Neurochem. Int. Vol. 7, No. 1, pp. 111–123, 1985
Printed in Great Britain.

0197-0186/85 $3.00 + 0.00

COMPARISON OF ENDOGENOUS PHOSPHORYLATION OF HEN AND RAT SPINAL CORD PROTEINS AND PARTIAL CHARACTERIZATION OF OPTIMAL PHOSPHORYLATION CONDITIONS FOR HEN SPINAL CORD*

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(*Received* 30 *January* 1984; *revised* 25 *May* 1984)

Abstract—The optimal conditions for the endogenous phosphorylation of hen spinal cord cytosolic and membrane proteins with 5 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, were determined by 10% SDS-polyacrylamide gel electrophoresis, autoradiography, and microdensitometry. Phosphate incorporation increased linearly with concentrations ranging from 35–75 μg/100 μl for cytosolic proteins and 21–125 μg/200 μl for membrane proteins. Optimal incubation times, temperatures, and pH values were 60 s, 30°C, and 6.0, respectively, for spinal cord cytosolic proteins and 15 s, 45°C, and 8.0, respectively, for spinal cord membranes. Prominent species differences in protein phosphorylation between these fractions in hens and similarly prepared fractions in rats, co-electrophoresed, include 80K and 30K protein phosphate acceptors unique to rat spinal cord cytosol, 60K and 16K protein phosphate acceptors characteristic of rat spinal cord membranes, a 50K protein phosphate acceptor present only in hen spinal cord membranes, and greater phosphorylation of a more abundant 20K protein in both hen spinal cord fractions. The functional significance of these differences is presently unclear. However, their characterization provides a basis from which to launch future investigations of the biochemistry, pharmacology, and toxicology of spinal cord protein phosphorylation and indicates that caution should be exercised in the choice of an animal model with characteristics appropriate to those of the system it is representing.

One of the many regulatory mechanisms of the central nervous system is protein kinase-catalysed protein phosphorylation utilizing ATP as the phosphate donor. It is known to mediate physiological responses for a number of neuronal second messengers. The most widely known response is that to cyclic nucleotides, for which it is the primary, if not exclusive, type of response. For example, the synaptic vesicle-associated protein, Synapsin I (Protein I, Kennedy and Greengard, 1981) is a substrate for cAMP-dependent protein kinase (Ueda *et al.*, 1979), as is the regulatory subunit of the type II cAMP-dependent protein kinase (Treiman *et al.*, 1983).

However, calcium also elicits this type of protein phosphorylation response, among other physiological responses which might require the intervention of the calcium-binding regulatory protein calmodulin (Kennedy, 1983). The previously mentioned Synapsin I is a substrate for calcium (Ca^{2+}) and calmodulin-activated as well as cAMP-activated protein phosphorylation (Kennedy and Greengard, 1981). Other nervous system substrates for Ca^{2+}-dependent phosphorylation include three of the four subunits of the acetylcholine receptor (δ and γ more than β; Smilowitz *et al.*, 1981) and two synaptic vesicle-associated proteins whose phosphorylation is inhibited by the anticonvulsant phenytoin (DeLorenzo, 1976), DPH-M (M_r 52K–54K) and DPH-L (M_r 60K–64K) which have subsequently been shown to be composed primarily of α and β-tubulin (Burke and DeLorenzo, 1982).

The *in vitro* assay for endogenous protein phosphorylation, using [γ-^{32}P]ATP as the phosphate donor, is a convenient tool for investigating certain aspects of normal physiological functioning in the nervous system. Furthermore, it lends itself to

*A preliminary account of this work has been presented (Patton *et al.*, 1983b).

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exploring the effects of perturbation of the protein phosphorylation system by xenobiotics. One should, however, be aware that proper interpretation of the results generated by this assay is often not straightforward, for the following reasons. In the extrapolation from *in vitro* to *in vivo* data, caution must be exercised. A net increase in the incorporation of radiolabelled phosphate *in vitro* has been postulated to result from a net dephosphorylation of available substrate protein sites *in vivo* (Bar *et al.*, 1981). It could be concluded from the aforementioned studies that ^{32}P incorporation seen in nervous tissue proteins is the result of the interplay of at least three types of enzymes. These enzymes include the protein kinases of various cofactor specificities, phosphoprotein phosphatases, and ATPases. Although quantitative differences in *in vitro* versus *in vivo* phosphorylation occur, qualitative differences have not been observed. Substrate proteins for *in vitro* phosphorylation were demonstrated to co-migrate by SDS-polyacrylamide gel electrophoresis with similarly prepared phosphoproteins which had been radiolabelled *in vivo* (Mitrius *et al.*, 1981).

The technique of *in vitro* [γ-^{32}P]ATP phosphorylation of neuronal proteins has been most commonly applied to rat brain proteins. Other nervous system tissues have not been as extensively studied. In this report, we have characterized the *in vitro* phosphorylation of the adult hen spinal cord cytosolic and membrane fractions. In addition, we have compared the phosphorylation of these proteins to similarly prepared mammalian rat spinal cord cytosolic and membrane fractions. This comparison parallels our previous comparison of hen brain with rat brain phosphorylation of proteins from cytosolic and membrane fractions prepared in the same may. Accordingly, similarities and differences in protein phosphorylation between different areas of the central nervous system may be directly contrasted in both of these species, with rat brain as the reference point. The adult hen is particularly vulnerable, while the rat and other rodents are quite insensitive, to organophosphorus-induced delayed neurotoxicity, a distal axonopathy characterized by neuropathological lesions in the dorsal columns and spinocerebellar tracts in the cervical region and in the corticospinal tract in the lumbosacral region of the spinal cord (Abou-Donia, 1981). Studies on the phosphorylation of spinal cord proteins should provide added insight into the development of this neuropathy, which has already been demonstrated to alter protein phosphorylation of chicken brain synaptosomes (Patton *et al.*, 1983a; Abou-Donia *et al.*, 1984). The characterization of protein phosphorylation differences in hen and rat spinal cord should be useful in facilitating further mechanistic investigations into this neuropathy.

