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Effects of glycyl-L-glutamine and methylprednisolone on maintenance of acetylcholinesterase of transected rat sciatic nerves

(neurotrophic factor/trophic factor)

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ABSTRACT **Under anesthesia with sodium pentobarbital, the sciatic nerves of rats were transected bilaterally, and a catheter was inserted into the central end of the left renal artery. After an initial flush, an Alzet pump was attached to the catheter, containing various concentrations of glycyl-L-glutamine (Gly-Gln), methylprednisolone sodium succinate (MePred), or both. Rats were sacrificed at intervals of 2, 4, or 6 days; the peripheral portions of the sciatic nerves were excised, homogenized, and centrifuged, and the supernates were assayed for acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) and protein. Significantly higher contents of AcChoEase over untreated transected controls were obtained (*i*) at 4 days posttransection in rats infused with 0.015 M Gly-Gln and (*ii*) at 6 days posttransection in rats infused with MePred at 3.0 mg·kg^{-1}·hr^{-1} after an initial dose of 120 mg/kg with or without Gly-Gln.**

An investigation initiated some years ago (1) culminated in the demonstration that glycyl-L-glutamine (Gly-Gln), delivered appropriately, maintains temporarily the acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) content of the preganglionically denervated cat superior cervical ganglion (summarized in ref. 2). It was postulated that Gly-Gln enhances the transcription of the DNA encoding AcChoEase into the corresponding mRNA, in a manner analogous to that proposed for triiodothyronine in the regulation of protein synthesis (3). It was then found that Gly-Gln also maintains the choline aceyltransferase (ChoAcTr; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) content of the denervated cat superior cervical ganglion (4). Since ChoAcTr, in contrast to AcChoEase, is located exclusively presynaptically, this observation suggested the possibility that Gly-Gln maintains the viability of transected axons. The present study was designed to test this hypothesis, using AcChoEase content as an index of axonal viability.

The procedure used was similar to that used earlier in a study of the effect of Gly-Gln on the regeneration of AcChoEase in the rat gastrocnemius after its inactivation by diisopropyl phosphorofluoridate (5). In brief, under anesthesia with sodium pentobarbital the sciatic nerves were transected bilaterally. The left renal artery was then exposed, catheterized, and flushed, and an Alzet miniosmotic pump containing the solution under test was attached. Rats were sacrificed 2, 4, or 6 days later by an overdose of sodium pentobarbital and the peripheral portions of the sciatic nerves were excised, homogenized, and assayed for AcChoEase and protein. In view of reports that methylprednisolone (MePred) enhances recovery after damage to the spinal cord (6), that compound was also studied, both alone and in combination with Gly-Gln.

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METHODS

Rats were anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneally). Both sciatic nerves were exposed high in the leg via dorsal approaches, ligated centrally, and transected, and the operative wounds were sutured. (After recovery from anesthesia, this operation results in surprisingly little impairment of ambulation.) The left kidney was exposed, and the renal artery was carefully separated from the vein and catheterized with Intramedic polyethylene tubing (no. 7400; o.d., 0.61 mm; Clay Adams) extended to the aorta. The catheter was flushed with 0.4 ml of the solution under test, also containing 50 units of heparin, and an Alzet miniosmotic pump (model 2 ML1; total content, 2.0 ml; delivery rate, 10 μl/hr) containing the test solution was attached. The minipump was loosely implanted intraperitoneally, the wound was sutured, and the rat was given 6.0 ml of 0.9% NaCl solution intraperitoneally, to maintain flow from the pump, and 0.3 ml of Combiotic (penicillin plus dihydrostreptomycin) subcutaneously. At intervals of 2, 4, or 6 days, rats were sacrificed by an overdose of sodium pentobarbital. The peripheral portions of both sciatic nerves were excised, trimmed of extraneous tissue, weighed, and homogenized in AcChoEase extraction medium (1 M NaCl/1% Triton X-100/0.05 M $MgCl_2$/0.02 M Tris·HCl, pH 7.4) at 0.1 ml/mg (wet weight). Homogenization was done in an ice-water bath in a ground glass tube with a motor-driven ground glass pestle. Homogenates were centrifuged for 60 min at full speed in a Dynac centrifuge (Clay Adams), and the faintly opalescent supernate was assayed for AcChoEase by a modification (7) of the method of Ellman *et al.* (8) and for protein by the bicinchoninic acid method (9).

