DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: 01 HL 00009-13

PERIOD COVERED
October 1, 1986 - September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cell Recognition and Synapse Formation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Marshall Nirenberg, Chief, LBG, NHLBI
Hemin Chin, Staff Fellow, LBG, NHLBI
Patricia Bray, Biologist, LBG, NHLBI
Li-Shan Hsieh, Visiting Fellow, LBG, NHLBI
Wu-Hong Tsai, Visiting Fellow
Maria Giovanni, Staff Fellow, NLBG, NHLBI
David Trisler, Guest Worker, LBG, NHLBI
Haruhiro Higashida, Visiting Scientist, LBG, NHLBI

COOPERATING UNITS *(if any)*
Gerald Aurbach, Chief, MDB, NIADDK
Allen Spiegel, MDB, NIADDK
Bruce Schrier, LDN, NICHD
Lou Hirsch, Dept. of Biochem., U. of Texas, Dallas, Texas

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section of Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11	9	2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mouse neuroblastoma-rat retina hybrid cell lines were established to rescue the expression of retinal genes. A monoclonal antibody that binds to cells from one hybrid line recognizes an antigen expressed by few cell types in retina and brain. cDNA corresponding to the neural antigen was cloned and sequenced. Additional monoclonal antibodies were obtained that bind to antigens that are markers of cell types in retina. Clones for 4 species of α_s cDNA and 1 α_s genomic DNA were obtained that correspond to the α_s subunit of G_s signal transduction protein. Two α_i-1 cDNA clones were obtained and sequenced. In addition, DNA clones for rat and human α_o genes were obtained. Monospecific antibodies to an α-subunit of voltage-sensitive Ca^{2+} channels from rat T-tubules were shown to activate voltage-sensitive calcium channels of parathyroid cells. Both cDNA and genomic DNA clones were obtained that correspond to species of poly A^+ RNA that increase or decrease in abundance when cells are treated with dibutyryl cAMP. TOP, a cell membrane protein that is distributed in a dorsal-ventral topographic gradient in chick retina was shown to be distributed in an inverted but matching ventral-dorsal gradient in chick embryo tectum. Proteins rich in lysine markedly stimulate the activity of a protein kinase in Xenopus oocyte membranes and NG108-15 membranes. NG108-15 cells respond to bradykinin by increased hydrolysis of phosphatidylinositol-4,5-bisphosphate and accumulation of inositol-1,4,5-trisphosphate, which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C. Cytoplasmic Ca^{2+} activates a Ca^{2+}-dependent K^+ channel leading to cell hyperpolarization. The activation of protein kinase C by bradykinin leads to an increase in the phosphorylation of 4 proteins and the inhibition of M-channels. Activation of protein kinase C greatly potentiates Ca^{2+} dependant acetylcholine secretion from NG108-15 cells.

Major Findings:

N18TG-2 mouse neuroblastoma cells were fused with 18-day rat embryo retina cells, and somatic cell hybrid lines then were established to obtain clonal cell lines that continue to express genes characteristic of cells in retina. One of the hybrid cell lines generated, N18RE-103 possesses high tyrosine hydroxylase activity and synthesizes dopamine; whereas, tyrosine hydroxylase is not expressed by the parental N18TG-2 cells. N18RE-103 cells also possess voltage-sensitive Na^+, K^+, and Ca^{2+} channels. N18RE-103 cells were used as an immunogen for the production of hybridoma cell lines that synthesize monoclonal antibodies to antigens expressed by N18RE-103 cells and retina. Thirty-three of the 374 hybridoma cell lines obtained synthesize antibodies to antigens expressed by N18RE-103 cells and rat retina cells, but not with cell lines from other tissues. Some of the antigens recognized by the monoclonal antibodies are expressed by different cell types in adult rat retina. For example, antibody 41C5 binds to antigen expressed by some, but not all, retinal ganglion neurons. Antibody 41A4 binds to antigen associated with the ganglion neuron layer. Antibody 89A5 binds to molecules restricted predominantly to the outer segments of photoreceptor cells. Antibody 38B6 binds to an abundant antigen on photoreceptor cell bodies and to less abundant antigen associated with horizontal, bipolar, and amacrine neuron soma. In contrast, antibody 35F8 recognizes antigen distributed in a punctate manner in the inner nuclear layer, the inner synaptic layer, and ganglion neuron layer of retina.

