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Comparison of Labeled Heat Shock Proteins in Neuronal and Non-neuronal Cells of *Aplysia californica*[1]

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Abstract

Aplysia californica has been used to study the protein synthetic response of nervous tissue to stress induced by elevated temperatures. The abdominal and pleural ganglia as well as associated connectives were exposed to various temperatures for 30 min, labeled with [^{35}S]methionine at room temperature, and then analyzed by sodium dodecyl sulfate gel electrophoresis. All cells examined responded to temperatures of greater than 31°C by a reduction in levels of labeled actin, as well as by the enhanced labeling of proteins with apparent M_r of 70,000 and 110,000. Two-dimensional electrophoresis indicated that the molecular weight and isoelectric focusing properties are similar to the heat shock proteins (HSPs) observed in other systems. In addition to these major HSPs, heat-induced proteins with molecular weights ranging from 70,000 to 90,000 were highly labeled in the neurosecretory bag cells. Further cell type-specific differences in the protein synthetic response to elevated temperatures were revealed by quantitation of the major HSPs. Levels of labeled HSPs were significantly lower in ganglion cells as compared to the non-neuronal connective cells. In addition, the decrease in actin levels appeared to be less dramatic in the ganglion cells. Analysis of the cellular compartmentalization of HSPs suggests that both neurons and glia are capable of HSP synthesis.

Studies in the squid have demonstrated that HSPs are transferred from adaxonal glia into the axoplasm (Tytell, M., S. G. Greenberg, and R. J. Lasek, unpublished observation). This finding, coupled with the low levels of labeled HSPs in ganglion cells, supports the possibility that glia-axon protein transfer may play an important role in nerve cell survival during periods of stress. Preliminary findings of this study have been reported previously (Greenberg, S. G., P. F. Drake, and R. J. Lasek (1983) J. Cell Biol. 97: 152).

A variety of cell types from a number of organisms respond to elevated temperatures by a decrease in levels of normal protein synthesis as well as the enhanced synthesis of a small number of heat shock proteins (HSPs) (see Schlesinger et al., 1982b, for review). Although many of these HSPs are highly conserved, studies in *Drosphila hydei* have indicated that there may be tissue or cell type differences in the relative synthesis of specific HSPs (Sondermeijer and Lubsen, 1978).

Nervous tissue responds to elevated temperatures, as well as to other stressful stimuli, by the enhanced synthesis of proteins with apparent M_rs of 71,000, 73,000, and 88,000 in the rat brain (Currie and White, 1981; Pearce et al., 1983), 74,000 in the rabbit brain and retina (Brown et al., 1982), and 70,000 in the goldfish retina (unpublished observations). A differential response to stressful situations by neurons and non-neuronal cells within the brain was suggested by the finding that the 71,000-dalton stress protein induced during *in vitro* incubations of brain slices is highly enriched in the brain microvascular fraction (White, 1980, 1981). Although cerebellar cell cultures enriched in either neuronal or glial cell types respond to elevated temperatures by the induction of the 71,000-dalton stress protein (Pearce et al., 1983), the relative contribution that these cell types make to the synthesis of HSPs in nervous tissue is not clear.

Many features of the heat shock response, including the synthesis of the highly conserved HSPs, occur after exposure of cells to a variety of stressful situations (see Ashburner and Bonner, 1979, for review). The heat shock response may therefore be used as a model to understand general mechanisms which different cell types use to deal with stressful situations. However, specific cell types may respond to heat shock in qualitatively and quantitatively different ways. Therefore, in order to understand the general stress response in the nervous system, it is useful to identify specific features of the heat shock response in defined cell types of nervous tissue.

We have used the nervous system of the invertebrate sea mollusc, *Aplysia californica*, to investigate further the relative protein synthetic response of neuronal and non-neuronal cells to elevated temperatures. The use of this organism offered the opportunity to selectively analyze the response of specific neuronal and non-neuronal cells to elevated temperatures.

In this study we analyzed two different populations enriched in neuronal cells, including the neurosecretory bag cells located at the base of the abdominal ganglion, as well as isolated neurons with their adherent glial sheath from the abdominal and pleural ganglia. The non-neuronal cells were obtained from the abdominal-pleural connectives. In addition, further analysis entailed dividing the samples into specific cellular compartments (Drake and Lasek, 1984). The connectives were dissected to obtain a desheathed connective containing enriched axons surrounded by adaxonal glia as well as the connective sheath, containing connective tissue, muscle cells, and blood cells. The ganglion cells were also further dissected so that the neuronal cytoplasm could be analyzed free of the adherent glial sheath.