The concentrations of Gly-Gln tested were chosen as follows. In the earlier study cited (5), a maximal effect on AcChoEase regeneration was obtained with 0.05 M Gly-Gln, using a minipump (model 2001) that delivered at one-tenth the rate of those used in the present study (model 2 ML1). The initial concentration tested here was therefore 0.005 M. It was found to have no significant effect. When the concentration was increased 3-fold to 0.015 M, a maximal effect was obtained at 4 days posttransection; at 3.3 times the latter dose (0.05 M), the effect was reduced. This was consistent with results obtained previously (2, 4, 5, 10): a diminished response at concentrations above the optimal level. If it is assumed that the optimal dose of Gly-Gln should remain the same with the present procedure, this can be accounted for by the presumably greatly reduced delivery of blood to the sciatic nerve in comparison with that to the gastrocnemius muscle. It was demonstrated that intraaortic administration of a drug results in its effective delivery to the sciatic nerve by the finding that the injection of 1.0 ml of 0.01 M diisopropyl

Abbreviations: AcChoEase, acetylcholinesterase; MePred, methylprednisolone sodium succinate; ChoAcTr, choline acetyltransferase.

phosphorofluoridate via similarly introduced catheters in two rats caused inactivation of essentially all the AcChoEase of the sciatic nerves.

The dose of MePred used was based on reports in the literature (6, 11) that to obtain significant improvement after injury to the spinal cord it was necessary to give an initial dose of 15–60 mg/kg i.v., followed by repeated hourly doses of 6.0 mg/kg i.v. With the procedure used here, it was not feasible to deliver that high a maintenance dose. Consequently, the concentration of MePred (Solu-Medrol, Upjohn) used (10%) was calculated to deliver an initial intraaortic dose of ≈120 mg/kg, followed by an hourly dose of ≈3.0 mg/kg. In retrospect, a lower initial dose would probably have resulted in an improved survival rate (see below). MePred was administered separately or in combination with Gly-Gln; in all cases, heat-inactivated (60°C for 6 min) horse serum was included to enhance penetration of Gly-Gln across neuronal membranes (12).

Experiments were conducted initially with large (500–600 g) male rats to facilitate catheterization of the renal artery. It was then found that effects of Gly-Gln were more readily detectable in smaller animals. All results reported here were obtained with female rats weighing 320–370 g. In studies in which only Gly-Gln was used, fatalities prior to the planned time of sacrifice rarely occurred. However, with the dose of MePred used, approximately half the animals expired before the scheduled time of sacrifice, with or without the inclusion of Gly-Gln. Nearly all deaths occurred during the first 24 hr.

RESULTS

All results are summarized in Table 1.

Transected Controls. After transection of the sciatic nerves, the AcChoEase contents of the peripheral portions fell to ≈80% (2 days), 64% (4 days), and 47% (6 days) of the control values. These figures are similar to those reported by Sawyer (13) many years ago.

Gly-Gln. At 2 days posttransection, the infusion of Gly-Gln over the range 0.005–0.05 M produced insignificant increases in AcChoEase content over controls. Four days after transection, 0.015 M Gly-Gln resulted in a significantly higher content of AcChoEase (77.4%) in comparison with controls (63.6%). The lower dose was without apparent effect, and the higher dose produced a lesser, insignificant effect. At 6 days posttransection, 0.015 M Gly-Gln produced an increase in AcChoEase (51.7%) but this was no longer significant.

MePred. MePred alone or in combination with 0.015 M Gly-Gln resulted in a small but statistically insignificant increase in AcChoEase at 4 days; only a limited number of animals were tested at this interval. However, at 6 days posttransection, MePred, alone or in combination with 0.015 M Gly-Gln, resulted in markedly higher levels of AcChoEase than in the corresponding controls; in fact, the levels sustained (≈70%) were essentially the same as those at 4 days with similar treatment.

