In Vitro Actions of Thyroid Hormone on Protein Phosphorylation in a Nucleus-free Subcellular Fraction from Adult Rat Brain.

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Introduction

Several converging lines of evidence point to a unique mechanism of action of thyroid hormones (TH) in adult brain. As revealed by debilitating neurological signs and symptoms associated with thyroid disorders in adults (Laurberg, 1990; Utiger, 1987), TH play fundamental roles in the central nervous system. In contrast to the neurological disorders related to a developmental imbalance in TH, most of the CNS signs and symptoms of adult-onset thyroid dysfunction are reversible with proper adjustment of the circulatory TH (Dratman and Gordon, 1996; Henley and Koehnle, 1997; Bunevicius and Prange, 2000; Sarkar 2002). In developing nervous system, the major effect of thyroid hormones is generally considered to be the result of stimulation of a nuclear receptor which is also found in adult peripheral tissues (Oppenheimer et al., 1999). High levels of 3,5,3'-L-triiodothyronine (T3) have been found in nerve terminals from adult brain, suggesting a synaptic action of TH (Dratman, 1974; Mason, et al., 1993; Sarkar and Ray, 1994; Dratman and Gordon, 1996).

In recent years, the possibility of direct nongenomic actions of TH in certain types of cells, including mature neurons, is gaining much attention (Martin et al., 1996; Sarkar and Ray, 2003; see Dratman and Gordon, 1996; Sarkar 2002). However, the mechanisms of action of TH in the regulation of synaptic functions in adult neurons are poorly understood. The present study examines the possibility of a nongenomic action of L-T3 to modulate protein phosphorylation in synaptosomes from adult rat brain.

Methods

Animals

Young adult male Sprague-Dawley rats (250-300 g) were decapitated and their brains immediately dissected in ice-cold isotonic saline.

Preparation of Synaptosomes

Synaptosomes were purified from cerebral cortical tissue using the method of Hajos (1975). A P_2 pellet was prepared by differential centrifugation in 0.32 M sucrose and layered on 0.8 M sucrose. Subsequent to an additional centrifugation at 9,000 x g for 30 min, the 0.8 M layer was diluted to 0.4 M. The pellet sedimenting after 20 min at 12,000 x g (purified synaptosomes) was lysed with hypotonic shock and used for the incubations.

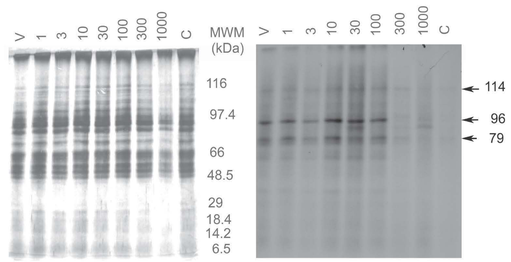
Incubation

The resulting preparation of soluble and membrane protein was incubated in 10 mM $MgCl_2$, 0.1 mM EGTA, and 50 mM HEPES (pH 7.4) with various concentrations of 3,5,3'-L-triiodothyronine (T3) and 3 μCi of γ-^{32}P-ATP for varying times at 37°C. The reaction was terminated by addition of SDS-Sample Buffer (Laemmli, 1970).

SDS-PAGE and Autoradiography

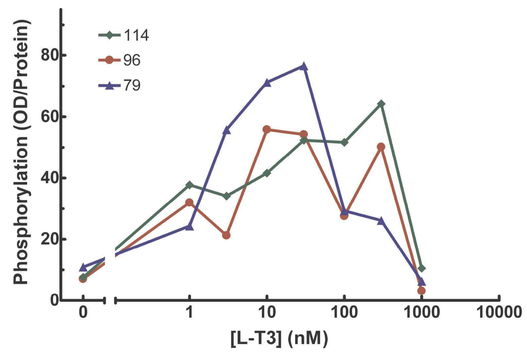
The samples were denatured for 3 minutes at 100°C, subjected to electrophoresis on 7.5 % SDS-PAGE at constant current, and stained with silver nitrate. The dried gels were then exposed to x-ray film at -80°C for 48 hours and the optical densities of bands in the gel and the resulting autoradiogram were quantified using the ONE-DSCAN program (Scanalytics, Inc., Fairfax, VA)

