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Biochemistry of Synaptogenesis. Clonal lines of hybrid cells derived by fusion of neuroblastoma cells with other cell types were shown previously to form synapses with striated muscle cells with high frequency. The formation of synapses between clonal cells of neural origin, such as NBr10A or NG108-15 hybrid cells, and rat striated muscle cells was found to be regulated. Exposure of hybrid cells for 3-7 days to PGE₁, which results in activation of adenylate cyclase, or exposure to various cyclic nucleotide phosphodiesterase inhibitors markedly increases the number of synapses formed. The effects of putative neurotransmitters or hormones on intracellular cyclic AMP or cyclic GMP levels, voltage-sensitive Ca²⁺ channel activity, and acetylcholine secretion were determined. Receptor-mediated increases in intracellular cyclic AMP or cyclic GMP levels had no immediate effect on K⁺-dependent ⁴⁵Ca²⁺ uptake by cells or on acetylcholine secretion from cells. However, prolonged exposure of hybrid cells to PGE₁ results both in an increase in cellular cyclic AMP and the gradual acquisition by cells of functional voltage-sensitive Ca²⁺ channels. Concomitantly cells acquire the ability to secrete acetylcholine in response to a depolarizing stimulus and can then form functional synapses with muscle cells. These results show that the acquisition of voltage-sensitive Ca²⁺ channels is regulated and that this reaction in turn controls the formation of synapses.

D600 inhibits ⁴⁵Ca²⁺ uptake dependent on 80 mM K⁺ (IC₅₀ = 2 x 10⁻⁷ M), but has little or no effect on ⁴⁵Ca²⁺ uptake in the presence of 5 mM K⁺. ⁴⁵Ca²⁺ uptake also is inhibited by 10 mM La³⁺, Co²⁺, Ni²⁺, Mn²⁺, Sr²⁺, or Ba²⁺, but not by 10 μM tetrodotoxin, 20 mM tetraethylammonium, or 1 mM 3,4-diaminopyridine.

Other cell lines were found that synthesize acetylcholine but do not form synapses with striated muscle cells. Various types of synapse defects were detected; including defects in voltage-sensitive ⁴⁵Ca²⁺ channels, vesicles, and an additional unidentified reaction that is required for acetylcholine secretion.

To identify molecules required for synaptogenesis or communication across the synapse, hybrid cell lines which synthesize mono-specific antibodies were obtained by fusion of clonal myeloma cells with spleen cells immunized against cells from the nervous system. Some of the hybridoma cell lines that were obtained synthesize monospecific antibodies of high titre directed against membrane antigens found on some cells from the nervous system that were not detected with cells from other tissues. One of these cell lines, A2B5, synthesizes antibody directed against an antigen that was shown by indirect immunofluorescence to be associated with plasma membranes of most, or all, neuron cell bodies in chick retina; however, the antigen was not detected on axons or dendrites of neurons, on retina Müller cells, or pigment cells, or on cells from non-neural tissues.

Antigen A2B5 activity is relatively stable at 100°C, is insensitive to trypsin, exhibits the solubility properties of a ganglioside, and is destroyed by neuraminidase. Antibody A2B5 cytotoxicity against retina cells is inhibited by a tetrasialo GQ ganglioside fraction from bovine brain (estimated half-maximal inhibition, 0.2 μM), or N-acetylneuraminic acid (half-maximal inhibition, 5,000

μM), but not by other purified gangliosides tested. These results suggest that the antigen is a GQ ganglioside in plasma membranes of retina neuron cell bodies but not membranes of axons or dendrites.

A solid-phase ^{125}I -Protein A radioassay for anti-cell surface antibodies was devised which employs target cell monolayers cultured on fenestrated polyvinyl chloride 96-well plates ("transfer plates"). The calibrated aperture in the bottom of each well is small enough to retain fluid contents by surface tension during monolayer growth, but also permits fluid to enter the wells when transfer plates are lowered in receptacles containing washing buffer or test sera. To assay for antibodies directed against target cell surface antigens, transfer plates bearing monolayers are inserted into microculture plates with corresponding 96-well geometry, thereby simultaneously sampling 96 wells. This assay allows rapid screening of hundreds of hybrid cell colonies for production of antibodies with desired specificity.

