This project has examined the biochemical mechanisms regulating the response of the rat adrenal medulla and chromaffin tissue. The responsivity of the tissue will change depending upon the treatment that the animal has received and the time after the treatment. The alteration in reactivity appears to be correlated with changes in tissue levels of the catecholamine neurotransmitters. Tissues that demonstrate altered reactivity have profound morphological changes that return to control with the return of normal reactivity. Additional biochemical factors that may regulate reactivity are currently being examined in a variety of model systems of the chromaffin cell.
1. SUMMARY:

This project has examined the biochemical mechanisms regulating the response of the rat adrenal medulla and chromaffin tissue. We have found that the responsivity of the tissue will change depending upon the treatment that the animal has received and the time after the treatment. The alteration in reactivity appears to be correlated with changes in tissue levels of the catecholamine neurotransmitters. Tissues that demonstrate altered reactivity have profound morphological changes that return to control with the return of normal reactivity. Additional biochemical factors that may regulate reactivity are currently being examined in a variety of model systems of the chromaffin cell.

2. RESEARCH OBJECTIVES:

1. Optimize Conditions to provide maximal alterations in the bioreactivity of the adrenal medulla.

2. Evaluate disassociated chromaffin cells isolated from animals exhibiting altered adrenal reactivity.

3. Compare the localization (compartamentalization) and activity of protein kinases and their substrates in control tissues and adrenal glands demonstrating altered reactivity or from disassembled cells derived from these tissues.

4. Quantitate the biosynthesis and number of chromaffin granules, catecholamine content, and evaluate the morphological characteristics of adrenal chromaffin vesicles exposed to treatments that alter the bioreactivity.

5. Purify and evaluate chromaffin vesicles from the adrenal medullary cells with respect to catecholamine content, protein content, protein kinase substrate content and ability to sequester calcium.

6. Quantitate the degree of phosphorylation and specific sites of phosphorylation of functionally significant proteins such as tyrosine hydroxylase, protein III, protein F1, and vesicle associated phosphoproteins from control, hyper- and hypo-reactive adrenal glands.
3. STATUS OF RESEARCH:

The research efforts from 3/1/86 to 3/1/88 have focused on identifying factors that regulate the responsivity of the rat adrenal medulla and biochemical mechanisms that regulate the PC12 cells. The specific areas of research include: a) Development of the isolated perfused rat adrenal gland preparation b) Examination of the morphological changes that accompany changes in bioreactivity of the rat adrenal medulla c) Development of the perfused rat adrenal slice technique d) Identification of a novel protein kinase activity in PC12 cells e) Investigation of the role of protein kinase C in regulating neurite outgrowth f) Application of monoclonal antibodies for the immunocytochemical localization of tyrosine hydroxylase and g) Development of techniques for quantitating TH mRNA. Each of these areas will be briefly discussed individually below.

A. Development of the isolated perfused rat adrenal gland preparation:

The ability of the rat adrenal sympathoadrenal axis to alter its reactivity in vivo had been previously reported. To determine if this change in reactivity occurred at the level of the adrenal gland, we employed the isolated perfused adrenal gland. This preparation enabled the investigator to perform experimental manipulations on the intact animal and then assess whether molecular or biochemical changes had occurred in the isolated perfused adrenal gland. We found with experimental manipulation that the amount of catecholamines released by a maximal dose of acetylcholine could be decreased by up to 50 percent or increased by 40 percent when compared with controls. Following chronic periods of insulin induced hypoglycemia, the adrenal glands isolated from the animals were found to have altered responsivity to standard doses of acetylcholine. The changes in responsivity was found to parallel the tissue content of catecholamines.

This change in reactivity was not only dependent upon the type of experimental manipulation, but also the time after the experiment treatment. The amount of epinephrine and norepinephrine released from the isolated perfused rat adrenal gland by a fixed dose of acetylcholine was found to be decreased at one day. The responsivity of the gland gradually returned to normal values at day 5. Initial experiments were performed using immobilization stress which increased the amount of catecholamine release by up to 60 percent above controls. Hypoglycemic stress caused a decrease in both the catecholamine content of the adrenal gland and the amount that was released by acetylcholine mediated depolarization. These series of experiments clearly demonstrated that the reactivity of the adrenal gland could be altered by physiological conditioning in the laboratory rat.

