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Glycyl-L-glutamine, a precursor, and glycyl-L-glutamic acid, a neurotrophic factor for maintenance of acetylcholinesterase and butyrylcholinesterase in the preganglionically denervated superior cervical ganglion of the cat *in vivo*

(sympathetic nervous system)

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ABSTRACT **L. W. Haynes and M. E. Smith have reported [(1985) *Biochem. Soc. Trans.* 13, 174–175] that glycyl-L-glutamine (Gly-Gln) increases the A_{12} and G_4 forms of acetylcholinesterase (AcChoEase) in cultured embryonic rat skeletal muscle. Since Gly-Gln meets the criteria established for the neurotrophic factor (NF) in extracts of central nervous system/sciatic nerves that maintains AcChoEase and butyrylcholinesterase (BtChoEase) in the denervated cat superior cervical ganglion (SCG) *in vivo*, it was tested by the latter procedure. Solutions of Gly-Gln (10^{-7}–10^{-3} M) in 0.9% NaCl solution were infused for 24 hr via the right common carotid artery of cats with preganglionically denervated SCG, following ligation of the external carotid and lingual arteries. At 48 hr postdenervation, the AcChoEase and BtChoEase contents of the right SCG were within the range of similarly treated controls infused with 0.9% NaCl solution; the AcChoEase and BtChoEase contents of the left SCG, where the infused solutions arrived by way of a much more circuitous route, were significantly elevated at concentrations of Gly-Gln of 10^{-5} M and higher. This suggested that the neurotrophic effect on the left SCG was produced by a metabolite of Gly-Gln. Accordingly, glycine, L-glutamine, and glycyl-L-glutamic acid (Gly-Glu) were then tested. Glycine and L-glutamine were inactive; Gly-Glu, 10^{-6}–10^{-5} M, exerted a significantly positive neurotrophic effect at both the right and left SCG; at 10^{-4} M, the effect was absent. The method employed currently for preparation of extracts of SCG for assay of AcChoEase, BtChoEase, and protein contents (homogenization of scissor-minced ganglia in water) was compared with homogenization in molar NaCl/1% Triton X-100. Values obtained by the former procedure, in comparison with the latter, were 91% ± 7% for AcChoEase and 83% ± 7% for BtChoEase, expressed as substrate hydrolyzed per mg of protein per min.**

Preganglionic denervation of the cat superior cervical ganglion (SCG) results in a loss of 80% of its acetylcholinesterase (AcChoEase, acetylcholine acetylhydrolase, EC 3.1.1.7) and 30% of its butyrylcholinesterase (BtChoEase, acylcholine acylhydrolase, EC 3.1.1.8) contents within 3 days (1–3). Electron microscopic histochemical examination showed that the loss of AcChoEase is associated with its disappearance from both pre- and postsynaptic membranous sites; BtChoEase, which is present in normal SCG only at the dendritic and perikaryonal membranes of the ganglion cells, shows a partial loss (4, 5). It was therefore postulated that the preganglionic fibers release a neurotrophic factor (NF) that is essential for the maintenance of postsynaptic membranous AcChoEase and the full component of BtChoEase (6). In an investigation of this proposal it was found that the intraarterial infusion of an aqueous extract of cat brain, spinal cord, and sciatic nerves, from 24 to 48 hr following preganglionic denervation, resulted in a marked decrease in the loss of both enzymes that otherwise occurred 48 hr postdenervation (3, 7). The NF responsible for this effect was shown to be a molecule of low (<1000) molecular weight, heat-stable, and probably a peptide (8). It was postulated (9) that the NF might act by regulating the conversion of the monomeric globular G_1 forms of the enzymes, present in the cytoplasm, to the tetrameric membranous G_4 and asymmetric synaptic A_{12} forms (10) or by delaying or preventing degeneration of the preganglionic fibers.

Haynes *et al.* (11) showed recently that β-endorphin inhibits the formation of A_{12} AcChoEase from smaller aggregates in cultured immature rat muscle. On the other hand, the dipeptide glycyl-L-glutamine (Gly-Gln), which is formed by endopeptidase cleavage of the terminal of β-endorphin (12), was found by Haynes and Smith (13) to enhance markedly the formation of A_{12} and G_4 AcChoEase in cultured embryonic rat and chicken skeletal muscle. Since Gly-Gln meets the criteria for the endogenous NF established for the cat SCG *in vivo* (8), it was examined by this procedure. Present results indicated that Gly-Gln itself, by the cat *in vivo* assay, is inactive as a NF, but that a metabolite is probably highly active. The three most likely metabolites were therefore tested: glycine, L-glutamine, and glycyl-L-glutamine acid (Gly-Glu). The first two were found to be inactive, the last (Gly-Glu) highly active.

