NEUROTRANSMITTER RECEPTORS

Mechanisms of Action and Regulation

Edited by
Shozo Kito
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PREFACE

This meeting was held commemorating Dr. Kito's 10th Anniversary as Professor of the Third Department of Internal Medicine, Hiroshima University School of Medicine. Dr. Kito was born in 1927 in Nagoya, graduated from Tokyo University School of Medicine and received his M.D. in 1951. He spent his first academic years as a research associate (1952 - 1968) at the Third Department of Internal Medicine, Tokyo University School of Medicine. During this period he studied for one year (1952 - 1953) at Illinois University School of Medicine, and acquired his Ph.D. in 1959. In 1968 he became Instructor and in 1971 he was appointed as Assistant Professor of Tokyo Women's Medical College. In 1973, he became Professor of the Third Department of Internal Medicine, Hiroshima University School of Medicine. Dr. Kito is a clinician but he is always enthusiastic about basic medicine. His major research field concerns neurotransmitters and their receptors in the central nervous system. He prefers a combination of neurotransmitter immunohistochemistry and receptor autoradiography as research techniques. He is also engaged in biochemical studies on amyloid proteins. When the Eighth International Congress of Pharmacology was held in Tokyo in 1981, Dr. Segawa, Dr. Yamamura, and Dr. Kuriyama organized a Satellite Symposium on Neurotransmitter Receptors in Hiroshima. Dr. Kito attended this meeting and was deeply impressed by the active presentations and discussions.

In order to make some contribution to the progress of neurosciences, Dr. Kito chose to have a scientific meeting commemorating his 10th Anniversary as Professor, instead of just having a routine party. For this purpose he asked Dr. Segawa to join in organizing this symposium. The symposium was held in Hiroshima on October 6-8, 1983, and entitled "Neurotransmitter Receptor Regulation, Interactions, and Coupling to the Effectors." Twenty-three invited speakers from five countries and a poster session covered the latest advances in research on virtually all of the neurotransmitter receptors. Emphasis was on effector coupling, especially the receptors which negatively regulate adenylate cyclase, but other

areas included interactions of receptors with each other and ion channels, and new techniques such as autoradiography and molecular biology of receptors. In all likelihood, these subjects will remain at the cutting edge of neuroscience for the next several years.

Shozo Kito
Tomio Segawa
Kinya Kuriyama
Henry I. Yamamura
Richard W. Olsen

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It was Dr. Eiko Itoga, former assistant professor of the Third Department of Internal Medicine, Hiroshima University, who first had the idea of having this symposium during her stay at Dr. Yamamura's laboratory in Tucson, and Dr. Yamamura agreed with her. She had devoted herself to establishment of the Department and morphological studies on neurotransmitters and their receptors until she left the laboratory recently. We tender our warmest acknowledgements to her.

Thanks are also expressed to Miss Masae Inokawa, instructor of Hiroshima University for her excellent secretarial work. Nancy Morrison, at UCLA, had the monumental task of assembling this volume and did a superb job in very rapid fashion.

Finally, this symposium was financially supported by the Japanese Educational Ministry and the Commemorative Association for the Japan World Exposition (1970).

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SELECTIVE BLOCKAGE BY ISLET-ACTIVATING PROTEIN, PERTUSSIS TOXIN, OF NEGATIVE SIGNAL TRANSDUCTION FROM RECEPTORS TO ADENYLATE CYCLASE

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OVERVIEW

The adenylate cyclase-cyclic AMP system is one of the signal transduction processes that has been the subject of extensive studies in recent years. Stimulation of some membrane receptors ("activatory" receptors) by particular agonists produces, via activation of adenylate cyclase, cyclic AMP, which acts as an intracellular second messenger of the receptors by activating one of the several kinds of cytosolic protein kinases. The receptor-linked adenylate cyclase system is now known to consist of three components; the guanine nucleotide-binding protein usually referred to as N, G, or G/F, has been identified as the functional communicator between receptors (R) and the catalytic unit of the cyclase (C). A growing body of evidence has accumulated in support of the concept that N plays the pivotal role in receptor-mediated activation of adenylate cyclase, as reviewed by Ross and Gilman (1), Limbird (2) and Spiegel and Downs (3). When no extracellular signal is available to the cell, the species of the guanine nucleotide bound to N is GDP, which allows functional dissociation of the three components, R, N, and C. If information is transmitted by an agonist (A) which occupies R, the ternary complex, A-R-N is formed as the "turn-on" reaction which leads to replacement of GDP by intracellular GTP at the specific binding sites on N. The adenylate cyclase enzyme is then activated as a result of association of the GTP-bound N with C. Synthesis of cyclic AMP continues until GTP is hydrolyzed by the GTPase "turn-off" reaction, leaving GDP on N.