Materials and Methods

Aplysia californica, weighing 150 to 200 gm, were purchased from Marine Specimens Unlimited (Pacific Palisades, CA). They were maintained at 17°C in individual chambers containing circulating artificial sea water (Instant Ocean, Aquarium Systems Inc., Eastlake, OH). The nervous system of

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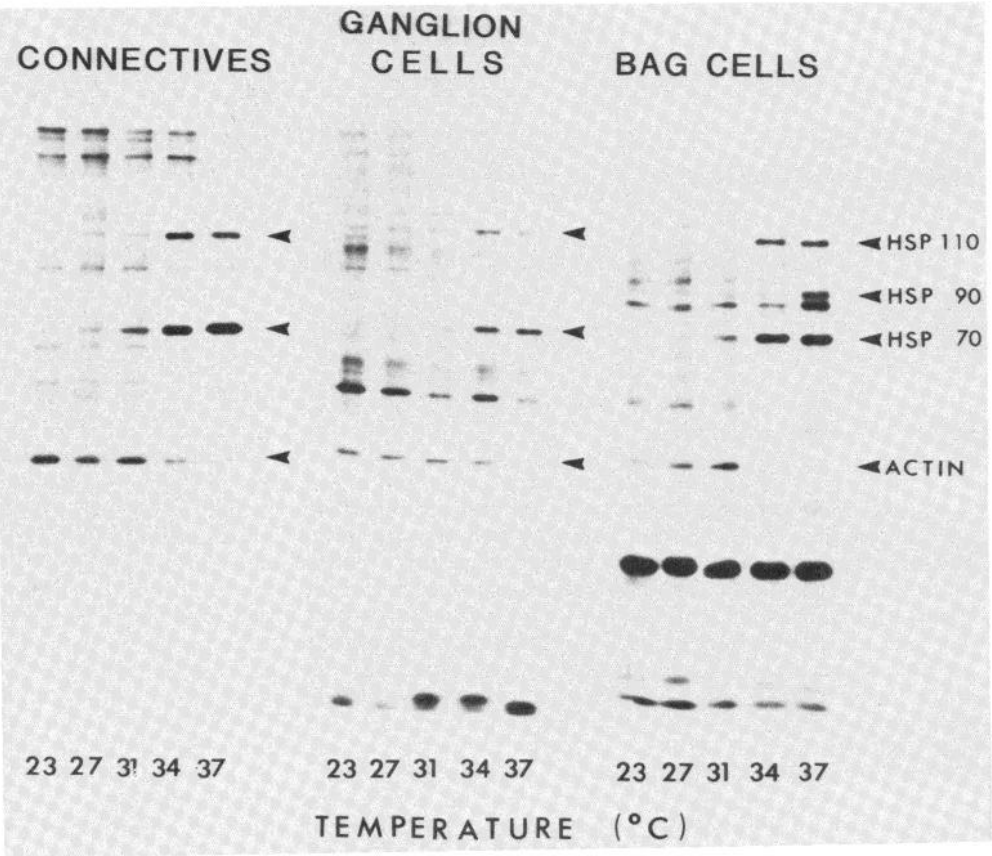

Figure 1. Fluorograph of labeled proteins in bag cells, ganglion cells, and non-neuronal connectives after various heat treatments. Samples were treated at the indicated temperatures for 30 min and then labeled with [^{35}S]methionine for 2 hr. Equal total trichloroacetic acid-precipitable counts per minute were loaded onto each lane (10,000) of a 10% SDS gel. In all samples analyzed, heat treatments of greater than 31°C lead to the enhanced labeling of 70,000- and 110,000-dalton proteins. In bag cells which were treated at 37°C, a protein with an apparent M_r of 90,000 is also prominently labeled. Heat treatments also lead to a relative decrease in the labeling of proteins which are normally synthesized at 23°C. The relative labeling of HSP 70 and HSP 110 in ganglion cells also appears dramatically lower than in the non-neuronal connective cells.