We have also included a comparison of the currently employed method of extraction of SCG by homogenization in distilled water for assay of AcChoEase, BtChoEase, and protein contents with a procedure widely used at present in which the enzymes are solubilized with molar NaCl/1% Triton X-100 (e.g., ref. 14).

METHODS

Anesthetic and surgical procedures and the methods for homogenization of ganglia and for determination of their AcChoEase, BtChoEase, and protein contents and 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, Pfizer, 0.5 ml, intramuscularly) was given. The following day cats were again anesthetized as before and atropinized (1.0

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Abbreviations: AcChoEase, acetylcholinesterase; BtChoEase, butyrylcholinesterase; EC, external carotid; Gly-Gln, glycyl-L-glutamine; Gly-Glu, glycyl-L-glutamic acid; L, lingual; NF, neurotrophic factor; SCG, superior cervical ganglion.

Table 1. Control data from previous and present studies

Group	Procedure	Substrate hydrolyzed, nmol/mg of protein per min: AcChoEase	BtChoEase
1	Normal cat SCG	449 ± 42 (8)	590 ± 61 (8)
2	SCG preganglionically denervated 48 hr previously	122 ± 5 (10)	349 ± 27 (10)
3	SCG denervated 48 hr previously; reanesthetized and EC and L arteries ligated 24 hr postdenervation	177 ± 8 (12)	390 ± 22 (12)
4	SCG denervated 48 hr previously; continuous anesthesia until time of sacrifice; without EC and L arterial ligations	250 ± 23 (8)	444 ± 41 (8)
5	Same as group 4, except EC and L arteries ligated, and cat infused with 0.9% NaCl 24–48 hr postdenervation	R 246	253
		L 199	180
		R 289	162
		L 258	213
		R 274	349
		L 258	347
	Mean ± SEM	254 ± 13 (6)	251 ± 33 (6)

Numbers of SCG are in parentheses. Mean ± SEM values of groups 1–4 are from previous studies (3, 7, 8); values of group 5 are from the present study. R and L, right and left SCGs, respectively.

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 (EC) and lingual (L) arteries and was repeated every 8 hr. Infusion of the solution under test in 0.9% NaCl solution was begun ≈24 hr after denervation and continued until the time of sacrifice, with a total volume of 300–400 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 removed, weighed, and frozen until homogenization a few minutes later. AcChoEase and BtChoEase were assayed by a modification (15) of the method of Ellman *et al.* (16), and protein was determined according to Lowry *et al.* (17).

To obtain absolute controls, three cats were infused for 24 hr as above with 0.9% NaCl solution only. Gly-Gln (prepared by Bachem Fine Chemicals, Torrance, CA) was provided by Lawrence W. Haynes. Glycine, L-glutamine, and Gly-Glu were obtained from Sigma. They were dissolved in 0.9% NaCl solution immediately prior to use.

The method we have employed for homogenization of ganglia in aqueous solution was compared with that reported for complete solubilization of AcChoEase and BtChoEase, by homogenization in 1% Triton X-100/molar NaCl/0.01 M Tris·HCl buffer, pH 7.00 (14), as follows. Two cat stellate ganglia, frozen immediately after excision, were pooled, scissor minced, and homogenized initially for 60 sec in half (0.125 ml/mg) of the usually employed volume of water. Equal volumes of the suspension were transferred immediately to two homogenization tubes. To one aliquot was added an equal volume of twice the above concentration of buffer, and it was homogenized for 60 sec plus 45 sec; the same total volume of straight buffer was then added and it was homogenized for an additional 15 sec, after which it was strained through two layers of gauze and frozen until assay. The second aliquot was treated in exactly the same manner, except that the same volumes of distilled water were added

Table 2. Effects of infusion of Gly-Gln on AcChoEase and BtChoEase contents of right and left cat SCG 48 hr postdenervation