Numerous kinds of approaches have been employed successfully to afford experimental evidence for the above-outlined sequence of

receptor-linked activation of adenylate cyclase. One of these approaches is the use of cholera toxin (see reference 4 for review). Exposure of a variety of mammalian cells to cholera toxin resulted in enormous increases in adenylate cyclase activity of membranes prepared therefrom, even in the absence of receptor agonists or guanine nucleotides. It has been established that the active fragment of the toxin oligomeric protein is an enzyme catalyzing ADP-ribosylation of a subunit of the N protein. N loses its GTPase activity upon ADP-ribosylation. Consequently, GTP continues to occupy the guanine nucleotide-binding sites on N even after agonists have detached from R. Thus, the adenylate cyclase enzyme retains its high activity irreversibly in membranes from cholera toxin-treated cells.

Apart from the above mentioned activation of adenylate cyclase, stimulation of several other receptors leads to inhibition, rather than activation, of adenylate cyclase (5-7). These "inhibitory" receptors include muscarinic cholinergic, alpha(2)-adrenergic, adenosine (A1) and opiate receptors. GTP is also an essential factor for the inhibition to occur in membrane preparations, suggesting that guanine nucleotide binding proteins are involved in receptor-mediated inhibition as well as activation of the cyclase. It is likely, for reasons described later, that the guanine nucleotide regulatory protein mediating the inhibition is an entity distinct from the N protein involved in the activation. The former is now referred to as N_i and the latter as N_s, where subscripts "s" and "i" stand for "stimulatory" and "inhibitory", respectively. As to the mechanism whereby the signal given to receptors is transferred to the cyclase enzyme via N, much more remains to be solved for N_i than for N_s. An agent that could modify the function of N_i specifically, if any, would contribute to solving the problem. Islet-activating protein (IAP) (8,9), the exotoxin produced by Bordetella pertussis, is the most promising probe in this regard, since our recent studies have shown that receptor-mediated inhibition of adenylate cyclase is reversed by exposure of a variety of cells to IAP (10-13). The present review will summarize the experimental data that IAP directly interacts with N_i in such a fashion as to block the negative signal transduction. It contrasts sharply with cholera toxin that is a specific modifier of N_s.

A BRIEF HISTORY LEADING TO DISCOVERY OF ISLET-ACTIVATING PROTEIN

The heat-labile exotoxin present in the culture medium of multiplying Bordetella pertussis, the pathogenic bacteria for whooping cough, has long been known for its potent biological activities (14). Among these are increases in the number of circulating lymphocytes and increases in the histamine-induced death (induction of histamine hypersensitivity) that are observed upon the injection of pertussis vaccine into mice; a putative factor involved

often has been referred to as a lymphocytosis-promoting factor (LPF) or a histamine-sensitizing factor (HSF). In addition, the injection of pertussis vaccine into rats was very effective in suppressing epinephrine-induced hyperglycemia; the catecholamine was no longer hyperglycemic in rats that had been injected with the vaccine 2 to 7 days before. The earlier attempts to unify these diverse actions of pertussis vaccine resulted in an hypothesis that a toxin present in the vaccine blocks beta-adrenergic receptors; it was based on both the notion that stimulation of beta-receptors by injected epinephrine is responsible for hyperglycemia and the experimental data that the histamine-sensitizing activity of the vaccine was somehow mimicked by beta-adrenergic antagonists.

The first stimulus that urged us to start our own study of pertussis toxin in 1977 was our earlier interest in epinephrine hyperglycemia. Up to that time, we had noticed that both alpha- and beta-adrenergic receptors are responsible for hyperglycemia following epinephrine injection (15,16) and had found that shift of the balance between the two classes of adrenergic receptors in favor of either one was very effective in attenuating hyperglycemia. The balance was effectively shifted by changes in pH of the body fluids (17,18), by injection of adrenocortical hormones (19), by induction of the hypo- or hyperthyroid state (20), or by forced exercise (21); epinephrine-induced hyperglycemia was markedly blunted under these particular conditions. The injection of pertussis vaccine into rats was an additional means effective in suppressing the epinephrine action to cause hyperglycemia (22,23). This action of pertussis vaccine was characterized by its long duration; the rat was unresponsive to epinephrine over a week after a single vaccination, indicating irreversible modification of certain bodily functions. In any case, a shift in balance between alpha- and beta-adrenergic receptor functions was postulated to occur in the animals treated with pertussis vaccine as our working hypothesis. We used insulin secretion as a much better index of adrenergic functions than glycemic changes.