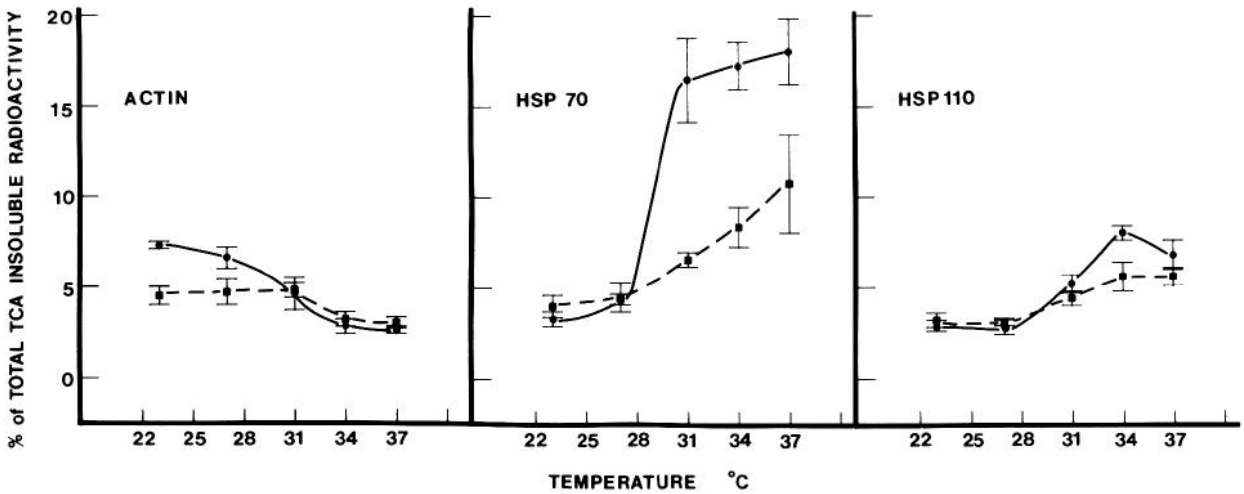

Figure 2. Comparison of the relative levels of [^{35}S]methionine-labeled actin, HSP 70, and HSP 110 after various temperature treatments. Samples were treated at the indicated temperatures for 30 min prior to labeling with [^{35}S]methionine for 2 hr. Samples were then run on one-dimensional SDS gels, and after fluorography, the indicated bands were cut from gels and the resulting radioactivity from (n)[3] samples was determined and is expressed as the mean ± SEM. Significant differences between ganglion cells and connectives at their maximal levels of expression[4] were $p < 0.05$ for HSP 70 and $p < 0.01$ for HSP 110 as analyzed by the Student's t test.

Aplysia was exposed through an incision in the foot. The abdominal and pleural ganglia with their associated connectives were removed from the animal and placed in artificial sea water.

Heat shock treatment and labeling. Samples were either subjected to elevated temperatures of 27, 31, 34, or 37°C by placing them in a water bath maintained at the indicated temperatures (± 1°C) or left at room temperature (~23°C) for 30 min. Unless otherwise indicated, samples were then labeled for 2 hr at room temperature in artificial sea water containing 5 mCi/ml of [^{35}S]methionine (New England Nuclear, Boston, MA; ~1000 Ci/mmol). Samples were then isolated and homogenized in SUB (0.5% SDS, 8 M urea, and 2% β-mercaptoethanol). To determine the total amount of radioactivity incorporated into samples, sample aliquots were trichloroacetic acid were precipitated on Whatman 3MM filters, washed, and then counted in Formula 963.

Isolation of neuronal and non-neuronal samples. Neuronal and non-

[3] Two animals were used for each determination, with two connectives from each animal and three ganglion cell groups—pooled ganglion cells, R2, and LPI—from each animal. One to three gels were analyzed for each sample.

[4] Maximum temperature of HSP 70 synthesis was 37°C in ganglion cells and connectives. Maximum temperature of HSP 110 synthesis was 34°C in non-neuronal connective cells and 34 or 37°C in ganglion cells.