Cat	Concentration infused, M	Substrate hydrolyzed, nmol/mg of protein per min: AcChoEase Right	AcChoEase Left	BtChoEase Right	BtChoEase Left
A	10^{-3}	239	457		
N-1	10^{-3}	279	317	352	145
	Mean ± SEM	259 ± 20	387 ± 70		
B	10^{-4}	280	345	211	234
C	10^{-4}	145	143	307	268
	Mean ± SEM	213 ± 68	244 ± 101	259 ± 48	251 ± 17
D	3×10^{-5}	252	331	352	518
E	3×10^{-5}	205	464	206	423
	Mean ± SEM	229 ± 24	398 ± 67*	279 ± 73	471 ± 48*
K	10^{-5}	309	369	293	312
R	10^{-5}	188	459	129	265
B-1	10^{-5}	201	281	304	405
	Mean ± SEM	233 ± 38	370 ± 51*	242 ± 57	327 ± 41
J	3×10^{-6}	316	364	282	355
G	3×10^{-6}	299	255	437	258
	Mean ± SEM	308 ± 9	310 ± 55	360 ± 78	307 ± 49
L	10^{-6}	226	154	325	242
M	10^{-7}	282	242	352	291

Mean ± SEM = $[\Sigma d^2/n(n-1)]^{1/2}$; where $n = 2$, SEM is equivalent to the range.
*Greater than controls (Table 1, group 5), $P < 0.025$.

Table 3. Effects of infusion of glycine and L-glutamine on AcChoEase and BtChoEase contents of right and left cat SCG 48 hr postdenervation

Cat	Compound	Concentration infused, M	Substrate hydrolyzed, nmol/mg of protein per min: AcChoEase Right	AcChoEase Left	BtChoEase Right	BtChoEase Left
A-1	Glycine	10^{-5}	245	267	—	305
G-1	Glycine	10^{-5}	297	366	310	327
H-1	Glycine	10^{-5}	260	244	315	272
		Mean ± SEM	280 ± 19		306 ± 9	
Y	L-Glutamine	10^{-5}	227	239	258	262
Z	L-Glutamine	10^{-5}	241	225	213	223
		Mean ± SEM	233 ± 4		239 ± 12	
X	L-Glutamine	3×10^{-6}	284	259	238	314
W	L-Glutamine	10^{-6}	269	138	349	—

Table 4. Effects of infusion of Gly-Glu on AcChoEase and BtChoEase contents of right and left cat SCG 48 hr postdenervation

Cat	Concentration infused, M	Substrate hydrolyzed, nmol/mg of protein per min			
		AcChoEase		BtChoEase	
		Right	Left	Right	Left
O-1	10^{-4}	237	249	195	220
P-1	10^{-4}	181	214	184	221
Q-1	10^{-4}	247	308	227	272
	Mean ± SEM	239 ± 17		220 ± 12	
C-1	10^{-5}	339	316	435	322
D-1	10^{-5}	316	254	481	394
E-1	10^{-5}	344	327	333	302
	Mean ± SEM	316 ± 13*		378 ± 29†	
I-1	3×10^{-6}	467	333	477	440
K-1	3×10^{-6}	197	250	230	258
	Mean ± SEM	312 ± 59		351 ± 63	
J-1	10^{-6}	366	338	234	161
L-1	10^{-6}	307	332	361	431
M-1	10^{-6}	313	306	385	379
	Mean ± SEM	327 ± 9‡		325 ± 43	

*Greater than controls (Table 1, group 5), $P < 0.01$.
†Greater than controls (Table 1, group 5), $P < 0.025$.
‡Greater than controls (Table 1, group 5), $P < 0.001$.

instead of the concentrated or straight buffer. AcChoEase and BtChoEase assays were conducted as usual (15), with a sample containing the inhibitors of both enzymes serving as a control for spontaneous hydrolysis of substrate in the presence or absence of Triton X-100/molar NaCl. For the protein assays (17) in the presence of Triton X-100, a standard curve with bovine serum albumen was prepared with the same concentration of Triton X-100 as in the samples to be assayed; following the addition of the phenol reagent, the precipitate formed in both the standard and assay solutions was removed by centrifugation (Dynac centrifuge, speed 80, 5 min) prior to measuring absorbance at 650 nm. Seven such experiments were performed.

RESULTS

Occasionally, cats failed to show complete relaxation of the nictitating membranes (Horner's syndrome) 24 hr following preganglionic denervation; in such cases the AcChoEase contents of the SCG were consistently abnormally high, indicating that sympathetic denervation of the ganglia was incomplete. Accordingly, such animals were excluded from the study.

Under identical conditions of treatment, values sometimes showed extreme variation (e.g., Table 4, cats I-1 and K-1). Thus, individual values obtained by the present assay can be misleading, and reliance can be placed only on mean values with sufficient numbers of observations to show significant differences between control and treated groups.