Extraordinary modification of adrenergic functions by pertussis treatment was revealed by perfusion experiments in which the perfusate of rat pancreas was supplemented with epinephrine with or without further addition of an alpha- or beta-adrenergic antagonist to stimulate either class of receptors selectively (24). It was found that alpha-receptors are predominant over beta-receptors in the normal (not treated with vaccine) rat pancreas in such a fashion as to render the infused epinephrine inhibitory to insulin secretion, whereas beta-adrenergic stimulation of insulin secretion is the only event observed in pancreas isolated from pertussis-treated rats. Alpha-adrenergic receptors did not function any longer in pancreas after pertussis treatment of the organ-donor rats. In response to other stimuli such as infusion of glucose, amino acids, or glucagon, much more insulin was also released from the pancreas

of pertussis-treated rats than from pancreas of non-treated rats (24). A search for the factor responsible for this unique action in pertussis vaccine resulted in successful purification of a protein with a Mr value of 117,000 from the 2-day culture supernatant of B. pertussis (Phase I, Tohama strain) (8). This protein, referred to as islet-activating protein (IAP) after its extraordinary action to enhance insulin secretory responses of pancreatic islets, possesses LPF and HSF activities and suppresses epinephrine-induced hyperglycemia due to accompanying hyperinsulinemia, when injected into rodents (9,25). Thus, IAP should be the same entity as the exotoxin termed pertussis toxin (26) or pertussigen (27).

IAP AS A SPECIFIC MODIFIER OF THE RECEPTOR-ADENYLATE CYCLASE LINKAGE

Receptor mediated changes in insulin release from isolated rat islets are usually associated with changes in the cellular cyclic AMP content in the same direction, reflecting the fact that cyclic AMP is the second messenger of these receptors in islet cells (28-30). Modification by IAP of receptor-linked insulin release was also accompanied by the same-directional modification of cyclic AMP changes in islets even when the degradation of cyclic AMP was prevented by methylxanthines or Ro-20-1724. This finding, together with the failure of IAP to affect dibutyryl cyclic AMP-induced insulin release (29), indicates that the process(es) responsible for receptor-linked regulation of adenylate cyclase activity should be the sole site of IAP action. Actually, membrane adenylate cyclase was markedly inhibited by the addition of epinephrine via alpha(2)-adrenergic receptors (31) when the membranes had been prepared from control islet cells, while this inhibition was of much less magnitude when membrane donor cells had been exposed to IAP (12). Moreover, glucagon- or adenosine (A2)-induced activation of adenylate cyclase in islet cell membranes was enhanced, though only slightly, by prior treatment of the cells with IAP (11,32).

The IAP-sensitive cell is not restricted to islets. Exposure of rat heart (13), fat (33) or C6 glioma cells (34), mouse 3T3 fibroblasts (35) or rat glioma x mouse neuroblastoma hybrid NG108-15 cells (36) to IAP resulted either in enhancement of increases in the cellular cyclic AMP content (or activation of membrane adenylate cyclase) via beta-adrenergic, glucagon or prostaglandin E receptors or in abolition (or marked attenuation) of decreases in cellular cyclic AMP (or inhibition of membrane adenylate cyclase) via alpha(2)-adrenergic, muscarinic cholinergic, adenosine (A1), or opiate receptors. It is noteworthy that, in all the IAP-sensitive cell types studied so far, neither the baseline content of cyclic AMP in the cell nor the basal activity of membrane adenylate cyclase that is assessed without stimulation of coupled receptors was altered by IAP treatment; it was their responses to receptor stimu-

lation that was modified by IAP. Hence, IAP is unlikely to interact directly with the adenylate cyclase catalytic protein (C). Furthermore, IAP-induced modification (enhancement or attenuation) of the cellular cyclic AMP (or membrane adenylate cyclase) response to receptor agonists is invariably observed in such a manner that the agonist concentration-response curve is shifted "upwards" rather than "to the left"; i.e., the degree of response to agonists is modified, without changes in the affinity of agonists for receptors, by IAP treatment (11-13,32-36). It is thus unlikely that the receptor protein (R) is a direct target of IAP action. As direct evidence for this notion, the affinity for antagonist binding to membrane receptors was not affected by the treatment of membrane-donor cells with IAP (36,37). In certain cases, GTP-induced activation of membrane adenylate cyclase even in the absence of receptor agonists was enhanced by IAP (34,35), suggesting that IAP could interact with the guanine nucleotide regulatory protein (N).