A λgt11 cDNA expression library was prepared and the recombinants were screened with antibody 41C5. One positive recombinant clone, λ41C5, was detected, which directs the synthesis of a 41C5-β galactosidase fusion protein recognized by antibody 41C5. Northern blot analysis with N18RE-103 poly A^+ RNA revealed one species of 41C5 poly A^+ RNA with a chain length of approximately 1 Kb. The nucleotide sequence of the λ41C5 insert was determined. One open reading frame was found, but no homology was detected between the predicted amino acid sequence of 41C5 and other proteins.

The distribution of 41C5 protein was shown by indirect immunofluorescence to be highly restricted to certain cells in rat retina and brain; the antigen was not detected in striated muscle, cardiac muscle, liver, or kidney. The distribution of 41C5 mRNA also was shown by *in situ* hybridization with a synthetic labeled oligodeoxynucleotide probe to be highly restricted in rat retina

and brain. 41C5 cDNA will be used as a probe to study the mechanisms that enable only a few cell types in the nervous system to express the 41C5 gene.

Hybridoma cell lines were generated that synthesize antibodies directed against cells from rat or rabbit retina that were used as immunogens. One monoclonal antibody detects an antigen that is most abundant in the outer layer of axons in the optic nerve. An antibody specific for the inner and outer synaptic layers of the retina was found as well as antibodies that bind only to Muller cells. Still other antibodies are specific for astrocytes in the retinal ganglion neuron layer. Some of the antigens that are markers of cell types were found to be proteins and were partially purified.

As described previously, 4 species of α_s cDNA clones were found that differ in nucleotide sequence in the region that corresponds to amino acid residues 71-88 in α_s protein, a subunit of α_s signal transduction protein. A mechanism was proposed for generating 4 species of α_s mRNA from a common precursor RNA transcribed from a single gene by 2 types of alternative splicing. Further work on α_s mRNA revealed that half the normal amount of α_s mRNA is present in fibroblasts of pseudo- hypoparathyroid patients. Levels of α_s mRNAs that code for low and high molecular weight forms of α_s protein were reduced with no apparent change in the ratio of the different species of α_s mRNA to one another. These results suggest that the genetic lesion in pseudohypoparathyroidism type Ia either decreases the rate of synthesis of a common precursor of the 4 species of α_s mRNA or increases equally the rates of turnover of the four species of α_s mRNA.

Two α_i-1 cDNA clones also were obtained from a human brain cDNA library that correspond to α_i protein subunits of G_i signal-transduction proteins. One of the cDNA inserts was sequenced completely, the other partially. The nucleotide sequence of human brain α_i-1 is similar to that of bovine brain α_i-1, but differs significantly from α_i-2 cDNAs from human monocytes, rat glioma cells, and mouse macrophages in nucleotide and amino acid sequences (88% and approximately 73% homology, respectively). The 3'-untranslated region of human brain α_i-1 also differs markedly from that of human monocyte α_i-2.

Five genomic DNA clones for α genes were obtained. One clone was identified as a gene for α_s, and 2 clones, 1 from a human genomic DNA library, the other from a rat genomic DNA library, were identified as α_o genes. Further work is needed to identify the 2 remaining α genomic DNA clones. The focus in current

studies is to define the nucleotide sequences of the 5'-upstream regulatory regions of α genes.

A λgtll cDNA library was constructed from poly A^+ RNA from 14 day chick embryo retina and screened for recombinants with antibodies to β-subunits. One recombinant was detected with 9 out of 10 monospecific rabbit antibody preparations directed against the β-subunit of bovine transducin. The nucleotide sequence of the cDNA insert was determined and the predicted amino acid sequence was compared to that of the bovine β-subunit. Only a small region of homology was observed; hence, the cloned DNA is not related to the β Six of 10 amino acid residues predicted for the recombinant DNA were identical to amino acid residues near the N-terminus of the β-subunit and 2 glutamine residues of the β-subunit were replaced by glutamic acid residues. A synthetic decapeptide corresponding to the amino-terminal residues of β blocked the binding of anti β antibodies to β-subunits. These results show that the amino terminal decapeptide of the β- subunit is a major antigenic site of the native protein.