EXPERIMENTAL PROCEDURES

Materials

[γ-^{32}P]ATP (4500 Ci/mmol) was obtained from ICN (Irvine, CA). Tris, ultrapure, was from Schwarz-Mann, Inc. (Spring Valley, NY) and glycerol was from Mallinkrodt (Paris, KY). All other materials used in the preparation of the polyacrylamide gels and associated buffers were of electrophoresis grade and were purchased from Bio-Rad Laboratories (Richmond, CA), as were Coomassie brilliant blue G-250 (protein assay dye reagent), bovine plasma gamma globulin, and Chelex 100. Ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), piperazine-*N,N'-bis*-(2-ethane-sulfonic acid) (PIPES), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St Louis, MO). Bovine brain calmodulin was provided by Dr Thomas C. Vanaman, Duke University Medical Center (Durham, NC). Trifluoperazine was the gift of Dr Leon C. Greene of Smith, Kline and French Laboratories (Philadelphia, PA).

Care and treatment of animals

Adult leghorn laying hens (*Gallus gallus* domesticus), each 14-months old and weighing approx 1.5 kg, were obtained from Featherdown Farm (Raleigh, NC). The hens were anesthetized with CO_2 and sacrificed by decapitation. In some experiments, young adult male rats, weighing approx 200 *g*, were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and were stunned and then also sacrificed by decapitation.

Preparation of subcellular fractions

Immediately following sacrifice, spinal cords were removed by the forceful injection of ice-cold 0.9% saline in the vertebral canal (Meikle and Martin, 1981) and each was immersed in ice-cold 0.32 M sucrose, 0.3 mM PMSF, 50 mM Tris–HCl, pH 7.4. Motor-driven Teflon-glass homogenization (six strokes) in fresh aliquots of the same buffer at 10% w/v, followed by differential centrifugation of the homogenate at 1000 *g* and then the S_1 supernatant at 10,000 *g*, yielded the crude mitochondrial fraction (P_2). Each P_2 fraction was subjected to hypoosmotic shock in 250 μl of 5.0 mM Tris, pH 8.0, 0.3 mM PMSF, per gram original tissue for 60 min with occasional Vortex mixing, as described previously (Patton *et al.*, 1983a). The resulting lysate was centrifuged at 140,000 *g* for 30 min, and the pellet and supernatant obtained were used as sources of membranes and cytosol, respectively. The membranes were stored frozen at -70°C for 1 wk prior to assay. In some experiments, the protease inhibitor PMSF (0.3 mM) was added to all preparative buffers based on its ability to preserve the Ca^{2+}-calmodulin tubulin kinase in rat brain (Burke and DeLorenzo, 1981).

Phosphorylation assays

The standard phosphorylation reaction was initiated by the addition of a 25 μl protein aliquot containing 35 (range = 25–75, when varied) μg of hen or rat spinal cord cytosol or 100 (range = 25–170, when varied) μg of crude P_2

membranes, in 5.0 mM Tris, pH 8.0, to the reaction mixture. The standard reaction mixture also contained, in a final volume of 200 μl: 50 mM PIPES, pH 6.5 (range = 6.0–7.0, when varied; with 50 mM HEPES for pH 7.5, 8.0), 10 mM $MgCl_2$, 0.3 mM PMSF (only where indicated), and 5.0 μM [γ-^{32}P]ATP (4500 Ci/mmol) at 30°C (range = 25–50, when varied). Additionally, cytosolic proteins were incubated in the absence or presence of 5 or 50 μM $CaCl_2$, 100 μM EGTA, or 50 μM $CaCl_2$ and 50 μM trifluoperazine, whereas membrane proteins were incubated in the absence or presence of 300 μM $CaCl_2$ alone or in combination with 1 μg calmodulin. The reaction was terminated after 60 (range = 30–120, when varied) seconds for cytosol or 20 (range = 5–60, when varied) seconds for membranes by the addition of 100 μl of "stop solution", containing 0.125 M Tris–HCl, pH 6.8, 4.5% SDS, 20% glycerol and 10% β-mercaptoethanol, followed by heating for 3 min in a 90°C water bath. Protein concentrations were determined by the method of Bradford (1976) using Coomassie brilliant blue G-250 with bovine plasma gamma globulin as a standard. The deionized water used in the phosphorylation assays as well as in the homogenization, lysis, and electrophoresis gel buffers was passed through a Chelex 100 column to ensure thorough removal of Ca^{2+}. The assays were performed in polyethylene test tubes, and all solutions in contact with the tissue samples before the addition of "stop solution" were stored in plastic containers which do not contribute exogenous calcium to their contents.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

The cytosolic and membrane proteins were resolved on 20 well SDS-polyacrylamide vertical slab gels (12 × 16 × 0.15 cm) (Bio-Rad Laboratories, Richmond, CA). Electrophoresis was performed using the stacking, resolving, and running gel buffers described by Laemmli (1970) with 6 and 10% acrylamide in the stacking and resolving gels, respectively (36.5:1 acrylamide–bisacrylamide). Aliquots (50 μl) of the samples were subjected to electrophoresis under conditions of constant current (30 mAmp/gel through the stacking gel and 40 mAmp/gel through the resolving gel) at 13–15°C until the tracking dye reached the bottom of the gels (3.5–4.0 h). The apparent molecular weights of the resolved proteins were determined from M_r (electrophoretic mobility) standards (Bio-Rad Laboratories, Richmond, CA) which had been subjected to electrophoresis under conditions identical to those used for the tissue samples and are presented in kilodaltons (K). Following electrophoresis, the gels were fixed and stained for protein overnight with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid. The gels were destained with 50% methanol and 10% acetic acid (2-3 h) in a diffusion destainer employing activated charcoal (Bio-Rad Laboratories, Richmond, CA) and then soaked (1 h) in 10% acetic acid and 1% glycerol (to prevent cracking) before drying for 2 h between two sheets of dialysis membrane (Bio-Rad Laboratories, Richmond, CA) at 80°C using a water aspirator to withdraw the vapor.

Autoradiography

Autoradiographs of the ^{32}P-labelled proteins were obtained by placing the dried gels in close contact with Kodak X-OMAT RP X-ray film (0.5–3.0 days) at −70°C (Laskey and Mills, 1977) using intensify screens (Dupont Cronex Lighting Plus, CGR Corp., Raleigh, NC.