Methyltransferases can be inhibited by S-adenosyl homocysteine or by analogs which either increase S-adenosyl homocysteine levels or inhibit methyltransferases directly such as 3-deazaadenosine (DZA), adenosine-2',3'-diazido-5'-carboxamide (744-99), 5'-deoxy-5'-isobutylthioadenosine (SIBA), and 5'-deoxy-5'-isobutylthio-3-deazaadenosine (DZ-SIBA). In collaboration with P. Chiang and G. Cantoni the effects of these and other compounds on synapses between dissociated chick embryo retina neurons and cultured rat striated muscle cells were investigated. The frequency of spontaneous synaptic responses of muscle cells was markedly reduced by these compounds; half-maximal inhibition was obtained with 1.5×10^{-6} M DZ-SIBA, 1.5×10^{-5} M DZA, 3×10^{-5} M SIBA, or 1×10^{-4} M 744-99. Homocysteine thiolactone, 5-deoxy-adenosine, or tubercidin, which do not increase levels of S-adenosine homocysteine or inhibit methyltransferase activity, do not affect the frequency of spontaneous synaptic responses of muscle cells. These results suggest that a transmethylation reaction may be required for acetylcholine secretion or vesicle cycling in synaptic terminals of neurons.

Regulation of Adenylate Cyclase of Cell Lines From The Nervous System. The inhibition of adenylate cyclase by morphine and the gradual increase in adenylate cyclase activity that results when NG108-15 cells are incubated for 12 or more hours in the presence of morphine was previously proposed as a model for the analgesic action of opiates and for the phenomena of opiate dependence and tolerance. We now find that linoleic acid or serum lipids are required for the morphine-dependent increase in adenylate cyclase activity, but not for inhibition of the enzyme. Similar results were obtained with norepinephrine which activates α -receptors of NG108-15 cells. In this model system, therefore, the inhibition of NG108-15 adenylate cyclase by morphine or norepinephrine can be dissociated from the acquisition of dependence upon opiates or norepinephrine.

Ten μM morphine or norepinephrine completely inhibit the activation of adenylate cyclase by Ca^{2+} ions, but inhibit basal or PGE_1 -activated adenylate cyclase by no more than 55 percent in NG108-15 homogenates. The extent of inhibition of adenylate cyclase by morphine or norepinephrine thus is a function of the Ca^{2+} ion concentration and the proportion of adenylate cyclase molecules that are activated by Ca^{2+} ions.

Activation of serotonin receptors of NG108-15 or NCB-20 hybrid cells by serotonin results in cell depolarization, action potentials, and secretion of acetylcholine into the medium. These responses desensitize in less than 15 sec

and are not inhibited or mimicked by LSD. Serotonin also stimulates adenylate cyclase activity of NCB-20 hybrid cells, but the effect of serotonin does not desensitize. Eadie-Scatchard analysis suggests a bimolecular interaction and reveals no evidence of receptor heterogeneity. The Hill interaction coefficient is 1.0, indicating independent, noncooperative reactions. LSD activates adenylate cyclase ($K_{act} = 12$ nM) and also inhibits the activation of the enzyme by serotonin ($K_i = 10$ nM). In addition, mianserin and cyproheptadine inhibit serotonin activation of adenylate cyclase ($K_i = 43$ nM and 95 nM, respectively) and LSD activation of adenylate cyclase ($K_i = 100$ nM and 64 nM, respectively). These results show that serotonin and LSD interact during activation of adenylate cyclase.

Binding sites for [3 H]LSD were detected in NCB-20 homogenates; the K_{Dapp} was 36 nM, the Hill coefficient was 1.0, and the receptor concentration was 385 fmol/mg of protein. [3 H]LSD was displaced by serotonin ($K_i = 110$ -180 nM). These results agree well with those found to be mediated by a serotonin receptor responsive to LSD that mediates activation of adenylate cyclase. Two binding sites for [3 H]serotonin were detected in NCB-20 homogenates [$K_{Dapp} = 200$ nM and 3750 nM] and serotonin-LSD interactions also were detected.

We conclude that NCB-20 hybrid cells possess two species of serotonin receptors, one coupled to activation of adenylate cyclase, the other to cell depolarization and acetylcholine release; that activation of adenylate cyclase does not affect the rate of acetylcholine release, and, conversely, that serotonin-dependent cell depolarization does not affect intracellular levels of cAMP or cGMP in the hybrid cells tested.

Muscarinic Acetylcholine Receptors. [3 H]-Quinuclidinyl-benzilate (QNB) was used to study muscarinic acetylcholine receptors in NG108-15 membrane preparations. The apparent dissociation constant of [3 H]-QNB is 1×10^{-10} M; the average NG108-15 cell possesses 30,000 specific sites for [3 H]-QNB. Activation of the receptors with acetylcholine or carbachol results in cell depolarization, a small increase in cellular cGMP, and inhibition of adenylate cyclase. Cell depolarization and rise in cGMP levels desensitize in 30 sec; whereas, the inhibition of adenylate cyclase does not desensitize. Scatchard analysis revealed only one homogeneous class of [3 H]-QNB binding sites; however, biphasic rates of [3 H]-QNB association with and dissociation from receptors were found. Evidence was obtained for the formation of a dissociable [[3 H]-QNB-Receptor] complex which then is converted to a form which dissociates only slowly. Hill coefficients of approximately 1.0 were found for receptor antagonists and approximately 0.5 for receptor activators. A sequential series of reactions were proposed to account for these observations and for the various states of the muscarinic acetylcholine receptor that were detected.