L-Type voltage-sensitive calcium channels are sensitive to dihydropyridine agonists and antagonists. Activation of the L-type voltage-sensitive calcium channels of parathyroid cells opens the calcium channels and permits the entry of extracellular calcium ions into the parathyroid cells, which paradoxically, inhibits parathyroid hormone release. Calcium channel antagonists that block calcium entry into cells, stimulate secretion of the parathyroid hormone. Polyclonal, monospecific mouse antibodies to highly purified preparations of the α-subunit of voltage-sensitive calcium channels from rat T-tubules were obtained and were tested for their effects on parathyroid hormone secretion by cells. Three mouse antibody preparations blocked the secretion of parathyroid hormone from cells. Parathyroid cell protein was solublized and fractionated by SDS gel electrophoresis. The antibodies bound to only one major band of protein with an apparent M_r of 150,000. Incubation of a mouse antibody with parathyroid cells resulted in the activation of voltage-sensitive calcium channels and markedly increased Ca^{2+} uptake by parathyroid cells and inhibited parathyroid hormone release. These results suggest that the antibodies bind to α subunits of voltage-sensitive calcium channels of parathyroid cells and activate the calcium channels.

Previously we showed that elevation of cAMP levels of NG108-15 neuroblastoma-glioma hybrid cells or neuroblastoma cells for several days results in marked increases in the activities of voltage-sensitive Na^+, K^+, and Ca^{2+} channels, and the rate of spontaneous secretion of acetylcholine at synapses between the

hybrid cells and cultured striated muscle cells. We obtained cDNA clones for species of poly A^+ RNA that increase in abundance when NG108-15 or NS20-Y cells are treated for 5 days with dibutyryl cAMP, as well as cDNA clones for other species of mRNA that decrease in abundance. The dibutyryl cAMP-dependent increases in poly A^+ RNA range from 5- to 90-fold above those of control cells, depending upon the species of mRNA examined. The levels of most species of poly A^+ RNA were not affected by treatment of cells with dibutyryl cAMP. Northern blot analysis showed that some independently isolated recombinant clones hybridize to the same species of mRNA; however, cDNA clones were obtained for approximately 15 species for dibutyryl cAMP responsive species of mRNA. A mouse genomic DNA library in Charon 30 was screened with cDNA probes for some dibutyryl cAMP responsive species of mRNA. Fragments of the cloned genomic DNA will be tested for promoter and/or enhancer activities.

TOP is a cell surface protein that is distributed topographically in a 35 fold gradient from the dorsal margin of chicken retina, which contains the highest concentration of TOP, to the ventral margin of the retina, which contains little TOP. An inverted gradient of TOP was detected in the tectum of 3 to 5 day chick embryos. The highest concentration of TOP is present in ventral tectum and the lowest is in dorsal tectum. The topographic map of cell position in the avian retina is inverted in its projection to the optic tectum. Dorsal retinal ganglion neuron axons project to ventral tectum and ventral retinal ganglion neurons axons project to dorsal tectum. Gradients of TOP are present in chick embryo retina and tectum before retinal axons arrive in the tectum. After 10 days of embryonic development, the number of antigenic TOP sites in the tectum decreases markedly; whereas, TOP levels in the retina remain relatively constant. The presence of corresponding TOP gradients in retina and tectum at the time of innervation of the tectum by retinal ganglion neuron axons suggests a possible role for TOP in orienting the dorsal-ventral axis of the retinal projection onto the tectum by homophilic interactions between TOP molecules.

The half-life of TOP in cultured retinal cells in the presence of cycloheximide or actinomycin D was 5 or 6 hr, respectively. Ablation of cells at the poles of the gradient in 60 hr chick embryos altered TOP expression during subsequent retinal development. Cells at the dorsal pole of the 13-day embryo retinas, 11.5 days after dorsal ablation, expressed 50% less TOP than normal and those at the ventral pole, after ventral ablation, expressed 300% more TOP than normal. Cells in other regions of the retina expressed normal levels of TOP.

Polypeptides rich in lysine markedly stimulate the phosphorylation of some membrane proteins catalyzed by a protein kinase in Xenopus oocyte membranes or membranes from NG108-15 neuroblastoma-glioma hybrid cells. A synthetic peptide containing the last 14 amino acid residues of human Ki-ras 2 protein (KKKKKKSKTKCVIM),which is rich in lysyl-residues, also stimulates protein phosphorylation. These effects were not observed with polyarginine. Polylysine peptides including the synthetic c-Ki-ras 2 peptide also stimulate the in vitro phosphorylation of membrane inositolphospholipids, resulting in the synthesis primarily of phosphatidylinositol 4-phosphate and to a lesser extent, phosphatidylinositol 4,5-bisphosphate.

Hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) produces two prospective intracellular messengers: inositol-1,4,5-trisphosphate ($InsP_3$), which releases Ca^{2+} from intracellular stores; and diacylglycerol (DG), which activates protein kinase C. The formation of these two substances triggered by one external messenger, bradykinin, leads to the appearance of two different sequential membrane conductance changes in NG108-15 neuroblastoma-glioma hybrid cells. The addition of bradykinin to these cells rapidly stimulates hydrolysis of PtdIns(4,5)P_2 to $InsP_3$ and DG, raises intracellular Ca^{2+} and hyperpolarizes and then depolarizes the cell membrane. By voltage-clamp recording hyperpolarization was shown to result from the activation of a pharmacologically-identifiable species of Ca^{2+}-dependent K^+ current. This is also activated by intracellular injections of Ca^{2+} or $InsP_3$ so may be attributed to the formation and action of $InsP_3$. The subsequent depolarization results primarily from the inhibition of a different, voltage-dependent K^+ current, the M-current that is also inhibited by DG activators. Hence a dual, time-dependent role is described for these two intracellular messengers in the control of neuronal signalling by a peptide.

Iontophoretic injections of inositol 1,4,5-trisphosphate inside NG108-15 cells evoked an outward K^+ current across the outer cell membrane, probably activated by the release of intracellular Ca^{2+}. No such current was produced by equivalent intracellular injections of inositol 1,3,4-trisphosphate or inositol 1,3,4,5-tetrakisphosphate. Instead, these compounds evoked an inward current with a reversal potential of about -20 mV, which may therefore be due to a non-specific cation conductance. This suggests that these compounds are unable to release sufficient Ca^{2+} to activate the Ca^{2+} -dependent K^+ current in these cells.

The role of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) as possible mediators of the membrane current

responses of NG108-15 cells to bradykinin (BK) has been tested using intracellular iontophoresis of $InsP_3$ and external application of phorbol dibutyrate (PDBu) and 1-oleoyl-2-acetylglycerol (DAG). Intracellular iontophoresis of $InsP_3$ into cells clamped at -30 to -50 mV produced (i) a transient outward current, (ii) a transient outward current followed by an inward current, or (iii) an inward current. All currents were accompanied by an increased input conductance. The transient outward current reversed at between -80 and -90 mV. The reversal potential was shifted to more positive potentials on raising extracellular $[K^+]$, suggesting that it resulted from an increased K^+ -conductance. The outward current was inhibited by apamin (0.4 uM) or d-tubocurarine (0.2-0.5 mM); these drugs also inhibit the outward current produced by BK or by intracellular Ca^{2+} injections. The outward current was also slowly reduced in the absence of external Ca^{2+} or in the presence of a solution containing 0.5 mM Cd^{2+} and 2 mM Co^{2}. Iontophoretic injection of inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, guanosine trisphosphate or inorganic phosphate did not evoke an outward current but produced only an inward current with an increased conductance, reversing at between -10 and -20 mV. Bath-application of PDBu (10 nM - 1 uM) or DAG (1 - 10 μM) produced an inward current with a fall in input conductance. The inward current was voltage-dependent and was accompanied by an inhibition of the time-dependent current relaxations associated with activation or deactivation of the voltage-dependent K^+-current. PDBu did not clearly reduce the Ca^{2+}-current or the Ca^{2+}-dependent K^+-current recorded in these cells. During superfusion with PDBu, the outward current produced by intracellular iontophoresis of $InsP_3$ was greatly enhanced. Analysis of homogenates of ^{32}P-labelled NG108-15 cells by two-dimensional gel electrophoresis revealed more than 260 phosphoproteins. Four proteins were phosphorylated due to the addition of both BK and PDBu, including the 80,000 M_r substrate for protein kinase C. The results support the view that the two membrane current responses to BK might result from accelerated membrane phosphatidylinositide hydrolysis. One product, $InsP_3$, releases Ca^{2+} and activates an apamin/curare sensitive outward K^+ current; this effect is imitated by intracellular $InsP_3$ iontophoresis. The second product, DAG, activates protein kinase C to inhibit the voltage-dependent K^+-current and generate an inward current; this effect is imitated by external application of PDBu or DAG, and is associated with increased protein phosphorylation.