Microdensitometry

The amount of phosphoprotein in each autoradiographic band was quantified by integration of the area under the corresponding peak in the densitometric scan obtained by using a Quick-Scan (Helena Laboratories, Beaumont, TX) Transmission Microdensitometer or a Zeineh Soft Laser Densitometer (BioMed Laboratories, Inc., Fullerton, CA) interfaced with a PDP-11 Computer (Digital Equipment Corp., Maynard, MA) programmed for integration or an LKB Ultroscan Laser Densitometer interfaced with a Recording Integrator (LKB Instruments, Gaithersburg, MD). All data which were to be directly compared were scanned on the same instrument, taking the background darkness of the autoradiographs as the baseline. Since the darkness of the autoradiographic bands, and therefore the peak areas in the densitometric scans, depend not only on the level of protein phosphorylation and specific radioactivity of the [γ-^{32}P]ATP but also on the duration and temperature of film exposure, during which the radioactivity of the incorporated ^{32}P may decrease significantly, all phosphorylation results are reported in arbitrary units.

Statistics

Statistical comparisons between hen and rat spinal cord protein phosphorylation were based on the Student's unpaired two-tailed t-test. Statistical comparisons of effects of cofactor concentration within the same animal were based on the Student's paired two-tailed t-test.

RESULTS

In order to describe the optimal *in vitro* protein phosphorylation assay conditions for hen spinal cord cytosol and membranes (shocked P_2), the protein concentration, incubation time, temperature, and pH were each varied in turn while the other three were held constant. The ^{32}P-labelled proteins from each combination of assay conditions were electrophoresed on polyacrylamide gels in the presence of SDS. Protein phosphorylation, expressed in arbitrary units of area under the peaks in the densitometric scans of the autoradiographs of the dried gels, was plotted versus each varying parameter for the major peaks, identified by their electrophoretic mobilities (M_r) in the four preparations (Figs 1–4).

Protein concentrations

Phosphate incorporation was found to increase linearly with protein concentration, at least over a range of 35–75 μg/200 μl for the spinal cord cytosolic 20K band (Fig. 1a). The increase in phosphate incorporation with increasing protein concentration was greater above than below 50 μg/200 μl for the 55K and 70K bands in spinal cord cytosol, for which low yield placed limitations on the amount of assays which could be performed with a single animal. Protein phosphorylation increased in nearly linear

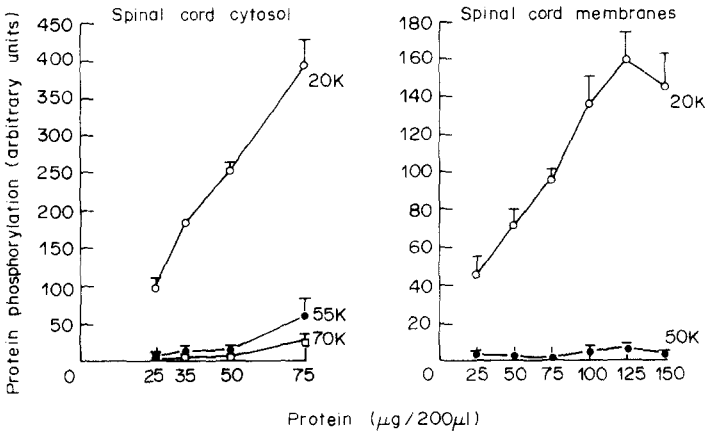

Fig. 1. Protein concentration curves for phosphorylation in hen spinal cord cytosol and membranes. ^{32}P Incorporation *in vitro* from [γ-^{32}P]ATP, under the conditions described in the Methods section into various concentrations of neuronal proteins is plotted as the corresponding density ± SEM (in arbitrary units) of the autoradiographs of the SDS–PAGE bands with indicated electrophoretic mobilities (n = 5).

fashion to a maximum of 125 μg/200 μl for both spinal cord membrane bands (Fig. 1b).

Incubation time

Phosphate incorporation into spinal cord cytosolic proteins fell slightly after 60 s at which time it was maximal for the 20K band and before which it was still greater for the two higher M_r bands (Fig. 2c). The optimal time was 20 s for the spinal cord membrane protein.

Incubation temperature

Different proteins responded differently to variations in incubation temperature. In spinal cord cytosol, the *in vitro* phosphorylation of the 55K and 70K bands exhibited no significant alterations up to 45°C, while the ^{32}P incorporation into the 20K band plummeted sharply above its maximum at 30°C (Fig. 3a). In contrast, phosphate incorporation increased fairly linearly at least up to 45°C in the 20K band of spinal cord membranes (Fig. 2b).

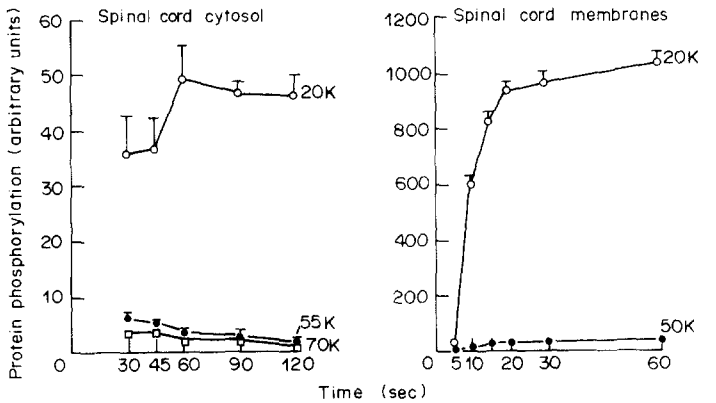

Fig. 2. Incubation time curves for phosphorylation in hen spinal cord cytosol and membranes. Protein ^{32}P incorporation, over various times of incubation *in vitro* with [γ-^{32}P]ATP under the conditions described in the Methods section is plotted as the corresponding density ± SEM (in arbitrary units) of the autoradiographs of the SDS–PAGE bands with indicated electrophoretic mobilities (n = 5).

