	Gly-Gln, μM	Right			Left	Right			Left
U	1000	43			58	50			68
			Mean	51 ± 8			Mean	59 ± 9	
I	100	158			146	220			211
K	100	116			162	161			275
			Mean	146 ± 10*			Mean	217 ± 23†	
A	30	210			221	503			519
B	30	198			210	481			505
			Mean	210 ± 5			Mean	502 ± 8‡	
W	10	311			314	346			352
Q	10	350			410	616			677
W_1	10	320			319	351			349
			Mean	337 ± 16‡			Mean	449 ± 63	
Y	3	370			374	606			573
Z	3	438			346	372			423
A_1	3	310			270	497			366
			Mean	351 ± 24‡			Mean	473 ± 42	
E	1	228			230	154			153
G	1	302			282	560			462
			Mean	260 ± 19§			Mean	332 ± 105	
S	0.3	287			276	540			531
U	0.3	300			267	547			521
			Mean	282 ± 7‡			Mean	535 ± 6¶	

Means are expressed ± SEM. Controls: AcChoEase, 206 ± 10; BtChoEase, 409 ± 21 (Table 2, cats W_2, G_2, V).
*Less than controls, $P < 0.005$.
†Less than controls, $P < 0.001$.
‡Greater than controls, $P < 0.001$.
§Greater than controls, $P < 0.05$.
¶Greater than controls, $P < 0.005$.

Table 2. Effects of intracarotid infusion of Gly-Gln preincubated with heat-treated plasma on AcChoEase and BtChoEase contents of cat SCG 48 hr after preganglionic denervation

		Substrate hydrolyzed, nmol/mg of protein per min							
		AcChoEase				BtChoEase			
Cat	Gly-Gln, μM	Right			Left	Right			Left
W_2	0 (control)	205			221	417			490
G_2	0 (control)	188			247	349			441
V	0 (control)	173			204	381			378
			Mean	206 ± 10			Mean	409 ± 21	
K_1	10	246			177	621			416
M	10	238			210	547			515
			Mean	218 ± 16			Mean	525 ± 42*	
O	3	300			382	385			524
P	3	373			366	495			643
Q†	3	226			230	333			353
R	3	418			443	613			562
			Mean	380 ± 20‡			Mean	537 ± 38*	
H	1	311			319	416			440
I_2	1	373			347	527			487
J†	1	246			156	310			220
L	1	320			308	487			508
			Mean	330 ± 10‡			Mean	478 ± 17*	
S_1	0.3	321			247	461			386
X	0.3	314			311	414			283
			Mean	298 ± 17§			Mean	386 ± 38	

Means are expressed ± SEM.
*Greater than controls, $P < 0.05$.
†Omitted from calculations of means since these values for AcChoEase and BtChoEase are >3 SEM outside respective means for remaining six SCG in each set. If these values are included, the means for AcChoEase (3 μM, 342 ± 29; 1 μM, 298 ± 24) differ from that of controls (206 ± 10) at $P < 0.025$ and $P < 0.05$, respectively.
‡Greater than controls, $P < 0.001$.
§Greater than controls, $P < 0.005$.

DISCUSSION

The forgoing results are consistent with the proposal that Gly-Gln must combine with some component of fresh or heat-treated plasma to allow it to exert its previously considered "indirect" neurotrophic action (6).

At high concentrations (1000 or 100 μM) Gly-Gln treated with fresh plasma was found to have an inhibitory effect on the AcChoEase and BtChoEase contents of denervated SCG (Table 1). The same type of action was noted with infusions of 100 μM Gly-Glu (6). These effects were probably due to suppression of some stage of enzyme synthesis, since Gly-Gln was found here, as was Gly-Glu (6), to have no direct anticholinesterase action. The apparent lack of effect of Gly-Gln at 30 μM was due most likely to a combination of its inhibitory and positive effects, since the latter was marked at 10 μM and peaked at 3 μM. Treatment at 60°C for 60 min results in inactivation of several but not all enzymes that hydrolyze peptides (e.g., refs. 11, 12). When Gly-Gln was incubated with heat-treated plasma, no apparent effect was detectable with infusion of 10 μM Gly-Gln (comparable to the result with 30 μM Gly-Gln and untreated plasma), but a peak effect was again found at 3 μM (Table 2). This suggests that the heat-treated plasma preparations contained a somewhat higher concentration of Gly-Gln than in initially corresponding concentrations treated with fresh plasma, possibly because of the partial enzymatic conversion of Gly-Gln to its inactive hydrolylic products, glycine and L-glutamine (6), in the latter.