DISCUSSION

Two statistically significant results were obtained in the present study: (*i*) a moderately higher content of AcChoEase in transected sciatic nerves in 0.015 M Gly-Gln-infused rats over corresponding controls at 4 days posttransection, and (*ii*) a markedly higher content in MePred-treated animals (*a*) without or (*b*) with added a Gly-Gln, at 6 days posttransection. Since the mean values for *a* and *b* were nearly identical, it can be assumed that the effect was due chiefly to MePred.

The mechanism of action of Gly-Gln is unknown. Its action in maintaining the AcChoEase of the preganglionically denervated cat superior cervical ganglion was hypothesized to be due to regulation of the transcription of the gene(s) for AcChoEase to the corresponding mRNA (2). However, this type of action would not account for the subsequent finding that Gly-Gln also maintains the ChoAcTr of the denervated ganglion, since the latter enzyme is restricted to the presynaptic nerve terminals (4). Other reported actions of Gly-Gln include inhibition of neurons of the brainstem after iontophoretic application (14), modulation of immune functions (10), and release of arachidonic acid and its metabolites from cultured embryonic cortical neurons by activation of phospholipase A_2 (L. W. Haynes, personal communication). In essentially all these effects, Gly-Gln exhibited an optimal concentration–response effect above or below which no response was obtained. At present, no common mechanism can be suggested for the production of all these various effects. It is likely that more than one mechanism is involved.

In designing the present investigation of MePred, we used a higher initial dose (120 mg/kg) than that recommended for clinical use (15–60 mg/kg), since it was feasible to deliver a maintenance dose of only 3.0 mg/kg rather than the recommended dose of twice this amount (6, 11). The high initial dose was probably responsible for the high incidence of fatalities encountered, since they generally occurred during the first 24 hr. Whether this was due to gastrointestinal hemorrhage or some other cause could not be determined.

In reviewing the actions of high doses of MePred and other cortical steroids that might contribute to their beneficial effects in the treatment of spinal cord injury, Hall and Braughler (6) emphasized three likely mechanisms: facilitation of neuronal excitability (15), improved blood flow (16), and preservation of neuronal ultrastructure through reduction of lipid peroxidation (17, 18, 19). Each of these actions requires far higher doses than is necessary to activate standard corticosteroid receptors.

The primary contribution of the present study to our understanding of the mechanism of MePred action is that it localizes the effect to the axon itself, since both the neuronal perikarya and the axonal terminals were excluded from the

Table 1. Effect of Gly-Gln and/or MePred on maintenance of AcChoEase of rat sciatic nerves at 2, 4, and 6 days posttransection

Alzet infusion	% control AcChoEase		
	2 days	4 days	6 days
Transected control	79.7 ± 2.9 (16)	63.6 ± 3.7 (14)	46.5 ± 2.4 (14)
Gly-Gln (0.005 M)	83.4 ± 3.7 (10)	64.7 ± 5.7 (4)	
Gly-Gln (0.015 M)	81.6 ± 2.7 (12)	77.4 ± 3.7* (14)	51.7 ± 2.1 (12)
Gly-Gln (0.05 M)	84.4 ± 5.2 (5)	70.8 ± 3.1 (6)	
MePred (120 mg/kg) + 3.0 $mg \cdot kg^{-1} \cdot hr^{-1}$		70.1 ± 1.9 (6)	69.4 ± 2.4† (10)
Gly-Gln (0.015 M) + MePred (as above)		74.3 ± 4.8 (6)	71.5 ± 3.7† (14)

Number of sciatic nerves is in parentheses. Values are expressed as percent of a mean of seven control sciatic nerves (3.85 ± 0.16 nmol of acetylthiocholine hydrolyzed per mg of protein per hr) ± SEM.
*Greater than mean of transected controls; $P < 0.025$.
†Greater than mean of transected controls; $P < 0.001$.

samples taken. As a therapeutic consideration, these findings suggest that continuation of treatment with MePred for 6 days or longer might offer a distinct advantage over the 1-day treatment used in the recent collaborative clinical study (11). Findings also suggest, but do not prove, that the beneficial effects of MePred might be enhanced by the addition of Gly-Gln.

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