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pH-sensitive, Ca^{2+}/calmodulin-dependent phosphorylation of unique protein in molluscan nervous system

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Intracellular pH and Ca^{2+} are prominent co-regulators of neuron excitability that act on ion channels. In looking for a possible mechanism of their action, we tested their combinatorial effect on the phosphorylation state of nervous system proteins. $^{32}PO_4$ labelling in endogenous phosphorylation reactions of homogenates of nervous tissue of the sea-slug *Pleurobranchaea* showed steep pH sensitivity in protein migrating at a molecular mass of 108 kDa with p*I* 6.9–7.0 (pp108). Phosphorylation of pp108 was highest below reaction pH 7.0 and declined steeply as pH rose to 7.4. pp108 phosphorylation was Ca^{2+}/calmodulin-dependent. pp108 constituted a significant part of the total protein (0.15%) and phosphoprotein (8.9%) of the nervous system. The specifically and uniquely combinatorial pH and Ca^{2+} sensitivity of the phosphorylation of pp108, and its relative abundance, suggest that it could mediate integrated actions of H^+ and Ca^{2+} in the molluscan neuron.

Introduction

Changes in intracellular pH (pH_i) accompany, potentiate, or actually trigger changes in cell state in many systems. Egg and sperm activation, the mitotic cycle, protein synthesis, cell differentiation and the membrane conductance of excitable cells are examples where changing pH_i acts in a regulatory intracellular messenger role [5,11,16].

In certain neurons of the molluscan nervous system pH_i has an important role in regulating a cyclic AMP-dependent Na^+ current, a role it shares with the calcium ion [4,7]. Regulation of the current by pH_i is complex and may occur through two separate mechanisms: either acidification or alkalinization of the neuron augments the current, and it has been suggested that the current amplitude adapts to the resting pH_i through a balance of these mechanisms [7]. A possibly related phenomenon is steep pH sensitivity of the distribution of calmodulin between cytosolic and membrane-cytoskeletal fractions of the molluscan nervous system [19]. Ca^{2+} also regulates the ion current itself by multiple mechanisms, both potentiating and suppressing it through different pathways [6,7].

Protein phosphorylation is an aspect of many regulatory pathways within the cell where intracellular messengers such as Ca^{2+}, cyclic AMP, and H^+ could interact to exert an integrated influence over cell function, and thus we have considered its potential combinatorial regulation in the molluscan neuron. In the present study our goals were to assess the possibility that protein phosphorylation could be sensitive to the effects of altered $[H^+]$ within the range of intracellular pH, and to determine potential interactions with other intracellular messengers. We studied the effects of altered pH upon phosphorylation in endogenous reactions in nervous system homogenates. We present results that are evidence for acute pH sensitivity of Ca^{2+}/calmodulin-dependent phosphorylation of a unique phosphoprotein.

Materials and Methods

Central nervous systems, consisting of cerebropleural, pedal, and buccal ganglia were removed from specimens of the seaslug *Pleurobranchaea californica* (100–600 g; provided by Pacific BioMarine, Santa Monica, CA, and Sea-Life Supply, Sand City, CA) and homoge-

Abbreviations: pp108, phosphoprotein migrating at a molecular mass of 108 kDa; pH_i, interacellular pH; PMSF, phenylmethylsulfonyl fluoride; EF-2, peptide synthesis elongation factor.

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nized in 10 vol. of buffer (5 mM Hepes, 5 mM EDTA, 0.5 mM EGTA, 5.7 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at pH 7.5) at 4°C. The homogenate was spun two times at 15000 × *g* and the supernatant was used in protein phosphorylation reactions.