Nicotinic Acetylcholine Receptors. An α BT-horseradish peroxidase conjugate was used to study the distribution of nicotinic acetylcholine receptors in developing chick retina. Incubation of the retina *in vitro* with the conjugate allowed quantitative comparison of developmental stages. α BT-binding synapses were found at the early stages of synapse formation and comprised between 5 and 11% of the inner plexiform layer synapse population during *in ovo* development.

The acetylcholine receptor aggregation factor from neuroblastoma x glioma hybrid cells was partially purified by ion exchange chromatography, gel filtration, and

preparative isoelectric focusing. Factors with similar activity were detected in embryonic brain and cultures of sympathetic ganglion neurons and spinal cord neurons, but not in liver, adult brain, or embryonic glial cell cultures.

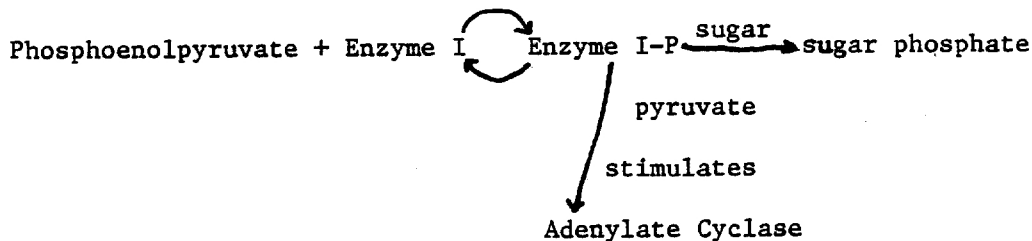
Detergent treatment under appropriate conditions removed most lipid and soluble protein from cultures skeletal muscle cells, but left the cytoskeleton and bound components intact. This extraction was used to distinguish tightly bound and loosely bound populations of acetylcholine receptors, which may be correlated with the degree of receptor aggregation.

Endorphin Synthesis and Secretion. AtT-20 mouse pituitary tumor cells were shown to synthesize and secrete β -endorphin (β -lipotropin₆₁₋₉₁). The cells contain at least 1 nmole β -endorphin equivalents of opioid peptides per mg cell protein. Analysis of cell extracts by gel filtration and high pressure liquid chromatography indicate that the activity is due to β -endorphin, α -endorphin (β -lipotropin₆₁₋₇₆), and γ -endorphin (β -lipotropin₆₁₋₇₇), in the approximate proportions 70%, 24%, and 6%, respectively. Subcellular fractionation indicated that most of the activity is located in the granular fraction. Electron microscopy revealed the presence of osmiophilic granules resembling the secretory granules of corticotrophs of the anterior pituitary. These granules were positive for β -endorphin/ β -lipotropin immunoreactivity, when assayed. Thus, AtT-20 tumor cells possess a mechanism similar to that of normal endocrine cells for packaging peptides destined for secretion.

In the absence of serum, basal secretion of β -lipotropin/ β -endorphin immunoreactivity is 20-30 pmoles per mg protein per hr and secretion is linear for at least 12 hr. Fifty to 70% of the immunoreactivity secreted is due to β -lipotropin-like peptides and the rest to β -endorphin-like peptides. Thus much β -lipotropin is secreted with further processing.

Secretion is stimulated 5-8 fold by brief exposure of cells to elevated K^+ ion concentration; this stimulation is dependent on Ca^{++} ions. Glucocorticoids, such as dexamethasone, reduce the secretion of β -lipotropin/ β -endorphin within 2 hr; for example, secretion is reduced by 33% or 67% after 2 or 8 hrs, respectively. During this time the intracellular content remains the same, however, intracellular content diminishes after 24 hr of treatment. The half-maximally effective dexamethasone concentration is 2 nM. The effect of dexamethasone on secretion is abolished by cycloheximide or actinomycin D which inhibit protein synthesis and RNA synthesis, respectively. This suggests that glucocorticoids act at the transcriptional level to induce the synthesis of protein(s) which inhibit the secretion of ACTH and β -endorphin. Other workers have shown recently that corticotropin/ β -lipotropin mRNA is gradually reduced by glucocorticoid treatment for 1-4 days. The present results with AtT-20 cells suggest that glucocorticoids have an earlier different effect on secretion than the slower reduction in mRNA for the prohormone.