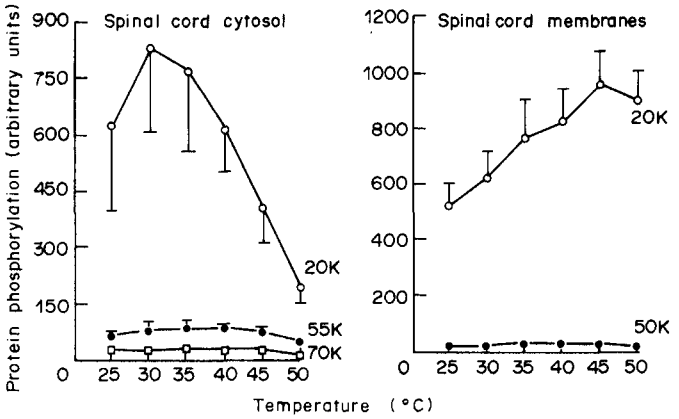

Fig. 3. Incubation temperature curves for phosphorylation in hen spinal cord cytosol and membranes. Protein ^{32}P incorporation, at various incubation temperatures, with the presence of [γ-^{32}P]ATP under the conditions described in the Methods section, is plotted as the corresponding density ± SEM (in arbitrary units) of the autoradiographs of the SDS–PAGE bands with indicated electrophoretic mobilities ($n = 5$).

Incubation pH

The protein phosphorylation response to pH varied considerably with the protein (Fig. 4). The spinal cord cytosolic 20K protein was preferentially phosphorylated at pH 6.0 or less, while the spinal cord membrane 20K protein was preferentially phosphorylated at pH 8.0 or greater. The higher molecular weight proteins in both spinal cord fractions seemed to be phosphorylated relatively independently of pH.

Effect of trifluoperazine

Calmodulin is a cytosolic protein capable of transducing many of the cellular actions of Ca^{2+} including its role as a second messenger for protein phosphorylation. Accordingly, an inhibitor of calmodulin activity, trifluoperazine, was employed to investigate the involvement of calmodulin in hen spinal cord cytosolic protein phosphorylation. *In vitro* addition of 50 μM trifluoperazine inhibited the phosphory-

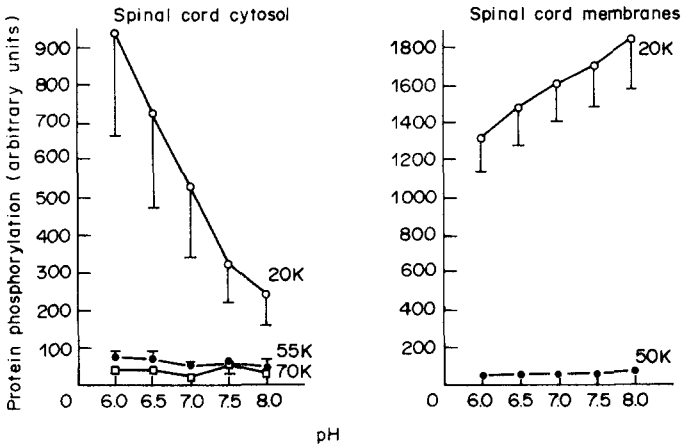

Fig. 4. Incubation pH curves for phosphorylation in hen spinal cord cytosol and membranes. Protein ^{32}P incorporation *in vitro* in the presence of [γ-^{32}P]ATP under the conditions described in the Methods section, except at various pHs, is plotted as the corresponding density ± SEM (in arbitrary units) of the autoradiographs of the SDS–PAGE bands with indicated electrophoretic mobilities ($n = 5$).

Table 1. Effect of calmodulin inhibitor trifluoperazine on the *in vitro* phosphorylation of hen spinal cord cytosolic proteins

M_r(K)	Control (arbitrary units)*	Trifluoperazine (arbitrary units)*	% Of control†	Significance‡
70	39.3 ± 3.3	2.8 ± 1.5	7.1	$P < 0.001$
55	242.2 ± 46.3	9.5 ± 2.3	4.0	$P < 0.005$
20	905.8 ± 162.8	30.6 ± 6.8	3.4	$P < 0.005$

*The net *in vitro* incorporation of ^{32}P from [γ-^{32}P]ATP in the presence of 50 μM Ca^{2+} under standard assay conditions, into hen spinal cord cytosolic proteins, separated by SDS–PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels and is expressed as the mean ± SEM of independent observations from 6 hens in the absence of *in vitro* addition of 50 μM trifluoperazine and 4 hens in its presence (phosphorylation was below the threshold for detection in the other 2 hens under these conditions).

†The percentage that the mean *in vitro* protein phosphorylation (for 5 hens) in the presence of trifluoperazine is of that in its absence.

‡Significance of the difference between ^{32}P incorporation in the presence and that in the absence of trifluoperazine, based on the Student's unpaired two-tailed *t*-test.

lation of all detectable hen spinal cord cytosolic proteins (70K, 55K and 20K), in the presence of 50 μM Ca^{2+}, to less than 10% of control. This suggests a significant dependence on calmodulin and implies a requirement for some level of Ca^{2+}, either exogenous or endogenous, for the phosphorylation of proteins with each of these electrophoretic mobilities.

Effect of phenylmethylsulfonyl fluoride (PMSF)

The effect of addition of the protease inhibitor phenylmethylsulfonyl fluoride to all preparative (homogenization, lysis and resuspension) buffers was investigated. We found such addition to have a significant protective effect (an increase in ^{32}P incorporation in the presence of PMSF to 149.5 ± 10.9% of that in the absence of PMSF) only on the Ca^{2+}-independent phosphorylation of the 20K band, in hen spinal cord membranes, tentatively identified as myelin basic protein, on the basis of its size and co-electrophoresis with a hen brain myelin protein (Patton *et al.*, 1984). Unlike the cytosolic fractions which were used only when fresh, these membrane fractions were resuspended and stored at −80°C for 1 wk prior to phosphorylation. The addition of PMSF to the actual reaction medium to a final concentration of 300 μM, after either its inclusion or exclusion from the preparative buffers, was not observed to significantly enhance the phosphorylation of any proteins examined. Indeed, it actually inhibited the phosphorylation of the 20K spinal cord cytosolic protein to 75.7 ± 5.3% of control.

Comparisons between hen and rat spinal cord protein phosphorylation

Comparisons of staining and autoradiographic patterns of spinal cord phosphorylated proteins from the hen and the rat, which were co-electrophoresed on the same gel slab, disclose several striking differences.