For assay of pH dependence, homogenate aliquots (about 7–10 μg protein) were preincubated with reaction buffer (final conc. 50 mM Hepes, 10 mM $MgCl_2$, 2.2 μM $CaCl_2$, 2 mM EGTA, 1.25 mM EDTA, 1.42 mM β-mercaptoethanol, 0.025 mM PMSF) for 1 min at 20°C. The pH of the individual reaction mixtures was set between 6.6–8.0 with preadjusted stock solutions of Hepes. For assays of Ca^{2+} dependence, Ca^{2+} was omitted from reaction mixtures. The calmodulin blocker trifluoperazine was added in some experiments at concentrations of 25–100 μM. Calmodulin dependence was assessed by depleting calmodulin through precipitation of homogenate protein in 55% $(NH_4)_2SO_4$, followed by resuspension and dialysis of the centrifuged pellet (15000 × *g*, 15 min) in homogenate buffer [2,21]. Exogenous bovine calmodulin was added to some reactions at 50 or 100 nM. Protein labelling was initiated by addition of [^{32}P]ATP (final conc. 5–10 μM, 5 μCi) and allowed to proceed for 2 min. The reaction was terminated by the addition of Laemmli stop buffer (final conc. 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 62.5 mM Tris, 0.002% Bromphenol blue at pH 6.8) and samples were boiled for 2 min. Samples were then run on 8–15 or 6–12% SDS-PAGE. Gels were stained with Coomassie blue, photographed and dried. Dried gels were exposed to X-ray film at −70°C for autoradiography. Marker proteins of known molecular weights were loaded on adjacent lanes in some experiments for calibration of molecular weights. The standards used were carbonic anhydrase (M_r 29000), ovalbumin (45000), bovine serum albumin (66000), phosphorylase B (97,400), β-galactosidase (116000) and myosin (205000). Protein amounts in homogenates were measured according to Lowry.

Relative estimates of radioactivity incorporation into specific phosphoprotein bands were made by densitometric scans of autoradiograms. Alternatively, radioactivity incorporated was quantitated by liquid scintillation of gel slices of the protein band located initially by autoradiography and cut out by hand.

For two-dimensional separations, samples were treated with DNase and RNase, snap-frozen and shipped on dry ice to Protein Databases, where they were counted and assayed for specific activity. Lyophilized samples were dissolved in isoelectric focusing buffer (9.5 M urea, 2% NP-40, 100 mM DTT and 2% basic ampholines) at 37°C for 30 min. Two-dimensional gel electrophoresis was performed following the method of Garrels [3]. 10–20 μl of sample containing 2.5–4 · 10^5 dpm and 20–30 μg protein were loaded onto a narrow bore isoelectric focusing tube (0.8 mm diameter; 20 cm long). The tube contained 2.9% acrylamide, 2% NP-40, 9.5 M urea and 2% 3–10 pH ampholines. Proteins were focused at 19000 V/h overnight. The SDS-equilibrated isoelectric focusing gel was mounted on the second dimension gel (24 × 24 cm × 1 mm) and an acrylamide concentration of 7.5%. The second dimension was electrophoresed at constant 60 W. Gels with corresponding radioactive calibration strips containing known amounts of radioactive protein were then silver stained [10,12] and autoradiograms were prepared [3]. Gels and films were scanned with a photodiode array camera at a resolution of 200 μm. Images were processed with PDQUEST software. After scanning, merging and spot detection, a matchset was assembled having two components: the standard (reference) image and the individual gel (sample) images. The majority of well-resolved spots and the individual gels were analyzed and matched to the standard. Estimation of phosphorylation stoichiometry was done as:

$$\text{(mol } PO_4 \text{ incorporated/mol pp108)} = \frac{\left(\text{specific activity } {}^{32}PO_4\right)^{-1} \times \text{(total activity of pp108)}}{\text{(pp108 fraction of total protein (g)/molecular weight pp108)}}$$

Results

pH sensitivity of phosphorylation

Incubation of [^{32}P]ATP in homogenates of *Pleurobranchaea* CNS at various pH in the presence of excess Ca^{2+} demonstrated marked pH dependence of phosphorylation in a single phosphoprotein band. The phosphoprotein migrated at an estimated molecular mass of 108 kDa (Fig. 1), and is hereafter called pp108. In the range pH 7.0–8.0 labelling was most intense at acidic pH and decreased rapidly with alkalinity. Other, more minor, bands showed occasional variations in labelling intensity at different reaction pH; however, the pH sensitivity of labelling of these bands was not consistent.

In three experiments the autoradiographed bands were analyzed densitometrically. The incorporated radioactivity declined from the values measured for pH 7.0 reaction conditions to 30% for pH 7.5 (13% S.E.M.) and 22% (9% S.E.M.) for pH 8.0. The pH sensitivity of pp108 labelling was prominent even at very short reaction times (10 s) where the amount of phosphorylated protein was so low that the origin of pH sensitivity in a phosphatase may have been unlikely. In another case, the pH sensitivity of pp108 phosphorylation was determined as reaction pH was varied in steps from pH