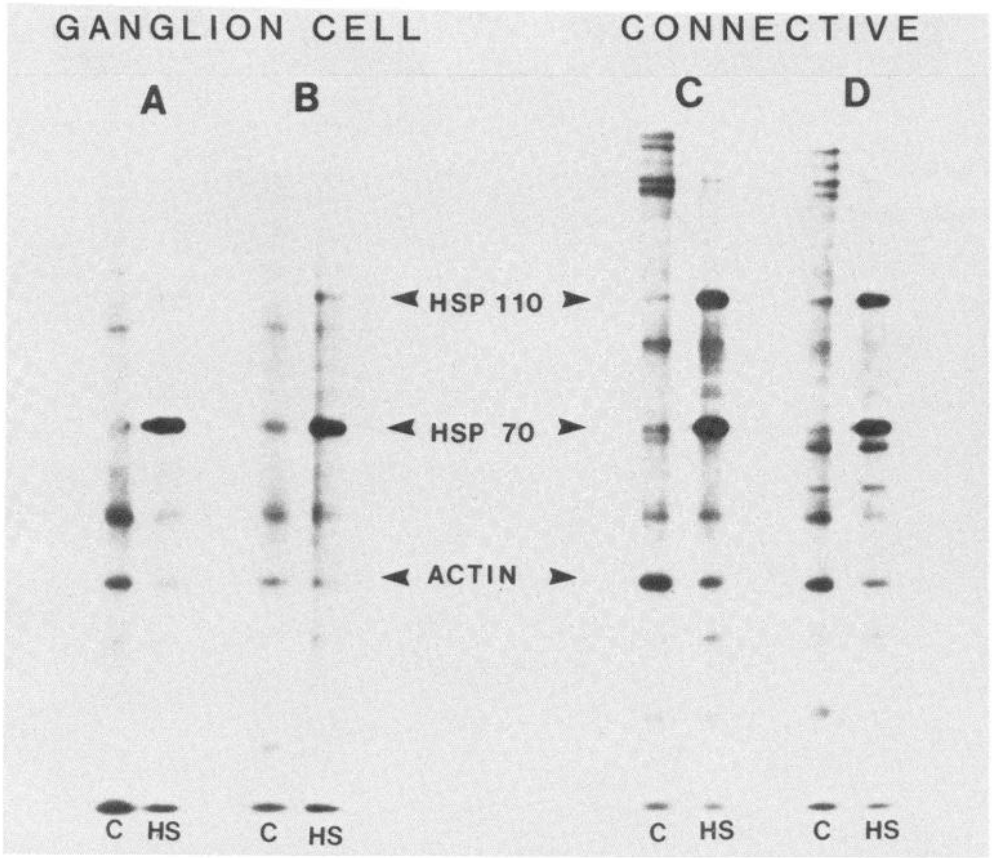

Figure 3. Fluorograph of labeled proteins in different cellular compartments after exposure to a heat shock. Samples were either maintained at room temperature (control) or exposed to a heat treatment at 34°C for 30 min prior to labeling for 2 hr. After labeling, the ganglion cell LPl was slit open and the neuronal components were extruded into artificial sea water to give a neuronal cytoplasm fraction (*B*) and the remaining remnant (*A*). In addition, the connectives were further analyzed by desheathing the connectives to obtain a sheath fraction (*C*), as well as the desheathed connective, enriched in adaxonal glia (*D*). The results demonstrate that the major HSPs are present in all fractions analyzed.

neuronal samples were obtained by the methods of Drake and Lasek (1984). Ganglia were pinned through the connective tissue sheath in a dish lined with Sylgard (Dow Corning) and containing a phosphate-stabilizing buffer (0.1 M sodium phosphate, pH 7.5, 1 mM $MgCl_2$, 2 mM EGTA, 2 M glycerol). Bag cells were obtained after removing the connective tissue sheath at the base of the abdominal ganglion. To obtain ganglion cells, the connective tissue sheath lying over the cell bodies was removed by microdissection, and individual cells were removed using microforceps. Cell bodies could be isolated free of adherent neuropil and axons, although the adherent glial sheath surrounding the neural cell bodies still remained.

Separation of the neuronal cytoplasmic constituents from the adherent glial sheath was done by transecting the neuronal membranes from large ganglion cells (LPl or R2) and extruding the cytoplasmic constituents into artifical sea water. The two fractions obtained therefore included a neuronal fraction containing the neuronal cytoplasm and nucleus, as well as the remnant which contained the glial sheath, neuronal membranes, and unextruded neuronal cytoplasm.

Connectives were desheathed by transecting the connective sheath layer while holding the axon bundle with microforceps and pulling the sheath away. The desheathed connective contained primarily axons surrounded by adaxonal glia, in addition to possible remaining material from the connective sheath, whereas the connective sheath contained connective tissue and blood vessels, as well as muscle cells.

Polyacrylamide gel electrophoresis. One-dimensional SDS gel electrophoresis was performed either by a modification of the method of Laemmli (1970), using a gradient of 6 to 17.5% with a 4% stacking gel, or by using a 10% gel with a 4% stacking gel. Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). Prior to isoelectric focusing, samples were mixed with lysis buffer (9 M urea, 8% Triton X-100, 5% β-mercaptoethanol, and 2% Ampholines (1.6%, pH 5 to 7 and 0.4%, pH 3 to 10; LKB Instruments, Gaithersburg, MD). Proteins were stained with 1% Sorva Blue. After destaining, the gels were subjected to fluorography by dehydration with dimethylsulfoxide (DMSO) and impregnated with 2,5-diphenyloxazole (PPO) (Bonner and Laskey, 1974; Laskey and Mills, 1975). The gels were then vacuum dried onto dialysis membrane and fluorographs were made using XAR-5 x-ray film (Kodak) at −70°C.

Quantitation of proteins. Fluorographs were used to locate the labeled bands corresponding to actin (43,000 daltons) or proteins whose synthesis was apparently enhanced by the heat treatments. The bands were then cut from the gels and solubilized by incubation in 0.75 ml of 30% hydrogen peroxide at 60°C for 48 hr, and the radioactivity was determined by liquid scintillation counting in Formula 963.