Controls. In the three denervated control cats infused for 24 hr with 0.9% NaCl solution following ligation of the EC and L arteries (Table 1, group 5), the mean value for the AcChoEase contents of the SCG was practically identical with that of cats continuously anesthetized with sodium pentobarbital from the time of denervation to 48 hr later, but without the arterial ligations (group 4). The mean value for denervated cats that were temporarily reanesthetized at 24 hr for ligation of the EC and L arteries (group 3) was considerably lower, although it was still significantly higher than that for denervated controls that were otherwise untreated (group 2). It therefore appears that continuous anesthesia with sodium pentobarbital is the critical factor in reducing or delaying the loss of ganglionic AcChoEase following denervation and that it is not significantly influenced by ligation of the EC and L arteries. It is also apparent that group 5 constitutes the appropriate controls against which the effects of infusion of putative NF must be compared, and not group 3 as was assumed previously. The modification of conclusions drawn in earlier studies of this series (3, 7, 8) entailed by these findings is noted in the *Discussion*.

As noted previously (7), values for BtChoEase showed extreme variation in control and most subsequent test series, as indicated by the high SEMs in comparison with those for AcChoEase.

Gly-Gln and Its Possible Metabolites. Results obtained with infusions of Gly-Gln at first appeared inconsistent. Then, as more data were accumulated, a distinct pattern emerged: at concentrations of 10^{-5} M and above, values for right SCG were within the control range, whereas those for left SCG were considerably elevated in all experiments except one (Table 2, cat C), in which results were unexplainably aberrant. In the two cats infused with 3×10^{-5} M (Table 2, cats D and E) and the three infused with 10^{-5} M (Table 2, cats K, R, and B-1) Gly-Gln, mean values for AcChoEase of the left SCG were greater than those of controls (Table 1, group 5) at $P < 0.025$; in the former group, BtChoEase was likewise significantly elevated. Of two infused with 3×10^{-6} M, a high value was obtained in the left SCG of one (Table 2, cat J); results were negative in the single cats infused with 10^{-6} and 10^{-7} M (Table 2, cats L and M).

Since infusions were made into the right common carotid artery, they reached the right SCG directly, whereas they arrived at the left SCG by way of a more circuituous route, probably chiefly through the anastomoses of the right and left

Table 5. Comparison of AcChoEase and BtChoEase activities of cat stellate ganglia homogenized with distilled water (Aq) or 1% Triton X-100/molar NaCl (TrX)

Cat	Protein, mg/ml			Substrate hydrolyzed, nmol/mg of protein per min					
				Aq		TrX		Aq/TrX	
	Aq	TrX	Aq/TrX	AcChoEase	BtChoEase	AcChoEase	BtChoEase	AcChoEase	BtChoEase
Q	0.112	0.165	0.679	260	342	292	391	0.890	0.875
R	0.175	0.197	0.888	169	284	235	434	0.719	0.647
S	0.142	0.168	0.842	186	215	249	339	0.747	0.636
T	0.133	0.190	0.700	266	—	310	—	0.857	—
U	0.099	0.148	0.669	316	382	303	419	1.043	0.912
V	0.127	0.198	0.641	211	268	171	246	1.234	1.089
Y	0.124	0.162	0.765	195	277	228	329	0.855	0.842
Mean	0.130	0.175	0.741	229	295	255	360	0.906	0.834
±SEM	±0.009	±0.007	±0.036	±20	±24	±19	±28	±0.068	±0.070

internal carotid arteries. This suggested that the neurotrophic effect of Gly-Gln at the left SCG might be due to a metabolite formed en route. We therefore tested glycine and L-glutamine as the two most likely candidates that might be formed by enzymatic hydrolytic cleavage of Gly-Gln but obtained only values within the range of the controls (Table 3). On the assumption that Gly-Gln might be deaminated by glutaminase in the same manner as glutamine, we then tested Gly-Glu. Within the range of 10^{-5}–10^{-6} M results were distinctly positive in both right and left SCG (Table 4). In spite of one low value (cat D-1, L-SCG), the difference between the means for AcChoEase contents of SCG from cats infused with 10^{-5} M Gly-Glu and controls was significant at $P < 0.01$, and the difference was significant for BtChoEase at $P < 0.025$. As noted above, the two cats infused with 3×10^{-6} M Gly-Glu (Table 4; cats J-1 and K-1) showed extreme variation; in cat K-1 values for both enzymes were also markedly low in the stellate ganglia. However, in the denervated SCG of three cats infused with 10^{-6} M Gly-Glu (Table 4; cats J-1, L-1, and M-1) values for AcChoEase were consistently high; the mean was greater than that of the controls (Table 1, group 5) at $P < 0.001$. Values for BtChoEase again showed marked variation. In most cats within these groups, values for both enzymes in the right SCG were somewhat higher than in the corresponding left SCG, in contrast with results obtained with Gly-Gln. When Gly-Glu was infused at 10^{-4} M, mean values for both AcChoEase and BtChoEase were somewhat below corresponding control values, in contrast with results obtained with infusions at lower concentrations. This unexpected finding is considered in the *Discussion*. Gly-Glu exhibited no anticholinesterase activity *in vitro* following incubation of homogenates of stellate ganglia for 1 hr with concentrations up to 10^{-3} M prior to addition of substrate.