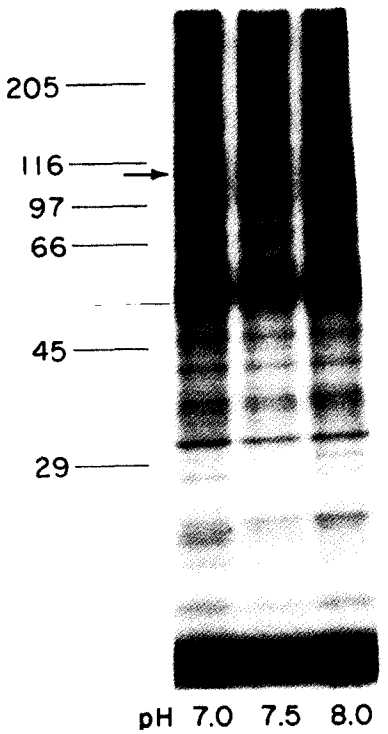

Fig. 1. pH sensitivity of endogenous phosphorylation. Shown here are autoradiograms of $^{32}PO_4$ incorporated in endogenous phosphorylation reactions in *Pleurobranchaea* CNS homogenates. Proteins were separated on 8–15% SDS-PAGE gels. Phosphorylation reactions were run at pH 7.0, 7.5 and 8.0 in Ca^{2+}-containing media. A single band at molecular mass 108 kDa was consistently labelled at pH 7.0, less intensely at 7.5, and much less so at pH 8.0. Similar results were obtained in ten separate experiments.

6.6 to 8.0. Results of liquid scintillation measurements of gel slices containing the pp108 band are shown in Fig. 2. They indicate that phosphorylation of pp108 was greatest under these experimental conditions below pH 7, that its phosphorylation was rapidly reduced between pH 7–7.4, and that it was least phosphorylated above pH 7.6.

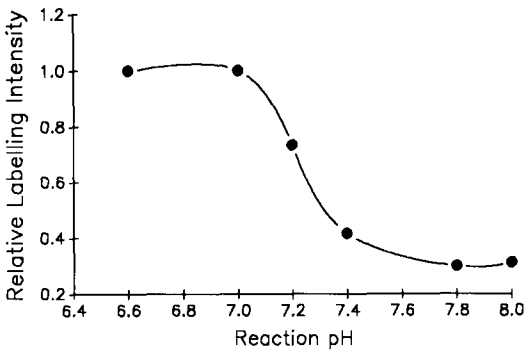

Fig. 2. pH sensitivity of endogenous phosphorylation of pp108. Reactions were carried out at pH values between 6.6–8.0. The pp108-containing gel region was located by autoradiography, cut out, dissolved and counted by liquid scintillation for 5 min. Corrected for background, counts per minute ranged from 140 to 256 (S.D. 0.18–2.12). The data are normalized to the highest value. From a plateau in phosphorylation at pH 7.0 and below, a steep drop occurs in the pH range 7.0–7.4. The relation appears sigmoid within the range of physiological pH, with least phosphorylation at reaction conditions pH 7.6 and above.

Phosphorylation of pp108 was compared at different pH in three two-dimensional gel separations. The estimation of molecular mass was consistent at 108 kDa; the p*I* was found to lie close to 7.0 (Fig. 3). Three pH sensitive spots were resolved, migrating adjacently along the gel pH gradient, suggesting either a protein with multiple phosphorylation sites, three closely related protein isoforms, or degradation of basic residues during handling. Each of the three spots showed pH sensitivity: labelling intensity declined as reaction pH went from 6.8 to 7.6. Phosphorylated at pH 6.8, pp108 contained 8.9% of total radioactivity; at pH 7.2, 3.3%; and at pH 7.6, 0.8%.

Out of 263 total phosphoprotein spots observed in the autoradioradiograms, 15 others showed monotonic pH sensitivity. However, these were distinguished from pp108 in two ways. First, they were relatively minor