Results

One-dimensional electrophoresis of labeled proteins after various temperature treatments. Incubation of *Aplysia* neuronal and non-neuronal cells at temperatures greater than 31°C for 30 min resulted in a relative decrease in the labeling of proteins normally synthesized at 23°C as well as an increase in [^{35}S]methionine incorporation into at least two proteins having M_rs of approximately 70,000 and 110,000, where the 70,000-dalton protein was the most abundant protein induced. An increase in label was also observed in a 90,000-dalton protein after a 30-min heat treatment at 37°C in the neurosecretory bag cells. A robust increase in the labeling of this protein was not apparent in either the ganglion cells or the connectives as analyzed by one-dimensional SDS gel electrophoresis (Fig. 1). Figure 1 also reveals a difference in the relative protein synthetic response of neuronal and non-neuronal cells to elevated temperatures.

Quantitation of labeled HSPs. The relative difference in this protein synthetic response is more clearly observed in Figure 2. In general, the temperature dependence of the heat shock response is similar in both the neuronal ganglion cells and the non-neuronal connectives. However, in connectives the relative level of HSP 70 labeling is approximately twice that observed in ganglion cells at temperatures above 31°C. The relative level of HSP 110 synthesis in ganglion cells is also significantly different from that observed in connectives, and the decrease in the labeling of actin (M_r = 43,000) appears to be more dramatic in the connective cells than in the ganglion cells.

Comparison of the protein synthetic response of different neural populations indicated that, although the relative levels of HSP 70 and HSP 110 were higher in the bag cells, the maximum levels observed were not significantly different from those of the mixed population of ganglion cells. The data for ganglion cells were derived from the analysis of a pooled population of cells from the abdominal