The action of bradykinin (BK), inositol 1,4,5-trisphosphate ($InsP_3$), and phorbol dibutyrate (PDBu) on the release of acetylcholine (ACh) was studied electrophysiologically on

short-distance (<20 μm) synapses formed between cultured NG108-15 cells and rat muscle cells. Intophoretic application of BK onto the somatic surface of an NG108-15 cell produced an increase in frequency of m.e.p.p.s for 40-50 s in the paired myotube. Some m.e.p.p.s were evoked during BK-induced hyperpolarization (10-20 sec) of the hybrid cell soma. A few m.e.p.p.s also were elicited during BK-induced depolarization. Iontophoretic injection of Ca^{2+} into an NG108-15 cell soma generated m.e.p.p.s for a very brief period (less than 3 sec), coincident with somatic hyperpolarization. No increase was observed during a subsequent somatic depolarization induced by a larger current of Ca^{2+}. Iontophoretic injection of $InsP_3$ into the cytoplasm of an NG108-15 cell soma transiently evoked m.e.p.p.s during the $InsP_3$-induced hyperpolarizing phase. A large $InsP_3$ injection caused sustained generation of m.e.p.p.s for 2-4 min, associated with $InsP_3$-evoked depolarization. Within 3-5 min after exposure of NG108-15-myotube pairs to 1 μM PDBu, the m.e.p.p. frequency increased by 2-5 times and reached a plateau after 8 min. The increase continued after washout of the drug. The PDBu-induced increase of m.e.p.p.s was still observed when the menmbrane potential of the NG108-15 cell was clamped at -30 mV. The data suggest that the BK-induced facilitation results from the action of two intracellular second messengers: an $InsP_3$-dependent release of Ca^{2+} from the intracellular storage sites and protein phosphorylation by DAG-activated protein kinase C.

Publications:

1. Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. and Nirenberg, M.: Human cDNA Clones for Four Species of $G_{\alpha s}$ Signal Transduction Protein. Proc. Natl. Acad. Sci., USA 83, 8893-889 (1986).

2. Bray, P., Carter, A., Guo, V., Puckett, C., Kamholz, J., Spiegel, A., and Nirenberg, M.: Human cDNA Clones for an α Subunit of G_i Signal-transduction Protein. Proc. Natl. Acad. Sci., USA 84, 5115-5119 (1987).

3. Carter, A., Bardin, C., Collins, R., Simons, C., Bray, P. and Spiegel, A.: Reduced Expression of Multiple Forms of G_s alpha in Pseudohypoparathyroidism Type Ia. Proc. Natl. Acad. Sci., USA, In Press.

4. Gatica, M., Allende, C. C., Antonelli, M. and Allende, J.E.: Polylysine-containing Peptides, Including the Carboxyl-terminal Segment of the Human c-Ki-ras 2 Protein, Affect the Activity of Some Key Membrane Enzymes. Proc. Natl. Acad. Sci., USA 84,

324-328 (1987).

5. Trisler, D. and Collins, F.: Corresponding Spatial Gradients of TOP Molecules in the Developing Retina and Optic Tectum. Science, In Press.

6. Trisler, D.: Synapse Formation in Retina is Influenced by Molecules that Identify Cell Position. Current Topics in Devel. Biol. 21, 277-308 (1987).

7. Trisler, D.: Molecular Markers of Cell Position in Avian Retina are Involved in Synapse Formation. American Zoologist 27, 189-206 (1987).

8. Higashida, H. and Brown, D.A.: Two Polyphosphatidylinositide Metabolites Control Two K^+ Currents in a Neuronal Cell. Nature 323, 333-335 (1986).

9. Higashida, H. and Brown, D.A.: Membrane Current Responses to Intracellular Injections of Inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate in NG108-15 Hybrid Cells. FEBS Letters 208, 283-286 (1986).

10. Brown, D.A. and Higashida, H.: A Note on Voltage- and Ca-activated K-currents in Mouse Neuroblastoma x Rat Glioma Hybrid Cells. J. Physiol., In Press.

11. Brown, D.A. and Higashida, H.: Membrane Current Responses of NG108-15 Mouse Neuroblastoma x Rat Glioma Hybrid Cells to Bradykinin. J. Physiol., In Press.

12. Brown, D.A. and Higashida, H.: Inositol 1,4,5-trisphosphate and Diacylglycerol Mimic Bradykinin effects on Mouse Neuroblastoma x Rat Glioma Hybrid Cells. J. Physiol., In Press.

13. Higashida, H.: Acetylcholine Release Induced by Bradykinin, Inositol 1,4,5-Trisphosphate and Phorbol Dibutyrate in Mouse Neuroblastoma x Rat Glioma Hybrid Cells. J. Physiol., In Press.