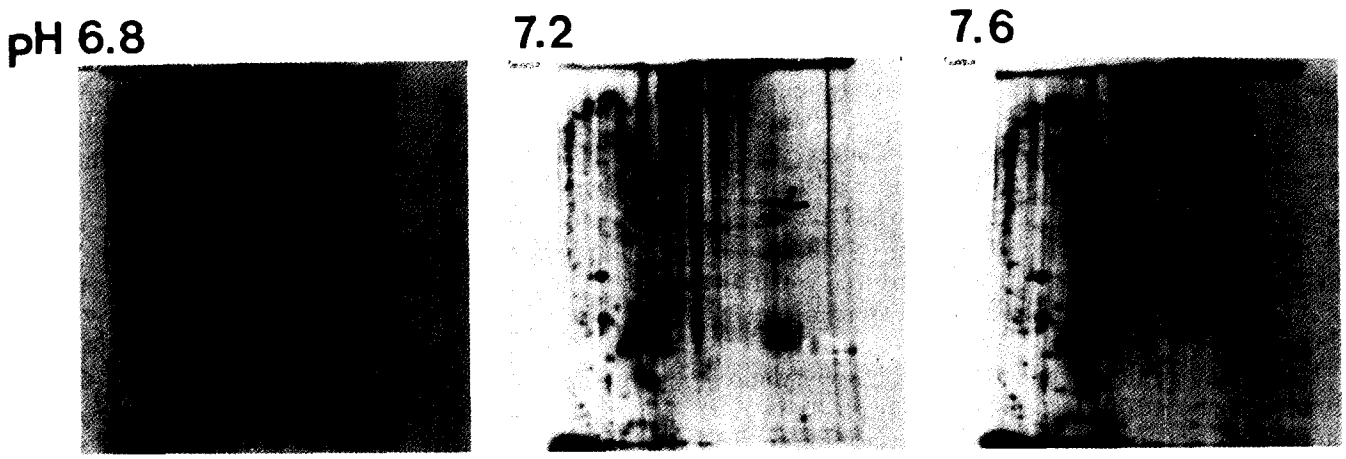

Fig. 3. Two-dimensional gel autoradiograms of CNS aliquots reacted at pH 6.8 (left), 7.2 (middle), and 7.6 (right) and separated by mobility in SDS (vertical axis) and isoelectric point (horizontal axis). pH sensitivity is seen in the diminishing label of phosphoprotein at molecular weight 108000, p*I* 6.9–7.0 (arrowheads) as the reaction pH is increased.