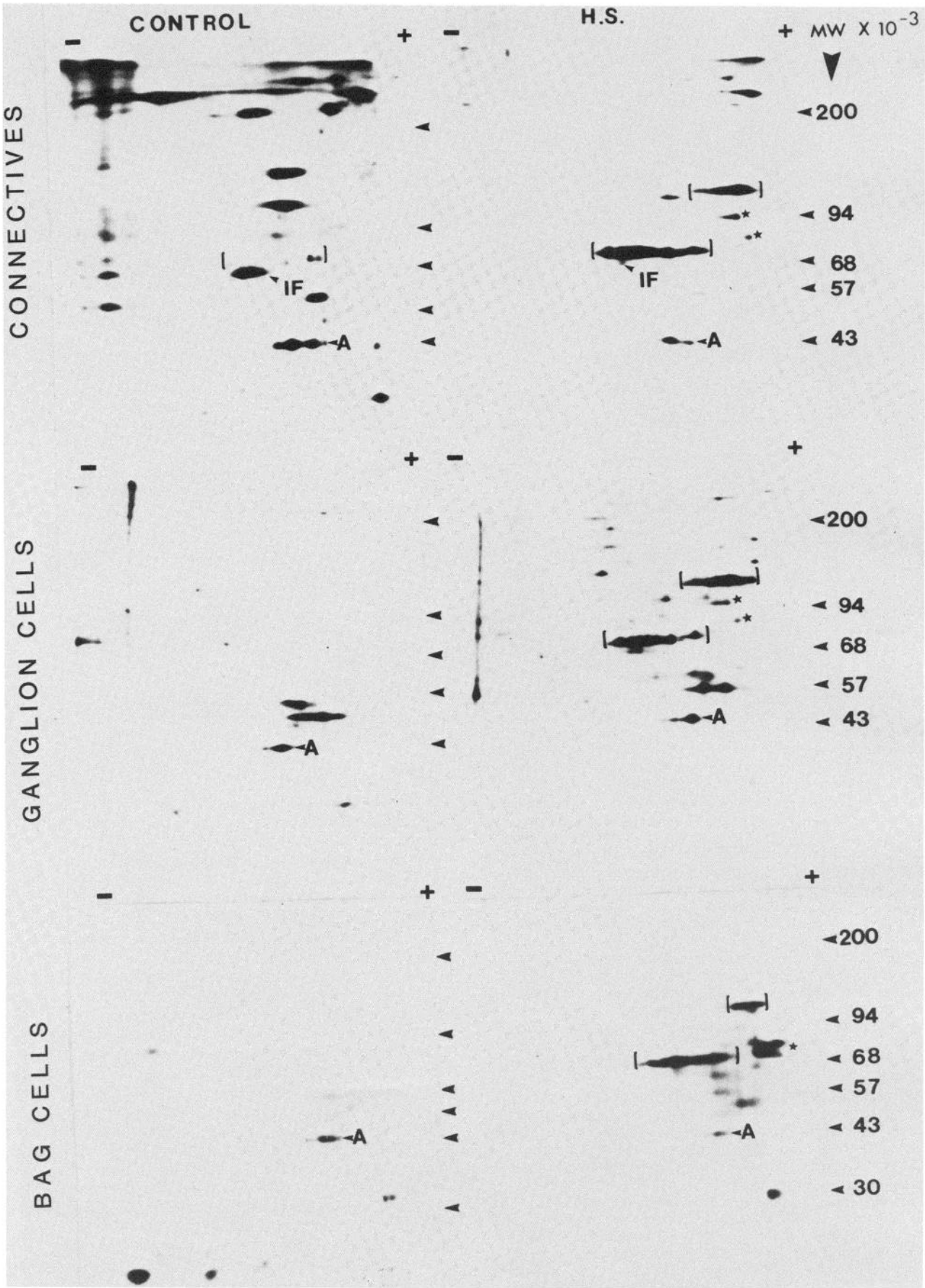

Figure 4. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled proteins in bag cells, ganglion cells, and non-neuronal connectives after a 30-min exposure to 34°C (*H.S.*) or 23°C (*CONTROL*). The major labeled HSPs (*spots* between *brackets*) are similar in both neuronal and non-neuronal cells. In addition, other heat-induced proteins are also seen (★). For example, the bag cells contain additional highly labeled HSPs with M_r ranging from approximately 70,000 to 90,000. In addition, some minor heat-induced proteins can be noted in both ganglion cells and connectives. Actin (*A*) and the intermediate filament protein (*IF*), which runs just beneath HSP 70 on two-dimensional gels of connective cells (Drake and Lasek, 1984), are also indicated.

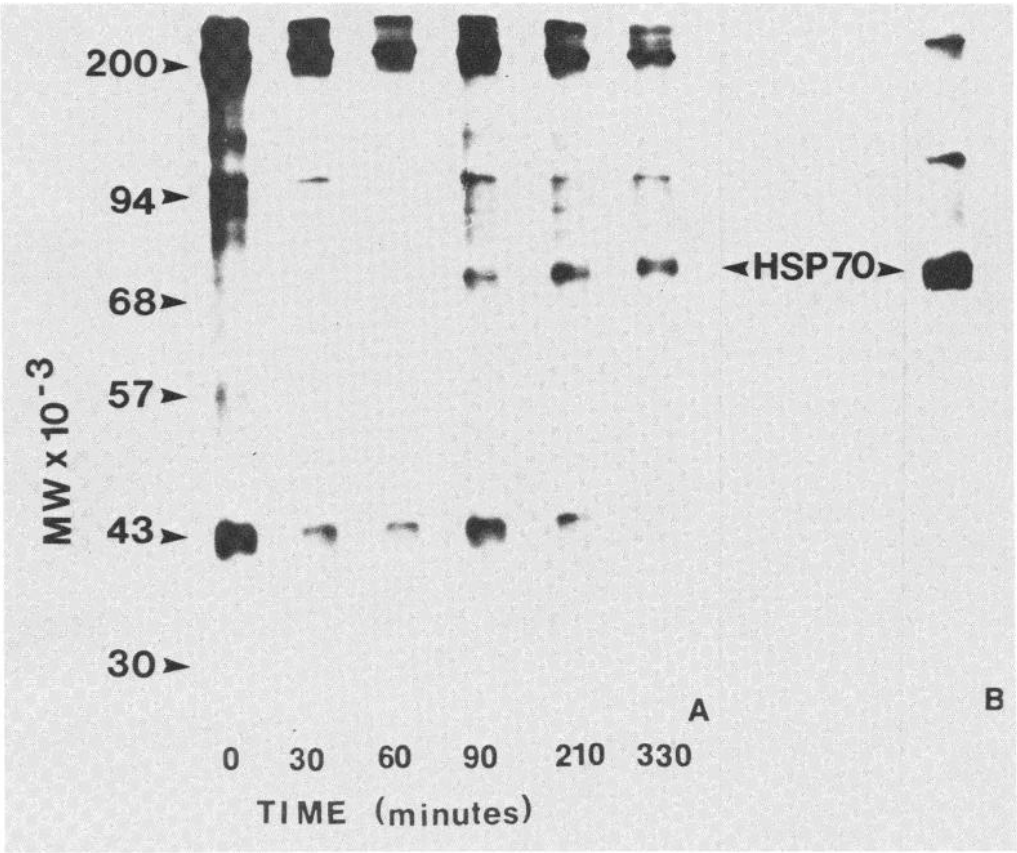

Figure 5. Fluorogram illustrating the production of HSP 70 in connectives by *in vitro* incubation conditions. *A*, Connectives were removed from the animal and at the indicated times were pulse labeled with [^{35}S]methionine for 30 min before subjecting to SDS gel electrophoresis and fluorography. At increasing times *in vitro*, an enhanced labeling of a 70,000-dalton protein occurs. *B*, Connective which was heat shocked for 37°C for 30 min and then labeled for 30 min with [^{35}S]methionine is shown for comparison. The level of labeled HSP 70 synthesis which is induced by heat shock is much greater than that induced by the *in vitro* incubation conditions.