ADP-RIBOSYLATION OF A MEMBRANE PROTEIN BY IAP AS A MECHANISM FOR MODIFICATION OF THE RECEPTOR-ADENYLATE CYCLASE SYSTEM

All the data mentioned above were obtained with intact cells exposed to IAP for times as long as 2-24 h. Direct addition of IAP by itself to the cell-free membrane preparations failed to exert any influence on the membrane adenylate cyclase system even after a long incubation, suggesting that a cytosolic factor would be essential for membranes to be affected by IAP. Such a factor was later identified as NAD. GTP-dependent (and isoproterenol-stimulated) adenylate cyclase activity of membranes from C6 cells was markedly enhanced after the membranes were incubated for a short while with IAP only if the incubation medium was further fortified with NAD and ATP (38). NAD served as the substrate of an IAP-catalyzed novel reaction; labeled NAD was incorporated into a membrane protein with an Mr value of 41,000 upon incubation of membranes with IAP.

This IAP-catalyzed labeling of the membrane Mr = 41,000 protein exhibited the following characteristics (39). 1. The radioactivity was detected in this protein when the ADP-ribose moiety of NAD was labeled with ^{32}P, ^{14}C or ^{3}H, whereas no radioactivity was incorporated from [carbonyl-^{14}C]NAD. Moreover, [α-^{32}P]NAD once incorporated into the Mr = 41,000 protein in the presence of IAP was liberated upon subsequent incubation of membranes with snake venom phosphodiesterase as 5'-AMP. Thus, IAP displays ADP-ribosyltransferase activity with the Mr = 41,000 protein as substrate. 2. Tryptic digestion of the IAP-labeled Mr = 41,000 protein was markedly interfered with by the prior incubation of membranes with Gpp(NH)p or NaF, specific ligands of the N protein, probably due to a change in the peptide conformation. Thus, the IAP specific substrate, the Mr = 41,000 protein, must be one of the subunits of the N protein. 3. Incubation of membranes with labeled NAD in the presence of the

A_1 (active) component of cholera toxin resulted in ADP-ribosylation of membrane proteins with Mr values of 45,000 and 48,000/49,000 (doublet), proteins distinct from the substrate protein of the IAP-catalyzed reaction. 4. The degree of IAP-catalyzed ADP-ribosylation of the Mr = 41,000 protein was strictly correlated with IAP-induced enhancement of receptor-dependent adenylate cyclase activity under various conditions. Such was also the case when intact cells were exposed to IAP.

The higher the concentration of IAP in the medium for incubation of cells, the smaller the amount of ADP-ribose incorporated into the Mr = 41,000 protein during the subsequent incubation with IAP of membranes isolated therefrom. No ADP-ribose was incorporated any longer into the membrane Mr = 41,000 protein, if membrane-donor cells had been exposed to a saturating concentration of IAP. Enhancement of receptor-mediated adenylate cyclase activity in membranes as induced by the prior treatment of the membrane-donor cells with IAP was inversely correlated in magnitude with [^{32}P]ADP-ribosylation of the Mr = 41,000 protein caused by IAP during the incubation of another batch of the same membranes. This is because the ADP-ribosyl moiety of the intracellular NAD is transferred to the membrane Mr = 41,000 protein during exposure of intact cells to IAP; as a result, no further incorporation of [^{32}P]ADP-ribose occurs to the same protein molecule during the subsequent incubation of the isolated membranes. Thus, ADP-ribosylation of the Mr = 41,000 protein is invariably associated with the occurrence of the unique action of IAP on the membrane receptor-adenylate cyclase system either in intact cells or in isolated membranes.

These characteristics afford convincing evidence for the idea that IAP exerts its unique influence on the membrane signal transmission processes as a result of ADP-ribosylation of a subunit of the guanine nucleotide regulatory protein (N) which differs from the peptide serving as the substrate of the cholera toxin-catalyzed similar reaction.

BLOCKADE BY IAP OF N_i-MEDIATED NEGATIVE SIGNAL TRANSDUCTION

The function of the N protein is bidirectional; in one direction, it associates with the receptor protein (R) when R is occupied by an agonist and N is bound with GDP, while, in another direction, it activates (in the case of N_s as a positive signal) or inhibits (in the case of N_i as a negative signal) the adenylate cyclase enzyme (C) when bound GDP is replaced by GTP subsequent to agonist binding to R. We have studied how these functions are affected by IAP, and concluded that IAP blocks the negative signal transduction via N_i. The experimental basis for this conclusion is as follows.

1. Adenylate cyclase of adipocyte membranes exhibits biphasic