In spinal cord cytosol, the Ca^{2+}-dependent phosphorylation of the protein triplet at approx 55K is only statistically significant in the hen, but it is nonetheless quite visually apparent for the highest M_r band in the rat (Fig. 5, Tables 2 and 3). There is an

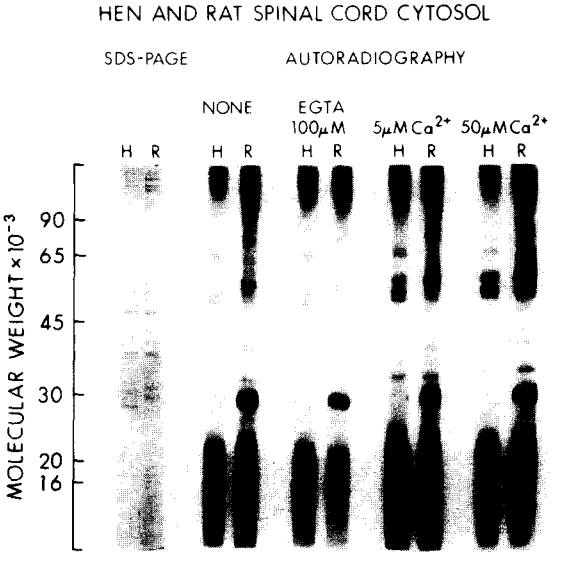

Fig. 5. Comparison of hen and rat spinal cord cytosolic protein phosphorylation: dependence on calcium. Representative 10% SDS–PAGE Coomassie Blue R-250 staining profiles and autoradiographs of the *in vitro* ^{32}P incorporation from [γ-^{32}P]ATP in the presence of indicated cofactor concentrations and other conditions described under Methods, into polypeptides from hens (H) or rats (R).

Table 2. Comparison of hen and rat spinal cord cytosolic protein phosphorylation (in arbitrary units)

Cofactors	M_r(K)	Hen	Rat	Hen/Rat	Significance†
None added	80	NP	2.7 ± 0.6	—	S
	70	3.5 ± 0.8	2.1 ± 1.1	1.65	NS
	55	9.9 ± 3.5	16.5 ± 9.9	0.60	NS
	30	0.9 ± 0.3	32.4 ± 10.3	0.03	$P < 0.02$
	20	76.4 ± 23.2	26.0 ± 7.8	2.94	NS
	16	29.9 ± 12.4	55.7 ± 14.3	0.54	NS
100 μM EGTA	80	NP	1.5 ± 0.4	—	S
	70	2.6 ± 0.2	1.6 ± 0.5	1.61	NS
	55	11.7 ± 6.4	7.5 ± 2.3	1.55	NS
	30	9.2 ± 8.5	22.5 ± 9.6	0.41	NS
	20	56.1 ± 12.8	19.4 ± 6.4	2.88	$P < 0.05$
	16	27.4 ± 6.7	48.3 ± 6.5	0.57	NS
5 μM Ca^{2+}	80	NP	2.9 ± 1.0	—	S
	70	4.2 ± 0.7	2.1 ± 0.6	2.04	$P < 0.05$
	55	13.3 ± 3.0	18.1 ± 5.2	0.74	NS
	30	1.7 ± 0.6	36.3 ± 11.4	0.05	$P < 0.02$
	20	80.5 ± 10.9	41.6 ± 14.0	1.93	NS
	16	46.0 ± 21.0	71.1 ± 14.7	0.65	NS
50 μM Ca^{2+}	80	NP	3.9 ± 0.2	—	S
	70	3.9 ± 0.7	4.2 ± 0.9	0.92	NS
	55	17.4 ± 4.1	26.5 ± 4.2	0.66	NS
	30	1.4 ± 0.3	37.6 ± 9.3	0.04	$P < 0.005$
	20	79.9 ± 9.8	43.6 ± 7.3	1.83	$P < 0.02$
	16	36.3 ± 9.9	79.8 ± 7.8	0.45	$P < 0.02$

*The net *in vitro* incorporation of ^{32}P from [γ ^{32}P]ATP, in the presence of various concentrations of calcium, into specific brain cytosolic proteins, separated by SDS–PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels and is expressed as the mean ± SEM of independent observations from 5 hens and 5 rats.

†Significance of the difference between hens and rats in ^{32}P incorporation, at the same calcium concentration, into a band of similar electrophoretic mobility in both species, as determined by the Student's unpaired two-tailed *t*-test.

NP Band not present or undetectable in one species.

S *A priori* significant difference from a band with no counterpart in the other species.

NS Not significantly different.

80K band in rat, but not hen, spinal cord cytosol whose phosphorylation is not significantly dependent on Ca^{2+}, while a 70K band is phosphorylated in the spinal cord cytosol of both species, though only in a significantly Ca^{2+}-dependent manner (tripled by Ca^{2+}) in the rat, despite its calmodulin-dependence in the hen. One of the most dramatic contrasts between hen and rat CNS protein phosphorylation concerns the 30K band in rat spinal cord cytosol whose Ca^{2+}-independent phosphorylation is at least 20–30 times greater than the near-threshold level at the corresponding M_r in hen spinal cord cytosol. A more quantitative, rather than qualitative, species difference is evident in the lower molecular weight phosphorylated proteins and is most easily visualized in the presence of 100 μM EGTA at the X-ray film exposure chosen to permit resolution of minor bands (Fig. 5). Both the 16K and 20K bands are present in both species; however, in the rat, relatively more 16K band is phosphorylated than 20K band while the reverse is true in the hen (Table 2).