Homogenization Procedure. In the seven experiments in which the two homogenization procedures were compared, there was considerable variation with respect to the relative amounts of both protein and enzyme activities obtained (Table 5). The mean value for the ratios of protein recovered by aqueous compared with Triton X-100/molar NaCl homogenization was 0.741 ± 0.036. The means for the ratios of AcChoEase and BtChoEase, in terms of substrate hydrolyzed per mg of protein per min, were 0.906 ± 0.068 and 0.834 ± 0.070, respectively.

DISCUSSION

Present findings indicate that Gly-Gln is the precursor of a NF for the maintenance of AcChoEase and BtChoEase in the preganglionically denervated SCG of the cat. Of its possible metabolites tested, glycine and L-glutamine were found to be inactive. Gly-Glu showed no effect, or possibly an inhibitory effect at 10^{-4} M, but showed a distinctly positive effect at 10^{-5}–10^{-6} M. This dual action is difficult to explain. In the *in vivo* studies that led to the present investigation, it will be recalled that β-endorphin had an inhibitory effect on the conversion of G_1 to A_{12} AcChoEase in cultured skeletal muscle (11), whereas its metabolite, Gly-Gln, had a stimulant effect (13). As a pharmacological analogy, acetylcholine and its derivatives produce excitation in low and inhibition at high doses at nicotinic receptor sites.

Although Gly-Glu has been demonstrated to act as a NF when infused at concentrations of 10^{-5}–10^{-6} M, it is not necessarily the major metabolite of Gly-Gln that is responsible for its indirect neurotrophic effect. It is also possible that Gly-Gln exerts its indirect effect by releasing an endogenous NF. It also remains to be determined whether Gly-Gln or Gly-Glu is the agent in extracts of the central nervous system that maintains the levels of AcChoEase and BtChoEase in SCG following preganglionic denervation (3) or is released by preganglionic fibers to maintain the enzymes at postsynaptic membranous sites under physiological conditions. Another question that awaits resolution is the site of action of the NF of central nervous system extracts and of Gly-Gln and Gly-Glu—i.e., prevention or delay of degeneration of preganglionic fibers or direct maintenance of postsynaptic AcChoEase and BtChoEase.

The finding of the maintenance of relatively high levels of AcChoEase and BtChoEase in denervated SCG following ligation of the EC and L arteries and infusion of saline solution from 24 to 48 hr postdenervation [i.e., equivalent to the values obtained previously (7) in continuously anesthetized, nonligated cats] requires some modifications of conclusions drawn from earlier studies in this series (3, 7, 8). By comparison with the new control series, previous reports of positive results obtained with infusion for 24 hr of extracts of central nervous system (3) and its dialysates at molecular weight cutoff 1000 (8) are confirmed. However, the results obtained with infusion of extract for periods of 12 hr or less (7) and for 24-hr infusions of aprotinin (Trasylol) alone (3), and of nerve growth factor, and carboxypeptidase-treated dialysates (8) now appear to be negative; those obtained with cyclic AMP and extracts of small intestine become borderline (8).

It has been demonstrated that aqueous homogenates of SCG, employed throughout this series of experiments, provide values of ≈91% for AcChoEase and ≈83% for BtChoEase of those obtained by homogenization with 1% Triton X-100/molar NaCl.

Although attention in the current studies has been focused on the effects of endogenous and synthetic NFs on the maintenance of AcChoEase, and to a lesser extent of BtChoEase, in the denervated cat SCG, it is of course possible that these agents also affect significantly other components of the cholinergic system and of other systems as well. These aspects likewise remain to be explored.

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