B. Examination of the morphological changes that accompany changes in bioreactivity of the rat adrenal medulla:

When adrenal glands isolated from these animals were examined by electronmicroscopy, profound differences were observed. The most striking difference was in the cellular content of chromaffin granules. At day one of treatment, the number of granules was significantly decreased. At day one of
recovery, the granule depletion was so severe that the cells contained only a few granules. The granule content of the glands isolated from the hypoglycemic treated animals slowly returned to normal by day 7. The Golgi apparatus was markedly affected by the hypoglycemic treatment. During the early stages of cellular response, the Golgi apparatus is enlarged and appeared to be in an active secretory stage. Numerous multivesiculate bodies were observed in the cytosol, suggesting that a massive secretory event had occurred. Currently, a complete morphological analysis is being performed on these tissue to more fully quantitate the observed differences.

C. Development of the perfused rat adrenal slice technique.

To more fully characterize functional changes that occur to the adrenal medulla at the biochemical level, we have developed a novel preparation using adrenal glands isolated from the treated animals. The glands are removed and sliced in 300 um slices, perfused with an oxygenated physiological buffer solution for 30 minutes. The slices are then depolarized with acetylcholine, the endogenous secretagogue, and the amount of epinephrine or norepinephrine released from this preparation is quantitated by HPLC/EC methods. We find that the patterns of altered reactivity with this preparation are similar to those observed with the perfused rat adrenal gland.

D. Identification of a novel protein kinase activity in PC12 cells.

During the investigation of the regulation of tyrosine hydroxylase, the rate limiting enzyme in the biosynthesis of the catecholamine neurotransmitters, we discovered that this enzyme was phosphorylated in a novel site that would not be phosphorylated by any other known protein kinase. We have developed methods for the assay of this kinase activity using a synthetic peptide that is homologous with the N-terminal region of TH. We have succeeded in partially purifying this kinase activity and find that it has a molecular weight of approximately 50,000 daltons. Additional research using intact cells labelled with radioactive phosphate suggests that this kinase may play an important role in mediating some of the actions of the growth factors.

E. Investigation of the role of protein kinase C in regulating neurite outgrowth

While investigating the regulation of cell phosphorylation in PC12 cells following treatment with nerve growth factor (NGF), we observed that the NGF-mediated neurite outgrowth was potentiated by simultaneous treatment with phorbol esters. Inclusion of a specific inhibitor of protein kinase C blocked the outgrowth of these neurite processes, suggesting that protein kinase C activity is essential for neurite outgrowth. The role of other protein phosphorylation in regulating the responsivity of the PC12 cells is currently being investigated.
F. Application of monoclonal antibodies for the immunocytochemical localization of tyrosine hydroxylase.

During the earlier stages of this project, we developed a monoclonal antibody specifically directed against rat tyrosine hydroxylase. TH was purified from rat pheochromocytoma and injected into mice and lymphocytes from the spleen isolated and fused with myeloma cells. We screened and cloned a cell line that produces a monoclonal antibody that is specific for tyrosine hydroxylase. We have used this antibody to localize TH in the growth cone of developing neurites. In other laboratories, this antibody has been used to identify and localize TH in caudate nucleus, the regenerating rat spinal cord, the retina of the fish eye, and a variety of other tissues.

G. Development of techniques for quantitating TH mRNA.

We have recently obtained a copy of cDNA coding for tyrosine hydroxylase. This DNA is in the Bluescript plasmid and also contains the endogenous promoter for the rat gene. This segment of DNA has been used to generate specific fragments that are radioactively labelled that have used to quantitate the amount of TH mRNA present in the adrenal gland. We find that during periods of chronic stress, the level of TH message increases nearly two fold and that this increase precedes the appearance of increased TH activity.

4. WRITTEN PUBLICATIONS:


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