The situation with the low molecular weight proteins in spinal cord membranes (Fig. 6) bears some resemblance to that in spinal cord cytosol, in terms of the greater phosphorylation of a 20K band in hens than in rats. However, the almost total absence in the hen membranes, even after replenishment with exogenous calmodulin, of the slight but significant Ca^{2+}-dependence which the bands with these mobilities evidence in spinal cord cytosol, together with the relatively reduced performance for phosphorylation of the 16K over the 20K band in rat spinal cord membranes (Tables 4 and 5) and the replacement of a distinctly resolved, lightly phosphorylated 16K band in hens with a heavily phosphorylated "tail" on the 20K band, visible also in the protein staining patterns (Fig. 6), corroborate the conclusion that the low molecular weight proteins in the spinal cord cytosol of both species are not the same as those with comparable electrophoretic mobilities in the spinal cord membranes. This conclusion is further supported by notable differences in

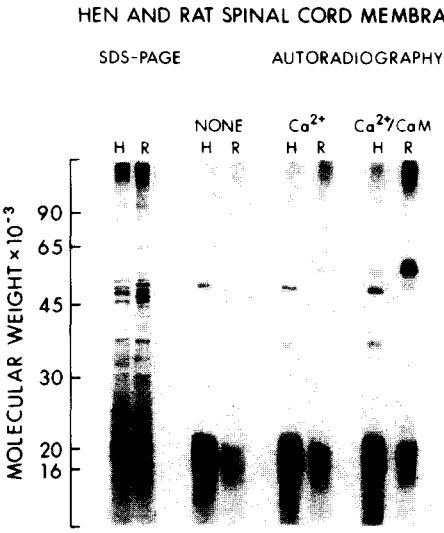

Fig. 6. Comparison of hen and rat spinal cord membrane protein phosphorylation: dependence on calcium and calmodulin. Representative SDS–PAGE Coomassie Blue R-250 staining profiles and autoradiographs of the *in vitro* ^{32}P incorporation from [γ-^{32}P]ATP in the presence of indicated cofactor concentrations and other conditions described under Methods, into polypeptides from hens (H) or rats (R).

pH- and temperature-dependence of the hen 20K band between spinal cord cytosol, in which temperature and pH at the low ends of the test ranges favor phosphorylation, and spinal cord membranes, in which a higher temperature and pH are preferable (Figs 3a, b; 4a, b).

There are also species differences in the higher molecular weight spinal cord membrane proteins. For instance, a 37K band in hen spinal cord membranes was scarcely phosphorylated at all in rat spinal cord membranes (Tables 4 and 5; Fig. 6). Exogenous calcium and calmodulin were required to detect the phosphorylation of a 50K band in rat spinal cord membranes at approximately one-third the level observed in hen spinal cord membranes. On the other hand, both the basal and the Ca^{2+}-calmodulin-dependent phosphorylation of the 60K doublet are much greater in rat than in hen spinal cord membranes (Tables 4 and 5; Fig. 6).

DISCUSSION

Identically prepared synaptosomal cytosolic and membrane fractions from rat and hen spinal cords were used to compare species differences in protein phosphorylation as well as to investigate intraspecies tissue differences in *in vitro* protein phosphorylation between spinal cord and brain. The extent of subcellular fractionation chosen was designed to minimize the preparation time delay-related tendency for decreased incorporation of ^{32}P into certain proteins as a result of kinase lability or proteolysis (Rodnight, 1979; DeLorenzo, 1982) while achieving approximately the same pattern and behavior of phosphate acceptor proteins, except for the notable addition of

Table 3. Stimulation of hen and rat spinal cord cytosolic protein phosphorylation by calcium (mean % of control)*

Calcium concentration	M_r(K)	Hen	Rat
	80	NP	133.7 ± 57.6
	70	143.4 ± 31.8	205.5 ± 64.0
5 μM Ca^{2+}	55	181.0 ± 62.4	265.4 ± 131.9
	30	200.2 ± 53.8	132.8 ± 53.6
	20	135.0 ± 27.0	298.8 ± 162.7
	16	199.4 ± 54.7	145.1 ± 34.7
	80	NP	177.7 ± 36.0
	70	123.8 ± 20.4	633.0 ± 355.5
50 μM Ca^{2+}	55	203.1 ± 30.8†	362.7 ± 118.2
	30	245.6 ± 85.0	137.2 ± 35.4
	20	152.6 ± 55.9	244.2 ± 67.4
	16	256.6 ± 146.8	187.3 ± 49.4

*The net *in vitro* incorporation of ^{32}P from [γ-^{32}P]ATP into specific spinal cord cytosolic proteins, separated by SDS–PAGE, was quantified from the densitometric scans of the autoradiographs of the same dried gels as in Table 2 and is expressed as the percentage each mean ± SEM of independent observations from 5 hens or 5 rats, of the percentage of phosphorylation in the presence of exogenous calcium is of the phosphorylation of the same sample in the absence of exogenous calcium.

NP Band not present or undetectable in one species.

†Significantly stimulated by indicated calcium concentration, based on paired *t*-test, $P < 0.02$.

Table 4. Comparison of hen and rat spinal cord membrane protein phosphorylation* (in arbitrary units)

Cofactors	M_r	Hen	Rat	Hen/Rat	Significance†
None Added	60K	NP	1.4 ± 0.3	—	S
	50K	4.6 ± 0.6	NP	—	S
	37K	4.8 ± 0.7	NP	—	S
	20K	168.9 ± 10.5	12.7 ± 2.8	13.32	$P < 0.001$
	16K	NP	16.9 ± 4.4	—	S
300 μM Calcium	60K	0.1 ± 0.1	6.3 ± 0.7	0.02	$P < 0.001$
	50K	6.1 ± 0.7	0.1 ± 0.1	53.54	$P < 0.001$
	37K	6.2 ± 0.8	NP	—	S
	20K	164.5 ± 17.7	18.7 ± 4.7	8.78	$P < 0.001$
	16K	NP	25.8 ± 7.3	—	S
300 μM Calcium, 5 μg/ml Calmodulin	60K	4.2 ± 0.4	31.9 ± 2.2	0.132	$P < 0.001$
	50K	8.2 ± 1.2	2.9 ± 0.6	2.86	$P < 0.005$
	37K	7.9 ± 1.0	0.1 ± 0.1	79.11	$P < 0.001$
	20K	173.7 ± 16.8	19.1 ± 4.5	9.12	$P < 0.001$
	16K	NP	21.8 ± 6.3	—	S

*The net *in vitro* incorporation of ^{32}P from [γ-^{32}]ATP, in the absence or presence of cofactors at indicated concentrations, into specific spinal cord membrane proteins, separated by SDS–PAGE, was quantified from the densitometric scans of the autoradiographs of the dried gels, and is expressed as the mean $\pm$ SEM of independent observations from 5 hens and 5 rats.