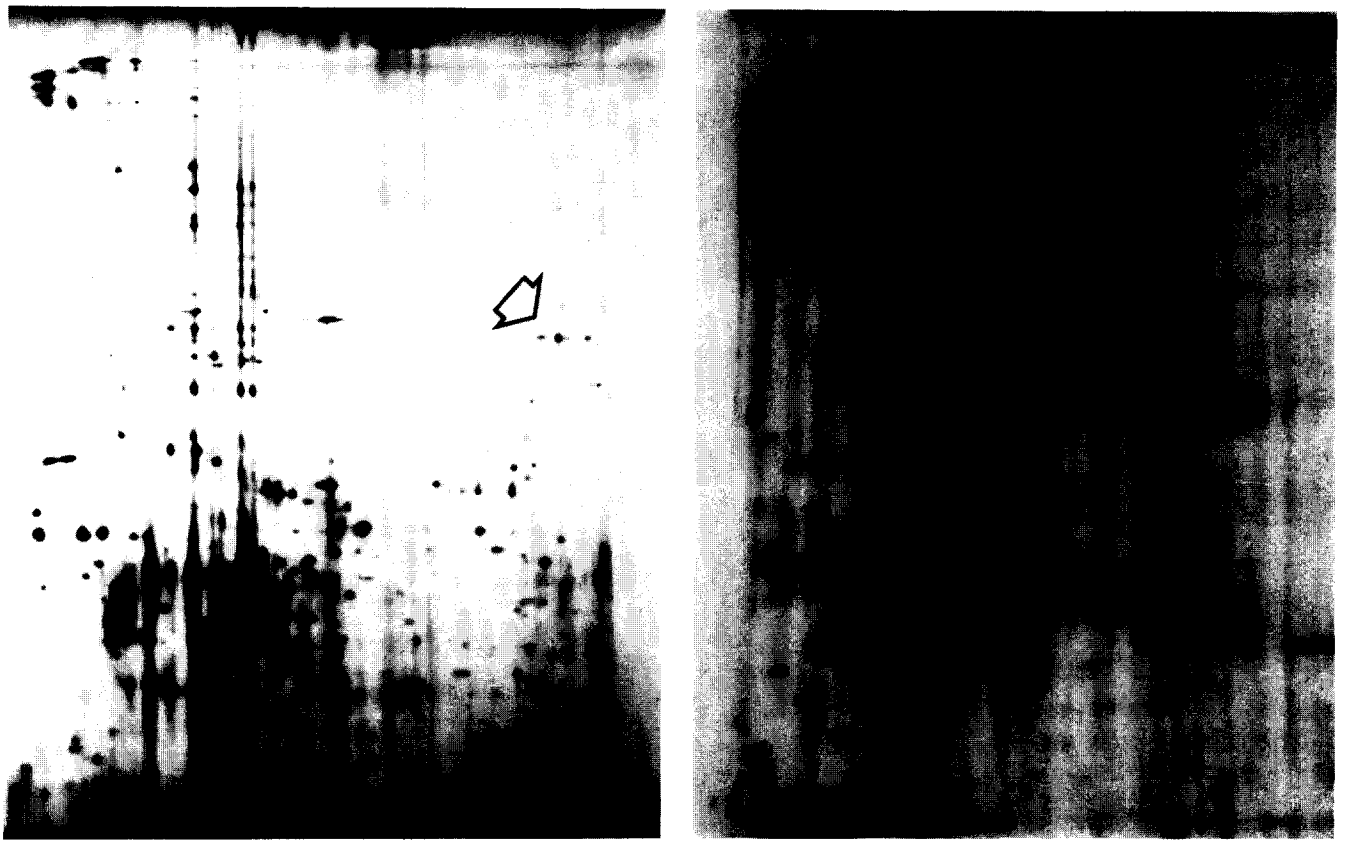

Fig. 4. Left panel; a silver-stained two-dimensional gel showing prominent staining of the pp108 triad (arrow). Right panel; The corresponding autoradiogram (reaction pH 6.8).

phosphoproteins with less than 0.9% of total radioactivity at fullest phosphorylation. Second, the direction of their pH sensitivity was opposite to that of pp108; phosphorylation increased with increasing pH. Thus, pp108 appears unique in being a moderately abundant phosphoprotein that is increasingly phosphorylated with acidifying pH within the physiological range.

In Coomassie blue-stained one-dimensional gels a stained band was visible at the position of the 108 kDa phosphoprotein (data not shown). This suggested that the 108 kDa protein is not only a moderately abundant phosphoprotein, but could be a significant fraction of cell protein. This was corroborated on silver stained two-dimensional gels (Fig. 4). Quantitation of the three spots corresponding in position to the pH sensitively phosphorylated spots indicated that pp108 constituted 0.15% of the total protein. Thus, the pp108 is a significant portion of the total protein, as well as a moderately abundant phosphoprotein.

Calculation of the stoichiometry of phosphorylation from the radioactivity incorporated into pp108 in the endogenous reaction run at pH 6.8 (greatest phosphorylation) yielded a value of 0.43 mol of phosphate incorporated per mol of protein. This may reflect an appreciable basal phosphorylation state of the native protein in vivo. Thus, these data suggest a minimum phospnorylation of 1 PO_4 per protein molecule.

Ca^{2+} dependence of pH-sensitive phosphorylation

EGTA-chelation of free Ca^{2+} in the reaction medium caused disappearance of a number of phosphoprotein bands from the autoradiogram, among them pp108 (Fig. 5; $n = 10$). The pH dependence of the 108 kDa phosphorylation was not enhanced by addition of phosphatidylserine and diacylglycerol or phorbol esters to the reaction medium, although these cofactors for Ca^{2+}-dependent kinase C phosphorylation did stimulate label incorporation into other protein bands (data not shown; see Ref. 7). Addition of trifluoperazine, an inhibitor of both Ca^{2+}/calmodulin- and Ca^{2+}/phospholipid-dependent phosphorylation, to the phosphorylation reaction blocked label incorporation into pp108 (Fig. 5; $n = 6$). In separate experiments cyclic AMP stimulated phosphorylation of a variety of different proteins, but not of pp108 [9].

Calmodulin dependence was confirmed by precipitation of homogenate protein in 55% ammonium sulfate ($n = 2$), a procedure that leaves low molecular weight, acidic proteins like calmodulin in the supernatant and suppresses calmodulin-dependent phosphorylation

[2,21]. Addition of exogenous calmodulin to undepleted homogenates had no significant effect on phosphorylation; soluble calmodulin in similar homogenates of *Pleurobranchaea* CNS has been estimated by radioimmunoassay as 0.6–1.2% of total protein [19]; thus, the concentration of endogenous calmodulin may have been in excess of 80 nM and well over saturating levels. In homogenates depleted of endogenous calmodulin by ammonium sulfate precipitation, the phosphorylation of pp108 was suppressed; addition of 50–100 nM exogenous bovine heart calmodulin restored the phosphorylation of pp108 (Fig. 6).