Cyclic Nucleotides In E. Coli. Our previous studies led to the development of a model for the regulation of adenylate cyclase involving the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The proposal has been made that Enzyme I of the PTS interacts in a regulatory sense with the catalytic unit of adenylate cyclase:



The phosphoenolpyruvate (PEP)-dependent phosphorylation of Enzyme I is assumed to be associated with a high activity state of adenylate cyclase. The pyruvate or sugar-dependent dephosphorylation of Enzyme I is correlated with a low activity state of adenylate cyclase. Evidence in support of the proposed model involves the observation that Enzyme I mutants have low cAMP levels and that PEP increases cellular cAMP levels and, under certain conditions, activates adenylate cyclase. Kinetic studies indicate that various ligands have opposing effects on adenylate cyclase. While PEP activates the enzyme, either glucose or pyruvate inhibit it. The unique relationships of PEP and Enzyme I to adenylate cyclase activity provide further support for the model outlined above.

The interaction of adenylate cyclase with sugars that are transported by systems other than the PTS also were explored. Sugars such as lactose are transported without modification by a mechanism involving proton cotransport; this mechanism requires a proton motive force across the cell membrane. We have been able to show that uptake of sugars through the lactose transport system results in inhibition of adenylate cyclase activity if the proton symport mechanism is also active. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone also inhibits adenylate cyclase activity. These data suggest that the steady-state electrochemical proton gradient regulates the activity of adenylate cyclase. We propose that sugar-dependent inhibition of adenylate cyclase activity may occur by either of two mechanisms. Sugars transported by the PTS inhibit adenylate cyclase activity by dephosphorylation of a regulatory protein, while sugars transported by the proton motive force system inhibit adenylate cyclase activity as a result of collapse of the proton electrochemical gradient.

Metabolism of Thyrotropin Releasing Hormone. Previously we described an enzyme (pyroglutamate aminopeptidase) in brain extracts that converts TRH to histidyl-prolineamide which spontaneously cyclizes to histidyl-proline diketopiperazine. We also presented evidence of the presence in hypothalamic extracts of an enzyme (TRH deamidase) that converts TRH to pyroglutamyl-histidyl proline. Further studies have led to the isolation from brain of an imidopeptidase for histidyl-prolineamide not previously described. The enzyme was found in extracts of porcine brain acetone powder and purified by conventional column chromatography on DEAE cellulose resulting in the separation of the enzyme from other enzymes that metabolize TRH. The best substrates for the imidopeptidase contain an α -amino group on histidine and a blocked carboxyl group on proline, as is found in histidyl-prolineamide. Other polypeptide hormones were shown to inhibit imidopeptidase activity. Inhibition of the enzyme by adrenocorticotrophic hormone (1-24) is noncompetitive. These studies have led us to propose that pituitary hormones may stimulate the production of histidyl-proline diketopiperazine by inhibiting alternate routes of TRH metabolism.

The Biological Activity of Histidyl-Proline Diketopiperazine. Previously we showed that injection of radioactive TRH into rat brain led to the formation of radioactive histidyl-proline diketopiperazine, establishing this compound as a naturally occurring brain peptide. While TRH could antagonize the effects of ethanol in inducing sleep in rats, the dipeptide diketopiperazine was substantially more active than TRH. We therefore suggested that the activity of TRH in antagonizing ethanol narcosis may require its conversion to histidyl-proline diketopiperazine.

We have continued to explore the biological activities of histidyl-proline diketopiperazine and find that it plays a role in thermoregulation and in the regulation of brain cyclic nucleotide levels.

Intraventricular administration of histidyl-proline diketopiperazine to rats produces a dose-dependent hypothermia at 4° or 24°, but not at 31°. At 4° administration of TRH elicits a dose-dependent hypothermia up to 0.1 µmole/Kg which is not evoked at higher doses. At 24°, TRH administration results in no change in temperature, whereas it induces hypothermia at 31°. At 4°, TRH antagonizes and TRH antiserum potentiates the hypothermic effects of histidyl-proline diketopiperazine, suggesting opposing effects of TRH and histidyl-proline diketopiperazine on thermoregulation.

Intraperitoneal administration of thyrotropin releasing hormone (50 µmole/Kg) produced an approximately 2-fold increase in rat brain cGMP concentration within 15 min. Histidyl-proline diketopiperazine produced a similar effect, but the response was faster and shorter-lasting. Intraperitoneal administration of ethanol (1.5 g/Kg) decreased brain cGMP concentration approximately 50% within 10-15 min; thyrotropin releasing hormone or histidyl-proline diketopiperazine, injected 5 min after ethanol, antagonized the ethanol-induced increase in cGMP.