†Significance of the difference between hens and rats in ^{32}P incorporation under the same cofactor conditions, into a band of similar electrophoretic mobility in both species, as determined by the Student's unpaired two-tailed t-test.

NP Band not present.

S *A priori* significant difference from a band with no counterpart in the other species.

Table 5. Stimulation of hen and rat spinal cord membrane protein phosphorylation by calcium alone or with calmodulin (mean % of control)*

Cofactors	M_r	Hen	Rat
300 μM Calcium	60K	A	515.9 ± 98.6***
	50K	136.9 ± 15.0	C
	37K	133.2 ± 9.9*	NP
	20K	96.6 ± 5.5	143.7 ± 5.6*
	16K	NP	190.7 ± 41.4*
300 μM Calcium, 5 μg/ml Calmodulin	60K	B	2679.5 ± 552.2***
	50K	181.1 ± 17.0	D
	37K	170.5 ± 21.2	E
	20K	102.0 ± 5.4	149.8 ± 10.3
	16K	NP	133.6 ± 33.5

*The net *in vitro* incorporation of ^{32}P from [γ-^{32}P]ATP into specific spinal cord membrane proteins, separated by SDS–PAGE, was quantified from the densitometric scans of the autoradiographs of the same dried gels as in Table 2 and is expressed as the mean $\pm$ SEM of independent observations from 5 hens or 5 rats, of the percentage of the phosphorylation in the presence of calcium $\pm$ calmodulin is of the phosphorylation of the same sample in the absence of exogenous cofactors.

NP Band not present or undetectable.

A Stimulated to 0.1 ± 0.1 arbitrary units (see Table 4); not detectable in the absence of calcium.

B Stimulated to 4.2 ± 0.4 arbitrary units (see Table 4); not detectable in the absence of calcium.

C Stimulated to 0.1 ± 0.1 arbitrary units (see Table 4); not detectable in the absence of calcium.

D Stimulated to 2.9 ± 0.6 arbitrary units (see Table 4); not detectable in the absence of calcium.

E Stimulated to 0.1 ± 0.1 arbitrary units (see Table 4); not detectable in the absence of calcium.

* Significantly stimulated by indicated cofactor(s), based on paired t-test, $P < 0.05$.

**Significantly stimulated by indicated cofactor(s), based on paired t-test, $P < 0.02$.

***Significantly stimulated by indicated cofactor(s), based on paired t-test, $P < 0.005$.

myelin basic protein as would be attained upon further purification of the synaptosomes away from contaminating myelin and mitochondria by density gradient centrifugation (Krueger *et al.*, 1977). We have also characterized the response of hen spinal cord cytosol and membranes with respect to optimal protein concentration and incubation time, temperature, and pH in the *in vitro* protein phosphorylation assay.

In hen spinal cord membranes, phosphorylation was found to increase fairly linearly with protein concentration up to 125 μg/200 μl, before decline began. This contrasts with the observed onset of decline just above 75 μg/200 μl in hen brain membranes (Patton *et al.*, 1984). No limit to the direct increase in phosphate incorporation per band was seen for spinal cord cytosol over the range of protein concentrations we were limited to as a consequence of low yields obtained with this fraction. Phosphorylation was complete and net dephosphorylation had already begun by 60 s in hen spinal cord cytosol. This time is shorter than the continued increase up to 90 s for all bands and to 120 s or beyond for some bands in hen brain cytosol (Patton *et al.*, 1984). Yet the times for which net increases in ^{32}P incorporation were observed for the hen membranes were shorter still: 20 s for the spinal cord and only 10 s for the brain (Patton *et al.*, 1984). Perhaps this discrepancy between the spinal cord and the brain is due to fewer acceptor proteins in spinal cord membranes generating less competition for the limited supply of ATP which is rapidly being hydrolyzed by the high level of membrane-bound ATPases in synaptic plasma membranes (Wiegant *et al.*, 1978).

The only spinal cord proteins whose phosphorylation varies with temperature and pH over the ranges tested are the cytosolic and membrane 20K bands. The sharp contrast between the decrease in net phosphorylation with pH and with temperatures greater than 30°C in the cytosol and the increase in net phosphorylation with pH and with temperatures less than 50°C in the membranes suggests that either these are two completely different proteins or that translocation between membranous and soluble environments is accompanied by great alterations in physical properties.

The selective benefit of PMSF for phosphorylation of membrane-bound rather than soluble proteins of hen spinal cord P_2 lysate might be attributable to the relative longevity of the preparation and storage of the membranes. Burke and DeLorenzo (1982) have noted that prolonged handling, multiple washes, or increases in temperature decreased the Ca^{2+}-dependent phosphorylation of their vesicle fraction proteins.

There are several interesting differences as well as similarities in the characteristics of various phosphate acceptor proteins between the rat and hen. Both have a major staining triplet in the cytosol, comprising bands of M_r approx 50K, 55K and 60K, which undergoes Ca^{2+}-activated phosphorylation, though of greater magnitude in the rat than in the hen. Subsequent experiments have also shown this phosphorylation to be calmodulin-dependent on the basis of its susceptibility to inhibition by the widely used calmodulin inactivator trifluoperazine (Levin and Weiss, 1979). Due to incomplete resolution by microdensitometry, this triplet has been collectively designated "55K" for quantitative purposes. Breithaupt and Babitch (1979) have found several baby chick brain synaptoplasmic polypeptides in the 53,000–56,000 Dalton range, including α- and β-tubulin as shown by 2-D electrophoresis, which do incorporate ^{32}P, although the calcium and calmodulin dependence of that phosphorylation could not be easily ascertained in their whole synaptosome system (Breithaupt *et al.*, 1980). Furthermore rat brain synaptic vesicle α- and β-neurotubulin phosphorylation has been shown to be Ca^{2+}- and calmodulin dependent (Burke and DeLorenzo, 1981).