Figure 1. SDS-PAGE of Synaptosomal Proteins incubated *In Vitro* with γ-^{32}P-ATP and Varying Concentrations of T3



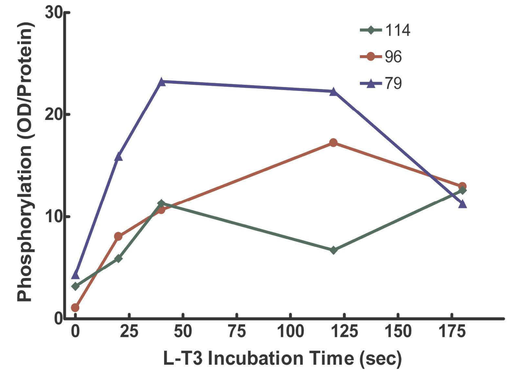
A preparation of soluble and membrane protein from purified synaptosomes which had been lysed osmotically was incubated in 10 mM $MgCl_2$, 0.1mM EGTA, and 50 mM HEPES (pH 7.4) with various doses of T3 and 3 μCi of γ-^{32}P-ATP for 1 min at 37°C. The proteins were subjected to SDS-PAGE followed by autoradiography as described in Methods. A silver-stained gel is depicted on the left and the corresponding autoradiogram on the right. Lanes are labeled according to the concentration of T3 in nM. The middle column indicates the positions of the molecular weight markers (MWM). Three bands of 114, 96 and 79 kDa (labeled with arrows on the right) were quantitated.

Figure 2. Influence of Concentration of L-T3 on *In Vitro* Modulation of Protein Phosphorylation



A preparation of soluble and membrane protein from purified synaptosomes which had been lysed osmotically was incubated in 10 mM $MgCl_2$, 0.1 mM EGTA, and 50 mM HEPES (pH 7.4) with various doses of T3 and 3 μCi of γ-^{32}P-ATP for 1 min at 37°C. The proteins were subjected to SDS-PAGE followed by autoradiography as described in Methods. The integrated OD from bands on the autoradiograms corresponding to three phosphorylated proteins were quantitated and corrected for the integrated lane density from the corresponding silver-stained gel. Results are the combined normalized values from two separate experiments.

Figure 3. Time Course oif Influence of L-T3 on *In Vitro* Modulation of Protein Phosphorylation



A preparation of soluble and membrane protein from purified synaptosomes which had been lysed osmotically was incubated in 10 mM $MgCl_2$, 0.1 mM EGTA, and 50 mM HEPES (pH 7.4) with 3 μCi of γ-^{32}P-ATP for a total of 5 minutes at 37°C. At varying times, 10 nM T3 was added to the incubation so that the incubation time with hormone varied as indicated. The proteins were subjected to SDS-PAGE followed by autoradiography as described in Methods. The integrated OD from bands on the autoradiograms corresponding to three phosphorylated proteins were quantitated and corrected for the integrated lane density from the corresponding silver-stained gel.

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Summary and Conclusion

(1) A dramatic enhancement of incorporation of ^{32}P per unit amount of protein was demonstrated in three proteins of molecular weight 114, 96 and 79 kDa, following incubation with concentrations of T3 from 10-100 nM. Physiological concentrations of T3 in nerve terminals are ~15 nM (Mason et al., 1993; Sarkar and Ray, 1994). The degree of phosphorylation of the 96 and 79 kDa proteins stimulated by these concentrations of T3 was higher than the 114 kDa protein. However, the higher doses of T3 (300-1000 nM) showed a lesser stimulation of phosphorylation of all three proteins, indicating a biphasic action. Overall, T3 induced varying levels of phosphorylation of several proteins in adult rat brain cerebrocortical synaptosomes, in vitro, within 1 min.

(2) During a fixed 5-min incubation with γ-^{32}P-ATP, the optimal incubation time with 10 nM T3 to maximally stimulate protein phosphorylation was between 40-120 sec. The 96 and 79 kDa proteins responded more to this T3-induced phosphorylation as compared to the 114 kDa protein.

(3) In conclusion, brain physiological concentrations of T3 rapidly enhanced protein phosphorylation in lysed synaptosomes, a nucleus-free subcellular fraction from young adult rat brain cerebral cortex, in vitro. This finding suggests a novel nongenomic signal transduction pathway for T3 in adult mammalian brain.

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