ganglia, as well as from two individually analyzed cells: R2 from the abdominal ganglion and LPl from the left pleural ganglion. In general, there was no significant difference in the relative synthesis of HSP 70 or HSP 110 in the individual cells as compared to the pooled cell population. However, in one R2 cell, treatment at 37°C for 30 min resulted in a robust stress response, not significantly different from that observed in non-neuronal cells. It is not clear whether this example represents a peculiarity with R2 or whether other individual neurons may also occasionaly undergo a vigorous stress response.

Further analysis of the cell type specificity of the heat shock response. The use of nervous tissue from *Aplysia* allowed for further analysis of the localization of specific HSPs in specific cellular compartments. Identified ganglion cells could be dissected into an exclusive neuronal fraction, containing the neuronal cytoplasm and nucleus, as well as a remnant fraction, containing the glial sheath, neuronal membranes, and unextruded neuronal cytoplasm. Moreover, connectives could be desheathed so that the axons containing desheathed connectives were enriched in adaxonal glia, whereas the sheath fraction consisted primarily of connective tissue, blood vessels, and muscle cells. Localization of labeled HSPs in these cellular compartments is illustrated in Figure 3. The results demonstrate that labeled HSPs are located in all of these cellular compartments, suggesting that both neurons and adaxonal glia are capable of HSP synthesis.

Two-dimensional gel electrophoresis. Further analysis of the proteins synthesized in response to heat stress by two-dimensional gel electrophoresis demonstrate that the 70,000- and 110,000-dalton proteins possess similar isoelectric points, in both the ganglion cell R2 and the connective samples (Fig. 4), indicating that these HSPs are probably the same in both cell types. In addition to these major HSPs, a few minor heat stress-induced proteins can be seen by two-dimensional electrophoresis. Two-dimensional electrophoresis of the bag cells indicates that there is also an abundant increase in the labeling of more acidic proteins with M_rs ranging from approximately 70,000 to 90,000.

The 70,000-dalton protein consists of a complex pattern of spots, which is characteristic of the 70,000-dalton HSPs of other organisms (Mirault et al., 1977). Both post-translational modifications, such as phosphorylation (Schlesinger et al., 1982a) or methylation (Wang et al., 1981), and the presence of closely related gene products (see Schlesinger et al., 1982a) may contribute to the charge heterogeneity of the major HSPs.

In vitro labeling of HSPs. Although HSP 70 synthesis could be detected at low levels in control samples, it is possible that this low level of synthesis may have been due to the *in vitro* incubation conditions used in this study. An experiment in which connective cells were pulse labeled for 30-min intervals at times after placing connectives *in vitro* demonstrates that the 70,000-dalton protein can be detected on one-dimensional SDS gels after 90 to 120 min *in vitro* (Fig. 5). No labeling of the HSP 70 protein could be detected when connective cells were labeled *in vitro* at room temperature for 30 min (unpublished observations). The induction of HSP 70 by *in vitro* incubation conditions is consistent with numerous other studies which have demonstrated the induction of HSPs under a variety of stressful conditions (see Ashburner and Bonner, 1979; Thomas et al., 1982, for reviews).

Discussion

General features of the heat shock response, involving the preferential synthesis of a small number of proteins, have been conserved throughout evolution. Organisms which have shown this response include bacteria, plants, yeast, insects, and avian as well as a number of mammalian organisms (see Schlesinger et al., 1982b, for review). We have demonstrated that cells in the invertebrate mollusc *Aplysia* also undergo a similar response to elevated temperatures. Treatment of *Aplysia* neuronal and non-neuronal cells at temperatures of greater than 31°C results in the relative decrease in actin labeling, as well as a relative increase in the labeling of a small number of proteins, with the 70,000-dalton HSP being the most abundant protein labeled.

Antibody studies have demonstrated that the 70,000-dalton HSPs are conserved in organisms ranging from yeast to mammalian species (Kelly and Schlesinger, 1982). Dot blot hybridization analysis of *Aplysia* connective cell mRNA with a cDNA probe prepared from *Drosophila* HSP 70 mRNA (Craig et al., 1979) has indicated that the heat shock-induced mRNAs from *Aplysia* and the *Drosophila* cDNA probe crosshybridize (S. G. Greenberg, unpublished observations), illustrating the conservation of HSP mRNAs between these organisms.