Table 3. Effects of intracarotid infusion of 3.0 μM Gly-Gln preincubated with dialysate or retentate of heat-treated plasma on AcChoEase and BtChoEase contents of cat SCG 48 hr after preganglionic denervation

		Substrate hydrolyzed, nmol/mg of protein per min			
		AcChoEase		BtChoEase	
Cat	Plasma fraction	Right	Left	Right	Left
C	Dialysate	337	353	611	579
E	Dialysate	283	257	332	262
J	Dialysate	410	404	731	786
		Mean	341 ± 25*	Mean	550 ± 86
D	Retentate	328	337	684	663
G	Retentate	292	240	607	376
I	Retentate	356	361	583	593
		Mean	319 ± 19*	Mean	584 ± 45†

Means are expressed ± SEM.
*Greater than controls (Table 2), $P < 0.001$.
†Greater than controls, $P < 0.005$.

The results of the dialysis experiments indicate that Gly-Gln combines with at least two components of plasma, with M_rs above and below 10,000, to allow its penetration of ganglion cells where it is presumed to exert its neurotrophic effect. No further attempts have been made to establish their identifications.

It was noted in the Introduction that the difference in the physical chemical properties of Gly-Gln, on one hand, and of L-glutamic acid and Gly-Glu, on the other, would favor the passive penetration of the latter compounds to the cytoplasm of the ganglion cells. Additional factors might be involved. It is generally considered that the excitatory and inhibitory neurotransmitter actions of L-glutamic acid and γ-aminobutyric acid, respectively, are terminated primarily by their active uptake by presynaptic terminals and glial cells (13). Recently a convincing alternative case has been presented for the active transport of the latter compound into postsynaptic neurons (14). It can be speculated that L-

glutamic acid and its glycyl analog, in contrast to Gly-Gln, might also be transported actively into ganglion cells.

A direct neurotrophic effect for the maintenance of AcChoEase and BtChoEase in the preganglionically denervated cat SCG has now been demonstrated for three authentic compounds: Gly-Gln, Gly-Glu (6), and L-glutamic acid (7). It is not known whether any of these is identical with the endogenous compound of the central nervous system that shows the same effect (3, 4) or with the hypothetical neurotrophic factor that is believed to be released by preganglionic fibers to maintain the postsynaptic membranous AcChoEase and BtChoEase of ganglion cells under physiological conditions (15). Gly-Gln is present in the central nervous system (16), and L-glutamic acid is found in high concentration both there (13) and in the rat SCG (17). There are apparently no reports of the physiological occurrence of Gly-Glu. Of the three, Gly-Gln appears to be the most effective as a neurotrophic factor. Recent results suggest that Gly-Gln acts by regulating the conversion of an inactive precursor to G_1 AcChoEase (G.B.K. and J. Massoulié, unpublished observations) but further work is required to confirm this finding.

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1. Sawyer, C. H. & Hollinshead, W. H. (1945) *J. Neurophysiol.* **8,** 137–153.
2. Koelle, G. B., Davis, R. & Koelle, W. A. (1974) *J. Histochem. Cytochem.* **22,** 244–251.
3. Koelle, G. B. & Ruch, G. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3106–3110.
4. Koelle, G. B., Sanville, U. J., Rickard, K. K. & Williams, J. E. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6539–6542.
5. Haynes, L. W. & Smith, M. E. (1985) *Biochem. Soc. Trans.* **13,** 174–175.
6. Koelle, G. B., Sanville, U. J. & Wall, S. J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5213–5217.
7. Koelle, G. B., Sanville, U. J., Thampi, N. S. & Wall, S. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2751–2754.
8. Koelle, G. B., Ruch, G. A., Rickard, K. K. & Sanville, U. J. (1982) *J. Neurochem.* **38,** 1695–1698.
9. Ellman, G. L., Courtney, K. D., Andres, V., Jr., & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7,** 88–95.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
11. Trapeznikova, S. S. & Paskhina, T. S. (1968) *Biokhimiya* **33,** 1012–1022.
12. McDonald, J. K., Zeitman, B. B. & Ellis, S. (1972) *Biochem. Biophys. Res. Commun.* **46,** 62–70.
13. McGeer, E. G., McGeer, P. I. & Thompson, S. (1983) in *Glutamine, Glutamate, and GABA in the Central Nervous System*, eds. Hertz, L., Kvamme, E., McGeer, E. G. & Schousboe, A. (Liss, New York), pp. 3–17.
14. Cupello, A. & Hyden, H. (1986) *Cell. Mol. Neurobiol.* **6,** 1–16.
15. Davis, R. & Koelle, G. B. (1981) *J. Cell Biol.* **88,** 581–590.
16. Parish, D. C., Smyth, D. G., Normanton, J. R. & Wolstencroft, J. H. (1983) *Nature (London)* **306,** 267–269.
17. Nagata, Y., Yokoi, Y. & Tsukada, Y. (1966) *J. Neurochem.* **13,** 1421–1431.