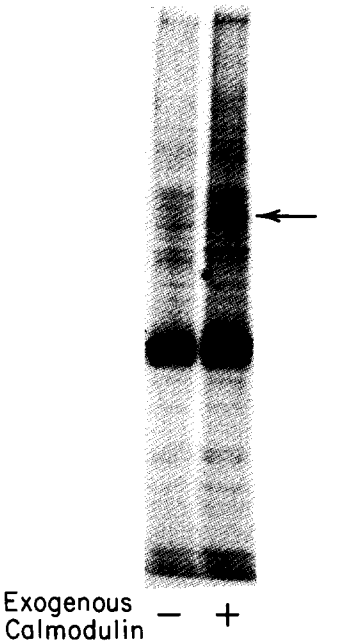

Fig. 6. Calmodulin dependence of pp108 phosphorylation. Endogenous reaction media made deficient in calmodulin by ammonium sulfate precipitation do not support phosphorylation of pp108. Exogenous calmodulin (50 nM) restores phosphorylation.

Discussion

The evidence for pH sensitive Ca^{2+}/calmodulin-dependent phosphorylation is a novel documentation of regulation of phosphorylation state. It is observed in an in vitro system derived from tissue showing prominent and integrated physiological regulation by intracellular H^+ and Ca^{2+}. The parameters of pp108 phosphorylation are consistent with potential intracellular function in the neurons of *Pleurobranchaea*. Measured pH_i in healthy, quiescent neurons lies in the range 7.3–7.45; during electrical activity similar to normal function, pH_i may acidify up to 0.07 pH unit [4,5,7]. In that range such a slight change of pH_i may significantly alter the amplitude of the cyclic AMP-dependent Na^+ current that regulates excitability and electrical activity in many neurons of the feeding-motor and locomotor networks [7]; a similar pH_i change caused a more than 10% increase in phosphorylated pp108 in vitro (Fig. 2). The high degree of specificity of phosphorylation of pp108 and the abundance of the phosphoprotein are also consistent with a significant role in the neurons of the molluscan nervous system.

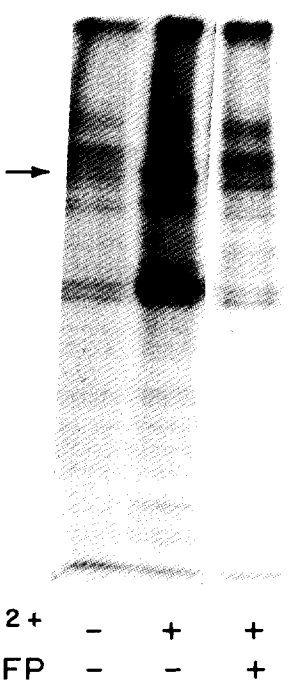

Fig. 5. Ca^{2+} dependence of phosphorylation and suppression by added trifluoperazine. Omission of excess Ca^{2+} from the reaction mixture, or addition of 25 μM trifluoperazine (TFP), suppressed labelling of pp108.

The exact origin of the pH sensitivity of phosphorylation of pp108 is presently unresolved. The pH sensitivity of phosphorylation could originate from either kinase or phosphatase activity. However, there are well-known precedents for pH sensitivity in two other calmodulin-containing kinase holoenzymes, phosphofructokinase [8] and phosphorylase kinase [18], and we speculate that pH sensitivity will be found to reside in kinase action.

In searching for a possible identity for pp108, we noticed that it superficially resembles the identified phosphoprotein EF-2 from vertebrate tissue. EF-2, the peptide synthesis elongation factor, has similar molecular weight and isoelectric point [13], and is also phosphorylated by a highly specific, Ca^{2+}/calmodulin-stimulated kinase [13,17]. Phosphorylation of EF-2 inhibits protein synthesis [15,17]. However, while EF-2 phosphorylation was also found to have a certain pH sensitivity [14], the pH sensitivity of pp108 is significantly different in its requirement of calmodulin for its expression and in its pH range. Nevertheless, the possibility that pp108 in molluscan nervous system is an invertebrate variant of an EF-2 type protein is an

interesting speculation; indeed, protein synthesis is pH sensitive in several systems [1,20].

Withal, the cellular function of pp108 and its possible connection with the pH- and Ca^{2+}-sensitive cyclic AMP-dependent Na^+ current of molluscan neurons are as yet untested. The data collectively indicate its potential for transducing and integating fluctuations of H^+ and Ca^{2+} into physiological action. The present observations invite future studies to determine whether the phosphorylation state of pp108 is regulated by pH_i in vivo and to determine the functional effects of its perturbation.

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