Since many features of the heat shock response have been observed following a variety of stressful situations (see Ashburner and Bonner, 1979, for review), the heat shock response can be used to understand basic molecular mechanisms which cells use to deal with stressful situations. Molecular events involved in the heat shock response have been most thoroughly studied in *Drosophila melanogaster* (see Ashburner and Bonner, 1979, for review). The decreased production of proteins normally synthesized at 25°C in this organism is due to a decrease in the translation of mRNAs coding for normally synthesized proteins, whereas the enhanced production of HSPs is due to both increased transcription and preferential translation of heat shock mRNAs (McKenzie et al., 1975; Spradling et al., 1975; Mirault et al., 1977). Studies in the mammalian brain have also demonstrated that nervous tissue responds to heat shock by an increase in the transcription of heat shock-induced mRNAs (Brown et al., 1983).

While different cell types may use similar basic mechanisms to deal with stressful situations, different cell types may also display characteristic features of a general stress response. Cell- and tissue type-specific differences in the heat shock response have been described in a few systems. Atkinson (1981) has found that, as avian cells progress through myogenesis, their ability to respond to similar temperature shifts is reduced. In addition, the induction of HSP 25, which was observed in myoblasts, was not detectable in myotubes. Tissue-specific differences in the expression of specific HSPs have also been described in *Drosophila hydei* (Sondermeijer and Lubsen, 1978).

In *Aplysia* we found that, although basic features of the heat shock response are similar in neuronal and non-neuronal cell populations, a few differences were also observed. Although all of the cells analyzed responded to heat stress by the induction of 70,000- and 110,000-dalton proteins, the bag cells also showed enhanced levels of other labeled proteins in the range of M_r = 70,000 to 90,000. In addition, relative levels of labeled HSPs in the ganglion cells were, in general, significantly lower than in the connective cells. This finding is consistent with previous studies describing an enhanced synthesis of the 71,000-dalton stress protein in the microvascular fraction of mammalian nervous tissue (White, 1980, 1981).

The ganglion cells used in this study consisted of the neuronal cell body, as well as an adherent glial sheath. The ability of neurons to respond to elevated temperatures by the induction of HSPs is suggested by the finding that labeled HSPs could be detected in a ganglion cell fraction containing the neuronal cytoplasm and nucleus after being dissected away from the glial sheath and neuronal membranes. Although this result demonstrates that HSPs are located in neurons, it is possible that some of the labeled HSPs present in the neuronal cytoplasm may have been transferred from the surrounding glia sheath. The possibility that glia cells produce HSPs is supported by the presence of labeled HSPs in desheathed connectives which are enriched in adaxonal glia.

In vitro studies have demonstrated that specific proteins are selectively transferred from adaxonal glia into the squid giant axon (Gainer et al., 1977; Lasek et al., 1977; Lasek and Tytell, 1981). Although the function of most of the transferred proteins is not clear, it has been suggested that these proteins serve a supportive role in the axon (Lasek et al., 1977). Traversin, a 70,000-dalton protein, is an abundantly labeled protein transferred from the adaxonal glia into the squid axoplasm (Lasek and Tytell, 1981). The current results, which suggest that the 70,000-dalton HSP may be synthesized by adaxonal glia, as well as the finding that this HSP is prduced during *in vitro* incubation conditions, prompted us to test the possibility that HSPs are among the group of glia-axon transfer proteins. The results indicated that HSPs are indeed transferred into the squid giant axon from the surrounding glia sheath (M. Tytell, S. G. Greenberg, and R. J. Lasek, unpublished observation).

The highly conserved nature of the HSPs, as well as their high level of synthesis during a variety of stressful situations, argues for an important protective role of these proteins during the cell's response to stress (see Ashburner and Bonner, 1979; Thomas et al., 1982, for reviews). Moreover, a few studies have implicated a possible role for these proteins in conferring thermotolerance to heat-stressed cells (McAlister and Finkelstein, 1980; Petersen and Mitchell, 1981). Although the specific function of HSPs is not clear, there is some evidence which suggests that HSPs are associated with the cytoskeletal network of cells (Schlesinger et al., 1982a; Weller and Solomon, 1983), thereby indicating a potentially important role of these proteins in the cytoplasm during periods of stress.

A recent report (Clark and Brown, 1984) has indicated that HSPs may be provided to axons by slow axonal transport. However, the time required for HSPs to reach distal regions of the axon by slow axonal transport may not be favorable for a protective role of these proteins in the axon. Glia-axon protein transfer may therefore be an important mechanism for supplying HSPs to axons during periods of stress.

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