Hen and rat spinal cord membranes show very minor phosphorylation at M_r 55K but major Ca^{2+} and calmodulin-dependent phosphorylatable substrates at M_r 50K uniquely in the hen and at 60K uniquely in the rat (Fig. 6). This species difference is limited to the spinal cord, as brain membranes from both species have both 50K and 60K Ca^{2+} and calmodulin-dependent phosphorylatable substrates (Patton *et al.*, 1984). Interestingly, O'Callaghan *et al.* (1980) have noted that the polypeptides with apparent electrophoretic mobilities of 50K and 60K in rat brain synaptic plasma membranes purified on discontinuous sucrose density gradients had matching counterparts in synaptosomal cytosol. Similar phosphoproteins can be seen on our spinal cord cytosolic autoradiographs for both hens and rats Fig. 5).

Synapsin Ia, b, is a doublet of M_r 86K and 80K, associated principally with synaptic vesicles (Kennedy, 1983). In the electrophoretic mobility range of 80–86K we observed a band phosphorylated in a Ca^{2+}-dependent manner, which may be a poorly resolved doublet in the rat spinal cord cytosolic fraction, but which is undetectable in the hen. Sorenson and Babitch (1982) have reported that baby chicks have Synapsin Ia but lack Synapsin Ib. Using

a specific radioimmunoassay, Kennedy (1983) reported low concentrations of Synapsin I-like proteins with divergent properties in non-mammalian species, including the chicken, and concluded that Synapsin I has been less conserved during evolution than has the cAMP-dependent protein kinase. This could, conceivably, account for the lack of an observable phosphorylated band with that M_r in the hen.

The hen and rat both have a prominent band with an M_r of 70K in spinal cord cytosolic but not membrane autoradiographs. This 70K band was also visible in both brain cytosolic and membrane autoradiographs of hens but not of rats (Patton *et al.*, 1984). Nonetheless, its presence in even one rat fraction argues against the possibility that it may be an avian variation on the 80K band unique to rat spinal cord and brain cytosol. Accordingly, another possible identity worth considering for this 70K autoradiographic band is that of the 68K neurofilament subunit which is known to be phosphorylated as a single, identical site both *in vivo* and *in vitro*, although to a much greater extent *in vitro*, by a kinase which copurifies with it from rat spinal cord and brainstem (Julien and Mushynski, 1981). That rat spinal cord cytosol would be the most likely fraction for it to show up in is consistent with our observations, yet its far greater phosphorylation in hens and its appearance in three hen fractions remain anomalous.

Further inspection of the autoradiographs for spinal cord cytosol presented here (Fig. 5) reveals a minor, slightly Ca^{2+}-dependent phosphorylated protein in hens and rats with an M_r slightly above 30K, while the rat spinal cord cytosol, alone, also possesses a major Ca^{2+}-independent phosphate acceptor with an M_r slightly below 30K. Bovine brain synaptic vesicles have been reported to display Ca^{2+}- and calmodulin-dependent phosphorylation of their 53K and 55K proteins which is inhibitable by an antibody against their 30K protein. Phosphorylation of this 30K protein is stimulated only by cAMP (Moskowitz *et al.*, 1983). The slight, but apparent, Ca^{2+}-dependence of the phosphorylation of the minor band with $M_r \geqslant 30K$ and the exclusive location of the band with $M_r \leqslant 30K$ in rat spinal cord cytosol belie any identification of either of them with the putative 30K synaptic vesicle Ca^{2+}-calmodulin kinase. Rat sciatic nerve and cauda equina myelin have the P_o glycoprotein of M_r 28K (32K and a different amino acid sequence in chick sciatic nerve, Mezei and Verpoorte, 1981) whose phosphorylation is half-maximally stimulated by 5 μM free Ca^{2+} but inhibited by higher (> 100 μM) calcium concentrations (Petrali and Sulakhe, 1979). The slight reduction in ^{32}P incorporation seen in our prominent $M_r \leqslant 30K$ rat spinal cord cytosolic band incubated with EGTA, coupled with the lack of increase in ^{32}P incorporation upon addition of exogenous calcium, is compatible with these observations of P_o. If such a protein were to be phosphorylated at all in the central nervous system, it would be in the spinal cord, as it is not phosphorylated in the rat brain (Singh *et al.*, 1976; Steck and Appel, 1974). However, it is quite unlikely that a myelin membrane glycoprotein would be found exclusively in spinal cord cytosol without being in either spinal cord or brain P_2 membrane fractions.

The same pattern of Ca^{2+}-dependence described for the rat peripheral myelin P_o protein applies to the phosphorylation of its large (P_1, 19K–20K) and small (P_2, 16K) basic protein (Steck and Wuthrich, 1979). Slight stimulation of ^{32}P incorporation into both the 16K and 20K rat spinal cord membrane proteins by Ca^{2+} was observed, strongly suggesting that they should be identified as myelin basic proteins and contrasting with the lack of any calcium effect on the heavier phosphorylation of a slightly larger (22K) counterpart in hens. We have tentatively identified this 22K hen polypeptide as hen myelin basic protein on the basis of its specificity to the myelin fraction when hen P_2 membranes are subfractionated on discontinuous sucrose gradients according to the procedures of Jones and Matus, 1974 (Patton *et al.*, unpublished).

Effective phosphorylation conditions for hen spinal cord proteins *in vitro* have been documented to facilitate biochemical mechanistic investigations in settings for which the hen is the experimental model of choice. In order to ascertain the degree to which extrapolation of results from hens to mammals is justified, rat spinal cord proteins were phosphorylated under identical conditions to those determined for hen spinal cord proteins. Several differences in protein phosphorylation between rats and hens have been discussed. While the functional significance of these differences and of the proteins involved is not yet clear, the possibility of a relationship between differences in rodent and avian phosphorylation of neuronal proteins and differences in their susceptibility to neurotoxic compounds, such as the organophosphorus esters, should be investigated.

Acknowledgements—The authors wish to thank Dr Thomas C. Vanaman for his permission to use the Zeineh Soft Laser Microdensitometer belonging to the Duke University Department of Microbiology and Immunology for part of this study. The statistical programming of the Apple II Com-

puter by Dr Clark D. Carringon and the data processing assistance of Karen L. Patton and Kathy Y. Jones are greatfully acknowledged. The secretarial work of Mrs Erna S. Daniel is much appreciated. This study was supported in part by NIEHS Grant ESO-2717 and NIOSH Grant No. 0H02003.

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